EFFECTS OF AGE ON NICOTINE CONSUMPTION AND
ENZYME ACTIVITY FOLLOWING METHOXSALEN
ADMINISTRATION IN C57BL/6J MALE MICE

A Dissertation in
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by
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ABSTRACT

With tobacco use and nicotine addiction reaching epidemic proportions worldwide, the race to find effective smoking cessation aids and methods for all populations of individuals is in full swing. There are several drugs on the market today specific for this purpose; however, many of these pharmacotherapies are not universally helpful, particularly for adolescents. Age, along with interindividual genetic polymorphisms in the enzyme responsible for nicotine metabolism (CYP2A6), may contribute to these disparities. Mimicking the genetic variants of this enzyme with known CYP2A6 inhibitors (e.g., methoxsalen) in adolescents and adults could provide crucial evidence for the role of these biological and genetic influences on cigarette consumption and nicotine dependence.

This dissertation is aimed to understand the effects of age on nicotine consumption and metabolism following acute and chronic methoxsalen administration in adolescent and adult male C57BL/6J mice. Experiment I was a 1-day study in which adolescent (n=60) and adult (n=60) male C57BL/6J mice received either no injection or a single subcutaneous injection of either physiological saline, vehicle (Emulphor, distilled water and ethyl alcohol) or one of two doses of methoxsalen (5 or 10 mg/kg), followed by access to a 3-bottle oral nicotine paradigm [tap water, 50 (LOW NIC) and 200 (HIGH NIC) ug/ml (-)-freebase nicotine dissolved in tap water] for 12 hours.

To further investigate the age differences in nicotine consumption and drug effects on serum cotinine (ng/ml/g), an 8-day two-phase study with adolescent (n=32) and adult (n=32) male C57BL/6J mice (Experiment II) was conducted. For the first four days (Phase I), animals received 24-hour unlimited access to the 3-bottle oral nicotine
paradigm (tap water, LOW NIC and HIGH NIC). During the four days of Phase II, animals continued to have access to the water and nicotine bottles, but were also given daily subcutaneous injections of either vehicle or 10 mg/kg 8MOP at the end of the light cycle. For Experiment I and II, nicotine exposure continued until sacrifice when livers were removed and trunk blood was collected to assess hepatic CYP2a5 and CYP2e1 gene expression and serum cotinine levels, respectively.

In Experiment I, adults consumed more nicotine (mL, mg/kg, % total fluid intake) than did adolescents, regardless of drug treatment. Results for Experiment II regarding nicotine consumption were not conclusive. For both studies, however, methoxsalen was found to be an age-dependent CYP2a5 inhibitor, such that serum cotinine levels (ng/ml/g) were reduced with increasing dosages of methoxsalen in adults, but not adolescents. Drug treatment and age did not alter relative gene expression levels for CYP2a5 or CYP2e1.

Results of these two experiments provide significant contributions to the small pool of scientific literature regarding the effects of methoxsalen on nicotine consumption and pharmacokinetics in mouse models. Alternative methods for future studies that are indicated below are necessary to test explanations for the effects observed in this dissertation as well as to begin to understand what effect other biological determinants (e.g., sex) have on nicotine consumption and metabolism. Preclinical studies, such as Experiment I and II, are important stepping stones to answer the overarching question of whether or not smoking cessation aids can be tailored to the individual.
# TABLE OF CONTENTS

LIST OF TABLES ................................................................................................................. xv
LIST OF FIGURES ............................................................................................................. xviii
ABBREVIATIONS ............................................................................................................. xxv
ACKNOWLEDGEMENTS ................................................................................................. xxvi
CHAPTER I: INTRODUCTION .................................................................................... 1

- Tobacco Smoking: Incidence and Significance .................................................. 2
- Age, Smoking Onset and Cessation ........................................................................ 3
- Nicotine Pharmacology ....................................................................................... 4
  - Nicotine Pharmacokinetics ............................................................................. 4
    - Absorption .................................................................................................. 5
    - Distribution ................................................................................................. 6
    - Metabolism .................................................................................................. 6
    - Elimination .................................................................................................. 9
  - Nicotine Pharmacodynamics ........................................................................ 9
    - Effects of nicotine on the central nervous system .................................... 10
    - Effects of nicotine on the peripheral nervous system ............................... 11
- Side Effects and Toxicity of Nicotine .................................................................... 12
  - Second- and third-hand smoke ........................................................................ 13
- The Need for Smoking Cessation Aids ............................................................... 15
- Current Smoking Cessation Aids ......................................................................... 16
  - Nicotine Pharmacotherapies for Smoking Cessation .................................... 16
    - Long-acting NRTs ...................................................................................... 17
Side Effects and Toxicity of Methoxsalen………………………………………37
Methoxsalen Sans UV Radiation………………………………………………37
PUVA .................................................................................................38
Rodent in vitro and in vivo studies ........................................39
Human in vitro studies .................................................................40
Clinical studies .............................................................................40
Current Clinical and Experimental Data on Methoxsalen as a Potential
Pharmacotherapy for Smoking Cessation .......................................41
Clinical Data on the Effects of Methoxsalen on Smoking ...............42
Preclinical Studies on the Effects of Methoxsalen on Nicotine
Consumption ..................................................................................42
Gaps in the Literature ...................................................................43
Age Differences .............................................................................43
Summary ......................................................................................44
Short-Term Goals of Dissertation ..................................................45
Long-Term Goals of Dissertation ...................................................45
CHAPTER II: EXPERIMENT I............................................................50
Overview .......................................................................................51
Study Design ................................................................................52
Methods ........................................................................................52
Animals .......................................................................................52
Drugs ............................................................................................52
Freebase (-)-nicotine .................................................................52
Methoxsalen .................................................................................................................. 53

Study Procedure ............................................................................................................. 53

Acclimation (3 days) & baseline (2 days) ................................................................. 53

Methoxsalen and nicotine treatment test (1 day) ..................................................... 54

Blood collection and tissue harvest ......................................................................... 54

Analytical Methods ...................................................................................................... 55

Serum cotinine assessment ......................................................................................... 55

Quantitative real-time PCR (qPCR) ........................................................................... 55

CYP2a5 and CYP2e1 gene expression calculations .............................................. 56

Nicotine Calculations ................................................................................................. 57

Treatment of Data ....................................................................................................... 58

Statistical Analyses ..................................................................................................... 59

Hypotheses ..................................................................................................................... 60

Results ............................................................................................................................ 65

Baseline (2 days) .......................................................................................................... 65

Body weight (g), food intake (g) and water consumption (mL) …........................... 65

Twelve Hours Post-Methoxsalen Injection ................................................................. 66

Body weight and food intake (g) ............................................................................... 66

Total fluid intake (mL) ............................................................................................... 66

Total nicotine consumption (mL) .............................................................................. 66

Total nicotine intake as a percent of total fluid intake (%) .......................................... 66

Total nicotine dosage (mg/kg) .................................................................................. 67

Liver weight (g) and ratio of liver weight to body weight ........................................ 67
CHAPTER III: EXPERIMENT II

Overview ................................................................. 96
Study Design ............................................................. 97
Methods ................................................................. 97

Animals ................................................................. 97

Drugs ................................................................. 97

Freebase (-)-nicotine .............................................. 97

Methoxsalen .......................................................... 98

Study Procedure ..................................................... 98

Acclimation (3 days) & baseline (2 days) ......................... 98

Phase I: Oral voluntary nicotine consumption (choice) treatment
test (4 days)........................................................................99

Phase II: Methoxsa...len treatment test (4 days)......................99

Blood collection and tissue harvest........................................100

Analytical Methods...............................................................100

  Serum cotinine assessment..............................................100

  Quantitative real-time PCR (qPCR).................................100

  CYP2a5 and CYP2e1 gene expression calculations.............102

  Nicotine Calculations......................................................102

  Treatment of Data...........................................................103

  Statistical Analyses........................................................104

Hypotheses ........................................................................105

Results ................................................................................111

  Baseline (2 days)..............................................................111

    Body weight (g), food intake (g) and water consumption (ml)...111

Phase I: Oral voluntary nicotine consumption (choice) treatment test

  (4 days) ........................................................................111

    Body weight (g)............................................................111

    Food intake (g)............................................................112

    Water consumption (ml)..............................................114

    Total fluid consumption (ml).....................................115

    Total nicotine consumption (mL).................................115

    Nicotine intake as a percent of total fluid intake (%).......116

    Total nicotine dosage (mg/kg).....................................117
Review of Experiments ..........................................................179
Contributions to Literature.....................................................181
  Effects of Methoxsalen on Nicotine Consumption in C57BL/6J Mice...182
  Effects of Nicotine and Methoxsalen on Body Weight in Adolescent and
  Adult Mice.................................................................183
  Nicotine Consumption in Adolescent and Adult Mice....................186
  Effects of Methoxsalen on Nicotine Metabolism..........................189
Mouse Data Summary...........................................................193
Clinical Implications............................................................194
  Dosing instructions.........................................................194
  Length of exposure........................................................195
  Route of administration.................................................196
  Side effects................................................................196
  Contraindications..........................................................196
  Smoking while using methoxsalen.......................................198
Alternative Methods and Future Directions.................................199
Study..................................................................................200
  Length of study..............................................................200
  Sex..............................................................................200
  Age..............................................................................201
  Strain............................................................................201
  Nicotine naivety, dependence, and withdrawal..........................202
  Methoxsalen without nicotine............................................203
Methoxsalen and other tobacco compounds……………………203
Genetically altered mouse models……………………………...203
Nicotine…………………………………………………………………205
Other tobacco constituents……………………………………...205
Methoxsalen…………………………………………………………….206
Type of inhibitor used…………………………………………..206
Non-specificity……………………………………
Photosensitivity………………………………………………208
Toxicity…………………………………………………………208
Outcome Measures……………………………………………………...209
CYP2a5 and CYP2e1……………………………………209
CYP location……………………………………………………210
Analyses on CYP enzymes……………………………………210
Techniques to assess nicotine exposure…………………………..211
Conclusion……………………………………………………………………...211
REFERENCES………………………………………………………………………..213
APPENDICES………………………………………………………………………….243
APPENDIX A: Methoxsalen MSDS Sheet…………………………………….244
APPENDIX B: Nicotine Preparation Protocol…………………………………252
APPENDIX C: Experiment I Methoxsalen Preparation Protocol………………254
APPENDIX D: Liver Preparation and Dissection Protocol…………………...257
APPENDIX E: Enzyme Immunoassay Protocol for Serum Cotinine…………259
APPENDIX F: Quantitative Real-time PCR – RNA Extraction Protocol………264
APPENDIX G: Quantitative Real-time PCR – Quality Control Protocol……..267

APPENDIX H: Quantitative Real-time PCR – Reverse Transcription Protocol……………………………………………………………………269

APPENDIX I: Quantitative Real-time PCR – Real Time PCR Protocol……..271

APPENDIX J: Primer sequences for RT-PCR……………………………………..275

APPENDIX K: Quantitative Real-time PCR – Absolute Quantification Protocol…………………………………………………………………………277

APPENDIX L: Experiment II Methoxsalen Preparation Protocol……………….279
List of Tables

Table 1. Experiment I Design.................................................................78

Table 2. Mean body weight (g) for all animals prior to (PRE) and after (POST) drug treatment among adolescent (n=60) and adult (n=60) male C57BL/6J mice (means ± standard error of the mean).........................................................79

Table 3. Mean food consumption (g) for all animals prior to (PRE) and after (POST) drug treatment among adolescent (n=60) and adult (n=60) male C57BL/6J mice (means ± standard error of the mean)..........................................................80

Table 4. Mean total fluid intake (mL) for all animals prior to (PRE) and after (POST) drug treatment among adolescent (n=60) and adult (n=60) male C57BL/6J mice (means ± standard error of the mean)..........................................................81

Table 5. Mean total nicotine consumption (mL) 12 hours after drug treatment among adolescent (n=60) and adult (n=60) male C57BL/6J mice (means ± standard error of the mean)..........................................................82

Table 6. Mean total nicotine intake as a percent of total fluid intake (%) 12 hours after drug treatment among adolescent (n=60) and adult (n=60) male C57BL/6J mice (means ± standard error of the mean)..........................................................83

Table 7. Mean total nicotine dosage (mg/kg) 12 hours after drug treatment among adolescent (n=60) and adult (n=60) male C57BL/6J mice (means ± standard error of the mean)..........................................................84

Table 8. Relative CYP2a5 and CYP2e1 gene expression among adolescent (n=18) and adult (n=18) male C57BL/6J mice (means ± standard error of the mean)......................85
Table 9. Experiment II Design………………………………………………………………………………140

Table 10. Mean body weight (g) for all animals during nicotine-only treatment (Phase I) among adolescent (n=32) and adult (n=32) male C57BL/6J mice (means ± standard error of the mean)……………………………………………………………………………………………………………………141

Table 11. Mean food consumption (g) for all animals during nicotine-only treatment (Phase I) among adolescent (n=32) and adult (n=32) male C57BL/6J mice (means ± standard error of the mean)……………………………………………………………………………………………………………………142

Table 12. Mean water intake (mL) and total fluid intake (mL) for all animals during nicotine-only treatment (Phase I) among adolescent (n=32) and adult (n=32) male C57BL/6J mice (means ± standard error of the mean)……………………………………………………………………………………………………………………143

Table 13. Mean total nicotine consumption (mL) during nicotine-only treatment (Phase I) in adolescent (n=32) and adult (n=32) male C57BL/6J mice (means ± standard error of the mean)……………………………………………………………………………………………………………………144

Table 14. Mean total nicotine intake as a percent of total fluid intake (%) during nicotine-only treatment (Phase I) in adolescent (n=32) and adult (n=32) male C57BL/6J mice (means ± standard error of the mean)……………………………………………………………………………………………………………………145

Table 15. Mean total nicotine dosage (mg/kg) during nicotine-only treatment (Phase I) in adolescent (n=32) and adult (n=32) male C57BL/6J mice (means ± standard error of the mean)……………………………………………………………………………………………………………………146

Table 16. Mean body weight (g) for all animals during methoxsalen and nicotine treatment (Phase II) in adolescent (n=32) and adult (n=30) male C57BL/6J mice (means ± standard error of the mean)……………………………………………………………………………………………………………………147
Table 17. Mean food consumption (g) for all animals during methoxsalen and nicotine treatment (Phase II) in adolescent (n=32) and adult (n=30) male C57BL/6J mice (means ± standard error of the mean)………………………………………………………………..148

Table 18. Mean water intake (mL) and total fluid intake (mL) for all animals during methoxsalen and nicotine treatment (Phase II) in adolescent (n=32) and adult (n=30) male C57BL/6J mice (means ± standard error of the mean)…………………………………...149

Table 19. Mean total nicotine consumption (mL) for all animals during methoxsalen and nicotine treatment (Phase II) in adolescent (n=32) and adult (n=30) male C57BL/6J mice (means ± standard error of the mean)……………………………………………………………………………..150

Table 20. Mean total nicotine intake as a percent of total fluid intake (%) for all animals during methoxsalen and nicotine treatment (Phase II) in adolescent (n=32) and adult (n=30) male C57BL/6J mice (means ± standard error of the mean)……………………………………………………………………………..151

Table 21. Mean total nicotine dosage (mg/kg) for all animals during methoxsalen and nicotine treatment (Phase II) in adolescent (n=32) and adult (n=30) male C57BL/6J mice (means ± standard error of the mean)……………………………………………………………………………..152
List of Figures

Figure 1. Numbered carbon nicotine molecule.........................................................46

Figure 2. Mechanism of nicotine metabolism............................................................47

Figure 3. Numbered carbon methoxsalen molecule....................................................48

Figure 4. Theoretical model of age and nicotine metabolism on the onset of smoking.................................................................49

Figure 5. Experiment I timeline.................................................................................86

Figure 6. Total nicotine consumption (mL) among adolescent (n=60) and adult (n=60) male C57BL/6J mice exposed to control (n=24), saline (n=24), vehicle (n=24), 5 mg/kg 8MOP (n=24) and 10 mg/kg 8MOP (n=24) treatment conditions (means ± standard error of the mean)........................................................................87

Figure 7. Total nicotine intake as a percent of total fluid intake (%) among adolescent (n=60) and adult (n=60) male C57BL/6J mice exposed to control (n=24), saline (n=24), vehicle (n=24), 5 mg/kg 8MOP (n=24) and 10 mg/kg 8MOP (n=24) treatment conditions (means ± standard error of the mean)........................................................................88

Figure 8. Total nicotine dosage (mg/kg) among adolescent (n=60) and adult (n=60) male C57BL/6J mice exposed to control (n=24), saline (n=24), vehicle (n=24), 5 mg/kg 8MOP (n=24) and 10 mg/kg 8MOP (n=24) treatment conditions (means ± standard error of the mean)........................................................................89

Figure 9. Liver weight (g) among adolescent (n=60) and adult (n=60) male C57BL/6J mice exposed to control (n=24), saline (n=24), vehicle (n=24), 5 mg/kg 8MOP (n=24) and 10 mg/kg 8MOP (n=24) treatment conditions (means ± standard error of the mean)........................................................................90
Figure 10. Serum cotinine levels adjusted for liver weight (ng/ml/g) among adolescent (n=60) and adult (n=60) male C57BL/6J mice exposed to control (n=24), saline (n=24), vehicle (n=24), 5 mg/kg 8MOP (n=24) and 10 mg/kg 8MOP (n=24) treatment conditions (means ± standard error of the mean)…………………………………………………………………………………..90

Figure 11. Correlation between serum cotinine levels adjusted for liver weight (ng/ml/g) and total nicotine dosage (mg/kg) for adolescent (n=60) and adult (n=60) male C57BL/6J mice exposed to control (n=24), saline (n=24), vehicle (n=24), 5 mg/kg 8MOP (n=24) and 10 mg/kg 8MOP (n=24) treatment conditions (means ± standard error of the mean)……………………………………………………………………………………..91

Figure 12. Relative CYP2a5 gene expression corrected for 18S rRNA quantity among adolescent (n=18) and adult (n=18) male C57BL/6J mice exposed to vehicle (n=12), 5 mg/kg 8MOP (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean)……………………………………………………………………………………………92

Figure 13. Relative CYP2e1 gene expression corrected for 18S rRNA quantity among adolescent (n=18) and adult (n=18) male C57BL/6J mice exposed to vehicle (n=12), 5 mg/kg 8MOP (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean)……………………………………………………………………………………………93

Figure 14. Experiment II timeline……………………………………………………...153

Figure 15. Average total nicotine consumption (mL) during nicotine-only treatment (Phase I) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=32) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean)………………………………………………………………………………………154
Figure 16. Total nicotine intake as a percent of total fluid intake (%) during nicotine-only treatment (Phase I) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=32) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean).…………………………………………………………………………………..155

Figure 17. Total nicotine dosage (mg/kg) during nicotine-only treatment (Phase I) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=32) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean).…156

Figure 18. Total nicotine consumption (mL) during methoxsalen and nicotine treatment (Phase II) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean).…………………………………………………………………………….157

Figure 19. Comparison of average total nicotine consumption (mL) for adolescent (n=48) and adult (n=48) male C57BL/6J mice exposed to vehicle (n=48) and 10 mg/kg 8MOP (n=48) treatment conditions during Phase I (mean days 1-4) and adolescent (n=30) and adult (n=30) C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=30) treatment conditions during Phase II (mean days 5-8) (means ± standard error of the mean).………………………………………………………………………………158

Figure 20. Total nicotine intake as a percent of total fluid intake (%) during methoxsalen and nicotine treatment (Phase II) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean)………………………………………………………………………………..159

Figure 21. Comparison of average total nicotine intake as a percent of total fluid intake (%) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=32) and 10 mg/kg 8MOP (n=32) treatment conditions during Phase I (mean days 1-4) and adolescent (n=32) and adult (n=30) C57BL/6J mice exposed to vehicle (n=30) and 10
mg/kg 8MOP (n=32) treatment conditions during Phase II (mean days 5-8) (means ± standard error of the mean). .......................................................... 160

Figure 22. Total nicotine dosage (mg/kg) during methoxsalen and nicotine treatment (Phase II) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean). .......................................................... 161

Figure 23. Comparison of average total nicotine dosage (mg/kg) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=32) and 10 mg/kg 8MOP (n=32) treatment conditions during Phase I (mean days 1-4) and adolescent (n=32) and adult (n=30) C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions during Phase II (mean days 5-8) (means ± standard error of the mean). .......................................................... 162

Figure 24. Liver weight (g) for adolescent (n=32) and adult (n=30) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean). .......................................................... 163

Figure 25. Serum cotinine levels adjusted for liver weight (ng/ml/g) for adolescents (n=32) and adults (n=30) adult male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean) ........ 164

Figure 26. Relative gene expression of CYP2a5 corrected for the quantity of 18S rRNA for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) ........................................................................................................ 165

Figure 27. Relative gene expression of CYP2e1 corrected for the quantity of 18S rRNA for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12)
and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean)……………………………………………………………………………………166

Figure 28. Correlation between relative CYP2a5 gene expression corrected for 18S rRNA quantity and total nicotine dosage (mg/kg) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=32) and 10 mg/kg 8MOP (n=32) treatment conditions during Phase I (mean days 1-4)……………………………………167

Figure 29. Correlation between relative CYP2a5 gene expression corrected for 18S rRNA quantity and total nicotine dosage (mg/kg) for adolescent (n=32) and adult (n=30) C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions during Phase II (mean days 5-8)……………………………………………168

Figure 30. Comparison of total nicotine consumption (ng/ml/g) for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=32) and adult (n=30) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions from Experiment II (means ± standard error of the mean)…………………………………………………………………………….169

Figure 31. Comparison of total nicotine intake as a percent of total fluid intake (%) for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=32) and adult (n=30) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions from Experiment II (means ± standard error of the mean)…………………………………………………………………………….170

Figure 32. Comparison of total nicotine dosage (mg/kg) for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=32) and adult (n=30) male C57BL/6J mice exposed to vehicle (n=30) and
10 mg/kg 8MOP (n=32) treatment conditions from Experiment II (means ± standard error of the mean)….

Figure 33. Comparison of liver weight (ng/ml/g) for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=32) and adult (n=30) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions from Experiment II (means ± standard error of the mean)….

Figure 34. Comparison of serum cotinine levels adjusted for liver weight (ng/ml/g) for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=32) and adult (n=30) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions from Experiment II (means ± standard error of the mean)….

Figure 35. Comparison of relative gene expression of CYP2a5 corrected for the quantity of 18S rRNA for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions from Experiment II (means ± standard error of the mean)….

Figure 36. Comparison of relative gene expression of CYP2e1 corrected for the quantity of 18S rRNA for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions from Experiment II (means ± standard error of the mean)….
Figure 37. Correlation between relative gene expression of CYP2a5 corrected for the quantity of 18S rRNA and total nicotine dosage (mg/kg) for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions from Experiment II (means ± standard error of the mean).
Abbreviations

8MOP = methoxsalen
CNS = central nervous system
CYP = cytochrome P450
nAChR = nicotinic acetylcholine receptor
NRT = nicotine replacement therapy
NIC = nicotine solution [freebase (-)-nicotine dissolved in tap water]
PND = post natal day
PNS = peripheral nervous system
s.c. = subcutaneous
WTR = tap water without nicotine
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Dedication

This dedication blossoms from the Hawaiian phrase, “ohana” meaning “family”, or “nobody gets left behind…or forgotten” (Disney’s 2002 movie, “Lilo and Stitch”)…

To my immediate – Michael, Yvonne, Nicole, and Melissa Kapelewski – and extended family: Eternal thank you’s for your endless love, support and guidance from the day I was born, through trials and tribulations of adolescence and college, and most of all, throughout the hell that most call “graduate school”.

To my many past and present beta fish: Life is so much better when you can come home to an animal that loves you for you, even if it has a limited memory.

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To my new family – Lisa, Louis, Lexie, Cassie and the Kinkades, but especially Matt: You’ve shown me immediate kindness, warmth, love and affection just for being me, and taken me in without question. Matt, you search everyday for that special someone, the person who makes you laugh without trying, cry without sadness, glow without effort, and love without thought. I found that person in you and for that, I’ll always love you.

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And finally, to a worldwide family that is dedicated to banning smoking and reducing tobacco use via a multitude of mandates, treaties, projects, and laws. Through these endeavors, I hope that we will better our nation’s current health and that of future generations as “smoking is a custom loathsome to the eye, hateful to the nose, harmful to the brain, dangerous to the lungs, and in the black, stinking fume thereof nearest resembling the horrible Stygian smoke of the pit that is bottomless” -- James I of England
Epigraph

“It is easy to think of smoking as an adult problem. It is adults who die from tobacco-related diseases…[however,] a person who hasn’t started smoking by age 19 is unlikely to ever become a smoker. Nicotine addiction begins when most tobacco users are teenagers, so let’s call this what it really is: a pediatric disease”

-- David Kessler, FDA commissioner

What a weird thing smoking is and I can't stop it. I feel cozy, have a sense of well-being when I'm smoking, poisoning myself, killing myself slowly. Not so slowly maybe. I have all kinds of pains I don't want to know about and I know that's what they're from. But when I don't smoke I scarcely feel as if I'm living. I don't feel as if I'm living unless I'm killing myself.

-- Russell Hoban, Turtle Diary, 1975
CHAPTER I

INTRODUCTION
INTRODUCTION

Tobacco Smoking: Incidence and Significance

Following its introduction in the 20th century, the cigarette has become the most common method to self-administer nicotine, the principal psychoactive component found in tobacco [United States Department of Health and Human Services (USDHHS), 1988]. Nearly 1 billion men and 250 million women smoke to administer this addictive compound, regardless of the health consequences (Shafey, Eriksen, Ross, & Mackay, 2009; USDHHS, 1988). In order to capitalize on these individuals and their nicotine addiction, tobacco companies will produce and distribute more than 6 trillion cigarettes worldwide in 2010 alone, the consumption of which will lead to more than 6 million tobacco-related deaths in one year (Shafey et al., 2009). During this century, fatalities attributed to smoking are estimated to reach 1 billion, a 100-fold increase over the number of tobacco-related deaths in the previous century [Shafey et al., 2009; World Health Organization (WHO), 2009]. Unfortunately, roughly 500 million tobacco users worldwide will die from this initially voluntary habit (WHO, 2009), making tobacco use the most preventable cause of illness and death worldwide (WHO, 2010). Approximately 80% of these deaths will occur in developing countries due to a lack of political mandates preventing individuals from smoking in public places, a paucity of smoking cessation programs, and numerous advertisements that are directed at adolescents and women and intended to glorify smoking (WHO, 2008). Because of the shocking number of deaths from previous years and those estimated for the future, dozens of organizations and government agencies are dedicated to banning smoking from communal areas, improving public education about the harmful effects of smoking, and developing new techniques to
help smokers quit smoking. It is only by working together that society can hope to get tobacco use under control to improve the current and future health and well being of each individual around the world, regardless of an individual’s age, sex, race/ethnicity, socioeconomic status, education level, location, or smoking status.

**Age, Smoking Onset and Cessation**

Aggressive advertising, easily obtainable and inexpensive tobacco products, parental or peer smoking, feelings of rebellion, impulsivity, independence, depression or stress, other drug use, weight control and/or social pressures (Kaufman & Augustson, 2008; for review, see Lenney & Enderby, 2008; Shafey et al., 2009; O’Loughlin, Karp, Koulis, Paradis, & DiFranza, 2009; USDHHS; 1994) lure approximately 1,460,000 adolescents in the United States to smoke every year before the age of 18 [Substance Abuse and Mental Health Services Administration (SAMHSA), 2008b]. Roughly 25% of these individuals will smoke their first cigarette by the age of ten (Shafey et al., 2009).

This early age of onset is cause for alarm because the consumption of exogenous substances, including nicotine, significantly alters the natural course of neurological maturation, potentially increasing a young person’s vulnerability to abuse other drugs (Spear, 2000; 2007; USDHHS, 1988; Zickler, 2004), and decreasing the ability to quit smoking in the future (Breslau & Peterson, 1996; Chen & Millar, 1998; McQuown, Belluzzi, & Leslie, 2007; USDHHS, 1994). In 2004, more than 20% of high school students (ages 14-18) and 8% of middle school-aged children (ages 11-14) smoked cigarettes [Center for Disease Control and Prevention (CDC), 2005b], and the percentage of current cigarette smoking among high school students has remained constant at 21.9% from 2003 to 2007 (CDC, 2008b). Moreover, the probability of dependence increases
two-fold for individuals who initiated smoking between 14 and 16 years of age compared to those who take up smoking later in life (Breslau, Fenn, & Peterson, 1993). To the extent that these children and adolescents represent a highly vulnerable audience, the ones that do succumb are placed at a substantially greater risk for poor health outcomes and increased mortality associated with a lifelong smoking habit (Pierce & Gilpin, 1996).

**Nicotine Pharmacology**

Cultivation of the *Nicotina tabacum* plant to manufacture tobacco has led to a multitude of detrimental effects on the lives of billions of people worldwide, and will continue to do so for centuries to come, assuming its current trajectory. These life-altering effects are, in part, due to the addictive nature of the small psychoactive alkaloid: Nicotine (see Figure 1). In conjunction with alcohol and caffeine, smoking tobacco to self-administer nicotine is one of the top three most common addictions worldwide, ranking above any illicit drug (USDHHS, 1994). The health risks and number of deaths directly and indirectly caused by tobacco smoke is the driving force that compels drug addiction scientists to combat this growing epidemic with a full understanding of the drug’s pharmacology.

**Nicotine Pharmacokinetics**

Throughout history, mankind has developed a variety of techniques and devices to effectively deliver nicotine: Buccal/nasal membrane absorption via chewing tobacco (“dip”), wet oral snuff (“snus”) or dry nasal snuff, and the most common of all, inhalation (for review, see Hukkanen, Jacob III, & Benowitz, 2005; McKim, 2003) via cigarettes, cigars, bidis, kreteks, sticks and pipes (for review, see Lenney & Enderby, 2008; Shafey et al., 2009). Specifically, cigarettes provide a unique opportunity to rapidly deliver a
controlled amount of nicotine into the body at a rate chosen by the smoker (Britton et al., 2000; Henningfield & Heishman, 1998), making it the most widely used tobacco delivery device in the world.

**Absorption**

On average, roughly 1.0 mg of nicotine is absorbed from a single cigarette, although, nicotine absorption can range from 0.3 to 3.2 mg, depending on how the cigarette is smoked (Benowitz & Jacob, 1984; for review, see Hukkanen et al., 2005; Jacob, Benowitz, & Shulgin, 1988; McKim, 2003; Taylor, 2001; Tutka, Mosiewicz, & Wielosz, 2005). The dosage and rate of nicotine delivery for cigarettes depends on the depth and rate of inhalation, in addition to puffing intensity (Benowitz, 1999; Britton et al., 2000; Jacob III et al., 1988; USDHHS, 2001). Deeper inhalations of smoke, longer drags on the cigarette (as is customary with “low tar” cigarettes), and blocking air holes on the filter introduce more nicotine for absorption. Nicotine is readily absorbed into the body; however, this process is altered by the pH of tobacco smoke, as well as blood flow to and volume of the area of absorption. The pH of tobacco smoke is dependent on how it is cured: Flue-cured tobacco produces acidic smoke (pH 5.5), while the smoke of tobacco cured by the air is basic (pH 8.5). Because nicotine has a pKa of 8.0 (Gori, Benowitz, & Lynch, 1986; Benowitz, 1998b; Busto, Bendayan, & Sellers, 1989), acidic smoke produced by cigarettes causes a large percentage of nicotine to remain ionized due to the protonation of the nitrogen in the pyrrolidine ring. In this state, nicotine cannot traverse buccal membranes easily (Benowitz, 1998a), and therefore must be deeply inhaled into the lungs where the pH is 7.4 in order to absorb an adequate amount of nicotine (Benowitz, Hukkanen, & Jacob, 2009; Britton et al., 2000; Gori et al., 1986;
Busto et al., 1989). Once in the lungs, absorption of nicotine into the blood vessels is immediate due to the large surface area of the alveoli, and extensive blood flow (Henningfield & Keenan, 1993; for review, see Hukkanen et al., 2005). Conversely, nicotine is converted into a non-ionized state in basic environments, which allows the compound to easily travel through buccal membranes, as is the case with cigar smoke (Benowitz, 1998a). Thus, instead of being inhaled, cigar smoke need only be kept in the mouth to absorb enough nicotine into the blood.

**Distribution**

Once in the bloodstream, an arterial bolus carrying nicotine travels through the blood-brain barrier and into the brain 10 to 20 seconds after the first puff of a cigarette (Benowitz, 1996). A large percentage of the bolus remains in the brain for 20 to 30 minutes, while the rest of the nicotine is distributed to other organs (Britton et al., 2000; for review, see Hukkanen et al., 2005; Julien, 2005; Le Houezec, 2003; Schmitz, Schneider & Jarvik, 1997; Wilkinson, 2001), depending on blood flow to the organ, output from the heart, and the volume of the tissue (Wilkinson, 2001). Organs with the highest concentration of nicotine are the brain, liver, spleen, lungs and kidney, compared to adipose tissue, muscle and skin, which have the lowest accumulation (Wilkinson, 2001; for review, see Hukkanen et al., 2005; USDHHS, 1988). In addition to organs, nicotine migrates into bodily fluids, such as breast milk, sweat, and saliva (for review, see Hukkanen et al., 2005; McKim, 2003).

**Metabolism**

The metabolism of nicotine occurs soon after absorption into the bloodstream, ultimately leading to approximately 50% of serum nicotine being biotransformed into
metabolites within the first 2 hours (Benowitz, 1998a). Based on total nicotine concentration, roughly 70 to 90% is metabolized by hepatic enzymes; however, other organs, such as the lungs, nasal mucosa and brain, also contribute to a small percentage (Britton et al., 2000; for review, see Hukkanen et al., 2005; USDHHS, 1988). There are several pathways (e.g., N-demethylation, C-oxidation, methylation, glucuronidation, 2’-Hydroxylation; for review, see Hukkanen et al., 2005; see Figure 2) that produce the primary metabolites of nicotine; however, the major metabolic strategy is C-oxidation (oxidation of the 5’ carbon on the pyrrolidine ring; Nakajima et al., 1996), converting 70-90% of nicotine into cotinine (Jacob et al., 1988; McClure & Swan, 2006). Nicotine is initially oxidized by the cytochrome (CYP) P450 enzyme, CYP2A6, to create nicotine-Δ-1′(5′)iminium ion. This unstable intermediate is immediately converted into cotinine by aldehyde oxidase (for review, see Benowitz, 1999; Hukkanen et al., 2005; Murphy, Raulinaitis, & Brown; 2005; Mwenifumbo & Tyndale, 2009; USDHHS, 1988). CYP2A6 then further metabolizes cotinine into 5 metabolites (Jacob et al., 1988), the primary one being trans-3-hydroxycotinine (for review, see Hukkanen et al., 2005).

Compared to nicotine, cotinine is found in much higher venous blood concentrations: 250-300 ng/ml for nicotine versus 10-50 ng/ml for cotinine (Gori & Lynch, 1985; for review, see Hukkanen et al., 2005; USDHHS, 1988). Additionally, cotinine has a longer half-life compared to nicotine (about 16 versus 2 hours, respectively; Benowitz, 1998b; McClure & Swan, 2006) and is detectable in urine up to 72 hours after initial nicotine use (Britton et al. 2000; USDHHS 1988). These features, in addition to the fact that cotinine can also be found in blood, saliva, and hair, make cotinine the primary biomarker for nicotine use (Benowitz et al., 2009; Galeazzi,
Daenens, & Gugger, 1985; for review, see Hukkanen et al., 2005). However, the most prominent urinary biomarker for nicotine is trans-3-hydroxycotinine because its concentration in urine is far greater than that of cotinine (Jacob et al., 1988). A ratio of trans-3-hydroxycotinine (3HC) to cotinine (3HC/COT) also can be used as a marker for metabolic activity of hepatic CYP2A6 (Dempsey et al., 2004; for review, see Benowitz et al., 2009).

Although some of the metabolites are processed to a small extent by a variety of other enzymes (e.g., flavin-containing monooxygenase 3, UDP-glucuronosyltransferase, CYP2A13, CYP2A7, CYP2B6; for review, see Hukkanen et al., 2005; Mwenifumbo & Tyndale, 2007; 2009; USDHHS, 1988), the vast majority of nicotine is metabolized by CYP2A6. Several factors can alter nicotine metabolism, including musculature, exercise (McClure & Swan, 2006), diet, specifically gastric acid secretion, protein binding (Bonate, 1991; Fletcher, Acosta, & Strykowski, 1994; Meibohm, Beierle, & Derendorf, 2002), tobacco smoking (Benowitz & Jacob, 1993), organ damage (especially liver and kidney), age, race/ethnicity, sleep, pregnancy (Dempsey, Jacob, & Benowitz, 2002; for review, see Hukkanen et al., 2005; Benowitz et al., 2009), sex hormones (Bonate, 1991; Fletcher, Acosta, & Strykowski, 1994; Meibohm, Beierle, & Derendorf, 2002), use of oral contraceptives (Benowitz, Swan, Jacob, Lessov-Schlaggar, & Tyndale, 2006; Berlin, Gasior, & Moolchan, 2007; Johnstone et al., 2006), menstrual cycle (Bonate, 1991; Fletcher, Acosta, & Strykowski, 1994; Meibohm, Beierle, & Derendorf, 2002), smoking (Benowitz & Jacob, 1993), genetic polymorphisms of CYP2A6 (Sellers, Tyndale, & Fernandes, 2003; Tyndale, Pianezza, & Sellers, 1999; Tyndale & Sellers, 2001), and taking known CYP2A6 inhibitors (e.g., methoxsalen) or inducers (e.g., dexamethasone).
**Elimination**

The kidneys are responsible for nicotine excretion, which is dependent on urine pH and flow (for review, see Benowitz et al., 2009; Busto et al., 1989; for review, see Hukkanen et al., 2005; Le Houezec, 2003; Tutka et al., 2005; USDHHS, 1988). Unchanged nicotine and large amounts of metabolites are found in acidic urine (i.e., pH \( \leq 6.9 \)), whereas basic urine (i.e., pH \( \geq 7.1 \)) contains low amounts of nicotine and its metabolites (for review, see Benowitz et al., 2009; Busto et al., 1989). In addition to urine, a small percentage of nicotine and cotinine are excreted via feces and sweat (for review, see Hukkanen et al., 2005).

Excretion of nicotine only represents physical elimination from the body, but not all aspects of nicotine’s physiological presence are gone. Because the body must always have an optimum level of serum nicotine to maintain pleasurable effects and avoid negative effects of nicotine withdrawal, a significant drop in this concentration induces the aversive mental (e.g., irritability, anxiety, depression, difficulty concentrating) and physical (e.g., increased appetite, decreased heart rate, weight gain) discomfort of nicotine withdrawal (O’Brien, 2001). So, therein lies the vicious cycle: Attempting to quit reduces nicotine levels, thus, increasing these withdrawal symptoms. The individual will therefore begin smoking again to replenish serum nicotine to avoid these negative effects. Therefore, in addition to pharmacokinetics, the pleasurable and aversive effects of nicotine (or pharmacodynamics) play a large role in its dependence-producing nature.

**Nicotine Pharmacodynamics**

Because of its high lipid solubility and small molecular weight, nicotine is capable of crossing physiological membranes, including the blood-brain barrier and that
of other organs to produce its effects on the central (CNS) and peripheral (PNS) nervous system. Following transport through the membrane barriers within the CNS and PNS, nicotine binds to nicotinic acetylcholine receptors (nAChRs). These ligand-gated ion channels consist of a variety of α and β subunit combinations (α2 – α10 and β2 – β4), the most physiologically common with a high binding affinity to nicotine include α4β2, α3β2, α3β4, α6β2 and α7 receptors (Morley & Rodriguez-Sierra, 2004). Specific subunit amalgamations are important to influence certain behaviors; for example, the combination of alleles of α5 and α3 nAChR subunits genetically predisposes individuals to become heavy smokers (Berrettini et al., 2008), and the presence of α3β4 nAChRs is important to promote nicotine’s physical effects on the heart (Benowitz, 1998b). Metabolites of nicotine, such as nornicotine, also may have some effect on nAChR (e.g., α6 and 7 receptors; Papke, Dwoskin, & Crooks, 2007). Following chronic nicotine administration, the quantity of nAChRs significantly increases, resulting in tolerance to the drug (Sparks & Pauly, 1999; for review, see Govind, Vezina, & Green, 2009; Wüllner et al., 2008); a phenomenon that has also been reported after smoking a single cigarette (Walsh et al., 2008). Recent evidence indicates that these receptor up-regulations may depend on the length and concentration of nicotine: Chronic nicotine exposure is found to upregulate α4β2 receptors (Wüllner et al., 2008), whereas α6 receptors increase in quantity as a result of acute nicotine exposure (Walsh et al., 2008).

**Effects of nicotine on the central nervous system**

Within the central nervous system (CNS), nicotine binds to specific nAChRs in several areas of the brain (e.g., nucleus accumbens, ventral tegmental area, hippocampus prefrontal cortex), leading to an influx of sodium and calcium, and the eventual release of
several neurotransmitters (e.g., dopamine, serotonin, acetylcholine, epinephrine, norepinephrine; for review, see Balfour, 1989) and hormones [e.g., cortisol, adrenocorticotropic hormone (ACTH), prolactin] to induce a multitude of physiological, behavioral, and cognitive effects (for review, see Benowitz, 1999). For example, stimulation of presynaptic nAChR on dopaminergic neurons in the ventral tegmental area activates the release of dopamine, which induces the positive and negative self-reinforcing behaviors that can lead to nicotine dependence (e.g., reward, relief from withdrawal symptoms; Balfour, 1989; Benowitz, 1996; 1998b; 1999; Clarke & Pert, 1985; Clarke, Schwartz, Paul, Pert, & Pert, 1985; Corrigall, Coen, & Adamson, 1994; Koob & Bloom, 1988; Pidoplichko et al., 2004; Pontieri, Tanda, Orzi, & Chiara, 1996). The stimulated release of serotonin and norepinephrine may reduce depression symptoms and regulate mood, whereas acetylcholine release in the brainstem increases attention and cognition (Benowitz, 1998b; McKim, 2003). Appetite suppression is regulated and controlled by several neurotransmitters, including dopamine, norepinephrine, and serotonin (Benowitz, 1998b). The primary inhibitory neurotransmitter, GABA, is essential for relaxation and relieving tension (Benowitz, 1998b), whereas nicotinic stimulation of areas within the medulla oblongata can result in vomiting (Taylor, 2001).

**Effects of nicotine on the peripheral nervous system**

The effects of nicotine extend beyond that of the CNS to the periphery. For instance, even low doses of nicotine leads to the release of epinephrine, which increases blood pressure, heart rate, coronary and skin blood vessel constriction, vascular blood vessel dilation, bowel movements, decreased appetite, and reduced skin temperature (Benowitz, 1996; 1998a; McKim, 2003). Conversely, high doses of nicotine have
physiological depressant effects, including hypotension, tremors and convulsions, and reduced reactivity of voluntary muscles due to the over-stimulation of cholinergic receptors in neuromuscular junctions (Benowitz, 1998b; McKim, 2003; Taylor, 2001). Many of these physical symptoms (e.g., increased blood pressure and blood clots) significantly enhance the risk of more detrimental or fatal health problems that commonly are associated with smoking (Benowitz, 1996).

**Side Effects and Toxicity of Nicotine**

The majority of the smoking-attributed health risks are life-threatening, making cigarette smoking the leading cause of illness and death in the United States (WHO, 2009) and the world (WHO, 2010). To date, smoking causes more than 440,000 – or one in five – deaths per year in the United States alone (CDC, 2002a; 2008a). Worldwide, more than 5 million deaths per year are attributable to smoking, and this figure is estimated to surpass 8 million per year by 2030 (WHO, 2009). Together, these numbers overwhelm the number of deaths attributable to the combined effects of infectious agents, toxins, alcohol use, suicide, homicide and motor vehicle accidents (McGinnis & Foege, 1993).

Because nicotine is rapidly absorbed, direct skin contact with concentrated nicotine or wet tobacco leaves leads to nicotine poisoning (also known as green tobacco sickness). Headache, nausea, vomiting, and reductions in blood pressure, breathing and mental clarity are all symptoms, and these are usually followed by death within minutes (Taylor, 2001). In contrast, tobacco-related fatalities due to smoking or use of other tobacco products are protracted, as they are attributed to diseases that steadily affect life-sustaining organs, including the heart and lungs. Therefore, the three most common
diseases associated with smoking are lung cancer, chronic obstructive pulmonary disease (COPD) and ischemic heart disease (IHD; CDC, 2005a). Lung cancer occurs in approximately 90% of adult men and 80% of women smokers (USDHHS, 2004). In addition, smoking is directly correlated with 30% of all cancers, including acute myeloid leukemia as well as cancer of the mouth, throat (pharyngeal, laryngeal, and esophageal), lung, stomach, pancreas, kidney, bladder, and cervix [American Cancer Society (ACS), 2009; USDHHS, 2004].

In addition to cancer, smoking also causes heart, lung, and circulatory problems, including coronary and ischemic heart disease, stroke, atherosclerosis, peripheral and cardiovascular disease, abdominal aortic aneurysms, respiratory problems (e.g., pneumonia, bronchitis, influenza) and COPD (USDHHS, 2004). Reductions in the sense of smell and taste, compromised immune system, wrinkles, macular degeneration, Type-2 diabetes, gingivitis, periodontitis, stomach ulcers, and cataracts are also common health risks associated with smoking (Shafey et al., 2009; USDHHS, 2004). Women who smoke are at a higher risk of becoming infertile, having complications during the pregnancy (e.g., abruptio placentae, placenta previa, miscarriage), with the neonate (e.g., early delivery, stillbirth, low birth weight), or after birth [e.g., premature death via sudden infant death syndrome (SIDS; USDHHS, 2004)]. Smokers who are post-menopausal are likely to have osteoporosis, increasing the risk of bone fractures (USDHHS, 2001).

**Second- and third-hand smoke**

The direct effects to the smoker via cigarette smoke ranges from life-altering to life-threatening; however, these toxic effects can also affect innocent bystanders. Unfortunately, over 126 million non-smokers, incorporating close to 22 million children,
are exposed to secondhand smoke, or environmental tobacco smoke (ETS; USDHHS, 2006). ETS significantly increases the risk for heart and lung disease (by 25-30%, and 20-30%, respectively) in adults (USDHHS, 2006). The increased danger of such life-threatening diseases also causes 3,400 individuals – 2200 of them women – to prematurely die each year [California Environmental Protection Agency (EPA), 2005]. Children exposed to ETS are at a greater risk for ear diseases (e.g., otitis media) and respiratory problems (e.g., pneumonia, bronchitis and asthma attacks), leading to a reduction in lung growth (USDHHS, 2006). In tandem with these physical effects, smoking in front of children increases their potential to initiate their own smoking habit.

It is also important to briefly highlight recent public health attention to third-hand smoke, or the exposure to residual contaminants and toxins (e.g., cyanide, lead) left behind after smoking tobacco (Ueta, Saito, Teraoka, Miura, & Jinno, 2010), as an important new line of nicotine addiction research intended to improve public health and knowledge on the detriments of smoking. In particular, infants and children are the intended targets to protect based on their likelihood of coming into contact with porous surfaces that absorb these residues (i.e., carpets). However, smokers themselves also carry these residues on their clothing; therefore, although minute, potentially dangerous toxins, such as cyanide, can be affect the health of other individuals besides children. Additional research is necessary to fully understand the extent to which these toxins can have detrimental consequences; however, potential dangers of smoking to the smoker and to others even after the cigarette is consumed are becoming more apparent.
The Need for Smoking Cessation Aids

Since the Surgeon General distributed the first report on the detrimental effects of smoking in 1964 (USPHS, 1964), the public has been aware of the harm that accompanies cigarette smoking, and yet, the number of persons who smoke worldwide is staggering. Millions of adults are daily smokers, and over 1000 adolescents are becoming part of this statistic each day (SAMHSA, 2008b). For these individuals, their life expectancy is prematurely reduced by 13 to 14 years compared to that of non-smokers (CDC, 2002a). Fortunately, health risks are reduced and years can be added onto a smoker’s life simply by quitting (USDHHS, 1990).

There are a series of significant health benefits associated with smoking cessation. Life expectancy is increased due to a reduction in the risk of stroke or developing cancer and coronary heart disease for men and women (USDHHS, 1990; 2001). For example, two weeks to three months after quitting, the risk of a myocardial infarction (MI) decreases and following one year of abstinence, a smoker’s risk of dying from coronary heart disease (CHD) is reduced by 50% (USDHHS, 1990). For those who have already developed a disease associated with smoking – for example, pneumonia or CHD – quitting leads to improvements in the time course of the disease and longer survival rates (e.g., decreased risk of heart attacks; USDHHS, 1990).

Efforts thus far to persuade smokers to abandon the habit are effective in theory; in practice, however, smokers find it very difficult to quit indefinitely, as is evidenced by large recidivism rates for all age groups. For example, of the millions of smokers worldwide, about 70% have a desire to quit (CDC, 2002b); however, only 39.8% actually make the attempt. This number of quit attempts has decreased for all age groups since
1993 (CDC, 2008c), but there are a greater number of quit attempts in the younger age groups (CDC, 2008c). More than 50% of smokers 18-25 years old quit for one or more days, which is the largest percentage of quit rates of any age group over 18 years old (CDC, 2008c). However, quit attempts are correlated with the severity of addiction: Young adults are less likely to quit, or attempt to, if they smoke a cigarette within minutes of waking or consume more than 20 cigarettes over the course of a day (Fagan et al., 2007). Among smokers under the age of 18, 60.9% of high school aged adolescents who smoke – with the highest majority in female and African American students – attempt to quit smoking. Of these individuals, only 12.2% succeed, with higher quit percentages in the lower grades (9th grade; CDC, 2009). For those who quit early in life, there are more health benefits, and lower risks of life-threatening diseases associated with smoking (USDHHS, 1990). Therefore, in order to protect the health and well-being of future generations as well as those individuals presently willing to quit, scientists are developing effective smoking cessation treatments, and hopefully in the near future, prevention techniques.

**Current Smoking Cessation Aids**

With the ever-increasing demand for smoking cessation pharmacotherapies, scientists have successfully developed several first-line medications, altered the purposes of currently available drugs (“second-line medications”), and are in the process of synthesizing new drugs and vaccines for smoking cessation.

**Nicotine Pharmacotherapies for Smoking Cessation**

Other than quitting “cold turkey,” use of Food and Drug Administration (FDA)-approved nicotine replacement therapies (NRT) has been around for nearly 20 years as
the primary aid for smoking cessation in the United States. Although seemingly counterintuitive, NRTs operate by adding the drug back into the system, thereby reducing withdrawal symptoms, and making the process of “getting clean” considerably less painful. This is true not only of nicotine addiction treatments, but also of treatments for illicit drugs of abuse; for example, methadone treatments for opioid addictions.

**Long-acting NRTs**

Scientists have developed two types of NRT delivery devices: Long- and short-acting. Following FDA-approval in 1991, the long-acting delivery device – transdermal patch (Nicoderm CQ®, Habitrol®, Nicotrol®) – is designed to release nicotine consistently over 16 or 24 hours, depending on the dose, to reduce cigarette cravings (for review, see Fant, Buchhalter, Buchman, & Henningfield, 2009). For those fighting very strong cravings, this product can be combined with other smoking cessation aids (for review, see Nides, 2008) that will be discussed in a later section (see Non-Nicotine Pharmacotherapies for Smoking Cessation section of dissertation).

**Short-acting NRTs**

The FDA-approved short-acting delivery devices include the nasal spray (Nicotrol NS®), vapor inhaler (Nicotrol®), lozenge (Commit®), sublingual tablet (Ariva®, Nicorette Microtab®), and nicotine gum (Nicorette®). In contrast to the long-acting devices, the short-acting interventions are used on an “as needed” basis, and are absorbed through buccal or nasal membranes. Short-acting NRTs are available in different dosages, and can be used separately or in combination with the transdermal patch (for review, see Fant et al., 2009; Nides, 2008) for added efficacy.
NRT benefits

Regarding both sustained and acute dosing delivery devices, there are a series of benefits that enable these products to remain on the market. In general, the overall odds of quitting smoking significantly doubles (1.5-2 fold) when using these medications (Eisenberg et al., 2008; Moore et al., 2009; Silagy, Lancaster, Stead, Mant, & Fowler, 2004; Stead, Perera, Bull, Mant & Lancaster, 2009), and the percentage further increases with a combination of NRTs (Stead, Perera, Bull, Mant & Lancaster, 2009). Also, the abuse liability of these products is low due to a slower speed of nicotine delivery (Tutka et al., 2005). With the variety of different dosages and devices available, there is ample opportunity to find a dosage and method or combination of devices that works for the individual and severity of their nicotine addiction. Additionally, NRTs do not include the array and quantity of carcinogens or supplementary agents found in tobacco that severely increase the abuse potential of the drug. Finally, the harmful effects while taking NRTs and after cessation of these methods are minimal at best (Moore et al., 2009).

Non-Nicotine Pharmacotherapies for Smoking Cessation

Despite the popularity of NRTs, scientists have, or are in the process of developing pharmacotherapies that do not contain nicotine, including varenicline (Chantix®, Champix®), bupropion (Wellbutrin®, Zyban®), clonidine (Catapres®, Dixarit®), nortripyline (Sensoval®, Aventyl®, Pamelo®, Norpress®, Allegron®, Nortrilen®), rimonabant (Acomplia®), mecamylamine (Inversine®), SB-277011A, and monoamine oxidase inhibitors (MAOIs; selegiline, moclobemide, lazabemide). Many of these medications have already found their way into the hands of the public as solitary smoking cessation treatments (e.g., first-line pharmacotherapies) while others are more
useful as “last ditch efforts” (e.g., second-line pharmacotherapies) or in conjunction with NRTs. Regardless of their status, the development of these medications or restoration of old drugs for a new function is progressive, a giant step toward alleviating the millions of smokers worldwide of their habitual captivity.

First-line pharmacotherapies

**Bupropion.** Other than NRTs, only two non-nicotine drugs – bupropion and varenicline – have been approved by the FDA specifically for their use as smoking cessation aids. Smoking is commonly associated with a variety of co-morbidities, including depression, particularly major depressive disorder (MDD; Brown, Lewinsohn, Seeley, & Wagner, 1996; O’Brien, 2001). In fact, individuals, specifically adolescents, with MDD consume more cigarettes, and therefore, are at a considerably greater risk for dependence, compared to adolescents without MDD (Fergusson, Goodwin, & Horwood, 2003). Thus, because of its history as an anti-depressant, bupropion is a frequently prescribed medication to help individuals quit smoking while treating any underlying depression, specifically atypical depression. Irrespective of its use as an anti-depressant, bupropion has been found to be more effective in promoting smoking cessation compared to placebo for those with and without a history of depression. This finding indicates that the mechanism of action to combat depression may not necessarily correlate with that of a smoking cessation aid (Eisenberg et al., 2008). It is important to note that specific details concerning the mechanisms by which bupropion helps to treat either depression or a smoking habit are not yet fully understood (for review, see Siu & Tyndale, 2007).

However, for functioning as an effective smoking cessation aid (for review, see
Frishman, 2009), bupropion is theorized to block dopamine and norepinephrine reuptake, thereby reducing nicotine’s rewarding effects (for review, see Fant et al., 2009).

**Varenicline.** Varenicline is a competitive α4β2 nAChR partial agonist that significantly increases continuous quit and abstinence rates, while reducing withdrawal symptoms, cravings, and reward during relapse in clinical trials (for review, see Nides, 2008). This drug surpasses abstinence rates of bupropion (for review, see Eisenberg et al., 2008; Frishman, 2009; Garrison & Dugan, 2009), while producing a handful of relatively minor side effects for most individuals (e.g., nausea, sleep problems, skin irritations; for review, see Fant et al., 2009; Frishman, 2009; Pfizer, 2009). However, it is extremely important to note that very recently, individuals taking varenicline have reported extreme behavioral alterations that deviate from the norm. These negative behaviors range from mild (e.g., agitation and confusion) to intense (e.g., hostility, anger, anxiety, panic, depression, suicidal thoughts, hallucinations and paranoia; Pfizer, 2009). Investigations into the effects of this drug are ongoing to understand the mechanism of action behind these behavior changes. Although varenicline is under scrutiny for its adverse effects, the existence of FDA-approved non-nicotine smoking cessation aids that affect different biological aspects of nicotine addiction gives smokers a broader variety of options to choose from when deciding to quit smoking. Additionally, the development of second-line smoking cessation aids may be beneficial to those that are exceptions to the efficacy of first-line pharmacotherapies.

**Second-line pharmacotherapies**

Although second-line pharmacotherapies (e.g., clonidine and nortripyline) are FDA-approved to treat other health problems (e.g., hypertension caused by opiate
withdrawal/anxiety/Tourette’s syndrome, and major depression/chronic fatigue syndrome, respectively), these drugs are not FDA-approved for smoking cessation. However, despite the lack of government approval, clonidine and nortripyline are still considered “last resort” smoking cessation treatments and should only be used if NRTs, bupropion, and varenicline are not effective.

Although primarily used as a treatment for opiate withdrawal, clonidine, an α2 adrenergic receptor agonist, has shown some promising results towards alleviating nicotine withdrawal symptoms (for review, see Frishman, 2009; Siu & Tyndale, 2007). Regarding the mechanism of action, the administration of clonidine is thought to induce a sedative effect to dampen the "fight-or-flight” response, which is initiated by the release of epinephrine and norepinephrine from the sympathetic-adrenal-medullary (SAM) axis.

Similar to bupropion, tricyclic anti-depressants, such as nortripyline, have had success as pharmacotherapies for smoking cessation (for review, see Frishman, 2009; Nides, 2008). Nortripyline significantly increases abstinence (for review, see Nides, 2008) and can be combined with the transdermal patch. Details concerning the mechanism of action are still unknown, especially since other types of anti-depressants – for example, selective serotonin reuptake inhibitors (SSRIs) – failed to be considered effective as a smoking cessation in the vast majority of clinical studies.

**Pharmacotherapies still under investigation**

To date, there are several drugs currently under investigation as smoking cessation aids: A dopamine (D3) receptor antagonist (SB-277011), a cannabinoid (CB1) receptor antagonist (rimonabant), an α3β4 nAChR antagonist (mecamylamine), and monoamine oxidase inhibitors (MAOIs), including selegiline, lazabemide, and
moclobemide. At this point, scientists are still scrutinizing the potential of each of these drugs for their role as smoking cessation aids in both preclinical and clinical trials. Initial findings are positive for some of these drugs; for example, SB-277011A reduces preference due to the environment, and decreases relapse-like behaviors in the first phases of preclinical studies (Pak et al., 2006; for review, see Fant et al., 2009).

**Vaccines**

Immunological strategies, including vaccines, have recently become of great interest as potential smoking cessation aids or even as future prevention techniques. Using different combinations of immunogen carriers, several biopharmaceutical companies are developing vaccines that inject nicotine-specific antibodies into the body to bind to nicotine within seconds of administration. The addition of the antibody increases the size of the nicotine molecule beyond that which can cross through the blood brain barrier, thereby preventing nicotine from inducing its rewarding effects (for review, see Fant et al., 2009; Frishman, 2009; Siu & Tyndale, 2007; Nides, 2008). Within the last two years, several of the clinical trials for each of the companies’ vaccines were successful in increasing abstinence rates of smokers. With the positive effects of these vaccines, there may be a possibility in the future to have vaccines available for smoking cessation as many of these findings have led to the FDA to label some of these products as a “priority”. Vaccinations for non-smokers to be used as a smoking prevention technique in the future have yet to be tested.

**Problems Concerning Current Smoking Cessation Aids**

Many of the pharmacotherapies previously mentioned are quite effective; in fact, millions of individuals have quit using many of these drug options. That said, the current
options for smoking cessation treatments focus on smoking as a problem that impinges on everyone equally, which is by no means applicable to past, present or future societies. In general, as with all treatment medications for drug addictions, there are certain aspects of these drugs that may affect everyone’s ability to quit. For example, side effects, ranging from minor (e.g., nausea, gas, sleep disturbances) to major (e.g., hypotension, suicidal thoughts, hallucinations) may be severe enough to cause an individual to cease taking the drug or even prevent them from using smoking cessation medications altogether (Vogt, Hall, & Marteau, 2008).

Perceived inefficacy of the medications to work in general or only in some cases may also prevent individuals from using NRTs and other smoking cessation aids (Vogt et al., 2008). Many individuals regard medications as a “crutch” as if the smoker is completely incapable of battling the addiction themselves. Others are of the opinion that these medications or devices would subject them to more harm (e.g., cancer, addiction), or would not treat their specific addiction because they regard themselves as “exceptions” (Vogt et al., 2008). Therefore, the risk of relapse for all smokers is ever present as these are treatments, not cures.

By taking the smoking population as a whole, the ability for medications to effectively treat nicotine addictions is significantly reduced because the specific needs and biological makeup of the individual or groups of people is not taken into account. Unfortunately, the majority of the problems with current smoking cessation aids stem from variations among populations and between individuals.
**Efficacy of Smoking Cessation Aids Based on Age**

Age poses a variety of obstacles that prevents the development of universally effective smoking cessation aids. Adolescence is a time frame that most individuals find difficult to define; however, one thing is clear: Adolescents are not merely a smaller version of adults, but rather, they are developing – biologically, psychologically, and behaviorally – into adults (Dahl, 2004). Similar to the original reasons for not including females into basic and clinical research (e.g., variable behavior and hormone levels), adolescents are proving to be significantly more variable compared to adults (Dahl, 2004), with regard their biology (e.g., neurological maturation, differences in enzymatic metabolism and elimination of nicotine, hormonal changes; Paus, 2005; Spear, 2000), psychology (e.g., cognition, decision making ability, impulsivity; Dahl, 2004; Spear, 2000; Steinberg, 2004; 2005), CYP2A6 activity (O’Loughlin et al., 2004; Karp, O’Loughlin, Hanley, Tyndale, & Paradis, 2006), and behavior (e.g., knowledge of current smoking cessation aids and interventions, risk factors for becoming smokers, smoking patterns, withdrawal symptoms, relapse; Audrain-McGovern et al., 2009; Karp et al., 2006; Dahl, 2004; Messer, Trinidad, Al Delaimy, & Pierce, 2008; O’Loughlin et al., 2004; Solberg, Boyle, McCarty, Asche, & Thoele, 2007; Spear, 2000).

**Biology and psychology**

One of the defining characteristics of adolescence is the continual development of the brain (Paus, 2005). Although the majority of the brain’s neuro- and synaptogenesis (with the exception of the olfactory bulbs, and subventricular zone of the dentate gyrus in the hippocampus; for review, see Ming & Song, 2005) reaches peak development during adolescence, the prefrontal cortex, an area of the brain that controls executive functions
and personality, is not fully developed until an individual’s early twenties (i.e., before the age of 25; Giedd et al., 1999; Steinberg, 2004; Sowell, Thompson, Holmes, Jernigan, & Toga, 1999). Before this landmark age, adolescents naturally experience hormonal and physical changes, in conjunction with intermittent emotional outbursts of rebellion and independence during their journey towards self-identity (Dahl, 2004). Continual neural construction towards a mature brain is, in part, responsible for the emotional roller coasters and personality changes associated with adolescence (Steinberg, 2004; 2005), that can lead to increases in risky behaviors, including experimentation with drugs of abuse (e.g., nicotine in the form of tobacco use; Dahl, 2004). For 13-15 year old girls, cigarettes are generally the first drug of choice during adolescence; boys, on the other hand, tend to experiment with alcohol before any other drug (Kandel, Yamaguchi, & Chen, 1992). Beginning drug use at such an early age significantly alters an individual’s normal developmental trajectory to potentially increase a young person’s vulnerability to abuse illicit drugs (e.g., marijuana, cocaine, heroin; Kandel et al., 1992; Spear, 2000; Spear, 2007; USDHHS, 1988; Zickler, 2004), and decrease the ability to quit smoking in the future (Breslau & Peterson, 1996; Chen & Millar, 1998; McQuown et al., 2007; USDHHS, 1994).

**Genetics**

Although the general neural development of a human is relatively universal, the genetic makeup, and subsequent behavior of each individual is unique. Genetic polymorphisms of CYP2A6 that alter nicotine metabolism are commonplace among all groups of people, regardless of race/ethnicity, age or sex; however, specific phenotypic behaviors following nicotine exposure for those with these genetic mutations may be age-
dependent. Although there is evidence to indicate the contrary (Audrain-McGovern et al., 2009), recent findings suggest that the risk of becoming nicotine-dependent increases for middle- and high-school aged adolescents with slow nicotine metabolisms (O’Loughlin et al., 2004; for review, see Malaiyandi, Sellers, & Tyndale, 2005) and depression (Karp et al., 2006), regardless of sex. These findings directly contrast those repeated by Audrain-McGovern and colleagues (2009), and the conclusions of adult studies (Pianezza, Sellers, & Tyndale, 1998; Rao et al., 2000; Sellers, Tyndale, & Fernandes, 2003; Tyndale et al., 1999; Tyndale & Sellers, 2001; 2002), whereby adults with one or two variant CYP2A6 alleles consume fewer cigarettes over their lifetime, and therefore, are less likely to become dependent on cigarettes compared to smokers without these genetic variants (Pianezza et al., 1998; Rao et al., 2000; Sellers et al., 2003b; Tyndale et al., 1999; Tyndale & Sellers, 2001; 2002).

**Behavior**

In addition to physical and emotional changes, adolescence is a period of time for discovery, uncovering one’s identity and personal style, while establishing personality, belief systems, and lifestyles. Focusing on daily routines, many adolescents experiment with different lifestyles, especially when it comes to smoking: Heavy or light smoker, daily, weekly or infrequent smoking, or better yet, current or former smoker. Unfortunately, because adolescents are just starting to test the waters of a smoker’s lifestyle, it is difficult to compare the smoking habits (e.g., puff volume, velocity and duration, length of time between each puff and throughout entire cigarette consumption, cigarettes consumed per day, nicotine yields) of adolescents (18 years and younger) to adults whom have firmly established their tobacco dependence and smoking style.
However, progressing just beyond adolescence into “young adulthood” (18 to 25 years of age), there are still significant smoking behaviors and trends that separate these young adults from the 25-and-over population. Young adults are more likely to become smokers, or infrequent “chippers”, whereas older adult populations are more likely to consist of daily smokers that smoke within minutes of waking and consume over 10 cigarettes per day, as well as those who intend to quit the habit, leading to a large number of former smokers (Solberg et al., 2007). Additionally, despite being more likely to become smokers and less likely to use any form of smoking aid, younger adults are still more likely to attempt to quit (Solberg et al., 2007) and successfully quit smoking for at least six months compared to older adults (Messer et al., 2008). This finding may indicate that the 18-25 year age group either does not believe that current smoking cessation pharmacotherapies are effective, and are therefore, more apt to not use any aids (Hammond, McDonald, Fong, & Borland, 2004), or their withdrawal symptoms are more tolerable and can be managed without the assistance of drugs. The environment is also more influential towards younger adults, as they tend to reduce the amount of cigarettes consumed due to increased taxes on cigarettes, smoking bans in public restaurants or bars (Solberg et al., 2007), and smoke-free homes (Messer et al., 2008).

All of these age differences amount to a series of obstacles for researchers to develop smoking cessation interventions for adolescents and young adults while catering to the limitless variability of these developmental stages and modifying current smoking cessation aids or methods used for older adults. Needless to say, current smoking cessation techniques and clinical interventions have experienced little success to date, possibly due to the fact that the majority of the pharmacotherapies out on the market for
smoking cessation are not technically approved for adolescents (Rosen & Maurer, 2008). Several reviews have examined the efficacy of smoking cessation trials for adolescents and young adults (for review, see Backinger, Fagan, Matthews, & Grana, 2003; Bancej, O’Loughlin, Platt, Paradis, & Gervais, 2007; Curry, Mermelstein, & Sporer, 2009; Grimshaw & Stanton, 2006; Moolchan, Monique, & Henningfield, 2000; Rosen & Maurer, 2008; Schepis & Rao, 2008; Sussman, Lichtman, Ritt, & Pallonen, 1999; Sussman, Sun, & Dent, 2006; Wiehe, Garrison, Christakis, Ebel, & Rivara, 2005). Yet, despite scrutiny of the reviewed studies, there is not one specific type of intervention that is highly effective for these age ranges, which poses a major obstacle towards preventing more adolescents from smoking or ceasing the habit in its early stages.

**Efficacy of Smoking Cessation Aids Based on the Individual**

Delving beyond age, individuals, including identical twins, are behaviorally and genetically unique, and the variety of smoking cessation pharmacotherapies or devices currently on the market is an attempt to address these individual differences, at least as far as behavior is concerned. Failure to quit using one device may motivate the individual to try another method. However, new questions then arise inquiring about why the individual failed to quit in the first place. Disregarding a basic underlying desire to remain a smoker, scientists are investigating biological and genetic reasons for the inability to quit the habit. At present, the main research focus is CYP2A6 (EC 1.14.14.1), the enzyme primarily responsible for nicotine metabolism in humans. The existence of over 30 allelic variants of CYP2A6 (for review, see Di, Chow, Yang, & Zhou, 2009) is the result of a series of DNA turnover and gene conversion events (e.g., insertions, deletions, frameshift mutations in the DNA) that eventually led to the dispersal
of genetic variants throughout populations, a concept known as molecular drive (for review, see Dover & Tautz, 1986; Gonzalez & Nebert, 1990; Ohta & Dover, 1984). These variants encode for altered CYP2A6 protein or activity levels, possibly leading to changes in nicotine metabolism (Ingelman-Sundberg, Daly, & Nebert, 2009; Mwenifumbo & Tyndale, 2007; Nakajima, Kuroiwa, & Yokoi, 2002; Tricker, 2003; Malaiyandi, Sellers, & Tyndale, 2005; Oscarson, 2001) and vulnerability towards dependence or addiction. In adults, polymorphisms that encode for a null enzyme or an enzyme with reduced activity renders the individual unable to metabolize large quantities of nicotine, thereby reducing the clearance of nicotine and cotinine, and diminishing their risk of nicotine dependence. For example, compared to the wild type genotype (CYP2A6*1), individuals with at least one or two variant alleles have altered responses to nicotine and later, risk of dependence. Certain polymorphisms, for example CYP2A6*1/*12 and CYP2A6*1/*9 genotypes, lead to partial reductions (approximately 20%) in enzymatic activity (e.g., increase in total and non-renal clearance of nicotine), increases in the half lives of nicotine and cotinine, and low 3HC/COT ratios (Benowitz et al., 2006). These polymorphisms frequently are detected in Chinese and Native Canadian populations (for review, see Hukkanen et al., 2005). Presence of other variant allelic genotypes, CYP2A6*1/*2, CYP2A6*1/*4, CYP2A6*9/*12, CYP2A6*9/*4, and CYP2A6*9/*9, can lead to a 40 to 50% reduction in nicotine clearance, considerable increases in nicotine and cotinine half lives, and very low 3HC/COT ratios (Benowitz et al., 2006). Populations of Korean and Japanese descent have a high prevalence of these allelic frequencies (for review, see Hukkanen et al., 2005), making these groups of individuals very important to pharmacogenetic research of these polymorphisms and
nicotine dependence. As yet, no current mainstream drugs – including smoking cessation pharmacotherapies – account for these genetic differences.

**Modulating CYP2A6 Activity: The Future of Smoking Cessation Aids**

One’s ability to quit smoking is dependent on several factors, including environmental cues, sex, age, and race/ethnicity; however, there is a strong genetic component with the presence of CYP2A6 polymorphisms detected amongst all individuals (Ho & Tyndale, 2007; Lee & Tyndale, 2006). Researchers are examining the administration of CYP2A6 inhibitors as a potential method to inevitably reduce the need for more cigarettes. Essentially, these drugs mimic the genetic polymorphisms associated with variant alleles that lower the risk of dependence and cigarette consumption (Sellers & Tyndale, 2000; Sellers et al., 2003b; Tyndale & Sellers, 2002), potentially enabling a greater number of smokers to abandon their smoking habit. In addition to the metabolism of nicotine, coumarin (for review, see Riveiro et al., 2010) and several pharmaceuticals (e.g., disulfiram), CYP2A6 is known to activate carcinogenic nitrosamines [e.g., aflatoxin B1, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanal (NNAL), N-nitrosodimethylamine (NDEA)], many of which induce tobacco-related cancers. However, individuals whom are poor metabolizers of nicotine have lower incidences of tobacco-related lung cancer (Miyamoto et al., 1999; for review, see Oscarson, 2001). Because CYP2A6 inhibitors mimic these polymorphisms, these medications will similarly slow the breakdown of carcinogenic nitrosamines to potentially reduce the risk of cancer (Kamataki, Fujieda, Kiyotani, Iwano, & Kunitoh, 2005; Sellers, Ramamoorthy, Zeman, Djordjevic, & Tyndale, 2003a; Strasser et al., 2007; Takauchi et al., 2006; Tutka et al., 2005).
Potential Smoking Cessation Aids: Methoxsalen

One of the most promising examples of CYP2A6 inhibitors to have the potential as a smoking cessation aid is the photoactive compound, methoxsalen (8MOP; methoxypsoralen; 8-methoxypsoralen; ammoidin; xanthotoxin).

History of Methoxsalen Use

Methoxsalen has been used to treat dermatoses and pigment disorders since antiquity (circa 1500 B.C.; Pathak, 1986; Wyatt, Sutter, & Drake, 2001); however, the biological mechanism of action for this compound was not questioned for another 3,000 years. This photosensitive furocoumarin is naturally found in a number of plant families, including Family Rutaceae (lemons, limes), Moraceae (figs), Umbelliferae (parsley, celery, trosøpalm, parsnips, carrots, fruit of Ammi majus, roots of the Heracleum candicans), and Leguminosae (malaytea scurf-pea) (for review, see Pathak, 1986; Pathak & Fitzpatrick, 1992). Following its isolation in 1948 by Fahmy and colleagues (Fahmy & Abu-Shady, 1948), research has focused on understanding the full extent of methoxsalen’s dermatological functions. Successful clinical trials using methoxsalen for vitiligo treatment began in the 1950s, followed by present-day treatments for psoriasis starting in 1960s (for review, see Bond, Grant, & Boh, 1981), and persistent cutaneous T-cell lymphoma (CTCL) in the 1970s (specifically mycosis fungoides; Bleehen, Briffa, & Warin, 1978; for review, see Pathak & Fitzpatrick, 1992). Although infrequently used, methoxsalen treatments successfully improve other dermatoses, including but not limited to, atopic dermatitis, lichen planus, alopecia areata totalis and universalis, eczema, palmoplantar pustolosis (Wyatt et al., 2001), prurigo nodularis, sclerosing skin diseases (e.g., scleromyxedema), cutaneous graft-versus-host disease (for review, see Pathak &
Fitzpatrick, 1992), urticaria pigmentosa (Christophers, Hönigsmann, Wolff, & Langner, 1978), acrodermatitis continua of Hallopeau (Durmazlar, Akpinar, Eren, Eskioglu, & Tatlican, 2009), livedo reticularis, livedoid vasculitis (Choi & Hann, 1999), telangiectasia macularis eruptive perstans (Sotiriou, Apalla, & Ioannides, 2010), nephrogenic fibrosing dermopathy, nephrogenic systemic fibrosis and solid organ transplant rejection (for review, see Kapelewski & Klein, under review; Knobler et al., 2009). In addition to dermatological functions, however, it has recently been discovered that methoxsalen has other activities, including inhibition of enzymes important for nicotine metabolism and the ability to mimic genetic polymorphisms of those enzymes. This inhibitory function places this drug high on a select list of potential pharmacotherapies for smoking cessation. Although only a handful of preclinical and human studies examining methoxsalen’s prospective role as a smoking cessation aid have been published at this time, there is still much to understand about this drug’s pharmacology, including the pharmacokinetics, pharmacodynamics, as well as side effects and toxicity associated with its use to find a niche as a tobacco addiction cessation technique.

**Methoxsalen Pharmacokinetics**

### Absorption

Despite a lengthy history of use, little is known about the pharmacokinetics of methoxsalen. Methoxsalen is rapidly absorbed indirectly via handling or ingestion of specific foods that naturally contain this psoralen (e.g., lemons, limes, figs, parsley; for review, see Pathak, 1986; Pathak & Fitzpatrick, 1992). However, within the last 60 years, researchers have conceived more reliable methods to dispense precise dosages of the drug. In the United States, direct administrations are accomplished using several
routes: Hard crystalline (8MOP®) or soft liquid (Oxsoralen-Ultra®) capsules to be taken orally, topical lotions (Oxsoralen®), and intravenous injections of a sterile solution (for CTCL treatment only; Uvadex®).

**Distribution and metabolism**

Although the majority of the drug is bound to serum albumin, free (unbound) methoxsalen is rapidly distributed throughout the body (Artuc, Stuettgen, Schalla, Schaeffer, & Gazith, 1979; Busch, Schmid, Koss, Zipp, & Zimmer, 1978; for review, see de Wolff & Thomas, 1986), with the majority migrating to the skin to be taken up by melanocytes and concentrated in the epidermal layers (Artuc et al., 1979). The specific steps of the metabolism of methoxsalen are still under investigation with several proposed theories; however, the most accepted view involves Phase I cytochrome (CYP) P450 enzymes, specifically CYP2A6 (Koenigs, Peter, Thompson, Rettie, & Trager, 1997). CYP enzymes oxidize the outer double bond of methoxsalen’s furan ring (Mays et al., 1986; Schmid, Prox, Reuter, Zipp, & Koss, 1980), leading to the creation of an unstable epoxide (Koenigs & Trager, 1998) and then metabolites. The majority of the metabolite structures have yet to be characterized (Bickers & Pathak, 1984); however, studies have determined that the major reactive metabolite may be a dihydrodiol (Koenigs & Trager, 1998), in conjunction with the presence of minor metabolites, including 8-hydroxypsoralen (Busch, Schmid, Koss, Zipp, & Zimmer, 1978; for review, see de Wolff & Thomas, 1986). Once formed, the metabolites irreversibly inactivate CYP enzymes, including CYP2A6 (Kharasch, Hankins, & Taraday, 2000), by covalently binding to the enzyme’s active site to induce a mechanism-based (“suicide”) inactivation (Koenigs et al., 1997; Koenigs & Trager, 1998; Mays, Hilliard, Wong & Gerber, 1989;
Sharp et al., 1984). This irreversible bond decreasing monooxygenase activities and CO-binding abilities of the enzyme for up to 24 hours in human microsomes (Tinel et al., 1987), despite a two-hour half-life of methoxsalen. The time frame for which the metabolites inactivate CYP2A6 parallels the half-life of the methoxsalen’s metabolites (Mays et al., 1990), as well as the turnover rate for these enzymes (Fouin-Fortunet et al., 1986; Labbe et al., 1989).

**Elimination**

With regard to drug elimination, relatively little of the parent drug is excreted unchanged through urine (Ehrsson, Nilsson, Ehrnebo, Wallin, & Wennersten, 1979). However, the metabolites of methoxsalen (approximately 95%) are eliminated within 24 hours (Busch et al., 1978; Muni et al., 1984). Small traces of metabolites also can be traced in feces and bile (Mays et al., 1986).

**Methoxsalen Pharmacodynamics**

In comparison to pharmacokinetics, researchers are more knowledgeable about the mechanisms that can result in the symptoms, side effects and toxicity associated with the use of methoxsalen. To date, there are two known mechanisms of action for this drug: The more understood mechanism involves photoactivation using UV radiation (known as Photochemotherapy or PUVA, and the sister therapy, extracorporeal photopheresis or ECP). The combination of light and photoactive psoralens is a modified technique used by the Ancient Egyptians, which consisted of the direct application of juice from the fruit of the *Ammi majus* plant to the ailing area of the skin followed by prolonged exposure to natural light (Pathak & Fitzgerald, 1992; Pathak, 1986). The chemical structure of methoxsalen [9-Methoxy-7H-furo[3,2-g][1]benzopyran-7-one
(C_{12}H_8O_4); see Figure 3] plays a key role in the process of photoactivation (known as 2 + 2 cycloaddition; for review, see de Wolff & Thomas, 1986; Gasparro, Liao, Foley, Wang, & McNiff, 1998; Schmitt, Chimenti, & Gasparro, 1995; Vigny, Gaboriau, Voituriez, & Cadet, 1985). Following the absorption of photons, the C_5=C_6 double bond of a thymine DNA base pair can create a monoadduct in one of two ways: A covalent bond with the pyrone ring C_3=C_4 double bond, leading to a non-fluorescent monofunctional adduct, or to the furan ring C_4=C_5 double bond to produce a fluorescent monoadduct, also known as a cyclobutane (C_4) dimer (for review, see Kitamura, Kohtani, & Nakagaki, 2005; Rodighiero, & Dall’Aqua, 1984). Subsequent exposure to long-wave ultraviolet radiation (UVA; generally in 320 – 400 nm range) electronically stimulates these adducts (Pathak, 1986) to form DNA cross-linkages (for review, see Gasparro, Liao, Foley, Wang, & McNiff, 1998; Gasparro, Felli, & Schmitt, 1997; Park, Amici, & Gasparro, 1995; Vigny et al., 1985). These cross-linkages inhibit DNA synthesis (Epstein & Fukuyama, 1975; Fritsch et al., 1979; Walter, Voorhees, Kelsey, & Duell, 1973) and replication, thereby reducing abnormal cell proliferation (Lüftl, Röcken, Plewig, & Degitz, 1998). Although common during PUVA treatments, DNA cross-linkages are dependent on several factors, including the light wavelengths, sequence of DNA that is being irradiated, and the light sourced used (e.g., UVA or UVB; Moor & Gasparro, 1996).

PUVA also is an effective therapy for persistent cutaneous T-cell lymphoma (CTCL; Bleehen et al., 1978); however, extracorporeal photopheresis (ECP) is regarded as a more valid technique comparatively because a reduction in toxic effects generally is associated with PUVA (for review, see Gasparro, Dall’Amico, Goldminz, Simmons, & Weingold, 1989; Geskin, 2007; Taylor & Gasparro, 1992). Although both treatments
involve the combination of methoxsalen and UVA light, ECP is more invasive as it involves a blood transfusion. Following ingestion of a therapeutic dose of methoxsalen, red blood cells are separated from white blood cells, and immediately returned to the body. The white blood cells, on the other hand, then are radiated with UVA light and infused back into the patient (for review, see Gasparro et al., 1997; Gasparro et al., 1989). As some of the side effects can be attributed to the ingestion of methoxsalen, recent ECP treatments involve the exposure of the white blood cells to a sterile solution of methoxsalen (Uvadex®), which is then irradiated with UVA light and returned to the body (Geskin, 2007).

The second and less familiar mechanism of action of methoxsalen involves the inhibition of CYP2A6. As previously mentioned, active metabolites of methoxsalen irreversibly inhibit the enzyme, thereby reducing nicotine metabolism and increasing bioavailability. Stable serum nicotine levels achieved via CYP2A6 inhibitors reduce cigarette cravings, and in turn, cigarette consumption (Sellers, Kaplan, & Tyndale, 2000), which contrasts with the oscillating serum nicotine levels normally caused by cigarette smoking. Additionally, the inhibition of CYP2A6 by methoxsalen also helps reduce the risk of lung tumorigenesis and adenomas via a slower breakdown of carcinogenic nitrosamines (e.g., NNK) in murine models (Takeuchi et al., 2006; Kamataki, Fujieda, Kiyotani, Iwano, & Kunitoh, 2005) and humans (Sellers et al., 2003a; Tutka et al., 2005).

Although there are a variety of benefits stemming for methoxsalen’s potential use as a smoking cessation aid and as a current treatment for dermatoses, certain aspects of the drug’s mechanisms of action (e.g., interactions with DNA and effects on liver enzyme
activity) may enhance the risk of more detrimental or fatal health problems (e.g., cancer and hepatotoxicity) in the future.

**Side Effects and Toxicity of Methoxsalen**

There are a broad range of physiological effects that accompany the body’s ability to process a drug, and methoxsalen is no exception. The toxic effects of methoxsalen range from mild (e.g., light sensitivity) to life-threatening (e.g., cancer; see Appendix A for MSDS sheet). Although the majority of the known symptoms and side effects of methoxsalen are associated with PUVA treatments, there are a handful of studies that have examined the toxic effects of methoxsalen alone (Bridges & Mottershead, 1977; Diawara, Allison, Kulkoisky, & Williams, 1997; Diawara, Williams, Oganesian, & Spitsbergen, 2000; Dunnick, Davis, Jorgenson, Rosen, & McConnell, 1984). However, the number and severity of these effects are minuscule in comparison to those associated with PUVA therapy.

**Methoxsalen Sans UV Radiation**

In addition to mutagenic effects in *Escherichia coli* and *Salmonella typhimurium* (Bridges & Mottershead, 1977), male and female Wistar rats chronically treated with methoxsalen mixed into their diet endure significant reductions in body weight and birth rates (Diawara et al., 1997). Regarding liver enzyme activity and content, the effects of methoxsalen may be strain-dependent. Although there are some studies that suggest the opposite effect in adult male and female C57BW mice (Diawara et al., 2000), oral methoxsalen increases the level and activity of hepatic CYP enzymes and other drug-metabolizing enzymes (e.g., aryl hydrocarbon hydroxylase, ethylmorphine N-de-methylase) in male CD-1 mice (Bickers, Mukhtar, Molica, & Pathak, 1982). Similar
doses of oral, but not topical, administration of methoxsalen significantly increase the levels and activity of these same enzymes in adult female Sprague Dawley rats (Bickers et al., 1982); however, these findings were not confirmed in adult female Wistar rats (Tsambaos, Vizethum, & Goerz, 1978). Recent studies have also determined that methoxsalen incorporated into the diet of adult male and female C57BW mice induces histological alterations of the liver (i.e., enlarged centrolobular hepatocytes as well as eosinophilic cytoplasm; Diawara et al., 2000). Comparable hepatic alterations in the liver, testes, and adrenal glands of male and female Fischer 344 rats, along with increased mortality rates and reduced weight gain, have been identified as effects of chronic methoxsalen exposure via gavage (Dunnick et al., 1984). Acute administration of methoxsalen alone, however, has not resulted in any toxic effects in a multitude of other studies, both in vitro (Burger & Simons, 1979; Schenley & Hsie, 1981), and in vivo using rodents (Damaj, Siu, Sellers, Tyndale & Martin, 2007; Raunio et al., 2008) or humans (Sellers et al., 2000).

**PUVA**

In combination with UV light, temporary side effects of methoxsalen include nausea and light sensitivity, lasting several hours after treatment (Geskin, 2007). More enduring adverse dermatological effects may include, but are not limited to, reddening of the skin (erythema), blistering, delayed inflammation, burning and peeling (sunburn), itching (pruritus), hives (urticaria), rash (miliaria), hypopigmentation, tenderness, premature aging of the skin (photoaging), metastasis of the disease (Bergfeld, 1977; Fitzpatrick, Hopkins, Blickenstaff, & Swift, 1955; Grange & Parrish, 1984; Gschnait et al., 1980; Henseler, Wolff, Hönigsmann, & Christophers, 1981; Melski et al., 1977;
Pathak, Mosher, & Fitzpatrick, 1984; Shannon, Wilson, & Stang, 2004; Wolff, 1990; Wolff & Hönigsmann, 1981; 1984; Wyatt et al., 2001), pigmented lesions, wrinkles, and actinic elastosis (Stern, 2003), lichen planus (Nanda, Grover, & Reddy, 2003), freckles, lentigines, mottling, actinic keratosis (ephlides; Henseler, Christophers, Honigsmann, & Wolff, 1987; for review, see Kapelewski & Klein, under review). Although it is the skin that is mainly affected, chronic methoxsalen treatment along with UVA radiation is systemically toxic, as some individuals have experienced other non-skin related effects including damage to the eye (e.g., cataracts; Wolff, 1990), dizziness, headaches, nose bleeds, olfactory dysfunction (Vernassière et al., 2006), immunosuppression (Wolff, 1990), hypotension, anemia, fevers, depression, vertigo, gastrointestinal issues, nausea, fluid retention (edema), decreased motor coordination, drug fever, and leg cramps (Geskin, 2007; Henseler, Wolff, Hönigsmann, & Christophers, 1981; Herfst & de Wolff, 1982; for review, see Kapelewski & Klein, under review; Melski et al., 1977; Pathak, Mosher, & Fitzpatrick, 1984; Shannon et al., 2004). Albeit unpleasant, most of the above-mentioned side effects are non-life threatening. However, because of an interaction with DNA, methoxsalen can cause severely detrimental or potentially fatal genetic alterations (e.g., teratogenicity, mutations, cancer) in both rodents and humans.

**Rodent in vitro and in vivo studies.** Several studies have examined mutagenicity, hepatotoxicity and carcinogenicity following PUVA treatments in rodent models both in vitro and in vivo (for review, see Young, 1990). Genetic alterations of complex genetic structures (e.g., rearrangement of sister chromatids) are documented in Chinese hamster cells (Burger & Simons, 1979), specifically ovarian cells and hypoxanthine-guanine phosphoribosyl transferase system (Burger & Simons, 1978; Schenley & Hsie, 1981),
mouse embryonic cells (Besaratinia & Pfeifer, 2004), and *Escherichia coli* (Ohta et al., 2001). First and second degree burns, a greater incidence and size of melanomas (Aubin, Donawho, & Kripke, 1991), and the development of other large benign and malignant tumors (e.g., papillomas, keratoacanthomas and squamous cell carcinomas) that increase in number are observed following long term PUVA treatments in rodents (Hannuksela, Stenbäck, & Lahti, 1986).

**Human in vitro studies.** With regard to humans, the risk of several life-threatening events (e.g., genetic mutations, cancer) are greater in individuals using photosensitive drugs (Karagas et al., 2007), or receiving PUVA or photopheresis treatments (Stern, Nichols, & Väkevä, 1997; Stern, Väkevä, & the PUVA Follow-up Study, 1997). Several studies reported significant genetic mutations (e.g., chromosomal abnormalities, sister chromatid exchanges; see Gasparro et al., 1997) in human skin fibroblasts (Bredberg, Lambert, Lindblad, Swanbeck, & Wennersten, 1983; Chiou & Yang, 1995), blood lymphocytes (Bredberg et al., 1983) and lymphoblasts (Papadopoulo & Moustacchi, 1990) following methoxsalen and UVA treatments.

**Clinical studies.** In a 20-year long study (1976 – 1996) with 1,380 individuals, scientists report that PUVA patients are also more susceptible to malignant melanomas, squamous skin cell carcinoma, and basal cell carcinomas up to 5 years following treatment (Nijsten & Stern, 2003; Stern, Liebman, Väkevä, & the PUVA Follow-up Study, 1998; Stern & the PUVA Follow-up Study, 2001; Stern, 1998; for review, see Stern & Lunder, 1998; Young, 1990). Stern, and Väkevä along with individuals involved with the PUVA Follow-up Study (1997) also determined that, although there was no significant increase in non-cutaneous malignant tumors in those two decades, 3 of the 16
sites that were monitored had significant increases in the risk for specific cancers (thyroid and breast), as well as CNS neoplasms. The increase in basal cell carcinoma following PUVA treatment in children also has been reported (Stern, Nichols & the PUVA Follow-Up Study, 1996).

Within the last few decades, scientists have dedicated their time and effort to developing a full understanding of PUVA as well as methoxsalen without UV radiation. This knowledge is important to gain insight into how current pharmacotherapies for these dermatoses work. In addition, to bolster the development of other drug derivatives capable of the same efficacy sans toxic side effects as well as supplementary applications of PUVA and/or ECP for other disorders (for review, see Moor & Gasparro, 1996). To date, biochemical discoveries with regard to methoxsalen’s structure have given rise to several editions of the drug [e.g., 5-methoxypsoralen (5-MOP), 4,5,8-trimethylpsoralen (TMP), 3-carbethoxypsoralen (3-CP), 4,5’-dimethylangelicin, 5-methylangelicin; Wolff & Hönigsmann, 1984; 3,4,8-trimethylpsoralen, 3,4-dimethylpsoralen; Gia et al., 1992]. These and other derivatives are currently in the preliminary stages of investigation to determine their pharmacology and future applications (Willis & Menter, 1984).

Current Clinical and Experimental Data on Methoxsalen as a Pharmacotherapy for Smoking Cessation

Due to recent interest in methoxsalen as a cessation aid among scientists in the drug addiction community, there have been a handful of studies that examine the drug and its relationship with smoking in humans (Sellers et al., 2000), and nicotine consumption in rodents (Damaj et al., 2007; Raunio et al., 2008) at this time. All studies to date suggest a potential for the drug as a smoking cessation aid (Zickler, 2000).
**Clinical Data on the Effects of Methoxsalen on Smoking**

To date, only two experiments (one *in vitro*, one *in vivo*) have been completed examining the effects of methoxsalen on nicotine pharmacokinetics and cigarette consumption in 17 male and female adult (20-52 years old) smokers with active CYP2A6 alleles. The first experiment examined blood samples from smokers that orally administered one of three methoxsalen capsules or placebo, with a nicotine bitartrate mixture, to examine changes in nicotine pharmacokinetics (Sellers et al., 2000). In addition to self-report surveys, the second study instructed all participants to smoke one cigarette, ingest a combination of methoxsalen (or placebo) and nicotine (or placebo) capsules during a one-hour abstinence period, then smoke during a videotaped 90-minute session, followed by abstinence for an additional 30-minute period (Sellers et al., 2000). Sellers and colleagues (2000) discovered that the combination of methoxsalen and nicotine increases plasma nicotine levels, and subsequently, increasing bioavailability of nicotine *in vitro*. These increases significantly reduced cigarette consumption and carbon monoxide concentrations (24% and 47%, respectively) following the three-hour laboratory experiment (Sellers et al., 2000).

**Preclinical Studies on the Effects of Methoxsalen on Nicotine Consumption**

Similar to the paucity of clinical studies, only three murine studies have examined behavioral and pharmacological effects of methoxsalen on nicotine: One *in vitro*, and two *in vivo* studies. Initially, Damaj, Siu, Sellers, Tyndale and Martin (2007) determined that methoxsalen also inhibits nicotine C-oxidation in adult male ICR mouse microsomes, a process that primarily is accomplished by examining the structurally and functionally similar mouse orthologue, CYP2a5. Going one step further, Damaj et al. (2007)
discovered that a pretreatment of subcutaneous methoxsalen followed by a subcutaneous injection of nicotine decreases plasma cotinine levels (ng/ml), and increased the half-life of nicotine in adult male ICR mice.

Using gavage administration of several reversible (e.g., 2, 7-dimethylnaphthalene) and irreversible (e.g., methoxsalen) CYP2A6 inhibitors followed by nicotine 30 minutes later, Raunio et al. (2008) examined these effects of CYP2A6 inhibitors on nicotine metabolism and elimination in adult female CD2F1 mice. Out of all the inhibitors, only methoxsalen increased the amount of urinary nicotine and nicotine-N-oxide (another metabolite of nicotine), while decreasing levels of cotinine (Raunio et al., 2008). Additionally, metabolite-to-parent drug ratios (cotinine N-oxide/nicotine and trans-3-hydroxycotinine/nicotine) were also reduced in urine after 24-hours (Raunio et al., 2008). These findings, in combination with those of Damaj et al. (2007), indicate that the pretreatment of methoxsalen increases bioavailability of nicotine by decreasing metabolism and elimination in vitro and in vivo in adult male and female mice.

**Gaps in the Literature**

**Age Differences**

Regarding age, adult animals and humans are more commonly studied compared to adolescents. There is no doubt that investigations into why adults maintain their smoking habit and effective quitting strategies for this age range are very important; however, these studies do not account for the 4,000 adolescents that begin to smoke each day (SAMHSA, 2008b), many of whom will become chronic smokers. Although the quantity of published scientific literature examining the overall effects of nicotine on adolescent subjects is growing, these studies are still few and far between. To date, no
studies have been done in adolescents (rodent or human) to determine if treatments with methoxsalen will curb adolescent smoking or, more importantly, if pretreatment of methoxsalen will prevent adolescents from initiating the habit.

Adolescents are understudied at all levels of animal and clinical studies. Therefore, it is essential that researchers begin to understand adolescent versus adult initiation, development and maintenance of their smoking habit.

Summary

The end of the 1960s ended centuries of unbridled tobacco use with the Surgeon General’s 1964 and 1967 reports identifying tobacco use as a major public health concern (USPHS, 1964; 1967). From there, the field of tobacco dependence research blossomed with a new mission to understand the pharmacology of the drug, which culminated in the 1988 Surgeon General’s report recognizing the addictive nature of tobacco and isolating nicotine as the primary psychoactive component in tobacco (USDHHS, 1988). As more and more findings arose revealing the addictive nature of nicotine and harmful effects of tobacco, researchers began to question reasons for smoking initiation, development and maintenance, as well as to generate devices and drugs to help smokers abandon the habit.

Psychosocial factors have been found to represent a substantial portion of the source of initiation; however, researchers are now beginning to unearth the underlying biological determinants that lead to the development and maintenance of a smoking habit. One possible variable is age-related differences in the enzymes responsible for nicotine metabolism, specifically CYP2A6 that may alter the bioavailability of nicotine, and in turn, smoking habits (see Figure 4).
**Short-Term Goals of Dissertation**

In order to fully understand the effects of age on nicotine consumption and metabolism, mouse models are employed to mimic nicotine’s biological, as well behavioral, effects (e.g., nicotine consumption) as isolated from the effects of psychological or social influences of human society. The present doctoral dissertation aims to understand one factor that may influence the consumption and metabolism of nicotine – age – in mice as a means of understanding the sustained smoking habits of adolescent humans. Specifically, the short-term objective is to examine the effects of age differences on nicotine consumption and metabolism following subcutaneous administration of methoxsalen on naïve adolescent and adult male C57BL/6J mice.

**Long-Term Goals of Dissertation**

Findings concerning biological determinants of the smoking habits of adolescents can help scientists develop tailored and more effective aids to stop smoking. Therefore, the overarching goals of this doctoral dissertation are to 1) provide preclinical information that will lead to tailored pharmacological smoking cessation techniques based on age, 2) utilize a combination of CYP2A6 inhibitors, such as methoxsalen, and NRTs to further tailor smoking cessation techniques derived from an individual’s genetic profile (e.g., CYP2A6 polymorphisms), and 3) develop prevention techniques that will prevent future adolescents from joining the millions of smokers around the world.
Figure 1. Numbered carbon nicotine molecule (Source: Arnaud et al., 2007)
Figure 2. Mechanism of nicotine metabolism (Source: Hukkanen, Jacob, & Benowitz, 2005)
Figure 3. Numbered carbon methoxsalen molecule (Source: Mays et al., 1986)
Figure 4. Theoretical model of age and nicotine metabolism on the onset of smoking
CHAPTER II

EXPERIMENT I
EXPERIMENT I

Overview

To date, none of the available smoking cessation aids account for differences in CYP2A6 activity between populations or genetic polymorphisms among separate individuals. However, pharmacogenetic research has begun investigating CYP2A6 inhibitors, such as methoxsalen, to understand how these enzymatic differences can alter an individual’s ability to quit smoking. Although there are three studies examining the effects of methoxsalen (8MOP) on cigarette consumption/nicotine pharmacokinetics in adult humans and rodents, there are no investigations assessing nicotine consumption in adult C57BL/6J mice, and more importantly, adolescent mice of any strain. Therefore, this experiment examined the dose-dependent effects of 8MOP on nicotine volume (ml), nicotine intake as a percent of total fluid intake (%), and nicotine dosage (mg/kg) as well as serum cotinine levels adjusted for liver weight (ng/ml/g) and hepatic CYP enzyme mRNA levels in C57BL/6J mice. Adolescent (PND 42) and adult (8 weeks) male mice received either no injections (control) or a single subcutaneous (s.c.) injection of physiological saline, vehicle, or 8MOP (5 and 10 mg/kg) followed by 12-hour access to a 3-bottle choice in their home cage: Tap water without nicotine (WTR), and two solutions of nicotine [50 (LOW NIC) and 200 (HIGH NIC) ug/ml dissolved in tap water]. The next day, animals were sacrificed via cervical dislocation, and trunk blood and liver were collected for assessment of cotinine and CYP enzymes, respectively. This study was approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University (#28691) and the safety and care of all laboratory animals was upheld throughout the study (National Research Council, 1985).
**Study Design**

Experiment I was a 2 (age; adolescent and adult) X 5 (treatment; control, saline, vehicle, 5, and 10 mg/kg 8MOP) between-subjects design with 3-bottle oral nicotine consumption as a within-subjects factor.

**Methods**

**Animals**

One hundred twenty (60 adolescents, 60 adults) male C57BL/6J mice (Jackson Labs, Bar Harbor, Maine) were used with 12 animals per group (see Table 1). Animals were housed in a climate controlled room with a 12-hour light/dark cycle (lights on at 0700 hours and off at 1900 hours), where the humidity was maintained at 60% and the temperature at 21 ± 2 °C. Each animal was individually housed under standard conditions in conventional cages without filter tops filled with ¼ of an inch laboratory animal bedding (Bed-o’Cobs, The Andersons Agriservices, Inc., Champaign, IL). Throughout the experiment, animals were provided with unrestricted access to food (LabDiet 5001 Rodent Diet; PMI Nutrition International, Brentwood, MO) and daily measurements of body weight, food consumption and fluid intake were taken.

**Drugs**

Freebase (-)-nicotine. Animals were provided access to 50 (LOW NIC) and 200 (HIGH NIC) ug/ml freebase (-)-nicotine (Sigma-Aldrich; St. Louis, MO) dissolved in tap water (see Appendix B for procedure). The oral nicotine consumption model and the two nicotine concentrations were selected based on previous findings that periadolescent and adult C57BL/6J mice will voluntarily consume nicotine in solution at these concentrations, and in doing so, produce reliable cotinine levels similar to those found in
human smokers (Klein, Stine, Vandenbergh, Whetzel, & Kamens, 2004; Klein, Kapelewski, Bennett, & Guaderrama, 2008). In addition, two nicotine concentrations were provided to allow for different concentration preferences.

**Methoxsalen.** Methoxsalen (5 and 10 mg/kg; Sigma-Aldrich, St. Louis, MO; see Appendix A for MSDS sheet) was dissolved in a solution of Emulphor (Rhodia, Inc.; Cranbury, NJ), 200 proof ethyl alcohol and single distilled water in a 1:1:18 ratio (see Appendix C for solution procedure). Because the stability of 8MOP exposed to light is unknown, these solutions were prepared on the day of injections, stored in amber bottles that were wrapped in aluminum foil and placed in a cardboard box. Control groups included mice receiving no injections, or s.c. injections of physiological saline (VWR; Bridgeport, NJ) or a drug-free solution of Emulphor, 200 proof ethyl alcohol and distilled water (vehicle). Three control groups were used to ensure that observed effects were due to methoxsalen exposure, and not the stress of an injection or the components of the vehicle solution. The methoxsalen solution was administered via s.c. injection at two dosages – 5 and 10 mg/kg – with the 10 mg/kg dosage being the same as previously reported in adult ICR male mice (Damaj, Siu, Sellers, Tyndale, & Martin, 2007). No adverse effects have been previously reported with the dosages of methoxsalen in adults (Damaj et al., 2007).

**Study Procedure**

**Acclimation (3 days) & baseline (2 days)**

Figure 5 presents a timeline of Experiment I. After arrival, animals (PND 37; 60 animals; 8 weeks, 60 mice) were allowed three days to acclimate to their new environment. Standard food and 3 bottles of tap water were available ad libitum, and
mice were left undisturbed in their home cages. Next, food consumption, water intake and body weight were measured shortly after the lights are turned on for 2 days (PND 40–42/8 weeks). These baseline measurements were used to assign mice to the control groups (no injections, saline or vehicle) or one of two treatment groups (5 or 10 mg/kg 8MOP) to ensure that the groups did not differ on these measures at the start of the study.

**Methoxsalen and nicotine treatment test (1 day)**

Body weight, food consumption and fluid intake were monitored and recorded when the lights were turned at 0700 hours on the day of injections. Just before the dark cycle (0600 hours), animals (PND 42/8 weeks) received either no injection (control) or one of four subcutaneous injections – physiological saline, vehicle (solution of Emulphor, 200 proof ethyl alcohol and single distilled water) or methoxsalen (5 or 10 mg/kg) – underneath the skin amid the scapulas. This specific time frame of injections was used because of the higher concentration of CYP2a5 in the dark (Lavery et al., 1999), and the fact that rodents drink the majority of their fluid during the dark cycle when they are the most active (Pietilä, Laakso, & Ahtee, 1995). Thirty minutes after the injection, all mice were provided 12-hour access to tap water, LOW NIC and HIGH NIC bottles.

**Blood collection and tissue harvest**

Animals (PND 43/9 weeks) were sacrificed the next morning via cervical dislocation. Trunk blood was removed via cardiac puncture. Blood was allowed to sit at room temperature for 15 minutes and centrifuged at 3000 x g for 15 minutes. Following centrifugation, serum was aliquoted and stored at -80°C for later assessment of cotinine, the primary active metabolite of nicotine. Livers were removed, weighed, dissected, submerged in RINAlater (a solution necessary to stabilize and preserve RNA; Applied
Biosystems, Carlsbad, CA), flash frozen in liquid nitrogen, and stored at -80°C (see Appendix D for procedural details).

**Analytical methods**

**Serum cotinine assessment.** Serum cotinine was analyzed using a commercially available enzyme immuno sorbant assay (EIA; Immunalysis, Los Angeles, CA; see Appendix E for assay procedure). The assay sensitivity, based on the minimum cotinine concentration required to produce a three standard deviation from assay A0, was 1 ng/ml. All samples were tested in duplicate in a single assay batch in our laboratory using a Synergy 2 96-well plate reader (Biotek Instruments, Winooski, VT). Duplicate test values that varied by more than 5% error were subject to repeat testing. Following the initial run, samples that exceeded the highest standard (>105.00 ng/ml) were diluted 1 in 10 fold [10 ul sample: 90 ul phosphate buffered solution (PBS)] prior to subsequent assay.

**Quantitative real-time PCR (qPCR).** Quantitative real-time PCR was used to examine the effect of age and methoxsalen exposure on hepatic CYP2a5 and CYP2e1 RNA levels (18 adolescents, 18 adults). Based on the results, 36 mice (N=6/group) were chosen at random from six out of the 10 groups (adolescent vehicle, adolescent 5 mg/kg 8MOP, adolescent 10 mg/kg 8MOP, adult vehicle, adult 5 mg/kg 8MOP, adult 10 mg/kg 8MOP) to run these analyses. These groups were chosen based on significant findings found only between the vehicle, 5 mg/kg 8MOP and 10 mg/kg 8MOP groups regarding serum cotinine levels adjusted for liver weight. Isolation of total RNA was completed using TRI-reagent (Sigma-Aldrich, Inc., Saint Louis, MO; see Appendix F for RNA extraction protocol). Following the assessment of the quantity and quality of RNA using
a ND-1000 spectrophotometer and Nanodrop 3.31 software (Thermo Fisher Scientific, Inc., Wilmington, DE; see Appendix G for quality control procedure), RNA was translated into cDNA using a high capacity cDNA reverse transcriptase kit (Applied Biosystems Inc., Foster City, CA, USA; see Appendix H for reverse transcription protocol). SYBR Green Master Mix (Applied Biosystems Inc., Foster City, CA) was used to detect the RNA amplification of the samples (see Appendix I for RT-PCR protocol). The housekeeping gene, 18 Svedberg Units (S) ribosomal RNA subunit (18S; Applied Biosystems Inc., Foster City, CA), was used as an internal control, specifically to correct for RNA quality. In contrast to other commonly used housekeeping genes (e.g., β-actin), 18S was used because of the consistent gene expression regardless of drug treatment (Selvey et al., 2001). The optimal primer concentration in the master mix is set at 300 nM for this gene. Primers for 18s were gifts from the laboratory of John Vanden Heuvel, Ph.D. at The Pennsylvania State University (University Park, PA; see Appendix J for primer sequence). CYP2a5 and CYP2e1 primers, listed in Appendix J, were chosen based on previously published literature (Muguruma et al., 2006; Kashida et al., 2006, respectively) and synthesized at the Genomics Core Facility at The Pennsylvania State University (University Park, PA). Samples were run in Dr. Vanden Heuvel’s laboratory using ABI Prism® 7000 Sequence Detection System with 7000 Sequence Detection System (SDS) Software (Version 1.2.3.; Applied Biosystems Inc., Foster City, CA).

**CYP2a5 and CYP2e1 gene expression calculations.** Quantity of each gene was determined relative to a standard curve [logarithm of known cDNA concentration versus unknown cycle at threshold (ct) value] (see Appendix K for absolute quantification protocol). Quantity of relative gene expression for CYP2a5 and CYP2e1 was then based
on the quantity of the housekeeping gene, 18S. For each gene of interest, the quantity of CYP2a5 gene expression was divided by the quantity of 18S, such that:

\[
\frac{\text{Quantity of CYP2a5 [or CYP2e1] gene expression}}{\text{Quantity of 18S gene expression}}
\]

yielded corrected value of gene expression for CYP2a5 and CYP2e1.

**Nicotine Calculations.** Raw values for nicotine consumption (ml) for LOW and HIGH NIC were used in the following calculations. Total nicotine consumption (ml) was calculated by adding the nicotine consumption (ml) values for LOW and HIGH NIC together.

Nicotine intake as a percent (%) of total fluid intake was calculated separately for the LOW and HIGH NIC bottles. Specifically, volume of nicotine consumption (ml) from each bottle was divided by the total fluid volume (water plus nicotine solutions) consumed (ml). This value was multiplied by 100 to achieve a percent, such that:

\[
\frac{\text{volume of nicotine consumed [ml] for each bottle [LOW or HIGH]}}{\text{total fluid [water plus nicotine] consumed [ml]}} \times 100
\]

yielded the total nicotine intake of the LOW and HIGH bottle as a percent of total fluid intake (%). The same equation was used to determine the total nicotine intake as a percent (%), except the sum of nicotine consumption (ml) values from LOW and HIGH NIC solutions was used instead. Specifically:

\[
\frac{\text{volume of nicotine consumed [ml] for LOW + HIGH bottle}}{\text{total fluid [water plus nicotine] consumed [ml]}} \times 100
\]
Nicotine dosage (mg/kg) was calculated separately for the LOW and HIGH NIC solutions. For LOW NIC, nicotine consumption (ml) was multiplied by 0.05 and then divided by the product of body weight of each animal multiplied by 0.001, such that:

\[
\frac{(\text{volume of nicotine consumed [ml]) \times (0.05)}}{(\text{body weight of each animal [g]) \times (0.001)}}
\]

yielded the total LOW NIC consumption adjusted for body weight (mg/kg). For HIGH NIC, nicotine consumption (ml) was multiplied by 0.2 and then divided by the product of body weight of each animal multiplied by 0.001. Specifically:

\[
\frac{(\text{volume of nicotine consumed [ml]) \times (0.2)}}{\text{(body weight of each animal [g]) \times (0.001)}}
\]

Total nicotine dosage (mg/kg) was calculated by adding the nicotine dosages for the LOW and HIGH NIC bottles together. These seven nicotine consumption values (total ml, % and mg/kg) along with the raw values of nicotine consumption (ml) for LOW and HIGH NIC were used in the following analyses.

**Treatment of Data.** Adolescent and adult mice differ significantly in liver weight and overall body weight (see Results section of dissertation); therefore, two variables were adjusted to account for these differences. Serum cotinine levels (ng/ml) were adjusted for liver weight (ng/ml/g) and nicotine consumption (mL) was adjusted for body weight to calculate nicotine dosage (mg/kg; mg nicotine/kg body weight). Due to abnormal distributions, natural logarithmic transformations were applied to serum cotinine levels adjusted for liver weight (ng/ml/g). Following confirmation that this transformation led to a normal distribution (e.g., assessment of skewness and kurtosis of the variable), analyses were then conducted on these adjusted values. Unless otherwise
noted, raw adjusted means (± standard error of the mean) were used in tables and graphs (Klein et al., 2004).

Because of a statistically significant positive correlation between liver weight (g) and body weight (g), a ratio of liver weight to body weight on sacrifice day was calculated and used as an indicator of the toxic effects of methoxsalen. Statistical analyses then were conducted on these ratios.

CYP2a5 and CYP2e1 gene expression levels were adjusted for the quantity of gene expression for 18S rRNA to control for RNA quality throughout the qPCR process. Natural logarithmic transformations were applied to normalize the distribution of relative gene expression of CYP2a5 and CYP2e1. Following confirmation that this transformation led to a normal distribution of the data (e.g., assessment of skewness and kurtosis of the variables), analyses then were conducted on these adjusted values. Adjusted means (± standard error of the mean) were used in graphs for clarity unless otherwise noted.

Statistical Analyses. Two-way ANOVAs were used to assess the effects of age and methoxsalen on 1) food intake, 2) body weight, 3) nicotine volume (ml), 4) nicotine intake as a percent (%) of total fluid intake, 5) nicotine dosage (mg/kg), and 6) serum cotinine levels adjusted for liver weight (ng/ml/g). Log-transformed serum cotinine levels adjusted for liver weight (Klein et al., 2004) were analyzed in a two-way ANOVA (see Figure 10). Separate one-way ANOVAs were used to test statistically significant interactions and Bonferroni post hoc tests were used where appropriate.

Bi-variate correlations also were conducted to assess the relationship between total nicotine dosage (mg/kg), serum cotinine levels (ng/ml/g) and CYP2a5 and CYP2e1
gene expression levels. PASW (Predictive Analytics SoftWare Version 18.0) (SPSS Inc., Chicago, IL) was used to complete these statistical analyses. All tests were two-tailed with statistical significance determined at $\alpha = 0.05$.

**Hypotheses**

**Hypothesis 1 Overview.** With regard to body weight (g), I hypothesized a main effect for methoxsalen and age. Because of the lack of published data, an age X methoxsalen interaction was not predicted. Specifically:

**Hypothesis 1A.** Mice treated with 8MOP would gain less weight (g) compared to control mice.

  **Rationale.** Wistar rats that ingested a powdered diet infused with methoxsalen (250, 1250 and 2500ppm) had a reduced weight gain compared to animals that received a diet infused with bergapten and control mice (Diawara et al., 1997).

**Hypothesis 1B.** Adults would have a higher body weight (g) compared to adolescents.

  **Rationale.** Adult C57BL/6 mice (Meliska, Bartke, McGlacken, & Jensen, 1995) have a higher baseline body weight compared to adolescent C57BL/6J mice (Klein et al., 2004).

**Hypothesis 2 Overview.** With regard to nicotine volume (ml), I hypothesized a main effect for methoxsalen and age, and a two way interaction for age X methoxsalen.

Specifically:

**Hypothesis 2A.** Control mice would consume more nicotine (ml) compared to mice treated with 8MOP.

  **Rationale.** Sellers and colleagues (2000) reported that the combination of methoxsalen and nicotine increases plasma nicotine levels, and subsequently, increasing
bioavailability of nicotine. These increases significantly reduced cigarette consumption and carbon monoxide concentrations (24% and 47%, respectively) in adult smokers (Sellers et al., 2000).

In addition, data on CYP2A6 polymorphisms also indicate that individuals with at least one or two variant alleles have altered responses to nicotine and later, risk of dependence compared to the wild type genotype (CYP2A6*1). For example, CYP2A6*1/*12 and CYP2A6*1/*9 genotypes lead to partial reductions (approximately 20%) in enzymatic activity (e.g., increase in total and non-renal clearance of nicotine), increases in the half lives of nicotine and cotinine, and low 3HC/COT ratios (Benowitz et al., 2006). A 40 to 50% reduction in nicotine clearance, considerable increases in nicotine and cotinine half-lives, and very low 3HC/COT ratios are observed in individuals with other variant allelic combinations; for example, CYP2A6*1/*2, CYP2A6*1/*4, CYP2A6*9/*12, CYP2A6*9/*4, and CYP2A6*9/*9 (Benowitz et al., 2006). Furthermore, two clinical studies determined that the presence of CYP2A6 genetic variants that reduce the rate of nicotine metabolism along with recent cigarette consumption puts middle- and high-school aged adolescents at an increased risk of becoming dependent on tobacco and maintaining their dependence compared to those without these polymorphisms (Karp et al., 2006; O’Loughlin et al., 2004).

**Hypothesis 2B.** Adolescent mice would consume more nicotine (ml) than would adult mice, regardless of sex or methoxsalen treatment.

**Rationale.** An unpublished study conducted by Klein, Stine and Vandenbergh in 2003 determined that adolescent C57BL/6J mice consumed more nicotine (ml) compared to adults.
**Hypothesis 2C.** Age would interact with methoxsalen exposure such that, among adolescents, 8MOP-exposed mice would consume more nicotine (ml) compared to vehicle-exposed mice. In contrast, adult mice treated with 8MOP would consume less nicotine (ml) compared to vehicle-treated adult mice.

**Rationale.** Two clinical studies determined that the presence of CYP2A6 genetic variants that reduce the rate of nicotine metabolism and recent cigarette consumption puts middle- and high-school aged adolescents at an increased risk of becoming dependent on tobacco and maintaining their dependence compared to those without these polymorphisms (Karp et al., 2006; O’Loughlin et al., 2004). However, regarding adults, recent evidence suggests that CYP2A6 genetic variants may be protective against tobacco dependence for adults (Pianezza et al., 1998; Rao et al., 2000; Sellers et al., 2003b; Tyndale et al., 1999; Tyndale & Sellers, 2001; 2002). These findings indicate that adolescent mice treated with a CYP2A6 inhibitor that is intended to mimic polymorphisms encoding for a null CYP2A6 enzyme (methoxsalen) would consume more nicotine (ml) than their control counterparts, whereas adult mice treated with the same CYP2A6 inhibitor would consume less nicotine (ml) than control adult mice.

**Hypothesis 3 Overview.** With regard to nicotine dosage [nicotine volume adjusted for body weight (mg/kg)], I hypothesized main effects for methoxsalen and age, and a two-way age X methoxsalen interaction. Specifically:

**Hypothesis 3A.** Mice treated with 8MOP would consume more nicotine (mg/kg) compared to control mice.

**Rationale.** Sellers and colleagues (2000) discovered that the combination of methoxsalen and nicotine increases plasma nicotine levels, and subsequently, increasing
bioavailability of nicotine in vitro. These increases significantly reduced cigarette consumption and carbon monoxide concentrations (24% and 47%, respectively) in adult smokers (Sellers et al., 2000).

Because methoxsalen mimics CYP2A6 polymorphisms, clinical data also indicate that individuals with at least one or two variant alleles have altered responses to nicotine and later, risk of dependence compared to the wild type genotype (CYP2A6*1). For example, CYP2A6*1/*12 and CYP2A6*1/*9 genotypes lead to partial reductions (approximately 20%) in enzymatic activity (e.g., increase in total and non-renal clearance of nicotine), increases in the half lives of nicotine and cotinine, and low 3HC/COT ratios (Benowitz et al., 2006). A 40 to 50% reduction in nicotine clearance, considerable increases in nicotine and cotinine half lives, and very low 3HC/COT ratios are observed in individuals with other variant allelic combinations; for example, CYP2A6*1/*2, CYP2A6*1/*4, CYP2A6*9/*12, CYP2A6*9/*4, and CYP2A6*9/*9 (Benowitz et al., 2006). Furthermore, two clinical studies determined that the presence of CYP2A6 genetic variants that reduce the rate of nicotine metabolism along with recent cigarette consumption puts middle- and high-school aged adolescents at an increased risk of becoming dependent on tobacco and maintaining their dependence compared to those without these polymorphisms (Karp et al., 2006; O’Loughlin et al., 2004).

**Hypothesis 3B.** Adolescent mice would consume more nicotine (mg) per kg of body weight, regardless of sex or methoxsalen treatment.

**Rationale.** An unpublished study done by Klein, Stine and Vandenbergh in 2003 determined that adolescent C57BL/6J mice consumed more nicotine (mg) per kg of body weight (mg/kg) compared to adults.
Hypothesis 3C. Age would interact with methoxsalen exposure such that, among adolescent mice, 8MOP exposure would result in greater consumption of nicotine (mg) per kg of body weight compared to vehicle. In contrast, 8MOP exposure would result in less consumption of nicotine (mg) per kg of body weight compared to vehicle in adult mice.

Rationale. Two clinical studies determined that the presence of CYP2A6 genetic variants that reduce the rate of nicotine metabolism and recent cigarette consumption puts middle- and high-school aged adolescents at an increased risk of becoming dependent on tobacco and maintaining their dependence compared to those without these polymorphisms (Karp et al., 2006; O’Loughlin et al., 2004). However, among adults, recent evidence suggests that CYP2A6 genetic variants may be protective against tobacco dependence in humans (Pianezza et al., 1998; Rao et al., 2000; Sellers et al., 2003b; Tyndale et al., 1999; Tyndale & Sellers, 2001; 2002). These findings indicate that adolescent mice treated with a CYP2A6 inhibitor that is intended to mimic polymorphisms encoding for a null CYP2A6 enzyme (methoxsalen-treated) would consume more nicotine (mg) per kg of body weight than their control counterparts, whereas adult mice treated with the same CYP2A6 inhibitor would consume less nicotine (mg) per kg of body weight than control adult mice.

Hypothesis 4 Overview. With regard to levels of serum cotinine adjusted for liver weight (ng/ml/g), I hypothesized a main effect for methoxsalen treatment. Because of the lack of published data, a main effect of age, and an age X methoxsalen interaction were not predicted. Specifically:
Hypothesis 4A. 8MOP exposure would reduce serum cotinine levels (ng/ml/g) compared to the control.

Rationale. Several studies have determined that methoxsalen increases nicotine half-life and decrease cotinine levels *in vivo* in mice (Damaj et al., 2007; Raunio et al., 2008).

Hypothesis 5 Overview. With regard to levels of CYP RNA levels, I hypothesized main effects for methoxsalen treatment. Because of the lack of published data, a main effect for age, and an age X methoxsalen interaction were not predicted. Specifically:

Hypothesis 5A. 8MOP exposure would increase CYP RNA levels compared to vehicle.

Rationale. Bickers and Pathak (1984) determined that the oral administration of methoxsalen over 6 days to adult male CD-1 mice increased hepatic protein levels, enzyme activity [e.g., aryl-hydrocarbon hydroxylase (AHH), ethylmorphine N-deethylase], and cytochrome p450 levels. Therefore, a larger quantity of CYP2a5 protein would suggest a higher level of CYP2a5 RNA in females as opposed to males.

Results

Baseline (2 days)

Body weight (g), food intake (g) and water consumption (mL). Baseline body weight (g), food consumption (g), and water intake (mL) were averaged across the 3-day baseline period. Overall, adult mice weighed more, consumed more food and drank more water than did adolescent mice \[F(1,110)=294.41, \ p<0.05,\ F(1,106)=434.64, \ p<0.05,\] and \[F(1,92)=59.65, \ p<0.05,\] respectively. As expected, there was no Treatment group or Age X Treatment group differences in these baseline measures.
**Twelve Hours Post-Methoxsalen Injection**

**Body weight and food intake (g).** Tables 2 and 3 presents mean body weight (g) and food consumption (g), respectively. Twelve hours following the injection/no injection procedure, adult mice continued to weigh more compared to adolescent mice \( F(1,110)=211.93, p<0.05 \). However, adult and adolescent mice consumed similar amounts of food. There were no main effects for Treatment group and no Age X Treatment group interactions with respect to body weight and food consumption.

**Total fluid intake (mL).** Table 4 presents mean total fluid intake (mL). Adult mice consumed more fluid (i.e., nicotine and water intake combined) than did adolescent mice \( F(1,105)=31.97, p<0.05 \). There were no main effects for Treatment group and no Age X Treatment group interactions with respect to total fluid intake (mL).

**Total nicotine consumption (mL).** Table 5 and Figure 6 presents mean total nicotine consumption (mL). Adult mice drank more LOW NIC, HIGH NIC and total nicotine (mL) (i.e., LOW NIC and HIGH NIC combined) than did adolescent mice \( F(1,107)=31.14, p<0.05 \), \( F(1,108)=84.98, p<0.05 \), and \( F(1,105)=62.97, p<0.05 \), respectively. Because adults consumed more LOW NIC, HIGH NIC and total nicotine, only total nicotine (mL) was used in subsequent analyses. There were no main effects for Treatment group and no Age X Treatment group interactions with respect to nicotine consumption (mL).

**Total nicotine intake as a percent of total fluid intake (%).** Table 6 and Figure 7 presents mean total nicotine intake as a percent of total fluid intake (%). Similar to that observed with nicotine consumption (mL) and dosage (mg/kg), adults drank significantly more nicotine as a percent of total fluid intake (%) compared to adolescents.
[F(1,105)=51.95, p<0.05]. There were no main effects for Treatment group and no Age X Treatment group interactions with respect to nicotine intake as a percent of total fluid intake (%).

**Total nicotine dosage (mg/kg).** Table 7 and Figure 8 present mean total nicotine dosage (mg/kg) in adolescent and adult mice. Compared to adolescents, adult mice consumed more LOW NIC, HIGH NIC, and total nicotine per unit body weight (mg/kg) [F(1,107)=20.52, p<0.05, F(1,108)=58.11, p<0.05, and F(1,105)=61.81, p<0.05, respectively]. Because adults consumed more LOW NIC, HIGH NIC and total nicotine (mg/kg), only total nicotine (mg/kg) was used in subsequent analyses. There were no main effects for Treatment group and no Age X Treatment group interactions with respect to total nicotine dosage (mg/kg).

**Liver weight (g) and ratio of liver weight to body weight.** Figure 9 presents mean liver weights (g) for adolescent and adult mice exposed to nicotine and methoxsalen. As expected, adults displayed significantly larger livers compared to adolescents [F(1,110)=12.813, p<0.05]. Adolescents displayed a higher liver to body weight ratios compared to adults [F(1,66)=45.64, p<0.05]. There was no significant main effect for Drug and no Age X Drug interactions for liver weight or the liver to body weight ratio.

**Serum cotinine (ng/ml/g).** Figure 10 presents mean serum cotinine levels adjusted for liver weight (ng/ml/g). Consistent with greater nicotine intake, adults displayed significantly higher cotinine levels (ng/ml/g) than did adolescents [F(1,110)=24.52, p<0.05]. There was no main effect for Drug and no Age X Drug group interaction on serum cotinine levels (ng/ml/g).
Figure 11 presents the correlation total nicotine dosage (mg/kg) and mean serum cotinine levels adjusted for liver weight (ng/ml/g). Serum cotinine levels (ng/ml/g) were positively correlated with total nicotine dosage (mg/kg) \([r(113)=0.46, \ p<0.05]\). These positive correlations were observed in adolescents \([r(55)=0.49, \ p<0.05]\), but not in adults.

A significant reduction in serum cotinine values (ng/ml/g) between animals receiving vehicle versus those receiving 5 and 10 mg/kg 8MOP was observed (see Figure 10). Further, prior reports by Damaj and colleagues (2007) suggest that the vehicle condition may be a more appropriate control as opposed to saline and no injection control groups. Therefore, post hoc analyses were conducted with age and 3 levels of drug treatment group (vehicle, 5 mg/kg 8MOP and 10 mg/kg 8MOP) as independent variables.

**Post-Hoc Analyses**

**Body weight and food intake (g).** Adult mice weighed more than did adolescent mice \([F(1,66)=134.44, \ p<0.05]\); however, there was no Age difference in food consumption. Similarly, there were no main effects for Treatment group and no Age X Treatment group interactions with respect to body weight and food consumption.

**Total fluid intake (mL).** Adult mice consumed more fluid (i.e., nicotine and water intake combined) than did adolescent mice \([F(1,64)=21.06, \ p<0.05]\). There were no main effects for Treatment group and no Age X Treatment group interactions with respect to total fluid intake (mL).

**Total nicotine consumption (ml).** Adult mice consumed more LOW NIC, HIGH NIC and total nicotine (i.e., LOW NIC and HIGH NIC combined) than did adolescent mice \([F(1,66)=25.35, \ p<0.05, F(1,64)=34.005, \ p<0.05, \text{and } F(1,64)=41.26, \ p<0.05, \text{respectively}](see Figure 6). Because adults consumed more LOW NIC, HIGH NIC and
total nicotine, only total nicotine (mL) was used in subsequent analyses. There were no
main effects for Treatment group and no Age X Treatment group interactions with
respect to nicotine consumption (mL).

**Total nicotine intake as a percent of total fluid intake (%).** Adults drank
significantly more nicotine as a percent of total fluid intake (%) compared to adolescents
\[F(1,64)=36.97, p<0.05\] (see Figure 7). There were no main effects for Treatment group
and no Age X Treatment group interactions with respect to nicotine intake (%).

**Total nicotine dosage (mg/kg).** Compared to adolescents, adult mice consumed
more nicotine per unit body weight (mg/kg) \[F(1,64)=32.63, p<0.05\] (see Figure 8).
There were no main effects for Treatment group and no Age X Treatment group
interactions with respect to total nicotine dosage (mg/kg).

**Liver weight (g) and ratio of liver weight to body weight.** As expected, adults
displayed significantly larger livers compared to adolescents \[F(1,66)=8.67, p<0.05\] (see
Figure 9). Adolescents displayed higher liver to body weight ratios \[F(1,66)=45.64,
p<0.05\] compared to adults. There was no significant main effect for Drug and no Age X
Drug interactions for liver weight or the liver to body weight ratio.

**Serum cotinine (ng/ml/g).** Adults displayed significantly higher cotinine levels
(ng/ml/g) than did adolescents \[F(1,66)=20.67, p<0.05\] (see Figure 10). In addition,
there was a significant Age X Treatment group interaction \[F(2,66)=3.345, p<0.05\].
Among adolescents, there was no main effect for Treatment group. In contrast, adults in
the vehicle group had the highest cotinine levels (ng/ml/g), followed by the 5 mg/kg
8MOP group, and then 10 mg/kg 8MOP group \[F(2,33)=3.21, p=0.05\]. Adults in the
vehicle and 5 mg/kg groups had higher cotinine levels (ng/ml/g) when compared to
adolescents \[F(1,70)=21.77, p<0.05 \text{ and } F(1,22)=11.82, p<0.05, \text{ respectively}\]; however, there was no main effect for Age within the 10 mg/kg 8MOP group. There was no significant main effect for Drug with respect to serum cotinine levels (ng/ml/g).

Serum cotinine levels (ng/ml/g) also were positively correlated with total nicotine dosage (mg/kg) \[r(68)=0.40, p<0.05\] (see Figure 11).

**Hepatic CYP gene expression.** Figures 12 and 13 presents gene expression levels for CYP2a5 and CYP2e1 adjusted for 18S gene expression levels, respectively. After adjusting for the RNA quantity of 18S, there were no significant main effects for Age or Drug and no Age X Drug interaction on the relative gene expression of CYP2a5 or CYP2e1.

**Confirmation of Hypotheses**

**Hypothesis 1: Age, Methoxsalen Exposure and Body Weight (g)**

The first hypothesis stating that mice receiving methoxsalen would not gain as much weight (g) as control mice was **not supported**. There was no difference in weight gain between drug treatments.

An additional hypothesis stating that adults would weigh more (g) than adolescents was **supported**.

**Hypothesis 2: Age, Methoxsalen Exposure and Nicotine Consumption (mL)**

It was hypothesized that mice receiving methoxsalen would consume more nicotine compared to control mice; however, this hypothesis was **not supported**, as there were no differences in nicotine consumption between drug treatment groups.
Similarly, the hypothesis that adolescents would consume more nicotine than adults (mL) was also not supported. In fact, adults consumed significantly more nicotine (mL) than did adolescents.

The third hypothesis involved an Age X Drug interaction, such that adolescents exposed to methoxsalen would consume more nicotine (mL) compared to control mice, whereas adult mice that receive methoxsalen will consume less nicotine (mL). Both of these hypotheses were not supported. There were no differences between drug treatment groups among either age group.

Hypothesis 3: Age, Methoxsalen Exposure and Nicotine Dosage (mg/kg)

The hypothesis that mice treated with methoxsalen will consume more nicotine per unit body weight when compared to control mice was not supported. There was no difference in nicotine consumption among drug treatment groups.

Similarly, the second hypothesis stating that adolescents will consume more nicotine than adults, even after adjusting for body weight (mg/kg) was also not supported. In fact, adults consumed significantly more nicotine (mg/kg) than did adolescents.

The hypothesis that adolescents exposed to methoxsalen would consume more nicotine (mg/kg) compared to control mice, whereas adult mice that receive methoxsalen will consume less nicotine (mg/kg) was also not supported. There were no differences between drug treatment groups among either age group.

Hypothesis 4: Methoxsalen Exposure and Serum Cotinine Levels (ng/ml/g)

It was hypothesized that methoxsalen would reduce serum cotinine levels compared to the control; this hypothesis was partially supported. Among adults, mice
receiving vehicle had the highest serum cotinine levels (ng/ml/g), followed by mice in the
5 mg/kg 8MOP group and then those receiving the 10 mg/kg 8MOP. This effect was not
observed in adolescents. There was no significant drug treatment effect when all five
groups were examined in either age group.

Hypothesis 5: Age, Methoxsalen Exposure and RNA Levels of Hepatic CYP enzymes

The hypothesis that methoxsalen would increase CYP RNA levels was not
supported. There was no effect of age or drug treatment on relative gene expression
levels of CYP2a5 or CYP2e1.

Conclusions

The purpose of Experiment I was to examine nicotine consumption following
acute administration of 8MOP in adolescent and adult male C57BL/6J mice. To our
knowledge, this is the first study to examine the effects of methoxsalen on nicotine
consumption and metabolism in C57BL/6J mice, and in both adult and adolescent mice,
regardless of strain.

As expected, adults weighed more and had larger livers than did adolescents. An
age comparison of liver to body weight ratios revealed that adolescents had a higher ratio
compared to adults, a finding consistent with human data (Urata et al., 1995). Because
there was no effect of methoxsalen on the liver to body weight ratio, this finding indicates
that methoxsalen does not significantly increase liver weight in relation to overall body
weight.

Because no murine studies have examined the effects of methoxsalen on nicotine
consumption and metabolism in adolescents alone or in comparison to adults, this is the
first study to demonstrate an age difference in nicotine consumption and cotinine levels
following administration of this CYP2a5 inhibitor. As expected, adults had larger livers and weighed more overall during baseline and after treatment compared to adolescents. Additionally, adults drank more water during baseline and total fluid (nicotine and water) following drug treatment than did adolescents. Interestingly, the age difference in food consumption during baseline disappeared following treatment. More specifically, adolescents significantly increased their food consumption from baseline to treatment, whereas only a slight increase in the consumption of food was observed in adults. This food consumption increase among adolescent mice is consistent with published reports from our lab (Klein et al., 2003; Klein et al., 2004).

Among those animals that did not receive any drug (control, saline, vehicle), adults consumed significantly more total nicotine (mL) than did adolescents. A similar age difference also was found when nicotine consumption was adjusted for body weight (mg/kg) and calculated as a percent of total fluid intake (%). This finding suggests that, regardless of body weight, naïve adult male mice consume more nicotine in 12-hours compared to adolescents, which is a new contribution to the nicotine addiction literature.

When comparing all five drug treatment groups, adults continued to consume more nicotine, both in volume (mL) and dosage (mg/kg) than did adolescents, regardless of methoxsalen administration. The lack of a main effect for drug treatment was not expected. It is possible that the length of time for nicotine access after the injection was not long enough to observe an effect of methoxsalen or that acute methoxsalen exposure is too short to influence acute nicotine intake. The next step would be to extend methoxsalen injections and nicotine access to examine the effects of age on nicotine consumption and metabolism following chronic methoxsalen administration.
Interestingly, adults had higher cotinine levels per unit liver weight (ng/ml/g) compared to adolescents. Because adults consumed more nicotine and had higher cotinine levels compared to adolescents, this finding suggests that adults either have a faster nicotine metabolism or slower nicotine and nicotine metabolite (e.g., cotinine) elimination rates. A valuable next step would be to examine the primary metabolite of cotinine, 3-hydroxycotinine via gas chromatography coupled with mass spectrosopy. This assay method provides concentrations of nicotine, cotinine and 3-hydroxycotinine. Concentration ratios of nicotine to cotinine (NIC/COT) or 3-hydroxycotinine to cotinine (3HT/COT) are useful to examine activity levels of human CYP2A6 (Dempsey et al., 2004; for review, see Benowitz et al., 2009) or mouse CYP2a5. A low NIC/COT ratio indicates a fast rate of nicotine metabolism whereas a high 3HT/COT signifies a fast rate of nicotine clearance. Determining these ratios is another step towards our understanding of how age alters nicotine pharmacokinetics. Additionally, collection of urine using metabolic cages would also be an important future study to assess nicotine elimination (Raunio et al., 2008). Although these analyses were not completed in this experiment, subsequent studies should utilize these methods to fully understand the rate of nicotine metabolism elimination in adolescents and adult mice.

Further investigation of serum cotinine data (ng/ml/g) indicated a significant reduction in the cotinine levels among animals exposed to 5 and 10 mg/kg 8MOP compared to vehicle. When comparing these three groups, adults weighed, ate, and drank more water and nicotine than did adolescents. Adults also had higher serum cotinine levels adjusted for liver weight (ng/ml/g). Among mice in both the vehicle and 5 mg/kg 8MOP groups, adults had higher cotinine levels (ng/ml/g); however, no age difference
was found among mice in the 10 mg/kg 8MOP group. Among adults, there was a significant difference in serum cotinine levels (ng/ml/g), with animals in the vehicle group having the highest level, followed by those in the 5 mg/kg 8MOP, and then 10 mg/kg 8MOP. These findings indicate that methoxsalen can successfully inhibit the metabolic activity of CYP2a5 in adults in 12 hours, at least among male C57BL/6J mice. Interestingly, this methoxsalen inhibition was not observed in adolescents, thereby suggesting that methoxsalen may not be effective during adolescence. It is possible that additional exposure time to nicotine and methoxsalen is needed to see an effect of methoxsalen on serum cotinine levels (ng/ml/g) in adolescents or a greater effect of methoxsalen on serum cotinine levels (ng/ml/g) in adults. Future studies should therefore increase the time for nicotine access as well as methoxsalen exposure to assess this variable.

In addition, investigations into whether the effects of methoxsalen on nicotine consumption (mL, % total fluid, mg/kg) and serum cotinine levels observed in this experiment are specific to methoxsalen or are generalized to all CYP2A6 inhibitors would be advantageous, as selectivity and specificity of other CYP2A6 inhibitors, such as tranylcypromine and tryptamine, vary significantly. For example, in addition to inhibiting CYP2A6, tranylcypromine is an irreversible inhibitor of monoamine oxidase (MAO)-A and –B, the enzymes responsible for the metabolism of dopamine, serotonin and norepinephrine (Herraiz & Chaparro, 2005; Fowler, Logan, Wang, & Volkow, 2003; Lewis, Miller, & Lea, 2007). Tryptamine, on the other hand, inhibits the amino acid transporter, PAT1 (Metzner, Kottra, Neubert, Daniel, & Brandsch, 2005) and induces phosphoinositide hydrolysis (Ishitani, Kimura, Takeichi, & Chuang, 1994), a process that
produces second messengers that control calcium release, and cell growth and fertilization (Berridge, 1993). Separate future studies should examine nicotine consumption following methoxsalen, tranylcypromine and tryptamine to compare effects and determine which would be a better smoking cessation aid. In addition, subsequent studies should investigate and compare derivatives of methoxsalen (e.g., 5-methoxypsoralen) that are thought to be more effective without the toxic effects associated with methoxsalen, at least in terms of dermatological improvement (Willis & Mentor, 1984).

Taken together, the results of Experiment I provide evidence that age significantly alters the effects of methoxsalen on nicotine self-administration (mL, % total fluid intake, mg/kg) and serum cotinine levels (ng/ml/g). It is important to keep in mind that this experiment only was conducted in a single strain of mice: C57BL/6J. Because different mouse strains vary significantly in responses to nicotine (e.g., Collins, Miner, & Marks, 1988), behaviors related to nicotine consumption, tolerance (e.g., Collins, Miner, & Marks, 1988), genetic differences (e.g., Collins, Marks, & Pauly, 1989) and nicotine pharmacokinetics (e.g., Siu & Tyndale, 2007), future studies should examine other strains of mice to document any differences, and parallel individual differences found in human populations. On the same note, this study was only conducted with male mice. It has been well documented that adolescent and adult female rodents and humans respond to nicotine in considerably different ways, spanning from the molecular level (e.g., hormone and neurotransmitter levels) to behavior (e.g., smoking habits/nicotine consumption), from their male counterparts. Therefore, additional studies are needed to examine age and sex differences in nicotine consumption and metabolism following methoxsalen.
administration, as it is currently unknown how females respond to nicotine subsequent to exposure to methoxsalen.

Among male C57BL/6J mice, methoxsalen appears to be more effective at reducing nicotine metabolism in adults compared to adolescents. The interaction between age and drug treatment, specifically between the vehicle, 5 mg/kg and 10 mg/kg 8MOP groups, was an exciting finding, indicating that effects on metabolic CYP enzymes could be observed after just 12 hours of access to nicotine following a single dose of methoxsalen. It currently is unknown whether this effect can be replicated after chronic methoxsalen administration, given that the two published studies in mouse models only lasted one day (Damaj et al., 2007; Raunio et al., 2008). Interestingly, these drug treatment effects on metabolism did not affect nicotine consumption for either age group, an unexpected result that demands further long-term investigation to see if these effects are delayed.

The collective interpretations of these results led to Experiment II, which was designed to expand these results by 1) pretreating male mice with nicotine prior to methoxsalen exposure, and 2) extending methoxsalen exposure from 12 hours to 4 days.
<table>
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3 Bottle Choice
(tap water, 50 and 200 ug/ml NIC solutions)

Total N=120

Table 1. Experiment I Design
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Table 2. Mean body weight (g) for all animals prior to (PRE) and after (POST) drug treatment among adolescent (n=60) and adult (n=60) male C57BL/6J mice (means ± standard error of the mean).

*Adult mice > Adolescent mice, p<0.05
### Table 3

Mean food consumption (g) for all animals prior to (PRE) and after (POST) drug treatment among adolescent (n=60) and adult (n=60) male C57BL/6J mice (means ± standard error of the mean).

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Control (n=12)</th>
<th>Saline (n=12)</th>
<th>Vehicle (n=12)</th>
<th>5 mg/kg 8MOP (n=12)</th>
<th>10 mg/kg 8MOP (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRE</td>
<td>2.92 ± 0.40</td>
<td>3.44 ± 0.45</td>
<td>6.38 ± 0.37</td>
<td>2.62 ± 0.34</td>
<td>3.16 ± 0.30</td>
</tr>
<tr>
<td>POST</td>
<td>7.57 ± 0.20</td>
<td>6.78 ± 0.36</td>
<td>7.30 ± 0.23</td>
<td>7.85 ± 0.24</td>
<td>7.57 ± 0.45</td>
</tr>
</tbody>
</table>
### Drug Treatment

<table>
<thead>
<tr>
<th>Total fluid intake (mL)</th>
<th>Control</th>
<th>Saline</th>
<th>Vehicle</th>
<th>5 mg/kg 8MOP</th>
<th>10 mg/kg 8MOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adolescent (n=12)</td>
<td>5.95 ± 0.24</td>
<td>7.18 ± 0.22*</td>
<td>6.12 ± 0.50</td>
<td>6.96 ± 0.19*</td>
<td>6.01 ± 0.19</td>
</tr>
<tr>
<td>Adult (n=12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Mean total fluid intake (mL) for all animals prior to (PRE) and after (POST) drug treatment among adolescent (n=60) and adult (n=60) male C57BL/6J mice (means ± standard error of the mean).

1 Adult mice > Adolescent mice, p<0.05
### Drug Treatment

<table>
<thead>
<tr>
<th></th>
<th>Control (n=12)</th>
<th>Saline (n=12)</th>
<th>Vehicle (n=12)</th>
<th>5 mg/kg 8MOP (n=12)</th>
<th>10 mg/kg 8MOP (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adolescent</strong></td>
<td>2.09 ± 0.53</td>
<td>1.94 ± 0.45</td>
<td>1.65 ± 0.19</td>
<td>1.49 ± 0.21</td>
<td>1.89 ± 0.48</td>
</tr>
<tr>
<td><strong>Adult</strong></td>
<td>4.05 ± 0.33*</td>
<td>4.13 ± 0.43*</td>
<td>4.25 ± 0.55*</td>
<td>4.04 ± 0.62*</td>
<td>3.68 ± 0.37*</td>
</tr>
</tbody>
</table>

Table 5. Mean total nicotine consumption (mL) 12 hours after drug treatment among adolescent (n=60) and adult (n=60) male C57BL/6J mice (means ± standard error of the mean).

*Adult mice > Adolescent mice, p<0.05
<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Control</th>
<th>Saline</th>
<th>Vehicle</th>
<th>5 mg/kg 8MOP</th>
<th>10 mg/kg 8MOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adolescent (n=12)</td>
<td>Adult (n=12)</td>
<td>Adolescent (n=12)</td>
<td>Adult (n=12)</td>
<td>Adolescent (n=12)</td>
<td>Adult (n=12)</td>
</tr>
<tr>
<td>33.77 ± 6.54</td>
<td>56.65 ± 5.00*</td>
<td>31.33 ± 6.83</td>
<td>59.76 ± 6.62*</td>
<td>27.00 ± 2.64</td>
<td>57.67 ± 5.91*</td>
</tr>
</tbody>
</table>

Table 6. Mean total nicotine intake as a percent of total fluid intake (%) 12 hours after drug treatment among adolescent (n=60) and adult (n=60) male C57BL/6J mice (means ± standard error of the mean).

*Adult mice > Adolescent mice, p<0.05
<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Control (n=12)</th>
<th>Adult (n=12)</th>
<th>Saline (n=12)</th>
<th>Adult (n=12)</th>
<th>Vehicle (n=12)</th>
<th>Adult (n=12)</th>
<th>5 mg/kg 8MOP (n=12)</th>
<th>Adult (n=12)</th>
<th>10 mg/kg 8MOP (n=12)</th>
<th>Adult (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adolescent</td>
<td>10.48 ± 2.26</td>
<td>9.98 ± 1.45</td>
<td>20.55 ± 1.65*</td>
<td>10.75 ± 1.68</td>
<td>20.14 ± 2.26*</td>
<td>9.45 ± 2.18</td>
<td>19.00 ± 2.10*</td>
<td>9.28 ± 1.65</td>
<td>17.54 ± 1.54*</td>
<td></td>
</tr>
</tbody>
</table>

Table 7. Mean total nicotine dosage (mg/kg) 12 hours after drug treatment among adolescent (n=60) and adult (n=60) male C57BL/6J mice (means ± standard error of the mean).

*Adult mice > Adolescent mice, p<0.05
<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Vehicle</th>
<th>5 mg/kg 8MOP</th>
<th>10 mg/kg 8MOP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adolescent (n=6)</td>
<td>Adult (n=6)</td>
<td>Adolescent (n=6)</td>
</tr>
<tr>
<td>CYP2a5</td>
<td>1.12 ± 0.11</td>
<td>1.92 ± 0.37</td>
<td>1.44 ± 0.19</td>
</tr>
<tr>
<td>CYP2e1</td>
<td>1.15 ± 0.28</td>
<td>1.17 ± 0.24</td>
<td>1.28 ± 0.23</td>
</tr>
</tbody>
</table>

Table 8. Relative CYP2a5 and CYP2e1 gene expression among adolescent (n=18) and adult (n=18) male C57BL/6J mice (means ± standard error of the mean).
Figure 5. Experiment I timeline.
Figure 6. Total nicotine consumption (mL) among adolescent (n=60) and adult (n=60) male C57BL/6J mice exposed to control (n=24), saline (n=24), vehicle (n=24), 5 mg/kg 8MOP (n=24) and 10 mg/kg 8MOP (n=24) treatment conditions (means ± standard error of the mean).
Figure 7. Total nicotine intake as a percent of total fluid intake (%) among adolescent (n=60) and adult (n=60) male C57BL/6J mice exposed to control (n=24), saline (n=24), vehicle (n=24), 5 mg/kg 8MOP (n=24) and 10 mg/kg 8MOP (n=24) treatment conditions (means ± standard error of the mean).
Figure 8. Total nicotine dosage (mg/kg) among adolescent (n=60) and adult (n=60) male C57BL/6J mice exposed to control (n=24), saline (n=24), vehicle (n=24), 5 mg/kg 8MOP (n=24) and 10 mg/kg 8MOP (n=24) treatment conditions (means ± standard error of the mean).
Figure 9. Liver weight (g) among adolescent (n=60) and adult (n=60) male C57BL/6J mice exposed to control (n=24), saline (n=24), vehicle (n=24), 5 mg/kg 8MOP (n=24) and 10 mg/kg 8MOP (n=24) treatment conditions (means ± standard error of the mean).
Figure 10. Serum cotinine levels adjusted for liver weight (ng/ml/g) among adolescent (n=60) and adult (n=60) male C57BL/6J mice exposed to control (n=24), saline (n=24), vehicle (n=24), 5 mg/kg 8MOP (n=24) and 10 mg/kg 8MOP (n=24) treatment conditions (means ± standard error of the mean).
Figure 11. Correlation between serum cotinine levels adjusted for liver weight (ng/ml/g) and total nicotine dosage (mg/kg) for adolescent (n=60) and adult (n=60) male C57BL/6J mice exposed to control (n=24), saline (n=24), vehicle (n=24), 5 mg/kg 8MOP (n=24) and 10 mg/kg 8MOP (n=24) treatment conditions (means ± standard error of the mean).
Figure 12. Relative CYP2a5 gene expression corrected for 18S rRNA quantity among adolescent (n=18) and adult (n=18) male C57BL/6J mice exposed to vehicle (n=12), 5 mg/kg 8MOP (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean).
Figure 13. Relative CYP2e1 gene expression corrected for 18S rRNA quantity among adolescent (n=18) and adult (n=18) male C57BL/6J mice exposed to vehicle (n=12), 5 mg/kg 8MOP (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean).
CHAPTER III

EXPERIMENT II
EXPERIMENT II

Overview

Experiment 1 was the first 12-hour study to use C57BL/6J adolescent and adult male mice to examine the effect of age on nicotine consumption following methoxsalen administration. Adults consumed more nicotine than did adolescents, and a drug treatment effect in serum cotinine levels (ng/ml/g) among adults, but not adolescents, was observed. In order to further investigate the duration of these effects following chronic methoxsalen and nicotine exposure, Experiment II examined the effects of age (adolescent vs. adult) and repeated methoxsalen exposure on nicotine consumption (ml), nicotine intake as a percent of total fluid intake (%), and nicotine dosage (mg/kg), serum cotinine levels adjusted for liver weight (ng/ml/g) and hepatic CYP2a5 RNA levels in male C57BL/6J mice over the course of 8 days of nicotine exposure. Adolescent (PND 35-38; N=32) and adult (8-9 weeks; N=32) male C57BL/6J mice were provided with access to a 3-bottle choice [tap water without nicotine (WTR), and two solutions of nicotine: 50 (LOW NIC) and 200 (HIGH NIC) ug/ml] in their home cage for four days. Next, daily subcutaneous (s.c.) injections of vehicle (N=32) or 10 mg/kg 8MOP (N=32) along with continued access to the 3-bottle choice paradigm, were provided to all mice for an additional four days (PND 39-42; 9-10 weeks). Animals (PND 43; 10 weeks) then were sacrificed via cervical dislocation, and trunk blood and liver were collected for assessment of cotinine and CYP enzymes, respectively. This study was approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University (#28692) and the safety and care of all laboratory animals was upheld throughout the study (National Research Council, 1985).
**Study Design**

Experiment II used a 2 (age; adolescent vs. adult) X 2 (drug; vehicle vs. 10 mg/kg 8MOP) between-subjects design with daily 3-bottle oral nicotine consumption as the within-subjects factor.

**Methods**

**Animals**

Sixty-four (32 adolescents, 32 adults) male C57BL/6J mice (Jackson Labs, Bar Harbor, Maine) were used with 16 animals per treatment group (age; drug) (see Table 9). Animals were housed in a climate controlled room with a 12-hour light/dark cycle (lights on at 0700 hours), where humidity was maintained at 60% and temperature at 21 ± 2 °C. Each animal was individually housed under standard conditions in conventional cages without filter tops filled with ¼ of an inch laboratory animal bedding (Bed-o’Cobs, The Andersons Agriservices, Inc., Champaign, IL). Throughout the experiments, animals were provided with unrestricted access to food (LabDiet 5001 Rodent Diet; PMI Nutrition International, Brentwood, MO) and daily measurements of body weight, food consumption and fluid intake were taken.

**Drugs**

Freebase (-)-nicotine. Animals were provided access to 50 (LOW NIC) and 200 (HIGH NIC) ug/ml freebase (-)-nicotine (Sigma-Aldrich; St. Louis, MO) dissolved in tap water (see Appendix B for procedure). The oral nicotine consumption model and the two nicotine concentrations were selected based on previous findings that periadolescent and adult C57BL/6J mice will voluntarily consume nicotine in solution at these concentrations, and in doing so, produce reliable cotinine levels similar to those found in
human smokers (Klein et al., 2004; Klein et al., 2008). In addition, two nicotine concentrations were provided to allow for different concentration preferences among mice.

Methoxsalen. Methoxsalen (10 mg/kg; Sigma-Aldrich, St. Louis, MO; see Appendix A for MSDS sheet) was dissolved in a solution of Emulphor (Rhodia, Inc.; Cranbury, NJ), 200 proof ethyl alcohol and single distilled water in a 1:1:18 ratio (see Appendix L for solution preparation procedure). Because the stability of 8MOP is unknown, these solutions were prepared on each injection day no more than 4 hours prior to administration. Solutions were kept in dark brown medicine bottles and placed in a box to avoid exposure to light. Control group mice received an s.c. injection of drug-free solution of Emulphor, 200 proof ethyl alcohol and distilled water (vehicle), the volume of which was based on body weight measurements taken that day. Methoxsalen was administered via s.c. injection at one dosage, 10 mg/kg, which was the same dosage previously reported in adult male ICR mice (Damaj, Siu, Sellers, Tyndale, & Martin, 2007) and was one of two dosages of methoxsalen used in Experiment I (see Chapter II of this dissertation). No adverse effects have been previously reported with this dosage of methoxsalen in adults (Damaj et al., 2007) and adolescents (see Chapter II of this dissertation).

Study Procedure

Acclimation (3 days) & baseline (2 days)

Figure 13 represents a timeline of Experiment II. After arrival, animals (PND 30; 8 weeks) were allowed three days to acclimate to their new environment. Standard food and 3 bottles of tap water were available ad libitum, and mice were left undisturbed in
their home cages. Next, food consumption, water intake and body weight were measured shortly after the lights are turned on for 2 days (PND 33-35; 8 weeks). Baseline measurements were used to assign mice to the control (vehicle) or 8MOP treatment group to ensure that the groups did not differ on these measures at the start of the study.

**Phase I: Oral voluntary nicotine consumption (choice) treatment test (4 days)**

Following baseline, all animals (PND 35-38; 9 weeks) were provided with 24-hour access to tap water, LOW NIC and HIGH NIC solutions for four days. Each day, body weight, food consumption and fluid intake were monitored and recorded, and placement of the bottles were switched to prevent place preference (Klein et al., 2003; Klein et al., 2004).

**Phase II: Methoxsalen treatment test (4 days)**

Following body weight, food consumption and fluid intake measurements, animals (PND 39-42; 9 weeks) were given continued 24-hr access to tap water, LOW NIC and HIGH NIC bottles. One hour before the beginning of the dark cycle (0600 hrs), each animal (PND 39-42; 9 weeks) received an s.c. injection of either vehicle or 8MOP underneath the skin amid the scapulas for four days. Consistent with Experiment I, this specific time frame for injections was used because of the higher concentration of CYP2a5 in the dark (Lavery et al., 1999), and the fact that rodents drink the majority of their fluid during the dark cycle when they are the most active (Pietilä, Laakso, & Ahtee, 1995). After injection, all animals were returned to their home cages for continued access to the 3 bottles. Injection order was randomized across treatment groups and rotated daily.
**Blood collection and tissue harvest**

On the last day of the experiment, animals (PND 43; 10 weeks) were sacrificed in the morning via cervical dislocation. Trunk blood was removed via cardiac puncture. Blood was allowed to sit at room temperature for 15 minutes and centrifuged at 3000 x g for 15 minutes. Following centrifugation, serum was aliquoted and stored at -80°C for later assessment of cotinine, the primary active metabolite of nicotine. Livers were removed, weighed, dissected, submerged in RNAlater (a solution necessary to stabilize and preserve RNA; Applied Biosystems, Carlsbad, CA), flash frozen in liquid nitrogen, and stored at -80°C (see Appendix D for procedure). Sacrifice order was counterbalanced across drug treatment condition.

**Analytical methods**

**Serum cotinine assessment.** Serum cotinine was analyzed using a commercially available enzyme immunosorbant assay kit (EIA; Immunalysis, Los Angeles, CA; see Appendix E for assay procedure). The assay sensitivity, based on the minimum cotinine concentration required to produce a three standard deviation from assay A₀, was 1 ng/ml. All samples were tested in duplicate in a single assay batch in our laboratory using a Synergy 2 96-well plate reader (Biotek Instruments, Winooski, VT). Duplicate test values that varied by more than 5% error were subject to repeat testing. Following the initial run, samples that exceeded the highest standard (>105.00 ng/ml) were diluted 1 in 10 fold [10 ul sample: 90 ul phosphate buffered solution (PBS)] prior to subsequent assay.

**Quantitative real-time PCR (qPCR).** Quantitative real-time PCR was used to examine the effect of age and methoxsalen exposure on hepatic CYP2a5 and CYP2e1...
RNA levels (12 adolescents, 12 adults). Based on the results, 24 mice (N=6/experimental group) were chosen at random from each of the four treatment groups (adolescent vehicle, adolescent 8MOP, adult vehicle, adult 8MOP) to conduct these assays. Isolation of total RNA was completed using TRI-reagent (Sigma-Aldrich, Inc., Saint Louis, MO; see Appendix F for RNA extraction protocol). Following the assessment of the quantity and quality of RNA using a ND-1000 spectrophotometer and Nanodrop software (Version 3.31; Thermo Fisher Scientific, Inc., Wilmington, DE; see Appendix G for quality control protocol), RNA was translated into cDNA using a high capacity cDNA reverse transcriptase kit (Applied Biosystems Inc., Foster City, CA, USA; see Appendix H for reverse transcription protocol). SYBR Green Master Mix (Applied Biosystems Inc., Foster City, CA) was used to detect the RNA amplification of the samples (see Appendix I for RT-PCR protocol). The housekeeping gene, 18 Svedberg Units (S) ribosomal RNA subunit (Applied Biosystems Inc., Foster City, CA), was used as an internal control, specifically to correct for RNA quality. In contrast to other commonly used housekeeping genes (e.g., β-actin), 18S was used because of the consistent gene expression regardless of drug treatment (Selvey et al., 2001). The optimal primer concentration in the master mix is set at 300 nM for this gene. Primers for 18s were gifts from the laboratory of John Vanden Heuvel, Ph.D. at The Pennsylvania State University (University Park, PA; see Appendix J for primer sequence). CYP2a5 and CYP2e1 primers, which are listed in Appendix J, were chosen based on previously published literature (Muguruma et al., 2006; Kashida et al., 2006, respectively) and synthesized at the Genomics Core Facility at The Pennsylvania State University (University Park, PA). The samples were run in singlet in Dr. Vanden Heuvel’s laboratory using ABI Prism®
7000 Sequence Detection System with 7000 Sequence Detection System (SDS) Software (Version 1.2.3.; Applied Biosystems Inc., Foster City, CA).

**CYP2a5 and CYP2e1 gene expression calculations.** Quantity of each gene was determined relative to a standard curve [logarithm of known cDNA concentration versus unknown cycle at threshold (ct) value] (see Appendix K for absolute quantification protocol). Quantity of relative gene expression for CYP2a5 and CYP2e1 was then based on the quantity of the housekeeping gene, 18S. For each gene of interest, the quantity of CYP2a5 gene expression was divided by the quantity of 18S, such that:

\[
\frac{\text{Quantity of CYP2a5 [or CYP2e1] gene expression}}{\text{Quantity of 18S gene expression}}
\]

yielded corrected value of gene expression for CYP2a5 and CYP2e1.

**Nicotine Calculations.** Raw values for nicotine consumption (ml) for LOW and HIGH NIC were used in the following calculations. Total nicotine consumption (ml) was calculated by adding the nicotine consumption (ml) values for LOW and HIGH NIC together for sacrifice day and each treatment day. Total nicotine consumption (ml) values were combined for treatment days 1-4 and 5-8 to examine the amount of nicotine consumed before and after methoxsalen administration. Average nicotine consumption (ml) was also calculated separately for treatment days 1-4 and 5-8.

Nicotine intake as a percent of total fluid intake (%) was calculated by dividing the sum of LOW NIC and HIGH NIC consumption (ml) by the total fluid volume (water plus nicotine solutions) consumed (ml). This value was multiplied by 100 to achieve a percent, such that:
(volume of nicotine consumed [ml] for LOW NIC + HIGH NIC)  
\[ \frac{\text{TOTAL NiC}}{\text{TOTAL FLUID}} \times 100 \]

yielded the total nicotine intake as a percent of total fluid intake (%). Average nicotine intake as a percent also was calculated for treatment days 1-4 and 5-8 to examine any change in nicotine intake before and after methoxsalen administration.

Total nicotine dosage (mg/kg) was calculated for sacrifice day and each treatment day. Nicotine consumption (ml) for LOW NIC and HIGH NIC were added together and then divided by the product of body weight of each animal multiplied by 0.001, such that:

\[ \frac{\text{TOTAL NiC}}{\text{BODY WT} \times 0.001} \]

yielded the total nicotine consumption adjusted for body weight (mg/kg). Total nicotine dosage (mg/kg) was calculated for treatment days 1-4 and 5-8 by combining nicotine dosages for each group of four days. Average nicotine dosage for total nicotine dosage was calculated separately for treatment days 1-4 and 5-8. These nicotine consumption values (total ml, % total fluid intake, mg/kg) were used in the following statistical analyses.

**Treatment of Data.** Two adult mice from the vehicle group were removed from analyses of Phase II. One mouse died during the injection procedure and the other was inadvertently injected with 8MOP on the third day of the experiment.

Adolescent and adult mice differed significantly in liver weight and overall body weight (see Results section); therefore, two variables were created to account for these age-related differences: Serum cotinine levels (ng/ml) were adjusted for liver weight (ng/ml/g) and nicotine consumption (mL) was adjusted for body weight to calculate
nicotine dosage (mg/kg; mg nicotine/kg body weight; see Klein et al., 2004). Natural logarithmic transformations were applied to normalize the distribution of serum cotinine levels adjusted for liver weight (ng/ml/g). Following confirmation that this transformation led to a normal distribution of the data (Klein et al., 2004), analyses then were conducted on these adjusted values. Unless otherwise noted, raw adjusted means (± S.E.M) were used in tables and graphs for clarity (Klein et al., 2004).

Because of the positive correlation between liver weight (g) and body weight (g), a ratio of liver weight to body weight on sacrifice day was calculated and used as an indicator of toxic effects of methoxsalen. Analyses then were conducted on these ratios.

CYP2a5 and CYP2e1 gene expression levels were adjusted for the quantity of gene expression for 18S rRNA to control for RNA quality throughout the qPCR process. Natural logarithmic transformations were applied to normalize the distribution of relative gene expression of CYP2a5. Following confirmation that this transformation led to a normal distribution of the data (Klein et al., 2004), analyses then were conducted on these adjusted values. Because relative gene expression of CYP2e1 was normally distributed, no transformations were applied to these values. Adjusted means (± standard error of the mean) were used in graphs for clarity unless otherwise noted.

Statistical Analyses. After entering the data into PASW (Predictive Analytics Software Version 18.0; SPSS Inc.; Chicago, IL), separate, repeated-measures analyses of variance (RM-ANOVA), with age (2 levels) and drug treatment (2 levels) as the independent variables and time as the within-subjects variable, were used to examine 1) body weight, 2) food intake, 3) water consumption, 4) nicotine volume (ml), 5) nicotine dosage (mg/kg), and 6) nicotine intake as a percent of total fluid intake (%) from Phase I
and Phase II data. Nicotine consumption (mL), nicotine intake as a percent of total fluid intake (%), and nicotine dosage (mg/kg) data were separately averaged for each phase, and compared using an RM-ANOVA with age (2 levels) and drug treatment (2 levels) as the independent variables and time (2 time points) as the within-subjects variable, to examine any group differences in nicotine consumption before and after methoxsalen administration. Separate two-way ANOVAs were used to assess any interactions. Bonferroni post hoc tests were used where appropriate.

Additionally, separate two-way ANOVAs, with age (2 levels) and drug (2 levels) as independent variables were also completed to assess 1) liver weight, 2) serum cotinine levels adjusted for liver weight (ng/ml/g) and relative gene expression for 3) 18S, and the corrected values for 4) CYP2a5 and 5) CYP2e1. Interactions were further examined for statistical significance using separate one-way ANOVAs.

Bi-variate correlations were conducted to assess the relationship between total nicotine dosage (mg/kg), serum cotinine levels (ng/ml/g) and CYP2a5 and CYP2e1 gene expression levels. Bonferroni post hoc tests were used where appropriate. All tests were two-tailed with statistical significance determined at $\alpha = 0.05$.

**Hypotheses**

**Hypothesis 1 Overview.** With regard to body weight (g), I hypothesized a main effect for methoxsalen and age. Because of the lack of published data, age X methoxsalen and age X methoxsalen X time interactions were not predicted. Specifically:

**Hypothesis 1A.** Mice treated with 8MOP would gain less weight (g) compared to vehicle mice.
Rationale. Wistar rats that ingested a powdered diet infused with methoxsalen (250, 1250 and 2500ppm) had a reduced weight gain compared to animals that received a diet infused with bergapten and control mice (Diawara et al., 1997).

**Hypothesis 1B.** Adults would have a higher body weight (g) compared to adolescents.

Rationale. Adult C57BL/6 mice (Meliska, Bartke, McGlacken, & Jensen, 1995) have a higher baseline body weight compared to adolescent C57BL/6J mice (Klein et al., 2004).

**Hypothesis 2 Overview.** With regard to nicotine volume (ml), I hypothesized a main effect for methoxsalen and age, and a two way interaction for age X methoxsalen. Because of the lack of published data, an age X methoxsalen X time interaction was not predicted. Specifically:

**Hypothesis 2A.** Vehicle mice would consume more nicotine (ml) compared to mice treated with 8MOP.

Rationale. Sellers and colleagues (2000) discovered that the combination of methoxsalen and nicotine increases plasma nicotine levels, and subsequently, increasing bioavailability of nicotine. These increases in concentration significantly reduced cigarette consumption and carbon monoxide concentrations (24% and 47%, respectively) in adult smokers (Sellers et al., 2000).

In addition, data on CYP2A6 polymorphisms also indicates that individuals with at least one or two variant alleles have altered responses to nicotine and later, risk of dependence compared to the wild type genotype (CYP2A6*1). For example, CYP2A6*1/*12 and CYP2A6*1/*9 genotypes lead to partial reductions (approximately 20%) in enzymatic activity (e.g., increase in total and non-renal clearance of nicotine),
increases in the half lives of nicotine and cotinine, and low 3HC/COT ratios (Benowitz et al., 2006). A 40 to 50% reduction in nicotine clearance, considerable increases in nicotine and cotinine half lives, and very low 3HC/COT ratios are observed in individuals with other variant allelic combinations; for example, CYP2A6*1/*2, CYP2A6*1/*4, CYP2A6*9/*12, CYP2A6*9/*4, and CYP2A6*9/*9 (Benowitz et al., 2006).

Furthermore, two clinical studies determined that the presence of CYP2A6 genetic variants that reduce the rate of nicotine metabolism along with recent cigarette consumption puts middle- and high-school aged adolescents at an increased risk of becoming dependent on tobacco and maintaining their dependence compared to those without these polymorphisms (Karp et al., 2006; O’Loughlin et al., 2004).

**Hypothesis 2B.** Adolescent mice would consume more nicotine (ml) than would adult mice, regardless of methoxsalen treatment.

**Rationale.** An unpublished study done by Klein, Stine and Vandenbergh in 2003 determined that adolescent C57BL/6J mice consumed more nicotine (ml) compared to adults.

**Hypothesis 2C.** Age would interact with methoxsalen exposure such that, among adolescents, 8MOP-exposed mice would consume more nicotine (ml) compared to vehicle-exposed mice. In contrast, adult mice exposed to 8MOP would consume less nicotine (ml) compared to vehicle-exposed mice.

**Rationale.** Two clinical studies determined that the presence of CYP2A6 genetic variants that reduce the rate of nicotine metabolism and recent cigarette consumption puts middle- and high-school aged adolescents at an increased risk of becoming dependent on tobacco and maintaining their dependence compared to those without these
polymorphisms (Karp et al., 2006; O’Loughlin et al., 2004). However, regarding adults, recent evidence suggests that CYP2A6 genetic variants may be protective against tobacco dependence for adults (Pianezza et al., 1998; Rao et al., 2000; Sellers et al., 2003b; Tyndale et al., 1999; Tyndale & Sellers, 2001; 2002). These findings indicate that adolescent mice treated with a CYP2A6 inhibitor that is intended to mimic polymorphisms encoding for a null CYP2A6 enzyme (methoxsalen) would consume more nicotine (ml) than their control counterparts, whereas adult mice treated with the same CYP2A6 inhibitor would consume less nicotine (ml) than control adult mice.

**Hypothesis 3 Overview.** With regard to nicotine dosage [nicotine volume adjusted for body weight (mg/kg)], I hypothesized main effects for methoxsalen and age, and a two-way age X methoxsalen interaction. Because of the lack of published data, an age X methoxsalen X time interaction was not predicted. Specifically:

**Hypothesis 3A.** Mice treated with 8MOP would consume more nicotine (mg/kg) compared to vehicle mice.

**Rationale.** Sellers and colleagues (2000) discovered that the combination of methoxsalen and nicotine increases plasma nicotine levels, and subsequently, increasing bioavailability of nicotine in vitro. These increases significantly reduced cigarette consumption and carbon monoxide concentrations (24% and 47%, respectively) in adult smokers (Sellers et al., 2000).

Because methoxsalen mimics CYP2A6 polymorphisms, clinical data also indicates that individuals with at least one or two variant alleles have altered responses to nicotine and later, risk of dependence compared to the wild type genotype (CYP2A6*1). For example, CYP2A6*1/*12 and CYP2A6*1/*9 genotypes lead to partial reductions
(approximately 20%) in enzymatic activity (e.g., increase in total and non-renal clearance of nicotine), increases in the half lives of nicotine and cotinine, and low 3HC/COT ratios (Benowitz et al., 2006). A 40 to 50% reduction in nicotine clearance, considerable increases in nicotine and cotinine half lives, and very low 3HC/COT ratios are observed in individuals with other variant allelic combinations; for example, CYP2A6*1/*2, CYP2A6*1/*4, CYP2A6*9/*12, CYP2A6*9/*4, and CYP2A6*9/*9 (Benowitz et al., 2006). Furthermore, two clinical studies determined that the presence of CYP2A6 genetic variants that reduce the rate of nicotine metabolism along with recent cigarette consumption puts middle- and high-school aged adolescents at an increased risk of becoming dependent on tobacco and maintaining their dependence compared to those without these polymorphisms (Karp et al., 2006; O’Loughlin et al., 2004).

**Hypothesis 3B.** Adolescent mice would consume more nicotine (mg) per kg of body weight, regardless of methoxsalen treatment.

**Rationale.** An unpublished study done by Klein, Stine and Vandenbergh in 2003 determined that adolescent C57BL/6J mice consumed more nicotine (mg) per kg of body weight (mg/kg) compared to adults.

**Hypothesis 3C.** Age would interact with methoxsalen exposure such that, among adolescent mice, 8MOP exposure would result in greater consumption of nicotine (mg) per kg of body weight compared to vehicle. In contrast, 8MOP exposure would result in less consumption of nicotine (mg) per kg of body weight compared to vehicle in adult mice.

**Rationale.** Two clinical studies determined that the presence of CYP2A6 genetic variants that reduce the rate of nicotine metabolism and recent cigarette consumption puts
middle- and high-school aged adolescents at an increased risk of becoming dependent on tobacco and maintaining their dependence compared to those without these polymorphisms (Karp et al., 2006; O’Loughlin et al., 2004). However, regarding adults, recent evidence suggests that CYP2A6 genetic variants may be protective against tobacco dependence for adults (Pianezza et al., 1998; Rao et al., 2000; Sellers et al., 2003b; Tyndale et al., 1999; Tyndale & Sellers, 2001; 2002). These findings indicate that adolescent mice treated with a CYP2A6 inhibitor that is intended to mimic polymorphisms encoding for a null CYP2A6 enzyme (methoxsalen) would consume more nicotine (mg) per unit body weight than their control counterparts, whereas adult mice treated with the same CYP2A6 inhibitor would consume less nicotine (mg) per unit body weight than control adult mice.

**Hypothesis 4 Overview.** With regard to levels of serum cotinine adjusted for liver weight (ng/ml/g), I hypothesized a main effect for methoxsalen treatment. Because of the lack of published data, age X methoxsalen and age X methoxsalen X time interactions were not predicted. Specifically:

**Hypothesis 4A.** 8MOP exposure would reduce serum cotinine levels (ng/ml/g) compared to vehicle.

**Rationale.** Several studies have determined that methoxsalen increases nicotine half-life and decrease cotinine levels in vivo in mice (Damaj et al., 2007; Raunio et al., 2008).

**Hypothesis 5 Overview.** With regard to levels of CYP2a5 RNA levels, I hypothesized main effects for methoxsalen treatment. Because of the lack of published data, a main
effect for age, and age X methoxsalen and age X methoxsalen X time interactions were not predicted. Specifically:

**Hypothesis 5A.** 8MOP exposure would increase CYP2a5 RNA levels compared to vehicle.

**Rationale.** Bickers and Pathak (1984) determined that the oral administration of methoxsalen over 6 days to adult male CD-1 mice increased hepatic protein levels, enzyme activity [e.g., aryl-hydrocarbon hydroxylase (AHH), ethylmorphine N-deethylase], and cytochrome p450 levels.

**Results**

**Baseline (2 days)**

**Body weight (g), food intake (g) and water consumption (ml).** Compared with adolescents, adults weighed more on baseline day 1 \( F(1,60)=553.71, p<0.05 \) and 2 \( F(1,60)=389.78, p<0.05 \). Adults ate more than did adolescents on baseline day 1 \( F(1,60)=55.02, p<0.05 \), but not on baseline day 2. Average water consumption from all three bottles over both baseline days, on the other hand, was higher in adolescents compared to adults \( F(1,60)=38.95, p<0.05 \). Although drug treatment groups were not assigned until the end of Phase 1, it is important to note that there were no significant Drug effects or Age X Drug interactions for these three dependent variables on either baseline day prior to the start of nicotine consumption testing.

**Phase I: Oral voluntary nicotine consumption (choice) treatment test (4 days)**

**Body weight (g).** Table 10 presents mean body weight (g) for adolescent and adult mice in the two drug treatment groups during Phase I of the experiment. In order to assess changes in body weight due to nicotine consumption, body weight on treatment
days 2, 3, 4 and 5 were used in the following analyses (instead of days 1, 2, 3 and 4, because animals were not given access to nicotine until after body weight measurements on day 1). An Age effect was observed over the span of all four treatment days with adults continuing to weigh more than adolescents [F(1,60)=364.51, p<0.05].

There also was a significant Time effect where all mice gained weight across the 4-day nicotine exposure period [F(3,180)=8.21, p<0.05]. Treatment days 2, 3, and 4 were different from each other (p<0.05). Body weight on treatment day 4 was the highest for all animals.

Additionally, there was a Time X Age interaction on body weight during nicotine-only treatment [F(3,180)=4.53, p<0.05]. Specifically, adults had a higher body weight on all treatment days than did adolescents [F’s(1,60)>325.04, p’s<0.05]. A significant Time effect was observed among adolescents [F(3,90)=10.23, p<0.05], such that body weight increased linearly throughout Phase I. Treatment day 2 was significantly different from each day (p<0.05); however, days 3, 4 and 5 were not different from each other.

Similarly, there was a significant a Time effect for adults [F(3,90)=5.31, p<0.05]; however, the pattern of weight gain was cubic. Treatment day 4 was the highest, followed by day 2, 5 and then day 3. Body weight was significant only different between treatment days 3 and 4, and days 4 and 5 (p’s<0.05).

Interestingly, there also was a Time X Drug interaction [F(3,90)=4.43, p<0.05], but only among adults. Note that groups were not treated with vehicle or methoxsalen until after measurements were taken on the morning of treatment day 5. No effect of drug treatment for body weight on treatment days 2, 3, 4, or 5 was observed. There was, however, an effect of Time in the vehicle [F(3,45)=3.01, p<0.05] and 8MOP group [F(3,
Among adults in the vehicle group, body weight on treatment day 2 was the highest, followed by day 4, 3 and then day 5. In contrast, body weight for adults in the 8MOP group increased for the first three days and then decreased on treatment day 5. Body weight in the 8MOP group was the highest on treatment day 4, followed by day 5, 3 and then day 2. Significant differences in body weight were observed only between treatment days 1 and 3, and days 2 and 3 (p's < 0.05).

Drug treatment groups were assigned at the end of Phase 1; it is important to note that there was no significant effect of Drug or Age X Drug interactions for body weight on any treatment day prior to group assignments.

Food intake (g). Table 11 presents mean food consumption (g) for adolescent and adult mice in the two drug treatment groups during Phase I of the experiment. Similar to that of body weight, there was a significant Time effect \( F(3,177)=53.82, \ p<0.05 \) as well as a Time X Age interaction \( F(3,177)=5.02, \ p<0.05 \). Additionally, adults consumed more food throughout Phase I compared to adolescents \( F(1,59)=19.00, \ p<0.05 \).

With the exception of days 2 and 4, food consumption was significantly different throughout Phase I. Specifically, animals consumed the most food on treatment day 1, followed by day 4, 2 and then 3. There was no main effect for age on treatment day 1; however, adults consumed more food on treatment day 2 through 4 compared to adolescents \( F'(s(1,59)>5.89, \ p'<0.05 \). Among adolescents, a Time effect was observed \( F(3,87)=139.51, \ p<0.05 \), with the most food consumed on treatment day 1, followed by day 4, day 2 and then day 3. Food consumption on all days differed from one another except for days 2 and 4 (p<0.05). Among adults, there was a main effect for Time \( F(3,90)=10.62, \ p<0.05 \) resulting in a similar pattern: Treatment day 1 > day 4 >
day 2 > day 3. Specifically, food intake on treatment day 1 was significantly different from days 2 and 3, and consumption on day 3 was significantly different from day 4 ($p$’s<0.05).

Animals that eventually would be placed in the vehicle group consumed more food on treatment day 1 compared to their 8MOP counterparts [$F(1,59)=4.98$, $p<0.05$]. There also was an Age X Drug interaction [$F(1,59)=5.27$, $p<0.05$] on treatment day 1. Further examination revealed a significant main effect for drug treatment for adults [$F(1,30)=9.06$, $p<0.05$], but not adolescents, with animals in the vehicle group consuming more food than those in the 8MOP group. These drug treatment effects on food consumption only were observed on treatment day 1.

**Water consumption (ml).** Table 12 presents mean water (mL) for adolescent and adult mice in the two drug treatment groups during Phase I of the experiment. Overall, adolescents consumed significantly more water compared to adolescents during Phase I [$F(1,60)=14.51$, $p<0.05$]. Additionally, there was a significant Time X Age interaction [$F(3,180)=2.68$, $p<0.05$], such that adolescents consumed more water than did adults across all treatment days [$F’s(1,60)=4.47$, $p$’s<0.05] (Table 12). When analyzed separately, there were no significant Time effect or Time X Drug interactions for adolescents or adults.

Drug treatment groups were assigned at the end of Phase I; there was no significant effect of Drug or Age X Drug interactions for water consumption on any treatment day prior to group assignments. This finding meant that water consumption did not need to be controlled for in Phase II nicotine intake analyses.
Total fluid consumption (mL). Table 12 presents mean total fluid consumption (mL) for adolescent and adult mice in the two drug treatment groups during Phase I of the experiment. Throughout the entire nicotine pre-treatment phase, adolescents consumed more water and nicotine (mL) than did adults \(\text{[F(1,60)=15.66, } p<0.05\]}. Additionally, there was a Time effect \(\text{[F(3,180)=27.97, } p<0.05\]}, such that animals consumed the most fluid on treatment day 3, followed by day 2, 1 and then 4. Treatment days 1 and 2, days 1 and 3, days 2 and 4, and days 3 and 4 were significantly different from each other \(p's<0.05\). Adolescents consumed more total fluid (mL) compared to adults on all treatment days \(\text{[F'(s(1,60)=4.47, } p's<0.05\]}. Among adolescents, there was a significant Time effect \(\text{[F(3,90)=14.00, } p<0.05\]}; Treatment day 3 > day 2 > day 1 > day 4. Treatment day 1 was different from 3, day 2 was different from 4, and day 3 was different from 4 \(p<0.05\). Adults consumed the most fluid on treatment day 3, followed by day 2, 1 and then 4. Treatment days 1 and 2, days 1 and 3, days 2 and 4, and days 3 and 4 were significantly different from each other \(p's<0.05\).

Similar to body weight and water consumption, there was no significant effect of Drug or Age X Drug interactions for total fluid consumption on any treatment day prior to assigning groups.

Total nicotine consumption (mL). Table 13 and Figure 15 present mean total nicotine consumption (mL) for adolescent and adult mice in the two drug treatment groups during Phase I of the experiment. Adults and adolescents consumed similar amounts of total nicotine (mL) over all four days. However, there was a main effect for Time \(\text{[F(3,180)=24.54, } p<0.05\]}. Animals consumed the most nicotine (mL) on treatment
day 3, followed by day 2, 1 and then day 4. All treatment days were significantly different from each other with the exception of days 2 and 3 ($p$'s<0.05).

In addition, a Time X Age interaction was observed $[F(3,180)=3.81, p<0.05]$. There were no main effects for Age on treatment days 1, 2, and 4; however, adults consumed significantly more nicotine (mL) compared to adolescents on treatment day 3 $[F(1,60)=5.02, p<0.05]$. Among adolescents, there was a significant effect for Time: Treatment day 3 > day 2 > day 1 > day 4. Treatment day 1 was different from day 3, and day 4 was different from all three treatment days ($p$'s<0.05). Similarly, there was a main effect for Time for adults $[F(3,90)=8.52, p<0.05]$. Adult mice consumed the most nicotine (mL) on day 2, followed by day 3, 1 and then day 4.

There was no significant effect of Drug or Age X Drug interactions for total nicotine consumption (mL) on any treatment day prior to drug group assignments.

Nicotine intake as a percent of total fluid intake (%). Table 14 and Figure 16 present mean total nicotine intake as a percent of total fluid intake (%) for adolescent and adult mice in the two drug treatment groups during Phase I of the experiment. Overall, adults consumed more nicotine (%) compared to adolescents $[F(1,60)=4.00, p<0.05]$. Additionally, a main effect of Time was observed $[F(3,180)=14.87, p<0.05]$, with the highest percent nicotine intake on treatment day 3, followed by day 2, 1 and then day 4.

There also was a Time X Age interaction $[F(3,180)=5.12, p<0.05]$. Adults consumed more nicotine (%) than did adolescents on treatment days 1 $[F(1,60)=4.51, p<0.05]$, 2 $[F(1,60)=5.26, p<0.05]$ and 4 $[F(1,60)=5.19, p<0.05]$, but not on treatment day 3. Among adolescents, there was a significant main effect of Time $[F(3,90)=41.60, p<0.05]$, such that nicotine consumption (%) steadily increased for the first three days
and then decreased on day 4. Each treatment day was significantly different from each other (p’s<0.05). Adults, on the other hand, increased their nicotine consumption (%) from day 1 to day 2; however, consumption decreased for the rest of treatment.

**Total nicotine dosage (mg/kg)**. Table 15 and Figure 17 present mean total nicotine dosage (mg/kg) for adolescent and adult mice in the two drug treatment groups during Phase I of the experiment. When nicotine intake was adjusted for body weight (mg/kg), adolescents consumed more nicotine than did adults [F(1,60)=6.41, p<0.05]. Overall, there was a Time effect [F(3,180)=20.55, p<0.05], such that nicotine consumption increased for the first three days, and then decreased significantly on day 4.

There was no significant effect of Drug or Age X Drug interactions for nicotine consumption, dosage or intake as a percent on any treatment day prior to drug treatment group assignments.

**Phase II: Methoxsalen Treatment Test (4 days)**

**Body weight (g)**. In order to assess changes in body weight due to nicotine consumption and methoxsalen, body weight on treatment days 6, 7, 8 and sacrifice day were used in the following analyses, instead of days 5, 6, 7 and 8, because animals were not given 8MOP/vehicle until the beginning of the dark cycle on day 5.

Table 16 presents mean body weight (g) for adolescent and adult mice in the two drug treatment groups during Phase II of the experiment. There was a significant main effect of Time on body weight during Phase II [F(3,174)=25.80, p<0.05]. Body weight increased over the four days. No Time X Age interaction was observed on body weight during this treatment phase. Adults had a higher body weight on all 4 treatment days.
There was no main effect for drug on body weight and no Time X Age, Time X Drug, or Time X Age X Drug interactions.

To understand how methoxsalen altered each dependent variable [consumption of food, total fluid (water and nicotine), nicotine (mL, % total fluid, mg/kg)] over time, averages of each measure from Phase I (treatment days 1-4) and Phase II (treatment days 6-8 and sacrifice day) were created for all dependent measures. Treatment days 2-5 (Phase I) and days 6-8 plus sacrifice day (Phase II) were averaged for body weight. Across both phases, there was no main effect of drug treatment or an Age X Drug interaction; however, adults weighed more (23.00 ± 0.20g) than did adolescents (17.88 ± 0.20g) \[F(1,59)=338.20, p<0.05\].

There was a significant Time effect \[F(1,59)=89.48, p<0.05\], Time X Age \[F(1,59)=4.01, p<0.05\] and Time X Age X Drug interaction on body weight \[F(1,59)=89.48, p<0.05\]. Both adolescents \[F(1,30)=103.86, p<0.05\] and adults \[F(1,29)=19.98, p<0.05\], weighed more during Phase II (18.13 ± 0.16g and 23.16 ± 0.23g, respectively) than they did during Phase I (17.63 ± 0.16g and 22.83 ± 0.23g, respectively) \[F(1,59)=89.48, p<0.05\]. All mice exposed to vehicle \[F(1,30)=39.23, p<0.05\] and 8MOP \[F(1,30)=50.85, p<0.05\] weighed more during Phase II (20.67 ± 0.22g and 20.62 ± 0.19g, respectively) compared to Phase I (20.27 ± 0.20g and 20.19 ± 0.19g, respectively). Adolescents in the vehicle \([F(1,15)=101.33, p<0.05]\ and 8MOP group \([F(1,15)=26.00, p<0.05]\ weighed more during Phase II (18.35 ± 0.25g and 17.90 ± 0.22g, respectively) compared to Phase I (17.75 ± 0.24g and 17.50 ± 0.21g, respectively). Similarly, adults in the 8MOP group weighed more during Phase II (23.33 ± 0.31g).
compared to Phase I (22.87 ± 0.31g) \[F(1,15)=25.29, \ p<0.05\]. However, body weight among adults in the vehicle group did not change between Phase I and II.

**Food intake (g).** Table 17 presents mean food consumption (g) for adolescent and adult mice in the two drug treatment groups during Phase II of the experiment. Throughout Phase II, there was a significant main effect for Age \[F(1,57)=15.16, \ p<0.05\], such that adults consumed more food than did adolescents. Additionally, a Time effect was observed \[F(3,171)=48.76, \ p<0.05\]. Food consumption increased from treatment day 5 through day 7 but decreased on day 8. No main effect of Drug or no Age X Drug, Time X Drug or Time X Age X Drug interactions were observed for food consumption during Phase II.

There also was a Time X Age interaction \[F(3,171)=5.97, \ p<0.05\] on food consumption. Adults consumed more food compared to adolescents on treatment day 6 \[F(1,57)=22.17, \ p<0.05\] and 8 \[F(1,58)=21.84, \ p<0.05\], but not on days 5 or 7. Among adolescents, a significant main effect for Time was observed \[F(3,87)=49.77, \ p<0.05\], such that food consumption on treatment day 7 was the greatest, followed by day 6, day 8 and then day 5. Similarly, there was a Time effect among adults \[F(3,84)=17.56, \ p<0.05\]. Food consumption increased from days 5 to 7 but decreased on day 8. There was no effect of Age or Drug, and no Age X Drug, Time X Drug, or Time X Drug X Age interactions when comparing food consumption from Phase I to Phase II.

**Water consumption (mL).** Table 18 presents mean water intake (mL) for adolescent and adult mice in the two drug treatment groups during Phase II of the experiment. Although there is no overall Age effect for Phase II water consumption, a main effect for Time was observed \[F(3,174)=4.37, \ p<0.05\]. Water consumption was the
highest on treatment day 8, followed by day 5, 7 and then day 6. Treatment day 5 was significantly different from 6, and day 6 was significantly different from days 7 and 8 ($p<0.05$). There were no Time X Age or Time X Drug interactions for water consumption during Phase II.

Upon averaging water consumption separately during Phase I and II, animals consumed more water during Phase I than they did during Phase II [$F(1,58)=6.47$, $p<0.05$]. Additionally, there is a significant Age effect [$F(1,58)=8.28$, $p<0.05$], such that adolescents consumed more water over both phases compared to adults. A Time X Age interaction also was observed [$F(1,58)=6.66$, $p<0.05$]. Adolescents consumed more water during Phase I compared to adults [$F(1,60)=14.51$, $p<0.05$]; however, there was no age difference during Phase II. Although there was no Time X Drug interaction, adolescents consumed more water during Phase I than during Phase II. In contrast, there was no Time effect or Time X Drug interaction among adults.

No main effects of Drug or Age X Drug, Time X Drug or Time X Age X Drug interactions were observed for water intake (mL) during Phase II and when comparing average water consumption from Phase I to that of Phase II.

**Total fluid consumption (mL).** Table 18 presents mean total fluid intake (mL) for adolescent and adult mice in the two drug treatment groups during Phase II of the experiment. There was a main effect for Time [$F(3,174)=4.37$, $p<0.05$], but no overall Age effect and no Time X Age or Time X Drug interactions, on total fluid intake during Phase II. Total fluid consumption was highest on treatment day 8, followed by day 5, day 7, and then day 6. Treatment day 6 was significantly different from the other treatment days ($p<0.05$); however, there was no statistically significant difference
between the other days. Although there were no age or drug treatment differences in total fluid consumption on treatment days 5, 6, and 8, adults consumed more fluid on treatment day 7 compared to that of adolescents \([F(1,58)=6.24, p<0.05]\).

A comparison of average total fluid consumption during Phase I and II revealed a significant Time effect \([F(1,58)=57.62, p<0.05]\), such that animals consumed more fluid overall during Phase II compared to Phase I. There also was an overall Age effect, where adolescents consumed more fluid than did adults \([F(1,58)=3.91, p=0.05]\).

**Total nicotine consumption (mL).** Table 19 and Figure 18 present mean total nicotine consumption (mL) for adolescent and adult mice in the two drug treatment groups during Phase II of the experiment. There was a significant main effect for Time regarding the consumption of LOW and HIGH NIC combined \([F(3,174)=5.82, p<0.05]\). Animals reduced nicotine intake (mL) for the first three days of Phase II until treatment day 8 when nicotine consumption increased. Treatment days 5 and 7, and 7 and 8 were significantly different from each other \((p<0.05)\). Adults and adolescents consumed similar amounts of nicotine on treatment days 5, 6 and 8; however, adults consumed more nicotine overall compared to adolescents on treatment day 7 \([F(1,58)=11.59, p<0.05]\).

Figure 19 presents average total nicotine consumption (mL) for adolescent and adult mice in the two drug treatment groups during Phase I and II of the experiment. Upon averaging total nicotine consumption separately during Phase I and II, animals, both adolescents \([F(1,30)=38.54, p<0.05]\) and adults \([F(1,28)=49.47, p<0.05]\), consumed more nicotine overall during Phase II than they did during Phase I \([F(1,58)=84.73, p<0.05]\). There were no Age or Drug treatment differences during Phase I or II, no
overall Age or Time effect or Age X Drug (for either phase), Time X Age, Time X Drug, or Time X Age X Drug interactions.

**Nicotine intake as a percent of total fluid intake (%).** Table 20 and Figure 20 present mean total nicotine intake as a percent of total fluid intake (%) for adolescent and adult mice in the two drug treatment groups during Phase II of the experiment. Adolescents and adults consumed similar amounts of nicotine (%) during Phase II. There was a significant effect of Time with nicotine intake (%) on day 8 being the highest, followed by days 5, 6 and then day 7 $[F(3,174)=15.53, p<0.05]$. There was also a significant Time X Age interaction $[F(3,174)=3.32, p<0.05]$. Adolescents and adults consumed similar amounts of nicotine (%) on all treatment days except for treatment day 7, where adults consumed significantly more nicotine (%) compared to adolescents $[F(1,58)=7.89, p<0.05]$. Adolescents consumed the most nicotine (%) on treatment day 8, followed by day 6, 5 and then day 7 $[F(3,90)=12.88, p<0.05]$. Significant differences were found between treatment days 1 and 3, days 2 and 3, and days 3 and 4 ($p$’s<0.05). Adults consumed the most nicotine (%) on treatment day 8, followed by day 5, 6 and then day 7 $[F(3,84)=6.37, p<0.05]$. Treatment day 4 was significantly different from days 2 and 3 ($p$’s<0.05).

Figure 21 presents average total nicotine intake as a percent of total fluid intake (%) for adolescent and adult mice in the two drug treatment groups during Phase I and II of the experiment. Upon averaging nicotine intake (%) separately during Phase I and II, animals consumed more nicotine (%) during Phase II than they did during Phase I $[F(1,58)=6.47, p<0.05]$. No overall Age effects or Time X Age, Time X Drug or Time X Age X Drug interactions were observed in nicotine intake (%) when comparing Phase I
and II. There were no main effects for Age or Drug and no Age X Drug interactions in Phase I or II.

**Total nicotine dosage (mg/kg).** Table 21 and Figure 22 present mean total nicotine dosage (mg/kg) for adolescent and adult mice in the two drug treatment groups during Phase II of the experiment. Over the course of Phase II, adolescents consumed more nicotine overall compared to adults $[F(1,58)=5.37, p<0.05]$. There also was a significant Time effect $[F(3,174)=4.64, p<0.05]$, such that animals consumed more nicotine (mg/kg) on treatment day 5, followed by day 8, day 6 and then day 7. Treatment days 1 and 3, and days 4 and 3 are significantly different from each other ($p's>0.05$).

Figure 23 presents mean total nicotine dosage (mg/kg) for adolescent and adult mice in the two drug treatment groups during Phase I and II of the experiment. Animals consumed more nicotine (mg/kg) overall during Phase II than they did during Phase I $[F(1,58)=55.01, p<0.05]$. There was an overall main effect for Age across both phases $[F(1,58)=7.03, p<0.05]$, such that adolescents consumed more nicotine (mg/kg) compared to adults. There were no Drug treatment differences during Phase I or II, and no overall Age or Time effect or Age X Drug (for either phase), Time X Age, Time X Drug or Time X Age X Drug interactions.

**Liver weight (g) and ratio of liver weight to body weight.** Figure 24 presents mean liver weights (g) for adolescent and adult mice exposed to nicotine and methoxsalen. Adults had larger livers $[F(1,58)=28.30, p<0.05]$; however, adolescents have a higher liver to body weight ratio than did adult mice $[F(1,58)=50.19, p<0.05]$. Additionally, animals exposed to methoxsalen had a higher liver to body weight ratio compared to animals in the vehicle group $[F(1,58)=5.91, p<0.05]$. There was no
significant main effect for Drug on liver weight and no Age X Drug interactions for liver weight or the liver to body weight ratio.

**Serum cotinine (ng/ml/g).** Figure 25 presents mean serum cotinine levels adjusted for liver weight (ng/ml/g). Adults had higher serum cotinine adjusted for liver weight (ng/ml/g) \[F(1,58)=18.02, \ p<0.05\] compared to adolescents. Adult mice in the vehicle group had marginally statistically-significantly higher cotinine levels after adjusting for liver weight in comparison to the 8MOP group \[F(1,28)=3.56, \ p=0.07\]. No such trend was found among adolescents. There were no significant main effects for Drug and no Age X Drug interactions for cotinine levels after adjusting for liver weight.

**Hepatic CYP gene expression.** Figures 26 and 27 present mean gene expression levels for CYP2a5 and CYP2e1 based on 18S gene expression levels, respectively. After adjusting for the RNA quantity of 18S, adults have higher levels of CYP2a5 gene expression compared to adolescents \[F(1,20)=5.76, \ p<0.05\]. There also was no effect for Drug and no Age X Drug interaction on the relative gene expression of CYP2a5. There was no effect for Age or Drug and no Age X Drug interaction on the relative gene expression of CYP2e1.

Figures 28 and 29 present the correlations between total nicotine dosage (mg/kg) and mean gene expression levels for CYP2a5 based on 18S gene expression levels for Phase I and Phase II, respectively. Among adolescents, CYP2a5 gene expression levels were positively correlated with average total nicotine dosage during Phase I \[r(10)=0.82, \ p<0.05\] and Phase II \[r(10)=0.69, \ p<0.05\]. Among adults, CYP2a5 gene expression levels were negatively correlated with average total nicotine dosage during Phase II \[r(10)=-0.71, \ p<0.05\], but not during Phase I.
Statistical Analyses Comparing Experiment I to Experiment II

Nicotine consumption (mL), nicotine intake as a percent of total fluid intake (%), nicotine dosage (mg/kg), serum cotinine levels (ng/ml/g), liver weight (g) and gene expression levels of CYP2a5 and CYP2e1 were outcome measures for both experiments. Therefore, the last day of Experiment I and II were compared using three-way ANOVAs with age, drug treatment and experiment (3 levels) as independent variables, in order to examine effects of 12-hour (Experiment I) and 4-day (Experiment II) methoxsalen exposure in adolescent and adult mice on the dependent measures.

Total nicotine consumption (mL). Figure 30 presents mean total nicotine consumption (mL) for mice at the end of Experiment I and II. Adults consumed more nicotine (mL) at the end of both studies compared to adolescents \(F(1,100)=27.50, p<0.05\). More nicotine was consumed at the end of Experiment II compared to Experiment I \(F(1,100)=51.98, p<0.05\). An Age X Experiment interaction also was observed \(F(1,100)=16.91, p<0.05\). Adolescents \(F(1,50)=69.73, p<0.05\) and adults \(F(1,50)=4.45, p<0.05\) consumed more nicotine (mL) at the end of Experiment II in contrast to Experiment I. During Experiment I, adults consumed more nicotine (mL) than did adolescents \(F(1,42)=26.39, p<0.05\). There was no main effect for Drug or Drug X Age, Experiment X Drug, Experiment X Age, or Experiment X Age X Drug interactions for total nicotine consumption (mL).

Nicotine intake as a percent of total fluid intake (%). Figure 31 presents mean total nicotine intake as a percent of total fluid intake (%) for mice at the end of Experiment I and II. Adults consumed more nicotine (%) at the end of both studies
compared to adolescents $[F(1, 100)=25.76, \ p<0.05]$. There also was a main effect for Experiment, such that animals consumed more nicotine (%) at the end of Experiment II compared to Experiment I $[F(1, 100)=5.66, \ p<0.05]$.

An Age X Experiment interaction also was observed $[F(1, 100)=13.14, \ p<0.05]$. Although % nicotine intake did not differ at the end of the 2 experiments among adults, adolescents consumed more nicotine (%) at the end of Experiment II compared to Experiment I $[F(1, 50)=15.59, \ p<0.05]$. At the end of Experiment I, adults consumed more nicotine (%) did than adolescents $[F(1, 42)=18.91, \ p<0.05]$. There was no main effect for Drug or Drug X Age, Experiment X Drug, Experiment X Age, or Experiment X Age X Drug interactions for total nicotine intake (%).

**Total nicotine dosage (mg/kg).** Figure 32 presents mean total nicotine dosage (mg/kg) for mice at the end of Experiment I and II. A main effect for Experiment was observed, such that animals consumed more nicotine (mg/kg) at the end of Experiment II $[F(1, 100)=108.17, \ p<0.05]$. There was no overall main effect for age; however, adolescents $[F(1, 50)=107.41, \ p<0.05]$ and adults $[F(1, 50)=12.51, \ p<0.05]$ consumed more nicotine (mg/kg) at the end of Experiment II compared to Experiment I $[F(1, 100)=37.29, \ p<0.05]$. During Experiment I $[F(1, 42)=23.46, \ p<0.05]$ and II $[F(1, 58)=14.31, \ p<0.05]$, adults consumed more nicotine (mg/kg) compared to adolescents $[F(1, 42)=20.20, \ p<0.05]$. There was no main effect for Age or Drug and no Drug X Age, Experiment X Drug, Experiment X Age, or Experiment X Age X Drug interactions for total nicotine dosage (mg/kg).

**Liver weight (g).** Figure 33 presents mean liver weight (g) for mice at the end of Experiment I and II. Overall, adults had larger livers compared to adolescents
There also was an Experiment X Age interaction $[F(1,102)=26.49, \ p<0.05]$. Although Experiment did not affect liver weight in adolescents, adults had larger livers in Experiment I $[F(1,44)=4.54, \ p<0.05]$ and II $[F(1,58)=28.30, \ p<0.05]$ than did adolescents. There was no main effect for Drug or Experiment and no Drug X Age, Experiment X Drug, or Experiment X Age X Drug interactions for liver weight (g).

**Ratio of liver weight to body weight.** Adolescents had a higher liver to body weight ratio compared to adults $[F(1,102)=84.97, \ p<0.05]$. Additionally, animals in Experiment II had a higher liver to body weight ratio than did those in Experiment I, regardless of age or methoxsalen exposure $[F(1,102)=14.91, \ p<0.05]$. There was also an Experiment X Drug interaction $[F(1,102)=4.99, \ p<0.05]$. Although Drug treatment did not affect the liver to body weight ratio at the end of Experiment I, animals exposed to methoxsalen had a higher liver to body weight ratio compared to the vehicle group at the end of Experiment II $[F(1,58)=5.91, \ p<0.05]$. Further, animals in the methoxsalen group had a higher liver to body weight ratio after Experiment II compared to Experiment I $[F(1,52)=21.37, \ p<0.05]$. There was no main effect for Drug and no Experiment X Age, Age X Drug, or Experiment X Age X Drug interactions.

**Serum cotinine levels (ng/ml/g).** Figure 34 presents mean serum cotinine levels (ng/ml/g) for mice at the end of Experiment I and II. Regardless of experiment, there was a significant Age effect, such that adults had higher serum cotinine levels (ng/ml/g) compared to adolescents $[F(1,102)=27.59, \ p<0.05]$. Overall, serum cotinine levels (ng/ml/g) in vehicle group were higher compared to levels in the 8MOP group $[F(1,102)=3.882, \ p=0.05]$. In addition, there was a Drug X Age interaction across both
experiments for serum cotinine levels (ng/ml/g) \( [F(1,102)=8.15, p<0.05] \). More specifically, adults in the 8MOP group had higher serum cotinine levels after adjusting for body weight compared to animals in the vehicle group \( [F(1,50)=9.18, p<0.05] \). This result was not observed in adolescents. An Age effect was absent in the 8MOP group; however, adults in the vehicle group had higher serum cotinine levels after adjusting for liver weight compared to their adolescent counterparts \( [F(1,50)=42.23, p<0.05] \). There was no main effect for Experiment and no Experiment X Drug, Experiment X Age, or Experiment X Age X Drug interactions for serum cotinine levels (ng/ml/g).

**Hepatic CYP gene expression.** Figures 35 and 36 present mean gene expression levels for CYP2a5 and CYP2e1 for adolescent and adult mice in the vehicle and 8MOP groups at the end of Experiment I and II, respectively. Across studies, adults had higher levels of CYP2a5 compared to adolescents \( [F(1,39)=7.164, p<0.05] \). Animals had higher levels of CYP2a5 following Experiment I compared to that of Experiment II \( [F(1,39)=14.626, p<0.05] \). CYP2e1 gene expression, on the other hand, did not differ by age or experiment.

There was no main effect for Drug and no Age X Experiment, Drug X Experiment or Drug X Age X Experiment interactions for CYP2a5 levels when comparing Experiment I to II. No main effects for Age, Drug or Experiment and no Drug X Age, Experiment X Drug, Experiment X Age, Experiment X Drug X Age interactions were observed for CYP2e1 levels when comparing Experiments I and II.

Figure 37 presents the correlation between total nicotine dosage (mg/kg) and mean gene expression levels for CYP2a5 based on 18S gene expression levels at the end of Experiment I and II. CYP2a5 gene expression levels were negatively correlated with
total nicotine dosage \[r(45)=-0.33, \ p<0.05\], both in adolescents \[r(22)=-0.42, \ p<0.05\]
and adults \[r(21)=-0.45, \ p<0.05\].

**Confirmation of Hypotheses**

**Hypothesis 1: Age, Methoxsalen Exposure and Body Weight (g)**

The first hypothesis stating that mice receiving methoxsalen would not gain as much weight (g) as control mice was **not supported**.

An additional hypothesis stating that adults would weigh more (g) than adolescents was **supported**.

**Hypothesis 2: Age, Methoxsalen Exposure and Nicotine Consumption (mL)**

It was hypothesized that mice receiving methoxsalen would consume more nicotine compared to control mice; however, this hypothesis was **not supported**, as there were no differences in nicotine consumption between drug treatment groups.

Similarly, the hypothesis that adolescents would consume more nicotine than adults (mL) was also **not supported**. In fact, adults consumed significantly more nicotine (mL) than did adolescents.

The third hypothesis involved an Age X Drug interaction, such that adolescents exposed to methoxsalen would consume more nicotine (mL) compared to control mice, whereas adult mice that receive methoxsalen will consume less nicotine (mL). Both of these hypotheses were **not supported**. There were no differences between drug treatment groups among either age group.
Hypothesis 3: Age, Methoxsalen Exposure and Nicotine Dosage (mg/kg)

The hypothesis that mice treated with methoxsalen will consume more nicotine per unit body weight when compared to control mice was not supported. There was no difference in nicotine consumption among drug treatment groups.

Similarly, the second hypothesis stating that adolescents will consume more nicotine than adults, even after adjusting for body weight (mg/kg) was supported in Phase I and Phase II.

The hypothesis that adolescents exposed to methoxsalen would consume more nicotine (mg/kg) compared to control mice, whereas adult mice that receive methoxsalen will consume less nicotine (mg/kg) was also not supported. There were no differences between drug treatment groups among either age group.

Hypothesis 4: Methoxsalen Exposure and Serum Cotinine Levels (ng/ml/g)

It was hypothesized that methoxsalen would reduce serum cotinine levels compared to the control; this hypothesis was not supported; however, it is important to note that the drug treatment effect on serum cotinine levels (ng/ml/g) was trending toward significance among adults. No such trend was observed in adolescents.

Hypothesis 5: Age, Methoxsalen Exposure and RNA Levels of Hepatic CYP enzymes

The hypothesis that methoxsalen would increase CYP RNA levels was not supported. There was no effect of age or drug treatment on relative gene expression levels of CYP2a5 or CYP2e1.

Conclusions

The purpose of Experiment II was to expand upon Experiment I and examine nicotine consumption following chronic methoxsalen administration in adolescent and
adult male C57BL/6J mice. To my knowledge, this is the first study to examine the effects of long-term methoxsalen exposure on nicotine consumption and metabolism in C57BL/6J mice, and in both adult and adolescent mice, regardless of strain.

Adolescents had higher liver to body weight ratios compared to adults, which was expected according to a study done by Urata and colleagues in 1995 to determine liver volumes for liver transplant patients. In addition to age, drug treatment also affected liver to body weight ratios. More specifically, animals receiving methoxsalen had higher liver weight to body weight ratios compared to those receiving vehicle treatments. Oral methoxsalen given over 4 weeks has been previously found to induce histological alterations of the liver of adult C57BW mice (i.e., enlarged centrolobular hepatocytes as well as eosinophilic cytoplasm; Diawara et al., 2000). Therefore, it is possible that methoxsalen can induce hepatomegaly even after 4 days of exposure. Additional studies are necessary to detect how quickly toxic effects of methoxsalen affect the liver and other organs, and the extent of the toxicity following long-term methoxsalen exposure.

As expected, adult mice weighed more and consumed more food (g) than did adolescents during Phase I and II. Interestingly, adolescents consumed more water compared to adults during Phase I, but an age difference in water consumption was absent during Phase II. Because adults did not increase water consumption between Phase I and II, the lack of an age difference during Phase II indicates that adolescents, but not adults, reduced their water intake. It is important that future studies investigate the validity of this claim; a reduction in water consumption increases the risk for dehydration, which presents a health risk for younger patients taking methoxsalen as a smoking cessation aid.
Nicotine consumption (mL) was not affected by age or drug treatment. Given that methoxsalen is a CYP2a5 inhibitor, the lack of an effect of methoxsalen on nicotine consumption (mL) was unexpected, especially in adults. The surprise concerning these findings was primarily focused on adults because no studies examining the effects of methoxsalen on nicotine consumption have been conducted in adolescents. There are several possibilities as to why methoxsalen did not induce the predicted effects on nicotine consumption. First, nicotine pretreatment (Phase I) may have altered the effects of methoxsalen on nicotine consumption during Phase II. The design of Experiment II was based on a study conducted by Sellers, Kaplan and Tyndale in 2000 that concluded that oral methoxsalen reduced nicotine cravings and cigarette consumption by 24% in chronic adult male and female smokers (Sellers et al., 2000). Although subjects in Experiment II and in the Sellers study (2000) consumed nicotine before methoxsalen exposure, there are several factors that could account for the different findings between the two studies. The study design used by Sellers and colleagues (2000) examined the effects of a single oral dose of encapsulated methoxsalen on cigarette consumption in adult male and female chronic smokers. Experiment II, on the other hand, assessed the effects of chronic, subcutaneous injections of methoxsalen on nicotine consumption in adolescent and adult male C57BL/6J mice. Subcutaneous route of drug administration was chosen because this technique previously was used to administer methoxsalen in mice (Damaj et al., 2007) and has less of a chance of injury and/or death compared to oral gavage, the administration route used in the study conducted by Raunio and colleagues (2008). Even though human CYP2A6 and mouse CYP2a5 are functionally and structurally similar, human behaviors following exposure to methoxsalen could differ
from that of mice. For example, participants in Sellers and colleagues’ (2000) study were tobacco users. Tobacco has 4000+ compounds beyond that of nicotine, including nitrosamines, that are metabolized by methoxsalen (e.g., NNK; Kamataki, Fujieda, Kiyotani, Iwano, & Kunitoh, 2005; Sellers et al., 2003a; Takeuchi et al., 2006; Tutka et al., 2005). Use of tobacco, as opposed to pure nicotine, may therefore have an altered tobacco smoking behavior in response to methoxsalen exposure, leading to a reduction in nicotine consumption not observed in mice. The methods in which methoxsalen was prepared in the two studies [i.e., 8MOP capsules in Sellers et al., (2000) versus subcutaneous injection in the current study] also may have resulted in significantly different pharmacokinetic factors, which also could alter the effects of methoxsalen on nicotine intake. In short, future studies should examine these variables before any conclusions are made as to the validity of methoxsalen as a smoking cessation aid.

Another potential reason for the lack of an effect of drug treatment on nicotine consumption (mL) is that the exposure period for methoxsalen, 4 days, may have been too short (or too long), to influence nicotine consumption in this experiment. Because the other two murine studies (e.g., Damaj et al., 2007; Raunio et al., 2008) that examined the relationship between methoxsalen and nicotine exposed mm to these drugs for less than a day, there was no precedent for how many times methoxsalen would have to be administered to achieve or maintain an effect on nicotine intake. Therefore, additional studies should determine if once-a-day methoxsalen treatments are appropriate and, if not, how many times per day or week would achieve the desired effect of reducing nicotine consumption (mL).
Third, the methoxsalen dosage used may have been too low or too high to observe an effect on nicotine intake. Although the methoxsalen dosage was selected on previous research (Damaj et al., 2007) and results from Experiment I, Damaj et al. (2007) used adult male ICR mice. Therefore, it may be possible that this dosage was optimal to cause nicotine-induced behaviors in ICR mice, but may not be the most effective dosage for C57BL/6J male mice. This hypothesis indicates that a higher (> 10 mg/kg) dosage should be included to evaluate a dose-response effect on nicotine consumption. Future studies also should include lower methoxsalen dosages in order to fully delineate a dose-response curve. Because there is no precedent for using dosages above 10 mg/kg of methoxsalen in mouse studies, investigators conducting these studies should be alert for any potential toxic effects, with special attention to hepatic pathology. Subcutaneous injections of 10 mg/kg for 4 days did not induce any pathological changes in liver physiology. I observed some hair loss among most mice during the course of the study; however, the amount fell within the normal range for C57BL/6J mice, according to David Bienus, Research Support Specialist at the Pennsylvania State University (personal communication, September 22, 2010) and it did not appear to be any greater among methoxsalen-exposed mice. Along these lines, the photosensitivity of methoxsalen can be a limiting factor in these studies, especially long-term experiments. Patients taking methoxsalen as a dermatologic treatment are instructed wear long sleeves, dark sunglasses, and suntan lotion when going outside or even sitting by a sunny window because sunlight can react with methoxsalen and cause additional problems (e.g., sunburn). Although the effects of methoxsalen seem to occur with only sunlight or UV
radiation, areas of the body constantly exposed to any kind of light should be closely monitored in any future studies to prevent undo harm to the animal or human.

Finally, the strain and sex of the mice used in this experiment could have resulted in a lack of methoxsalen effect on nicotine consumption. According to previous studies, adult male ICR (Damaj et al., 2007) and adult female CD2F1 mice (Raunio et al., 2008) respond as expected to methoxsalen, at least on a pharmacokinetic level. Therefore, additional studies should conduct this study again in ICR and CD2F1 mice as well as other strains of mice (e.g., DBA/2) to not only see if the effects on nicotine consumption (mL, % total fluid intake, mg/kg) shown here are observed in different strains, but also to mirror individual differences among human populations. Additionally, females should also be involved in this study, as pharmacokinetics (Harrod, Booze, & Mactutus, 2007), pharmacodynamics (Dluzen & Anderson, 1997; Rosecrans, 1971; 1972) and behaviors associated with nicotine vary between the sexes (Chaudhri et al., 2005; Donny et al., 2000; Klein et al., 2003, Klein et al., 2004; Roth, Cosgrove, & Carroll, 2004).

Regardless of the lack of age differences in nicotine intake (mL), adults consumed more nicotine (%) than did adolescents. Because % nicotine intake is based on the combination of nicotine and water consumption (mL), the increase in nicotine consumption (%) for adults is probably due to adolescents consuming significantly more water comparatively during Phase I. Additional evidence for this hypothesis lies within Phase II data, where adolescents and adults consumed similar amounts of nicotine (%) and water. Adolescents consumed significantly more nicotine per unit body weight (mg/kg) than did adults, regardless of drug treatment during Phase I and II. Adults and adolescents consumed similar volumes of nicotine (mL); however, due to the smaller
body weight, adolescents drank more nicotine per unit body weight than did adults. Additional studies on nicotine-induced behaviors, and nicotine pharmacodynamics and pharmacokinetics between adolescents and adults would be essential to determine what an increase in nicotine exposure during adolescence might do to increase nicotine use later in life. This information also may provide information as to why individuals who begin smoking during adolescence have a more difficult time quitting compared to those that initiate the habit during adulthood.

Experiment II also replicated the effect of drug treatment on serum cotinine levels (ng/ml/g) observed in Experiment I, such that adult mice in the vehicle group displayed higher serum cotinine levels (ng/ml/g) than did adult mice receiving 8MOP; this drug effect on cotinine levels was not observed among adolescent mice. The most important aspect about this finding is that it was replicated across the two experiments with different experimental designs. Serum cotinine levels were reduced in adults, but not adolescents, when methoxsalen was given before (Experiment I) and after nicotine access (Experiment II). This finding suggests that the age-dependent inhibition of methoxsalen on CYP2a5 activity is very robust. The reduction of serum cotinine levels in adults also replicates findings from Damaj et al. (2007) and Raunio et al. (2008) in the plasma of adult male ICR and urine of adult female CD2F1 mice, respectively.

Another intriguing finding is the effect of age, but not drug treatment, on relative gene expression levels of CYP2a5. Specifically, adults had higher CYP2a5 levels than did adolescents. This is the first study to demonstrate age-dependent levels of CYP2a5 following chronic nicotine self-administration in male C57BL/6J mice. It currently is unknown how levels of CYP2a5 gene expression and activity change with age. If
CYP2a5 levels are higher in adults, this finding indicates that gene expression for this enzyme increases with age, which could translate to individuals being able to metabolize nicotine more effectively as the duration of nicotine exposure increases. Interestingly, human data suggest that nicotine clearance and metabolism actually decrease with age (Molander et al., 2001; Sotaniemi et al., 1996). Clearance of nicotine was not examined in this experiment; therefore, methods, such as gas chromatography-mass spectrometry and use of metabolic cages, should be employed to assess nicotine elimination rate changes over time in future murine studies. Regarding enzyme levels, no age differences in CYP2A6 protein levels have been observed (Messina et al., 1997; Shimada et al., 1994). Protein levels of CYP2a5 were not examined in Experiment I or II; longitudinal studies should therefore investigate changes in CYP2a5 gene expression and protein levels over time in mice, specifically C57BL/6J mice, respectively, both with and without nicotine exposure.

Age and drug treatment did not alter gene expression levels of CYP2e1, which is interesting because this enzyme is weakly inactivated by methoxsalen and plays a minor role in nicotine metabolism. It is possible that a weak inhibition of CYP2e1 was not enough to induce any change in gene expression. It currently is unknown whether other enzymes beyond these two are affected by methoxsalen and nicotine exposure. Albeit to a far lesser extent, other CYP enzymes, including CYP2A7, -2D6 and -2A13 (Mwenifumbo & Tyndale, 2009), as well as flavin-containing monooxygenase 3 (FMO3), amine N-methyltransferase, UDP-glucuronosyltransferases and aldehyde oxidase, are responsible for a small percentage of nicotine metabolism (for review, see Hukkanen, Jacob, & Benowitz, 2005). Therefore, future studies should examine activity
levels of these enzymes to develop a better understanding of how these enzymes are involved with nicotine metabolism. Along those lines, it is important to know how the activity level of any of these enzymes changes over time. Therefore, a long-term study where liver samples are examined in a subset of animals exposed to nicotine only, methoxsalen only, and a combination of methoxsalen and nicotine every couple of days to assess potential change over time would be advantageous.

When examined together, the sum of these findings are exciting. Adolescents consumed more nicotine (mg/kg) compared to adults; however, adults displayed higher serum cotinine levels (ng/ml/g) that also were affected by methoxsalen. These two findings are new and make an important contribution to the literature. Together, these results suggest that adults have a faster nicotine metabolic rate or a slower rate of nicotine elimination. Relative gene expression levels of CYP2a5 were higher in adults compared to adolescents, which suggests that adults do have a higher rate of nicotine metabolism compared to adolescents. This is the first study to demonstrate age-dependent levels of CYP2a5 with and without methoxsalen in male C57BL/6J mice. Addition of methoxsalen back into the equation suggests that this drug affects CYP2a5 on a functional level, but does not appear to change gene expression or behaviors associated with changes in this enzyme activity, namely nicotine consumption, at least among male C57BL/6J mice. Additional studies are needed to investigate the effects of methoxsalen in female mice, both of this and other strains (e.g., DBA/2, ICR, CD2F1) to assess the reliability and validity of these preliminary findings.

Regardless of methoxsalen exposure, it appears that adolescent male C57BL/6J mice consumed significantly more nicotine (mg/kg), yet had lower levels of CYP2a5
gene expression, compared to adult counterparts. Although methoxsalen did not affect nicotine intake behavior, serum cotinine levels (ng/ml/g) were significantly reduced in adults, but not adolescents. The juxtaposing findings, whereby age significantly affects how nicotine is metabolized and methoxsalen produces age-dependent effects on CYP2a5, but only at a functional level, are intriguing. These effects need to be replicated in different mouse strains, as previous mouse studies used ICR (Damaj et al., 2007) and CD2F1 mice (Raunio et al., 2008) and the present research is the first to use C57BL/6J mice. In addition, because of sex differences in ability to quit smoking (for review, see Perkins, Donny & Caggiula, 1999), future studies should include females of different mouse strains. Similarly, because the only published study in adult human smokers lasted one day (Sellers et al., 2000), it is currently unknown whether these effects can be replicated in humans, both adolescent and adult. Therefore, these age and drug treatment effects on nicotine consumption and metabolism demands further long-term investigation to see methoxsalen could become a smoking cessation aid.
<table>
<thead>
<tr>
<th>Age</th>
<th>Vehicle</th>
<th>10 mg/kg 8MOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adolescent</td>
<td>N = 16</td>
<td>N = 16</td>
</tr>
<tr>
<td>Adult</td>
<td>N = 16</td>
<td>N = 16</td>
</tr>
</tbody>
</table>

3 Bottle Choice  
(tap water, 50 and 200 ug/ml NIC solutions)

Total N = 64 animals

Table 9. Experiment II Design.
<table>
<thead>
<tr>
<th></th>
<th>Day 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th>Day 3&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th>Day 4&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th></th>
<th>Day 5</th>
<th></th>
</tr>
</thead>
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<tr>
<td></td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td></td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td></td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td></td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td></td>
</tr>
<tr>
<td>Adolescent</td>
<td>17.55 ± 0.28</td>
<td>17.35 ± 0.21</td>
<td>17.71 ± 0.24</td>
<td>17.50 ± 0.21</td>
<td>17.84 ± 0.23</td>
<td>17.57 ± 0.21</td>
<td>17.89 ± 0.24</td>
<td>17.59 ± 0.22</td>
</tr>
<tr>
<td>Adult*</td>
<td>22.88 ± 0.34</td>
<td>22.70 ± 0.31</td>
<td>22.68 ± 0.29</td>
<td>22.78 ± 0.31</td>
<td>22.87 ± 0.30</td>
<td>23.06 ± 0.34</td>
<td>22.59 ± 0.31</td>
<td>22.93 ± 0.33</td>
</tr>
</tbody>
</table>

Table 10. Mean body weight (g) for all animals during oral voluntary nicotine consumption (choice) treatment test (Phase I) among adolescent (n=32) and adult (n=32) male C57BL/6J mice (means ± standard error of the mean).

<sup>a</sup>Bonferroni post hoc tests: Differs from 2
<sup>b</sup>Bonferroni post hoc tests: Differs from 3
<sup>c</sup>Bonferroni post hoc tests: Differs from 4
<sup>d</sup>Bonferroni post hoc tests: Differs from 5

*Overall main effect for age: Adults > Adolescents; p<0.05
<table>
<thead>
<tr>
<th></th>
<th>Day 1&lt;sup&gt;b,c,d&lt;/sup&gt;</th>
<th>Day 2&lt;sup&gt;a,c&lt;/sup&gt;</th>
<th>Day 3&lt;sup&gt;a,b,d&lt;/sup&gt;</th>
<th>Day 4&lt;sup&gt;a,c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle 10 mg/kg</td>
<td>5.29 ± 0.12</td>
<td>5.30 ± 0.11</td>
<td>4.32 ± 0.08</td>
<td>4.54 ± 0.10</td>
</tr>
<tr>
<td>8MOP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle 10 mg/kg</td>
<td>3.57 ± 0.09</td>
<td>3.65 ± 0.08</td>
<td>4.42 ± 0.12</td>
<td>4.63 ± 0.16</td>
</tr>
<tr>
<td>8MOP</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adolescent</td>
<td>5.64 ± 0.16</td>
<td>5.06 ± 0.10</td>
<td>4.73 ± 0.11</td>
<td>4.72 ± 0.16</td>
</tr>
<tr>
<td>Adult*</td>
<td>4.36 ± 0.21</td>
<td>4.35 ± 0.28</td>
<td>5.20 ± 0.27</td>
<td>5.17 ± 0.28</td>
</tr>
</tbody>
</table>

Table 11. Mean food consumption (g) for all animals during oral voluntary nicotine consumption (choice) treatment test (Phase I) among adolescent (n=32) and adult (n=32) male C57BL/6J mice (means ± standard error of the mean).

<sup>a</sup>Bonferroni post hoc tests: Differs from 1
<sup>b</sup>Bonferroni post hoc tests: Differs from 2
<sup>c</sup>Bonferroni post hoc tests: Differs from 3
<sup>d</sup>Bonferroni post hoc tests: Differs from 4

*Overall main effect for age: Adults > Adolescents; p<0.05
Table 12. Mean water intake (mL) and total fluid intake (mL) for all animals during oral voluntary nicotine consumption (choice) treatment test (Phase I) among adolescent (n=32) and adult (n=32) male C57BL/6J mice (means ± standard error of the mean).

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>10 mg/kg 8MOP</td>
<td>Vehicle</td>
<td>10 mg/kg 8MOP</td>
</tr>
<tr>
<td><strong>Water intake (mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adolescent</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.20 ± 0.38</td>
<td>5.61 ± 0.12</td>
<td>5.13 ± 0.35</td>
<td>5.37 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>5.18 ± 0.36</td>
<td>5.32 ± 0.11</td>
<td>5.17 ± 0.43</td>
<td>5.59 ± 0.14</td>
</tr>
<tr>
<td>Adult*</td>
<td>4.37 ± 0.30</td>
<td>4.04 ± 0.29</td>
<td>4.13 ± 0.37</td>
<td>4.48 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>4.86 ± 0.17</td>
<td>4.65 ± 0.22</td>
<td>4.36 ± 0.30</td>
<td>4.53 ± 0.32</td>
</tr>
<tr>
<td><strong>Total fluid intake (mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adolescent*</td>
<td>7.39 ± 0.21</td>
<td>7.61 ± 0.25</td>
<td>7.68 ± 0.19</td>
<td>8.34 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>8.59 ± 0.23</td>
<td>8.69 ± 0.15</td>
<td>6.90 ± 0.25</td>
<td>7.22 ± 0.24</td>
</tr>
<tr>
<td>Adult</td>
<td>6.67 ± 0.19</td>
<td>6.58 ± 0.14</td>
<td>7.31 ± 0.20</td>
<td>7.43 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>7.54 ± 0.18</td>
<td>7.68 ± 0.33</td>
<td>6.38 ± 0.20</td>
<td>6.76 ± 0.35</td>
</tr>
</tbody>
</table>

*Bonferroni post hoc tests: Differs from 1
*bBonferroni post hoc tests: Differs from 2
*cBonferroni post hoc tests: Differs from 3
*dBonferroni post hoc tests: Differs from 4

*Overall main effect for age: Adults > Adolescents; p<0.05
(2)*Overall main effect for age: Adolescents > Adults; p<0.05
<table>
<thead>
<tr>
<th></th>
<th>Day 1&lt;sup&gt;b,c,d&lt;/sup&gt;</th>
<th>Day 2&lt;sup&gt;a,d&lt;/sup&gt;</th>
<th>Day 3&lt;sup&gt;a,d&lt;/sup&gt;</th>
<th>Day 4&lt;sup&gt;b,c,d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>10 mg/kg 8MOP</td>
<td>Vehicle</td>
<td>10 mg/kg 8MOP</td>
</tr>
<tr>
<td><strong>Adolescent</strong></td>
<td>2.20 ± 0.30</td>
<td>2.00 ± 0.22</td>
<td>2.55 ± 0.23</td>
<td>2.98 ± 0.68</td>
</tr>
<tr>
<td><strong>Adult</strong></td>
<td>2.27 ± 0.25</td>
<td>2.53 ± 0.27</td>
<td>3.18 ± 0.32</td>
<td>2.95 ± 0.16</td>
</tr>
</tbody>
</table>

Table 13. Mean total nicotine consumption (mL) during oral voluntary nicotine consumption (choice) treatment test (Phase I) in adolescent (n=32) and adult (n=32) male C57BL/6J mice (means ± standard error of the mean).

<sup>a</sup>Bonferroni post hoc tests: Differs from 1  
<sup>b</sup>Bonferroni post hoc tests: Differs from 2  
<sup>c</sup>Bonferroni post hoc tests: Differs from 3  
<sup>d</sup>Bonferroni post hoc tests: Differs from 4
<table>
<thead>
<tr>
<th>Day</th>
<th>Vehicle 10 mg/kg 8MOP</th>
<th>Vehicle 10 mg/kg 8MOP</th>
<th>Vehicle 10 mg/kg 8MOP</th>
<th>Vehicle 10 mg/kg 8MOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>30.38±4.98</td>
<td>25.62±2.00</td>
<td>34.11±4.23</td>
<td>32.41±3.14</td>
</tr>
<tr>
<td>Day 2</td>
<td>34.42±4.21</td>
<td>38.62±4.24</td>
<td>43.95±4.89</td>
<td>39.69±2.01</td>
</tr>
<tr>
<td>Day 3</td>
<td>35.00±1.61</td>
<td>38.84±2.75</td>
<td>32.07±4.34</td>
<td>33.79±3.83</td>
</tr>
<tr>
<td>Day 4</td>
<td>30.06±3.14</td>
<td>38.58±1.33</td>
<td>25.77±5.06</td>
<td>22.11±1.80</td>
</tr>
</tbody>
</table>

Table 14. Mean total nicotine intake as a percent of total fluid intake (%) during oral voluntary nicotine consumption (choice) treatment test (Phase I) in adolescent (n=32) and adult (n=32) male C57BL/6J mice (means ± standard error of the mean).

- Bonferroni post hoc tests: Differs from Day 1
- Bonferroni post hoc tests: Differs from Day 2
- Bonferroni post hoc tests: Differs from Day 3
- Bonferroni post hoc tests: Differs from Day 4

*Overall main effect for age: Adults > Adolescents; p<0.05
<table>
<thead>
<tr>
<th></th>
<th>Day 1&lt;sup&gt;b,c&lt;/sup&gt;</th>
<th>Day 2&lt;sup&gt;a,d&lt;/sup&gt;</th>
<th>Day 3&lt;sup&gt;a,d&lt;/sup&gt;</th>
<th>Day 4&lt;sup&gt;b,c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total nicotine dosage (mg/kg)</td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td>Vehicle 10 mg/kg 8MOP</td>
</tr>
<tr>
<td>Adolescent*</td>
<td>12.96 ± 1.97</td>
<td>13.31 ± 2.58</td>
<td>17.72 ± 1.06</td>
<td>24.17 ± 7.14</td>
</tr>
<tr>
<td>Adult</td>
<td>10.12 ± 1.26</td>
<td>10.79 ± 0.85</td>
<td>15.53 ± 1.11</td>
<td>15.62 ± 0.86</td>
</tr>
</tbody>
</table>

Table 15. Mean total nicotine dosage (mg/kg) during oral voluntary nicotine consumption (choice) treatment test (Phase I) in adolescent (n=32) and adult (n=32) male C57BL/6J mice (means ± standard error of the mean).

<sup>a</sup>Bonferroni post hoc tests: Differs from Day 1
<sup>b</sup>Bonferroni post hoc tests: Differs from Day 2
<sup>c</sup>Bonferroni post hoc tests: Differs from Day 3
<sup>d</sup>Bonferroni post hoc tests: Differs from Day 4

*Overall main effect for age: Adults > Adolescents; p<0.05
<table>
<thead>
<tr>
<th></th>
<th>Day 6&lt;sup&gt;c,d&lt;/sup&gt;</th>
<th>Day 7&lt;sup&gt;c,d&lt;/sup&gt;</th>
<th>Day 8&lt;sup&gt;a,b,d&lt;/sup&gt;</th>
<th>Sacrifice&lt;sup&gt;a,b,c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td>Vehicle 10 mg/kg 8MOP</td>
</tr>
<tr>
<td>Adolescent</td>
<td>18.20 ± 0.26</td>
<td>17.84 ± 0.23</td>
<td>18.29 ± 0.24</td>
<td>17.76 ± 0.22</td>
</tr>
<tr>
<td>Adult*</td>
<td>22.79 ± 0.36</td>
<td>23.11 ± 0.32</td>
<td>22.92 ± 0.38</td>
<td>23.14 ± 0.31</td>
</tr>
</tbody>
</table>

Table 16. Mean body weight (g) for all animals during methoxsalen treatment test (Phase II) in adolescent (n=32) and adult (n=30) male C57BL/6J mice (means ± standard error of the mean).

<sup>a</sup>Bonferroni post hoc tests: Differs from 6  
<sup>b</sup>Bonferroni post hoc tests: Differs from 7  
<sup>c</sup>Bonferroni post hoc tests: Differs from 8  
<sup>d</sup>Bonferroni post hoc tests: Differs from sacrifice  

*Overall main effect for age: Adults > Adolescents; p<0.05
<table>
<thead>
<tr>
<th></th>
<th>Day 5&lt;sup&gt;b,c,d&lt;/sup&gt;</th>
<th>Day 6&lt;sup&gt;b,c,d&lt;/sup&gt;</th>
<th>Day 7&lt;sup&gt;b,c,d&lt;/sup&gt;</th>
<th>Day 8&lt;sup&gt;b,c,d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td>Vehicle 10 mg/kg 8MOP</td>
<td>Vehicle 10 mg/kg 8MOP</td>
</tr>
<tr>
<td>Adolescent</td>
<td>4.08 ± 0.15</td>
<td>4.05 ± 0.25</td>
<td>4.45 ± 0.08</td>
<td>4.40 ± 0.18</td>
</tr>
<tr>
<td>Adult*</td>
<td>4.21 ± 0.32</td>
<td>4.38 ± 0.24</td>
<td>5.70 ± 0.28</td>
<td>4.97 ± 0.24</td>
</tr>
</tbody>
</table>

Table 17. Mean food consumption (g) for all animals during methoxsalen treatment test (Phase II) in adolescent (n=32) and adult (n=30) male C57BL/6J mice (means ± standard error of the mean).

<sup>a</sup>Bonferroni post hoc tests: Differs from 5  
<sup>b</sup>Bonferroni post hoc tests: Differs from 6  
<sup>c</sup>Bonferroni post hoc tests: Differs from 7  
<sup>d</sup>Bonferroni post hoc tests: Differs from 8

<sup>*</sup>Overall main effect for age: Adults > Adolescents; p<0.05
<table>
<thead>
<tr>
<th></th>
<th>Day 5&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Day 6&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Day 7&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Day 8&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>10 mg/kg</td>
<td>Vehicle</td>
<td>10 mg/kg</td>
</tr>
<tr>
<td></td>
<td>8MOP</td>
<td>8MOP</td>
<td>8MOP</td>
<td>8MOP</td>
</tr>
<tr>
<td>Water intake (mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adolescent</td>
<td>5.10 ± 0.15</td>
<td>4.61 ± 0.26</td>
<td>4.53 ± 0.17</td>
<td>4.17 ± 0.10</td>
</tr>
<tr>
<td>Adult</td>
<td>4.35 ± 0.30</td>
<td>4.65 ± 0.35</td>
<td>4.23 ± 0.29</td>
<td>4.17 ± 0.28</td>
</tr>
<tr>
<td>Total fluid intake (mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adolescent</td>
<td>9.39 ± 0.53</td>
<td>8.73 ± 0.32</td>
<td>8.32 ± 0.23</td>
<td>9.27 ± 1.33</td>
</tr>
<tr>
<td>Adult</td>
<td>9.11 ± 1.22</td>
<td>8.78 ± 0.53</td>
<td>7.87 ± 0.27</td>
<td>7.59 ± 0.23</td>
</tr>
</tbody>
</table>

Table 18. Mean water intake (mL) and total fluid intake (mL) for all animals during methoxsalen treatment test (Phase II) in adolescent (n=32) and adult (n=30) male C57BL/6J mice (means ± standard error of the mean).

<sup>a</sup>Bonferroni post hoc tests: Differs from 5
<sup>b</sup>Bonferroni post hoc tests: Differs from 6
<sup>c</sup>Bonferroni post hoc tests: Differs from 7
<sup>d</sup>Bonferroni post hoc tests: Differs from 8
<table>
<thead>
<tr>
<th>Day</th>
<th>Adolescent</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.29 ± 0.56</td>
<td>4.75 ± 1.19</td>
</tr>
<tr>
<td></td>
<td>4.12 ± 0.33</td>
<td>4.13 ± 0.36</td>
</tr>
<tr>
<td>6</td>
<td>3.79 ± 0.25</td>
<td>3.65 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>5.10 ± 1.32</td>
<td>3.42 ± 0.28</td>
</tr>
<tr>
<td>7</td>
<td>2.98 ± 0.20</td>
<td>3.55 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>2.87 ± 0.18</td>
<td>3.67 ± 0.22</td>
</tr>
<tr>
<td>8</td>
<td>4.41 ± 0.19</td>
<td>4.99 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>4.44 ± 0.34</td>
<td>4.39 ± 0.23</td>
</tr>
</tbody>
</table>

Table 19. Mean total nicotine consumption (mL) for all animals during methoxsalen treatment test (Phase II) in adolescent (n=32) and adult (n=30) male C57BL/6J mice (means ± standard error of the mean).

*aBonferroni post hoc tests: Differs from 5
*bBonferroni post hoc tests: Differs from 6
*cBonferroni post hoc tests: Differs from 7
*dBonferroni post hoc tests: Differs from 8
<table>
<thead>
<tr>
<th>Day 5&lt;sup&gt;c,d&lt;/sup&gt;</th>
<th>Day 6&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Day 7&lt;sup&gt;a,b,d&lt;/sup&gt;</th>
<th>Day 8&lt;sup&gt;a,c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>10 mg/kg 8MOP</td>
<td>Vehicle</td>
<td>10 mg/kg 8MOP</td>
</tr>
<tr>
<td>Adolescent</td>
<td>43.87 ± 2.62</td>
<td>46.76 ± 3.11</td>
<td>45.10 ± 2.26</td>
</tr>
<tr>
<td>Adult</td>
<td>47.95 ± 3.99</td>
<td>46.88 ± 2.59</td>
<td>46.52 ± 2.94</td>
</tr>
</tbody>
</table>

Table 20. Mean total nicotine intake as a percent of total fluid intake (%) for all animals during methoxsalen treatment test (Phase II) in adolescent (n=32) and adult (n=30) male C57BL/6J mice (means ± standard error of the mean).

<sup>a</sup>Bonferroni post hoc tests: Differs from 5
<sup>b</sup>Bonferroni post hoc tests: Differs from 6
<sup>c</sup>Bonferroni post hoc tests: Differs from 7
<sup>d</sup>Bonferroni post hoc tests: Differs from 8
<table>
<thead>
<tr>
<th>Day 5&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Vehicle</th>
<th>10 mg/kg 8MOP</th>
<th>Day 6</th>
<th>Vehicle</th>
<th>10 mg/kg 8MOP</th>
<th>Day 7&lt;sup&gt;a,d&lt;/sup&gt;</th>
<th>Vehicle</th>
<th>10 mg/kg 8MOP</th>
<th>Day 8&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Vehicle</th>
<th>10 mg/kg 8MOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adolescent*</td>
<td>32.28 ± 6.07</td>
<td>30.56 ± 3.11</td>
<td>26.80 ± 2.15</td>
<td>40.84 ± 15.35</td>
<td>18.78 ± 1.47</td>
<td>17.82 ± 1.17</td>
<td>29.64 ± 1.40</td>
<td>30.78 ± 2.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>28.40 ± 9.62</td>
<td>22.65 ± 2.80</td>
<td>19.67 ± 1.04</td>
<td>18.34 ± 1.04</td>
<td>16.13 ± 1.10</td>
<td>16.75 ± 1.27</td>
<td>25.58 ± 1.17</td>
<td>22.61 ± 0.97</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 21. Mean total nicotine dosage (mg/kg) for all animals during methoxsalen treatment test (Phase II) in adolescent (n=32) and adult (n=30) male C57BL/6J mice (means ± standard error of the mean).

<sup>a</sup>Bonferroni post hoc tests: Differs from 5  
<sup>b</sup>Bonferroni post hoc tests: Differs from 6  
<sup>c</sup>Bonferroni post hoc tests: Differs from 7  
<sup>d</sup>Bonferroni post hoc tests: Differs from 8

*Overall main effect for age: Adolescent > Adults; p<0.05
Figure 14. Experiment II timeline.
Figure 15. Average total nicotine consumption (mL) during nicotine-only treatment (Phase I) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=32) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean).
Figure 16. Total nicotine intake as a percent of total fluid intake (%) during nicotine-only treatment (Phase I) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=32) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean).
Figure 17. Total nicotine dosage (mg/kg) during nicotine-only treatment (Phase I) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=32) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean).
Figure 18. Total nicotine consumption (mL) during methoxsalen and nicotine treatment (Phase II) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean).
Figure 19. Comparison of average total nicotine consumption (mL) for adolescent (n=48) and adult (n=48) male C57BL/6J mice exposed to vehicle (n=48) and 10 mg/kg 8MOP (n=48) treatment conditions during Phase I (mean days 1-4) and adolescent (n=30) and adult (n=30) C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=30) treatment conditions during Phase II (mean days 5-8) (means ± standard error of the mean).
Figure 20. Total nicotine intake as a percent of total fluid intake (%) during methoxsalen and nicotine treatment (Phase II) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean).
Figure 21. Comparison of average total nicotine intake as a percent of total fluid intake (%) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=32) and 10 mg/kg 8MOP (n=32) treatment conditions during Phase I (mean days 1-4) and adolescent (n=32) and adult (n=30) C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions during Phase II (mean days 5-8) (means ± standard error of the mean).
Figure 22. Total nicotine dosage (mg/kg) during methoxsalen and nicotine treatment (Phase II) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean).
Figure 23. Comparison of average total nicotine dosage (mg/kg) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=32) and 10 mg/kg 8MOP (n=32) treatment conditions during Phase I (mean days 1-4) and adolescent (n=32) and adult (n=30) C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions during Phase II (mean days 5-8) (means ± standard error of the mean).
Figure 24. Liver weight (g) for adolescent (n=32) and adult (n=30) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean).
Figure 25. Serum cotinine levels adjusted for liver weight (ng/ml/g) for adolescents (n=32) and adults (n=30) adult male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions (means ± standard error of the mean).
Figure 26. Relative gene expression of CYP2a5 corrected for the quantity of 18S rRNA for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean).
Figure 27. Relative gene expression of CYP2e1 corrected for the quantity of 18S rRNA for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean).
Figure 28. Correlation between relative CYP2a5 gene expression corrected for 18S rRNA quantity and total nicotine dosage (mg/kg) for adolescent (n=32) and adult (n=32) male C57BL/6J mice exposed to vehicle (n=32) and 10 mg/kg 8MOP (n=32) treatment conditions during Phase I (mean days 1-4).
Figure 29. Correlation between relative CYP2a5 gene expression corrected for 18S rRNA quantity and total nicotine dosage (mg/kg) for adolescent (n=32) and adult (n=30) C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions during Phase II (mean days 5-8).
Figure 30. Comparison of total nicotine consumption (ng/ml/g) for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=32) and adult (n=30) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions from Experiment II (means ± standard error of the mean).
Figure 31. Comparison of total nicotine intake as a percent of total fluid intake (%) for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=32) and adult (n=30) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions from Experiment II (means ± standard error of the mean).
Figure 32. Comparison of total nicotine dosage (mg/kg) for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=32) and adult (n=30) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions from Experiment II (means ± standard error of the mean).
Figure 33. Comparison of liver weight (ng/ml/g) for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=32) and adult (n=30) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions from Experiment II (means ± standard error of the mean).
Figure 34. Comparison of serum cotinine levels adjusted for liver weight (ng/ml/g) for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=32) and adult (n=30) male C57BL/6J mice exposed to vehicle (n=30) and 10 mg/kg 8MOP (n=32) treatment conditions from Experiment II (means ± standard error of the mean).
Figure 35. Comparison of relative gene expression of CYP2a5 corrected for the quantity of 18S rRNA for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions from Experiment II (means ± standard error of the mean).
Figure 36. Comparison of relative gene expression of CYP2e1 corrected for the quantity of 18S rRNA for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions from Experiment II (means ± standard error of the mean).
Figure 37. Correlation between relative gene expression of CYP2a5 corrected for the quantity of 18S rRNA and total nicotine dosage (mg/kg) for adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions (means ± standard error of the mean) from Experiment I and adolescent (n=12) and adult (n=12) male C57BL/6J mice exposed to vehicle (n=12) and 10 mg/kg 8MOP (n=12) treatment conditions from Experiment II (means ± standard error of the mean).
CHAPTER IV

DISCUSSION
DISCUSSION

Overview

A former or current smoker dies approximately every 7 seconds (WHO, 2005). In a single year, the combination of direct and indirect effects (e.g., second and third hand smoke) of smoking kills 5 million people, a statistic that is projected to increase almost 2-fold by 2030 (WHO, 2009). Regardless of the mortality rates, diseases (e.g., cancer) and non-life threatening, yet detrimental, effects (e.g., discolored skin on the fingers) associated with smoking, over 1 million adolescents initiate the habit each day (SAMHSA, 2008b) to join over 1 billion smokers worldwide (Shafey, Eriksen, Ross, & Mackay, 2009). Effective smoking cessation aids are therefore in high demand to help reduce these staggering statistics. Medications to aid in smoking cessation that are available today include first-line FDA-approved [e.g., nicotine replacement therapies (NRTs), Varenicline (Chantix®), and Bupropion (Wellbutrin®, Zyban®)] and second-line non-FDA-approved pharmacotherapies (e.g., clonidine and nortripyline). Although effective in reducing withdrawal symptoms and increasing the ability to quit smoking, these drugs are manufactured for the general population, and therefore, do not take into account differences between populations and among individuals.

One such biological variable – age – can alter smoking cessation rates, as well as initiation, development and maintenance of the smoking habit. Roughly 90% of smokers began smoking before the age of 18 (USDHHS, 1994), and because early age of smoking initiation is inversely proportional to the ability to quit (Breslau & Peterson, 1996; Chen & Millar, 1998; McQuown, Belluzzi, & Leslie, 2007; USDHHS, 1994), these individuals are less likely to become former smokers. Based on these statistics alone, it is extremely
important to understand the mechanisms behind how age can predispose an individual to begin smoking and even prevent them from quitting.

The effects of age also extend beyond differences in populations to affect smokers on an individual level. For example, reduced activity levels of CYP2A6 or a null CYP2A6 enzyme significantly reduces the risk of dependence on nicotine, and cigarette cravings if the individual does become a smoker, in adults (Pianezza, Sellers, & Tyndale, 1998; Rao et al., 2000; Sellers, Tyndale, & Fernandes, 2003; Tyndale et al., 1999; Tyndale & Sellers, 2001; 2002). In contrast, adolescents with reduced CYP2A6 activity are at a higher risk to becoming dependent (Karp et al., 2006; O’Loughlin et al., 2004; for review, see Malaiyandi, Sellers, & Tyndale, 2005). These findings further strengthen the need to understand how age alters the initiation, development, maintenance and even cessation of a smoking habit, not only to protect the health and well-being of future generations, but also to develop smoking cessation aids that are tailored to the individual.

At this point, the mechanism behind age differences among individuals with varying degrees of CYP2A6 activity remains unknown. Therefore, the present doctoral dissertation aimed to examine the effects of age on nicotine consumption and metabolism following subcutaneous acute (1-day) and chronic (4-day) administration of a CYP2A6 inhibitor, methoxsalen, in drug-naïve adolescent and adult male C57BL/6J mice.

**Review of Experiments**

Experiment I was a 1-day study where 120 adolescent (PND 42-43) and adult (9 weeks old) male C57BL/6J mice received either no injection or a single subcutaneous injection of either physiological saline, vehicle (Emulphor, distilled water and ethyl alcohol) or one of two doses of methoxsalen (5 or 10 mg/kg), followed by access to a 3-
bottle oral nicotine paradigm [tap water, 50 (LOW NIC) and 200 ug/ml (HIGH NIC) (-)-freebase nicotine dissolved in tap water] for 12 hours.

This dissertation was the first 12-hour murine study to investigate nicotine consumption using a 3-bottle oral nicotine self-administration paradigm following a single subcutaneous injection of methoxsalen in male C57BL/6J mice. Additionally, this was the first study to examine and compare nicotine consumption and metabolism following administration of methoxsalen in adolescent and adult male mice, regardless of strain. The comparison of age groups revealed that adults consumed more nicotine (mL, mg/kg, % total fluid intake) than did adolescents, and that methoxsalen is an effective CYP2a5 inhibitor, as demonstrated by a reduction in serum cotinine levels (ng/ml/g) with increasing dosages of methoxsalen, in adult, but not adolescent, male C57BL/6J mice.

To further investigate the age differences in nicotine consumption and drug effects on serum cotinine (ng/ml/g), an 8-day two-stage study with 64 adolescent (PND 35-43) and adult (8-10 weeks old) male C57BL/6J mice (Experiment II) was conducted. For the first four days (Phase I), animals received 24-hour unlimited access to the 3-bottle oral nicotine paradigm (tap water, LOW NIC and HIGH NIC). During the four days of Phase II, animals continued to have access to the water and nicotine bottles, but were also given daily subcutaneous injections of either vehicle or 10 mg/kg 8MOP at the end of the light cycle.

Including Experiment I, previously published murine studies that have examined the effects of methoxsalen on nicotine-induced behaviors or nicotine pharmacokinetics have lasted 1 day. However, Experiment II is the first long-term report to examine and compare age differences (adolescent vs. adult) in nicotine consumption and metabolism
with and without the administration of methoxsalen over multiple days. Neither adolescents nor adults consistently consumed more nicotine (mL, mg/kg, %) throughout the eight day study. Unexpectedly, animals consumed more total nicotine during Phase II compared to Phase I across the age groups. Levels of serum cotinine (ng/ml/g) and CYP2a5 gene expression were higher in adults compared to adolescents. Additionally, serum cotinine levels were marginally affected by drug treatment in adults, but not adolescents, with higher serum cotinine levels (ng/ml/g) in the vehicle group compared to the 10 mg/kg 8MOP group. There was no effect of age or drug treatment on CYP2e1 gene expression levels.

These experiments are the first steps to understanding biological determinants, specifically age, and their contributions toward nicotine addiction in males. A summary of alternative methods for these experiments and future directions for subsequent studies and analyses are indicated below.

**Contributions to Literature**

Results of these two experiments provide significant contributions to the small pool of scientific literature regarding the effects of methoxsalen on nicotine consumption and pharmacokinetics in mice. Compared to the two previously published murine studies (Damaj et al., 2007; Raunio et al., 2008), Experiments I and II are the first to 1) use C57BL/6J mice, 2) use an oral nicotine self-administration method to administer nicotine in a methoxsalen study, 3) examine nicotine consumption and metabolism following methoxsalen administration in adolescents, and compare these results with that of adults, and 4) assess hepatic CYP2a5 and CYP2e1 enzymes following methoxsalen exposure.
Furthermore, Experiment II was the first long-term study to examine nicotine consumption with and without methoxsalen in mice, regardless of age, strain, or species.

**Effects of Methoxsalen on Nicotine Consumption in C57BL/6J Mice**

Of the other two preclinical murine studies examining the effects of methoxsalen on nicotine-induced behaviors and nicotine pharmacokinetics, this is the first study to use C57BL/6J mice. C57BL/6J mice were chosen based on their wide spread use in nicotine studies as both adolescent and adults (Abreu-Villaça, Queiroz-Gomes, Dal Monte, Filgueiras, & Manhães, 2006; Klein et al., 2003; Klein et al., 2004; Marks, Stitzel, & Collins 1989; Siu & Tyndale, 2007; Sparks & Pauly, 1999), willingness to consume drugs of abuse, including nicotine (The Jackson Laboratory; Hatchell & Collins, 1980; Middaugh, Kelley, Bandy, & McGroarty, 1999; Klein et al. 2004), and use in prior studies in our laboratory (Klein et al., 2003; Klein et al., 2004). Additionally, inbred mice, like the C57BL/6J mouse strain, are genetically identical; any changes in behavior can then be associated with the environment. ICR mice (i.e., used in Damaj et al., 2007) are outbred strains, the behavior of which is more sensitive to genetic differences between individual mice. Similarly, hybrids like CD2F1 mice (i.e., used in Raunio et al., 2008), which are crosses between BALB/C and DBA/2 mice, are subject to a variety of behaviors associated with both of the parent strains. Because only three mouse strains have been used in the methoxsalen-nicotine literature (Damaj et al., 2007; Raunio et al., 2008; current studies), future studies should use these mouse strains to replicate the findings in these three studies as well as add to the small pool of articles. In addition to these strains, subsequent studies should be conducted in several inbred and outbred strains (e.g., DBA/2) because, similar to that of human populations, different mouse
strains vary significantly in behavioral responses to nicotine (Collins, Miner, & Marks, 1988), nicotine consumption and tolerance (Collins et al., 1988), nicotine pharmacokinetics (Siu & Tyndale, 2007), and genetic differences (Collins, Marks, & Pauly, 1989). For example, adult DBA/2 mice have a tendency to avoid consuming drugs of abuse, like nicotine, which may be due to a slower rate of nicotine and cotinine clearance, longer elimination half life for cotinine, and higher area under the curve (AUC) values for both cotinine and nicotine (Siu & Tyndale, 2007). Because of their opposing nicotine consumption behaviors as adults and a single change in amino acid sequence, a comparison of nicotine consumption between these strains would be beneficial to examine individual differences in nicotine consumption. It is important to examine several strains of mice to mimic population and individual differences in smoking found among humans.

**Effects of Nicotine and Methoxsalen on Body Weight in Adolescent and Adult Mice**

Scientists have studied the effects of nicotine on body weight for over 100 years (for review, see Grunberg, 1990). Nicotine administered via osmotic pumps reduces body weight in adolescent (Faraday, Elliott, & Grunberg, 2001) and adult male and female rats (Faraday, Blakeman, & Grunberg, 2005; Grunberg, 1982; Grunberg, Bowen, & Morse, 1984; Grunberg, Winders, & Popp, 1987). These published results parallel body weight differences observed in current versus ex- or non-smokers (Munafo, Tilling, & Ben-Shlomo, 2009). It is important to note that these previously published experiments compared nicotine-induced changes in body weight to that of age-matched animals that did not receive nicotine. There was no pure control in either Experiment I or
II; therefore, no conclusions can be drawn regarding the effects of nicotine only on body weight in adolescent and adult mice.

In contrast, this study was well equipped to examine and compare body weight changes due to methoxsalen. There were no effects on body weight from drug treatment in adolescents, as both animals in the vehicle and 8MOP groups increased in body weight. Injections of methoxsalen in adults, on the other hand, significantly increased body weight. There were no significant changes in body weight for adults receiving vehicle, which suggests that methoxsalen may result in age-dependent body weight changes. This the first study to examine the effects of methoxsalen in conjunction with nicotine on body weight in adolescent and adult mice. Contradicting evidence states that body weight was reduced in male and female rats (age unknown) consuming a powdered diet infused with methoxsalen (250, 1250 and 2500ppm) compared to a diet without methoxsalen (Diawara et al., 1997). Several differences between the Diawara et al. (1997) study and the present experiments could account for opposing findings. First, administration of methoxsalen (oral versus subcutaneous injection) could alter the bioavailability of methoxsalen, thereby changing the physiological effects. Secondly, only three rats (2 male, 1 female) were used in the Diawara study (1997) compared to 30-32 adult mice in Experiment II. Such a small sample size can make it difficult to examine large main effects. Thirdly, nicotine, a substance known to induce body weight effects, was administered in Experiment II, but not in the Diawara study (1997). Although body weight usually decreases following exposure to nicotine in adults, the opposite effect occurred when methoxsalen was introduced. Nicotine alone and in combination with methoxsalen did not alter food consumption during our study; however,
there may have been a drug-drug interaction that caused the increase in body weight.

Fourthly, Diawara et al. (1997) used Wistar rats compared to male C57BL/6J mice used in Experiment I and II. Responses to nicotine and methoxsalen can differ between species, including rodents. According to a recent study by Visoni, Meireles, Monteiro, Rossini and Pinto (2008), the mechanisms by which methoxsalen inhibits mouse CYP2a5 and an enzyme responsible for nitrosamine activation in the rat, CYP2a3, are different. More specifically, methoxsalen functions as a mechanism-based inhibitor for CYP2a5, whereas the drug is a non-competitive inhibitor for CYP2a3 (Visoni et al., 2008).

Although these two enzymes are 95% structurally similar to each other, mouse CYP2a5 and rat CYP2a3 are categorized into different subsets of the CYP2a family (see Honkakoski & Negishi, 1997). Mouse CYP2a5 and human CYP2a6 classified as part of Subset 3, which are characterized by a Valine, Phenylalanine, and Alanine at positions 117, 209, and 481, respectively (see Honkakoski & Negishi, 1997). With an Alanine at position 117, rat CYP2a3 is classified as part of Subset 4. Differences in the inhibitory mechanism of methoxsalen between the two species could have led to opposing body weight findings. In addition, enzymes responsible for nicotine metabolism in the mouse and rat are in different gene families: CYP2A versus CYP2B and 2C subfamilies, respectively (Nakayama, Okuda, Nakashima, Imaoka, & Funae, 1993). Cytochrome P450 gene families are categorized by structural similarity; therefore, use of different enzyme families to metabolize nicotine may lead to altered behavioral responses to nicotine. Finally, male and female rats were used in the Diawara et al. (1997) study, whereas male C57BL/6J mice were used in Experiments I and II. Beyond the fact that there were only 3 rats (2 male, 1 female) used throughout the study, the combined use of
males and females could alter the observed results. Additional studies are necessary to further understand into the potential causes for these body weight gain changes in adolescent and adult male and female mice and rats exposed to methoxsalen.

**Nicotine Consumption in Adolescent and Adult Mice**

Use of oral nicotine self-administration, specifically in mouse models, has grown in popularity over the past decade to mirror behaviors associated with nicotine dependence in humans, such as preference (Adriani, Macri, Pacifici, & Laviola, 2002; Flynn, Webster, & Ksir, 1989; Klein et al., 2003), consumption (Klein et al., 2004; Siu & Tyndale, 2007), nicotine withdrawal (Gäddnäs, Pietilä, & Ahtee, 2000), locomotion (Gäddnäs, Pietilä, Piepponen, & Ahtee, 2001) and tolerance (Grabus et al., 2005; Robinson, Grun, Pauly, & Collins, 1996). Additionally, this technique also (1) allows for continuous access to nicotine without unnecessary distress or pain, (2) can be safely used with adolescents and adults, (3) minimizes the need to teach a behavior (e.g., lever pressing) that could take time to establish, and 4) has been successfully established in previous studies conducted by our laboratory (Klein et al., 2003; Klein et al., 2004).

Experiments in this dissertation are the first to provide access to nicotine using oral nicotine self-administration with a 3-bottle choice paradigm following acute and chronic methoxsalen administration, regardless of age. The other two published articles that have examined the relationship between methoxsalen and nicotine used drug administration techniques that involuntary administered nicotine directly into the mouse’s system. Gavage (Raunio et al., 2008) and subcutaneous injection (Damaj et al., 2007) administration techniques used in these studies are more practical to assess the effects of
nicotine pharmacokinetics and nicotine-induced behaviors (e.g., antinociception) following methoxsalen administration.

As with other drug administration techniques, the majority of oral nicotine self-administration studies in mouse models have used adults. With over 1.2 billion adults being chronic smokers, it is important to understand how smokers maintain their smoking habit and to develop additional effective smoking cessation aids. The larger problem lies with adolescents initiating smoking and continuing the habit for several decades. In view of this, adolescent rodents are taking a more central role in nicotine addiction research (Laviola, Macri, Morley-Fletcher, & Adriani, 2003; O’Dell et al., 2006; Park, Belluzzi, Han, Cao, & Leslie, 2007; Slotkin, 2002; Smith, 2003; Spear & Brake, 1983). With the emergence of adolescent rodent models, it is imperative to examine and compare any differences in nicotine consumption or related drug-taking behaviors in adults and adolescents.

Adults in Experiment I consumed more nicotine (mL, mg/kg, %) compared to adolescents, regardless of drug treatment. Adults and adolescents consumed the same amount of total nicotine (mL) with and without methoxsalen administration. This age-difference indicates that acute nicotine exposure without methoxsalen could be more rewarding and that tolerance could contribute to the blunted dopamine response following repeated nicotine exposure for adults, but not adolescents. Findings from a study completed by Badanich and Kirstein (2004) support this theory. Specifically, male and female adult rats (PN 60) had significantly higher dopaminergic levels in the nucleus accumbens, the area of the brain associate with reward, compared to adolescents (PN 35 and 45) following an acute nicotine exposure via injection. However, upon being given
access to repeated nicotine challenges, accumbal dopaminergic levels were similar for adolescents and adults (Badanich & Kirstein, 2004).

Based on published findings that methoxsalen decreases smoking in adult men and women (Sellers et al., 2000), the lack of an effect on nicotine consumption by methoxsalen in adult mice is puzzling. Because of vast differences between adolescents and adults with regard to smoking in humans and nicotine-related behaviors in animals, it was interesting that a drug treatment effect, in one direction or another, was not observed in adults and adolescents. This is the first study to examine nicotine consumption following methoxsalen administration in mice, regardless of strain, age or sex, so there are a number of potential future studies that could help ascertain the reason for lack of drug treatment differences. For example, the dosages of methoxsalen may have been too low to see a significant difference in nicotine consumption between drug treatment groups, therefore a dose-dependent study with higher (e.g., > 10 mg/kg) or lower (e.g., <5 mg/kg) should be conducted to test this hypothesis. The mode of administration may also be a factor. Although I was careful to ensure that the drug was successfully injected into the skin, there is always the potential that the entire amount of injected drug did not make it into the body, which would reduce any behavioral effects induced by the drug. Therefore, replication of this technique is necessary. Studies that use other forms of methoxsalen administration (e.g., gavage or infusion into food) would be useful to compare pharmacokinetics of methoxsalen should administration and absorption of the drug be a cause for concern. Because C57BL/6J mice readily consume nicotine, perhaps the motivation to drink nicotine was so intense that the effect could not be diminished by methoxsalen. Other strains, such as ICR, CD2F1 and DBA/2, should be examined to
assess their nicotine consumption. The addition of other strains would also be useful to parallel individual behavioral and genetic differences found in humans.

**Effects of Methoxsalen on Nicotine Metabolism**

One of the more interesting findings resulting from this dissertation was an age-dependent reduction in serum cotinine levels (ng/ml/g). For adolescents, there was no difference in serum cotinine levels (ng/ml/g) across drug treatment groups following Experiment I or II. In contrast, drug treatment significantly affected serum cotinine levels (ng/ml/g) in adults after both studies. In Experiment I, adults in the vehicle group had the highest levels, followed by 5 mg/kg 8MOP and then 10 mg/kg 8MOP groups. The reduction in serum cotinine levels (ng/ml/g) across drug treatment groups in adults was in the same direction as observed in Experiment II (vehicle > 8MOP); however, the drug treatment effect was only marginal at p=0.068. These findings suggest that methoxsalen is effective at reducing nicotine metabolism in adults, but not in adolescents.

In Experiment II, adults had higher serum cotinine levels (ng/ml/g) and relative gene expression levels of CYP2a5 compared to adolescents. These findings provide strong evidence that high levels of CYP2a5 metabolize nicotine faster to create high levels of serum cotinine following several days of oral nicotine consumption in adults. Interestingly, no age-related changes in nicotine consumption (mL) were observed during Experiment II; however, adolescents consumed more nicotine per unit body weight than did adults. Additional studies are necessary to explain how these behavioral and metabolic observations fit together.

Although there were no changes in nicotine consumption (mL) or CYP2a5 gene expression levels, methoxsalen induced significant reductions in serum cotinine levels
(ng/ml/g) in adults, but not adolescents. Methoxsalen did not alter gene expression levels of CYP2a5 at the end of Experiment I, indicating that exposure to methoxsalen and nicotine for 12 hours was not long enough to elicit a response in CYP2a5 gene expression. An age effect was observed in CYP2a5 at the end of Experiment II; however, there was no change in CYP2e1 levels. It is possible that gene expression levels change at different rates for different hepatic enzymes. Future studies should investigate the time it takes for CYP2a5, CYP2e1 and other enzymes involved at any degree of nicotine metabolism gene expression levels to change following oral nicotine consumption.

Because methoxsalen is a strong mechanism-based inhibitor of human CYP2A6 (Koenigs et al., 1997; Koenigs & Trager, 1998; Mays, Hilliard, Wong & Gerber, 1989; Sharp et al., 1984) and mouse CYP2a5 (Damaj et al., 2007; Raunio et al., 2008), it also was noteworthy that gene expression for CYP2a5 and CYP2e1 relative to 18S did not change in response to 1- and 4-day methoxsalen exposure. Based on reports using mice, monkeys and human subjects, there are four explanations for the lack of change for CYP2a5 gene expression. First, in a study by Schoedel and colleagues (2003), nicotine reduced mRNA levels of a CYP2A6-like enzyme (CYP2A6agm) in African green monkeys. Regarding methoxsalen, Bickers and Pathak (1984) determined that methoxsalen administered orally over 6 days increased hepatic protein levels, enzyme activity [e.g., aryl-hydrocarbon hydroxylase (AHH), ethylmorphine N-deethylase], and cytochrome p450 levels in adult male CD-1 mice. Therefore, it is plausible that the levels of CYP enzymes, including CYP2a5, were initially reduced because of chronic
exposure to nicotine and then increased to homeostatic levels by methoxsalen administration, thereby showing no net change in expression.

In contrast, a recently published article using adult male and female smokers by Hukkanen, Jacob, Peng, Dempsey and Benowitz in 2010 determined that nicotine has no effect on CYP2A6 levels (Hukkanen, Jacob, Peng, Dempsey, & Benowitz, 2010). Although this information could explain why animals in the vehicle group presented no change in CYP2a5 levels, it does not clarify why no change in CYP2a5 gene expression was observed in mice receiving methoxsalen, especially since metabolites of methoxsalen act directly on CYP2a5. A potential reason for this discrepancy may simply be that four consecutive days of methoxsalen administration is not long enough to alter CYP2a5 gene expression levels. Additional studies are necessary to determine the validity of these two scenarios.

Additionally, there is evidence to suggest that CYP2a5 is more highly expressed in kidney and liver of females compared to males (Hrycay & Bandiera, 2009; Squires & Negishi, 1988). Higher coumarin 7-hydroxylase activity has been found in 129, DBA/2 and CBA/Ca female mice compared to their male counterparts (van Iersel, Walters, Price, Lovell, & Lake, 1994). With only males being used in Experiment I and II, it is possible that a lower gene expression in males would not show significant age or drug treatment effects compared to a higher gene expression in females. Future studies should therefore include adolescent and adult female mice not only to assess these differences in gene expression, but also to compare nicotine consumption and metabolism across sex and different age groups.
Adult mice in Experiment I and II arrived at the same age (8 weeks old), but the adult mice in Experiment I and II were sacrificed one week apart (9 weeks versus 10 weeks, respectively). This one-week difference in age for the adult mice between the two experiments may alter gene expression of CYP2a5. Although it is currently unknown if CYP gene expression changes within short time frames (i.e., days or weeks) during adulthood, scientists have found age-related changes when comparing these developmental stages to adults (Yun et al., 2010). Therefore, it is possible that a single week could change gene expression; additional studies would be necessary for confirmation.

In addition to CYP2a5, a different hypothesis may explain the lack of change in CYP2e1 gene expression. Several studies suggest that low-dose chronic nicotine administration induces CYP2E1 in the brains of monkeys (Joshi & Tyndale, 2006a), the liver (Micu et al., 2003) and brain of rats (Howard, Miksys, Hoffmann, Mash, & Tyndale, 2003; Miksys, Hoffmann & Tyndale, 2000; Joshi & Tyndale, 2006b), as well as human neuroblastoma cells (IMR-32 cell line) (Howard, Miksys, Hoffmann, Mash, & Tyndale, 2003). Methoxsalen, on the other hand, is a weak inactivator of several CYP enzymes, including CYP2E1 (Koenigs et al., 1997). It is possible that methoxsalen inactivated CYP2e1, thereby cancelling out any increase in gene expression potentially caused by nicotine exposure.

Hukkanen and colleagues (2010), on the other hand, suggest that nicotine does not affect CYP2E1, at least in humans. If the inactivation of methoxsalen has no significant effects on CYP2e1, there would be no resultant net change in gene expression levels. Additional studies are necessary to determine the validity of these two scenarios.
To date, there are no reports comparing CYP2a5, CYP2e1 or any other CYP enzyme levels in animals receiving no drug, methoxsalen alone, nicotine alone, and the combination of methoxsalen and nicotine. This is the first study to examine CYP2a5 and CYP2e1 gene expression levels in mice receiving nicotine only (vehicle groups) and the combination of methoxsalen and nicotine (5 and 10 mg/kg 8MOP groups). Because the goal of this dissertation was determine the effect of methoxsalen on nicotine consumption and metabolism, these experiments did not include a pure control for which only water was provided (as in no nicotine or methoxsalen). Future studies should expand upon the experiments in this dissertation and compare the data collected to mice receiving methoxsalen alone and those not receiving any drug to fully understand how CYP2a5, CYP2e1 and other CYP enzymes altered by nicotine and/or methoxsalen changes with the administration of these two drugs, separately and in combination with each other.

**Mouse Data Summary**

In short, regardless of strain or sex, methoxsalen increases nicotine levels and reduces parent/metabolite ratios in urine (Raunio et al., 2008), as well as increases nicotine half-life in plasma (Damaj et al., 2007). Cotinine levels are significantly reduced in plasma (Damaj et al., 2007), urine (Raunio et al., 2008), and serum (Experiment I) following acute methoxsalen exposure in adults, but not adolescents. Similarly, serum cotinine levels in adults were reduced following chronic exposure as well (Experiment II). Thus, methoxsalen inhibits CYP2a5 and should, theoretically, alter nicotine consumption. However, nicotine consumption was not affected by acute or chronic methoxsalen exposure in either adult or adolescent male C57BL/6J mice, regardless of drug exposure time. Based on the lack of effect on nicotine consumption in addition to
indications for toxicological effects on the liver (e.g., a larger liver-to-body weight ratio in animals receiving methoxsalen compared to the vehicle), there is little indication that methoxsalen should be used as a smoking cessation aid. Consequently, because drug treatment significantly reduced serum cotinine levels (ng/ml/g), methoxsalen could be used as a tool to further examine CYP2A6 polymorphisms in humans and assess underlying biological mechanisms.

**Clinical Implications**

In contrast, positive results from a 3-hour laboratory test conducted by Sellers and colleagues (2000) on adult male and female smokers suggest that methoxsalen has potential to become a smoking cessation aid. More specifically, Sellers and colleagues (2000) determined that a single dose of methoxsalen (3x10mg capsules) administered orally to adult male and female smokers significantly reduced cigarette consumption and carbon monoxide levels by 24 and 48%, respectively. Based on these findings and from current protocols for methoxsalen use in dermatology, instructions for methoxsalen dosing, length of exposure, routes of administration and smoking indications are hypothesized and listed below.

**Dosing instructions.** Dosing instructions are determined by the attending physician and generally depend on the route of drug administration, skin type and color, body weight, type and severity of dermatoses (oral versus topical) to be treated and responsivity to treatment (8-MOP® package insert, 1954; Oxsoralen-Ultra® package insert, 1986). For example, 0.6mg/kg of methoxsalen in the form of a hard capsule (8MOP®) is used to treat mycosis fungoides or psoriasis in adults, whereas individuals seeking treatment for vitiligo are prescribed 20 mg 8MOP® per day.
With respect to smoking cessation, the 30mg dose (3x10mg capsules of Oxsoralen-Ultra®) used in the study by Sellers and colleagues (2000) was sufficient to reduce cigarette consumption by 24%. Because this is the only study to examine the effects of methoxsalen on cigarette consumption in adults and that this dose was effective in adult smokers, 30mg of methoxsalen serves as a good starting point for future studies using methoxsalen as a smoking cessation aid. Body weight of the participants was not indicated in the article; however, in the future, methoxsalen doses should be adjusted for body weight (Kharasch, Hankins, & Taraday, 2000), so as to avoid unwanted toxic effects over time. As there are currently no studies examining the effect of methoxsalen on cigarette consumption in adolescents, additional studies should be done to find the range of effective doses or formula to determine the effective dosage while avoiding toxicity for this age group.

**Length of exposure.** Regardless of the half-life of methoxsalen, individuals generally undergo PUVA treatments 2 to 3 times a week for up to 12 weeks. PUVA treatments are not given every day to avoid over-exposure to UV radiation and to give time for the skin to heal between treatments. With respect to methoxsalen as a smoking cessation aid, it would be best to examine dosing regimens for current smoking cessation aids that do not contain nicotine [e.g., varenicline (Chantix®) and bupropion (Wellbutrin®)]. Following oral administration, competitive inhibitors, Chantix® and Wellbutrin®, achieve peak plasma concentrations between 3-4 hours and have an approximate elimination half-life of 21-24 hours. Thus, other than the once-a-day schedule for the first three days, physicians recommend that both Chantix® and Wellbutrin® are taken twice-a-day for approximately 12 weeks. Methoxsalen, on the
other hand, reaches peak concentrations between 0.5 and 4 hours and has an unknown elimination half-life. However, CYP2A6 is not inhibited by the parent drug, but by the active metabolites using mechanism-based inhibition. Because of the irreversible nature of “suicide” inhibitors, methoxsalen may not have to be administered as frequently as other smoking cessation aids. However, similarly to Chantix® and Wellbutrin®, there is evidence to suggest that methoxsalen also functions as a noncompetitive (Zhang et al., 2001), and even as a competitive, inhibitor at certain concentrations (Visoni et al., 2008). Therefore, methoxsalen may have to be administered as often as the other two FDA-approved medications.

**Route of administration.** For dermatological treatments, methoxsalen can be administered topically via lotions or ointments, or orally via hard and soft capsules. A topical route of administration is not relevant for smoking cessation; therefore, methoxsalen would be administered orally, as was the case in the Sellers study (2000) where the participants were given liquid-filled, soft capsules (Oxsoralen-Ultra®). Intravenous administration of the drug could also be an option (Billiard et al., 1995).

**Side effects.** At this point, the vast majority of the scientific literature concerning the side effects and toxicity of methoxsalen is also associated with the combination of methoxsalen and UV radiation. Therefore, it is difficult to distinguish the side effects of methoxsalen alone in humans without the additional variable. This said, the physical effects of methoxsalen particularly regarding the skin (e.g., skin cancer, hyperpigmentation) would probably be enhanced by UV radiation. The **Side Effects and Toxicity of Methoxsalen** section of this dissertation gives a broad overview of the scientific literature on PUVA treatments using methoxsalen. The section, **Methoxsalen**
Sans UV Radiation, of this dissertation explicitly focuses on published information concerning the effects of methoxsalen without UV radiation in rodents. For details on these literature summaries, it is best to consult these sections of the dissertation as well as the review on methoxsalen by Kapelewski and Klein (under review).

**Contraindications.** Because there is no evidence to support the contrary, there is no reason to believe that the contraindications for PUVA treatments are any different than those for smoking cessation. As is the case for PUVA, any regimen involving the use of methoxsalen should be monitored by a trained physician. Photosensitivity (Uvadex® package insert, 1999), alcohol use, and the possibility of being pregnancy or are currently breast feeding (8-MOP® package insert, 1954; Oxsoralen-Ultra® package insert, 1986; Uvadex® package insert, 1999) should be brought to the attention of the physician. Use of prescriptions, over-the-counter medications or herbal supplements (e.g., anthralin, soaps, coal tar, dyes, griseofulvin, nalidixic acid, phenothaizines, sulfa or tetracycline antibiotics, “water pills”; Uvadex® package insert, 1999), any previously diagnosed or history of medical problems [e.g., infections, lupus erythematosus, porphyria cutanea tarda, erythropoietic protophyria, variegate porphyria, xeroderma pigmentosum, albinism, skin cancer (melanoma, basal or squamous cell carcinoma), gastrointestinal problems, problems concerning the skin or eyes (e.g., cataracts, aphakia), diseases of the liver, kidney, heart or blood vessels] and current or prior psoriasis treatments involving radiation, arsenic, tar, x- or grenz-rays, UVB radiation, methotrexate (MTX), cyclosporine, mycophenolate mofetil (MMF) or immunosuppressants (e.g., alefacept, efalizumab, etanercept, infliximab, adalimumab) should also be immediately brought to the doctor’s attention (8-MOP® package insert, 1954; Oxsoralen-Ultra®

While undergoing treatment, regular appointments should be kept with the attending physician, especially for organ function tests as well as eye and skin examinations (8-MOP® package insert, 1954; Oxsoralen-Ultra® package insert, 1986). Burns may occur upon contact with direct or even indirect sunlight (e.g., light through window glass); therefore, protective clothing covering all exposed skin, wrap-around sunglasses or UVA opaque goggles (for review, see Glew & Nigra, 1984; See & Weller, 1993; Wolff & Hönigsmann, 1981), hats and sunblock over SPF 15 (if approved by the physician) should be worn at all times, but especially before and 24 hours after treatment (8-MOP® package insert, 1954; Oxsoralen-Ultra® package insert, 1986). If possible, tanning booths, going outdoors or near windows and sunlamps, alcohol and foods that naturally contain methoxsalen (e.g., celery, figs, parsnip, limes, mustard, carrots, and parsley) should be avoided to prevent increased skin sensitivity, and potentially, burns. The skin should be checked everyday for sores or growths that are new, have not healed or have recently changed (know the “ABCD’s of skin cancer”), specifically looking for asymmetry (“A”), borders that are not uniform (“B”), multiple colors (“C”), diameters larger than ¼ of an inch (“D”), and raised or uneven surfaces (AMF, 2006).

Smoking while using methoxsalen. In the Sellers and colleagues (2000) study, participants were allowed to smoke a cigarette 10 minutes before and 60 minutes after receiving one of four treatments (placebo, methoxsalen only, nicotine only, methoxsalen
and nicotine). Unfortunately, participants were only given a single dose of methoxsalen; therefore, it is currently unknown how smoking will be tolerated following long-term methoxsalen exposure. Additionally, there was only a difference between the methoxsalen only treatment and placebo in latency to smoke between the first and second cigarette. In contrast, those individuals receiving methoxsalen/nicotine treatment had significantly lower carbon monoxide levels, consumed the fewest number of cigarettes, had the highest latency between cigarettes, and took the fewest number of puffs compared to placebo (Sellers et al., 2000). These findings indicate that the addition of nicotine in combination with methoxsalen and smoking is a more effective treatment than methoxsalen alone. Similar to the instructions for Chantix® and Wellbutrin®, individuals using methoxsalen should be allowed to smoke, at least for the first week or two, before quitting entirely. Additionally, nicotine replacement therapies, such as the patch, may also be helpful to increase efficacy.

**Alternative Methods and Future Directions**

To date, there have only been a handful of experiments investigating the potential for CYP2A6 inhibitors, specifically methoxsalen, to serve as smoking cessation aids regardless of the target age group. Therefore, there is ample opportunity for additional studies to be conducted in order to fully understand age differences in nicotine consumption and metabolism, as well as the role of methoxsalen in the reduction of nicotine (or cigarette) consumption beyond what information has been learned from these experiments. A variety of alternative methods and techniques to expand the results of this dissertation are discussed below.
Study

Length of study. Future studies should extend the length of each experiment. In Experiment I, a single injection of methoxsalen followed by access to nicotine for 12 hours demonstrated that a change in enzyme activity is apparent even after a single exposure to the inhibitor. The length of time for nicotine access should be expanded to investigate the duration of the washout period, or the length of time it takes for the effects from a single exposure to wear off before another dose of methoxsalen is administered. This information would be important to determine how many times per week would an individual have to administer methoxsalen to prevent nicotine consumption. These studies could even be applied to current smokers to observe how long the individual would take methoxsalen as a smoking cessation aid, rather than a prevention method.

Sex. Based on the limited number of studies that have been conducted so far, there is little information concerning the effects of methoxsalen on adult male or female mice. Both of the published in vivo studies were completed using adult mice (one used males, the other used females) of two different outbred strains (ICR and CD2F1, respectively). Historically, the progression of scientific investigations with regard to specific populations of subjects generally begins with adult males (for review, see Taylor et al., 2000). Compared to males, scientific research using females must take into account more variables (e.g., sex hormones, menstrual cycle phases, pregnancy), and the involvement of these hurdles can complicate the study design and its findings [for review, see Institute of Medicine (IOM), 2001; Taylor et al., 2000]. Therefore, males typically are included in studies because of the lack of hormonal fluctuations associated with estrus cycling. I chose to initially include only males to keep the study size limited to 2 or 3
independent variables. However, it is imperative that future studies continue to investigate both sexes, as males and female rodents and humans respond to stimuli in considerably different ways, spanning from the molecular level (e.g., hormone and neurotransmitter levels) to behavior (e.g., smoking habits/nicotine consumption) (for review, see IOM, 2001). For example, Klein and colleagues (2004) reported that female C57BL/6J mice consumed more nicotine (mg/kg) compared to males. Understanding the differences between factors that prevent males and females from quitting smoking will help guide the development of interventions that are tailored to an individual’s sex in order to reduce the incidence of initiating and developing a smoking habit effectively for men and women.

Age. Because adolescence is a vulnerable period of time when over 1 million adolescents begin smoking per day, it is imperative for studies to examine this vulnerable age range. This was the first study to examine nicotine consumption and metabolism in adolescent and adult mice, both with and without methoxsalen exposure. Age was a major contributing factor to pattern of nicotine metabolism following methoxsalen exposure; therefore, future studies should further investigate these findings to understand the underlying biological reason for these differences. Also, because adolescents were not permitted to mature into adulthood, an important bridge study would be to examine the effects of puberty by examining nicotine consumption from adolescence into adulthood.

Strain. Of the published literature examining the relationship between methoxsalen and nicotine in mice, three strains were used: ICR, CD2F1, and now, C57BL/6J mice. Mouse strains differ significantly (for review, see Crawley et al., 1997).
in their behavior (e.g., consumption of drugs of abuse; Robinson, Marks, & Collins, 1996; Stolerman, Naylor, Elmer, & Goldberg, 1999), stress sensitivity, xenobiotic metabolism, and genetic polymorphisms within receptor sequences (Butt, King, Hutton, Collins, & Stitzel, 2005; Dobelis et al., 2002). Additionally, there are significant differences between amino acid sequences and hepatic CYP enzyme activity levels between strains (van Iersel, Walters, Price, Lovell, & Lake, 1994; Wood & Conney, 1974; Wood & Taylor, 1979). For example, although sex-dependent, coumarin 7-hydroxylase is highest among DBA/2 and 129 mouse strains, whereas CBA/Ca and C57BL/6J mice have some of the lowest activity levels (van Iersel, Walters, Price, Lovell, & Lake, 1994; Wood & Taylor, 1979). These findings indicate that significant variations in the expression levels of CYP2a5 alters coumarin 7-hydroxylase activity among different mouse strains.

**Nicotine naivety, dependence, and withdrawal.** Experiment I, as well as the studies conducted by Damaj et al. (2007) and Raunio et al. (2008), were conducted using nicotine-naïve mice, such that a mice were given an injection of methoxsalen followed by access to, a subcutaneous injection of, or administration of nicotine via gavage, respectively. Each of these studies lasted less than 24-hours. Experiment II, on the other hand, was completed using nicotine-dependent mice; however, mice were only given 4 days of nicotine exposure before methoxsalen was administered. At this point, there have been no studies have been done on mice in nicotine withdrawal. Because of the short time frame for adolescence, this study would not be able to be done using adolescents; however, this type of study should be done to examine the effect of methoxsalen on withdrawal symptoms. These studies are important to understand if methoxsalen should
prescribed as a prevention medicine, a way to quit smoking or as a drug that can curb withdrawal symptoms (e.g., cravings).

**Methoxsalen without nicotine.** The studies included in this dissertation were well equipped to examine the effects of methoxsalen on nicotine consumption and metabolism; however, a treatment group was not included that were administered methoxsalen without nicotine access. It is important to examine the effects of methoxsalen alone to understand how methoxsalen alters body weight, food consumption, water intake, drug metabolism, and hepatic pathology. Although it is important to understand how methoxsalen affects nicotine metabolism for the purposes of smoking cessation, future studies should also compare the effects of methoxsalen alone with combined effects of methoxsalen and nicotine.

**Methoxsalen and other tobacco compounds.** Interestingly, nicotine consumption was not altered in Experiment I or II, whereas cigarette consumption was significantly reduced in the study conducted by Sellers and colleagues (2000). One potential reason for the difference in results is the use of nicotine versus tobacco. Because of its addictive properties (USDHHS, 1998), nicotine is the most commonly investigated tobacco compound in murine studies. However, nicotine is only one of over 4000 compounds found in tobacco. It is currently unknown how methoxsalen interacts with the compounds in tobacco other than nicotine (e.g., acetaldehyde). Future studies are necessary to determine if there is a drug-drug interaction between methoxsalen and any one of the tobacco compounds or a three-way interaction with nicotine.

**Genetically altered mouse models.** Within the last decade, one of the most recent advances in drug research is the development of genetically altered mice, specifically
chimeric (Katoh et al., 2005; for review, see Strom, Davila, & Grompe, 2010) and transgenic mice. Specifically for nicotine, mouse models with human CYP2A6 liver enzymes will be crucial in our understanding of this CYP enzyme and its responsibility towards nicotine metabolism, as well as providing scientists with the ability to study nicotine pharmacokinetics and toxicity without endangering human lives. For example, following transplantation of human hepatocytes into young, immune-deficient mice, a chimeric mouse with “humanized livers” was developed to examine CYP2A6 and CYP2a5 activity and expression levels as well as the effects of a variety of different compounds (e.g., coumarin) and inhibitors (e.g., methoxsalen) on these levels (Aoki et al., 2006). More recently, Zhang and colleagues (2005) used embryonic stem cell implantation to develop a viable, transgenic mouse with a C57BL/6 and 129/Sv background that specifically expresses human CYP2A6 in the liver. In the future, scientists should utilize these genetically altered mice for nicotine research to the fullest extent.

Scientists have also developed CYP2a5 knockout mice to study nicotine metabolism. In particular, Zhou and colleagues (2010) developed a viable CYP2a5-null mouse. Although there were no physiological differences between wild-type mice and CYP2a5 KO mice, nicotine metabolism and clearance, and cotinine clearance was decreased when hepatic CYP2a5 was conditionally knocked out (Zhou et al., 2010). Additionally, the half-lives of nicotine and cotinine were significantly increased in CYP2a5-null mice compared to their wild-type counterparts (Zhou et al., 2010). Future studies should also investigate nicotine consumption and metabolism in mice where CYP2a5 is knocked out throughout the body (constitutive knockout) or in other organs.
beside the liver, such as the kidneys, brain, lungs or nasal mucosa (conditional knockout). In addition, it is worthwhile to use CYP2a5-null mice as a means to investigate humans with non-functional CYP2A6 enzymes, as is the case with the CYP2A6*4 genetic polymorphism.

**Nicotine**

*Other tobacco constituents.* One of the more obvious differences between animal models and clinical studies of tobacco dependence is the route of nicotine administration. Because of the sensitivity of the olfactory system, the majority of animal models of nicotine dependence will inject nicotine directly into a multitude of body parts, such as the skin, veins, stomach and brain. Human smoke tobacco to deliver nicotine via tobacco smoke in the mouth and lungs, whereas nicotine is most commonly delivered subcutaneously, intraperitoneally, intravenously, via gavage or osmotic minipumps in rodents. Therefore, the comparison between rodent and human studies is complicated because of the other 4000+ constituents found in tobacco. In other words, animal studies only examine nicotine’s effects, whereas human studies examine nicotine’s effects in the presence of tobacco’s compounds. These differences may be one reason why methoxsalen is an effective inhibitor for adult human smokers, but not for mice. Out of the 4000+ compounds in tobacco, a portion of the more well known include, nicotine, menthol, carbon monoxide, tar, nitrogen oxides, metals (i.e., nickel, cadmium, lead), acetaldehyde, formaldehyde, nitrosamines, aromatic hydrocarbons, acetone, and hydrogen cyanide. Some of these compounds may have a significant effect on nicotine metabolism. For example, when healthy adults were given mentholated and non-mentholated cigarettes, researchers determined that smoking menthol cigarettes reduces
the metabolism of nicotine into cotinine (Benowitz, Herrera, & Jacob, 2004). Cadmium increases CYP2a5 mRNA, protein and activity levels in mice (Abu-Bakar, Satarug, Marks, Lang, & Moore, 2004) and humans (Satarug et al., 2004), thereby potentially altering nicotine metabolism. Although it is currently unknown if nicotine metabolism is affected, acetaldehyde significantly increases HPA activation (Cao et al., 2007) and nicotine-induced behaviors, such as self-administration (Belluzi et al., 2004) and locomotion (Cao et al., 2007). Therefore, additional studies should be done to investigate the effects of constituents of tobacco, other than nicotine, on nicotine metabolism. These studies would help to better translate findings from animal models of nicotine dependence to human clinical studies.

**Methoxsalen**

**Type of inhibitor used.** Methoxsalen is a known CYP2A6 and CYP2a5 inhibitor; however, Zhang and colleagues (2001) determined that other drugs may be better inhibitors of these enzymes. For example, tranylcypromine, an irreversible inhibitor of monoamine oxidase (MAO)-A and –B, and tryptamine, a neuromodulator, have greater selectivity at lower concentrations for CYP2A6 compared to methoxsalen (Zhang et al., 2001). Beyond a lack of precedence in the literature, tranylcypromine and tryptamine were not chosen for these studies because the primary functions of these drugs may induce unintentional neurochemical effects that could alter the study results in unexpected ways. More specifically, because MAO is an enzyme that metabolizes dopamine, serotonin and norepinephrine, inhibition of this process by tranylcypromine would increase extracellular levels of these monoamines, causing a multitude of effects (e.g., increased nicotine consumption). Tryptamine, on the other hand, inhibits the amino
acid transporter, PAT1 (Metzner, Kottra, Neubert, Daniel, & Brandsch, 2005) and
induces phosphoinositide hydolysis (Ishitani, Kimura, Takeichi, & Chuang, 1994), a
process that produces second messengers that control calcium release, and cell growth
and fertilization (Berridge, 1993). Regardless, methoxsalen was chosen based on
published literature regarding its clinical uses in dermatology, indicating a relative safety
level for humans, as well as previous research using humans and mouse models in
conjunction with nicotine for use as a potential smoking cessation aid. Separate future
studies should examine nicotine consumption following methoxsalen, tranylcypromine
and tryptamine to compare effects and determine which would be a better smoking
cessation aid. In addition, subsequent studies should also investigate and compare
derivatives of methoxsalen (e.g., 5-methoxypsoralen) that are thought to be more
effective without the toxic effects, at least in terms of dermatological improvement
(Willis & Mentor, 1984). To date, there are no studies examining the effects of these
drug derivatives on nicotine consumption or metabolism.

Non-specificity. In addition to irreversibly inactivating CYP2A6, methoxsalen is
also a strong inhibitor of CYP1A2 (Zhang et al., 2001) and a weak inactivator of
CYP2C8/9, -2C19, -2D6, -2E1, and -3A4 (Koenigs et al., 1997). Although many of these
CYP enzymes are responsible for the metabolism of xenobiotics, drugs other than
nicotine are the primary substrates for these enzymes. For example, CYP2D6
metabolizes opioids (Tyndale, Droll, & Sellers, 1997), while CYP2E1 breaks down
ethanol (Lieber, 1997). Additionally, although nicotine metabolism is primarily
accomplished via CYP2A6, CYP2D6 and -2E1 can play very minor roles in the process.
Future studies should take this non-specificity into account when administering
methoxsalen, especially if there is the potential for an adverse reaction with a second
drug (e.g., ethanol).

**Photosensitivity.** Due to its photosensitive properties, methoxsalen has a
successful history as a treatment for a large variety of dermatoses, including but not
limited to, psoriasis, vitiligo, CTCL, atopic dermatitis, lichen planus, alopecia areata, and
eczema (for review, see Kapelewski & Klein, under review). Although this trait has
proven to be an asset in dermatology, photosensitivity is a limitation to nicotine addiction
research. To treat dermatoses, methoxsalen is usually combined with UV radiation to
produce a significant effect. However, patients taking methoxsalen are instructed wear
long sleeves, dark sunglasses, and suntan lotion when going outside or even sitting by a
sunny window because sunlight can react with methoxsalen and cause additional
problems (e.g., sunburn). Although the effects of methoxsalen seem to occur with only
sunlight or UV radiation, areas of the body constantly exposed to any kind of light should
be closely monitored in any future studies to prevent undo harm to the animal or human.

**Toxicity.** To date, the short- and long-term effects and toxicity reports for
methoxsalen stem from the collective history as a dermatological treatment. Because
methoxsalen has been used as a treatment for dermatoses for several decades, there are
multitudes of studies indicating the intended, as well as detrimental, effects that can occur
when receiving PUVA therapy, and to a lesser extent, methoxsalen sans UV radiation
(See Side effects and Toxicity section of this dissertation). At this point, no studies have
been published on the side effects or toxicity levels of methoxsalen when combined with
nicotine. Therefore, if methoxsalen is to become a smoking cessation aid, toxicological
studies in humans and rodent models are extremely important to ensure the health and
safety of the drug’s recipient. The photosensitivity of methoxsalen and the abundant exposure of light, both natural and manufactured, that most individuals come in contact with on a daily basis places these studies high on the priority list.

**Outcome Measures**

**CYP2a5 and CYP2e1.** Because of the ethyl alcohol component of the vehicle, CYP2e1 was examined mainly to rule out any effects of this enzyme on the results of the study. In contrast, CYP2a5 was chosen as the primary target enzyme for this dissertation due to the structural and functional similarities with human CYP2A6, the enzyme primarily responsible for nicotine metabolism in humans. As is usually the case, investigation of a single enzyme does not paint the entire picture, as there are more enzymes involved in this process beyond that of CYP2A6 or CYP2a5. Albeit to a far lesser extent, other CYP enzymes, including CYP2A7, -2D6 and -2A13 (Mwenifumbo & Tyndale, 2009), as well as flavin-containing monoxygenase3 (FMO3), amine N-methyltransferase, UDP-glucuronosyltransferases and aldehyde oxidase, are responsible for a small percentage of nicotine metabolism (for review, see Hukkanen, Jacob, & Benowitz, 2005). Therefore, future studies should examine activity levels of these enzymes to formulate a complete picture of what is happening to all of the enzymes involved with nicotine metabolism. It also is important to know how the activity level of these enzymes changes over time in general, but also over time of methoxsalen and nicotine exposure. Therefore, a long term study where liver samples are examined in a subset of animals every couple of days of drug exposure to examine potential change over time would be advantageous.
CYP location. Although a small percentage of nicotine metabolism transpires in the lungs, nasal mucosa and brain, the majority occurs in the liver (Britton et al., 2000; for review, see Hukkanen et al., 2005; USDHHS, 1988) via CYP2A6 in humans and CYP2a5 in mice. This location specificity is the primary reason for examining liver enzymes; however, CYP2a5 and CYP2e1 are, in fact, located throughout the body. In addition to the liver, CYP2a5 is also found in the kidney, lung, brain (e.g., cerebellum, main olfactory bulb, hippocampus), nasal mucosa, esophagus, and small intestine (for review, see Hrycay & Bandiera, 2009). Similarly, CYP2e1 is found in the kidney, lung, nasal mucosa, small intestine, brain (e.g., cerebellum, main olfactory bulb, hippocampus), but also in the heart, skeletal muscle, brain, spleen, skin, testes and ovaries (for review, see Hrycay & Bandiera, 2009). Additional studies should not only examine these areas of the body to understand how gene expression and activity level of the enzyme changes when nicotine and methoxsalen are administered separately and together. It would also be interesting to understand the function of CYP2a5 within other body regions that are not directly involved with nicotine metabolism, such as the heart and spleen.

Analyses on CYP enzymes. There are three levels to the central dogma of molecular biology: DNA → RNA → protein. Differences in transcript levels (RNA) between treatment groups in adolescents and adults were assessed using RT-PCR; however, future studies also should assess disparities in gene expression among different strains. Investigations into potential changes DNA sequences, protein expression or protein activity levels were not completed. Therefore, future experiments should include techniques to assess any differences in alleles, protein levels or protein expression between males and females, and among an assortment of strains. Furthermore, additional
studies should compare levels of gene and protein expression, enzyme activity levels and allelic differences in mice that receive no drugs (control) to those that receive methoxsalen, nicotine, and the combination of the two drugs.

**Techniques to assess nicotine exposure.** The primary metabolite of nicotine, cotinine, was used to assess nicotine exposure. Specifically, cotinine levels were detected in serum via enzyme immunoassay (EIA). Using serum cotinine levels as a nicotine biomarker is a commonly used method because the half-life of cotinine is much longer compared to that of nicotine (about 16 versus 2 hours, respectively; Benowitz, 1998b; McClure & Swan, 2006). EIA were run on cotinine; however, effects on concentrations of the parent drug and other important metabolites (e.g., trans-3-hydroxycotinine) were left unknown. Future studies should utilize other methods, including gas chromatography coupled with mass spectrometry (GC-MS), to observe concentrations of nicotine, all of the metabolites of nicotine, including cotinine, and the metabolites of cotinine, (e.g., trans-3-hydroxycotinine). From this information, concentration ratios, for example cotinine (COT) to nicotine (COT/NIC) or trans-3-hydroxycotinine (3HC) to cotinine (3HC/COT), can be determined, which can be used as markers for metabolic activity of CYP2A6 (Dempsey et al., 2004; for review, see Benowitz et al., 2009) or CYP2a5 for mice.

**Conclusion**

Methoxsalen has an interesting future based on the results of Experiment I and II, and the previously published findings from Damaj et al. (2007), Raunio et al. (2008), and Sellers et al. (2000). With regard to the mouse studies, methoxsalen is a strong inhibitor of CYP2a5, as indicated by a reduction in cotinine levels in serum, plasma and urine.
Interestingly, the reduction in metabolite level did not change nicotine consumption in mice following acute or chronic methoxsalen exposure (Experiment I and II); however, cigarette consumption was reduced in adult smokers after a single administration of the inhibitor (Sellers et al., 2000). The resultant disparity between these three studies therefore leaves ample room for additional investigations into the potential for methoxsalen to become a smoking cessation aid.
REFERENCES
REFERENCES


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Lavery, D.J., Lopez-Molina, L., Margueron, R., Fleury-Olela, F., Conquet, F., Schibler, U., & Bonfils, C. (1999). Circadian expression of the steroid 15 a-hydroxylase (Cyp2a4) and coumarin 7-hydroxylase (Cyp2a5) genes in mouse liver is regulated by the PAR leucine zipper transcription factor DBP. Molecular and Cellular Biology, 19(10), 6488-6499.


Systematic Reviews, 3, CD000146.


http://www.who.int/topics/tobacco/facts/en/index.html


http://www.who.int/tobacco/health_priority/en/


APPENDICES
Appendix A: Methoxsalen MSDS Sheet
Material Safety Data Sheet
Methoxsalen MSDS

Section 1: Chemical Product and Company Identification

Product Name: Methoxsalen
Catalog Codes: SLM1885
CAS#: 298-81-7
RTECS: LV1400000
TSCA: TSCA 8(b) Inventory: Methoxsalen
Cf#: Not available.
Synonym: 8-MoP, 8-MP, Ammodin, Geroxal, eladinin, Meladamine, Meloxine, Methoxa-dome, New-meladomin, Oxsonal, Oxyxoralen, Proralone-mop, Xanthotoxin; 8-Methoxyxoralen; 5-Benzofuranacrylic acid, 6-hydroxy-7-methoxy-6,7-dimethoxy-7-methoxy-5-benzoquinone acid delta-lactone; 8-Methoxy-(furano-3,2,1-d,7,6,7-oxooumarin); 8-Methoxy-3,2,7,6,7-furocoumarin; 8-Methoxy-4,3,2,7,6,7-furocoumarin; 8-Methoxysporalene; 9-Methoxysporalene
Chemical Name: 7H-Furo(3,2-g)(1)benzopyran-7-one, 9-methoxy-
Chemical Formula: C12-H8-O4

Contact Information:
ScienceLab.com, Inc.
14025 Smith Rd.
Houston, Texas 77396

US Sales: 1-800-901-7247
International Sales: 1-281-441-4400

Order Online: ScienceLab.com

CHEMTREC (24HR Emergency Telephone), call:
1-800-424-9300
International CHEMTREC, call: 1-703-527-3887
For non-emergency assistance, call: 1-281-441-4400

Section 2: Composition and Information on Ingredients

Composition:

<table>
<thead>
<tr>
<th>Name</th>
<th>CAS #</th>
<th>% by Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoxsalen</td>
<td>298-81-7</td>
<td>100</td>
</tr>
</tbody>
</table>

Toxicological Data on Ingredients: Methoxsalen: ORAL (LD50): Acute: 791 mg/kg [Rat], 423 mg/kg [Mouse].

Section 3: Hazards Identification

Potential Acute Health Effects:
Very hazardous in case of skin contact (irritant), of eye contact (irritant), of ingestion, or inhalation. Hazardous in case of skin contact (corrosive), of eye contact (corrosive). Slightly hazardous in case of skin contact (sensitizer). The amount of tissue damage depends on length of contact. Eye contact can result in corneal damage or blindness. Skin contact can produce inflammation and blistering. Inhalation of dust will produce irritation to
Section 4: First Aid Measures

Eye Contact:
Check for and remove any contact lenses. In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Get medical attention immediately.

Skin Contact:
In case of contact, immediately flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Cover the irritated skin with an emollient. Wash clothing before reuse. Thoroughly clean shoes before reuse. Get medical attention immediately.

Serious Skin Contact:
Wash with a disinfectant soap and cover the contaminated skin with an anti-bacterial cream. Seek immediate medical attention.

Inhalation:
If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

Serious Inhalation:
Evacuate the victim to a safe area as soon as possible. Loosen tight clothing such as a collar, tie, belt or waistband. WARNING: It may be hazardous to the person providing aid to give mouth-to-mouth resuscitation when the inhaled material is toxic, infectious or corrosive. Seek immediate medical attention.

Ingestion:
Do NOT induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. If large quantities of this material are swallowed, call a physician immediately. Loosen tight clothing such as a collar, tie, belt or waistband.

Serious Ingestion: Not available.

Section 5: Fire and Explosion Data

Flammability of the Product: May be combustible at high temperature.

Auto-Ignition Temperature: Not available.

Flash Points: Not available.

Flammable Limits: Not available.

Products of Combustion: These products are carbon oxides (CO, CO2).

Fire Hazards In Presence of Various Substances:
**Explosion Hazards In Presence of Various Substances:**
Slightly explosive in presence of open flames and sparks.
Non-explosive in presence of shocks.

**Fire Fighting Media and Instructions:**
SMALL FIRE: Use DRY chemical powder.
LARGE FIRE: Use water spray, fog or foam. Do not use water jet.

**Special Remarks on Fire Hazards:**
COMBUSTIBLE.
When heated to decomposition it emits toxic fumes.
As with most organic solids, fire is possible at elevated temperatures.

**Special Remarks on Explosion Hazards:**
Fine dust dispersed in air in sufficient concentrations, and in the presence of an ignition source is a potential dust explosion hazard.

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**Section 6: Accidental Release Measures**

**Small Spill:** Use appropriate tools to put the spilled solid in a convenient waste disposal container.

**Large Spill:**
Corrosive solid.
Stop leak if without risk. Do not get water inside container. Do not touch spilled material. Use water spray to reduce vapors. Prevent entry into sewers, basements or confined areas; dike if needed. Eliminate all ignition sources. Call for assistance on disposal.

---

**Section 7: Handling and Storage**

**Precautions:**
Keep locked up. Keep container dry. Keep away from heat. Keep away from sources of ignition. Empty containers pose a fire risk, evacuate the residue under a fume hood. Ground all equipment containing material.
Do not ingest. Do not breathe dust. Never add water to this product. In case of insufficient ventilation, wear suitable respiratory equipment. If ingested, seek medical advice immediately and show the container or the label. Avoid contact with skin and eyes. Keep away from incompatibles such as oxidizing agents.

**Storage:** Keep container tightly closed. Keep container in a cool, well-ventilated area.

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**Section 8: Exposure Controls/Personal Protection**

**Engineering Controls:**
Use process enclosures, local exhaust ventilation, or other engineering controls to keep airborne levels below recommended exposure limits. If user operations generate dust, fume or mist, use ventilation to keep exposure to airborne contaminants below the exposure limit.

**Personal Protection:**
Splash gogglies. Synthetic apron. Vapor and dust respirator. Be sure to use an approved/certified respirator or equivalent. Gloves.

**Personal Protection In Case of a Large Spill:**
Splash gogglies. Full suit. Vapor and dust respirator. Boots. Gloves. A self-contained breathing apparatus should be used to avoid inhalation of the product. Suggested protective clothing might not be sufficient; consult a specialist BEFORE handling this product.

**Exposure Limits:** Not available.
### Section 9: Physical and Chemical Properties

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical state and appearance</td>
<td>Solid</td>
</tr>
<tr>
<td>Odor</td>
<td>Odorless</td>
</tr>
<tr>
<td>Taste</td>
<td>Bitter</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>216.19 g/mole</td>
</tr>
<tr>
<td>Color</td>
<td>White to yellowish, Off-white</td>
</tr>
<tr>
<td>pH (1% soln/water)</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Boiling Point</td>
<td>Not available</td>
</tr>
<tr>
<td>Melting Point</td>
<td>145°C (299.4°F)</td>
</tr>
<tr>
<td>Critical Temperature</td>
<td>Not available</td>
</tr>
<tr>
<td>Specific Gravity</td>
<td>Not available</td>
</tr>
<tr>
<td>Vapor Pressure</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Vapor Density</td>
<td>Not available</td>
</tr>
<tr>
<td>Volatility</td>
<td>Not available</td>
</tr>
<tr>
<td>Odor Threshold</td>
<td>Not available</td>
</tr>
<tr>
<td>Water/Oil Dist. Coeff.</td>
<td>Not available</td>
</tr>
<tr>
<td>Ionicity (in Water)</td>
<td>Not available</td>
</tr>
<tr>
<td>Dispersion Properties</td>
<td>See solubility in water, acetone</td>
</tr>
<tr>
<td>Solubility</td>
<td>Soluble in acetone</td>
</tr>
<tr>
<td></td>
<td>Very slightly soluble in hot water, diethyl ether.</td>
</tr>
<tr>
<td></td>
<td>Insoluble in cold water</td>
</tr>
<tr>
<td></td>
<td>Sparingly soluble in liquid petrolatum</td>
</tr>
<tr>
<td></td>
<td>Soluble in boiling alcohol, acetic acid, vegetable fixed oils, propylene glycol, benzene.</td>
</tr>
<tr>
<td></td>
<td>Freely soluble in chloroform</td>
</tr>
</tbody>
</table>

### Section 10: Stability and Reactivity Data

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stability</td>
<td>The product is stable</td>
</tr>
<tr>
<td>Instability Temperature</td>
<td>Not available</td>
</tr>
<tr>
<td>Conditions of Instability</td>
<td>Excess heat, dust generation, Incompatible materials</td>
</tr>
<tr>
<td>Incompatibility with various substances</td>
<td>Reactive with oxidizing agents</td>
</tr>
<tr>
<td>Corrosivity</td>
<td>Not available</td>
</tr>
<tr>
<td>Special Remarks on Reactivity</td>
<td>Sensitive to light, May decompose on exposure to light.</td>
</tr>
<tr>
<td>Special Remarks on Corrosivity</td>
<td>Not available</td>
</tr>
<tr>
<td>Polymerization</td>
<td>Will not occur</td>
</tr>
</tbody>
</table>
Section 11: Toxicological Information

Routes of Entry: Inhalation. Ingestion.

Toxicity to Animals: Acute oral toxicity (LD50): 423 mg/kg [Mouse].

Chronic Effects on Humans:
CARCINOGENIC EFFECTS: Classified 2 (Some evidence.) by NTP. 3 (Not classifiable for human.) by IARC.
MUTAGENIC EFFECTS: Mutagenic for mammalian somatic cells. Mutagenic for bacteria and/or yeast.

Other Toxic Effects on Humans:
Very hazardous in case of skin contact (irritant), of ingestion, of inhalation.
Hazardous in case of skin contact (corrosive), of eye contact (corrosive).
Slightly hazardous in case of skin contact (sensitizer).

Special Remarks on Toxicity to Animals: Not available.

Special Remarks on Chronic Effects on Humans:
May affect genetic material (mutagenic).
May cause adverse reproductive effects and birth defects (teratogenic).
May cause cancer

Special Remarks on other Toxic Effects on Humans:
Acute Potential Health Effects:
Skin: Causes severe skin irritation and burns. Causes photosensitivity. Exposure to light can result in allergic reactions resulting in dermatologic lesions, which can vary from sunburn like responses to edematous, vesiculated lesions or bullae.
Eyes: Causes severe irritation and burns.
Inhalation: Harmful if inhaled. Destruuctive to tissue of mucous membrane. Causes respiratory tract and mucous membrane irritation and burns. Symptoms may include burning sensation, coughing, wheezing, bronchitis, laryngitis (Inflammation and edema of the larynx and bronchi), shortness of breath, chemical pneumonitis. Inhalation may be fatal as a result of spasm inflammation and edema of the larynx and bronchi, and chemical pneumonitis. Possible allergic reaction to material if inhaled.
Ingestion: Harmful if swallowed. Causes gastrointestinal tract irritation and burns with nausea, vomiting, diarrhea, hypermotility. May affect behavior/central nervous system (convulsions, ataxia, nervousness, insomnia, mental depression, dizziness, headache), liver. Possible allergic reaction if material is ingested.
Chronic Potential Health Effects:
Repeated or prolonged ingestion may affect the liver (hepatitis, liver damage), blood (hemorrhage), and gastrointestinal tract (nausea, vomiting, hypermotility, diarrhea), endocrine system (pituitary gland, prostate gland). Skin: Repeated or prolonged skin contact may cause premature aging of skin and skin cancer. Prolonged or repeated exposure may cause hypersensitization.

Section 12: Ecological Information

Ecotoxicity: Not available.

BOD5 and COD: Not available.

Products of Biodegradation:
Possibly hazardous short term degradation products are not likely. However, long term degradation products may arise.

Toxicity of the Products of Biodegradation: The products of degradation are less toxic than the product itself.

Special Remarks on the Products of Biodegradation: Not available.

Section 13: Disposal Considerations

Waste Disposal:
Waste must be disposed of in accordance with federal, state and local environmental control regulations.

### Section 14: Transport Information

**DOT Classification:** Class 8: Corrosive material

**Identification:** Corrosive solid, n.o.s. (8-Methoxysporalen) UNNA: 1759 PG: III

**Special Provision for Transport:** Not available.

### Section 15: Other Regulatory Information

**Federal and State Regulations:**
California prop. 65: This product contains the following ingredients for which the State of California has found to cause cancer, birth defects or other reproductive harm, which would require a warning under the statute: Methoxsalen.
California prop. 65: This product contains the following ingredients for which the State of California has found to cause cancer which would require a warning under the statute: Methoxsalen.
California Director's List of Hazardous Substances: Methoxsalen.
TSCA 8(b) Inventory: Methoxsalen.

**Other Regulations:**
EINECS: This product is on the European Inventory of Existing Commercial Chemical Substances.

**Other Classifications:**
WHMIS (Canada): Not controlled under WHMIS (Canada).

**DSCCL (EEC):**
R22- Harmful if swallowed.
R34- Causes burns.
R43- May cause sensitization by skin contact.
R45- May cause cancer.
R46- May cause heritable genetic damage.
S22- Do not breathe dust.
S26- In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S36/37/39- Wear suitable protective clothing, gloves and eye/face protection.
S46- If swallowed, seek medical advice immediately and show this container or label.
S53- Avoid exposure - obtain special instructions before use.

**HMIS (U.S.A.):**
- Health Hazard: 3
- Fire Hazard: 1
- Reactivity: 0
- Personal Protection:

**National Fire Protection Association (U.S.A.):**
### Health: 3

**Flammability:** 1  
**Reactivity:** 0  

**Specific hazard:**

**Protective Equipment:**  
Gloves.  
Synthetic apron.  
Vapor and dust respirator. Be sure to use an approved/certified respirator or equivalent.  
Splash goggles.

## Section 16: Other Information

**References:** Not available.  

**Other Special Considerations:** Not available.  

**Created:** 10/09/2005 06:07 PM  

**Last Updated:** 10/09/2005 06:07 PM

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APPENDIX B: Nicotine Preparation Protocol
Nicotine Preparation Protocol

Concentrated Stock Solutions
For a final 50 ug/ml concentration (make a 10 fold stock solution – 500 ug/ml)

10 X 50 ug/ml = 500 ug/ml X 400ml = 200,000ug = 0.2g

\[
\frac{0.2g}{1.01g} = 0.198ml = 198 \text{ ul of nicotine / 400 ml of water}
\]

This solution needs to be diluted 10-fold to be at 50 ug/ml (e.g. 400 ml of above in 3,600 ml water to make 4 liters of 50 ug/ml)

For a final 200 ug/ml concentration (make a 10 fold stock solution – 2,000 ug/ml)

10 X 200 ug/ml = 2000 ug/ml X 400ml = 800,000ug = 0.8g

\[
\frac{0.8g}{1.01g} = 0.792ml = 792 \text{ ul of nicotine / 400 ml of water}
\]

This solution needs to be diluted 10-fold to be at 200 ug/ml (e.g. 400 ml of above in 3,600 ml water to make 4 liters of 200 ug/ml)

Diluted Solutions (Final or Working Solutions)

\[
\frac{100 \text{ ml of concentrated solutions}}{900 \text{ ml of water}} = 1000 \text{ ml of stock solution}
\]

100 ml bottle (provided by CBL) X amount of mice = total volume (mls) of solution needed
APPENDIX C: Experiment I Methoxsalen Preparation Protocol
Experiment I Methoxsalen Preparation Protocol

Methoxsalen Solution:
A 40 ml solution consists of methoxsalen dissolved in a solution of 1 part (ethyl alcohol): 1 part (Emulphor): 18 parts (distilled water)
- $1/20$ of 40 = 2ml 200 proof ethyl alcohol
- $1/20$ of 40 = 2ml Emulphor
- $18/20$ of 40 = 36ml distilled water

Vehicle:
1. In a beaker, add 2 ml of 200 proof ethyl alcohol into the beaker
2. Insert stir bar
3. Slowly begin adding Emulphor until the solution is homogenous
4. Drop by drop, slowly add 36 ml of distilled water until you reach total volume (40 mls) and the solution has a yellowish haze

Drug Solution for Adolescents:
Maximum weight of mouse: 25 grams
Maximum volume to be injected: 0.5ml

Ingredients of the stock solution (1-fold solution) of 8MOP:
For when the desired dosage is 10 mg/kg (i.e. 0.01 mg/g)
$0.01 \text{ mg/g} \times 25 \text{g (maximum mouse weight)} = 0.25 \text{mg} / 500 \text{ul} = 0.35 \text{mg} = 0.5 \text{mg/ml}$
500 ul (desired injection volume) 0.5ml
Want:
500 ug/ml (0.5mg/ml) of 8MOP into 40ml of vehicle, sufficient for injecting 80 mice.

$40\text{ml X 0.5 mg/ml} = 20\text{mg of 8MOP into 40ml (total)}$
*Note: this small amount will not alter the volume so you won’t have to account for a change in volume with this addition

Procedure:
1. Tare a 40 ml beaker
2. Measure out methoxsalen into the beaker
   a. For 10 mg/kg dosage: 20 mg (0.020g)
   b. For 5 mg/kg dosage: 10 mg (0.010g)
3. Using a stir bar, slowly add 2 ml of 200 proof ethyl alcohol into the beaker
4. Slowly begin adding Emulphor until the solution is homogenous
5. Drop by drop, slowly add 36 ml of distilled water until you reach total volume (40 mls) and the solution has a yellowish haze

Injection Volume:
$10 \text{mg/kg}$: Mouse weight (g) $\times 0.01 \text{ mg/g} = \text{Weight (g)} \times 0.02 \text{ml/g} = \text{injection mls}$
$0.5\text{mg/ml}$
$5 \text{mg/kg}$: Mouse weight (g) $\times 0.005 \text{ mg/g} = \text{Weight (g)} \times 0.02 \text{ml/g} = \text{injection mls}$
$0.25\text{mg/ml}$
**Drug Solution for Adults:**
Maximum weight of mouse: 35 grams
Maximum volume to be injected: 0.5ml

**Ingredients of the stock solution (1-fold solution) of 8MOP:**
For when the desired dosage is 10 mg/kg (i.e. 0.01 mg/g)

\[
0.01 \text{ mg/g} \times 35 \text{g (maximum mouse weight)} = 0.35 \text{mg} / 500 \text{ ul} = 0.7 \text{ mg/ml} \\
500 \text{ ul (desired injection volume)} \\
\]

Want:
500 ug/ml (0.7 mg/ml) of 8MOP into 40ml of vehicle, sufficient for injecting 80 mice.

40ml \(\times 0.7 \text{ mg/ml} = 28\text{mg of 8MOP into 40ml (total)}\)

*Note: this small amount will not alter the volume so you won’t have to account for a change in volume with this addition*

**Procedure:**
1. Tare a 40 ml beaker
2. Measure out methoxsalen into the beaker
   a. For 10 mg/kg dosage: 28 mg (0.028g)
   b. For 5 mg/kg dosage: 14 mg (0.014g)
3. Using a stir bar, slowly add 2 ml of 200 proof ethyl alcohol into the beaker
4. Slowly begin adding Emulphor until the solution is homogenous
5. Drop by drop, slowly add 36 ml of distilled water until you reach total volume (40 mls) and the solution has a yellowish haze

**Injection Volume:**
10 mg/kg: Mouse weight (g) \(\times 0.01 \text{ mg/g} = \text{Weight (g)} \times 0.14286 \text{ ml/g} = \text{injection mls} \times 0.7 \text{ mg/ml}\)

5 mg/kg: Mouse weight (g) \(\times 0.005 \text{ mg/g} = \text{Weight (g)} \times 0.14286 \text{ ml/g} = \text{injection mls} \times 0.35 \text{ mg/ml}\)
APPENDIX D: Liver Preparation and Dissection Protocol
Liver Preparation and Dissection Protocol

1. Using forceps, lift the liver up slightly and cut connecting tendons without cutting the liver
2. Remove intact liver from the mouse
3. Tare piece of aluminum foil on scale
4. Weigh the entire liver on the aluminum foil and record
5. Cut and weigh out 2 separate 0.1g sections (for later assessment of RNA levels) and record
6. After cutting and weighing each section, place liver section into a labeled (mouse ID number) screw top cryovial filled with 500 ul of RNAS later (for preservation of RNA)
7. Flash freeze each cryovial in liquid nitrogen
   a. Put on safety apparel (protective eye wear, -80°C freezer gloves)
   b. Using the long tongs, drop the cryovial into the liquid nitrogen for four seconds
   c. Remove cryovial from liquid nitrogen using long tongs and place inside labeled cryovial box that is sitting in dry ice
8. Once the experiment is complete, store the labeled cryovial box in a -80°C freezer for later experiments
APPENDIX E: Enzyme Immunoassay Protocol for Serum Cotinine
COTinine Direct ELISA Kit

Ver: 01/2004

Immunalysis Corporation:
Catalog Number: 217-0096 1 x 96 well plates
217-0480 5 x 96 well plates

THE IMMUNALYSIS COTININE DIRECT ELISA KIT IS INTENDED FOR RESEARCH USE ONLY.

EXPLANATION OF THE TEST

The Immunalysis Cotinine Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of cotinine in serum and urine. Exposure to tobacco smoke can be detected by measuring nicotine and its metabolites. Nicotine has a short half life and is not used as a marker for tobacco smoke exposure. Cotinine due to its longer half life has been used in research as a reliable marker for smoking status and smoking cessation studies.

PRINCIPLES OF THE PROCEDURE

The Immunalysis Cotinine Direct ELISA Kit is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture.

A 10 μl aliquot of a diluted unknown specimen is incubated with a 100 μl dilution of enzyme (Horseradish peroxidase) labeled Cotinine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 1 ng/ml.

REAGENTS

Immunalysis COTinine Direct ELISA Kit Contents:

<table>
<thead>
<tr>
<th>Component</th>
<th>96 test Kit Cat#217-0096</th>
<th>480 test Kit Cat#217-0480</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well Micro-plate</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Cotinine Conj.</td>
<td>15 mL</td>
<td>60 mL</td>
</tr>
<tr>
<td>Neg Serum Std</td>
<td>1 mL</td>
<td>2 x 1 mL</td>
</tr>
<tr>
<td>Cotinine 5 ng/mL</td>
<td>1 mL</td>
<td>2 x 1 mL</td>
</tr>
<tr>
<td>Cotinine 10 ng/mL</td>
<td>1 mL</td>
<td>2 x 1 mL</td>
</tr>
<tr>
<td>Cotinine 25 ng/mL</td>
<td>1 mL</td>
<td>2 x 1 mL</td>
</tr>
<tr>
<td>Cotinine 50 ng/mL</td>
<td>1 mL</td>
<td>2 x 1 mL</td>
</tr>
<tr>
<td>Cotinine 100 ng/mL</td>
<td>1 mL</td>
<td>2 x 1 mL</td>
</tr>
<tr>
<td>Neg Control Std</td>
<td>2 mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>Cotinine Pos Std</td>
<td>2 mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>TMB Substrate</td>
<td>30 mL</td>
<td>2 x 30 mL</td>
</tr>
<tr>
<td>Stop Reagent</td>
<td>25 ml</td>
<td>55 ml</td>
</tr>
</tbody>
</table>

96 well micro-plate. The micro-plate is coated with polyclonal antibody to Cotinine via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a desiccant.

Cotinine-Enzyme Conjugate. The conjugate solution contains a Cotinine derivative labeled with horseradish peroxidase in a buffered, protein solution with stabilizers, pH 7.6 containing non azide preservatives.

Negative Serum Standard. This bottle contains drug free rabbit serum containing azide free preservatives.
Serum Cotinine Standards. These bottles contain 5, 10, 25, 50 and 100 ng/mL of Cotinine dissolved in a rabbit serum non azide preservatives.

Negative Urine Standard. This bottle contains drug free synthetic urine matrix containing azide free preservatives.

Urine Cotinine Positive Standard. This bottle contains 500 ng/mL of cotinine in a synthetic urine matrix containing azide free preservatives.

TMB chromogenic substrate. The color reagent contains 3,3',5,5' tetramethylbenzidine and urea peroxidase in buffer.

Stop Reagent. This contains 1 N hydrochloric acid.

MATERIALS AND EQUIPMENT

Materials and equipment required but not supplied with the Immunoanalysis Cotinine Direct ELISA Kit are itemized below. 12x75 mm Disposable Glass or Plastic Culture Tubes to predilute samples (if required).

Manual or electronic micropipets (single channel or multichannel) or automated pipetting stations.

Refrigerator (for kit storage).

Interval Timer.

Wash bottle or Plate Washer.

Microplate reader capable of reading at 450 nm. And 650 nm.

Precautions

1. Not for Internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. Do not add sodium azide to samples as preservative.
6. Do not use external controls containing sodium azide.
7. Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle stop reagent with care, since it is corrosive.
13. Bring all reagents to room temperature.
14. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pipetting.
15. Ensure the bag containing the micro-plate strips and dessicant is well sealed if only a few strips are used.

General. Precise pipetting is the essence of successful immunoassay. It is critical to pipet right at the center and bottom of each well to ensure good replicates and coefficients of variation Micropipets supplied by "Eppendorf" or "SMI" with disposable tips are excellent when used carefully according to instructions to insure the necessary accuracy. New automatic dispensers improve reliable delivery.

Storage. The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 – 4°C.

Indications of Deterioration. A drop of greater than 50% in the A9 (zero-standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

SPECIMEN COLLECTION

Precautions.

The Immunoanalysis Cotinine Direct ELISA Kit is to be used with human urine or serum. Immunoanalysis has not tested all possible applications of this assay. Cutoff criteria are important in deciding the sample dilution.
Additives.
Specimens to which sodium azide has been added affect the assay.

DETAILS OF THE PROCEDURE.

All reagents must be brought to room temperature (20-25°C) before use.

The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipettor. Use urine calibrators for urine and serum calibrators for serum. Depending on the cutoffs a sample dilution may be required for urine applications.

1. Add 10 μl of calibrators and standards to each well in duplicate.
2. Add 10 μl of the specimens in duplicate (recommended) to each well.
3. Add 100 μl of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
4. Incubate for 60 minutes at room temperature (20-25°C) preferably in the dark, after addition of enzyme conjugate to the last well.
5. Wash the wells 6 times with 350 μl distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells.
6. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
7. Add 100 μl of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
8. Incubate for 30 minutes at room temperature, preferably in the dark.
9. Add 100 μl of Stop Solution to each well, to change the blue color to yellow.
10. Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
11. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response serum cotinine curve.

<table>
<thead>
<tr>
<th>Cotinine ng/ml</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.759</td>
</tr>
<tr>
<td>5</td>
<td>1.075</td>
</tr>
<tr>
<td>10</td>
<td>0.865</td>
</tr>
<tr>
<td>25</td>
<td>0.691</td>
</tr>
<tr>
<td>50</td>
<td>0.495</td>
</tr>
<tr>
<td>100</td>
<td>0.419</td>
</tr>
</tbody>
</table>

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run. A dose response curve or a cutoff calibrator should be run with every plate.

RESULTS

If the average sample absorbance is equal to or less than the average absorbance of the laboratory positive reference standard the sample is POSITIVE for Cotinine. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called NEGATIVE for Cotinine.

Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy.
20 urine samples from non-smokers were screened with the Immunalysis Cotinine ELISA method. All 20 samples screened negative with the ELISA method. 15 samples from smokers which contained various amounts of Cotinine were screened with the Immunalysis Cotinine Direct ELISA Kit. All 15 samples showed a presence of cotinine at a level greater than 500 ng/ml.
Three urine samples submitted by individuals exposed to passive inhalation for over 30 days all showed levels of 5 to 10 mg/mL of cotinine when extrapolated of a dose response curve.

**Sensitivity**
Assay sensitivity based on the minimum Cotinine concentration required to produce a three standard deviation from assay Ao is 1 ng/mL.

**Specificity**
The specificity of the Immunalysis Cotinine ELISA was determined by generating inhibition curves for each of the compounds listed below. The antisera cross-reactivities are listed below.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Approx. ng/ml equivalent to 100 ng Cotinine/ml</th>
<th>Cross-reactivities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotinine</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Nicotine</td>
<td>&gt;10000</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>&gt;10000</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Nicotic Acid</td>
<td>&gt;10000</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

**Cross-Reactivity with Unrelated Drugs**
Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 50,000 ng/ml. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level.

Acetaminophen, Acetylsalicylic acid, Aminopyrine, Aminopyrine, Ampicillin, Amobarbital, Ascorbic acid, Atropine, Barbital, Butabarbital, Caffeine, Cocaine, Carbamazepine, Codeine, Chloroquine, Chlorpromazine, Carbromal, Desipramine, Dextromethorphan, Dextropropoxyphene, 5,5-Diphenylhydantoin, 10-11-Dihydrocarbamazine, Diazepam, Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimide, Hexobarbital, Ibuprofen, Imipramine, Lidocaine, LSD, Methadone, Methadone-primary metabolite, Methaqualone, Methamphetamine, Metharbital, Mephenytoin, Mepobarbital, Methyl PEMA, Methsuximide, 4-Methylprimidone, Morphine, Meperidine, Niacinamide, Norethindrone, N-Normethsuximide, Phenobarbital, Phensuximide, PEMA, Primidone, Phencyclidine, Pentobarbital, Phenothiazine, Phenylpropanolamine, Procaine, Quinine, Secobarbital, Tetracycline, Tetrahydrozoline

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APPENDIX F: Quantitative real time PCR (qPCR) – RNA Extraction Protocol
Quantitative real time PCR (qPCR) – RNA Extraction Protocol

1. Pipette 250 ul Tri-reagent into each newly labeled 1.5 ml centrifuge tube
2. Using sterilize forceps, remove a 50-100 mg piece of liver
   a. Keep forceps in ethyl alcohol when not in use
   b. Wipe off excess ethyl alcohol with a new Kim wipe each time
3. Gently blot liver section on a Kim wipe to remove excess RNA later
   a. Use a new Kim wipe for blotting for each sample
4. Place liver section into Tri-reagent
5. Place tube on ice until all samples are done
6. Insert a sterilized pestle into the homogenizer
   a. Keep pestle in ethyl alcohol between samples
   b. Remove any excess ethyl alcohol with a new Kim wipe
7. Without turning on the homogenizer, gently mash any large pieces of liver
8. Slowly homogenize liver for 30s to 1 minute
   a. Turn on homogenizer once it is in the Tri-reagent
   b. Can move the pestle up and down slightly
   c. Should have a cloudy brownish red appearance when finished
9. When finished, remove pestle from homogenizer
   a. Make sure to scrape the pestle against the lip of the tube to remove any excess liver pieces or liquid before placing the pestle in ethyl alcohol
10. Once homogenized, add 750 ul Tri-reagent to centrifuge tube
    a. Keep tube on ice until all samples are done
11. Centrifuge samples at 12,000 x g for 10 minutes at 4ºC
    a. When inserting the centrifuge tubes into the centrifuge, make sure the hinge of the centrifuge tube top is pointed up
12. Pipette out the pink supernatant into a new set of labeled 2.0 ml centrifuge tubes
    a. Avoid pipetting any potential fat layer on the surface, clear or deep red layers near the pellet, or the actual pellet at the bottom of the tube
    b. Use a new pipette tip for each sample
    c. Once the supernatant is pipetted out, throw away the remaining pellet
13. Pipette 200 ul chloroform into the pink supernatant
14. Vortex for 15 seconds
    a. Should have a cloudy pink appearance when finished
15. Let tube sit for 2-15 minutes
16. Centrifuge at 12,000 x g for 15 minutes at 4ºC
17. While samples are centrifuging, pipette 500 ul isopropanol into a new set of labeled 2.0 ml centrifuge tubes
18. Pipette the top layer (clear upper aqueous phase) into the isopropanol
    a. Avoid pipetting white (DNA interphase) and pink (organic phase) layers
    b. Pour out remaining white and pink layers into a waste container and throw out tube
19. Close tube containing isopropanol and aqueous layer and invert four times
    a. Should have a milky white appearance when finished
20. Let sample sit at room temperature for 5-10 minutes
21. Centrifuge at 12,000 x g for 10 minutes at 4°C
   a. RNA pellet is at the bottom when centrifuging is finished
22. Carefully pour off clear supernatant, making sure not to lose the pellet
23. Pipette in 750 ul 75% ethanol to wash
   a. Good stopping point! Freeze at -20 ºC if stopping here is necessary
24. Vortex sample for 5 seconds to get pellet off of the bottom
25. Centrifuge at 7,500 x g for 5 minutes at 4ºC
   a. RNA pellet is at the bottom when centrifuging is finished, directly
      below the hinge when centrifuging is finished
26. Carefully pour off clear supernatant making sure not to lose the pellet
27. Pipette in 750 ul 100% ethanol to wash
28. Vortex sample for 5 seconds to get pellet off of the bottom
29. Centrifuge at 7,500 x g for 3 minutes at 4ºC
   a. At this point, put 3 centrifuge tubes (1ml each) of ddH2O on the
      heating block at 65ºC for later use
30. Very carefully pour off clear supernatant, making sure not to lose the pellet
31. Using a smaller centrifuge, centrifuge pellet to make sure any liquid is now at the
    bottom
32. Pipette off any remaining liquid
   a. Avoid pipetting the pellet at the bottom
33. Allow pellet to air-dry with the cap open at room temperature for 10 minutes in
    the hood
   a. Flick the tube to see if the pellet is dry, making sure not to drop the
      tube or accidently open the cap
   b. When dry, pellet should have a flaky, chalk-like appearance that does
      not stick to the sides of the tube
   c. Close tube if dry; if not, keep open
   d. Be careful not to over-dry
34. Once dry, pipette 200 ul ddH2O that has been heated to 65ºC
35. Give the tube a couple of flicks with your fingers, making sure not to drop the
    tube or accidently open the cap
36. Vortex for a second to make sure the pellet freely comes off the bottom of the
    tube
37. Leave tubes on a heating block at 65ºC for 10-15 minutes or until dissolved
APPENDIX G: Quantitative real time PCR (qPCR) – Quality Control Protocol
Quantitative real time PCR (qPCR) – Quality Control Protocol

1. Using Nanodrop 3.3.1 software, click on “Nucleic Acids”
2. Pipette a 2 ul droplet of double distilled water (ddH₂O) on the spectrophotometer pedestal
3. Wipe both pedestals with a fresh Kim wipe
4. Pipette a 2 ul droplet of ddH₂O on the spectrophotometer pedestal
5. Click “Ok” to initialize the instrument
6. Immediately click “Blank” to blank the machine with ddH₂O
7. When finished, wipe both pedestals with fresh Kim wipe
8. Select sample type “RNA-40”
9. Enter sample ID number
10. Pipette a 2 ul droplet of sample on the spectrophotometer pedestal
11. Click “Measure”
   a. Detection Limitations
      i. Concentration: between 2-3000 ng/ul
      ii. 260/280 (RNA/protein) ratio ~ 2.0
         iii. 260/230 (RNA/contaminants) ratio ~ 1.8-2.2
12. When finished, wipe off both pedestals before adding the next sample
Quantitative real time PCR (qPCR) – Reverse Transcription Protocol

1. Calculate and print out ddH₂O and RNA concentrations for a total volume of 10ul/tube
   a. RNA being reverse transcribed: 1.5 ug
   b. \[\text{Concentration} \times \text{Volume}]_{\text{given}} = \[\text{Concentration} \times \text{Volume}]_{\text{desired}}
2. Calculate concentration of the reagents/enzymes needed to create master mix
3. Keep metal block and samples in numerical order on ice
4. Place labeled tube strips with caps onto metal block
5. Pipette previously calculated ddH₂O concentrations into each tube
6. Pipette previously calculated RNA concentrations into each tube, rounding to the nearest tenth
7. Pipette the following into a new labeled 1.5 ml centrifuge tube to create master mix (20 ul reaction size):
   a. 132 ul 10X RT Buffer
   b. 52.80 ul 25X dNTP (100 mM)
   c. 132 ul 10X RT Random Primers
   d. 66 ul MultiScribe Reverse Transcriptase (50 U/ul)
   e. 3.30 ul RNase inhibitor
   f. 273.90 ul Nuclease-free ddH₂O
      i. NOTE: these numbers are dependent on number of reactions (in this case 60 + 10% for error = 66 reactions)
8. Vortex master mix
9. Centrifuge tube containing master mix for 2 seconds to make sure all of the liquid is at the bottom
10. Pipette 10 ul of master mix into each tube
    a. After securing the caps, vortex each strip
    b. Centrifuge each strip for 2 seconds using the Galaxy Mini centrifuge
    c. Insert strips with caps into the MJ Research PTC-200 Peltier Thermal cycler to convert RNA into cDNA
       i. 25°C for 10 minutes
       ii. 37 °C for the next 2 hours
APPENDIX I: Quantitative real time PCR (qPCR) – real time PCR Protocol
Quantitative real time PCR (qPCR) – real time PCR Protocol

1. Following cDNA synthesis, remove strips from MJ Research PTC-200 Peltier Thermal cycler
2. Pipette 55 ul ddH2O into each tube to get to 20 ng/ul cDNA
3. Vortex each strip for 2 seconds
4. Centrifuge each strip for 2 seconds
5. Make 1:100 dilutions from cDNA reactions
   a. Label 8 new strips
   b. Pipette 198 ul ddH2O into each tube
   c. Pipette 2 ul of the sample into each tube
   d. Invert to mix
   e. Centrifuge for 2 seconds
6. Make top tier for standard curve
   a. Label 1 new strip
   b. Pipette 2 ul of each sample into a single tube
7. Make standards
   a. Label 1 new strip
   b. Pipette 80 ul ddH2O into 7 of the 8 tubes (1st tube should be left empty)
   c. 1st tube: Transfer the entire contents from the top tier standard tube into this tube
   d. 2nd tube: Pipette 20 ul from 1st tube
      i. Mix by sucking up and blowing out contents 3 times using a pipetter
   e. 3rd tube: Pipette 20 ul from 2nd tube
      i. Mix by sucking up and blowing out contents 3 times using a pipetter
   f. 4th tube: Pipette 20 ul from 3rd tube
      i. Mix by sucking up and blowing out contents 3 times using a pipetter
   g. 5th tube: Pipette 20 ul from 4th tube
      i. Mix by sucking up and blowing out contents 3 times using a pipetter
   h. 6th tube: Pipette 20 ul from 5th tube
      i. Mix by sucking up and blowing out contents 3 times using a pipetter
   i. 7th tube: Pipette 20 ul from 6th tube
      i. Mix by sucking up and blowing out contents 3 times using a pipetter
   j. 8th tube: Only 80 ul ddH2O should be in this tube
8. Centrifuge standards and 1:100 dilutions
9. Make primer dilution mix (300mM solution)
   a. In a new labeled 1.5 ml centrifuge tube, combine:
      i. 20 ul of the forward primer of the specific gene (10mM)
      ii. 20 ul of the reverse primer of the specific gene (10mM)
      iii. 360 ul ddH2O
10. Make PCR Master mix for real time PCR for the housekeeping gene
   a. NOTE: Amounts used are dependent on number of reactions
      i. 60 samples + 8 standards + 10% error = 74.8 or 75 well
   b. Pipette the following in a new labeled 2.0 ml centrifuge tube:
      i. 935 ul of 2X SYBR Green master mix
      ii. 112.2 ul of 18S primer dilution (300mM solution)
      iii. 748 ul ddH₂O
   c. Invert to mix
   d. Centrifuge for 2 seconds
11. In a 96-well plate, pipette 24 ul of the corresponding PCR Master mix into each well
12. Pipette 1 ul of each standard into corresponding well in the first row of the plate
13. Pipette 1 ul of the 1:100 dilution of each sample into the additional wells
    Calculation to achieve a 25 ul IX reaction for 18S:
    \[
    \begin{align*}
    &12.5 \text{ ul 2X SYBR Green Master Mix} \\
    &1.5 \text{ ul primer dilution mix} \\
    &24.0 \text{ ul of PCR Master Mix} \\
    &1 \text{ ul sample or standard} \\
    &\text{25 ul reaction}
    \end{align*}
    \]
14. Skip to Step #18 if only running 18S

Specific for gene of interest (Steps 14-17): CYP2a5 and CYP2e1
15. To make PCR Master mix for real time PCR for genes of interest
   a. Note: Amounts used are dependent on number of reactions
      i. 60 samples + 8 standards + 10% error = 74.8 or 75 wells
   b. Pipette the following into a new labeled 2.0 ml centrifuge tube:
      i. 935 ul of 2X SYBR Green master mix
      ii. 112.2 ul of gene of interest primer dilution (300nM each)
      iii. 448.8 ul ddH₂O
   c. Invert to mix
   d. Centrifuge for 2 seconds
16. In a 96-well plate, pipette 20 ul of the corresponding PCR Master mix into each well
17. Pipette 5 ul of each standard into corresponding well in the first row of the plate
18. Pipette 5 ul of the 1:100 dilution of each sample into the additional wells
    Calculation to achieve a 25 ul IX reaction for a gene of interest:
    \[
    \begin{align*}
    &12.5 \text{ ul 2X SYBR Green Master Mix} \\
    &1.5 \text{ ul primer dilution mix} \\
    &20.0 \text{ ul of PCR Master Mix} \\
    &5 \text{ ul sample or standard} \\
    &\text{25 ul reaction}
    \end{align*}
    \]
19. Regardless of which gene you are examining, once you have finished plating, carefully center optical film lightly over top of the plate
20. Once its perfectly centered, lightly “brush” on the plate cover using an adhesive film applicator
21. Once its lightly on without any creases or air bubbles, firmly “brush” on the plate cover, rotating the plate to make sure that the plate cover is securely adhered to the plate
22. Seal the edges of film to the plate by running the tool close to the edges of the wells
23. Rip off white tabs at the edges of film
24. Centrifuge the plate using a Labnet Mini Plate Spinner and the appropriate balance for 5 seconds
25. When using 7000 Sequence Detection System Software, be sure to:
   a. Label plate
   b. Select and add the appropriate detection gene name with SYBR
   c. Indicate which wells are unknowns, standards, and blanks (NTC)
   d. Under the “Instrument” tab:
      i. Delete first cycle phase
      ii. Add dissociation cycle
      iii. Change reaction volume to 25 ul
26. Insert plate into Applied Biosystems 7000 Sequence Detection System
27. Place the compression pad on top with the brown side facing up
28. Close Applied Biosystems 7000 Sequence Detection System
29. Press start ➔ takes approximately 2 hours to run
APPENDIX J: Primer sequences for RT-PCR
Primer sequences for RT-PCR

### Housekeeping gene

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Organism</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>Ribosomal RNA</td>
<td>mouse</td>
<td>CTACCACATCCAAGGAAGGCA</td>
<td>GGGTGCGGAGTGGGTAATTT</td>
</tr>
</tbody>
</table>

### Gene of Interest (from Muguruma et al., 2006)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Organism</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2a5</td>
<td>Cytochrome P450 Family 2 Subfamily a Polypeptide 5</td>
<td>mouse</td>
<td>ACCAGACAAGTCAGGGTTG</td>
<td>TTTCCCTCTTTGGCTACC</td>
</tr>
</tbody>
</table>

### Genes of Interest (from Kashida et al., 2006)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Organism</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2e1</td>
<td>Cytochrome P450 Family 2 Subfamily e Polypeptide 1</td>
<td>mouse</td>
<td>TCAAAAAGACCAAAGGCCAG</td>
<td>TCCGCAATGACATTGCAG</td>
</tr>
</tbody>
</table>


Appendix K: Absolute Quantification Protocol
Absolute Quantification Protocol

1. Import data into 7000 Sequence Detection System Software
2. Under the “Dissociation Curve” tab:
   Make sure that a single amplicon is represented (1 peak)
3. Under the “Amplification Plot” tab:
   a. Set data view to “Rn vs. cycle”
      i. Start cycle should be left as default (3 cycles)
      ii. End cycle is 2 cycles prior to exponential amplification phase (e.g.,
          determine where amplification begins to increase exponentially
          and subtract 2 cycles)
   b. Set data view to “delta Rn vs. cycle”
      Threshold bar should be placed in the middle 1/3 of the linear
      range where amplification plots are parallel to each other
4. Under the “Standard Curve” tab:
   a. Slope should be approximately -3.33
   b. R^2 should be 0.99 or higher
5. Export results into Excel spreadsheet
APPENDIX L: Experiment II Methoxsalen Preparation Protocol
Experiment II Methoxsalen Preparation Protocol

**Drug Solution for Adults & Adolescents:**
- Maximum weight of mouse: 30 grams
- Maximum volume to be injected: 0.5ml

**Ingredients of the stock solution (1-fold solution) of 8MOP:**
For when the desired dosage is 10 mg/kg (i.e. 0.01 mg/g)
0.01 mg/g \(\times\) 30g (maximum mouse weight) = 0.30mg / 500 ul = \[\frac{0.30 \text{ mg}}{0.5\text{ ml}}\]

Want:
1. 500 ug/ml (0.6mg/ml) of 8MOP into 40ml of vehicle, sufficient for injecting 80 mice.

40ml \(\times\) 0.6 mg/ml = 24mg of 8MOP into 40ml (total)
* Note: this small amount will not alter the volume so you won’t have to account for a change in volume with this addition

**Procedure:**
6. Tare a 40 ml beaker
7. Measure out methoxsalen into the beaker
   - c. For 10 mg/kg dosage: 24 mg (0.024g)
   - d. For 5 mg/kg dosage: 12 mg (0.012g)
8. Using a stir bar, slowly add 2 ml of 200 proof ethyl alcohol into the beaker
9. Slowly begin adding Emulphor until the solution is homogenous
10. Drop by drop, slowly add 36 ml of distilled water until you reach total volume (40 mls) and the solution has a yellowish haze

**Injection Volume:**
**10 mg/kg:** Mouse weight (g) \(\times\) 0.01 mg/g = Weight (g) \(\times\) 0.016667 ml/g = injection mls
\(\frac{0.6 \text{ mg/ml}}{\text{0.6 mg/ml}}\)
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Books & Publications


Scientific Presentations
Guaderrama, M., Kapelewski, C.H., Bennett, J.M., & Klein, L.C. (2008 October). Stress exposure results in elevated cotinine levels but does not alter 24-hr oral nicotine intake in periadolescent male and female mice. Pennsylvania State University’s Social Science Research Institute’s Stress and Health Research Day, University Park, PA.