DISCOVERY OF NOVEL Se-ASPIRIN MOLECULES AS POTENTIAL THERAPEUTICS FOR GASTROINTESTINAL CANCERS

A Dissertation in
Molecular Toxicology
by
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ABSTRACT

In today’s era there have been a lot of advances in cancer screening, surgery, and chemotherapy, however, gastrointestinal (GI) cancer remains the second leading cause of cancer related deaths. Among all the GI cancers, colorectal cancer (CRC) (New cases: 134,490 deaths: 49,190) and pancreatic cancer (PC) (New cases: 53,070 deaths: 41,780) have high mortality rates. Current therapies result in minimal survival advantage and are linked to multiple adverse events and drug resistance. Further, inflammation has been linked towards initiation and metastasis of both PC and CRC. Literature reports show that the inflammatory pathways like NF-κB and STAT3 are constitutively active and have shown to play an important role in resistance towards chemotherapeutic agents. Therefore, there is an unmet need for novel chemotherapeutic agents, that target such inflammatory pathways, with enhanced potency and reduced toxicity to enhance survival and improve patient’s quality of life.

Non-steroidal anti-inflammatory drugs (NSIADs), specifically aspirin (ASA), have been highlighted for their chemopreventive properties and anti-inflammatory activities. Recent studies suggest that long term use of ASA reduces the risk of both CRC and PC. However, long term use, as is required in a prevention setting, and the high dose, lead to GI toxicity; e.g. GI bleeding and ulcer formation. Therefore, many efforts are being made to generate novel ASA analogs with reduced GI toxicity, so as not only to achieve a preventive effect at lower doses but increased efficacy towards killing cancer cells to extend their use as therapeutics. Further, selenium containing molecules have gained a lot of attention for their chemopreventive potential and anticancer activities. Selenium
containing molecules have also demonstrated to have gastro-protective effects like accelerating ulcer healing as well as reducing NSAIDs induced ulcer formation. Hence, we hypothesize that combining both ASA and selenium will lead to discovery of novel agents with potent anti-cancer activities while having minimal associated GI toxicity. Through extensive structure-activity relationship studies of novel compounds designed by incorporating selenium into the NSAIDs, we recently identified two novel ASA-selenium hybrid molecules (AS-10 and Comp 8), that showed potent anti-cancer activities against both CRC and PC.

Comp 8, was designed by incorporating ethyl selenocyanate moiety into carboxylic acid moiety of ASA. AS-10 on the other hand, was generated via novel synthesis pattern to contain a cyclic selenazolidine ring flanked by two acetyl salicylate moieties similar to ASA. Our mechanistic studies show that both agents exhibit similar mechanism of action in PC and CRC. They both inhibited the activation of pro-inflammatory and pro-survival NF-κB pathway, when PC cells were stimulated with inflammatory stimuli (TNF-α), at a similar potency. They also reduced the expression of NF-κB down-stream anti-apoptotic targets like Bcl-xL, Mcl-1 and survivin. Further, they induced intrinsic form of apoptosis via caspase 9 and caspase 3 activation. Notably, AS-10 was more effective at inhibiting PC growth, and hence we explored its mechanism of action further. AS-10 increased the expression of p21 and p27 at early time points (compared to Comp 8) which arrested cell cycle at G1/G0 and G2/M phase. Cell cycle arrest was later translated to apoptosis. AS-10 also increased reactive oxygen species (ROS) levels, and quenching of ROS via N-acetylcysteine, led to reduction of AS-10’s anti-cancer activity. Moreover, similar
mechanism of action was seen in CRC, where both agents were able to induce cell cycle arrest and activation of apoptosis.

Our findings show that both agents are more potent at inhibiting cancer cell growth compared to current first line therapies for both PC and CRC. Notably, AS-10 also potentiated the effectiveness of gemcitabine, first line therapy for PC, in gemcitabine resistant PC cell line. Additionally, both agents are more selective towards cancer cells than normal cells, making them clinically more significant. Moreover, we also tested the broad spectrum activity of AS-10 and Comp 8 in different cancer types. Effectiveness of AS-10 was further tested in an even broader range of cancers via National Cancer institute’s (NCI’s) drug screening program in 60 human cancer cell lines which contains cell lines from nine different types of cancer; AS-10 was effective in different cancer types, out of which renal cancer cells were most sensitive. Taken together, our in vitro results suggest both Comp 8 and AS-10 as promising novel chemotherapeutic agents for treatment of CRC and PC. However, based on our preliminary MTD studies in nude mice, AS-10 was found ~5 times more tolerable, and hence could prove to be a potent anti-cancer agent with high therapeutic index.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-amino-actinomycin D</td>
</tr>
<tr>
<td>ADMET</td>
<td>Absorption, distribution, metabolism, excretion and toxicity</td>
</tr>
<tr>
<td>ASA</td>
<td>Aspirin</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electro mobility shift assay</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase complex</td>
</tr>
<tr>
<td>IκBα</td>
<td>inhibitor of κB proteins</td>
</tr>
<tr>
<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MOA</td>
<td>Mechanism of action</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MTD</td>
<td>maximum dose tolerable</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl cysteine</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear transcription factor-kappa B</td>
</tr>
<tr>
<td>nHDFs</td>
<td>Normal human dermal fibroblasts</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>PC</td>
<td>Pancreatic cancer</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
</tbody>
</table>
PE  Plating efficiency
PI  Propidium iodide
PP  Polypeptide producing cells
PS  Phosphatidylserine
ROS Reactive oxygen species
SAR Structure activity relationship studies
Se-CN  Selenocyanate
Sec  selenocysteine
SRB  sulphorhodamine B
TNF-α  Tumor necrosis factor α
VEGF  Vascular endothelial growth factor
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Chapter 1

Review of Literature

Gastrointestinal Cancer

Gastrointestinal (GI) cancers are the cancers of GI tract and its accessory organs such as stomach, pancreas, esophagus, small intestine, large intestine, biliary system, rectum and anus. Despite the advancement of medical inventions in the treatment, GI cancers still remain the second leading cause of cancer related death in USA.\(^1\) Among all GI cancers, colorectal (CRC) and pancreatic (PC) cancers are the most aggressive forms.

According to the cancer statistical data from NCI’s Surveillance, Epidemiology, and End Results (SEER) program, total number of new cases of CRC is projected to be 134,490, and an estimated death of 49,190 during the year of 2016. Out of all CRC cases detected, about 20\% of the cases are familial CRC, while the remaining cases have been linked to environmental causes.\(^2\) Familial adenomatous polyposis (FAP) and lynch syndrome are two hereditary form of familial CRC.\(^3\) Epithelial cells have been proposed to lead to CRC due to their high proliferative rates and their direct contact with intestinal microflora, pathogens and toxic substances, all of which could lead to spontaneous mutations.\(^4\) There are several risk factors associated with CRC such as environmental and food-borne mutagens, age, intestine specific pathogens, familial mutations, and chronic
intestinal inflammations. Moreover, it has been shown that patients with ulcerative colitis or high levels of inflammation in the colon are highly pre-disposed to CRC.

Among GI cancers PC is considered one of the deadliest, as the ratio of death vs survival is very high. As stated by NCI SEER statistics, 53,070 new cases of PC are estimated to occur, with an estimated 41,780 deaths in 2016. Although substantial progress has been made over the past 25 years, the five year survival rate for PC is still extremely poor, with a rate of less than 7%. More than 80% of the PC patients show an advanced disease stage at diagnosis, which accounts for its poor prognosis. Factors such as cigarette smoking; environmental pollutants; occupational exposure; chronic pancreatitis; diabetes mellitus; familial syndromes like Peutz-Jaegers (STK11 / LKB1), familial pancreatitis (PRSS / TRY1), familial atypical multiple mole melanoma (CDK2A / p16 / MTS1); and demographic host factors such as old age, low social class status, race play an important role in the pathogenesis of this dreaded disease.

The GI related cancers and estimated number of deaths are shown in Table 1-1.
Table 1-1. Inflammation related GI cancer and associated estimated deaths in US for year 2016. ¹, ¹⁰

<table>
<thead>
<tr>
<th>Organ</th>
<th>Tumor type</th>
<th>Chronic inflammation</th>
<th>Estimated deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal</td>
<td>Colorectal cancer</td>
<td>Ulcerative colitis, Crohn’s disease</td>
<td>49,190</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Pancreatic ductal adenocarcinoma</td>
<td>Chronic pancreatitis</td>
<td>41,780</td>
</tr>
<tr>
<td>Liver</td>
<td>Hepatocellular carcinoma</td>
<td>HBV, HCV and cirrhosis (alcohol, NAFLD)</td>
<td>27,170</td>
</tr>
<tr>
<td>Esophagus</td>
<td>Squamous cell carcinoma and Adenocarcinoma</td>
<td>Cigarette smoking, alcohol and hot beverages and GERD</td>
<td>15,690</td>
</tr>
<tr>
<td>Stomach</td>
<td>Adenocarcinoma / MALT lymphoma</td>
<td>H. pylori, autoimmune, HCV</td>
<td>10,730</td>
</tr>
<tr>
<td>Biliary system</td>
<td>Gallbladder carcinoma / Cholangiocarcinoma</td>
<td>Chronic cholecystitis / PSC, chronic cholangitis and liver cirrhosis</td>
<td>3,710</td>
</tr>
</tbody>
</table>

**Etiology of CRC and PC**

One of the risk factors, which plays an important role in pathogenesis of both CRC and PC is chronic inflammation.¹¹-¹³ Inflammatory bowel disease (IBD) and pancreatitis, both inflammatory diseases, have been linked to genesis of CRC and PC, respectively.¹⁴, ¹⁵ Additionally, presence of constitutive activation of inflammatory signaling pathways (such as NF-κB and STAT3) and high concentration of pro-inflammatory cytokines in the tumor microenvironment, demonstrate the role of chronic inflammation in the progression of CRC and PC.⁹, ¹¹, ¹⁴-²⁰ This argument is further supported by the studies showing that NSAIDs delay tumor incidences in both CRC and PC.²¹-²⁶
Colorectal Cancer (CRC)

CRC can be divided into two subtypes based on the initiation of CRC (Figure 1-1 and Table 1-2). The first subtype of CRC develops by sporadic mutations in the key oncogenes (K-Ras, PIK3CA, CMYC etc.), and tumor suppressors (p53, APC, PTEN, etc.) (Table 1-3); while the second subtype, known as colitis-associated cancer (CAC), is linked to IBD. More than 20% of IBD patients develop CAC, which accounts for half of the deaths in these patients. Thus, IBD plays a crucial part in the initiation of CAC. Importantly, samples from CRC patients with the sporadic form of CRC also show infiltration of inflammatory cells and high expression of pro-inflammatory cytokines, implying that inflammation plays a part in genesis of both subtypes of CRC.
Figure 1-1. Role of inflammation in CRC formation via sporadic or by colitis associated cancer (CAC). Figure modified from


In the sporadic form of CRC, mutations in key tumor suppressors (p53, APC, PTEN, etc.) or oncogenes (KRAS, CMYC, PIK3CA etc.), shifts the normal epithelium towards high grade dysplasia to in situ carcinoma. As shown in Figure 1-1, mutations in tumor suppressor genes such as APC or p53, followed by mutations in oncogenes like KRAS, leads to increase in pro-survival and pro-inflammatory pathways such as NF-κB and STAT3, thereby displacing normal epithelial cells, and leading to adenoma formation, carcinoma and eventually metastatic CRC.27 During progression of CAC, there is robust inflammatory cells infiltration (like macrophages, neutrophils, mast cells, dendritic cells, etc.) and high expression of inflammatory cytokines.27 This could be due to damage of the
intestinal wall by intestinal specific pathogen or external agents. Moreover, the intestinal microflora presence further intensifies the inflammatory response. Hence, the continuing presence of activated inflammatory cells produces high levels of ROS and reactive nitrogen species which can cause DNA damage in the epithelial cells and lead to mutations (Figure 1-1). Though CRC and CAC are two subtypes of colon cancer, there are some similarities and differences in the progression of these tumor. (Table 1-2)

**Table 1-2.** Similarities and differences between sporadic CRC and CAC. Table modified from 14

<table>
<thead>
<tr>
<th>Differences</th>
<th>Sporadic CRC</th>
<th>CAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 mutation occurs late</td>
<td>p53 mutation occurs early</td>
<td></td>
</tr>
<tr>
<td>APC mutation occur early</td>
<td>APC mutation occurs late</td>
<td></td>
</tr>
<tr>
<td>Detected in patients with median age of 50 years</td>
<td>Detected in patients with median age of 65 years</td>
<td></td>
</tr>
<tr>
<td>Polypoid tumors</td>
<td>Flat tumors</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Similarities</th>
<th>Both represent dysplasia- cancer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both have high expression of COX2 protein and lipoxygenase</td>
<td></td>
</tr>
<tr>
<td>Multi-step mutations are required for generation of CRC in both forms</td>
<td></td>
</tr>
<tr>
<td>Both cancers have shown to be preventable with NSAIDs use</td>
<td></td>
</tr>
<tr>
<td>Both cancers have similar mutations but they occur in different sequences</td>
<td></td>
</tr>
<tr>
<td>Strong presence of inflammation in both subtypes of CRC</td>
<td></td>
</tr>
</tbody>
</table>

Reprinted from Trends in Molecular Medicine, 8(1), Jonathan M. Rhodesemail and Barry J. Campbell, Inflammation and colorectal cancer: IBD-associated and sporadic cancer compared, p 10-16, © 2002, with permission from Elsevier

The most common mutations found in CRC are mutations in APC, KRAS and p53 genes. Mutations in KRAS and p53 have been reported to be associated with poor prognosis in CRC.28, 29 Table 1-3 lists the name of genes and their frequencies in which they are found to be mutated in CRC patients. Mutations in the APC gene leads to stabilization of \(\beta\)-catenin, and hence, \(\beta\)-catenin in combination with DNA binding T cell factor/lymphoid
enhancer family proteins, can translocate to the nucleus to activate different target genes. These target genes are involved in cell proliferation, differentiation, and morphogenesis.\textsuperscript{19} However, studies have shown that constitutive activation of Wnt/\(\beta\)-catenin signaling in a sterile environment showed two-fold lower formation of adenomas compared to mice grown in normal conditions. These studies concluded that along with constitutive Wnt/\(\beta\)-catenin signaling, activation of inflammatory pathways via gut microflora was essential, for expeditious adenoma formation.\textsuperscript{19}

**Table 1-3.** List of genes commonly mutated in CRC. Taken from Fearon 2011.\textsuperscript{19}

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type of Mutation</th>
<th>Approximate frequency of alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oncogenes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS</td>
<td>Point mutations (activating)</td>
<td>40%</td>
</tr>
<tr>
<td>NRAS</td>
<td>Point mutations (activating)</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>Point mutations (activating)</td>
<td>15-25%</td>
</tr>
<tr>
<td>BRAF</td>
<td>Point mutations (activating)</td>
<td>5-10%</td>
</tr>
<tr>
<td>EGFR</td>
<td>Gene amplification</td>
<td>5-15%</td>
</tr>
<tr>
<td>CDK8</td>
<td>Gene amplification</td>
<td>10-15%</td>
</tr>
<tr>
<td>CMYC</td>
<td>Gene amplification</td>
<td>5-10%</td>
</tr>
<tr>
<td>CCNE1</td>
<td>Gene amplification</td>
<td>5%</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>Stabilizing point mutations</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>NEU (HER2)</td>
<td>Gene amplification</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>MYB</td>
<td>Gene amplification</td>
<td>&lt;5%</td>
</tr>
<tr>
<td><strong>Tumor suppressor genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>Point mutation, allele loss</td>
<td>60-70%</td>
</tr>
<tr>
<td>APC</td>
<td>Frameshift, deletion, allele loss or point mutations</td>
<td>70-80%</td>
</tr>
<tr>
<td>FBXW7</td>
<td>Nonsense, missense, deletion</td>
<td>20%</td>
</tr>
<tr>
<td>PTEN</td>
<td>Nonsense, deletion</td>
<td>10%</td>
</tr>
<tr>
<td>SMAD4</td>
<td>Nonsense, missense, allele loss</td>
<td>5-10%</td>
</tr>
<tr>
<td>SMAD3</td>
<td>Nonsense, deletion</td>
<td>5%</td>
</tr>
<tr>
<td>TGF(\beta)IIIR</td>
<td>Frameshift, nonsense</td>
<td>10-15%</td>
</tr>
<tr>
<td>TCF7L2</td>
<td>Frameshift, nonsense</td>
<td>5%</td>
</tr>
<tr>
<td>ACVR2</td>
<td>Frameshift</td>
<td>10%</td>
</tr>
<tr>
<td>BAX</td>
<td>Frameshift</td>
<td>5%</td>
</tr>
</tbody>
</table>
As mentioned above, CRC is associated with a high presence of inflammatory cytokines and chemokines (Table 1-4), which can lead to the activation of inflammatory pathways such as NF-κB and STAT3 (pathways discussed in later section). Activation of both of these pathways leads to increases in anti-apoptotic proteins (e.g. Bcl-2 family proteins), pro-inflammatory cytokines, expression of pro-inflammatory proteins (like COX-2), proliferation, and overall progression of tumor growth and metastasis. Additionally, both of the pathways are activated by the mutations in oncogenes like K-Ras. Hence therapies which can target the cytokines activating these pathways, or inhibitors which can directly inhibit the signaling within cells, have been proposed to be effective therapeutic strategies for CRC. In fact, targeting either of the STAT3 or NF-κB pathways in mouse models of CRC has shown to reduce CRC tumor burden. In support of this, patient derived tumor samples exhibited high expression of STAT3 and NF-κB pathways. These studies strongly suggest that activation of inflammatory pathways may play a vital role in progression of CRC.
Table 1-4. Major inflammatory cytokines in PC and CRC.\textsuperscript{11, 30, 34}

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Survival, activation, recruitment. Growth. Activation of AP-1, MAPK and NF-κB factors</td>
</tr>
<tr>
<td>IL-6</td>
<td>Survival, growth, T cell survival and differentiation. Activation of STAT3, ERK and Akt</td>
</tr>
<tr>
<td>IL-11</td>
<td>Survival, growth. Activation of STAT3, STAT1, ERK</td>
</tr>
<tr>
<td>IL-23</td>
<td>T-cell differentiation and interference with Tregs, production of IL-17 and IL-22 by immune cells. Activation of STAT3</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Survival, growth, cytokines, chemokines, T cell activation and differentiation, Activation of MAPK, NF-κB</td>
</tr>
<tr>
<td>IL-22</td>
<td>Survival, mucosal integrity, chemokines. Activation of STAT3</td>
</tr>
<tr>
<td>IL-17A,F</td>
<td>Survival, chemokines, T-cell regulation. Activation of MAPK, NF-κB</td>
</tr>
<tr>
<td>EGF</td>
<td>Survival, proliferation. Activation of MAPK, STAT3</td>
</tr>
<tr>
<td>IL-10</td>
<td>Anti-inflammatory., Treg stimulation. Activation of STAT3 and MAPK</td>
</tr>
<tr>
<td>IL-8</td>
<td>Promotes angiogenesis, migration/metastasis and is associated with aggressiveness and invasion</td>
</tr>
<tr>
<td>MIF</td>
<td>Enhances proliferation, induces EMT and associated with poor progression</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Promotion of immune evasion, associated with poor prognosis</td>
</tr>
</tbody>
</table>

Pancreatic Cancer (PC)

The pancreas is made up of various types of cells. The exocrine pancreas is composed of acinar and duct cells, while the endocrine pancreas consist of α-cells, β-cells, δ-cells and pancreatic polypeptide producing (PP) cells.\textsuperscript{35} Cancer of exocrine pancreas is the most common form of PC, accounting for approximately 95% of PC diagnoses. Further,
infiltrating ductal adenocarcinoma comprises more than 90% of PC, and therefore our studies on PC focuses on infiltrating ductal adenocarcinoma. The exact cell of cancer origin in pancreatic adenocarcinoma is not clear, however the candidate cells proposed to be the origin of pancreatic adenocarcinoma are epithelium, acinar, and islet cells. A recent publication from Maddipati et al. strongly suggests that acinar cells are likely cells of PC origin.6, 7

The key genetic lesions, which play critical roles in the progression of PC, are shown in Figure 1-2.8 Pancreatic intraepithelial neoplasia (PanINs) arises from normal pancreatic ductal epithelium and gradually turn into in situ carcinoma and finally into invasive PC.9 KRAS mutation is proposed to occur first, driving cell proliferation, and is followed by mutations of tumor suppressor genes such as p16, p53 and DPC4.10, 11 Major mutations in PC are identified to be in KRAS, TP53, PALB2, BRCA2 and other DNA repair genes. PC also demonstrates an amplification of ERBB2 (HER2/neu) gene and deletions of SMAD4 and CDKN2A genes.12 Error! Reference source not found. shows the list of important proteins, which contribute towards the progression of PC.13
Figure 1-2. Genetic progression model of pancreatic adenocarcinoma. PanIN: Pancreatic intraepithelial neoplasia.

Image modified from [35] and [44].

*KRAS* mutation is thought to occur first and is known as the driving force behind PC formation. However, Guerra et al. showed that *KRAS*\(^{G12V}\) mutation expressed in pancreatic acinar cells cooperated with experimentally induced chronic pancreatitis to induce PC.\(^{45}\) These experiments indicate that chronic pancreatitis is an essential step for induction of PC by *KRAS* oncogene in mice.\(^{45}\) Further, *KRAS* mutation alone induces permanent proliferative arrest, also known as premature senescence, mostly mediated by proliferation inhibitors like p53, p21, p16 and p19.\(^{46, 47}\) Both studies together, reveal that pre-existing inflammation, along with *KRAS* mutation, leads to the formation of PC. In PC
tumor samples, compared to normal tissue, markers of inflammation like COX-2, nitric oxide synthase, IL-1, IL-6, IL-8, TNF-α, and activated NF-κB were highly expressed.48

Table 1-5. Therapeutic targets in PC.43

<table>
<thead>
<tr>
<th>Target</th>
<th>Frequency of mutation or expression in PC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholecystokinin B and gastrin receptor</td>
<td>95</td>
</tr>
<tr>
<td>Ras</td>
<td>95</td>
</tr>
<tr>
<td>Telomerase</td>
<td>95</td>
</tr>
<tr>
<td>VEGF</td>
<td>93</td>
</tr>
<tr>
<td>Gastrin precursors and gastrin</td>
<td>23-91</td>
</tr>
<tr>
<td>COX-2</td>
<td>90</td>
</tr>
<tr>
<td>Hepatocyte growth factor receptor</td>
<td>78</td>
</tr>
<tr>
<td>Notch3</td>
<td>70</td>
</tr>
<tr>
<td>SHH</td>
<td>70</td>
</tr>
<tr>
<td>Src</td>
<td>70</td>
</tr>
<tr>
<td>EGFR</td>
<td>69</td>
</tr>
<tr>
<td>β-catenin</td>
<td>65</td>
</tr>
<tr>
<td>Insulin-like growth factor I receptor</td>
<td>64</td>
</tr>
<tr>
<td>Activated Akt</td>
<td>59</td>
</tr>
<tr>
<td>SMAD4</td>
<td>50</td>
</tr>
<tr>
<td>Focal adhesion kinase</td>
<td>48</td>
</tr>
<tr>
<td>AKT2</td>
<td>20</td>
</tr>
<tr>
<td>TGFBR2</td>
<td>4</td>
</tr>
<tr>
<td>TGFBR1</td>
<td>1</td>
</tr>
</tbody>
</table>


As there is a great deal of crosstalk between oncogenes and inflammatory pathways, the transcription factors, STAT3 and NF-κB, have been shown to be the common denominator in PC.13, 20, 49 Animal experiments show that genetic inhibition of STAT3 leads to reduced infiltration of inflammatory cells and cytokine expression, and hence, deaccelerated inflammation-mediated PanIN formation.50, 51 Further, experiments in PC animal models and human cell lines revealed that higher expression of phospho-STAT3 correlated with sensitivity towards JAK inhibitors.52 Overall, animal experiments and data
from clinical studies suggest the STAT3 signaling pathway as a key target for PC. Considering the NF-κB pathway, expression of mutant IKK2/β (inhibition of the NF-κB pathway) in an animal model of PC that mimics rapid growth (which has mutant KRAS and conditional deletion of Cdkn2a), failed to develop pancreatic ductal adenocarcinoma (PDAC) or PC.⁵³ In a different mouse model, featuring Pdx1-Cre; Kras<sup>G12D</sup>/+; IKK2/β<sup>fl/fl</sup> mutations, there was an inhibition of infiltration of macrophages, B and T cells, and neutrophils near the tumor site.¹³ These observations further imply the importance of NF-κB and inflammation in PC growth and progression.

**Current Therapeutic Regimes for CRC and PC:**

**Current Treatments for CRC**

Colonoscopy is considered to be the best preventive measurement and has proven an effective technique for detecting polyps (early stages of CRC).⁵⁴ The treatments of CRC include surgery, radiation and chemotherapy. CRC tumors, which are enclosed in the epithelial lining in colon, can be surgically removed, however, once they have spread, chemotherapy remains the only option. Among the FDA approved drugs for CRC treatment, 5-fluorouracil (5-FU) is considered the gold standard. Hence, many combinational therapeutic regimes use 5-FU as the main agent. Other agents, such as DNA damaging agents (such as Oxaliplatin or Irinotecan Hydrochloride), vascular endothelial growth factor (VEGF) (e.g. ramucirumab, and ziv-Aflibercept) and Epidermal growth factor (EGF) receptors inhibitors (e.g. panitumumab, and cetuximab) are also approved by
FDA for CRC use. The list of all FDA approved chemotherapeutic agents are shown in Error! Reference source not found.

Because chronic inflammation plays a critical role in CRC, non-steroidal anti-inflammatory drugs (NSAIDs) specifically aspirin (acetylsalicylic acid, ASA), have been shown to be effective chemopreventive agents for CRC. Along these lines, encouraging reports of various clinical trials, suggest that ASA could be used as a chemopreventive agent for CRC. Recently, the studies by Drew et al. suggest that ASA reduces the risk of CRC by more than 29%.

Table 1-6. List of major drugs approved by FDA for CRC treatment.

<table>
<thead>
<tr>
<th>Compounds approved</th>
<th>Mechanisms of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avastin (Bevacizumab)</td>
<td>Angiogenesis inhibitor (Inhibits VEGF-A)</td>
</tr>
<tr>
<td>Irinotecan Hydrochloride (Camptosar)</td>
<td>Topoisomerase I inhibitor</td>
</tr>
<tr>
<td>5-FU (Capecitabine, Xeloda)</td>
<td>irreversible inhibition of thymidylate synthase</td>
</tr>
<tr>
<td>Cetuximab (Erbitux)</td>
<td>EGFR inhibitor</td>
</tr>
<tr>
<td>Ramucirumab (Cyramza)</td>
<td>VEGFR2 inhibitor, angiogenesis inhibitor</td>
</tr>
<tr>
<td>Oxaliplatin (Eloxatin)</td>
<td>DNA damaging agent</td>
</tr>
<tr>
<td>Trifluridine/tipiracil (Lonsurf)</td>
<td>DNA damaging agents</td>
</tr>
<tr>
<td>Panitumumab (Vectibix,</td>
<td>EGFR inhibitor</td>
</tr>
<tr>
<td>Ramucirumab (Cyramza)</td>
<td>VEGFR2 inhibitor, angiogenesis inhibitor</td>
</tr>
<tr>
<td>CAPOX</td>
<td>Folinic Acid + 5-FU + Oxaliplatin</td>
</tr>
<tr>
<td>FOLFIRI</td>
<td>Folinic Acid + 5-FU + Irinotecan Hydrochloride</td>
</tr>
<tr>
<td>FOLFIRI-BEVACIZUMAB</td>
<td>Folinic Acid + 5-FU + Irinotecan Hydrochloride + bevacizumab</td>
</tr>
<tr>
<td>FOLFIRI-CETUXIMAB</td>
<td>Folinic Acid + 5-FU + Irinotecan Hydrochloride + cetuximab</td>
</tr>
<tr>
<td>FOLFOX</td>
<td>Folinic Acid + 5-FU + oxaliplatin</td>
</tr>
<tr>
<td>FU-LV</td>
<td>5-FU + Leucovorin Calcium</td>
</tr>
<tr>
<td>XELIRI</td>
<td>Xeloda (5-FU) + Irinotecan Hydro chloride</td>
</tr>
<tr>
<td>XELOX</td>
<td>Xeloda (5-FU) + oxaliplatin</td>
</tr>
</tbody>
</table>
For more than a decade, gemcitabine still holds as the first line therapy for PC.\textsuperscript{59} This agent has been also used in combination with erlotinib or nab-paclitaxel for advanced PC patients.\textsuperscript{60, 61} For localized, resectable PDAC, pancreaticoduodenectomy is performed followed by adjuvant gemcitabine or 5-FU, and radiation. Locally advanced unresectable PC treatment options are limited to 5-FU or gemcitabine or capecitabine along with radiation.

For metastatic PC, gemcitabine alone is administered, but depending on patient performance status, combinational treatments are prescribed (e.g. Gemcitabine + capecitabine (GX), Gemcitabine + nab-paclitaxel, Gemcitabine + Taxotere + capecitabine (GTX), Gemcitabine + erlotinib or FOLFIRINOX). Combinational treatment, FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan and oxaliplatin), has recently shown to increase overall survival in comparison to gemcitabine (11.1 months vs. 6.8 months) and thus, is used as first line therapy for PC patients, who are fit to handle the toxicity associated with it.\textsuperscript{62} Several phase III clinical trials are currently being carried out comparing gemcitabine vs. gemcitabine plus a second agent (e.g. angiogenesis inhibitor, EGFR inhibitors, etc.).\textsuperscript{63} The second agent varies from specific kinase inhibitors to monoclonal antibodies against certain proteins which are expressed in PC cells. Another three-drug regimen of gemcitabine, docetaxel and capecitabine (GTX) has shown to improve median survival but is associated with an increased amount of toxicity. GTX is currently used as a first line therapy for patients who are in their 50’s or patients who can better tolerate this therapy.\textsuperscript{64}
**Limitations of Current Treatments**

Molecular and cellular tumor heterogeneity of both PC and CRC, make it difficult to design targeted chemotherapeutic agents.\textsuperscript{65} As mentioned above, DNA damaging agents, 5-FU and gemcitabine, although not satisfactory, still remain the first line therapies for CRC and PC, respectively. Both gemcitabine or 5-FU are associated with different toxic effects such as hair loss, diarrhea, nausea, mouth sores, sensitivity to light, metallic taste in mouth, discoloration along vein through which the medication is given, etc. Another key issue in both PC and CRC is that the cancer cells build drug resistance very fast to these agents and hence different drug regimen needs to be given. The only approach that could avoid/delay drug resistance is multi targeted drugs as combination therapy, but the concern with currently used combinations is the severe systemic toxicity. For example, FOLFIRINOX can cause febrile neutropenia, diarrhea and sensitivity towards cold things.\textsuperscript{66} Hence, although this combination regimen may provide a marginal survival benefit for PC patients, their quality of life deteriorates significantly. Therefore, there is an urgent need for novel potent and safer agents that target different pathways, either as a single agent or in combination, to effectively kill cancer cells while improving the patient’s quality of life.

**Inflammation Driven Pathways: a Potential Target**

As discussed above, inflammation plays a major role in the initiation and maintenance of both CRC and PC. Further, the mutations and released cytokines activate two major inflammatory activated transcription factors, NF-κB and STAT3. As mentioned earlier,
inhibition of either of these pathways leads to reduced tumor growth and elimination of the associated inflammatory pathways. Further, depending on the cancer type, inhibition of one or both of them can lead to sensitization of the first line therapy drugs like 5-FU or gemcitabine.\textsuperscript{67-69} Hence, this section focuses on the activation of these pathways as well as genes expressed via both pathways, which leads to cancer cell survival, proliferation and metastasis.

\textit{NF-κB Pathway}

The NF-κB pathway mediates major stress signaling pathways which promotes cell growth, cell proliferation, protects cells from apoptosis, and increase inflammatory markers for recruiting more inflammatory cells.\textsuperscript{70} In cancer cells, NF-κB pathway is manipulated by the oncogenes and inflammatory cytokines, which keep the pathway constitutively activated for cancer cell survival and metastasis.\textsuperscript{70-73}
The mammalian transcription factor NF-κB consists of either homo- or hetero protein dimer of Rel-family proteins. As shown in **Figure 1-3**, NF-κB can be activated via two different pathways, namely canonical and non-canonical pathways. During inflammation, NF-κB activation occurs through canonical pathway and hence this pathway has been extensively studied in inflammation and cancer. RelA (also known as p65) and p50 complex is the most abundant and ubiquitously expressed form of NF-κB found in cells, and is activated in presence of different inflammatory chemokines and cytokines like TNF-α, IL-1, LPS, CD40, BAFF, Lymphotoxin-β.
TNF-α, ROS, IL-1 and LPS. It plays an important role in regulating cellular response to different environmental stresses like inflammation and infection. RelA and p50 protein complex (NF-κB), is present in cytoplasm bound to inhibitor of κB protein α (IκBα). IκBα inhibits NF-κB from translocating to the nucleus. Upon activation of canonical NF-κB pathway in presence of inflammatory stimuli (like TNF-α), IκBα gets phosphorylated via a downstream IκB kinase complex (IKK), and is targeted for degradation via poly ubiquitination. As shown in the Figure 1-3, once IκBα gets degraded, NF-κB can localize to the nucleus and upregulates its target genes. Table 1-7 list few target genes of NF-κB associated with proliferation (cyclin D1, c-Myc), inflammation (TNF-α, COX-2), cell invasion (VEGF, MMPS, etc.) and anti-apoptosis (Blc-2, Bcl-xL, Mcl-1, etc.). One of the major targets of NF-κB, COX-2, is highly expressed in many cancer types including CRC and PC. Further, constitutive activation of NF-κB pathway has been associated with drug resistance, in both CRC and PC. As mentioned above, animals studies have shown that inhibition of this pathway via mutant IKK showed delayed tumor progression and reduced tumor size, implying the importance of the pathway in driving tumor progression in both PC and CRC. There are several inhibitors being developed targeting different components of the pathway (For a literature review, please refer to the references"
Table 1-7. STAT3 and NF-κB target genes 79

<table>
<thead>
<tr>
<th>Gene</th>
<th>Role in Cancer</th>
<th>NF-κB Regulation</th>
<th>STAT3 Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-xL</td>
<td>Survival</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>MYC</td>
<td>Proliferation</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>BIRC5 (encodes survivin)</td>
<td>Anti-apoptotic</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>MMP9</td>
<td>Invasion</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>MMP2</td>
<td>Invasion</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>CCND1 (cyclin D1)</td>
<td>Proliferation</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Survival/angiogenesis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Invasion</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>TWIST1</td>
<td>Metastasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>VIM (vimentin)</td>
<td>Survival/EMT</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>MCL1</td>
<td>Survival</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>HSP70 and HSP90</td>
<td>Survival</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>IL-10</td>
<td>Immune suppression</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>Angiogenesis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>FGF2 or BFGF</td>
<td>Enzymes</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>COX2</td>
<td>Inflammation</td>
<td>x</td>
<td>x</td>
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<tr>
<td>CXCL12/CXCR4</td>
<td>Metastasis</td>
<td>x</td>
<td>x</td>
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<td>IL-11</td>
<td>Inflammation</td>
<td>x</td>
<td>x</td>
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<td>IL-23</td>
<td>Inflammation</td>
<td>x</td>
<td>x</td>
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<td>IL-21</td>
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</tr>
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<td>CD80</td>
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<td>x</td>
</tr>
<tr>
<td>CD86</td>
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<td>x</td>
</tr>
<tr>
<td>CXCL10 (IP-10)</td>
<td>Inflammation</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Dual role survival/apoptosis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>IFNβ</td>
<td>Dual role survival/apoptosis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>CCL5 (RANTES)</td>
<td>Inflammation</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>NOS2</td>
<td>Metastasis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>IL-8</td>
<td>Inflammation</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Inflammation</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>CCL2 (MCP1)</td>
<td>Inflammation</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

STAT3

The second most common inflammatory pathway which is associated to cancer growth and highlighted for its anti-cancer and drug resistance is STAT3 pathway.\textsuperscript{80} STAT3 pathway has been shown to be up-regulated in both CRC and PC, and has been reported to account for the drug resistance.\textsuperscript{67, 81} It has been shown that inhibition of STAT3 pathway in CRC or PC models leads to inhibition of tumor growth.\textsuperscript{31, 33, 50, 51}

\textbf{Figure 1-4.} STAT3 pathway

STAT3 belongs to a class of proteins transcription factors known as signal transduction and activator of transcription (STAT) proteins. STAT3 was discovered as a
DNA binding protein in presence of IL-6 and epidermal growth factor signaling.\textsuperscript{82,83} STAT3 gets activated via phosphorylation of single tyrosine residue (Tyr705) by JAK kinase in presence of inflammatory stimuli or cytokine stimuli (e.g. cytokines like IL-6, IL-10 or IL-11, also via growth factors like EGF, VEGF or FGF).\textsuperscript{84} STAT3 can be also phosphorylated at Ser727 via MAPK or mTOR pathway,\textsuperscript{85} which is located in the transactivation domain. Phosphorylation of Ser727 has been proposed to be necessary for its full activation.\textsuperscript{86} Upon phosphorylation, STAT3 forms a dimer with corresponding activated STAT3 and localizes to the nucleus (\textbf{Figure 1-4}). In the nucleus it activates genes associated with proliferation (c-MYC, cyclin D1), pro-inflammation (COX-2) and anti-apoptosis (Bcl-xL, Survivin, Mcl-1).\textsuperscript{79}

In normal cells, STAT3 activity is short lived ranging from few minutes to several hours, however in tumor cells, STAT3 activity has been found to be constitutively active and leads to carcinogenesis by both preventing apoptosis and increasing cell proliferation. NF-κB pathway and STAT3 pathways can activate each other via release of different cytokines like IL-6 and TNF-α, which further increases the complexity of signaling networks in inflammation and cancer. In conclusion, both of these pathways are up-regulated in many cancers including PC and CRC, and play an important role in growth and metastasis of cancer cells.
Chemopreventive Role of NSAIDs in CRC and PC

Different clinical studies have shown that NSAIDs, particularly ASA, are effective chemopreventive agents, with most evidence of efficacy in CRC prevention.\textsuperscript{22, 56, 57} It has been suggested by different meta-analyses and systematic reviews that ASA and other NSAIDs consumption has been associated with reduced incidence of colonic adenomas, CRC, and metastatic CRC.\textsuperscript{21, 87-94} Chemoprevention of CRC by ASA has been extensively reviewed by Drew et al.,\textsuperscript{58} describing different clinical trials that have taken place since the discovery of ASA and future clinical trials exploring the role of ASA in combination with chemotherapeutic drugs for CRC treatment.\textsuperscript{58} Further, with the evidence of ASA reducing CRC rate in clinic, different animal models of CRC have also depicted the role of ASA in inhibiting CRC growth and induction of apoptosis.\textsuperscript{95-99}

Additionally, there have been several reports of clinical trials showing ASA to be clinically effective in reducing PC growth.\textsuperscript{22, 56, 57} Recent clinical studies showed that high ASA (>600 mg) decreased PC incidence.\textsuperscript{21} Furthermore, ASA has also demonstrated its effects in different animal models of PC.\textsuperscript{95, 100-105} Table 1-8 shows the animal models of PC in which ASA has shown to inhibit its genesis. However, ASA had significantly less effect once the tumor was already established. Authors from this study suggested that ASA has chemopreventive effects but at similar concentration does not show any significant effects on established PC tumors.\textsuperscript{103} Other than CRC and PC, ASA has also shown to have chemopreventive effects in other cancers including lung, skin, ovarian and breast cancers.\textsuperscript{106-110} These studies indicate that ASA has broad spectrum of effects on different kinds of cancers and represents itself as a unique drug for cancer chemoprevention.
**Table 1-8.** Effects of ASA in different animal models of PC

<table>
<thead>
<tr>
<th>Drug</th>
<th>Animal model</th>
<th>Dose</th>
<th>Findings</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>PDX-1-Cre; LSL-Kras(^{G12D}); mimics slow progression from PanIN 1 over PanIN 2 and PanIN 3 to invasive PC</td>
<td>20 mg/kg/body weight</td>
<td>Intraperitoneal injection</td>
<td>105</td>
</tr>
<tr>
<td>Aspirin</td>
<td>LSL-Kras(^{G12D})/; LSL-Trp53(^{R172H})/; Pdx-1-Cre; PDAC, accelerated PanIN, metastatic PC</td>
<td>20 mg/kg/body weight</td>
<td>Intraperitoneal injection</td>
<td>105</td>
</tr>
<tr>
<td>Aspirin</td>
<td>DMBA implanted in Black 6 mice; carcinogen induced PDAC</td>
<td>20 mg/kg/body weight; Mixed with diet</td>
<td>Aspirin inhibited MDSC and M2; inhibited PDAC formation</td>
<td>111</td>
</tr>
<tr>
<td>Aspirin + gem</td>
<td>Orthotopic; Panc02</td>
<td>20 mg/kg Asp (mixed with diet); Gemcitabine100 mg/kg (i.p.)</td>
<td>Potentiated gemcitabine effects</td>
<td>111</td>
</tr>
<tr>
<td>Aspirin + gem</td>
<td>LSL-Kras(^{G12D})/; LSL-Trp53(^{R172H})/; Pdx-1-Cre;</td>
<td>Gemcitabine100 mg/kg (i.p.); Aspirin (i.p.) 40 mg/kg/body</td>
<td>Potentiated gemcitabine effects; increased survival by 10 days</td>
<td>104</td>
</tr>
<tr>
<td>Aspirin</td>
<td>Orthotopic; Panc-1 cells</td>
<td>Aspirin 200 mg/kg (i.p.)</td>
<td>Inhibited tumor formation when treated 6 days before cells injection</td>
<td>103</td>
</tr>
<tr>
<td>Aspirin + Gem</td>
<td>Orthotopic; Panc-1</td>
<td>Aspirin 200 mg/kg; gem 12.5 mg/kg</td>
<td>Increased survival + inhibited CSC</td>
<td>101</td>
</tr>
</tbody>
</table>

**Mechanism of Action of ASA**

Classical mechanism of ASA is via inhibition of COX proteins, however ASA is proven unselective COX-1 and COX-2 inhibitor. Since, COX-2 protein has been shown to be up-regulated in CRC and PC, it has been hypothesized that ASA induces its effects via inhibition of COX-2. However, there are several other COX independent mechanisms reported by which ASA is able to show its chemopreventive and chemotherapeutic
Another mechanism reported in recent literature showed that ASA may prevent CRC metastasis by inhibiting COX-1 in platelets. These observations resulted from the finding that CRC cells incubated with ASA treated platelets were not able to metastasize as efficiently as CRC cells incubated with untreated platelets.

Moreover, ASA has several other mechanisms through which it can inhibit cancer growth and induce apoptosis. ASA is suggested to modulate NF-κB pathway; it has been reported that ASA can inhibit IKK activity and hence inhibit NF-κB activation in presence of TNF-α. ASA has also been shown to inhibit extracellular-signal-regulated kinase (ERK) signaling via prevention of c-Raf binding to Ras in vitro. It could be possible that ASA can inhibit this pathway in CRC and PC cells and work in combination with inhibition of COX activity to show its chemopreventive action. Other mechanisms of ASA include apoptosis via increasing mitochondrial permeability through Bcl-2 family proteins and release of cytochrome c, followed by caspase activation and apoptosis. Further, ASA treatment has shown to inhibit proteasomal activity and increase in ubiquitylated protein levels, which eventually leads to mitochondrial cell death. ASA has also been reported to inhibit Wnt/beta catenin pathway and activation of several transcription factors, like specific protein (SP) 1, SP2, SP3 and SP4, involved in cell proliferation, survival and angiogenesis. Other mechanisms include inhibition of angiogenesis, modulation of insulin-related neoplastic pathways affecting glucose uptake and apoptosis induction, inhibition of the mammalian target of rapamycin (mTOR) signaling and modulation of cellular metabolism through the AMP-activated protein kinase (AMPK). Recently, Ai et al. showed that ASA can directly acetylate mutant p53 and wild-type p53 and activation of
p21 in CRC cells and in vitro settings,\textsuperscript{125} indicating another potential mechanism by which ASA may be inhibiting CRC growth. ASA has been shown to acetylate many other proteins and many new targets are still being discovered.\textsuperscript{126} These studies also imply that the acetyl group of ASA is vital for its mechanism of action.

\textit{Limitations of ASA}

As mentioned above, long-term use of ASA has shown to have chemopreventive effects in both CRC and PC. However, long-term and high dose use of ASA has been reported to have serious side effects like gastrointestinal bleeding and renal toxicity.\textsuperscript{58} ASA is well-known to induce gastric damage or ulcer formation even when used in a low amount.\textsuperscript{127, 128} Two mechanisms are proposed in ASA mediated gastric damage: (i) direct damage by acidic properties of the compound and (ii) inhibition of the formation of mucosal layer in the stomach lining, which protects the stomach from the gastric juices, because of its effects on reduced production of prostaglandins.\textsuperscript{129, 130} Therefore, there are continuous efforts by many research groups to make an optimized ASA analog which is more potent at killing cancer cells and has gastro-protective effects.

\textbf{Modification of NSAIDs for Better Efficacy}

Several different NSAID scaffolds have been modified in an effort to achieve a favorable gastrointestinal tolerance while maintaining or enhancing the anti-inflammatory properties of the parent compound.\textsuperscript{131, 132} Two modification of ASA with nitro oxide (NO) and
nitroxyl (HNO) have been widely studied and characterized. These compounds were designed with the rational that combining two different scaffolds, will yield a molecule more potent than ASA, with reduced GI, renal and hepatic toxicities. Different studies have shown that NO- and HNO-releasing NSAIDs have greater potency towards killing cancer cells and chemopreventive effects in different cancers including CRC and PC.

Further they have shown to have lower toxicity profile than the corresponding the parent molecule alone.

More recently, ASA derivatives containing hydroxyapatite have been designed with a rational that hydroxyapatite have shown to increase PGE2 levels, hence reducing the gastric toxicity of ASA. In the study performed by Zhen et al. Ca-Asp (novel ASA hydroxyapatite derivative) showed very less gastro-damage compared to ASA alone. Gastro-protective effect of the compound is due to higher inhibition of COX-1 compared to COX-2. However, the anti-cancer activity of this modification has not been studied yet. Another modification of NSAIDs that has been performed is via addition of hydrogen sulfide. Hydrogen sulfide-releasing NSAIDs (HS-NSAIDs) were synthesized by conjugation of the parent NSAID with a dithiolethione moiety. The attached moiety releases hydrogen sulfide. It has been shown that HS-NSAIDs have less GI toxicity and have an increased anti-inflammatory activity and potency in animal model of colitis. HS-NSAIDs also displayed a broad spectrum of growth inhibitory activity in different cancer cell lines.

Additionally, novel agents containing both NO- and HS- moieties in ASA have shown to be potent at inhibiting CRC growth and anti-inflammatory activity. These NO-
and HS-releasing ASA display potent anti-tumor activity in CRC xenograft models.\textsuperscript{146, 148} Lastly, another modification which has been shown to possess anti-cancer activity is phospho-NSAIDs. These compounds have displayed potent chemotherapeutic and chemopreventive properties in different cancers.\textsuperscript{149, 150} Hence, novel agents are being continuously designed over the NSAID scaffolds to yield more potent compounds. However, these molecules are still in their early phases of clinical development, and hence, developing novel agents using NSAIDs scaffolds represent a valuable opportunity for cancer drug discovery.

**Selenium Molecules and its Potential as an important Micro Nutrient**

Selenium, an essential trace element in diet, has been linked to reduce risk of many cancers, including PC and CRC.\textsuperscript{151-154} Selenium was discovered by Jöns Berzelius in 1817 and was named after the Greek goddess of moon “Selene” (Figure 1-5).

![Figure 1-5](image)

**Figure 1-5.** Greek goddess of moon - "Selene"
Selenium in body is incorporated in selenoproteins in the form of selenocysteine (Sec) amino acid. Structure of Sec is nearly similar to the structure of cysteine amino acid, except sulfur in cysteine is replaced with selenium in Sec. As shown in Table 1-9, selenoproteins are involved in a wide range of processes ranging from anti-oxidant to anti-inflammatory. Proteins belonging to glutathione peroxidase (GPX1) and thioredoxin reductase (TRXR)s are mainly involved in cellular antioxidant systems. Hence, they play a major role in protecting cells from oxidative & nitrosative stress, and reduce DNA damage and inflammation. Therefore, individuals with less selenium content in their diet have less turnover of selenoproteins and are thought to be more prone towards cancer risk. Different laboratories and ecological studies have found that deficiency of selenium is associated with many human diseases such as central nervous system disease, cardiovascular diseases, inflammatory diseases, diabetes mellitus and cancer.
Table 1-9. Selenoproteins and their function

<table>
<thead>
<tr>
<th>Selenoprotein</th>
<th>Functions</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPX1</td>
<td>phospholipid hydroperoxides, antioxidant enzymes, and plays role in inflammation</td>
<td>167</td>
</tr>
<tr>
<td>Iodothyronine deiodinase (DIO)</td>
<td>During metabolism of thyroid hormones, cleave iodine-carbon bonds</td>
<td>168</td>
</tr>
<tr>
<td>TRXRs</td>
<td>NADPH for reduction of TRX (thioredoxin) in cellular redox pathways</td>
<td>169</td>
</tr>
<tr>
<td>Selenoprotein P (Se1P)</td>
<td>Secreted by liver for transporting Se to different body regions</td>
<td>170</td>
</tr>
<tr>
<td>Selenoprotein W (Se1W)</td>
<td>Similar to GPX family proteins, binds to glutathione, antioxidant</td>
<td>171</td>
</tr>
<tr>
<td>Selenoprotein H (Se1H)</td>
<td>DNA binding may act as transcription factor. Increases GSH levels and GPX activity</td>
<td>172</td>
</tr>
<tr>
<td>Selenoprotein I (Se1I)</td>
<td>phospholipid-synthesizing enzyme, ethanolamine phosphotransferase</td>
<td>173</td>
</tr>
<tr>
<td>Selenoprotein R (Se1R)/Selenoprotein X (Se1X)</td>
<td>Member of methionine sulfoxide reductase, plays important role in reduction of sulfoxymethyl groups</td>
<td>174</td>
</tr>
<tr>
<td>Selenoprotein N (Se1N)</td>
<td>Found in ER, necessary for muscle development</td>
<td>174</td>
</tr>
<tr>
<td>Selenoprotein S (Se1S)</td>
<td>Found in ER, necessary for removal of misfolded proteins</td>
<td>175</td>
</tr>
<tr>
<td>15kDa selenoprotein, Sep15, Se1K, Se1M, and Se1T</td>
<td>ER proteins with unknown functions</td>
<td>176</td>
</tr>
<tr>
<td>Se1O and Se1V</td>
<td>Unknown functions</td>
<td>176</td>
</tr>
</tbody>
</table>

Role of Selenium in GI

Selenium is an attractive element not only because of its anti-cancer activities, but also for its ability to protect against gastric ulcers. It was reported for the first time in 1988 by Parmar et al., that upon pre-treatment with sodium selenite in albino rats, reduced the gastric ulcer formation via NSAIDs like aspirin and indomethacin. These findings were tested by Kim et al., reporting that indomethacin formed ulcers were reduced by selenium treatment for 3 days. They showed that selenium decreased lipid peroxidation induced
by indomethacin treatment and increased the activities of superoxide dismutase, catalase and GSH in a dose-dependent manner.\textsuperscript{178} In another study, it was shown that selenium treatment reduced ulcer formation via decreasing TNF-α and malondialdehyde levels, and increasing anti-oxidants and PGE2 levels.\textsuperscript{179} Most recently, selenium was shown to heal gastric ulcer similar to current drug omeprazole, via increasing anti-oxidant levels, reducing lipid peroxidation, and altering mucus secretion response.\textsuperscript{180} Overall these studies describe the important role of selenium compounds in protecting ulcer formation in presence of ulcer causing agents like NSAIDs.

**Challenges with Developing Selenium Containing Drugs**

Knowing the different roles of selenoproteins, and a direct link of selenium levels and risk of different cancers, led to many different chemoprevention trials using selenium supplements.\textsuperscript{181} Major clinical trial which established the link between selenium supplementation and cancer prevention was the Nutritional Prevention of Cancer (NPC) clinical trial.\textsuperscript{182} Selenium-enriched yeast (SeY) was used for NPC trial as a selenium supplement. The trial was focused on selenium supplementation and risk of skin cancer incident. End point results were not significant for skin cancer. However, the trial became famous for the second end point, which measured the incidences of prostate cancer. It was shown that selenium enriched yeast (SeY) consumption reduced prostate cancer cases by nearly 63\%.\textsuperscript{182} This encouraging study provoked several clinical trials including Selenium and Vitamin E Cancer Prevention Trial (SELECT).\textsuperscript{183} SELECT trial focused on chemoprevention of prostate cancer, using selenomethionine (SeMet), the major
component of SeY. Results showed that there was no significant difference in the placebo group vs SeMet in prostate cancer prevention. However, there was an increase in type 2 diabetes incidences in patients supplemented with SeMet.\textsuperscript{183} Since SELECT trail was in a large population pool, and the constraints on how the data was evaluated, made the results very significant. Hence, failure of SeMet in clinic to inhibit prostate cancer, caused a negative impact on developing selenium containing preventive agents.

Recently, Cai \textit{et al.} investigated the association between selenium exposure and cancer risk from 69 different clinical studies.\textsuperscript{181} Their finding was that reduced selenium levels were associated with different cancers (including breast, colorectal, esophageal, bladder, gastric, lung, skin and other cancers).\textsuperscript{181} They also suggested that the use of different selenium supplementation in SELECT trial vs NPC may present distinct effects on human health. Further, different selenoproteins require different amount of selenium to be produced, likewise selenoprotein P needs more selenium intake compared to expression of GPXs.\textsuperscript{184} This indicates that selenium provided by the supplements may not be enough to achieve selenium protective effects. SELECT trial was failure of only SeMet and not all selenium containing compounds, since every molecule shows a different activity based on the position of selenium atom and overall structure of the molecule. Further, there was lack of convincing pre-clinical studies with SeMet even prior to SELECT.\textsuperscript{185} Hence, selenium containing small molecules, similar to any other chemical compound, must be rigorously evaluated before taking to the clinical trial.

Despite the negative waves of selenium as a chemopreventive agent, generated by SELECT trial, there has been a rising interest in development of novel selenium containing
agents with chemotherapeutic potential. Over the past decade, several organoselenium molecules have been developed which show potent anticancer activity in vitro and in vivo models against different cancer types.\textsuperscript{186-190} Further, selenium containing agents have shown to have synergistic or additive effects with known chemotherapeutic agents.\textsuperscript{191-195}

**Figure 1-6.** Novel Se molecules and their parent compounds. Modified from \textsuperscript{196}

Our lab and others have shown that rational incorporation of selenium in place of sulfur in known natural compounds, well-known synthetic chemotherapeutic agents, and existing drugs in clinic, yielded novel compounds which are more effective than parent compounds, e.g. in terms of anticancer activity.\textsuperscript{196} **Figure 1-6** shows the modification of well-known anti-cancer agents with selenium leading to potent analogs.\textsuperscript{196} **Table 1-10** represents examples of the novel organoselenium molecules developed in our laboratories as well as by other groups which have better activity against cancer than their parent
molecule. Interestingly, selenium incorporation into the different molecules exhibited different activity and potency, and hence the effect seen is not just due to selenium alone, but the position of active groups and the location of selenium atom within the structure of the compound.

Table 1-10. Summary of efficacy and molecular targets of selenium compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Targets</th>
<th>Efficiency</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISC-4</td>
<td>PI3K/Akt</td>
<td>Induces apoptosis in many different cancer cell lines and inhibits tumor growth in melanoma and CRC xenografts</td>
<td>196</td>
</tr>
<tr>
<td>Isatin-Se derivatives</td>
<td>Tubulin polymerization</td>
<td>Inhibits growth of CRC, melanoma, lung and breast cancer cells</td>
<td>196</td>
</tr>
<tr>
<td>PBISe</td>
<td>Akt, iNOS, MAPK</td>
<td>Inhibits tumor growth in melanoma xenograft</td>
<td>196</td>
</tr>
<tr>
<td>TMZ-Se</td>
<td>Induces autophagic response, Beclin-1</td>
<td>Effect against TMZ resistant cells, inhibits tumor growth in mouse glioma and melanoma models</td>
<td>196</td>
</tr>
<tr>
<td>SelSa-1 and SelSa-2</td>
<td>HDAC, MAPK, PI3K</td>
<td>Inhibits lung cancer and melanoma cell growth, effective chemopreventive potential in melanoma models</td>
<td>196</td>
</tr>
<tr>
<td>Selenocoxibs</td>
<td>COX-2, iNOS, TNF</td>
<td>Inhibits prostate tumor growth more effectively than celecoxib in vivo</td>
<td>196</td>
</tr>
<tr>
<td>EG-Se/Pt (Selenium-Platinum small molecule)</td>
<td>ROS generation</td>
<td>Inhibits liver, breast colon and lung cancer cell growth, inhibited liver tumor growth in xenograft studies. Selective killing of cancer cells over normal cells.</td>
<td>197</td>
</tr>
<tr>
<td>Se-REPS (Se-exoploysaccharide obtained from Rhizobium sp. N613)</td>
<td>-NA</td>
<td>Inhibits liver cancer cells and tumor growth of murine cancer cells in xenografts</td>
<td>198</td>
</tr>
<tr>
<td>Heterocycle-Selenourea derivatives</td>
<td>-NA</td>
<td>Inhibits cancer growth and incudes apoptosis in different cancer cell lines</td>
<td>199</td>
</tr>
</tbody>
</table>

The mechanistic aspects of how novel selenium molecules induce anti-cancer effects differ with the overall structure of the compound. As listed in Table 1-10, different anti-cancer mechanisms of selenium compounds have been identified, including inhibition
of the Akt pathway, inhibition of COX-2, inhibition of iNOS, cell cycle arrest, ROS generation or inhibition of ROS, induction of apoptosis, induction of autophagy and inhibition of angiogenesis.\textsuperscript{200-202}

**Genesis of Synthesis of Novel Agents (AS-10/Comp 8)**

To the best of our knowledge, the only NSAID framework that has been modified with selenium is celecoxib scaffold. The selenium modified celecoxib (selenocoxib) showed greater anti-inflammatory activity as well as greater anti-cancer activity compared to parent molecule.\textsuperscript{203, 204} Second generation of the selenocoxib derivative yielded another agent, the glutathione analog of selenocoxib-1, which maintained the anti-cancer potency of the parent compound. Mechanism of action of selenocoxib-1 GSH is reported to be via inhibition of COX-2 activity and Akt pathway.\textsuperscript{200}

In view of the well-established chemopreventive activity of ASA and gastro-protective properties of selenium molecules, *we hypothesized that a rationally designed selenium analog of ASA should not only maintain its anti-inflammatory properties but provide protection against gastrointestinal bleeding and ulcers due to presence of selenium*. In addition, presence of selenium was expected to make the resulting hybrid molecule additional potency so as to reduce the dosing requirements, thus further eliminating the possibilities of toxicity. Hence, with that rationale, we designed novel agents which contained NSAIDs (mainly ASA) and selenium. Chapter 2 discusses the
design and synthesis of novel agents, and the anti-cancer activity and mechanisms of the lead Compound 8 (Comp 8, Figure 1-7) in CRC and PC.

**Figure 1-7.** Novel Se-ASA molecules Comp 8 and AS-10.

Synthesis of all the NSAID-selenium analogs for SAR study and the biological investigations were published recently in the Journal of Medicinal Chemistry, however, the synthesis is not the part of this thesis. For design and synthesis details, readers are advised to read the original manuscript. Our overarching goal was to identify the potential mechanism of action (MOA) of the lead molecules identified (Comp 8 and AS-10). Our overall hypothesis was that these agents may modulate inflammatory pathways, such as NF-κB pathway, in cancer cells and hence inhibit their growth. Therefore, Chapter 2 focuses on Comp 8 anti-cancer activity in different cancer types including CRC and PC; and its effects on modulating the NF-κB pathway. Chapter 3 focuses on the effects of second lead molecule, AS-10 (Figure 1-7), in inhibiting NF-κB pathway and induction of apoptosis in PC and CRC cells. Chapter 3 also includes studies on the cancer cell growth inhibitory activity of AS-10 in broad spectrum of cancer types. Chapter 4 summarizes the overall findings of the whole project, and chapter 5 focuses on future directions for the current work and conclusions.
Chapter 2

Design, Synthesis, and Biological Evaluation of Novel Selenium (Se-NSAI) Molecules as Anticancer Agents\(^1,2\)

Introduction

A cohort of studies supports the indisputable promise of aspirin (ASA) and other non-steroidal anti-inflammatory drugs (NSAIDs) as chemopreventive agents, especially in colorectal cancer (CRC). Meta-analyses and systematic reviews suggest that ASA and other NSAIDs use is associated with a decreased incidence of colonic adenomas, CRC, and metastatic CRC.\(^{87-94}\) Furthermore, several other studies suggest that daily dosing of ASA decreases the risk of great variety of cancer types, including lung, breast, skin, pancreas and ovarian cancers.\(^{106-110}\) Although routine ASA use may not be warranted in the general population, an international panel advocated additional research in high-risk

\(^1\) Reprinted (adapted) with permission from the original article published in Journal of medicinal Chemistry.

\(^2\) Dr. Plano contributed equally by synthesizing all the agents required for this study. Therefore, the details of chemical synthesis work is not part of this thesis. Readers are referred to the original publication cited above.
populations. In fact, a randomized clinical trial in carriers of hereditary CRC, named CAPP2 (Colorectal Adenoma/carcinoma Prevention Program), has demonstrated a substantial protection by ASA against CRC and other Lynch syndrome cancers. The long-term results showed that 600 mg of ASA per day for a mean of 25 months substantially reduced cancer incidence after 55.7 months in Lynch syndrome patients, which is the major form of hereditary CRC.

Additionally, NSAIDs have demonstrated their intestinal antineoplastic effects in animal models, showing their effects in more than 90% of the 110 individual studies of various animal intestinal cancer models. A key mechanism for NSAIDs efficacy is cyclooxygenase (COX) inhibition and reduced production of prostaglandins. Nonetheless, several other mechanisms of action have been proposed, including the induction of apoptosis, inhibition of angiogenesis, activation of nuclear factor kappa B (NF-κB), modulation of insulin-related neoplastic pathways, inhibition of the mammalian target of rapamycin (mTOR) pathway, and modulation of cellular metabolism through the AMP-activated protein kinase (AMPK).

Several modifications, which maintained the anti-inflammatory effect of the parental NSAIDs with favorable gastrointestinal tolerance or with combined immune-modulatory and antioxidant activities, have been carried out over different NSAID scaffolds. The combination of reactive nitrogen species, e.g., nitric oxide (NO) and nitroxyl (HNO), with different NSAIDs, particularly ASA, has been widely studied and characterized. The rationale behind the generation of these molecules is that the combined effect of both the scaffolds will exceed those of each structural component individually,
along with a reduction of the renal and hepatic toxicities associated with chronic NSAID use. Several reports have demonstrated that the NO- and HNO-releasing NSAIDs possess stronger cytotoxicity and chemopreventive effects, mainly against CRC, together with a lower toxicity than the corresponding NSAID alone.\textsuperscript{134-139} Notably, hydrogen sulfide-releasing NSAIDs (HS-NSAIDs) are formed by the conjugation of the parent NSAID with a dithiolethione moiety, which releases hydrogen sulfide. These scaffolds were less toxic to the GI tract and exhibited a marked increase in the anti-inflammatory activity and potency in a murine model of colitis than their parental NSAIDs.\textsuperscript{141-144} Additionally, several HS-NSAIDs evinced growth inhibitory effects in a wide variety of human cancer cells.\textsuperscript{145-147} Furthermore, dual NO- and HS-releasing ASA derivatives have been synthesized, showing potent anti-inflammatory activity and inhibition of cancer cell growth \textit{in vitro} along with a marked reduction of tumor volume in colon cancer xenograft models.\textsuperscript{146, 148} Other modified NSAIDs extensively reported in the literature are the phospho-NSAIDs, which have shown preclinical chemotherapeutic and chemopreventive properties in several cancer models.\textsuperscript{149, 150}

Selenium compounds have attracted a vast interest in the last decades as promising chemopreventive agents, and several epidemiological studies have reported an inverse association between the nutritional selenium status and cancer risk, although considerable controversy has arisen mainly due to the negative outcome of the SELECT trial.\textsuperscript{196, 213} Contrary to the skepticism generated by the diverse clinical trials on selenium as a chemopreventive agent, a rising interest in its potential cancer therapeutic effects has been developed in the past few years. Several organoselenium compounds have been shown to inhibit cancer cell growth in various xenograft rodent models for different cancers types.\textsuperscript{186-}
as well as to have synergistic effects in combination with chemotherapeutic drugs. The mechanisms of action by which seleno compounds exert their anti-tumor effects differ with the overall structure of the molecule and have not been completely characterized. However, some of the representative anti-cancer mechanisms of seleno compounds are the induction of apoptosis, inhibition of angiogenesis, and modulation of AKT and COX pathways. To our knowledge, the only NSAID framework modified to date with selenium functionalities is the celecoxib scaffold, yielding greater anti-inflammatory and cancer cell growth inhibition, for the novel selenocoxib derivatives. Further development of these selenocoxib derivatives afforded the glutathione analog of selenocoxib-1, which elicited potent inhibition of tumor development in melanoma xenografted mice.

Considering the aforementioned chemopreventive effects of NSAIDs and the biological effects of organoselenium compounds, along with the previous reports that support the modification of NSAID scaffolds to obtain more potent and less systemically toxic antitumor agents, we designed novel hybrid Se-NSAID derivatives. This drug design is additionally supported by the reported protective effects of selenium compounds against gastric toxicity induced by indomethacin, along with the potential synergistic effects of selenium and NSAIDs.

In the present study, we report the design and synthesis of several Se-NSAID hybrid compounds, their cytotoxic activity in a panel of cancer cell lines, and the preliminary characterization of the mechanism of action for the lead compound 8 (Comp 8) in the HCT116 CRC cell line. Two shared mechanisms implicated in the biological effects of ASA and seleno compounds on cancer cells are apoptosis and inhibition of
inflammatory pathways.\textsuperscript{23, 203} In this chapter, we focused on the anti-proliferative effects of the lead Comp 8 and report insights into its mechanism of action in inhibiting CRC and PC cell growth.

**Materials and Methods**

*Chemistry.*

For detailed explanations and methods regarding the chemistry section, reader is advised to see the original manuscript.\textsuperscript{205}

*Reagents and antibodies for biological evaluation.*

Antibodies for Western blot purpose were ordered from following sources: cell signaling (anti-PARP (9542s), cyclin E1 (4129), cyclin B1 (12231), caspase 3 (9668s), Mcl-1 (5453s) and Bcl-xL (2764s)); Abcam (Bcl-2 (ab59348)); Sigma-Aldrich (β-actin (A5316)); and Santa Cruz Biotechnology (p21 (sc6246)). Thiazolyl Blue Tetrazolium Bromide (MTT) (M5655-500MG), Triton X-100 (93443), RNase A (R6513), and propidium iodide (PI) (P4170) were purchased from Sigma-Aldrich.

*Cell culture conditions.*

MDA-MB-231, Panc-1, BxPC3, MEFs and 1205Lu cell lines were maintained in DMEM medium; UACC903, DU145, and H460 cells were maintained in RPMI 1640 medium; HT29, SKOV3, HCT116 and RKO cells were maintained in McCoy's 5A medium; HEPG2 cells were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml of penicillin and streptomycin at 37°C and 5% CO\textsubscript{2}. nHDFs were grown in fibroblast basal medium (ATCC® PCS-201-030™)
supplemented with Fibroblast Growth Kit–Serum-Free (ATCC PCS-201-040) and penicillin-streptomycin-amphotericin B Solution (ATCC PCS-999-002). All the cell lines were obtained from ATCC.

Cell viability assay.

The effect of the compounds on cancer cell viability was determined by the MTT assay uptake method as described before.215 Briefly, 3000 cells were incubated with different concentrations of the test compounds in triplicate in a 96-well plate for 24, 48 and 72 h at 37 °C. Three hours prior to experiment termination, MTT solution (20 µL of 5.0 mg/mL solution) was added to each well and incubated at 37 °C. At the termination time point, the resultant formazan crystals were dissolved in DMSO, and the optical densities were measured at 570 nm and 630 nm using a 96-well multiscanner (Dynex Technologies, MRX Revelation; Chantilly, VA, USA). Graphpad Prism software was used to calculate the appropriate IC$_{50}$ values calculated by non-linear regression analysis.

Live and dead assay.

To determine dead cell population, we used LIVE/DEAD Viability/Cytotoxicity Kit (Life technologies, USA). HCT116 cells were treated with either DMSO, ASA (1 mM) or Comp 8 (15 µM) for 24 h. Cells were trypsinized and subjected to live and dead analysis using LIVE/DEAD Viability/Cytotoxicity Kit according to manufacturer’s protocol. The kit contains two components, calcein-AM and ethidium homodimer-1. Green-fluorescent calcein-AM indicates intracellular esterase activity (live cells), while red-fluorescent ethidium homodimer-1 indicates loss of plasma membrane integrity (dead cells). Stained cells were pipetted on the slides and covered with cover slip. Pictures were taken using a
fluorescence microscope (Zeiss - Axio Scope.A1) with a 20X objective lens. Percentage of live and dead cells were calculated.

Cell cycle analysis.

Cell cycle analyses were determined by following a flow cytometry protocol as described earlier.216 In brief, HCT116 cells were serum deprived for 72 h to get them synchronized. At the end of 72 h, cells were supplied with fresh medium or fresh medium with 5 μM of Comp 8 for 12 and 24 h time points. After each time point, the cells were fixed in ice cold 70% ethanol. Fixed cells were washed with 1X PBS and suspended in PI staining solution. PI staining solution was made by adding 0.1% (v/v) triton X-100, 20 mg DNAse-free RNAse A and 2 mg of PI in 100 mL of PBS. After staining for 15 min, the cells were analyzed by a BD FACS Calibur (BD Biosciences) for total DNA content.

Western blot analysis.

Whole-cell lysates were made by subjecting Comp 8 treated CRC cells to lysis in RIPA lysis buffer (Thermo Scientific, USA) containing protease (Roche, USA) and phosphatase inhibitor cocktails (Sigma-Aldrich, USA) as described before.217 The resulting lysates were spun at 15,000 rpm for a duration of 10 min to remove any insoluble debris. The resulting supernatant was stored at -80°C until use. NuPAGE gel 4-12% (Life Technologies, Carlsbad, CA) was used to resolve the lysates. After electrophoresis/SDS-PAGE, the resolved proteins were electro-transferred to PVDF membrane and blotted with the different antibodies. Enhanced Chemiluminescent reagent (Life technologies, USA) was used to detect the protein of interest. In order to save time and samples, consecutive detections of different proteins were achieved by using Restore Western blot stripping buffer (Thermo Scientific, USA).
**Annexin V assay**

In order to evaluate the ability of Comp 8 to induce apoptosis, HCT116 (5 x 10^5) cells were treated with increasing dose of Comp 8 or DMSO for 24 h. Different stages of apoptosis, in both floating and adherent cells, were measured using the Muse Annexin V & Dead Cell kit with Muse cell analyzer (EMD Millipore, Billerica, MA, USA), according to manufacturer’s protocol. The Muse Annexin V & Dead cell assay uses Annexin V to identify phosphatidylserine (PS) on the external surface of apoptotic cells. The kit also has a dead cell marker, 7-amino-actinomycin D (7-ADD), which does not enter live, healthy and early apoptotic cells, with intact cell membrane (Millipore, Catlog No, MCH100105). Data obtained from the equipment were analyzed using Muse 1.4 software.

**Caspase 3/7 activity assay**

Activation of caspase 3/7 in HCT116 cells treated with Comp 8 was detected using Muse Caspase-3/7 Assay kit with Muse cell analyzer (EMD Millipore, Billercia, MA. USA) according to manufacturer’s protocol. In brief, the Muse Caspase 3/7 kit uses a reagent namely, NucView, for detection of activated caspase 3/7. This reagent is cell membrane permeable and possesses no known toxicity towards cells. This reagent contains a DNA binding dye that is linked to a DEVD peptide substrate. DEVD inhibits the dye from binding to the DNA. Upon cleavage of the DEVD in presence of active caspase 3/7, DNA binding dye is released and hence gives high fluorescence (ref Millipore, Catlog No. MCH100108). The kit also contains a dead cell marker, 7-ADD, for detection of dead cells with compromised cell membrane but excludes cells with intact cell membrane. Data from Muse cell analyzer were analyzed using Muse 1.4 software.
**ROS measurement**

Measurement of total ROS levels in HCT116 cells, treated with Comp 8, was performed by using the Muse Oxidative Stress Kit (EMD Millipore, Billerica, MA) as per manufacturer’s protocol. The kit utilizes the Muse Oxidative Stress Reagent for detection of ROS inside the cells. The kit identifies two different cell population ROS (-) cells and ROS (+) cells. The ROS (-) population is represented by M1 peak, while ROS (+) cells represented by M2 peak in the graph. H₂O₂ (0.9 M) treated HCT116 cells were used as positive control for identification of M1 and M2 peaks. Percentage of ROS (-) and ROS (+) cells were measured using Muse cell analyzer.

**Electrophoretic mobility shift assay (EMSA)**

To determine NF-κB presence inside the nucleus upon inflammatory stimuli, Panc-1 (1 x 10⁶) cells were treated with different doses of AS-10 and Comp 8 for 6 h. Panc-1 cells were stimulated with 100 ng/mL (3.9 nM) TNF-α for 30 min before termination. At the end of 30 min, plates were placed on ice. Nuclear and cytosolic lysates were prepared as previously described. Following the previously described protocol, nuclear lysates were subjected to EMSA. Resulting gels were dried using a gel dryer and were exposed to X-rays films at -80 °C.
**Results and Discussion**

**Design**

Several studies have pointed out the chemopreventive effects of NSAIDs and the anti-tumor and chemopreventive effects of multiple selenium species in various cancers. In addition, several selenium compounds seem to possess a protective effect against indomethacin-induced gastric toxicity, as well as a synergistic effect with sulindac to inhibit intestinal tumorigenesis. Considering all the above-mentioned information, we hypothesized that assembly of both groups in the same molecule could be a valid approach in the development of potent and safe cancer preventive and therapeutic agents. Therefore, novel molecules were designed by the incorporation of an appropriate selenium moiety into various NSAIDs according to the general structure shown in **Figure 2-1**. In order to identify the NSAID that formed the most effective anti-cancer hybrid molecule with Se, three different NSAIDs [ASA, ibuprofen (Ibu) and naproxen (Nap)] were selected for Se incorporation. On the bases of our past experience and literature evidence on the structure-activity relationship of organoselenium compounds, 2-selenocyanoethanamine (Compound 1) was used as an appropriate Se moiety to introduce into ASA, Ibu and Nap scaffolds. The *in vitro* cytotoxic results in a panel of four cancer cell lines (**Table 2-1**) indicated that only in the case of ASA the IC$_{50}$ value of the resulting hybrid Se-NSAID derivative (comp 8) was decreased significantly as compared to the parent NSAID scaffold. Accordingly, the hybrid Se-ASA (Comp 8) was selected for further structural modifications with various selenium moieties.
In a second approach, several structural modulations were performed in two different regions of the Se-ASA scaffold: (i) The functional group used to link the ASA with the Se moiety was modulated, being amide, ester or selenoester as the selected conjugate groups (Figure 1); and (ii) the functional groups bearing the Se atom were selected among selenocyanate (-SeCN), monoselenide (-Se-ASA), and methylselenocysteinyl (-CO₂H-CH₂SeCH₃) (Figure 2-1). The methylselenocysteine moiety was incorporated into ASA structure to evaluate the influence of its anti-cancer properties as well as the demonstrated selective protection it offers against organ-specific toxicity induced by clinically active agents. Taking into account the IC₅₀ values obtained for the six Se-ASA hybrid molecules (Comp 8, 10, 12-15), Comp 8 emerged as the most potent and promising anticancer agent. To gain further knowledge about the importance of the selenium atom in this scaffold, its isosteric sulfur analog (Comp 16) was also synthesized and tested.
Figure 2-1. Structural design of the novel hybrid Se-NSAID derivatives (A) and the structures of the molecules synthesized (B).


Chemistry

For Chemistry section readers are referred to the original publication.  

Evaluation of the Antiproliferative Activities of the Novel Molecules.

The three NSAID-SeCN compounds (4, 5, and 8), the corresponding parent NSAIDs (Ibu, Nap, and ASA), and all the selenium (Comp 8, 10, and 12-15)- and S (Comp 16)-derivatives of ASA were examined for their effects on cancer cell viability. To determine the effect of –NH-CH$_2$CH$_2$-SeCN incorporation into the various NSAIDs, compounds 4, 5, and 8 were screened against a panel of four different cancer cell lines: HT-29 (human colon carcinoma), MDA-MB-231 (human breast adenocarcinoma), PANC-1 (human pancreatic carcinoma) and UACC903 (human malignant melanoma) using an MTT assay.

As reported in Table 2-1, parent NSAIDs (Ibu, Nap, and ASA) had no effect on cancer cell viability up to the maximum tested dose of 50 µM. The IC$_{50}$ values obtained for the hybrid SeCN derivatives 4, 5, and 8, compared to their corresponding parent NSAIDs, showed that only the introduction of the Se moiety in ASA resulted in a significantly more potent analogue 8, while similar modification of Ibu and Nap had no effect on cancer cell viability (Table 2-1). Cell viability curves for Comp 8 at 48 h time point are shown in Figure 2.

This clearly demonstrated that the enhanced cytotoxicity may not simply be the result of incorporation of the Se-moiety into the NSAID but that a unique combination of the selenium moiety with ASA is required for cytotoxicity. On the basis of this observation, we generated several selenium analogues of ASA and screened them against same panel
of the cells as used in Table 2-1. Table 2-2 summarizes the effects of all agents on cancer cell viability, which were designed by further modifying Comp 8.

Table 2-1. Effect of Se-NSAID hybrid compounds (4, 5 and 8) compared to their respective parent NSAIDs on different cancer cell lines.

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<td>&gt;50</td>
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<td>2.1 ±1.3</td>
<td>&gt;50</td>
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<td></td>
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<td>&gt;50</td>
<td>&gt;50</td>
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<td>12.1 ±2.2</td>
<td>&gt;50</td>
</tr>
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*Data represent mean IC$_{50}$ values (± SD) of % Cell viability determined by the MTT assay in triplicates. *b* Human colon carcinoma cell line (HT29). *c* Human breast adenocarcinoma cell line (MDA-MB231). *d* Human pancreatic carcinoma cell line (Panc-1). *e* Human malignant melanoma cell line (UACC903). A complete dose-dependent response of Comp 8 on cell viability for 48 h is plotted as Figure 2. IC$_{50}$ was calculated by non-linear regression (variable slope) analysis using GraphPad Prism.
Table 2-2. The effects of Se-ASA and S-ASA hybrid derivatives on cancer cell viability.

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<td>&gt;50</td>
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<tr>
<td></td>
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<td>17.6 ± 8.7</td>
<td>10.2 ± 5.0</td>
<td>3.0 ± 0.5</td>
<td>12.7 ± 3.0</td>
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</table>

*Listed data show the IC₅₀ values (± SD) of different agents in inhibiting cell viability determined by the MTT assay in triplicates. bHuman colon carcinoma cell line (HT29). cHuman breast adenocarcinoma cell line (MDA-MB231). dHuman pancreatic carcinoma cell line (Panc-1). eHuman malignant melanoma cell line (UACC903). IC₅₀ was calculated by non-linear regression (variable slope) analysis using GraphPad Prism.
An overview analysis of the IC$_{50}$ values obtained and summarized in Table 2 showed that 10 and 12 presented moderate potency against the four cancer cell lines evaluated. Compound 14 was effective at 48 and 72 h time points, while 15 and 16 were moderately cytotoxic only to Panc-1 cells. Compounds 8 and 13 showed a potent effect on cancer cell viability in all cancer cell lines; with Comp 8 being the most potent (Table 2-2 and Figure 2-2). Notably, in comparison to selenocoxib-1 and its glutathione conjugate (selecoxib-1-GSH), the only Se-NSAID hybrid compounds reported in the literature,$^{200, 204}$ Comp 8 possessed up to 5-fold lower IC$_{50}$ values as compared to selecoxib-1-GSH after 48 and 72 h of treatment against UACC903 cells.$^{200}$ Comp 8 also exhibited IC$_{50}$ values comparable with selenocoxib-1 in prostate cancer cells, the cytotoxic assays, however, were performed in different cell lines (DU145 for Comp 8 and PC3M for selenocoxib-1).$^{61}$

Figure 2-2. Se-ASA hybrid Comp 8 potently inhibits different cancer cells growth.

CRC (HT29), breast cancer (MDA-MB231), pancreatic cancer (Panc-1) and melanoma (UACC903) cells were treated with increasing doses of Comp 8 for 48 h. Cell viability was determined by the MTT assay. Growth curves were obtained by using nonlinear regression analysis
using GraphPad Prism software. Results were normalized to vehicle control. Calculated IC₅₀ values for 48 h are shown in Table 2-1.

Considering the potency of these Se-ASA hybrid molecules on cancer cell viability, some structure-activity relationship (SAR) can be inferred: (i) the most favorable functional group used to link the ASA scaffold with the selenium is the amide group (Comp 8); the replacement of the amide group forming an ester (Comp 10, Figure 2-1) or selenoester (Comp 14, Figure 2-1) reduced the potency of the compounds on cancer cell viability; (ii) the introduction of a carboxylic acid group to the methylene group adjacent to the amide, along with the replacement of selenocyanate by a methylseleno group yielded inactive comp 15 (Figure 2-1) in most of the cancer cell lines tested, with the exception of Panc-1 where comp 15 displayed a moderate effect on cancer cell viability; (3) the selenium atom in the hybrid ASA analogs seems to be necessary for the anti-cancer activity of the Se-ASA derivatives. This conclusion is inferred from the fact that the isosteric substitution of the selenium atom with sulfur causes a dramatic reduction in or the total loss of effect on cancer cell viability of the ASA-modified scaffold (Comp 8 versus Comp 16; Table 2-2, Figure 2-1).

On the basis of the potency of Comp 8 in reducing cancer cell viability, it was selected as the lead compound for further evaluation in six different cancer cell lines [DU 145 (human prostate carcinoma), NCI-H460 (human large-cell lung carcinoma), HEPG2 (human liver hepatocellular carcinoma), RKO and HCT116 (human colon carcinoma), and SKOV3 (human ovarian carcinoma)]. Comp 8 was effective in reducing cell viability of all of the above-mentioned cancer cell lines with IC₅₀ values in the low μM range at 48 and 72 h of treatment (Table 2-3 and Figure 2-3). The cytotoxic effect of Comp 8 was
particularly striking in all three colon cancer cell lines, HT29, RKO, and HCT116, where it showed cytotoxicity as early as at 24 h time point (Figure 2-3E-G). We further confirmed this observation by using another method known as Live and Dead assay. HCT116 cells were treated with 15 µM of Comp 8 for 24 h and were compared with 1 mM of ASA. As shown in Figure 2-3H, ASA had no significant effect on population of dead cells even at 1 mM concentration, while Comp 8 increased dead cells population to about 51%. These data were in concordance with the MTT assay. It is interesting to note that even at nearly 67 times lower dose, Comp 8 is >5 times more potent in causing HCT116 cell death than parent ASA.

Table 2-3. Anti-proliferative effects of Comp 8 on different cancer cell lines

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<td>2.9 ±0.2</td>
</tr>
<tr>
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<td>2.1 ±0.2</td>
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<tr>
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<td>2.9 ±0.5</td>
</tr>
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<td>Lung d</td>
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<td>1.3 ±0.2</td>
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<tr>
<td>Liver e</td>
<td>16.0 ±6.7</td>
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<td>4.3 ±0.4</td>
</tr>
<tr>
<td>Ovarian f</td>
<td>&gt;50</td>
<td>5.3 ±1.6</td>
<td>4.4 ±0.4</td>
</tr>
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</table>

aData represent mean IC₅₀ values (± SD) of Comp 8 in inhibiting cell viability, determined by the MTT assay in triplicates. bHuman colon carcinoma cell line (RKO). cHuman colon carcinoma cell line (HCT116). dHuman prostate adenocarcinoma cell line (DU 145). eHuman non-small cell lung cancer cell line (NCI-H460). fHuman liver hepatocellular carcinoma cell line (HEPG2). gHuman ovarian carcinoma cell line (SKOV3). Representative curves are shown in Figure 2-3. IC₅₀ was calculated by non-linear regression (variable slope) analysis using GraphPad Prism.
Figure 2-3. Comp 8 reduced cell viability of different cancer cells (A-C), including CRC cells (E-G). (A) Prostate cancer (DU 145), (B) liver cancer (HEPG2), (C) ovarian cancer (SKOV3) and (E-G) CRC cells HT29, RKO, and HCT116 were treated for 24, 48, and 72 h with different doses (0.5-50 μM) of Comp 8. Cell viability was determined using the MTT assay. Growth curves were obtained by using nonlinear regression analysis using variable slope equation by GraphPad Prism software. Results for ovarian cancer cell line at 24 h did not follow non-linear regression curve and hence a line was plotted. Results were normalized to vehicle control. IC₅₀ values are shown in Table 2-1 (for HT29 cells) and Table 2-3 (for the rest of the cell lines). (H) HCT116 cells were treated with IC₅₀ concentration of 8 (at 24 h, 15 μM) and 1mM ASA.
for 24 h. Cells were subjected to LIVE/DEAD analysis using microscopy. Calcein-AM (Green) stained cells represent live cells, while ethidium bromide (red) stained cells represent dead cells. (I) Percentage of dead cells were calculated. Data represents mean value ± SD.

Comp 8 was More Selective towards Cancer Cells in Reducing Cell Viability and Showed Greater Potency Compared to Current CRC Therapy.

The observed uniform cytotoxicity of Comp 8 to various cancer cell lines warranted its evaluation on normal cells to determine if it was selectively toxic to cancer cells. Since cytotoxicity towards healthy cells is frequently associated with unwanted side effects, it was essential to test Comp 8 against normal cells as a surrogate experiment to access its systemic toxicity in vivo. A human dermal fibroblast (nHDF) and mouse embryonic fibroblasts (MEFs) were used as a model of normal cells for the cytotoxicity study. Interestingly, nHDF and MEFs were not affected at doses of Comp 8, which are toxic towards CRC cells at 48h (Figure 2-4A), and for that matter, nor were all other cancer cells tested (see Tables 1 and 2). These data demonstrated that Comp 8 is selectively toxic to cancer cells but not transformed cells. Furthermore, we compared Comp 8 with the current therapy for CRC, 5-FU, in a cell viability assay. Comp 8 was more effective in inhibiting CRC cell viability than 5-FU or ASA (Figure 2-4B). Therefore, Comp 8 represents a truly novel Se-ASA molecule, which is more potent than the parent compound ASA and the current CRC therapy, at inhibiting cancer cell viability. In addition, Comp 8 showed high selectivity toward cancer cells suggesting that this compound may have a very high therapeutic window.
Figure 2-4. Comp 8 has more selectivity towards cancer cells and is more potent than the current CRC therapy in reducing cell viability.

(A) MEFs, nHDF and CRC cells (HCT116 and HT29) were treated with increasing concentration of 8 for 48h. Cell viability was measured by the MTT assay. Comp 8 reduced HCT116 and HT29 cell viability significantly more potently than the normal MEFs or nHDF cells (B) CRC cells (HCT116) were treated with increasing concentration of 8, ASA and 5-FU for 48 h. Compound 8 was more effective at inhibiting cell viability as compared to 5-FU and ASA. Data represents mean value ± SD; (*) p < 0.005.

Comp 8 Inhibited CRC Cell Growth by Inducing Cell Cycle Arrest.

To explore the underlying mechanism for the reduced cell viability of CRC cells by Comp 8, we investigated effect of Comp 8 on the cell cycle by flow cytometry. Treatment of HCT116 cells with Comp 8 effectively arrested cells in G1 and G2/M phase of cell cycle. The population of cells in S phase was drastically reduced compared to vehicle (DMSO) treated cells (Figure 2-5 (A and B)).
The cyclins B1, E1, and p21 play important roles in regulation of the cell cycle. Cyclin B1 is required for cells to enter the mitosis phase, while cyclin E1 is required for cell to transit from G1 to the synthesis (S phase) phase. The activation of cyclin dependent kinase inhibitor p21 has been associated with G1/S and G2/M cell cycle arrest. Hence, the effect of Comp 8 on these cell regulatory proteins was investigated. Treatment of CRC cells with Comp 8 for 24 h showed an increasing p21 expression in a dose dependent manner (Figure 2-5C). Both Cyclins B1 and E1 were also inhibited at similar doses where p21 expression was induced. These results suggest that Comp 8 up-regulates p21 expression, which leads to inhibition of Cyclin B1 and E1 expression and arresting CRCs in the G1 and G2/M phases of the cell cycle.

Figure 2-5. Comp 8 inhibited the CRC cell cycle.
HCT116 were serum starved for 72 h, followed by treatment with Comp 8 (5 μM) in media with serum as described in the Experimental Section. (A) Cell cycle phase distribution was determined at 12 and 24 h by flow-cytometry. (B) Bar diagram representing distribution of cells in different phases of cell cycle. (C) CRC cells were treated with Comp 8 at indicated doses for 24 h. Whole cell lysates were prepared and subjected to Western blot analysis. Subsequent blots were probed for cell cycle related proteins.

**Comp 8 induced Apoptosis in CRC cells**

Since CRC cells treated with Comp 8 showed a profound G1 and G2/M arrest accompanied by activation of p21, we investigated whether the inhibition in the cell cycle leads to apoptosis. HCT116 cells were treated with indicated concentrations (**Figure 2-6**) of Comp 8 for 24 h. Apoptosis was measured by using Muse Annexin V & Dead cell assay kit following manufacturer’s protocol. As shown in Figure 6A, cells treated with vehicle (DMSO) were mostly in bottom left quadrant (BLQ). As Comp 8 dose was increased, cells shifted from healthy state to apoptotic. At 2.5 μM concentration of Comp 8, >20% cells were apoptotic, and as the dose increased, cells shift from bottom right quadrant (BRQ) to upper right quadrant (URQ). More than 60% cells were detected to be apoptotic at 5 μM concentration of Comp 8, and at 10 μM >80% cells were apoptotic. Further, none of the cells were necrotic as there were no cells detected in upper left quadrant (ULQ), which were 7-ADD positive, while Annexin-V and Caspase 3/7 negative. In a similar manner, Comp 8 increased caspase 3/7 activity in a dose dependent manner (**Figure 2-6B**). **Figure 2-6C** and **6D** quantify the amount of total percent of apoptotic cells determined by both Annexin-V and caspase 3/7 assays, respectively.
We further confirmed caspase 3/7 activity by subjecting the whole cell lysates of treated HCT116 cells with compound Comp 8 to western blot analysis. As shown in Figure 2-6E, compared to the vehicle treated cells, exposure to Comp 8 significantly induced the cleavage of PARP, which is one of the main cleavage targets of caspase 3 and a marker of cells undergoing apoptosis. Overall, our results confirm that Comp 8 inhibits growth of CRC cells by inducing apoptosis.

**Figure 2-6.** Comp 8 induced apoptotic death in CRC cells.

HCT116 cells were treated with Comp 8 for 24 h. (A-B) Cells were subjected to Annexin-V and caspase 3/7 activity assay using Muse Cell analyzer. The method led to four different population of...
cells: healthy cells (Annexin-V negative, caspase 3/7 and 7-ADD negative (lower right quadrant)), early apoptotic cells (positive for Annexin-V, caspase 3/7 and negative for 7-ADD (lower Right corner)), late apoptotic/dead cells (both Annexin V, Caspase 3/7 and 7-ADD positive (upper right quadrant)) and Necrotic cells (only 7-ADD positive (upper left quadrant)). (C-D) Quantification of total apoptotic cells (early apoptosis + late apoptotic cells) determined by Annexin-V positivity or positive for caspase 3/7 activity. (E) HCT116 cells treated for 24 h with Comp 8 were subjected to Western blot analysis and probed for PARP protein. \(\beta\)-actin was used as the loading control.

**Comp 8 increased ROS Production in CRC Cells in a Time Dependent Manner.**

Studies have shown that anti-cancer effect of Se containing molecules are associated with increase in total ROS levels.\(^{224}\) To investigate whether induced cytotoxic effect of Comp 8 is related with ROS production, we measured total ROS levels in Comp 8 treated CRC cells following a previously reported method.\(^ {225}\) The CRC cells (HCT116) were treated with Comp 8 for 3, 6, and 24 h and total ROS level was measured. As shown in the **Figure 2-7**, the total ROS levels did not change significantly with the treatment of Comp 8 at early time points (3 and 6 h); however, at 24 h ROS levels increased to about 66% in Comp 8 treated cells. These results strongly suggest that Se containing Comp 8 is not able to induce ROS production at early time points. However, longer exposure of cells with Comp 8 induces ROS production which is correlated with an increase in apoptosis, suggesting that the induction of cell death by Comp 8 may be due the ROS production.
Figure 2-7. Effect of Comp 8 on ROS levels.

HCT116 cells were treated with 8 for 3, 6, and 24 h and subjected to Muse flow cytometry based oxidative stress analysis for total ROS levels measurement. The histogram shows two different populations of cells: ROS (-) (M1 peak/blue color) and ROS (+) (M2 peak/Red color). The gray peak is the overlay of DMSO M1 peak to compare the shift of the M1 peak with treatment of Comp 8. H$_2$O$_2$ was used as a positive control.

Comp 8 Inhibited Activation of NF-κB Pathway in Presence of TNF-α in PC.

NF-κB pathway, as described in chapter 1, has been shown to be responsible for cell proliferation, pro-inflammation and cancer cell survival in both PC and CRC. As reported earlier, NF-κB complex is inhibited from entering the nucleus by forming a complex with IκBα in cytoplasm. However, when cells are stimulated with TNF-α, IKK kinase gets activated and phosphorylates IκBα. Phosphorylated IκBα is targeted for ubiquitin mediated
proteasomal degradation and hence NF-κB complex can freely migrate to the nucleus and bind its target DNA sites. Here, we tested Comp 8’s ability to inhibit NF-κB pathway activation in presence of TNF-α, in PC. EMSA was used test Comp 8’s ability to inhibit NF-κB pathway. Secondly, expression levels of IκBα were also monitored using Western blot upon treatment with Comp 8 and TNF-α. As shown in Figure 2-8, Comp 8 inhibited activation of NF-κB in presence of TNF-α. Additionally, Comp 8 hindered the degradation of IκBα, further confirming our findings in EMSA results. Our results reveal that Comp 8, can inhibit NF-κB activation in presence of inflammatory stimuli. Selenium containing molecules have shown to inhibit NF-κB.\textsuperscript{226} Specially, organoselenocyanates similar to Comp 8, e.g. p-XSC, have shown to inhibit NF-κB through covalent modification of Cys(62) of the p50 subunit of the complex.\textsuperscript{227} Hence, it is very likely that selenium-containing moiety has a role to play in Comp 8’s activity. Future metabolism studies may provide more insights on the active moieties involved in inhibition of this pro-inflammatory pathway.
Figure 2-8. Comp 8 inhibits the NF-κB activation in presence of inflammatory stimuli.

(A) Panc-1 cells were treated Comp 8 at indicated concentration for 6 hr and stimulated with TNF-α (100 ng/µL) for 30 minutes. NF-κB activation was detected by EMSA in the nuclear lysates of the treated cells. (B) Cytosolic lysates of the treated cells in (A) were subjected to western blot analysis and probed with antibody against IκBα. β-actin was used as loading control. Blots in A and B are part of the same gel, the middle lanes between 0 and 1 µM were removed as they represented a different agent.
**Comp 8 Activates Intrinsic Apoptotic Pathway in PC**

We investigated the ability of Comp 8 to induce intrinsic apoptosis in PC cells after inhibition of NF-κB pathway. Panc-1 cells were treated with Comp 8 in a time- and dose-dependent manner and the expression of caspase 8 and 9 was monitored. Comp 8 induced activation of intrinsic apoptosis, as seen by presence of cleaved caspase 9 (Figure 2-9). Further, the Western blot study, showed that Comp 8 activated caspase 3, downstream of caspase 9, and induced PARP cleavage in a dose dependent manner. From these studies, we concluded that Comp 8 induced apoptosis in PC cells was via activation of intrinsic apoptotic pathway. Comp 8’s effects on CRC cells may also be in a similar manner as PC, since Comp 8 also induces Caspase 3 activation and PARP cleavage at similar doses in CRC. However, it remains to be determined.
Figure 2-9. Comp 8 induces apoptosis via activation of intrinsic apoptotic pathway

(A) Panc-1 cells were treated with 5 µM of Comp 8 at indicated time points. Whole cell lysates were subjected to Western blot analysis for detection of indicated antibodies. (B) Panc-1 cells were treated in an increasing dose with Comp 8 and a single 10 µM dose of ASA for 24 h. Whole cell lysates were analyzed using western blot for caspase 3 activation and PARP degradation. GAPDH was used as loading control in both (A) and (B).
**Conclusion**

In conclusion, the addition of Se moieties into NSAID structures led to the discovery of novel Se-ASA analogs as potential cancer therapeutics. Of the new Se-ASA derivatives, Comp 8 emerged as the most potent anti-cancer agent, particularly against CRC and PC cells. Comp 8 showed more selectivity towards cancer cells and was >10 fold potent than current CRC therapeutic agent 5-FU. Our studies revealed that Comp 8 inhibited CRC growth via cell cycle arrest leading to apoptosis. Moreover, Comp 8 induced apoptotic cell death evident by Caspase 3/7 activity and positivity of phosphatidylserine (PS) on outer membrane of CRC cells. Further, Comp 8 also increased ROS levels in correlation with apoptosis at 24 h. In PC cells Comp 8 inhibited the activation of pro-inflammatory NF-κB pathway and induced intrinsic mode of apoptosis. Overall, Comp 8 was identified as a potential selenium containing cancer therapeutic compound with a solid mechanistic basis of its activity. While developing ASA related analogs as therapeutics, it is important to consider the major adverse effect of ASA, which is gastrointestinal bleeding. However, these effects are generally associated with long term and high dosage use of ASA.\(^{128}\) Such issues with compound Comp 8 are not anticipated since (i) it is several fold more potent than ASA, and therefore, a much lower concentration of Comp 8 would be required, and (ii) it is being developed for cancer patients who currently have very low survival rates. Thus, the ASA like adverse effects, if any, related to long-term usage of Comp 8 would be a negligible problem as compared to the benefits. Future studies warrant evaluation of the *in vivo* efficacy, toxicity, and greater detailed mechanism of action of lead compound Comp 8.
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Chapter 3

A Novel Small Molecule, AS-10, Modulates the NF-κB Pathway and ROS Levels in Pancreatic Cancer Cells to Induce Apoptosis, and has a Broad Spectrum Activity Against Different Cancer Types.

Introduction

Pancreatic cancer (PC) has a high tendency for loco-regional invasion and forming metastatic tumors, making it one of the deadliest cancers, with a median survival of 6 months and an unsatisfactory 5-year survival rate of less than 5%. Pancreatic ductal adenocarcinoma comprises greater than 90% of PC. Poor prognosis and disease stage at diagnosis contributes to low survival rate. Chemotherapy remains the current standard of care for advanced PC patients, which include gemcitabine alone or in combination with erlotinib or a highly toxic regimen FOLFIRINOX (oxaliplatin, irinotecan, fluorouracil, and leucovorin). The current standard of care only improves survival rate on average by 6 months, but is associated with poor quality of life due to high toxicity. Molecular and cellular heterogeneity of PC tumors contribute to resistance to current standard of chemotherapy. Therefore, there is an urgent need to develop novel approaches to battle metastatic PC.
Many signaling pathways have been suggested to be associated with chemo-resistance, including NF-κB, PI3k-Akt and NOTCH pathways in cancer. However, chemo-resistance in PC has mainly been associated with aberrant activation of the NF-κB pathway. Further, the NF-κB pathway has been known to be induced in presence of chemotherapeutic drugs as well as radiation in PC cells, hence making this pathway one of the hot targets for combinational treatments with current drugs in the clinic. The mammalian transcription factor NF-κB is formed by homo- or hetero-protein dimer complex of Rel-family proteins. The most abundant form of NF-κB exists as a heterodimer consisting of RelA (also known as p65) and p50 complex. The RelA and p50 NF-κB complex is inhibited in the cytoplasm from entering the nucleus by inhibitor of κB proteins (IκBα). In classical activation of NF-κB pathway, upon inflammatory stimuli (like TNF-α), downstream IκB kinase complex (IKK), which is made up of two catalytic kinases (IKKα and IKKβ) and a regulatory component IKKγ, is activated via phosphorylation. Upon activation of IKK, it phosphorylates IκBα. Phosphorylated IκBα is polyubiquitinated and degraded, hence RelA and p50 complex can freely localize to the nucleus and interact with their corresponding gene targets. Upon activation, NF-κB upregulates the transcription of anti-apoptotic proteins (like Bcl-2, Bcl-xL, Mcl-1, etc), proliferative proteins (cyclin D), proteins involved in cell invasion (VEGF, MMPS, etc.) and pro-inflammatory cytokines (TNF-α, and different interleukins).

Over the past decade, NSAIDs have emerged as potent chemopreventive agents. Out of all the NSAIDs, aspirin (ASA) has received more attention for its chemopreventive ability in different cancers, specifically colorectal cancer (CRC).
Further, there have been different clinical trials showing ASA to be clinically effective in reducing PC growth. Recent clinical studies showed that high ASA decreased PC incidence, however high doses or long term usage of ASA are also related to gastrointestinal (GI) toxicity. Hence, efforts have been made by different research groups to optimize ASA structure for improving its potency and ultimately reducing its toxicities.

Interest in designing selenium containing small molecules intensified after the link between selenium and cancer prevention strengthened based on preclinical, epidemiological and clinical investigations. In the recent literature, several new selenium compounds have been developed including several from our laboratories and many of them have shown promising cancer preventive and/or therapeutic activity. The mechanisms by which selenium containing molecules inhibit cancer growth differ according to the structure of the overall compound. The most commonly described mechanisms by which selenium compounds exerts their anti-cancer ability are: induction of reactive oxygen species (ROS) or quenching of ROS; inhibition of different pro-survival proteins (like Bcl-xL and Survivin); induction of apoptosis; inhibition of angiogenesis; modulation of AKT, COX, p38 and NF-κB-signaling pathways. Further, selenium compounds have shown to have protective effects against gastric toxicity induced by indomethacin, making another potential reason of combining NSAIDs and selenium. Because selenium molecule possesses anti-cancer properties and NSAIDs have been effectively used in clinic, we combined and synthesized these two moieties to make a novel agent organoselenium agents. In continuation of our pursuits in developing
organoselenium agents, we have now identified a novel compound, AS-10 (Figure 3-1), through extensive structure-activity relationship (SAR) studies based on incorporation of appropriate selenium moieties into the NSAIDs structures. The synthesis of AS-10 followed a unique chemistry (unpublished data) resulting in a cyclic selenazolidine ring substituted by two acetyl salicylate groups similar to aspirin. Herein, we evaluated the effect of AS-10 to inhibit the viability of and induce apoptosis in PC cells, alone and in combination with clinically used first-line therapy, gemcitabine, and began establishing the underlying mechanism of therapeutic action.

AS-10 was further subjected to National Cancer Institute’s-60 (NCI-60) cell line screening. In NCI-60 cell line screening, AS-10 was most effective in renal cancer cells, while most cytostatic in leukemia cells. Additionally, removal of acetyl groups from AS-10’s structure or replacing selenium with sulfur ameliorated its effects on cancer cell viability, implying the importance of those groups to be involved in its growth inhibitory activity in cancer cells.

**Materials and Methods**

**Reagents.**

AS-10, ASD-171 and ASD-123 compounds were synthesized in laboratory. AS-10 stock was dissolved in DMSO at 10 mM concentration and store at –20 °C. Antibodies were ordered from following companies: Cell signaling (Acetyl H3 (lys9) (9649), Acetyl H4 (Lys8) (2594), cyclin B1 (4138s), caspase 3 (9662s), caspase 9 (9508s), Caspase 8 (9746), IκBα (9242s), Bcl-xL (2762s), Mcl-1 (5453s) and PARP (9542s)), Abcam (PCNA...
(ab18197), p100/p50 (ab31412), GAPDH (5174p) and p65 (ab7970)), Santa Cruz Biotechnology (p21 (sc-397) and p27 (sc-528)), Jackson Immuno Research (Alexa fluor 680 (711-625-152) and Donkey serum (017-000-002)) and Sigma-Aldrich (β-actin (a-5441) and Tubulin-β (c-4585)). Rabbit IgG-chip grade protein was ordered from abcam (ab37415), Propidium Iodide (P4170-10MG), Ribonuclease A (RNAse A, R6513-10MG) and Crystal Violet (C6158-50G) from Sigma.

Cell culture

Panc-1, BxPC3, MEFs, 1205Lu, MDA-MB-231, cell lines were cultured and maintained in DMEM medium; HT29, HCT116, RKO, and SKOV3 cell lines were maintained in McCoy’s 5A medium, H460, UACC903, and A549 cell lines were maintained in RPMI 1640; HEPG2 cell lines were maintained in Eagle’s minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL of penicillin and streptomycin at 37 °C in 5% CO₂ chamber. All cell lines were obtained from American Type Culture Collection (ATCC).

Cell viability assay.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay was performed to determine the effect of AS-10 on cancer cell viability, as described earlier. Briefly, 3 x 10³ cells were treated with different concentrations of AS-10 in triplicate in a 96-well plate for different time points. MTT solution (20 µL of 5.0 mg/mL solution) was added to each well, three hours prior to termination time point and incubated at 37 °C. Resultant formazan crystals, at termination time point, were dissolved in 50 µL DMSO. The plate was subjected to a 96-well multiscanner (Dynex Technologies, MRX Revelation;
Chantilly, VA, USA) for measuring the optical densities at 570 nm and 630 nm. IC$_{50}$ values were determined by performing non-linear regression using Graphpad Prism software.

Colony formation assay.

Crystal violet staining method was utilized for performing colony formation assay.\(^{245}\) In brief, Panc-1 cells were treated with vehicle control (DMSO), 2.5 μM or 10 μM of AS-10 for 48 h. Subsequently, cells were trypsinized and live cells were counted using tryphan blue dye exclusion assay. Tissue culture plates of size 60 x 15 mm were seeded with 100 live cells from vehicle treated and 5000 live cells from AS-10 treated cells. Cells were allowed to form colonies for 17 days in DMEM free from DMSO or AS-10 at 37 ºC in a 5% CO$_2$ chamber. After 17 days colonies were stained with 0.5% alcoholic crystal violet. Percentage Plating efficiency (PE) was calculated accordingly: % PE=number of colonies*100/number of cells plated. Data plotted as % relative PE.

Cell cycle analysis.

Flow cytometry was used to determine the cell cycle phases according to a previously described protocol.\(^{205}\) In brief, Panc-1 or HT29 cells were serum starved for 72 h, followed by treatment with different doses of AS-10 in 10% serum containing media for different time points. Treated cells were fixed in 70% cold ethanol at the end of each time point. Cells were fixed for a minimum of 4 h at 4 ºC. Following fixation, cells were washed with 1X PBS and were re-suspended in Propidium iodide (PI) staining solution (0.1% (v/v) triton X-100, 20 mg DNAse-free RNAse A and 2 mg of PI in 100 mL PBS). Cells were incubated in the staining solution for 15 minutes at room temperature and then analyzed by
a BD FACS Calibur (BD Biosciences) for total DNA content. Modfit software (BD Biosciences) was used to obtain the histograms.

**Western blot analysis.**

Whole-cell lysates were made by subjecting AS-10 treated Panc-1 cells to RIPA lysis buffer (Thermo Scientific, USA) containing protease (Roche, USA) and phosphatase inhibitor cocktails (Sigma-Aldrich, USA) as described before. Lysates were spun at 15,000 rpm for 10 minutes. Resulting supernatant was stored at -80 °C until use. NuPAGE gel 4-12% (Life Technologies, Carlsbad, CA) was used to resolve the lysates, followed by electro-transfer to PVDF membrane. After the transfer membrane was blocked with 5% non-fat milk and probed with different antibodies. Protein of interest were detected by using enhanced Chemiluminescent reagent (Life technologies, USA). Blots were stripped using Restore Western blot stripping buffer (Thermo Scientific, USA) to save time and samples.

**Annexin V assay.**

Muse Annexin V & Dead Cell kit was used to determine the apoptosis cells in AS-10 treated Panc-1 cells as previously described. The Muse Annexin V & Dead cell assay contains Annexin V to detect phosphatidylserine (PS) on the outer surface of cells. The kit also contains a dead cell marker, 7-amino-actinomycin D (7-AAD). 7-AAD molecule does not enter live or early apoptotic cells which have not lost their cell membrane integrity (Millipore, MCH100105). In brief, Panc-1 (1.5x10^5) cells were treated with a given dose of AS-10 or DMSO for appropriate time (0, 6, 12, 24, 36, or 48 h). Both floating cells as well as adherent cells were collected and subjected for Annexin V staining using Muse
Annexin V & Dead Cell kit with Muse cell analyzer (EMD Millipore, Billerica, MA, USA). The obtained data were analyzed using Muse 1.4 software.

*Caspase 3/7 activity assay.*

To evaluate the efficacy of AS-10 in inducing caspase 3, Panc-1 cells were treated with AS-10 at different concentrations for different time points. Caspase 3/7 activity was measured using Muse Caspase-3/7 Assay kit with Muse cell analyzer (EMD Millipore, Billerica, MA. USA) according to manufacturer’s protocol and as previously described.\textsuperscript{205} In brief, the Muse Caspase 3/7 kit contains a reagent namely NucView for detection of activated caspase 3/7. The reagent is cell membrane permeable and contains a DNA binding dye that is linked to a DEVD peptide substrate. In presence of activated caspase 3/7, DEVD is cleaved and DNA binding dye is released. Therefore, cells with active caspase 3/7 activity will yield high fluorescence. (Ref Millipore, Catlog No. MCH100108). The kit also contains a dead cell marker, 7-AAD, for detection of cells with compromised cell membrane. Data from Muse cell analyzer were analyzed using Muse 1.4 software.

*Electrophoretic mobility shift assay (EMSA).*

To determine NF-κB activation, Panc-1 (1 x 10\textsuperscript{6}) cells were treated with different doses of AS-10 for 6 h. Thirty minutes before termination cells were stimulated with 100 ng/mL TNF-α (which is about 3.9 nM in media after addition). At the end of 30 min, cells were immediately stored on ice. Nuclear and cytosolic extracts were prepared as described previously.\textsuperscript{218} Nuclear lysates were subjected to EMSA following the protocol.\textsuperscript{218} The dried gels were exposed to X-ray films at -80 °C for exposure.
**Immunocytochemistry for NF-κB p65 localization.**

Immunocytochemistry was used to determine the translocation of p65 from cytoplasm to nucleus in presence of inflammatory stimuli either with vehicle or AS-10 (10 μM). In a 12 well plate, Panc-1 cells (1.6 x 10^5) were seeded on top of a small cover slip in DMEM medium. Cells were allowed to attach to the cover slip overnight at 37 ºC incubator with 5% CO₂. Next day, cells were treated with 10 μM AS-10 for 6 h and further stimulated with TNF-α (100 ng/mL, 3.9 nM) for 30 min. At the end of 30 min plates were kept on ice and cells were fixed with 4% Formaldehyde Solution made in PBS for 15 min. Cells were washes three times with 1 x PBS, followed by 1 h incubation in blocking medium (0.3% Triton X-100, 5% donkey serum, made in 1 x PBS) at room temperature in dark. After blocking coverslips were incubated with primary antibody solution (primary antibody diluted 1:500 in 1% BSA solution containing 0.3% Triton X-100) over night at 4 ºC. Next day, primary antibody was removed and cover slips were washed three times with 1 x PBS. Following the wash with PBS, the cover slips were incubated with 0.03% H₂O₂ for 15 min at room temperature in dark. After the incubation, secondary antibody (Alexa fluor 680) was added at dilution of 1:500 in 1% BSA containing 0.3% triton X-100 and further incubated for 1 hr. After incubation, coverslips were washed and mounted on glass slides with mounting medium containing DAPI (17985-50, Electron Microscopy Sciences). Slides were allowed to dry for 2 days and pictures were taken using the DELTA VISION microscope at 60X. Imaris 8.2 software was used for image processing.
Live/Dead assay.

To determine the dead cell population, we used LIVE/DEAD Viability/Cytotoxicity Kit (L3224, Life technologies, USA). Panc-1 cells were treated with either DMSO, gemcitabine (50 µM), or AS-10 (2.5 µM) or in combination for 48 h. Live and dead cells were determined by subjecting treated cells to LIVE/DEAD Viability/Cytotoxicity Kit as previously described.\(^{205}\) Calcein-AM can freely travel through the plasma membrane of cells and fluoresce green in presence of intracellular esterase activity, hence green fluorescence indicates live cells. While ethidum homodimer-1 can only enter cells who have lost their plasma membrane integrity or dead cells and fluoresce red. Stained cells were visualized using a fluorescence microscope (Zeiss - Axio Scope.A1) with a 20× objective lens. Percentage values of live and dead cells were calculated.

ROS measurement.

Measurement of total ROS levels in AS-10 treated Panc-1 cells was performed by using Muse Oxidative Stress Kit (EMD Millipore, Billerica, MA, MCH10011) as per the manufacturer’s protocol. ROS inside the cells are detected by Muse Oxidative Stress Reagent provided in the kit. Two different cell populations of ROS (-) and ROS (+) are identified using the kit. The ROS (−) population is characterized by the M1 peak, while the ROS (+) cells is characterized by M2 peak in the graph. Panc-1 cells treated with H\(_2\)O\(_2\) (0.9 M) were used as the positive control for the classification of M1 and M2 peaks. Percentages of ROS (−) and ROS (+) cells were measured using Muse cell analyzer.
NCI 60 cell line screening.

The effects of on cancer cell growth was tested by NCI which screens 60 different cancer cell lines of nine different cancer types like colon, CNS, lung, prostate, renal, breast, melanoma, ovarian, and leukemia. Detailed procedure regarding the NCI screening is available through their webpage http://dtp.nci.nih.gov/branches/btb/ivclsp.html. In brief, cells were seeded in 96 well plate and allowed to settle overnight at 37 °C in 5% CO₂ chamber. The next day, one set of 96 well plates from all cell lines were fixed with tricholoroacetic acid and labelled as time 0h (T₀). Zero hour plates were subjected to sulphorhodamine B (SRB) assay to quantify the cells. Other set of plates were either treated with single dose of 10 µM AS-10 or range of five doses (0.01, 0.1, 1, 10 and 100 µM) for 48 h (Tᵢ). At the end of 48 h the cells were fixed in a similar manner at T₀ and SRB assay was performed. Percentage growth inhibition was calculated by using the measurements obtained via SRB assay using the formula:

\[
% \text{Growth} = \left( \frac{T_i - T_z}{C - T_z} \right) \times 100
\]

for concentrations in which \(T_i \geq T_z\)

\[
% \text{Growth} = \left( \frac{T_i - T_z}{T_z} \right) \times 100
\]

for concentrations in which \(T_i < T_z\)

Cell growth is indicated by values which are between 0% and 100%, 0% indicates no growth, and values < 0% indicate cell death.
Results

AS-10 Inhibited PC Cell Growth.

The cytotoxic response of AS-10 on PC cell lines was evaluated using MTT cell viability assay. The PC cell lines, Panc-1 and BxPC3, were treated with AS-10 at different doses (0-50 µM) for 24, 48, and 72 h time points. As shown in Figure 3-1(B and C), AS-10 effectively inhibited the cell viability of both cell lines, with an IC50 of 0.7±0.1 µM for Panc-1 and 2.1±0.3 µM for BxPC3 cells at 48 h (Table 3-1). Panc-1 cell line was more sensitive to AS-10 and hence further studies were performed on this cell line.

To evaluate the long term growth inhibitory activity of AS-10 in Panc-1 cells, we performed colony formation assay. Panc-1 cells were treated with different doses of AS-10 for 48 h. After 48 h, live cells were counted and plated in a new petri dish. Cells were allowed to form colonies for 17 days. After 17 days, the colonies were stained, counted, and colony forming abilities were calculated in reference to untreated control.245 As shown in Figure 3-1D, AS-10 decreased the amount of colonies formed, compared to vehicle treated cells, indicating that the live cells remaining after 48 h of treatment had lost their proliferative ability compared to untreated cells. Overall, our results suggest that AS-10 potently inhibits the growth and proliferation of PC cells in vitro.
Figure 3-1. AS-10 inhibited PC cell growth.

(A) AS-10 structure. (B-C) Panc-1 and BxPC3 cells were treated with AS-10 for 24, 48 and 72 h in a dose dependent manner. Treated cells were subjected to cell viability MTT assay. Growth curves obtained by performing non-linear regression using variable slope equation in GraphPad prism software. Data represented as mean ± SD. (D) Panc-1 cells were treated with AS-10 for 48 h. After 48 h live cells were counted and plated in new petri dish and were allowed to form colonies for 17 days. After 17 days, colonies were stained with crystal violet and counted. Data represents mean ± SD; * (p<0.0001).
**AS-10 was more Efficacious than Current Standard of Care and was Selectively Active Against PC Cells.**

To test whether AS-10 is more efficacious than gemcitabine, which is the first line therapy for PC, we compared the % cell viability in presence of gemcitabine to AS-10 treatment utilizing MTT assay. Cells were exposed to AS-10 or gemcitabine at different doses for 48 h and resulting cells were subjected to MTT assay. As shown in **Figure 3-2A**, Panc-1 cells were resistant to gemcitabine treatment even at the highest tested dose of 500 μM for 48 h. In contrast, AS-10 was potent with an IC\textsubscript{50} in the range of 1-2.5 μM at 48 h. Hence, AS-10 proved to be >500 times more potent than gemcitabine.

To determine whether AS-10 is selectively toxic to cancer cells, we treated mouse embryonic fibroblast cells (MEFs) with AS-10 for 48 h and MTT assay was performed. The cell viability data is plotted against Panc-1 cells for comparison (Panc-1 cells were treated under similar experimental conditions) (**Figure 3-2B**). The dose that caused 50% growth inhibition in PC cells, had no effect on MEFs growth. Therefore, AS-10 possesses a large therapeutic window *in vitro*. Taken together, our results strongly suggest that AS-10 is significantly more effective than the current first line therapy while being safe at effective doses.
Figure 3-2. AS-10 is more potent than Gemcitabine and selective towards cancer cells.

(A) Panc-1 cells were treated with increasing dose of AS-10 or Gemcitabine for 48 h. Cells were subjected to cell viability MTT assay at end of 48 h. (B) MEFs and Panc-1 cells both were treated with AS-10 for 48 h in a dose dependent manner and subjected to MTT assay. Curves in both (A) and (B) were obtained by performing non-linear regression with variable slope equation using GraphPad Prism software. Curve for Gemcitabine in (A) did not converge and hence a line was obtained. Data represents mean ± SD.
**AS-10 Induced G1/G0 and G2/M Cell Cycle Arrest in PC Cells.**

The data obtained thus far indicated that AS-10 inhibited colony formation ability and induced cell growth arrest. Hence, we investigated the effect of AS-10 on the cell cycle of PC cells. Panc-1 cells were serum starved for 72 h and then exposed to AS-10 at different doses and time points. At the end of each time point, Panc-1 cells were fixed in cold ethanol and stained with propidium iodide (PI). Stained cells were subjected to cell cycle analysis using flow cytometry.\(^{205}\) As shown in Figure 3-3A and 3-3B, Panc-1 cells treated with AS-10 were not able to transit from G1 to synthesis phase (S phase), and from G2/M to G1 phase. This data suggests that AS-10 is inducing cell cycle arrest in both G1/G0 phase and G2/M phase, and a complete inhibition of S phase. Further, we investigated different markers associated with cell cycle arrest (p21 and p27) using Western blot to confirm our findings. The expression of both the markers, p21 and p27, increased in a time-dependent manner starting as early as 6 h for p21 and 12 h for p27 (Figure 3-3C). Moreover, we also investigated the cyclin levels associated with M phase and S phase. As indicated by Figure 3-3C, there was an increase in cyclin B1, a marker for G2 phase to M phase, indicating cells arrested in G2 phase of the cell cycle. The expression of PCNA has been shown to increase when cells are going from G1 into S phase of the cell cycle,\(^{246}\) however in our studies expression of PCNA did not change further confirming the absence of S phase when cells are treated with AS-10. Overall, these results show that AS-10 inhibits PC cell growth by arresting cells in G1/G0 and G2/M phase.
Figure 3-3. AS-10 induced G1/G0 and G2/M cells cycle arrest.

Panc-1 cells were serum starved for 72 h and then treated with AS-10 for given time points. At end of each time point cells were fixed and stained with PI. Stained cells were subjected to cell cycle analysis using flow cytometry. (A) Histogram shows three cell population: G1 (first peak), S (second flat peak) and G2 (last peak on the left) (B) Quantification of different cell cycle phases at different time points with their DMSO control respectively. (C) Panc-1 cells were treated with 10 µM AS-10 for given time points. Whole cell lysate was subjected to Western blot analysis. Blots were probed for given proteins. β-actin was used as loading control.
**AS-10 Induced Apoptotic Cell Death in PC Cells.**

To confirm AS-10 induced cell growth inhibition results in apoptotic cell death, Panc-1 cells were treated with a dose of 5 µM for indicated time points (Figure 3-4A and 3-4B). Apoptosis was detected by using Muse caspase 3/7 activity assay and Muse Live/Dead Annexin V assay. Caspase 3/7 activity assay kit contains a reagent which is cell permeable and in presence of active caspase 3/7 gets cleaved to a fluorescence molecule that can be detected via Muse cell analyzer. One of the hallmarks of apoptosis is the presence of phosphatidylserine (PS) on the outer surface of the plasma membrane. Muse Live/dead kit contains Annexin V dye, which binds to PS on the outer surface of the plasma membrane and detects apoptotic cells. Both assays have a common reagent, 7-ADD, which stains for cells that have lost the integrity of their plasma membrane. Hence, the population of cells which are negative for all three markers (Caspase 3/7 (-), Annexin V (-) and 7-ADD (-)) are healthy cells and will show up in the bottom left quadrant of the graph. Cell population which are positive for caspase 3/7 activity or Annexin V and negative for 7-ADD, are considered as early apoptotic cells, showed up in the bottom right quadrant of the graph. Cell population positive for caspase 3/7, Annexin and 7-ADD will show up in the top right quadrant of the graph, which represents cells that died of apoptosis. Cell population which are just positive for 7-ADD and negative for either caspase 3/7 or Annexin V are considered necrotic cells (shown in top left quadrant). As shown in the Figure 3-4A and 3-4B, upon increasing the exposure time of AS-10 to PC cells, the cell population in the later stages of apoptosis (Bottom and top right quadrant of the graph) increased compared to vehicle
treated cells. This data indicates that upon treatment with AS-10, PC cells die in an apoptotic manner, as detected by the presence of caspase 3/7 activity and Annexin V binding on the outer surface of the plasma membrane. Further, AS-10 did not induce necrosis, as there were no cells detected in the top left quadrant (cells only positive for 7-ADD). Quantification of total apoptotic cells is shown in Figure 3-4C and 3-4D, obtained from Caspase 3/7 activity assay and Annexin V assay, respectively.

We confirmed our findings by investigating the levels of activation of caspase (cleaved caspase 3) and degradation poly ADP ribose polymerase (PARP) (target of caspase 3). As shown in Figure 3-4F, increasing the dose of AS-10, increased caspase 3 activation and PARP degradation. These results are in accordance with our finding from Muse caspase 3/7 activity assay and Muse Live/dead Annexin V assay. Further, we also investigated whether the apoptosis induction in presence of AS-10 was mediated through intrinsic or extrinsic apoptotic pathway. Cleavage/activation of caspase 9 is a hallmark for intrinsic apoptosis, while cleavage/activation of caspase 8 is the hallmark for extrinsic apoptosis. As shown in Figure 3-4E, we observed a profound activation of caspase 9 (cleaved caspase 9), but no activation of caspase 8 (cleaved caspase 8) in presence of AS-10. Thus, our finding suggests that AS-10 induces cell death through the induction of intrinsic apoptotic pathways.
Figure 3-4. AS-10 induced intrinsic apoptosis pathway in PC cells.

(A-B) PC cells (Panc-1) were treated with AS-10 for given time points. At the end of last time point, cells were subjected to apoptosis detection using Muse caspase 3/7 activity assay and Muse Live/dead Annexin V assay. Bottom left quadrant represents healthy cells; bottom right quadrant represents early apoptotic cells (Caspase 3/7 (+), Annexin V (+) and 7-AAD (+)); top right quadrant represents late apoptotic/dead cells (Caspase 3/7 (+), Annexin V (+) and 7-AAD (+)); Top left
quadrant represents cells which have died of necrosis (Caspase 3/7 (-), Annexin V (-) and 7-ADD (+). (C) and (D) represents the total apoptotic cells (early + late apoptotic cells) measured by caspase 3/7 activity assay and Live/dead annexin V assay respectively. Error bars represents mean ± SD (E) Panc-1 cells were treated with AS-10, 5 µM, for given time points. Whole cell lysate was subjected to Western blot analysis. Resulting blots were probed for caspase 9. GAPDH was used as loading control. (F) Panc-1 cells were treated with different concentrations of AS-10 for 24 h. Whole cell lysates were made and subjected to Western blot analysis. Blots were probed with caspase 3 and PARP antibodies. GAPDH was used as loading control.

**AS-10 Inhibited Activation of NF-κB in Presence of Pro-Inflammatory Stimuli.**

Since the structure of AS-10 contains selenium and partial fragment of ASA, both of which are well-known for their anti-inflammatory properties and targeting NF-κB pathway, we sought to determine if AS-10 would modulate this pro-inflammatory pathway more effectively. In order to test the ability of AS-10 to inhibit NF-κB pathway in presence of an inflammatory stimuli (TNF-α), electro mobility shift assay (EMSA) was performed. Panc-1 cells were pre-treated with AS-10 for 6 h and then stimulated with TNF-α for 30 min prior to termination. After incubation, PC cells were subjected for nuclear and cytosolic lysis. Nuclear lysates were subjected to electro mobility shift assay (EMSA) using p32 labelled NF-κB binding oligonucleotides, while cytosolic lysates were subjected to Western blot studies, for detection of IκBα. As shown in **Figure 3-5A**, treatment of AS-10 inhibited NF-κB DNA binding induced by TNF-α, in a dose dependent manner. Further,
AS-10 treatment inhibited the degradation of IκBα in presence of TNF-α stimulation (Figure 3-5B).

To confirm that the restoration of IκBα in AS-10 treated cells inhibited NF-κB translocation from cytoplasm to nucleus, we performed an immunocytochemistry assay. Panc-1 cells were treated for 6 h with AS-10 and stimulated with TNF-α for 30 min before termination. Cells were fixed and probed for p65 protein (sub unit of NF-κB complex). As shown in Figure 3-5D, when vehicle treated cells were stimulated with TNF-α, most of the p65 translocated to the nucleus. However, in presence of AS-10 and TNF-α, p65 was unable to translocate to the nucleus. Overall, based on the three above described experiments, it can be concluded that AS-10 inhibits the activation of NF-κB pathway in Panc-1 cells stimulated with TNF-α. To confirm that these effects were not due to the cell death, Panc-1 cells were treated with 10 µM AS-10 for 6 h and subjected to live and dead analysis using calcein-AM and ethidium bromide. We did not observe any increase in the dead cell population at 6 h time point in presence of AS-10 (Figure 3-5E), indicating that the effect seen on NF-κB was independent of cell death. AS-10 also inhibited NF-κB mediated anti-apoptotic proteins like Bcl-xL and Mcl-1 (Figure 3-5F)
Figure 3-5. AS-10 inhibited the translocation of NF-κB to nucleus in presence of inflammatory stimuli.
(A) Panc-1 cells were treated with AS-10 at indicated concentration for 6 hr and stimulated with TNF-α (100 ng/µL, 3.9 nM in total cell culture media) for 30 minutes. Nuclear lysates were assayed for NF-κB activation by EMSA. (B) Cytosolic lysates of Panc-1 cells treated in (A), were subjected to western blot and probed with antibody against IκBα. β-actin was used as loading control. (C) NF-κB complex has p50 and p65 subunits. Nuclear lysates from TNF-α (100 ng/mL) treated cells and untreated cells were incubated with the indicated antibodies, or mutant (MT) unlabeled oligos or unlabeled/cold (WT) NF-κB oligos. They were than subjected to EMSA to confirm that the binding seen is NF-κB specific (D) Panc-1 cells were seeded on glass coverslip and treated with AS-10 for 6 hr and stimulated with TNF-α (100 ng/mL) for 30 minutes. Resultant cells were fixed and probed with antibody against p-65 protein (green). DAPI (blue) was used to stain the nucleus. (E) Panc-1 cells were treated with 10 µM AS-10 for 6 hr and subjected to live and dead analysis using calcein-AM and ethidium bromide. Green indicates live cells and red indicates dead cells. (F) Panc-1 cells were treated with AS-10, 5 µM, for given time points. Whole cell lysate was subjected to Western blot analysis. Resulting blots were probed for MCl-1 and Bcl-xL. GAPDH was used as loading control.

**AS-10 Potentiated the Anti-Cancer Effects of Gemcitabine.**

Since AS-10 inhibited NF-κB pathway, and it is known that activation of NF-κB pathway increases resistance of PC cells towards gemcitabine, we sought to investigate the effect of combining AS-10 with gemcitabine on PC cells. We hypothesize that in presence of AS-10, NF-κB pathway will be inhibited thus minimizing gemcitabine resistance. PC cells (Panc-1) were treated with low dose AS-10 (2.5 µM) or gemcitabine (50 µM) for 48 h. After treatment cells were subjected to Live and dead assay using Muse Annexin V assay
(Figure 3-6A) as well as microscopy live/dead assay using calcein-AM and ethidium bromide (Figure 3-6B). Using the Muse Live and dead Annexin V assay (Figure 3-6A and 3-6C), we observed about ~25% apoptotic cells with either gemcitabine or AS-10 alone, while in combination total apoptotic cells rose to 75%. Further, in live and dead assay, about 10% dead cells were detected in cells treated gemcitabine or AS-10 alone, however in combination treatment, more than 75% dead cells were seen (Figure 3-6B and 3-6D). As shown in Figure 3-6A, we did not observe significant cell population in top left quadrant (necrotic cell quadrant), indicating that the cell death observed is due to apoptosis. Hence, AS-10 does indeed potentiate the effects of gemcitabine synergistically, when treated together. Results were further confirmed by subjecting the whole cell lysates of the treated cells to Western blot analysis. As shown in Figure 3-6E, low dose AS-10 or low dose gemcitabine, had no effect on PARP cleavage, however, combination treatment induced PARP cleavage (Figure 3-6E) further confirming our findings shown in Figure 3-6A-D.
Figure 3-6. AS-10 potentiated the anti-cancer effects of gemcitabine towards PC cells.

Panc-1 cells were treated with AS-10 and Gemcitabine (Gem) for 48 h. After 48 h cells were subjected to Muse Live/dead Annexin V assay, Live/dead calcein-AM & ethidium bromide staining and Western blot analysis. (A) Histograms show four quadrants, bottom left quadrant (Healthy cells (7-ADD (-), Annexin V (-))); bottom right quadrant (Early apoptotic (7-ADD (-), Annexin V (+))); top right quadrant (Late apoptotic/dead cells (7-ADD (+), Annexin V (+))); top left quadrant (necrotic (7-ADD (+), Annexin V (-))). (B) Microscopic images of calcein-AM stained live cells (green color) and ethidium bromide stained (red color). (C-D) Quantification of total apoptotic cells
and total dead cells obtained from (A) and (B), respectively. Data represents mean ± SD; * (p < 0.05); ** (p < 0.08); *** (p < 0.005); **** (p < 0.0001). (E) Whole cell lysates of treated Panc-1 cells were subjected to Western blot analysis. Resulting blot was probed for PARP. Tubulin was used as a loading control.

**ROS Quencher, NAC, Antagonized AS-10’s Anti-Apoptotic Effects on PC Cells.**

Selenium containing compounds are known to increase ROS levels in cancer cells and it has been hypothesized that the effect on cell viability may be due to the compound’s ability to generate ROS.\(^{197,224}\) We measured the levels of ROS in AS-10 treated PC cells both at early time points of 3 h and 6 h and a late time point of 24 h. As shown in Figure 3-7A, AS-10 induced ROS levels by ~23% at all-time points compared to vehicle treated cells. Whether these increases in ROS levels by AS-10 were significant to induce any effects on cell death was further investigated, with a ROS quencher, N-acetyl cysteine (NAC), treatment. PC cells were incubated with AS-10, NAC or both for 48 h. After 48 h cells were subjected to MTT assay, Muse Annexin-V staining assay and Muse caspase 3/7 activity assay. As shown in the Figure 3-7B, MTT results showed that NAC antagonized AS-10’s effects and hence, the cell viability was similar to that of control in NAC and AS-10 treated cells. Further, PC cells morphology resembled more to apoptotic cells when treated alone with AS-10, while when treated in combination (AS-10 + NAC) PC cell morphology resembled to vehicle treated cells. To further confirm our finding, we performed Muse Annexin-V and Muse Caspase 3/7 activity assay as they have been known to be more sensitive than MTT assay. Figure 3-7D and 3-7E, clearly show that upon
addition of NAC, the effect of AS-10 was reduced, indicating that ROS induction may have preliminary role in AS-10 induced cytotoxicity. Therefore, AS-10 has dual function of killing cell death (i) By reducing the pro-survival NF-κB pathway, and (ii) increasing ROS over time to further activate the apoptotic pathway in PC cells.

**Figure 3-7.** AS-10’s anti-apoptotic effects on PC cells were antagonized by ROS quencher, NAC.
Panc-1 cells were treated with DMSO, H$_2$O$_2$ or AS-10 for 3 and 6 h time points and subjected to Muse oxidative stress assay. M1 peak represents reactive oxygen species (ROS) negative, while M2 represents ROS positive cells (A). Panc-1 cells were treated with DMSO, AS-10. NAC or in combination for 48 h. After 48 h cells were subjected to (B) MTT assay (C) Light microscopy (D) Muse Caspase 3/7 activity assay (E) Muse Live/dead Annexin V assay. (D-E) Histograms show four quadrants, bottom left quadrant (Healthy cells (7-ADD (-), Annexin V (-) or Caspase 3/7 (-))); bottom right quadrant (Early apoptotic (7-ADD (-), Annexin V (+) or Caspase 3/7 (+))); top right quadrant (Late apoptotic/dead cells (7-ADD (+), Annexin V (+), Caspase 3/7 (+))); top left quadrant (necrotic (7-ADD (+), Annexin V (-), Caspase 3/7 (-))).

**AS-10 Inhibits Cell Cycle in CRC Cells, and Induces Apoptosis.**

In order to understand that similar MOA for AS-10 exists in different cancer types, we tested AS-10’s ability to inhibit CRC cell growth. As shown in Figure 3-8A, AS-10 potently inhibited the cell viability of three different CRC cell lines. Since, AS-10 inhibited PC cell cycle in S phase, we investigated AS-10’s effect on CRC cells (HT29). AS-10 reduced the population of cells S phase cells in the CRC cell line (Figure 3-8B and 3C). Further, CRC cells were treated with AS-10 in a dose dependent manner and markers associated with cell cycle arrest, p21, and apoptosis were observed. AS-10 induced p21 expression in CRC cells and at similar dose PARP cleavage was also observed Figure 3-8D.
Figure 3-8. AS-10 inhibits CRC cell viability via cell cycle arrest, followed by apoptosis.  

(A) MTT assay was performed on CRC cell lines (HT29, HCT116 and RKO) treated with increasing dose of AS-10 for 24, 48 and 72 h. Curves were obtained by performing non-linear regression analysis using variable slopes. Data represents Mean ± SD. (B) HT29 cells were serum starved for 72 h, followed by addition of media with serum and AS-10. Cells were harvested, fixed
and stained with PI (details in method and material section). Cell cycle phases were analyzed using Flow cytometer. (C) Quantification of the different phases of cell cycle from A. (D) HT29 cells were treated with AS-10 for 24 h in a dose dependent manner. Whole cell lysates were subjected to western blot analysis for PARP and p21 expression. GAPDH was used as loading control.

**AS-10 is Effective at Inhibiting Cancer Cell Viability of Various Types of Cancer in Addition to CRC and PC.**

We tested the ability of AS-10 on different cancer cell lines, including Melanoma: UACC903 and 1205Lu; Breast: MDA-MB-231; Lung: H460; Liver: HEPG2; and Ovarian: SKOV3 cells. MTT assay was used to measure the cell viability in presence of either agent for 24, 48 and 72 h at different concentrations. Figure 3-9 represent the % cell viability curves for all cell lines tested. Table 3-1 represents the IC_{50} values calculated by performing non-linear regression studies. From these results it can be concluded that AS-10 is effective in wide range of cancer cell lines.
Figure 3-9. AS-10 is effective at reducing cell viability of different cancer cell lines.

Cancer cell lines representing different types of cancers were treated with an increasing dose of AS-10 for 24, 48 and 72 h. Cell viability was performed using MTT assay. Data obtained was normalized with vehicle control. GraphPad Prism software was used to obtain the growth curves via nonlinear regression analysis using variable slope. For 24 h time point for H460 cells and SKOV3 cells best fit graph using non-linear regression did not converge and hence a line was obtained. IC\textsubscript{50} values of AS-10 in all cell lines are listed in Table 3-1.
Table 3-1. IC<sub>50</sub> of AS-10 in different cancer cell lines using MTT assay.

<table>
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<tr>
<th>Cancer Type</th>
<th>Cell Line</th>
<th>AS-10 (µM)</th>
<th></th>
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<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>72 h</td>
<td></td>
</tr>
<tr>
<td>CRC</td>
<td>HT29</td>
<td>4.2 ± 1.7</td>
<td>1.5 ± 0.2</td>
<td>1.4 ± 0.3</td>
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<td></td>
<td>HCT116</td>
<td>26.9±6.3</td>
<td>4.5±0.8</td>
<td>2.5±0.2</td>
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<tr>
<td></td>
<td>RKO</td>
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<td>2.1±0.3</td>
<td>2.2±0.2</td>
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<tr>
<td>PC</td>
<td>Panc-1</td>
<td>5.5 ± 0.7</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BxPC3</td>
<td>5.5±1.3</td>
<td>2.1±0.3</td>
<td>2.1±0.2</td>
<td></td>
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<tr>
<td>Melanoma</td>
<td>UACC903</td>
<td>25.8 ± 2.5</td>
<td>2.1 ± 1.5</td>
<td>1.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1205Lu</td>
<td>34.4±5.9</td>
<td>6.4±1.5</td>
<td>2.5±0.5</td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>MDA-MB-231</td>
<td>17.2±8.0</td>
<td>2.4±0.5</td>
<td>1.9±0.3</td>
<td></td>
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<tr>
<td>Lung</td>
<td>H460</td>
<td>&gt;50</td>
<td>1.9±0.6</td>
<td>0.7±0.1</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>HEPG2</td>
<td>37.6±15.4</td>
<td>4.6±0.6</td>
<td>2.8±0.5</td>
<td></td>
</tr>
<tr>
<td>Ovarian</td>
<td>SKOV3</td>
<td>&gt;50</td>
<td>5.5±1.1</td>
<td>3.3±1.2</td>
<td></td>
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AS-10 is Effective on most of the NCI’s 60 Human Cancer Cell Lines.

To further authenticate our findings and to test the effect of AS-10 in various cancers, AS-10 was submitted to ‘NCI-60 Human Tumor Cell Lines Screen’ for evaluating its cytotoxicity and growth inhibitory activity. The ‘NCI-60 Human Tumor Cell Lines Screen’ includes cell lines from nine different cancer types including breast, renal, prostate, kidney, liver, melanoma, leukemia, central nervous system (CNS) cancer and colon cancer. The results from the NCI screening studies are shown in Figure 3-10 (growth curves) and Table 3-2. Optical densities obtained from the SRB assay for cancer cells at time 0 h was considered as the base line for the studies, and is represented by 0% growth for all the graphs (Figure 3-10). Therefore, if the cells number increased, then the optical density in SRB assay would increase (compared to 0 h) and therefore positive values will be obtained.
for percent growth. In case of cell death, the optical density obtained from SRB assay would be lower compared to 0 h time point or base line and hence, negative percent growth will be obtained (cytotoxic response). However, if the optical density obtained from SRB assay does not change from the 0 h time point/base line, it can be considered as a cytostatic response. In a scenario, when the percent growth value of compound treated cancer cells are positive, but lower than vehicle treated, then a different assay (such as Muse Live/dead assay, tryphan blue exclusion assay, cell cycle analysis, etc.) need to be adapted to figure out growth inhibitory response (cytostatic) vs cell death. (Figure 3-10). Results suggest that the most sensitive cancer types towards AS-10 are renal cancer, and CNS cancer. Renal cancer cells were furthermore sensitive as almost all cell lines showed close to 100% cell death (cytotoxic) at 10 µM dose of AS-10. AS-10 was cytostatic in leukemia cells at the highest dose and hence they display resistance towards AS-10’s cytotoxicity. Table 3-2, lists three types of values represented by GI_{50}, TGI, and LC_{50}. GI_{50} values represent the concentration of AS-10 required to inhibit 50% cell growth. LC_{50} values represent the dose of AS-10 at which 50% cell death is observed (-50% cell growth). TGI (Total growth inhibition) values represent the dose at which the maximum response of AS-10 is seen regardless of growth inhibition or cell death. The bar graph in the table shows the deviation in the dose of AS-10 required for a particular response (GI_{50}, TGI or LC_{50}) from its mean concentration for similar response in all cancer cell types. Overall, AS-10 was effective at inhibiting cancer cell growth and inducing cell death in a broad spectrum of cancer cell lines.
Figure 3-10. AS-10 has a broad spectrum growth inhibitory activity in different cancer cell types.

Different cancer cell lines were treated with increasing dose of AS-10 for 48 h. At termination cells were fixed and SRB assay was performed to quantify the amount of cells in control vs treated. Initial absorbance at time 0 h was considered as base line (0%). Any absorbance detected above the initial concentration was considered as cell growth (positive values, vehicle at 48 h considered as 100%), while any absorbance lower than base line was considered cell death (negative values, total cell death as -100%).
Table 3-2. Report of AS-10’s growth inhibitory activity and cytotoxicity on NCI 60 cancer cell line screening.
Acetyl Groups and Selenium Atom both are Necessary for AS-10’s Ability to Reduce Cell Viability of Cancer Cells.

AS-10 was identified through a SAR study based on the novel substituted cyclic selena-/thia-zolidine ring compounds (will be published elsewhere). In order to understand the role of different moieties of AS-10 in inhibiting viability of cancer cell, we focused on two closely related agents, ASD-171 (that lacked the acetyl groups) and ASD-123(with selenium replaced with sulfur), respectively (Figure 3-11A). Both the agents were tested for reducing cancer cell viability using MTT assay. ASD-171 was tested in lung cancer cell lines and ASD-123 was tested on PC cells. As shown in Figure 3-11B, ASD-123 (analogue of AS-10 with selenium replaced by sulfur) was about 20 times less potent than AS-10. ASD-171 (analogue of AS-10 without acetyl groups) completely lost the cell viability inhibitory activity (Figure 3-11C), implying that both selenium and acetyl groups together in AS-10 structure play an important role in inhibiting cancer cell viability. Since, acetyl groups of AS-10 were required for its activity, we investigated the acetylation levels of histones H3 and H4 in presence of AS-10. As demonstrated in Figure 3-11D, AS-10 increased acetylation of both histones in a time dependent manner.
Figure 3-11. Loss of acetyl groups or replacement of selenium by sulfur in AS-10 leads to inhibition of its anti-cancer activity.

(A) Structures of the two new AS-10 analogues ASD-123 and ASD-171. PC cells (Panc-1) (B) and Lung cancer cells (A549) (C) were treated for 48 h with increasing dose of AS-10. MTT assay was performed to measure cancer cell viability. Bar graph showed mean ± SD. (C) Panc-1 cells were
treated with 10 µM AS-10 for increasing time points. At termination, whole cell lysates were subjected to Western blot analysis for monitoring the expression of acetylated histones H3 and H4. GAPDH was used as a loading control.

Discussion

PC is one of the deadliest cancer among all the gastrointestinal cancers.\textsuperscript{65} Chemotherapy remains the current standard of care for advanced PC patients, which includes gemcitabine alone or in combination with erlotinib or a highly toxic regime FOLFIRINOX (oxaliplatin, irinotecan, fluorouracil, and leucovorin).\textsuperscript{228, 229} Current standard of care improves survival rate on average by only few weeks, and therefore discovery of more effective and safe novel therapeutics is highly desired. Our encouraging results with newly designed AS-10 provide a ray of hope in this direction. AS-10 was identified as a result of extensive SAR studies that were focused towards developing an effective small molecule for PC therapy, in our laboratory. The chemical composition of AS-10 consists of a selanazolidine ring flanked by to acetyl salicylate (aspirinyl) functionalities leading to a unique compound that is selectively toxic to PC cells.

In the literature, it is well established that inflammation plays a major role in PC initiation, progression and metastasis.\textsuperscript{2, 13, 15} Patients who show resistance to gemcitabine, have high expression of NF-κB in their tumor sites, and is correlated with less survival rates.\textsuperscript{248-250} AS-10 was designed to possess strong anti-inflammatory properties as expected from its structural features containing both selenium and ASA moiety. ASA has emerged as a wonder drug for not only alleviating pain but also reducing/preventing cancer
development. Several clinical trials have suggested that daily consumption of ASA can reduce different kinds of cancer risk, including PC. However, prolonged usage of ASA is associated with GI toxicity. Hence, efforts are being made to make ASA safer and more potent, thereby reducing the dose required to achieve its anti-cancer activity. AS-10 was more effective than ASA in killing cancer cells and thus the lower dose requirements of AS-10 are expected to overcome GI toxicity issues.

In our screening results, AS-10 potently inhibited PC growth, while at similar doses ASA, did not have any effects on these cancer cell lines. Colony formation assay tests the growth inhibitory activity of a compound over long period of time. Therefore, Panc-1 cells pre-treated with AS-10, were subjected to colony formation assay in absence of AS-10 over a period of 17 days. AS-10 pre-treated Panc-1 cells had lost their ability to proliferate. Further, we demonstrate that AS-10, is more effective at reducing cell viability of cancer cells compared to normal MEFs, implying that AS-10 has more selectivity towards cancer cell over normal cells, and hence AS-10 is expected to have minimal systemic toxicity. It is noteworthy that the current therapies, including first line therapeutic gemcitabine, are associated with serious side effects. We compared the effects of AS-10 to gemcitabine; our data shows that AS-10 is more effective at reducing PC cell viability than gemcitabine. Gemcitabine is a nucleoside that exhibits its toxicity via DNA damage and hence it is toxic to most highly proliferative normal cells. AS-10 thus may have an edge over gemcitabine as it is not only significantly more potent, but is selective towards cancer cells.

We investigated the growth inhibition ability of AS-10, by performing cell cycle studies of PC cells treated with AS-10. Our findings indicate that AS-10 induces G1/G0
and G2/M phase cell cycle arrest. At later time points there was an increase in sub G₀ peak indicative of apoptotic cells. Since ASA has been known to have multiple effects in cancer cells, it may be possible that AS-10 may also affect different pathways to inhibit PC growth. AS-10 up-regulated the cell cycle arrest markers such as p21 and p27, which indicates the activation of DNA checkpoint markers in both G1 and G2/M phase. This marker is activated in the presence of DNA damage or apoptosis. Literature reports suggest that histone deacetylase (HDAC) inhibitors can activate p21 and p27 expression. ASA also has been known to induce expression of both proteins and could increase histone acetylation to show its effects. Therefore, AS-10 may not be a DNA damaging agent, but might instead induce expression of p21 and p27 by acetylating the histones at the promoter regions. These mechanisms remain to be elucidated. Another possibility could be proteasomal activity inhibition, which has been suggested as the cause of ASA’s ability to increase p21 and p27 expression.

We also explored the downstream effect of cell cycle inhibition, which is apoptosis or senescence. Our data indicate that AS-10 induced apoptotic death in PC cells. The apoptotic pathway can be activated via extrinsic or intrinsic apoptotic signals. Activation of caspase 8 is a hallmark of extrinsic apoptotic pathway activation mediated by activation of death receptors, while activation of caspase 9 is a hallmark of intrinsic apoptotic pathway activation mediated by mitochondrial mediated death pathway. In our Western blot studies, caspase 8 was never activated, however AS-10 was able to activate caspase 9, as indicated by the cleavage of pro-caspase 9. Further, caspase 3 activation was detected and at the same concentration of AS-10, the down-stream target of caspase 3, PARP was also
cleaved in the presence of AS-10 (Figure 3-4). In CRC cells, AS-10 similar to PC, induced cell cycle arrest, which was confirmed by expression of cell cycle inhibitory kinase p21. Further, AS-10 activated apoptosis in this cell line, as specified by PARP cleavage, indicating that mechanism in PC and CRC are identical for AS-10.

One of the common mechanisms of action of ASA and most selenium compounds is inhibition of pro-inflammatory NF-κB pathway. Therefore, we investigated the effects of AS-10 on NF-κB activation in presence of inflammatory stimuli like TNF-α. As shown in our EMSA assay, in the presence of inflammatory stimuli, NF-κB binds to its consensus DNA sequence and a strong band shift in the vehicle treated Panc-1 cells is observed. However, when the dose of AS-10 is increased in cells stimulated with TNF-α, NF-κB associated lagging/shifting band disappears. These assays clearly demonstrate that AS-10 has the ability to inhibit NF-κB-DNA binding. Further, Western blot analysis, of the cytosolic lysates of Panc-1 cells revealed that IκBα was degraded in presence of inflammatory stimuli, however in presence of AS-10, the degradation of IκBα was inhibited. Further, to confirm whether the restoration of IκBα leads to inhibition of NF-κB translocation to the nuclei, we implemented confocal microscopy to look at the translocation patterns of p65 (a subunit of NF-κB) in presence of inflammatory stimuli and AS-10. Confocal microscopy results distinctly demonstrate that in presence of AS-10 and inflammatory stimuli, p65 was unable to translocate to the nuclei. Taken together, based on results from EMSA, Western blot, and confocal microscopy assays, it can be concluded that AS-10 is able to prevent the activation of NF-κB pathway in presence of inflammatory stimuli. It has been shown that both ASA and selenium containing compounds can inhibit
activity of IKK kinase, by either competing with ATP at the activation cite or by covalently binding and inhibiting the activation loop.\textsuperscript{226} It has also been shown that acetylation of the threonine amino acid inside IKK activation loop can inhibit its activity.\textsuperscript{261} Therefore, if the acetyl groups in AS-10 behave in a similar manner then IKK can be a potential target of AS-10 for this pathway. Further studies are needed to identify the target of AS-10 in these pathways. Additionally, restoration of IκBα levels as shown in our Western blot analyses suggests that either IKK kinase activity or proteasomal activity has been inhibited by AS-10. Therefore, future studies should also be carried out towards examining the activity of the proteasome in presence of AS-10. Moreover, AS-10 also downregulated the NF-κB target proteins like Bcl-xL and Mcl-1, and hence making cells sensitive towards cell death stimuli.

Studies have suggested that cancer cells acquire resistance to gemcitabine through aberrant activation of NF-κB pathway.\textsuperscript{248-250} It has been demonstrated that inhibition of this pathway using si-RNA or small molecule inhibitors can sensitize PC cells towards gemcitabine.\textsuperscript{76, 249} Hence, several NF-κB inhibitors alone or in combination with gemcitabine are in different phases of clinical trials.\textsuperscript{77} Here we show that AS-10 has the ability to inhibit NF-κB activation in presence of inflammatory stimuli, as well as to sensitize PC cells towards gemcitabine treatment (Figure 3-6). The results obtained in our study are in accordance with literature reports.\textsuperscript{68, 69, 262}

In normal cells, the major source of ROS is mitochondria.\textsuperscript{263} ROS are very unstable and can damage DNA and proteins.\textsuperscript{264} Cancer cells show high basal ROS levels because of mitochondrial activity as compared to their normal parent cells.\textsuperscript{264} Hence, ROS inducing
agents have been proposed to kill cancer cells selectively over normal cells by increasing the amount of ROS enough to tip the balance towards cancer cell death.\textsuperscript{265} Drawback of the ROS inducing agents is that the cancer cells also have upregulation of stress pathways like NF-κB,\textsuperscript{266} which can upregulate not only anti-apoptotic proteins but anti-oxidant enzymes which can counteract the increased ROS levels in cancer cells.\textsuperscript{264, 267-270} Hence, it has been proposed that compounds that have dual action of increasing ROS levels and further inhibiting the NF-κB resistance pathway may play a major role in killing the cancer cells.\textsuperscript{268} Further, ASA derived molecules like NO-aspirin are known to induce ROS species in cancer cells,\textsuperscript{271} while selenium containing compounds can either be a pro-oxidant or anti-oxidant depending on the types of active metabolites formed.\textsuperscript{205} As our data indicates, AS-10 induced ROS in PC cells by about 20\%. The contribution that this \~20\% increase in ROS levels may have on anti-cancer activity of AS-10 was further investigated by examining the anti-cancer effects of AS-10 in presence of ROS quencher, NAC. Our data show that AS-10 partially lost its activity in presence of a ROS quencher, suggesting that it has the ability to increase ROS. These experiments clearly demonstrate that AS-10 has a dual effect of increasing ROS and inhibition of NF-κB pathway, since quenching ROS only partially reduced its anti-cancer activity. Further studies are required to understand the mechanisms by which AS-10 increases ROS. NF-κB is a major stress related pathway, and one of the targets of NF-κB are anti-oxidant proteins.\textsuperscript{272, 273} Hence, it is likely that increase in ROS is due to NF-κB inhibition, however, it remains to be elucidated.

Further, AS-10 was found to be effective at early time points in all cell lines except lung (H460), ovarian (SKOV3), liver (HEPG2) and melanoma (UACC903) cell lines.
However, at 48 h and 72 h AS-10 potently inhibited cell viability of these cell lines. This effect of AS-10 not being potent at early time points in most cell types may be attributed to its cell cycle inhibitory properties, since in our hands the SKOV3, HEPG2, UACC903 and H460 cells grew slower (doubling time more than 24 h) compared to rest of the cell lines. Limitation of MTT assay is that it does not distinguish cell cycle arrest vs cell death. Different assays such as Calcein-AM and ethidium bromide could answer the question as to whether AS-10 is able to induce cell death. Hence, effects seen in our MTT assay by AS-10 could be both via cell death and/or cell growth inhibition. Overall, AS-10 was effective at reducing cell viability of different cancer cell types.

NCI-60 human tumor cell line screening was introduced by NCI in 1980’s, with the goal to study the effects of novel agents on cancer cells from leukemia to solid tumors like melanoma. As mentioned above the NCI screening has cell lines from nine different cancer types, and hence the screening can help determine the sensitivity towards some cancers over others. Further, the screening can also help to elucidate potential mechanism of action by looking at the pattern of sensitive cancer cell lines vs resistant cancer cell lines and associated mutations or gene expression. AS-10 showed cytotoxic effects in different cancer cell types, out of which renal cancer cells were the most sensitive. Second most sensitive cancer type was CNS followed by melanoma. Most resistant cancer types were found to be leukemia, ovarian, and breast cancer. In colon cancer cell lines, AS-10 was mostly cytotoxic except in HT29 cells which showed a cytostatic response. This result was contradictory to our reports of AS-10 treatment in HT29 cells. In our studies, AS-10 did induce cell cycle arrest, but it translated to apoptosis at higher dose. This could be due to the difference in assays used. Further, the assay used in the NCI screening studies does not
account for any floating cells/ dead cells. Once the dead cells are lifted from the plate, they are washed out and the effect translates as growth inhibitory activity, therefore, the results from NCI screening should be verified with different technique on the cell line of interest. Since AS-10 was effective in all cell lines, it is hard to pin-point which mutations are more likely to make the cancer cells sensitive, or which proteins expression could make cell lines more resistant.

Our preliminary data on an AS-10 analogue, ASD-171, analogue without the acetyl groups, showed that upon removal of the acetyl moieties, the compound completely loses its anti-cancer activity. In literature, ASA has been reported to acetylate different proteins, and further it has also been observed that ASA can synergistically work with Histone deacetylase (HDAC) inhibitors. HDACs remove acetyl groups off proteins, and hence they can silence different genes (via histone tail modification) as well as modulate protein activities (either activate them or inhibit them). Hence, in cell lines with less HDAC protein expression, ASA can overcome the effects by acetylating more proteins, than HDACs can remove. These acetylated proteins may be involved in cancer growth inhibition or apoptosis. Implying, higher the expression of HDAC in a given cell, more resistant it will be towards ASA’s mode of action. In NCI-60 cancer cell lines, renal cancer cells have lower HDAC expression compared to leukemia and others cancer cell types. Hence, acetylation of different proteins/histones may be a potential anti-cancer mechanism for AS-10. Further, our data shows that AS-10 is able to induce p21 expression in p53 mutant cell lines (HT29 and Panc-1), which is also similar to HDAC inhibitors where p21 expression is also p53 independent. Further, in our preliminary results, we have seen an increase in histone acetylation in AS-10 treated cells. However, whether AS-
10 directly effects acetylation needs to be further evaluated. Further, the role of selenium in AS-10 also needs to be further explored because replacement of selenium with sulfur completely lost the anti-cancer activity of AS-10’s analogue, ASD-123.

Conclusions

In summary, we have identified a novel Se-aspirin hybrid compound with potent activity against PC, and has a broad spectrum of activity against different cancer cell types. Our in vitro studies showed that AS-10 is more than 100 times potent than gemcitabine and selectivity toxic to PC cells, while sparing the normal MEF cells. AS-10’s mechanism of action relies on inhibition of NF-κB pathway, cell cycle inhibition, and induction of apoptosis. In addition, inhibition of NF-κB pathway by AS-10 potentiates the effect of first line therapy drug for PC, gemcitabine. Our data suggests that the anti-cancer activity of AS-10 may, at least partially, be due to induction of ROS, since ROS quencher (NAC) was partially able to inhibit the activity of AS-10. Further, AS-10 has greater potency compared to ASA, hence low doses of AS-10 could potentially reduce the GI toxicity that aspirin causes. Further, NCI-60 cell line screening showed that AS-10 has a broad spectrum activity against different cancer types. From these studies, it was concluded that renal cancer cells were the most sensitive towards AS-10. This could, at least partially, be due to lower HDAC expression in these cell lines as compared to others present in the NCI-60 human tumor cells library. Additionally, our results show that both the acetyl groups and selenium in AS-10 are essential for its activity. Overall, our results indicate that AS-10 may be an effective treatment option for PC patients, both as a single agent and particularly
in combination with gemcitabine. However, further investigation of its \textit{in vivo} efficacy, mechanism of action, and toxicity profile will determine AS-10’s fate for further development for clinical use.

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Chapter 4

Summary

Cancer in general is a deadly disease and further, the chemotherapeutic agents (such as 5-FU, platinum based agents, Gemcitabine, etc.) used in clinic are associated with toxic side effects (such as mouth sores, GI toxicities, neutropenia, etc.). Hence, there is an unmet need for developing small-molecule agents which can target different cellular pathways in cancer cells to kill them and minimizes toxic side effects. Targeting a single pathway to inhibit cancer growth has been shown to be an ineffective therapy. The classic example of single targeted drug is mutant BRAF inhibitor, vemurafenib, to which resistance builds within 6 months and cancer relapses.\textsuperscript{281} Hence, targeting multiple pathways in cancer has become a more transient approach.\textsuperscript{282} For example, a single agent targeting multiple pathways or a combination therapy where two agents targeting two or more different pathways can both synergistically kill the cancer cells.

Gastrointestinal (GI) cancers are the second leading cause of death among all cancer related deaths.\textsuperscript{1} As highlighted in chapter 1, inflammation plays an important role in GI cancer initiation and metastasis. The important inflammatory pathways, NF-κB and STAT3, have been shown to be highly expressed in these cancers and contribute towards cell proliferation, pro-survival, pro-inflammatory, and most importantly, resistance towards different chemotherapeutic agents.\textsuperscript{67, 75, 76, 81} Therefore, targeting these pathways have always been a hot topic in the cancer field. Further, mutations in key oncogene such
as KRAS in CRC and PC, are also known to prolong the activation of these pathways for cell growth and proliferation.  

NSAIDs and specifically aspirin (ASA), have emerged as chemopreventive agents and have been proposed to be potential chemotherapeutic agents for different cancers.\textsuperscript{22, 56, 57, 106-110} The association of ASA as chemopreventive agent for CRC has been explored in many clinical trials and has been clearly defined. Further, the latest meta-analysis which evaluated the past 12 different clinical trials of ASA in PC, shows a clear link of ASA as a chemopreventive agent for PC.\textsuperscript{284} In addition, different in vitro and animal studies have shown that ASA can inhibit CRC and PC cell growth.\textsuperscript{95-105} However, the dosages required for ASA to achieve its chemotherapeutic effects are very high. As discussed in chapter 1, the major side effect of ASA is GI bleeding and stomach ulcer formation.\textsuperscript{58}

Many different groups in the field have modified ASA, by attaching/altering different functional groups around the ASA skeleton. The modified ASA molecules have been shown to have gastro-protective effects, as well as more potent anti-cancer effects than ASA.\textsuperscript{101, 134-140, 145-150} Nonetheless, these molecules are still in their early phases of clinical development, and hence, developing novel agents using NSAIDs represents a valuable opportunity for cancer drug discovery.

Selenium has gained a lot of attention because of increased cancer risk in population with selenium deficiency.\textsuperscript{151-154} In the past decade selenium molecules have emerged as potent anti-cancer compounds.\textsuperscript{196} Replacement of sulfur in known anticancer molecules to selenium increased compounds anti-cancer activity and hence reduced the compounds toxicity.\textsuperscript{196} Further, selenium containing molecules have been shown to have gastro-protective effects and to increase the speed of ulcer healing compared to current drugs for
Therefore, novel agents generated by combining selenium and NSAIDs may prove to be less toxic and potent at inhibiting cancer growth.

Here we report the discovery of novel agents which contain rationally incorporated NSAIDs and selenium moieties. The study was based on the rationale that combining NSAIDs and selenium, will yield novel agents that may have lower toxicity and potent anti-cancer activities. From our initial screening studies, we discovered two novel compounds from this innovative approach of synthesizing small drug-like molecules using key fragments from common NSAIDs (like ASA, ibuprofen, and naproxen) and selenium. Chapter 2 describes the synthesis, development, and screening of these novel molecules. Readers are referred to our publication in the Journal of Medicinal Chemistry for details regarding the chemical synthesis of the best identified novel agent (Comp 8) from this SAR study. Out of the first set of compounds generated using the three NSAIDs scaffolds and appropriate selenium moieties, we identified that the compound (Comp 8) having ASA and selenocyanate (SeCN) moiety was most effective at killing cancer cells. These studies show that only certain appropriate fusions of ASA and selenium fragments can lead to potent anti-cancer agents. It is interesting to note that SeCN incorporated only with ASA molecule had effect on cancer cell viability, but not in ibuprofen or naproxen. Hence, the potency of Comp 8 may not be due solely to the presence of SeCN but the total molecular structure. This was further evident from the fact that none of the different analogs designed by incorporating different selenium moieties into were better than Comp 8.

The anti-cancer activities of Comp 8 were examined in different cancer types as described in chapter 2. Overall it was effective in most cancer cell lines tested. Since our focus was on CRC and PC, characterization of mechanism of action (MOA) of Comp 8
was limited to CRC and PC. The MOA of Comp 8 included cancer cell growth inhibition mediated via cell cycle arrest and cell death. Further, the cell death mechanism studies reported in chapter 2 yield that Comp 8 activated the intrinsic form of apoptosis via caspase 9 and caspase 3 activation. PARP degradation was also observed which further confirms the activation of caspase 3. We demonstrated that Comp 8 can inhibit NF-κB pathway activation in the presence of inflammatory stimuli. Therefore, Comp 8 represents itself as an anti-inflammatory and anti-cancer agent. However, its anti-inflammatory activities remain to be characterized.

The second compound in our studies was AS-10. The synthesis of AS-10 followed a unique chemistry (to be published later) resulting in a cyclic selenazolidine ring substituted by two acetyl salicylate groups similar to ASA. AS-10 potently reduced cell viability of different types of cancer cell lines (Chapter 3). Its MOA was characterized in PC and CRC. AS-10 was able to induce its anti-cancer effects via inhibition of NF-κB pathway, which led to reduced expression of anti-apoptotic proteins like Bcl-xL, Mcl-1 and survivin. Moreover, AS-10 caused cell cycle inhibition via increasing the expression of p21 and p27 cell cycle inhibitory kinases. Additionally, AS-10 activated intrinsic apoptosis via caspase 9 and caspase 3 activation. We showed that AS-10’s effects were partially mediated via ROS generation, as ROS quencher, NAC, partially inhibited the anti-cancer activity of AS-10. Most importantly, AS-10 via inhibition of NF-κB pathway, potentiated the anti-cancer activity of first line PC therapy drug, gemcitabine. Our findings also suggest that AS-10 has the ability of increasing acetylation of histones H3 and H4, however this observation needs to be further evaluated. NCI-60 cell line screening suggested that AS-10 was most cytotoxic in renal cancer cell lines, while in other cancers
it showed a mixed response of either being cytostatic or cytotoxic. Leukemia cell lines were the most resistant in terms of cell death, as all the leukemia cell lines showed to be growth arrested rather than cell death. Together, AS-10 represents itself as a novel agent with anti-cancer activity via dual activity of modulating NF-κB pathway and ROS.

We also demonstrate that the cytotoxic effects of both agents are more selective towards cancer cells than normal cells. In our preliminary MTD studies, we found AS-10 to be ~5 times more tolerable as compared to Comp 8. Further our *in vitro* MTT studies demonstrate AS-10 to have a wider therapeutic window than Comp 8. In conclusion, we have developed two novel agents, with potent anti-cancer effects and with more selectivity towards cancer cells than normal cells. They represent a first of their kind where ASA and selenium have been combined to generate innovative molecules which are much more potent than the first line therapies for CRC and PC.
Chapter 5

Future Directions and Conclusions

Future directions

Most of the future directions regarding both agents MOA have been discussed in chapter 2 and 3, however following key future studies will ultimately define the future path for both AS-10 and Comp 8 as potential therapeutic agents in clinical setting.

Target Identification Studies for AS-10 and Comp 8

Target identification of both agents will help find more susceptible cancer types towards AS-10 and Comp 8. Since both AS-10 and Comp 8 have been synthesized with novel chemistry, and have a unique structure, their protein targets need to be elucidated. We report that both agents have the ability to inhibit NF-κB pathway, induce cell cycle arrest and induce cell death. First, from our NF-κB studies we can speculate that both the agents may be inhibiting the IKK kinase upstream of the pathway. Hence, we can perform whole genome kinase profiling to see which kinases are inhibited in presence of AS-10 and Comp 8. These testing must be performed in a range of concentrations from low to high, for examining the selectivity of both the agents towards certain pool of kinases and also to understand their off target effects. Commercially available high-through-put kinase screening services like KINOMEscaN™ via DrugDiscoverX may help identify potential targets of AS-10 and Comp 8.
Further techniques like Drug Affinity Responsive target stability (DARTS) assay or cellular thermal shift assay (CETSA) can be used for identification of proteins that can directly interact with AS-10 and Comp 8.\textsuperscript{285, 286}

- DARTS assay is based on the principle that when a target protein interacts with a small molecule, its thermodynamic stability changes and hence these interaction may either increase or decrease resistance to proteolysis.\textsuperscript{285} In brief, this technique is performed by taking aliquots of cell lysates and incubating them with compound of interest (AS-10 or Comp 8), followed by partial digestion of proteins with proteases (degradation of proteins with a range of different protease concentrations). After partial digestion, cell lysates are separated via electrophoresis and stained. Proteins bands with different pattern of degradation than controls are potential binding partners of our agents. The detected protein bands can then be subjected to mass spectrometry (MS) analysis for identification.

- CETSA assay is based on a similar principle that when a protein is bound to an agent, it will either make the protein thermodynamically more stable or less stable at different temperatures.\textsuperscript{286} Hence, when cell lysates treated with a small molecule are subjected to different temperatures, unbound proteins will be denatured faster and precipitate out of solution compared to agent bound proteins, or the agent bound proteins may precipitate faster than unbound proteins. Therefore, when the lysates at different temperatures are separated via electrophoresis, proteins which interact directly with the compounds, will have a different precipitation pattern than the control proteins present in control lysates. These proteins can be separated out
and given for MS analysis for identifying the potential binding partners of our compound.

Thus the targets for AS-10 and Comp 8 can be elucidated using one of the above described techniques.

**Pharmacokinetics and Toxicity Studies of AS-10 and Comp 8**

The major reasons for drug failure in clinical setting are due to poor efficacy and increased toxicity. Therefore, it is very critical that all the novel agents undergo pre-clinical pharmacokinetic studies to eliminate the poorly bioavailable and systemically toxic compounds at early stages of drug development and consequently more positive results can be obtained in clinic. Absorption, distribution, metabolism, excretion and toxicity (ADMET) studies altogether defines pharmacokinetics and toxicity profile of a given agent. When a compound is introduced inside the body, the body undertakes different actions to excrete it out of the system. In the process of excretion, different metabolites of the compound are formed, majority by liver enzymes known as cytochrome P450’s. The resultant metabolites may be active or inactive. Hence, *in vitro* metabolism studies will help identify different metabolites of both AS-10 and Comp 8, and whether one or more of these metabolites are active needs to be tested via *in vitro* experiments. Further, from *in vitro* studies on cytochrome P450’s and their inhibitors, we can also identify which enzymes are responsible for metabolism of AS-10 or Comp 8, and hence drug-drug interaction can be predicted when administrated with different drugs. Once metabolism studies are done, other studies such as plasma stability, plasma protein binding studies, rate
of metabolism and clearance will help understand the half-life of the compound or the active metabolite in the system and hence accordingly the dose regime can be assessed for tumor inhibition studies. Maximum tolerated dose (MTD) studies can then be performed for understanding the dose regime and associated toxicities. Therefore, ADMET studies will provide valuable information on agent’s future as a potential drug candidate.

**In Vivo Tumor Inhibition Studies and GI Toxicity**

Our findings provide strong rationale that Comp 8 and AS-10, are very active against CRC and PC. Further, our *in vitro* cell viability studies demonstrate that both agents are more selective towards cancer cells, than MEFs, which imply that our agents may be effective at killing cancer cells and show less toxicity *in vivo*. To bring AS-10 and/or Comp 8 a step closer to the clinical trials, their efficacy and toxicity *in vivo* needs to be elucidated. For understanding *in vivo* tumor efficacy, xenograft studies should be conducted to get an idea of the dose at which our agents may inhibit tumor growth. Tumor growth inhibition studies in animals will provide valuable information on whether the *in vitro* activities of the compounds can be translated in animals. Further, treated tumors can be tested for markers of proliferation, cell cycle arrest, apoptosis and NF-κB status. As discussed in chapter 1, NF-κB inhibition shows less metastasis of tumors, hence orthotopic models for CRC and PC may also be adapted in future studies for understanding the role of our agents in metastasis. Additionally, AS-10 and gemcitabine combinational treatments should also be investigated in different PC models, due to their cooperation in killing PC cells *in vitro*. 
These results will have more clinical relevance and will accelerate the development of AS-10.

Since Comp 8 and AS-10 were designed based on the rational to reduce GI toxicity, at the dose where we observe tumor inhibition, GI toxicity should also be addressed. Based on our in vitro studies, it may be reasoned that the concentration of AS-10 or Comp 8 required to achieve overall anti-cancer effects may be very less compared to ASA in vivo. Further, our agents contain selenium atom, and selenium containing agents have shown to be gastro-protective. Therefore, to lower dose requirement and gastro protective effect of selenium, GI toxicity of these compounds should be eradicated or reduced substantially. However, these are just speculations, future in vivo studies will answer the questions regarding efficacy and GI toxicity.

**Plausible use of AS-10 and Comp 8 in Diseases other than Cancer**

Potentially both the agents (Comp 8 and AS-10) can be used for all other diseases where ASA is already being used. Due to their more potent role in inhibiting inflammatory pathways than ASA, their effects in chronic inflammatory diseases (e.g. pancreatitis, IBD, arthritis, Alzheimer’s, etc.) can be studied. Further, analgesic effects of both Comp 8 and AS-10 can be explored. Due to the presence of acetyl groups in AS-10 and Comp 8, their role as an anti-thrombotic agent can also be investigated. Overall, AS-10 and Comp 8, represent themselves with a lot of potential as a future chemotherapeutic agents for CRC and PC. Future ADMET studies will project the use of both agents in clinic for different diseases.
Limitation of AS-10 and Comp 8 as Potential Anti-Cancer Therapeutics

Our studies have shown both AS-10 and Comp 8 to be selectively toxic to cancer cells. In addition, computational studies reveal both the compounds to satisfy Lipinski’s rule-of-five for drug-likeness requirements. However, limitation of both compounds use will rely upon how well they are tolerable and bioavailable in preclinical \textit{in vivo} models and in the clinic. One limitation that can be conjectured is their effect on overall immune system. NF-κB pathway plays a pivotal role in genesis of innate immune system cells and host defense system. Prolonged inhibition of NF-κB may lead to suppression of host immune system and hence, making the host more susceptible to pathogens. Therefore, the dose and the length of the regimen will shed more lights on the suppression of immune system. Such immune suppression problem, if caused by the compounds, would limit their use in a chemopreventive setting where continuous dosing is required over a prolonged period of time. However, for cancer patients, particularly for PC patients for whom the survival time currently is short, issues related to long-term use may be ignored as long as the survival time and quality of life can be enhanced significantly.

Clinical Significance

AS-10 and Comp 8 exemplify a novel class of compounds, with selenium and ASA activities integrated in one. Both the agents represent stand-alone treatments for PC and CRC which have limited treatment options. Further, AS-10 synergistically enhanced the potency of first line chemotherapeutic agent for PC, gemcitabine, at a lower dose than the
dose required for its cytotoxicity activity. Hence, it will be clinically very relevant to reduce the dose of gemcitabine, and therefore, the associated toxicity, and improve quality of life of PC patients.

Conclusions

In conclusion, we have developed two unique novel small molecules, Comp 8 and AS-10, that target pro-inflammatory pathways to selectively kill cancer cells, and are highly effective against CRC and PC. Additionally, both agents have a broad spectrum of activity in different cancer types. Our results show that although both AS-10 and Comp 8 were equipotent on inhibiting the growth of cancer cells, AS-10 was ~ 5 times more tolerable in mice. Hence, AS-10 in particular, is an ideal drug candidate to be developed further. Successful outcomes of future studies are expected to identify a novel agent that could be used alone or in combination with existing chemotherapy to effectively treat various cancers and will provide a solid rationale for initiating clinical trials in cancer patients.
Appendix

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Publications

1. **Karelia D.**, Pandey M.K., Plano D., Amin S.G., Sharma A.K. A novel small molecule, AS-10, inhibits pancreatic cancer cells survival and induces apoptosis by modulating the NF-κB pathway and ROS levels. [In prep]

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