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Design, Synthesis, and Biological Evaluation of Novel Selenium (Se-NSAID) Molecules as Anticancer Agents

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ABSTRACT

The synthesis and anti-cancer evaluation of novel Selenium-Non-steroidal anti-inflammatory drugs (Se-NSAIDs) hybrid molecules are reported. The Se-aspirin analog **8**, was identified as the most effective agent in reducing viability of different cancer cell lines, particularly colorectal cancer (CRC) cells, was more selective towards cancer cells than normal cells, and was >10 times more potent than 5-FU, the current therapy for CRC. Compound **8** inhibits CRC growth via inhibition of cell cycle in G1 and G2/M phase, and reduces the cell cycle markers like cyclin E1 and B1 in a dose dependent manner; the inhibition of the cell cycle may be dependent on the ability of **8** to induce p21 expression. Furthermore, **8** induces apoptosis by activating caspase 3/7 and PARP cleavage, and its longer exposure causes increase in intracellular ROS levels in CRC cells. Taken together, **8** has the potential to be developed further as a chemotherapeutic agent for CRC.

INTRODUCTION

A cohort of studies supports the indisputable promise of aspirin (ASA) and other non-steroidal anti-inflammatory drugs (NSAIDs) as chemo-preventive 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.[1-8] 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. [9-13] Although routine ASA use may not be warranted in the general population, an international panel advocated additional research in high-risk populations.[14] 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.[15] 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.[15]

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.[16] A key mechanism for NSAIDs efficacy is cyclooxygenase (COX) inhibition and reduced production of prostaglandins.[17] Nonetheless, several other mechanisms of action have been proposed, including induction of apoptosis,[18, 19] inhibition of angiogenesis,[20] activation of nuclear factor kappa B (NF-κB),[21, 22] modulation of insulin-related neoplastic pathways,[23] inhibition of the mammalian target of rapamycin (mTOR) pathway[24] and modulation of cellular metabolism through the AMP-activated protein kinase (AMPK).[25]

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.[26, 27] 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 chemo-preventive effects, mainly against CRC, together with a lower toxicity than the corresponding NSAID alone.[28-33] 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.[34-37] Additionally, several HS-NSAIDs evinced growth inhibitory effects in a wide variety of human cancer cells.[38-40] Furthermore, dual NO- and HS-releasing ASA derivatives have been synthesized, showing potent anti-inflammatory activity and inhibition of cancer cell growth *in vitro* along with a marked reduction of tumor volume in colon cancer xenograft models.[41, 42] Other modified NSAIDs extensively reported in the literature are the phospho-NSAIDs, which have shown preclinical chemotherapeutic and chemopreventive properties in several cancer models.[43, 44]

Selenium (Se) compounds have attracted a vast interest in the last decades as promising chemo-preventive agents and several epidemiological studies have reported an inverse association between the nutritional Se status and cancer risk, although considerable controversy

has arisen mainly due to the negative outcome of the SELECT trial.[45, 46] Contrary to the skepticism generated by the diverse clinical trials on Se as a chemo-preventive 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,[47-51] as well as to have synergistic effects in combination with chemotherapeutic drugs.[52-56] 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 induction of apoptosis, inhibition of angiogenesis and modulation of AKT and COX pathways.[57-59] To our knowledge, the only NSAID framework modified to date with Se functionalities is the celecoxib scaffold, yielding greater anti-inflammatory and cancer cell growth inhibition; for the novel selenocoxib derivatives.[60, 61] Further development of these selenocoxib derivatives afforded the glutathione analog of selenocoxib-1, which elicited potent inhibition of tumor development in melanoma xenografted mice.[57]

Considering the aforementioned chemo-preventive 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 Se compounds against gastric toxicity induced by indomethacin,[61, 62] along with the potential synergistic effects of Se and NSAIDs.[63]

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**) in the HCT116 CRC cell line. Two shared mechanisms implicated in the biological effects of ASA and seleno compounds on cancer cells are apoptosis[59, 64] and inhibition of inflammatory pathways.[60, 65] In this paper, we focused on the anti-proliferative effects of the lead compound **8** and report insights into its mechanism of action in inhibiting CRC cell growth.

RESULTS AND DISCUSSION

Design. Several studies have pointed out the chemo-preventive effects of NSAIDs and the anti-tumor and chemo-preventive effects of multiple Se species in various cancers. In addition, several Se 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, 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 Se moiety into various NSAIDs according to the general structure shown in Figure 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)] where selected for Se incorporation. Based on our past experience and literature evidences on the structure-activity relationship of organoselenium compounds, 2-selenocyanoethanamine (**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 1) indicated that only in the case of ASA the IC_{50} value of the resulting hybrid Se-NSAID derivative (compound **8**) was decreased significantly as compared to the parent NSAID scaffold. Accordingly, the hybrid Se-ASA (**8**) was selected for further structural modifications with various Se 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 ($-CO_2H-CH_2SeCH_3$) (Figure 1). The methylselenocysteine moiety was incorporated into ASA structure to evaluate the influence of its anti-cancer properties[66-68] as well as the demonstrated selective protection it offers against organ-specific toxicity induced by clinically active agents.[66] Taking into account the IC_{50} values obtained for the six Se-ASA hybrid molecules (**8**, **10**, **12**-**15**), compound **8** emerged as the most potent and promising anti-cancer agent. To gain further knowledge about the importance of the Se atom in this scaffold, its isosteric sulfur analog (**16**) was also synthesized and tested.

Chemistry. The Se derivatives of Ibu (**4**) and Nap (**5**) were prepared from the corresponding acyl chloride and 2-selenocyanoethanamine (**1**) by a nucleophilic addition/elimination reaction in the presence of triethylamine (TEA) to neutralize the hydrochloric acid formed in the reaction mixture (Scheme 1). The SeCN derivative **1** was obtained by the nucleophilic substitution of -Br atom in 2-bromoethanamine by -SeCN, using KSeCN as nucleophilic donor, in acetonitrile as solvent and under a nitrogen atmosphere (Scheme 1). The acyl chloride (**3**) of Ibu was synthesized by the reaction of (*RS*)-2-(4-(2-methylpropyl)phenyl)propanoic acid (**2**) with oxalyl chloride in methylene chloride.

Surprisingly, the reaction of the acyl chloride derivative of ASA (**6**) with 2-selenocyanoethanamine (**1**), under the reaction conditions used for compounds **4** and **5**, did not yield the expected ASA analog (**8**) (Scheme 1). Modifications of the reaction conditions including changes of solvent, reaction temperature, and time failed to yield compound **8**. The

chemical reasons for this surprising behavior are unknown. We hypothesize that an intramolecular cyclization is occurring for this specific reaction, however, further investigations are warranted to prove this hypothesis. An alternative synthetic route was thus developed for compound **8**. In this case, the SeCN group was inserted in 2-((2-bromoethyl)carbamoyl)phenyl acetate (**7**), obtained by the reaction of 2-bromoethylamine hydrobromide with acid chloride **6**, by refluxing in dry acetonitrile with KSeCN with a 41% yield (Scheme 1).

The hybrid SeCN-ASA derivative (**10**) with the ester linker was synthesized by reaction of the aspirinyl chloride (**6)** and 2-selenocyanatoethanol (**9**) in the presence of TEA under a nitrogen atmosphere at room temperature in methylene chloride with a 79% yield (Scheme 2). Compound **9** was synthesized by the replacement of the bromine atom in 2-bromoethanol by a SeCN group using KSeCN in dry acetonitrile (Scheme 2).

The synthesis of the aspirinyl selenoester derivatives (**12-14**) was carried out by first reacting aspirinyl chloride (**6**) with an aqueous solution of sodium hydrogen selenide, following a previously published method,[69] followed by the treatment of the resulting sodium salt of aspirinyl selenoester (**11**) in situ with 1,2-dibromoethane in a mixture of distilled water and THF, yielding a mixture of the mono- and di-substituted derivatives (**12** and **13**, respectively) (Scheme 3). Both of the compounds were easily isolated by silica gel column chromatography and the ratio for both compounds could be modulated using different proportions of 1,2-dibromoethane and different reaction times. Finally, the selenocyanate derivative **14** was obtained by the reaction of compound **12** with KSeCN in dry acetonitrile at room temperature with a 72% yield (Scheme 3).

The methylseleno derivative **15** was obtained by a nucleophilic addition/elimination reaction between the aspirinyl chloride (**6**) and Se-(methyl)selenocysteine hydrochloride (Scheme 4) in the presence of TEA. The synthesis of the sulfur analog (**16**) of the lead Se-ASA compound (**8**) was carried out by following the similar methods as described in Scheme 1 using NH_4SCN instead of KSeCN (Scheme 5).

Evaluation of the anti-proliferative 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 Se- (**8**, **10**, and **12-15**) and S- (**16**) derivatives of ASA were examined for their effects on cancer cell viability. To determine the effect of $–NH-CH_2CH_2-SeCN$ 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 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 analog **8**, while similar modification of Ibu and Nap had no effect on cancer cell viability (Table 1). Cell viability curves for **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 a unique combination of the Se moiety with ASA is required for cytotoxicity. Based on this observation, we generated several Se analogs of ASA and screened them against same panel of the cells as used in Table 1. Table 2 summarizes the effects of all agents on cancer cell viability, which were designed by further modifying **8**.

An overview analysis of the IC_{50} values obtained and summarized in Table 2 evinced 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 **8** being the most potent (Table 2, and Figure 2). Notably, in comparison to selenocoxib-1 and its glutathione conjugate (selecoxib-1-GSH), the only Se-NSAID hybrid compounds reported in the literature,[57, 61] compound **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.[57] Compound **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 **8** and PC3M for selenocoxib-1). [61]

Considering the potency of these Se-ASA hybrid molecules on cancer cell viability, some structure-activity relationship (SAR) can be inferred: 1) the most favorable functional group used to link the ASA scaffold with the Se is the amide group (**8**); the replacement of the amide group forming an ester (**10**) or selenoester (**14**) reduced the potency of the compounds on cancer cell viability; 2) 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 compound **15** in most of the cancer cell lines tested, with the exception of PANC-1 where **15** displayed a moderate effect on cancer cell viability; 3) the Se 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 Se atom with S causes a dramatic reduction in or the total loss of effect on cancer cell viability of the ASA-modified scaffold (**8** versus **16;** Table 2).

Based on the potency of **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)]. Compound **8** was effective in reducing cell viability of all of the above mentioned cancer cell lines with IC_{50} values in the low μM range at 48 and 72 h of treatment (Table 3 and Figure 3). The cytotoxic effect of **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 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 **8** for 24 h and were compared with 1 mM of ASA. As shown in Figure 3H, ASA had no significant effect on population of dead cells even at 1 mM concentration, while **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, **8** is >5 times more potent in causing HCT116 cell death than parent ASA.

Compound 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 **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 **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 **8**, which are toxic towards CRC cells at 48 h (Figure 4A), and for that matter, all other cancer cells

tested (see Tables 1 and 2). These data demonstrated that **8** is selectively toxic to cancer cells but not transformed cells. Furthermore, we compared **8** with the current therapy for CRC, 5-FU, in a cell viability assay. Compound **8** was more effective in inhibiting CRC cell viability than 5-FU or ASA (Figure 4B). Therefore, **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, **8** showed high selectivity towards cancer cells suggesting that this compound may have a very high therapeutic window.

Compound 8 inhibited CRC cell growth by inducing cell cycle arrest.

To explore the underlying mechanism for the reduced cell viability of CRC cells by **8**, we investigated effect of **8** on the cell cycle by Flow cytometry. Treatment of HCT116 cells with **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 5A-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.[70] Hence, the effect of **8** on these cell regulatory proteins was investigated. Treatment of CRC cells with **8** for 24 h, showed an increasing p21 expression in a dose dependent manner (Figure 5C). Both Cyclins B1 and E1 were also inhibited at similar doses where p21 expression was induced. These results suggest that **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.

Compound 8 induced apoptosis in CRC cells.

Since CRC cells treated with **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 6) of compound **8** for 24 h. Apoptosis was measured by using Muse Annexin V & Dead cell assay kit following manufactures protocol. As shown in Figure 6A, cells treated with vehicle (DMSO), were mostly in bottom left quadrant (BLQ). As compound **8** dose was increased, cells shifted from healthy state to apoptotic. At 2.5 µM concentration of compound **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 compound **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, **8** increased caspase 3/7 activity in a dose dependent manner (Figure 6B). Figures 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 **8** to western bot analysis. As shown in Figure 6E, compared to the vehicle treated cells, exposure to **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.[71] Overall, our results confirm that compound **8** inhibits growth of CRC cells by inducing apoptosis.

Compound 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.[72] To investigate whether induced cytotoxic effect of **8** is related

with ROS production, we measured total ROS levels in **8** treated CRC cells following a previously reported method.[73] The CRC cells (HCT116) were treated with **8** for 3, 6, and 24 h and total ROS level was measured. As shown in the Figure 7, the total ROS levels did not change significantly with the treatment of **8** at early time points (3 and 6 h), however, at 24 h ROS levels increased to about 66% in **8** treated cells. These results strongly suggest that Se containing **8** is not able to induce ROS production at early time points. However, longer exposure of cells with **8** induces ROS production which is correlated with an increase in apoptosis, suggesting that the induction of cell death by **8** may be due the ROS production.

Conclusions

In conclusion, 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, **8** emerged as the most potent anti-cancer agent, particularly against CRC cells. Compound **8** showed more selectivity towards cancer cells and was >10 fold potent than current CRC therapeutic agent, 5-FU. Our studies revealed that **8** inhibited CRC growth via cell cycle arrest leading to apoptosis. In addition, **8** induced apoptotic cell death evident by Caspase 3/7 activity and positivity of phosphatidylserine (PS) on outer membrane of CRC cells. Further, **8** also increased ROS levels in correlation with apoptosis at 24 h. Overall, **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.[74] Such issues with compound **8** are not anticipated since (i) it is several fold more potent than ASA and therefore much lower concentration of **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 **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 **8**.

EXPERIMENTAL SECTION

Chemistry

General. All reactions were conducted in round-bottom flasks equipped with a Teflon-coated magnetic stirring bar. Experiments involving moisture and/or air sensitive components were performed under a nitrogen atmosphere in oven-dried glassware. Reagents, starting materials, and anhydrous solvents were purchased from commercial suppliers and were used as received. Reaction courses were monitored by thin-layer chromatography (TLC) on pre-coated silica gel 60 F_{254} aluminum sheets (Merck, Darmstadt, Germany), and the spots were visualized under UV light. The crude reaction products were purified by silica gel column chromatography using silica gel 60 Å (Merck, 230-400 mesh), and hexane/ethyl acetate was used as the elution solvent. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Advance 500 instrument in either D_2O or $CDCl_3$, operating at 500 and 125 MHz, respectively. Chemical shifts are reported in δ values (ppm) and J values are reported in hertz (Hz). The signals are quoted as s (singlet), d (doublet), t (triplet), sept (septet), q (quadruplet), m (multiplet), dd (doublet of doublets), and td (triplet of doublets). High resolution (ESI) MS were carried out at the Chemistry Instrumentation Center, State University of New York at Buffalo, NY. The purity of the final compounds (≥98%) was quantified by high performance liquid chromatography analysis.

Synthesis of 2-selenocyanatoethanamine (**1**). KSeCN (3.0 g, 14.64 mmol) was added to a mixture of 2-bromoethylamine hydrobromide (2.1 g, 14.64 mmol) in anhydrous acetonitrile (40 mL) under a nitrogen atmosphere. The reaction mixture was stirred at room temperature for 14 h and the solid formed in the reaction was filtered, washed with diethyl ether and dried under vacuum. The isolated solid was used without purification for further reactions. Yield, 91%. ^{1}H NMR (500 MHz, D_2O) δ 3.59 (t, 2H, J = 7.5 Hz, CH_2-SeCN); 3.95 (d, 2H, J = 7.5 Hz, CH_2-NH).

General procedure for the preparation of NSAID-SeCN derivatives (**4** and **5**). To a mixture of 2-selenocyanatoethanamine (**1**, 0.45 g, 3.0 mmol) and TEA (0.68 ml, 4.9 mmol) in anhydrous methylene chloride (25 mL) was added the corresponding acyl chloride (1.96 mmol). The reaction mixture was stirred at room temperature under a nitrogen atmosphere for 18-24 h, and then filtered. The filtrate was concentrated in vacuo and partitioned between water and methylene chloride. The organic layer was dried with magnesium sulfate and concentrated in vacuo. The crude product was purified using silica gel chromatography with an hexane/ethyl acetate gradient (starting from 10% up to 40% ethyl acetate) to afford the desired product.

2-(4-isobutylphenyl)-N-(2-selenocyanatoethyl)propanamide ***(4)****.* The title compound was synthesized from **1** and the acyl chloride **3** (0.4 g, 1.96 mmol) according to the general procedure described above. Yield 44%; mp 145−147 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.92 (d, 6H, J = 6.5 Hz, 2-CH_3); 1.55 (d, 3H, J = 7.5 Hz, CH_3); 1.87 (sept, 1H, J = 7.0 Hz, CH); 2.47 (d, 1H, J = 7.0 Hz, CH_2-Ph); 3.18 (td, 2H, J = 8.0 and 1.0 Hz, CH_2-SeCN); 3.68 (q, 1H, J = 7.5 Hz, CH); 3.84 (td, 2H, J = 8.0 and 2.5 Hz, CH_2-NH); 7.13 (d, 2H, J = 8.0 Hz, H-3, H-5); 7.20 (d, 2H, J = 8.0 Hz, H-2, H-6). ^{13}C NMR (125 MHz, $CDCl_3$) δ 18.4 (CH_3), 22.4 ($2CH_3$), 26.7 (CH), 30.1 (CH_2-

Se), 45.0 (CH_2-NH), 47.4 (CH), 55.7 (CH_2-Ph), 127.4, 129.8, 137.2, 141.1 (aryl), 158.4 (SeCN), 175.6 (C=O). HRMS (ESI) calcd for $C_{16}H_{23}N_2OSe$ $[M + H]^+$: 339.0970. Found: 339.0971.

*(S)-2-(6-methoxy-2-naphthyl)-N-(2-selenocyanatoethyl)propanamide (**5**).* The title compound was synthesized from **1** and (*S*)-2-(6-methoxy-2-naphthyl)propionyl chloride (0.49 g, 1.96 mmol) according to the general procedure described above. Yield 29%; mp 157−158 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.64 (d, 3H, J = 7.0 Hz); 3.18 (td, 2H, J = 7.5 and 3.0 Hz); 3.81 (m, 2H); 3.87 (q, 1H, J = 7.5 Hz); 3.95 (s, 3H); 7.13 (d, 1H, J = 2.5 Hz); 7.17 (dd, 1H, J = 9.0 and 2.5 Hz); 7.38 (dd, 1H, J = 8.5 and 1.5 Hz); 7.71 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 18.3 (CH_3), 26.2 (CH_2-Se), 47.8 (CH_2-NH), 54.6 (CH), 55.3 (OCH_3), 105.7 (SeCN), 119.2, 126.1, 126.4, 127.7, 129.0, 129.3, 133.9, 135.2, 157.8, 160.5 (aryl), 176.1 (C=O). HRMS (ESI) calcd for $C_{17}H_{18}N_2O_2Se$ $[M + H]^+$: 363.0606. Found: 363.0609.

Synthesis of 2-((2-bromoethyl)carbamoyl)phenyl acetate (**7**). To a mixture of 2-bromoethylamine hydrobromide (2.94 mmol, 1.5 equiv) and TEA (4.9 mmol, 2.5 equiv) in anhydrous methylene chloride (25 mL) was added *O*-acetylsalicyloyl chloride (**6**, 1.96 mmol, 1 equiv). The reaction mixture was stirred at room temperature under a nitrogen atmosphere for 6 h. The mixture was then filtered. The filtrate was concentrated in vacuo and then partitioned between water and methylene chloride. The organic layer was dried over magnesium sulfate and concentrated in vacuo. The crude product was purified using silica gel chromatography with an hexane/ethyl acetate gradient (starting from 10% up to 60% ethyl acetate) to afford the desired product in 51% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.42 (s, 3H, CH_3), 3.62 (t, 2H, J = 6.0 Hz, CH_2); 3.89 (q, 2H, J = 6.0 Hz, CH_2); 6.83 (br s, 1H, NH); 7.16 (d, 1H, J = 8.0, H-6); 7.36 (dt, 1H, J = 7.5 and 1.0 Hz, H-5); 7.52 (dt, 1H, J = 7.5 and 1.5 Hz, H-4); 7.78 (dd, 1H, J = 8.0 and 2.0 Hz, H-3).

Synthesis of 2-((2-selenocyanatoethyl)carbamoyl)phenyl acetate (**8**). To a mixture of potassium selenocyanate (1.15 mmol, 1 equiv) in anhydrous acetonitrile (25 mL) was added 2-((2-bromoethyl)carbamoyl)phenyl acetate (**7**, 1.15 mmol, 1 equiv). The reaction mixture was stirred at room temperature under a nitrogen atmosphere for 16 h and filtered. The filtrate was concentrated in vacuo and then partitioned between water and methylene chloride. The organic layer was dried with magnesium sulfate and concentrated in vacuo. The crude product was purified using silica gel chromatography with a hexane/ethyl acetate gradient (starting from 10% up to 40% ethyl acetate) to afford the desired product. Overall yield 41%; mp 103-104 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.38 (s, 3H, CH_3); 3.33 (t, 2H, J = 6.2 Hz, CH_2); 3.89 (q, 2H, J = 6.1 Hz, CH_2); 6.81 (s, 1H, NH); 7.15 (dd, 1H, J = 8.2 and 1.0 Hz, H-6); 7.34 (m, 1H, H-5); 7.53 (m, 1H, H-4); 7.78 (dd, 1H, J = 7.7 and 1.7 Hz, H-3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.2 (CH_3), 28.9 (CH_2-Se), 40.3 (CH_2-NH), 101.2 (SeCN), 123.3, 126.3, 127.3, 129.5, 132.4, 148.2 (aryl), 166.5, 169.0 (C=O). HRMS (ESI) calcd for $C_{12}H_{12}N_2NaO_3Se$ $[M + Na]^+$: 334.9905. Found: 334.9905.

Synthesis of 2-selenocyanatoethyl 2-acetoxybenzoate (**10**). The title compound was synthesized from 2-selenocyanatoethan-1-ol (**9**, 0.38 g, 2.5 mmol) and *O*-acetylsalicyloyl chloride (**6**, 0.50 g, 2.5 mmol**)** according to the procedure described above for the preparation of NSAID-SeCN derivatives, stirring the reaction mixture for 22 h. Overall yield 79%; mp 48-50 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.37 (s, 3H, CH_3); 3.39 (t, 2H, J = 6.5 Hz, CH_2); 4.69 (t, 2H, J = 6.5 Hz, CH_2); 7.14 (dd, 1H, J = 8.0 and 1.0 Hz, phenyl H-3); 7.36 (td, 1H, J = 8.0 and 1.0 Hz, phenyl H-5); 7.62 (td, 1H, J = 8.0 and 1.5 Hz, phenyl H-4); 8.07 (dd, 1H, J = 8.0 and 1.5 Hz, phenyl H-6). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.0 (CH_3), 27.1 (CH_2-Se), 63.1 (CH_2-O), 100.5 (SeCN),

122.3, 124.0, 126.2, 131.8, 134.5, 151.6 (aryl), 163.8, 169.6 (C=O). HRMS (ESI) calcd for $C_{12}H_{11}NNaO_4Se$ $[M + Na]^+$: 335.9746. Found: 335.9747.

General procedure for the preparation of selenoester derivatives (**12** and **13**). To a water solution, previously filtered through celite, of sodium 2-acetoxybenzoselenate (**11**, 3.36 mmol, 1 equiv), obtained *in situ* following the procedure reported by Sanmartin *et al.*,[66] 1,2-dibromoethane (3.36 mmol, 1 equiv) dissolved in THF (20 mL) was added. The reaction mixture was stirred at room temperature for 30 min, filtered through celite and extracted with methylene chloride (3 x 20 mL). The organic layers were dried with magnesium sulfate and concentrated in vacuo. The crude product contained a mixture of the compounds **12** and **13**, which was separated and purified using silica gel chromatography with a hexane/ethyl acetate gradient (starting from 5% up to 15% ethyl acetate).

2-(((2-Bromoethyl)selenyl)carbonyl)phenyl acetate (12). Overall yield 31%; mp 156−158 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.37 (s, 3H, CH_3); 3.45 (t, 2H, J = 10.0 Hz, CH_2-Se); 3.61 (t, 2H, J = 10.0 Hz, CH_2-Br); 7.14 (d, 1H, J = 8.0 Hz, phenyl H-3); 7.35 (t, 1H, J = 8.0 Hz, phenyl H-5); 7.59 (td, 1H, J = 8.0 and 1.5 Hz, phenyl H-4); 7.89 (dd, 1H, J = 8.0 and 1.5 Hz, phenyl H-6). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.2 (CH_3), 27.1 (CH_2-Se), 30.7 (CH_2-Br), 124.1, 126.4, 129.8, 131.3, 134.2, 147.4 (aryl), 169.1, 190.6 (C=O). HRMS (ESI) calcd for $C_{11}H_{11}O_3BrNaSe$ $[M + Na]^+$: 372.8949. Found: 372.8950.

((Ethane-1,2-diylbis(selanediyl))bis(carbonyl))bis(2,1-phenylene) diacetate (13). Overall yield 3%; mp 154-158 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.38 (s, 6H, 2(CH_3)); 3.39 (s, 4H, 2(CH_2)); 7.15 (dd, 1H, J = 8.0 and 1.0 Hz, phenyl H-3); 7.37 (td, 1H, J = 8.0 and 1.0 Hz, phenyl H-5); 7.59 (td, 1H, J = 8.0 and 1.5 Hz, phenyl H-4); 7.93 (dd, 1H, J = 8.0 and 1.5 Hz, phenyl H-

6). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.2 (CH_3), 26.1 (CH_2-Se), 124.1, 126.3, 129.8, 131.7, 133.9, 147.3 (aryl), 169.2, 191.6 (C=O). HRMS (ESI) calcd for $C_{20}H_{18}O_6NaSe_2$ $[M + Na]^+$: 536.9326. Found: 536.9328.

Synthesis of 2-(((2-selenocyanatoethyl)selenyl)carbonyl)phenyl acetate (**14**). The title compound was synthesized by reacting potassium selenocyanate (0.11 g, 0.76 mmol) and **12** (0.25 g, 0.71 mmol) according to the procedure described for the preparation of compound **8**, stirring the reaction mixture for 20 h. Overall yield 72%; mp 34−35 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.36 (s, 3H, CH_3); 3.36 (dd, 2H, J = 10.0 and 6.0 Hz, CH_2-SeCN); 3.48 (dd, 2H, J = 10.0 and 6.0 Hz, CH_2-Se-C(=O)); 7.14 (d, 1H, J = 8.0 Hz, phenyl H-3); 7.35 (t, 1H, J = 8.0 Hz, phenyl H-5); 7.59 (td, 1H, J = 8.0 and 1.5 Hz, phenyl H-4); 7.88 (dd, 1H, J = 8.0 and 1.5 Hz, phenyl H-6). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.2 (CH_3), 27.1 (CH_2-SeCN), 28.9 (CH_2-Se), 100.8 (SeCN), 124.2, 126.4, 129.8, 131.0, 134.4, 147.5 (aryl), 169.1, 190.6 (C=O). HRMS (ESI) calcd for $C_{12}H_{11}O_3NNaSe_2$ $[M + Na]^+$: 399.8962. Found: 399.8959.

Synthesis of 2-(2-acetoxybenzamido)-3-(methylselenyl)propanoic acid (**15**). The title compound was synthesized from *Se*-(methyl)selenocysteine hydrochloride (1.09 g, 5.0 mmol) and *O*-acetylsalicyloyl chloride (**6**, 1.00 g, 5.0 mmol**)** according to the procedure described for the preparation of NSAID-SeCN derivatives, stirring the reaction mixture for 32 h. Overall yield 10%; mp 105−107 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.05 (m, 3H, $SeCH_3$); 2.45 (s, 3H, CH_3); 3.16 (t, 2H, J = 6.0 Hz, CH_2-Se); 5.12 (dd, 1H, J = 11.0 and 6.0 Hz, CH-NH); 7.18 (d, 1H, J = 8.0 Hz, phenyl H-3); 7.35 (td, 1H, J = 8.0 and 1.0 Hz, phenyl H-5); 7.52-7.56 (m, 1H, phenyl H-4); 7.60 (d, J = 7.0 Hz, 1H, COOH); 7.96 (dd, 1H, J = 8.0 and 1.5 Hz, phenyl H-6). ^{13}C NMR (125 MHz, $CDCl_3$) δ 5.5 (Se-CH_3), 21.3 (CH_3), 26.7 (CH_2-Se), 52.6 (CH-NH), 123.4, 126.2,

126.4, 130.7, 132.6, 148.3 (aryl), 165.2, 169.1, 174.2 (C=O). HRMS (ESI) calcd for $C_{13}H_{15}O_5NNaSe$ $[M + Na]^+$: 368.0008. Found: 368.0005.

Synthesis of 2-((2-thiocyanatoethyl)carbamoyl)phenyl acetate (**16**). The title compound was synthesized from ammonium thiocyanate (0.08 mg, 1.1 mmol) and **7** (0.31 g, 1.1 mmol) according to the procedure described for the preparation of compound **8**, stirring the reaction mixture for 18 h. Overall yield 54%; mp 37−40 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.39 (s, 3H, CH_3); 3.25 (t, 2H, J = 6.0 Hz, CH_2-S); 3.84 (dd, 2H, J = 12.0 and 6.0 Hz, CH_2-NH); 6.79 (s, 1H, NH); 7.16 (d, 1H, J = 8.0 Hz, phenyl H-3); 7.35 (t, 1H, J = 8.0 Hz, phenyl H-5); 7.51-7.55 (m, 1H, phenyl H-4); 7.81 (dd, 1H, J = 8.0 and 1.5 Hz, phenyl H-6). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.2 (CH_3), 33.2 (CH_2-S), 39.5 (CH_2-NH), 111.6 (SCN), 123.3, 126.3, 127.4, 129.6, 133.3, 148.2 (aryl), 163.3, 169.0 (C=O). HRMS (ESI) calcd for $C_{12}H_{12}O_3N_2NaS$ $[M + Na]^+$: 287.0461. Found: 287.0461.

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_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.[75] Briefly, 3000 cells were incubated with different concentrations of the test compounds in triplicate in a 96-well plate for 24 h, 48 h 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 **8** (15 µM) for 24 h. Cells were trypsinized and subjected to live and dead analysis using LIVE/DEAD Viability/Cytotoxicity Kit according to manufactures protocol. 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) at 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.[76] 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 **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 PBS. After staining for 15 minutes, 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 **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.[77] The resulting lysates were spun at 15,000 rpm for a duration of 10 minutes 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 wasachieved by using Restore Western blot stripping buffer (Thermo Scientific, USA).

Annexin V assay: In order to evaluate the ability of **8** to induce apoptosis, HCT116 (5×10^5) cells were treated with increasing dose of compound **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 compound **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 **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. ROS (-) population represented by M1 peak, while ROS (+) cells represented by M2 peak

in the graph. H_2O_2 (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.

ASSOCIATED CONTENT

Supporting Information. Copies of the ^{1}H NMR and ^{13}C NMR spectra for new compounds **4, 5, 8, 10 and 12-16,** and SMILES and IC_{50} data (CSV). This material is available free of charge via the internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

ASA, Aspirin; Ibu, Ibuprofen; Nap, Naproxen; CRC, colorectal cancer; mTOR, mammalian target of rapamycin; AMPK, AMP-activated protein kinase; MEFs, mouse embryonic fibroblasts; nHDF, human dermal fibroblast; PS, phospatidylserine; ROS, Reactive Oxygen Species.

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

		IC_{50} (μM)[a]					
	Hr.	**4**	**Ibu**	**5**	**Nap**	**8**	**ASA**
Colon cancer[b]	**24**	>50	>50	>50	>50	3.4 ±1.1	>50
	48	>50	>50	>50	>50	2.1 ±1.0	>50
	72	>50	>50	>50	>50	2.1 ±1.3	>50
Breast cancer[c]	**24**	>50	>50	>50	>50	21.1 ±0.4	>50
	48	>50	>50	>50	>50	5.5 ±1.0	>50
	72	>50	>50	>50	>50	6.0 ±1.9	>50
Pancreatic cancer[d]	**24**	>50	>50	>50	>50	12.1 ±2.2	>50
	48	>50	>50	>50	>50	2.1 ±0.5	>50
	72	>50	>50	>50	>50	2.6 ±1.0	>50
Melanoma[e]	**24**	>50	>50	>50	>50	24.3 ± 5.8	>50
	48	>50	>50	>50	>50	4.0 ±0.6	>50
	72	>50	>50	>50	>50	2.9 ±0.7	>50

[a]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 **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. The effects of Se-ASA and S-ASA hybrid derivatives on cancer cell viability.

		IC_{50} (μM)[a]						
	Hr.	**8**	**10**	**12**	**13**	**14**	**15**	**16**
Colon cancer[b]	**24**	3.4 ± 1.1	10.2 ± 13.3	28.5 ± 13.3	18.6 ± 3.8	>50	>50	>50
	48	2.1 ± 0.9	30.9 ± 17.0	27.7 ± 23.2	3.5 ± 1.0	27.5 ± 9.6	>50	>50
	72	2.1 ± 1.3	20.2 ± 9.4	19.3 ± 13.6	5.5 ± 4.8	17.7 ± 9.3	>50	>50
Breast cancer[c]	**24**	21.1 ± 0.4	>50	23.7 ± 10.6	18.6 ± 3.8	>50	>50	>50
	48	5.5 ± 1.0	23.8 ± 7.8	18.7 ± 10.8	11.0 ± 3.4	34.2 ± 8.4	>50	>50
	72	6.0 ±1.9	34.3 ± 30.4	8.7 ± 3.4	7.6 ± 2.2	18.6 ± 3.2	>50	>50
Pancreatic cancer[d]	**24**	12.1 ±2.2	38.7 ± 16.8	39.3 ± 32.5	33.4 ± 0.8	>50	>50	>50
	48	2.1 ±0.5	36.1 ± 1.6	>50	13.5 ± 0.3	18.9 ± 5.3	26.1 ± 9.4	17.7 ± 6.9
	72	2.6 ±1.0	15.1 ± 5.1	>50	3.4 ± 0.7	9.6 ± 2.8	27.6 ± 13.4	8.7 ± 2.1
Melanoma[e]	**24**	24.3 ± 5.8	33.9 ± 12.1	22.7 ± 7.9	17.2 ± 2.1	>50	>50	>50
	48	3.9 ±0.6	14.5 ± 3.2	19.4 ± 9.6	3.6 ± 3.8	15.8 ± 3.2	>50	>50
	72	2.9 ±0.7	17.6 ± 8.7	10.2 ± 5.0	3.0 ± 0.5	12.7 ± 3.0	>50	>50

[a]Listed data show the IC_{50} values (± SD) of different agents in inhibiting 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). IC_{50} was calculated by non-linear regression (variable slope) analysis using GraphPad Prism.

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

	IC_{50} (μM)[g]		
	24	**48**	**72**
Colon[a]	13.3 ±2.3	2.2 ±0.2	2.9 ±0.2
Colon[b]	16.2 ±2.1	2.5 ±0.3	2.1 ±0.2
Prostate[c]	8.2 ±3.0	3.4 ±0.7	2.9 ±0.5
Lung[d]	>50	2.4 ±0.8	1.3 ±0.2
Liver[e]	16.0 ±6.7	5.0 ±0.8	4.3 ± 0.4
Ovarian[f]	>50	5.3 ±1.6	4.4 ±0.4

[g]Data represent mean IC_{50} values (± SD) of **8** in inhibiting cell viability, determined by the MTT assay in triplicates. [a]Human colon carcinoma cell line (RKO). [b]Human colon carcinoma cell line (HCT116). [c]Human prostate adenocarcinoma cell line (DU 145). [d]Human non-small cell lung cancer cell line (NCI-H460). [e]Human liver hepatocellular carcinoma cell line (HEPG2). [e]Human ovarian carcinoma cell line (SKOV3). Representative curves are shown in Figure 3. IC_{50} was calculated by non-linear regression (variable slope) analysis using GraphPad Prism.

FIGURES:

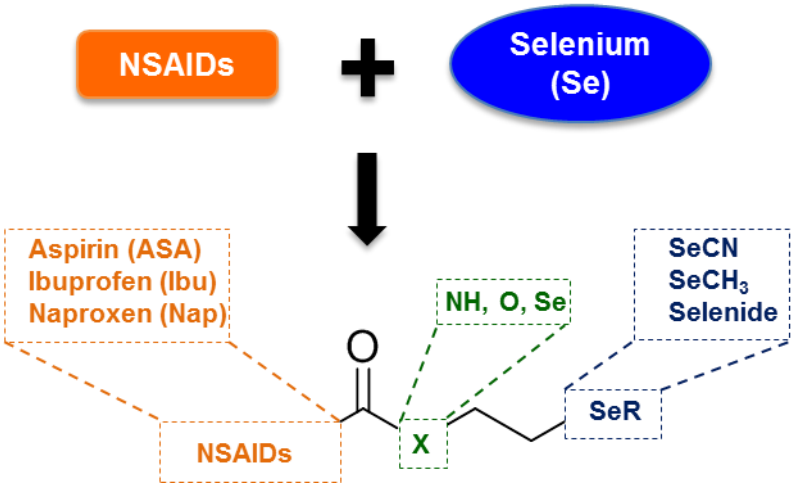


Figure 1. Structural design of the novel hybrid Se-NSAID derivatives

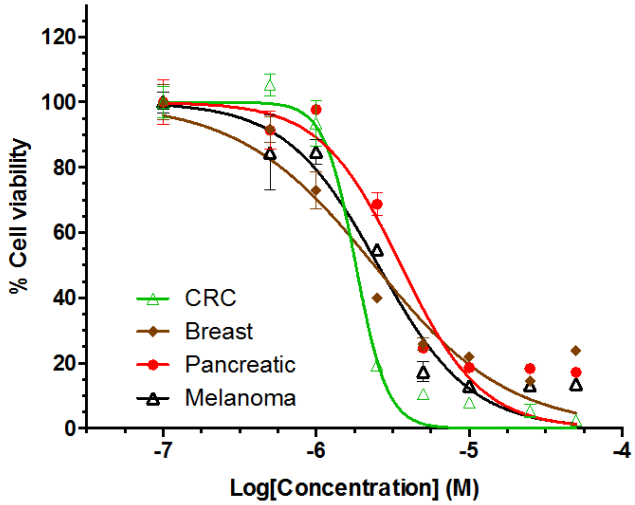


Figure 2. Se-ASA hybrid compound **8** potently inhibits different cancer cells growth. CRC (HT29), Breast cancer (MDA-MB231), Pancreatic cancer (PANC-1) and Melanoma (UACC903) cells were treated with increasing doses of **8** for 48 h. Cell viability was determined by the MTT assay. Growth curves were obtained by using non-linear regression analysis using GraphPad Prism software. Results were normalized to vehicle control. Calculated IC_{50} values for 48 h are shown in Table 1.

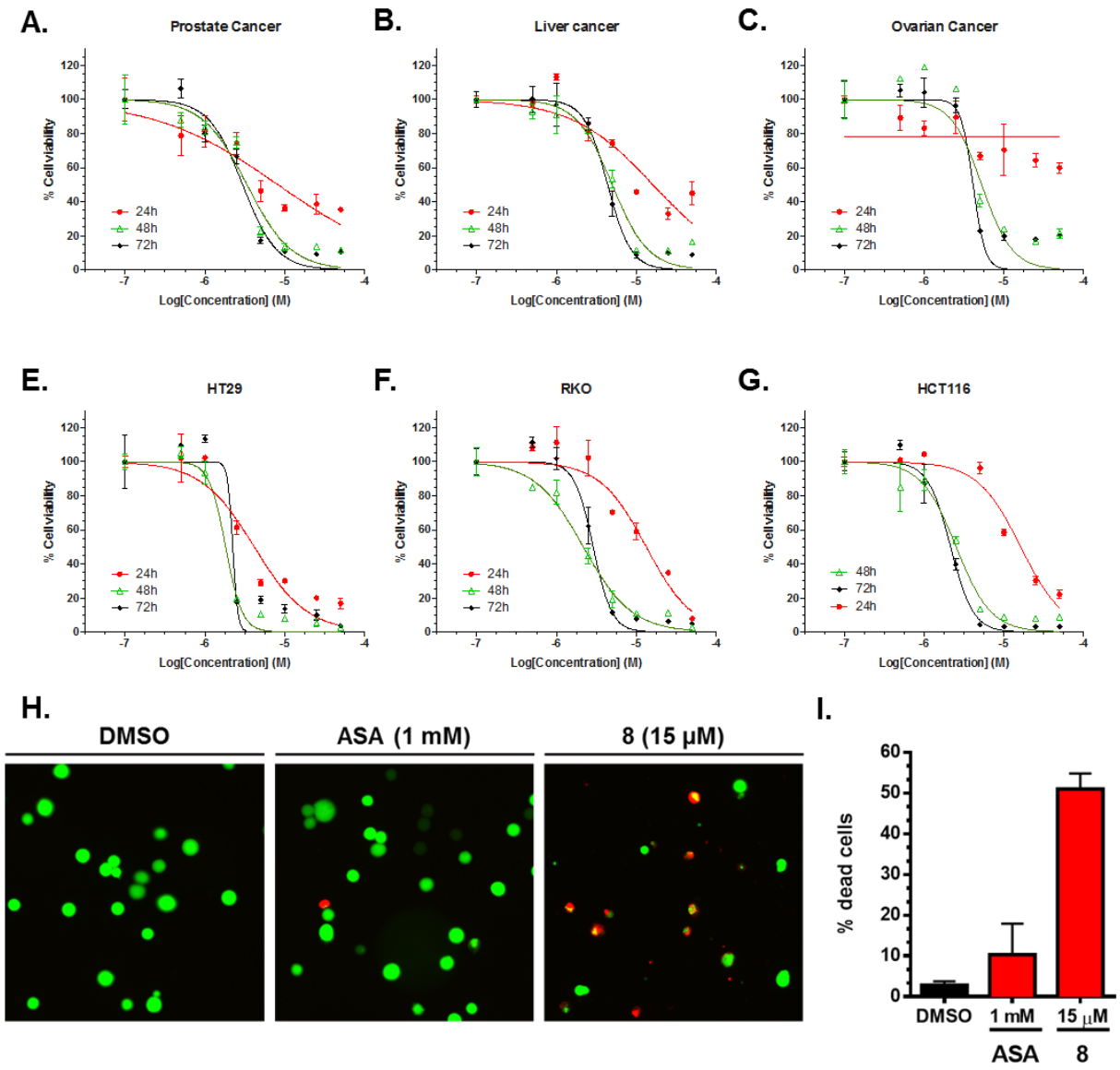


Figure 3. Compound **8** reduced cell viability of different cancer cells (**A-C**), including CRC cells (**E-G**). **(A)** Prostate cancer (DU 145), **(B)** liver cancer (HEPG2), **(C)** ovarian cancer (SKOV3) and (**E-G**) CRC cells HT29, RKO, and HCT116 were treated for 24, 48 and 72 h with different doses (0.5-50 µM) of **8**. Cell viability was determined using the MTT assay. Growth curves were obtained by using non-linear regression analysis using GraphPad Prism software. Results were normalized to vehicle control. IC_{50} values are shown in Tables 1 (for HT29 cells) and 3 (for the rest of the cell lines). (**H**) HCT116 cells were treated with IC_{50} concentration of **8** (at 24 h**,** 15 µM) and 1 mM ASA for 24 h. Cells were subjected to

LIVE/DEAD analysis using microcopy. 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.

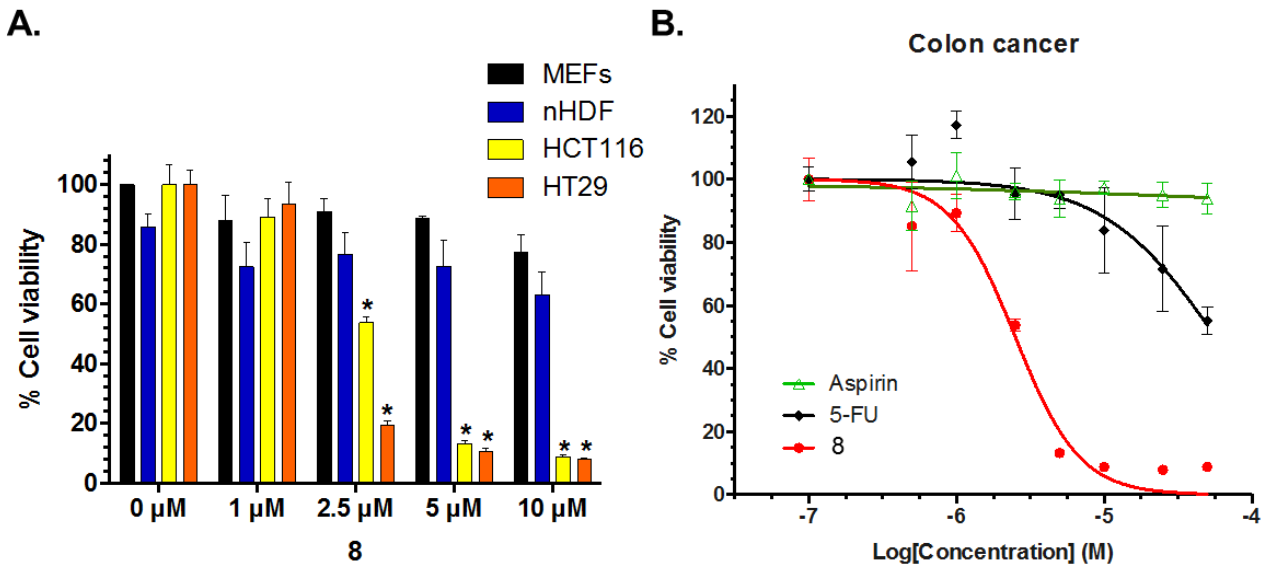


Figure 4. Compound **8** has more selectivity towards cancer cells and is more potent than current CRC therapy in reducing cell viability. **(A)** MEFs, nHDF and CRC cells (HCT116 and HT29) were treated with increasing concentration of **8** for 48 h. Cell viability was measured by the MTT assay. Compound **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; (*) $p < 0.005$.

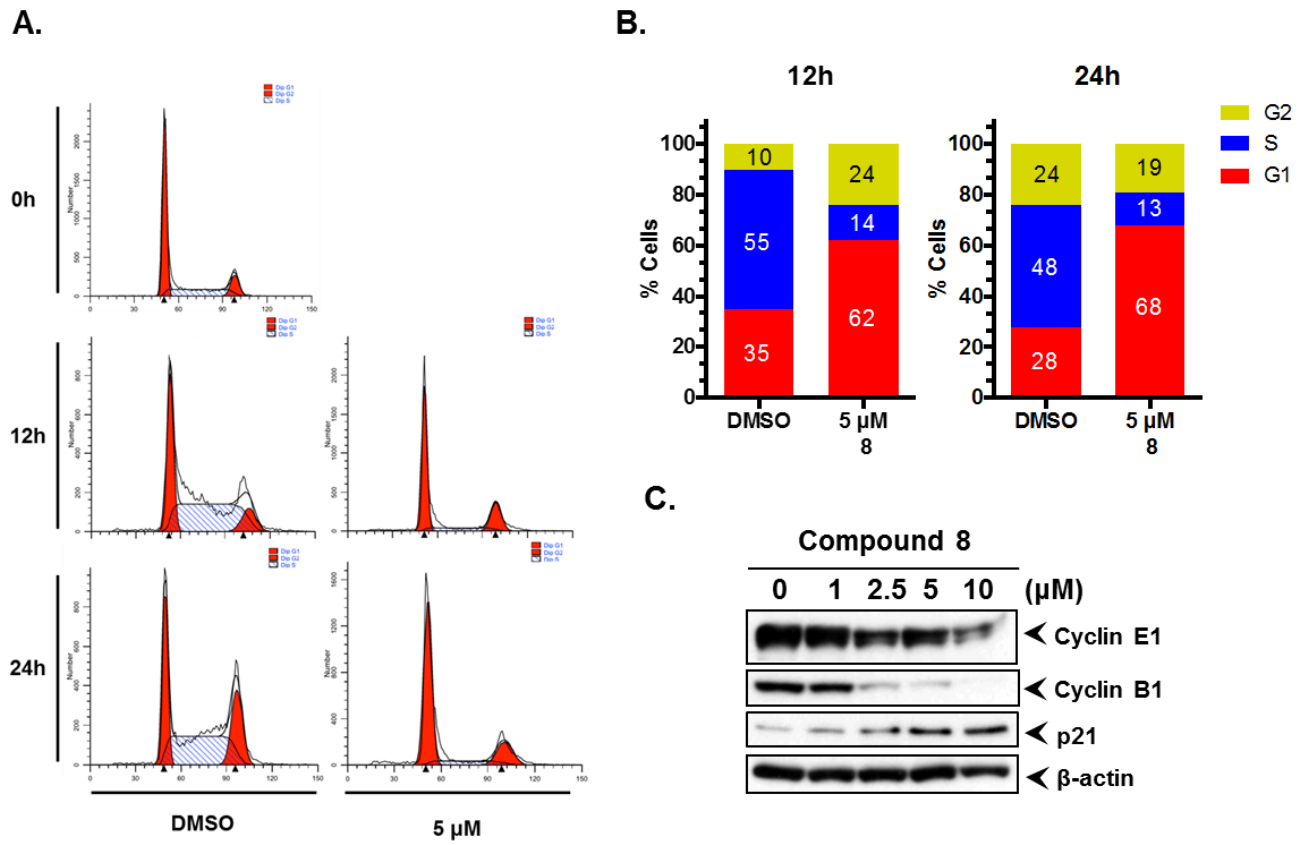


Figure 5. Compound **8** inhibited the CRC cell cycle**.** HCT116 were serum starved for 72 h, followed by treatment with **8** (5 μM) in media with serum as described in the Experimental Section. **(A)** Cell cycle phase distribution was determined at 12 h and 24 h by flow-cytometry. **(B)** Bar diagram representing distribution of cells in different phases of cell cycle. **(C)** CRC cells were treated with **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.

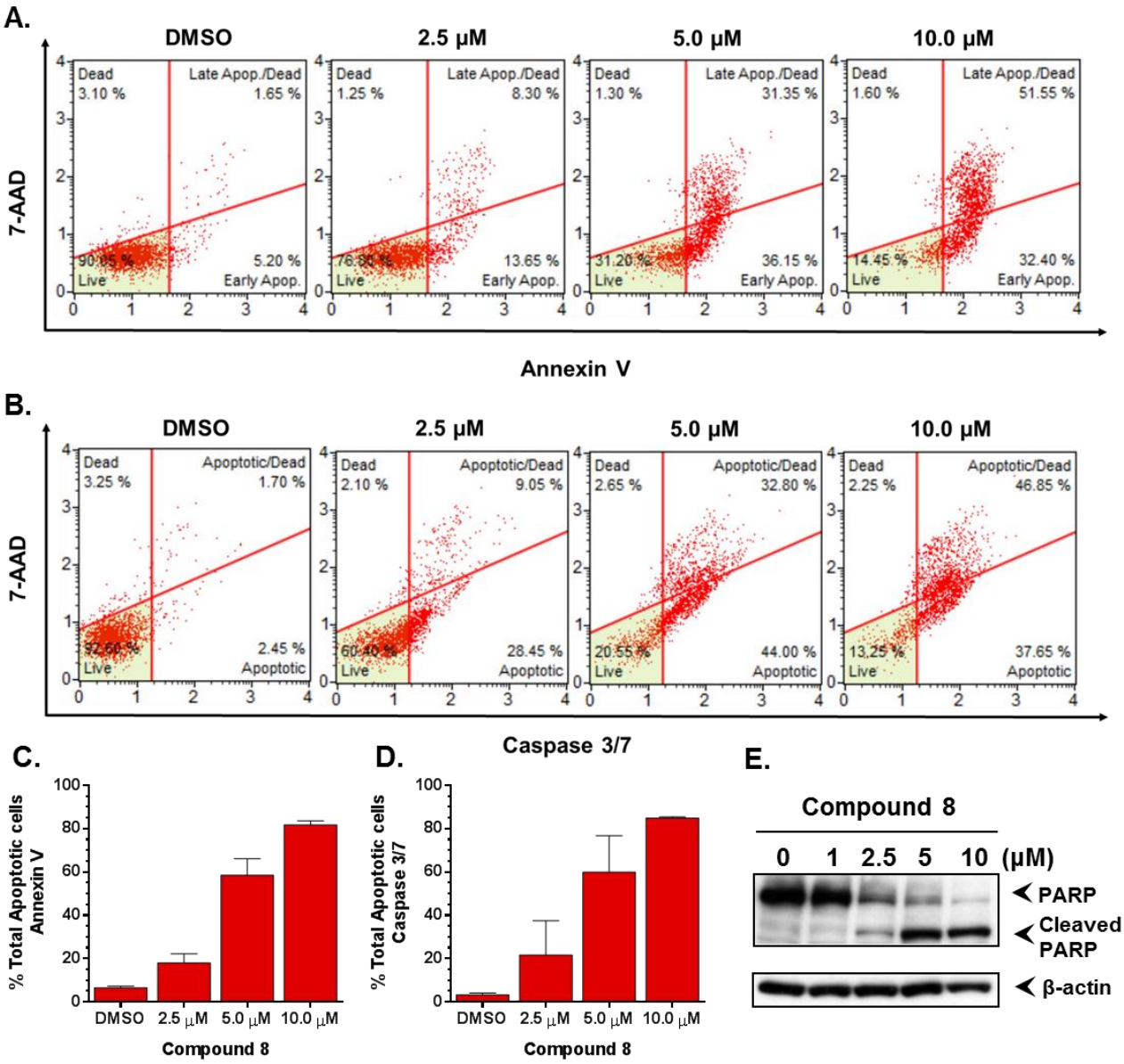


Figure 6. Compound **8** induced apoptotic death in CRC cells. HCT116 cells were treated with compound 8 for 24 h. **(A-B)** Cells were subjected to Annexin-V and caspase 3/7 activity assay using Muse Cell analyzer. The method lead to four different population of cells: Healthy cells (Annexin-V negative, 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 compound **8** were subjected to western blot analysis and probed for PARP protein. β-actin was used as loading control.

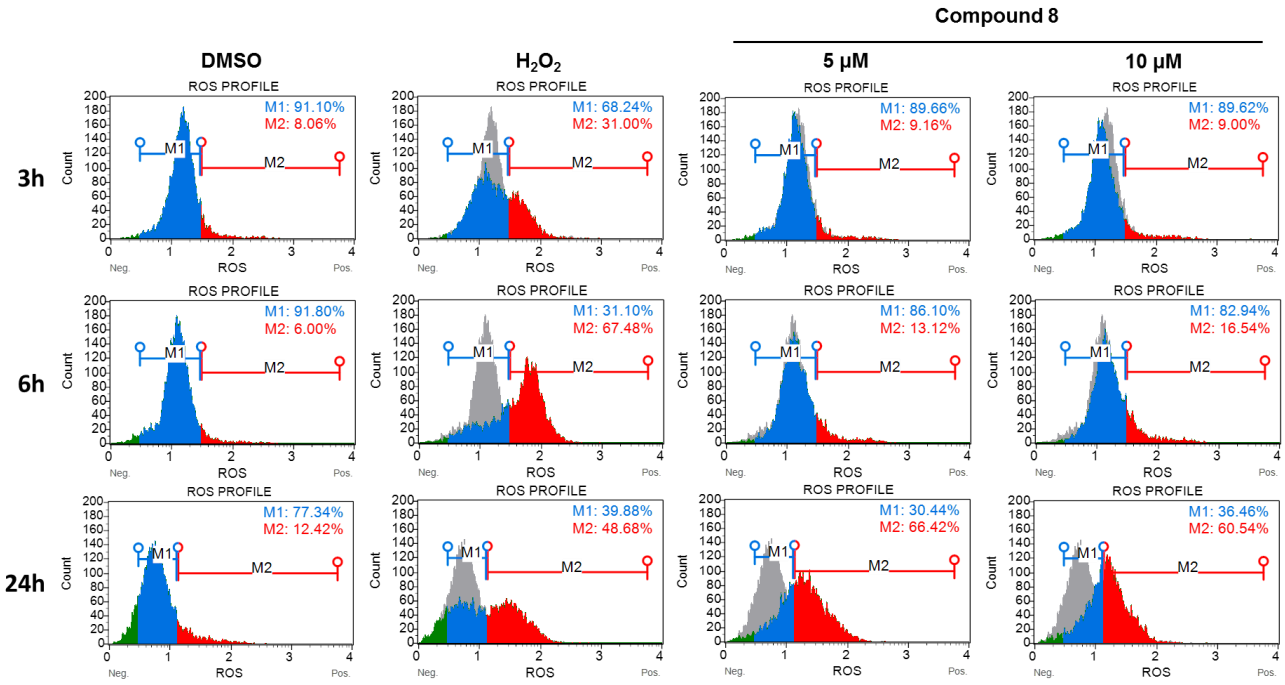
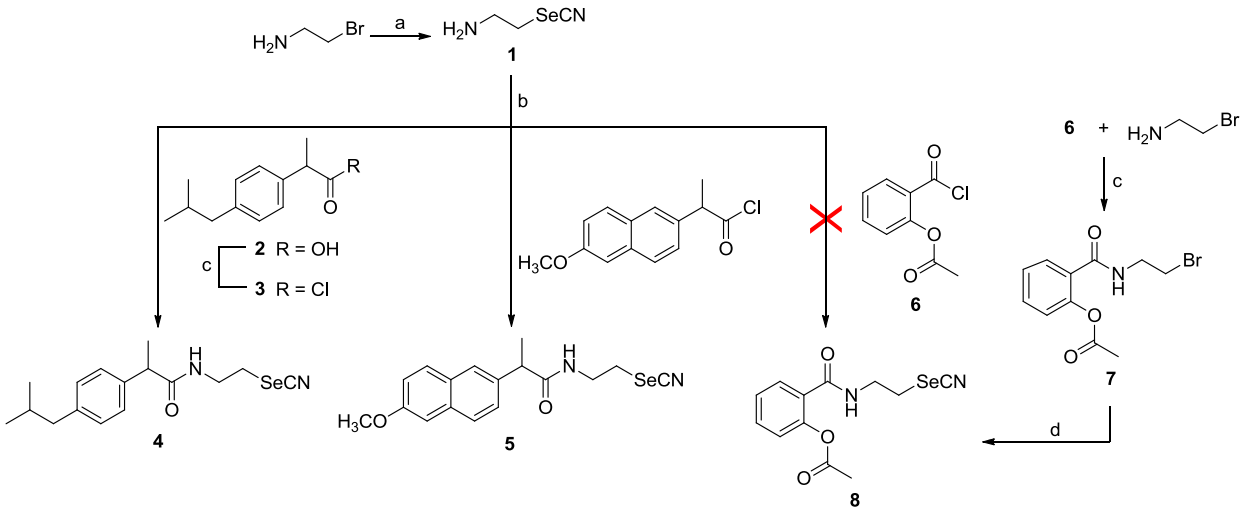


Figure 7. Effect of Compound **8** on ROS levels. HCT116 cells were treated with **8** for 3, 6 and 24 h and subjected to Muse flow cytometry based oxidative stress analysis for total ROS levels measurement. The histogram shows two different populations of cells: ROS (-) (M1 peak/blue color) and ROS (+) (M2 peak/Red color). Gray colored peak is the overlay of DMSO M1 peak to compare the shift of the M1 peak with treatment of **8**. H_2O_2 was used as a positive control.

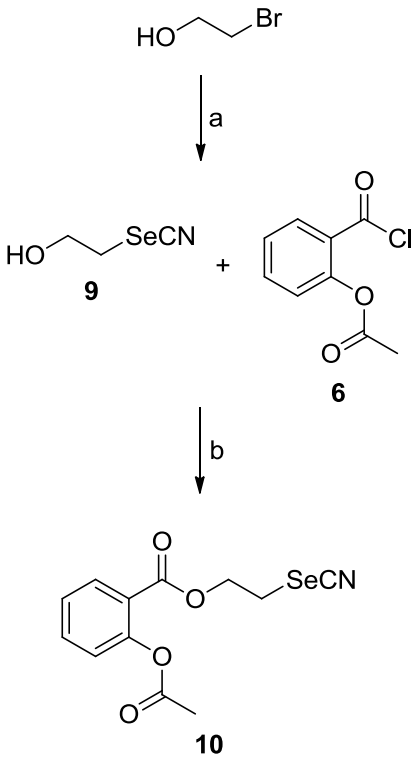
SCHEMES:

Scheme 1. Synthesis of the hybrid NSAID-SeCN derivatives (4, 5, and 8)[a]



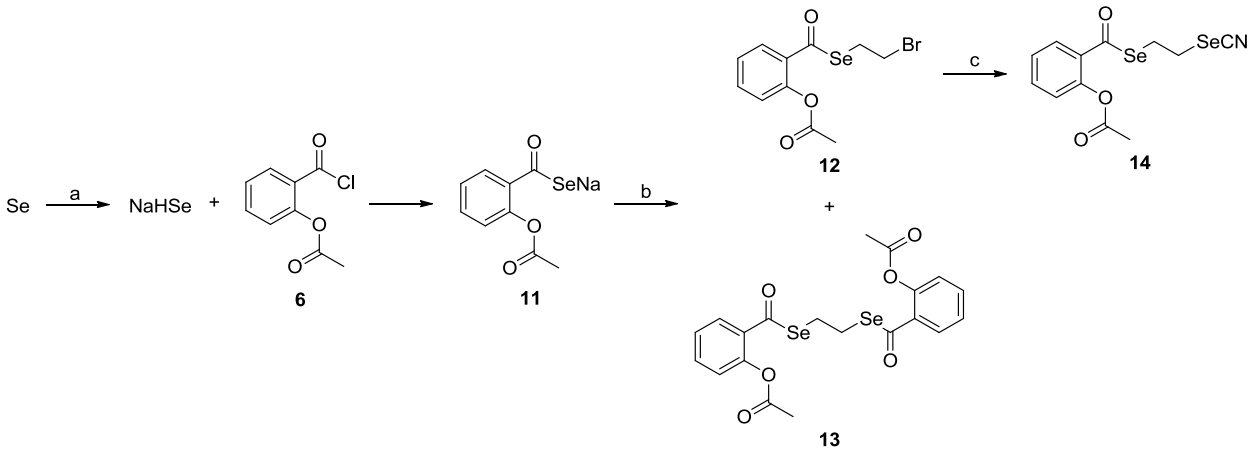
[a]*Reagents and conditions:* (a) KSeCN, CH_3CN, N_2 atm, 24 h, room temp; (b) TEA, CH_2Cl_2, N_2 atm, 18 h, room temp; (c) TEA, CH_2Cl_2, N_2 atm, 6 h, room temp; (d) KSeCN, CH_3CN, N_2 atm, 10 h, reflux.

Scheme 2. Synthesis of 2-selenocyanatoethyl 2-acetoxybenzoate (10)[a]



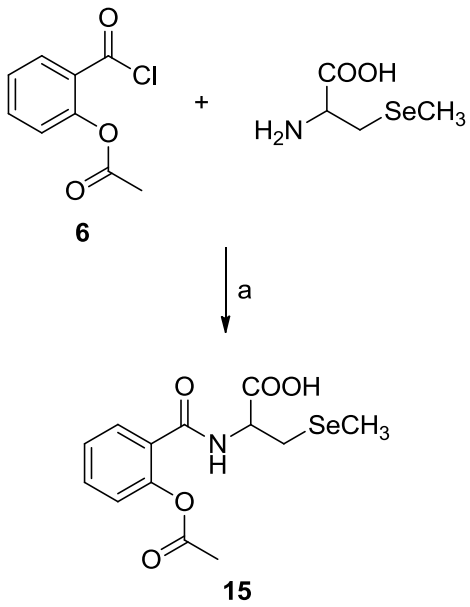
[a]*Reagents and conditions:* (a) KSeCN, CH_3CN, N_2 atm, 24 h, room temp; (b) TEA, CH_2Cl_2, N_2 atm, 22 h, room temp.

Scheme 3. Synthesis of monoselenoester derivatives (12-14) of hybrid Se-ASA compounds[a]



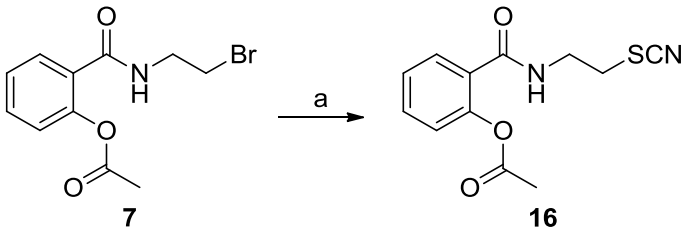
[a]*Reagents and conditions:* (a) $NaBH_4$, H_2O (dist), room temp; (b) Br-CH_2-CH_2-Br, THF/H_2O (dist), room temp; (c) KSeCN, CH_3CN, N_2 atm, 20 h, room temp.

Scheme 4. Synthesis of 2-(2-acetoxybenzamido)-3-(methylselanyl)propanoic acid (15)[a]



[a]*Reagents and conditions:* (a) TEA, CH_2Cl_2, N_2 atm, 32 h, room temp.

Scheme 5. Synthesis of aspirinyl sulfur analog 16[a]



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[a]*Reagents and conditions:* (a) NH_4SCN, CH_3CN, N_2 atm, 24 h, room temp.

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