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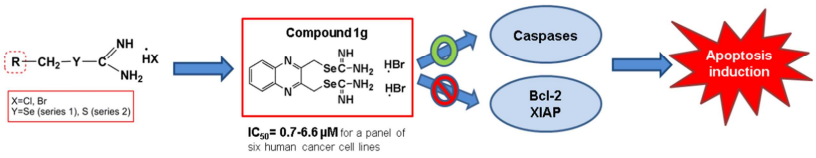

Novel seleno- and thio-urea derivatives with potent in vitro activities against several cancer cell lines

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Abstract

A series of novel selenourea derivatives and corresponding thiourea analogs were synthesized and tested against a panel of six human cancer cell lines: melanoma (1205Lu), lung carcinoma (A549), prostatic carcinoma (DU145), colorectal carcinoma (HCT116), pancreatic epithelioid carcinoma (PANC-1) and pancreatic adenocarcinoma (BxPC3). In general, we found that the selenium-containing derivatives were more potent than their isosteric sulfur analogs. Four selenourea derivatives (**1e**, **1f**, **1g** and **1i**) showed IC_{50} values below 10 μM in all of tested cell lines at 72 h. On the basis of its potent activity, compound **1g** was selected for further biological evaluation in different colon cancer cell lines. Our results indicated that compound **1g** induced apoptosis by caspase activation, along with inhibition of anti-apoptotic proteins.

Keywords: selenium, selenourea, cytotoxicity, apoptosis

1. Introduction

Cancer is known to be one of the leading causes of death in the world [1]. A considerable amount of progress has been made in the field of cancer treatment. However, the current chemotherapeutic agents are associated with unwanted side effects and poor quality of life. Moreover, drug resistance is also a rising issue with current therapies. Therefore, there is an unmet need to develop novel cancer therapeutic agents which are effective against the disease and can improve quality of life [2].

In the last decade, a wide range of novel agents have emerged as potential anti-cancer agents. Among them, selenium (Se) containing molecules have generated a growing interest for their anti-cancer activity and safety [3-6]. The chemical form in which selenium is used and the dose are the most critical factors in its activity [7].

Guanidine group has been emphasized over the years for its anti-cancer properties. The anti-cancer activity of this framework is generally associated with its ability to bind to various substituents on the DNA helix via the three nitrogen atoms present in its structure [8,9]. Besides, guanidines can act as urokinase [10] or cyclin kinase inhibitors [11]. Recently, some guanidine compounds have been reported to sensitize prostate cancer cells towards known chemotherapeutic agents such as staurosporine [12]. Moreover, compounds including the isothiourea group, for example the compound *S,S*-(1,4-phenylenebis[1,2-ethanediyl])bisisothiourea (PBIT), have been described as potent and selective inhibitors of the inducible nitric oxidase synthase (iNOS) [13]. This kind of inhibitors has been shown in several studies as potential chemopreventive agents [14,15]. The isosteric selenium analog (PBISe) was found superior to PBIT as apoptosis inductor, iNOS inhibitor and cell growth inhibitor in several cancer cell lines [16-18].

On the basis of these results, we have designed new hybrid compounds by introducing selenium in the form of isoselenourea, into different carbo- and hetero-cyclic scaffolds which have previously been shown to possess antitumor properties. These scaffolds include nitrofuran [19,20], isoxazole [21-23], 4-phenylpyrrole [24], imidazolidone [25-27], quinoline [28,29], quinoxaline [30-32], coumarin [33,34], acridine [35,36], anthraquinone [37] and benzodioxole [38,39]. We have selected variable mono, bi or tri-cyclic carbo- and hetero-cycles in order to confer different volume, rigidity, and electronic and lipophilic characteristics to the target molecules.

In addition, to evaluate the differences in the anti-cancer activities between two similar chemical elements, selenium and sulfur, when attached to above referred carbo/heterocyclic scaffolds, the isosteric isothiourea analogs were also synthesized. Literature reports, both from our group [40-42] and others [43], have demonstrated the enhanced cytotoxicity of selenium compounds as compared to corresponding sulfur isosteres. In spite of these two elements are in the same column of the periodic table, selenium is a larger atom with more labile electrons that likely contributes to the observed differences in activity. This study examines the effect of newly synthesized hybrid molecules on various cancer cell lines and begins to evaluate the underlying mechanism of action.

2. Results and discussion

2.1. Synthesis of seleno- and thio-urea derivatives

The twenty novel compounds synthesized are summarized in **Figure 1**. The compounds were prepared according to previously published procedures [13] with some

modifications (**Scheme 1**), starting from the commercially available alkyl halides and selenourea (compounds **1a-j**) or thiourea (compounds **2a-j**). The reactions were carried out under different conditions, depending on the alkyl halide used (more information in experimental section), in a 1:1.1 (alkyl halide:selenourea) molar ratio using absolute ethanol as solvent. After isolation and purification of the compounds, a yield of 15-92 % range was achieved. This variability in yield cannot be explained by chemical properties of the reagents. No correlation was found between yields and chemical features as nucleophilic activity of seleno-/thio-urea or the ligand, kind of halogen or ligand volume. Therefore, the differences might be ascribed to other physical factors. The unalike reaction times (0.5-6 h) and temperature conditions (0 ºC, r.t., reflux), along with the varying isolation and purification procedures (wash, recrystallization, precipitation) could be proposed as the main causes of the diverse yield values obtained.

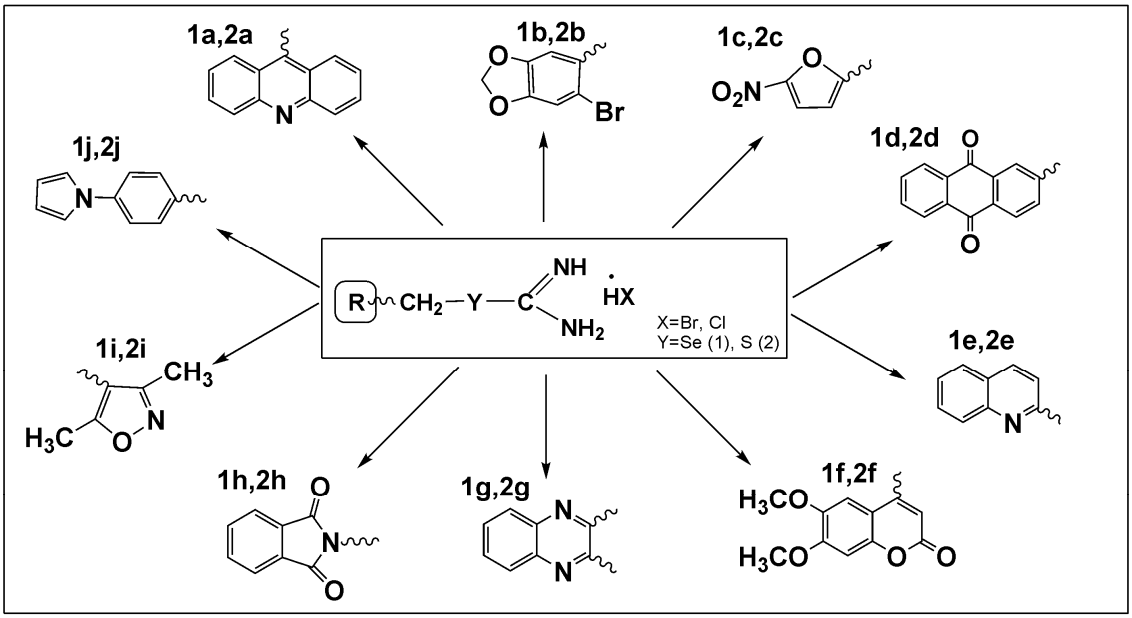
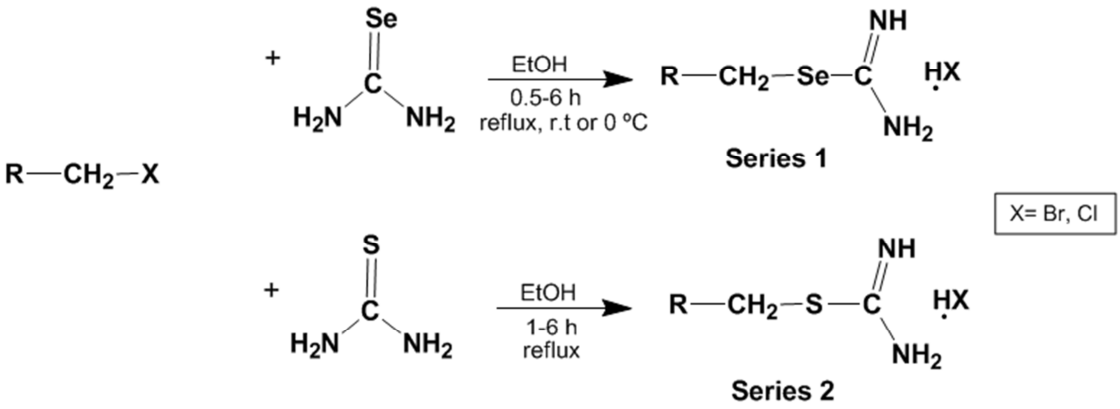


Figure 1. General structures of compounds. The wavy bond indicates the position where the seleno- or thio-urea group is attached.



Scheme 1. General schematic for synthesis of selenoureas (series 1) and thioureas (series 2)

Mechanistically, these reactions follow is a second-order nucleophilic substitution. The lone electron pair of Se or S atom attacks the methylene group and simultaneously the halogen atom leaves. Subsequently, the C=Se bond recovers the pi electron pair and one of the amino groups loses a proton, leading to the imine group formation.

The purity of all the products was determined by thin layer chromatography (TLC) and elemental analysis. Purity of all final compounds was 95 % or higher and their structures were confirmed by IR, ^{1}H and ^{13}C NMR, mass spectrometry (MS) and elemental analysis.

2.2. Biology

2.2.1. Effect on cancer cell viability

The effects of the novel compounds on cancer cell viability were evaluated in a panel of six human cancer cell lines: 1205Lu (melanoma), A549 (lung), DU145 (prostate), HCT116 (colon), PANC-1 (pancreas) and BxPC3 (pancreas). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay was performed in order

to measure cancer cell viability as previously described [45]. Results are expressed as half maximal inhibitory concentration (IC_{50}), the concentration that produces 50 % growth inhibition. The anti-cancer effect of each agent was tested at seven different concentrations between 0.5 and 50 µM and three different time points (24, 48 and 72 h). The results are summarized in **Tables 1A and 1B**.

Table 1A. Screening of novel seleno- and thio-urea derivatives on cancer cell viability in melanoma (1205Lu), lung (A549) and prostate (DU145) cancer cell lines

Comp.	**IC_{50} (µM)**								
	Cell lines								
	1205Lu			A549			DU145		
	24 h	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*
1a	13.0 ± 4.0	16.7 ± 1.7	11.6 ± 1.2	34.4 ± 4.1	14.9 ± 1.8	6.2 ± 0.7	31.8 ± 4.0	14.9 ± 2.9	12.9 ± 1.5
1b	>50.0	>50.0	21.8 ± 3.0	>50.0	>50.0	36.3 ± 1.4	>50.0	>50.0	>50.0
1c	19.1 ± 2.3	15.7 ± 2.1	13.0 ± 1.0	22.9 ± 0.9	10.2 ± 0.9	9.0 ± 1.0	>50.0	>50.0	15.6 ± 4.7
1d	17.4 ± 3.1	14.5 ± 2.4	11.3 ± 1.5	8.9 ± 1.2	2.2 ± 0.5	1.9 ± 0.4	21.4 ± 10.2	6.2 ± 1.6	8.2 ± 2.3
1e	14.4 ± 1.7	9.9 ± 1.2	7.1 ± 0.3	39.6 ± 16.2	15.1 ± 0.7	8.1 ± 0.4	19.6 ± 1.2	11.6 ± 0.8	7.3 ± 0.5
1f	21.0 ± 2.5	4.4 ± 0.8	1.9 ± 0.3	15.7 ± 1.6	5.2 ± 0.6	4.5 ± 0.3	11.4 ± 1.9	8.4 ± 0.6	6.8 ± 0.3
1g	3.3 ± 0.5	2.8 ± 0.3	2.2 ± 0.1	3.0 ± 0.8	2.0 ± 0.4	1.0 ± 0.2	2.3 ± 0.9	2.8 ± 0.3	1.0 ± 0.3
1h	19.0 ± 3.7	13.2 ± 1.2	9.3 ± 1.2	11.2 ± 1.5	7.8 ± 0.8	5.5 ± 0.2	18.8 ± 2.5	14.0 ± 1.5	6.9 ± 0.4
1i	>50.0	>50.0	3.4 ± 1.3	>50.0	2.8 ± 1.0	1.2 ± 0.3	>50.0	22.0 ± 8.4	7.4 ± 1.4
1j	>50.0	>50.0	>50.0	25.3 ± 6.4	16.3 ± 3.0	12.8 ± 1.9	>50.0	>50.0	>50.0
2a	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0
2b	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0
2c	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0
2d	9.4 ± 0.8	8.5 ± 0.8	6.3 ± 0.5	9.7 ± 1.3	8.7 ± 0.6	7.6 ± 0.5	29.8 ± 23.5	22.9 ± 4.7	31.2 ± 5.4
2e	>50.0	>50.0	39.2 ± 1.4	>50.0	>50.0	>50.0	>50.0	>50.0	31.6 ± 11.5
2f	>50.0	>50.0	37.4 ± 7.6	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0
2g	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0
2h	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0
2i	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0
2j	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	31.7 ± 3.8

Table 1B. Screening of novel seleno- and thio-urea compounds in colon (HCT116) and pancreatic (PANC-1 and BxPC3) cancer cell lines

Comp.	IC_{50} (μM)								
	Cell lines								
	HCT116			PANC-1			BxPC3		
	24 h	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*
1a	31.8 ± 3.0	16.7 ± 2.1	14.4 ± 2.6	12.1 ± 1.9	11.6 ± 1.3	9.8 ± 1.1	20.4 ± 2.7	10.5 ± 0.7	8.2 ± 0.5
1b	>50.0	31.8 ± 3.8	23.9 ± 3.4	>50.0	24.7 ± 3.8	16.5 ± 2.7	>50.0	>50.0	36.9 ± 5.8
1c	14.6 ± 3.3	3.7 ± 1.1	3.8 ± 0.4	>50.0	15.6 ± 1.6	13.6 ± 1.8	42.9 ± 4.6	39.4 ± 3.8	15.6 ± 2.2
1d	>50.0	12.6 ± 3.9	5.9 ± 1.4	>50.0	>50.0	31.4 ± 7.2	>50.0	>50.0	>50.0
1e	29.7 ± 6.4	19.6 ± 2.2	10.6 ± 0.9	18.0 ± 3.3	15.9 ± 3.8	9.6 ± 1.2	36.6 ± 2.3	17.0 ± 3.4	7.8 ± 0.7
1f	26.7 ± 5.6	8.9 ± 1.9	6.6 ± 1.4	20.6 ± 2.5	19.4 ± 1.5	10.0 ± 1.5	>50.0	>50.0	6.6 ± 2.1
1g	2.9 ± 2.1	0.7 ± 0.6	0.7 ± 0.4	6.6 ± 3.1	2.9 ± 1.1	2.0 ± 0.5	3.6 ± 1.4	2.9 ± 0.5	2.2 ± 0.2
1h	29.2 ± 2.7	17.9 ± 2.4	15.6 ± 1.7	23.5 ± 4.2	15.3 ± 2.2	12.6 ± 1.0	19.8 ± 3.0	19.1 ± 4.2	18.8 ± 2.5
1i	>50.0	45.0 ± 1.0	4.6 ± 1.1	>50.0	37.4 ± 10.1	13.6 ± 3.5	>50.0	6.8 ± 1.5	2.4 ± 0.6
1j	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	14.7 ± 4.8	8.3 ± 2.1
2a	>50.0	>50.0	45.1 ± 24.3	>50.0	>50.0	>50.0	>50.0	>50.0	46.3 ± 1.8
2b	>50.0	34.8 ± 7.6	22.1 ± 1.7	>50.0	40.5 ± 5.3	34.3 ± 6.0	>50.0	>50.0	44.3 ± 6.2
2c	>50.0	30.8 ± 9.2	7.0 ± 7.7	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0
2d	20.2 ± 2.5	10.7 ± 3.6	10.3 ± 2.8	7.6 ± 0.6	7.4 ± 0.7	6.8 ± 0.5	4.1 ± 0.7	9.0 ± 1.5	7.9 ± 1.1
2e	>50.0	28.5 ± 6.6	22.9 ± 3.5	>50.0	>50.0	>50.0	>50.0	37.3 ± 2.1	25.7 ± 1.7
2f	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0
2g	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0
2h	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0
2i	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	47.2 ± 2.6
2j	>50.0	>50.0	31.5 ± 3.8	>50.0	>50.0	40.6 ± 17.8	>50.0	>50.0	24.6 ± 2.6

As shown in **Tables 1A** and **1B**, the isosteric replacement of sulfur by selenium was correlated with a significant decrease in cancer cell viability, with the exception of the anthracene-9,10-dione scaffold (compound **2d**) in 1205Lu, HCT116, PANC-1 and BxPC3 cell lines. In fact, the activity of sulfur-containing derivatives remained over the maximum studied concentration (50 μM) in almost all cases. A more in-depth analysis of the cell viability effects showed that seven out of the ten selenium-containing compounds possessed IC_{50} values below 20 μM after 72 h of treatment in all of the tested cell lines. On the contrary, only one of the sulfur-containing derivatives (compound **2d**) showed IC_{50} values below 32 μM in all cell lines screened at the same time-point. Taken together, these results suggest that the presence of the selenium atom is crucial for the biological effects of these derivatives.

Compounds **1e**, **1f**, **1g** and **1i** were found to be the most active, with IC_{50} values below 10 µM in all of tested cell lines at 72 h. From our screening, compound **1g** emerged as the most potent agent with low IC_{50} values (0.7-6.6 µM) in all cancer cell lines tested. The potent activity of compound **1g** may be related to the presence of two selenourea groups.

Taking into account that compound **1g** was the only compound that had single digit IC_{50} values even at 24 h treatment in all the cancer cell lines tested, it was selected for further biological evaluation. Compound **2d** was the only sulfur-containing compound that showed a decrease on cell viability in all cancer cell lines screened. Therefore, compound **2d** was chosen as the most promising compound from series 2.

2.2.2. Compounds 1g and 2d reduced cell viability in different colon cancer cell lines

As observed from **Tables 1A** and **1B**, compound **1g** was most potent in HCT116, colon cancer, cell line. Therefore, we focused our experiments to evaluate the activity of **1g** in other human colon cancer cell lines. Compound **2d**, the most potent sulfur containing compound from series 2, was also evaluated since the thiourea analog PBIT has been shown to be an effective colon cancer chemopreventive agent [15]. As shown in **Table 2** and **Figure 2**, compound **1g** was almost equally effective in reducing the viability of all three colon cancer cell lines (HCT116, HT29 and RKO) with IC_{50} ranging from 0.7-5.0 µM at different treatment time points. This observation is unique in the essence that all the three cell lines harbor different mutations. As expected, compared to **1g**, the thiourea analog **2d** was much less effective in reducing cancer cell viability in all three colon cancer cell lines tested (**Figure 2** and **Table 2**).

We also compared the potency of compound **1g** with 5-FU, a first line therapeutic agent for colon cancer. As shown in **Figure 2** and **Table 2**, compound **1g** was up to >50 times more potent than 5-FU. Taken together, these results indicated that **1g** not only emerged as the most potent agent from the series of novel agents synthesized, but it was also superior to the current therapy, 5-FU, for colon cancer. Hence, compound **1g** was narrowed down as the most promising agent for further biological evaluations.

Table 2. Effects of compounds 1g, 2d and 5-FU on cell viability of different colon cancer cell lines.

	IC_{50} (μM)								
	Cell lines								
Comp.	HCT116			HT29			RKO		
	24 h	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*	*24 h*	*48 h*	*72 h*
1g	2.9 ± 2.0	0.7 ± 0.7	0.7 ± 0.4	5.0 ± 1.0	4.1 ± 1.7	2.2 ± 1.9	3.8 ± 2.9	1.9 ± 1.6	2.5 ± 1.9
2d	20.2 ± 2.5	10.7 ± 3.6	10.3 ± 2.8	17.0 ± 3.28	8.1 ± 2.7	7.4 ± 0.6	28.9 ± 7.3	20.4 ± 1.3	21.0 ± 2.4
5-FU	>50.0	>50.0	27.8 ± 4.8	>50.0	25.0 ± 2.0	6.3 ± 0.7	>50.0	41.1 ± 12.6	6.8 ± 0.8

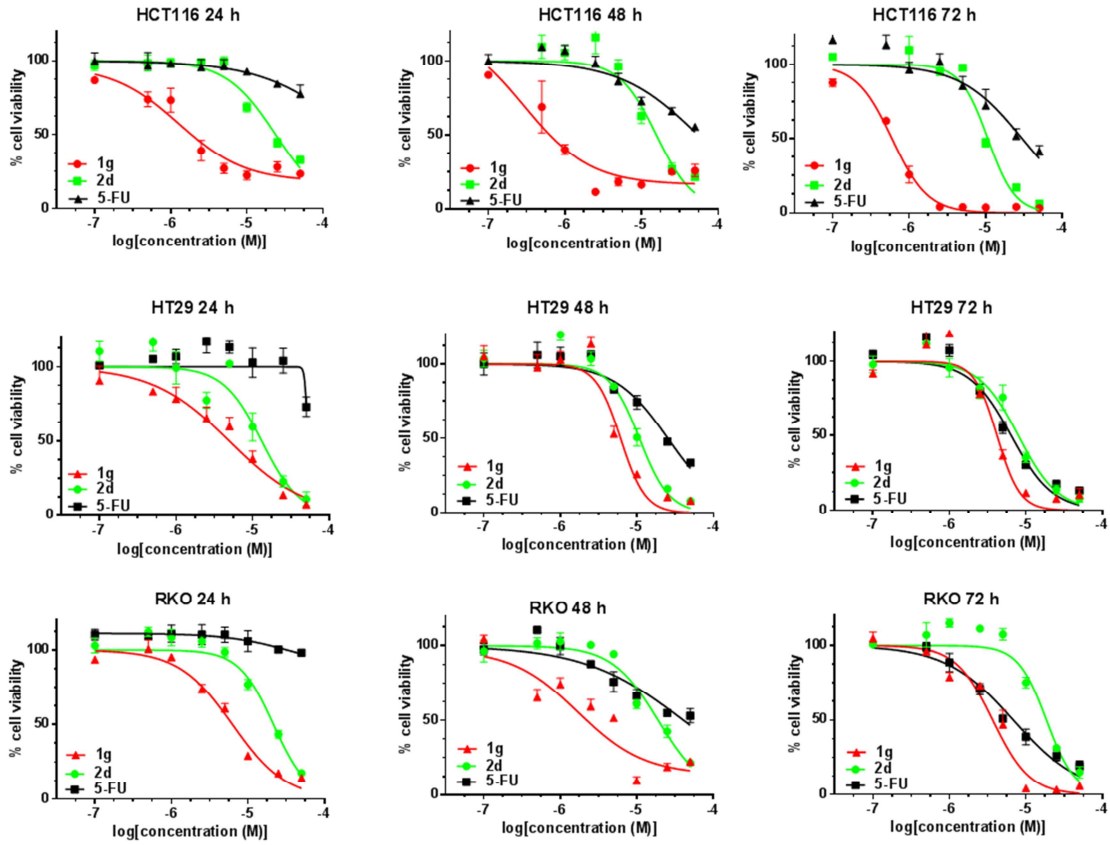


Figure 2. Compound 1g and 2d reduced cancer cell viability in three different colon cancer cell lines. Colon cancer cells were treated with different concentrations of the compounds for the given time points. Cell viability was evaluated by MTT assay. Mean values are represented with their respective standard deviation (± SD).

2.2.3. Compound 1g induced cell death in HCT116 cells at 24 h

The MTT assays performed above demonstrated that **1g** substantially reduces the viability of various cancer cell lines. However, to understand whether the reduction in cell viability is due the cell death and not due to the inhibition of cell growth, HCT116 cells were treated with **1g** for 24 h and were subjected to Live and Dead assay as described in the experimental section. As shown in **Figure 3**, treatment of HCT116 cells with compound **1g** increased the amount of cells positive for Ethidium Homodimer staining (dead cells, Upper left quadrant), while reduced the cells with Calcein staining

(live cells, Lower right quadrant). Dose of 5 µM showed more than 28 % cell death compared to vehicle. Hence, the inhibitory effect of compound **1g** on cancer cell viability was primarily due to cancer cell death.

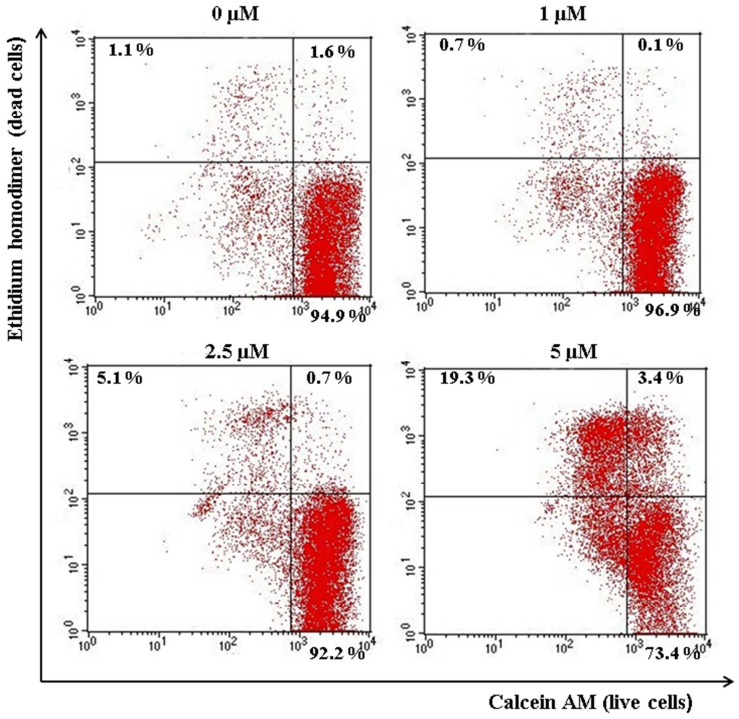


Figure 3. Compound 1g induced cell death. HCT116 cells were treated with compound **1g** (1, 2.5 or 5 µM) or DMSO (control) for 24 h and stained with Calcein AM and Ethidium Homodimer.

2.2.4. Compound 1g induced apoptosis in colon cancer cells

In order to determine if **1g** induces cell death through apoptosis, the Muse™ Annexin V & Dead Cell assay was carried out. In this assay, Annexin V is employed to detect the externalization of phosphatidylserine (PS) to the cell surface, a process occurring in early apoptosis [46]. This kit also included a dead cell marker (7-ADD). Only the cells which have lost their cell membrane integrity are stained by 7-ADD. Hence, cells positive for 7-ADD marker only, are indicative of necrosis (Upper left quadrant). Cells

positive for both markers (Annexin V and 7-ADD) are indicative of apoptotic cell death (Upper right quadrant), while cells only positive for Annexin V are considered to be in early apoptosis (Lower right quadrant). Cells which are negative for both markers are present in the Lower left quadrant (healthy cells). As shown in **Figure 4A**, the treatment with compound **1g** increased the number of apoptotic cells even at low concentration (1 µM). When the dose of **1g** was increased to 5 µM, population of early apoptotic cells as well as late apoptotic cells also increased, indicating the dose dependent effect of **1g** in induction of apoptosis in colon cancer cells. These results are in concordance with the data obtained from the MTT assay and Live & Dead assay.

To confirm these results, the activation of the effector Caspases 3 and 7 was studied using Muse^{TM} Caspase 3/7 kit. The kit included a reagent with a DNA binding dye which contains an effector Caspase 3/7 recognizing sequence (DEVD). Upon the presence of Caspase 3/7 activity, this sequence is cleaved and DNA binding dye is released from the reagent, hence staining the DNA of cells having positive Caspase 3/7 activity. This kit also included the dead cell marker 7-ADD. The distribution of cells in quadrants is the same as in the Annexin V assay: viable (both dyes negatives, Lower left quadrant), early apoptotic (only positive for Caspase 3/7 reagent, Lower right quadrant), late apoptotic/dead (positive for both markers, Upper right quadrant) and necrotic cells (only positive for 7-ADD, Upper left quadrant). As shown in **Figure 4B**, the amount of cells positive for the Caspase 3/7 reagent increased dramatically after the treatment with **1g**. At the dose of 5 µM, more than 50 % of the cells were positive for the apoptosis marker (Lower right and Upper right quadrants), suggesting that compound **1g** is a potent apoptosis inducer. The above results clearly demonstrate that compound **1g**

induced cell death through apoptosis, as indicated by presence of PS on outer leaflet of cell membrane and presence of Caspase 3/7 activity.

To evaluate the mechanism by which **1g** induces apoptosis, we investigated its effect on anti-apoptotic proteins, XIAP and Bcl-2, by Western blot analysis. As shown in **Figure 4C**, the levels of both XIAP and Bcl-2 were reduced in a dose dependent manner. Further, we evaluated the effect of **1g** at the protein levels of pro-Caspase 3 and full-length PARP (**Figure 4D**). At doses similar to at which the reduction in anti-apoptotic proteins (Bcl-2 and XIAP) was observed, there was also a decrease in the pro-Caspase 3 and whole PARP levels. The full-length Caspase 3 (pro-Caspase 3) is inactive, whereas its cleaved form is active. The active form of Caspase 3 is able to cleave other substrates as PARP [46]. Therefore, the reduction of both proteins in this experiment indicated the activation of Caspase 3. The expression of both pro-Caspase 3 and whole PARP decreased significantly at 2.5 µM dose of **1g** and were undetectable after treatment with 5 µM dose. These results are in concordance with the data obtained in the Annexin V and Caspase 3/7 assays.

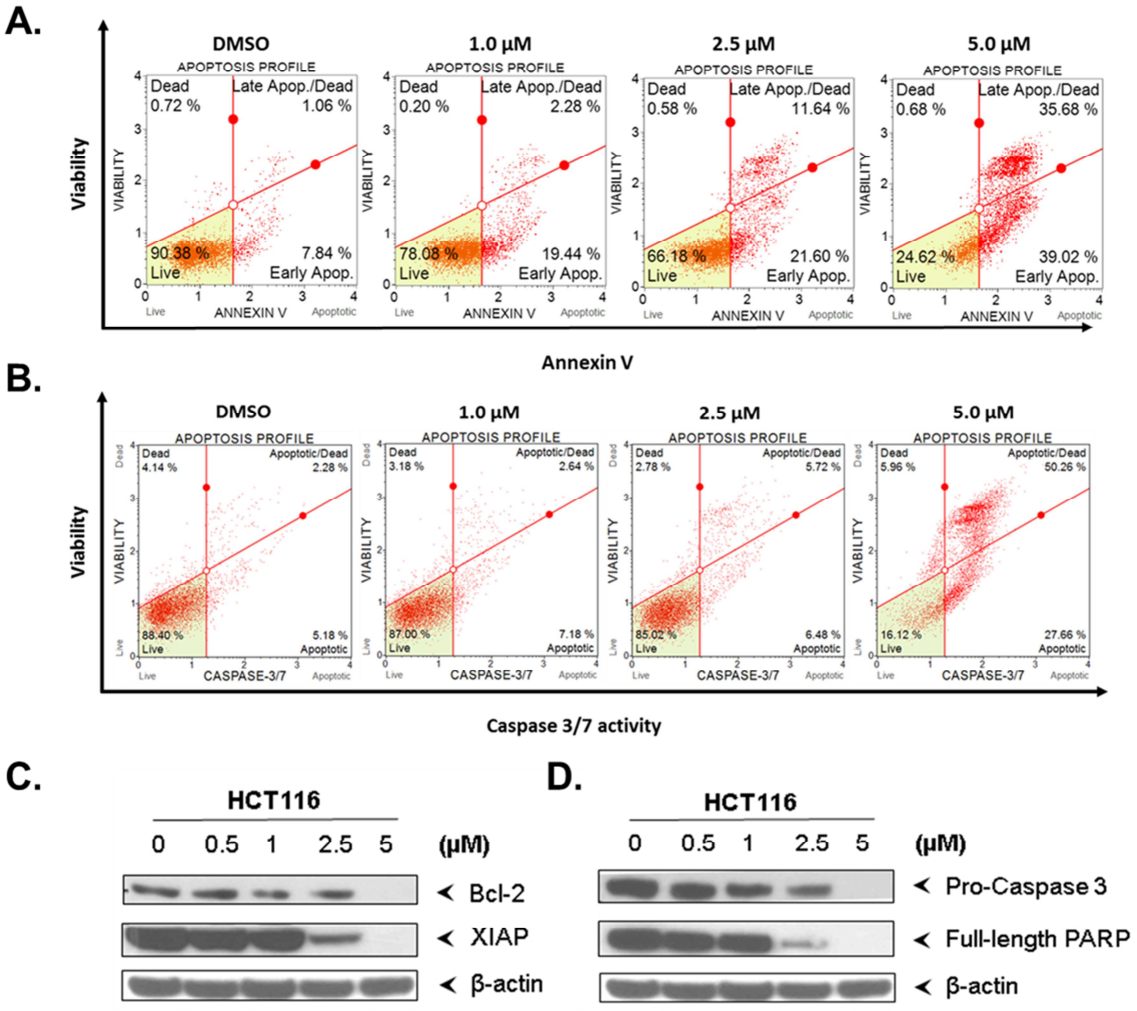


Figure 4. Compound 1g induced apoptosis and inhibited anti-apoptotic proteins. After 24 h of treatment with the indicated amounts of compound **1g**, HCT116 cells were analyzed with a Muse™ automated cell analyzer using the Muse™ Annexin V & Dead Cell Kit according to manufacturer's instructions (A). Analogous independent experiments were analyzed with Muse™ Caspase 3/7 Kit to confirm the results (B). The effects of compound **1g** on the expression of two anti-apoptotic proteins, Bcl-2 and XIAP (C), as well as on the protein levels of pro-Caspase 3 and full-length PARP (D) were evaluated by Western blot analysis after 24 h of treatment with increasing concentrations of **1g.** β-Actin was used as loading control.

3. Conclusion

In this work, a novel series of selenourea derivatives and its corresponding thiourea analogs were designed and synthesized. The MTT assay confirmed that the selenium-

containing derivatives were much more potent than their corresponding sulfur analogs. Therefore, the replacement of sulfur by selenium seems to be a valid approach to obtain new agents with more potent anti-cancer activity. After screening in a panel of six human cancer cell lines, **1g** was identified as the most promising compound. This compound showed low IC_{50} values in all cancer cell lines and time points tested and it was found to be up to >50 times more potent than 5-FU in three different colon cancer cell lines. Live and Dead, Annexin V, Caspase 3/7 and Western blot assays demonstrated that compound **1g** induced apoptosis by caspases activation, along with inhibition of the anti-apoptotic proteins Bcl-2 and XIAP. Hence, **1g** has the potential to be developed as a chemotherapeutic agent for colon cancer treatment and warrants further in depth *in vitro* and *in vivo* studies in colon cancer models.

4. Experimental

4.1. Chemistry

4.1.1. Material and methods

Melting points (mp) were determined with a Mettler FP82+FP80 apparatus (Greifense, Switzerland). Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a Bruker 400 Ultrashield^{TM} spectrometer (Rheinstetten, Germany) using DMSO-d_6 as solvent. Chemical shifts are given in parts per million (ppm) (δ relative to residual solvent peak for ^{1}H and ^{13}C). IR spectra were recorded on a Thermo Nicolet FT-IR Nexus spectrophotometer. Elemental analysis was performed on a LECO CHN-900 Elemental Analyzer. Purity of all final compounds was 95 % or higher. Chemicals were purchased from E. Merck (Darmstadt, Germany), Panreac Química S.A. (Montcada i Reixac, Barcelona, Spain), Sigma-Aldrich Quimica, S.A. (Alcobendas, Madrid, Spain) and Acros Organics (Janssen Pharmaceuticalaan, Geel, Belgium).

4.1.2. General procedure of synthesis of compounds

The corresponding alkyl halide reagent (1 mmol) was added to a mixture of selenourea (1.1 mmol) (compounds **1a-j**) or thiourea (compounds **2a-j**) in absolute ethanol (20 mL). The mixture was stirred at reflux, room temperature or 0 ºC for 0.5-6 h. The product was isolated by filtration or by rotatory evaporation of the solvent under vacuum and purified by recrystallization or washing.

4.1.3. Acridin-9-ylmethyl carbamimidoselenoate hydrobromide (1a)

Conditions: 1 h at 0 ºC. The precipitate was washed with dichloromethane and hexane (25 mL of each). A pink powder was obtained; Yield: 15 %; mp: 222 ºC (direct combustion). ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.58 (s, 2H, -CH_2-), 7.01-7.04 (m, 4H, H2 + H3 + H6 + H7), 7.31 (t, 2H, *J*= 7.6 Hz, H4 + H5), 7.39 (d, 2H, *J*= 7.8 Hz, H1 + H8), 9.32 + 9.63 + 9.71 (bs + bs + bs, 3H, NH + NH_2), 11.06 ppm (bs, 1H, HBr). ^{13}C NMR (100 MHz, DMSO-d_6): δ 44.1 (-CH_2-), 69.1 (C9), 115.4 (C8a + C9a), 120.7 (C1 + C8), 121.2 (C2 + C7), 126.3 (C4 + C5), 130.0 (C3 + C6), 138.2 (C4a + C10a), 171.5 ppm (Se-C-(NH)(NH_2). IR (KBr): $\tilde{\nu}$ 3293-3198 (s; N-H, N-H_2), 3052 (s; C-H_{arom}), 1636 (s; C=N), 1578-1484 cm^{-1} (m; C-C_{arom}). MS (*m/z* (% abundance)): 194(100). Elemental analysis calculated (%) for $C_{15}H_{13}N_3Se{\cdot}HBr{\cdot}H_2O$: C: 43.58, H: 3.39, N: 10.17; found: C: 43.22, H: 3.25, N: 9.85

4.1.4. (6-Bromobenzo[*d*][1,3]dioxol-5-yl)methyl carbamimidoselenoate hydrobromide (1b)

Conditions: 6 h at reflux. The precipitate was filtered and purified by recrystallization from ethanol. A pink powder was obtained. Yield: 49 %; mp: 182-183 ºC. ^{1}H NMR (400

MHz, DMSO-d_6): δ 4.51 (s, 2H, -CH_2-Se), 6.09 (s, 2H, O-CH_2-O), 7.19 (s, 1H, H4), 7.28 (s, 1H, H7), 9.26 + 9.34 ppm (bs + bs, 4H, NH_2 + NH + HBr). ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.4 (-CH_2-Se), 103.2 (O-CH_2-O), 111.4 (C4), 113.7 (C6), 115.7 (C7), 129.2 (C5), 148.2 (C7a), 149.1 (C3a), 166.8 ppm (Se-C-(NH)(NH_2)). IR (KBr): $\tilde{\nu}$ 3233-3078 (s; N-H, N-H_2), 2889 (w; C-H_{aliph}), 1644 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 294(11), 213(100), 157(11), 124(16), 75(21). Elemental analysis calculated (%) for $C_9H_9N_2BrO_2$ Se·HBr: C: 25.90, H: 2.40, N: 6.72; found: C: 26.02, H: 2.37, N: 6.75.

4.1.5 (5-Nitrofuran-2-yl)methyl carbamimidoselenoate hydrobromide (1c)

Conditions: 1h at reflux. The precipitate was washed with ethyl ether (50 mL). A white powder was obtained. Yield: 80 %; mp: 223 (direct combustion). ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.67 (s, 2H, -CH_2-), 6.81 (d, 1H, *J*= 3.7 Hz, H3), 7.69 (d, 1H, *J*= 3.8 Hz, H4), 9.25 + 9.35 ppm (bs + bs, 4H, NH_2 + NH + HBr). ^{13}C NMR (100MHz, DMSO-d_6): δ 22.4 (-CH_2-), 113.6 (C3), 115.2 (C4), 152.0 (C5), 156.4 (C2), 166.1 ppm (1C, -Se-C-(NH)(NH_2)). IR (KBr): $\tilde{\nu}$ 3241-3093 (s; N-H, N-H_2), 1661 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 207(72), 160(39), 126(80), 80(79), 52(100). Elemental analysis calculated (%) for $C_6H_7N_3O_3$ Se·HBr: C: 21.90, H: 2.45, N: 12.77; found: C: 21.64, H: 2.48, N: 12.69.

4.1.6. (9,10-Dioxo-9,10-dihydroanthracen-2-yl)methyl carbamimidoselenoate hydrochloride (1d)

Conditions: 4 h at reflux. The precipitate was washed with dichloromethane and ether (25 mL of each). A green powder was obtained; Yield: 75 %; mp: 221-223 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.73 (s, 2H, -CH_2 -), 7.92-7.96 (m, 3H, H3 + H5 + H8), 8.20-

8.21 (m, 3H, H1 + H6 + H7), 8.28 (bs, 1H, H4), 9.37 + 9.45 ppm (bs + bs, 4H, NH + NH_2 + HCl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.1 (-CH_2-), 127.5 (C5 + C8), 127.9 (C4), 128.1 (C1), 132.8 (C4a), 133.8 (C6 + C7), 134.0 (C3), 135.3 (C8a + C10a), 135.6 (C9a), 146.1 (C2), 166.7 (Se-C-(NH)(NH_2)), 182.8 (C10), 183.1 ppm (C9). IR (KBr): $\tilde{\nu}$ 3192-3075 (s; N-H, N-H_2), 1661 (s; C=O), 1539 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 302(14), 221(100), 193(49), 165(29), 139(7), 82(7). Elemental analysis calculated (%) for $C_{16}H_{12}N_2O_2Se$ ·HCl: C: 50.61, H: 3.45, N: 7.38; found: C: 50.15, H: 3.46, N: 7.40.

4.1.7. Quinolin-2-ylmethyl carbamimidoselenoate hydrochloride (1e)

The commercially available 2-(chloromethyl)quinoline hydrochloride was treated with basic water (50 mL) in order to obtain the free base, which was used for the synthesis of **1e**. The reaction was carried out at 0 ºC for 30 minutes. Then, the mixture was filtered and the solvent was removed under vacuum by rotatory evaporation. The residue was precipitated with ether (10 mL) and washed with acetone, dichloromethane and hexane (25 mL of each). A dark brown powder was obtained. Yield: 48 %; mp: 113-114 ºC. ^1H NMR (400 MHz, DMSO-d_6): δ 4.77 (s, 2H, -CH_2 -), 7.65 (d, 2H, *J*=8.35 Hz, H3 + H8), 7.80 (t, 1H, *J*= 7.6 Hz, H6), 7.98-8.04 (m, 2H, *J*= 7.6 Hz, H5 + H7), 8.46 (d, 1H, *J*= 8.2 Hz, H4), 9.54 + 9.89 ppm (bs + bs, 4H, NH + NH_2 + HCl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.4 (-CH_2-), 121.3 (C3), 127.5 (C6 + C4a), 128.9 (C5 + C8), 131.4 (C7), 138.8 (C4), 145.7 (C8a), 158.8 (C2), 168.9 ppm (Se-C-(NH)(NH2)). IR (KBr): $\tilde{\nu}$ 3254-3105 (s; N-H, N-H_2), 1645 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 223(6), 143(100), 128(16), 115(21). Elemental analysis calculated (%) for $C_{11}H_{11}N_3S$ ·HCl·1/2H_2O: C: 42.63, H: 4.20, N: 13.56; found: C: 42.36, H: 3.94, N: 13.95.

4.1.8. (6,7-Dimethoxy-2-oxo-2*H*-chromen-4-yl)methyl carbamimidoselenoate hydrobromide (1f)

Conditions: 3 h at reflux. The precipitate was washed with ethyl ether (50 mL). A yellow powder was obtained. Yield: 79 %; mp: 221-222 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.85 (s, 6H, (-O-CH_3) ×2), 4.68 (s, 2H, -CH_2-), 6.33 (s, 1H, H3), 7.11 (s, 1H, H5), 7.42 (s, 1H, H8), 9.26 + 9.36 ppm (bs + bs, 4H, NH_2 + NH + HBr). ^{13}C NMR (100 MHz, DMSO-d_6): δ 27.6 (-CH_2-), 57.6 + 57.1 ((-OCH_3) ×2), 101.4 (C8), 107.6 (C5), 110.6 (C3), 112.7 (C4a), 146.8 (C6), 150.3 (C8a), 152.6 (C7), 153.7 (C4), 160.9 (C2), 165.7 ppm (Se-C-(NH)(NH_2)). IR (KBr): $\tilde{\nu}$ 3302-3220 (m; N-H, N-H_2), 2977 (s; C-H_{aliph}), 1687 (s; C=O), 1648 (s, C=N), 1611-1467 cm^{-1} (m; C-C_{arom}). MS (*m/z* (% abundance)): 300(47), 220(98), 205(22), 191(100), 177(26), 147(29), 124(27), 80(37). Elemental analysis calculated (%) for $C_{13}H_{14}N_2O_4Se \cdot HBr$: C: 36.99, H: 3.58, N: 6.64; found: C: 36.56, H: 3.53, N: 6.47.

4.1.9. Quinoxaline-2,3-diylbis(methylene)dicarbamimidoselenoate dihydrobromide (1g)

Conditions: 2 h at room temperature. The precipitate was washed with ethyl ether (50 mL). A brown powder was obtained. Yield: 82 %; mp: 175 ºC (direct combustion). ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.03 (s, 4H, ((-CH_2-) ×2), 7.89-7.90 (m, 2H, H6 + H7), 8.03-8.05 (m, 2H, H5 + H8), 9.24 + 9.38 ppm (bs + bs, 4H, NH_2 + NH + HBr). ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.4 ((-CH_2-) ×2), 128.4 (C5 + C8), 131.7 (C6 + C8), 140.5 (C4a + C8a), 152.1 (C2 + C3), 167.7 ppm ((-Se-C-(NH)(NH_2)) ×2). IR (KBr): $\tilde{\nu}$ 3360-3257 (s; N-H, N-H_2), 2991 (s; C-C_{aliph}), 1632 (s; C=N), 1550-1487 cm^{-1} (m; C-H_{arom}). MS (*m/z* (% abundance)): 258(100), 230(8), 156(66), 129(22), 103(29), 76(31).

Elemental analysis calculated (%) for $C_{12}H_{14}N_6Se_2 \cdot 2HBr$: C: 25.64 H: 2.87 N: 14.95; found: C: 25.60 H: 2.68 N: 15.14.

4.1.10. (1,3-Dioxoisoindolin-2-yl)methyl carbamimidoselenoate hydrobromide (1h)

Conditions: 45 min at 0 ºC. The precipitate was washed with acetone and ether (25 mL of each). A pink powder was obtained. Yield: 50 %; mp: 202-203 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.44 (s, 2H, -CH_2-), 7.89-7.91 (m, 2H, H4 + H7), 7.94-7.96 (m, 2H, H5 + H6), 9.32 + 9.42 ppm (bs + bs, 4H, NH_2 + NH + HBr). ^{13}C NMR (100 MHz, DMSO-d_6): δ 34.2 (-CH_2-), 124.4 (C4 + C7), 132.4 (C3a + C7a), 135.9 (C5 + C6), 165.1 (Se-C-(NH)(NH_2)), 167.4 ppm (C1 + C3). IR (KBr): $\tilde{\nu}$ 3314-3172 (s; N-H, N-H_2), 3020 (s; C-H_{arom}), 1774-1717 (s; C=O), 1648 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 241(6), 160(100), 133(10), 104(13), 76(14). Elemental analysis calculated (%) for $C_{10}H_9N_3O_2Se \cdot HBr \cdot 1/2H_2O$: C: 32.26, H: 2.78, N: 11.57; found: C: 31.91, H: 2.59, N: 11.31.

4.1.11. (3,5-Dimethylisoxazol-4-yl)methyl carbamimidoselenoate hydrochloride (1i)

Conditions: 2.5h at reflux. After this time, the mixture was filtered and the solvent was removed under vacuum by rotatory evaporation. The brown powder was washed with acetone (25 mL). Yield: 76 %; mp: 168-170 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.23 (s, 3H, C3-CH_3), 2.40 (s, 3H, C5-CH_3), 4.41 (s, 2H, -CH_2-), 9.50 ppm (bs, 4H, NH_2 + NH + HCl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 10.7 ((C5)-CH_3), 11.8 ((C3)-CH_3), 19.5 (-CH_2-), 110.6 (C4), 160.1 (C5), 166.5 (C3), 167.7 ppm (Se-C-(NH)(NH_2)). IR (KBr): $\tilde{\nu}$ 3220-3100 (s; N-H, N-H_2), 1655 cm^{-1} (s, C=N). MS (*m/z* (% abundance)): 228(13), 213(22), 190(24), 156(43), 110(98), 68(100), 43(100). Elemental analysis calculated

(%) for $C_7H_{11}N_3OSe \cdot HCl$: C: 31.30, H: 4.50, N: 15.64; found: C: 30.94 H: 4.59, N: 15.53.

4.1.12. 4-(1*H*-Pyrrol-1-yl)benzyl carbamimidoselenoate hydrobromide (1j)

Conditions: 2 h at reflux. Then, the mixture was filtered and the solvent was removed under vacuum by rotatory evaporation. The pink powder was washed with acetone and ether (25 mL of each). Yield: 78 %; mp: 176-177 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.55 (s, 2H, -CH_2-), 6.26 (t, 2H, *J*= 2.2 Hz, H3' + H4'), 7.37 (t, 2H, *J*= 2.2 Hz, H2' + H5'), 7.48 (d, 2H, *J*=8.0 Hz, H2 + H6), 7.57 (d, 2H, *J*=8.0 Hz, H3 + H5), 9.17 + 9.28 ppm (bs + bs, 4H, NH_2 + NH + HBr). ^{13}C NMR (100 MHz, DMSO-d_6): δ 22.9 (-CH_2-), 110.8 (C3' + C4'), 120.4 (C2' + C5'), 122.7 (C3 + C5), 129.6 (C2 + C6), 135.8 (C1), 137.4 (C4), 168.1 ppm (Se-C-(NH)(NH_2)). IR (KBr): $\tilde{\nu}$ 3213-3139 (s; N-H, N-H_2), 1654 cm^{-1} (s, C=N). MS (*m/z* (% abundance)): 156(100), 124(27), 80(29), 43(45). Elemental analysis calculated (%) for $C_{12}H_{13}N_3Se \cdot HBr$: C: 40.13, H: 3.93, N: 11.70; found C: 39.57, H: 3.93, N: 11.45.

4.1.13. Acridin-9-ylmethyl carbamimidothioate hydrobromide (2a)

Conditions: 2h at reflux. The precipitate was recrystallized from ethanol. An orange powder was obtained. Yield: 16 %; mp: 180-181 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.62 (s, 2H, -CH_2-), 6.99-7.04 (m, 4H, H2 + H3 + H6 + H7), 7.32 (t, 2H, *J*= 7.6 Hz, H4 + H5), 7.39 (d, 2H, *J*= 7.8 Hz, H1 + H8), 9.39 + 9.70 + 9.82 (bs +bs + bs, 3H, NH_2 + NH), 11.1 ppm (s, 1H, HBr). ^{13}C NMR (100 MHz, DMSO-d_6): δ 48.6 (-CH_2-), 68.6 (C9), 115.4 (C8a + C9a), 120.3 (C1 + C8), 121.2 (C2 + C7), 130.2 (C3 + C6), 126.5 (C4 + C5), 138.3 (C4a + C10a), 171.8 ppm (Se-C-(NH)(NH_2)). IR (KBr): $\tilde{\nu}$ 3289-3200 (s; N-H, N-H_2), 3047 (s; C-H_{arom}), 1639 cm^{-1} (s; C=N). MS (*m/z* (% abundance)):

267(27), 220(100), 205(15), 193(16), 179(45), 152(16), 110(15). Elemental analysis calculated (%) for $C_{15}H_{13}N_3S{\cdot}HBr$: C: 51.72, H: 4.02, N: 12.07; found: C: 52.12, H: 4.22, N: 11.97.

4.1.14. (6-Bromobenzo[*d*][1,3]dioxol-5-yl)methylcarbamimidothioate hydrobromide (2b)

Conditions: 6 h at reflux. The solvent was removed under vacuum by rotatory evaporation and the residue was recrystallized from ethanol. The obtained yellow oil was precipitated with ether (10 mL). A white powder was obtained. Yield: 79 %; mp: 180-181 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.52 (s, 2H, -CH_2-S), 6.13 (s, 2H, O-CH_2-O), 7.22 (s, 1H, H4), 7.31 (s, 1H, H7), 9.28 ppm (bs, 4H, NH_2 + NH + HBr). ^{13}C NMR (100 MHz, DMSO-d_6): δ 36.5 (-CH_2-S), 103.3 (O-CH_2-O), 111.7 (C4), 113.7 (C6), 115.9 (C7), 127.2 (C5), 148.2 (C7a), 149.4 (C3a), 169.9 ppm (S-C-(NH)(NH_2)). IR (KBr): $\tilde{\nu}$ 3222-3088 (s; N-H, N-H_2), 2889 (m; C-H_{aliph}), 1648 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 294(8), 213(100), 209(32), 192(10), 166(9), 157(14), 76(44). Elemental analysis calculated (%) for $C_9H_9N_2BrO_2S{\cdot}HBr$: C: 29.19, H: 2.70, N: 7.57; found C: 29.27 H: 2.95 N: 7.74.

4.1.15. (5-Nitrofuran-2-yl)methyl carbamimidothioate hydrobromide (2c)

Conditions: 1 h at reflux. The precipitate was filtered and washed with ether (25 mL). A pink powder was obtained. Yield: 41 %; mp: 171-172 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.78 (s, 2H, -CH_2-), 6.86 (s, 1H, H3), 7.77 (s, 1H, H4), 9.17 + 9.35 ppm (bs + bs, 4H, NH_2 + NH + HBr). ^{13}C NMR (100 MHz, DMSO-d_6): δ 27.8 (-CH_2-), 114.1 (C3), 114.9 (C4), 152.2 (C5), 154.2 (C2), 168.8 ppm (-S-C-(NH)(NH_2)). IR (KBr): $\tilde{\nu}$ 3219-3078 (s; N-H, N-H_2), 1655 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 205(1), 170(15), 159(13), 154(58), 140(19), 126(75), 114(33), 80(100), 60(31), 52(30). Elemental

analysis calculated (%) for $C_6H_7N_3O_3S \cdot HBr$: C: 25.53, H 2.83, N: 14.89; found: C: 25.61, H: 2.80, N: 14.96.

4.1.16. (9,10-Dioxo-9,10-dihydroanthracen-2-yl)methyl carbamimidothioate hydrochloride (2d)

Conditions: 3 h at reflux. The precipitate was filtered and washed with dichloromethane and ether (25 mL of each). A yellow powder was obtained. Yield: 57 %; mp: 233-234 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.77 (s, 2H, -CH_2-), 7.92-7.95 (m, 2H, H5 + H8), 7.99 (d, 1H, *J*=8.0Hz, H3), 8.19-8.23 (m, 3H, H1 + H6 + H7), 8.29-8.30 (m, 1H, H4), 9.29 + 9.44 ppm (bs, 4H, NH + NH_2 + HCl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 34.2 (-CH_2-), 127.6 (C5 + C8), 127.8 (C4), 128.2 (C1), 133.2 (C3), 133.8 (C6 + C7), 134.0 (C4a), 135.4 (C8a + C10a), 135.6 (C9a), 143.8 (C2), 169.5 (S-C-(NH)(NH_2)), 182.8 (C10), 183.1 ppm (C9). IR (KBr): $\tilde{\nu}$ 3274-3195 (m; N-H, N-H_2), 3025 (s; C-H_{arom}), 1668(s; C=O), 1650 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 296(4), 254(57), 221(100), 193(55), 165(23), 152(8), 139(6), 82(10). Elemental analysis calculated (%) for $C_{16}H_{12}N_2O_2S \cdot HCl$: C: 57.74, H: 3.94, N: 8.42; found: C: 57.60, H: 4.02, N: 8.17.

4.1.17. Quinolin-2-ylmethyl carbamimidothioate hydrochloride (2e)

The commercially available 2-(chloromethyl)quinoline hydrochloride was treated with basic water (50 mL) in order to obtain the free base, which was used for the synthesis of **2e**. The reaction was carried out at reflux for 3 hours. The solvent was removed under vacuum by rotatory evaporation and the residue was recrystallized from ethanol. The precipitate was filtered and washed with dichloromethane and hexane (25 mL of each). A brown powder was obtained. Yield: 34 %; mp: 178-179 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.83 (s, 2H, -CH_2-), 7.64-7.67 (m, 2H, H3 + H8), 7.82 (t, 1H, *J*=8.5 Hz, H6), 8.02 (t, 2H, *J*=8.4 Hz, H5 + H7), 8.46 (d, 1H, *J*=8.4 Hz, H4), 9.50 ppm (bs, 3H,

NH + NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ 37.3 (-CH_2-), 122.2 (C3), 127.8 (C6 + C4a), 128.9 (C5 + C8), 131.2 (C7), 138.7 (C4), 147.4 (C8a), 157.1 (C2), 171.1 ppm (S-C-(NH)(NH2)). IR (KBr): $\tilde{\nu}$ 3382 (s; N-H, N-H_2), 3035 (s; C-H_{arom}), 1676-1641 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 221(30), 193(100), 165(33), 142(36), 73(44), 55(54). Elemental analysis calculated (%) for $C_{11}H_{11}N_3S$·HCl: C: 52.07, H: 4.77, N: 16.56; found: C: 51.59, H: 4.80, N: 16.47

4.1.18. (6,7-Dimethoxy-2-oxo-2*H*-chromen-4-yl)methyl carbamimidothioate hydrobromide (2f)

Conditions: 3 h at reflux. The precipitate was filtered and washed with ether (25 mL). A yellow powder was obtained. Yield: 92 %; mp: 253-254 ºC. ^1H NMR (400 MHz, DMSO-d_6): δ 3.88 (s, 6H, (-OCH_3) ×2), 4.78 (s, 2H, -CH_2-), 6.39 (s, 1H, H3), 7.13 (s, 1H, H5), 7.34 (s, 1H, H8), 9.14 + 9.33 ppm (bs + bs, 4H, NH_2 + NH + HBr), ^{13}C NMR (100 MHz, DMSO-d_6): δ 31.9 (-CH_2-), 57.2 + 57.4 ((-OCH_3) ×2), 101.3 (C8), 107.3 (C5), 110.6 (C3), 112.7 (C4a), 146.8 (C6), 150.1 (C8a), 150.8 (C7), 153.7 (C4), 160.9 (C2), 168.9 ppm (Se-C-(NH)(NH_2)). IR (KBr): $\tilde{\nu}$ 3310-3214 (s; N-H, N-H_2), 2975(s; C-H_{aliph}), 1692 (s; C=O), 1651 (s; C=N). MS (*m/z* (% abundance)): 300(21), 294(26), 277(29), 252(100), 237(42), 219(26), 209(16), 191(94), 177(9), 147(19), 76(30). Elemental analysis calculated (%) for $C_{13}H_{14}N_2O_4S$·HBr: C: 41.61 H: 4.03 N: 7.47; found: C: 41.36, H: 3.79, N: 7.24.

4.1.19. Quinoxaline-2,3-diylbis(methylene) dicarbamimidothioate dihydrobromide (2g)

Conditions: 3.5 h at reflux. The precipitate was filtered and washed with ether (25 mL). A gray powder was obtained. Yield: 16 %; mp: 221 ºC (direct combustion). ^1H NMR

(400 MHz, DMSO-d_6): δ 5.07 (s, 4H, (-CH_2-) ×2), 7.89-7.91 (m, 2H, H6 + H7), 8.04-8.07 (m, 2H, H5+ H8), 9.01 + 9.26 ppm (bs + bs, 4H, NH_2 + NH + HBr). ^{13}C NMR (100 MHz, DMSO-d_6): δ 35.9 ((-CH_2-) ×2), 129.1 (C5 + C8), 131.6 (C6 + C8), 140.5 (C4a + C8a), 150.1 (C2 + C3), 170.6 ppm ((-Se-C-(NH)(NH_2)) x2). IR (KBr): $\tilde{\nu}$ 3378-3243 (s; N-H, N-H_2), 3091 (s; C-H_{arom}), 2992 (s; C-H_{aliph}), 1631 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 272(2), 220(9), 187(31), 156(9), 102(9), 76(100), 60(27). Elemental analysis calculated (%) for $C_{12}H_{14}N_6S_2 \cdot 2HBr$: C: 30.78, H: 3.44, N: 17.95; found: C: 30.43, H: 3.26, N: 18.27.

4.1.20. (1,3-Dioxoisoindolin-2-yl)methyl carbamimidothioate hydrobromide (2h)

Conditions: 2 h at reflux. The precipitate was washed with ethyl ether (50 mL). A white powder was obtained. Yield: 77 %; mp: 217-219 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.40 (s, 2H, -CH_2-), 7.90-7.92 (m, 2H, H4 + H7), 7.95-7.97 (m, 2H, H5 + H6), 9.30 ppm (bs, 4H, NH_2 + NH + HBr). ^{13}C NMR (100 MHz, DMSO-d_6): δ 39.4 (-CH_2-), 124.5 (C4 + C7), 132.2 (C3a + C7a), 135.9 (C5 + C6), 167.5 (C1 + C3), 168.1 ppm (S-C-(NH)(NH_2)). IR (KBr): $\tilde{\nu}$ 3322-3129 (s; N-H, N-H_2), 3009 (s; C-H_{arom}), 1774-1716 (s; C=O), 1655 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 233(40), 191(36), 163(91), 110(49), 75(54), 57(85), 43(100). Elemental analysis calculated (%) for $C_{10}H_9N_3O_2S \cdot HBr$: C: 37.99, H: 3.19, N: 13.29; found: C: 37.87, H: 3.22, N: 13.36.

4.1.21. (3,5-Dimethylisoxazol-4-yl)methyl carbamimidothioate hydrochloride (2i)

Conditions: 2.5 h at reflux. The solvent was removed under vacuum by rotatory evaporation. The yellow solid was washed with acetone and ether (25 mL of each). Yield: 91 %; mp: 179-181 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.24 (s, 3H, C3-CH_3), 2.40 (s, 3H, C5-CH_3), 4.45 (s, 2H, -CH_2-), 9.42 ppm (bs, 3H, NH_2 + NH). ^{13}C NMR

(100 MHz, DMSO-d_6): δ 9.7 ((C5)-CH_3), 10.8 ((C3)-CH_3), 23.6 (-CH_2-), 108.0 (C4), 159.2 (C5), 167.5 (C3), 168.8 ppm (-S-C(NH)(NH_2)). IR (KBr): $\tilde{\nu}$ 3228 (s; N-H, N-H_2), 3066 (s; C-H_{arom}), 1652 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 160(16), 144(56), 110(58), 76(53), 68(95), 43(100). Elemental analysis calculated (%) for $C_7H_{11}N_3OS$ ·HCl: C: 37.92, H: 5.46, N: 18.95; found: C: 37.67, H: 5.62, N: 19.52.

4.1.22. 4-(1*H*-Pyrrol-1-yl)benzyl carbamimidothioate hydrobromide (2j)

Conditions: reflux for 2 h. The precipitate was filtered and the solvent was evaporated under vacuum by rotatory evaporation. The residue was precipitated with hexane (10 mL) and washed with acetone and ether (25 mL of each). A brown powder was obtained. Yield: 75 %; mp: 195-196 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.52 (s, 2H, -CH_2-), 6.27 (t, 2H, *J*=2.0 Hz, H3' + H4'), 7.38 (t, 2H, *J*=2.0 Hz, H2' + H5'), 7.50 (d, 1H, *J*=8.5Hz, H2 + H6), 7.60 (d, 1H, *J*=8.5 Hz, H3 + H5), 9.01 + 9.20 ppm (bs + bs, 4H, NH_2 + NH + HBr). ^{13}C NMR (100 MHz, DMSO-d_6): δ 33.6 (-CH_2-), 110.6 (C3' + C4'), 118.9 (C2' + C5'), 119.4 (C3 + C5), 130.3 (C2 + C6), 131.7 (C1), 139.4 (C4), 168.9 ppm (S-C-(NH_2)(NH)). IR (KBr): $\tilde{\nu}$ 3453-3233 (s; N-H + N-H_2), 3088 (s; C-H_{arom}), 1639 cm^{-1} (s; C=N). MS (*m/z* (% abundance)): 231(59), 156(100), 128(27), 43(44). Elemental analysis calculated (%) for $C_{12}H_{13}N_3S$·HBr: C: 46.16, H: 4.52, N: 13.46; found: C: 45.90, H: 4.76, N: 13.20.

4.2. Biological evaluation

4.2.1. Cell cultures

PANC-1, BxPC3 and 1205Lu cell lines were grown in DMEM medium; DU145 and A549 cells in RPMI 1640 medium; HT29, HCT116 and RKO cells were grown in McCoy's 5A medium (Corning # 10-051-CI) at 37 °C and 5 % CO_2. All used mediums

were supplemented with 10 % fetal bovine serum (FBS) and 100 units/mL of penicillin and streptomycin (Corning # 30-002-CI). Cell lines were purchased from ATCC.

4.2.2. Reagents and anti-bodies

Dimethyl Sulfoxide (DMSO) was purchased from Fisher (#BP231-100) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma (#2128). The antibodies for Caspase 3 (#9668S), PARP (#9542S) and XIAP (#14334s) were obtained from Cell Signaling and Bcl-2 (#ab7973) from abcam. β-Actin antibody was purchased from Sigma-Aldrich (A5316).

4.2.3. Cell viability

A total of 3,000 cells/well were treated with either DMSO or increasing concentrations (0.5-50 µM) of the new seleno- and thio-urea derivatives for 24, 48 or 72 hours. Three hours prior the termination point, 20 µL of MTT (5 mg/mL stock) was added. Resultant formazan crystals were dissolved in 50 µL of DMSO and absorbance was measured at 570 nm and 630 nm wavelength. IC_{50} values were calculated using GraphPad Prism version 6.01.

4.2.4. Live and Dead assay

HCT116 cells (7×10^4 /well) were seeded in a 6 well plate, grown overnight and treated with the different concentrations of **1g** for 24 h. Then they were trypsinized and stained with Calcein AM and Ethidium Homodimer-1 (LIVE/DEAD viability/cytotoxicity kit for mammalian cells, Life Technologies) according to the manufacturer's instructions. Data were analyzed by flow cytometry (BD FACSCalibur)

4.2.5. Annexin V assay

The Muse™ Annexin V & Dead Cell kit (#MCH100105) was employed for this essay according to manufacturer's instructions. HCT116 cells (5×10^5 cells/well) were plated in a 6 well plate and treated with DMSO (control) or different amounts of **1g**. After 24 h, cells were collected and stained with 100 µL of Muse™ Annexin V & Dead Cell Reagent. After 20 minutes of incubation at room temperature in dark, samples were analyzed on the Muse™ Cell Analyzer (Millipore).

4.2.6. Caspase 3/7 assay

HCT116 cells (4.5×10^5 cells/well) were plated and treated with DMSO (negative control) or increasing concentrations of compound **1g** for 24 h. 50 µL of cells suspension were stained with the Muse™ Caspase 3/7 Reagent working solution (Muse™ Caspase 3/7 Kit, #MCH100108, Millipore) and incubated for 30 minutes in a 37 ºC incubator with 5 % CO_2. After incubation, 150 µL of Muse™ Caspase 7-ADD solution was added to each sample. After another 5 minutes of incubation in dark at room temperature, results were obtained by Muse™ Cell Analyzer (Millipore).

4.2.7. Western blot analysis

A total of 6×10^5 HCT116 cells/well were treated with different concentrations of **1g** for 24 h. Whole cell lysates were prepared by incubating the cells in RIPA buffer (Thermo Scientific #89900) supplemented with 1 % phosphatase inhibitor cocktail 2 (Sigma #P5726-5ML), 1 % protease inhibitor (Complete mini, Roche #11836170001) and 0.5 % of 200 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma #P7626-250MG). Cell lysates were spun at 15,000 *g* for 10 min to remove any insoluble cell debris. Resultant

supernatants were collected and stored at -80 °C. The whole cell lysates were resolved by SDS-page. Proteins were transferred to Immobilon®-P PVDF Membrane (Millipore # IPVH304F0) and blotted with the corresponding antibodies overnight in cold. Then the membrane was incubated with the corresponding peroxidase linked secondary antibody for 1-4 h at room temperature. Antibodies were detected by an enhanced chemiluminescence reagent (Thermo Scientific #1856135 and #1856136).

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Highlights

- A series of 20 novel selenourea and thiourea derivatives was synthesized
- Compounds were tested against a panel of six human cancer cell lines
- The selenourea derivatives showed lower IC_{50} than the sulfur-containing analogs
- IC_{50} values for **1g** were below 7.0 µM in all the cell lines and time points tested
- **1g** induced apoptosis by modulation of Caspases and anti-apoptotic proteins