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Synthesis and antiproliferative activity of novel symmetrical alkylthio- and alkylseleno-imidocarbamates

Elena Ibáñez [a], Daniel Plano [a], María Font [b], Alfonso Calvo [c], Celia Prior [c], Juan Antonio Palop [a,*], Carmen Sanmartín [a]

[a] Synthesis Section, Department of Organic and Pharmaceutical Chemistry, University of Navarra, Irunlarrea, 1, E-31008 Pamplona, Spain
[b] Molecular Modeling Section, Department of Organic and Pharmaceutical Chemistry, University of Navarra, Irunlarrea, 1, E-31008 Pamplona, Spain
[c] Oncology Division, Center for Applied Medical Research, CIMA, University of Navarra, Pío XII, 53, E-31008 Pamplona, Spain

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ABSTRACT

The study described here concerns the synthesis of a series of thirty new symmetrically substituted imidothiocarbamate and imidoselenocarbamate derivatives and their evaluation for antitumoral activity *in vitro* against a panel of five human tumor cell lines: breast adenocarcinoma (MCF-7), colon carcinoma (HT-29), lymphocytic leukemia (K-562), hepatocarcinoma (Hep-G2), prostate cancer (PC-3) and one non-malignant mammary gland-derived cell line (MCF-10A). The GI_{50} values for eighteen of the compounds were below 10 μM in at least one cell line. Two cancer cells (MCF-7 and HT-29) proved to be the most sensitive to five compounds (**1b**, **2b**, **3b**, **4b** and **5b**), with growth inhibition in the nanomolar range, and compounds **1b**, **3b**, **7b**, **8b** and **9b** gave values of less than 1 μM. In addition, all of the aforementioned compounds exhibited lower GI_{50} values than some of the standard chemotherapeutic drugs used as references. The results also reveal that the nature of the aliphatic chain (methyl is better than benzyl) at the selenium position and the nature of the heteroatom (Se better than S) have a marked influence on the antiproliferative activity of the compounds. These findings reinforce our earlier hypothesis concerning the determinant role of the selenomethyl group as a scaffold for the biological activity of this type of compound. Considering both the cytotoxic parameters and the selectivity index (which was compared in MCF-7 and MCF-10A cells), compounds **2b** and **8b** (with a selenomethyl moiety) displayed the best profiles, with GI_{50} values ranging from 0.34 nM to 6.07 μM in the five cell lines tested.

Therefore, compounds **2b** and **8b** were evaluated by flow cytometric analysis for their effects on cell cycle distribution and apoptosis in MCF-7 cells. **2b** was the most active, with an apoptogenic effect similar to camptothecin, which was used as a positive control. Both of them provoked cell cycle arrest leading to the accumulation of cells in either G_2/M and S phase.

These two compounds can therefore be considered as the most promising candidates for the development of novel generations of antitumor agents.

1. Introduction

Cancer is still a major health problem and it represents the main cause of death in many countries. Although major advances have been made in the chemotherapeutic management of some patients, the continued commitment to the laborious task of discovering new anticancer agents remains critically important [1,2]. In the last three years, among the wide range of compounds tested as potential anticancer agents, several structurally diverse derivatives that contain a selenium template have been reported and have generated growing interest [3–13]. At present, our main focus in the laboratory is the synthesis and evaluation of the cytotoxic activity of selenium-containing compounds. Taking into account the results obtained in previous studies [14–18], we present here twenty new derivatives that contain this trace element. In addition, based on a rational bioisosteric approach, the differences in the anticarcinogenic activity between two similar chemical elements, selenium and sulfur, were compared. The previous hypothesis that isosteric replacement of sulfur by selenium would result in more effective anticancer agents [14], is reinforced by the higher activity shown by selenium derivatives as compared to the activity of the sulfur analogs [19–22]. With the aim of finding further support for the starting hypothesis, ten methylthio analogs were synthesized and screened. It is well known that certain alkylthio groups have

* Corresponding author. Tel.: +34 948 425 600; fax: +34 948 425 649.
E-mail address: jpalop@unav.es (J.A. Palop).

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proven to be key structural components for antitumoral agents [23–28].

Considering the information given above, as well as the chemical structural patterns previously described by our group [14,15] and the structures of chemotherapeutic drugs with recognized efficacy in cancer treatment, we propose a general structure (Fig. 1) for the new compounds described here. The following characteristics were taken into consideration: (1) Molecular symmetry, as a broad concept, because this is a structural property that is frequently present in many cytotoxic drugs [29] and some reported sulfur [24–28] and selenium derivatives have this property [6,9,13]. (2) A central scaffold consisting of a methyl imidothiocarbamate (methylisothiourea) or methyl (methylselenourea) and benzyl (benzylselenourea) imidoselenocarbamates connected by a carbonyl group onto two identical lateral heteroaromatic rings. The methyl group was selected on the basis of data in the literature that show this moiety is present (Fig. 2) in selenoderivatives with anticancer activity [30,31]. There is increasing evidence that anticarcinogenic effects for some seleno compounds have been consistently associated with methylselenol, an active metabolite of methylseleno derivatives. The benzyl template is present (Fig. 2) in 1,4-phenylenebis(methylene)selenocyanate (*p*-XSC) and in *p*-xylylbis(methylselenide) (*p*-XMS), two organoselenium compounds with relevant biological properties since they act as regulators of cell cycle, growth factor signaling, angiogenesis and cell invasion and metastasis [32].

The choice of lateral rings was based on data that support the antitumor activity of mono-, bi- or tri-cyclic heteroaromatic systems following a model based on a fragmental approach. Among the many moieties identified, the following are included in our series of chemical structures: furyl [33,34], thienyl [35,36], isoxazole [37,38], benzothiophene [39], benzodioxole [40], quinoline [41,42], phenylquinoline [43,44] and acridine [45,46].

Finally, in the design of the new series of compounds we considered the potential adjustment to the Lipinski rules, which is taken as an estimation of the potential bioavailability. According to the aforementioned rules, an orally active drug must not violate more than one of the following criteria: ≤5 hydrogen donors (nOHNH), ≤10 hydrogen acceptors (nON), MW ≤ 500, clog*P* ≤ 5.

In view of the rational outline above and our previous work related to finding new structures with potential chemotherapeutic activities, we report here the synthesis of thirty previously unreported alkylthio- and alkylseleno-imidocarbamate derivatives and their biological evaluation as potential antitumoral agents.

2. Results and discussion

2.1. Chemistry

The synthesis of the bisacylimidocarbamates **1**–**10a**, **1**–**10b** and **1**–**10c** was carried out according to Scheme 1, starting from the appropriate *S*-methyl imidothiocarbamate (**a**), *Se*-methyl imidoselenocarbamate (**b**) or *Se*-benzyl imidoselenocarbamate (**c**) as hydrohalides, and the corresponding heteroaryl acyl chloride (**1**–**10**) in a 1:2 M ratio in chloroform in the presence of pyridine as a catalyst at room temperature for 48 h. The compounds were

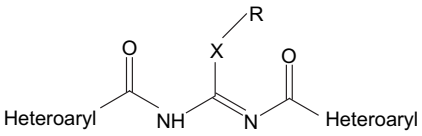


Fig. 1. General structure of compounds.

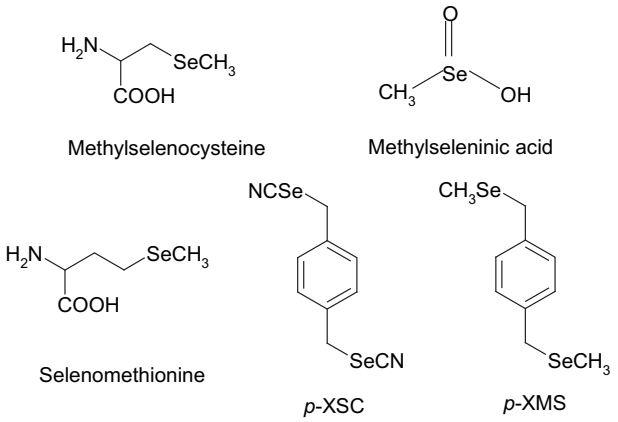
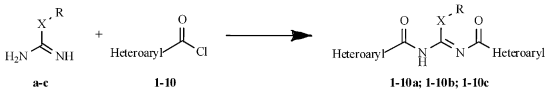


Fig. 2. Some selenoderivatives with anticancer activity.

obtained in yields ranging from 2 to 56%. The purity of each compound was assessed by TLC and elemental analysis and the structures were identified by spectroscopic methods such as IR, ^{1}H NMR and MS. In the IR spectra, the compounds showed absorption



	a	b	c
1	1a	1b	1c
2	2a	2b	2c
3	3a	3b	3c
4	4a	4b	4c
5	5a	5b	5c
6	6a	6b	6c
7	7a	7b	7c
8	8a	8b	8c
9	9a	9b	9c
10	10a	10b	10c

Scheme 1. Synthesis of bisacylimidocarbamates **1**–**10a**, **1**–**10b** and **1**–**10c**.

Table 1
Average GI_{50}, TGI and LC_{50} values (μM) for compounds for compounds **1**–**10a**, **1**–**10b** and **1**–**10c**.

	MCF-7[a]			HT-29[b]			K-562[c]			Hep-G2[d]			PC-3[e]			MCF-10A[f]		
Comp.	GI_{50}[g]	TGI[h]	LC_{50}[i]	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
1a	56.08	>100	>100	77.14	>100	>100	64.84	>100	>100	>100	>100	>100	56.18	>100	>100	8.22	46.52	85.69
2a	44.49	88.96	>100	77.92	>100	>100	72.59	>100	>100	>100	>100	>100	39.02	98.25	>100	19.70	55.10	90.50
3a	10.28	57.10	>100	35.58	76.52	>100	36.95	78.89	>100	65.25	>100	>100	24.68	57.38	90.08	0.09	23.72	64.11
4a	44.65	78.07	>100	51.82	91.96	>100	56.85	98.68	>100	63.41	>100	>100	41.48	82.45	>100	1.55	21.35	75.14
5a	77.83	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	99.24	>100	>100	16.85	>100	>100
6a	9.51	43.40	78.48	16.76	52.46	88.16	34.66	66.63	98.60	15.81	52.99	75.84	8.58	39.36	72.55	0.02	5.60	54.56
7a	24.55	59.69	94.83	46.42	>100	>100	67.99	>100	>100	61.42	>100	>100	28.71	54.54	80.38	1.01	5.03	9.05
8a	23.90	>100	>100	>100	>100	>100	65.75	>100	>100	57.57	>100	>100	76.30	>100	>100	0.50	42.17	82.19
9a	43.08	87.70	>100	23.69	>100	>100	34.39	>100	>100	6.24	48.15	>100	7.48	>100	>100	0.04	0.54	48.81
10a	27.68	>100	>100	69.17	>100	>100	45.02	75.98	>100	90.25	>100	>100	51.08	87.26	>100	1.00	>100	>100
1b	0.03	88.92	>100	5×10^{-5}	>100	>100	8.78	>100	>100	0.76	8.66	>100	0.36	56.67	>100	5.94	40.16	98.07
2b	0.0034	5.64	>100	3.4×10^{-4}	>100	>100	6.07	>100	>100	0.63	1.50	12.51	1.34	63.03	>100	9.26	41.14	73.51
3b	0.30	31.18	>100	0.0049	38.80	>100	4.81	28.46	90.03	0.69	4.02	8.67	0.97	7.03	>100	3.16	5.49	7.82
4b	7.6×10^{-4}	28.14	>100	6.3×10^{-4}	>100	>100	4.98	>100	>100	0.62	1.55	10.04	0.10	7.09	>100	1.95	4.74	7.53
5b	9×10^{-5}	57.52	>100	7.9×10^{-4}	>100	>100	0.87	45.63	>100	0.51	9.35	>100	0.09	39.32	>100	2.89	15.41	63.94
6b	5.07	32.66	80.64	5.62	40.14	>100	32.43	60.99	89.55	9.59	41.01	73.29	37.16	65.59	94.02	0.49	34.65	73.21
7b	0.99	52.77	>100	0.53	>100	>100	9.23	46.30	85.20	5.84	23.62	73.14	4.24	36.79	>100	5.58	45.97	>100
8b	0.66	8.03	60.70	0.20	>100	>100	3.80	29.92	74.75	4.16	23.68	87.77	2.33	43.64	>100	20.34	52.10	83.86
9b	0.49	7.52	>100	0.46	32.59	90.69	5.80	30.58	73.05	4.54	10.13	65.42	3.97	40.22	>100	0.71	20.05	76.90
10b	73.92	>100	>100	7.63	63.95	>100	27.16	60.98	94.79	22.30	52.38	82.46	41.28	80.12	>100	18.25	53.28	88.31
1c	8.80	57.98	>100	7.19	38.08	89.11	9.53	44.18	80.59	7.65	39.45	76.51	30.48	57.63	84.78	8.80	57.98	>100
2c	72.52	>100	>100	55.07	>100	>100	29.54	58.48	87.42	34.71	58.09	81.46	49.86	>100	>100	72.52	>100	>100
3c	3.68	28.33	>100	6.51	>100	>100	7.63	95.82	>100	4.42	45.46	>100	52.70	>100	>100	3.68	28.33	>100
4c	24.44	54.10	83.76	9.25	54.65	>100	23.48	55.84	88.20	34.08	62.84	91.59	35.91	69.19	>100	24.44	54.10	83.76
5c	7.80	>100	>100	6.82	>100	>100	19.97	57.34	94.72	9.12	58.92	>100	53.12	>100	>100	7.80	>100	>100
6c	37.94	62.92	87.90	6.51	53.40	>100	14.94	42.79	70.63	21.01	49.00	76.99	30.53	58.42	86.31	37.94	62.92	87.90
7c	55.34	>100	>100	57.27	>100	>100	97.84	>100	>100	>100	>100	>100	>100	>100	>100	55.34	>100	>100
8c	23.55	>100	>100	26.12	>100	>100	29.34	72.61	>100	8.54	48.85	90.28	34.84	>100	>100	23.55	>100	>100
9c	8.46	45.56	97.04	6.07	>100	>100	9.06	47.39	88.86	25.81	52.37	78.94	39.26	97.07	>100	8.46	45.56	97.04
10c	53.69	88.14	>100	26.92	>100	>100	9.60	44.53	80.65	30.42	64.46	98.51	42.30	77.38	>100	53.69	88.14	>100
Doxorubicin[j]	0.02	5.01	19.95	0.20	6.31	25.40	0.10	5.01	19.95	n.d.[k]	n.d.	n.d.	0.25	3.16	19.95	n.d.	n.d.	n.d.
Camptothecin[j]	0.01	0.16	>100	0.05	0.79	31.62	0.06	50.12	>100	n.d.	n.d.	n.d.	0.06	>100	>100	n.d.	n.d.	n.d.
Etoposide[j]	19.95	>100	>100	31.62	>100	>100	12.59	>100	>100	n.d.	n.d.	n.d.	0.63	3.98	79.43	n.d.	n.d.	n.d.
Taxol[j]	0.003	>100	>100	0.003	0.20	79.43	0.004	15.85	>100	n.d.	n.d.	n.d.	0.004	12.59	>100	n.d.	n.d.	n.d.
Cisplatin[j]	3.16	>100	>100	7.95	>100	>100	5.01	>100	>100	n.d.	n.d.	n.d.	5.01	50.12	>100	n.d.	n.d.	n.d.
Methotrexate[j]	0.06	>100	>100	0.04	>100	>100	0.03	>100	>100	n.d.	n.d.	n.d.	0.1	>100	>100	n.d.	n.d.	n.d.

[a] Breast adenocarcinoma.
[b] Colon carcinoma.
[c] Leukemia.
[d] Liver carcinoma.
[e] Prostate carcinoma.
[f] Nontumorigenic breast epithelial cells.
[g] Concentration that inhibits 50% of cell growth.
[h] Concentration that inhibits 100% of cell growth.
[i] Concentration that kills 50% of cells.
[j] NCI data (http://dtp.nci.nih.gov).
[k] No data.

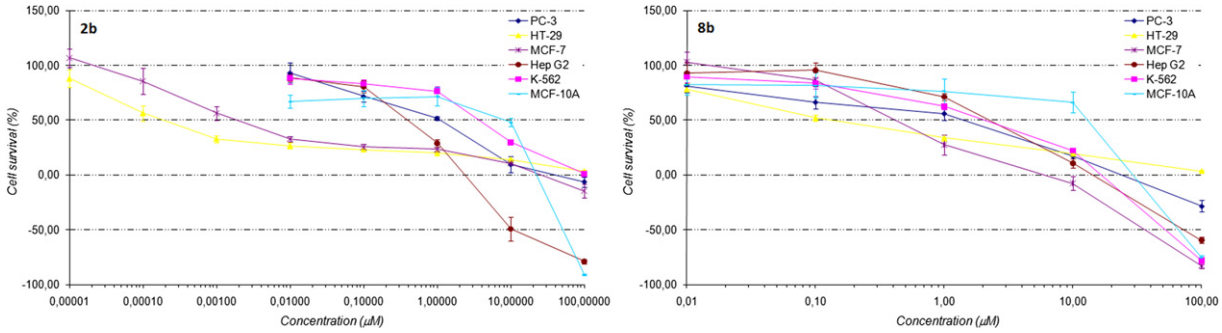


Fig. 3. Cytotoxic effect of compounds **2b** and **8b** on MCF-7, HT-29, K-562, Hep-G2, PC-3 and MCF-10A cells. Data are expressed as the percentage of growth ± SEM of at least 3 independent experiments performed in quadruplicate.

bands around 3400–3450 cm^{-1} originated from the N–H stretching vibration. The strong bands between 1646 and 1776 cm^{-1} corresponded to carbonyl vibrations. In most of the ^1H NMR spectra, one sharp peak from 13.77 to 15.11 ppm was observed due to the presence of the proton in secondary amide NH. The protons on heteroaryl rings were split into multiple peaks from 6.08 to 9.86 ppm. The chemical shift of methyl group attached to the heteroatom sulfur or selenium appeared at δ 2.59–2.82 and δ 2.44–2.67 respectively.

2.2. Biological evaluation

2.2.1. Cytotoxicity

All thirty of the synthesized compounds were screened for their cytotoxic and antiproliferative activities against a panel of five human tumor cell lines: breast adenocarcinoma (MCF-7), colon carcinoma (HT-29), lymphocytic leukemia (K-562), hepatocarcinoma (Hep-G2) and prostate cancer (PC-3) as well as the non-malignant mammary gland cell line MCF-10A. These cell lines represent common cancer tumor types. Regardless of the type of tumor cell, several common features were observed in the antiproliferative activities of the compounds. Cytotoxicity assays were based on the reactivity of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] according to a method previously described by Denizot and Lang [47]. Results are expressed as GI_{50}: the concentration that reduces by 50% the growth of treated cells with respect to untreated controls; TGI: the concentration that completely inhibits cell growth, and LC_{50}: the concentration that kills 50% of the cells. The cytotoxic effect of each substance was tested at five different concentrations between 0.01 and 100 μM, or at lower levels when the GI_{50} was less than 10 nM. Mean GI_{50}, TGI and LC_{50} values are summarized in Table 1. Doxorubicin, camptothecin, etoposide, taxol, cisplatin and methotrexate were used as controls. In order to assess the selectivity of the compounds for tumor cells, cytotoxicity was tested in MCF-10A normal cells and the results were compared to those of MCF-7 cancer cells (Table 1). The analyses were all carried out with a minimum of three independent experiments and values were calculated after 72 h exposure.

The methylthio derivatives (**1**–**10a**) exhibited comparable and weak antiproliferative activities in all cancer cells tested, as shown by the results in Table 1. Compound **6a** in MCF-7 and in PC-3 and compound **9a** in Hep-G2 and in PC-3 had GI_{50} values lower than 10 μM. However, when sulfur was replaced by selenium all of the methylseleno compounds (**1**–**10b**) presented GI_{50} values below 10 μM in all the cell lines tested, with the exception of **6b** in PC-3 (GI_{50} value of 37.16 μM) and K-562 (32.43 μM), and **10b** in all cell lines apart from HT-29. Among the cancer cell lines tested, MCF-7 and HT-29 were the most susceptible ones, with GI_{50} values in the nanomolar ranges for **2b**, **4b** and **5b** in MCF-7 and **1b**, **2b**, **3b**, **4b** and **5b** in HT-29. Moreover, GI_{50} values for **1b**, **3b**, **7b**, **8b** and **9b** in MCF-7, and the values for **7b**, **8b** and **9b** in HT-29, were below 1 μM.

Comparison of the results with the cytotoxicity values for the standard drugs showed that three (**2b**, **4b**, **5b**) of the compounds had GI_{50} values lower than those found for doxorubicin, nine lower than those obtained for etoposide (**1b**, **2b**, **3b**, **4b**, **5b**, **6b**, **7b**, **8b** and **9b**) and two (**4b** and **5b**) lower than those observed for taxol in MCF-7 cells. The results reveal that the selenomethyl derivatives are more antiproliferative than the corresponding benzyl derivatives. In addition, the results indicate that the replacement of the methyl group by benzyl had less effect than the replacement of sulfur by selenium. For example, GI_{50} values in MCF-7 for compounds **2b**, **4b** and **5b** were at least four orders of magnitude lower than those determined for **2c**, **4c** and **5c** in the same cell line. Similar effects were observed in HT-29 and, to a lesser extent, in K-562, Hep-G2 and PC-3. The likely explanation for this finding is that compounds **1**–**10b**, with a selenomethyl moiety, act as methylselenol precursors. It has been suggested that methylselenol is a critical selenium metabolite for anticancer activity [48–50].

In spite of this, compounds **1c**, **3c**, **5c** and **9c** showed pronounced inhibitory effects (GI_{50} < 10 μM) on at least three

Table 2
Average GI_{50} values (μM) for compounds **1**–**5b**, **8b** and **9b** in PC-3 and HT-29 cell lines using CV method.

Comp.	PC-3[a] GI_{50}[c]	HT-29[b] GI_{50}
1b	0.16	1.0×10^{-4}
2b	0.63	9.0×10^{-4}
3b	0.24	0.0059
4b	0.11	7.1×10^{-4}
5b	0.03	4.9×10^{-4}
8b	1.42	0.46
9b	3.72	0.85

[a] Prostate carcinoma.
[b] Colon carcinoma.
[c] Concentration that inhibits 50% of cell growth.

Table 3
Effects of **2b** and **8b** on apoptosis in MCF-7 cells at a concentration of 15 μM for 24 and 48 h using the *Apo-Direct* kit (BD Pharmingen) based on the TUNEL technique.

Compound	24 h	48 h
Control	9.77 ± 2.34	19.61 ± 0.75
2b	29.35 ± 3.19**	71.08 ± 1.88**
8b	7.70 ± 1.63	27.19 ± 1.89**
Camptothecin	21.25 ± 6.16	71.45 ± 7.80**

Results are expressed as percent (%) of apoptotic cells and data are representative of three experiments (mean ± SEM). **$p < 0.01$ with respect to the control.

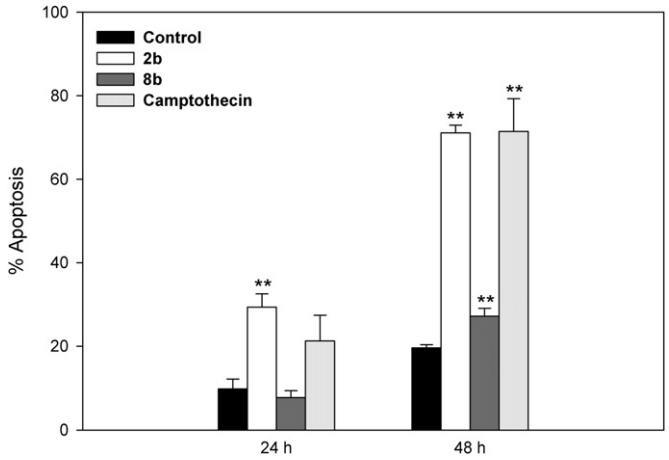


Fig. 4. Compounds **2b** and **8b** induce an apoptotic effect on MCF-7 cells at 48 h. MCF-7 cells were incubated either with 15 μM of the indicated compound or vehicle (control cells) for 24 and 48 h. The results are presented as the mean ± SEM of three independent experiments (duplicate wells). $^{**}p < 0.01$ with respect to the control.

tumoral cell lines. In addition, these chemical structures were more active than the positive etoposide against HT-29 cell growth (with the exception of **2c** and **7c**). Once again, the test compounds were more active against MCF-7 and HT-29 cell growth.

In the present study a precise structure–activity relationship cannot be defined, although some general trends related to the heteroaromatic rings can be highlighted. When the size of the heteroaryl group was increased (from five members to a fused-ring system) a loss of antiproliferative activity was observed. This suggests that the bulk of the ring is critical for the antitumor activity. Compounds **1**–**5b** showed GI_{50} values between 5×10^{-5} μM in HT-29 and 8.78 μM in K-562, whereas compounds **6**–**10b** gave values that ranged from 0.20 in HT-29 to 73.92 μM in MCF-7. A relationship between the presence of substituents with strongly deactivating electron-withdrawing groups (e.g., nitro) in the thienyl ring and increased activity in seleno compounds seems to exist. For example, the unsubstituted derivatives **2b** and **2c** are less active than the nitro-derivatives **4b** and **4c**, mainly in MCF-7, HT-29 and PC-3 cell lines.

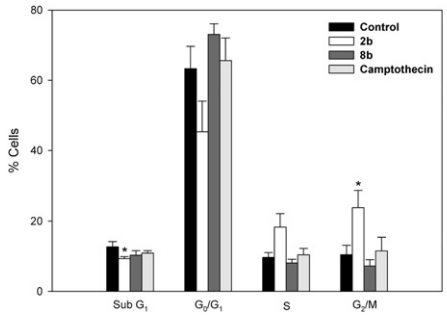
The tested compounds also affected the proliferation of the normal mammary gland cell line MCF-10A, although GI_{50} and TGI values were usually higher than those obtained for cancer cell lines. The ratio between the cytotoxic parameters found in MCF-10A and those observed in MCF-7 can be considered as a measurement of compound selectivity. Ratios between 3 and 6 refer to moderate selectivity, ratios greater than six indicate high selectivity, while compounds that do not fulfill either of these criteria are rated non-selective [51]. If we consider the results as a whole, the most promising candidates are **2b** and **8b**. These compounds were highly selective for MCF-7 cell growth inhibition compared to normal mammary cells, as evidenced by the ratios of their GI_{50} ($GI_{50\ MCF\text{-}10A}/GI_{50\ MCF\text{-}7}$ ranged from 31 for **2b** to 2723 for **8b**) and TGI ($TGI_{MCF\text{-}10A}/TGI_{MCF\text{-}7}$ ranged from 6.5 for **2b** to 7.3 for **8b**). Fig. 3 shows exemplary curves with the original data from which the GI_{50}, TGI and LC_{50} values for compounds **2b** and **8b** were calculated.

Although MTT test is the most usual method for evaluating the cytotoxic effects of chemicals in cultured cells, this assay could be questioned when testing selenium-containing compounds. Selenium could have an influence on these results due to modification of redox processes in cells. In order to validate the GI_{50} values obtained from the MTT assay, crystal violet (CV) assays were performed for the seven most active compounds (**1**–**5b**, **8b** and **9b**) in two representative cell lines (PC-3 and HT-29). The GI_{50} values obtained from the CV method (Table 2) validated those obtained from the MTT assay. Therefore, we consider the MTT assay as a valid cytotoxicity method for the organoselenium compounds studied in this paper.

Analysis of the Lipinski descriptors for bioavailability estimation [52,53] using the freely accessible program OSIRIS Explorer Properties was also carried out. Compounds **2b** (clogP = 3.15; MW = 357; nOHNH = 1; nON = 4) and **8b** (clogP = 4.09; MW = 447; nOHNH = 1; nON = 6) met the Lipinski criteria. However, the reference drugs for pharmacological testing, doxorubicin, etoposide and taxol, which were also analyzed with this program, did not fulfill the Lipinski criteria. For example, doxorubicin (clogP = 0.48; MW = 543; nOHNH = 7; nON = 12) did not fulfill three out of four conditions and etoposide (clogP = 0.53; MW = 588; nOHNH = 3; nON = 13) and taxol (clogP = 3.64; MW = 853; nOHNH = 4; nON = 15) did not fulfill two of them.

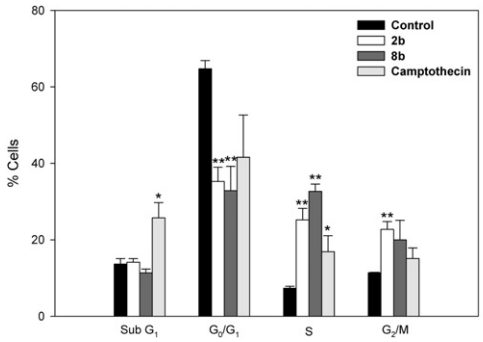
2.2.2. Apoptosis and effects on cell cycle progression

Mounting evidence indicates that apoptosis is a critical mechanism for cancer prevention by Se compounds [15,54–56]. In addition, cell cycle arrest is one of the targets of many anticancer drugs, including doxorubicin and camptothecin. To gain further insight into the mechanisms of action of these new active compounds (**2b** and **8b**) and as a preliminary study, we decided to assess whether this activity was related to their ability to induce apoptosis and affect cell cycle progression in MCF-7 cells, due to the remarkable cytotoxic activity of the compounds **2b** and **8b** in this cell line. The cells were treated with 15 μM of the corresponding compound for 24 and 48 h and the apoptotic status (Table 3 and Fig. 4) and cell cycle progression (Figs. 5 and 6) were determined by flow cytometry analysis [57]. The apoptotic status of the cells after 24 and 48 h of treatment with 15 μM of the corresponding compound was determined using the *Apo-Direct* kit (BD Pharmingen) [57] based on the TUNEL technique. Camptothecin was used as



Compound	Sub G_1	G_0/G_1	S	G_2/M
Control	12.66 ± 1.51	63.32 ± 6.32	9.65 ± 1.41	10.43 ± 2.69
2b	9.31 ± 0.56*	45.32 ± 8.73	18.30 ± 3.86	23.85 ± 4.91*
8b	10.29 ± 1.30	73.06 ± 3.02	8.10 ± 1.03	7.21 ± 1.81
Camptothecin	10.92 ± 0.64	65.62 ± 6.40	10.40 ± 1.82	11.51 ± 3.89

Fig. 5. Effects of **2b** and **8b** on cell cycle distribution in MCF-7 cells. Exponentially growing cells were treated with 15 μM of the indicated compound or vehicle (control) for 24 h. The changes of cell cycle phase distribution were assessed by DNA flow cytometric analysis. Results are expressed as percentages of total cell counts. Camptothecin was used as a positive control. Results are presented as the mean ± SEM of three independent experiments (duplicate wells). * $0.05 > p > 0.01$ with respect the control.



Compound	Sub G_1	G_0/G_1	S	G_2/M
Control	13.64 ± 1.46	64.74 ± 2.18	7.33 ± 0.53	11.38 ± 0.12
2b	14.14 ± 0.94	35.25 ± 3.74**	25.21 ± 3.00**	22.74 ± 2.05**
8b	11.34 ± 1.01	32.87 ± 6.34**	32.68 ± 1.93**	19.97 ± 5.12
Camptothecin	25.73 ± 3.99*	41.61 ± 11.03	16.91 ± 4.17*	15.15 ± 2.72

Fig. 6. Effects of **2b** and **8b** on cell cycle distribution in MCF-7 cells. Exponentially growing cells were treated with 15 μM of the indicated compound or vehicle (control) for 48 h. The changes of cell cycle phase distribution were assessed by DNA flow cytometric analysis. Results are expressed as percentages of total cell counts. Camptothecin was used as a positive control. Results are presented as the mean ± SEM of three independent experiments (duplicate wells). * $0.05 > p > 0.01$ with respect the control.

a positive control. The results obtained are shown in Table 3 and Figs. 4–6. As can be seen, **2b** led to a marked increase in the proportion of apoptotic cells in the cultures accompanied of accumulation in S and G_2/M phases and a diminution of cell proportion in G_0/G_1 phase at both times. On the other hand, **8b** only exhibited a light apoptotic effect at 48 h and had a similar behaviour on cell cycle distribution.

In summary, considering both the biological activities and the Lipinski parameters, the two most active and selective compounds (**2b** and **8b**) should be considered as novel candidates for further studies to understand the exact mechanism of action in cancer cells. Work is currently in progress in our laboratory with this aim and results will be reported in due course.

3. Conclusions

The biological activities of the designed compounds confirm our hypothesis that molecular symmetry and the presence of a selenomethyl moiety is a valid approach to obtain potent new antitumor agents. Eighteen compounds exhibited inhibitory effects (<10 μM) on at least one cell line. MCF-7 and HT-29 were the most susceptible lines, with GI_{50} values in the nanomolar range for three (**2b**, **4b** and **5b**) and five (**1b**, **2b**, **3b**, **4b** and **5b**) compounds, respectively. In addition, **1b**, **3b**, **7b**, **8b** and **9b** showed GI_{50} values lower than 1 μM in these same cell lines. It is noteworthy that some of these compounds were more cytotoxic than the reference drugs currently used in the treatment of cancer patients. As a result of the preliminary SAR, it was revealed that the nature of the aliphatic chain (methyl > benzyl) at the selenium site and the nature of the heteroatoms (Se > S) have a critical influence on the antiproliferative activity of the molecules. Considering all of the biological results and the predicted values for Lipinski parameters, compounds **2b** and **8b** were found to be the most appropriate as candidates for further testing with the aim of finding more selective and active anticancer drugs. For these reasons, they were evaluated by flow cytometric analysis for their effects on cell cycle distributions and apoptosis induction in MCF-7 cells. Results indicated that both of them effectively induced cell cycle accumulation at S and G_2/M phases and arrest at G_0/G_1, which consequently trigger apoptosis.

4. Experimental protocols

4.1. Chemistry

Melting points were determined with a Mettler FP82 + FP80 apparatus (Greifense, Switzerland) and are uncorrected. The ^{1}H NMR spectra were recorded on a Bruker 400 Ultrashield™ spectrometer (Rheinstetten, Germany) using TMS as the internal standard. The IR spectra were obtained on a Thermo Nicolet FT-IR Nexus spectrophotometer with KBr pellets. Elemental microanalyses were carried out on vacuum-dried samples using a LECO CHN-900 Elemental Analyzer. Alugram® SIL G/UV_{254} (Layer: 0.2 mm) (Macherey–Nagel GmbH & Co. KG. Postfach 101352, D-52313 Düren, Germany) was used for Thin Layer Chromatography. Chemicals were purchased from E. Merck (Darmstadt, Germany), Scharlau (F.E.R.O.S.A., Barcelona, Spain), Panreac Química S.A. (Montcada i Reixac, Barcelona, Spain), Sigma–Aldrich Química S.A. (Alcobendas, Madrid, Spain), Acros Organics (Janssen Pharmaceuticalaan 3a, 2440 Geel, België) and Lancaster (Bischheim-Strasbourg, France).

4.1.1. General procedure for the synthesis of compounds ***a*–*c***

The appropriate alkyl halide (45.0 mmol) was added dropwise to a cooled (0 °C), stirred mixture of thiourea (2.47 g, 32.5 mmol) or selenourea (4.0 g, 32.5 mmol) in dry ethanol (25 mL). The mixture was heated under reflux for 90 min. The solvent was removed *in vacuo* and the product was recrystallized from ethanol.

*4.1.1.1. Methyl imidothiocarbamate hydroiodide (**a**).* IR (KBr): 3500–3200, 1634 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6, δ): 2.24 (t, 3H, S–CH_3); 8.87 (bs, 3H, NH·HI, NH_2). Anal. Calcd. for $C_2H_6N_2S$·HI (%): C, 11.01; H, 3.21; N, 12.85. Found: C, 11.16; H, 3.12; N, 12.92.

*4.1.1.2. Methyl imidoselenocarbamate hydroiodide (**b**).* IR (KBr): 3319–3102, 1635 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6, δ): 2.45 (s, 3H, Se–CH_3); 8.90 (bs, 3H, NH·HI, NH_2). Anal Calcd. for $C_2H_6N_2Se$·HI (%): C, 9.45; H, 2.76; N, 11.03. Found: C, 9.02; H, 2.51; N, 10.66.

*4.1.1.3. Benzyl imidoselenocarbamate hydrobromide (**c**).* IR (KBr): 3219–3086, 1657 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6, δ): 4.54 (s, 2H, Se–CH_2); 7.28 (t, 1H, $J_{3-4} = J_{4-5} = 7.5$ Hz, H_4); 7.35 (t, 2H, $J_{2-3} = J_{5-6} = 7.5$ Hz, 2H, H_3, H_5); 7.41 (d, 2H, H_2, H_6); 9.16 (s, 2H, NH_2); 9.28 (s, 2H, NH·HBr). Anal Calcd. for $C_8H_{10}N_2Se$·HBr (%): C, 32.66; H, 3.74; N, 9.53. Found: C, 32.50; H, 3.76; N, 9.36.

4.1.2. General procedure for the synthesis of compounds ***1*–*10***

Compounds **1**, **2**, **3**, **4** and **5** were commercially available. Compounds **6**, **7**, **8**, **9** and **10** were prepared from the corresponding carboxylic acid (9.18 mmol) and thionyl chloride (30 mL) with heating under reflux for 2 h. The thionyl chloride was removed *in vacuo*. The resulting acyl chloride was used without further purification.

4.1.3. General procedure for the synthesis of compounds ***1*–*10a*, *1*–*10b* and *1*–*10c***

A solution of the corresponding acyl chloride **1**–**10** (9.18 mmol) in chloroform (25 mL) was slowly added dropwise to a stirred

solution of compounds **a**–**c** (4.59 mmol) in dry chloroform (40 mL) and pyridine (5 mL). The mixture was stirred for 48 h at room temperature. Solvents were removed under vacuum by rotatory evaporation and the residue was treated with water (100 mL) and purified.

*4.1.3.1. Methyl N,N′-di(fur-2-ylcarbonyl)-imidothiocarbamate (**1a**)* . From methyl imidothiocarbamate hydroiodide **a** and 2-furoyl chloride **1**. Recrystallized from ethanol/DMF. Yield: 38%. m.p.163–164 °C. IR (KBr): 3398, 1676 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, δ): 2.63 (s, 3H, SCH_3); 7.19–7.21 (m, 2H, H_4, $H_{4'}$); 7.65 (dd, 1H, $J_{5'-3'}$ = 0.9 Hz, $J_{5'-4'}$ = 4.9 Hz, $H_{5'}$); 7.72 (dd, 1H, J_{5-3} = 0.8 Hz, J_{5-4} = 5.0 Hz, H_5); 7.84 (dd, 1H, J_{3-4} = 3.8 Hz, H_3); 7.98 (dd, 1H, $J_{3'-4'}$ = 3.7 Hz, $H_{3'}$); 14.37 (s, 1H, NH). MS (*m*/*z*, % abundance): 278 (M^+, <1); 111 (100). Anal Calcd. for $C_{12}H_{10}O_4N_2S\cdot 0.9HCl$ (%): C, 46.32; H, 3.22; N, 9.00. Found: C, 46.46, H, 3.52; N, 9.03.

*4.1.3.2. Methyl N,N′-di(thien-2-ylcarbonyl)-imidothiocarbamate (**2a**)* . From methyl imidothiocarbamate hydroiodide **a** and 2-thiophenecarbonyl chloride **2**. Recrystallized from ethanol/DMF. Yield: 14%. m.p. 153–154 °C. IR (KBr): 3447, 1673 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, δ): 2.62 (s, 3H, SCH_3); 7.19–7.22 (m, 2H, H_4, $H_{4'}$); 7.65 (dd, 1H, $J_{5'-3'}$ = 1.2 Hz, $J_{5'-4'}$ = 4.9 Hz, $H_{5'}$); 7.72 (dd, 1H, J_{5-3} = 1.1 Hz, J_{5-4} = 4.9 Hz, H_5); 7.84 (dd, 1H, J_{3-4} = 3.8 Hz, H_3); 7.98 (dd, 1H, $J_{3'-4'}$ = 3.8 Hz, $H_{3'}$); 14.37 (s, 1H, NH). MS (*m*/*z*, % abundance): 310 (M^+, 17); 111 (100). Anal Calcd. for $C_{12}H_{10}O_2N_2S_3$ (%): C, 46.45; H, 3.23; N, 9.03. Found: C, 46.70; H, 3.31; N, 9.39.

*4.1.3.3. Methyl N,N′-di(3-chlorothien-2-ylcarbonyl)-imidothiocarbamate (**3a**).* From methyl imidothiocarbamate hydroiodide **a** and 3-chloro-2-thiophenecarbonyl chloride **3**. Recrystallized from ethanol/DMF. Yield: 18%. m.p.165–166 °C. IR (KBr): 3426, 1662 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, δ): 2.59 (s, 3H, SCH_3); 7.06 (d, 1H, $J_{4'-5'}$ = 5.3 Hz, $H_{4'}$); 7.08 (d, 1H, J_{4-5} = 5.3 Hz, H_4); 7.51 (d, 1H, $H_{5'}$); 7.63 (d, 1H, H_5); 13.77 (s, 1H, NH). MS (*m*/*z*, % abundance): 379 (M^+, 1); 145 (100). Anal Calcd. for $C_{12}H_8N_2O_2Cl_2S_3$ (%): C, 37.99; H, 2.11; N, 7.39. Found: C, 37.84; H, 2.37; N, 7.17.

*4.1.3.4. Methyl N,N′-di(5-nitrothien-3-ylcarbonyl)-imidothiocarbamate (**4a**).* From methyl imidothiocarbamate hydroiodide **a** and 5-nitro-3-thiophenecarbonyl chloride **4**. Recrystallized from ethanol/DMF. Yield: 27%. m.p. 225–226 °C. IR (KBr): 3430, 1702 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, δ): 2.66 (s, 3H, SCH_3); 8.33 (s, 1H, $H_{5'}$); 8.37 (s, 1H, H_5); 8.45 (s, 1H, H_3); 8.48 (s, 1H, $H_{3'}$); 14.51 (s, 1H, NH). MS (*m*/*z*, % abundance): 110 (100). Anal Calcd. for $C_{12}H_8N_4O_6S_3$ (%): C, 36.00; H, 2.00; N, 14.00. Found: C, 36.24; H, 2.26; N, 13.80.

*4.1.3.5. Methyl N,N′-di(5-isoxazol-5-ylcarbonyl)-imidothiocarbamate (**5a**).* From methyl imidothiocarbamate hydroiodide **a** and isoxazole-5-carbonyl chloride **5**. Purified by washed with water and diethylether. Yield: 50%. m.p. 177–178 °C. IR (KBr): 3387, 1706 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, δ): 2.67 (s, 3H, SCH_3); 7.09 (s, 1H, $H_{4'}$); 7.18 (s, 1H, H_4); 8.41 (s, 1H, H_3); 8.47 (s, 1H, $H_{3'}$); 14.14 (s, 1H, NH). MS (*m*/*z*, % abundance): 96 (100). Anal Calcd. for $C_{10}H_8O_4N_4S$ (%): C, 42.86; H, 2.86; N, 20.00. Found: C, 42.90; H, 2.92; N, 20.30.

*4.1.3.6. Methyl N,N′-di(thienaphthen-2-ylcarbonyl)-imidothiocarbamate (**6a**).* From methyl imidothiocarbamate hydroiodide **a** and thianaphthene-2-carbonyl chloride **6**. Recrystallized from ethanol/DMF. Yield: 43%. M.p. 226–227 °C. IR (KBr): 3436, 1685 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, δ): 2.72 (s, 3H, SCH_3); 7.49–7.51 (m, 4H, H_6, H_7, $H_{6'}$, $H_{7'}$); 7.93–7.95 (m, 4H, H_5, H_8, $H_{5'}$, $H_{8'}$); 8.11 (s, 1H, H_3); 8.27 (s, 1H, $H_{3'}$); 14.68 (s, 1H, NH). MS (*m*/*z*, % abundance): 410 (M^+, 9); 161 (100). Anal Calcd. for $C_{20}H_{14}N_2O_2S_3\cdot 0.5HCl$ (%): C, 56.04; H, 3.39; N, 6.54. Found: C, 56.16; H, 3.40; N, 6.43.

*4.1.3.7. Methyl N,N′-di(3,4-methylenedioxybenzoyl)-imidothiocarbamate (**7a**).* From methyl imidothiocarbamate hydroiodide **a** and 3,4-methylenedioxy benzoyl chloride **7**. Recrystallized from ethanol/DMF. Yield: 56%. m.p. 195–196 °C. IR (KBr): 3430, 1694 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, δ): 2.63 (s, 3H, SCH_3); 6.08 (s, 2H, CH_2); 6.11 (s, 2H, CH_2'); 6.90 (d, 1H, J_{2-3} = 8.2 Hz, H_3); 6.95 (d, 1H, $J_{2'-3'}$ = 8.2 Hz, $H_{3'}$); 7.51 (d, 1H, J_{2-6} = 1.7 Hz, H_6); 7.61 (dd, 1H, H_2); 7.79 (d, 1H, $J_{2'-6'}$ = 1.4 Hz, $H_{6'}$); 8.02 (dd, 1H, $H_{2'}$); 14.53 (s, 1H, NH). MS (*m*/*z*, % abundance): 386 (M^+, 8); 149 (100). Anal Calcd. for $C_{18}H_{14}N_2O_6S$ (%): C, 55.96; H, 3.63; N, 7.25. Found: C, 55.71; H, 4.03; N, 7.11.

*4.1.3.8. Methyl N,N′-di(quinolin-3-ylcarbonyl)-imidothiocarbamate (**8a**).* From methyl imidothiocarbamate hydroiodide **a** and quinoline-3-carbonyl chloride **8**. Recrystallized from ethanol/DMF. Yield: 40%. m.p. 206–207 °C. IR (KBr): 3416, 1703 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.78 (s, 3H, SCH_3); 7.66 (t, 1H, J_{8-7} = J_{8-9} = 8.0 Hz, H_8); 7.72 (t, 1H, $J_{8'-7'}$ = $J_{8'-9'}$ = 8.4 Hz, $H_{8'}$); 7.87 (t, 1H, J_{7-6} = 8.0 Hz, H_7); 7.92 (t, 1H, $J_{7'-6'}$ = 8.4 Hz, $H_{7'}$); 8.02 (d, 1H, H_9); 8.10 (d, 1H, $H_{9'}$); 8.20 (d, 1H, H_6); 8.23 (d, 1H, $H_{6'}$); 8.83 (d, 1H, J_{4-2} = 2.2 Hz, H_4); 9.12 (d, 1H, $J_{4'-2'}$ = 1.6 Hz, $H_{4'}$); 9.56 (d, 1H, H_2); 9.83 (d, 1H, $H_{2'}$); 14.96 (s, 1H, NH). MS (*m*/*z*, % abundance): 400 (M^+, 1); 57 (100). Anal Calcd. for $C_{22}H_{16}N_4O_2S$ (%): C, 66.00; H, 4.00; N, 14.00. Found: C, 65.86; H, 4.16; N, 14.32.

*4.1.3.9. Methyl N,N′-di(2-phenylquinolin-3-ylcarbonyl)-imidothiocarbamate (**9a**).* From methyl imidothiocarbamate hydroiodide **a** and 2-phenylquinoline-3-carbonyl chloride **9**. Recrystallized from ethanol/DMF. Yield: 31%. m.p. 201–202 °C. IR (KBr): 3430, 1701 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.74 (s, 3H, SCH_3); 7.57–7.59 (m, 7H, H_{13}, H_{14}, H_{15}, $H_{8'}$, $H_{13'}$, $H_{14'}$, $H_{15'}$); 7.71 (t, 1H, H_8); 7.79 (t, 1H, H_7); 7.87 (t, 1H, $H_{7'}$); 8.29–8.31 (m, 6H, H_6, H_{12}, H_{16}, $H_{6'}$, $H_{12'}$, $H_{16'}$); 8.33 (s, 1H, H_3); 8.50 (d, 1H, H_9); 8.76 (s, 1H, $H_{3'}$); 8.89 (d, 1H, $H_{9'}$); 14.34 (s, 1H, NH). MS (*m*/*z*, % abundance): 65 (100). Anal Calcd. for $C_{34}H_{24}N_4O_2S$ (%): C, 73.91; H, 4.35; N, 10.14. Found: C, 74.01; H, 4.55; N, 10.30.

*4.1.3.10. Methyl N,N′-di(acridin-9-ylcarbonyl)-imidothiocarbamate (**10a**).* From methyl imidothiocarbamate hydroiodide **a** and 9-acridinecarbonyl chloride **10**. Recrystallized from ethanol/DMF. Yield: 6%. m.p. >300 °C. IR (KBr): 3423, 1776 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.82 (s, 3H, SCH_3); 6.30 (s, 1H, NH); 6.71 (d, 2H, H_6, H_{10}); 6.96 (t, 2H, H_5, H_{11}); 7.08 (d, 2H, $H_{6'}$, $H_{10'}$); 7.20 (t, 2H, $H_{5'}$, $H_{11'}$); 7.50 (t, 2H, H_4, H_{12}); 7.73 (t, 2H, $H_{4'}$, $H_{12'}$); 7.78 (d, 2H, H_3, H_{13}); 8.24 (bs, 2H, $H_{3'}$, $H_{13'}$). MS (*m*/*z*, % abundance): 500 (M^+, 1); 57 (100). Anal Calcd. for $C_{30}H_{20}N_4O_2S\cdot 0.1HCl$ (%): C, 71.48; H, 3.99; N, 11.12. Found: C, 71.29; H, 4.11; N, 11.46.

*4.1.3.11. Methyl N,N′-di(fur-2-ylcarbonyl)-imidoselenocarbamate (**1b**).* From methyl imidoselenocarbamate hydroiodide **b** and 2-furoyl chloride **1**. Recrystallized from ethanol. Yield: 48%. m.p. 131–132 °C. IR (KBr): 3436, 1684 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.44 (s, 3H, $SeCH_3$); 6.58 (dd, 1H, $J_{4'-3'}$ = 1.7 Hz $J_{4'-5'}$ = 3.5 Hz, $H_{4'}$); 6.62 (dd, 1H, J_{4-3} = 1.7 Hz, J_{4-5} = 3.5 Hz, H_4); 7.40 (dd, 2H, J_{5-3} = $J_{5'-3'}$ = 0.8 Hz H_5, $H_{5'}$); 7.66 (dd, 1H, H_3); 7.69 (dd, 1H, $H_{3'}$); 14.23 (s, 1H, NH). MS (*m*/*z*, % abundance): 326 (M^+, 5); 231 (100). Anal Calcd. for $C_{12}H_{10}N_2O_4Se$ (%): C, 44.31; H, 3.08; N, 8.61. Found: C, 44.44; H, 3.15; N, 8.71.

*4.1.3.12. Methyl N,N′-di(thien-2-ylcarbonyl)-imidoselenocarbamate (**2b**).* From methyl imidoselenocarbamate hydroiodide **b** and 2-thiophenecarbonyl chloride **2**. Recrystallized from ethanol/DMF. Yield: 47%. m.p. 161–162 °C. IR (KBr): 3442, 1668 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.47 (s, 3H, $SeCH_3$); 7.17 (dd, 1H, $J_{4'-3'}$ = 3.8 Hz, $J_{4'-5'}$ = 4.9 Hz, 1H, $H_{4'}$); 7.20 (dd, 1H, J_{4-3} = 3.8 Hz, J_{4-5} = 4.9 Hz, H_4);

7.65 (dd, 1H, $J_{5'-3'} = 1.2$ Hz, $H_{5'}$); 7.73 (dd, 1H, $J_{5-3} = 1.1$ Hz, H_5); 7.84 (dd, 1H, H_3); 7.98 (dd, 1H, $H_{3'}$); 14.49 (s, 1H, NH). MS (*m*/*z*, % abundance): 358 (M^+, 1); 111 (100). Anal Calcd. for $C_{12}H_{10}N_2O_2S_2Se$ (%): C, 40.34; H, 2.80; N, 7.84. Found: C, 40.38; H, 2.96; N, 7.88.

*4.1.3.13. Methyl N,N′-di(3-chlorothien-2-ylcarbonyl)-imidoselenocarbamate (**3b**).* From methyl imidoselenocarbamate hydroiodide **b** and 3-chloro-2-thiophenecarbonyl chloride **3**. Recrystallized from ethanol/DMF. Yield: 4%. m.p. 174–175 °C. IR (KBr): 3426, 1654 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.44 (s, 3H, $SeCH_3$); 7.06 (d, 1H, $J_{4'-5'} = 5.3$ Hz, $H_{4'}$); 7.09 (d, 1H, $J_{4-5} = 5.3$ Hz, H_4); 7.53 (d, 1H, $H_{5'}$); 7.66 (d, 1H, H_5); 13.96 (s, 1H, NH). MS (*m*/*z*, % abundance): 426 (M^+, 1); 161 (100). Anal Calcd. for $C_{12}H_8N_2O_2Cl_2S_2Se$ (%): C, 33.80; H, 1.88; N, 6.57. Found: C, 34.14; H, 1.85; N, 6.50.

*4.1.3.14. Methyl N,N′-di(5-nitrothien-3-ylcarbonyl)-imidoselenocarbamate (**4b**).* From methyl imidoselenocarbamate hydroiodide **b** and 5-nitro-3-thiophenecarbonyl chloride **4**. Purified by washed with water and diethylether. Yield: 18%. m.p. 199–200 °C. IR (KBr): 3430, 1690 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.51 (s, 3H, $SeCH_3$); 8.34 (s, 1H, $H_{5'}$); 8.37 (s, 1H, H_5); 8.46 (s, 1H, H_3); 8.50 (s, 1H, $H_{3'}$); 14.64 (s, 1H, NH). MS (*m*/*z*, % abundance): 448 (M^+, 1); 156 (100). Anal Calcd. for $C_{12}H_8N_4O_6S_2Se$ (%): C, 32.21; H, 1.79; N, 12.53. Found: C, 32.58; H, 1.91; N, 12.47.

*4.1.3.15. Methyl N,N′-di(5-isoxazol-5-ylcarbonyl)-imidoselenocarbamate (**5b**).* From methyl imidoselenocarbamate hydroiodide **b** and isoxazole-5-carbonyl chloride **5**. Recrystallized from ethanol. Yield: 14%. m.p. 162–163 °C. IR (KBr): 3416, 1703 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.54 (s, 3H, $SeCH_3$); 7.20 (bs, 2H, H_4, $H_{4'}$); 8.45 (bs, 2H, H_3, $H_{3'}$); 14.25 (s, 1H, NH). MS (*m*/*z*, % abundance): 327 (M^+, 1); 96 (100). Anal Calcd. for $C_{10}H_8O_4N_4Se$ (%): C, 36.70; H, 2.45; N, 17.12. Found: C, 36.64; H, 2.36; N, 17.24.

*4.1.3.16. Methyl N,N′-di(thienaphthen-2-ylcarbonyl)-imidoselenocarbamate (**6b**).* From methyl imidoselenocarbamate hydroiodide **b** and thianaphthene-2-carbonyl chloride **6**. Recrystallized from ethanol/DMF. Yield: 2%. m.p. 203–204 °C. IR (KBr): 3430, 1678 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.57 (s, 3H, $SeCH_3$); 7.49–7.51 (m, 4H, H_6, H_7, $H_{6'}$, $H_{7'}$); 7.94–7.96 (m, 4H, H_5, H_8, $H_{5'}$, $H_{8'}$); 7.98 (s, 1H, H_3); 8.11 (s, 1H, $H_{3'}$); 14.68 (s, 1H, NH). MS (*m*/*z*, % abundance): 161 (100). Anal Calcd. for $C_{20}H_{14}N_2O_2S_2Se$ (%): C, 52.52; H, 3.06; N, 6.13. Found: C, 52.72; H, 3.14; N, 6.12.

*4.1.3.17. Methyl N,N′-di(3,4-methylenedioxybenzoyl)-imidoselenocarbamate (**7b**).* From methyl imidoselenocarbamate hydroiodide **b** and 3,4-methylenedioxy benzoyl chloride **7**. Recrystallized from ethanol/DMF. Yield: 30%. m.p. 178–179 °C. IR (KBr): 3427, 1685 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.47 (s, 3H, $SeCH_3$); 6.08 (s, 2H, CH_2); 6.10 (s, 2H, CH_2'); 6.89 (d, 1H, $J_{2-3} = 7.9$ Hz, H_3); 6.94 (d, 1H, $J_{2'-3'} = 8.1$ Hz, $H_{3'}$); 7.50 (s, 1H, H_6); 7.60 (d, 1H, H_2); 7.78 (s, 1H, $H_{6'}$); 8.03 (d, 1H, $H_{2'}$); 14.65 (s, 1H, NH). MS (*m*/*z*, % abundance): 433 (M^+, 1); 95 (100). Anal Calcd. for $C_{18}H_{14}N_2O_6Se$ (%): C, 49.88; H, 3.01; N, 6.02. Found: C, 50.01; H, 3.27; N, 6.16.

*4.1.3.18. Methyl N,N′-di(quinolin-3-ylcarbonyl)-imidoselenocarbamate (**8b**).* From methyl imidoselenocarbamate hydroiodide **b** and quinoline-3-carbonyl chloride **8**. Recrystallized from ethanol. Yield: 23%. m.p. 181–182 °C. IR (KBr): 3446, 1686 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.63 (s, 3H, $SeCH_3$); 7.64 (t, 1H, $J_{8-7} = J_{8-9} = 7.8$ Hz, H_8); 7.71 (t, 1H, $J_{8'-7'} = J_{8'-9'} = 7.8$ Hz, $H_{8'}$); 7.86 (t, 1H, $J_{7-6} = 7.8$ Hz, H_7); 7.91 (t, 1H, $J_{7'-6'} = 7.8$ Hz, $H_{7'}$); 8.00 (d, 1H, H_9); 8.08 (d, 1H, $H_{9'}$); 8.23 (d, 1H, H_6); 8.25 (d, 1H, $H_{6'}$); 8.82 (d, 1H, $J_{4-2} = 2.1$ Hz, H_4); 9.18 (d, 1H, $J_{4'-2'} = 1.7$ Hz, $H_{4'}$); 9.56 (d, 1H, H_2); 9.84 (d, 1H, $H_{2'}$); 15.09 (s, 1H, NH). MS (*m*/*z*, % abundance): 128 (100). Anal Calcd. for $C_{22}H_{16}N_4O_2Se$(%): C, 59.06; H, 3.58; N, 12.53. Found: C, 58.87; H, 3.71; N, 12.59.

*4.1.3.19. Methyl N,N′-di(2-phenylquinolin-3-ylcarbonyl)-imidoselenocarbamate (**9b**).* From methyl imidoselenocarbamate hydroiodide **b** and 2-phenylquinoline-3-carbonyl chloride **9**. Recrystallized from ethanol/DMF. Yield: 24%. m.p. 179–182 °C. IR (KBr): 3449, 1694 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.59 (s, 3H, $SeCH_3$); 7.58–7.60 (m, 7H, H_{13}, H_{14}, H_{15}, $H_{8'}$, $H_{13'}$, $H_{14'}$, $H_{15'}$); 7.71 (t, 1H, H_8); 7.79 (t, 1H, H_7); 7.87 (t, 1H, $H_{7'}$); 8.29–8.31 (m, 6H, H_6, H_{12}, H_{16}, $H_{6'}$, $H_{12'}$, $H_{16'}$); 8.33 (s, 1H, H_3); 8.51 (d, 1H, H_9); 8.81 (s, 1H, $H_{3'}$); 8.91 (d, 1H, $H_{9'}$); 14.46 (s, 1H, NH). MS (*m*/*z*, % abundance): 128 (100). Anal Calcd. for $C_{34}H_{24}O_2N_4Se$ (%): C, 68.10; H, 4.01; N, 9.36. Found: C, 68.20; H, 4.22; N, 9.09.

*4.1.3.20. Methyl N,N′-di(acridin-9-ylcarbonyl)-imidoselenocarbamate (**10b**).* From methyl imidoselenocarbamate hydroiodide **b** and 9-acridinecarbonyl chloride **10**. Recrystallized from ethanol/DMF. Yield: 18%. m.p. 232–233 °C. IR (KBr): 3398, 1692 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 2.67 (s, 3H, $SeCH_3$); 6.32 (s, 1H, NH); 6.73 (d, 2H, $J_{5'-6'} = J_{10'-11'} = 7.4$ Hz, $H_{6'}$, $H_{10'}$); 6.97 (t, 2H, $J_{4-5} = J_{5-6} = J_{10-11} = J_{11-12} = 7.0$ Hz, H_5, H_{11}); 7.08 (d, 2H, H_6, H_{10}); 7.20 (t, 2H, $J_{4'-5'} = J_{11'-12'} = 7.4$ Hz, $H_{5'}$, $H_{11'}$); 7.52 (t, 2H, $J_{3-4} = J_{12-13} = 7.0$ Hz, H_4, H_{12}); 7.74–7.76 (m, 4H, H_3, H_{13}, $H_{4'}$, $H_{12'}$); 8.34 (d, 2H, $J_{3'-4'} = J_{12'-13'} = 8.8$ Hz, $H_{3'}$, $H_{13'}$). MS (*m*/*z*, % abundance): 57 (100). Anal Calcd. for $C_{30}H_{20}N_4O_2Se \cdot 0.1HCl$ (%): C, 65.38; H, 3.65; N, 10.17. Found: C, 65.55; H, 4.02; N, 9.61.

*4.1.3.21. Benzyl N,N′-di(fur-2-ylcarbonyl)-imidoselenocarbamate (**1-c**).* From benzyl imidoselenocarbamate hydrobromide **c** and 2-furoyl chloride **1**. Recrystallized from ethanol. Yield: 56%. m.p. 146–147 °C. IR (KBr): 3447, 1684 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$, δ): 4.51 (s, 2H, $SeCH_2$); 6.58 (bs, 1H, $H_{4'}$); 6.62 (bs, 1H, H_4); 7.26 (t, 1H, $J_{3''-4''} = J_{4''-5''} = 7.3$ Hz, $H_{4''}$); 7.32 (t, 2H, $J_{2''-3''} = J_{5''-6''} = 7.3$ Hz, $H_{3''}$, $H_{5''}$); 7.40–7.42 (m, 4H, H_5, $H_{5'}$, $H_{2''}$, $H_{6''}$); 7.66 (bs, 1H, H_3); 7.71 (bs, 1H, $H_{3'}$); 14.26 (s, 1H, NH). MS (*m*/*z*, % abundance): 401 (M^+, 1); 95 (100). Anal Calcd. for $C_{18}H_{14}O_4N_2Se$ (%): C, 53.86; H, 3.49; N, 6.98. Found: C, 54.06; H, 3.26; N, 6.97.

*4.1.3.22. Benzyl N,N′-di(thienyl-2-carbonyl)-imidoselenocarbamate (**2c**).* From benzyl imidoselenocarbamate hydrobromide **c** and 2-thiophenecarbonyl chloride **2**. Recrystallized from ethanol. Yield: 44%. m.p. 183–184 °C. IR (KBr): 3423, 1676 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 4.57 (s, 2H, $SeCH_2$); 7.19 (dd, 1H, $J_{4'-3'} = 3.8$ Hz, $J_{4'-5'} = 4.9$ Hz, $H_{4'}$); 7.21 (dd, 1H, $J_{4-3} = 3.9$ Hz, $J_{4-5} = 4.9$ Hz, H_4); 7.26 (t, 1H, $J_{3''-4''} = J_{4''-5''} = 6.9$ Hz, $H_{4''}$); 7.33 (ddd, 2H, $J_{2''-3''} = J_{5''-6''} = 7.6$ Hz, $H_{3''}$, $H_{5''}$); 7.67 (dd, 1H, $J_{3'-5'} = 1.2$ Hz, $H_{5'}$); 7.73 (dd, 1H, $J_{3-5} = 1.1$ Hz, H_5); 7.85 (dd, 1H, H_3); 8.02 (dd, 1H, $H_{3'}$); 14.54 (s, 1H, NH). MS (*m*/*z*, % abundance): 434 (M^+,1); 111 (100). Anal Calcd. for $C_{18}H_{14}N_2O_2S_2Se$ (%): C, 49.88; H, 3.23; N, 6.47. Found: C, 49.64; H, 3.33; N, 6.57.

*4.1.3.23. Benzyl N,N′-di(3-chlorothien-2-ylcarbonyl)-imidoselenocarbamate (**3c**).* From benzyl imidoselenocarbamate hydrobromide **c** and 3-chloro-2-thiophenecarbonyl chloride **3**. Recrystallized from ethanol/DMF. Yield: 11%. m.p. 175–177 °C. IR (KBr): 3430, 1646 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 4.52 (s, 2H, $SeCH_2$); 7.08 (d, 2H, $J_{4-5} = J_{4'-5'} = 5.3$ Hz, H_4, $H_{4'}$); 7.27–7.29 (m, 1H, $H_{4''}$); 7.33 (t, 2H, $J_{2''-3''} = J_{3''-4''} = J_{4''-5''} = J_{5''-6''} = 7.4$ Hz, $H_{3''}$, $H_{5''}$); 7.42 (d, 2H, $H_{2''}$, $H_{6''}$); 7.54 (bs, 1H, $H_{5'}$); 7.64 (bs, 1H, H_5); 14.00 (s, 1H, NH). MS (*m*/*z*, % abundance): 145 (100). Anal Calcd. for $C_{18}H_{12}N_2O_2Cl_2S_2Se$ (%): C, 43.03; H, 2.39; N, 5.58. Found: C, 43.00; H, 2.61; N, 5.40.

*4.1.3.24. Benzyl N,N′-di(5-nitrothien-3-ylcarbonyl)-imidoselenocarbamate (**4c**).* From benzyl imidoselenocarbamate hydrobromide **c** and 5-nitro-3-thiophenecarbonyl chloride **4**. Recrystallized from

ethanol. Yield: 26%. m.p. 156–157 °C. IR (KBr): 3426, 1692 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 4.54 (s, 2H, $SeCH_2$); 7.30–7.32 (m, 1H, $H_{4''}$); 7.36 (t, 2H, $J_{2''-3''} = J_{3''-4''} = J_{4''-5''} = J_{5''-6''} = 7.3$ Hz, $H_{3''}$, $H_{5''}$); 7.43 (d, 2H, $H_{2''}$, $H_{6''}$); 8.41–8.43 (m, 4H, H_5, H_3, $H_{3'}$, $H_{5'}$); 14.65 (s, 1H, NH). MS (*m*/*z*, % abundance): 91 (100). Anal Calcd. for $C_{18}H_{12}N_4O_6S_2Se$ (%): C, 38.60; H, 2.32; N, 10.01. Found: C, 38.09; H, 2.30; N, 9.78.

*4.1.3.25. Benzyl N,N′-di(5-isoxazol-5-ylcarbonyl)-imidoselenocarbamate (**5c**).* From benzyl imidoselenocarbamate hydrobromide **c** and isoxazole-5-carbonyl chloride **5**. Recrystallized from ethanol. Yield: 19%. m.p. 117–119 °C. IR (KBr): 3393, 1706 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 4.58 (s, 2H, $SeCH_2$); 7.14 (bs, 2H, H_4, $H_{4'}$); 7.28 (t, 1H, $J_{3''-4''} = J_{4''-5''} = 7.5$ Hz, $H_{4''}$); 7.34 (t, 2H, $J_{2''-3''} = J_{5''-6''} = 7.5$ Hz, $H_{3''}$, $H_{5''}$); 7.44 (d, 2H, $H_{2''}$, $H_{6''}$); 8.45 (bs, 2H, H_3, $H_{3'}$); 14.28 (s, 1H, NH). MS (*m*/*z*, % abundance): 401 (M^+, 1); 91 (100). Anal Calcd. for $C_{16}H_{12}N_4O_4Se \cdot 0.8HCl$ (%): C, 44.42; H, 2.78; N, 12.96. Found: C, 44.59; H, 2.84; N, 12.92.

*4.1.3.26. Benzyl N,N′-di(thienaphthen-2-ylcarbonyl)-imidoselenocarbamate (**6c**).* From benzyl imidoselenocarbamate hydrobromide **c** and thianaphthene-2-carbonyl chloride **6**. Recrystallized from ethanol/DMF. Yield: 36%. m.p. 192–193 °C. IR (KBr): 3425, 1677 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 4.65 (s, 2H, $SeCH_2$); 7.31 (t, 1H, $J_{3''-4''} = J_{4''-5''} = 7.4$ Hz, $H_{4''}$); 7.38 (t, 2H, $J_{2''-3''} = J_{5''-6''} = 7.4$ Hz, $H_{3''}$, $H_{5''}$); 7.49 (m, 6H, H_6, H_7, $H_{2''}$, $H_{6''}$, $H_{6'}$, $H_{7'}$); 7.96–7.99 (m, 4H, H_5, H_8, $H_{5'}$, $H_{8'}$); 8.10 (s, 1H, H_3); 8.29 (s, 1H, $H_{3'}$); 14.71 (s, 1H, NH). MS (*m*/*z*, % abundance): 91 (100). Anal Calcd. for $C_{26}H_{18}N_2O_2S_2Se$ (%): C, 58.54; H, 3.38; N, 5.25. Found: C, 58.22; H, 3.64; N, 5.17.

*4.1.3.27. Benzyl N,N′-di(3,4-methylenedioxybenzoyl)-imidoselenocarbamate (**7c**).* From benzyl imidoselenocarbamate hydrobromide **c** and 3,4-methylenedioxy benzoyl chloride **7**. Recrystallized from ethanol/DMF. Yield: 31%. m.p. 203–205 °C. IR (KBr): 3448, 1673 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 4.55 (s, 2H, $SeCH_2$); 6.08 (s, 2H, CH_2); 6.11 (s, 2H, CH_2'); 6.90 (d, 1H, $J_{2-3} = 8.2$ Hz, H_3); 6.95 (d, 1H, $J_{2'-3'} = 8.1$ Hz, $H_{3'}$); 7.27–7.29 (m, 1H, $H_{4''}$); 7.33 (t, 2H, $J_{2''-3''} = J_{3''-4''} = J_{4''-5''} = J_{5''-6''} = 7.4$ Hz, $H_{3''}$, $H_{5''}$); 7.43 (d, 2H, $H_{2''}$, $H_{6''}$); 7.49 (s, 1H, H_6); 7.60 (d, 1H, H_2); 7.79 (s, 1H, $H_{6'}$); 8.05 (d, 1H, $H_{2'}$); 14.70 (s, 1H, NH). MS (*m*/*z*, % abundance): 149 (100). Anal Calcd. for $C_{24}H_{18}N_2O_6Se$ (%): C, 56.58; H, 3.54; N, 5.50. Found: C, 56.53; H, 3.86; N, 5.48.

*4.1.3.28. Benzyl N,N′-di(quinolin-3-ylcarbonyl)-imidoselenocarbamate (**8c**).* From benzyl imidoselenocarbamate hydrobromide **c** and quinoline-3-carbonyl chloride **8**. Recrystallized from ethanol/DMF. Yield: 19%. m.p. 178–179 °C. IR (KBr): 3417, 1684 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 4.70 (s, 2H, $SeCH_2$); 7.31 (t, 1H, $J_{3''-4''} = J_{4''-5''} = 7.4$ Hz, $H_{4''}$); 7.38 (t, 2H, $J_{2''-3''} = J_{5''-6''} = 7.4$ Hz, $H_{3''}$, $H_{5''}$); 7.50 (d, 2H, $H_{2''}$, $H_{6''}$); 7.70 (t, 1H, $J_{7-8} = J_{8-9} = 7.7$ Hz, H_8); 7.73 (t, 1H, $J_{7'-8'} = J_{8'-9'} = 7.7$ Hz, $H_{8'}$); 7.93–7.96 (m, 2H, H_7, $H_{7'}$); 8.03 (d, 1H, H_9); 8.10 (d, 1H, $H_{9'}$); 8.24 (d, 1H, $J_{6-7} = 8.5$ Hz, H_6); 8.32 (d, 1H, $J_{6'-7'} = 8.3$ Hz, $H_{6'}$); 8.83 (d, 1H, $J_{2-4} = 1.8$ Hz, H_4); 9.20 (d, 1H, $J_{2'-4'} = 1.0$ Hz, $H_{4'}$); 9.54 (d, 1H, H_2); 9.86 (d, 1H, $H_{2'}$); 15.11 (s, 1H, NH). MS (*m*/*z*, % abundance): 524 (M^+, 1); 91 (100). Anal Calcd. for $C_{28}H_{20}N_4O_2Se$ (%): C, 64.24; H, 3.82; N, 10.71. Found: C, 63.80; H, 3.72; N, 10.67.

*4.1.3.29. Benzyl N,N′-di(2-phenylquinolin-3-ylcarbonyl)-imidoselenocarbamate (**9c**).* From benzyl imidoselenocarbamate hydrobromide **c** and 2-phenylquinoline-3-carbonyl chloride **9**. Recrystallized from ethanol/DMF. Yield: 22%. m.p. 180–181 °C. IR (KBr): 3426, 1698 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 4.63 (s, 2H, $SeCH_2$); 7.30–7.32 (m, 3H, $H_{3''}$, $H_{4''}$, $H_{5''}$); 7.44 (d, 2H, $H_{2''}$, $H_{6''}$); 7.60–7.62 (m, 8H, H_8, H_{13}, H_{14}, H_{15}, $H_{8'}$, $H_{13'}$, $H_{14'}$, $H_{15'}$); 7.70–7.72 (m, 2H, H_7, $H_{7'}$); 8.23–8.25 (m, 7H, H_3, H_6, H_{12}, H_{16}, $H_{6'}$, $H_{12'}$, $H_{16'}$); 8.48 (d, 1H, H_9); 8.75 (s, 1H, $H_{3'}$); 8.88 (d, 1H, $H_{9'}$); 14.49 (s, 1H, NH). MS (*m*/*z*, % abundance): 204 (100). Anal Calcd. for $C_{40}H_{28}N_4O_2Se \cdot 0.25HCl$ (%): C, 70.16; H, 4.13; N, 8.19. Found: C, 70.42; H, 4.17; N, 7.98.

*4.1.3.30. Benzyl N,N′-di(acridin-9-ylcarbonyl)-imidoselenocarbamate (**10c**).* From benzyl imidoselenocarbamate hydrobromide **c** and 9-acridinecarbonyl chloride **10**. Recrystallized from ethanol/DMF. Yield: 29%. m.p. 144–146 °C. IR (KBr): 3606, 1709 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$, δ): 4.62 (s, 2H, $SeCH_2$); 6.46 (s, 1H, NH); 6.71 (d, 2H, $J_{5'-6'} = J_{10'-11'} = 8.0$ Hz, $H_{6'}$, $H_{10'}$); 6.94 (t, 2H, $J_{4-5} = J_{5-6} = J_{10-11} = J_{11-12} = 7.7$ Hz, H_5, H_{11}); 7.03 (d, 2H, H_6, H_{10}); 7.19 (t, 2H, $J_{4'-5'} = J_{11'-12'} = 8.0$ Hz, $H_{5'}$, $H_{11'}$); 7.36–7.37 (m, 3H, $H_{3''}$, $H_{4''}$, $H_{5''}$); 7.49–7.52 (m, 2H, $H_{2''}$, $H_{6''}$); 7.56 (t, 2H, $J_{3-4} = J_{12-13} = 7.7$ Hz, H_4, H_{12}); 7.74–7.77 (m, 4H, H_3, H_{13}, $H_{4'}$, $H_{12'}$); 8.33 (d, 2H, $J_{3'-4'} = J_{12'-13'} = 8.0$ Hz, 2H, $H_{3'}$, $H_{13'}$). MS (*m*/*z*, % abundance): 237 (100). Anal Calcd. for $C_{36}H_{24}N_4O_2Se \cdot 0.75HCl$ (%): C, 66.42; H, 3.81; N, 8.61. Found: C, 66.78; H, 4.16; N, 8.36.

4.2. Cytotoxic and antiproliferative activities

Cell culture materials were obtained from BD Bioscience. Stock solutions of the different compounds were prepared as follows: All compounds were dissolved in DMSO at a concentration between 0.01 and 0.005 M. Sterile filtration of the compounds was achieved using 0.2 μm filter disks. Serial dilutions with supplemented medium were prepared daily to a final concentration of less than 2% DMSO in cell culture.

The cytotoxic effect of each substance was tested at five different concentrations between 0.01 and 100 μM and viability was determined according to the protocol of Denizot and Lang [47].

Hep-G2 (liver carcinoma), HT-29 (colon carcinoma), K-562 (leukemia), MCF-7 (breast carcinoma) and PC-3 (prostate carcinoma) cell lines were routinely maintained in RPMI medium (Lonza/12-702) supplemented with 10% fetal bovine serum and 1% antibiotics in tissue culture flasks at 37 °C and 5% CO_2. MCF-10A (nontumorigenic breast epithelial cells) were routinely maintained in MEBM medium (Lonza/CC-3150) supplemented with 1% antibiotics and a supplement cocktail containing human Epithelial Growth factor, Bovine Pituitary Extract, Insulin, Hydrocortisone and Gentamicin A (Lonza/CC-4136) in tissue culture flasks at 37 °C and 5% CO_2. Culture medium was exchanged at 3-day intervals. MTT-assays were performed with 1×10^4 Hep-G2 cells/well, 4×10^3 HT-29 cells/well, 4×10^3 K-562 cells/well, 1.2×10^4 MCF-7 cells/well, 7×10^3 MCF-10A cells/well and 2×10^3 PC-3 cells/well in 96-well plates. Briefly, cells were incubated in the presence of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and analyzed for their ability to generate a purple formazan dye. After incubation for 72 h, the absorbance was measured at a wavelength of 590 nm and the ratio of viable cells was calculated. Results are expressed as GI_{50}, the concentration that reduces by 50% the growth of treated cells with respect to untreated controls, TGI, the concentration that completely inhibits cell growth, and LC_{50}, the concentration that kills 50% of the cells. Data were obtained from at least 3 independent experiments performed in quadruplicate.

The CV method was performed according to a modification of the protocol described by Kueng et al. [58]. Each compound was tested at eight different concentrations ranging from 0.01 to 100 μM. After incubation for 72 h, cells were fixed with 100 μL of formaldehyde (4%) and were left for at least 20 min. The plates were stained with 0.1% crystal violet solution for 30 min. After being washed with water, 200 μL of acetic acid was added and the absorbance at 590 nm was measured by an automatic microplate reader. Data were obtained from at least three independent experiments performed in quadruplicate.

4.3. Evaluation of cell cycle progression and apoptosis induction

For breast adenocarcinoma MCF-7 cells, the apoptotic status and cell cycle analysis of the cells were determined using the Apo-Direct kit (BD Pharmingen), based on the TUNEL technique, under the conditions described by the manufacturer. Briefly, for fixation step cells were suspended in 1% paraformaldehyde in PBS (pH 7.4) at a concentration of 1×10^6 cells/mL, incubated on ice for 1 h, collected by centrifugation, washed, adjusted to 1×10^6 cells/mL in 70% ice cold ethanol and incubated at −20 °C for 30 min. After fixation, cells were recovered by centrifugation, washed, resuspended in FITC dUTP-DNA labelling solution and incubated for 1 h at 37 °C. Cells were then rinsed, resuspended in PI/RNase staining buffer, incubated in the dark for 30 min at RT and analyzed using a Coulter Epics XL flow cytometer.

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