

Novel quinazoline and pyrido[2,3-*d*]pyrimidine derivatives and their hydroselenite salts as antitumoral agents

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Dedicated to Rosa M. Claramunt Vallespí on the occasion of her 65th anniversary

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Abstract

A series of 22 quinazolines, pyrido[2,3-*d*]pyrimidines and their hydroselenite salts were synthesized with the aim of evaluating *in vitro* their cytotoxicity against PC-3 cell line and their antioxidant properties related to DPPH (1,1-diphenyl-2-picrylhydrazylradical) activity, showing some of them better profile than the respective controls. Three of these derivatives (**5d**, **6d** and **7f**) were selected in order to gain preliminary insights to establish the mechanism of action. Caspase-3 activity and cell cycle regulation studies revealed that compound **6d** provoked an increase in caspase-3 level accompanied by cell cycle perturbation in a time-dependent manner.

Keywords: antioxidant, cytotoxicity, pyridopyrimidine, quinazoline, selenite.

Introduction

Although the development of novel targeted antitumor drugs have obtained important progress in recent years, cancer remains the major leading cause of death in the world due to drug resistance or undesirable toxic effects. Quinazoline derivatives have been utilized extensively in medicinal chemistry due to its privileged structure that shows various pharmacological activities, such as antifungal,¹ antibacterial,² anti-inflammatory,³ anticonvulsant⁴, antihypertensive⁵ and anticancer activities⁶⁻⁸ among others. This scaffold has been identified as new class of cancer chemotherapeutic agents acting as potent inhibitors for epidermal growth factor receptor (EGFR),⁹ or dihydrofolate reductase.¹⁰ Furthermore, its ability to induce apoptosis,^{11,12} or

modulate caspases¹³ as well as its capacity to bind the DNA have also been reported.¹⁴ Moreover, the anticancer activity of photoactivable drugs containing the quinazoline moiety has been evaluated.¹⁵ On the other hand, pyrido[2,3-*d*]pyrimidine derivatives are of great interest due to their anticancer or antiproliferative activities.¹⁶⁻¹⁸ They exhibited very diverse biological profiles. Some members of this family inhibited tyrosine kinases¹⁹ whereas others inhibited non-receptor tyrosine kinases such as PI3K and mTOR.²⁰ Thus, pyridopyrimidine derivatives produced dihydrofolate reductase inhibition²¹ as well as cell cycle modulation.²²

Selenium (Se) is an essential trace element that has been identified as an anticarcinogenic agent, with supporting evidence from epidemiological studies, clinical intervention trials, preclinical intervention studies (animal cancer models) and cell culture studies. Natural organic and inorganic sources of Se as well as synthetic organoselenium compounds have been shown to be effective and safe in the treatment and prevention of cancer.²³⁻²⁶

The observations outlined above, along with our experience in the synthesis and antitumoral studies related to quinazoline and pyrido[2,3-*d*]pyrimidines,²⁷⁻³¹ so as a continuation with our efforts in the search of new selenium derivatives with antitumoral properties³²⁻³⁸ enthused us to develop new 2,4-disubstituted quinazoline and pyrido[2,3-*d*]pyrimidine derivatives. The linkages investigated were (–NH–), which was inserted in positions 2 and/or 4, and sulfur which was introduced in the 2-position. Bonded to these linkages, aliphatic chains of variable length with aryl rings at the end of the chain were placed. In addition, in the aryl rings different chemical functions that are present in a number of antineoplastic agents³⁹⁻⁴¹ such as methylthio, methylseleno or selenocyanate were introduced in order to verify their possible influence on the biological activity. The choice of different or identical substituents in 2 and 4 positions was done with the aim of corroborating the importance of molecular symmetry in the biological activity. Many literature reports include molecules that possess symmetry as cytotoxic agents.⁴² Finally, to improve the low water solubility and bioavailability, that has limited the development of some of these compounds, some derivatives were formulated as hydroselenite salts.

All the derivatives were screened by cell viability assays against a human prostate cancer cell line (PC-3) and as antioxidants using the DPPH test. In order to gain preliminary insights related to the mechanism of action, some of the most promising compounds were also evaluated as caspase modulators and cell cycle regulators.

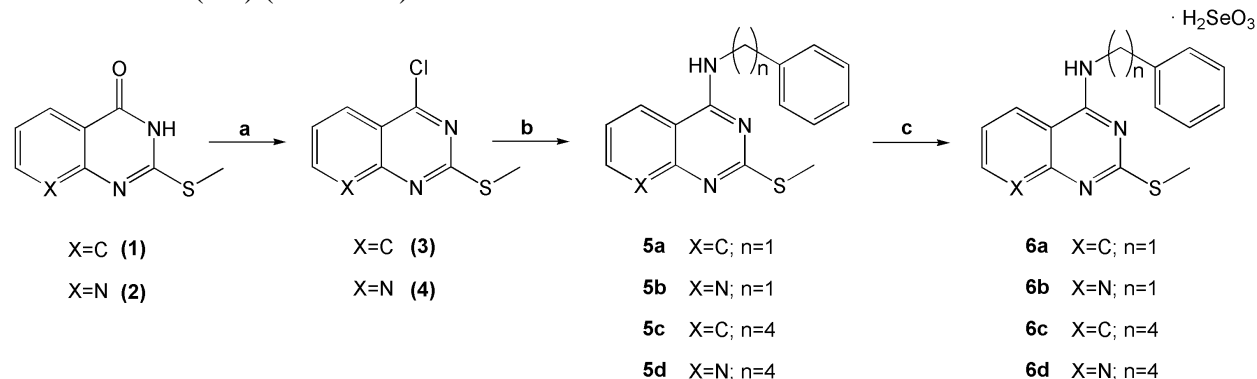
Results and Discussion

Chemistry

Schemes 1 and 2 outline the synthetic pathway used to obtain compounds described in this paper. In order to prepare these molecules, the most appropriate starting materials were some compounds which were prepared previously by us, such as 4-hydroxy-2-methylthioquinazoline **1**³¹ and 2-methylthio-4-oxo-3,4-dihydropyrido[2,3-*d*]pyrimidine **2**²⁹ (Scheme 1) and the commercially available 2,4(1*H*,3*H*)-quinazolinone **A** (Aldrich, 86-96-4) (Scheme 2) and the

pyrido[2,3-*d*]pyrimidine-2,4(1*H*,3*H*)-dione **B** (Shanghai Richem International Co., Ltd) (Scheme 2).

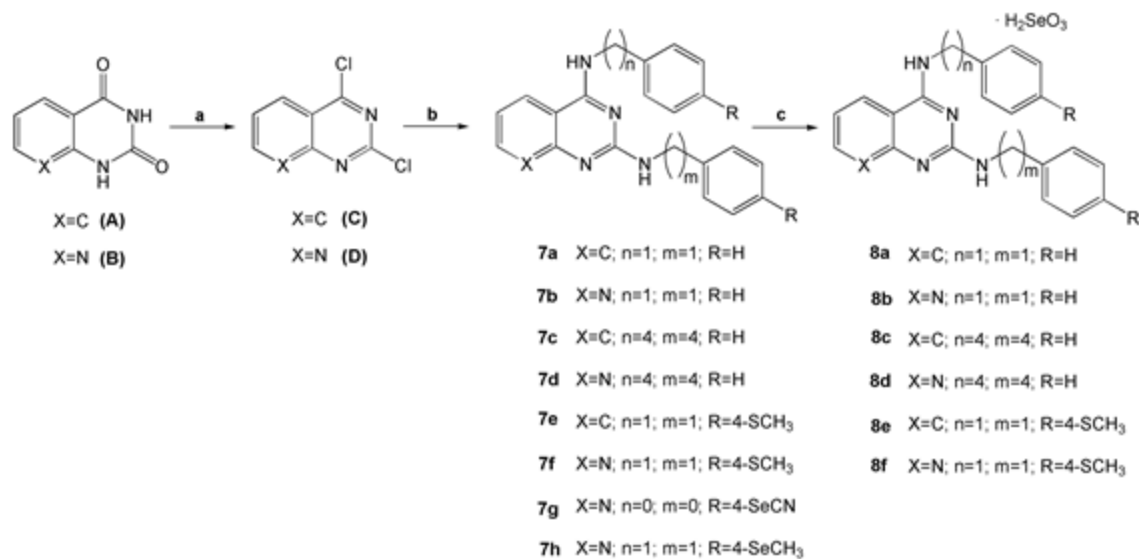
The substituted 4-alkylamino-2-methylthio derivatives of quinazoline and pyrido[2,3-*d*]pyrimidine have been synthesized in two steps (Scheme 1) starting from compounds **1** and **2**, respectively. Upon refluxing with freshly distilled phosphoryl chloride (POCl₃) in presence of dimethylformamide (DMF), compounds **1** and **2** yielded the corresponding key 4-chloro intermediates **3** and **4**, which were used without purification. Reaction of **3** and **4** with the appropriate amine in a molar ratio 1:1.1 respectively, in ethanol by refluxing gave the desired compounds **5a-5d** in yields ranging from 4 to 60 %. Compounds **5a-5d** were converted in hydroselenite salts (**6a-6d**) by reaction with selenium dioxide in heating in a mixture ethanol:water (1:1) (Scheme 1).



a: POCl₃/DMF; **b:** phenylalkylamine, EtOH; **c:** SeO₂, EtOH:H₂O (1:1).

Scheme 1. Synthetic routes for the preparation of compounds **5a-5d** and **6a-6d**.

On the other hand, the commercially available compounds **A** or **B** (Scheme 2) reacted with phosphoryl chloride in the presence of DMF in one step to give the corresponding dihalides which were further reacted with the appropriated amines in ethanol using triethylamine (TEA) as catalyst to afford the target compounds (**7a-7h**) with good yields. The corresponding salts (**8a-8f**) were obtained by treating selenium dioxide with the organic free bases quinazoline and pyridopyrimidine, respectively. Salts (**6a-6d** and **8a-8f**) were characterized by a broad singlet in ¹H NMR spectrum (Scheme 2).



a: POCl₃/DMF; b: The corresponding amine, TEA, EtOH; c: SeO₂, EtOH:H₂O (1:1).

Scheme 2. Synthetic route for the preparation of compounds **7a-7h** and **8a-8f**.

Biological Evaluation

a) Cytotoxic activity in PC-3

Initially, the new compounds were evaluated for their *in vitro* cytotoxic activity against a human prostate cancer cell line (PC-3, ATCC, Manassas, VA) using the MTT assay.⁴³ After an extensive literature review, this cell line was selected considering that many clinical trials suggest a role of some selenium compounds in the reduction of prostate cancer.⁴⁴⁻⁴⁶ Results are tabulated as IC₅₀ values. IC₅₀ was defined as the concentration of tested compound that cause 50% inhibition of cell growth, as compared to the untreated control. IC₅₀ values were calculated by log-linear interpolation of data points. All experiments were independently performed at least three times and values calculated after 72 hours exposure (drug concentrations of 2, 5, 7 and 10 μM). These concentrations have been chosen with an exigent criteria in order to select very potent compounds (with IC₅₀ < 10 μM). To place the data in perspective, topotecan and methylseleninic acid were selected as positive controls.

The results presented in Table 1 show that twelve compounds **5d**, **6a**, **6c**, **6d**, **7b**, **7e**, **7f**, **7h**, **8b**, **8c**, **8e** and **8f** with IC₅₀ < 8 μM are more potent than methylseleninic acid (IC₅₀ = 8.4 μM) exhibiting seven of them (**6a**, **6c**, **7e**, **7f**, **7h**, **8e** and **8f**) higher cytotoxicity than topotecan (IC₅₀ = 4.0 μM). A detailed SAR analysis is not feasible taking into account the relatively small number of derivatives tested, but some observations and comparisons can be made. This primary screening reveals that the pyridopyrimidine nucleus is better than the corresponding quinazoline, mainly when the substituents in 2 and 4 positions are different. For example, if we compare derivatives **5c** (IC₅₀ > 10 μM) with **5d** (IC₅₀ = 5.8 μM) or compound **7a** (IC₅₀ > 10 μM) with **7b** (IC₅₀ = 8.0 μM). In contrast, for symmetrical derivatives **7c** and **7d**, or **7e** and **7f** the activities are similar. Moreover, it has been confirmed that molecular symmetry improved the cytotoxic

activity in comparison with non-symmetrical derivatives. For example, only four compounds (**5d**, **6a**, **6c** and **6d**) of the non-symmetrical molecules (**5a-5d** and **6a-6d**) were active. However, from the rest of the tested derivatives (14), that presented molecular symmetry, ten of them showed activity. The length of the alkylamino side chains is not crucial for the cytotoxicity when the linkages inserted in positions 2 and 4 are identical. Compounds **7a** ($m = 1$) versus **7c** ($m = 4$) indicated that the cytotoxicity may not be affected by the length of alkyl substituents. Another aim of this study was to test the importance of the presence of hydroselenite salt in the formulation. Previously, some studies of Arsenyan *et al.*⁴⁷⁻⁴⁹ have reported the cytotoxic activity against hepatoma, fibrosarcoma, melanoma, neuroblastoma of some heterocycles formulated as hydroselenites. Taking into account the above aforementioned we concluded that the effect of hydroselenite salts formulation depends of the central scaffold. So, hydroselenite salts enhanced the cytotoxicity of quinazoline heterocycle framework compounds: **5a** versus **6a**, **5c** versus **6c**, **7a** versus **8a**, **7c** versus **8c** but they do not modified it or have a weak effect in pyridopyrimidine derivatives: **5b** versus **6b**, **5d** versus **6d**, **7b** versus **8b**.

The presence of substituents SCH₃ or SeCH₃ located at *para* position of the aryl moiety improved the cytotoxicity independently of the formulation as salt or of the central scaffold. This point is corroborated by the fact that if we compared the activities of **7e**, **7f**, **7h**, **8e**, and **8f**, they are similar (IC₅₀ from 1.5 to 3.2 μM). To our surprise, **7g** with SeCN group at *para* position did not show as marked cytotoxic activity on this cell line (IC₅₀ = 9.4 μM). This fact may be due to the replacement of the flexible aliphatic side chains (n and $m = 1$) by a rigid chain (n and $m = 0$).

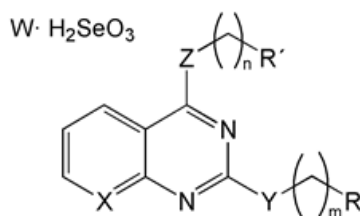


Table 1. Cytotoxic activity of compounds in PC-3 cell line

Comp.	X	Y	m	R	Z	n	R'	W	IC ₅₀ (μM)
5a	C	S	0	CH ₃	NH	1	C ₆ H ₅	0	>10
5b	N	S	0	CH ₃	NH	1	C ₆ H ₅	0	>10
5c	C	S	0	CH ₃	NH	4	C ₆ H ₅	0	>10
5d	N	S	0	CH ₃	NH	4	C ₆ H ₅	0	5.8
6a	C	S	0	CH ₃	NH	1	C ₆ H ₅	1.97	3.4
6b	N	S	0	CH ₃	NH	1	C ₆ H ₅	0.45	>10
6c	C	S	0	CH ₃	NH	4	C ₆ H ₅	1.25	3.8
6d	N	S	0	CH ₃	NH	4	C ₆ H ₅	1	7.0

Tabel 1 (continued)

7a	C	NH	1	C ₆ H ₅	NH	1	C ₆ H ₅	0	>10
7b	N	NH	1	C ₆ H ₅	NH	1	C ₆ H ₅	0	8
7c	C	NH	4	C ₆ H ₅	NH	4	C ₆ H ₅	0	>10
7d	N	NH	4	C ₆ H ₅	NH	4	C ₆ H ₅	0	>10
7e	C	NH	1	4-SCH ₃ -C ₆ H ₄	NH	1	4-SCH ₃ -C ₆ H ₄	0	1.7
7f	N	NH	1	4-SCH ₃ -C ₆ H ₄	NH	1	4-SCH ₃ -C ₆ H ₄	0	1.5
7g	C	NH	0	4-SeCN-C ₆ H ₄	NH	0	4-SeCN-C ₆ H ₄	0	9.4
7h	N	NH	1	4-SeCH ₃ -C ₆ H ₄	NH	1	4-SeCH ₃ -C ₆ H ₄	0	3.2
8a	C	NH	1	C ₆ H ₅	NH	1	C ₆ H ₅	1	8.2
8b	N	NH	1	C ₆ H ₅	NH	1	C ₆ H ₅	1.5	7.9
8c	C	NH	4	C ₆ H ₅	NH	4	C ₆ H ₅	1	6.3
8d	N	NH	4	C ₆ H ₅	NH	4	C ₆ H ₅	0.75	>10
8e	C	NH	1	4-SCH ₃ -C ₆ H ₄	NH	1	4-SCH ₃ -C ₆ H ₄	1.85	1.6
8f	N	NH	1	4-SCH ₃ -C ₆ H ₄	NH	1	4-SCH ₃ -C ₆ H ₄	0.4	1.8
Topotecan									4.0
MSA^b									8.4

^a PC-3 cell line; ^b Methylseleninic acid.

b) Antioxidant activity

Since antioxidants are gaining attention as a potential means of treating a large number of lifestyle diseases like cancer, it is of immense significance to establish some new antioxidants via a convenient synthetic methodology. In addition, selenium derivatives are being studied due to their antioxidant properties.^{50,51} The DPPH radical has been widely used to test the ability of compounds to behave as free radical scavengers or hydrogen donors. Briefly, the assay measures the decrease in absorbance of the DPPH radicals at a characteristic wavelength after 60 min incubation of the DPPH radical with different concentrations (from 62.5 μ M to 312.5 μ M) of the antioxidant compound according to the method of Koparir *et al.*⁵² The absorbance of the reaction mixture was recorded at 517 nm using a UV visible spectrophotometer (Jasco V-630). Ascorbic acid was used as standard. Results are expressed as the percentage of the DPPH free radical scavenging at five concentrations, as shown in Table 2. Each value is expressed as the average of three experiments per concentration \pm SD. Although all the compounds were tested, Table 2 only shows the data obtained for the derivatives that exhibited some activity. **6d** showed the best

DPPH radical scavenging activity in comparison with ascorbic acid used as reference, followed by compounds **7f**, **5c**, **6c**, **7g**, **5a** and **8e**. In general, analogues with quinazoline moiety presented better DPPH radical scavenging activity at the concentrations tested than compounds with pyridopyrimidine moiety with the exception of **6d** and **7f**.

Table 2. Percentages of free radical scavenging activity (DPPH radical) obtained for the compounds at different concentrations

Compound	62.5 μ M	125 μ M	187.5 μ M	250 μ M	312.5 μ M
5a	25.9 \pm 0.1	32.9 \pm 0.2	36.7 \pm 0.1	48.3 \pm 0.1	55.6 \pm 0.1
5b	3.4 \pm 0.1	11.9 \pm 0.1	12.2 \pm 0.1	15.3 \pm 0.2	16.3 \pm 0.1
5c	37.9 \pm 0.2	59.5 \pm 0.1	68.1 \pm 0.1	70.5 \pm 0.1	71.9 \pm 0.1
6c	27.1 \pm 0.2	38.2 \pm 0.2	45.6 \pm 0.1	51.1 \pm 0.1	56.7 \pm 0.2
6d	52.9 \pm 0.1	63.6 \pm 0.4	72.4 \pm 0.2	87.6 \pm 0.4	95.7 \pm 0.5
7f	32.3 \pm 0.1	62.3 \pm 0.1	76.8 \pm 0.2	77.2 \pm 0.2	82.1 \pm 0.1
7g	39.2 \pm 0.1	49.8 \pm 0.1	56.9 \pm 0.2	63.6 \pm 0.1	68.4 \pm 0.1
7h	9.2 \pm 0.1	14.2 \pm 0.2	17.2 \pm 0.1	20.1 \pm 0.1	22.4 \pm 0.1
8e	16.3 \pm 0.1	31.4 \pm 0.1	41.1 \pm 0.1	43.6 \pm 0.3	50.6 \pm 0.1
8f	16.1 \pm 0.1	22.9 \pm 0.2	24.6 \pm 0.1	27.6 \pm 0.3	28.1 \pm 0.1
Ascorbic acid^a	52.7 \pm 0.4	63.4 \pm 0.1	69.2 \pm 0.2	70.4 \pm 0.1	72.8 \pm 0.1

^aAscorbic acid (reference antioxidant compounds) was used as a standard. The scavenging capacities were represented as percentage inhibition and values were the means of three replicates (mean \pm SD, n = 3).

c) Mechanism Studies

Since some compounds had strong inhibitory effects on the growth of the cancer cell line tested, and/or antioxidant activity, we decided to study the effect of three compounds (**5d**, **6d** and **7f**) in greater detail in order to know their mode of action. **6d**, formulated as hydroselenite salt, because it is active against PC-3 and exhibited a marked antioxidant activity being better than ascorbic acid used as positive control; its analogue **5d** with the same chemical structure in order to explore the importance of the presence of hydroselenite group in the mechanism of action and **7f**,

which was the most active as cytotoxic agent ($IC_{50} = 1.5 \mu\text{M}$) and one of the most potent derivative as DPPH radical scavenging.

First, we investigated the effects of these three compounds on caspase activity. We focused on caspase-3, which is activated by a great number of apoptotic signals. This enzyme is the main executor of apoptosis playing a central role in its biological processing. It has been reported that activation of caspase-3 is an essential event for the induction of oligonucleosomal DNA fragmentation.⁵³

We analysed the effect of treatment with **5d**, **6d** and **7f** on caspase-3 activation in PC-3 at a concentration of $15 \mu\text{M}$ after 24 and 48 h of treatment. As shown in Figure 1, the activity of caspase-3 in cells exposed to compound **6d** increased slightly comparing to control after 24 h, although this effect disappeared after 48 h. However, the analogue without hydroselenite salt (**5d**) and compound **7f** did not modify the caspase-3 level at the times tested.

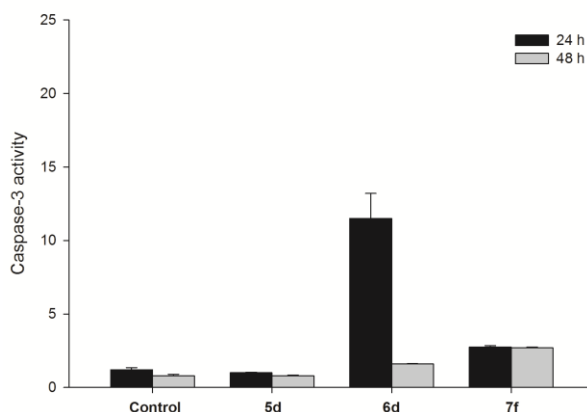


Figure 1. Assessment of caspase-3 activity in PC-3 cell line.

Then, these compounds were selected for further evaluation on their effects of PC-3 cell cycle distribution by flow cytometric analysis using propidium iodide at 24 and 48 h. It is known that cell cycle dysregulation is a hallmark of anticancer drug-induced cell death and many anticancer drugs interact with cells leading to cell growth arrest.⁵⁴ To determine whether the anticancer effects of the compounds were caused by cell cycle modulation, the effects of compounds **5d**, **6d** and **7f** on cell cycle progression were examined in PC-3 cell line at a concentration of $15 \mu\text{M}$. After 24 h and 48 h of treatment, compound **5d** did not induce any specific phase arrest of the cell cycle related to control. In contrast, **6d** at 24 h provoked an increase in phases subG₀/G₁ and G₂/M with a significant reduction of G₀/G₁. On the other hand, **7f** induced a slightly decrease in the G₀/G₁ population with a weak increase in other phases (S and G₂/M). In all the cases these effects disappear at 48 h. Cell accumulation in G₂/M phase, accompanied by a diminution of cell proportion in G₀/G₁ phase accompanied by a significant

increase in subG₀/G₁ phase (which is representative of cell with fragmented DNA) could suggest a mitotic arrest prior to metaphase. Figure 2 summarizes the results of cell cycle distribution.

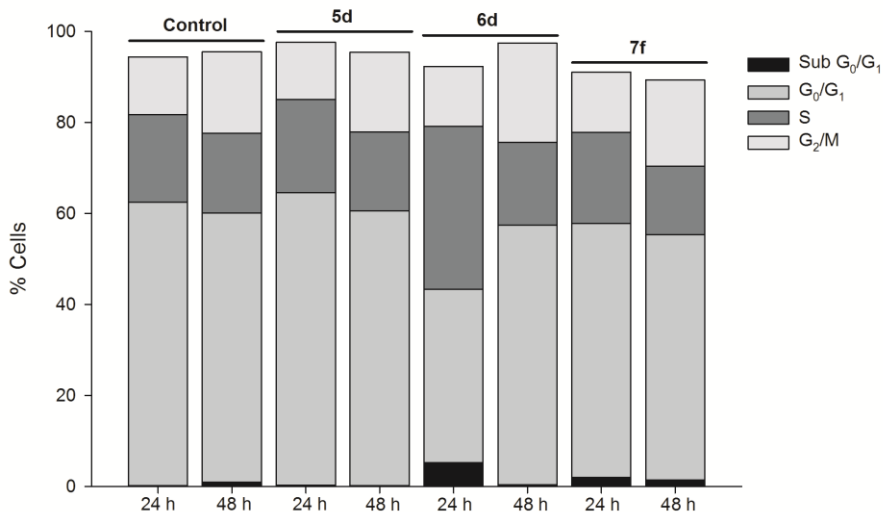


Figure 2. Effects of **5d**, **6d** and **7f** on cell cycle distribution in PC-3 cells.

d) Theoretical evaluation of ADME properties

To better understand the properties of the compounds, the theoretical ADME properties such as molecular weight, log *P*, TPSA (a predictive indicator of membrane penetration), volume and number of hydrogen donors and acceptors were calculated (Table 3) using the Molinspiration property calculation program.⁵⁵

From the obtained results, it is important to point out that the three selected compounds (**5d**, **6d** and **7f**) possess acceptable log *P* values, which mean that these derivatives could be able to cross membranes. Furthermore, values obtained from TPSA are also found to be positive. It can also be observed no violations of Lipinski's rule (Table 3). It is remarkable that this freely accessible program do not allow to include the hydroselenite salt formulation in the structure so the values obtained for compound **6d** were determined by protonation of the amine group.

Table 3. Theoretical structural properties of the selected compounds

Comp.	log <i>P</i>	MW	TPSA	n-OH acceptors	n-OHNH donors	Volume
5d	4.69	324.45	50.70	4	1	301.27
6d	1.67	453.45	55.28	4	2	302.25
7f	4.88	433.61	62.73	5	2	386.17

Conclusions

The current study reports the synthesis of twenty-two quinazolines, pyrido[2,3-*d*]pyrimidines and their hydroselenite salts and the *in vitro* growth inhibitory activity against tumoral PC-3 cell line as well as their antioxidant activity considered as the interaction with the free radical DPPH. It was observed from the results of biological assays that twelve compounds (**5d**, **6a**, **6c**, **6d**, **7b**, **7e**, **7f**, **7h**, **8b**, **8c**, **8e** and **8f**) showed potent growth inhibitory activity ($IC_{50} < 8.0 \mu M$) and were more potent than standard drug methylseleninic acid ($IC_{50} = 8.4 \mu M$). In addition, seven of them (**6a**, **6c**, **7e**, **7f**, **7h**, **8e** and **8f**) were more active than topotecan with IC_{50} below than $4.0 \mu M$.

On the ground of the biological results, some structural features were inferred to be beneficial to the antitumor activity of such compounds. Cytotoxic data confirmed that molecular symmetry is a valid approach to obtain potent antitumor agents. In general, the hydroselenite salt formulation had a beneficial effect on the cytotoxic activity for 2 and 2 and 4-phenylalkylamino derivatives when the aryl ring was not functionalized. Meanwhile, the modification of the length in the alkyl chain substituent was not accompanied by alteration of antitumor activity. The effect of the replacement of quinazoline by pyridopyrimidine scaffold was dependent of the nature of the substituents.

Two compounds **6d** and **7f** exhibited strong cytotoxic and antioxidant activities in comparison to the positive controls and they were selected for further studies related to caspase-3 activity and cell cycle regulation. Compound **6d** provoked caspase-3 activation and cell cycle arrest in a time-dependent manner being these effects less marked for **7f**. In addition, the described compounds seem to present desirable ADME properties that could be an important information about the promising potential of these derivatives.

These compounds may become a promising class of cytotoxic and antioxidant agents and the results provide an insight for future direction in the development of new molecules.

Experimental Section

Chemistry

General. Melting points were determined with a Mettler FP82+FP80 apparatus (Greifense, Switzerland) and have not been corrected. The 1H NMR spectra were recorded on a Bruker 400 UltrashieldTM 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. Silica gel 60 (0.040–0.063 mm) 1.09385.2500 (Merck KGaA, 64271 Darmstadt, Germany) was used for Column Chromatography and Alugram[®] SIL G/UV₂₅₄ (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 Pharmaceuticaaan 3a, 2440 Geel, Belgium), Shanghai Richem International Co., Ltd and Lancaster (Bischheim-Strasbourg, France).

General procedure for compounds 5a-5d

A solution of the corresponding 2-methylthio-4-chloroquinazoline (**3**) or the suitable pyrido[2,3-*d*]pyrimidine (**4**) (4.0 mmol) in ethanol (25 mL) was cooled to 4 °C and the proper amine (4.4 mmol) was added. The mixture was stirred at room temperature for 5-6 h and after heating at 75 °C during 15 h, it was concentrated. The residue was treated with water and the precipitate was collected by filtration, washed with Et₂O (3 × 15 mL) and recrystallized from the appropriate solvent.

Compounds 4-benzylamino-2-methylthioquinazoline (**5a**), 4-benzylamino-2-methylthiopyrido[2,3-*d*]pyrimidine (**5b**) and 2-methylthio-4-(4-phenylbutyl)aminoquinazoline (**5c**) were synthesized according to the procedure previously reported by us and the spectroscopical data obtained were congruent.³¹

4-(4-Phenylbutylamino)-2-methylthiopyrido[2,3-*d*]pyrimidine (5d). From 4-chloro-2-methylthiopyrido[2,3-*d*]pyrimidine and 4-phenylbutylamine. Recrystallized from ethanol. Yield 43%, mp 175-176 °C; IR (ν_{\max} , cm⁻¹): 3247 (w, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_{H} 1.64 (s, 4H, CH₂-CH₂-CH₂-CH₂), 2.49 (s, 3H, SCH₃), 2.62 (s, 2H, CH₂-Ph), 3.53 (s, 2H, NH-CH₂), 7.14-7.25 (m, 5H, H_{2'}, H_{3'}, H_{4'}, H_{5'}, H_{6'}), 7.38 (s, 1H, H₆), 8.59-8.61 (bs, 2H, H₅, NH), 8.84 (s, 1H, H₇). MS (m/z, %) 324 (M⁺, 97), 219 (100). Anal. Calcd for C₁₈H₂₀N₄S·0.3H₂O: C, 65.57; H, 6.16; N, 17.00. Found: C, 65.65; H, 6.17; N, 16.67.

General procedure for compounds 6a-6d. The corresponding derivatives **5a-5d** (1 mmol) were dissolved in ethanol:water (1:1, 50 mL) and selenium dioxide (1.5 mmol) was added carefully. The resulting mixture was heated and stirred during 12 h and the solvent was removed under reduced pressure. The residue was washed with ethyl ether (4 × 25 mL), dried and recrystallized from ethanol.

4-Benzylamino-2-methylthioquinazoline·1.97 hydroselenite (6a). From 4-benzylamino-2-methylthioquinazoline and selenium dioxide. Yield 50 %, mp 106-107 °C; IR (ν_{\max} , cm⁻¹): 3228 (m, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_{H} 2.45 (s, 3H, SCH₃), 4.75 (d, 2H, NH-CH₂, $J_{\text{CH}_2\text{-NH}} = 5.8$ Hz), 5.55 (bs, 1H, NH-H₂SeO₃), 7.24 (dd, 1H, H₇, $J_{7-8} = 8.3$ Hz, $J_{7-6} = 7.1$ Hz), 7.36-7.41 (m, 6H, H₈, H_{2'}, H_{3'}, H_{4'}, H_{5'}, H_{6'}), 7.54 (d, 1H, H₆), 8.22 (dd, 1H, H₅, $J_{5-7} = 1.8$ Hz), 8.93 (t, 1H, NH). MS (m/z, %) 281 (M⁺, 100). Anal. Calcd for C₁₆H₁₅N₃S·1.97H₂SeO₃: C, 35.97; H, 3.60; N, 7.70. Found: C, 63.52; H, 5.15; N, 19.68.

4-Benzylamino-2-methylthiopyrido[2,3-*d*]pyrimidine·0.45 hydroselenite (6b). From 4-benzylamino-2-methylthiopyrido[2,3-*d*]pyrimidine and selenium dioxide. Yield 94 %, mp 197-198 °C; IR (ν_{\max} , cm⁻¹): 3254 (m, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_{H} 2.47 (s, 3H, SCH₃), 3.51 (bs, 1H, NH-H₂SeO₃), 4.75 (d, 2H, NH-CH₂, $J_{\text{CH}_2\text{-NH}} = 4.8$ Hz), 7.34-7.38 (m, 5H, H_{2'}, H_{3'}, H_{4'}, H_{5'}, H_{6'}), 8.66 (d, 1H, H₆, $J_{6-5} = 8.4$ Hz, $J_{6-7} = 4.1$ Hz), 8.86 (dd, 1H, H₅, $J_{5-7} = 2.1$ Hz), 9.20 (dd, 1H, H₇). MS (m/z, %) 156 (100). Anal. Calcd for C₁₅H₁₄N₄S·0.45H₂SeO₃: C, 52.93; H,

4.38; N, 16.47. Found: C, 52.84; H, 4.15; N, 16.39.

4-(4-Phenylbutylamino)-2-methylthioquinazoline-1.25 hydroselenite (6c). From 4-(4-phenylbutylamino)-2-methylthioquinazoline and selenium dioxide. Yield 48 %, mp 166-167 °C; IR (ν_{\max} , cm^{-1}): 3207 (m, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_{H} 1.68-1.69 (m, 4H, NH-CH₂-(CH₂)₂-CH₂-Ph), 2.57 (s, 3H, SCH₃), 2.63-2.65 (m, 2H, NH-(CH₂)₃-CH₂-Ph), 3.63-3.66 (m, 2H, NH-CH₂), 5.55 (bs, 1H, NH-H₂SeO₃), 7.20-7.22 (m, 5H, H_{2'}, H_{3'}, H_{4'}, H_{5'}, H_{6'}), 7.49 (t, 1H, H₇, $J_{7-8} = 8.3$ Hz, $J_{7-6} = 7.8$ Hz), 7.60 (d, 1H, H₈), 7.80 (t, 1H, H₆, $J_{6-5} = 7.8$ Hz), 8.35 (d, 1H, H₅), 9.45 (bs, 1H, NH). MS (*m/z*, %) 323 (M⁺, 100). Anal. Calcd for C₁₉H₂₁N₃S·1.25H₂SeO₃: C, 47.01; H, 4.61; N, 8.67. Found: C, 46.69; H, 5.01; N, 8.32.

4-(4-Phenylbutylamino)-2-methylthiopyrido[2,3-*d*]pyrimidine hydroselenite (6d). From 4-(4-phenylbutylamino)-2-methylthiopyrido[2,3-*d*]pyrimidine and selenium dioxide. Yield 86%, mp 165-166 °C; IR (ν_{\max} , cm^{-1}): 3240 (w, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_{H} 1.69 (s, 4H, CH₂-CH₂-CH₂-CH₂), 2.56 (s, 3H, SCH₃), 2.63 (s, 2H, CH₂-Ph), 3.66 (s, 2H, NH-CH₂), 4.35 (bs, 1H, NH-H₂SeO₃), 7.16-7.26 (m, 5H, H_{2'}, H_{3'}, H_{4'}, H_{5'}, H_{6'}), 7.64 (s, 1H, H₆), 8.82 (s, 1H, H₅), 8.92 (s, 1H, H₇), 9.73 (s, 1H, NH). MS (*m/z*, %) 324 (M⁺, 72), 219 (100). Anal. Calcd for C₁₈H₂₀N₄S·H₂SeO₃: C, 47.68; H, 4.86; N, 12.36. Found: C, 48.00; H, 4.83; N, 12.43.

General procedure for compounds 7a-7h

To a solution of the corresponding 2,4-dichloroquinazoline or 2,4-dichloropyrido[2,3-*d*]pyrimidine (5.0 mmol), and equimolecular amounts of triethylamine in ethanol (25 mL), the corresponding amine (11 mmol) was added. The mixture was stirred for 12 h at 70 °C. The solvent was removed under vacuum. Then, the residue was treated with water (75 mL) and the corresponding solid was purified by recrystallization from ethanol.

N,N'-Dibenzylaminoquinazolin-2,4-diamine (**7a**), *N,N'*-dibenzylaminopyrido[2,3-*d*]pyrimidin-2,4-diamine (**7b**), *N,N'*-bis(4-phenylbutyl)quinazolin-2,4-diamine (**7c**) and *N,N'*-bis(4-phenylbutyl)pyrido[2,3-*d*]pyrimidin-2,4-diamine (**7d**) were synthesized according to the procedure previously reported by us.³⁰

***N,N'*-Bis(4-methylthiobenzyl)quinazoline-2,4-diamine hydrochloride (7e).** From 2,4-dichloroquinazoline and 4-methylthiobenzylamine. Yield 41%, mp 165-166 °C; IR (ν_{\max} , cm^{-1}): 3292 (w, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_{H} 2.43 (s, 6H, 2 SCH₃), 4.52 and 4.67 (d + d, 4H, 2(CH₂) $J_{\text{CH}_2\text{-NH}} = 5.8$ Hz), 7.15 (bs, 8H, 2H_{2'}, 2H_{3'}, 2H_{5'}, 2H_{6'}), 7.24 (bs, 1H, H₆), 7.33 (bs, 1H, H₇), 7.59 (bs, 1H, H₅), 8.13 (bs, 1H, H₈), 9.72 (bs, 2H, 2NH). MS (*m/z*, %) 432 (M⁺, 33), 137 (100). Anal. Calcd for C₂₄H₂₄N₄S₂·HCl: C, 61.47; H, 5.34; N, 11.95. Found: C, 61.65; H, 5.70; N, 11.84.

***N,N'*-Bis(4-methylthiobenzyl)pyrido[2,3-*d*]pyrimidin-2,4-diamine-0.9 hydrochloride (7f).** From 2,4-dichloropyrido[2,3-*d*]pyrimidine and 4-methylthiobenzylamine. Yield 42%, mp 218-220 °C; IR (ν_{\max} , cm^{-1}): 3268 (w, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_{H} 2.44 (s, 6H, 2 SCH₃), 4.56 (bs, 2H, CH₂), 4.69 (bs, 2H, CH₂), 7.12-7.24 (m, 8H, 2H_{2'}, 2H_{3'}, 2H_{5'}, 2H_{6'}), 7.30 (bs, 1H, H₆), 8.25 (bs, 1H, NH), 8.63 (bs, 1H, H₅), 8.71 (bs, 1H, H₇), 9.80 (bs, 1H, NH). MS

(m/z, %) 433 (M⁺, 58), 105 (100). Anal. Calcd for C₂₃H₂₃N₅S₂·0.9 HCl: C, 59.25; H, 5.13; N, 15.03. Found: C, 59.56; H, 5.08; N, 15.05.

***N,N'*-Bis(4-selenocyanatophenyl)quinazoline-2,4-diamine·0.8 hydrochloride (7g).** From 2,4-dichloroquinazoline and 4-aminophenylselenocyanate. 4-aminophenylselenocyanate was prepared according to the method reported by Kachanov *et al.*⁵⁶ Yield 45%, mp 208-209 °C; IR (ν_{max}, cm⁻¹): 3190 (w, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_H 7.56-7.59 (m, 2H, H₆, H₇), 7.66-7.71 (m, 4H, 2H_{2'}, 2H_{6'}), 7.77-7.83 (m, 4H, 2H_{3'}, 2H_{5'}), 8.70 (d, 1H, H₈, J₈₋₇ = 8.0 Hz), 10.71 (bs, 1H, NH), 11.25 (bs, 1H, NH). MS (m/z, %) 57 (100). Anal. Calcd for C₂₂H₁₄N₆Se₂·0.8HCl: C, 48.07; H, 2.69; N, 15.29. Found: C, 48.35; H, 2.99; N, 15.18.

***N,N'*-Bis(4-methylselenobenzyl)pyrido[2,3-*d*]pyrimidin-2,4-diamine·0.5 hydrochloride (7h).** From 2,4-dichloropyrido[2,3-*d*]pyrimidine and 4-methylselenobenzylamine. 4-methylselenobenzylamine was prepared according to the procedure previously reported by us.³¹ Yield 38%, mp 137-139 °C; IR (ν_{max}, cm⁻¹): 1611 (m, C=N). ¹H NMR (400 MHz, DMSO-*d*₆): δ_H 2.32 (s, 6H, 2 SeCH₃), 4.78 (d, 4H, 2CH₂, J_{CH₂-NH} = 5.6 Hz), 7.21-7.37 (m, 8H, 2H_{2'}, 2H_{3'}, 2H_{5'}, 2H_{6'}), 7.56 (dd, 1H, H₆, J₆₋₇ = 8.0 Hz, J₆₋₅ = 4.5 Hz), 8.89 (d, 1H, H₅), 9.03 (d, 1H, H₇), 11.48 (bs, 1H, NH), 11.68 (bs, 1H, NH). MS (m/z, %) 163 (100). Anal. Calcd for C₂₃H₂₃N₅Se₂·0.5 HCl: C, 50.61; H, 4.31; N, 12.84. Found: C, 50.29; H, 4.53; N, 13.12.

General procedure for compounds 8a-8f

The corresponding derivatives **7a-7f** (1 mmol) were dissolved in ethanol:water (1:1, 50 mL) and selenium dioxide (1.5 mmol) was added. The resulting mixture was heated and stirred during 3 h and the solvent was removed under reduced pressure. Finally, the residue was washed with (4 × 25 mL), dried and recrystallized from ethanol.

***N,N'*-Dibenzylaminoquinazolin-2,4-diamonium hydroselenite (8a).** From *N,N'*-dibenzylaminoquinazolin-2,4-diamine and selenium dioxide. Yield 65 %, mp 190-192 °C; IR (ν_{max}, cm⁻¹): 3442 (s, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_H 3.92 (bs, H₂SeO₃), 4.61 (d, 2H, -NH-CH₂-Ph), 4.77 (d, 2H, -NH-CH₂-Ph), 7.21-7.24 (m, 10H, 2H_{2'}, 2H_{3'}, 2H_{4'}, 2H_{5'}, 2H_{6'}), 7.42 (t, 1H, H₆), 7.48 (t, 1H, H₇), 7.76 (d, 1H, H₅), 8.33 (d, 1H, H₈), 8.55 (bs, 1H, NH), 10.23 (bs, 1H, NH·H₂SeO₃). MS (m/z, %) 340 (M⁺, 25), 91(100). Anal. Calcd for C₂₂H₂₀N₄·H₂SeO₃: C, 56.29; H, 4.69; N, 11.94. Found: C, 56.32; H, 4.85; N, 12.02.

***N,N'*-Dibenzylaminopyrido[2,3-*d*]pyrimidin-2,4-diamonium·1.5 hydroselenite (8b).** From *N,N'*-dibenzylaminopyrido[2,3-*d*]pyrimidin-2,4-diamine and selenium dioxide. Yield 73 %, mp 227-229 °C; IR (ν_{max}, cm⁻¹): 3345 (s, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_H 4.82 (d, 4H, 2 -NH-CH₂-Ph), 7.33-7.43 (m, 10H, 2H_{2'}, 2H_{3'}, 2H_{4'}, 2H_{5'}, 2H_{6'}), 8.45-8.75 (m, 3H, H₅, H₆, H₇), 10.53 (bs, 2H, 2 NH·H₂SeO₃). MS (m/z, %) 91 (100). Anal. Calcd for C₂₁H₁₉N₅·1.5H₂SeO₃: C, 47.15; H, 4.11; N, 13.10. Found: C, 47.11; H, 4.35; N, 13.82.

***N,N'*-Bis(4-phenylbutyl)quinazolin-2,4-diamonium hydroselenite (8c).** From *N,N'*-bis(4-phenylbutyl)quinazolin-2,4-diamine and selenium dioxide. Yield 47 %, mp 148-149 °C; IR (ν_{max}, cm⁻¹): 3325 (s, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_H 1.90 (d, 4H, 2 -NH-(CH₂)₃-CH₂-Ph), 2.56-2.59 (m, 8H, 2 -NH-CH₂-(CH₂)₂-CH₂-Ph), 3.39-3.55 (m, 4H, 2 -NH-CH₂-(CH₂)₃-Ph),

7.18-7.24 (m, 11H, H₆, 2H_{2'}, 2H_{3'}, 2H_{4'}, 2H_{5'}, 2H_{6'}), 7.39 (t, 1H, H₇), 7.76 (d, 1H, H₅), 8.15 (bs, 1H, NH), 8.32 (d, 1H, H₈), 9.70 (bs, 1H, NH·H₂SeO₃). MS (m/z, %) 91(100). Anal. Calcd for C₂₁H₁₉N₅·1.5H₂SeO₃: C, 47.15; H, 4.11; N, 13.10. Found: C, 47.11; H, 4.35; N, 13.82.

***N,N'*-Bis(4-phenylbutyl)pyrido[2,3-*d*]pyrimidin-2,4-diamonium·0.75 hydroselenite (8d).** From *N,N'*-bis(4-phenylbutyl)pyrido[2,3-*d*]pyrimidin-2,4-diamine and selenium dioxide. Yield 32 %, mp 127-149 °C; IR (ν_{max}, cm⁻¹): 3325 (s, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_H 1.88 (d, 4H, 2 -NH-(CH₂)₃-CH₂-Ph), 2.52-2.57 (m, 8H, 2 -NH-CH₂-(CH₂)₂-CH₂-Ph), 3.35-3.55 (m, 4H, 2 -NH-CH₂-(CH₂)₃-Ph), 7.18-7.24 (m, 11H, H₆, 2H_{2'}, 2H_{3'}, 2H_{4'}, 2H_{5'}, 2H_{6'}), 7.88 (bs, 1H, NH), 8.63 (d, 1H, H₅), 8.85 (d, 1H, H₇), 9.70 (bs, 1H, NH·H₂SeO₃). MS (m/z, %) 91 (100). Anal. Calcd for C₂₁H₁₉N₅·1.5H₂SeO₃: C, 47.15; H, 4.11; N, 13.10. Found: C, 47.11; H, 4.35; N, 13.82.

***N,N'*-Bis(4-methylthiobenzyl)quinazoline-2,4-diamonium·1.85 hydroselenite (8e).** From *N,N'*-bis(4-methylthiobenzyl)quinazoline-2,4-diamine and selenium dioxide. Yield 83%, mp 153-154 °C; IR (ν_{max}, cm⁻¹): 3262 (w, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_H 2.43 (s, 6H, 2 SCH₃), 4.49 and 4.68 (s + s, 4H, 2(CH₂)), 7.15 (bs, 8H, 2H_{2'}, 2H_{3'}, 2H_{5'}, 2H_{6'}), 7.37 (bs, 2H, H₆, H₇), 7.62 (bs, 1H, H₅), 8.11 (bs, 1H, H₈), 9.45 and 9.63 (bs + bs, 2H, 2NH). MS (m/z, %) 432 (M⁺, 13), 137 (100). Anal. Calcd for C₂₄H₂₄N₄S₂·1.85H₂SeO₃: C, 42.90; H, 4.13; N, 8.34. Found: C, 42.65; H, 3.99; N, 8.32.

***N,N'*-Bis(4-methylthiobenzyl)pyrido[2,3-*d*]pyrimidin-2,4-diamonium·0.4 hydroselenite (8f).** From *N,N'*-bis(4-methylthiobenzyl)pyrido[2,3-*d*]pyrimidin-2,4-diamine and selenium dioxide. Yield 79%, mp 152-153 °C; IR (ν_{max}, cm⁻¹): 3340 (w, N-H). ¹H NMR (400 MHz, DMSO-*d*₆): δ_H 2.45 (s, 6H, 2 SCH₃), 3.81 (bs, H₂SeO₃), 4.63 and 4.73 (d + d, 4H, 2 (CH₂), *J*_{CH₂-NH} = 5.8 Hz), 7.12-7.24 (m, 8H, 2H_{2'}, 2H_{3'}, 2H_{5'}, 2H_{6'}), 7.48 (dd, 1H, H₆, *J*₆₋₇ = 7.7 Hz, *J*₆₋₅ = 4.7 Hz), 8.56 (s, 1H, NH), 8.75 (d, 1H, H₅), 8.87 (d, 1H, H₇), 10.59 (s, 1H, NH·H₂SeO₃). MS (m/z, %) 177 (100). Anal. Calcd for C₂₃H₂₃N₅S₂·0.4H₂SeO₃: C, 56.95; H, 4.91; N, 14.44. Found: C, 57.29; H, 4.96; N, 14.43.

Biological evaluation

Cytotoxic activity. PC-3 cells were seeded in 96-well plates (Millipore, Eschborn, Germany) at a density of 5 × 10³ cells per well. Plates were incubated at 37 °C under 5% CO₂ overnight prior to the addition of the compounds (diluted in complete medium). After 72 h of incubation, 10 μL of sterile MTT solution (5 mg/mL in PBS) was added to the cells in each well and these plates were stored for an additional 4 h at 37 °C. The absorbance of formazan crystals were measured at λ = 570 nm on a Polarstar Galaxy plate reader (BMG LabTechnologies GmbH). The percentage of viable cells was calculated to obtain IC₅₀-values.

PC-3 are human tumorigenic and metastatic prostate cancer cells and these were obtained from American Type Culture Collection (ATCC), Manassas, USA. The cells were cultured in Dulbecco's RPMI 1640 medium with GlutamaxTM 1 (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), Fetalclone III, SH30109.03, HYCLONE and 1% Penicillin-Streptomycin (Invitrogen).

Antioxidant activity. The antioxidant activity of the compounds was determined by the DPPH method. The free radical-scavenging activity of the title compounds was determined by spectrophotometric measurement of the change in the absorbance of DPPH at 517 nm. Stock solutions (500 mM) of the tested samples and DPPH were prepared in DMSO. DPPH solution (400 mM) was added to the sample solution at different concentrations (500, 1000, 1500, 2000 and 2500 mL) and appropriately diluted with DMSO to a total volume of 4.0 mL. A control was produced by diluting 400 μ L from DPPH stock solution was also diluted to 4.0 mL using DMSO solvent. For the control, only solvent was added. Ascorbic acid was used as a standard (using the reference antioxidant) for this test. For the standard, the sample was replaced with the same amount of ascorbic acid. The DPPH radical scavenging method was chosen to assess the antioxidant potential of the target compounds in comparison with the commercially available antioxidant ascorbic acid at the same concentrations. The reaction mixtures were thoroughly mixed by shaking the test tubes vigorously and incubated in a water bath at 25 °C for 60 min in darkness. Absorbance at 517 nm was measured and the solvent was corrected throughout. The scavenging effect was calculated using the following equation:

$$\text{Scavenging activity (\%)}: \frac{A_0 - A_S}{A_0} \times 100$$

where A_S is the absorbance of the DPPH in the presence of the tested compounds and standard and A_0 is the absorbance of the DPPH in the absence of the tested compound and standard (control).

Caspase-3 activity. Detection was carried out by means of flow cytometry (Coulter Epics XL), using the *Active-Caspase-3 FITC Mab apoptosis kit* from Pharmingen, which evaluates the number of cells (%) that are contained in the dimerized and caspase-3 activated form. Therefore, measurements were taken at 24 and 48 h and the values obtained were compared with the control cells.

Cell cycle analysis. Cells were plated at a density of 5×10^5 cells/well in six-well plates and they were incubated overnight at 37 °C under 5% CO₂. Then, cells were treated with 15 μ M concentration of the tested compounds for 24 and 48 h. After the incubation period with the compounds, the cells were collected, centrifuged and fixed with ice-cold ethanol (70%). Cells were stained with 0.5 g/L propidium iodide and analyzed using a Coulter Epics XL flow cytometer.

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