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Selenoesters and Selenoanhydrides as Novel Agents Against Resistant Breast Cancer

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Abstract. *Background/Aim: Selenium-containing compounds are becoming new alternatives in experimental chemotherapy in order to overcome multidrug resistance in cancer. The main goal of this study was to determine whether combined treatment with new Se-compounds would increase the effect of conventional doxorubicin chemotherapy in breast cancer cell lines. Materials and Methods: Se-compounds were evaluated regarding their cytotoxic and apoptosis-inducing effect on MCF-7 and ATP-binding cassette subfamily B member 1 (ABCB1)-overexpressing KCR breast cancer cell lines. Moreover, the interaction of Se-compounds with doxorubicin was assessed using the MTT assay. Results: Selenoanhydride exerted a selective activity towards the doxorubicin-resistant KCR cell line overexpressing ABCB1. Among the selenoesters, only ketone-containing selenoesters exerted significant cytotoxic activity against MCF-7 and KCR cell lines and the Se-compounds acted synergistically with doxorubicin on the KCR cell line. Conclusion: The importance of the* $COSeCH_2COCH_3$ *and* $COSeCH_2CO(CH_3)_3$ *moieties for the cytotoxic and adjuvant role of Se-compounds was highlighted.*

Selenium-containing compounds (Se-compounds) are becoming a novel and promising alternative approach in the fight against cancer: according to recent reviews in the field, many selenium derivatives have been reported to show antiproliferative, anticancer or cancer-chemopreventive activity in different biological assays (1, 2). The mechanisms of action of the Se-compounds against cancer are very diverse, as these derivatives can interact with key biological processes such as oxidative stress, angiogenesis, and apoptosis induction, among others (1, 2). Furthermore they possess chemopreventive properties (3, 4). Besides their intrinsic anticancer activity, specific selenium derivatives can inhibit certain cancer resistance mechanisms such as the function of multidrug resistance (MDR) efflux pumps (5, 6), or can modulate the activity of chemotherapeutic drugs (7, 8).

Previously our group synthesized a selenoanhydride and a series of selenoesters (Figure 1), finding that they were potent antiproliferative and anticancer agents (9). Subsequently, four of these selenium derivatives (selenoanhydride **1** and the ketone-containing selenoesters **9-11**) were described as very potent inhibitors of the ATP-binding cassette subfamily B member 1 (ABCB1; P-glycoprotein) efflux pump in the MDR subline of the mouse T-lymphoma cell line L5178Y (5) and in MDR Colo 320 colon adenocarcinoma cell line (6). In addition, they interacted synergistically with chemotherapeutic drugs such as vincristine, doxorubicin, cyclophosphamide, methotrexate, topotecan and 5-fluorouracil in checkerboard combination assay on L5178Y mouse T-lymphoma cells (10).

It has been reported that Se-compounds are less active against MCF-7 cells compared to other tumor cell lines such as A549, PC-3 and HT-29 (9). Herein, we aimed to determine whether combined treatment with Se-compounds and doxorubicin would overcome this previously observed resistance, and become thus a novel and promising approach to fight breast cancer.

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Key Words: Selenoesters, apoptosis, breast cancer, ABCB1 (P-glycoprotein), doxorubicin.

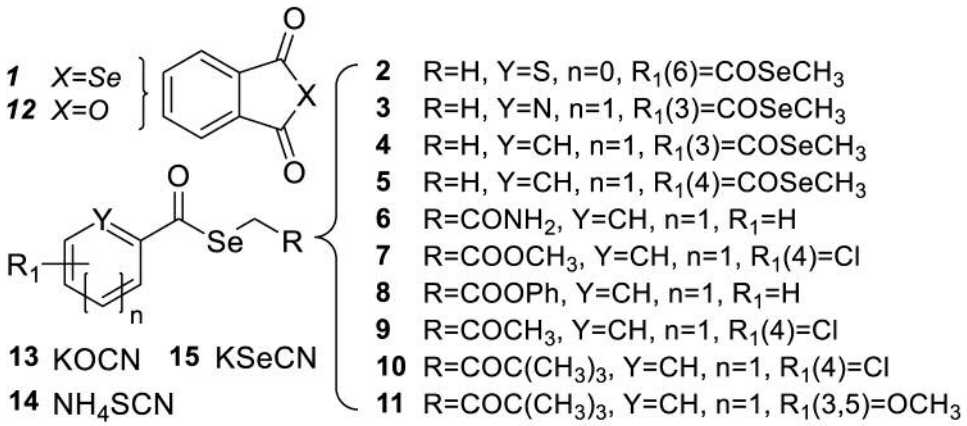


Figure 1. *Chemical structure of the tested compounds. The number in parentheses denotes the position at which R_1 is bound to the (hetero) aromatic ring.*

Materials and Methods

Compounds. The eleven Se-compounds tested (selenoanhydride **1** and selenoesters **2-11**, Figure 1) were kindly provided by Dr. Enrique Domínguez-Álvarez (Spanish National Research Council, Madrid, Spain) and by Professor Dr. Carmen Sanmartín (University of Navarra) (9). Se-compounds **1-11** were stable and their purity was assessed through spectroscopic techniques (elemental analysis, nuclear magnetic resonance, mass spectrometry and infrared spectroscopy). Compounds **12-15** were purchased from Sigma-Aldrich (Steinheim, Germany), respectively, to be used as non-selenium (**12**) isostere of selenoanhydride (**1**) and as inorganic chalcogen salts (**13-15**), for comparing their activity with the selenoesters. The compounds were dissolved in dimethyl sulfoxide (DMSO).

Cell lines. Breast cancer cell line MCF-7 (ATCC® HTB-22) was purchased from LGC Promochem (Teddington, Middlesex, UK). The MCF-7 cell line and its drug-resistant subline KCR were grown in Eagle's minimal Essential medium (EMEM), containing 4.5 g/l glucose supplemented with a non-essential amino acid mixture, a selection of vitamins and 10% heat-inactivated fetal bovine serum. The cell lines were incubated at 37˚C, in an atmosphere of 5% CO_2 and 95% air. On every third passage, 0.56 μg/ml doxorubicin (Teva Pharmaceuticals, Budapest, Hungary) was added to the medium in order to maintain ABCB1 expression in KCR cells.

Cytotoxicity assay. The cytotoxic effects of the Se-compounds were determined on MCF-7 and KCR breast cancer cell lines. The effects of increasing concentrations of Se-compounds on cell growth were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in 100 μl of medium.

The adherent breast cancer cell lines were cultured in 96-well flat-bottomed microtiter plates, using EMEM supplemented with 10% heat-inactivated fetal bovine serum. The density of the cells was adjusted to 1×10^4 cells in 100 μl per well, the cells were seeded for 24 h at 37˚C, with 5% CO_2 prior to the assay, then the medium was removed from the plates containing the cells, and dilutions of Se-compounds were previously made in a separate plate and added to the cells in 200 μl.

The culture plates were incubated at 37˚C for 24 h; at the end of the incubation period, 20 μl of thiazolyl blue tetrazolium bromide (MTT; Sigma) solution (from a stock solution of 5 mg/ml) were added to each well. After incubation at 37˚C for 4 h, 100 μI of sodium dodecyl sulfate (Sigma) solution (10% in 0.01 M HCI) were added to each well and the plates were further incubated at 37˚C overnight. Cell growth was determined by measuring the optical density (OD) at 540/630 nm with Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Inhibition of the cell growth was determined according to the formula below:

$$IC_{50}=100-\left[\frac{OD\,sample-OD\,medium\,control}{OD\,cell\,control-OD\,medium\,control}\right]\times100$$

Results are expressed in terms of IC_{50}, defined as the inhibitory dose that reduced the growth of the cells exposed to the tested compounds by 50%.

The selectivity was calculated using the selectivity index (SI), which is defined as the quotient of the IC_{50} value determined for the non-tumorous MRC-5 cell line described previously (6) to the IC_{50} value for the respective cancer cell line (MCF-7 or KCR). Following the criteria reported in bibliography (6), we considered a compound to be strongly selective when its SI was 6 or higher. Compounds with SI values of 1-3 and 3-6 were regarded as slightly and moderately selective, respectively.

Checkerboard combination assay. A checkerboard microplate method was applied to study the effect of drug interactions between the Se-compounds **1-11** and the chemotherapeutic drug doxorubicin. The assay was carried out on MCF-7 and KCR breast cancer cell lines. The adherent breast cancer cell lines were cultured in 96-well flat-bottomed microtiter plates, using EMEM supplemented with 10% heat-inactivated fetal bovine serum. The density of the cells was adjusted to 6×10^3 cells in 100 μl per well, the cells were seeded for 24 h at 37˚C with 5% CO_2 prior to the assay and then the medium was removed from the plates containing the cells.

The final concentration of the Se-compounds and doxorubicin used in the combination experiment was chosen in accordance with

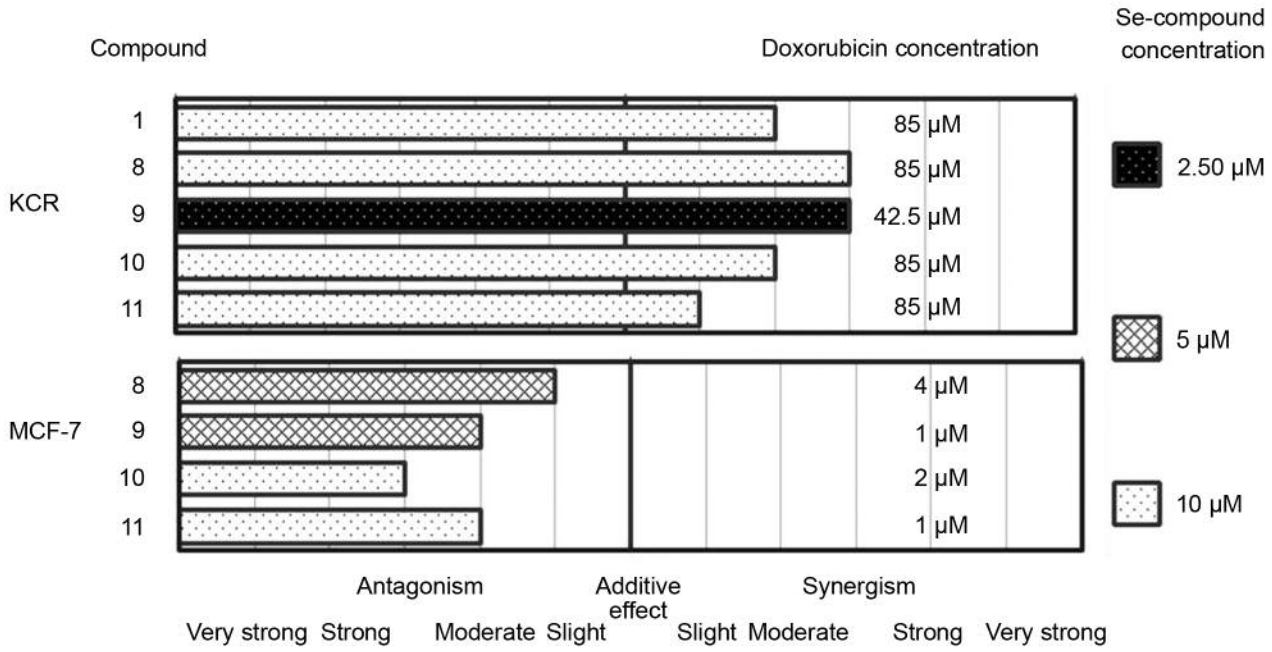


Figure 2. *Interactions of the active Se-compounds with doxorubicin in KCR and MCF-7 cells. The figure indicates, at the most effective interaction ratio (doxorubicin:Se-compound), the concentration of the Se-compound in the presence of doxorubicin at the concentration indicated; furthermore the type of interaction (antagonism, additive effect and synergism) is also presented.*

their cytotoxicity towards these cell lines. The dilutions of doxorubicin were made in a horizontal direction in 100 μl, and the dilutions of the Se-compounds vertically in the microtiter plate in 50 μl volume. The plates were incubated for 72 h at 37˚C in 5% CO_2 atmosphere. The cell growth rate was determined after MTT staining. At the end of the incubation period, 20 μl of MTT (Sigma) solution (from a stock solution of 5 mg/ml) were added to each well. After incubation at 37˚C for 4 h, 100 μl of SDS (Sigma) solution (10% in 0.01 M HCI) were added to each well and the plates were further incubated at 37˚C overnight. Optical density (OD) was measured at 540/630 nm with Multiscan EX ELISA reader (Thermo Labsystems). Combination index (CI) values at 50% of the growth inhibition dose (ED50) were determined using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA) to plot four to five data points at each ratio. CI values were calculated by means of the median-effect equation, according to the Chou–Talalay method, where CI<1, CI=1, and CI>1 represent synergism, additive effect (or no interaction), and antagonism, respectively (11, 12).

Apoptosis induction. The ability of the Se-compounds to induce apoptosis was determined on breast cancer cell lines. The apoptosis assays were performed using Annexin V-FITC Apoptosis Detection Kit from Calbiochem (EMD Biosciences, Inc. La Jolla, CA, USA), following the instructions provided by the manufacturer. This assay enables the quantification of early and late apoptotic events, as well as necrosis and cell death in the cell population exposed to the Se-compounds. The density of the cell suspension was adjusted to 1×10^6 cells/ml. The cell suspension was distributed into 0.5 ml aliquots (5×10^5 cells) to a 24-well microplate and incubated overnight at 37˚C in 5% CO_2. On the following day, the medium was removed, and fresh medium was added to the cells. The cells were then incubated in the presence of Se-compounds at 2 μM for 3 h at 37˚C. 12*H*-Benzo[α]phenothiazine M627 (13), which is a known early apoptosis inducer, was used as positive control. The samples were washed in PBS and fresh medium was added to the cells, followed by the incubation of the plate for 24 h at 37˚C, in 5% CO_2. After the incubation period, the cells were trypsinized. The harvested cells were centrifuged at 2,000 × *g* for 2 min. The cells were then re-suspended in fresh serum-free medium. Thereafter, the apoptosis assay was carried out according to the rapid protocol of the kit and the fluorescence was analyzed immediately using a ParTec CyFlow flow cytometer (Partec, Münster, Germany).

Results

The screening of the anticancer activity of Se-compounds in MCF-7 cells indicated that selenoanhydride **1** and selenoesters **2-7** were not cytotoxic towards this cell line (Table I), as all the IC_{50} values of these derivatives were above 100 μM. In contrast, the ketone-containing selenoesters **9-11** had a potent low-micromolar activity, as their IC_{50} values ranged from 1.04 to 1.70 μM, whereas the IC_{50} of the phenoxycarbonylmethyl selenoester **7** was 64.8 μM. Results were similar for the multidrug-resistant KCR cells except for two derivatives. Firstly, in this case the IC_{50} of selenoanhydride **1**, at a concentration as low as 2.35 μM, which was more than 40-fold lower than for MCF-7, suggesting that this compound acts directly on ABCB1 overexpressed by KCR cells. Secondly, compound **11** was close to 2-fold less active against KCR cells compared to MCF-7 cells. None of compounds **12-15**

Table I. *Cytotoxic activity of Se-compounds against MCF-7 and doxorubicin-resistant KCR breast cancer cell lines.*

Compound	MCF-7		KCR		SI MCF-7 /KCR	MRC-5*		SI	
	IC_{50} (μM)	±SD	IC_{50} (μM)	±SD		IC_{50} (μM)	±SD	MRC-5/MCF-7	MRC-5/KCR
1	>100	-	2.35	0.47	≥42	>100	-	-	≥42
2	>100	-	>100	-	-	4.26	0.65	≤0.04	≤0.04
3	>100	-	>100	-	-	17.9	0.00	≤0.18	≤0.18
4	>100	-	>100	-	-	28.4	0.70	≤0.28	≤0.28
5	>100	-	>100	-	-	61.5	2.16	≤0.62	≤0.62
6	>100	-	>100	-	-	76.6	0.92	≤0.77	≤0.77
7	>100	-	>100	-	-	33.4	3.08	≤0.33	≤0.33
8	64.8	16.7	82.2	15.7	0.79	>100	-	≥1.5	≥1.2
9	**1.04**	0.47	**0.96**	0.18	1.08	*5.35*	0.24	*5.2*	*5.6*
10	**1.70**	0.45	**1.75**	0.15	0.97	*8.10*	0.90	*4.8*	*4.6*
11	**1.45**	0.23	**2.37**	0.30	0.61	*5.04*	0.71	*3.5*	*2.2*

IC_{50}: 50% Inhibitory concentration; SI: selectivity index. For cytoxicity, IC_{50} values in bold denote IC_{50} values below 5 μM, and those in italics, values between 5 and 10 μM. In selectivity, values in bold denote a strong selectivity, and in italics, a moderate selectivity. Compounds **12-15** were not included as their IC_{50} values for the three cell lines were above 100 μM. *Values taken from a previous study (6).

evaluated for comparison studies exerted cytotoxic effects at concentrations below 100 μM on any of KCR, MCF-7 and MRC-5 cell lines evaluated. The anticancer effect of Se-compounds on MRC-5 was determined previously (6).

Regarding the selectivity of the selenoesters towards cancer cells, it was clearly observed that the ketone-containing selenoesters exerted a moderate selectivity towards MCF-7 and KCR cancer cells with respect to the non-tumorous MRC-5 lung fibroblast cells (6), with the exception of compound **11**, which was slightly selective towards KCR, exhibiting a SI of 2.2. The SI of compound **9** for KCR cells was approximately to 6 (SI=5.6), which was the threshold for considering that a compound is strongly selective. Remaining selenoesters lacked of selectivity due to their poor activity against MCF-7 and KCR.

In contrast, selenoanhydride (**1**) was strongly selective towards KCR cells in comparison to the non-tumorous fibroblast cells with SI of 42 (7-fold higher the threshold).

The five active compounds in the cytotoxicity assay were evaluated in combination with doxorubicin (Figure 2). Results were quite fascinating as they showed a marked difference between the two tested cell lines. All Se-compounds assayed exerted synergistic interactions with doxorubicin against the KCR cell line, whereas all the observed interactions of the selenoesters with doxorubicin against the MCF-7 cell line were antagonistic.

Against KCR cells, compound **9** was undoubtedly the most profitable in the combination assay, as it showed the highest grade of synergy among all evaluated compounds and at the lowest concentrations of both Se-compound (2.5 μM) and doxorubicin (42.5 μM). The remaining Se-compounds interacted in a synergistic manner with doxorubicin at a concentration of compound and drug four- and two-fold higher, respectively. Against MCF-7 cells, compound **9** interacted in a moderately antagonistic manner at higher concentration (5 μM). Slight antagonism was observed for compound **8** at the same concentration, but the concentration of doxorubicin was in this case four times higher.

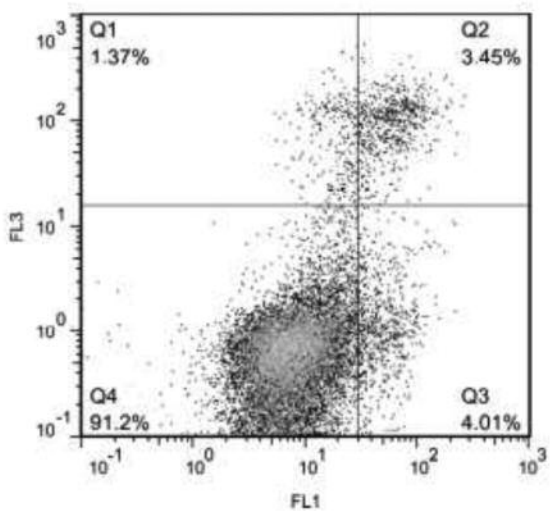
Finally, the compounds were not able to induce significant apoptotic events in MCF-7 and KCR cells; with the exception of the phenoxycarbonylmethyl selenoester **7** in MCF-7 cells. This derivative, at a low concentration (2 μM), triggered early apoptotic and late apoptotic/necrotic events in 16.9% and 7.85% of cells (Figure 3). This apoptosis-inducing activity was moderate, as reference compound M627 induced 20.8% and 67.1% events, respectively.

Discussion

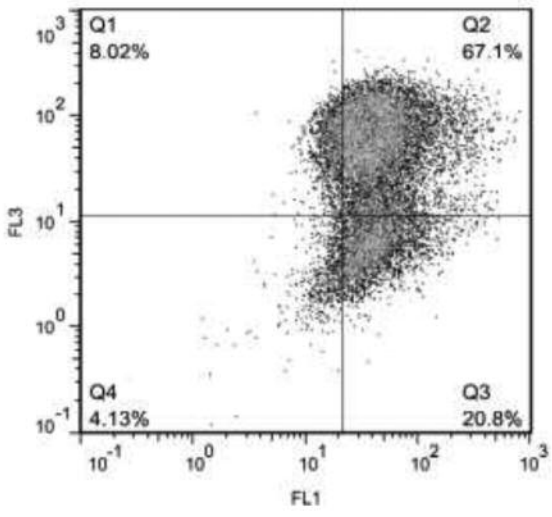
Conventional chemotherapy in the treatment of early and metastatic breast cancer is partly based on the administration of anthracycline drugs *e.g.* doxorubicin. Since these drugs provoke side-effects such as cardiotoxicity and myelosuppression (14, 15), there is an urgent need to minimize the side-effects. In order to reduce the adverse effect of anthracyclines, several alternatives could be applied, for example the use of liposomal doxorubicin (16), nanotechnology (17) and preparation of less toxic derivatives.

In this study, we investigated the cytotoxic properties of Se-compounds and their interaction with doxorubicin in order to find effective adjuvants for combination chemotherapy using doxorubicin with Se-compounds.

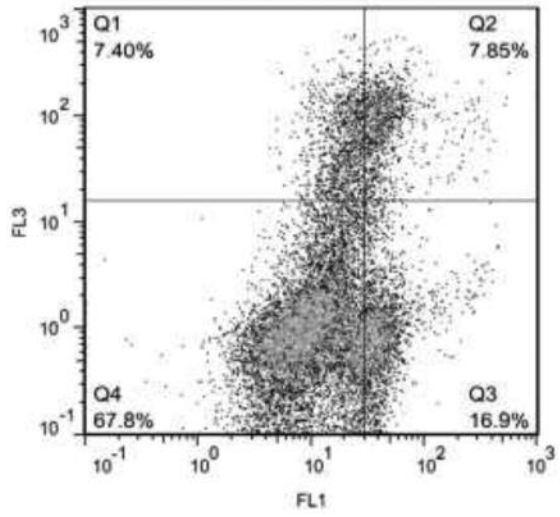
As commented in the previous section, selenoanhydride **1** exerted selective activity towards the resistant KCR cell line overexpressing ABCB1 (IC_{50}=2.35 μM), as it was



Untreated control



12*H*-[α]benzophenothiazine (20 μM)



Compound **7** (2 μM)

Figure 3. *Apoptosis induction by compound* ***7*** *in MCF-7 cells compared to the positive control 12H-[α]benzophenothiazine. Q1: dead cells; Q2: cells undergoing late apoptosis/necrosis; Q3: cells undergoing early apoptosis; Q4: healthy, living cells.*

ineffective against MCF-7 and MRC-5 (non-tumor lung fibroblast) cells. These results are in accordance with our previous data confirming that selenoanhydride **1** interacts directly with ABCB1 (5, 6). Surprisingly, this derivative was unable to trigger apoptotic events in the tested breast cancer cell lines, probably due to a dual inhibition of ABCB1 and multidrug resistance protein 1 efflux pumps, however, other resistance mechanisms are also involved.

Among the selenoesters, only the ketone-containing selenoesters **9-11** exerted significant cytotoxic activity against these two cell lines. Symmetrical dimethyl selenodiesters **2-5** were inactive, as were the amide-containing selenoester **6** and the methoxycarbonylmethyl selenoester **7**. In the latter, the replacement of the methyl moiety bound to the oxygen of the *O*-ester by a phenyl ring lowers the IC_{50} but still at a level between 60 and 100 μM. When this phenyl ester is replaced by a methylketone (**9**) or a *tert*-butylketone, then the activity increases dramatically, this time lowering the IC_{50} to low micromolar concentrations, pointing to the crucial role of this alkylketone moiety in the biological activity of ketone-containing selenoesters. Furthermore, these promising selenium derivatives exerted a noteworthy selectivity towards

the evaluated cancer cells (MCF-7, KCR) rather than the non-tumorous cell line MRC-5.

The results observed in combination assays are astonishing, in that they point to differential activity in the two cell lines, the resistant (KCR) one in this case being more sensitive to the action of the compounds. It has been shown that doxorubicin and methylseleninic acid act synergistically on MCF-7 cells, inducing apoptosis because doxorubicin and selenium cooperatively activate first apoptosis signal (FAS) pathway. Doxorubicin causes Fas oligomerization in a FasL-independent manner and methylseleninic acid increases FAS-associated death domain protein expression together triggering apoptosis (18). Out of our 11 Se-compounds, only methoxycarbonylmethyl *p*-chlorobenzoselenoate (**7**) induced apoptosis of MCF-7 cells, the other derivatives were not capable of provoking apoptosis of MCF-7 and KCR cells.

This is very relevant as it suggests that these derivatives might have the ability to overcome some aspects of resistance of KCR cells. Since the derivatives are proven ABCB1 modulators, their synergism with doxorubicin might be due to their interaction with this efflux pump overexpressed by KCR cells. On the contrary, the explanation of their antagonism with doxorubicin in MCF-7 cells is the involvement of other resistance mechanisms and cellular processes. This could open a new and straightforward approach to treat ABCB1-expressing resistant breast cancer that is resistant to the treatments currently in clinical use. The methylketone selenoester **9** would be in such cases the most promising compound. Its activity makes it worth investigating in more depth for potential applications of this compound and of closely related new derivatives (which could be synthesized in future work) with intrinsic anticancer activity as sensitizers of resistant cancer.

Overall, the results obtained herein highlight the importance for biological activity of the $-COSeCH_2COCH_3$ and $-COSeCH_2CO(CH_3)_3$ moieties in comparison with the remaining substituents considered ($-COSeCH_3$, $-COSeCH_2CONH_2$, $-COSeCH_2COOCH_3$, and $-COSeCH_2COPh$). The good cytotoxic activity, selectivity and ability to modulate the effect of doxorubicin found for the ketone-containing selenoesters **9-11** against the two breast cancer cell lines evaluated are in agreement with previous work of our group on mouse T-lymphoma cells and colonic adenocarcinoma cells (5, 6) and draw the attention to this privileged moiety. In future studies it will be necessary to obtain and evaluate more compounds with these moieties in order to ascertain what substituents in the phenyl ring bound to the carbonyl of the selenoester enhance activity, with the aim of designing more potent and selective anticancer agents.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

Authors' Contributions

Enrique Domínguez-Álvarez synthesized the compounds evaluated and wrote the article. Carmen Sanmartín synthesized the compounds evaluated. Gabriella Spengler conceived the experiments and wrote the article. Andrea Csonka, Annamária Kincses, Márta Nové and Zsófia Vadas performed the biological experiments.

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