

MULTIDRUG RESISTANCE REVERSING ACTIVITY OF ORGANOSELENIUM COMPOUNDS

Ph.D. Thesis

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I. INTRODUCTION

Cancer-related mortality is the second major cause of death, posing as a significant public health burden worldwide. In 2012, cancer accounted for 8.2 million deaths and 14.1 million new cancer cases across the globe, while 32.6 million people entered the period of 5-year remission (partial or complete). Among the EU member states, the incidence of lung and colon cancers and the mortality associated with these tumors is the highest in Hungary. Cancer refers to a broad group of diseases characterized by the conversion of healthy cells into malignant cells that are able to divide and grow uncontrollably, becoming a danger to the integrity of the rest of the organism. The causative agents of malignant disorders include genetic predisposition, lifestyle choices, environmental factors and other factors, such as immunosuppression and various medicinal drugs leading to the development of neoplasms.

The main objective of cancer treatment is the elimination of all tumor cells present in the patient's body. The three most commonly utilized strategies for the treatment of cancer are the following: surgery, radiotherapy and chemotherapy, which can be either used alone or in combination. Chemotherapy is the most frequently used method, both for hematological malignancies and solid tumors. The phenomenon whereby tumors show resistance to chemotherapeutic agents of different structure and mechanism of action is termed multidrug resistance (MDR), which can be mediated by a variety of mechanisms. For the purposes of this thesis, the importance of efflux pump-mediated MDR and the deficiencies in apoptosis induction of cancer cells are highlighted. The ATP-binding cassette (ABC) transporter superfamily is an extremely important and prevalent group of transport proteins in the human body, which bind and hydrolyze ATP to provide the energy needed to transport various substrates across the cell membranes. The first characterized ABC transporter was ABCB1, and the overexpression of this protein is often associated disadvantageous clinical outcome. Due to the flexible and non-specific substrate binding site of this transporter, it can expel a wide variety of chemotherapeutic agents. An emerging research approach and therapeutic strategy is to use efflux pump inhibitors (EPIs) as adjuvant compounds (so-called 'chemosensitizers') to improve the efficacy of standard antitumor therapy. One of the most important forms of cell death induced by antitumor chemotherapy is apoptosis, and MDR is a scenario where the apoptotic processes are defective, resulting in therapy refractory malignancy.

Many selenoderivatives, both organic and inorganic, have been studied as cancer chemopreventive agents and/or as cytotoxic compounds for tumor cells and these results are supported by epidemiological trials. The rationale behind the notion of Se-compounds as cancer therapeutic agents is associated with their redox modulating activity. Literature suggests that cancer cells are more vulnerable to exogenous oxidative stress than healthy, non-tumoral cells, because of increased levels of reactive oxygen species (ROS) and a maximum threshold for antioxidant capacity, due to the accelerated metabolism of these cells, contributing to successive cell division and proliferation. Initially, experimental studies tested inorganic selenium compounds for their cytotoxicity and their potential for enhancing the efficacy of chemotherapy as adjuvants, however, successive investigations discovered that organoselenium derivatives presented with superior biological activities and with less toxicity. These compounds were tested in a wide range of *in vitro* cancer cells model systems and in animal experiments, exhibiting potent anticancer and ROS-modulating properties, both as singular agents and in combination chemotherapy with clinically relevant anticancer drugs.

II. AIMS OF THE STUDY

The aim of our study was to evaluate the activity of novel organoselenium compounds synthesized by Domínguez-Álvarez *et al.* to find effective and selective anticancer derivatives using various cell lines (cell lines of murine and human origin, tumoral and non-cancerous cell lines) as *in vitro* model systems. In addition to their cytotoxic activity, their potency as multidrug resistance reversing compounds was studied against two resistance mechanisms namely inhibition of ABCB1-related efflux and induction of apoptosis in tumor model systems. Furthermore, their attributes as potential lead compounds for further derivatization and their potential for future *in vivo* use were also investigated.

The specific goals of the study were the following:

- 1. Determination of the cytotoxic activity and selectivity** of the compounds (a cyclic selenoanhydride, ten selenoesters and four inorganic chalcogen cyanates) on L5178Y parental (PAR) and *ABCB1*-transfected resistant (MDR) mouse T-cell lymphoma cell lines, Colo 205 (doxorubicin-sensitive) and resistant Colo 320 (*ABCB1*-overexpressing) human colonic adenocarcinoma cell lines, A549 human lung adenocarcinoma cells, NIH/3T3 mouse embryonic fibroblast, and MRC-5 human embryonic lung fibroblast cell lines by MTT method.
- 2. Evaluation of the efflux pump modulatory activity** of the compounds (a cyclic selenoanhydride, ten selenoesters and four inorganic chalcogen cyanates) on L5178Y parental (PAR) and *ABCB1*-transfected resistant (MDR) mouse T-cell lymphoma cell lines, Colo 205 (doxorubicin-sensitive) and resistant Colo 320 (*ABCB1*-overexpressing) human colonic adenocarcinoma cell lines using a flow cytometry-based rhodamine 123 retention assay.
- 3. Characterization of the selenocompounds** (a cyclic selenoanhydride, ten selenoesters and four inorganic chalcogen cyanates) **as apoptosis inducers** using Annexin V-FITC detection system on L5178Y parental (PAR) and *ABCB1*-transfected MDR mouse T-cell lymphoma cell lines and resistant Colo 320 (*ABCB1*-overexpressing) human colonic adenocarcinoma cell line.
- 4. *In silico* analysis of predicted physico-chemical and *in vivo* absorption properties** of the compounds using OSIRIS Molecular Property Explorer and PreADMET 2.0 software. Correlation-regression analysis to study the relationship between the predicted physico-chemical properties of the selenocompounds and their activity as efflux pump inhibitors.
- 5. Determination of structure-activity relationships (SAR)** of the selenocompounds based on experimental results.

III. MATERIALS AND METHODS

Compounds

The tested compounds include a cyclic selenoanhydride (**1**) and selenoesters (**2-11**) with various functional groups (**2-5**: methyl group; **6**: amide; **7-8**: carboxylic ester; **9-11**: ketone). The synthesis of the compounds was described previously by Domínguez-Álvarez *et al.* The stock solutions (in 10 mM concentration) of the compounds were prepared in DMSO. Phthalic anhydride (**12**; the oxygen isoster of compound **1**) and three inorganic chalcogen cyanates (**13-15**; **13**-KOCN, **14**-NH₄SCN, **15**-KSeCN) were included in the study (Sigma) and used as references in our experiments. The following compounds were used during the assays as reagents: rhodamine 123 (R123; Sigma, St. Louis, MO, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO, USA), sodium dodecyl sulfate (SDS; Sigma), verapamil (EGIS Hungarian Pharmaceutical Company, Budapest, Hungary), cisplatin (TEVA Pharmaceutical Company, Petah Tikva, Israel) and dimethyl sulfoxide (DMSO; Sigma). All the chemical compounds used in the apoptosis assay, except for the positive control M627 (12*H*-benzo[*a*]phenothiazine) were included in the Annexin V-FITC Apoptosis Detection Kit Cat. No. PF 032, purchased from Calbiochem (EMD Biosciences, Inc. La Jolla, CA). The positive control M627 was kindly provided by Prof. Dr. Noboru Motohashi (Meiji Pharmaceutical University, Kiyose, Tokyo, Japan) and dissolved in DMSO. Stock solution of R123 was prepared in PBS and verapamil was dissolved in water. All solutions were prepared on the day of assay.

Cell lines

L5178Y mouse T-cell lymphoma cells (PAR) (ECACC Cat. No. 87111908, obtained from FDA, Silver Spring, MD, USA) were transfected with pHa MDR1/A retrovirus, as previously described by Cornwell *et al.* The **ABCB1-expressing cell line (MDR)** was selected by culturing the infected cells with colchicine. **Human colonic adenocarcinoma cell lines (Colo 205 doxorubicin-sensitive and Colo 320/MDR-LRP multidrug resistant, overexpressing ABCB1 (MDR1)-LRP)**, ATCC-CCL-220.1 (Colo 320) and CCL-222 (Colo 205), **A549 human lung adenocarcinoma cell line** (ATCC CCL-185), **NIH/3T3 mouse embryonic fibroblast cell line** (ATCC CRL-1658) and **MRC-5 human embryonic lung fibroblast cell line** (ATCC CCL-171) were purchased from LGC Promochem, Teddington, UK.

The **L5178Y mouse T-cell lymphoma cells (PAR and MDR)** were cultured in McCoy's 5A medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich, St Louis, MO, USA), 200 mM L-glutamine (Sigma-Aldrich, St Louis, MO, USA), nystatin (Sigma-Aldrich, St Louis, MO, USA) and a penicillin-streptomycin mixture (Sigma-Aldrich, St Louis, MO, USA) in concentrations of 100 U/L and 10 mg/L, respectively. The **human colonic adenocarcinoma cell lines (Colo 205 and Colo 320)** were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine (Sigma-Aldrich, St Louis, MO, USA), 1 mM Na-pyruvate (Sigma-Aldrich, St Louis, MO, USA), 100 mM HEPES (Sigma-Aldrich, St Louis, MO, USA), nystatin (Sigma-Aldrich, St Louis, MO, USA) and a penicillin-streptomycin mixture (Sigma-Aldrich, St Louis, MO, USA) in concentrations of 100 U/L and 10 mg/L, respectively. The **NIH/3T3 mouse embryonic fibroblast cell line** was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco Life Technologies Co., UK), containing 4.5 g/L glucose, supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine (Sigma-Aldrich, St Louis, MO, USA), 1 mM Na-pyruvate (Sigma-Aldrich, St Louis, MO, USA), nystatin (Sigma-Aldrich, St Louis, MO, USA) and a penicillin-streptomycin mixture (Sigma-Aldrich, St Louis, MO,

USA) in concentrations of 100 U/L and 10 mg/L, respectively. **MRC-5 human embryonic lung fibroblast cell line** and **A549 human lung adenocarcinoma cell lines** were cultured in Eagle's Minimal Essential Medium (EMEM, Sigma-Aldrich, St Louis, MO, USA) supplemented with 1% of non-essential amino acid (NEAA) mixture (Sigma-Aldrich, St Louis, MO, USA), a selection of vitamins (in case of the MRC-5 cell line), 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine (Sigma-Aldrich, St Louis, MO, USA), 1 mM Na-pyruvate (Sigma-Aldrich, St Louis, MO, USA), nystatin (Sigma-Aldrich, St Louis, MO, USA) and a penicillin-streptomycin mixture (Sigma-Aldrich, St Louis, MO, USA) in concentrations of 100 U/L and 10 mg/L, respectively.

Assay for cytotoxic effect

The effects of increasing concentrations of the compounds on cell growth were tested in 96-well flat-bottomed microtiter plates. The two-fold serial dilutions of the tested compounds were made starting in the third row of the 96-well microtiter plate (4 μ L of the 10 mM stock solutions were added to 196 μ L of medium, then diluted in the respective wells). Then, 10^4 of mouse T-cell lymphoma and human colonic adenocarcinoma cells in 100 μ L of the corresponding medium were added to each well, except for the medium control wells, resulting in a final concentration of 100 μ M. The adherent mouse embryonic fibroblast cells (10^4 /well), human lung fibroblast cells (1.5×10^4 /well) and human lung adenocarcinoma cells (10^4 /well) were seeded in the corresponding medium in 96-well flat-bottomed microtiter plates for 4 hours before the assay. The serial dilutions of the compounds were made in a separate plate, and then transferred to the plates containing the adherent corresponding cell line. Culture plates were incubated at 37°C for 24 h; at the end of the incubation period, 20 μ L of MTT (thiazolyl blue tetrazolium bromide, Sigma) solution (from a 5 mg/mL stock solution) were added to each well. After incubation at 37°C for 4 h, 100 μ L of sodium dodecyl sulfate (SDS) (Sigma) solution (10% SDS in 0.01 M HCl) 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 550 nm (ref. 630 nm) with Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Inhibition of cell growth was expressed as IC₅₀ values, defined as the inhibitory dose that reduces the growth of the cells exposed to the tested compounds by 50%, determined according to the formula below:

$$IC_{50} = 100 - \left[\frac{OD_{sample} - OD_{medium\ control}}{OD_{cell\ control} - OD_{medium\ control}} \right] \times 100$$

Selectivity indices (SI) were calculated to determine the potency of the tested compounds towards the tumoral and non-malignant cell lines, respectively. The SI values were calculated as a quotient of the IC₅₀ values in the non-tumoral cells divided by the IC₅₀ values in the (sensitive or MDR, where applicable) cancer cell lines. The compounds are considered as strongly selective if the SI values are higher than 6, moderately selective if $3 < SI < 6$, slightly selective if $1 < SI < 3$ and non-selective if the SI values are lower than 1.

Assay for the inhibition of the ABCB1 efflux pump

The inhibition of the cancer multidrug efflux pump ABCB1 by the tested compounds was evaluated using flow cytometry, measuring the retention of rhodamine 123 by ABCB1 (P-glycoprotein) in MDR mouse T-lymphoma cells and Colo320 colonic adenocarcinoma cells, as both cell lines overexpress the ABCB1 transporter (P-glycoprotein). This method is a fluorescence-based detection system which uses verapamil (a first-generation, competitive EPI) as reference inhibitor. Briefly, cell number of mouse T-lymphoma and colonic adenocarcinoma cells were adjusted to 2×10^6 cells/mL, re-suspended in serum-free McCoy's 5A medium in case of mouse T-lymphoma cells and serum-free RPMI-1640 medium in case

of colonic adenocarcinoma cells and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The tested compounds were added at different concentrations (2 and 20 μM ; from 1 and 10 mM stock solutions, respectively) and the samples were incubated for 10 minutes at room temperature. Verapamil (EGIS Hungarian Pharmaceutical Company, Budapest, Hungary) was applied as positive control (20 μM final concentration) and DMSO was used as solvent control (at 2 V/V%). Next, 10 μL (5.2 μM final concentration) of the fluorochrome rhodamine 123 (Sigma, St. Louis, MO, USA) was added to the samples and the cells were incubated for 20 minutes at 37°C.

Rhodamine 123 (R123) is a non-toxic, lipophilic, cationic fluorescent dye ($\lambda_{\text{ex/em}} = 505/534 \text{ nm}$), which is a substrate of the ABCB1 transporter. As the compound is membrane-permeable, it is rapidly taken up by the cells, therefore it can be effectively used for the screening of efflux pump inhibiting compounds. After the incubation period, the cells were washed twice and re-suspended in 0.5 mL PBS for analysis. The fluorescence of the gated cell population was measured with a Partec CyFlow® flow cytometer (Partec, Münster, Germany). The percentage of mean fluorescence intensity was calculated for the treated MDR cells as compared with the untreated cells. The results were obtained from a representative flow cytometry experiment in which at least 20,000 individual cells of the overall population were evaluated for the rhodamine 123 retained inside the cells. The fluorescence activity ratio (FAR) was calculated based on the following equation which relates the measured fluorescence values:

$$\text{FAR} = \frac{\text{MDR}_{\text{treated}}/\text{MDR}_{\text{control}}}{\text{parental}_{\text{treated}}/\text{parental}_{\text{control}}}$$

Parameters evaluated from flow cytometric experiments were: Forward Scatter Count (FSC, provides information about cell size); Side Scatter Count (SSC, proportional to cell granularity or internal complexity); FL-1 (Mean fluorescence of the cells) and Fluorescence Activity Ratio (FAR), which was calculated by the equation given above. The efflux pump inhibitory activity of the tested compounds was compared to the activity of verapamil, additionally, a FAR-quotient was calculated, according to the following equation:

$$\text{Quotient} = 100 \times (\text{FAR}_{\text{compound}}/\text{FAR}_{\text{verapamil}})$$

Assay for apoptosis induction

The capacity of the selenocompounds to induce apoptosis was investigated by using L5178Y mouse T-cell lymphoma cells (parental and *ABCB1*-transfected) as well as multidrug resistant (MDR) Colo 320 colonic adenocarcinoma cells as *in vitro* model systems. The apoptosis induction assays were performed using Annexin V-FITC Apoptosis Detection Kit, following the manufacturer's instructions. This method enables the quantification and differentiation of early and late apoptotic events as well as necrosis and cell death in the cell population exposed to the respective selenocompounds. In the assay, Annexin V was used as phospholipid binding protein, to which fluorescein isothiocyanate (FITC) was conjugated as a fluorescent substrate ($\lambda_{\text{ex/em}}=495/519 \text{ nm}$). Because the Annexin-FITC conjugate also binds to the externalized phosphatidyl serine of the necrotic cells, propidium iodide (PI; $\lambda_{\text{ex/em}}=535/617 \text{ nm}$) staining was used to detect cells in distinct stages of cell death. These two stains can be used in parallel in this fashion, because they don't have overlapping wavelength ranges. The concentration of the cell suspension was adjusted to 1×10^6 cells/mL. The L5178Y mouse T-cell lymphoma cell suspension was distributed into 0.5 mL aliquots (5×10^5 cells) to Eppendorf tubes. The mouse T-lymphoma cells were then incubated in the presence of the compounds for 1 h at 37°C. In case of the Colo 320 cell line, the cell suspension was distributed

to 24-well plate and incubated overnight at 37°C in 5% CO₂ incubator. On the following day, the medium was removed, and fresh medium was added to the cells containing the tested compounds. The incubation period for colonic adenocarcinoma cells in the presence of the compounds was 3 h. 12*H*-benzo[*a*]phenothiazine M627 was used as positive control at the final concentration of 20 μM, which is a known early apoptosis inducer, whereas DMSO was used as solvent control (at 2 V/V%). The samples were washed in PBS and the harvested cells were centrifuged at 2000 × g for 2 minutes. Fresh medium was added to the cells, followed by the overnight incubation of the plate at 37°C, in 5% CO₂ atmosphere. After the incubation period, the Colo 320 cells were removed gently from the wells using a cell scraper. Following this step, 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, Munster, Germany).

Predictive *in silico* assay

A preliminary *in silico* assay was performed using the freely accessible software packages OSIRIS Molecular Property Explorer (Actelion Pharmaceuticals, Allschwil, Switzerland) and PreADMET 2.0 (Yonsei University, Seoul, Republic of Korea) to predict the physico-chemical and *in vivo* absorption properties of the tested compounds. OSIRIS works with a database of 2000-5000 drug molecules (depending on the model in question), while PreADMET 2.0 contains the experimentally measured physico-chemical attributes of more than 1 million molecules. The following properties of the tested compounds were assessed to verify their conformity with Lipinsky’s Rule of Five (RO5): molecular weight (*M*), number of hydrogen bond donors (n-OHNH), number of hydrogen bond acceptors (n-ON), base 10 logarithm of the octanol/water partition coefficient (*cLogP*), base 10 logarithm of water solubility (*logS*; expressed as mol/L) and topological polar surface area of the molecules (*TPSA*). Additionally, the programs were used to predict the pharmacokinetic (i.e., ADME) properties of the tested compounds, such as the percentage of plasma protein binding (PPB%), permeability on different model systems of cellular monolayers (Caco-2, MDCK), percentage of human intestinal absorption (HIA%) and interaction with various cytochrome P450 enzymes. To compare our data to clinically relevant anticancer agents, various, structurally and functionally different chemotherapeutic drugs (doxorubicin, gemcitabine, irinotecan, methotrexate, and 5-fluorouracil) were chosen as references. Additionally, a correlation-regression analysis was performed with the aim of assessing the relationship between the measured fluorescence ratios (i.e. efflux pump inhibitory activity) on mouse T-lymphoma and colonic adenocarcinoma cells and selected physico-chemical properties (*M*, *cLogP*, *logS* and *logTPSA*). The statistical analyses were performed using Past 3.16 statistical software: $p < 0.05$ was considered statistically significant, furthermore the coefficient of determination (R^2) was also calculated, which shows percentage variation in FAR values which is explained by the respective physico-chemical parameters.

IV. RESULTS

Cytotoxicity assay

The cyclic selenoanhydride (**1**) and the selenoesters **9-11** exerted remarkable cytotoxic activity on both the parental and multidrug resistant murine cell lines (0.94-3.97 μM and 0.43-4.65 μM), the latter three compounds were proven to be cytotoxic in nanomolar range, the IC_{50} value for the most active selenoester (**10**) was 430 nM in MDR cells. Compounds **3** and **6** also presented with strong cytotoxicity, but they exhibited their activity in concentrations 5-40 times higher, than the previous four compounds. Selenoesters **9-11** were also toxic on non-tumoral mouse embryonic fibroblast cells at low concentrations (IC_{50} : 0.62-1.35 μM), while compounds **2** and **7** (IC_{50} : 23.72 μM), **6** and **8** (IC_{50} : 69.69 and 74.47 μM) exhibited toxic properties in similar concentrations. Compound **10** was moderately selective ($\text{SI}=3.14$), while the selenoanhydride showed excellent ($\text{SI}\geq 25.19$) selectivity towards the cancerous cell lines. The assays on human colonic adenocarcinoma cells showed that selenoesters **9-11** had strong cytotoxic effect on both the sensitive (Colo 205) and multidrug resistant colonic adenocarcinoma (Colo 320) cell lines (IC_{50} : 1.19-5.48 μM and 0.35-0.96 μM , respectively), the IC_{50} value for the most active selenoester (**9**) was 350 nM in Colo 320 cells. It is worth noting that selenoesters **9** and **11** displayed moderate-strong selectivity (SI : 3.40-10.0) towards the Colo 320 cell line and colonic adenocarcinoma cells (SI : 4.2-14.40).

The activity of the cyclic selenoanhydride and two other selenoesters (**4** and **8**) were similarly encouraging, as they did not show cytotoxic activity on the Colo 205 cell line, but exerted activity on the resistant cells. The IC_{50} values of cisplatin were 11.36-108.29-times higher than that of selenoesters **9-11** and the selenocompounds were 2.07-6.09-times more selective towards colonic adenocarcinoma cells. Similarly, selenocompounds **9-11** emerged as potent cytotoxic agents on A549 lung carcinoma cells (IC_{50} : 5.91-15.22 μM), together with two other compounds (**2** and **8**) which were cytotoxic approximately at 50 μM . However, the compounds showed slight or no selectivity (0.09-1.95) in respect to lung adenocarcinoma/lung fibroblast cells. The reference compounds (**12-15**) did not have cytotoxic activity on any of the tested cell lines.

Inhibition of the ABCB1 efflux pump

Four derivatives (**1**, **9-11**) out of the eleven compounds exhibited remarkable ABCB1 inhibitory activity (the intracellular concentration of R123 was the highest) on the MDR mouse T-lymphoma and Colo 320 colonic adenocarcinoma cell lines at 20 μM concentration. The FAR quotients of the active compounds were 217.71-458.48% compared to the positive control's (verapamil, 20 μM) activity. The two most active compounds (cyclic selenoanhydride **1** and selenoester **9**) were more potent inhibitors of ABCB1 than verapamil in concentrations ten times lower (20 vs. 2 μM), with quotient values of 202.58% and 442.41%, respectively. Comparable results were observed on the ABCB1-overexpressing Colo 320 cells; the cyclic selenoanhydride **1** and selenoesters **9-11** were promising inhibitors of the efflux protein, outperforming verapamil ($\text{FAR}=2.85$) in 2 μM concentration (quotients ranging from 135.44-401.05 %). The other compounds investigated in our study (selenoesters **2-8** and reference compounds **12-15**) did not show efflux pump inhibitory activity similar to verapamil on either cell lines.

Apoptosis assay

The efficacy of the apoptosis-inducing properties of the selenocompounds was compared to 12*H*-benzo[α]phenothiazine. The tested selenocompounds were effective apoptosis-inducers on murine cell lines. The cyclic selenoanhydride (**1**) and the selenoesters **9-11** were the most noteworthy, compared to the positive control, when considering the total percentage of apoptotic events. The positive control only matched the activity of the abovementioned compounds at ten times higher concentrations (2 vs. 20 μ M). In the experiment on multidrug resistant mouse T-lymphoma cells, compound **1** had excellent activity, as it induced early apoptosis in 32.2% of the gated cell population (77.67% overall). In addition, the selenoesters **9-11** were also effective, although their activity was predominantly detected as late apoptosis/necrosis inducers. The compounds exhibited comparable apoptosis inducing effects on the parental mouse T-lymphoma cells. However, it is of interest that compounds **10-11** were notably more effective on the susceptible parental cell line (apoptotic events in 39.01% vs. 84.37% and 47.16% vs. 92.46% of the gated cell population, respectively). The roles of efflux transporters have been described in apoptosis evasion, mediated by dampening of the extrinsic apoptotic pathway and the stabilization of cell membrane; the inter-relatedness of overexpressed efflux pumps and programmed cell death may explain our observations in these experiments. Another possible explanation is the lower intracellular concentration of these compounds due to the operation of the ABCB1 efflux pump: while compounds **9-11** were all cytotoxic ($IC_{50} < 2 \mu$ M), thus contributing to their late apoptosis/necrosis inducing properties on murine cells, selenoester **9** was an effective efflux pump modulator at 2 μ M concentration, while compounds **10-11** were only effective as EPIs at 20 μ M. The fact that the compounds exhibited similar percentages of late apoptosis/necrosis on PAR cells, while compound **9** was disproportionally (2.8-3.9-times) more effective on MDR cells further validates this hypothesis.

The compounds (**1, 9-11**) showed a similar activity profile for multidrug resistant (Colo 320) human colon adenocarcinoma cells (apoptosis was induced in 64.6-80.5% of gated cell population overall). Comparing the results on all three cell lines, it can be observed that the cyclic selenoanhydride **1** was a potent inducer of early apoptosis (32.2-66.1%), while the active selenoesters (**9-11**) mainly induced late apoptosis or necrosis, which may be attributed to their strong cytotoxic properties. Considering our results, the selenocompounds exhibited more potent early apoptosis inducing effect in the human colonic adenocarcinoma system. Other selenocompounds (**2-8**) and reference compounds (**12-15**) did not have comparable apoptosis inducing properties.

Predictive *in silico* assay

The selenocompounds are in accordance with the evaluation criteria detailed in the thesis, regarding their physico-chemical properties, and they all complied with the Rule of Five without exception, as opposed to certain reference compounds (doxorubicin, methotrexate, irinotecan). The organoselenium compounds are expected to have excellent oral bioavailability (96.74-99.10%) and based on the predicted permeability on Caco-2 monolayers, the compounds have moderate penetration properties. The predicted plasma protein binding (PPB%) is nearly 100% for all respective selenocompounds, which can be attributed to the presence of the selenium atom in the biologically active molecules. The tested selenium compounds should be inhibitors of the CYP2C9 enzyme and excluding compound **6**, they are presumably substrates and inhibitors of the CYP3A4 enzyme, which may pose an issue, if these compounds were to be co-administered with anticancer drugs that are metabolized on these enzymes.

The efflux pump inhibitory activity (logFAR) of the tested selenocompounds showed significant association with their base 10 logarithm of the octanol/water partition coefficients (*cLogP*), both in the case of the measured data on MDR mouse T-lymphoma ($p=0.0034$; $R^2=0.6934$) and Colo 320 colonic adenocarcinoma cells ($p=0.0198$; $R^2=0.5117$). The molecular weight, the base 10 logarithm of topological polar surface area and the base 10 logarithm of water solubility did not show relevant correlation with the fluorescence (logFAR) data.

V. DISCUSSION

Malignant diseases present a significant public health burden, accounting for 8.2 million deaths and 14.1 million new cancer cases worldwide (according to the data of the WHO). Due to the ageing population in developed countries, the mortality rates related to cancer are expected to increase further. Hungary has the worst mortality rates of lung and colon cancer among EU member states. The goal of chemotherapy is the elimination or reduction of malignant cell mass and to improve the quality of life of the patient. The use of chemotherapy is complicated by its low bioavailability, disadvantageous side effects due to non-selective cytotoxic activity, in addition to the emergence of multidrug resistance, whereby tumors show resistance to chemotherapeutic agents of different structure and mechanism of action. The two mechanisms of MDR elucidated in this thesis are the overexpression of energy-dependent efflux pumps and failure of apoptosis induction. Considerable number of compounds have been described with the ability to inhibit the function of the ABCB1 efflux pump, an emerging therapeutic strategy of using chemosensitizers as adjuvants, reversing the MDR phenotype. Inorganic selenium salts and organoselenium compounds have been extensively studied for their anticancer activities. Organic compounds containing selenium are known modulators of the intracellular redox state of mammalian cells, therefore they may be effective and selective anticancer drugs, since neoplastic cells are thought to be sensitive to exogenous ROS. The present study focuses on the activity of novel organoselenium compounds as anticancer agents and their potency as MDR reversers with respect the epidemiology of cancer in Hungary. An additional aim was to establish structure-activity relationship for the selenocompounds.

Considering our results, selenocompounds with specific structures showed favorable activity in all assays, while others presented with limited or no activity. The compounds that were chosen as references (**12-15**) had no activity in any of the experiments. In the **cytotoxicity assays**, the chemical variation of the alkyl chain directly bound to the selenium atom in the selenoesters was key in modulating anticancer efficacy. A methyl (**9**) or *tert*-butylketone-substituent (**10,11**) was the most profitable for cytotoxic activity, in addition to the cyclic selenoanhydride (**1**). The substituents on the aromatic ring seem to be less important for the anticancer potency. In general, the selenocompounds were the least effective and selective on the A549 lung adenocarcinoma cells. The potency of the selenoesters **9-11** is further verified by the fact that they had IC_{50} values in the nanomolar range in many cases and showed SI values over 6, in respect to human adenocarcinoma cells. The selenoanhydride (**1**) presented with very high selectivity in murine cell lines ($SI>20$) and was not toxic on any of the non-tumoral cell lines. Results obtained from **the rhodamine 123 accumulation studies** are in concordance with those from the cytotoxicity assay.

The 4-chlorophenyl-substituted methyl-ketone-substituted selenoester (**9**) was the most active compound (surpassing any other tested molecule in 2 μ M concentration), followed by cyclic selenoanhydride and the two remaining acylmethyl-selenoesters (**10,11**), showing similarly potent ABCB1 inhibition at 20 μ M. The efflux pump modulatory activity of selenoesters **2-8** was not comparable to the effect of verapamil, due to the variable side chains in their structures. It is also important to note that the lipophilicity of the compounds influenced ABCB1 modulation, as the cLogP values showed significant ($p < 0.05$) correlation with the calculated FAR values.

In the **apoptosis assay**, the abovementioned four compounds (**1, 9-11**) induced apoptosis in 39.01-97.32% of the tested murine cells and 64.6-71.4% of human cells, respectively. However, the selenoanhydride (**1**) was the most effective inducer of early apoptosis (around one-third of the gated cell population of mouse T-lymphoma cells and two-thirds of the colonic adenocarcinoma cells), surpassing the activity of the positive control (M627) used in the experiments. On the other hand, the acylmethyl-selenoesters also had pronounced toxic activity, which predominantly presented as necrosis (up to 85.80% in murine cells). This distinction in the activity profiles is relevant because early apoptosis is the advantageous type of cell death for chemotherapy-treated malignant cells, as collateral damage will not occur in adjacent cells due to the release of inflammatory mediators compared to necrotic cell death processes. The remaining selenoesters (**2-8**) had slight or no capacity to induce apoptosis, although the human colonic adenocarcinoma cell line showed to be more sensitive to the treatment with the selenocompounds.

Organic compounds contacting chalcogenic elements (S, Se, Te) recently received substantial interest in experimental oncopharmacology. The literature suggests that the most active member of this group is tellurium (Te); but it has been shown that the Te-containing compounds are highly toxic and do not possess adequate selectivity. The advantage of Se-compounds is that they affect various cellular redox mechanisms and signal transduction pathways, which makes them especially appealing. The reference compound **12**, which showed no activity in any of the experiments, is the oxygen-isoster of the cyclic selenoanhydride, which suggests the significant role of Se atom for the activity of these organic molecules. The exact molecular mechanism of these compounds is yet to be described. According to the previous studies and hypotheses of Domínguez-Álvarez *et al.*, it is proposed that the activity of organoselenium derivatives lies in the hydrolysis of the selenoester group. This breakdown allows the liberation of selenium anions to the medium, allowing for these particles to take part in oxidation-reduction reactions due to the charged selenium atom. In other words, it is suggested that the novel selenoesters act as prodrugs that make the transport of the molecules possible through the cell cytoplasm. Inside the cells, the breakdown of the carrier molecule liberates the active ionic chemical forms of selenium with cytotoxic properties in cells. The inclusion of electron withdrawing groups, like a ketone or a carboxylic acid is thought to facilitate the breakdown for stabilizing the resulting fragments. However, it is not desirable to design derivatives whose breakdown occurs before the arrival of the compound to the cytoplasm of the target cells. In this case, the ionic species generated in the extracellular hydrolysis would not pass the cell membrane due to their hydrophilic properties. The rationale behind the activity of the cyclic selenoanhydride is presumably like those of compounds **9-11**, however, the degradation mechanisms and kinetics of this compound should be different, as it

contains the Se atom in a five-member ring system. Also, it is not clear whether the tested compounds are competitive inhibitors of the efflux pump or bind to a specific domain of the ABCB1 transporter.

Overall, four compounds were identified as promising candidates for further studies: the cyclic selenoanhydride (**1**; benzo[c]selenophene-1,3-dione), and the selenoesters **9-11**. All respective compounds exhibited remarkable anticancer and multidrug resistance reversing (ABCB1 pump-modulating and apoptosis inducing) properties. Considering the predicted data from the *in silico* assays, these four compounds show attractive properties for good *in vivo* bioavailability and potential for transitioning from the pre-clinical to clinical study phase (as presented by their conformity to the RO5 and predicted HIA percentages). As for future perspectives, the derivatization and synthesis of novel structural variants of these compounds are warranted. In addition, as there is abundant literature regarding various selenium compounds co-administered with various chemotherapeutic drugs, the survey of these agents *in vitro* could shed some light about their potency as combinational drugs.

VI. NEW FINDINGS

a. Selenocompounds as cytotoxic agents: Three selenoesters with ketone-containing alkyl groups showed potent cytotoxic activity on mouse T-lymphoma, human colon adenocarcinoma and human lung adenocarcinoma cell lines with IC₅₀ values in the nanomolar range. The cyclic selenoanhydride was highly active and selective towards malignant murine cell lines.

b. Selenocompounds as efflux pump inhibitors: The cyclic selenoanhydride and three selenoesters with ketone-containing alkyl groups showed potent efflux pump modulatory effects in the ABCB1-overexpressing subline of mouse T-lymphoma and human colon adenocarcinoma cell lines. The 4-chlorophenyl-substitution was the most beneficial for ABCB1-inhibiting activity on both murine and human cell lines.

c. Selenocompounds as apoptosis inducers: The cyclic selenoanhydride was an effective inducer of early apoptosis, while the selenoesters with ketone-containing alkyl groups predominantly induced late apoptosis/necrosis in parental and multidrug resistant mouse T-lymphoma and MDR human colon adenocarcinoma cell lines.

d. Prediction of ADME properties of the selenocompounds by *in silico* methods: All organoselenium compounds compiled with Lipinsky's Rule of Five and they are expected to have moderate intestinal penetration properties, very strong plasma protein binding and excellent oral bioavailability. The results suggest a link between the lipophilicity and efflux pump modulatory activity of the compounds.

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IX. PUBLICATIONS

I. Domínguez-Álvarez E, **Gajdács M**, Spengler G, Palop JA, Maré MA, Kieć-Kononowicz K, Amaral L, Molnár J, Jacob C, Handzlik J, Sanmartín C: Identification of selenocompounds with promising properties to reverse cancer multidrug resistance. *Bioorganic and Medicinal Chemistry Letters* 26(12): 2821-2824, 2016.

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II. **Gajdács M**, Spengler G, Sanmartín C, Maré MA, Handzlik J, Domínguez-Álvarez E: Selenoesters and selenoanhydrides as novel multidrug resistance reversing agents: A confirmation study in a colon cancer MDR cell line. *Bioorganic and Medicinal Chemistry Letters* 27(4): 797-802, 2017.

IF₂₀₁₇: 2.442, Citations: 10 (Independent citations: 4)

III. **Gajdács M**, Handzlik J, Sanmartín C, Domínguez-Álvarez E, Spengler G: [Organoselenium compounds as antitumor agents: *in vitro* evaluation on a colon cancer model system] (*article in Hungarian*). *Acta Pharmaceutica Hungarica* 88(2): 59-66, 2018.

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IV. **Gajdács M**, Handzlik J, Sanmartín C, Domínguez-Álvarez E, Spengler G: [Prediction of ADME properties for selenocompounds with anticancer and efflux pump inhibitory activity using preliminary computational methods] (*article in Hungarian*). *Acta Pharmaceutica Hungarica* 88(2): 67-74, 2018.

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