EFFLUX PUMP INHIBITORS AND POTENTIAL ADJUVANTS TO REVERSE MULTIDRUG RESISTANCE IN BACTERIA AND TUMOR CELLS

Ph.D. Thesis

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INTRODUCTION

Drug resistance is a natural process and it is based on the interaction between the organisms and their environment. However, during the last decades, the occurrence of drug resistant microbial infections has increased dramatically which can be explained by global spread of drug resistant microbes. In addition, multidrug resistance (MDR) has also been found to occur in cancer cells which also makes clinical treatments difficult. Despite the efforts and successes achieved in cancer therapy, resistance to anticancer drugs is one of the most crucial challenges in tumor therapy.

There are several mechanisms of drug resistance that are detected in bacteria and cancer cells as well. Some resistance mechanisms are specific, however, the phenomenon of increased drug efflux is observed in bacteria and tumor cells as well. This mechanism is due to the reduced drug accumulation which is a consequence of over-expressed efflux pumps (EPs). The inhibition of these EPs is a promising approach to overcome MDR. In order to reverse MDR, new synthetic efflux pump inhibitors (EPIs) could be designed (e.g., novel selenium (Se)-containing compounds) or „old" drugs (e.g., phenothiazines) could be repurposed and applied with new indications.

Se is an essential trace element in living organisms, in addition, selenocompounds are effective against different types of cancer, because these can modulate tumor growth, metastasis, angiogenesis, and drug resistance. In addition, these agents also possess antibacterial activity. Consequently, Se-containing agents could provide alternative and effective scaffolds to overcome MDR in both anticancer and antibacterial therapies. Phenothiazines have been reported as antiemetic, antipsychotic, antihistaminic, and anticholinergic compounds over the years. Nevertheless, many studies have demonstrated the ability of phenothiazines as EPIs to reverse MDR in bacteria, furthermore they are potent anticancer compounds. Phenothiazines are widely known and applied in clinical practice, furthermore the use of these as adjuvants may be a promising alternative to overcome the MDR phenotype in cancer by drug repositioning, where the ultimate goal is to find new uses for existing drugs.

Over the years several compounds have been discovered as EPIs but these are not suitable for clinical application in their current form. Inspite of this fact, specific EPIs can restore the efficacy of known drugs and may also be useful as feed additives to reduce the colonization of microbes in gastrointestinal tract of animals, thereby reducing infection transmission to humans.

AIMS OF THE STUDY

In our study novel selenocompounds were investigated on Gram-positive and Gram-negative bacteria. Regarding MDR cancer, various approaches can help to overcome the MDR phenotype. In the present work, selenocompounds were applied to enhance the effect of antitumor phenothiazines in cancer model systems *in vitro*.

The main goals of the study:

1. **Influence of the external pH (pH 5 and pH 7) on the AcrAB-TolC efflux pump (EP) of Gram-negative *Escherichia coli* K12 AG100 in the presence of the efflux pump inhibitor (EPI) promethazine (PMZ)**
   - **1.1.** Determination of minimum inhibitory concentration (MIC) of PMZ by microdilution method
   - **1.2.** Investigation of the efflux pump inhibitory effect of the PMZ using LightCycler real-time thermocycler.
   - **1.3.** PMZ induced changes in relative gene expression of EP genes *acrA*, *acrB* and their regulators *marA*, *marB*, *marR*, and *rob* and the stress gene *soxS* by RT-qPCR.
2. **Antibacterial activity of nine symmetrical selenoesters on Gram-negative and Gram-positive bacterial strains**
   - **2.1.** Determination of MICs of compounds by microdilution method on wild-type *Salmonella enterica* serovar Typhimurium SL1344 (SE01) expressing the AcrAB-TolC pump system, *S.* Typhimurium SL1344 strain (SE02; *ΔacrB*), *S.* Typhimurium SL1344 (SE03; *ΔacrA*) and *S.* Typhimurium SL1344 strain (SE39; *ΔtolC*) and methicillin-susceptible reference *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 strain and methicillin and ofloxacin-resistant *S. aureus* 272123 clinical isolate (MRSA).
   - **2.2.** Evaluation of the resistance modulating effect of selenocompounds with ciprofloxacin (CIP) and tetracycline (TET) on reference *S. aureus* ATCC 25923 and resistant *S. aureus* 272123 MRSA strains.
   - **2.3.** Anti-biofilm effect of selenocompounds on reference *S. aureus* ATCC 25923 and resistant *S. aureus* 272123 MRSA strains by crystal violet staining.
   - **2.4.** Efflux pump inhibiting effect of selenocompounds by real-time automated EB method using a CLARIOstar Plus plate reader.

2.5. QS inhibitory effect of selenocompounds on sensor bacterial strain *Chromobacterium violaceum* 026 and the *N*-acyl- homoserine lactone (AHL) producer *Enterobacter cloacae* 31298 strain by agar diffusion method.

3. **Interaction of selenocompounds and phenothiazines as antitumor adjuvants *in vitro* on mouse T-lymphoma cells**

3.1. Determination of cytotoxicity and selectivity of compounds on NIH/3T3 mouse embryonic fibroblast cells and sensitive and resistant mouse T-lymphoma cells.

3.2. Interaction of selenocompounds with phenothiazines on MDR mouse T-lymphoma cells by checkerboard combination assay.

MATERIAL AND METHODS

1. Compounds studied

Selenocompounds were pure and chemically stable on air and they were adequately characterized using NMR, MS, and IR techniques and their purity was assessed by elemental analysis by Dr. E. Domínguez-Álvarez and his coworkers (CSIC, Madrid, Spain). Before their use in biological assays the compounds were dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions of 10 mM concentration. The remaining chemicals used in the study were promethazine (PMZ), chlorpromazine (CPZ) and thioridazine (TZ), that were dissolved in DMSO, exception was PMZ that was dissolved in distilled water. The concentration of DMSO was kept below 1% in all the experiments.

1.1. Symmetrical selenoesters

Nine symmetrical selenodiesters or selenotriesters were synthesized by Dr. E. Domínguez-Álvarez and his coworkers (CSIC, Madrid, Spain). Three 2-oxopropyl selenoesters (briefly, ketone selenoesters, or methylketone selenoesters; compounds **Se-K1**, **Se-K2** and **Se-K3**), three methyloxycarbonylmethyl selenoesters (methylcarbonyl selenoesters or methyloxycarbonyl selenoesters; compounds **Se-E1**, **Se-E2,** and **Se-E3**) and three methylcyano selenoesters (cyano selenoesters; compounds **Se-C1**, **Se-C2,** and **Se-C3**). Their synthesis is described in the patent application EP17382693.

1.2. A selenoanhydride and selenoesters with previously confirmed anticancer activity

The resynthesis, purification and characterization of cyclic selenoanhydride **EDA1** and symmetrical-selenoesters **EDA2-EDA5** and non-symmetrical selenoesters **EDA6-EDA11**

were performed by Dr. E. Domínguez-Álvarez to gather the amount of selenocompounds needed for the performance of the assays (Domínguez-Álvarez, E. et al. *Eur J Med Chem* 2014). Their purity was assessed by elemental analysis.

2. Cell lines

pHa MDR1/A retrovirus was used to transfect L5178Y mouse T-cell lymphoma cells (PAR) (ECACC Cat. No. 87111908, acquired from FDA, Silver Spring, MD, USA) as formerly described by Cornwell et al. The ABCB1-expressing cell line L5178Y (MDR) was selected by culturing the infected cells with colchicine. The L5178Y parental cell line and its human *ABCB1*-transfected subline was cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, 200 mM L-glutamine and a penicillin-streptomycin mixture in concentrations of 100 U/L and 10 mg/L, respectively. All cell lines were incubated at 37°C, in a 5% CO_2, 95% air atmosphere.

NIH/3T3 mouse embryonic fibroblast cell line (ATCC CRL-1658) was purchased from LGC Promochem, Teddington, UK. The cell line was cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum and a penicillin-streptomycin mixture in concentrations of 100 U/L and 10 mg/L, respectively. The cell line was incubated at 37°C, in a 5% CO_2, 95% air atmosphere.

3. Bacterial strains

Compounds were evaluated against the following bacterial strains:

Gram-negative wild-type *Escherichia coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44] expressing the AcrAB-TolC efflux pump at its basal level. This strain was kindly provided by Prof. Dr. Hiroshi Nikaido (Department of Molecular and Cell Biology and Chemistry, University of California, Berkeley, CA, USA).

Gram-negative wild-type *Salmonella enterica* serovar Typhimurium SL1344 (SE01) expressing the AcrAB-TolC pump system and its *acrB* gene inactivated mutant *S.* Typhimurium SL1344 strain (SE02), *acrA* gene inactivated mutant *S.* Typhimurium SL1344 (SE03), and *tolC* gene inactivated mutant *S.* Typhimurium SL1344 strain (SE39) were used in the study. The strains were provided by Dr. Jessica Blair (University of Birmingham).

Gram-positive *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 was used as the methicillin-susceptible reference bacterial strain. The methicillin and ofloxacin-resistant *S. aureus* 272123 clinical isolate (MRSA), which was kindly provided by Prof. Dr.

Leonard Amaral (Institute of Hygiene and Tropical Medicine, Lisbon, Portugal), was used in the assays.

For QS tests *Chromobacterium violaceum* 026 (CV026) was used as a sensor strain and *Enterobacter cloaceae* 31298 as a *N*-acyl-homoserine lactone (AHL) producer clinical bacterial isolate. If *C. violaceum* reaches a high cell density, it produces violacein, which is a purple pigment.

4. Determination of minimum inhibitory concentrations by microdilution method

The minimum inhibitory concentrations (MICs) of PMZ and selenocompounds (**Se-K1**, **-K2**, **-K3**; **Se-E1**, **-E2**, **-E3**; **Se-C1**, **-C2**, **-C3**) were determined according to the Clinical and Laboratory Standard Institute guidelines (CLSI). MIC values of the compounds were determined by visual inspection. The solvent was also assayed to ensure that there was no antibacterial effect at the concentration (1 v/v%) applied in the assays**.**

5. Real-time ethidium bromide accumulation assay using a LightCycler real-time thermocycler at pH 5 and pH 7

The effect of PMZ on the real-time accumulation of EB in the presence and absence of glucose 0.4% against *E. coli* AG100 K-12 strain was assessed by an automated EB method as described previously (Viveiros, M. et al. *Int J Antimicrob Agents* 2008), using a LightCycler real-time thermocycler (LightCycler 1.5; Roche, Indianapolis, IN, USA). The final concentrations of PMZ and EB were 25 μg/ml and 1 μg/ml, respectively. The capillaries were placed into a carousel (Roche) and the fluorescence was monitored at the FL-2 channel every minute on a real-time basis. From the real-time data, the activity of the compound, namely the relative fluorescence index (RFI) of the last time point (minute 30) of the EB accumulation assay was calculated.

6. Total RNA isolation

E. coli AG100 K-12 strain was cultured overnight in LB broth at pH 5 and pH 7 at 37°C with shaking (OD_{600}: 0.6). Bacterial suspensions were prepared with and without PMZ (25 μg/ml) in 3.5 ml of LB medium at pH 5 and pH 7 and incubated at 37 ºC with shaking. The total RNA was isolated at various time points (0, 1, 2, 4, 8, and 18 hours). The RNA preparation was carried out in an RNase-free environment using NucleoSpin RNA kit (Macherey Nagel, Germany) according to the manufacturer's instructions. Purified RNA was stored in RNase-

free water in nuclease-free collection tubes and was maintained at −20 ºC until quantification was performed. The concentration of the extracted RNA templates was assessed by spectrophotometry at 260 nm (Bio-Rad, Hercules, CA, USA, SmartSpec™ Plus).

7. Relative gene expression analyses by real-time reverse transcriptase quantitative polymerase chain (RT-qPCR) reaction

Following total RNA preparation at different time points, the relative expression of efflux pump genes and their regulators was examined in the presence and absence of PMZ on *E. coli* strain AG100 K-12 using RT-qPCR. Real-time quantification of the RNA templates by one-step RT-qPCR was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad, USA) strictly adhered to the manufacturer's recommendations of the SensiFAST^{TM} SYBR No-ROX One-Step Kit (Bioline GmbH, Germany). Briefly, each well of the 96-well microtiter plate contained 20 µl as follows: 10 µl of the 2x SensiFAST^{TM} SYBR No-ROX One-Step Mix, 0.2 µl Reverse Transcriptase, 0.4 µl RiboSafe RNase Inhibitor, 5.4 µl Diethylpyrocarbonate (DEPC)-treated water, 500 nM of each primer and approximately 20 ng of total RNA in RNase-free water. Thermal cycling was initiated with a denaturation step of 5 min at 95°C, followed by 40 cycles each of 10 s at 95°C, 30 s at 57°C and 20 s at 72°C. The relative quantities of the mRNA of each gene of interest were determined by the use of the $\Delta\Delta C_T$ method. Gene transcript levels were normalized against the *E. coli* housekeeping gene *GAPDH* measured in the same sample. The equation $2^{-\Delta\Delta C_T}$ allows the relative quantification of differences of each gene's expression level between two samples, the sample of interest and a calibrator or reference sample.

8. Resistance modulation assay

The resistance modulation effect of compounds (**Se-K1, -K2, -K3**; **Se-E1, -E2, -E3**; **Se-C1, -C2, -C3**) with ciprofloxacin (CIP) and tetracycline (TET) antibiotics were evaluated by MIC reduction method on *S. aureus* strains. Briefly, CIP or TET was diluted in a 96-well microtiter plate by two-fold serial dilution in MH broth and then the compounds were added at subinhibitory concentrations (½ MIC). In this assay, only the tested compounds with well-defined MIC values were tested. Finally, 10^{-4} dilution of the overnight bacterial culture in MH was added to each well. The final volume was 200 µL in each well. The microtiter plates were incubated at 37°C for 18 h. At the end of the incubation period, 20 µL of MTT solution (from

a stock solution of 5 mg/mL) were added to each well. MIC values in the presence of the antibiotics alone and in combination with Se-compounds were determined by visual inspection.

9. Real-time ethidium bromide accumulation assay using a CLARIOstar Plus plate reader

The impact of selenocompounds (**Se-K1, -K2, -K3**; **Se-E1, -E2, -E3**; **Se-C1, -C2, -C3**) on EB accumulation was determined by the automated EB method using a CLARIOstar Plus plate reader (BMG Labtech, UK). Firstly, the bacterial strain was incubated until it reached an optical density (OD) of 0.6 at 600 nm. The culture was washed with phosphate buffered saline (PBS; pH 7.4) and centrifuged at 13,000 × *g* for 3 min, the cell pellet was re-suspended in PBS. The compounds were added at ½ MIC concentration to PBS containing a non-toxic concentration of EB (1 μg/mL). Then, 50 μL of the EB solution containing the compound were transferred into 96-well black microtiter plate (Greiner Bio-One Hungary Kft, Hungary), and 50 μL of bacterial suspension (OD_{600} 0.6) were added to each well. Then, the plates were placed into the CLARIOstar plate reader, and the fluorescence was monitored at excitation and emission wavelengths of 530 nm and 600 nm every minute for one hour on a real-time basis. From the real-time data, the activity of the compounds, namely the relative fluorescence index (RFI) of the last time point (minute 60) of the EB accumulation assay was calculated.

10. Inhibition of biofilm formation using crystal violet

The anti-biofilm effect of the tested compounds against *S. aureus* strains was measured using crystal violet (CV; 0.1% (v/v)). This dye is used to detect the total biofilm biomass formed. Overnight cultures were diluted to OD of 0.1 at 600 nm in TSB medium. Then, the bacterial cultures were added to 96-well microtiter plates and the compounds were added at ½ MIC concentration. The final volume was 200 μL in each well. The microtiter plates were incubated at 30°C for 48 h with gentle agitation (100 rpm). After the incubation period, TSB medium was discarded, and the plates were washed with tap water to remove unattached cells. Then 200 μL crystal violet were added to the wells and incubated for 15 min at room temperature. Then, CV was removed from the wells and the plates were washed again with tap water, and 200 μL of 70% ethanol were added to the wells. Finally, the biofilm formation was determined by measuring the OD at 600 nm using Multiscan EX ELISA plate reader (Thermo Labsystems, Cheshire, WA, USA). The anti-biofilm effect of compounds was expressed in the percentage (%) of decrease in biofilm formation.

11. Quorum sensing (QS) assay

The QS inhibitory effect of selenocompounds (**Se-K1, -K2, -K3**; **Se-E1, -E2, -E3**; **Se-C1, -C2, -C3**) was examined on the AHL producer *E. cloacae* strain and *C. violaceum* sensor bacterial strain. These strains were inoculated as parallel lines. The QS inhibition was monitored by agar diffusion method on LB* agar plate. Filter paper discs (7.0 mm in diameter) were placed between the parallel inoculated strains and impregnated with 10 μL compounds. The starting concentration of the compounds was ½ MIC. The agar plates were incubated at room temperature (20°C) for 24–48 h and the inhibition of violacein production was measured. PMZ was used as a positive control in 25 μg/ml concentration.

12. Cytotoxicity assay

The effects of increasing concentrations of the phenothiazines alone on cell growth were tested in 96-well microtiter plates. The cytotoxic activity of Se-compounds was previously determined on PAR and MDR mouse T-lymphoma cells (Domínguez-Álvarez, E. et al. *Bioorg Med Chem Lett* 2016).

The PAR and MDR mouse T-lymphoma cells were cultured using McCoy's 5A medium supplemented with 10% heat-inactivated horse serum.

The adherent NIH/3T3 mouse embryonic fibroblast cells were cultured using DMEM medium, supplemented with 10% heat-inactivated fetal bovine serum.

The density of the cells was adjusted to $1x10^4$ cells per well (in 100 μL of medium) and then added to the 96-well flat-bottomed microtiter plates containing the dilutions of the tested compounds. The culture plates were incubated at 37°C for 24 h; at the end of the incubation period, 20 μL of MTT (thiazolyl blue tetrazolium bromide) 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 sodium dodecyl sulfate (SDS) 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).

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

13. Checkerboard combination assay

A checkerboard microplate method was applied to study the effect of drug interactions between anticancer Se-compounds (**EDA1-11**) or reference compounds and phenothiazines. TZ and CPZ were dissolved in DMSO and PMZ was dissolved in distilled water on the day of the examinations. The assay was carried out using multidrug resistant mouse T-lymphoma cells overexpressing the ABCB1 transporter. The dilutions of phenothiazines 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 cells were re-suspended in culture medium and distributed into each well in 50 μL containing 6×10^3 cells each. The plates were incubated for 72 h at 37°C. The cell growth rate was determined after MTT staining. At the end of the incubation period, 20 μL of MTT 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 sodium dodecyl sulfate (SDS) (Sigma) solution (10% in 0.01 M HCl) 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, Cheshire, WA, USA) as described above. Combination index (CI) values at 50% of the growth inhibition dose (ED_{50}) were determined using CompuSyn software (ComboSyn, Inc., Paramus, NJ. 07652 USA) to plot four to five data points to 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.

14. Statistical analysis

The values are given as the mean ± standard deviation determined for three replicates from three independent experiments. The analysis of data was performed using SigmaPlot for Windows Version 12.0 software (Systat Software Inc, San Jose, CA, USA), applying the two-tailed *t*-test.

RESULTS

1. The activity of AcrAB-TolC efflux pump system at pH 7 and pH 5 in the presence of promethazine (PMZ)

1.1. Efflux pump inhibitory effect of PMZ

The real-time accumulation curves demonstrated higher intracellular EB concentration without glucose at pH 7 compared to the accumulation at pH 5. The intracellular concentration of EB increased in the presence of PMZ at neutral pH, however the PMZ treated sample exhibited lower EB accumulation at acidic pH. In case of PMZ treated sample the intracellular EB accumulation was significantly higher at pH 7 than at pH 5. The efflux pump inhibitor PMZ could exert more potent EPI effect at neutral pH.

1.2. Changes in relative gene expression of pump genes and their regulators

In case of acidic pH all genes except for *soxS* exhibited a decreased gene expression pattern in the first 1-2 h. After this period of time the gene expression levels started to increase. Increase in gene expression was detected in the case of the efflux pump genes *acrA* and *acrB*, as well as in *marR* regulator and *soxS* stress gene after the 18th hour. In case of neutral pH almost all genes except for *marB* and *marR* exhibited a decreased expression pattern in the first 1-2 h. Significant gene expression could be observed in the expression levels of *acrA*, *acrB*, and *marA* genes in the 18th hour, of *marB* in the 1st hour. Initially, the efflux pump genes *acrA* and *acrB* were downregulated, but at the end of the culturing period (18th hour) both genes were upregulated.

2. Antibacterial activity of novel symmetrical selenoesters

2.1. Determination of minimum inhibitory concentrations of selenocompounds

Based on the MIC values, the Se-compounds were more effective against *S. aureus* strains. The most effective compounds were the ketone selenoesters **Se-K1**, **Se-K2** and **Se-K3** on the reference *S. aureus* ATCC 25923, showing an MIC of 0.39 μM. These three derivatives share a common moiety, namely a methylketone group in the alkyl moiety bound to the selenium atom. The replacement of this methylketone by a cyano or by a methyloxycarbonyl moiety reduced the activity dramatically, as the MIC values are higher in *S. aureus* ATCC 25923, respectively, with the exception of the trisubstituted derivative **Se-C3**. The same tendency was observed in *S. aureus* MRSA 272123. The compounds showed slight antibacterial

effect on *Salmonella* strains. The most effective compound was **Se-C3** on SE01, SE02, and SE03 strains, showing an MIC of 12.5 μM.

2.2. *Evaluation of the resistance modulation effect of selenocompounds*

Selenocompound **Se-E3** showed synergism with tetracycline (TET) on the methicillin susceptible *S. aureus* ATCC 25923. All selenocompounds showed synergism with TET on the methicillin resistant *S. aureus* strain. **Se-E3** and **Se-C2** were the most effective ones in combination with TET against MRSA strain. Additionally, compounds **Se-E1** and **Se-C1** exerted also a noteworthy reduction of the MIC value of TET. On the other hand, **Se-K1** and **Se-E3** showed synergism with CIP on MRSA strain.

2.3. *Anti-biofilm effect of selenocompounds*

The biofilm inhibition (%) was calculated based on the mean of absorbance units (AUs). The absorbance expressed in AUs was the following on non-treated samples: reference *S. aureus* ATCC 25923 showed an absorbance of 2.4 ± 0.1, the resistant *S. aureus* MRSA 272123 strain exhibited 1.3 ± 0.1 AU, and the wild-type *S.* Typhimurium presented 2.2 ± 0.3 AU. Selenocompounds **Se-K1** (AU: 0.45 ± 0.17; inhibition: 64.5%), **Se-K3** (AU: 0.16 ± 0.06; inhibition: 84.7%), **Se-E3** (AU: 0.32 ± 0.07; inhibition: 74.6%), and **Se-C1** (AU: 0.72 ±- 0.15; inhibition: 43.7%) could inhibit efficiently the biofilm formation of *S. aureus* MRSA. In case of the reference *S. aureus* strain, the anti-biofilm effect was observed for **Se-K2** (AU: 1.67 ± 0.10; inhibition: 30.3%) and **Se-E3** (AU: 1.22 ± 0.17; inhibition: 74.6%). The compounds showed no significant anti-biofilm effect on *S.* Typhimurium SE01.

2.4. *Efflux pump inhibition by selenocompounds*

In case of *Salmonella* strains, the Se-compounds could increase the intracellular EB accumulation more efficiently on the *tolC* gene inactivated mutant *S.* Typhimurium SE39 after 60 min. In contrast, RFUs obtained in the presence of Se-compounds (½ MIC) were the lowest on the wild type *S.* Typhimurium SE01. CCCP, the reference efflux pump inhibitor (EPI) was the positive control in case of *Salmonella* and reference *S. aureus* strain. Concentration of CCCP was 50 μM on *S.* Typhimurion strains and 6.25 μM on *S. aureus* ATCC 25923 strain. In addition, verapamil was applied as reference EPI on *S. aureus* MRSA in 50 μg/ml concentration. The solvent DMSO served as a negative control in the experiments. **Se-E2** significantly increased the intracellular EB accumulation on *S.* Typhimurium SE02, -03, -39. In addition, a significant EB accumulation was observed for **Se-K3** on *S.* Typhimurium SE39. In case of the reference and MRSA strains, the highest RFUs were recorded in the presence of **Se-E3**, for this reason this compound exerted the most prominent EPI activity In addition,

methylcarbonyl selenoesters **Se-E1** and **Se-E2** were proved to be effective in both *S. aureus* strains.

2.5. *QS inhibitory effect of selenocompounds*

Selenocompounds **Se-K1**, **Se-K2** and **Se-E1** had QS inhibitory effect. In addition, **Se-K1** and **Se-K2** showed inhibition zones of 37 and 40 mm, respectively, whereas the methyloxycarbonyl selenoester **Se-E1** was the most effective QS inhibitor with an inhibition zone of 41 mm.

3. Interaction of selenocompounds with phenothiazines on mouse T-lymphoma cells

3.1. *Determination of cytotoxicity and selectivity of compounds on NIH/3T3 mouse embryonic fibroblast cells and mouse T-lymphoma cells*

Out of the fifteen tested compounds, seven showed toxicity against the NIH/3T3 cells (namely compounds **EDA2, EDA6-EDA11**) under 100 μM concentrations, while only three showed selectivity (**EDA1**: strongly selective, **EDA3** and **EDA10**: moderately selective) towards tumor cells, in perspective of the previous results on murine lymphoma cell lines (Gajdács, M. et al. *Bioorg Med Chem Lett* 2017).

3.2. *Interaction of selenocompounds and phenothiazines by checkerboard assay*

Compounds **EDA2-5** presented with the most advantageous interaction-profile (i.e. the highest CI scores were obtained); in fact, **EDA2** and **EDA5** showed synergism with all tested phenothiazines. This is further highlighted by the fact that these compounds exhibited synergism with the phenothiazines in low concentration ranges (1.46-11.25 μM). In contrast, compounds **EDA6-EDA8** and the reference Se-compounds showed antagonism with phenothiazines. The O-isostere of **EDA1** showed synergism interactions with PMZ and CPZ, as well as the oxygen salt (KOCN) showed synergism effect with TZ. The compounds **EDA1**, **EDA10,** and **EDA11** with low IC_{50} values showed additive (CI=1) or antagonistic (CI>1) interactions with the phenothiazines, the exception of **EDA9**, that exhibited synergism with CPZ and TZ.

DISCUSSION

The resistance among microbes to antimicrobials, in cancer cells towards chemotherapeutic drugs has emerged and created public health threads worldwide. The overexpression of EPs as an important resistance mechanism enables the cells to extrude several toxic agents. EPIs might be therapeutic options that may help to overcome MDR. EPIs could

be naturally-occurring bioactive agents, synthetic agents, and synergistic modulators. Novel compounds and „old" drugs may be used as antibacterial/antitumor adjuvants in combonation chemotherapy, as well as clinically approved drugs with well-known pharmacological profile may be considered as potential agents with new uses that are different compared to the original medical indication, according to the drug repurposing strategy.

The main goal of our study was to evaluate the MDR reversing effects of clinically approved phenothiazines and novel synthetic selenocompounds in different bacterial and tumor models.

1. Consideration of stress response in the application of bacterial EPIs

The virulence and adaptation of bacteria to the environmental conditions depend on the stress response induced by different factors, such as reduced nutrient source and starvation, pH, low and high osmolarity. The survival and colonization of enteric bacteria depend on extreme pH tolerance. In addition it has been described previously that several agents such as antibiotics showed pH-dependent antibacterial activity against certain organisms. Diversity of biological activities of phenothiazines has been highlighted in psychopharmacology and in novel therapeutic indications by several studies.

The role of phenothiazines as EPIs of the AcrAB-TolC pump has been studied at neutral pH under conditions that permit the phenothiazine to affect the activity of the pump, however, these compounds have not been studied previously at acidic pH.

In this study the inhibition of the AcrAB-TolC system of the *Escherichia coli* K-12 AG100 strain was investigated at pH 7 and pH 5 in the presence of the phenothiazine efflux pump inhibitor PMZ.

In the EB accumulation assay the EPI activity of PMZ was less effective at pH 5 compared to pH 7. Based on this result, it can be concluded that the EPI activity of PMZ is pH-dependent because the proton motive force (PMF) provides a more pronounced energy supply for the AcrB pump at acidic pH. At pH 5 the PMF is higher compared to the pH 7, for this reason the EB accumulation was lower at pH 5. Moreover, the expression of *marB, marR, acrA, acrB,* and *soxS* genes was up-regulated at acidic pH. The gene expression of *soxS* exhibited a continuous increase at pH 5, however, at pH 7 it was down-regulated until the end of the culturing period. The *rob* gene required for the initiation of replication has the highest expression rate in the 4th hour of culturing. The over-expression of the EP genes *acrA* and *acrB* at pH 5 and pH 7 indicates the continuous removal of toxic substances by efflux pumps. It can be stated that the acidic pH and PMZ treatment induced a stress response in *E. coli*.

2. Antibacterial activity of symmetrical selenoesters

Previously, novel selenocompounds were studied for antibacterial activity in different bacterial strains. In these studies a cyclic selenoanhydride, as well as symmetrical and non-symmetrical selenoesters were evaluated. The symmetrical compounds showed promising antibacterial effects, for this reason we examined novel second-generation symmetrical selenoesters as antibacterial agents. The antibacterial activity of three groups of selenocompounds such as methylketone selenoesters (**Se-K1, -K2, -K3**), methyloxycarbonylmethyl selenoesters (**Se-E1, -E2, -E3**) and methylcyano selenoesters (**Se-C1, -C2, -C3**) was determined on sensitive and resistant *S. aureus* strains, as well as on *S.* Typhimurium strains.

In case of MIC determination, the symmetrical selenoesters were more effective on Gram-positive *S. aureus* strains compared to the Gram-negative *S.* Typhimurium bacterial strains. This may suggest that these symmetrical Se-compounds are more active against Gram-positives than against Gram-negatives. In the resistance modulation assay all compounds were able to modulate the activity of tetracycline against *S. aureus* MRSA. The methylketone selenoesters **Se-K1**, **Se-K2**, and **Se-K3** were the most potent antibacterials on reference *S. aureus*. In contrast, the methyloxycarbonyl selenoesters **Se-E1**, **Se-E2**, and **Se-E3** and the cyano selenoesters **Se-C1** and **Se-C2** showed strong resistance modulating activity with tetracycline against the *S. aureus* resistant MRSA strain. In case of real-time EB accumulation assay the intracellular EB concentration was the highest in the Δ*tolC* mutant *S.* Typhimurium SE39 and the lowest accumulation was obtained in the wild type *S.* Typhimurium SE01 in the presence of methyloxycarbonyl selenoester **Se-E2**. This compound significantly increased the EB accumulation in the efflux pump gene inactivated Δ*acrA*, Δ*acrB*, Δ*tolC* mutant *S.* Typhimurium strains due to efflux independent mechanisms, e.g. membrane destabilizing effect. Moreover, methyloxycarbonyl selenoester **Se-E3** showed significantly effective EP inhibition on sensitive (p <0.001) and resistant (p = 0.001) *S. aureus* strains. Regarding the anti-biofilm effect, the methyloxycarbonyl selenoester **Se-E3** showed significant biofilm inhibition on both sensitive and resistant *S. aureus* strains. Furthermore, the methylketone selenoester **Se-K3** was the most effective anti-biofilm agent on the MRSA strain. In addition, **Se-K1** was also remarkable as it showed a biofilm inhibiting effect higher than 50% against MRSA. It is surprising that **Se-K2** promoted the biofilm formation of *S. aureus* MRSA, because it has the same chemical formula as **Se-K1** they only differ in the substitution pattern at the phenyl ring. In the case of the methyloxycarbonyl selenoesters, only the trisubstituted derivative **Se-E3** was capable to inhibit significantly the biofilm formation in *S. aureus* strains. According to QS assay the methylketone

selenoester **Se-K1** and **Se-K2** and the methyloxycarbonyl selenoester **Se-E1** were potent QS-inhibitors, **Se-E1** being the most effective inhibitor out of these three derivatives. It can be concluded that the symmetrical selenoesters have a potent antibacterial activity, mainly against *S. aureus* strains. The most potent derivatives were the methylketone selenoesters, followed by the cyano selenoesters and at the end by the methyloxycarbonyl selenoesters.

3. Interaction of selenocompounds with phenothiazines

As mentioned previously, the antibacterial effects of these selenocompounds were described in previous studies. Moreover, these compounds were studied formerly for anticancer and EP inhibitory activity in different tumor cells. The activity of Se-compounds in combination with a selection of anticancer drugs (vincristine, doxorubicin, cyclophosphamide, and methotrexate) and EP inhibitor verapamil has been studied on MDR mouse T-lymphoma cell line. According to the results some selenocompounds were highly effective adjuvants with previously mentioned chemotherapeutic agents. As a continuation of these antecedents, one cyclic selenoanhydride (**EDA1)**, four symmetric selenoesters (**EDA2-EDA5**), six non-symmetric selenoesters (**EDA6-EDA11**), as well as four reference compounds (**EDA12-EDA15**) were investigated in combination with three phenothiazines such as PMZ, TZ, and CPZ. Regarding the selectivity index (SI) the cyclic selenoanhydride **EDA1** showed the strongest selectivity, although it showed no significant effects in combination assay. In contrast, the phthalic anhydride that is the oxygen isostere of compound **EDA1** showed moderate synergism with PMZ and CPZ. In addition, the symmetrical selenoesters **EDA2** and **EDA5**, which contain two selenium atoms were synergistic with all three phenothiazines, however only the thiophene-derivative **EDA2** exhibited synergism with TZ. Regarding our results, TZ showed lower CI values in a lower concentration range compared to PMZ and CPZ, however, the presence of the chlorine atom in position 2 of CPZ was previously shown to enhance its biological activities, therefore it is not surprising that this compound showed more potent activity in our assays than the parental compound phenothiazine.

According to the results obtained in the present work, Se-compounds have the capacity to reverse multidrug resistance in both tumor cells and bacterial strains. Selenocompounds may be a noteworthy new class of potential adjuvants in antibacterial and anticancer therapy. Furthermore, phenothiazines are all already approved drugs with known pharmacological and toxicity profiles, therefore, their use as adjuvants in cancer may be considered as a potential useful approach as suggested by the drug repurposing strategy. However, based on the results of the bacterial response to the environmental factor, it is worth considering environmental

conditions for more effective therapy. It should be emphasized that these studies are preliminary and further research needs to be conducted for the more-in-depth exploration of the potential applications of selenocompounds and their derivatives. In addition, EPI compounds can influence virulence factors, and they should be studied using different bacterial model systems imitating the environmental conditions present in the host organism.

NEW FINDINGS

1. **Consideration of stress response in the application of bacterial EPIs**
   - The efflux pump inhibiting (EPI) activity of promethazine was less effective at pH 5 compared to pH 7 on *Escherichia coli* K-12 AG100 bacterial strain. It can be concluded that the efflux pump inhibiting activity of promethazine is pH-dependent.
   - The acidic pH and promethazine treatment induced a significant stress response in *E. coli*. For this reason, the expression of efflux pump genes (*acrA*, *acrB*) and their regulators (*marB*, *marR*), as well as stress gene (*soxS*) was up-regulated at pH 5 compared to the pH 7.
2. **Antibacterial activity of symmetrical selenoesters**
   - Symmetrical methylketone selenoesters (**Se-K1, -K2, -K3**), methyloxycarbonylmethyl selenoesters (**Se-E1, -E2, -E3**), and methylcyano selenoesters (**Se-C1, -C2, -C3**) have effective antibacterial activity on Gram-positive bacteria such as sensitive and resistant *S. aureus* strains.
   - The methyloxycarbonyl selenoesters **Se-E1**, **Se-E2**, and **Se-E3**, as well as the cyano selenoesters **Se-C1** and **Se-C2** were strong resistance modulators in combination with tetracycline against the methicillin resistant *S. aureus* strain.
   - The selenoesters were more effective efflux pump inhibitors on the Δ*tolC* mutant *Salmonella* Typhimurium SE39 strain compared to its wild-type counterpart. Noteworthy efflux pump inhibition was demonstrated in presence of methyloxycarbonyl selenoester **Se-E2** on Δ*acrA*, Δ*acrB*, Δ*tolC* mutant *S.* Typhimurium strains. In addition, the methyloxycarbonyl selenoester **Se-E3** presented effective efflux pump inhibition on sensitive and resistant *S. aureus* strains.
   - The methyloxycarbonyl selenoester **Se-E3** possessed significant anti-biofilm effect on sensitive and resistant *S. aureus* strains. Furthermore, the methylketone selenoester **Se-K3** had the strongest anti-biofilm effect on methicillin resistant *S. aureus* strain.

- The methylketone selenoester **Se-K1** and **Se-K2** and the methyloxycarbonyl selenoester **Se-E1** were able to inhibit the bacterial quorum sensing system, being **Se-E1** the most effective inhibitor.

3. Interaction of selenocompounds with phenothiazines

- The symmetrical selenoesters, the thiophene-derivative **EDA2** and the benzene-derivative **EDA5** exerted synergistic interaction with all three phenothiazines (promethazine, chlorpromazine, thioridazine) on multidrug-resistant (ABCB1-overexpressing) mouse T-lymphoma cells. The strongest synergism was observed in the case of the thiophene-derivative **EDA2**.

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PUBLICATIONS RELATED TO THE THESIS

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