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

Ph.D. Thesis

Márta Bozóki-Nové

Supervisor: Gabriella Spengler, Ph.D.

Department of Medical Microbiology

Albert Szent-Györgyi Health Center Faculty of Medicine

University of Szeged

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CONTENTS

PUBLICATIONS

1. Publications related to the thesis

I. **Nové Márta**, Kincses Annamária, Molnár József, Amaral Leonard, Spengler Gabriella. The Role of Efflux Pumps and Environmental pH in Bacterial Multidrug Resistance. *In Vivo.* **34**:65-71, 2020.

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II. **Márta Nové,** Annamária Kincses, Beatrix Szalontai, Bálint Rácz, Jessica M A Blair, Ana González-Prádena, Miguel Benito-Lama, Enrique Domínguez-Álvarez, Gabriella Spengler. Biofilm Eradication by Symmetrical Selenoesters for Food-Borne Pathogens. *Microorganisms* **8** (4) 566, 2020.

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III. Mário Gajdács, **Márta Nové**, Ákos Csonka, Borisz Varga, Carmen Sanmartin, Enrique Domínguez-Álvarez, Gabriella Spengler. Phenothiazines and Selenocompounds: A Potential Novel Combination Therapy of Multidrug Resistant Cancer. *Anticancer Res.* **40**(9):4921-4928, 2020.

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2. Publications not related to the thesis

I. Mottaghipisheh J, **Nové M**, Spengler G, Kúsz N, Hohmann J, Csupor D. Antiproliferative and cytotoxic activities of furocoumarins of *Ducrosia anethifolia. Pharm. Biol.* **56**(1): 658–664, 2018.

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II. Mouwakeh A, Kincses A, **Nové M**, Mosolygó T, Mohácsi-Farkas Cs, Kiskó G, Spengler G. Nigella sativa essential oil and its bioactive compounds as resistance modifiers against *Staphylococcus aureus*. *Phytotherapy Res*. **33**: 1010-18, 2019.

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VIII. Savchenko RG, **Nové M**, Spengler G, Hunyadi A, Parfenova LV. *In vitro* adjuvantantitumor activity of various classes of semi-synthetic poststerone derivatives. *Bioorg Chem.* **106**:104485, 2021.

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ABBREVIATIONS

ABC	ATP-binding cassette family
ABCB1	ATP-binding cassette subfamily B member 1
AHL	*N*-acyl-homoserine lactone
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazone
CI	combination index
CIP	ciprofloxacin
CV	crystal violet
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EB	ethidium bromide
ECM	extracellular matrix
eDNA	extracellular DNA
EP	efflux pump
EPI	efflux pump inhibitor
EPS	exopolysaccharides
EST	eukaryotic sterol transporter
FAR	fluorescence activity ratio
FDA	Food and Drug Administration
HAE	hydrophobe/amphiphile efflux
HME	heavy metal efflux
IR	infrared spectroscopy
LB	Luria-Bertani
MATE	multidrug and toxic compound extrusion family
MAR	multiple antibiotic resistance
MDR	multidrug resistant
MFS	major facilitator superfamily
MH	Mueller Hinton
MIC	minimum inhibitory concentration
MRP	multidrug resistance-associated protein
MRSA	methicillin resistant *Staphylococcus aureus*

MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBD	nucleotide-binding domain
NFE	nodulation factor exporter family
NMR	nuclear magnetic resonance
NSC	normal stem cell
OD	optical density
OM	outer membrane
PACE	proteobacterial antimicrobial compound efflux family
PAR	parental
PBS	phosphate buffered saline
PMF	proton motive force
PMZ	promethazine
QS	quorum sensing
RF	relative fluorescence
RFI	relative fluorescence index
RNA	ribonucleic acid
RND	resistance-nodulation-division family
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
Se	selenium
SI	selectivity index
SMR	small multidrug resistance family
TET	tetracycline
TMD	transmembrane domain
TSB	tryptic soy broth

INTRODUCTION

1. Drug resistance

Drug resistance is a natural process and is based on the interaction of organisms with their environment [1]. 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 (bacteria, fungi, viruses, and parasites) [2]. The multidrug resistant (MDR) phenotype is the consequence of selection pressure resulting from the widespread and inappropriate use of antimicrobials (antiseptics, antibiotics, antifungals, and anti-helminthics) [3, 4]. Furthermore, there are less therapeutic options because of the lack of new drug development by the pharmaceutical industry [5]. Accordingly, it is not surprising that the successful clinical use of therapeutic agents are compromised by the potential development of resistance [6]. Drug resistance causes reduction in effectiveness of a medication to cure infectious diseases [7, 8]. In spite of the fact that the development of resistance is a natural phenomenon, extensive rise in the number of immunocompromised conditions (e.g., HIV-infection, burn and diabetic patients) makes the human body more susceptible to nosocomial infections, thereby contributing to further spread of MDR [2]. As a result of the rapid emergence of MDR bacterial strains in public health, the Centers for Disease Control and Prevention (CDC) has classified a number of bacterial strains as serious and concerning threats [5]. Pathogens are categorized into three priority groups based on their resistance by World Health Organization (WHO). Based on this priority list Gram-negative bacteria (e.g., *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) are the most dominant because they can lead to infections that are untreatable with most available antibiotics [9].

In addition to antimicrobial MDR, similar resistance mechanism has been found to occur in cancer cells which also makes clinical treatments difficult [10]. Despite the efforts and successes made in cancer treatment, resistance to anticancer drugs is one of the most crucial challenges in tumor therapy [11]. Similarly to the antimicrobial drug resistance, the ability of cancer cells to resist a wide spectrum of structurally unrelated anticancer drugs is referred to as cancer MDR [12]. MDR in tumors is a complex process that arises from the innate (intrinsic) and/or acquired (extrinsic) ability of tumor cells to reduce drug absorption, thereby eliminating the effects of antitumor agents [12, 13]. Intrinsic resistance (primary) exists before drug treatment by innate molecular properties of tumor cells while the acquired resistance (secondary) is induced after prolonged tumor therapy [14]. Consequently, both intrinsic and

acquired resistance is responsible for most relapses in cancer [11]. Generally, cancer MDR is accountable for over 90% of deaths in tumor patients receiving traditional chemotherapeutic agents and/or novel targeted drugs [15]. In present clinical practice, anticancer drug resistance can only be recognized during the course of treatment, for this reason regular diagnostic monitoring is needed [16].

Regarding the resistance crisis coordinated efforts are much needed to implement new policies and renew research strategies [5]. Moreover, more information is required regarding the mechanisms of drug resistance to ensure effective clinical treatment and achieve better outcomes [11].

2. Resistance mechanisms

There are several mechanisms of drug resistance that are detected in bacteria and cancer cells as well. Because of the overuse of antibiotics, microorganisms have developed multiple mechanisms of resistance towards antibiotics [6]. Concerning the antibiotic resistance of bacteria, there are natural (intrinsic) and acquired (extrinsic) resistance mechanisms. In the former case, the cells can be considered to be resistant even before encountering the harmful agents and this resistance is a trait of a species or genus. In contrast, the acquired resistance arises from the mutations and horizontal gene transfer [1, 17]. The bacterium often acquires multiple resistance through resistance genes which can be carried by mobile elements [18, 19]. Over time bacteria have developed many different resistance strategies, which can be classified in two categories: **1)** preventing antibiotics from reaching their targets: **a)** destruction and/or modification of the toxic agents, **b)** reduction of membrane permeability and **c)** removal of antibiotics by efflux pumps (EPs). **2)** Modification or bypassing the target of the antibiotics: **a)** modification of the target, **b)** expression of alternative proteins (**Figure 1**) [20, 21].

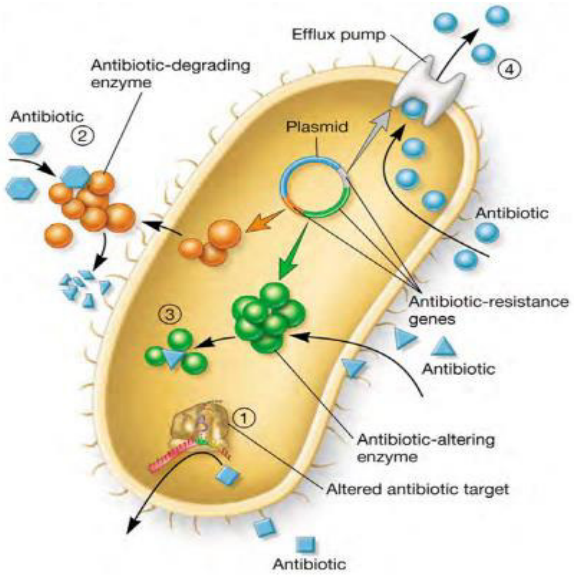


Figure 1 Mechanisms of antimicrobial resistance in bacteria [17]

Comparable to antibiotic resistance in bacteria MDR of cancer cells has also developed by different mechanisms during chemotherapy leading to reduction of the therapeutic efficacy of administered drugs [22]. Resistance

mechanisms of cancer cells include: **a)** inactivation of the drug, **b)** overexpression of EP proteins, **c)** inhibition of cell death, **d)** alteration in the drug metabolism and/or drug targets, **e)** target gene amplification, **f)** enhanced DNA-repair, **g)** epigenetic changes and **h)** drug compartmentalization (**Figure 2**) [23]. Furthermore, it is not uncommon in case of tumor cells to be resistant by multiple mechanisms present at once [15, 24].

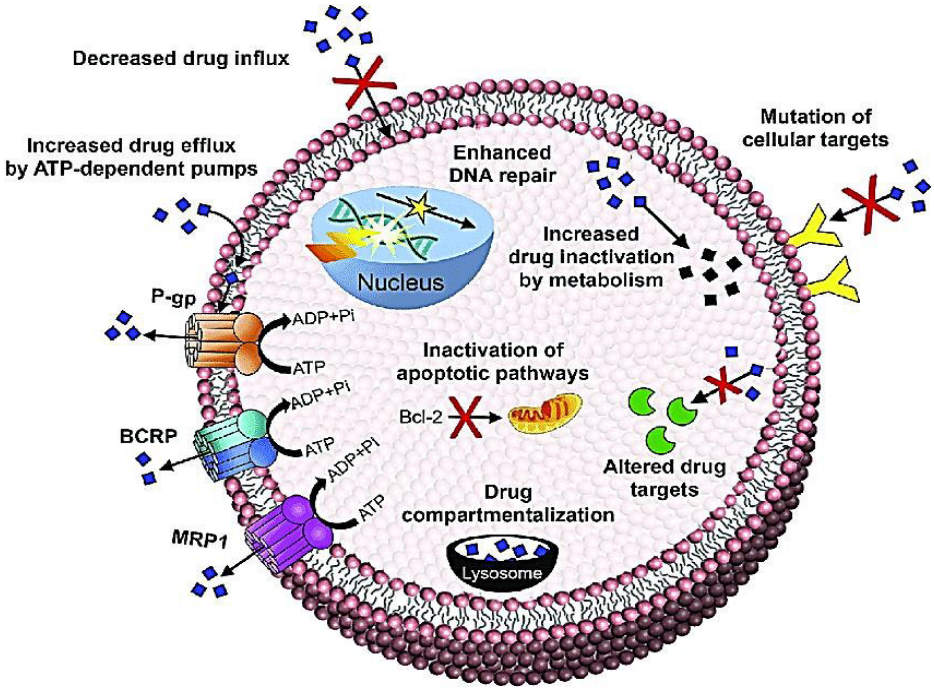


Figure 2 Resistance mechanisms in cancer cells [23]

Some resistance mechanisms are disease-specific, however, the phenomenon of increased drug efflux is observed in bacteria and tumor cells as well. This resistance mechanism is due to the reduced drug accumulation which is a consequence of overexpressed EPs [10].

3. Efflux pump-mediated resistance in prokaryotes

3.1. Efflux transporters and their physiological role

Bacterial EPs have the capacity to regulate the internal environment by removing harmful agents (e.g., antibiotics, toxic bile salts), metabolites as well as cell-cell communication (quorum sensing) signal molecules [25]. Expression of these EP proteins is subject to tight regulation by different local and global transcriptional regulators suggesting that drug EPs have essential physiological functions. Concerning the physiological functions, EPs are especially important in stress adaptation, pathogenesis, and virulence of bacteria [26]. Some EPs are expressed constitutively, whereas others are induced or overexpressed under environmental

stimuli [27]. Regarding the functions of EPs, it is reasonable to speculate that improper overexpression of pumps may cause unwanted efflux of metabolites and other signaling molecules, resulting in deleterious effects on cells physiology [26].

Nevertheless, efflux mechanisms are widely recognized as key components of drug resistance [28]. EPs are found in almost all Gram-positive and Gram-negative bacterial species. There are six EP families based on their membrane topology, energy coupling mechanisms as well as substrate specificities. These EP families are as follows: ATP-binding cassette (ABC) family, multidrug and toxic compound extrusion (MATE) family, small multidrug resistance (SMR) family, major facilitator superfamily (MFS), resistance nodulation division (RND) family and proteobacterial antimicrobial compound efflux (PACE) family [29, 30]. Gram-positive bacteria mainly express the members of the MATE and MFS families, whereas RND-type pumps have a great impact on the efflux mechanisms of Gram-negatives [27].

The members of the RND family obtain their energy using the proton motive force (PMF) and these pumps remove multiple, structurally distinct classes of antimicrobials from inside of the bacterial cells contributing to the development of MDR. Various resistance mechanisms rely on the intrinsic (innate) resistance conferred by RND-related efflux [31]. The RND pump connects the inner and outer membranes throughout the entire periplasm [32, 33]. The AcrAB-TolC is a tripartite multidrug EP system and constitutively expressed in *Escherichia coli* [34, 35]. The AcrAB-TolC is comprised of the RND type AcrB pump, the outer membrane protein TolC and the periplasmic adaptor protein AcrA [31]. The AcrB transporter recognizes and binds the toxic agent and releases it to the TolC channel to which it is connected. The AcrB is attached to the plasma membrane *via* the two fusion proteins whose peristaltic action drives water through the transporter and TolC channel, ultimately releasing the agent to the environment at the junction of TolC to the outer membrane (**Figure 3**) [26, 36-38]. The housekeeping AcrAB-TolC system serves as a major antibiotic resistance determinant and plays the key role in the MDR phenotype of *E. coli* clinical isolates [39].

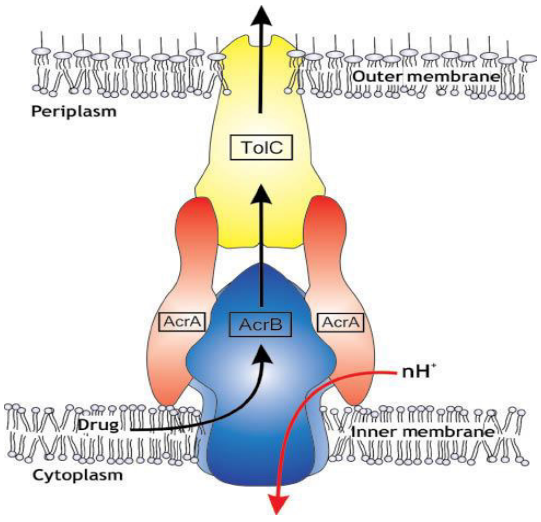


Figure 3 Structure of the AcrAB-TolC efflux pump system [32]

3.2. Connection between efflux pumps and other virulence determinants

EP systems are directly and indirectly related to other virulence determinants, thereby contribute to bacterial pathogenesis. For example, the pump systems are indirectly related to quorum sensing (QS) responses and biofilm formation [26]. Many Gram-negative and Gram-positive bacteria use the QS cell-cell communication system to coordinate the population density-dependent gene expression pattern [40-43]. QS involves the production, detection, as well as response to extracellular signaling autoinducer molecules such as oligopeptides, *N*-acyl homoserine lactones (AHLs), furanosyl borate diester, 3-hydroxy palmitic acid methyl ester (3-hydroxy PAME) and cis-11-methyl-2-dodecenoic acid [44-46]. These molecules passively diffuse through the cell wall and accumulate in the environment in case of increasing bacterial population density and bacteria can monitor this information and react accordingly [44]. Martinez *et al.* showed that EPs facilitate the shut-down of QS response by increasing the efflux of autoinducers and/or their precursors, thereby enabling the bacteria to quickly respond to environmental changes [47]. QS plays a major role in biofilm development and the bacterial community embedded in biofilm responds poorly to antibiotic treatment [48].

Biofilms are immobile microbial communities which adhere and grow on biotic or abiotic surfaces. Microbes produce an extracellular matrix (ECM) which consists of exopolysaccharides (EPS), extracellular DNA (eDNA), proteins, and amyloidogenic proteins. Cells of biofilm are embedded in ECM [49, 50]. Biofilm producing bacteria can cause serious problems regarding the antibiotic therapy, because many microbes can form biofilms on tissues, different surfaces, and artificial devices (e. g. catheters, contact lenses, implants) and these bacterial populations are resistant to antibiotics [51]. In addition, these bacteria are more resistant to antibiotics than their free-living, planktonic counterparts [52]. *S. aureus* and *Salmonella enterica* serovar Typhimurium are food-borne pathogens which are able to form biofilms on various surfaces [53-55]. Regarding the effects of EP systems on biofilm, many studies have evidenced to show that defect in efflux activity leads to impaired biofilm production. Baugh *et al.* showed that in *Salmonella enterica* serovar Typhimurium, genetic inactivation of any EP or chemical inhibition of the efflux activity by efflux pump inhibitors (EPIs) results in compromised ability of *Salmonella* to develop biofilm [56]. Moreover, they also demonstrated that EPIs known to be active against *Salmonella* also effectively prevented biofilm formation in other species, including *E. coli* and *S. aureus* [57]. These studies suggested a promising anti-biofilm strategy *via* inhibition of efflux activity.

4. Efflux pump-mediated resistance in eukaryotes

4.1. ABC transporters and their physiological role

ABC transporters are present in all living species from *Archaea* to *Homo sapiens* [58]. ABC transporters have physiological roles, however, they can also contribute to drug resistance [10]. Under physiological conditions, ABC transporters can be found in almost all tissues of the human body, and they are essential for the healthy functioning of barrier systems such as blood-brain, blood-testes, blood-thymus as well as the placenta, bronchi, sweat glands, intestinal absorption and the proximal tubules of the kidney [59, 60]. These transporter proteins are vital for the defense of healthy cells against harmful compounds, and they also eliminate the toxic endogenous metabolites as well as xenobiotics (e.g., chemical agents, medicines) [61]. Nevertheless, ABC transporters provide advantage to tumors in terms of their proliferation, metastasis, avoidance of apoptotic stimuli, and maintenance of poorly differentiated cell populations. The association of ABC proteins' expression with tumor aggressiveness has for most part been linked to their drug-efflux ability, leading to drug resistance [62]. The chemoresistance was first reported by Keld Dano as the active outward transport of Vinca alkaloids (vincristine and vinblastine) and anthracyclines (daunomycin) presumably via ABCB1 transporter from murine Ehrlich ascites tumor cells [61, 63]. The ABC superfamily includes seven subfamilies, that involves many transporter proteins. Three members of subfamilies, such as multidrug resistance protein 1 (MDR1, P-glycoprotein, P-gp or ABCB1), multidrug resistance-associated protein 1 (MRP1 or ABCC1) and breast cancer resistance protein (BCRP or ABCG2) play an essential role in cancer drug resistance [10, 61, 64]. It is important to mention a small population of cancer cells, cancer stem cells (CSCs) that are able to self-renew and differentiate similar to normal stem cells (NSCs). Moreover these cells are relatively quiescent and they have a slow cycling rate so CSCs are protected against chemotherapeutics that target rapidly dividing cells. In addition the tumor cells and the CSCs have the ability to overexpress the ABC transporter proteins such as ABCB1 [65]. The ABCB1 consists of two homologous halves, each containing a variable transmembrane domain (TMD) and a highly conserved nucleotide-binding domain (NBD).

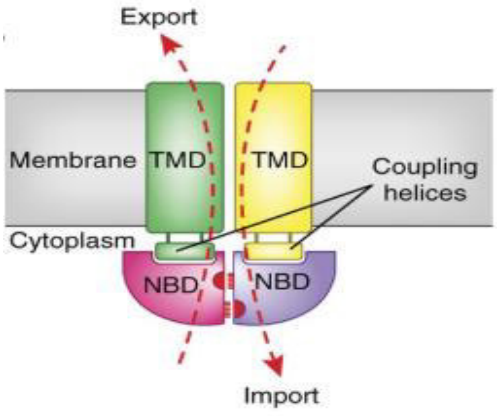


Figure 4 Schematic representation of ABC transporter structure [69]

While the TMD takes part in drug recognition and transport, the NBD is able to bind and hydrolyze ATP, that drives transport (**Figure 4**) [66-69]. ABCB1 is a mediator of drug efflux-based multidrug resistance in many cancers [67, 70].

5. Strategies of efflux inhibition

5.1. Efflux inhibition in bacteria

The inhibition of efflux mechanisms is a promising approach to increase the intracellular drug concentration and to restore the activity of drugs against the resistant strains, in addition it minimizes the further development of resistant strains [71]. There are three major strategies to overcome drug resistance by efflux mechanisms [72]. These strategies are as follows:

a) *Bypassing efflux mechanisms:*

In case of structural analogues of an antibiotic family, differences can be observed in transport processes [72]. Considering this fact, the newer agents developed from the main antibiotic classes are less susceptible to efflux compared to the older ones (e.g., third and fourth generation quinolones versus first and second generation quinolones) [73]. Consequently, optimizing the structure of a molecule within an antibiotic class could be an effective strategy [72].

b) *Biological inhibition of efflux:*

In case of this approach, EPs are inhibited by blocking the pump proteins, using neutralizing antibodies, or adequate genes by antisense oligonucleotides. In the latter case, antisense oligonucleotides or small interfering RNA or other nontraditional antisense molecules can interfere with the transcription and/or the translation [72].

c) *Pharmacological inhibition of efflux:*

This strategy involves the development of EPIs, which are intended for combined therapy with specific antibiotics [74, 75]. EPIs can inhibit the EPs by many different mechanisms [76]. These EPIs and their mode of action will be explained more in-depth in the next chapter.

5.2. Efflux inhibition in cancer

A potential way to overcome MDR in tumor cells and CSCs would be to target the function of ABC transporters. Regarding the inhibition of transporters many different approaches can be distinguished [65]. These strategies are as follows: **a)** inhibition by

competitive and non-competitive modulators, **b)** inhibition mediated by nanoparticle targeting, **c)** targeting transcriptional regulation of ABC transporters, **d)** small interfering RNA (siRNA) and microRNA (miRNA) therapeutics, **e)** targeting signaling pathways involved in the regulation of ABC transporters, **f)** inhibition using monoclonal antibodies (e.g., MRK16), **g)** design of new chemotherapeutics which are non-substrates for ABC transporters (e.g., tyrosine kinase inhibitors) and **h)** combinational targeting with CSC targeting agents and transporter modulating drugs or dual targeting with a single agent [65, 77, 78].

The first strategy involves designed or natural EPIs, including competitive and non-competitive inhibitors. Competitive inhibitors of ABC transporters exert their function by tightly binding and blocking the substrate binding pockets, while non-competitive inhibitors exert their function by binding to a non-substrate binding site thereby inhibiting the ATPase activity or modulating transporters function allosterically [77].

ABCB1 is a potential target to overcome MDR in cancer, however specific inhibition of this transporter is a major challenge due to its large binding pocket with low substrate specificity which enables ABCB1 to interact with many various known substrates (e.g., vinca alkaloids, taxanes) [77, 79].

6. Efflux pump inhibitors and their mode of action

In view of the fact that the most of the MDR pathogens express or overexpress EPs that are responsible for the extrusion of toxic agents such as antibiotics from the inside out, EPIs can interfere with EPs at different levels [74]. For this approach, the understanding of the structural and physiological mechanisms of the EPs is crucial. The abolishment of efflux mechanisms could be achieved by different ways: **1)** downregulation of the expression of EP genes **2)** re-design of antibiotics and development of new antibiotics **3)** inhibition of the assembly of functional pumps, **4)** inhibition of the substrate binding by competitive or non-competitive inhibitors, **5)** blocking the outer membrane channel as well as **6)** interference with the energy supply of pumps [30, 80, 81].

Classification of EPIs is a difficult task because some inhibitors are pump specific, while others are not [82, 83]. Based on their origin, microbial and mammalian inhibitors can be classified into two major groups: natural bioactive agents and synthetic compounds.

Microbial EPIs may involve the natural compounds such as berberine from plant species belonging to family *Berberidaceae* which is an antimicrobial alkaloid and known substrate for many MDR pumps (e.g., NorA pump in *Staphylococcus aureus*) [84, 85]. Berberine can be a

outstanding EPI as a hybrid molecule that combines both an antibiotic moiety and an efflux inhibitor (e.g., antimicrobial berberine and 5-nitro-2-phenyindole; INF55 inhibitor) [86]. In addition the synthetic microbial EPIs include the following compounds: **a)** membrane destabilizing agents (e.g., clinically known neuroleptics such as phenothiazines), **b)** selective inhibitors of serotonin re-uptake (e.g., paroxetine), **c)** calcium channel antagonists (e.g., verapamil), **d)** proton pump inhibitors (e.g., omeprazole), **e)** analogues of antimicrobial agents such as tetracyclines, aminoglycosides, and quinolones, **f)** energy uncouplers (e.g., carbonyl cyanide *m*-chlorophenyl hydrazone; CCCP) and **g)** newly designed and synthesized drugs (e.g., selenium-containing compounds) [72, 82]. Difficulties in antimicrobial treatment urges the development of new therapeutic strategies, as well as the discovery of a new generation of safe and effective EPI compounds. In view of this fact, many studies reported the importance of selenium and selenocompounds in several biological processes and its potential applications in infection diseases and cancer [87].

A major class of mammalian EPIs are inhibitors of the ABCB1 transporter that are classified into three generations based on their specificity, affinity, and toxicity. **a)** First generation ABCB1 inhibitors are pharmacologically active substances and some of them are in clinical use (e.g., reserpine) [88, 89]. **b)** Second generation inhibitors (e.g., valspodar, dexverapamil) are chemically modified derivatives or enantiomers of first-generation agents. They have a better pharmacologic profile than the first-generation compounds but these modulators still remained ABCB1 substrates and they significantly inhibit the metabolism and excretion of cytotoxic agents (e.g., substrates for the cytochrome P450 isoenzyme), thus leading to unacceptable toxicity [90]. **c)** Third generation ABCB1 inhibitors are under clinical development, they have higher specificity and lower toxicity compared to the previous generation (e.g., tariquidar) [91]. These compounds may be able to overcome the limitations of second generation modulators [90]. Moreover, it is important to note that many studies have demonstrated that phenothiazine derivatives can be effective ABCB1 inhibitors [92-94].

7. Old drugs versus new drugs

7.1. Phenothiazines as old drugs with new pharmacological effect

Since Paul Ehrlich published methylene blue as the ’poster child’ of heterocyclic phenothiazines in the late 19th century, many phenothiazine derivatives and their beneficial effects have been discovered [95, 96]. Considering their pharmacological profile, phenothiazines have been reported as antiemetic, antipsychotic, antihistaminic, and anticholinergic compounds over the years [96, 97]. In addition, antipsychotic phenothiazines

such as thioridazine (TZ) [98] and chlorpromazine (CPZ) [99] as well as antihistamine phenothiazines (e.g., PMZ) [100] have been applied in clinical practice since the 1940s and 1950s, respectively [101]. Researchers have paid increasing attention to the biological properties of phenothiazines as antiviral, antimalarial, antiplasmid, antibacterial, antitumor, and anti-neurodegenerative compounds. Many studies have demonstrated the ability of phenothiazines as EPIs to reverse multidrug resistance in bacteria [102]. Moreover, phenothiazines are potent anticancer compounds according to former studies [103-106]. Phenothiazines exert their anticancer activities via various mechanisms such as **a)** influence on cell cycle (e.g., PMZ) [107], **b)** induction of apoptosis (e.g., TZ) [108], **c)** interfering with DNA repair mechanisms [109], **d)** inhibition of MDR EPs [110], **e)** inhibition of angiogenesis [111], **f)** generation of reactive oxygen species (ROS) [112] and **g)** interfering of anti-cancer stem cell activity [113]. Phenothiazines may act as sensitizing adjuvants to conventional chemotherapeutic agents as well as radiotherapy [114].

Consequently phenothiazines are widely known and applied in clinical practice, for this reason there are further possibilities regarding drug repositioning/repurposing. In the drug repositioning/repurposing approach the ultimate goal is to find new uses of already existing drugs [115]. The strategy has a significant economic benefit as it can provide a solution to the difficulties caused by the productivity problem of drug discovery by pharmaceutical industry [114]. In addition, drug repurposing is becoming increasingly important due to high failure rates and costs as well as slow pace of *de novo* drug discovery (**Figure 6**) [114, 116, 117].

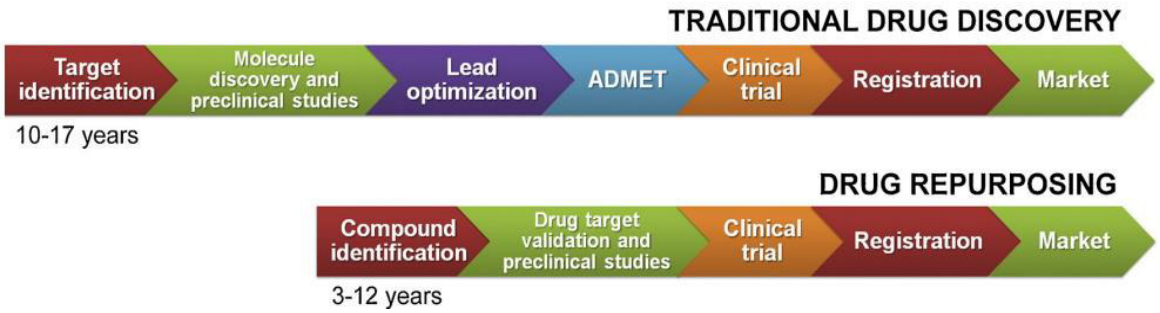


Figure 6 Schematic representation of the steps of traditional drug discovery and drug repurposing strategy [114]

Strategy of repositioning excludes the structural modification of the drugs, it implies a new indication of the biological properties such as formulation, a new dose or via a new route of administration, drug repositioning is based on common biological targets in some diseases and on the concept of pleiotropic drugs [115]. Based on previous studies, the use of

phenothiazines may be a promising alternative to overcome the MDR phenotype in cancer by drug repositioning.

7.2. Selenium-containing agents as novel resistance modulators

Selenium (Se) is an essential trace element in living organisms and is crucial for the nutrient supply and energy generation of bacteria [118, 119]. Se exists in inorganic and organic forms and both can be absorbed by the human body, but only organic Se-compounds, usually in the form of amino acids such as selenomethionine and selenocysteine, can be better retained [120, 121]. Se is an antioxidant that regulates cell metabolism, oxidative stress, as well as DNA- and RNA-protein-synthesis [122], however, is important to emphasize that overdoses of Se can be highly toxic. Interestingly, organic Se may have less toxicity compared to inorganic Se [118-120]. Se-containing agents can be effective against cancer, because these can modulate tumor growth, metastasis, angiogenesis, and drug resistance [122]. Several studies have demonstrated that selenium in combination with anticancer agents or other micronutrients (e.g., vitamin E, zinc) showed synergistic effect against cancer cells and various microorganisms [123-125]. Moreover, many studies have described that drug combinations can selectively kill the drug resistant cells, without affecting normal cells [126, 127]. In addition, beyond the anticancer effects, Se-containing compounds also possess antibacterial activity as shown by several studies [128, 129]. Selenoesters and selenoanhydrides exhibited anti-biofilm activity against *S. aureus* and *Salmonella* Typhimurium as described by former studies [130, 131].

Consequently, based on the anticancer and antibacterial effects of Se-containing agents, these compounds could provide alternative and effective scaffolds to overcome MDR in both anticancer and antibacterial therapies.

8. Practical relevance and challenges of efflux pump inhibitors

The possibility of using EPIs against bacterial infections has been in an experimental stage since the beginning of this century. Peptidomimetics, such as the first identified pump inhibitor, MC-207,110 or PAβN (Phe-Arg-β-napthylamide), which is a competitive inhibitor of EPs, are known inhibitors for EPs (such as MexAB pump) belonging to the RND family of *P. aeruginosa* [132]. In the case of this inhibition mechanism, the pumps recognize PAβN as a substrate instead of the target molecules (e.g., ciprofloxacin), while the EPs expel these inhibitors outside the cells, the drug remains intracellular reaching an increased concentration inside of the cells. However, their main disadvantage is their low affinity to the target that requires the use of higher doses for a longer period of time, and this fact is a significant barrier

to their *in vivo* applicability [133]. Furthermore their toxic properties hinder their clinical application and they are used to evaluate the various EP mechanisms expressed by different bacteria *in vitro* [81, 132].

Over the years several compounds have been discovered as bacterial EPIs when used alone or as adjuvants in combination with antibiotics. However, these compounds would have to go through a stringent checklist to fulfill the criteria of successful inhibitors. The requirements are as follows: **1)** the molecule must not be antibacterial, because it can lead to resistance, **2)** the molecule should be selective and not target any eukaryotic EPs, **3)** it should possess ideal pharmacological features: non-toxicity, high therapeutic and safety indices, good ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) profile and serum stability and **4)** the production of the EPI must be economically feasible. Moreover, in case of combination therapy, the pharmacokinetics of the inhibitor and the antibiotic must be precisely adjusted to achieve the desired activity [30, 80].

Nevertheless, it is important to note that bacterial EPIs are not suitable for clinical application in their current form. Despite this fact, specific EPIs can restore the efficacy of known drugs and may also be useful as feed additives to reduce microbes colonization in gastrointestinal tract of animals, thereby reducing infection transmission to humans [102].

Concerning clinical applicability, mammalian EPIs have similar challenges like bacterial EPIs. By identifying ABC transporters and their functions, new possibilities have opened up in cancer treatment. However, ABCB1 inhibitors are less selective and potent, differ in their *in vitro* and *in vivo* properties, and often cause severe side effects, so far no drugs that directly target or inhibit ABCB1 have been accepted for clinical use. It is important to note that the problems associated with NSCs and CSCs in pump inhibition. NSCs express ABC transporters to protect themselves from cytotoxic agents, however CSCs overexpress these pumps. Moreover, the main obstacle to inhibit ABC transporters is the similar properties of NSCs and CSCs, as CSCs are able to regenerate and differentiate like NSCs. As a result, inhibition of ABC transporters may also affect NSCs, causing severe side effects (e.g., hematopoietic disorders due to bone marrow dysfunction). Therefore, it is extremely important to take these difficulties into account when developing pump inhibition strategies. In view of this fact, ixabepilone is a promising inhibitor that leads to G2/M phase arrest by stabilizing microtubules and promoting tubulin polymerization. This compound is not a substrate for the ABCB1 therefore cannot be pumped out. As a result, the goal is currently to develop drugs that are not substrates for ABCB1 [134].

AIMS OF THE STUDY

The MDR phenotype is the consequence of selection pressure resulting from the widespread use of antimicrobials and antibiotics. For this reason, the design and development of new antibacterial agents could provide an alternative strategy to treat infections caused by MDR bacteria. 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 concentrations (MICs) of PMZ by microdilution method
   - **1.2.** Investigation of the EP 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; Δ*acrB*), *S.* Typhimurium SL1344 (SE03; Δ*acrA*) and *S.* Typhimurium SL1344 strain (SE39; Δ*tolC*) 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.

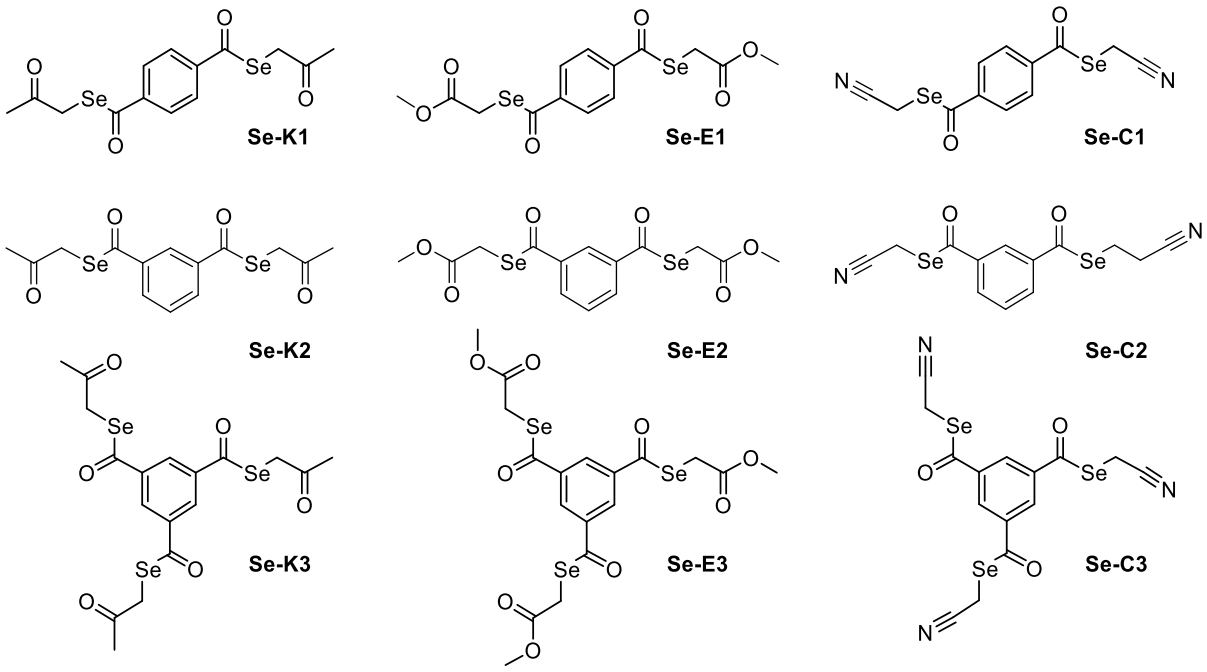
MATERIAL AND METHODS

1. Compounds studied

The selenoompounds 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 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 concentration of DMSO was kept below 1% in all the experiments.

1.1. Symmetrical selenoesters

Nine symmetrical selenodiesters or selenotriesters were synthesized by 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**) (see **Scheme 1** and **Appendix 1**). Their synthesis is described in the patent application EP17382693 [135].



Scheme 1 Chemical structure of the symmetrical selenoesters evaluated

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** (see **Appendix 2** and **Appendix 3**) were performed as described previously [136] to gather the amount of selenocompounds needed for the performance of the assays. Their purity was assessed by elemental analysis that is performed to confirm the structures of the different derivatives as reported previously [137].

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 [138]. 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 (DMEM, containing 4.5 g/L glucose) 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 [139]. 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 [140, 141].

4. Reagents and media

Four Se-containing reference compounds: phthalic anhydride (oxygen-isostere of **EDA1**; Sigma-Aldrich, Seelze, Germany), potassium cyanate (KOCN or oxygen-salt; Chempur, Piekary Śląskie, Poland), ammonium thiocyanate (NH_4SCN or sulfur-salt; Polskie Odczynniki Chemiczne, Gliwice, Poland) and potassium selenocyanate (KSeCN or selenium-salt; Sigma-Aldrich, Seelze, Germany), three phenothiazines: promethazine (PMZ; EGIS), chlorpromazine (CPZ; Sigma-Aldrich, St Louis, MO, USA), thioridazine (TZ; Sigma-Aldrich, St Louis, MO, USA). Phosphate-buffered saline (PBS; pH 7.4), verapamil, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), ethidium bromide (EB), ciprofloxacin-hydrochloride (CIP), tetracycline-hydrochloride (TET), crystal violet (CV), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA), Luria-Bertani (LB) broth and LB agar were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The modified LB agar (LB*) was prepared from bacteriological agar 20 g/L (Difco, Detroit, USA), tryptone 10 g/L, NaCl 10 g/L, yeast extract 5 g/L, K_2HPO_4 1 g/L, $MgSO_4 \times 7H_2O$ 0.3 g/L, and FeNaEDTA 36 mg/L. pH of the agar was adjusted to 7.2. Mueller–Hinton (MH) broth, tryptic soy broth (TSB), and tryptic soy agar was purchased from Scharlau Chemie S.A. (Barcelona, Spain). McCoy's 5A medium (Sigma-Aldrich, St Louis, MO, USA), Dulbecco's Modified Eagle's Medium (DMEM; Gibco Life Technologies Co., UK), fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO, USA), Na-pyruvate (Sigma-Aldrich, St Louis, MO, USA), nystatin (Sigma-Aldrich, St Louis, MO, USA), horse serum (Sigma-Aldrich, St Louis, MO, USA), penicillin-streptomycin (Sigma-Aldrich, St Louis, MO,

USA), L-glutamine (Sigma-Aldrich, St Louis, MO, USA), DMSO (Sigma-Aldrich, St Louis, MO, USA).

5. 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) [142]. MIC values of the compounds were determined by visual inspection. In case of PMZ, the solvent was distilled water. In case of selenocompounds, the solvent was DMSO that was assayed to ensure there was no antibacterial effect at the concentration (1 v/v%) applied in the assays.

6. 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 [143], 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 according to the following formula:

$$RFI = \frac{RF_{treated} - RF_{untreated}}{RF_{untreated}}$$

where $RF_{treated}$ is the relative fluorescence (RF) at the last time point of EB retention curve in the presence of an inhibitor, and $RF_{untreated}$ is the RF at the last time point of the EB retention curve of the untreated control having the solvent control (DMSO). The solvent was distilled water in case of PMZ [144].

7. 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 37ºC 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 −20ºC 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).

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

The relative gene expression levels were determined at pH 5 and pH 7 in the presence and absence of PMZ on *E. coli* AG100 K-12 strain. Bacteria were cultured in LB at pH 5 and pH 7 and total RNA was isolated at various time points (after 0, 1, 2, 4, 8, and 18 hours). The relative expression levels of the efflux pump genes, their regulators and stress genes were determined by RT-qPCR [110, 145] (see **Appendix 6**), using CFX96 Touch real-time PCR detection system (BioRad, Hercules, CA, USA) strictly following the manufacturer's recommendations for the SensiFAST^{TM} SYBR No-ROX One-Step Kit (Bioline GmbH, Luckenwalde, 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. The relative gene expression analysis was calculated according to the following formulas:

$$\Delta C_T = C_{T\,(\text{interested gene})} - C_{T\,(\text{reference gene})}$$

$$\Delta\Delta C_T = \Delta C_{T\,(\text{PMZ treated})} - \Delta C_{T\,(\text{PMZ untreated})}$$

9. 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 assay 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 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 (**Figure 7**).

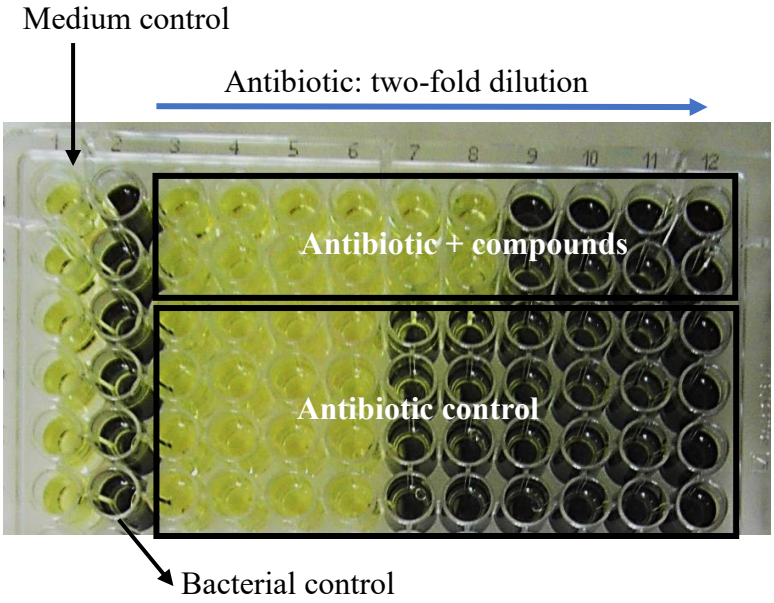


Figure 7 The layout of antibiotic combination plates

10. 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 according to the formula as given in section 6. The positive control was carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) on *S*. Typhimurium strains (50 µM) and *S. aureus* reference strain (6.25 µM), furthermore verapamil (50 µg/ml) was the positive control on *S. aureus* resistance MRSA strain.

11. 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.

12. Quorum sensing (QS) assay

The QS inhibitory effect of selenocompounds 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 as described previously [146]. Filter paper discs (7.0 mm in diameter) were placed between the parallel inoculated strains and impregnated with 10 µL compounds. The concentration of the selenocompounds 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 (**Figure 8**). PMZ was used as a positive control.

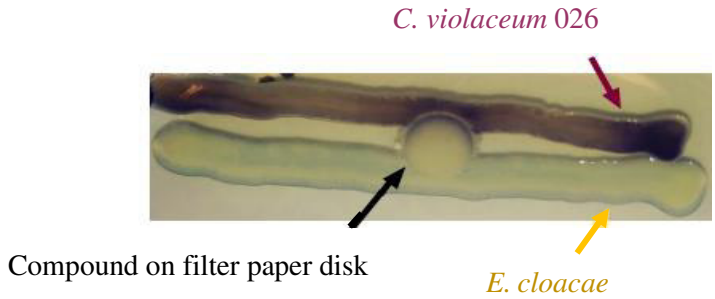


Figure 8 Layout of quorum sensing agar plates

13. 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 [147].

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, 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 sodium dodecyl sulfate (SDS; 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). 50% inhibition of the cell growth (IC_{50}) was determined according to the formula below:

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

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%.

14. 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 (see **Appendix 2** and **Appendix 3**) and phenothiazines (see **Appendix 4**). 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. 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 (**Figure 9**). Optical density (OD) was measured at 540/630 nm with Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA) as described above [148]. 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 [149]. 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 [149].

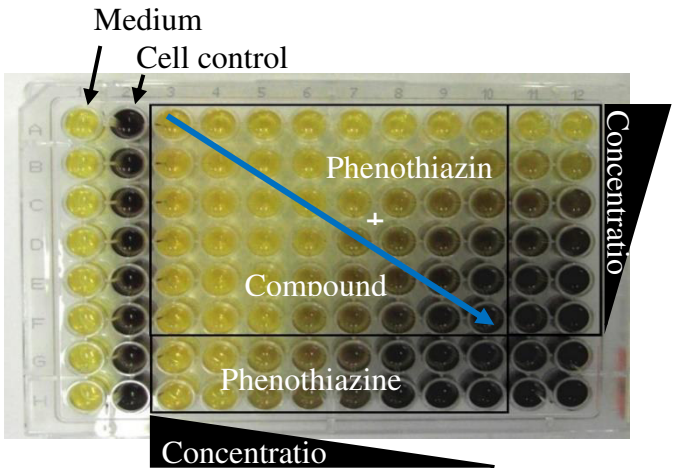


Figure 9 The layout of checkerboard plates

15. 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.

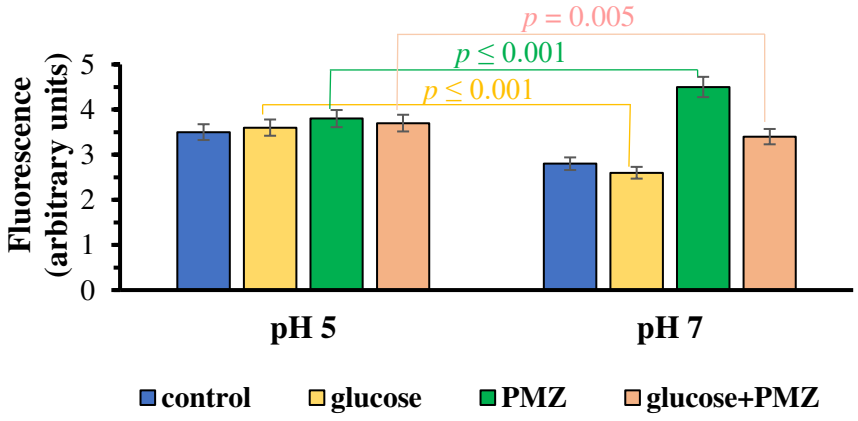
RESULTS

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 activity of the AcrAB-TolC system at neutral pH depends on metabolic energy whereas at pH of 5 no metabolic energy is needed. Moreover, the effect of antibiotics at acidic pH is less pronounced [150]. The effect of pH on the growth of bacteria is well known regarding the stress promoted at acidic conditions [151]. For this reason, efflux pump inhibition should be investigated considering these environmental factors to have a better understanding of the factors influencing the EPI activity.

The effect of PMZ on the real-time accumulation of EB was assessed by an automated EB method in the presence and absence of glucose 0.4%. 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 (**Figure 10**).



	Relative fluorescence index (RFI)		
glucose	0.03	- 0.07	$p \leq 0.001$
PMZ	0.09	0.61	$p \leq 0.001$
glucose+PMZ	0.06	0.21	$p = 0.005$

Figure 10 Accumulation of EB at pH 5 and pH 7 by *E. coli* K-12 AG100 in the presence and absence of glucose 0.4%, with and without 25 µg/ml of PMZ. The correlation is significant: $p \leq 0.001$ and $p = 0.005$

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

The influence of PMZ treatment was examined on the relative expression of the efflux pump genes (*acrA*, *acrB*) and their regulators at neutral and acidic pH. 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 (**Figure 11**).

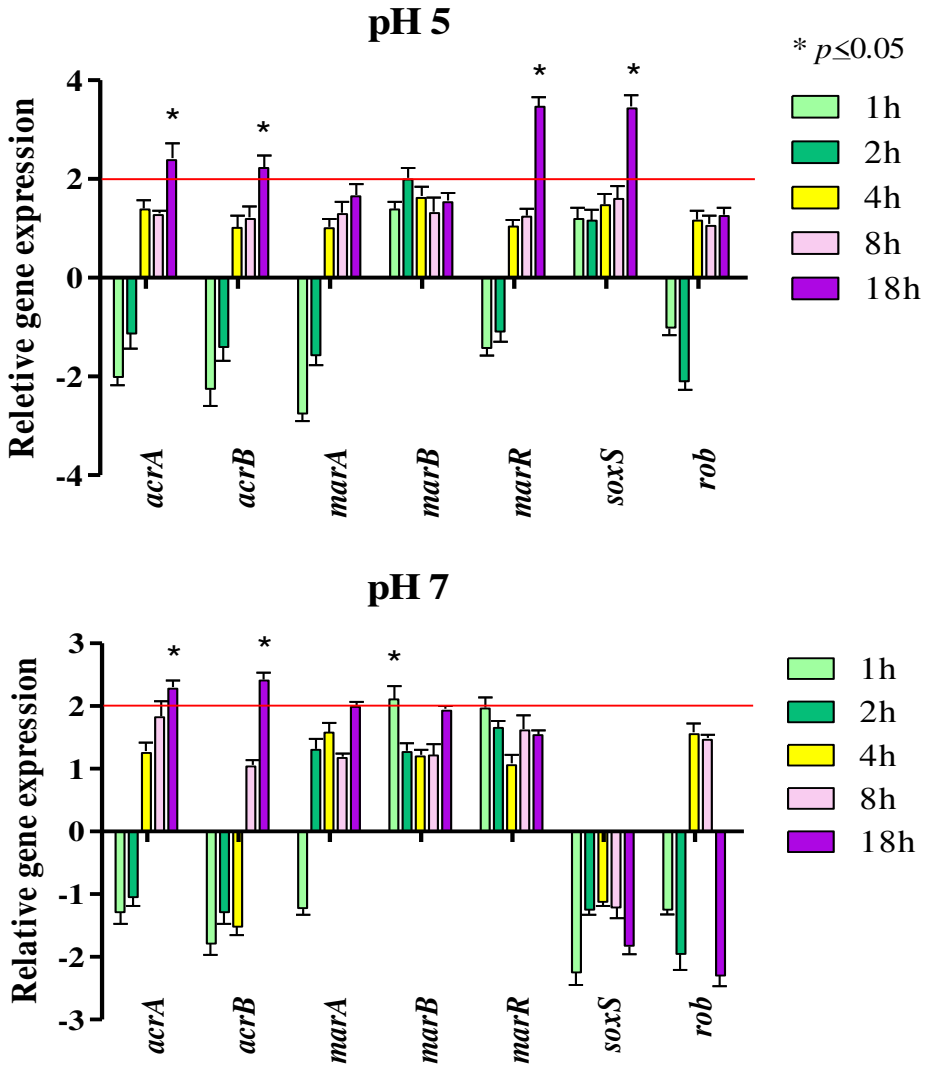


Figure 11 Relative gene expression level of efflux pump genes and their regulators in the presence of 25 µg/ml PMZ at pH 5 and pH 7 on *E. coli* K-12 AG100. The significant correlation is: $p \leq 0.05$.

2. Antibacterial activity of novel symmetrical selenoesters

2.1. Determination of minimum inhibitory concentrations of selenocompounds

It was reported in former studies that selenoesters and selenoanhydrides are bioactive selenium containing compounds with anticancer and MDR reversing effects [137, 152]. Moreover, the antibacterial activity of these derivatives was assessed highlighting their antibacterial and EPI activity against MRSA, *E. coli*, and *Chlamydia trachomatis* serovar D [128]. As a continuation to these studies, novel symmetrial selenoesters were synthesized and evaluated as antibacteria agents.

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. Interestingly, 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 MICs are 16- and 32-fold higher against *S. aureus* ATCC 25923, respectively; with the exception of the trisubstituted derivative **Se-C3**, because its MIC is only 4-fold higher than the MIC of the trisubstituted methylketone **Se-K3**. The same tendency, but accentuated, was observed in *S. aureus* MRSA 272123, where the MIC values of the methylketone derivatives were in the range of 64- to 128-fold lower than the equivalent methyloxycarbonyl derivatives and in the range of 16- to 32-fold lower than the equivalent nitrile-containing selenoesters. 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 (**Table 1**).

	MIC determination (μM)					
Compounds	***S. aureus* ATCC 25923**	***S. aureus* MRSA 272123**	***S.* Typhimurium SE01 wild-type**	***S.* Typhimurium SE02 Δ*acrB***	***S.* Typhimurium SE03 Δ*acrA***	***S.* Typhimurium SE39 Δ*tolC***
Se-K1	0.39	1.56	50	50	50	50
Se-K2	0.39	1.56	50	50	50	100
Se-K3	0.39	0.78	50	25	25	50
Se-E1	12.5	100	>100	>100	>100	>100
Se-E2	12.5	100	>100	>100	>100	>100
Se-E3	12.5	100	>100	>100	>100	>100
Se-C1	6.25	50	25	25	25	25
Se-C2	6.25	50	25	25	25	25
Se-C3	1.56	12.5	12.5	12.5	12.5	25

Table 1. Antibacterial activity of selenocompounds: the MICs were determined on *S. aureus* ATCC 25923, methicillin resistant *S. aureus* 272123 and *S.* Typhimurium strains.

Importantly, the MIC of the efflux pump gene deleted strains remained unchanged compared to the wild-type suggesting that the compounds are not substrates of the AcrAB-TolC efflux pump system.

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

Since the Se-compounds were more effective on *S. aureus* strains, these strains were selected for combination studies with reference antibiotics tetracycline (TET) and ciprofloxacin (CIP). Selenocompound **Se-E3** showed synergism with TET on the methicillin susceptible *S. aureus* ATCC 25923. Surprisingly, 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, as they reduced the MIC value of TET against this MRSA strain to a value 32-fold lower. Additionally, compounds **Se-E1** and **Se-C1** exerted also a noteworthy reduction of the MIC value, of 16-fold in this case. On the other hand, **Se-K1** and **Se-E3** showed synergism with CIP on MRSA strain, achieving a 2-fold reduction of the MIC value (**Table 2**).

Compounds	MIC reduction (μM)			
	S. aureus ATCC 25923 *with*		*S. aureus* MRSA 272123 *with*	
	TET	CIP	TET	CIP
-	**0.88**	**1.06**	**14.06**	**33.99**
Se-K1	0.88	1.06	3.51	16.99
Se-K2	0.88	1.06	7.03	33.99
Se-K3	0.88	1.06	7.03	33.99
Se-E1	0.88	1.06	0.88	33.99
Se-E2	0.88	1.06	1.76	33.99
Se-E3	0.44	1.06	0.44	16.99
Se-C1	0.88	1.06	0.88	33.99
Se-C2	0.88	1.06	0.44	33.99
Se-C3	0.88	1.06	3.51	33.99

Table 2 Resistance modulating effect of selenocompounds (½ MIC) in the presence of antibiotics on *S. aureus* strains. The resistance modulating effect of Se-compounds with ciprofloxacin (CIP) and tetracycline (TET) on the *S. aureus* bacterial strains were evaluated by MIC reduction assay.

2.3. *Anti-biofilm effect of selenocompounds*

The anti-biofilm effect of selenocompounds on biofilm formation of sensitive and resistant *S. aureus* strains and wild-type *S.* Typhimurium SE01 was evaluated. 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 (**Figure 11)**.

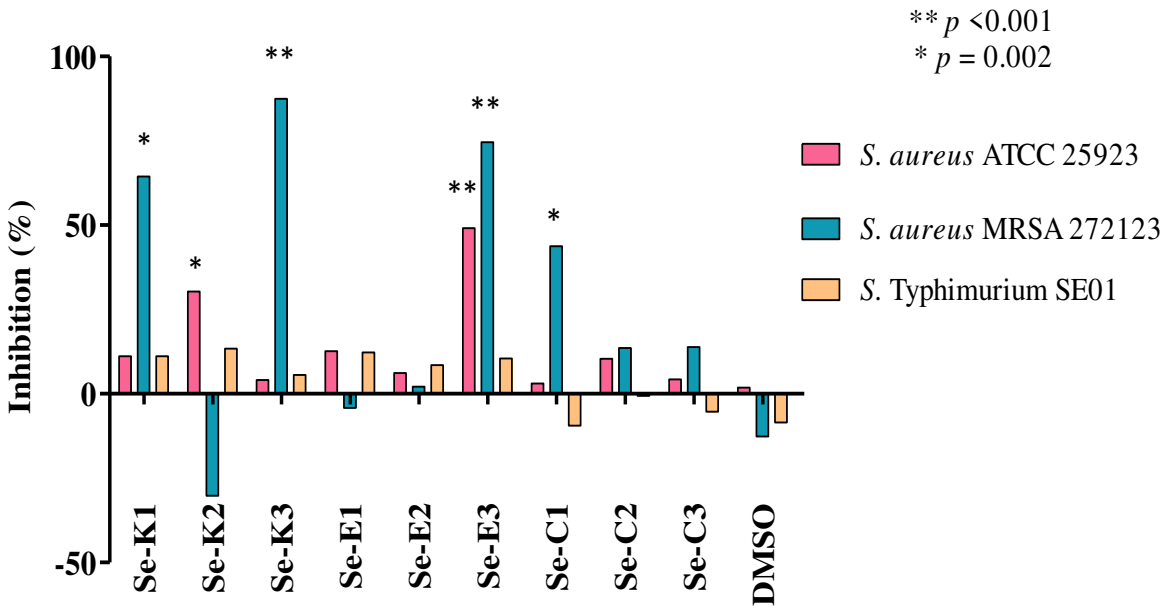


Figure 11 Anti-biofilm effect of Se-compounds (1/2 MIC) on *S.* Typhimurium SE01 wild type and on *S. aureus* ATCC 25923 and MRSA 272123 strains. The concentration of DMSO was kept below 1%. The levels of significance were $p < 0.001$ and $p = 0.002$,

2.4. *Efflux pump inhibition by selenocompounds*

The activity of the selenocompounds on EB accumulation was determined by the automated EB method on sensitive and resistant *S. aureus* and *S.* Typhimurium SE01, -02, -03, -39 strains. The relative fluorescence index was calculated based on the means of relative fluorescence units (RFUs) (**Table 3**).

Relative fluorescence index (RFI)						
Compounds	***S.* Typhimurium SE01 wild type**	***S.* Typhimurium SE02 *ΔacrB***	***S.* Typhimurium SE03 *ΔacrA***	***S.* Typhimurium SE39 *ΔtolC***	***S. aureus* ATCC 25923**	***S. aureus* MRSA 272123**
Se-K1	-0.16	0.10	0.17	0.27	0.1	-0.15
Se-K2	-0.04	0.13	0.20	0.26	0.11	-0.07
Se-K3	-0.20	0.08	0.28	0.44	0.16	-0.18
Se-E1	-0.10	-0.03	0.03	0.15	0.98	0.19
Se-E2	0.09	**0.70**	**0.56**	**0.59**	0.67	0.33
Se-E3	0.26	0.08	0.27	0.25	**4.15**	**0.47**
Se-C1	-0.08	0.06	0.04	0.13	0.14	-0.15
Se-C2	-0.10	0.03	0.09	0.25	0.08	-0.13
Se-C3	-0.07	-0.02	0.08	0.06	0.18	-0.05
CCCP	3.50	2.46	1.81	1.32	0.52	-
Verapamil	-	-	-	-	-	0.32

Table 3 Relative fluorescence indices based on real-time EB accumulation data on *S.* Typhimurium and *S. aureus* strains. Concentration of selenocompounds: ½ MIC, concentration of positive controls: CCCP: 50 μM on *S.* Typhimurium strains and 6.25 μM on *S. aureus* ATCC 25923 strain, verapamil: 50 μg/ml on *S. aureus* MRSA strain. The active compounds are presented in bold.

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 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* strains. In addition, verapamil was applied as reference EPI on *S. aureus* MRSA. 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 (**Figure 12**).

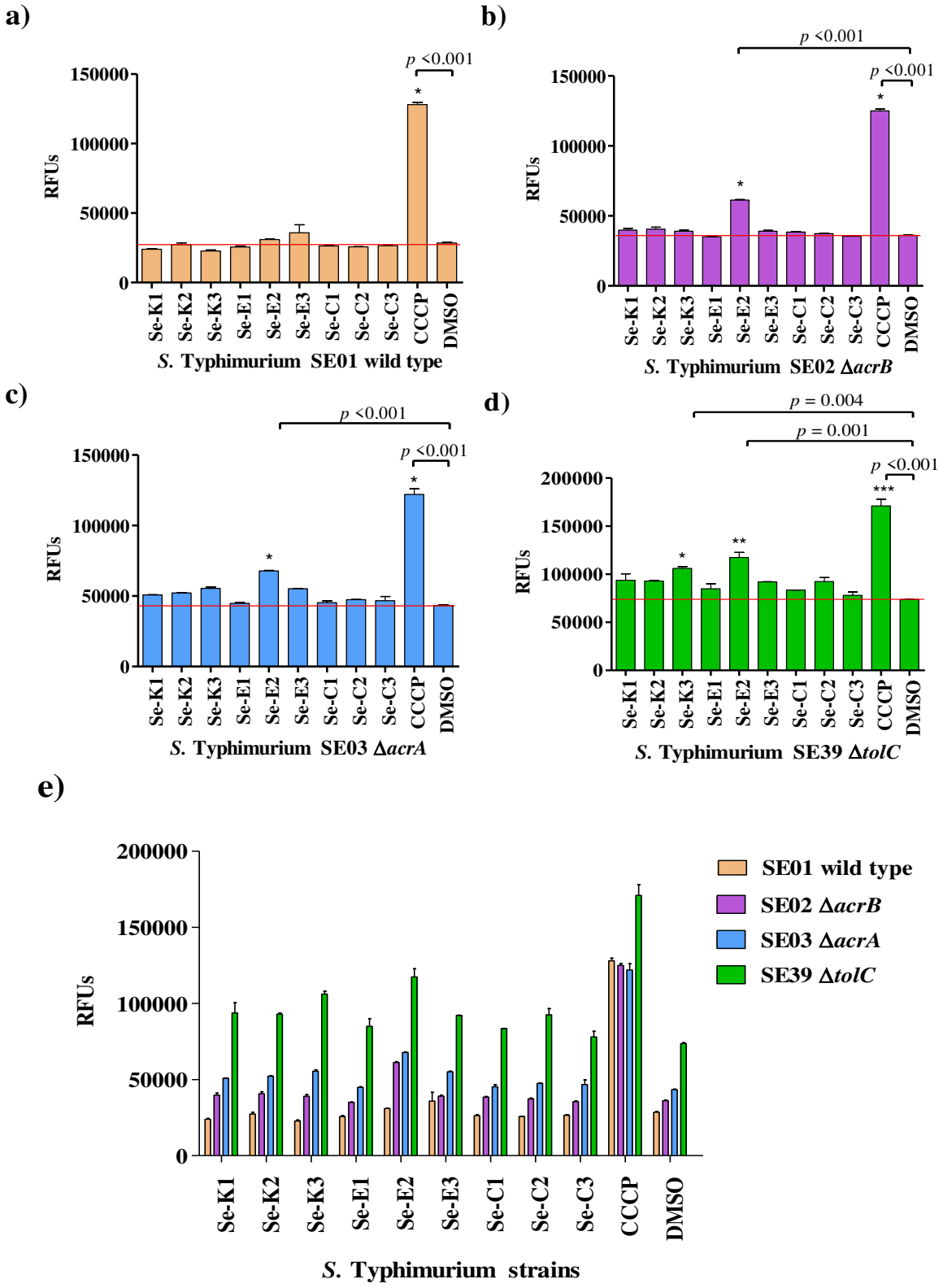


Figure 12 EB accumulation in *S.* Typhimurium strains in the presence of Se-compounds. The graphs show the RFUs (relative flurorescence units) of **a)** *S.* Typhimurium SE01 (wild-type), **b)** *S.* Typhimurium SE02 (Δ*acrB*), **c)** *S.* Typhimurium SE03 (Δ*acrA*), **d)** *S.* Typhimurium SE39 (Δ*tolC*) and **e)** all *S.* Typhimurium bacterial strains in the presence of the compounds in the 60th minute of the assay. The levels of significance were p <0.001, p = 0.001, and p = 0.004, respectively.

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 (**Figure 13**).

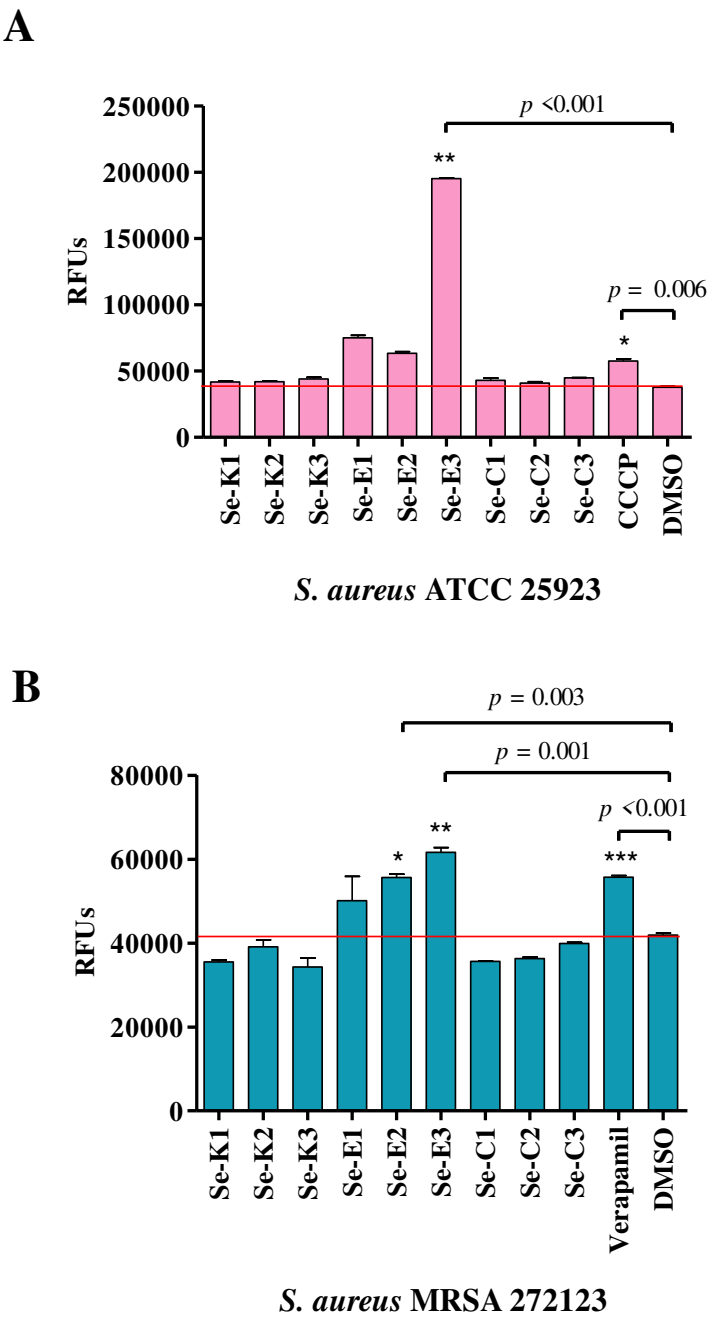


Figure 13 EB accumulation on *S. aureus* strains. The graphs show the RFUs of **A)** *S. aureus* ATCC 35923 **B)** *S. aureus* MRSA 272123 bacterial strains in the presence of selenocompounds in the 60th minute of the assay. The levels of significance were p <0.001 and p = 0.006, respectively.

2.5. QS inhibitory effect of selenocompounds

The sensor strain *C. violaceum* 026 and the AHL producer strains *E. cloacae* 31298 were inoculated as parallel lines. Interactions between strains and compounds were evaluated based on the size of the inhibition zone of pigment production in millimeters. PMZ was applied as QS inhibitor, its zone of inhibition was 46 mm. 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 (**Figure 14**).

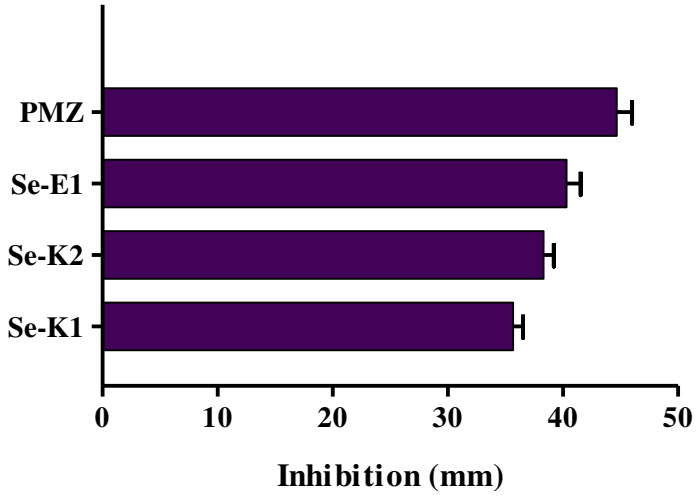


Figure 14 QS-inhibition by selenocompounds in ½ MIC concentration by disk diffusion method. The ineffective compounds are not shown. Promethazine (PMZ) was used as a positive control in 25 μg/ml concentration.

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

The cytotoxic activity of the Se-compounds and reference compounds has been previously determined on parental and multidrug resistant mouse T-lymphoma cells [147]. The final concentration of the compounds used in the combination experiment was chosen in accordance with the previous results, while the cytotoxicity of the phenothiazines was assessed before performing the checkerboard combination assay to determine their ideal concentrations for these experiments. Using the NIH/3T3 mouse embryonic fibroblast cell line, the toxicity on non-tumoral cells and the selectivity of the Se-compounds and phenothiazines was also

investigated, the results were expressed in terms of the selectivity index (SI) (**Table 5**) [137, 153].

Compounds	IC_{50} (μM)			Selectivity index (SI)	
	Previously reported [147]			NIH/3T3 / PAR	NIH/3T3 / MDR
	PAR	MDR	NIH/3T3		
EDA1	3.97	4.65	> 100	**≥ 25**	**≥ 22**
EDA2	>100	>100	23.7	0.24	0.24
EDA3	19.5	16.9	> 100	*5.1*	**5.9**
EDA4	>100	>100	> 100	≥ 1.0	≥ 1.0
EDA5	>100	>100	> 100	≥ 1.0	≥ 1.0
EDA6	>100	36.4	69.7	≤ 0.70	≤ 0.70
EDA7	>100	87.8	23.7	≤ 0.24	≤ 0.24
EDA8	>100	>100	74.5	≤ 0.75	≤ 0.75
EDA9	0.78	1.03	0.62	0.81	0.60
EDA10	0.94	0.43	1.35	1.44	**3.14**
EDA11	1.31	0.97	0.82	0.63	0.85
O-isostere	>100	>100	> 100	≥ 1.0	≥ 1.0
O-salt	>100	>100	> 100	≥ 1.0	≥ 1.0
S-salt	>100	>100	> 100	≥ 1.0	≥ 1.0
Se-salt	>100	>100	> 100	≥ 1.0	≥ 1.0

Table 5 Cytotoxicity and selectivity of tested compounds (see **Appendix 2**) against NIH/3T3 mouse embryonic fibroblast cells and PAR/MDR mouse T-lymphoma cells. Selectivity indices (SI): SI<1 values denote lack of selectivity, 1<SI<3 mean a slight selectivity and 3<SI<6 values indicate moderate selectivity and are signalled with orange; whereas values of SI>6 point that the compounds are strongly selective and are highlighted in red

Out of the fifteen tested compounds, seven showed toxicity against the mouse embryonic fibroblasts (namely compounds **EDA2, EDA6-EDA11**) under 100 μM concentrations, while only three showed selectivity (**EDA1**: strongly selective, compounds **EDA3** and **EDA10**: moderately selective) towards tumor cells, in perspective of the previous results on murine lymphoma cell lines [137].

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

The results of the combination experiments between the Se-compounds and PMZ, CPZ, and TZ are shown in **Figures 15** and **Appendix 5**, respectively. In addition, the concentrations that showed the most beneficial interactions in these experiments are also highlighted in **Appendix 5**. As a general rule, 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 compounds showed antagonism with phenothiazines. The O-isostere of **EDA1** showed synergistic interactions with PMZ and CPZ, as well as the oxygen salt (KOCN) showed synergism with TZ. Compounds **EDA1**, **EDA10,** and **EDA11** with low IC_{50} values showed additive (CI=1) or antagonistic (CI>1) interactions with phenothiazines, with the exception of **EDA9**, that exhibited synergism with CPZ and TZ (CI values: 0.58-0.82). If the results are compared among the different phenothiazines, a pronounced decreasing tendency may be observed for the majority of Se-compounds from PMZ to TZ with CI values going as low as CI_2=0.276 in case of TZ referring synergistic interaction between Se-compounds and phenothiazines.

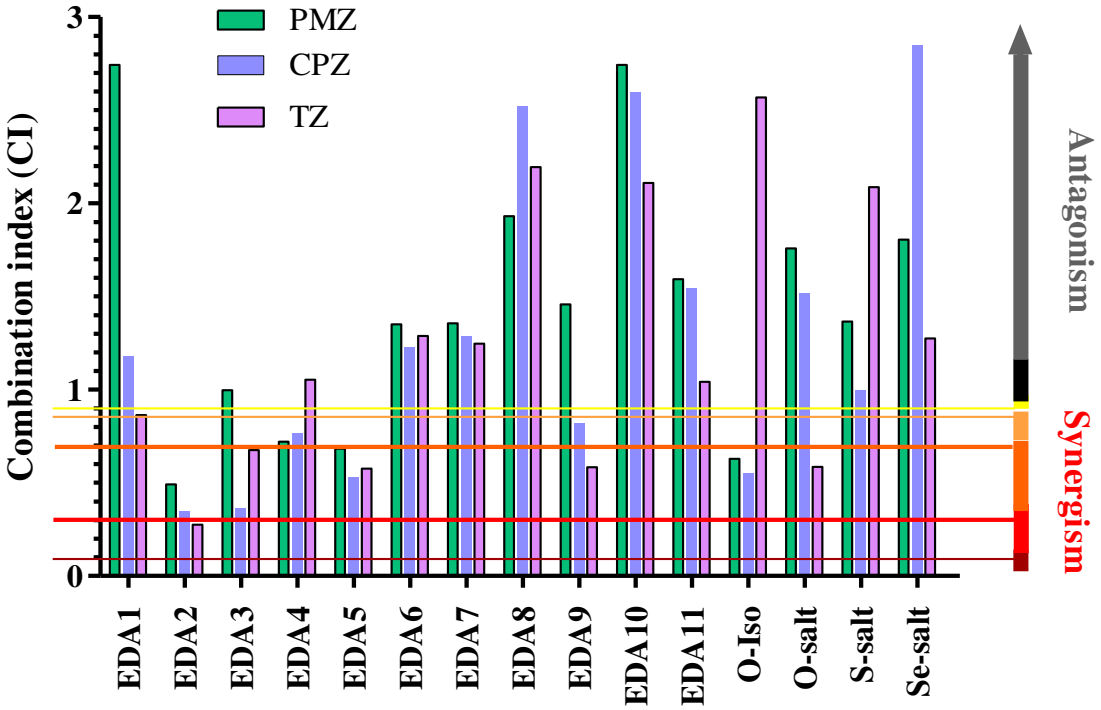


Figure 15 Interactions of the Se-compounds **EDA1-11** and the reference chalcogens with promethazine (PMZ), chlorpromazine (CPZ) and thioridazine (TZ) on MDR mouse T-lymphoma cells. Combination index (CI) values were calculated, where CI<1, CI=1, and CI>1 represent synergism, additive effect (or no interaction), and antagonism, respectively.

DISCUSSION

The resistance among microbes to antimicrobials, in cancer cells towards chemotherapeutic drugs has emerged and created public health threads worldwide. The overexpression of efflux pumps (EPs) as an important resistance mechanism enables the cells to extrude several toxic agents. Efflux pump inhibitors (EPIs) are 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 combination 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 multidrug resistance 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 [154]. The survival and colonization of enteric bacteria depend on extreme pH tolerance [155]. In addition, it has been described previously that several agents such as antibiotics showed pH-dependent antibacterial activity against certain organisms [156]. Diversity of biological activities of phenothiazines has been highlighted in psychopharmacology and in novel therapeutic indications by several studies [96, 102].

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 [157, 158].

In this study the inhibition of the AcrAB-TolC system of the *E. 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 had the highest expression rate in the 4th hour of culturing. The overexpression 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 [128, 130]. 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 *Salmonella* 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 [128, 130]. Moreover, these compounds were studied formerly for anticancer and EP inhibitory activity in different tumor cells [136, 137, 159, 160]. 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 [160]. According to the results some selenocompounds were highly effective adjuvants with reference 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 these compounds showed more potent activity in our assays than the parental compound phenothiazine [161].

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 [114]. However, based on the results of the bacterial response to the environmental factors, 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 [162], 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 *ΔacrA*, *ΔacrB*, *ΔtolC* 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**.

SUMMARY

Multidrug resistance (MDR) is an alarming problem in health care regarding microbial infections and cancer. Both bacterial and cancer cells are able to become drug resistant by different mechanisms. One of the most important phenomena is the overexpression of MDR efflux pumps (EPs) that extrude harmful agents out of the cells. The inhibition of these membrane transporters is a promising approach to overcome MDR. In order to reverse MDR, new synthetic efflux pump inhibitors (EPIs) could be designed or „old" drugs could be repurposed and applied as adjuvants. First, to monitor the stress response of the Gram-negative *E. coli* K-12 AG100, the EP inhibiting activity of the well-known phenothiazine-type drug promethazine (PMZ) was investigated at acidic pH (pH 5) and neutral pH (pH 7). The EP inhibiting effect of PMZ was determinated by real-time ethidium bromide accumulation, furthermore the efflux pump and regulator genes of the AcrAB-TolC efflux system were examined by quantitative PCR (qPCR) reaction. According to the results, the acidic pH and the PMZ treatment induced a significant stress response in the bacterium. The genetic system that regulates the activity of the EP is pH-dependent. Secondly, symmetrical methylketone (**Se-K1, -2, -3**), methyloxycarbonyl (**Se-E1, -2, -3**) and methylcyano (**Se-C1, -2, -3**) selenoesters were examined on *Salmonella* Typhimurium and *Staphylococcus aureus* strains. The antibacterial effect, the EPI activity, the anti-biofilm and quorum sensing (QS) inhibiting effects were assessed, furthermore the resistance modulating effects of selenocompounds was determined in the presence of antibiotics (tetracycline and ciprofloxacin). The symmetrical selenoesters showed a strong antibacterial and anti-biofilm activity, mainly against *S. aureus* strains. Methyloxycarbonyl selenoesters (**Se-E2**, -**3**) showed significant EPI activity in pump mutant *S.* Typhimurium and *S. aureus* strains, furthermore a strong QS inhibiting effect were observed for **Se-E1**. The last goal of this study was to investigate the combination of phenothiazines and anticancer selenocompounds on MDR cancer cells. The combination of anticancer Se-compounds (**EDA1-11**), four reference selenocompounds and three phenothiazines (promethazine, chlorpromazine, thioridazine) were investigated on MDR, ABCB1-overexpressing mouse T-lymphoma cells. Two symmetrical selenoesters (**EDA2** and **EDA5**) showed synergistic interaction with all three phenothiazines. The strongest synergism was obtained in case of the combination of **EDA2** and thioridazine. Consequently, in case of cancer therapy, phenothiazines could be applied as adjuvants in a drug repositioning approach. Based on our results selenocompounds might be valuable novel EPI compounds to reverse efflux related MDR in both bacteria and cancer cells.

ÖSSZEFOGLALÓ

A multidrog rezisztencia (MDR) aggasztó probléma az egészségügyben a mikrobiális fertőzések, valamint a rák tekintetében. Mind a bakteriális, mind a rákos sejtek különböző mechanizmusokkal képesek gyógyszerrezisztenssé válni. Az egyik legfontosabb jelenség az MDR efflux pumpák túlzott expressziója, amelyek képesek a káros anyagokat eltávolítani a sejtekből. Ezeknek a membrán transzportereknek a gátlása ígéretes megközelítés lehet az MDR leküzdésére. Az MDR visszafordítása érdekében új szintetikus efflux pumpa inhibítorokat lehetne megtervezni, vagy „régi" gyógyszereket újra felhasználni és adjuvánsként alkalmazni. Először megvizsgáltuk a Gram-negatív *E. coli* K-12 AG100 törzs stresszválaszát savas pH-n (pH 5) és semleges pH-n (pH 7) a jól ismert fenotiazin típusú efflux pumpa gátló prometazin (PMZ) jelenlétében. A PMZ gátló hatását valós idejű etídium-bromid akkumulációval határoztuk meg, továbbá kvantitatív PCR (qPCR) reakcióval vizsgáltuk az AcrAB-TolC efflux rendszert szabályozó és a pumpát kódoló géneket. Az eredményeink alapján elmondható, hogy a savas pH és a PMZ kezelés szignifikáns stresszreakciót váltott ki a baktériumban. Az efflux pumpa aktivitását szabályozó genetikai rendszer pH-függést mutatott. Második célként szimmetrikus metilketon (**Se-K1**, **-2**, **-3**), metiloxikarbonil (**Se-E1**, **-2**, **-3**) és metilciano (**Se-C1**, **-2**, **-3**) szelenoésztereket vizsgáltunk *Salmonella* Typhimurium és *Staphylococcus aureus* törzseken. Meghatároztuk az antibakteriális hatásukat, az efflux pumpa gátló aktivitásukat, az anti-biofilm és a quorum-sensing (QS) gátló hatásukat, valamint a rezisztenciát módosító hatásukat antibiotikumok (tetraciklin és ciprofloxacin) jelenlétében. A szimmetrikus szelenoészterek erős antibakteriális és anti-biofilm aktivitást mutattak, főleg *S. aureus* törzsekkel szemben. A metiloxikarbonil szelenoészterek (**Se-E2**, **-3**) szignifikáns pumpa gátló aktivitást mutattak a pumpa mutáns *S*. Typhimurium és *S. aureus* törzsekben, továbbá erős QS-gátló hatást figyeltünk meg **Se-E1** esetében. Harmadik célunk a fenotiazinok és a rákellenes szelénvegyületek kombinációjának vizsgálata volt MDR rákos sejteken. Rákellenes Se-vegyületek (**EDA1**-**11**), négy referencia szelénvegyület és három fenotiazin (prometazin, klórpromazin, tioridazin) kombinációját vizsgáltuk MDR egér T-lymphoma sejteken. Két szimmetrikus szelenoészter (**EDA2** és **EDA5**) szinergista kölcsönhatást mutatott mind a három fenotiazinnal. A legerősebb szinergizmust az **EDA2** és a tioridazin kombinációja esetén tapasztaltuk. Mindezeket figyelembe véve a fenotiazinok adjuvánsként alkalmazhatók lehetnek a gyógyszer újrapozicionálási megközelítés szerint a rákterápiában. Eredményeink alapján a szelénvegyületek értékes új efflux pumpa gátló vegyületek lehetnek az effluxhoz kapcsolódó MDR leküzdésében mind a baktériumok, mind a rákos sejtek esetén.

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APPENDIX

Appendix 1 IUPAC names of the selenocompounds

Se-K1: Se,Se-bis(2-oxopropyl) benzene-1,4-bis(carboselenoate)

Se-K2: Se,Se-bis(2-oxobutyl) benzene-1,3-bis(carboselenoate)

Se-K3: Se,Se,Se-tris(2-oxobutyl) benzene-1,3,5-tris(carboxyloselenoate)

Se-E1: dimethyl 2,2'-(terephthaloylbis(selanediyl))diacetate

Se-E2: dimethyl 2,2'-(isophthaloylbis(selanediyl))diacetate

Se-E3: trimethyl 2,2',2''-(benzenetricarbonyltris(selanediyl))triacetate

Se-C1: Se,Se-bis(cyanomethyl) benzene-1,4-bis(carboselenoate)

Se-C2: Se,Se-bis(cyanomethyl) benzene-1,3-bis(carboselenoate)

Se-C3: Se,Se,Se-tris(cyanomethyl) benzene-1,3,5-tris(carboxyloselenoate)

.

Appendix 2 IUPAC names of the selenocompounds

EDA1: Benzo[c]selenophen-1,3-dione (cyclic selenoanhidride)

EDA2: Dimethyl thiophene-2,5-dicarboselenoate (thiophene selenodiester)

EDA3: Dimethyl pyridine-2,6-dicarboselenoate (pyridine selenodiester)

EDA4: Dimethyl benzene-1,3-dicarboselenoate (meta-substituted benzene selenodiester)

EDA5: Dimethyl benzene-1,4-dicarboselenoate (para-substituted benzene selenodiester)

EDA6: Carbamoylmethyl benzoselenoate (carbamoylmethyl selenoester)

EDA7: Methoxycarbonylmethyl p-chlorobenzoselenoate (methoxycarbonylmethyl selenoester)

EDA8: Phenoxycarbonylmethyl benzoselenoate (phenoxycarbonyl selenoester)

EDA9: 2-Oxopropyl 4-chlorobenzoselenoate (methylketone selenoester)

EDA10: 3,3-Dimethyl-2-oxobutyl 4-chlorobenzoselenoate (chloro-substituted *tert*butylketone selenoester)

EDA11: 3,3-Dimethyl-2-oxobutyl 3,5-dimethoxybenzoselenoate (dimethoxy-substituted *tert*butylketone selenoester)

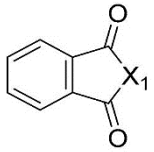
O-Isostere: phthalic anhydrid

O-salt: potassium cyanate

S-salt: ammonium thiocyanate

Se-salt: potassium selenocyanate

Anhydride



EDA1 X^1=Se

O-isostere X^1=O

Cyanates

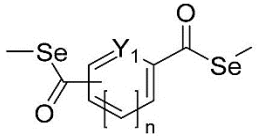
Y^2X^2

O-salt $Y^2=K^+$ $X^2=OCN^-$

S-salt $Y^2=NH_4^+$ $X^2=SCN^-$

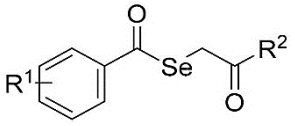
Se-salt $Y^2=K^+$ $X^2=SeCN$

Symmetrical selenoester



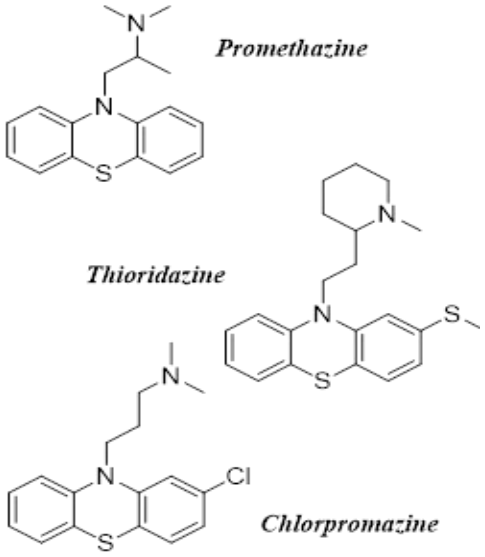
EDA2	Y^1=S	n=0	subst. 2,5
EDA3	Y^1=N	n=1	subst. 2,6
EDA4	Y^1=C	n=1	subst. 1,3
EDA5	Y^1=C	n=1	subst. 1,4

Non-symmetrical selenoester



EDA6	R^1= -H	R^2= - NH
EDA7	R^1= 4-Cl	R^2= -OCH_3
EDA8	R^1= -H	R^2= -OPh
EDA9	R^1= 4-Cl	R^2= -CH_3
EDA10	R^1= 4-Cl	R^2= -$C(CH_3)_3$
EDA11	R^1=C	R^2= -$C(CH_3)_3$

Appendix 3 Structure of the Se-compounds (**EDA1-11**) and the reference compounds (**O-isostere**, **O-salt**, **S-salt**, **Se-salt**) evaluated in this study



Appendix 4 Structure of the phenothiazines evaluated

	Best ratio [µM] phenothiazine:compounds		
	Promathazine	**Chlorpromazine**	**Thioridazine**
EDA1	2.8125 : 25	9.9 : 3.125	6.75 : 6.25
EDA2	**1.46025 : 50**	**2.475 : 12.5**	**1.6875 : 6.25**
EDA3	5.625 : 25	9.9 : 50	1.6875 : 50
EDA4	11.25 : 50	2.478 : 50	6.75 : 50
EDA5	**1.40625 : 50**	**9.9 : 50**	**1.6875 : 100**
EDA6	1.40625 : 100	2.475 : 100	1.6875 : 100
EDA7	11.25 : 100	1.2375 : 100	1.6875 : 100
EDA8	11.25 : 100	4.95 : 100	3.375 : 100
EDA9	11.25 : 2.5	4.95 : 10	0.84375 : 2.5
EDA10	5.625 : 1.25	1.2375 : 2.5	3.375 : 2.5
EDA11	11.25 : 2.5	4.95 : 2.5	0.84375 : 5
O-Isostere	2.8125 : 100	1.2375 : 100	3.375 : 100
O-salt	5.625 : 100	4.95 : 100	0.84375 : 100
S-salt	11.25 : 50	2.475 : 100	1.6875 : 100
Se-salt	11.25 : 100	9.9 : 25	3.375 : 100

Appendix 5 Best ratios of the combination assays.

Gene	Primer sequence (5'-3')	Amplicon size (bp)
marA	CATAGCATTTTGGACTGGAT	187
	TACTTTCCTTCAGCTTTTGC	
marB	ATAGCAGCTGCGCTTATTC	154
	ACTTATCACTGCCAGTACCC	
marR	AGCGATCTGTTCAATGAAAT	170
	TTCAGTTCAACCGGAGTAAT	
acrA	CTTAGCCCTAACAGGATGTG	189
	TTGAAATTACGCTTCAGGAT	
acrB	CGTACACAGAAAGTGCTCAA	183
	CGCTTCAACTTTGTTTTCTT	
soxS	CCATTGCGATATCAAAAATC	210
	ATCTTATCGCATGGATTGAC	
rob	GTCGTCTTTATCCTGACTCG	189
	TTTGTCACCCTGGAAGATAC	
GAPDH	ACTTACGAGCAGATCAAAGC	170
	AGTTTCACGAAGTTGTCGTT	

Appendix 6 Forward and reverse primers used in RT-qPCR reaction [145]

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.....	.....
Dátum	Dr. Gajdács Mária
	Szerző
	.....
	Dr. Spengler Gabriella
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The Role of Efflux Pumps and Environmental pH in Bacterial Multidrug Resistance

MÁRTA NOVÉ[1], ANNAMÁRIA KINCSES[1], JÓZSEF MOLNÁR[1], LEONARD AMARAL[1,2] and GABRIELLA SPENGLER[1]

[1]*Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Szeged, Hungary;*
[2]*Travel Medicine, Institute of Hygiene and Tropical Medicine, Universidade Nova de Lisboa, Lisbon, Portugal*

Abstract. *Background/Aim: One of the most studied bacterial resistance mechanisms is the resistance related to multidrug efflux pumps. In our study the pump activity of the Escherichia coli K-12 AG100 strain expressing the AcrAB-TolC pump system was investigated at pH 7 and pH 5 in the presence of the efflux pump inhibitor (EPI) promethazine (PMZ). Materials and Methods: The EPI activity was assessed by real-time fluorimetry. The influence of PMZ treatment on the relative expression of the pump genes acrA, acrB and their regulators marA, marB, marR, the stress genes soxS, rob, as well as the bacterial growth control genes ftsI, and sdiA were determined by RT-qPCR. Results: The EPI activity of PMZ was more effective at neutral pH. The PMZ treatment induced a significant stress response in the bacterium at acidic pH by the up-regulation of genes. Conclusion: The genetic system that regulates the activity of the main efflux pump is pH-dependent.*

Bacterial infections that are resistant to two or more classes of antibiotics are deemed multidrug resistant (MDR). The frequency of MDR clinical isolates is global and renders commonly available antibiotics useless. Among the many ways by which the MDR phenotype arises, the over-expression of MDR transporters promotes the expelling of antibiotics and other toxins from the bacterium prior to their reaching their targets (1). The main efflux pump of *E. coli* K-12 AG100 is the AcrAB-TolC system which is either transiently over-expressed in the MDR isolate or permanently over-expressed as a consequence of mutations of the regulatory genes that encode the pump (2).

The AcrAB-TolC efflux pump system consists of three proteins, the AcrB transporter and two fusion proteins that attach the AcrB to the plasma membrane. The AcrB transporter recognizes and binds the toxic agent (antibiotic) and releases it to the TolC channel to which it is connected. The AcrB is attached to the plasma membrane via the two fusion proteins whose peristaltic action drives water through the transporter and TolC channel, ultimately releasing the agent to the environment at the junction of TolC to the outer membrane, which consists partly of lipopolysaccharides (LPS). Transport of drugs from the cell to the outside by this system is coupled to proton motive force (PMF) from the periplasm to the cytoplasm. Proton binding and release takes place in the transmembrane domain. The energy transduction and substrate transport seem to be spatially separated (3-5). The activity of the AcrAB-TolC pump in *E. coli* K-12 AG100 is inhibited by all phenothiazines studied to date. Whether inhibition is direct or indirect is not fully understood. Nevertheless, because the inhibition of the over-expressed efflux pump renders the MDR bacterium susceptible to antibiotics to which it was initially resistant, phenothiazines may have a clinical use as adjuvants. Such examples are PMZ that reduced the frequency of pyelonephritis in children treated with gentamicin, and thioridazine that was effective against XDR-*Mycobacterium tuberculosis* in combination with antibiotics to which the isolate was initially resistant (6). The activity of the AcrAB-TolC system at neutral pH depends on metabolic energy whereas at pH of 5 no metabolic energy is needed. Moreover, the effect of antibiotics at acidic pH is less pronounced. The effect of pH on the growth of bacteria is well known regarding the stress promoted at acidic conditions (7). The expression of the AcrAB-TolC efflux pump system is controlled by transcriptional regulators, such as MarA, MarB, MarR and the stress proteins SoxS and Rob (8-11). In addition, the bacterial growth is controlled by SdiA and its over-expression results in the over-expression of the AcrAB-TolC system (12).

Correspondence to: Gabriella Spengler, Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Dóm tér 10, 6720 Szeged, Hungary. Tel: +36 0662545115, Fax: +36 0662545113, E-mail: spengler.gabriella@med.u-szeged.hu

Key Words: Multidrug resistance, efflux pump, promethazine, *Escherichia coli* K-12 AG100, efflux pump genes, pH-dependent.

The bacterial cell division is controlled by the transpeptidase FtsI required for synthesis of peptidoglycan (13). 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 at acidic pH (3, 14). It is the intent of the study to describe the expression of genes that are known to be affected at neutral and acidic pH and determine the effect of PMZ on the expression of these genes.

Materials and Methods

Reagents and media. Promethazine (EGIS), ethidium bromide (EB) and Luria-Bertani (LB) broth were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Mueller Hinton (MH) broth was purchased from Scharlau Chemie S. A. (Barcelona, Spain). Sterile glucose solution (40%) was applied in the experiments (PannonPharma, Hungary). The pH was adjusted to 5.0 and 7.0. In the case of PMZ the solvent for the stock solution was distilled water. Phenothiazines are affected by light, thus, PMZ was protected from it (15). The optical properties of the broth with and without PMZ were not altered.

Bacterial strain. The wild-type *Escherichia coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44] expresses the AcrAB-TolC efflux pump at its basal level (16, 17). This strain was kindly provided by Prof. Dr. Hiroshi Nikaido (Department of Molecular and Cell Biology and Chemistry, University of California, Berkeley, CA, USA).

Determination of minimum inhibitory concentrations by microdilution method. The minimum inhibitory concentration (MIC) of PMZ was determined according to Clinical and Laboratory Standard Institute (CLSI) guidelines using MH media at pH 5 and pH 7. Briefly, the bacterial strain was separately cultured in broth of pH 5 and pH 7 overnight at 37˚C. On the following day, the determination of MIC was carried out using broth dilution method in 96-well plates and the plates were incubated for 18 h at 37˚C.

Determination of growth curves at pH 5 and pH 7. The growth curves of the *E. coli* K-12 AG100 strain were determined by measuring the optical density (OD_{600}) at pH 5 and pH 7 in LB broth with and without 25 μg/ml of PMZ. The bacterial cultures were incubated for further 24 h at 37˚C with shaking (220 rpm) and the optical density was monitored at 600 nm. In addition, we determined the colony forming units (CFUs) at various time points (0, 2, 5, 8, and 24 h) on LB agar plates.

Phenothiazines promote the elongation and subsequent filamention of the bacterium (18). This morphological response affects the interpretation of the results that define growth and optical properties of the culture. From the MIC data obtained at pH 5 and pH 7, the concentration of PMZ that produced no obvious effect on the viability, elongation or filamentation of *E. coli* K-12 AG100 at both acidic and neutral pH was determined.

Real-time EB accumulation assay. The effect of PMZ on the real-time accumulation of EB in the presence and absence of glucose (0.4%) was assessed by an automated EB method as described previously (19), 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 according to the formula:

$$RFI = \frac{RF_{treated} - RF_{untreated}}{RF_{untreated}}$$

where $RF_{treated}$ is the relative fluorescence at the last time point of the EB retention curve in the presence of an inhibitor, and $RF_{untreated}$ is the relative fluorescence at the last time point of the EB retention curve of the untreated solvent control. The solvent was distilled water in the case of PMZ.

Total RNA isolation. The *E. coli* K-12 AG100 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 were incubated at 37˚C with shaking. The total RNA was isolated at various time points (0, 1, 2, 4, 8, and 18 h). The RNA preparation was carried out in an RNase-free environment using the 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˚C until quantification was performed. The concentration of the extracted RNA templates was assessed by spectrophotometry SmartSpec™ Plus at 260 nm (Bio-Rad, Hercules, CA, USA).

Relative gene expression analyses by real-time reverse transcriptase quantitative polymerase chain (RT-qPCR) reaction. The relative gene expression levels were determined at pH 5 and pH 7 in the presence and absence of PMZ. The *E. coli* K-12 AG100 strain was cultured in LB at pH 5 and pH 7 and total RNA was isolated at various time points (after 0, 1, 2, 4, 8, and 18 h). The relative expression levels of the efflux pump genes, their regulators, stress genes and genes involved in cell wall biosynthesis and quorum sensing were determined by RT-qPCR (20, 21) (Table I), using CFX96 Touch real-time PCR detection system (BioRad), strictly following the manufacturer's recommendations for the SensiFAST™ SYBR No-ROX One-Step Kit (Bioline GmbH, Luckenwalde, Germany). Briefly, each well of the 96-well microtiter plate contained 20 μl as follows: 10 μl of the 2x SensiFAST™ 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 in each gene's expression level between two samples, the sample of interest and a calibrator or reference sample. The relative gene expression analysis was calculated, according to the following formulas:

Table I. *Primers used in the RT-qPCR.*

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Reference
marA	CATAGCATTTTGGACTGGAT TACTTTCCTTCAGCTTTTGC	187	(20)
marB	ATAGCAGCTGCGCTTATTC ACTTATCACTGCCAGTACCC	154	(20)
marR	AGCGATCTGTTCAATGAAAT TTCAGTTCAACCGGAGTAAT	170	(20)
acrA	CTTAGCCCTAACAGGATGTG TTGAAATTACGCTTCAGGAT	189	(20)
acrB	CGTACACAGAAAGTGCTCAA CGCTTCAACTTTGTTTTCTT	183	(20)
soxS	CCATTGCGATATCAAAAATC ATCTTATCGCATGGATTGAC	210	(20)
rob	GTCGTCTTTATCCTGACTCG TTTGTCACCCTGGAAGATAC	189	(20)
ftsI	GGCGCTGGTTTATCGCGAAC CCAGCTTGGAAACACCGAC	380	(12)
sdiA	CTGATGGCTCTGATGCGTTTA TCTGGTGGAAATTGACCGTATT	163	This study
GAPDH	ACTTACGAGCAGATCAAAGC AGTTTCACGAAGTTGTCGTT	170	(20)

marA: Multiple resistance antibiotic protein A; *marB*: multiple resistance antibiotic protein B; *marR*: multiple resistance antibiotic protein R; *acrA*: acridin resistance protein A; *acrB*: acridin resistance protein B; *soxS*: superoxid stress protein; *rob*: right origin-binding protein; *ftsI*: peptidoglycan D,D-transpeptidase FtsI; *sdiA*: quorum-sensing transcriptional activator; *GAPDH*: glyceraldehyde-3-phospate dehydrogenase.

$$\Delta C_T = C_{T\ (\text{gene of interest})} - C_{T\ (\text{reference gene})}$$

$$\Delta\Delta C_T = \Delta C_{T\ (\text{PMZ treated})} - \Delta C_{T\ (\text{PMZ untreated})}$$

Results

The MIC of PMZ was determined using MH broth at pH 5 and pH 7 on *E. coli* K-12 AG100 strain expressing the AcrAB-TolC efflux pump system. The MIC of PMZ was 200 μg/ml at pH 5 and 7.

The growth of the *E. coli* K-12 AG100 strain at pH 5 and pH 7 in LB broth with and without the lowest concentration of PMZ that had a nominal effect on growth for 24 h is shown in Figure 1. Briefly, the growth of PMZ treated bacterial culture was slower at both pH, although the effect on growth was greater at pH 7.

The effect of PMZ on the real-time accumulation of EB was assessed using an automated EB method in the presence and absence of 0.4% glucose. The real-time accumulation curves demonstrated a higher intracellular EB concentration without glucose at pH 7 compared to the EB 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 the case of PMZ treated sample the intracellular EB accumulation was significantly higher at pH 7 compared to pH 5. The efflux pump inhibitor PMZ could exert a more potent EPI effect at neutral pH (Figure 2).

The influence of PMZ treatment was examined on the relative expression of the efflux pump genes (*acrA*, *acrB*) and their regulators at neutral and acidic pH. The RT-qPCR results showed that the external environment can influence the activity of efflux pumps. In the case of acidic pH every gene 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 cases of the efflux pump genes *acrA* and *B*, as well as in *marR* regulator, *soxS* stress gene and QS regulator *sdiA* after the 18th hour (Figure 3). At 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 and of *ftsI* after 4 h. Initially the efflux pump genes *acrA* and *acrB* were down-regulated, but at the end of the culturing period (18th hour) both genes were up-regulated (Figure 3).

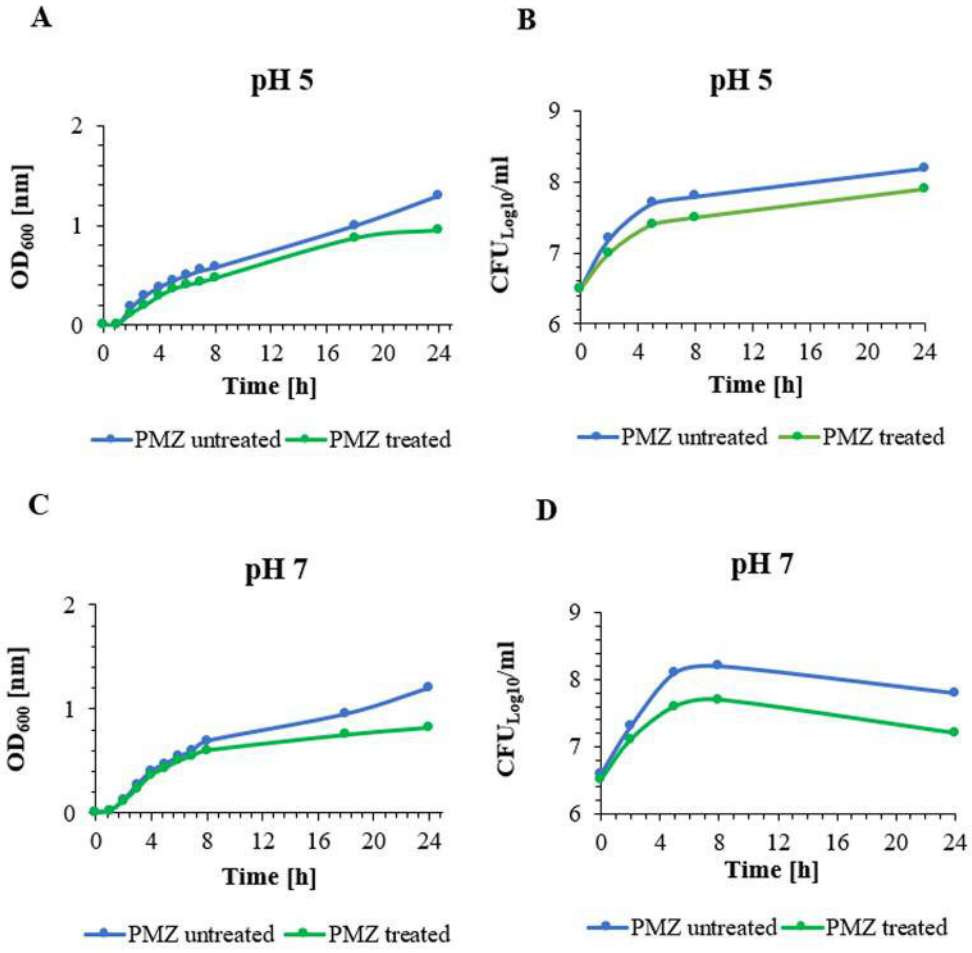


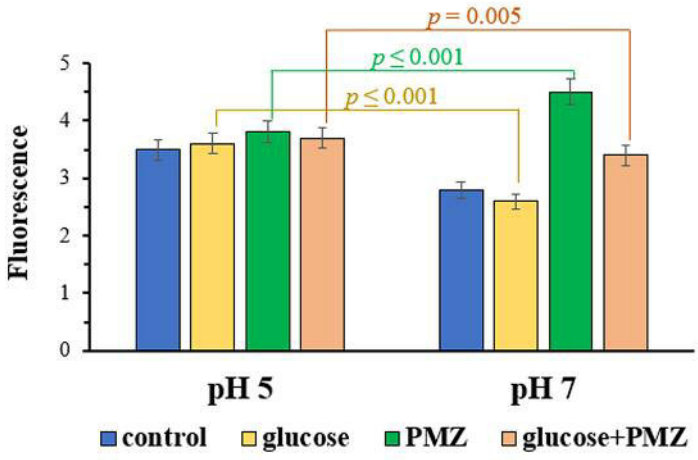
Figure 1. *The growth curves of E. coli K-12 AG100 strain were determined at pH 5 (A, B) and pH 7 (C, D) in LB broth in the presence and absence of 25 μg/ml of PMZ. Graphs A and C show the growth curves by measuring the optical density (OD_{600}) at pH 5 and pH 7, respectively. Graphs B and D show the growth curves by counting the CFUs at pH 5 and pH 7, respectively. The growth of PMZ treated bacterial culture was slower at pH 5 compared to pH 7. The growth of bacterial culture was more rapid at pH 7, moreover after 8 h of culturing the declination phase of the bacterial culture could be detected at pH 7 (Graph D).*

Discussion

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 (22). The survival and colonization of enteric bacteria depend on extreme pH tolerance (23). Before the pathogenic bacteria reach the alkaline pH of the small intestine they must survive the acidic pH of the stomach (24). According to our results, the pH can influence the accumulation and efflux of the RND pump substrate EB. Applying resistance modifiers in our experimental model, the consideration of different pH received special attention in order to mimic the physiological conditions in the gastrointestinal tract (25).

In order to restore survival of *E. coli* K-12 AG100 and maintain the conditions of cell growth, the acidic stress and PMZ treatment have to be overcome by different cellular mechanisms, such as proton pumps and induce a buffering effect through increasing the concentration of intracellular alkaline compounds (26). In addition, *E. coli* K-12 AG100 has two major energy sources, and the ATP synthesis. The latter is generated via the respiratory chain and is used mainly for ATP synthesis and various membrane transports (27). At acidic pH, the ATP supply is crucial for the survival of *E. coli*. At pH 7, *E. coli* cells are able to maintain a constant internal pH over a range of external pH values from 6.7 to 7.9 (28).

In the EB accumulation assay the EPI activity of PMZ was less effective at acidic pH compared to neutral pH. From this,



	Relative fluorescence index (RFI)		α = 0.05
glucose	0.03	- 0.07	$p \leq 0.001$
PMZ	0.09	0.61	$p \leq 0.001$
glucose+PMZ	0.06	0.21	$p = 0.005$

Figure 2. *Accumulation of EB at pH 5 and pH 7 by E. coli K-12 AG100 in the presence and absence of glucose 0.4%, with and without 25 μg/ml of PMZ. The real-time accumulation curves demonstrated a higher intracellular EB concentration without glucose at pH 7 compared to pH 5. The intracellular concentration of EB was significantly higher in the presence of PMZ at pH 7, in addition the PMZ treated sample exhibited lower EB accumulation at pH 5. The calculation of statistical significance and p-value was based on the relative fluorescence index (RFI) of the given sample. The correlation is significant: p≤0.001 and p=0.005.*

it can be concluded that the EPI activity of PMZ is pH-dependent because the proton motive force provides a more pronounced energy supply for the AcrB pump at pH 5. At acidic pH the PMF is higher compared to the neutral pH, for this reason the EB that accumulated in the presence of PMZ was lower at pH 5. It has been demonstrated by Mulkidjanian and coworkers that protons generated through metabolic pathways are transported as hydronium ions via channels to the surface of the cell where they are distributed and bound to reactive groups of LPS. These hydronium ions are then transported to the periplasm where they drive the efflux system, thus promoting the rapid extrusion of the substrate (29).

In addition, the acidic pH and PMZ treatment induced a significant stress response in *E. coli*, and this fact was confirmed by the up-regulation of *marB*, *marR*, *acrA*, *acrB*, *soxS, ftsI,* and *sdiA* genes at acidic pH compared to the neutral pH. Interestingly, *soxS* exhibited a continuous increase at pH 5, however, at pH 7 the *soxS* gene was down-regulated until the end of the culturing period. It has to be emphasized that the presented experimental setting applied PMZ at a very low concentration, for which at pH 7 it does not affect the stress genes in the wild type *E. coli*. For this reason, the *rob* and *ftsI* genes required for the initiation of replication and cell wall synthesis, respectively, have the highest expression rate in the 4th hour of culture. In addition, the expressions of *ftsI* and *sdiA* were higher at acidic pH compared to neutral pH. The over-expression of the efflux pump genes *acrA* and *B* at pH 5 and pH 7 indicates the continuous removal of toxic substances by efflux pumps. Finally, it can be concluded that the acidic pH and PMZ treatment induced a stress response in the bacterium.

Taking our results together we were able to demonstrate that efflux pump inhibitors are promising therapeutic options that may help overcome bacterial multidrug resistance, as well as improve the efficacy of combined antibacterial chemotherapy using conventional antibiotics and EPIs. EPI compounds can influence virulence factors (30), and should be further studied using different bacterial model systems imitating the environmental conditions present in the host organism.

Conflicts of Interest

None declared.

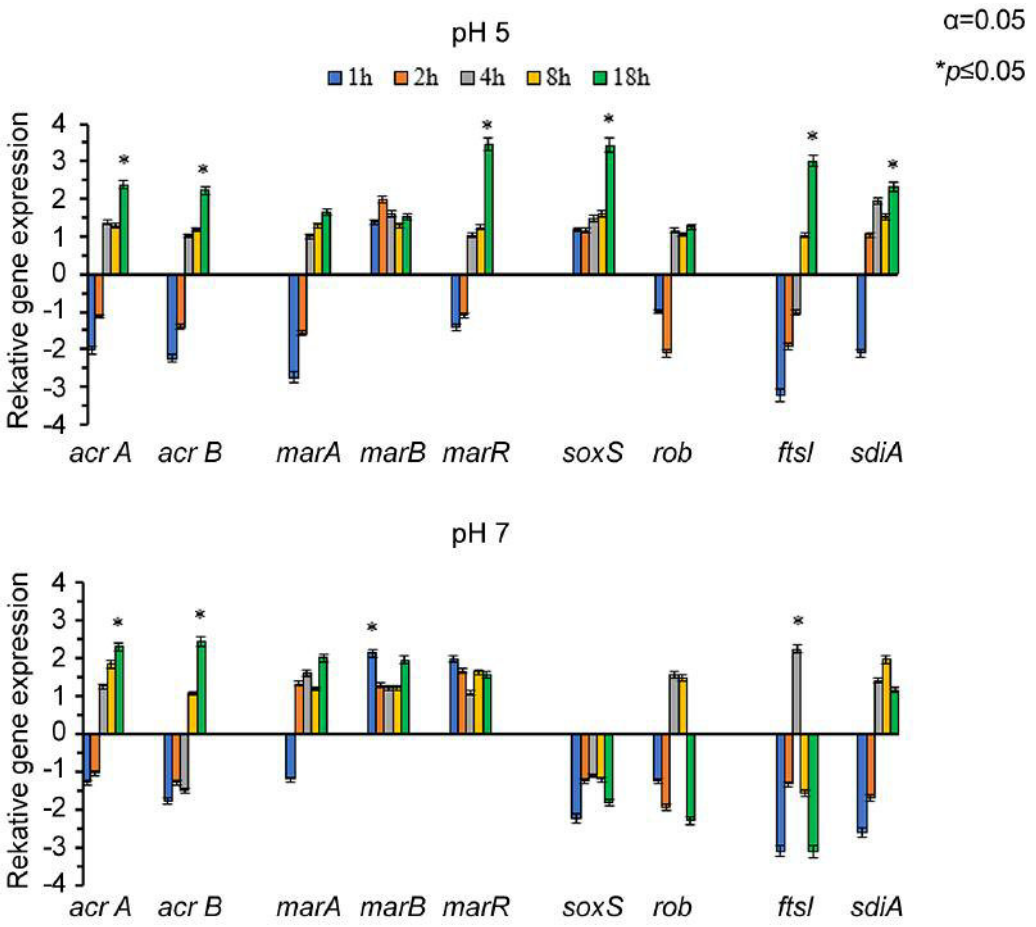


Figure 3. *Relative expression of genes involved in stress response on E. coli K-12 AG100 strain in the presence of 25 μg/ml of PMZ at pH 5 and pH 7 at different time points (1-18 h). At the beginning of the culturing period most of the studied genes showed a decreased expression pattern. Increase in gene expression level was detected in the cases of the acrA and B, marR, soxS, sdiA genes after the 18th hour at pH 5. 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 and of ftsI after 4 h. Initially the efflux pump genes acrA and acrB were down-regulated, but at the end of the culturing period (18th hour) both genes were up-regulated at pH 7. The significant correlation is: p≤0.05.*

Authors' Contributions

GS conceived and designed the study. MN and AK performed the laboratory work. MN, AK and GS wrote the article. JM and LA revised the manuscript critically. All authors read and approved the final manuscript.

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Biofilm Eradication by Symmetrical Selenoesters for Food-Borne Pathogens

Márta Nové [1], Annamária Kincses [1], Beatrix Szalontai [1], Bálint Rácz [1], Jessica M. A. Blair [2], Ana González-Prádena [3], Miguel Benito-Lama [3], Enrique Domínguez-Álvarez [3,*] and Gabriella Spengler [1,*]

1 Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Dóm tér 10, 6720 Szeged, Hungary; nove.marta@med.u-szeged.hu (M.N.); kincses.annamaria@med.u-szeged.hu (A.K.); beatrixszalontai@gmail.com (B.S.); balintracz95@gmail.com (B.R.)

2 Institute of Microbiology and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, UK; J.M.A.Blair@bham.ac.uk

3 Instituto de Química Orgánica General (IQOG-CSIC), Consejo Superior de Investigaciones Científicas, Juan de la Cierva 3, 28006 Madrid, Spain; anagonzalez.prad@gmail.com (A.G.-P.); m.benito@iqog.csic.es (M.B.-L.)

* Correspondence: e.dominguez-alvarez@iqog.csic.es (E.D.-Á.); spengler.gabriella@med.u-szeged.hu (G.S.); Tel.: +34-91-258-7661 (E.D.-Á.); +36-62-445-115 (G.S.)

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Abstract: Infections caused by *Salmonella* species and *Staphylococcus aureus* represent major health and food industry problems. Bacteria have developed many strategies to resist the antibacterial activity of antibiotics, leading to multidrug resistance (MDR). The over-expression of drug efflux pumps and the formation of biofilms based on quorum sensing (QS) can contribute the emergence of MDR. For this reason, the development of novel effective compounds to overcome resistance is urgently needed. This study focused on the antibacterial activity of nine symmetrical selenoesters (Se-esters) containing additional functional groups including oxygen esters, ketones, and nitriles against Gram-positive and Gram-negative bacteria. Firstly, the minimum inhibitory concentrations of the compounds were determined. Secondly, the interaction of compounds with reference antibiotics was examined. The efflux pump (EP) inhibitory properties of the compounds were assessed using real-time fluorimetry. Finally, the anti-biofilm and quorum sensing inhibiting effects of selenocompounds were determined. The methylketone and methyloxycarbonyl selenoesters were the more effective antibacterials compared to cyano selenoesters. The methyloxycarbonyl selenoesters (**Se-E2** and **Se-E3**) showed significant biofilm and efflux pump inhibition, and a methyloxycarbonyl selenoester (**Se-E1**) exerted strong QS inhibiting effect. Based on results selenoesters could be promising compounds to overcome bacterial MDR.

Keywords: *Salmonella* species; *Staphylococcus aureus*; multidrug resistance; antibacterial activity; symmetrical selenoesters

1. Introduction

The emergence of multidrug resistant pathogens is a major problem, leading to a progressive reduction in the efficiency of many antibacterial agents. This phenomenon is a serious challenge in public healthcare and medicine [1].

The most frequent multidrug resistance (MDR) mechanisms enable the resistant bacteria to achieve one or several of the following effects: (a) limited uptake of drug; (b) target modification; (c) drug inactivation; and (d) active efflux mediated by efflux pumps. Some efflux pumps are expressed

constitutively, whereas others are induced or over-expressed under environmental stimuli [2]. There are six families of the efflux pump systems: ATP-binding cassette (ABC) family, multidrug and toxic compound extrusion (MATE) family, small multidrug resistance (SMR) family, major facilitator family (MFS), resistance nodulation division (RND) family, and proteobacterial antimicrobial compound efflux (PACE) family [3,4]. Gram-positive bacteria mainly express the members of the MATE and MFS families, whereas Gram-negative bacteria also have transporters of the RND family [2]. The AcrAB-TolC efflux system is comprised of AcrB which belongs to the RND efflux transporters, the outer membrane protein TolC, and the periplasmic adaptor protein AcrA [5].

The formation of biofilms can also contribute to bacterial resistance. Biofilms have a dynamic structure involving a multicellular bacterial community and an extracellular polymeric matrix produced by the bacterial population. Biofilm-associated infections can lead to antibiotic resistant and persistent infections as this environment enhances the ability of the embedded bacteria to resist the action of the antibiotics [6].

One of the major food-borne illnesses is the salmonellosis caused by non-typhoidal *Salmonella enterica* [7]. In addition, the staphylococcal food poisoning (SFP) is a frequent food-born disease caused by staphylococcal enterotoxin (SE) producer enterotoxigenic *Staphylococcus aureus* strains [8]. *S. aureus* and *Salmonella enterica* serovar Typhimurium are food-borne pathogens capable of forming biofilms on various surfaces. Alkaline and acidic detergents, as well as iodophores, can be effective against biofilm. However, these substances damage surfaces, and the inappropriate use of biocides and disinfectants could lead to a quick and undesired emergence of resistant microbes [9]. Many bacteria use a cell–cell communication system, namely quorum sensing (QS), to coordinate the population density-dependent gene expression pattern [10]. This communication system plays a major role in biofilm development, as bacteria can produce new virulence factors and thanks to them this bacterial community responds poorly to antibiotic treatment [11].

Selenium(Se)-containing compounds could provide alternative and effective scaffolds to overcome MDR [12]. Se is an essential trace element in living organisms and is crucial for the nutrient supply and energy generation of bacteria. However, overdoses of Se can be highly toxic [13,14]. There is significant evidence about the pro-oxidant effect of Se, particularly in the form of sodium selenite (Na_2SeO_3), while selenomethionine and selenocysteine are less toxic [14]. It has been described previously that Se-containing agents have an antibacterial effect [15,16]. Selenoesters and selenoanhydrides have exhibited anti-biofilm activity against *S. aureus* and *S.* Typhimurium as described previously [17]. Furthermore, selenocompounds have been used as selenium nanoparticles (SeNPs) against *S. aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* strains [18,19].

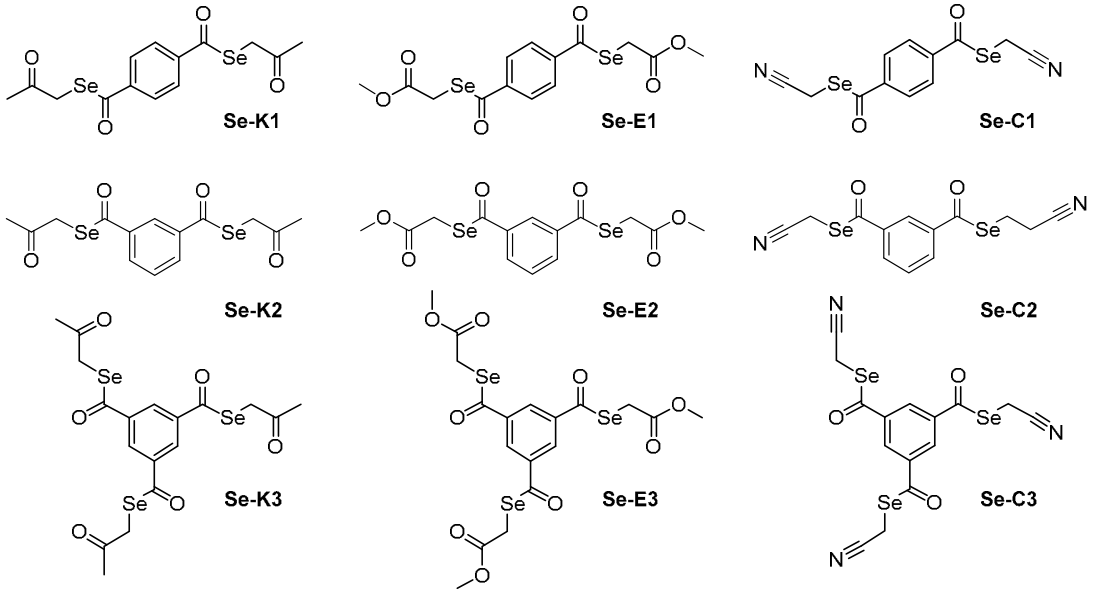
In the present study, and based in these antecedents, symmetrical 2-oxopropyl selenoesters, methyloxycarbonylmethyl selenoesters, and methylcyano selenoesters have been investigated against Gram-negative and Gram-positive bacterial strains to determine their antibacterial, efflux pump inhibiting, and anti-biofilm properties.

2. Materials and Methods

2.1. Compounds

Nine symmetrical selenodiesters or selenotriesters were synthesized and evaluated. Three were 2-oxopropyl selenoesters (briefly, ketone selenoesters, or methylketone selenoesters; compounds **Se-K1**, **Se-K2** and **Se-K3**). The next three selenocompounds were methyloxycarbonylmethyl selenoesters (methylcarbonyl selenoesters or methyloxycarbonyl selenoesters; compounds **Se-E1**, **Se-E2,** and **Se-E3**) [20]. The final three compounds were methylcyano selenoesters (cyano selenoesters; compounds **Se-C1**, **Se-C2,** and **Se-C3**). For each group of three compounds, the first is the symmetrical para-disubstituted derivative, the second is the symmetrical meta-substituted derivative, and the third is the symmetrical 1,3,5-trisubstituted derivative (Scheme 1). Their synthesis is described in the patent application EP17382693, and they were adequately characterized using nuclear magnetic

resonance spectroscopy (NMR), mass spectrometry (MS), and infrared spectroscopy (IR) techniques and their purity was assessed by elemental analysis [21]. Before their use in biological assays the selenocompounds were dissolved in dimethyl sulfoxide (DMSO), to obtain 10 mM concentration stock solutions.



Scheme 1. Chemical structure of the symmetrical selenoesters evaluated.

2.2. Reagents and Media

DMSO (Sigma-Aldrich, St Louis, MO, USA), phosphate-buffered saline (PBS; pH 7.4), promethazine (PMZ; EGIS), verapamil, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), ethidium bromide (EB), ciprofloxacin-hydrochloride (CIP) tetracycline-hydrochloride (TET), crystal violet (CV), Luria-Bertani (LB) broth, and LB agar were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The modified LB agar (LB*) was prepared from bacteriological agar 20 g/L (Difco, Detroit, USA), tryptone 10 g/L, NaCl 10 g/L, yeast extract 5 g/L, K_2HPO_4 1 g/L, $MgSO_4 \times 7H_2O$ 0.3 g/L, and FeNaEDTA 36 mg/L. pH of the agar was adjusted to 7.2. Mueller–Hinton (MH) broth, tryptic soy broth (TSB), and tryptic soy agar was purchased from Scharlau Chemie S.A. (Barcelona, Spain).

2.3. Bacterial Strains

Compounds were evaluated against the following bacterial strains:

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 [22–25].

Gram-positive *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 was used as the methicillin-susceptible reference bacterial strain, and 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 we used *Chromobacterium violaceum* 026 (CV026) as a sensor strain and *Enterobacter cloacae* 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 [26,27].

2.4. Cell Line

MRC-5 human embryonal lung fibroblast cell line (ATCC CCL-171) was purchased from LGC Promochem, Teddington, UK. The cells were cultured 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 a 5% CO_2, 95% air atmosphere.

2.5. Determination of Minimum Inhibitory Concentrations by Microdilution Method

The minimum inhibitory concentrations (MICs) of compounds were determined according to the Clinical and Laboratory Standard Institute guidelines (CLSI) [28]. MIC values of the compounds were determined by visual inspection. The solvent was also assayed to ensure there was no antibacterial effect and the concentration (1 *v/v*%) applied in the assays had no antibacterial activity. DMSO was used at subinhibitory concentration (1 *v/v*%) in the assays.

2.6. Cytotoxicity Assay

The adherent MRC-5 human embryonal lung fibroblast cells 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 overnight at 37 °C, 5% CO_2, then the medium was removed from the plates containing the cells, and the dilutions of selenocompounds previously made in a separate plate were 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 MTT (thiazolyl blue tetrazolium bromide, Sigma) solution (from a stock solution of 5 mg/mL) was 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 HCI) was 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:

$$IC_{50} = 100 - [(OD_{sample} - OD_{medium\ control})/(OD_{cell\ control} - OD_{medium\ control})] \times 100 \quad (1)$$

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%.

2.7. Resistance Modulation Assay

The resistance modulation effect of compounds with ciprofloxacin (CIP) and tetracycline (TET) antibiotics were evaluated by the checkerboard 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 ($\frac{1}{2}$ 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. MIC values in the presence of the antibiotics alone and in combination with Se-compounds were determined by visual inspection.

2.8. Real-Time Ethidium Bromide Accumulation Assay

The impact of compounds 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 $\frac{1}{2}$ 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 the 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 according to the following formula:

$$RFI = (RF_{treated} - RF_{untreated})/RF_{untreated} \quad (2)$$

where $RF_{treated}$ is the relative fluorescence (RF) at the last time point of EB retention curve in the presence of an inhibitor, and $RF_{untreated}$ is the RF at the last time point of the EB retention curve of the untreated control having the solvent control (DMSO).

2.9. Measuring Biofilm Formation Using Crystal Violet

The anti-biofilm effect of the tested compounds against *S. aureus* strains and wild-type *S.* Typhimurium SE01 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 $\frac{1}{2}$ 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 was 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 was 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.

2.10. Quorum Sensing (QS) Assay

The QS inhibitory effect of selenocompounds was examined on the AHL producer *E. cloacae* strain and *C. violaceum* sensor bacterial strain. These strains were inoculated in parallel. The QS inhibition was monitored by agar diffusion method on LB* agar plate as described previously [29]. Filter paper discs (7.0 mm in diameter) were placed between the parallel inoculated strains and impregnated with 10 μL compounds. Starting concentration of the compounds was $\frac{1}{2}$ MIC. The agar plates were incubated at room temperature (20 °C) for 24–48 h and the inhibition of violacein production was measured.

2.11. Statistical Analysis

The values are given as the mean ± standard deviation (SD) 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.

3. Results

3.1. Determination of Minimum Inhibitory Concentrations by Microdilution Method

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. Interestingly, 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 MICs were 16- and 32-fold higher against *S. aureus* ATCC 25923, respectively; with the exception of the trisubstituted derivative **Se-C3**, as its MIC was only 4-fold higher than the MIC of the trisubstituted methylketone **Se-K3**. The same tendency, but accentuated, was observed in *S. aureus* MRSA 272123, where the MIC values of the methylketone derivatives were in the range of 64- to 128-fold lower than the equivalent methyloxycarbonyl derivatives and in the range of 16- to 32-fold lower than the equivalent nitrile-containing selenoesters. The compounds showed a 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 (Table 1). Importantly, the MIC to the efflux knockout strains was unchanged suggesting that the compounds were not substrates of the AcrAB-TolC efflux pump.

Table 1. Antibacterial activity of selenocompounds. Minimum inhibitory concentrations (MICs) of compounds were determined on reference *Staphylococcus aureus* ATCC (American Type Culture Collection) 25923 and methicillin and ofloxacin-resistant *S. aureus* 272123 (MRSA) strains and *Salmonella* Typhimurium strains.

	MIC Determination (μM)					
Compounds	***S. aureus* ATCC 25923**	***S. aureus* MRSA 272123**	***S.* Typhimurium SE01 Wild-Type**	***S.* Typhimurium SE02 Δ*acrB***	***S.* Typhimurium SE03 Δ*acrA***	***S.* Typhimurium SE39 Δ*tolC***
Se-K1	0.39	1.56	50	50	50	50
Se-K2	0.39	1.56	50	50	50	100
Se-K3	0.39	0.78	50	25	25	50
Se-E1	12.5	100	>100	>100	>100	>100
Se-E2	12.5	100	>100	>100	>100	>100
Se-E3	12.5	100	>100	>100	>100	>100
Se-C1	6.25	50	25	25	25	25
Se-C2	6.25	50	25	25	25	25
Se-C3	1.56	12.5	12.5	12.5	12.5	25

3.2. Resistance Modulation Assay

As the Se-compounds were more effective on *S. aureus* strains, these strains were selected for combination studies with reference antibiotics. Selenocompound **Se-E3** showed synergism with TET on the methicillin-susceptible *S. aureus* ATCC 25923.

Surprisingly, all selenocompounds showed synergism with TET on the methicillin-resistant *S. aureus* strain. **Se-E3** and **Se-C2** were the most effective compounds in combination with TET, as they reduced the MIC value of TET against this MRSA strain to a value 32-fold lower. Additionally, compounds **Se-E1** and **Se-C1** also exerted a noteworthy reduction of the MIC value, of 16-fold in this case. On the other hand, **Se-K1** and **Se-E3** showed synergism with CIP on the MRSA strain, achieving a 2-fold reduction of the MIC value (Table 2).

Table 2. Resistance modulating effect of selenocompounds in the presence of antibiotics on *S. aureus* strains. The resistance modulation effect of Se-compounds with ciprofloxacin (CIP) and tetracycline (TET) antibiotics on the *S. aureus* bacterial strains were evaluated by the checkerboard method.

MIC Reduction (μM) In Brackets, the X-Fold Reduction of MIC Is Presented				
Compounds	***S. aureus* ATCC 25923 *with***		***S. aureus* MRSA 272123 *with***	
	TET	**CIP**	**TET**	**CIP**
–	**0.88**	**1.06**	**14.06**	**33.99**
Se-K1	0.88	1.06	3.51 (4)	16.99 (2)
Se-K2	0.88	1.06	7.03 (2)	33.99
Se-K3	0.88	1.06	7.03 (2)	33.99
Se-E1	0.88	1.06	0.88 (16)	33.99
Se-E2	0.88	1.06	1.76 (8)	33.99
Se-E3	0.44 (2)	1.06	0.44 (32)	16.99 (2)
Se-C1	0.88	1.06	0.88 (16)	33.99
Se-C2	0.88	1.06	0.44 (32)	33.99
Se-C3	0.88	1.06	3.51 (4)	33.99

3.3. Ethidium Bromide Accumulation Assay

The activity of the selenocompounds on EB accumulation was determined by the automated EB method on sensitive and resistant *S. aureus* and *S.* Typhimurium SE01, -02, -03, and -39 strains. The relative fluorescence index was calculated based on the means of relative fluorescence units (RFUs; Table 3).

Table 3. Relative fluorescence indices based on real-time ethidium bromide (EB) accumulation data on *S.* Typhimurium and *S. aureus* strains. The active compounds are presented in bold.

Relative Fluorescence Index (RFI)						
Compounds	***S.* Typhimurium SE01 Wild-Type**	***S.* Typhimurium SE02 Δ*acrB***	***S.* Typhimurium SE03 Δ*acrA***	***S.* Typhimurium SE39 Δ*tolC***	***S. aureus* ATCC 25923**	***S. aureus* MRSA 272123**
Se-K1	−0.16	0.10	0.17	0.27	0.1	−0.15
Se-K2	−0.04	0.13	0.20	0.26	0.11	−0.07
Se-K3	−0.20	0.08	0.28	0.44	0.16	−0.18
Se-E1	−0.10	−0.03	0.03	0.15	0.98	0.19
Se-E2	0.09	**0.70**	**0.56**	**0.59**	0.67	0.33
Se-E3	0.26	0.08	0.27	0.25	**4.15**	**0.47**
Se-C1	−0.08	0.06	0.04	0.13	0.14	−0.15
Se-C2	−0.10	0.03	0.09	0.25	0.08	−0.13
Se-C3	−0.07	−0.02	0.08	0.06	0.18	−0.05
CCCP	3.50	2.46	1.81	1.32	0.52	–
Verapamil	–	–	–	–	–	0.32

In case of *Salmonella* strains, the Se-compounds increased 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 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. In addition, verapamil was applied as reference EPI on *S. aureus* MRSA. The solvent DMSO served as a negative control in the experiments. **Se-E2** significantly increased the intracellular EB accumulation on *S.* Typhimurium SE02, -03, and -39. In addition, a significant EB accumulation was observed for **Se-K3** on *S.* Typhimurium SE39 (Figure 1).

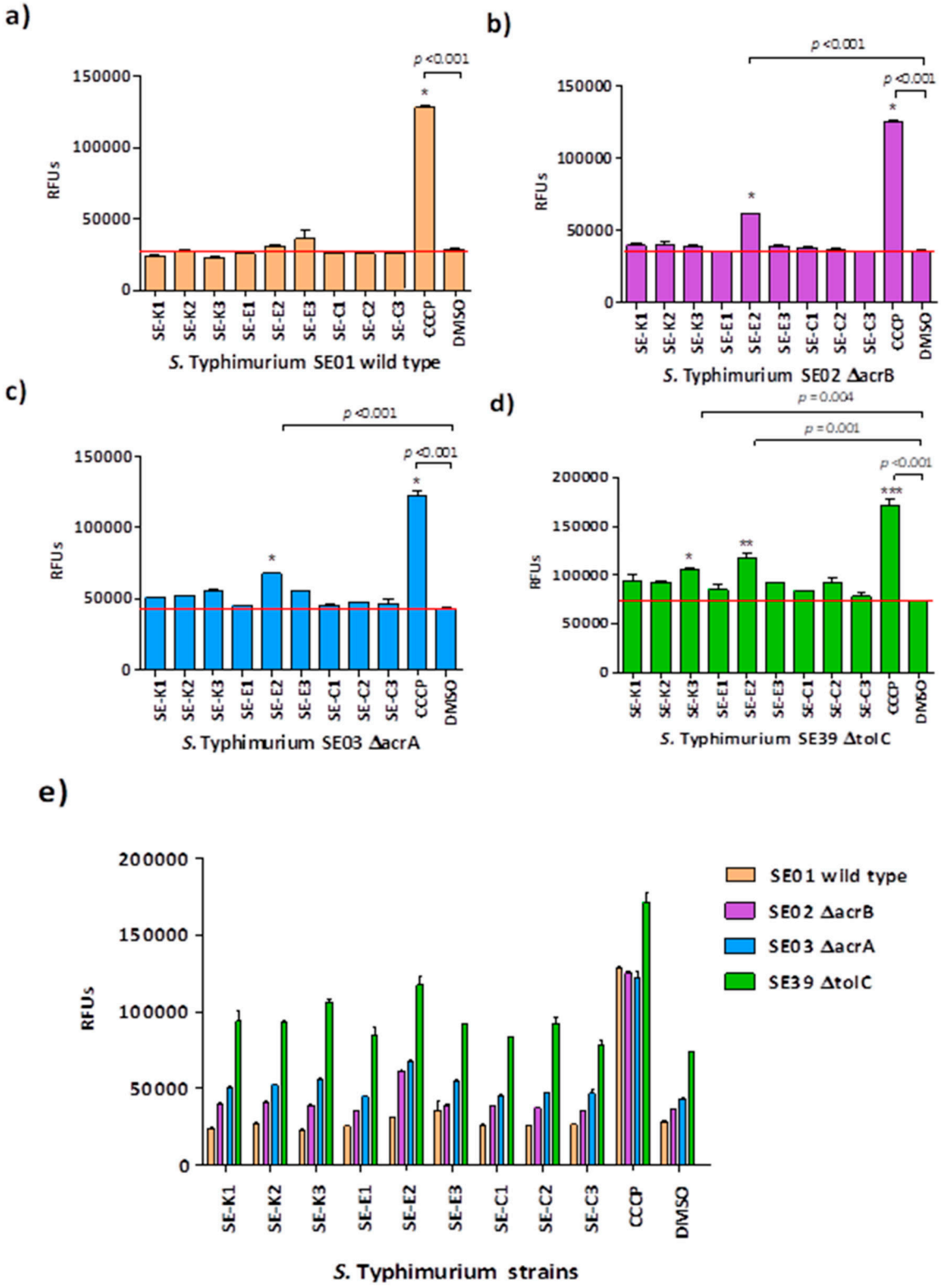


Figure 1. Ethidium bromide (EB) accumulation in *S.* Typhimurium strains in the presence of Se-compounds. The graphs show the relative fluorescence units (RFUs) of (**a**) *S.* Typhimurium SE01, (**b**) *S.* Typhimurium SE02, (**c**) *S.* Typhimurium SE03, (**d**) *S.* Typhimurium SE39, and (**e**) all *S.* Typhimurium bacterial strains in the presence of the compounds in the 60th minute of the assay. In case of *S.* Typhimurium SE01, -SE02 and -SE03 the level of significance was * $p < 0.001$. The levels of significance were * $p = 0.004$, ** $p = 0.001$, and *** $p < 0.001$ on *S.* Typhimurium SE39.

In case of the reference *S. aureus* and resistant MRSA strain 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 proven to be effective in both *S. aureus* strains (Figure 2).

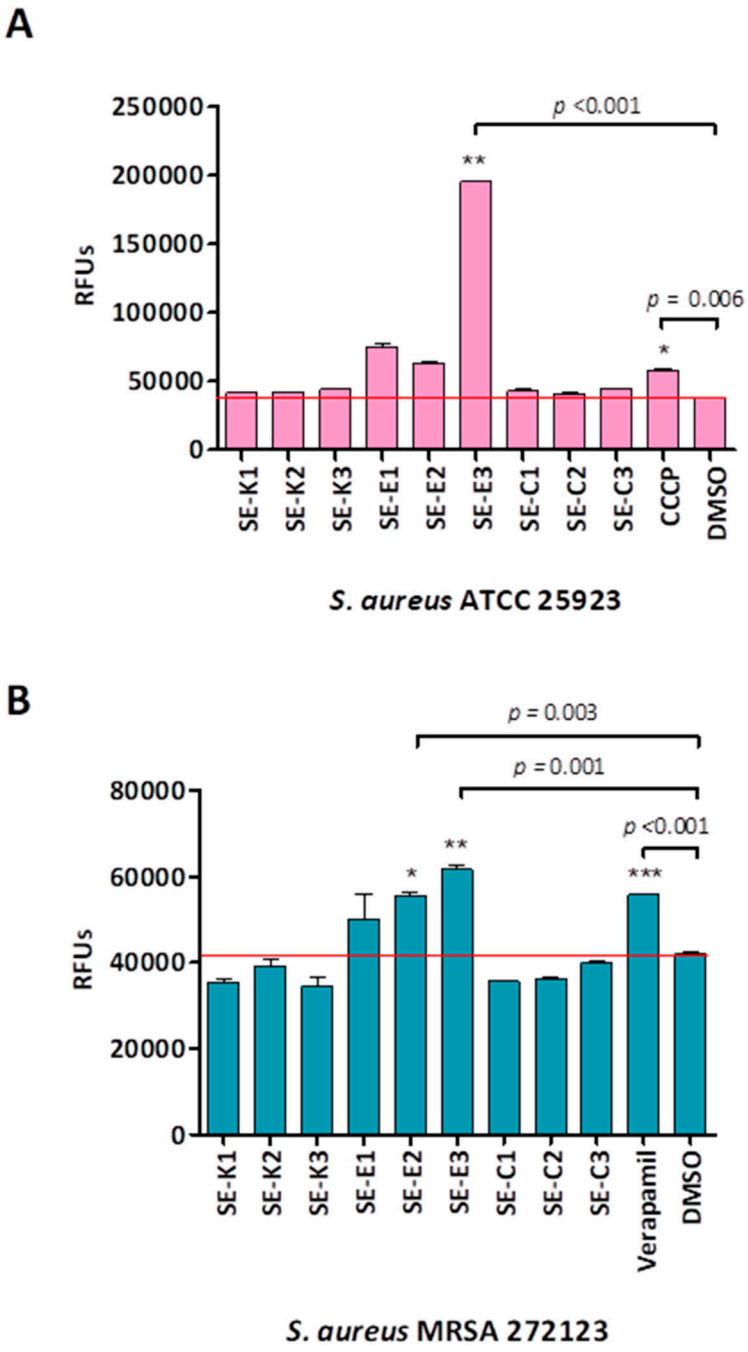


Figure 2. EB accumulation on *S. aureus* strains. The graphs show the RFUs of (**A**) *S. aureus* ATCC 25923 (**B**) *S. aureus* MRSA 272123 bacterial strains in the presence of the compounds in the 60th minute of the assay. In case of *S. aureus* ATCC 25923 the levels of significance were * $p = 0.006$ and ** $p < 0.001$. The levels of significance were * $p = 0.003$, ** $p = 0.001$, and *** $p < 0.001$ on *S. aureus* MRSA 272123.

3.4. Measuring Biofilm Formation Using Crystal Violet

The effect of selenocompounds on biofilm formation of sensitive and resistant *S. aureus* strains and wild-type *S.* Typhimurium SE01 was evaluated. 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* showed an absorbance of 2.4 ± 0.1, the resistant *S. aureus* 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 efficiently inhibit 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 (Figure 3).

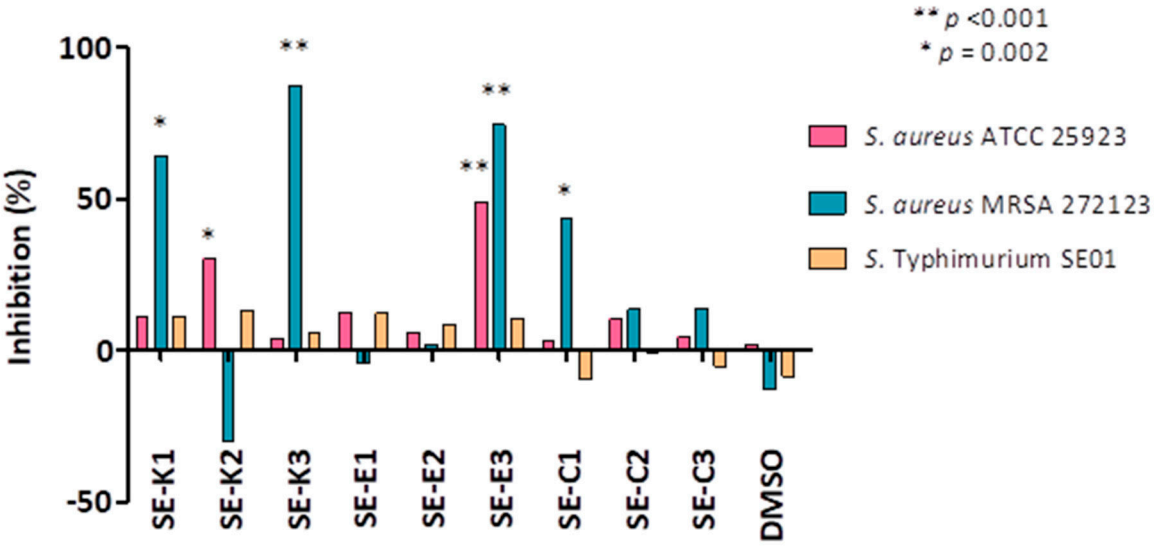


Figure 3. Anti-biofilm effect of Se-compounds on *S.* Typhimurium SE01 wild-type and on sensitive and resistant *S. aureus* strains. The levels of significance were ** $p < 0.001$ and * $p = 0.002$, respectively.

3.5. Quorum Sensing (QS) Assay

The sensor strain *C. violaceum* 026 and the AHL producer strains *E. cloacae* 31298 were inoculated as parallel lines. Interactions between the strains and compounds were evaluated for the reduction in the size of the zone of pigment production and the zone of growth inhibition of the affected strains, in millimeters. Promethazine (PMZ) was applied as a QS inhibitor and its zone of inhibition was 46 mm. Selenocompounds **Se-K1**, **Se-K2**, and **Se-E1** had QS inhibitory effect. In addition, **Se-K1** and **Se-K2** showed inhibition zones of 37 mm and 40 mm, respectively, whereas the methyloxycarbonyl selenoester **Se-E1** was the most effective QS inhibitor with an inhibition zone of 41 mm (Figure 4).

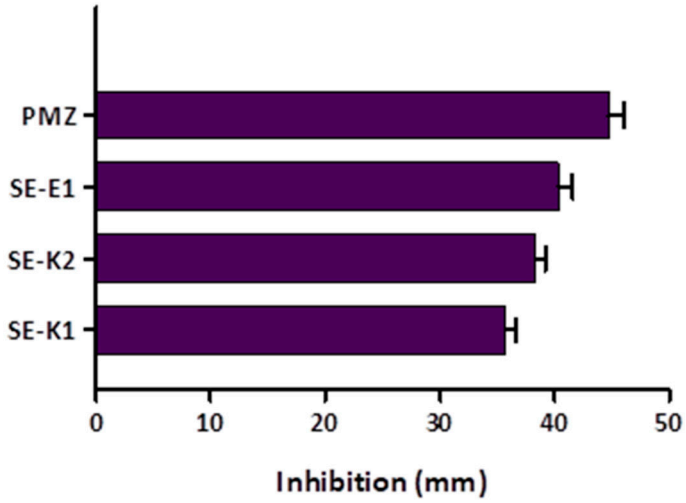


Figure 4. Quorum Sensing (QS) inhibition by selenocompounds. The QS-inhibition assay was performed using the parallel inoculation disk diffusion method. The ineffective compounds are not shown. Promethazine (PMZ) was used as a positive control.

3.6. Cytotoxicity Assay on Normal Human Fibroblasts

In order to determine the toxicity and safety of the selenocompounds on human cells, a cytotoxicity assay was performed using normal MRC-5 human embryonal lung fibroblast cells (Table 4).

Table 4. Cytotoxic activity of selenocompounds on MRC-5 human embryonal fibroblast cells, expressed in Inhibitory Concentration 50 (IC_{50}) and with the calculated standard deviation (SD).

Compound	MRC-5	
	IC_{50} (μM)	SD ±
Se-K1	0.54	0.00
Se-K2	1.34	0.16
Se-K3	0.74	0.04
Se-E1	77.91	15.86
Se-E2	>100	–
Se-E3	76.61	9.18
Se-C1	>100	–
Se-C2	>100	–
Se-C3	>100	–

Based on the data obtained, ketone selenoesters **Se-K1**, **Se-K2**, and **Se-K3** presented high toxicity on normal cells (IC_{50} between 0.5 and 1.5 μM). Fortunately, the methylcarbonyl selenoesters (**Se-E1**, **Se-E2**, and **Se-E3**) and the cyano selenoesters (**Se-C1**, **Se-C2**, and **Se-C3**) showed no toxicity on normal cells as all their IC_{50} values were above 75 μM.

4. Discussion

In case of MIC determination, the symmetrical selenoesters evaluated herein (whose selenium-bound alkyl moiety contains functional groups as a ketone, oxygen ester or nitrile) were more effective on sensitive and resistant *S. aureus* strains compared to the four *S.* Typhimurium bacterial strains. This suggests that these symmetrical selenoesters are more active against Gram-positive bacteria (as *Staphylococcus aureus*) than against Gram-negative bacteria (as *Salmonella enterica* serovar Typhimurium). This fact is in accordance with the antibacterial activity of non-symmetrical selenoesters, which were evaluated in a previous work of the group [27]; only three non-symmetrical ketone selenoesters

(**9–11** in [27]) were active against *S. aureus*, whereas none of them were active against *Escherichia coli*. Interestingly, all of them were active against *Chlamydia trachomatis* (Gram-negative), but since *Chlamydia* is an intracellular bacterium this may affect its sensitivity to the compounds [27].

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 MRSA strain. Comparing the antibacterial activity with the previously reported data [27], two observations are of interests. First, the symmetrical selenoesters are more potent antibacterials against *S. aureus* ATCC 25923 than the respective asymmetrical derivatives. This is observed when we compare the 0.39 μM MIC values of **Se-K1**, **Se-K2**, and **Se-K3** with the 3.12 μM MIC value of **9** in [27] (methylketone selenoesters), and the 12.5 μM MIC values of **Se-E1**, **Se-E2** and **Se-E3** with **7** in [27], which was not active at concentrations below 100 μM (methyloxycarbonyl selenoesters). Second, the symmetrical methyl selenoesters **2–5** in [27] were not active against *S. aureus* ATCC 25923 (MIC > 100 μM), whereas all the functionalized selenoesters evaluated in this work (-CH_2COCH_3, -CH_2COOCH_3, -CH_2CN) showed MIC values against this strain at 12.5 μM or lower. This indicates that these second-generation selenoesters have improved antibacterial activity compared with those that have been previously reported.

If we compare the antibacterial activity of the symmetrical selenocompounds with its toxicity against MRC-5 normal embryonal lung fibroblast cell line, we observe that the MIC values of the compounds against *S. aureus* ATCC 25923 were lower than the IC_{50} values against this cell line.

In the resistance modulation assay, the selenocompounds were tested at $\frac{1}{2}$ of their MIC in combination with tetracycline and ciprofloxacin in the two *S. aureus* strains (ATCC 25923 and MRSA 272123). As mentioned previously, all compounds were able to modulate the activity of tetracycline against *S. aureus* MRSA 272123. The results were somehow comparable with the antibacterial activity. Interestingly, the –CH_2COOCH_3 and –CN containing symmetrical selenoesters were more potent modulators than the –CH_2COCH_3 selenoesters (X-fold reductions of 2–4, 8–32, and 4–32, respectively). However, as MIC values of the selenocompounds were higher against this *S. aureus* strain, only **Se-C1** and **Se-C2** could be used at a safe concentration (25 μM, non-toxic in MRC-5 cells) with a noteworthy effect (16- and 32-fold reduction of MIC value of tetracycline).

Real-time EB accumulation was applied in order to monitor the EPI activity of the compounds. The intracellular EB accumulation was the highest on the *tolC* gene inactivated mutant *S.* Typhimurium SE39, and the lowest EB 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*, and Δ*tolC*) mutant *S.* Typhimurium strains due to efflux independent mechanisms, e.g., membrane destabilizing effect. In addition, methyloxycarbonyl selenoester **Se-E3** showed significantly effective pump inhibition on sensitive ($p < 0.001$) and resistant ($p = 0.001$) *S. aureus* strains. Unfortunately, these two Se-compounds have to be applied at a high concentration (50 μM, which is $\frac{1}{2}$ of their MIC) against *S.* Typhimurium (**Se-E2**) or *S. aureus* MRSA 272123 (**Se-E3**), respectively. Compound **Se-E3** could be used in this application against *S. aureus* ATCC 25923, as in this case its concentration would be 6.25 μM, much lower.

Regarding the anti-biofilm effect, the methyloxycarbonyl selenoester **Se-E3** showed significant biofilm inhibition on both of sensitive and resistant *S. aureus* strains. Furthermore, the methylketone selenoester **Se-K3** was the most effective anti-biofilm agent on resistant *S. aureus* MRSA. In addition, **Se-K1** was also interesting, as it showed a biofilm inhibiting effect higher than 50% against MRSA. It was surprising that **Se-K2** promoted the biofilm formation of *S. aureus* MRSA, because it has the same chemical formula as **Se-K1** (both are 2-oxopropyl selenodiesters); they only differ in the substitution pattern at the phenyl ring, such that **Se-K1** has a *para* substitution (1,4) and **Se-K2** has a *meta* substitution (1,3). It is interesting to see how such a small change in the substitution pattern at the core phenyl ring leads to completely different activities. What is more, in **Se-K2** the inclusion of a third –$COSeCH_2COCH_3$ at the position five of the core phenyl ring led to **Se-K3**, recovering

the biofilm inhibition in respect to **Se-K2** and enhancing it in respect to **Se-K1**. In the case of the methyloxycarbonyl selenoesters, only the trisubstituted derivative **Se-E3** was capable of significantly inhibiting the biofilm formation in both strains of *S. aureus* (reference and MRSA), whereas the two disubstituted ones were inactive. Methylcyano selenoesters showed a lower inhibition than the other two families of compounds, however, one of them (the *para*-disubstituted (**Se-C1**)) was close to exerting a 50% inhibition of *S. aureus* MRSA.

Finally, QS inhibiting effect of compounds was evaluated based on the inhibition of violacein production. The methylketone selenoester **Se-K1** and **Se-K2** and the methyloxycarbonyl selenoester **Se-E1** were potent QS-inhibitors, with **Se-E1** being the most effective QS inhibitor of these three derivatives by showing an inhibition close to the reference promethazine (positive control).

All these findings reveal that the symmetrical selenoesters have a potent antibacterial activity, mainly against *S. aureus* strains. Furthermore, the methylcyano selenoesters could be used as potential novel antibiotics. Additional studies to evaluate the ADME-Tox properties of these compounds is needed to evaluate their applicability in medicine more in-depth. Besides, the methylketone selenoesters, which are less selective, still could be used, for example, in disinfection of surfaces or in the coating of surfaces to prevent biofilm formation.

5. Conclusions

It can be concluded that all the symmetrical selenoesters evaluated have a potent antibacterial activity against *S. aureus* ATCC 25923. The most potent derivatives were the methylketone selenoesters (**Se-K1**, **Se-K2**, and **Se-K3**), followed by the cyano selenoesters (**Se-C1**, **Se-C2**, and **Se-C3**), and at the end by the methyloxycarbonyl selenoesters (**Se-E1**, **Se-E2**, and **Se-E3**). After determining the toxicity on normal fibroblasts, the more selective ones were the cyano selenoesters, followed by the methyloxycarbonyl selenoesters, and the ones by the methylketone selenoesters. Combining both the antibacterial activity and the cytotoxic activity, the most promising compound against *S. aureus* ATCC 25923 was **Se-C3**. The tested selenocompounds also showed antibacterial activity against *S. aureus* MRSA 272123 and against different strains of *S.* Typhimurium, although with higher MIC values.

In addition to the antibacterial activity, the methyloxycarbonyl selenoesters and two cyano selenoesters showed strong resistance reversing activity in the presence of tetracycline against the MRSA strain. Additionally, the methyloxycarbonyl selenoester **Se-E3** was the most effective compound concerning the reversal of resistance, efflux pump inhibition, and anti-biofilm activity on *S. aureus* strains.

6. Patents

This work explores the antibacterial activity of compounds covered by the patent EP18382693 [21] (filed on 28 September 2018 by Enrique Domínguez-Álvarez, Gabriella Spengler, Claus Jacob and Carmen Sanmartín) more in-depth.

Author Contributions: G.S. conceived and designed the study. A.G.-P., M.B.-L., and E.D.-Á. synthesized the selenocompounds used in the study. M.N., B.S., B.R., and A.K. performed the laboratory work. M.N., G.S., and E.D.-Á. wrote the article. J.M.A.B. revised the manuscript critically. All authors read and approved the final manuscript.

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III.

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Phenothiazines and Selenocompounds: A Potential Novel Combination Therapy of Multidrug Resistant Cancer

MÁRIÓ GAJDÁCS[1,2], MÁRTA NOVÉ[1], ÁKOS CSONKA[1,3], BORISZ VARGA[1,4], CARMEN SANMARTÍN[5,6], ENRIQUE DOMÍNGUEZ-ÁLVAREZ[7*] and GABRIELLA SPENGLER[1*]

[1]*Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Szeged, Hungary;*
[2]*Department of Pharmacodynamics and Biopharmacy, Faculty of Pharmacy, University of Szeged, Szeged, Hungary;*
[3]*Department of Traumatology, Faculty of Medicine, University of Szeged, Szeged, Hungary;*
[4]*Department of Rheumatology and Immunology, Szeged, Hungary;*
[5]*Department of Pharmaceutical Technology and Chemistry, School of Pharmacy and Nutrition, University of Navarra, Pamplona, Spain;*
[6]*Instituto de Investigación Sanitaria de Navarra (IdiSNA), Pamplona, Spain;*
[7]*Instituto de Química Orgánica General, Consejo Superior de Investigaciones Científicas (IQOG-CSIC), Madrid, Spain*

Abstract. *Background/Aim: Phenothiazines constitute a versatile family of compounds in terms of biological activity, which have also gained a considerable attention in cancer research. Materials and Methods: Three phenothiazines (promethazine, chlorpromazine and thioridazine) have been tested in combination with 11 active selenocompounds against MDR (ABCB1-overexpressing) mouse T-lymphoma cells to investigate their activity as combination chemotherapy and as antitumor adjuvants in vitro with a checkerboard combination assay. Results: Seven selenocompounds showed toxicity on mouse embryonic fibroblasts, while three showed selectivity towards tumor cells. Two compounds showed synergism with all tested phenothiazines in low concentration ranges (1.46-11.25 μM). Thioridazine was the most potent among the three phenothiazines. Conclusion: Phenothiazines belonging to different generations showed different levels of adjuvant activities. All the tested phenothiazines are already approved medicines with known pharmacological and toxicity profiles, therefore, their use as adjuvants in cancer may be considered as a potential drug repurposing strategy.*

Phenothiazines constitute a versatile family of compounds in terms of biological activity: though they were widely used, the interest towards these agents grew tremendously after promethazine (an antihistaminic agent) and chlorpromazine (an antipsychotic drug) were discovered in the 1940s (1). Since then, a wide variety of related compounds have been discovered and used in psychiatric patients, but recent studies indicate that these drugs could be also utilized in the treatment of other disorders, such as neurodegenerative diseases, tumors, and infections (2). Phenothiazines can inhibit efflux pumps causing multidrug resistance (MDR) (3). This inhibition is suggested by the observation that phenothiazines used as adjuvants in antibiotic therapy can re-sensitize resistant bacteria to certain antibiotics (3). Other studies have also shown that phenothiazines have a direct antibacterial effect, especially against MDR *Mycobacterium tuberculosis* infections, which is a pathogen of considerable morbidity and mortality (4).

Phenothiazines have also gained considerable attention in the field of cancer research (5). Their antitumor activity is exerted through different biological mechanisms, including the aforementioned MDR efflux pump inhibition (5). Several *in vitro* studies show that thioridazine can effectively inhibit P-glycoprotein (or ABCB1), therefore reversing resistance to cancer chemotherapy. This inhibition of MDR efflux pumps enables the reduction of the dosage for chemotherapy drugs without reducing their therapeutic effect, thus reducing their

*These Authors contributed equally to this work.

Correspondence to: Gabriella Spengler, Ph.D., Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Szeged, Hungary. E-mail: spengler.gabriella@med.u-szeged.hu

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side effects (2, 5). Besides, phenothiazines – especially thioridazine and trifluoperazine – can exert antitumor activity due to their capacity to trigger apoptosis through different mechanisms, *e.g.*, the inhibition of DNA protein kinases and DNA repair (6, 7), and inhibition of the binding of calcium to Ca^{2+}-dependent enzymes (8).

Selenium and selenocompounds (Se-compounds) have crucial roles in pivotal biological processes, and there is a growing body of evidence of the potential applications of organoselenium compounds as potent chemopreventive, antiproliferative and cytotoxic drugs (9, 10). Moreover, Se-compounds (Se-compounds) as sodium selenite and selenocystine have the ability to enhance the anticancer effects exerted by chemotherapy drugs currently used in clinical practice, such as cisplatin and doxorubicin, respectively (11, 12). In line with these findings, our group synthesized a series of 30 selenoesters and one cyclic selenoanhydride with marked antiproliferative and/or cytotoxic activity in selected prostate (PC-3), breast (MCF-7), lung (A549) and colon (HT-29) cancer cell lines. These compounds also showed potent chemopreventive activity (13). In subsequent studies, 10 selenoesters and 1 selenoanhydride of the abovementioned 31 Se-compounds were selected for performing a more-in-depth evaluation. Our results showed that they have: (i) a potent cytotoxic activity (even in submicromolar concentrations) in human colon adenocarcinoma and breast cancer cell lines, (ii) capacity to inhibit the ABCB1 efflux pump overexpressed in MDR sublines of these colon / breast cancer cell lines with higher potency than known reference inhibitors like verapamil or thioridazine, and (iii) the ability to trigger apoptosis (14, 15).

Based on these promising results, in the present study, three phenothiazines (namely promethazine, chlorpromazine and thioridazine) were combined with these 11 active Se-compounds against MDR mouse T-lymphoma cells to investigate their activity in combination with chemotherapy and as antitumor adjuvants *in vitro*. In addition, the toxicity of the tested compounds and phenothiazines was studied in non-cancerous NIH/3T3 mouse embryonic fibroblast cells.

Materials and Methods

Cell lines. pHa MDR1/A retrovirus was used to transfect L5178Y mouse T-cell lymphoma cells (PAR; parental cell line) (ECACC Cat. No. 87111908, acquired from the Food and Drug Administration, Silver Spring, MD, USA) as formerly described by Cornwell *et al*. (16). The ABCB1-expressing cell line L5178Y (MDR; multidrug resistant) was selected by culturing the infected cells with colchicine. The L5178Y human *ABCB1*-transfected subline was cultured in McCoy's 5A medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich), 200 mM L-glutamine (Sigma-Aldrich) and a penicillin-streptomycin (Sigma-Aldrich) mixture at concentrations of 100 U/l and 10 mg/l, respectively.

The NIH/3T3 mouse embryonic fibroblast cell line (ATCC CRL-1658) was acquired from LGC Promochem, (Teddington, UK). The cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM, containing 4.5 g/l glucose) supplemented with 10% heat-inactivated fetal bovine serum and a penicillin-streptomycin (Sigma-Aldrich) mixture at 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.

Compounds. Re-synthesis, purification and characterization of cyclic selenoanhydride (**1**) and the ten selenoesters **2-11** included in our study (Figure 1) were performed as described previously (13). All tested compounds were pure and chemically stable on air, according to the spectroscopic (IR, MS, ^{1}H- and ^{13}C-NMR) and the elemental analysis performed to confirm the structures of the different derivatives as reported previously (13). Additionally, four reference compounds **12-15** were obtained from the following providers: potassium selenocyanate (KSeCN) and phthalic anhydride (**15** and **12**, respectively; Sigma-Aldrich, Seelze, Germany), potassium cyanate (KOCN, **13**, Chempur, Piekary Śląskie, Poland), ammonium thiocyanate (NH_4SCN, **14**, Polskie Odczynniki Chemiczne, Gliwice, Poland) (Figure 1) (14). Compounds were dissolved in DMSO to obtain stock solutions. Working solutions were prepared by dilution in water, keeping the concentration of DMSO below 1% in all the experiments.

The remaining chemicals used in the study were purchased from Sigma-Aldrich, (St Louis, MO, USA). These were: promethazine (PMZ), chlorpromazine (CPZ), thioridazine (TZ), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulphate (SDS) and dimethyl sulfoxide (DMSO) (Figure 2). All solutions were prepared on the day of the assay.

Assay for cytotoxic effect. The effects of increasing concentrations of the phenothiazines alone on cell growth were tested in 96-well microtiter plates. The parental and multidrug resistant mouse T-lymphoma cells were cultured using McCoy's 5A medium supplemented with 10% heat-inactivated horse serum. The density of the cells was adjusted to 1×10^4 cells per well (in 100 μl of medium per well) and then added to the 96-well flat-bottomed microtiter plates containing the dilutions of the tested Se-compounds. The cells were incubated at 37˚C, in a 5% CO_2, 95% air atmosphere. The cytotoxic activity of the Se-compounds was previously determined on parental and multidrug resistant mouse T-lymphoma cells (17).

The adherent mouse embryonic fibroblast cells were cultured in 96-well flat-bottomed microtiter plates in DMEM supplemented with 10% heat-inactivated fetal bovine serum. The cells were incubated at 37˚C, in a 5% CO_2, 95% air atmosphere. In a separate plate, the respective dilutions of the tested compounds were prepared. The density of the cells was adjusted to 1×10^4 cells per well and the cells were seeded for 4 h at 37˚C, 5% CO_2. Then, the medium was removed and the cells were incubated with the various compounds at 37˚C for 24 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. 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 (14, 15, 18). Cell growth was determined by measuring the optical density (OD) at 540/630 nm with Multiscan

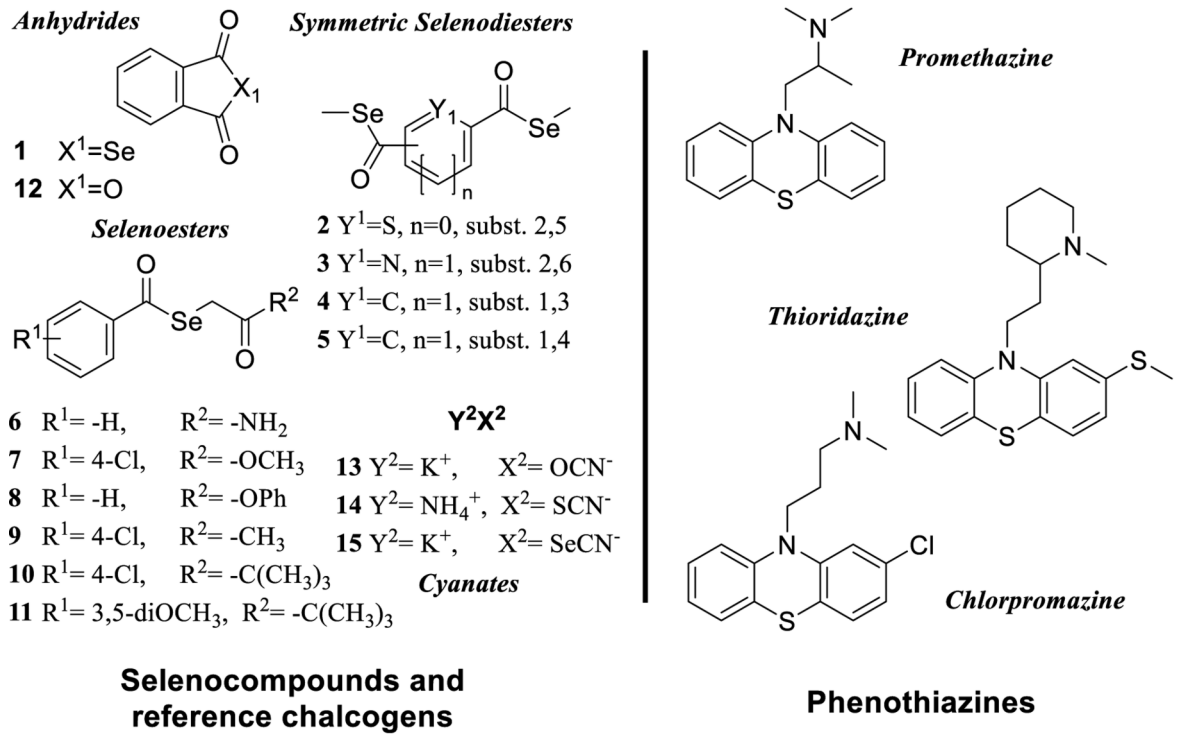

Figure 1. *Structure of the Se-compounds (**1-11**), reference chalcogens (**12-15**) and phenothiazines evaluated in this study.*

EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Inhibition of the cell growth was determined according to the formula below:

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

Checkerboard combination assay. A checkerboard microplate method was applied to study the effect of drug interactions between the Se-compounds (**1-11**), the reference chalcogens (**12-15**) and the phenothiazines (PMZ, CPZ and TZ). The assay was carried out using multidrug-resistant mouse T-lymphoma cells overexpressing the ABCB1 transporter (15). The dilutions of phenothiazines were made in the direction from left to right in 100 µl (stock solutions and final concentrations used in the assay are presented in Table I), and the dilutions of the Se-compounds from the top to bottom 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. The cells were incubated for 72 h. At the end of the incubation period the cell growth rate was determined by the MTT assay as described above. The combination index (CI) values at 50% of the growth inhibition dose (ED_{50}) were determined using CompuSyn software (ComboSyn Inc., Paramus, NJ, USA) to plot four to five data points to each ratio (19). 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 (19).

Table I. *Cytotoxicity of the tested compounds against NIH/3T3 mouse embryonic fibroblast cells and selectivity indices (SI).*

	NIH/3T3 A - IC_{50} (µM)	SI	
		A/B (31)	A/C (31)
1	>100	**≥25**	≥22
2	23.7	0.24	0.24
3	>100	*5.1*	*5.9*
4	>100	≥1.0	≥1.0
5	>100	≥1.0	≥1.0
6	69.7	≤0.70	≤0.70
7	23.7	≤0.24	≤0.24
8	74.5	≤0.75	≤0.75
9	0.62	0.81	0.60
10	1.35	1.44	*3.14*
11	0.82	0.63	0.85
12	>100	≥1.0	≥1.0
13	>100	≥1.0	≥1.0
14	>100	≥1.0	≥1.0
15	>100	≥1.0	≥1.0

PMZ: Promethazine; CPZ: chlorpromazine; TZ: thioridazine; B: IC_{50} of the tested compounds against parental (PAR) mouse T-lymphoma cells (14); C: IC_{50} of tested compounds against multidrug resistant (MDR) mouse T-lymphoma cells (14); SI: Selectivity Index; SI<1 values denote lack of selectivity, 1<SI<3 mean a slight selectivity and 3<SI<6 values indicate moderate selectivity and are signalled with italics; whereas values of SI<6 indicate that the compounds are strongly selective and are highlighted in bold (14, 19).

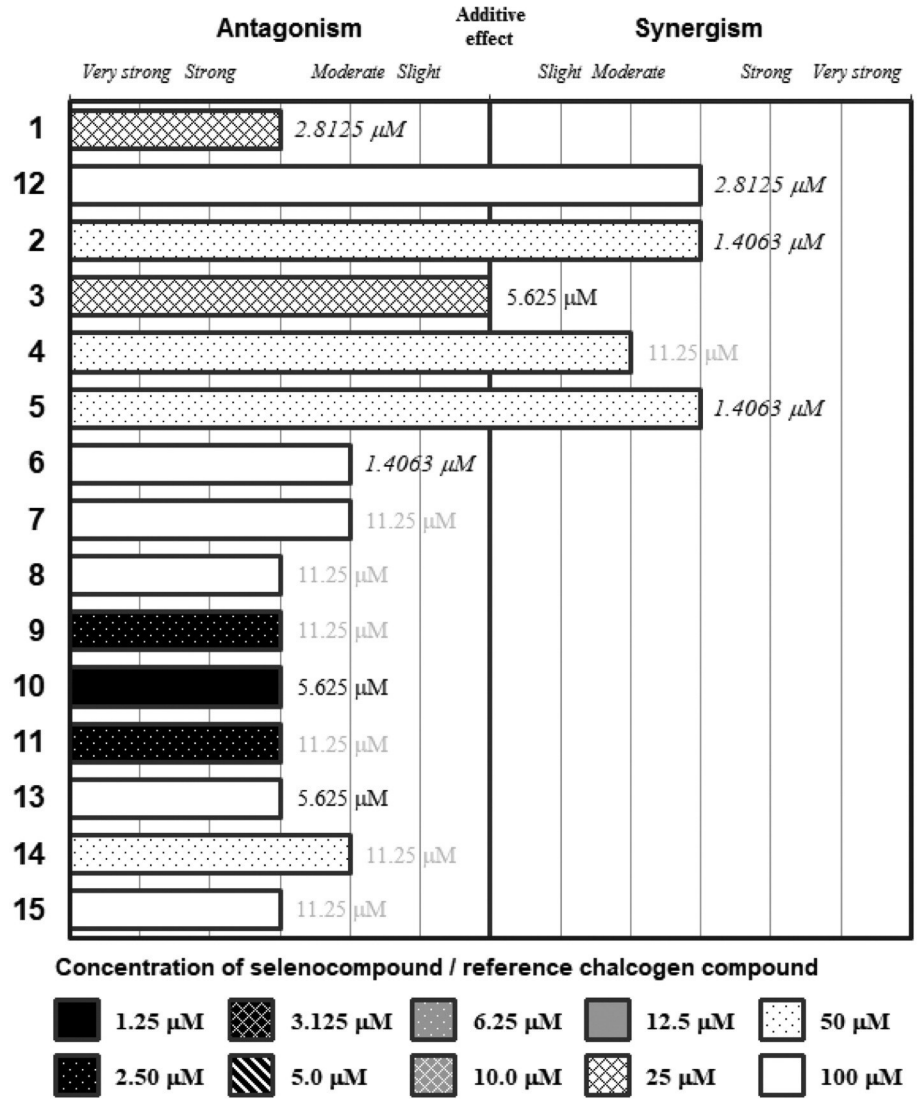

Figure 2. *Interactions of the Se-compounds **1-11** and the reference chalcogens **12-15** with promethazine. The concentration of the tested (O/S/Se)-compounds are presented below the graph, the concentration of promethazine is given inside graph (in bold: submicromolar concentration, in italics concentration between 1 and 5 μM, in plain text concentration between 5 and 10 μM, in grey concentration higher than 10 μM).*

Results

The cytotoxic activity of the Se-compounds and the reference chalcogens has been previously determined on parental and multidrug resistant mouse T-lymphoma cells (17). The final concentration of the Se-compounds used in the combination experiments was chosen in accordance with our previous results, while the cytotoxicity of the phenothiazines was assessed before performing the checkerboard combination assay to determine their ideal concentrations for these experiments (the concentrations of the stock solutions used and the final concentrations are presented in Table I). Using the NIH/3T3 mouse embryonic fibroblast cell line, the toxicity on non-tumoral cells and the selectivity of the Se-compounds and phenothiazines was also assessed and the results were expressed in terms of the selectivity index (SI) values (14, 20). Out of the fifteen tested compounds, seven showed toxicity against the mouse embryonic fibroblasts (namely compounds **2, 6, 7, 8, 9, 10** and **11**) at 100 μM concentrations, while only three showed selectivity (compound **1**: strongly selective, compounds **3** and **10**: moderately selective) towards tumor cells, in agreement with our previous results on murine lymphoma cell lines (Table I) (14).

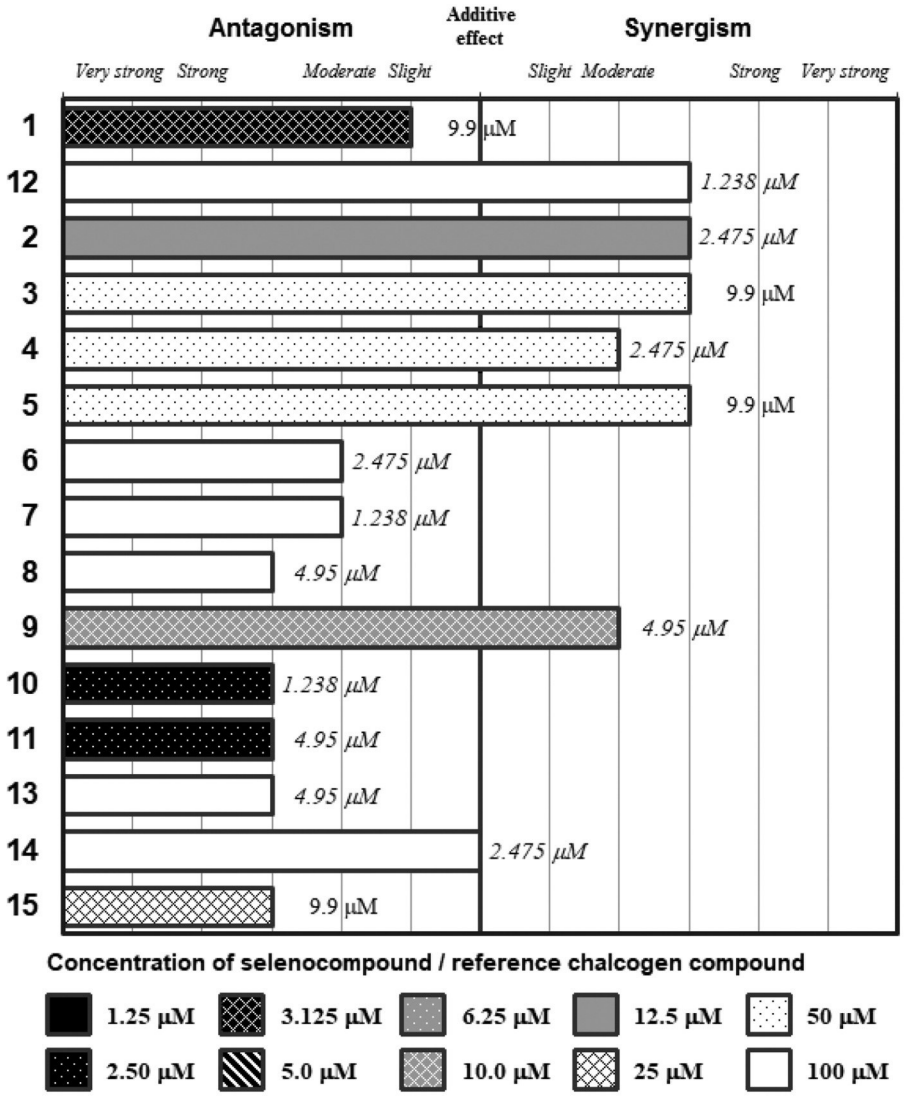

Figure 3. *Interactions of the Se-compounds* ***1-11*** *and the reference chalcogens* ***12-15*** *with chlorpromazine. The concentration of the tested (O/S/Se)-compounds are presented below the graph, the concentration of chlorpromazine is given inside graph (in bold: submicromolar concentration, in italics concentration between 1 and 5 μM, in plain text concentration between 5 and 10 μM, in grey concentration higher than 10 μM).*

The results of the combination experiments of Se-compounds and promethazine, chlorpromazine and thioridazine are shown in Figures 2, 3 and 4, respectively. In addition, the concentrations that showed the most beneficial interactions in these experiments are also highlighted. As a general rule, compounds **2-5** presented with the most advantageous interaction profile (*i.e.* the highest CI scores were observed). The compounds **2** and **5** showed synergism with all tested phenothiazines). This is further highlighted by the fact that these compounds showed synergism with the phenothiazines in low concentration ranges (1.46-11.25 μM). In contrast, compounds **6-8**, and the reference compounds **12-15** mainly exhibited antagonism with phenothiazines (Figures 2-4). The compounds with low IC_{50} values in the present study and previous reports (**1, 9, 10** and **11**) also showed additive or antagonistic interactions with the phenothiazines, with the exception of compound **9**, presenting synergism with chlorpromazine and thioridazine (CI values=0.58-0.82). The comparison of the results between the different phenothiazines indicated a pronounced tendency for decrease for the majority of Se-compounds from promethazine to thioridazine, with CI values going as low as Cl=0.276 in the case of thioridazine (Figures 2-4).

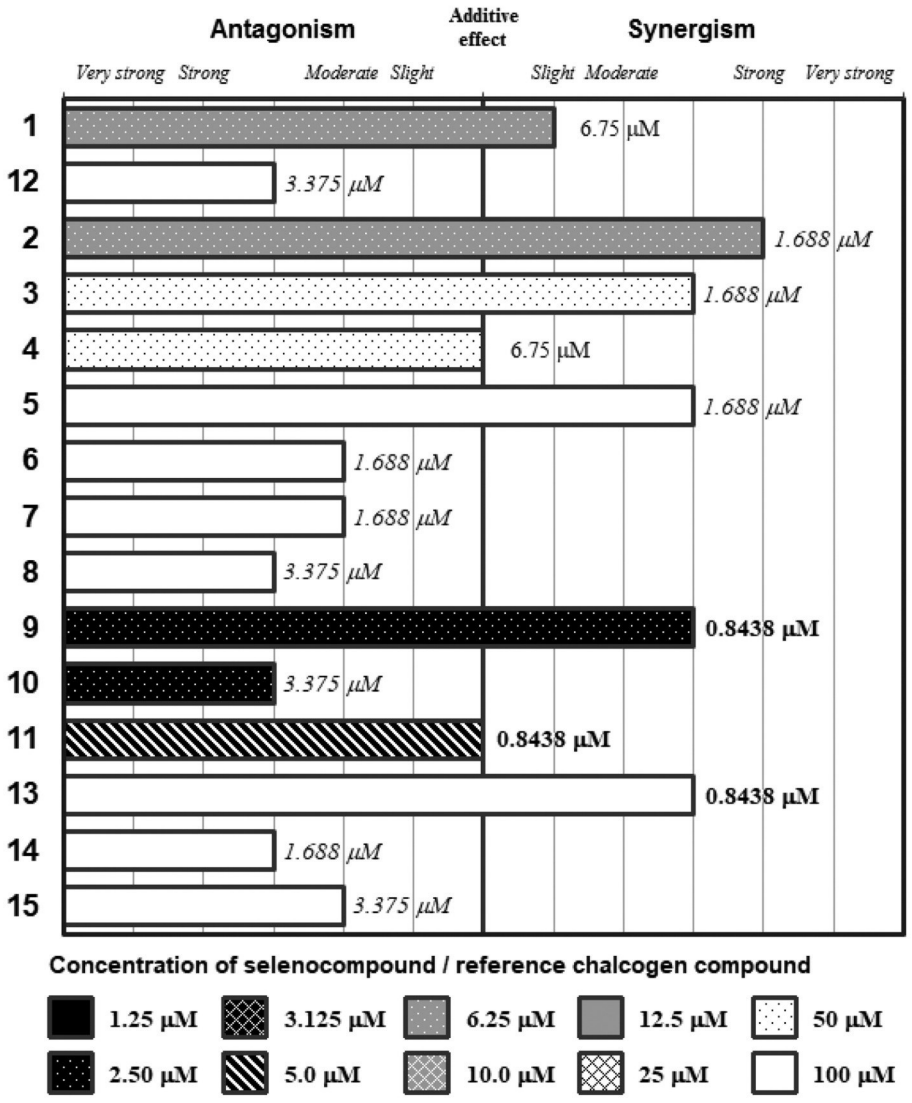

Figure 4. *Interactions of the Se-compounds* ***1-11*** *and the reference chalcogens* ***12-15*** *with thioridazine. The concentration of the tested (O/S/Se)-compounds are presented below the graph, the concentration of thioridazine is given inside graph (in bold: submicromolar concentration, in italics concentration between 1 and 5 μM, in plain text concentration between 5 and 10 μM, in grey concentration higher than 10 μM).*

Discussion

The diversity of biological activities by phenothiazines and related compounds has been highlighted by numerous studies, both in psychopharmacology and in potentially novel therapeutic indications (1-5). The pleiotropy in their biological activities has also been shown in the case of their antipsychotic effects. For instance, they can act as non-selective antagonists of the dopamine receptor, serotonin receptors, histamine receptors, α-adrenergic receptors and acetylcholine receptors (2). Certain phenothiazine compounds are currently used for the treatment of diseases: chlorpromazine as an antipsychotic drug (21); promethazine as antihistaminic; thioridazine as an antipsychotic, antitumor and antibacterial drug; trifluoperazine as a dopamine receptor-antagonist and methylene blue as an antioxidant and as a component of the therapy of cyanide intoxications and neurodegenerative diseases (1, 2, 21).

The interest in Se-compounds as anticancer agents and adjuvants in chemotherapy has increased substantially in the last 15-20 years (1-7, 22). The activity of the present Se-compounds in combination with a selection of chemotherapy drugs has been studied on a MDR mouse T-lymphoma cell line (23). It was found that the anticancer drugs that

interacted in a more synergistic manner with these Se-compounds were vincristine, doxorubicin, cyclophosphamide and methotrexate. In line to the results of this study, compounds **2-5** were shown to be highly effective adjuvants in very low concentrations (especially the thiophene-derivative compound **2**, showing strong synergism with all six tested chemotherapeutic drugs and verapamil). However, the cyclic selenoanhydride (**1**) and the methyl-ketone containing selenoesters (compounds **9-11**) interacted synergistically or antagonistically with the anticancer drugs and phenothiazine evaluated.

In line with previous studies, the inorganic chalcogen salts and the O-isoster of compound **1** also showed no synergistic potential (23). In subsequent experiments in microbial model systems, it has been suggested that the biological activity of Se-compounds is dependent on their degradation into biologically-active species in the presence of oxygen; thus, their potential as anticancer adjuvants may also be mediated by a similar, yet unexplained mechanism (24).

Considering these results, phenothiazines have potential to be used as adjuvants in cancer chemotherapy. Our results suggest that phenothiazines with different chemical modifications showed different levels of adjuvant properties: thioridazine (a second-generation phenothiazine) showed lower CI values in a lower concentration range compared to promethazine and chlorpromazine (first-generation phenothiazines). However, the presence of the chlorine atom in position 2 of chlorpromazine was previously shown to enhance its biological activities, therefore, it is unsurprising that these compounds showed more potent activity in our assays than the parental compound phenothiazine (4). The tested phenothiazines are all already approved medicines with known pharmacological and toxicity profiles, therefore, their use as adjuvants in cancer may be considered as a potential drug repurposing strategy. The synthesis of novel chemical compounds/libraries with phenothiazine-related structures and their biological screening as antitumor adjuvants may lead to potent lead compounds, capable of becoming relevant molecules for testing in animal experiments and human clinical trials.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

G.S., E.D.A. and M.G. conceived and designed the study. M.G. and M.N. performed the laboratory work. M.G., B.V., Á.C., G.S., E.D.A. wrote the article and prepared the graphs and tables. C.S. and E.D.A. revised the manuscript critically. All Authors read and approved the final manuscript.

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