SELENOCOMPOUNDS AS NOVEL ANTICANCER AGENTS AGAINST RESISTANT BREAST CANCER AND ANTIBACTERIAL AGENTS IN *CHLAMYDIA TRACHOMATIS* D

Ph.D. Thesis

Andrea Csonka, M.D.

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I. PUBLICATIONS

1. PUBLICATIONS RELATED TO THE THESIS

I. **Csonka A**, Kincses A, Nové M, Vadas Z, Sanmartín C, Domínguez-Álvarez E, Spengler G. Selenoesters and Selenoanhydrides as Novel Agents Against Resistant Breast Cancer. Anticancer Res. 2019 Jul;39(7):3777-3783. doi: 10.21873/anticanres.13526. PMID: 31262904.

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II. Mosolygó T, Kincses A, **Csonka A**, Tönki ÁS, Witek K, Sanmartín C, Marć MA, Handzlik J, Kieć-Kononowicz K, Domínguez-Álvarez E, Spengler G. Selenocompounds as Novel Antibacterial Agents and Bacterial Efflux Pump Inhibitors. Molecules. 2019 Apr 16;24(8):1487. doi: 10.3390/molecules24081487. PMID: 31014009; PMCID: PMC6514980.

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2. PUBLICATIONS NOT RELATED TO THE THESIS

I. Lentz F, Reiling N, Spengler G, Kincses A, **Csonka A**, Molnár J, Hilgeroth A. Dually Acting Nonclassical 1,4-Dihydropyridines Promote the Anti-Tuberculosis (Tb) Activities of Clofazimine. Molecules. 2019 Aug 8;24(16):2873. doi:10.3390/molecules24162873. PMID: 31398786; PMCID: PMC6720424.

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IF:0.869

TOTAL IF: 6.001

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II. LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ABCB1	ATP-binding cassette transporter B1 subfamily
ABs	aberrant bodies
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma-2 gene
BRCA	breast cancer susceptibility gene
CDH1	Cadherin-1
CHEK2	Checkpoint kinase 2 gene
CI	combination index
DDR	DNA damage response
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
doxorubicin	doxorubicin hydrochloride
EB	elementary body
EBS	ebselen
ED50	median effective dose
EMEM	Eagle' s minimal Essential medium
ER+/-	estradiol receptor positive /negative
EU	European Union
FAS	first apoptosis signal
FBS	foetal bovine serum
IGF 1	Insulin-like growth factor
LSP	lipopolysacharide
M627	12*H*-benzo[α]phenothiazine
MBC	metastatic breast cancer
MDR	multidrug resistance
miRNA	micro ribonucleic acid
MRI	magnetic resonance imaging
MRSA	methicillin resistant *Staphylococcus aureus*

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAATs	nucleic acid amplification tests
OD	optical density
p53	tumor suppressor protein p53
PALB2	partner and localizer of BRCA2 gene
PBPs	penicillin-binding proteins
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
P-gp	permeability glycoprotein
PTEN	Phosphatase and tensin homolog gene
RAD51C	DNA Repair Protein RAD 51 gen
RB	reticulate body
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
Se	selenium
SeNP	selenium nanoparticles
SI	selectivity index
SPG	sucrose-phosphate-glutamic acid buffer
STK11	Serine/threonine kinase 1
UV	ultraviolet
WHO	World Health Organization

III. INTRODUCTION

A. *EPIDEMIOLOGY OF BREAST CANCER*

The burden of the incidence and mortality of cancer is growing rapidly worldwide. It reflects both aging and growth of the population, as well as changes in the prevalence and distribution of the main risk factors for cancer, several of which are associated with socioeconomic development [1,2]

Breast cancer is an important health problem with an increasing global trend of prevalence and mortality rate [3,4]. It is the most frequently diagnosed neoplastic disease in women and one of the most important causes of death among them [3].

The incidence rate of breast cancer is estimated to reach 3.2 million by 2050 [4]. It is a major public health problem; therefore, it is alarming to note that GLOBOCAN statistics for 2020 show that breast cancer has surpassed lung cancer as the most frequently diagnosed cancer, with an estimated 2.3 million new cases (11.7 %), followed by lung cancer (11.4 %), colorectal cancer (10.0 %), prostate cancer (7.3 %), and stomach cancer (5.6 %). Lung cancer remained the leading cause of cancer deaths with an estimated 1.8 million deaths (18 %), followed by colorectal (9.4 %), liver (8.3 %), stomach (5.6 %), and female breast (6.9 %) cancers [5].

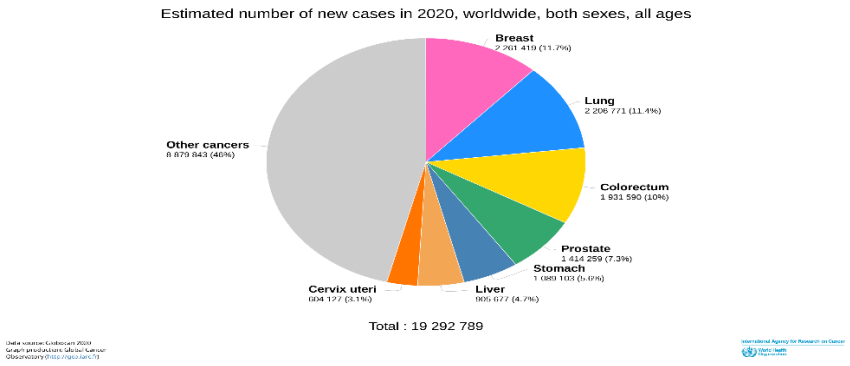


Figure 1. Estimated number of new cases in 2020, worldwide, both sexes, all ages [5]

Lung cancer is the most frequently occurring cancer and the leading cause of cancer deaths in men, followed by prostate and colorectal cancers for incidence and liver and colorectal cancers for mortality. In women, breast cancer is the most commonly diagnosed cancer and the leading cause of cancer deaths, followed by colorectal and lung cancers for incidence, and vica versa for mortality [5]. Among the total cancer cases worldwide, breast cancer incidence represented about 11 % in 2008, while this number elevated to 15.5 % in 2020 [5].

The epidemiological cancer burden of Hungary is among the highest in Europe [6] including the highest cancer-related overall mortality in men. Among 40 European countries, the incidence of lung and bronchial cancers, colon and rectal cancers, and oral cavity–pharyngeal cancers is the highest in Hungary in both males and females [7].

In the year 2020, a total of 66 874 new cancer cases were diagnosed in Hungary, 33 711 cases in men, and 33 163 cases in women. This number may be translated to approximately 183 new cancer cases diagnosed per day. In men, lung cancer, colorectal cancer, and prostate cancer accounted for 52 % of all cases. In women, breast, colorectal, and lung cancers accounted for 49.2 % of all cases diagnosed, and breast cancer alone was responsible for 22.8 % of the cases [8].

According to GLOBOCAN, 7565 new cases of breast cancer were recorded in women in Hungary with 2195 deaths in 2020 [8].

Etiology of breast cancer

The incidence, mortality, and survival rates of breast cancer vary considerably in various parts of the world, which is influenced by a number of factors, such as population structure, lifestyle, genetic factors, and environment [9].

Studies in recent years have shown that 20–30 % of newly diagnosed breast cancer cases are associated with a number of endogenous and exogenous factors that initiate or modify the process of tumor cell transformation. However, in 75–80 % of the affected women, no risk factors can be identified for developing breast cancer [10].

All the risk factors can be divided into two major groups. The first group would include inherent or endogenous factors, such as age, sex, race, and genetic makeup promoting familial occurrence of neoplastic diseases or the occurrence of benign proliferative lesions of the mammary gland. The second group would include extrinsic factors conditioned by lifestyle, diet, stress, or long-term medical interventions [10].

Inherent factors

The most significant risk factors are female gender, advanced age, and race. Accordingly, the disease is most frequently diagnosed in women around menopausal age, in their 50s [10,11].

A correlation can be observed between the age when the neoplastic disease is diagnosed and the expression of the estrogen receptor found in the examined tumor tissue. Neoplasms showing estrogen receptor overexpression ER (+) are characterized by a frequency increasing with age, as opposed to ER (–) tumors that occur more frequently up to 50 years of age and then reach a plateau. This phenomenon might explain an increased percentage of ER (+) tumors diagnosed in women after menopause [12].

Less than 1 % of all diagnosed breast cancers develop mainly in older men who have had hormonal imbalance, exposure to radiation, and hereditary factors [13].

Breast cancer develop mostly in white but non-Hispanic black, Asian American/Pacific Islander, Native American, and Hispanic women have higher proportions of invasive breast cancers at younger ages and at advanced stages and breast cancer deaths at younger ages than non-Hispanic white women[12,14].

Genetic factors may account for 5–10 % of breast cancer cases. Among BRCA mutations, 55–65 % of the carriers are of the BRCA1 mutation, and 45 % of the carriers of the BRCA2 mutation are by the age of 70 years. [3]. Other gene mutations that are significantly involved in the neoplastic transformation include genes, such as CHEK2, PTEN, PALB2, RAD51C, CDH1, STK11, p53, or genes determining Lynch Syndrome [3].

Family history without genetic predispositions is still an important risk factor even with relatives with an older age at diagnosis [15].

Women with mammographically dense breast tissue have 4 to 5 times as high risk of breast cancer as women of similar age with no dense breast tissue. The association between benign breast disease and breast density and the incident breast cancer was stronger among women with positive family history [15]. Whereas, the association between benign breast disease and breast cancer depends on the histological classification and family history of breast cancer[16].

Extrinsic factors

The cumulative number of periods is important because higher estrogen levels may increase the risk. Among them, menarche before the age of 12, the age of menopause over 50 years [3], being nulliparous [17], older age at first childbirth [3], and abortion [17] can increase the risk of breast cancer.

Using exogenous hormones, such as oral contraceptives (only formulation triphasic ethinyl estradiol with levonorgestrel) [18], ovulation-stimulating drugs for more than 6 months [19], postmenopausal hormone replacement therapy [20], using a levonorgestrel-releasing intrauterine device [21], in premenopausal women with elevated serum testosterone levels [22] significantly increases the risk of postmenopausal breast cancer.

Additional modifiable risk factors involve some lifestyle factors, such as obesity [23,24], smoking [24], high-fat diet [25], alcohol [26], deficiency of 25-hydroxicholecalciferol [27], low physical activity [28], high socioeconomic status [29], chest irradiation at a young age [30], night-shift work [31], and high IGF-1 among nondiabetic premenopausal and postmenopausal women [32] can result in high incidence of developing breast cancer.

The protective factors that may reduce breast cancer risk are long-term breastfeeding [33], physical activity [28], meal products containing a large amount of anti-oxidants, such as vitamin E [34] and Mediterranean diet [35].

Diagnosis of breast cancer

Cancer prevention is currently the most important strategy in the fight against breast cancer. Primary prevention includes the elimination of risk factors of developing breast cancer, such as inappropriate life style and environmental factors. Secondary prevention is a group of interventions leading to the discovery and control of cancerous or precancerous processes such as screening for early detection, i.e. regular mammography screening, ultrasonography, MRI, or breast self-examination [36]. For the organized mammography screening program, women aged 45–65 years have been invited every 2nd year since 2001 [37]. Additionally, core needle biopsies of suspicious breast findings are preferred over surgical biopsies [38], and a positive histological result is followed by further examinations and interventions, such as surgery or oncology care.

Therapy of breast cancer

If breast cancer is diagnosed in an early phase, the goal of the treatment is to cure the patient and prevent the risk of relapse of the condition. Therefore, a more aggressive approach is applied in the treatment in order to achieve this goal in the form of long-term survival, without signs of relapse, and without the existence of the disease [39]. Metastatic breast cancer (MBC) is still an incurable disease; therefore, the goal of treating MBC is to achieve disease control, as long survival as possible, with preserved quality of life, which should not be inadmissibly impaired [40]. The most commonly used methods of primary treatment of early breast cancer include local methods, such as surgical treatment and radiation. In the early forms of the disease (tumors of lower stage), radical interventions can replace sparing surgical treatment and radiation, thus achieving the same effect [41]. The most commonly used methods of systemic treatment are chemotherapy, antihormone therapy, targeted biological therapy, and more recently, immunotherapy [42]. The therapeutic approach is determined by the prevalence of the disease itself. If the tumor is found only in the breast, primary and definitive surgical treatment is usually used, and then, depending on the histological findings, additional (adjuvant) protective treatment with radiotherapy on the breast, and/or systemic chemotherapy, antihormone therapy, or targeted therapy is applied, depending on the biological profile of the tumor [39,43].

The neoadjuvant approach or the application of systemic therapy before surgery is used for a number of oncological and operative reasons, all of which should ensure better treatment results both in medical and technical terms (live monitoring of tumor response to therapy and achieving a complete response, reducing tumor mass, and enabling sparing surgery). If a complete response of the tumor to the applied systemic therapy is achieved, it is a proven sign of better survival [44].

B. DRUG RESISTANCE OVERVIEW

Drug resistance is a generally-known and widespread fact that develops when diseases become resistant to medical treatment. This impression was first detected when bacteria became resistant to antibiotics, but since then, similar mechanisms have been found in cancer diseases as well. There are many similar mechanisms between bacterial communities and tumor cells in their ability to win against the drugs designed to eliminate them, or they become less sensitive to the drug. But there are differences in the mechanisms as well. On the one hand, there is more variety in the cellular components that exist in a malignant tissue than a bacterial community. The coordinated interactions between endothelial cells, immune cells, fibroblasts, and epithelial cells are required for the formation of a malignancy and the development of drug resistance. Although bacterial cells have specialized functions within a bacterial community, the variety of cellular components is not so pronounced as in tumors. On the other hand, there are differences in the complexity of the two genomes. The mammalian genome has evolved sophisticated epigenetic controls that do not necessarily exist in the regulation of bacterial gene expressions [45].

Cells surrounding a tumor, such as fibroblasts, immune cells, and endothelial cells, are part of a tumor tissue, and they develop together with cancer cells. For example, stromal cells such as fibroblasts associated with cancerous tissues increase extracellular matrix production [46]. The increased matrix deposition reduces the effectiveness of chemotherapeutic drugs to penetrate a tumor [47–49]. In addition, the reduced amount of oxygen and nutrients reaching the center of a tumor in a similar way as the biofilm does for bacteria.

Another similarity to biofilms is that tumor cells may also switch to a fermentation pathway in the absence of oxygen; anaerobic glycolysis allows cells to produce adenosine triphosphate (ATP), but it accidentally leads to the acidification of the tumor microenvironment through the release and fermentation of lactate [50].

Many types of cancers are initially responsive to chemotherapy, with time they can become resistant through these and other mechanisms, such as DNA mutations and metabolic changes that promote drug inhibition and degradation. The most important factor of bacterial and cancer drug resistance is assigned to the multidrug resistance (MDR) efflux transporter proteins removing toxic materials and drugs out of the cells. These primary transporters derive their energy from the hydrolysis of ATP [51].

Drug resistance in tumors and in bacteria can be of intrinsic or of acquired origin [51]. Intrinsic resistance can be detected right as the tumors and bacteria fail to respond to the first-line agents, whereas acquired resistance is often observed when the malignancy and bacterial infection respond favorably to the initial treatment. However, on recurrence, the same therapeutic regimen has little or no effect [52].

MDR mechanisms are the following: limiting uptake of the drug, modification of a drug target, drug inactivation, overexpression of efflux pump proteins (it can occur only in cancer cells), inhibition of programmed cell death (apoptosis), enhancement of DNA repair, gene amplification, epigenetic alteration, and micro- ribonucleic acid (miRNA) in cancer drug resistance. It is not uncommon for cancer cells to be resistant to cytotoxic drugs because of multiple mechanisms present at the same time [53,54].

During drug inactivation, the metabolic activation of anticancer or antibiotic agent will be inhibited *in vivo*, and this can contribute to drug resistance. Drug inactivation happens by the cytochrome P450 system, glutathione S-transferase superfamily, and the uridine diphospho-glucuronosyltransferase superfamily [55].

Targeted therapies can block the growth of cancer cells by inhibiting the activity of specific target proteins involved in tumor development, thus being more selective and effective to cancer cells and less harmful to normal cells compared to traditional chemotherapy.

However, targeted therapy may also trigger the problem of resistance because of the alteration of drug targets. The alteration of drug targets may be either a secondary mutation in the target protein or changes in the expression levels due to epigenetic alterations. Quantitative or qualitative changes in these enzymatic targets can compromise drug efficacy. These changes have been demonstrated in several enzymes associated with drug resistant cells, including dihydrofolate reductase [56], thymidylate synthase [57], and topoisomerases I and II [58,59].

One of the most important and well-studied mechanisms of cancer drug resistance involves reduced drug accumulation by enhanced efflux because of the members of the ATP-binding cassette (ABC) transporter family proteins. ABC transporters are transmembrane proteins, which aim to transport a variety of substances across the cellular membranes. The transporter's structure varies from protein to protein, e.g., there are 49 known members of the ABC family in humans. These transporters are classified according to the presence of two distinct domains: a highly conserved nucleotide binding domain and a more variable transmembrane domain. When a given substrate binds to the transmembrane domain, ATP hydrolysis at the nucleotide binding site drives a conformational change that pushes the substrate out of the cell [60]. While efflux via ABC transporters is a normal physiological process, it is also a known mechanism of drug resistance in cancer cells. Three transporters, multidrug resistance protein 1 (MDR1) or ABCB1 (P-glycoprotein, P-gp), multidrug resistance-associated protein 1 (MRP1) or ABCC1 (ATP-binding cassette transporters), and breast cancer resistance protein (BCRP) or ABCG2 (ATP-binding cassette superfamily G member 2) are implicated in many drug resistant cancers. All three transporters have broad substrate specificity and are able to efflux many xenobiotics from cells, including vinca alkaloids, epipodophyllotoxins, anthracyclines, taxanes, and kinase inhibitors. MDR1 or ABCB1 (P-glycoprotein, P-gp) was the first of these transporters that was identified and has been studied extensively [61–64].

The repair of damaged DNA has a clear role in anticancer drug resistance in response to chemotherapeutic drugs that either directly or indirectly damage DNA. DNA damage response (DDR) mechanisms can reverse the drug-induced damage. For example, platinum-containing chemotherapeutic drugs such as cisplatin cause harmful DNA crosslinks, leading to apoptosis.

However, resistance to platinum-based drugs often arises due to nucleotide excision repair and homologous recombination and the primary DNA repair mechanisms involved in reversing platinum damage. The efficacy of DNA damaging cytotoxic drugs depends on the failure of the DDR mechanism of cancer cells. Inhibition of repair pathways used in conjunction with DNA damaging chemotherapy could sensitize cancer cells, and therefore increases efficacy of the therapy [65–67].

Inhibitors of apoptosis are a family of proteins that play a significant role in the control of programmed cell death. It is essential to maintain healthy cell turnover within the tissues but also to fight against diseases or infections. Uninhibitedly, inhibitors of apoptosis can suppress apoptosis and promote cell cycle progression. Therefore, cancer cells demonstrate significantly elevated expression levels of inhibitors of apoptosis, resulting in improved cell survival, enhanced tumor growth, and subsequent metastases [68,69].

Gene amplification is also a common basis for resistance to anticancer drugs. The observation that low level cytotoxic stress can cause rapid loss of amplified genes from cultured cell populations suggests that gene amplification may be a potential target for cancer chemotherapy [70].

Methylation and acetylation are two well-studied epigenetic events that are known to profoundly affect the expression of genes, resulting in activation of oncogenes and/or suppression of tumor suppressor genes, leading to the development of epigenetic alteration caused cancer drug resistance [71].

miRNAs are involved in the drug resistance of tumor cells by targeting drug-resistance-related genes or influencing genes related to cell proliferation, cell cycle, and apoptosis. A single miRNA often targets a number of genes, and its regulatory effect is tissue-specific [72].

C. ANTIMICROBIAL RESISTANCE

Infectious diseases are currently significant causes of morbidity and mortality worldwide. In a recent study, it has been reported that 700,000 deaths worldwide were attributed to infections associated with antimicrobial resistance contributing to increased healthcare costs [73,74].

Factors that have contributed to the growing resistance problem include the increased consumption of antimicrobial drugs both by humans and animals. Overuse of many common antimicrobials agents by physicians and improper prescribing of antimicrobials drugs such as the initial prescription of a broad-spectrum drug that is unnecessary, or ultimately found to be ineffective for the organism(s) causing the infection [75,76].

Natural resistance may be intrinsic (always expressed in the species) or induced (the genes are naturally occurring in the bacteria, but they are only expressed to resistance levels after exposure to an antibiotic). Intrinsic resistance may be defined as a trait that is shared universally within a bacterial species, is independent of previous antibiotic exposure, and is not related to horizontal gene transfer [77,78].

The most common bacterial mechanisms involved in intrinsic resistance are reduced permeability of the outer membrane, most specifically the lipopolysaccharide (LSP) in Gram negative bacteria.

Acquisition of genetic material that confers resistance is possible through all of the main routes by which bacteria acquire any genetic material: transformation, transduction, and conjugation (all termed horizontal gene transfer); in addition, bacteria may experience mutations to its own chromosomal DNA [79,80].

Mutations that aid antimicrobial resistance usually only occur in a few types of genes; the ones encoding drug targets, encoding drug transporters, encoding regulators that control drug transporters, or encoding antibiotic-modifying enzymes [77].

Antimicrobial resistance mechanisms fall into four main categories: (1) limiting uptake of a drug; (2) modifying a drug target; (3) inactivating a drug; or (4) active drug efflux [81].

Limiting drug uptake is a natural difference in the ability of bacteria to limit the uptake of antimicrobial agents. The structure and functions of the LPS layer in Gram negative bacteria provides a barrier to certain types of molecules. It gives bacteria innate resistance to certain groups of large molecules with antimicrobial activity [82].

Gram positive bacteria do not possess an outer membrane, and restricting drug access is not as prevalent. The porin channels in Gram negative bacteria generally allow access to hydrophilic molecules [82,83].

There are two main ways in which porin changes can limit drug uptake: a decrease in the number of porins present and mutations that change the selectivity of the porin channel [84]. Another widely seen phenomenon in bacterial colonization is the formation of a biofilm by a bacterial community. For pathogenic organisms, formation of a biofilm protects the bacteria from the attack by the host immune system, and it provides protection from antimicrobial agents [74,85–88].

Modification of drug targets in one mechanism of resistance to the β-lactam drugs used almost exclusively by Gram positive bacteria is via alterations in the structure and/or number of penicillin-binding proteins (PBPs). PBPs are transpeptidases involved in the construction of peptidoglycan in the cell wall. A change in the number of PBPs impacts the amount of drug that can bind to that target. A change in structure (e.g., PBP2a in *Staphylococcus aureus* by acquisition of the *mecA* gene) may decrease the ability of the drug to bind, or totally inhibit drug binding [88,89].

Resistance of Gram-negative bacteria to vancomycin has become a major issue in the enterococci (VRE: vancomycin-resistant enterococci) and in methicillin resistant *S. aureus* (MRSA). Resistance is mediated through acquisition of van genes, which results in changes in the structure of peptidoglycan precursors that cause a decrease in the binding ability of vancomycin [74,78].

For drugs that target nucleic acid synthesis (fluoroquinolones), resistance is via modifications in DNA gyrase (Gram negative bacteria, e.g., *gyrA*) or topoisomerase IV (Gram positive bacteria, e.g., *grlA*). These mutations cause changes in the structure of gyrase and topoisomerase, which decrease or eliminate the ability of the drug to bind to these components [90,91].

During drug inactivation there are two main ways in which bacteria inactivate drugs: by actual degradation of the drug or by transfer of a chemical group to the drug. The β-lactamases make up a very large group of drug hydrolyzing enzymes.

Another drug that can be inactivated by hydrolyzation is tetracycline via the TetX protein that is a flavin dependent monooxygenase [92,93].

Drug inactivation by transfer of a chemical group to the drug most commonly uses transfer of acetyl, phosphoryl, and adenyl groups. There is a large number of transferases that have been identified. Acetylation is the most diversely used mechanism, and it is known to be used against aminoglycosides, chloramphenicol, streptogramins, and fluoroquinolones. Phosphorylation and adenylation are known to be used primarily against the aminoglycosides [93–96].

Drug efflux is a mechanism when bacteria possess chromosomally encoded genes for efflux pumps. Some are expressed constitutively, and others are induced or overexpressed (high-level resistance is usually via a mutation that modifies the transport channel) under certain environmental stimuli or when a suitable substrate is present. The function of efflux pumps is primarily the removal of toxic substances, and many of these pumps transport a large variety of compounds [82].

Most bacteria possess several different types of efflux pumps. Until now, six families of bacterial efflux pumps have been reported, which consist of the ATP-binding cassette (ABC) family directly using ATP as energy source and other five secondary active transporters, i.e., the major facilitator superfamily (MFS), the multidrug and toxin extrusion (MATE) family, the small multidrug resistance (SMR) family, the resistance-nodulation-cell division (RND) superfamily, and the proteobacterial antimicrobial compound efflux (PACE) family (Fig.2) [97,98].

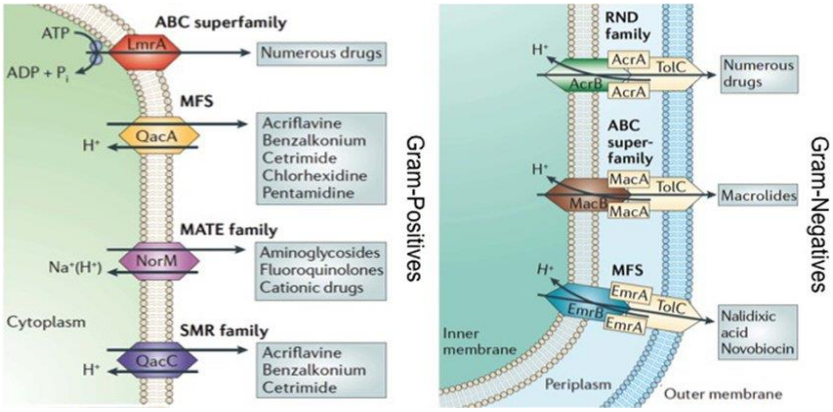


Figure 2. The representation of different types of efflux pumps in Gram-positive and Gram-negative bacteria [97].

D. ANTIMICROBIAL RESISTANCE IN CHLAMYDIAE

Chlamydiae are Gram-negative, obligate intracellular pathogens and symbionts of diverse organisms ranging from humans to amebae [99]. *Chlamydia trachomatis* can cause a wide spectrum of human infections, such as genitourinary infections in both genders and ocular infections, like neonatal conjunctivitis and trachoma. Acute infection caused by *Chlamydia pneumoniae* is a respiratory tract infection, whereas chronic infection has been linked to chronic bronchitis, asthma and atherosclerosis [100]. Respiratory and genital chlamydial pathogens can cause reactive arthritis (Reiter's syndrome) in long term infections, which can be resistant to antibiotic therapy [101].

Furthermore, chlamydiae have been implicated in neurological chronic diseases, such as multiple sclerosis and Alzheimer's disease, and in neurobehavioral diseases, such as autism and schizophrenia. Nonetheless, this evidence is contradictory [102].

Chlamydial infection of cells is initiated by an infectious but metabolically inactive, elementary body (EB). After EB enters the host cell, it forms inclusions and differentiate into a metabolically active but noninfectious, reticulate body (RB). Binary fission division by *C. trachomatis* RBs is synchronous until approximately 18–24 h post-infection, at which point dedifferentiation to infectious EBs can first be observed. During infection, a subset of host-derived vesicles is trafficked to the inclusions, where chlamydiae direct the modification of the inclusion membrane through secretion of proteins that facilitate vacuolar modification and manipulate host signaling pathways via interactions with other chlamydial or host cell proteins. Additional chlamydial proteins are secreted into the host cytosol, where they affect immune recognition and intracellular survival of the pathogen. Most chlamydial developmental cycles are complete in 24–72 h, when, in most cases, the host cell lyses and infectious progeny is released from the cell (Fig. 3) [103–108].

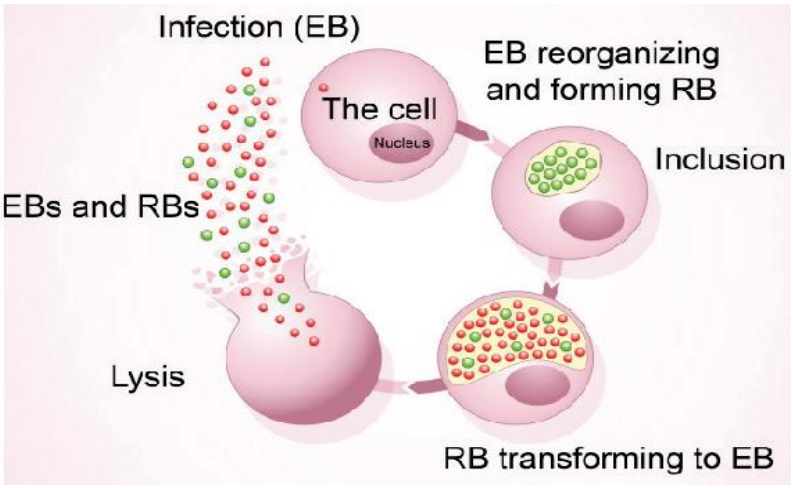


Figure 3. The life cycle of *C. trachomatis* [109].

EB = elementary body; RB= reticulate body

Host immune response, nutrient deficit, antibiotic exposure or co-infection with viruses or parasites, can result in the formation of aberrant bodies (ABs). ABs develop when RB replicative division and maturation of RB to EB is interrupted, resulting in abnormally large chlamydiae. This divergence from the typical developmental cycle constitutes a viable but noninfectious form of chlamydiae, and it is called persistence or chlamydial stress response and is able to cause chlamydial reinfection [105].

Several different stimuli can induce the persistence of chlamydiae *in vitro*: exposure to interferon-γ and antibiotics, heat shock, and depletion of essential nutrients. Persistence is reversible; once the inducer is removed, chlamydiae continue their productive replication [105].

Experimental and clinical data provide evidence for reactivation of persistent chlamydiae in vivo indicating that chlamydial recurrences were more likely due to the reactivations of persistent infections than to re-infections [110].

The diagnosis may be established by nucleic acid amplification tests (NAATs), which can detect only *C. trachomatis*, whereas ArrayTube microarray technique can detected single and mixed infections with *C. trachomatis*, *C. psittaci*, *C. suis*, *C. pecorum*, and *C. pneumoniae* [100]. Microimmunofluorescence testing is the standard for serodiagnosis of *C. pneumoniae* infections [111]

Recent polymerase chain reaction (PCR) based rapid diagnostic tests, however, are non-inferior to standard NAATs. Serology finds application in the diagnostic work-up of suspected chronic chlamydial infection but is inappropriate to diagnose acute infections. Detection of chlamydial antigens by enzyme immunoassay or rapid diagnostic tests is unsuitable because of the insufficient sensitivity and specificity [112].

Chlamydial infections can be managed by azithromycin, tetracyclines, fluoroquinolones, and in pregnant women, with amoxicillin [113]. However, it has been demonstrated that chlamydial persistence can be induced *in vitro* and *in vivo* when exposed to beta-lactam antibiotics [104]. Nevertheless, the prevalence of chlamydial infection is continuously raising, which is attributed to antimicrobial resistance, mainly as a result of chlamydiae being persistent. The alteration in the normal chlamydial developmental cycle can result in persistence and long-term infection, which is refractory to antibiotic therapy [114].

There are several molecular mechanisms for antibiotic resistance. In case of azithromycin resistance, there is a mutation on *rplD* gene [115]. In case of tetracycline, there are antibiotic resistance genes (*tetC, tetR*) acquired by horizontal gene transfer, which encode efflux pumps and regulatory repressors [116]. Regarding fluoroquinolone resistance, point mutation of the *gyrA* can be detected in the quinolone-resistance-determining region [117].

Treatment failure occurs because of re-infection from an untreated partner, mainly from persistent infection, or from acquired antibiotic resistance [100].

E. SELENIUM AND SELENIUM CONTAINING COMPOUNDS

Selenium (Se) is a trace non-metal element, but it is sometimes considered a metalloid. It is found in small amounts in the human body, but it is of essential importance to human biology and health. It was first isolated in 1817 by the Swedish chemist Jöns Jacob Berzelius. He named this element selenium after the Greek moon goddess Selene [118].

Se or Se-containing compounds can be grouped into three main categories: inorganic (selenomethionine and selenocysteine), organic (also known as the organoselenium compounds, such as selenomethionine and selenocysteine) compounds, and Se-containing nanoparticles [119].

Vegetables, such as turnip, peas, beans, carrot, tomato, beet, potato, cucumber, onion, asparagus, garlic, and brassicas (cabbage, broccoli, and mustard) are the primary sources of selenium compounds. Fruits generally contain only a small amount of selenium [120].

Currently, the recommended dietary allowance of Se for adults is set at 55 μg (0.7 μmol/day) [121]. Individuals with daily Se intake less than ~15 μg appear to be at risk of Se deficiency-related diseases, whereas those who consume over 400 μg/day are prone to Se toxicity. Although some studies have shown that safe levels of Se intake may be much lower than anticipated [121,122]. An intake of higher amounts of Se results in adverse effects that vary from being moderate at doses of 1.540–1.600 μg/day to the occurrence of selenosis and deoxyribonucleic acid (DNA) damage induction at doses 3.200–5.000 μg/day [123]. Interest in Se has arisen since the discovery of the fact that its deficiency can cause clinical disorders [124]. In 1979, in China, a congestive cardiac myopathy termed Keshan disease was the first reported human disease associated with Se deficiency [125]. Se is commonly used as a dietary supplement in the manufacture of pharmaceutical products for human purposes, in the treatment of autoimmune thyroiditis [126], and in the therapy of dandruff, seborrheic dermatitis, and other skin diseases [127]. In the 1970s, Se was found to be present in glutathione peroxidase as the amino acid selenocysteine, and the focus on selenium studies shifted to the field of molecular biology [128].

There are more than 25 human selenoproteins and enzymes that contain selenocysteine, many of them having an unknown function yet [129]. Se is a major structural component of several enzymes, such as glutathione peroxidase, thioredoxin reductase, and deiodinases. These enzymes play an important role in antioxidant defense, reproduction, the formation of thyroid hormones, muscle function, cancer prevention and in the humoral and cellular immune response [130]. Se exists in various oxidation states and forms including solid-state Se, selenate, and selenomethiones; in the natural environment, the most toxic form is known as ionic selenite.

Se compounds have chemopreventive, antiproliferative and cytotoxic activity against cancer [131], antioxidant or prooxidant activity, modulating inflammatory process [132], apoptosis induction [133] autophagy modulation [134], inhibition of multidrug efflux pump as permeability glycoprotein (P-gp) [135,136], inhibition of the cancer metastasis [137], angiogenesis inhibition [138], selective targeting of tumors and enhancement of the cytotoxic activity of chemotherapeutic drugs [139], cardiovascular protection, anti-diabetic properties, cerebral protection function in Parkinson's disease and in epilepsia, protection of reproductive system in both gender, thyreoid protection [140], antiviral, antimicrobial, anti-biofilm [141] and antifugal properties [142]

Se nanoparticles (SeNPs) are important formulations since they have low toxicity. SeNPs, as emerging Se species, are considered to be promising medical substances, according to their reported chemotherapeutical properties [143,144], nutritional effects [145], and relatively low toxicity.

F. ANTICANCER EFFECTS OF SELENIUM-CONTAINING COMPOUNDS

Selenocompounds have been reported by several studies as being antitumor agents because of their chemopreventive, antiproliferative, and cytotoxic activity against cancer [133,146,147]. The anticancer mechanism of Se-containing compounds is mainly based on the induction of oxidative stress [146] and promotion of apoptosis in cancer cells [148,149]. These compounds also affect gene expression and various intracellular signaling pathways, DNA repair/damage, as well as angiogenesis or metastasis, and they can be blocked by MDR efflux pumps overexpressed by cancer cells [150]. In addition, it has been observed that these compounds sensitize cancer cells to standard chemotherapy/radiotherapy synergistically enhancing their effectiveness and reducing their side effects [139,151]. Unfortunately, these mechanisms are multidirectional and are not fully understood [133], but improvement in chemotherapeutic drug activity has been described [152–154].

Se compounds, depending on the dose and forms, may have antioxidant or prooxidant properties.

At nutritional levels, redox-active Se-containing compounds have an antioxidant activity only after the incorporation of Se into selenoproteins, exhibiting a chemopreventive effect, for instance, selenocystein can balance the redox homeostasis and protect phagocytic cells from oxidative stress. At supranutritional doses, they manifest their prooxidant and anticancer properties because they can trigger the generation of reactive oxygen species (ROS) through the redox cycle in cancer cells leading to oxidative stress, which results in cytotoxic and genotoxic effects [132,133,150,155].

Se compounds may trigger other types of cell death besides apoptosis (Fig. 4). Non-apoptotic events may also occur, such as cell cycle arrest [133,156,157], necrosis [133,157], autophagy [133,156], ferroptosis [158], necroptosis [155], entosis [159], anoikis [146], NETosis [160], or mitotic catastrophe [133].

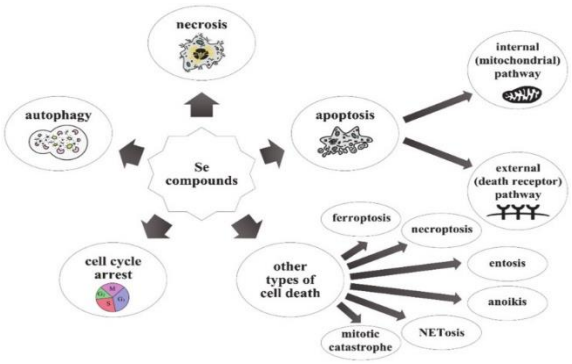


Figure 4. Types of cell death induced by Se-containing compounds. [150].

G. ANTIBACTERIAL EFFECTS OF SELENIUM-CONTAINING COMPOUNDS

Se-containing compounds are promising against certain bacteria and have shown remarkable anti-biofilm activity.

Ebselen (EBS) or 2-phenyl-1,2-benzoisoselenazol-3(2H) was one of the first effective bacterial urease inhibitors discovered against *Sporosarcina pasteurii* and *Helicobacter pylori* enzymes [161] . Sodium selenite can inhibit *H. pylori* [162]. Furthermore, EBS *in vitro* destroys bacteria by penetrating the cell membrane of Gram-negative bacteria such as *Escherichia coli* and showed excellent antibacterial action against MDR bacteria [163].

Derivatives of 1,2,3-selenediazole have also shown antibacterial activity against *E. coli* and *S. aureus* as well as antituberculotic activity against *Mycobacterium tuberculosis* [164]. *In vitro*, the selenizole analogues have shown remarkable action against *S. aureus* strains as well as Gram-negative pathogens, such as *Klebsiella pneumoniae*, *Acinetobacter* species, *Pseudomonas aeruginosa*, and *E. coli*. The effectiveness of selenazoles against bacteria is believed to be related to its interaction with glutathione and cysteine thiols of diverse proteins in the bacterial cells [163]. In addition, 2,20-diphenyl diselenide inhibited the growth of Gram-positive bacteria, such as *Enterococcus faecalis*, *S. aureus*, as well as the yeast *Candida albicans* with prooxidant activity [142].

Selenoesters have antibacterial activity against Gram-positive bacteria, such as MRSA, *E. faecalis*, *S. aureus*, and *S. aureus* HEMSA 5, furthermore they can inhibit *E. coli* by disrupting the proton motive force essential for the multidrug resistance efflux pump AcrAB-TolC [165].

Methylketone selenoesters and selenoanhydrides are effective against *S. aureus*, *E. faecalis*, and *C. trachomatis*. Selenoanhydride and diselenodiester have the capacity to inhibit the bacterial AcrAB-TolC efflux pump system [165].

Moreover, ketone-selenoesters exerted a potent antibacterial activity against *S. aureus* and MRSA, furthermore ketone- and cyano-selenoesters are able to prevent the formation of the biofilm by *S. aureus* and *P. aeruginosa* [166].

Se-NPs induce a higher production of ROS compared to selenite [167]; therefore, it has potent antibacterial activity against Gram-positive and Gram-negative bacteria, such as *S. aureus* [130], *Staphylococcus epidermidis*, *K. pneumoniae*, *Bacillus subtilis* [168], *E. coli*, *Acinetobacter baumannii* [169] *E. faecalis*, *Streptococcus mutans*, *Shigella sonnei*, and *P. aeruginosa*, and it exerts potent anti-biofilm activity against *P. aeruginosa* [170]. The combination of Se-NPs with antibiotics results in more pronounced antibacterial and anti-biofilm activity against MRSA [170] and it is a very effective microbial biofilm disruptor in case of *E. coli*, *P. aeruginosa*, and *S. aureus* [167]. Se-NPs exert an anti-biofilm activity against *P. aeruginosa* [170] and MRSA [171]; however, ketone-selenoesters are more potent disruptors of biofilms than the cyano-selenoesters against *P. aeruginosa* biofilms compared to the biofilms produced by *S. aureus* [166].

Selenocompounds have been inactive against anaerobes suggesting that the mechanism of action of these derivatives depends on the presence of oxygen [141].

Furthermore, selenium conjugated peptides, antibodies, and nanoparticles have been described as potent antimicrobial and anticancer therapeutics [172].

IV. AIMS OF THE STUDY

Multidrug resistance is becoming a serious problem in the treatment of cancer and bacterial infections. Therefore, the discovery of novel anticancer and antibacterial agents reversing multidrug resistance is of great importance. Selenium-containing compounds could provide new alternatives in experimental chemotherapy to overcome multidrug resistance in cancer and bacteria.

The aim of our study was to investigate the activity of eleven selenocompounds synthesized by Domínguez-Álvarez et al. on MCF-7 breast cancer cell line and its doxorubicin resistant subline KCR as *in vitro* model system [131]. Their potency in combination with doxorubicin was studied on MCF-7 and KCR breast cancer cell lines; furthermore, their activity as apoptosis inducers was studied in both breast cancer cell lines.

The antibacterial effects of selenocompounds were evaluated in *C. trachomatis* D, because there are several reports of clinical isolates exhibiting resistance to antibiotics and novel drugs could overcome this problem in the therapy.

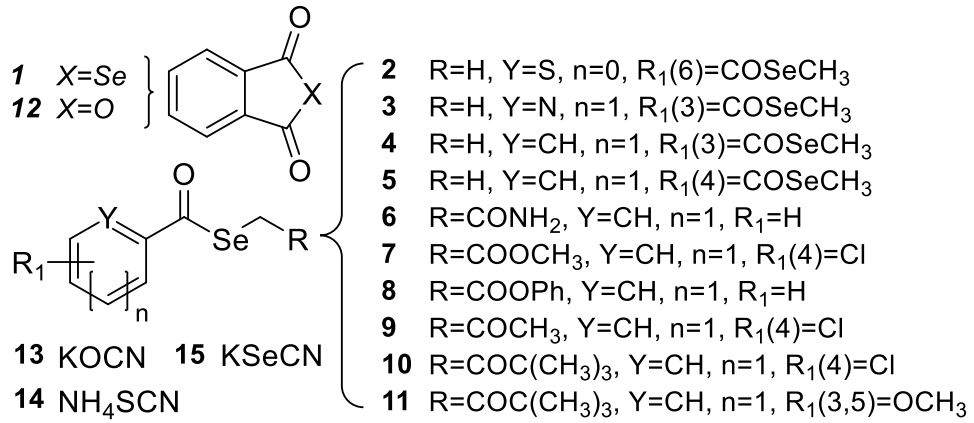
The main goals of the study were the following:

1. **Determination of the cytotoxic effects of selenocompounds** on doxorubicin sensitive MCF-7 and doxorubicin resistant KCR breast cancer cell lines by MTT assay.
2. **Characterization of the activity of selenocompounds in combination with doxorubicin** on MCF-7 and KCR breast cancer cells by checkerboard assay.
3. **Evaluation of the apoptosis inducing effect of selenocompounds** (a cyclic selenoanhydride, ten selenoesters, and four inorganic chalcogen cyanates) on MCF-7 and KCR breast cancer cell lines by Annexin V-FITC and propidium iodide double staining using flow cytometry.
4. **Determination of antibacterial activity of selenocompounds** on *C. trachomatis* D by indirect immunofluorescence.

V. MATERIALS AND METHODS

Compounds studied

The eleven selenocompounds including selenoanhydride (**1**) and selenoesters (**2–11**)(Fig. 5) were kindly provided by Dr. Enrique Domínguez-Álvarez (Spanish National Research Council, Madrid, Spain) and by Prof. Dr. Carmen Sanmartín (University of Navarra, Pamplona, Spain) [131]. All compounds were stable, and their purity was assessed by spectroscopic techniques (elemental analysis, nuclear magnetic resonance, mass spectrometry, and infrared spectroscopy). Compounds (**12–15**) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) to be used as non-selenium (**12**) isostere of selenoanhydride (**1**) and as inorganic chalcogen salts (**13–15**) for comparing their activity with the selenoesters. The stock solutions (in 10 mM concentration) of compounds were dissolved in dimethyl sulfoxide (DMSO).



***1**: Benzo[c]selenophen-1,3-dione; **2**: Dimethyl thiophene-2,5-dicarboselenoate; **3**: Dimethyl pyridine-2,6-dicarboselenoate; **4**: Dimethyl benzene-1,3-dicarboselenoate; **5**: Dimethyl benzene-1,4-dicarboselenoate; **6**: Carbamoylmethyl benzoselenoate; **7**: Methoxycarbonylmethyl 2-chlorobenzoselenoate; **8**: Phenoxycarbonylmethyl benzoselenoate; **9**: Methoxycarbonylmethyl 4-chlorobenzoselenoate; **10**: 3,3-Dimethyl-2-oxobutyl 4-chlorobenzoselenoate; **11**: 3,3-Dimethyl-2-oxobutyl 3,5-dimethoxybenzoselenoate; **12**: 2-Benzofuran-1,3-dione; **13**: Potassium cyanate; **14**: Ammonium thiocyanate; **15**: Potassium selenocyanate*

Figure 5. Chemical structure of the tested compounds. The numbers in parentheses denote the position at which R1 is bound to the (hetero) aromatic ring.

Reagents and media

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), and dimethyl sulfoxide (DMSO) were purchased from Merck KGaA (Darmstadt, Germany). Doxorubicin hydrochloride (doxorubicin) was purchased from Teva Pharma Kft. (Budapest, Hungary). 12*H*-benzo[α]phenothiazine (M627; positive control in the apoptosis assay) was kindly provided by Prof. Dr. Noboru Motohashi (Meiji Pharmaceutical University, Kiyose, Tokyo, Japan). All solutions were prepared on the day of assay.

Cell lines

Breast cancer cell line MCF-7 (ATCC® HTB-22) was purchased from LGC Promochem (Teddington, Middlesex, UK). The MCF-7 cell line and its drug-resistant subline KCR were grown in Eagle's minimal Essential medium (EMEM), containing 4.5 g/L glucose supplemented with a non-essential amino acid mixture, a selection of vitamins and 10 % heat-inactivated fetal bovine serum (FBS). The cell lines were incubated at 37 °C, in an atmosphere of 5 % CO2 and 95 % air. On every third passage, 0.56 μg/mL doxorubicin was added to the medium in order to maintain ABCB1 expression in KCR cells.

Bacterial strain

Chlamydia trachomatis reference strain (serovar D, UW-3/Cx, ATCC, VR-885D) was used in the anti-chlamydial assay.

Cytotoxicity assay

The cytotoxic effects of the Se-compounds were determined on MCF-7 and KCR breast cancer cell lines. The effects of increasing concentrations of Se-compounds on cell growth were tested in 96-well flat-bottomed microtiter plates. The stock solutions (10mM) of the compounds were diluted in 100 μL of EMEM medium.

The adherent breast cancer cell lines were cultured in 96-well flat-bottomed microtiter plates, using EMEM supplemented with 10 % heat-inactivated FBS.

The density of the cells was adjusted to 1×10^4 cells in 100 μL/well, the cells were seeded for 24 h at 37 °C, with 5 % CO_2 prior to the assay, then the medium was removed from the plates containing the cells, and dilutions of Se-compounds 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 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 SDS 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:

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

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

The selectivity was calculated by using the selectivity index (SI), which is defined as the quotient of the IC_{50} value determined for the non-tumorous MRC-5 cell line described previously [136] to the IC_{50} value for the respective cancer cell line (MCF-7 or KCR). Following the criteria reported in bibliography [136], we considered a compound to be strongly selective when its SI was 6 or higher. Compounds with SI values of 1–3 and 3–6 were regarded as slightly and moderately selective, respectively.

Cytotoxicity assay was also performed on HeLa cells by the use of the same method as described above, in order to evaluate the concentrations at which the Se-compounds exert no direct toxic effects to these cells.

Checkerboard combination assay

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

The final concentration of the Se-compounds and doxorubicin used in the combination experiment was chosen in accordance with their cytotoxicity towards these cell lines. The dilutions of doxorubicin were made in a horizontal direction in 100 µL, and the dilutions of the Se-compounds vertically in the microtiter plate in 50 µL volume. The plates were incubated for 72 h at 37 °C in 5 % CO_2 atmosphere. The cell growth rate was determined after MTT staining. At the end of the incubation period, 20 µL of MTT 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 SDS solution (10 % in 0.01 M HCI) was added to each well, and the plates were further incubated at 37 °C overnight. OD was measured at 540/630 nm with Multiscan EX ELISA reader. Combination index (CI) values at 50 % of the growth inhibition dose (ED50) were determined by using CompuSyn software (www.combosyn.com, ComboSyn, Inc., Paramus, NJ. 07652, USA) to plot 4 or 5 data points at each ratio. CI values were calculated by means of the median-effect equation according to the Chou-Talalay method, where CI<1, CI=1, and CI>1 represent synergism, an additive effect (or no interaction), and antagonism, respectively [173,174].

Apoptosis induction

The ability of Se-compounds to induce apoptosis was determined on breast cancer cell lines. The apoptosis assays were performed by using Annexin V-FITC Apoptosis Detection Kit from Calbiochem (EMD Biosciences, Inc. La Jolla, CA, USA), following the instructions provided by the manufacturer.

This assay enables the quantification of early and late apoptotic events, as well as necrosis and cell death in the cell population exposed to the Se-compounds. The density of the cell suspension was adjusted to 1×10^6 cells/mL. The cell suspension was distributed into 0.5 mL aliquots (5×10^5 cells) to a 24-well microplate and incubated overnight at 37 °C in 5 % CO_2. On the following day, the medium was removed, and a fresh medium was added to the cells. The cells were then incubated in the presence of compounds at 1 or 2 μM for 3 h at 37 °C. 12*H*-benzo[α] phenothiazine (M627) [175], which is a known early apoptosis inducer, was used as a positive control. The samples were washed in phosphate buffered saline (PBS), and fresh EMEM medium was added to the cells, followed by the incubation of the plate for 24 h at 37 °C in 5 % CO_2. After the incubation period, the cells were trypsinized. The harvested cells were centrifuged at 2000×g for 2 minutes. The cells were then re-suspended in fresh serum-free EMEM medium. Thereafter, the apoptosis assay was carried out according to the rapid protocol of the kit, and the fluorescence was analyzed immediately by using a Partec CyFlow flow cytometer (Partec, Münster, Germany).

Propagation of Chlamydia trachomatis D

C. trachomatis D was propagated on HeLa 229 cells (ATCC, CCL-2.1), as described earlier [176]. The titre of the infectious elementary bodies was determined by indirect immunofluorescence assay. Serial dilutions of the elementary bodies' preparation were inoculated onto HeLa monolayers, and after a 48 h culture, cells were fixed with acetone and stained with monoclonal anti-Chlamydia LPS antibody (AbD Serotec, Oxford, UK), and FITC-labelled anti-mouse IgG (Merck KGaA, Darmstadt, Germany). The inclusions of *C. trachomatis D* were enumerated under a UV microscope.

Anti-chlamydial assay

Elementary bodies of *C. trachomatis* D (4×10^3 IFU/mL) were incubated with compounds at selected concentrations (0.25 μM, 0.5 μM, 1.25 μM, and 2.5 μM) in sucrose-phosphate-glutamic acid buffer (SPG) for 2 h at 37 °C. As a control, *C. trachomatis* D was also incubated in SPG alone. To quantify the anti-chlamydial effects of compounds, HeLa cells were seeded in 24-well plates with 13 mm cover glasses.

The confluent cells were infected with selenocompound-treated *C. trachomatis* D or the non-treated controls. After 48 h, the cells were fixed with acetone at - 20 °C for 10 minutes. The titer of the infectious elementary bodies was determined by indirect immunofluorescence assay [177].

VI. RESULTS

Cytotoxicity assay

Eleven selenocompounds (**1–11**) and four reference compounds (**12–15**) were evaluated for their anticancer activity against MCF-7 and KCR breast cancer cell lines. The screening of the anticancer activity of Se-compounds in MCF-7 cells indicated that selenoanhydride (**1**) and selenoesters (**2–7**) were not cytotoxic towards this cell line (Table 1), as all the IC_{50} values of these derivatives were above 100 μM. In contrast, ketone-containing selenoesters (**9–11**) showed a potent low-micromolar activity, as their IC_{50} values ranged from 1.04 to 1.70 μM, whereas IC_{50} of phenoxycarbonylmethyl selenoester (7) was 64.8 μM. The results were similar for the resistant KCR cells except for two derivatives. First, in this case, IC_{50} of selenoanhydride (**l)** was at 2.35 μM, which was more than 40-fold lower than that for MCF-7, suggesting that this compound acts directly on ABCB1 overexpressed by KCR cells. Second, compound (**11**) was close to 2-fold less active on KCR cells compared to MCF-7. None of the compounds (**12–15**) evaluated for comparison studies exerted cytotoxic effects at concentrations below 100 μM on any of KCR, MCF-7, and MRC-5 cell lines evaluated. The anticancer effect of Se-compounds on MRC-5 human embryonic lung fibroblast cell line was determined previously [135].

Table 1. Cytotoxic activity of Se-compounds against MCF-7, doxorubicin-resistant KCR breast cancer, and MRC-5 lung fibroblast cell lines.

Cpd	A – MCF-7		B – KCR		SI	C - MRC-5*		SI	SI
	IC_{50} (μM)	±SD	IC_{50} (μM)	±SD	A/B	IC_{50} (μM)	±SD	C/A	C/B
1	>100	-	**2.35**	0.47	**≥42**	>100	-	-	**≥42**
2	>100	-	>100	-	-	4.26	0.65	≤0.04	≤0.04
3	>100	-	>100	-	-	17.9	0.00	≤0.18	≤0.18
4	>100	-	>100	-	-	28.4	0.70	≤0.28	≤0.28
5	>100	-	>100	-	-	61.5	2.16	≤0.62	≤0.62
6	>100	-	>100	-	-	76.6	0.92	≤0.77	≤0.77
7	>100	-	>100	-	-	33.4	3.08	≤0.33	≤0.33
8	64.8	16.7	82.2	15.7	0.79	>100	-	≥1.5	≥1.2
9	**1.04**	0.47	**0.96**	0.18	1.08	*5.35*	0.24	*5.2*	*5.6*
10	**1.70**	0.45	**1.75**	0.15	0.97	*8.10*	0.90	*4.8*	*4.6*
11	**1.45**	0.23	**2.37**	0.30	0.61	*5.04*	0.71	*3.5*	2.2
12	>100	-	>100	-	-	>100	-	-	-
13	>100	-	>100	-	-	>100	-	-	-
14	>100	-	>100	-	-	>100	-	-	-
15	>100	-	>100	-	-	>100	-	-	-

*IC50: 50 % inhibitory concentration; SI: selectivity index. For cytotoxicity, IC_{50} values in bold denote IC_{50} values below 5 M, and those in italics, values between 5 and 10 μM. In selectivity, values in bold denote a strong selectivity, and in italics, a moderate selectivity. *Values taken from a previous study* [136].

Regarding the selectivity of the selenoesters towards cancer cells, it was clearly observed that the ketone-containing selenoesters exerted a moderate selectivity towards MCF-7 and KCR cancer cells with respect to the non-tumorous MRC-5 lung fibroblast cells, with the exception of compound (**11**), which was slightly selective towards KCR, exhibiting a SI of 2.2.

The SI of compound (**9**) for KCR cells was close to 6 (SI=5.6), which was the threshold for considering a compound strongly selective. The remaining selenoesters lacked of selectivity due to their poor activity against MCF-7 and KCR.

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

Combination assay

The five active compounds in the cytotoxicity assay were evaluated in combination with doxorubicin on KCR and MCF-7 cells (Fig. 5). Results were quite fascinating showing a marked difference between the two tested cell lines. All Se-compounds assayed exerted synergistic interactions with doxorubicin in KCR cell line, whereas all the observed interactions of selenoesters with doxorubicin in MCF-7 cell line were antagonistic.

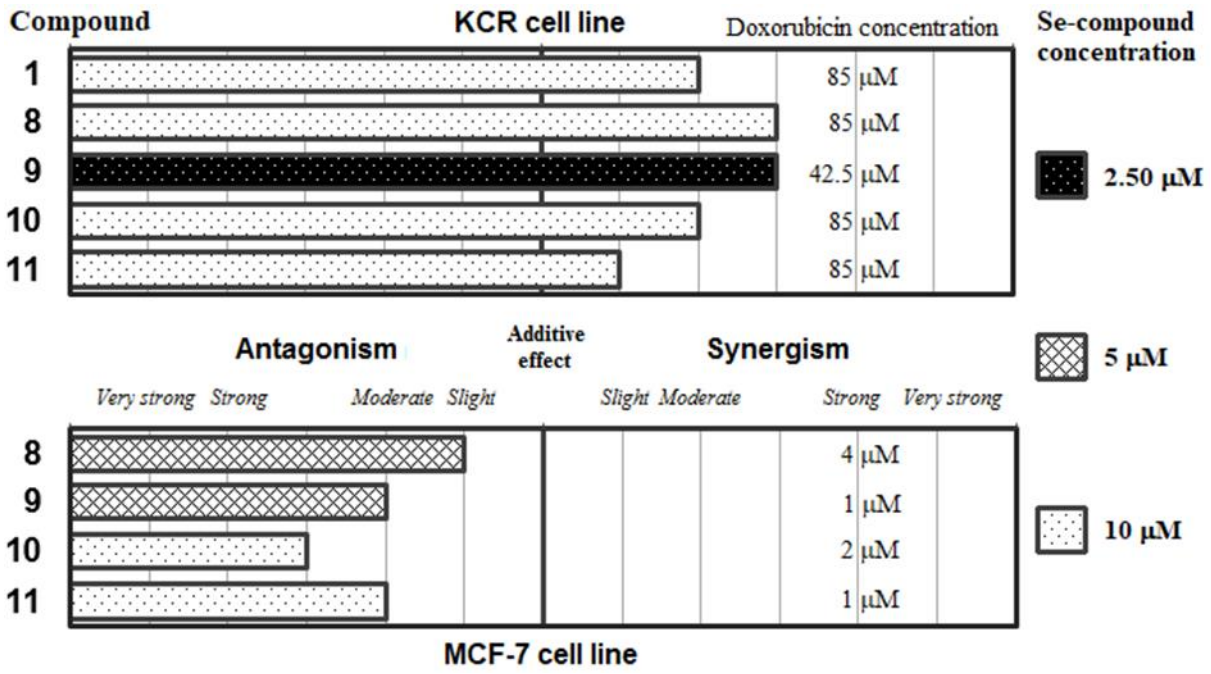


Figure 5. Interactions of the active Se-compounds with doxorubicin in KCR and MCF-7 cells. The figure indicates, at the most effective interaction ratio (Doxorubicin:Se-compound), the concentration of the Se-compounds in the presence of doxorubicin at the concentration indicated; furthermore, the type of interaction (antagonism, additive effect and synergism) is given.

Against KCR cells, compound (**9**) was undoubtedly the most profitable in the combination assay, as it exerted the highest grade of synergy among all evaluated compounds and at the lowest concentrations of both Se-compound (2.5 μM) and doxorubucin (42.5 μM). The remaining Se-compounds interacted in a synergistic manner with doxorubicin at a concentration of compound and drug 4-fold and 2-fold higher, respectively. Against MCF-7 cells, compound (**9**) interacted in a moderately antagonistic manner at higher concentration (5 μM). Slight antagonism was observed for compound (**8**) at the same concentration, but the concentration of doxorubicin was in this case 4 times as high.

Apoptosis assay

Se-compounds were not able to induce significant apoptotic events in MCF-7 and KCR cells (Table 2 and Table 3, respectively); with the exception of the selenoester (**7**) in MCF-7 cells. This derivative, at a low concentration (2 μM), triggered early apoptotic and late apoptotic/necrotic events in 16.9 % and 7.85 % of cells, respectively (Fig. 6). This apoptosis-inducing activity was moderate, as reference compound M627 induced early apoptosis in 20.8 % and late apoptosis in 67.1 % of the population.

According to previous studies, among the 11 selenocompounds evaluated, selenoanhydride (**1**)(benzoselenophene-diones) and ketone selenoesters (**9–11**) (2-oxoalkyl-benzoselenoesters) were able to induce apoptosis in resistant mouse T-lymphoma cells and in resistant Colo 320 colon adenocarcinoma cells, and they were able to reverse MDR because they proved to be potent inhibitors of the ABCB1 efflux pump. The structure activity relationship-analysis suggested that ketone substituents play a crucial role in the anticancer activity [135,136].

In addition, the three ketone selenoesters (**9–11**) were potent and strongly selective cytotoxic agents in resistant Colo 320 cells [136].

Table 2. Apoptosis induction in MCF-7 cell line by Se-compounds **(1–11)** after a 3 h incubation. Positive control: M627; negative control: DMSO.

Sample	Concentration (μM)	Gated events (%)		
		Early apoptosis	Late apoptosis, necrosis	Cell death
A-I-		0.02	0.03	0.05
A-I+		0.03	0.01	5.68
A+I-		4.00	0.00	0.01
A+I+		4.01	3.45	1.37
DMSO	5 %	2.62	1.56	0.47
M627	20	**20.8**	67.1	8.02
1	2	0.99	0.82	11.7
2	2	1.37	2.29	14.6
3	2	0.20	0.65	20.8
4	2	1.32	2.69	16.9
5	2	2.17	3.50	8.48
6	2	0.22	0.16	8.35
7	2	**16.9**	7.85	7.40
8	2	0.19	0.47	18.1
9	2	0.13	0.20	26
10	2	0.10	1.00	34.8
11	2	0.20	0.53	25.00

A-I-: annexin negative/propidium-iodide negative; A-I+: annexin negative/propidium-iodide positive; A+ I-: annexin positive/propidium-iodide negative; A+I+: annexin positive/propidium-iodide positive

Table 3. Apoptosis induction in ABCB1-expressing KCR cell line by Se-compounds (**1-11**) after a 3 h incubation. Positive control: M627; negative control: DMSO.

Sample	Concentration (μM)	Gated events (%)		
		Early apoptosis	Late apoptosis, necrosis	Cell death
A-I-		0.01	0.02	0.04
A-I+		0.01	0.01	2.80
A+I-		4.29	0.00	0.00
A+I+		3.07	2.70	0.46
DMSO	5 %	3.97	1.81	0.50
M627	20	2.96	6.93	1.1
1	1	4.09	1.15	0.28
2	1	4.96	1.70	0.57
3	1	4.23	1.89	0.59
4	1	4.53	1.82	0.53
5	1	3.79	1.18	0.49
6	1	3.16	1.23	0.47
7	1	3.39	1.34	0.46
8	1	3.92	1.36	0.48
9	1	3.65	1.51	0.35
10	1	3.58	1.37	0.34
11	1	3.18	1.55	0.41

A-I-: annexin negative/propidium-iodide negative; A-I+: annexin negative/propidium-iodide positive; A+ I-: annexin positive/propidium-iodide negative; A+I+: annexin positive/propidium-iodide positive

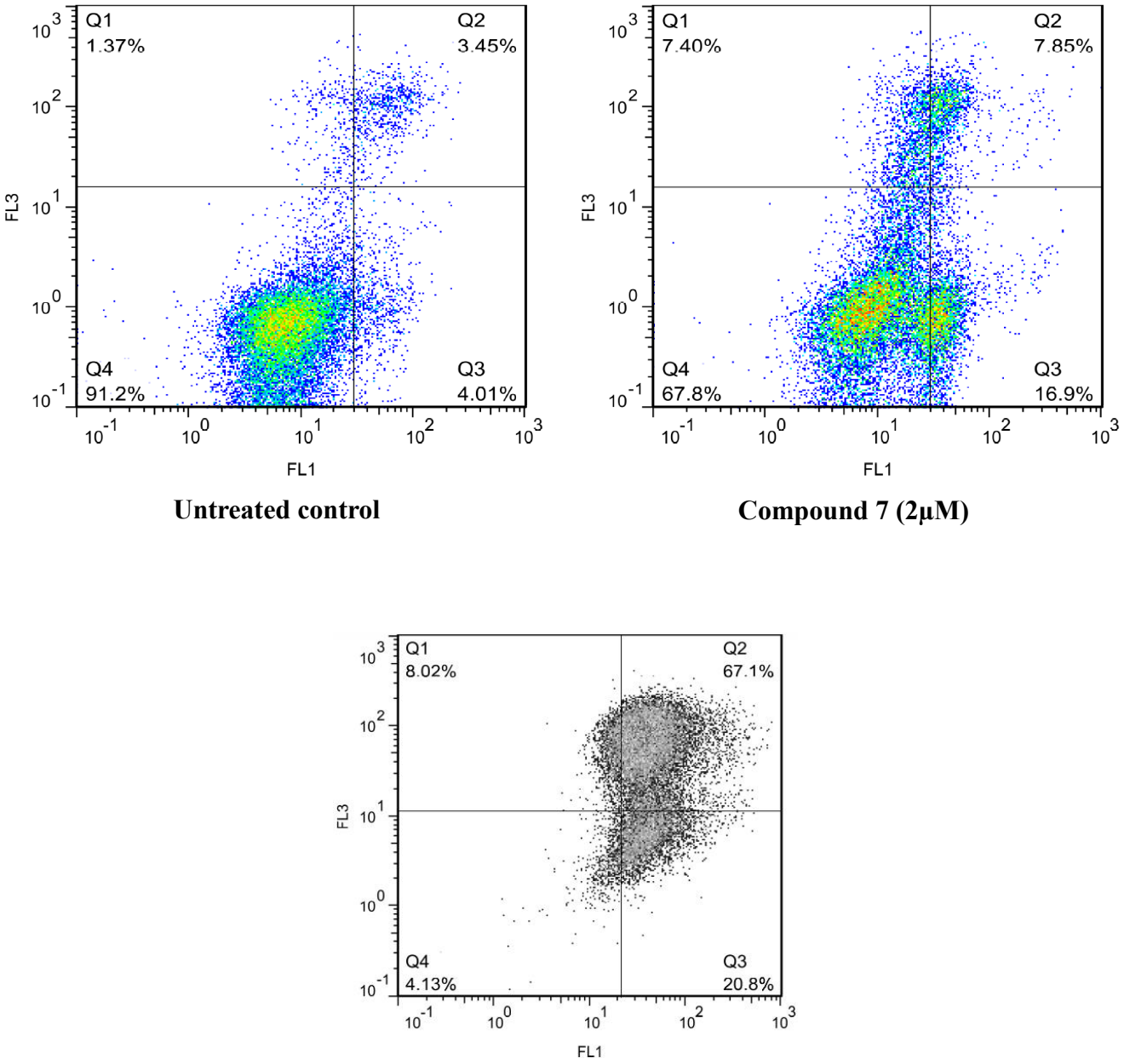


Figure 6. Apoptosis induction by compound (**7**) at 2 µM in MCF-7 cells compared to the positive control M627. Q4: healthy, living cells; Q3: cells undergone early apoptosis; Q2: cells undergone late apoptosis/necrosis; Q1: dead cells.

Anti-Chlamydial assay

Before the assessment of the anti-chlamydial activity of Se-compounds, a cytotoxicity assay was performed on HeLa cells to determine the ranges of concentrations at which Se-compounds can be evaluated without showing direct toxic effects to HeLa cells (Table 4).

Se-compounds (**2**, **3**, **5**, **7**) and (**9-11**) significantly inhibited the formation of chlamydial inclusions at selected concentrations (Fig. 7). Compounds (**2**) and (**7**) at 2.5 μM showed 82% and 71% inhibition, compared to the control, respectively. In addition, (**2**) and (**7**) were effective at 1.25 μM, whereas (**9**) and (**10**) inhibited the formation of inclusions at low submicromolar concentrations of 0.5 μM (Fig. 8). The most potent anti-chlamydial Se-compounds were (**9**) and (**11)**, as they inhibited more than 50% the growth of *C. trachomatis* D at the concentration of 0.25 μM (0.0689 μg/mL and 0.0858 μg/mL, respectively).

Table 4. Cytotoxic activity of Se-compounds against HeLa cells

Se-compounds	**IC50 (μM)**
1	26.57
2	>100
3	85.62
4	>100
5	>100
6	>100
7	>100
8	>100
9	6.68
10	10.27
11	9.48

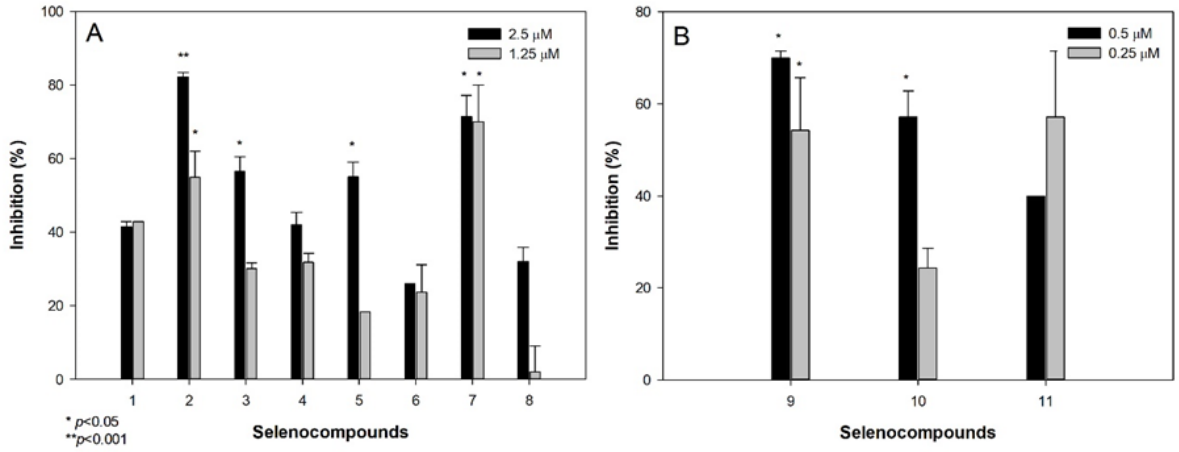


Figure 7. Anti-chlamydial effect of Se-compounds at 1.25 and 2.5 μM (**A**), and at 0.25 and 0.5 μM (**B**).

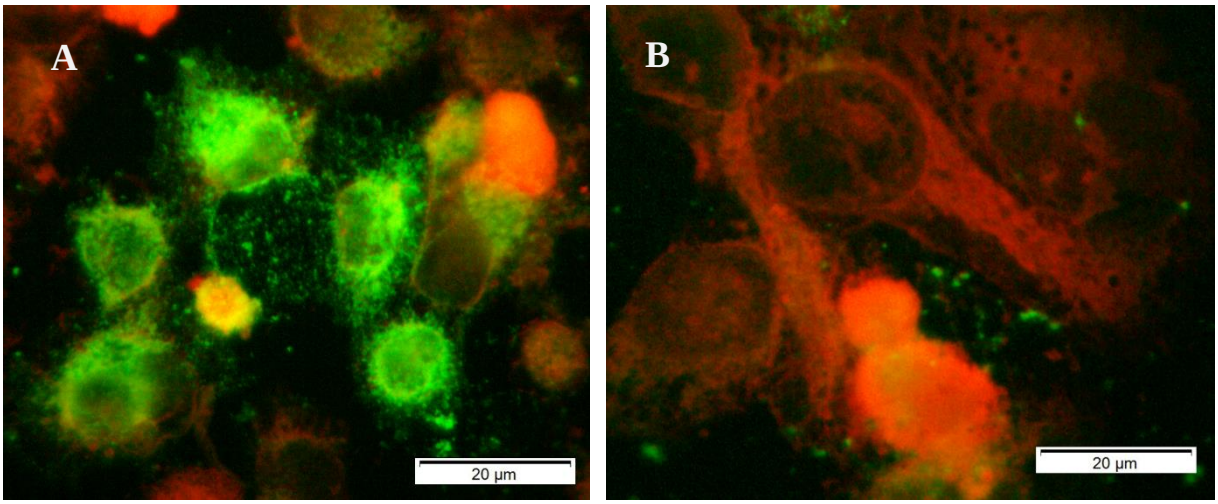


Figure 8. Immunofluorescence-stained inclusions of *C. trachomatis* D in HeLa cells. The cells were infected with *C. trachomatis* D alone (**A**) or with *C. trachomatis* D pre-incubated with Se-compounds (**9**) at a concentration of 0.5 μM (**B**). Pictures were acquired by a digital camera attached to a fluorescence microscope.

VII. DISCUSSION

In the treatment of early and metastatic breast cancer, conventional chemotherapy is partly based on the administration of anthracycline drugs, e.g., doxorubicin. Since these drugs provoke side-effects, such as cardiotoxicity and myelosuppression [178,179], there is an urgent need to minimize the side-effects. In order to reduce the adverse effect of anthracyclines, several alternatives could be applied, for example, the use of liposomal doxorubicin [180], nanotechnology [181], and the development of less toxic derivatives.

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

Chlamydia species can develop resistance for antibiotics, and this may show single- or multidrug resistance due to several molecular mechanisms. Nowadays, only a few antibiotics, including tetracyclines, macrolides, and quinolone [113] are in clinical use against these intracellular bacteria because this species is considered to be susceptible to antibiotics interfering with prokaryotic DNA-, RNA-, or protein synthesis [182]. The development of new antibacterials and MDR reversing compounds is required to overcome this problem and to find an effective therapeutic approach.

The activity of Se-compounds against Chlamydia was investigated in order to find effective drugs that inhibit the reproduction of these intracellular bacteria.

Antitumor activity

As commented in the Results section, selenoanhydride (**1**) exerted selective activity towards the resistant KCR cell line overexpressing ABCB1 (IC_{50}=2.35 μM); however, it was ineffective against MCF-7 and MRC-5 (non-tumorous lung fibroblast) cells. These results are in accordance with our previous data confirming that selenoanhydride (**1**) interacts directly with ABCB1 [135,136].

Surprisingly, this derivative was unable to trigger apoptotic events in the tested breast cancer cell lines, probably due to a dual inhibition of ABCB1 and multidrug resistance protein 1 efflux pumps; however, other resistance mechanisms could also be involved.

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

The results observed in combination assays are astonishing in that they point to differential activity in the two cell lines, the resistant (KCR) one, in this case, being more sensitive to the action of the compounds. It has been shown previously that doxorubicin and methylseleninic acid act synergistically on MCF-7 cells, inducing apoptosis because doxorubicin and selenium cooperatively activate first apoptosis signal (FAS) pathway. Doxorubicin causes Fas oligomerization in a FasL-independent manner, and methylseleninic acid increases FAS-associated death domain protein expression together triggering apoptosis [183]. Out of our 11 Se-compounds, only methoxycarbonyl-methyl *p*-chlorobenzoselenoate (**7**) induced apoptosis in MCF-7 cells, the other derivatives were not capable of provoking apoptosis of MCF-7 and KCR cells.

This is very relevant as it suggests that these derivatives might have the ability to overcome some aspects of resistance in KCR cells. Since the derivatives are proven ABCB1 modulators, their synergism with doxorubicin might be due to their interaction with this efflux pump overexpressed by KCR cells. On the contrary, the explanation of their antagonism with doxorubicin in MCF-7 cells is the involvement of other resistance mechanisms and cellular processes.

This could open a new and straightforward approach to treat ABCB1-expressing resistant breast cancer that is resistant to the current treatments in clinical use. Methylketone selenoester (**9**) would be in such cases the most promising compound. Its activity makes it worth to be investigated in more depth for potential applications and its closely related new derivatives (which could be synthesized in future work) with intrinsic anticancer activity as sensitizers of resistant cancer cells.

Overall, the results obtained herein highlight the importance for biological activity of the -$COSeCH_2COCH_3$ and -$COSeCH_2CO(CH_3)_3$ moieties in comparison with the remaining substituents considered (-$COSeCH_3$, -$COSeCH_2CONH_2$, -$COSeCH_2COOCH_3$, and -$COSeCH_2COPh$). The good cytotoxic activity, selectivity and ability to modulate the effect of doxorubicin found for the ketone-containing selenoesters (**9–11**) against the two breast cancer cell lines evaluated are in agreement with previous work of our group on mouse T-lymphoma cells and colonic adenocarcinoma cells [135,136] and draw the attention to this privileged moiety. In future studies, it will be necessary to obtain and evaluate more compounds with these moieties in order to ascertain what substituents in the phenyl ring bound to the carbonyl of the selenoester enhance activity, with the aim of designing more potent and selective anticancer agents.

Anti-chlamydial activity

Previous studies have reported that selected selenocompounds, such as certain selenocyanates, selenoureas, and diselenides, showed antiproliferative activities against the intracellular forms of *Leishmania* species [184,185]. Taking those results into account, this study provided a new line of evidence for the action of selenoanhydride/selenoesters on an obligate intracellular chlamydial strain. In particular, different selenoesters, such as (**2, 3, 5, 7**)**,** and (**9–11**), have exerted a noteworthy activity against *C. trachomatis* D. Furthermore, the activities of the methyl (**9**) and the *tert*-butyl (**11**) derivatives were very promising, as they inhibited the formation of more than 50 % of the chlamydial inclusions at a very low concentration (0.25 μM). However, their mode of action has not been ascertained in this study.

Regarding the observed structure activity relationships of the anti-chlamydial assays, ketone selenoesters (**9–11**) showed noteworthy activity at lower concentrations (0.25 μM, 0.5 μM) compared to the rest of the series (1.25 μM, 2.5 μM). Among the remaining selenoesters, the symmetric dimethyl selenodiester, which contains thiophene ring (**2**), and methyl oxoester derivative (7) showed a better activity, and the activities of the symmetric dimethyl selenodiesters (**3**) and (**5**) were also remarkable. This fact highlights the importance of the symmetry for the activity against intracellular pathogens [184].

Herein, we have reported the evaluation of the antitumor, multidrug resistance reversing and antibacterial activity of 11 novel selenocompounds. Selenoanhydride (**1**) exerted selective activity towards the doxorubicin-resistant KCR cell line overexpressing ABCB1. Among the selenoesters, only ketone-containing selenoesters promoted significant cytotoxic activity against MCF-7 and KCR cell lines, and the Se-compounds acted synergistically with doxorubicin on the KCR cell line. Methylketone selenoester (**9**) showed potential activity against *C. trachomatis* D at very low concentration (0.25 μM). Based on the results, the importance of the $COSeCH_2COCH_3$ and $COSeCH_2CO(CH_3)_3$ moieties for the cytotoxic, adjuvant role, and anti-chlamydial effect of Se-compounds was highlighted. Furthermore, it can be concluded that this group of compounds can be attractive potential antitumor and anti-chlamydial lead scaffolds, for further development of new chemical tools, to overcome multidrug resistance.

VIII. NEW FINDINGS

1. Ketone-containing selenoesters (**9–11**) exerted significant cytotoxic activity against the doxorubicin sensitive MCF-7 and doxorubicin resistant KCR breast cancer cell lines.

2. Ketone selenoesters (**9–11**) exerted a high or moderate selectivity towards the evaluated breast cancer cell lines (MCF-7, KCR) compared to non-tumorous MRC-5 cells.

3. Only methoxycarbonyl-methyl *p*-chlorobenzoselenoate (**7**) induced early and late apoptosis in MCF-7 cells.

4. Effective cytotoxic activity, selectivity, and synergistic activity with doxorubicin were found for the ketone-containing selenoesters (**9–11**) against the doxorubicin resistant KCR breast cancer cell line.

5. Different selenoesters, such as the methyl group selenoesters (**2, 3, 5**) **,** methoxycarbonyl-methyl *p*-chlorobenzoselenoate (**7),** and ketone-containing selenoesters (**9–11**), have exerted a noteworthy activity against *C. trachomatis* D.

6. Methylketone selenoester (**9**) showed the most potent antibacterial activity against *C. trachomatis* D at a very low concentration (0.25 μM).

IX. SUMMARY

Among the European Union member states, the incidence of lung and colon cancers and the mortality associated with these tumors is the highest in Hungary. The goal of chemotherapy is the elimination or reduction of malignant cell mass and to improve the quality of life of the patient. The use of chemotherapy could be complicated because of its low bioavailability, disadvantageous side effects due to non-selective cytotoxic activity, and the emergence of multidrug resistance, whereby tumors show resistance to chemotherapeutic agents of different structure and mechanism of action. The two main mechanisms of MDR investigated by our study are the failure of apoptosis induction and overexpression of energy-dependent efflux pumps. A considerable number of compounds have been described with the ability to inhibit the function of the ABCB1 efflux pump. An emerging therapeutic strategy is the use of chemosensitizers as adjuvants reversing the MDR phenotype. The aim of this study was to determine whether combined treatment with new Se-compounds would increase the effect of doxorubicin in doxorubicin sensitive and resistant breast cancer cell lines. Se-compounds were evaluated regarding their cytotoxic and apoptosis-inducing effect on MCF-7 and ABCB1-overexpressing KCR breast cancer cell lines. Moreover, the interaction of Se-compounds with doxorubicin was assessed by using checkerboard assay. A selenoanhydride exerted a selective activity towards the doxorubicin-resistant KCR cell line overexpressing ABCB1. Among selenoesters, only ketone-containing selenoesters exerted significant cytotoxic activity against MCF-7 and KCR cell lines; in addition, Se-compounds acted synergistically with doxorubicin on the KCR cell line. The importance of the $COSeCH_2COCH_3$ and $COSeCH_2CO(CH_3)_3$ moieties for the cytotoxic and adjuvant role of Se-compounds was highlighted.

According to the WHO, the estimate number of new chlamydial infections was 129 million in 2020. Recurrent or persistent infection occurs in 10 %–15 % of women who are treated for *C. trachomatis* infection. Several studies have shown potent antibacterial activity of the Se-compounds; therefore, the anti-chlamydial activity of se-compounds was investigated against *C. trachomatis* D. Among the Se-compounds, methylketone selenoester (**9**) exerted the most potent activity against *C. trachomatis* D at a very low concentration (0.25 μM).

Among the remaining selenoesters, symmetric dimethyl selenodiester, which contains a thiophene ring (**2**), and methyl oxoester derivative (**7**) showed a better activity, and the activities of symmetric dimethyl selenodiesters (**3**) and (**5**) were also remarkable. This fact highlights the importance of symmetry for the activity against intracellular pathogens.

X. ÖSSZEFOGLALÓ

Az Európai Unió tagállamai közül Magyarországon a legmagasabb a tüdőrák és a vastagbélrák előfordulása, illetve az ezen daganatokhoz kapcsolódó halálozás. A kemoterápia célja a rosszindulatú sejttömeg elpusztítása vagy csökkentése, valamint a beteg életminőségének javítása. A kemoterápia alkalmazását megnehezítheti az alacsony biohasznosulás, az alacsony szelektivitásból adódó kedvezőtlen mellékhatások, valamint a multidrog rezisztencia (MDR) kialakulása, amelynek során a daganatok rezisztenssé válhatnak a különböző szerkezetű és hatásmechanizmusú kemoterápiás szerekkel szemben. A kutatásunk során az MDR két fontos mechanizmusát vizsgáltuk: az apoptózis indukció gátlását és az energiafüggő, overexpresszált efflux pumpák gátlását. Számos olyan vegyületet írtak le, amelyek képesek gátolni az ABCB1 efflux pumpa működését. A kemoszenzitizáló molekulák adjuvánsként történő alkalmazása olyan új terápiás stratégia, amely visszafordíthatja az MDR fenotípust. A jelen vizsgálat célja annak meghatározása volt, hogy az új szelénvegyületekkel történő kombinált kezelés növeli-e a doxorubicin hatását doxorubicin érzékeny és rezisztens emlőrák sejtvonalakban. Az szelénvegyületeket citotoxikus és apoptózist indukáló hatásuk szempontjából vizsgáltuk MCF-7 és az ABCB1 pumpát túltermelő KCR emlőrák sejtvonalakon. Emellett a szelénvegyületek és a doxorubicin kölcsönhatását is vizsgáltuk checkerboard módszer segítségével. A szelenoanhidrid szelektív hatást fejtett ki a doxorubicin rezisztens, ABCB1-et overexpresszáló KCR sejtvonallal szemben. A szelenoészterek közül csak a keton-szelenoészterek fejtettek ki jelentős citotoxikus aktivitást MCF-7 és a KCR sejtvonalon, emellett szelénvegyületek szinergista kölcsönhatást mutattak doxorubicinnel a KCR sejtvonalon. Korábbi vizsgálatok kiemelték a $COSeCH_2COCH_3$ és a $COSeCH_2CO(CH_3)_3$ csoportok fontosságát a szelénvegyületek citotoxikus hatásában és adjuváns funkciójában.

A WHO 2020-ban 129 millióra becsülte az új *Chlamydia* fertőzések számát. A *C. trachomatis* fertőzés miatt kezelt nők 10–15%-ánál fordul elő visszatérő vagy tartós fertőzés. Számos tanulmány kimutatta a szelénvegyületek erős antibakteriális hatását, ezért a szelénvegyületek *Chlamydia* ellenes aktivitásának vizsgálatát végeztük el *C. trachomatis* D törzsön. A szelénvegyületek közül a metil-keton-szelenoészter (**9**) fejtette ki a legerősebb aktivitást a *C. trachomatis* D ellen nagyon alacsony koncentrációban (0,25 μM).

A többi szelenoészter közül a szimmetrikus dimetil-szelenodiészter, amely a tioféngyűrűt tartalmazó (**2**)-es számú vegyület és a metil-oxoészter (**7**) származék erősebb aktivitást mutatott, és a szimmetrikus dimetil-szelenoészterek (**3**) és (**5**) aktivitása is figyelemre méltó volt. Ez a tény rávilágít a szimmetria fontosságára az intracelluláris kórokozók elleni aktivitás szempontjából.

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XIV. APPENDIX

A. PUBLICATIONS

I.

Csonka A, Kincses A, Nové M, Vadas Z, Sanmartín C, Domínguez-Álvarez E, Spengler G. Selenoesters and Selenoanhydrides as Novel Agents Against Resistant Breast Cancer. Anticancer Res. 2019 Jul;39(7):3777-3783. doi: 10.21873/anticanres.13526. PMID: 31262904.

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Selenoesters and Selenoanhydrides as Novel Agents Against Resistant Breast Cancer

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

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

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

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

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

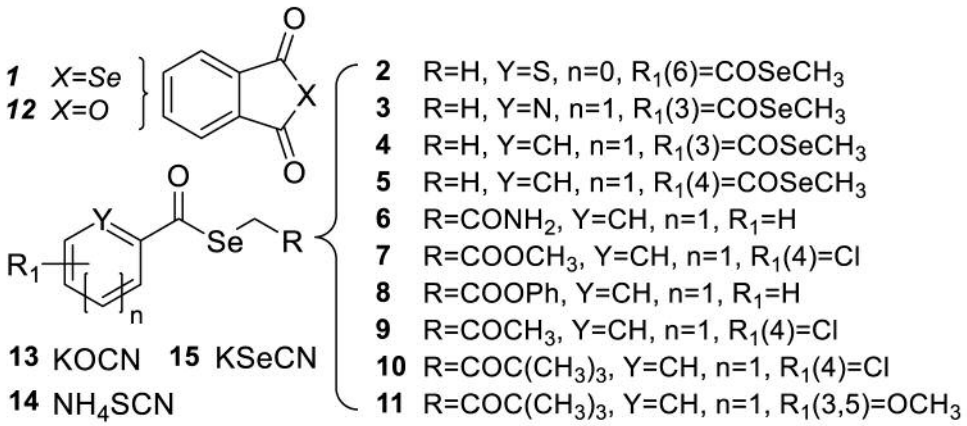


Figure 1. *Chemical structure of the tested compounds. The number in parentheses denotes the position at which R_1 is bound to the (hetero) aromatic ring.*

Materials and Methods

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

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

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

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

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

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

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

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

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

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

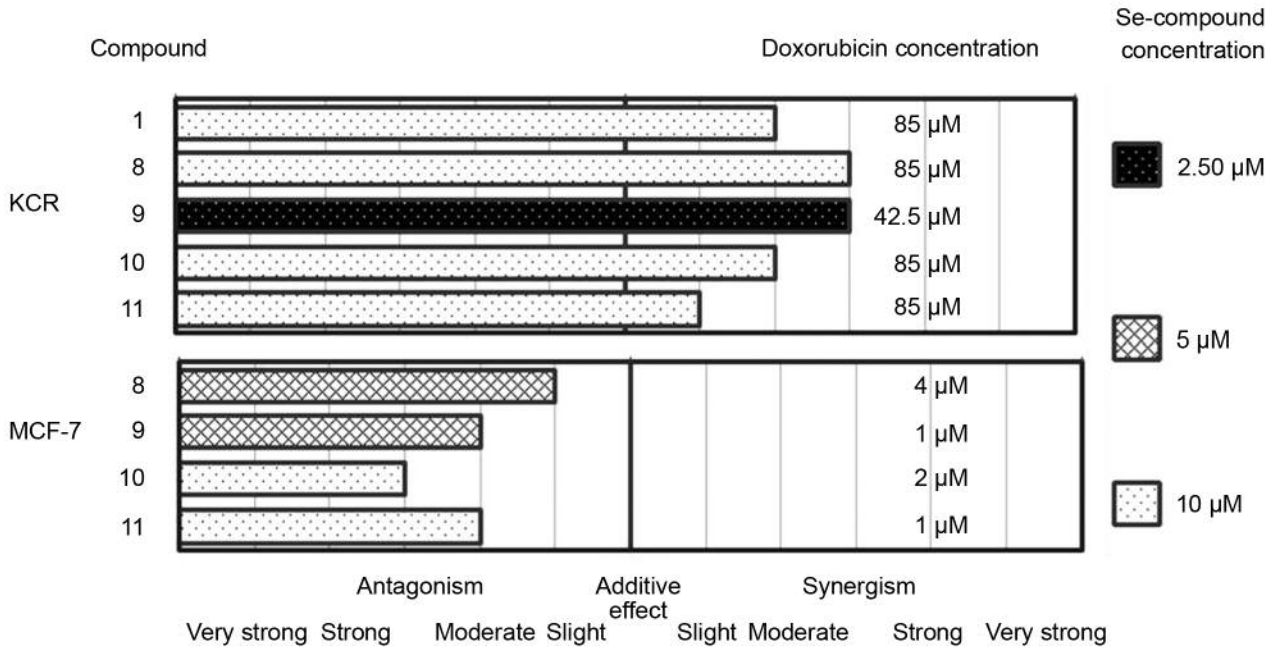


Figure 2. *Interactions of the active Se-compounds with doxorubicin in KCR and MCF-7 cells. The figure indicates, at the most effective interaction ratio (doxorubicin:Se-compound), the concentration of the Se-compound in the presence of doxorubicin at the concentration indicated; furthermore the type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

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

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

Results

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

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

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

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

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

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

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

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

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

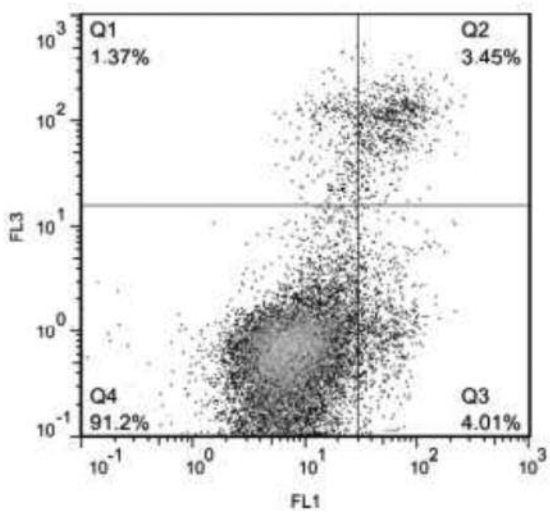
Finally, the compounds were not able to induce significant apoptotic events in MCF-7 and KCR cells; with the exception of the phenoxycarbonylmethyl selenoester **7** in MCF-7 cells. This derivative, at a low concentration (2 μM), triggered early apoptotic and late apoptotic/necrotic events in 16.9% and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

Discussion

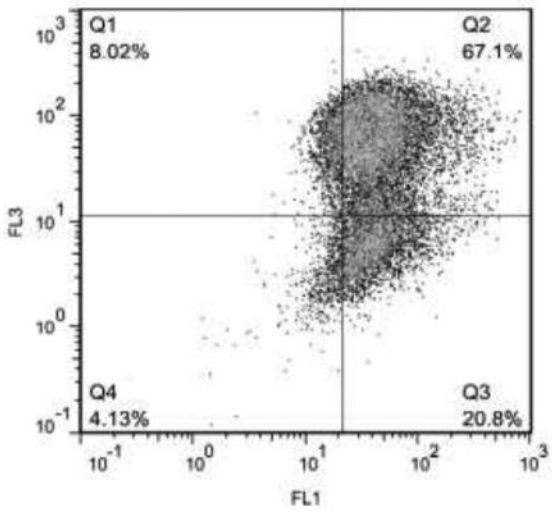
Conventional chemotherapy in the treatment of early and metastatic breast cancer is partly based on the administration of anthracycline drugs *e.g.* doxorubicin. Since these drugs provoke side-effects such as cardiotoxicity and myelosuppression (14, 15), there is an urgent need to minimize the side-effects.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

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

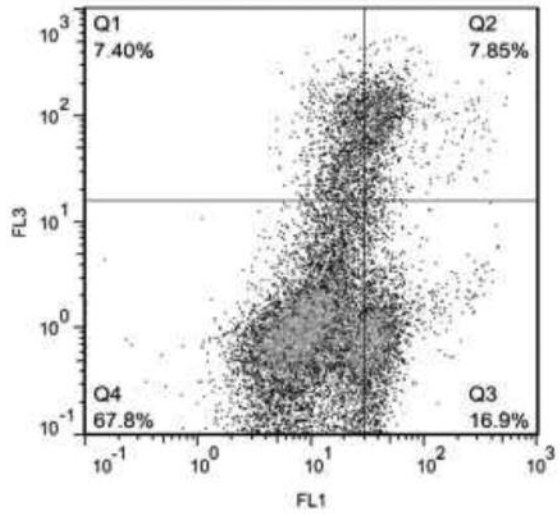
As commented in the previous section, selenoanhydride **1** exerted selective activity towards the resistant KCR cell line overexpressing ABCB1 (IC_{50}=2.35 μM), as it was



Untreated control



12*H*-[α]benzophenothiazine (20 μM)



Compound **7** (2 μM)

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

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

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

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

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

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

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

Conflicts of Interest

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

Authors' Contributions

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

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Selenocompounds as Novel Antibacterial Agents and Bacterial Efflux Pump Inhibitors

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Abstract: Bacterial multidrug resistance is becoming a growing problem for public health, due to the development and spreading of bacterial strains resistant to antimicrobials. In this study, the antibacterial and multidrug resistance reversing activity of a series of seleno-carbonyl compounds has been evaluated. The effects of eleven selenocompounds on bacterial growth were evaluated in *Staphylococcus aureus*, methicillin resistant *S. aureus* (MRSA), *Enterococcus faecalis, Escherichia coli*, and *Chlamydia trachomatis* D. The combination effect of compounds with antibiotics was examined by the minimum inhibitory concentration reduction assay. Their efflux pump (EP) inhibitory properties were assessed using real-time fluorimetry. Relative expressions of EP and quorum-sensing genes were studied by quantitative PCR. Results showed that a methylketone selenoester had remarkable antibacterial activity against Gram-positive bacteria and potentiated the activity of oxacillin in MRSA. Most of the selenocompounds showed significant anti-chlamydial effects. The selenoanhydride and the diselenodiester were active inhibitors of the AcrAB-TolC system. Based on these results it can be concluded that this group of selenocompounds can be attractive potential antibacterials and EP inhibitors. The discovery of new derivatives with a significant antibacterial activity as novel selenocompounds, is of high impact in the fight against resistant pathogens.

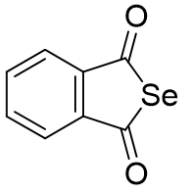
Keywords: selenocompounds; selenoesters; AcrAB-TolC efflux pump; *Chlamydia trachomatis* D; *Escherichia coli* K-12 AG100; *Staphylococcus aureus*

1. Introduction

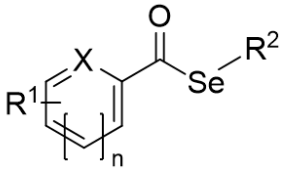
Multidrug resistance is becoming a serious problem in the treatment of resistant bacterial infections. The discovery of novel antibacterial or multidrug resistance reversing agents is extremely urgent as soon we may lack effective drugs to treat bacterial infections caused by the arising superbugs resistant to the majority of the clinically available antibiotics [1]. Selenium (Se)-containing molecules could be possible alternatives in the development of a new approach to combat infections caused by multidrug resistant (MDR) pathogens. Se is an important element in biological molecules in archea, bacteria, and eukaryotes [2]. In humans, Se is an essential trace element and also has chemopreventive effects [3].

In this context, a few studies have reported that certain selenocompounds have shown an interesting antibacterial activity. First, a series of selenides-bearing benzenesulfonamide moieties has been found to strongly inhibit the carbonic anhydrases VchCAα and VchCAβ of *Vibrio cholerae*, thus, exerting an inhibition on the growth and pathogenicity of this bacterium [4]. In addition, a degraded selenide polysaccharide, extracted from *Enteromorpha prolifera*, has been found to show antibacterial activity against *Escherichia coli* [5]. Additionally, a series of fused selenazolinium salts have been shown to have a potent activity against **ESKAPE** pathogens, which are: vancomycin-resistant **E**nterococci, methicillin-resistant ***S**taphylococcus aureus* (MRSA), ***K**lebsiella pneumoniae, **A**cinetobacter baumannii, **P**seudomonas aeruginosa*, and carbapenem-resistant ***E**nterobacteriaceae*. The majority of these compounds have minimum inhibitory concentration (MIC) values below 1 μg/mL, in resistant bacterial strains of MRSA, *K. pneumoniae*, *A. baumanii*, and *P. aeruginosa* [6]. This last work highlights the potential applications of selenocompounds, in the treatment of infections caused by the MDR bacterial strains. Our previous studies have demonstrated that the selenoanhydride **1** and selected selenoesters **2–11** (Table 1) have shown potent anticancer activity against ATP-Binding cassette sub-family B member 1 (ABCB1)-overexpressing MDR mouse T-lymphoma cells and MDR colon adenocarcinoma cells [7,8]. The ABC family of protein transporters also plays an important role in bacterial multidrug resistance [9]. Several members of the ABC family, e.g., MsrA in staphylococci [10,11] or Msr(D) in *Streptococcus pneumoniae* [12], significantly contribute to the efflux of antibiotics, and are considered as attractive protein targets in experimental chemotherapy. A major factor of bacterial and cancer drug resistance is assigned to the MDR efflux transporter proteins, expelling toxic compounds and drugs out of the cells. Based on the energy source of these pumps, the primary transporter derives their energy from the hydrolysis of ATP (ABC-transporters) and secondary transporters use proton or ion gradients to drive the extrusion of toxic compounds. Using selenocompounds, such as chemosensitizers, these compounds have been shown to inhibit the ABCB1 in cancer cells [7,8], and based on these results, our aim was to investigate the efflux pump inhibitory properties of these selenocompounds on the representative bacterial efflux system AcrAB-TolC. RND (Resistance–Nodulation–Division) family transporters are widespread, especially among Gram-negative bacteria, and catalyse the efflux of antibiotics and biocides. This tripartite efflux system consists of an outer membrane channel and periplasmic adaptor proteins, and the inner membrane transporter AcrB [13]. The MarR transcription factor regulates resistance to diverse antibiotics, organic solvents and oxidative stress agents by controlling the expression of efflux pumps (including AcrAB-TolC) through the repression of the operon that encodes the transcriptional activator MarA. The antibiotic resistance arises when the MarR protein is inactivated or the expression of *marR* genes is inhibited [14]. Although the expression of AcrAB-TolC efflux pump is regulated at several levels, the MarR the AcrR also regulates it negatively, meanwhile, the MarA, SoxS, and Rob are activators of this efflux pump [15]. In addition, the quorum sensing (QS) regulators, such as SdiA could also affect the expression of AcrAB-TolC efflux pump in *E. coli*, since AcrAB-TolC has been proposed to pump out QS signals [16].

Table 1. Selenocompounds evaluated as antibacterial and as multidrug resistance reversing agents—selenoanhydride (**1**) and selenoesters (**2**–**11**).



I. Cyclic selenoanhydride (**1**)



II. Selenoesters (**2**–**11**)

Compound	Group	R^1	X	n	R^2
1	**I**	-	-	-	-
2	**II**	5-$COSeCH_3$	S	0	-CH_3
3	**II**	6-$COSeCH_3$	N	1	-CH_3
4	**II**	3-$COSeCH_3$	C	1	-CH_3
5	**II**	4-$COSeCH_3$	C	1	-CH_3
6	**II**	-H	C	1	-CH_2CONH_2
7	**II**	4−Cl	C	1	-CH_2COOCH_3
8	**II**	-H	C	1	-CH_2COOPh
9	**II**	4−Cl	C	1	-CH_2COCH_3
10	**II**	4−Cl	C	1	-$CH_2COC(CH_3)_3$
11	**II**	3,5-diOCH_3	C	1	-$CH_2COC(CH_3)_3$

Furthermore, coating surfaces with Se could reduce the bacterial attachment to prosthetic devices [17], whereas sodium selenite exhibited ulcer healing and antibacterial activity against *Helicobacter pylori* [18]. Various studies have highlighted the antimicrobial properties of elemental Se, in the form of nanoparticles (SeNP) against *S. aureus* [19–22], *Staphylococcus epidermidis*, *K. pneumoniae*, *Bacillus subtilis* [23], *P. aeruginosa*, *E. coli*, and *A. baumannii* [24]. Additionally, biogenic SeNPs, synthesized by different non-pathogenic bacterial strains and stabilized with bacterial proteins, have shown activity against pathogenic bacteria [25,26].

In addition, there is an emerging evidence that *Chlamydia trachomatis* is developing resistance to antibiotics, as certain clinical isolates have shown single- or multidrug resistance [27,28]. Consequently, the development of new antibacterials and multidrug resistance reversing compounds is required to overcome this emerging problem. Although there are numerous studies that have investigated the antibacterial activity of Se-containing (in)organic compounds and SeNPs, according to our knowledge, no report has been described regarding anti-chlamydial activity of selenocompounds. Furthermore, the selenocompounds found as anticancer agents and cancer efflux pump inhibitors have not yet been tested on any bacterial strains.

Herein, we report the antibacterial effects of selenocompounds **1**–**11** on Gram-negative and Gram-positive bacteria, such as *E. coli*, *C. trachomatis* D, *Enterococcus faecalis*, and *S. aureus* (including methicillin resistant strain, MRSA).

2. Results

2.1. Antibacterial Activity: Determination of the MIC

The ketone-containing selenoesters **9**–**11** showed a potent antibacterial activity against the Gram-positive *S. aureus* ATCC 25923 and MRSA HEMSA 5. The methylketone selenoester **9** was the most active agent with noteworthy MIC values in the low micromolar range (3.12 and 3.91 μM). The *tert*-butylketone selenoesters **10** and **11** showed lower antibacterial activity than methylketone selenoester **9**, but was still significant (25 and 50 μM). The selenoanhydride **1** and the remaining selenoesters **2**–**8** evaluated were inactive as their MIC were equal or above 100 μM. The selenoester **9** showed also significant antibacterial activity towards *E. faecalis*, but this Gram-positive strain was less

sensitive to **9** than *S. aureus* and MRSA (MIC = 12.5 μM), and also was not sensitive to the rest of the selenocompounds tested (MIC > 100 μM), (Table 2).

In contrast, none of the eleven Se derivatives demonstrated antibacterial effects against the two Gram-negative strains evaluated in this study, which are the AcrAB-TolC-expressing *E. coli* AG100 and the AcrAB-TolC-deleted mutant *E. coli* AG100A. In these two strains, all compounds showed MIC values above 100 μM (data not shown).

Table 2. Minimum inhibitory concentration (MICs) of the selenocompounds on the Gram-positive bacteria. In bold—MIC values <10 μM.

Compounds	MIC (μM)		
	Staphylococcus aureus **ATCC 25923**	***Staphylococcus aureus*** **HEMSA 5**	***Enterococcus Faecalis*** **ATCC 29212**
1	>100	>125	>100
2	100	>125	>100
3	100	>125	>100
4	100	>125	>100
5	>100	>125	>100
6	>100	>125	>100
7	100	125	>100
8	100	>125	>100
9	**3.12**	**3.91**	12.5
10	25	>125	>100
11	50	>125	>100

2.2. Enhancement of the Activity of Antibiotics

In order to determine if selenocompounds **1**–**11** enhance the activity of antibiotics, they were tested in combination with antibiotics commonly used in clinical therapy, which are substrates of the AcrB pump—tetracycline [29] and ciprofloxacin [30]. The combined effects of selenocompounds and these antibiotics were tested on the AcrAB-TolC expressing Gram-negative *E. coli* AG100 strain. In addition, the chemosensitizing effects of selenocompounds on the Gram-positive MRSA HEMSA 5 strain were studied in combination with oxacillin. These antibiotics have been selected among the ones that are more widely used in clinical practice, in an attempt to cover different mechanisms of action, to see which ones are more affected by the selenocompounds. Gram-negative efflux pumps of the RND superfamily in Gram-negative bacteria are crucial to the cellular defence mechanisms, but the overexpression of these pumps can lead to multidrug resistance, which is an alarming problem for health care. The AcrAB-TolC system containing the RND type pump AcrB has been studied extensively, due to its importance in bacterial resistance. As an in vitro model system we used the AcrAB-TolC overexpressing *E. coli* AG100 strain and its pump-deleted mutant strain *E. coli* AG100A, in order to find effective efflux pump inhibitor (EPI) compounds. EPIs as chemosenzitizers could reverse the resistant phenotype, and in combination with antibiotics, they could enhance the activity of these conventional antibiotics. Moreover, the Gram-positive methicillin resistant *Staphylococcus aureus* (MRSA) is a major concern in healthcare facilities, for this reason our aim was to test the selenocompounds on reference ATCC and resistant MRSA strains. The enhancement of the activity of oxacillin was studied as the ability of compounds **1**–**11**, to reduce MIC of oxacillin against MRSA, whereas the enhancement of tetracycline or ciprofloxacin was studied analogously for AcrAB-TolC-expressing *E. coli* strain (Table 3).

In the absence of the selenocompounds, oxacillin showed MIC value of 374 μM (150 μg/mL) against MRSA, since this strain was highly resistant to this β-lactam antibiotic. The methylketone selenoester **9**, at a low concentration of 1.95 μM (0.537 μg/mL), exerted a noteworthy 64-fold reduction of the MIC value of oxacillin to 5.84 μM (2.34 μg/mL). Hence, this compound can be useful as a potent agent to reverse the resistance of MRSA to oxacillin. Interestingly, the rest of the compounds **1**–**8**, **10**, and **11** were not active, even at the concentration of 62.5 μM (MIC reduction of oxacillin ≤ 2).

However, none of the tested selenocompounds were able to improve the efficacy of antibiotics against the AcrAB-TolC-overexpressing strain of *E. coli* with the above-mentioned excellent 64-fold factor. The highest reductions observed in *E. coli* were a 2-fold reduction of the MIC values of tetracycline (compound **9**) and of ciprofloxacin (compounds **9, 10**).

Table 3. Numerical value of the reduction of the MICs of selected antibiotics in methicillin resistant *S. aureus* (MRSA) or in *E. coli* AG100 exerted by selenocompounds when administered in combination with antibiotics.

Cpd[1]	MRSA HEMSA 5		*Escherichia coli* AG100		
	Concentration of Compound [μM][2]	Reduction of Oxacillin MIC	Concentration of Compound [μM]	Reduction of Tetracycline mic	Reduction of Ciprofloxacin mic
1	62.5	no effect	50	no effect	no effect
2	ND	ND [3]	50	no effect	no effect
3	62.5	2-fold	50	no effect	no effect
4	62.5	no effect	50	no effect	no effect
5	62.5	no effect	50	no effect	no effect
6	62.5	no effect	50	no effect	no effect
7	62.5	≥ 2-fold	50	no effect	no effect
8	62.5	2-fold	50	no effect	no effect
9	1.95	64-fold	25	2-fold	2-fold
10	62.5	no effect	50	no effect	2-fold
11	62.5	no effect	50	no effect	no effect

[1] Cpd: Compound. [2] Starting concentration of tetracycline: 8.4 μM; ciprofloxacin: 1.4 μM; and oxacillin: 747 μM. [3] ND: Not determined.

2.3. Anti-Chlamydial Activity

Before the assessment of the anti-chlamydial activity of the selenocompounds, a cytotoxicity assay was performed on HeLa cells to determine the ranges of concentrations at which the selenocompounds can be evaluated without showing direct toxic effects to HeLa cells. Selenocompounds **2**, **3**, **5**, **7**, and **9**–**11**, significantly inhibited the formation of chlamydial inclusions at selected concentrations (Figure 1).

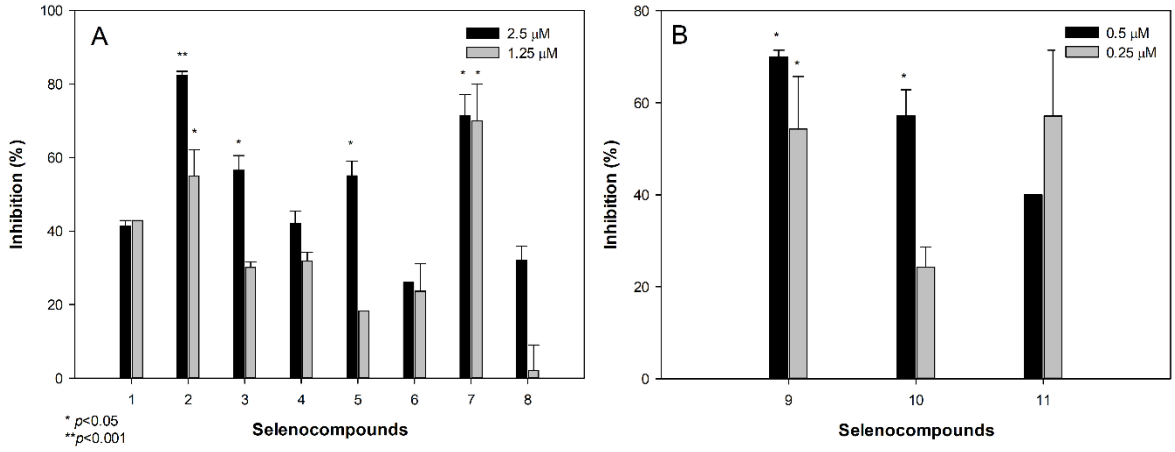


Figure 1. Anti-chlamydial effect of selenocompounds at 1.25 and 2.5 μM (**A**), and at 0.25 and 0.5 μM (**B**).

Compounds **2** and **7** at 2.5 μM, showed 82% and 71% inhibition, compared to the control, respectively. In addition, **2** and **7** were effective at 1.25 μM, whereas **9** and **10** inhibited the formation of inclusions at low submicromolar concentrations of 0.5 μM. The most potent anti-chlamydial selenocompounds were **9** and **11**, as they inhibited more than 50% of the growth of *C. trachomatis* D, at a concentration of 0.25 μM (0.0689 and 0.0858 μg/mL, respectively).

2.4. Real-Time Accumulation Assay

Since ethidium bromide (EB) is a substrate of the AcrB efflux pump, the intracellular accumulation of EB provides information about the inhibition of the AcrAB-TolC system, in the presence of selenocompounds, in a time-dependent manner. The assay records the real-time accumulation of EB, using a real-time thermocycler, by monitoring the fluorescence of EB inside the cells [31]. The activities of compounds **1**–**11** in the real-time EB accumulation assay, were given in terms of the relative fluorescence index (RFI) of the real-time accumulation curves (Table 4). In case of the real-time EB accumulation, the amount of EB accumulated by cells was higher if the difference between $RF_{treated}$ and $RF_{untreated}$ was greater, therefore, the degree of inhibition of the efflux pump system by the compound became greater. Compounds **9** and **10** possessed EPI activity and decreased the MIC of ciprofloxacin on *E. coli* AG100. However, the selenoanhydride **1** and the selenoester **4**, compared with the positive control promethazine (PMZ, RFI: 0.15), strongly inhibited the efflux of AcrAB-TolC in *E. coli* AG100; they had no effect in combination with the antibiotics, suggesting that other cellular mechanisms might also be involved in the mode of action, such as interaction with cell wall components, formation of reactive oxygen species (ROS), or membrane destabilizing effects. Without investigating the possible metabolites of the selenocompounds, no further conclusions can be drawn, for this reason we are planning to study the metabolites of these compounds in future works. Derivatives **7** and **9**–**11** caused moderate inhibitory action, whereas **2**, **3**, **5**, **6** and **8** showed weak or no activity on the intracellular EB accumulation in *E. coli* AG100. Among derivatives **7** and **9**–**11**, compound **7**, which contained a methyl oxoester in the alkyl moiety bound to Se, was the most active agent (RFI = 0.13).

Table 4. Relative fluorescence index (RFI) for the effect of selenocompounds and positive control promethazine (PMZ) on the AcrAB-TolC-expressing *Escherichia coli* AG100 strain.

Compound	RFI[a]	Compound	RFI[a]	Compound	RFI[a]
	***Escherichia coli* AG100**		***Escherichia coli* AG100**		***Escherichia coli* AG100**
1	0.28	**5**	0.04	**9**	0.11
2	0.03	**6**	0.06	**10**	0.12
3	0.04	**7**	0.13	**11**	0.11
4	0.18	**8**	0.08	**PMZ**	0.15

Nevertheless, no efflux pump inhibitory action of selenocompounds (**1**–**11**) was found in the *E. coli* AG100A strain.

2.5. Gene Expression Analysis by Quantitative PCR

For the effect of the selenocompounds on the relative expression of the efflux pump, antibiotic resistance and QS genes in *E. coli* AG100—the most effective compounds in the EB real-time accumulation assay—were examined (compounds **1**, **4**, **7**; Figure 2). In this assay, the genes of the multidrug efflux pump (AcrAB), the component of the *E. coli mar* locus (multiple antibiotic resistance), and the gene of SdiA were investigated. The changes in gene expression from reverse transcription quantitative PCR experiments were normalized to the expression of *gapdh* (internal control), in the same sample, and compared to the expression of the examined genes obtained from the untreated, control samples.

As shown in Figure 2A, compound **1** at 50 μM concentration significantly up-regulated the *acrB*, *marR*, and *sdiA* genes, after 4 h of exposure. However, after 18 h, the expression of the *acrB* gene returned to the basal levels and the expression of the *marR* and *sdiA* genes, significantly increased. Compound **4** up-regulated the expression levels of *acrB*, *marR*, and *sdiA*, after 4 h, although after 18 h, the expression levels of the *acrB* and *marR* genes decreased. The QS gene *sdiA* was significantly up-regulated after 18 h (Figure 2B).

Compound **7** also significantly up-regulated *marR*, after exposures of 4 h and of 18 h. After 18 h, the expression level of the RND transporters subunit genes (*acrA*, *acrB*) was significantly increased (Figure 2C).

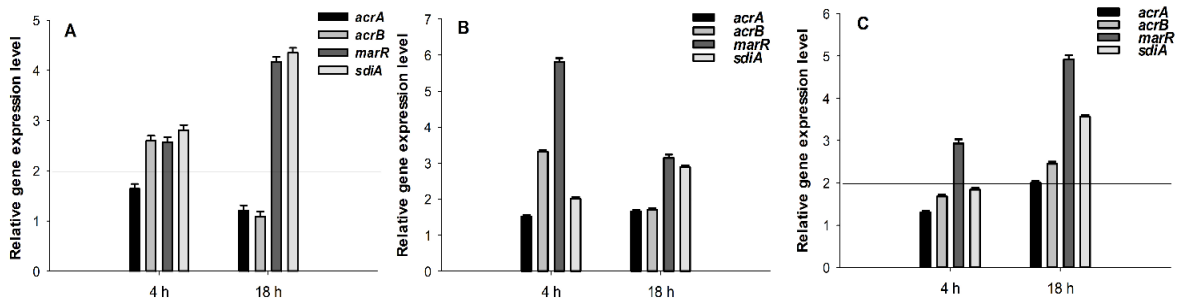


Figure 2. Relative gene expression levels of the genes of the *acrA*, *acrB*, *marR*, and *sdiA* in the presence of compounds **1** (**A**), **4** (**B**), and **7** (**C**), after 4 and 18 h exposure. The line denotes a threshold value, which was set at a two-fold increase in transcripts.

3. Discussion

Results of these studies indicate that selenoesters and selenoanhydrides, previously found as active anticancer or ABCB1 efflux pump inhibitors in cancer cells [7,8,32–35], also displayed a promising antimicrobial potential against the MDR bacterial strains.

3.1. Antibacterial Activity

The evaluation of the compounds proved that the ketone-containing selenoesters **9**–**11** showed an antibacterial activity against the Gram-positive reference *S. aureus* strain, whereas, the methylketone selenoester **9** was also active against the MRSA HEMSA 5 and *E. faecalis*. However, none of the compounds were active against the Gram-negative *E. coli* ag100. The background of the different antibacterial activity of **9** against the tested Gram-positives and Gram-negatives was unknown; further experiments are required to clarify whether the mechanism of action of the methylketone selenoester could be related with any kind of interaction between this compound and the bacterial cell wall that is typical for gram-positive bacteria. In contrast, the remaining alkyl groups (–CH_3) or alkyl-functionalized moieties (–CH_2CONH_2, –CH_2COOCH_3 and –CH_2COOPh, Table 1) bound to the Se atom rendered selenoesters that were ineffective against the tested strains. Interestingly, the compounds **9**–**11** were also the most potent anticancer agents in previous works [7,8,33], and they also showed a good selectivity towards cancer cells, with respect to non-tumour cell lines, as they showed selectivity indexes ranging from 8.4 to 14.4 [8].

In previous works, it was hypothesized that the possible mechanism of action of these compounds could be the hydrolysis of the compound and the subsequent liberation of the ionic species of Se, which could be responsible for the activity of the compounds [33]. In this case, this phenomenon enables us to hypothesize that the CH_3COCH_2SeH, or its anionic form, are the chemical forms of Se that could be behind the observed activities. The lack of activity of the non-ketone selenoesters, directs a special attention to this –$SeCH_2COCH_3$ ketone-containing moiety.

3.2. Enhancement of the Activity of Antibiotics

The activity of **9** on the MDR clinical isolate (MRSA) was very promising, because compound **9** reduced the MIC of oxacillin in 64-fold (from 374 μM to 5.84 μM). These results supported the potential applications of the methylketone selenoesters, such as antimicrobials, and the multidrug resistance reversing agents. These results were in accordance with the activity shown by these compounds as enhancers of the anticancer activity of chemotherapy drugs [35], suggesting that these selenium derivatives have the ability to effectively interact with the resistance mechanisms developed by the resistant bacterial strains and by the resistant cancer cells. This work intends to carry out a screening of the potential applications of the selenocompounds, and in future works we will attempt to ascertain the possible mechanisms of actions of the activities described herein, as this observed potential enhancement of the activity of oxacillin exerted by compound **9** in an MRSA clinical isolate.

3.3. Anti-Chlamydial Activity

Previous studies have reported that selected selenocompounds, such as certain selenocyanates, selenoureas, and diselenides, showed antiproliferative activities against the intracellular forms of *Leishmania spp.* [36,37]. Taking those results into account, this study provided a new line of evidence for the action of selenoanhydride/selenoesters on an obligate intracellular chlamydial strain. In particular, different selenoesters, such as **2**, **3**, **5**, **7**, and **9**–**11**, have exerted a noteworthy activity against *C. trachomatis* D. Furthermore, the activities of the methyl (**9**) and the *tert*-butyl (**11**) derivatives were very promising, as they inhibited the formation of more than 50% of the chlamydial inclusions, at a very low concentration (0.25 μM). However, their mode of action has not been ascertained in this study.

Regarding the observed structure activity relationships of the anti-chlamydial assays, the ketone selenoesters **9**–**11** showed noteworthy activity at lower concentrations (0.25 μM, 0.5 μM), compared to the rest of the series (1.25 μM, 2.5 μM). Among the remaining selenoesters, the symmetric dimethyl selenodiester, which contains a thiophene ring **2**, and the methyl oxoester derivative **7** showed a better activity, and the activities of the symmetric dimethyl selenodiesters **3** and **5** were also remarkable. These fact highlights the importance of the symmetry for the activity against intracellular pathogens [36].

3.4. Interaction of the Compounds with Bacterial Efflux Pumps

The resistance to the current antibacterial drugs is one of the major therapeutic challenges in the treatment of bacterial infections, and knowing the potential of these derivatives as multidrug resistance reversing agents (proved both by the capacity to enhance the activity of antibiotics described above and by the enhancement of anticancer drugs reported in previous works), we have studied here the procedure through which selenocompounds interact with the bacterial AcrAB-TolC system in the *E. coli* AG100 strain.

The results obtained revealed that the cyclic selenoanhydride **1** significantly inhibited this bacterial AcrAB-TolC efflux pump in the *E. coli* AG100 strain. Similarly, EP inhibiting activity has been found for compounds **4** and **7**. The second most potent inhibitor was the symmetrical benzene derivative 1,3-disubstituted with methylselenoester moieties (**4**). Interestingly, its 1,4-disubstituted analogue (**5**) showed an EP-inhibitory activity, 4.5-fold lower, suggesting the importance of the substituents' topology for the expected biological effect. Taking into account the distinct difference in electron density properties between *m*- and *p*-substituted phenyl rings, this factor seems to have affected the mechanisms of EP inhibition.

The well-characterized RND-type transporter, AcrB is associated with TolC and AcrA and is the major efflux pump of *E. coli* [38]. These efflux pumps recognize and extrude a large variety of antibiotics from the cytoplasm. The energy required for the operation of the efflux pump is provided by the proton motive force, created by the proton gradient resulting from electron transport [39]. This fact suggests that those selenocompounds, which possessed EP inhibitor activity, might interfere with the proton motive force. Surprisingly, compounds **1** and **4**, which inhibited the AcrAB-TolC system, influenced the expression of the gene *acrB*, which is a constituent of the AcrAB-TolC system. In addition, the compounds increased the expression of the QS gene *sdiA*, after 18 h of exposure, which suggests their roles in QS, although their QS inhibitory activities were not investigated in this study.

4. Materials and Methods

4.1. Chemistry

Eleven pure selenocompounds obtained as described earlier [33], were examined (**1**–**11**, Table 1). All compounds were stable in air and their purity was assessed by elemental analysis and ^{1}H and ^{13}C NMR, as reported in a previous work [35]. Before their use in biological assays, they were dissolved in dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany), to obtain stock solutions. Working solutions were prepared by dilutions in the culture medium.

4.2. Bacterial Strains

Wild-type *E. coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44] and its AcrAB-TolC-deleted mutant strain *E. coli* AG100A (a kind gift from Hiroshi Nikaido, Department of Molecular and Cell Biology and Chemistry, University of California, Berkeley, USA) were used for the evaluation of the EPI activity of the tested selenocompounds.

S. aureus ATCC 25923 and *E. faecalis* ATCC 29212 strains were used to determine the MIC. A methicillin-resistant *S. aureus* strain (MRSA HEMSA 5, a clinical isolate) was used in the combination assay, with oxacillin, to determine the capacity of compounds to enhance the antibacterial effect of this antibiotic. *C. trachomatis* reference strain (serovar D, UW-3/Cx, ATCC, VR-885D) was used in the anti-chlamydial assay.

4.3. Propagation of C. trachomatis D

C. trachomatis D was propagated on the HeLa 229 cells (ATCC, CCL-2.1), as described earlier [40]. The titre of the infectious elementary bodies was determined by an indirect immunofluorescence assay. Serial dilutions of the elementary bodies' preparation were inoculated onto the HeLa monolayers and, after a 48-h culture, the cells were fixed with acetone, and stained with monoclonal anti-*Chlamydia* LPS antibody (AbD Serotec, Oxford, UK) and FITC-labelled anti-mouse IgG (Merck KGaA, Darmstadt, Germany). The inclusions of *C. trachomatis* D were enumerated under a UV microscope.

4.4. Determination of MIC

The effects exerted by different concentrations of the compounds on the bacterial growth in *S. aureus*, *E. faecalis*, and *E. coli* AG100 were tested in 96-well plates. The MICs of selenocompounds were determined, according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [41]. The DMSO exerted no antibacterial effect. Alternatively, the MIC of the oxacillin in MRSA HEMSA 5 was determined by the broth microdilution method, in a cation-adjusted Mueller–Hinton Broth (MHB II), according to the recommendations of the CLSI. Results were recorded after a 20- or 24-hour incubation at 37 °C.

4.5. Enhancement of the Activity of Antibiotics

The chemosensitizing effect of the tested selenocompounds was evaluated through the determination of the MIC values of the antibiotics, in the presence of sub-inhibitory concentrations of the compounds (MIC/2 or MIC/4), in both Gram-negative (*E. coli* AG100) and Gram-positive (MRSA) strains. The MICs were evaluated in the *E. coli* strain, by a two-fold broth microdilution method in the 96-well plates, using serial dilutions of tetracycline (from 8.4 to 0.16 μM) and ciprofloxacin (from 1.4 to 2.7×10^{-3} μM). The first four rows contained two-fold dilutions of antibiotics, and the combinations of the antibiotics and tested compounds were added into the last four rows. 10^{-4} dilution of an overnight bacterial culture in 50 μL of MHB was then added to each well, with the exception of the medium control wells. The plates were then incubated at 37 °C for 18 h. MIC values of the antibiotics and their combination with the tested compounds were determined by naked eyes. In the assay with oxacillin in the MRSA HEMSA 5 bacterial strain, a microdilution method in MHB II was used.

4.6. Anti-Chlamydial Assay

Elementary bodies of *C. trachomatis* D (4×10^3 IFU/mL) were incubated with compounds at selected concentrations (0.25, 0.5, 1.25, 2.5 μM) in sucrose-phosphate-glutamic acid buffer (SPG) for 2 h at 37 °C. As a control, *C. trachomatis* D was also incubated alone in the SPG. To quantify the anti-chlamydial effects of the compounds, HeLa cells were seeded in 24-well plates with 13-mm cover glasses. The confluent cells were infected with compound-treated *C. trachomatis* D or with the non-treated controls. After 48 h, the cells were fixed with acetone at −20 °C for 10 min. The titre of the infectious elementary bodies was determined by the indirect immunofluorescence assay, as described earlier [42].

4.7. Real-Time Accumulation Assay

The effect of the studied selenocompounds on the real-time accumulation of ethidium bromide (EB) was assessed by an automated EB method [43], using a LightCycler real-time thermocycler (LightCycler 1.5; Roche). The compounds were added individually at different concentrations at MIC/2 to the EB solution in PBS. The final concentration of EB was 1 and 0.25 μg/mL for *E. coli* AG100 and AG100A, respectively. The method for the calculation of the relative fluorescence index (RFI) of the last time point (minute 30) was described earlier by Kincses et al. [44]. Promethazine (PMZ; EGIS) was applied as a positive control.

4.8. Expression Analyses of Genes by Quantitative PCR

Total RNA was isolated from *E. coli* AG100 (OD of 0.6 at 600 nm) using the NucleoSpin RNA kit (Macherey Nagel) according to the manufacturer's instructions. The concentration of the extracted RNA templates was assessed by spectrophotometry at 260 nm.

The expression of the *acrA*, the *acrB*, the multiple antibiotic resistance protein R (*marR*), and the quorum-sensing transcriptional activator (*sdiA*) genes was studied by reverse transcription quantitative PCR (RT-qPCR), as described earlier [44]. The real-time one-step PCR was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad), strictly adhering to the manufacturer recommendations of the SensiFAST™ SYBR No-ROX One-Step Kit (Bioline GmbH, Luckenwalde, Germany). The forward and reverse primers used in the experiment are shown in Table 5 [44,45]. The cycle threshold (Ct) values were determined with the Bio-Rad CFX Manager Software version 3.1. Relative quantification analysis was carried out using the Livak method [46]. The expression of *gapdh* was used as the internal control and the untreated *E. coli* AG 100 served as the external control. We have defined a threshold value—increases greater than 2-fold in the amount of transcripts relative to the control samples were considered significant.

Table 5. Primers used in the RT-qPCR.

Gene	Full Name	Primer Sequence (5′–3′)	Amplicon size (bp)	Ref.
acrA	Acridine resistance protein A	CTTAGCCCTAACAGGATGTG TTGAAATTACGCTTCAGGAT	189	[45]
acrB	Acridine resistance protein B	CGTACACAGAAAGTGCTCAA CGCTTCAACTTTGTTTTCTT	183	[45]
marR	Multiple antibiotic resistance protein R	AGCGATCTGTTCAATGAAAT TTCAGTTCAACCGGAGTAAT	170	[45]
sdiA	Quorum-sensing transcriptional activator	CTGATGGCTCTGATGCGTTTA TCTGGTGGAAATTGACCGTATT	163	[44]
gapdh	Glyceraldehyde-3-phospate dehydrogenase	ACTTACGAGCAGATCAAAGC AGTTTCACGAAGTTGTCGTT	170	[45]

5. Conclusions

Herein, we have reported the evaluation of the antibacterial and multidrug resistance reversing activity of 11 novel selenocompounds. The most active compound in the antibacterial assay, the methylketone selenoester **9**, showed potential antibacterial activity against the different strains of *S. aureus, E. faecalis*, and *C. trachomatis* D, even at very low concentrations (0.25 μM for *C. trachomatis* D). This selenocompound also enhanced the efficacy of antibiotics, namely it multiplied by 64-fold the antibacterial action of oxacillin, against the MDR clinical isolate of *S. aureus*. Alternatively, three compounds (the selenoanhydride **1** and the selenoesters **4** and **7**) inhibited the tripartite multidrug resistance efflux pump AcrAB-TolC in *E. coli*, and affected the expression of the different genes related to these resistance processes.

Based on these results, it can be concluded that this group of selenocompounds can be attractive potential EP inhibitors and antibacterial lead scaffolds, for further development of new chemical tools, to overcome bacterial multidrug resistance.

Author Contributions: E.D.-Á. and C.S. synthesized the compounds evaluated. T.M., G.S., J.H. and K.K.-K. conceived the experiments; T.M., A.K., A.C., Á.S.T., K.W., M.A.M. and G.S. performed the biological experiments; T.M., G.S. and E.D.-Á. wrote the paper; T.M., C.S., J.H., K.K.K., G.S. and E.D.-Á. revised the manuscript; T.M. and G.S. provided the funding for the biological evaluation; and E.D.-Á and M.A.M. provided the funding of the article publication.

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