University of Szeged

Albert Szent-Györgyi Medical School

Doctoral School of Interdisciplinary Sciences

# Ketone- and cyano-selenoesters as multi-target antibacterial and anticancer derivatives

PhD Thesis

**Nikoletta Szemerédi**

**Supervisor: Gabriella Spengler, PhD**

**Szeged**

**2024**

# INTRODUCTION

Multidrug resistance (MDR) is a significant challenge in bacterial infections and cancer, because MDR refers to the ability of microorganisms or tumor cells to withstand the effects of multiple drugs of different structural classes, making conventional therapies ineffective. Antibiotics, a significant achievement in treating infectious diseases, face challenges as bacteria develop resistance. The escalating rate of antibiotic resistance is linked to overuse, inadequate drug development focus, and economic factors. Antibiotic misuse, including in animals and international travel, contributes to resistance evolution. Antimicrobial resistance falls into categories like limited drug uptake, target modification, drug inactivation, and active drug efflux, furthermore the elevated expression of efflux pumps can contribute to increased virulence of bacteria and may influence bacterial communication (quorum sensing) and biofilm formation. Statistical data indicates that drug resistance is a major factor in the mortality of cancer patients, accounting for over 90% of cases. Chemotherapy resistance in cancer cells can be attributed to various mechanisms, for example increased drug efflux, genetic factors, influence of growth factors, enhanced DNA repair, and elevated metabolism of xenobiotics. Each of these mechanisms reduces the effectiveness of administered drugs, making the treatment of tumors more challenging. As a result of anticancer chemotherapy many patients develop MDR against anticancer drugs, making the treatment less effective. Understanding the mechanisms behind MDR in both bacteria and cancer is crucial for developing more efficient therapeutic strategies.

In recent times, there has been a growing interest in selenium (Se), Se-nanoparticles and selenocompounds as promising avenues for discovering potent antibacterials. This attention is especially directed towards combating multidrug-resistant bacteria and addressing tumor-related challenges. The functional characteristics of Se-compounds stem from their specific nature, enabling them to exhibit dual roles as antioxidants and prooxidants. As antioxidants, Se-compounds, particularly selenocysteine, play an important role in maintaining redox homeostasis and protecting phagocytic cells from oxidative stress induced by reactive oxygen species (ROS). Conversely, as prooxidants, Se-compounds can trigger a substantial generation of ROS through the redox cycle, leading to oxidative stress within the cell.

## AIMS OF THE STUDY

In the present study we aim to address some important resistance mechanisms in bacteria and cancer cells and provide a strategy to overcome MDR.

**The main goals of the study:**

1. **Antibacterial and MDR reversing activity of fifteen selenoesters on Gram-negative and Gram-positive bacterial strains**
   - **1.1.** Determination of MICs of compounds using microdilution method on *Staphylococcus aureus* ATCC 25923, the methicillin and ofloxacin-resistant clinical isolate *S. aureus* MRSA 272123, the methicillin and oxacillin-resistant *S. aureus* MRSA ATCC 4330, biofilm producing *Pseudomonas aeruginosa* CCM 3955/ATCC 27853, the multidrug resistant *P. aeruginosa* NEM 986, the wild-type *Salmonella enterica* serovar Typhimurium SL1344 (SE01) expressing the AcrAB-TolC pump system and its *acrB* gene inactivated mutant *S.* Typhimurium SL1344 strain (SE02), *acrA* gene inactivated mutant *S.* Typhimurium SL1344 (SE03), and *tolC* gene inactivated mutant *S.* Typhimurium SL1344 strain (SE39).
   - **1.2.** Efflux pump inhibition by Se-compounds using real-time ethidium bromide accumulation assay in *S. aureus* ATCC 25923, *S. aureus* MRSA ATCC 43300, *S.* Typhimurium SE01, SE02, SE03, and SE39 strains.
   - **1.3.** Quorum sensing by Se-compounds using *Vibrio campbellii* (ATCC BAA-1118™ and ATCC BAA-1119™).
   - **1.4.** Anti-biofilm activity of Se-compounds on *S. aureus* ATCC 25923 and *P. aeruginosa* CCM 3955 (ATCC 27853) using resazurin assay.
2. **Antitumor and MDR reversing activity of fifteen selenoesters in cancer cell lines**
   - **2.1.** Determination the cytotoxic effect of selenoesters using MTT and resazurin assays on different cancer cell lines and normal cells.
   - **2.2.** Combined activity of selenoesters in the presence of doxorubicin using checkerboard assay and Chou-Talalay method on ABCB1 expressing Colo 320 colon adenocarcinoma cell line.
   - **2.3.** Inhibition of the ABCB1 efflux pump using rhodamine 123 accumulation assay and flow cytometry.
   - **2.4.** Inhibition of Pgp ATPase in the presence of selenoesters using Pgp-Glo$^{TM}$ Assay System (Promega)
   - **2.5.** Apoptosis induction in the presence of selenoesters by Annexin V-FITC and propidium iodide staining on Colo 320 cell line using flow cytometry.

## MATERIALS AND METHODS

### 1. Compounds

The 15 selenoesters that were examined in this study were previously synthesized and assessed as outlined in the patent application EP17382693. Before each biological test, a stock solution of selenoesters (10 mM) was prepared in dimethyl sulfoxide (DMSO).

### 2. Bacterial strains

Gram-positive strains: *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 strain was used as methicillin-susceptible reference and biofilm producing strain; the clinical isolate *S. aureus* MRSA 272123 and the methicillin and oxacillin-resistant *S. aureus* MRSA ATCC 43300 strains were investigated in the study. Gram-negative strains: biofilm producing *Pseudomonas aeruginosa* CCM 3955/ATCC 27853*, multidrug resistant strain *P. aeruginosa* NEM 986*, the wild-type *Salmonella enterica* serovar Typhimurium SL1344 (SE01) expressing the AcrAB-TolC pump system and its *acrB* gene inactivated mutant *S.* Typhimurium SL1344 strain (SE02), *acrA* gene inactivated mutant *S.* Typhimurium SL1344 (SE03), and *tolC* gene inactivated mutant *S.* Typhimurium SL1344 strain (SE39) were used in the study. In case of QS tests the Gram-negative *Vibrio campbellii* ATCC BAA-1118 and ATCC BAA-1119 strains were applied: (*: microorganisms were obtained from the Czech Collection of Microorganisms (CCM, Masaryk University, Czech Republic) and the Collection of Laboratory of Medical Microbiology (NEM, Czech Laboratory, Inc.)).

### 3. Cell lines

The effect of selenoesters was investigated on several cell lines: the doxorubicin-sensitive human colonic adenocarcinoma cell line (Colo 205; ATCC-CCL-222) and the multidrug resistant P-gp expressing (MDR1)-LRP human colonic adenocarcinoma cell line (Colo 320; ATCC-CCL-220.1) (LGC Promochem, Teddington. UK); human embryonal lung fibroblast cell line (MRC-5; ATCC CCL-171; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany); hepatocellular carcinoma (HepG2; ATCC®, CCL-23TM, Manassas, VA, USA), cervical adenocarcinoma (HeLa; ATCC®, CCL-2TM), skin melanoma (B16; ATCC®, CCL-6322TM), human dermal fibroblasts (HDF; Sigma-Aldrich); human keratinocyte (HaCaT, Thermo Fisher Scientific, Waltham, MA, USA). Their culture conditions are the following ones: Colo 205 (ATCC-CCL-222) and Colo 320 expressing P-gp (MDR1)-LRP (ATCC-CCL-220.1 human colon adenocarcinoma cell lines were cultured in RPMI. MRC-5 human embryonal lung fibroblast cells were cultured in Eagle's Minimal Essential Medium (EMEM). HepG2, HeLa, B16 and HaCaT cell lines were cultivated in EMEM medium supplemented with 10% FBS, 2

mM L- glutamine and 1×antibiotic antimycotic solution. All cell lines were cultivated in a $CO_2$ incubator (5% $CO_2$, 37 °C, Thermo Fisher Scientific).

**4. Determination of minimum inhibitory concentrations (MIC) by microdilution method**

The study involved determining the minimum inhibitory concentrations (MICs) of ketone- and cyano-selenoesters, following the Clinical and Laboratory Standard Institute guidelines (CLSI).

**5. Real-time ethidium bromide (EB) accumulation assay**

The efflux pump inhibiting activity of Se-compounds was investigated on *S. aureus* ATCC 25923, *S. aureus* MRSA ATCC 43300, *S.* Typhimurium SE01, SE02, SE03, and SE39 strains. Real-time fluorimetry was used based on the intracellular accumulation of the efflux pump substrate EB. CLARIOstar Plus plate reader (BMG Labtech, UK) was used for this purpose. Reserpine (RES) was applied at 25 μM and carbonyl cyanide m-chlorophenyl hydrazine (CCCP) at 50 μM as a positive control, and DMSO at 1 v/v% as a negative control. The Se-compounds were added at ½ MIC concentration to PBS containing a non-toxic concentration of EB (2 μg/mL). The solutions were then pipetted into a 96-well black microtiter plate (Greiner Bio-One Hungary Kft, Hungary), and 50 μL of bacterial suspension (OD600 0.6) were added to each well. The plates were placed into the CLARIOstar plate reader, and the fluorescence was monitored every minute for one hour at excitation and emission wavelengths of 530 nm and 600 nm. The relative fluorescence index (RFI) of the last time point (minute 60) of the EB accumulation assay, was calculated.

**6. Assay for quorum sensing (QS) inhibition**

Anti-quorum sensing (QS) activity of the compounds was investigated in two commercial strains of *V. campbellii* (ATCC BAA-1118™ and ATCC BAA-1119™). The first strain responds to the autoinducer-1 through bioluminescence, while the second strain responds to the autoinducer-2. An overnight culture of the strains was diluted to $5x10^5$ CFU/mL in Autoinducer Bioassay medium and was split into 96-well plates, and treated with the compounds at 2-fold serial dilutions. Luminescence was recorded for 16 h using a microplate reader set up at 30°C. The effective concentration 50 ($EC_{50}$) of the compounds was determined based on the sum of luminescence, and the viability of the culture was checked by resazurin assay to calculate the $IC_{50}$ of the compounds. The compounds were compared based on their $EC_{50}$ (the concentration that halves the cell communication) and $IC_{50}$ (viability; represents the minimal concentration of a drug that is required for 50% inhibition *in vitro*).

## 7. Anti-biofilm activity

### 7.1. Inhibition of biofilm formation

The study investigated the impact of selenocompounds on biofilm formation in *S. aureus* ATCC 25923 and *P. aeruginosa* CCM 3955 (ATCC 27853) using 96-well microplates. The experiment began by diluting overnight bacterial in Brain Heart Infusion (BHI) broth to obtain an optical density of 0.5 McFarland, followed by distributing the suspension into 96-well plates in 100 µL aliquots per well. Next, Se-compounds were added to the cells in a concentration range of 100 µM to 0.19 µM. The plates were then incubated for 24 hours at 37°C. After incubation, the viability of adherent cells was assessed immediately using resazurin assay. Fluorescence was measured (560/590 nm, ex./em.) using the SpectraMax i3x Multi-Mode Detection Platform (Molecular Devices, USA), and the assays were carried out in four parallels, then the relative viability was evaluated.

### 7.2. Disruption of Mature Biofilm

The study aimed to examine the efficacy of Se-compounds in damaging mature biofilms formed by two bacterial strains: *S. aureus* ATCC 25923 and *P. aeruginosa* CCM 3955 (ATCC 27853). To determine the results resazurin assay was used in 96-well plates. Firstly, overnight cultures of the bacteria were diluted in BHI broth to an optical density of 0.5 McFarland, and then 100 µL aliquots of the bacterial suspension were added to the wells. After 24 hours of incubation at 37 °C, the old medium was discarded, and new BHI broth containing Se-compounds was added to the wells. The plates were then incubated for another 24 hours. 100 µL of resazurin in PBS (0.03 mg/L) was added to each well, and fluorescence was measured using the SpectraMax i3x Multi-Mode Detection Platform (Molecular Devices, USA) with excitation and emission wavelengths of 560/590 nm, respectively, then the $IC_{50}$ values were calculated.

## 8. Cytotoxicity assay

### 8.1. MTT assay

Two-fold serial dilutions of the compounds were prepared in a separate plate and then transferred to the corresponding (Colo 205, Colo 320, MRC-5) cell line plates. The culture plates were then incubated at 37 °C for 24 hours and stained by MTT. The optical density (OD) at 540 nm (ref. 630 nm) was measured using an ELISA reader to determine cell growth inhibition, expressed as $IC_{50}$ values. The $IC_{50}$ values and standard deviation (SD) were calculated using GraphPad Prism software version 5.00 for Windows with a non-linear regression curve fit.

**8.2. Resazurin assay**

In case of Hela, HepG2, B16, HDF and HaCaT cell line resazurin assay was used. For the experiment, cells were seeded into 96-well plates at a concentration of 1 x $10^5$ cells/mL to a final volume of 100 µL. After 24 hours, the plates were washed with PBS three times, the compounds were added at a final concentration range of samples was 0.625-20 µM. After 72 hours of incubation resazurin assay was carried out. The fluorescence signal was then measured (ex./em. 560/590 nm) using a SpectraMax i3x Multi-Mode Microplate reader with MiniMax Imaging Cytometer from Molecular Devices®, USA.

**9. Checkerboard combination assay**

Checkerboard microplate method was utilized to investigate the potential synergistic effects of the selenocompounds and doxorubicin on resistant Colo 320 colon adenocarcinoma cells expressing the ABCB1 transporter based on the Chou-Talalay method. The CalcuSyn software was used to plot 4 or 5 data points for each ratio, and the results were expressed as combination index (CI) values at 50% growth inhibition ($ED_{50}$). The median-effect equation was used to calculate the CI values, where a CI < 1 indicated synergism, CI = 1 indicated an additive effect or no interaction, and CI > 1 indicated antagonism. To carry out the experiment, doxorubicin dilutions were prepared in a 100 µL volume horizontally, while the Se-compound's dilutions were prepared vertically in a microtiter plate with a volume of 50 µL. The starting concentration of doxorubicin was 8.62 µM, the concentration of the Se-compounds was calculated based on their $IC_{50}$. The plates were incubated at 37 °C in a $CO_2$ incubator for 72 hours, and the cell growth rate was determined using MTT staining. The optical density (OD) was measured at 540/630 nm using a Multiscan EX ELISA reader.

**10. ABCB1 inhibition in the presence of selenoesters**

The retention of rhodamine 123 by ABCB1 was measured as an indicator of pump inhibition. The cells were distributed into Eppendorf centrifuge tubes, and the tested compounds were added at different concentrations (0.2 and 2 µM) from stock solutions. The fluorescence of the gated cell population was measured using a CyFlow® flow cytometer (Partec), and the results were obtained from a representative flow cytometry experiment in which at least 20,000 individual cells of the overall population were evaluated for the rhodamine 123 retained inside the cells. The fluorescence activity ratio (FAR) was calculated. Tariquidar was used as the positive control (0.2 µM final concentration), and DMSO was used as the solvent control (at 2 v/v%).

**11. P-gp ATPase activity assay**

The P-glycoprotein ATPase activity was assessed using the Pgp-Glo™ Assay System from Promega. Compounds were tested at a concentration of 25 μM. The emitted luciferase-generated luminescent signal was measured at 580 nm using a CLARIOstar Plus plate reader from BMG Labtech. The relative ATPase activity was determined by calculating the ratio between the luminescence measured for each compound's P-gp ATPase activity and the basal P-gp ATPase activity.

**12. Apoptosis induction**

Annexin V-FITC Apoptosis Detection Kit (Calbiochem, EMD Biosciences. Inc. La Jolla, CA) was used. Colo 320 cell suspension was adjusted to approximately $1 \times 10^6$ cells/mL and distributed into 1 mL aliquots in a 24-well microplate. The cells were incubated overnight at 37 °C with 5% $CO_2$. Se-compounds were added to the cells, and the cells were incubated for 3 h at 37 °C. The concentration of 2 μM was selected for apoptosis induction based on previous cytotoxicity results ($IC_{50}$ values). Additionally, 12*H*-benzo(α)phenothiazine (M627) was used as a positive control at a final concentration of 20 μM. The apoptosis assay was carried out according to the rapid protocol of the kit using Annexin V-FITC and propidium iodide staining. The fluorescence was immediately analyzed using a CyFlow® flow cytometer (Partec, Münster, Germany), and the results were obtained from a representative flow cytometry experiment evaluating at least 20,000 individual cells of the overall population in a sample. The FlowJo™ software (BD Biosciences, San Jose, NJ, USA) was used to analyze the data.

## RESULTS

**1. Antibacterial activity**

**1.1. Determination of minimum inhibitory concentrations (MICs) by microdilution method**

The results suggest that the ketone-selenoesters have a robust antibacterial effect against the Gram-positive bacteria studied. Notably, the derivatives K1, K7, and K8 exhibited the highest potency, being active against all three *S. aureus* strains examined, with MIC values ranging from 0.39 to 1.56 μM.

**1.2. Inhibition of bacterial efflux pumps**

Among the Salmonella strains tested, the ketone-selenoester K7 demonstrated the most potent efflux pump inhibitory (EPI) activity, as it could inhibit the AcrAB-TolC system. In case of the Δ*acrB* strain lower EB concentration and lower EPI activity was obeserved than in the wild type. However, regarding the Δ*acrA* and Δ*tolC* strains higher EB concentration was recorded

compared to the wild type strain with relative fluorescence intensities (RFI) of 1.15 and 1.67, respectively. Ketone-selenoesters K4 and K5 inhibited EB accumulation in the *tolC* inactivated mutant strain. The cyano-selenoesters N4 and N7 exhibited the most notable activity on the *tolC* inactivated mutant strain. For *S. aureus* ATCC MRSA 43300, only one derivative, the cyano-selenoester N4 exhibited potent efflux pump inhibition.

### 1.3. Assay for quorum sensing (QS) inhibition

To differentiate between the concentration that induces toxicity and the concentration that inhibits cell-to-cell communication (quorum sensing), the study compared the $IC_{50}$ (the concentration that reduces viability by 50%) with the $EC_{50}$ (the concentration that halves cell-to-cell communication). This comparison was essential in determining the efficacy of the tested compounds. The ability of selenocompounds to inhibit quorum sensing was tested using two strains of *V. campbellii*, the wild-type of these bacteria uses both autoinducer-1 (AI-1) and autoinducer-2 (AI-2) types of molecules for its communication, strain 1118 is deficient in communication on the basis of AI-2, while strain 1119 is deficient in AI-1 type communication. The impact of different concentrations of a compound on cell-to-cell communication ($EC_{50}$) and cell viability ($IC_{50}$) was examined, and compared to determine the selectivity index (SI) of each compound. The SI was calculated as the ratio of $IC_{50}$ and $EC_{50}$, allowing for differentiation between toxic and quorum sensing inhibiting concentrations. According to previous studies, a selectivity index (SI) higher than 10 is desirable for practical application. Based on this criterion, the most promising ketone-selenoesters for QS inhibition were K1, K2, and K8, while the most effective cyano-selenoester was N2.

### 1.4. Inhibition of biofilms

Biofilm inhibition can be achieved through several strategies, e.g. (a) preventing bacterial surface adhesion during the initial stages (anti-adhesion effect), (b) disrupting mature biofilms (anti-biofilm effect). All tested compounds showed efficacy against both stages of biofilm formation. However, Se-compounds were generally more effective against *P. aeruginosa* than against *S. aureus*.

## 2. Antitumor activity

### 2.1. Cytotoxicity

The ketone-selenoesters displayed strong cytotoxic activity against the sensitive Colo 205 and resistant Colo 320 cancer cell lines, with $IC_{50}$ values ranging from 1 to 4 μM for both cell lines. However, they also exhibited similar toxicity on normal lung fibroblast cells (MRC-5), suggesting that they lack selectivity towards cancer cells. On the other hand, cyano-selenoesters did not affect MRC-5 cells ($IC_{50}$ > 100 μM), but were highly toxic on both colon cancer cell

lines. The ketone-selenoesters showed a range of $IC_{50}$ values between 2.2 to 4.3 μM for HepG2 cells, 1.9 to 2.7 μM for HeLa cells, and 1.1 to 2.0 μM for B16 cells. The cyano-selenoesters, on the other hand, exhibited $IC_{50}$ values ranging from 5.2 to 11.8 μM for HepG2 cells, 1.3 to 5.2 μM for HeLa cells, and 1.4 to 2.6 μM for B16 cells.

### 2.2. Interaction of selenoesters with doxorubicin: *in vitro* model of combination chemotherapy

The combination of six ketone-selenoesters (K1, K3, K4, K5, K6, K8) with doxorubicin resulted in a synergistic interaction on Colo 320 cells, with K5 and K6 showing consistent synergistic effects at all ratios. Similar findings were observed for five cyano-selenoesters (N1, N2, N3, N4, N7), which exhibited a synergistic effect when combined with doxorubicin on Colo 320 cells.

### 2.3. ABCB1 inhibition in the presence of selenoesters

According to the results obtained by flow cytometry, some of the ketone-selenoesters exhibited potent inhibition on the ABCB1 transporter, with K1, K2, K3, K7, and K8 being the most effective ones. These compounds showed a FAR value of 45.73, 37.35, 39.17, 40.38, and 36.09 at 2 μM, respectively. K3 and K8 were effective at both concentrations (0.2 μM and 2 μM), with a FAR value of 3.99 and 3.38, respectively, at 0.2 μM. The cyano-selenoesters did not show any modulating activity towards the ABCB1 transporter.

### 2.4. Pgp ATPase activity assay

A lower relative ATPase activity indicated a more effective inhibitor. Only compounds that exhibited ABCB1 inhibitory activity were evaluated in this assay. K1, K4, K7, and K8 proved to be inhibitors of ABCB1 ATPase activity. On the other hand, the remaining compounds stimulated ATPase activity.

### 2.5. Induction of apoptosis

The results showed that K3 was the most potent compound, inducing early apoptosis in 18.6% of the cell population. K3 was also the most effective in inducing late apoptosis, with a rate of 25.6% in the cell population. It was interesting to note that all of the derivatives contributed to late apoptosis in the studied cell population, with activities ranging from 16.1% to 33.9% in the treated cells.

## DISCUSSION

### 1. Antibacterial activity

#### 1.1. Determination of minimum inhibitory concentrations (MICs) by microdilution method

The ketone-selenoesters demonstrated potent activity against both sensitive and methicillin-resistant *S. aureus* strains. Furthermore, cyano-selenoesters showed slightly greater activity than the ketone-selenoesters against the evaluated strains of *Salmonella enterica* serovar Typhimurium. Among the tested Se-compounds, K6 stood out as the most active compound. It contained a *tert*-butyl group in the *para*-position and displayed impressive MIC values of 1.56 μM against the sensitive strains and 0.39-3.13 μM against the methicillin-resistant *S. aureus* (MRSA) strains. Interestingly, K6 was the only compound in our study that had an electron-donating substituent. This is noteworthy since previous studies on selenoesters have indicated that electron-withdrawing substituents generally exhibit higher biological activity. The nitrile derivatives (N1-N7) demonstrated comparable activity against the sensitive *S. aureus* ATCC 25923 strain, with a MIC of 12.5 μM. Considering the results obtained from the MRSA and *S.* Typhimurium strains, the most potent compounds were identified as N1 (unsubstituted), N3 (4-Br-substituted), N6 (3-Cl-substituted), and N7 (3,5-bis(trifluoromethyl)-substituted). These findings indicate that among the monosubstituted compounds, those containing a bromine or chlorine atom attached to the ring exhibit superior activity compared to those with a fluoro or trifluoromethyl group.

#### 1.2. Efflux pump inhibition

In the case of the ketone- and cyano-selenoesters investigated in this study, only one cyano-selenoester, N4, exhibited potent EPI activity against methicillin-resistant *S. aureus*, surpassing the effect of the reference EPI reserpine. Moreover, ketone-selenoester K7 demonstrated effective EPI activity on *S.* Typhimurium strains, likely due to its ability to destabilize the bacterial membrane. Interestingly, all compounds containing at least one trifluoromethyl group (K4, K5, N4, and N7), except for N5, demonstrated moderate inhibitory effects on efflux pumps in the *S.* Typhimurium SE39 Δ*tolC* strain, as observed in the real-time ethidium bromide accumulation assay. Hence, the presence of the $-CF_3$ moiety and the $-C(CH_3)_3$ moiety in K7 appeared to be significant for the efflux pump inhibitory activity in the *S.* Typhimurium SE39 Δ*tolC* strain.

#### 1.3. Quorum sensing (QS) and biofilm inhibition

Based on our findings, we have identified several promising compounds that can be utilized as inhibitors of bacterial communication. Compound K2, in particular, displayed significant

selectivity in inhibiting communication rather than bacterial growth. Among the compounds evaluated, ketone-selenoester K1 emerged as the most promising inhibitor of AI-1-based communication, followed by N2, the only cyano-selenocompound capable of inhibiting this type of communication. Both K1 and N2 demonstrated inhibitory activity at remarkably low concentrations of 0.25 μM and 0.34 μM, respectively. Interestingly, both K2 (ketone-selenoester) and N2 (cyano-selenoester) share a common 2-fluorophenyl moiety attached to the selenoester, suggesting its importance in inhibiting AI-1 communication. Notably, the presence of fluorine atoms without other substituents proved to be beneficial for activity, as evidenced by the most active compound K8 with a 2,4,5-trifluoro substitution. However, the activity of the most potent inhibitor, K1, may not be solely attributed to the absence of substitution, as its nitrile equivalent (N1) displayed no activity. Compounds K1, K2, and K8 exhibited QS selectivity indexes above 10 and demonstrated significant inhibition of *P. aeruginosa* adhesion in the anti-biofilm assay. In contrast, the cyano-selenocompounds demonstrated superior efficacy in inhibiting AI-2-based communication. Among them, N3 and N7 emerged as the most potent compounds, with N3 exhibiting remarkable inhibition even at a concentration as low as 60 nM. Interestingly, these compounds featured distinct substitutions on the phenyl ring compared to the AI-1 inhibitors. The quorum sensing process in Gram-positive bacteria is typically mediated by peptide molecules, which differ from the communication signals utilized by *Vibrio* bacteria. Many of the tested compounds exhibited significant inhibition of *S. aureus* adhesion, indicating the potential of Se-compounds for modulating the activity of efflux pumps. AI-2 molecules are commonly utilized by both Gram-positive and Gram-negative bacteria. In the AI-2 system, these transporters are involved in the uptake of communication molecules. Interestingly, compounds K2, K4, K7, N3, N6, and N7 displayed notable inhibitory effects on this universal communication system, which is shared by both Gram-positive and Gram-negative bacteria. In the anti-biofilm assay, all tested compounds exhibited biofilm adhesion inhibition activity against the evaluated bacterial strains (*S. aureus* ATCC 25923 and *P. aeruginosa* CCM 3955) at concentrations below 4 μM. Notably, two compounds (K8 and N3) demonstrated remarkable inhibition of *P. aeruginosa* biofilm adhesion at nanomolar concentrations (0.86 μM and 0.92 μM, respectively). Additionally, the compounds showed higher effectiveness against *P. aeruginosa* biofilms compared to *S. aureus* biofilms.

## 2. Antitumor activity

### 2.1. Cytotoxicity

Oxoselenoesters, with a few exceptions, have shown greater cytotoxicity compared to cyano-selenoesters in Colo 320, HepG2, and B16 cancer cell lines. The cyano-selenoesters had no

effect on MRC-5 cells, they exhibited significant toxicity in both colon cancer cell lines, albeit less potent in resistant cells. The most active compound in each cell line, with the second and third most active compounds in brackets, were as follows: K4 in Colo 205 (K1, N4); K1 in Colo 320 (K4, K6); K2 in HepG2 (K1, K5); N6 in HeLa (K4, K3); and K5 in B16 cells (K2, K6). Interestingly, the majority of the most active compounds are oxoselenoesters, with the presence of substituents without halogens, such as K7, reducing activity. Compounds with a trifluoromethyl group or one or two halogens bound to the phenyl ring exhibited better activity. Furthermore, the recurrent appearance of K1 and K4 among the most active compounds against each cell line (three times each) suggests that the thionyl ring and the presence of a trifluoromethyl group in the *ortho* position of the phenyl ring enhance cytotoxicity compared to selenoesters. Notably, K5, which has a 3-$CF_3$ substituent on the ring, also displayed significant activity, while the cyano-selenoester N4, with a 2-$CF_3$ substituent on the ring, was perhaps the most active derivative among the cyano-selenoesters. These findings support the observation that the trifluoromethyl group, preferably in the ortho position, plays a crucial role in the cytotoxic activity, although the compound with this substituent in the meta position showed comparable activity in all cell lines, except for HepG2, where it was significantly less active. The selenoester containing ketone groups demonstrated toxicity against normal MRC-5 cells, whereas none of the derivatives containing cyano groups exhibited toxicity at concentrations below 100 μM. This suggests that all the cyano-selenoesters displayed strong selectivity towards cancer cells.

**2.2. Combination assay**

When studying the interaction between ketone-selenoesters, cyano-selenoesters, and the cytotoxic drug doxorubicin, it was found that eleven out of the fifteen evaluated selenoesters exhibited synergistic interactions with doxorubicin in at least one of the tested ratios. The selenoesters that demonstrated synergism were K1, K3, K4, K5, K6, K8, N1, N2, N3, N4, and N7. Interestingly, no clear structure-activity relationships (SARs) could be established, as compounds like K5 and K6 showed varying degrees of synergism with doxorubicin across all tested ratios, while their cyano-selenoester counterparts (N5 and N6) displayed antagonistic interactions in five out of the six ratios tested. Similarly, the thiophene cyano-selenoester N1 exhibited synergistic interactions with doxorubicin in four out of the six ratios, whereas its ketone analog, K1, only displayed synergism in one out of the five ratios.

### 2.3. Rhodamine 123 accumulation assay

It was found that the presence of a 2-oxopropyl group in ketone-selenoesters was crucial for their activity as ABCB1 inhibitors. All ketone-selenoesters, except for K4, exhibited greater potency as ABCB1 inhibitors, whereas none of the cyano-selenoesters demonstrated ABCB1 inhibitory activity. Among the compounds evaluated, K1 emerged as the most active compound, featuring a thiophene ring instead of a phenyl ring. Notably, the introduction of a bulky trifluoromethyl group at the two-position of the phenyl ring (K4) significantly diminished its activity. Interestingly, relocating this group to the three-position, thereby eliminating steric hindrance, resulted in a sixfold increase in activity (K5).

### 2.4. P-gp ATPase activity assay

The P-glycoprotein (P-gp) ATPase activity was assessed exclusively for the oxoselenoesters, as they were the only compounds that exhibited ABCB1 inhibitory activity. All the compounds, except for K4, demonstrated modulation of the ATPase activity. Remarkably, the most potent ABCB1 inhibitors, namely K1 and K7, significantly inhibited the ATPase activity, with K7 showing particularly strong inhibition. Additionally, K8 displayed a milder inhibition of the ATPase activity. Given that ABCB1 activity can protect cells from apoptosis, the inhibition of ATP supply to this pump can promote apoptosis, as supported by our finding. In fact, the aforementioned derivatives (K1, K7, and K8) induced late apoptosis in MDR Colo 320 cells, further confirming the link between ABCB1 inhibition and the induction of apoptosis.

### 2.5. Apoptosis induction assay

The connection between ABCB1 inhibition and the induction of apoptosis was explored in the case of ketone-selenoesters. Overall, the ketone-selenoesters exhibited notable capacity to trigger apoptotic events, except for K4. Notably, compound K3 outperformed the reference phenothiazine in inducing early apoptosis and demonstrated comparable potency to the reference when considering both early and late apoptosis induction. It was evident that the inclusion of a bromine atom at the four-position of the phenyl ring (K3) enhanced apoptosis induction, while the introduction of a bulky substituent at the two-position (such as the trifluoromethyl moiety) of the phenyl ring reduced the ability to trigger apoptotic events. Furthermore, the presence of selenium in the compounds can promote the generation of free radicals, leading to apoptosis and cell death in cancer cells.

## NEW FINDINGS

### 1. Selenoesters as antibacterial agents

- Ketone-selenoesters showed an outstanding antibacterial effect against methicillin susceptible and methicillin resistant *Staphyloccus aureus* strains. In addition, cyano-selenoesters showed also potent antibacterial activity on these Gram-positive strains. Ketone- and cyano-selenoesters had a slight antibacterial activity on the Gram-negative *Salmonella* Typhimurium SE01, SE02, SE03, SE 39, *Pseudomonas aeruginosa* ATCC 27853 and NEM 986 strains.

### 2. Modification of bacterial virulence: inhibition of efflux pumps, quorum sensing and biofilm formation

- The ketone-selenoester K7 could inhibit the AcrAB-TolC system of wild-type *Salmonella* Typhimurium.
- Cyano-selenoester N4 inhibited the function of the AcrAB-TolC system on *S.* Typhimurium, and the highest inhibition was achieved on the *tolC* deficient mutant SE39 strain of *S.* Typhimurium. Cyano-selenoester N4 inhibited the efflux pump activity of *S. aureus* MRSA 43300 strain.
- All ketone- and cyano-selenoesters, except one compound, were able to reduce bacterial communication of *Vibrio campbellii* strains lacking either the autoinducer-1 or autoinducer-2 types quorum sensing molecules. The most potent ketone selenoesters were K1, K2, K4, K7, and K8, among the cyano selenoesters compounds N2, N3 and N7 should be highlighted.
- All ketone- and cyano-selenoesters had an inhibitory effect on biofilm formation and could disrupt mature biofilms of *S. aureus* ATCC 25923 and *P. aeruginosa* CCM 3955 strains.

### 3. Antitumor and cytotoxic effects of selenoesters

- All ketone- and cyano-selenoesters had a cytotoxic effect on the tested colon adenocarcinoma (Colo 205 and Colo 320), hepatocellular carcinoma (HepG2), cervical adenocarcinoma (HeLa), and skin melanoma (B16) cell lines. An outstanding result is that the cyano-selenoesters were selective, this means that they had no cytotoxic effect on the normal MRC-5 lung fibroblast cell line.
- The ketone-selenoesters K1, K3, K4, K5, K6, K8 and the cyano-selenoesters N1, N2, N3, N4, N7 presented synergistic interaction with doxorubicin on resistant Colo 320 colon adenocarcinoma cells, as they were able to reduce the $IC_{50}$ value of doxorubicin.

**4. Reversal of cancer MDR by selenoesters**

- ❖ Among the selenoesters, only the ketone-selenoesters proved to be effective in the inhibition of ABCB1 transporter, and the most potent derivatives were K1, K2, K3, K7, and K8.
- ❖ Ketone-selenoesters K1, K4, K7, and K8 proved to be the inhibitors of ABCB1 ATPase activity.
- ❖ Ketone-selenoesters were able to induce late apoptosis in resistant Colo 320 colon adenocarcinoma cells, but only compound K3 induced early apoptosis.


## FINANCIAL SUPPORT

The work on which this thesis was based on was supported by the following organizations and grants:

- Szeged Foundation for Cancer Research (Szegedi Rákkutatásért Alapítvány)
- COST Action CA17104 (STRATAGEM) - STSM
- New National Excellence Programme (ÚNKP) (2021 - UNKP-21-3-SZTE-103; 2022 - ÚNKP-22-3 - SZTE-285; 2023 - ÚNKP-23-4 -SZTE-347)
- GINOP-2.3.2-15-2016-00038
- EFOP-3.6.3-VEKOP-16-2017-00009
- SZTE ÁOK-KKA 2018/270-62-2 (Selenium derivatives as novel promising antibacterial agents) of the University of Szeged, Faculty of Medicine
- 2018-2.1.15-TÉT-PT-2018-00017 Bilateral Portuguese-Hungarian Science & Technology Cooperation (FCT/NKFIH, 2019/2020).
- OTKA K132446
- International Visegrad Fund (No. 22010090)

## PUBLICATIONS RELATED TO THE THESIS

I. **Szemerédi N**, Dobiasová S, Salardón-Jiménez N, Kincses A, Nové M, Habibullah G, Sevilla-Hernández C, Benito-Lama M, Alonso-Martínez FJ, Viktorová J, Spengler G, Domínguez-Álvarez E. Cyano- and Ketone-Containing Selenoesters as Multi-Target Compounds against Resistant Cancers. Cancers (Basel). 2021 Sep 11;13(18):4563. doi: 10.3390/cancers13184563. PMID: 34572790; PMCID: PMC8465942.

**IF: 6.575**

II. **Szemerédi N**, Kincses A, Rehorova K, Hoang L, Salardón-Jiménez N, Sevilla-Hernández C, Viktorová J, Domínguez-Álvarez E, Spengler G. Ketone- and Cyano-Selenoesters to Overcome Efflux Pump, Quorum-Sensing, and Biofilm-Mediated Resistance. Antibiotics (Basel). 2020 Dec 11;9(12):896. doi: 10.3390/antibiotics9120896. PMID: 33322639; PMCID: PMC7763688

**IF: 4.639**

**Total IF: 11.214**