RESISTANCE REVERSING ACTIVITY OF PLANT-DERIVED AND NOVEL SYNTHETIC COMPOUNDS IN BACTERIAL MODELS

Ph.D. Thesis

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1. INTRODUCTION

Antimicrobial resistance (AMR) is the ability of a microorganism to survive treatment with an antibiotic or antimicrobial agent to which it was previously sensitive. Resistant bacteria are able to withstand the attack of antimicrobials, so that standard treatments become ineffective and infections persist and may spread to others. The rapid spread of AMR is a serious problem for the treatment of bacterial infections and increase the costs of therapy. The O'Neill report estimates that by 2050 10 million lives and 100 trillion USD of economic output are at risk from AMR.

Bacteria have evolved different mechanisms to prevent the harmful effect of antibacterial drugs and one of the most important mechanism is the increased expression of efflux pumps (EPs) that are able to export different classes of antimicrobial agents outside the bacterium before they reach their targets within the bacterial cell. EPs also play a role in biofilm formation and quorum sensing (QS) process. In bacteria the QS is a cell-to-cell communication and regulatory mechanism that controls gene expression depending on bacterial cell density. Bacterial biofilm is a microbial community consisting of sessile bacterial cells attached to each other and to a surface and is embedded in a self-produced extracellular matrix. In the biofilm bacterial cells adapt to environmental conditions and bacteria show increased (about 10-1000 times) resistance against antimicrobial agents compared to planktonic bacteria.

There are several studies in the literature providing evidence of the EP, biofilm formation and QS inhibitory potential of natural, plant-derived and synthetic compounds, furthermore, they can potentiate the efficacy of antibiotics as well. In this study plant-derived compounds from *Cleistochlamys kirkii* (Benth) Oliv. (Annonaceae), synthesized fluorinated β-diketo phosphorus ylides and selenocompounds were investigated. *C. kirkii* is an African medicinal plant traditionally used in Mozambique for the treatment of wound infections, tuberculosis, rheumatism and based on preliminary studies *C. kirkii* derivatives have antimicrobial activity. The EP modulating activity of phosphorus ylides has already been described regarding the ATP-binding cassette subfamily B member (ABCB1) pump of multidrug resistant (MDR) mouse T-lymphoma cells. Furthermore, selenium is an important trace element, which plays a role in the prevention of inflammatory diseases and cancer and selenium-containing compounds have a broad antimicrobial spectrum.

The spread of AMR is a global challenge and to overcome this problem the combination therapy could be a solution (antibiotic plus adjuvants: for example efflux pump inhibitor (EPI)) in order to improve the efficacy of antibacterial therapy, prevent the emergence of MDR

bacteria and decrease the costs of therapy. However, this requires the discovery of new efflux pump inhibitors that is a major challenge for drug development.

2. AIMS OF THE STUDY

The aim of our study was to investigate the resistance modifying activity of bioactive compounds of *C. kirkii*, synthesized fluorinated β-diketo phosphorus ylides and selenocompounds on Gram-positive and Gram-negative model bacterial strains.

The main goals of the study were the following:

1. Determination of the antibacterial activity of the compounds (five natural compounds, ten fluorinated β-diketo phosphorus ylides and eleven selenocompounds) on Gram-positive and Gram-negative strains by microdilution method.

2. Investigation of the EP inhibitory effect of the compounds (five natural compounds, ten fluorinated β-diketo phosphorus ylides and eleven selenocompounds) on Gram-positive and Gram-negative strains using real-time ethidium bromide (EB) accumulation assay.

3. Evaluation of the EP inhibitory effect of eleven selenocompounds on Gram-negative wild type *Salmonella enterica* serovar Typhimurium SL1344 strain expressing the AcrAB-TolC EP and its AcrB mutant L644 strain using EB efflux assay.

4. Determination of the anti-biofilm activity of eleven selenocompounds on Gram-negative, biofilm producing *S.* Typhimurium 14028s strain using crystal violet (CV).

5. Characterization of the activity of five natural compounds of *C. kirkii* as adjuvants in the presence of tetracycline (TET) and ciprofloxacin (CIP) on *Staphylococcus aureus* ATCC 25923 and *S. aureus* 27213 strains by checkerboard method.

6. Evaluation of the adjuvant role of eleven selenocompounds on *Escherichia coli* AG100 strain by a two-fold broth microdilution method in the presence of TET and CIP.

7. QS inhibition analysis of five natural compounds of *C. kirkii* and ten fluorinated β-diketo phosphorus ylides using the sensor strain *Chromobacterium violaceum* 026 and the N-acyl-homoserine lactone (AHL) producer strain *Enterobacter cloacae* 31298 by agar diffusion method.

8. Monitoring the changes in relative gene expression of efflux (*norA*, *mepA*, *acrA*, *acrB*), antibiotic resistance (*marR*) and QS (*sdiA*) genes in the presence of the most effective EPIs investigated in this study (natural compounds isolated from *C. kirkii*, fluorinated β-diketo phosphorus ylides and selenocompounds) by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR).

3. MATERIALS AND METHODS

3.1. Compounds

Five natural compounds (**CK1-5**) were isolated from the methanol extract of the root barks of *C. kirkii* (Benth) Oliv. (Annonaceae)*:* triterpene polycarpol (**CK1**), *C*-benzylated flavanones chamanetin (**CK2**), isochamanetin (**CK3**), dichamanetin (**CK4**) and the heptane derivative acetylmelodorinol (**CK5**) were kindly provided by Prof. Dr. Maria-José U. Ferreira (Universidade de Lisboa, Lisbon, Portugal). The stock solutions (in 10 mM concentration) of compounds were prepared in dimethyl sulfoxide (DMSO).

Ten synthesized fluorinated β-diketo phosphorus ylides (P-ylides; **PY1-10**) were kindly provided by Prof. Dr. Masami Kawase (Matsuyama University, Matsuyama, Japan). The stock solutions (in 10 mg/mL concentration) of compounds were prepared in DMSO.

Eleven selenocompounds including a cyclic selenoanhydride (**EDA1**), heteroaryl selenoesters (**EDA2**-**3**) and aryl selenoesters (**EDA4**-**11**) were kindly provided by Dr. Enrique Domínguez-Álvarez (Consejo Superior de Investigaciones Científicas, Madrid, Spain) and Prof. Dr. Carmen Sanmartín (University of Navarra, Pamplona, Spain). The stock solutions (in 10 mM concentration) of compounds were prepared in DMSO.

3.2. Bacterial strains

Wild-type *E. coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44], expressing the AcrAB-TolC EP at its basal level and its AcrAB-TolC deleted mutant *E. coli* AG100 A strain were used in the study. These strains were kindly provided by Prof. Dr. Hiroshi Nikaido (University of California, Berkeley, CA, USA). Wild-type *S.* Typhimurium SL1344 and its *acrB* gene inactivated mutant *S.* Typhimurium strain (L644), furthermore, the biofilm producing *S.* Typhimurium 14028s strain were used in the study. These strains were kindly provided by Dr. Jessica M. A. Blair (University of Birmingham, Birmingham, United Kingdom).

S. aureus ATCC 25923, was used as the methicillin susceptible reference strain, and the methicillin and ofloxacin resistant *S. aureus* 272123 clinical isolate, was kindly provided by

Prof. Dr. Leonard Amaral (Institute of Hygiene and Tropical Medicine, Lisbon, Portugal). In addition, *Enterococcus faecalis* ATCC 29212 strain was used in the assays.

For quorum sensing tests, the following strains were used: *C. violaceum* 026 (CV026) as sensor strain and *E. cloacae* 31298 as AHL producer strain (a clinical isolate from a wound).

3.3. Determination of minimum inhibitory concentrations by microdilution method

The minimum inhibitory concentrations (MICs) of all tested compounds were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The solvent DMSO had no antibacterial effect.

3.4. Real-time accumulation assay by Roche LightCycler real-time thermocycler

The activity of compounds isolated from *C. kirkii* (**CK1**-**5**), P-ylides (**PY1**-**10**) and selenocompounds (**EDA1**-**11**) on the real-time accumulation of EB was assessed by the automated EB method using a LightCycler real-time thermocycler (LightCycler 1.5, Roche, Indianapolis, USA). Briefly, an aliquot of an overnight culture of *S. aureus* strains (ATCC 25923 and MRSA 272123) in tryptic soy broth (TSB) medium was transferred to fresh TSB medium, and it was incubated until it reached an optical density (OD) of 0.6 at 600 nm. In case of *E. coli* AG100 and AG100 A, the medium used in the assay was LB broth. The cells were washed with phosphate buffered saline (PBS) and the compounds were added individually at different concentrations at ½ MIC, ⅓ MIC, ¼ MIC or ⅕ MIC (in double concentrated form) to the EB solution in PBS. In case of *S. aureus* strains, the concentration of EB was 0.5 μg/mL, for *E. coli* AG100 1 μg/mL, and in case of *E. coli* AG100 A it was 0.25 μg/mL. Then, 10 μL of the EB solution containing the compound were transferred into standard glass capillary tubes of 20 μL maximum volume (Roche, Indianapolis, USA), and 10 μL of bacterial suspension (OD of 0.6 at 600 nm) were added to the capillaries and the fluorescence was monitored at the FL-2 channel in every minute on a real-time basis.

From the real-time data, the activity of the compounds, namely the relative fluorescence index (RFI) of the last time point (minute 30) of the EB accumulation assay, was calculated according to the following formula:

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

Where $RF_{treated}$ is the relative fluorescence (RF) at the last time point of EB retention curve in the presence of an inhibitor, and $RF_{untreated}$ is the RF at the last time point of the EB retention

curve of the untreated control having the solvent control (DMSO). Verapamil was applied as a positive control on Gram-positive strains and promethazine (PMZ) was used on Gram-negative strains.

3.5. Efflux assay using ethidium bromide

The activity of selenocompounds (**EDA1-11**) on the efflux of EB was determined on wild type (SL1344) and *acrB* gene inactivated (L644) *S.* Typhimurium strains. Briefly, an aliquot of an overnight culture of *S.* Typhimurium strain in Luria-Bertani (LB) medium was transferred to fresh LB medium, and it was incubated until it reached an OD of 0.4 at 600 nm. The cultures were washed in 20 mM potassium phosphate buffer (PPB) with 1 mM $MgCl_2$ and the OD was adjusted to 0.2 at 600 nm. 100 μM of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was added to de-energize the cells and therefore EB was added at 50 μg/mL concentration. The cultures were incubated at 23°C with shaking at 150 rpm for 1 h. After the incubation period the cultures were centrifuged, and the pellet was re-suspended in 20 mM PPB with 1 mM $MgCl_2$ and 5% glucose to energize the cells. Following energization, 200 μL of the bacterial culture were transferred into black 96-well microtiter plate (Corning, Amsterdam) containing the compounds at 50 μM (½ MIC) and the fluorescence of EB was measured over 2 hours at excitation and emission wavelengths of 530 and 600 nm, respectively, using a FLUOstar Optima plate reader (BMG Labtech, United Kingdom). During the evaluation the exact time was determined when the fluorescence dropped by 25% and 50% of the starting value. CCCP was applied as a positive control and DMSO was used as a negative control.

3.6. Measuring biofilm formation using crystal violet

The biofilm forming ability of *S.* Typhimurium 14028s strain was studied in 96-well microtiter plates using LB broth without salt in the presence of selenocompounds (**EDA1-11**). Initially, overnight cultures were diluted to an OD of 0.1 at 600 nm and then added to each well with the exception of the medium control wells and compounds were added at 50 μM (½ MIC) concentration. The final volume was 200 μL in each well. Plates were incubated at 30°C for 48 h with gentle agitation (100 rpm). After the incubation period the medium was discarded, and the plate was washed with tap water. 200 μL CV (0.1% [v/v]) was added to the wells and incubated for 15 minutes at room temperature. CV was removed from the wells and the plate was washed again with tap water. 200 μL of 70% ethanol was added to each well and the biofilm formation was determined by measuring the OD at 600 nm using a FLUOstar Optima plate reader. The anti-biofilm effect of selenocompounds was expressed in the percentage (%) of

decrease in biofilm formation. The results were analyzed using t-test and *p*-values of <0.05 were considered significant.

3.7. Interaction between antibiotics and resistance modifiers using checkerboard method

The combined effect of chamanetin (**CK2**) and dichamanetin (**CK4**) and antibiotics (TET and CIP) on the growth inhibition of *S. aureus* ATCC 25923 and resistant *S. aureus* 272123 strains was evaluated by checkerboard method. Two-fold serial dilutions of antibiotics were prepared in Mueller Hinton (MH) broth on the horizontal rows of microtiter plate and then cross-diluted vertically by two-fold serial dilutions of the compounds. After the dilution of an overnight culture, bacterial cells were re-suspended in MH medium containing 1×10^4 cells and distributed into each well. The plates were incubated for 18 h at 37°C. The cell growth rate was determined after MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) staining. The combination index (CI) values at 90% growth inhibition (ED_{90}) were determined by CompuSyn software. CI values were calculated by means of the median-effect equation, where CI < 1, CI = 1 and CI > 1 represent synergism, an additive effect (or no interaction) and antagonism, respectively.

3.8. Interaction between antibiotics and resistance modifiers using minimum inhibitory concentration reduction assay

The chemosensitizing effect of the selenocompounds (**EDA1-11**) was evaluated by the determination of the MIC values of the antibiotics (TET and CIP), in the presence of sub-inhibitory concentrations of the compounds (½ MIC) in *E. coli* AG100 strain by a two-fold broth microdilution method in the 96-well plates, using serial dilutions of TET and CIP. 10^{-4} dilution of an overnight bacterial culture in 50 µL of MH 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.

3.9. Assay for quorum sensing inhibition

Modified LB agar (prepared from yeast extract 5 g/L, tryptone 10 g/L, NaCl 10 g/L, K_2HPO_4 1 g/L, $MgSO_4$ x $7H_2O$ 0.3 g/L, FeNaEDTA 36 mg/L and agar 20 g/L) was used for these experiments. The sensor strain *C. violaceum* 026 and the AHL producer strain *E. cloacae* 31298 were inoculated as parallel lines and incubated at room temperature (20°C) for 24–48 h. Filter paper discs were impregnated with 10 µL of stock solutions (10 mM or 10 mg/mL) of the CK and PY compounds in DMSO. The discs were placed between the parallel lines of the sensor and the AHL producer strains on the surface of the nutrient agar. The plates were

incubated at room temperature for another 24–48 h, and the interactions between the strains and compounds were evaluated for the reduction in the size of the zone of pigment production and the zone of growth inhibition of the affected strains, in millimeters. PMZ was applied as a positive control.

3.10. Expression analyses of genes by RT-qPCR reaction

E. coli AG100 strain was cultured in LB broth, *S. aureus* ATCC 25923 and MRSA 272123 strains were cultured in TSB broth. On the day of RNA isolation, the compounds were added to the bacterial suspension at 50 μM (for **EDA1**, **-4**, **-7**), 50 μg/mL (for **PY2**, **-4**, **-5**), 5 μM (for **CK2**) and 0.5 μM (for **CK4**) concentrations, which were incubated at 37°C. After 4 hours (for **CK2** and **-4**) or 4 and 18 hours (for **PY2**, **-4**, **-5** and **EDA1**, **-4**, **-7**) of culturing, the total RNA was isolated in an RNase-free environment using NucleoSpin RNA kit (Macherey Nagel, Germany) according to the manufacturer's instructions. The concentration of the extracted RNA templates was assessed by SmartSpec™ Plus Spectrophotometer at 260 nm (Bio-Rad, USA).

Expression of the efflux pump genes *norA* and *mepA* in the presence of chamanetin (**CK2**) and dichamanetin (**CK4**) was studied by reverse transcription of the total RNA of *S. aureus* ATCC 25923 and MRSA 272123 strains. The data obtained for gene targets were normalized against the *S. aureus 16S ribosomal RNA* gene measured in the same sample.

The effect of **PY2**, **-4**, **-5** and **EDA1**, **-4**, **-7** on the relative expression of the efflux pump (*acrA, acrB*), antibiotic resistance (*marR*) and QS (*sdiA*) genes were studied in *E. coli* AG100. The data obtained for gene targets were normalized against the *E. coli* house-keeping gene glyceraldehide-3-phosphate-dehydrogenase (*gapdh)* measured in the same sample.

Real-time quantification of the RNA templates by one-step RT-qPCR was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad, USA) strictly adhered to the manufacturer's recommendations of the SensiFAST™ SYBR No-ROX One-Step Kit (Bioline GmbH, Germany). The relative quantities of the mRNA of each gene of interest were determined by the $\Delta\Delta C_T$ method.

4. RESULTS

4.1. *In vitro* antibacterial activity of compounds

4.1.1. Bioactive compounds from C. kirkii

Concerning the antibacterial effect of the compounds, chamanetin (**CK2**) and dichamanetin (**CK4**) had a potent antibacterial effect on both *S. aureus* ATCC 25923 and *S.*

aureus 272123 strains. MIC of compound **CK2** was 12.5 μM on reference *S. aureus* ATCC strain; however, the MIC of the methicillin and ofloxacin resistant strain was 25 μM. Compound **CK4** was the most effective flavanone because its MIC on *S. aureus* ATCC 25923 strain was 0.8 μM; furthermore, on the methicillin resistant strain it exhibited the MIC of 1.56 μM. The compounds had no antibacterial effect on the Gram-negative *E. coli* AG100, AG100 A, *C. violaceum,* and *E. cloacae* strains (MIC: >100 or 100 μM).

4.1.2. Fluorinated β-diketo phosphorus ylides

Compounds **PY1-10** did not have any antibacterial effect on the AcrAB-TolC-expressing *E. coli* AG100, *C. violaceum* and *E. cloacae* strains and the AcrAB-TolC deleted *E. coli* AG100 A strain (MIC: >100 μg/mL), except for ethyl-4,4,4-trifluoro-3-oxo-2-(triphenylphosphoranylidene)butanoate (**PY6**), which had a mild effect on the EP deleted strain (MIC: 50 μg/mL).

4.1.3. Selenocompounds

The ketone-containing selenoesters (**EDA9**–**11**) showed a potent antibacterial activity against the *S. aureus* ATCC 25923. The methylketone selenoester **EDA9** was the most active compound with noteworthy MIC value (3.12 μM). The selenoanhydride **EDA1** and the remaining selenoesters **EDA2**–**8** evaluated were inactive as their MIC was equal or above 100 μM on *S. aureus* ATCC 25923 strain. **EDA9** showed antibacterial activity towards *E. faecalis* (MIC: 12.5 μM). The compounds had no antibacterial effect (MIC: 100 μM or >100 μM) on Gram-negative *E. coli* AG100, *E. coli* AG100 A, *S.* Typhimurium SL1344, *acrB* inactivated *S.* Typhimurium L644 and *S.* Typhimurium 14028s strains.

4.2. Efflux pump inhibiting activity (accumulation assay)

4.2.1. Bioactive compounds from C. kirkii

CK2 and **CK4** had EP inhibiting activity compared to verapamil (RFI: 0.13) on the *S. aureus* ATCC 25923 strain, and the most active compound was **CK2**. However, compounds **CK1–5** had no EP inhibitory activity on the methicillin resistant *S. aureus* strain at the concentrations applied in the assay. Concerning the inhibitory activity on Gram-negatives, triterpene polycarpol (**CK1**) and **CK5** could inhibit the AcrAB-TolC system of *E. coli* AG100 compared to PMZ (RFI: 0.15). Based on the real-time accumulation data, **CK1–5** had no effect on the *E. coli* AG100 A strain lacking the AcrAB-TolC pump.

4.2.2. Fluorinated β-diketo phosphorus ylides

The majority of the P-ylides were found to inhibit the AcrAB-TolC system of *E. coli* except **PY3**, **PY7** and **PY8**, which had little or no effect on the intracellular EB accumulation

in both strains. Among the P-ylide series, **PY2**, **-4**, and **-5** exhibited strong AcrAB-TolC pump inhibiting properties compared to the AcrAB-TolC pump deficient mutant strain. The most potent derivative was **PY4** and its effect was more pronounced on the multidrug resistant *E. coli* strain compared to the pump deleted *E. coli* strain.

4.2.3. Selenocompounds

EDA1 and **EDA4** strongly inhibited the efflux mediated by AcrAB-TolC in *E. coli* AG100 compared to the positive control PMZ (RFI: 0.15). **EDA7** and **EDA9–11** caused moderate inhibitory action on the intracellular EB accumulation in *E. coli* AG100. Nevertheless, no EP inhibitory action was found in the *E. coli* AG100 A strain in case of selenocompounds (**EDA1–11**).

4.3. Efflux pump inhibiting activity (efflux assay)

Each selenocompound showed a 25% and 50% decrease in fluorescence at an earlier time point, compared with positive control CCCP in *S.* Typhimurium SL1344. For the L644 strain only **EDA7** was able to prevent the efflux of EB more effectively than CCCP.

4.4. Anti-biofilm activity of selenocompounds

Except compounds **EDA6** and **-11** all derivatives showed significant (>45%; $p<0.05$) or higher biofilm inhibition at 50 μM on *S.* Typhimurium 14028s. The most potent selenocompounds with anti-biofilm effect were **EDA4** and **-5** at 50 μM showing 75% and 73% of inhibition, respectively.

4.5. Combined effects of chamanetin (CK2) and dichamanetin (CK4) with antibiotics

The combined effect of TET or CIP and compounds **CK2** or **CK4** on *S. aureus* ATCC 25923 resulted in synergism. On the methicillin resistant *S. aureus* strain **CK2** showed slight synergism with CIP. The interactions of **CK4** with TET on the resistant *S. aureus* strain was synergism.

4.6. Enhancement of the activity of antibiotics in the presence of selenocompounds

In the absence of selenocompounds, TET showed MIC value of 4.2 μM and CIP exhibited MIC of 0.02 μM. **EDA9** potentiated the effect of TET (MIC: 2.1 μM) and CIP (MIC: 0.01 μM), furthermore **EDA10** reduced the MIC value of CIP by two-fold in *E. coli* strain.

4.7. Anti-quorum sensing activity

4.7.1. Bioactive compounds from C. kirkii

Compounds **CK1** (51 mm), **-2** (50 mm), **-4** (53 mm), and **-5** (52 mm) were able to inhibit effectively the QS between *C. violaceum* 026 and *E. cloacae* compared to the positive control PMZ (46 mm).

4.7.2. Fluorinated β-diketo phosphorus ylides

P-ylides were not able to inhibit the QS (inhibition zone: 0 mm) in the applied systems compared to the positive control PMZ.

4.8. Relative expressions of genes related to antibiotic resistance and efflux pumps

4.8.1. Bioactive compounds from C. kirkii

CK2 at 5 μM significantly up-regulated the expression of *norA* and *mepA* genes after 4 h of exposure in *S. aureus* resistant strain. Compound **CK4** at 0.5 μM also significantly up-regulated both efflux pump genes after 4 h of exposure in *S. aureus* 272123. In *S. aureus* ATCC 25923 strain, the expression level of the *mepA* gene was not influenced. Nevertheless, the *norA* gene was significantly up-regulated by compounds **CK2** at 5 μM and **CK4** at 0.5 μM.

4.8.2. Fluorinated β-diketo phosphorus ylides

PY2 at 50 μg/ml up-regulated all the genes studied after 4 h of exposure, however, after 18 h the gene expression returned to basal levels. **PY4** also significantly up-regulated the secondary resistance-nodulation-division family (RND) transporter gene *acrB* after 4 h and 18 h exposures as well. **PY5** up-regulated the expression levels of *acrA* and *acrB* after 4 h.

4.8.3. Selenocompounds

EDA1 at 50 μM significantly up-regulated *acrB, marR* and *sdiA* genes studied after 4 h of exposure, however, after 18 h, the expression of *acrB* gene returned to basal level and the *marR* and *sdiA* genes increased significantly. **EDA4** up-regulated the expression of *acrB, marR* and *sdiA* after 4 h although after 18 h the expression levels of *acrB* and *marR* genes decreased. The QS gene *sdiA* was significantly up-regulated after 18h. **EDA7** also significantly up-regulated *marR* after 4 h and 18 h exposures. After 18 h the RND transporter subunit genes (*acrA, acrB*) were significantly upregulated in the presence of **EDA7**.

5. DISCUSSION

Multidrug resistance to antibiotics has become a serious problem in the treatment of infectious diseases. One of the most important mechanisms causing multidrug resistance is the

over-expression of EPs, whereby cells pump out toxic substances to the exterior of the cells. Infections caused by MDR bacteria lead to increased treatment costs and may result in fatal outcomes; consequently, it is a major challenge for drug development in order to discover new EPIs.

The natural, plant-derived or synthetic compounds may represent a valuable source of new antibacterial agents because they can inhibit the growth of bacteria and the activity of bacterial efflux systems which indirectly prevent the formation of biofilm and the bacterial cell-to-cell communication system, furthermore, they can potentiate the efficacy of antibiotics as well.

The main goal of our study was to evaluate the antibacterial and multidrug resistance reversing effects of bioactive compounds from *C. kirkii* (**CK1-5**), fluorinated β-diketo phosphorus ylides (**PY1-10**) and selenocompounds (**EDA1-11**) in different bacterial models. The following methods were used in the studies: MIC determination, EB accumulation and efflux assay, checkerboard combination method, MIC reduction assay, biofilm, QS inhibitory test and RT-qPCR.

5.1. Bioactive compounds from *C. kirkii* (CK1-5)

According to this study chamanetin (**CK2**) and dichamanetin (**CK4**) had the most pronounced antibacterial activity in case of methicillin sensitive and resistant *S. aureus* strains. Dichamanetin showed stronger effect than chamanetin, due to its higher lipophilic character resulting from the extra benzyl group at C-6. In contrast to chamanetin, isochamanetin (**CK3**), differing only in the position of benzyl substituents, was inactive at the concentration tested. Thus, besides the importance of lipophilicity, the presence of a benzyl moiety at C-8 appears to play a decisive role in the antibacterial activity of this type of compounds. In the combination assays, chamanetin and dichamanetin showed synergism with TET and CIP on the *S. aureus* reference strain. Furthermore, on the MRSA strain, chamanetin and dichamanetin, combined with TET and CIP, respectively, also showed synergism, which indicates that these compounds could be potential adjuvants in the therapy. Besides having an antibacterial effect, chamanetin and dichamanetin could inhibit the activity of EPs compared to the positive control on the *S. aureus* ATCC 25923. Furthermore, these compounds inhibited the QS between *C. violaceum* 026 and *E. cloacae* 31298, and they caused over-expression of EP genes (*norA* and *mepA*) after 4 h of exposure in the MRSA strain. The change in gene expression could be due to the stress response against chamanetin and dichamanetin because these compounds as potential noxious agents for *S. aureus* had to be extruded from the cytoplasm of the bacterium as soon as possible. This stress response can be the explanation for the up-regulation of the EP genes after 4 h.

Chamanetin and dichamanetin influenced the expression of *mepA* gene in the MRSA strain, however, these compounds did not have any effect on the expression level of *mepA* in the reference *S. aureus* strain. The difference between the methicillin resistant *S. aureus* and methicillin susceptible *S. aureus* strains is due to the over-expression of the *mepA* gene in the resistant strain and it has low expression level in the reference strain. It has been demonstrated by other studies that the over-expression of EPs confers a fitness cost for the organism, for example, a resistant isolate over-expressing EPs shows reduced production of virulence determinants. In Gram-negatives, compounds **CK1**–**5** did not show antibacterial activity. The highly lipophilic polycarpol (**CK1**) inhibited the RND transporter AcrAB-TolC transport system in *E. coli* AG100 strain because it could increase the membrane permeability of bacteria. In contrast polycarpol had no EPI effect on the pump mutant *E. coli* AG100 A strain confirming that it may have a direct EPI effect on the AcrAB-TolC EP. In addition, apart from chamanetin and dichamanetin, polycarpol and acetylmelodorinol (**CK5**) were also able to inhibit effectively the bacterial communication.

5.2. Fluorinated β-diketo phosphorus ylides (PY1-10)

In our previous study trifluoro-1-phenyl-2-(triphenylphosphoranylidene)butane-1,3-dione (**PY3**), 4,4,5,5,5-pentafluoro-1-phenyl-2-(triphenylphosphoranylidene)pentane-1,3-dione (**PY7**) and 4,4,5,5,6,6,6-heptafluoro-1-phenyl-2-(triphenylphosphoranylidene)hexane-1,3-dione (**PY8**) have been shown to have activity against the EPs of cancer cells, but did not show any activity against the EPs of *E. coli* strains. The aim of the previous work was to examine the primary ATP-binding cassette subfamily B member (ABCB1) EP inhibitory effect of PY compounds in cancer cells, in the present study the compounds have been tested against the secondary AcrAB-TolC system in bacteria. It is important to note that ATP-binding cassette (ABC) transporters derive their energy from the hydrolysis of ATP, however, the AcrAB-TolC system is a proton motive force (PMF)-dependent multidrug efflux system. The most effective compounds in *E. coli* AG100 were 1,1,1-trifluoro-3-oxo-1-methoxy-3-(triphenylphosphoranylidene)propane-2-one (**PY2**), 4,4,4-trifluoro-3-oxo-2-(triphenylphosphoranylidene)butanal (**PY4**), and 1,1,1-trifluoro-3-(triphenylphosphoranylidene)pentane-2,4-dione (**PY5**), which inhibited the AcrAB-TolC system and influenced the expression of the transporter genes *acrA* and *acrB*. In addition, although the compounds are not QS inhibitors, **PY4** increased the expression of *sdiA* after 18 h exposure. The compounds in the R^1 chain contain a trifluoromethyl ketone ($COCF_3$) and the R^2 chain is a methoxy (OMe; **PY2**), formyl (CHO; **PY4**), and acetyl (COMe; **PY5**) substituent.

Thus, some structurally related fluorinated β-diketo phosphorus ylides differ in their multidrug resistance reversal activities between cancer cells and bacterial strains, indicating that the compounds act differently as inhibitors of ABCB1 and AcrB efflux pumps because these pumps differ in their structure and energy source driving the pump (ATP and PMF, respectively).

5.3. Selenocompounds (EDA1-11)

These novel selenocompounds were studied previously for anticancer and ABCB1 EP inhibitory activity in different cancer cells. According to the results on bacteria it was found that the ketone-containing selenoesters **EDA9-11** showed antibacterial activity against *S. aureus* reference strain, furthermore the methylketone selenoester (**EDA9)** was effective on *E. faecalis* ATCC 29212. In the EB accumulation assay the cyclic selenoanhydride (**EDA1)** and the meta-substituted benzene selenodiester (**EDA4)** could inhibit the activity of the AcrAB-TolC efflux pump system and they were more potent than the positive control PMZ in *E. coli* AG100. Methylketone selenoester significantly inhibited the efflux mechanism of wild-type *S.* Typhimurium SL1344 strain. EP inhibiting activity has been found for methoxycarbonylmethyl selenoester (**EDA7)** in the *acrB* mutant *S.* Typhimurium strain. From the results obtained in the EB efflux assay, it can be concluded that the same compounds had efflux pump inhibitory activity in the wild type and mutant *S.* Typhimurium strains as well. The similarities observed in the efflux assay of compounds mean that the derivatives do not directly inhibit the AcrAB-TolC system of *S.* Typhimurium.

Results showed that the meta-substituted benzene selenodiester (**EDA4**) and para-substituted benzene selenodiester (**EDA5**), which contain a phenyl ring, were the strongest inhibitors of biofilm formation in *S.* Typhimurium 14028s. **EDA7-10** and the thiophene selenodiester (**EDA2**) and pyridine selenodiester (**EDA3**) also exerted a biofilm inhibiting activity higher than 50%. Methylketone selenoester reduced the MIC value of TET by 2-fold, furthermore this compound and chloro-substituted *tert*-butylketone selenoester potentiated the activity of TET and CIP on *E. coli* AG100. In the gene expression analysis, we observed that cyclic selenoanhydride, meta-substituted benzene selenodiester, and methoxycarbonylmethyl selenoester influenced the gene expression of resistance (and transporter) genes because these derivatives significantly up-regulated the *marR* gene after 4 and 18 h exposure, respectively. Furthermore, the QS gene *sdiA* was significantly up-regulated in the presence of cyclic selenoanhydride, after exposures of 4 h and 18 h.

According to the results these studies indicated that natural and synthetic compounds could be used as potential antibacterial agents alone or combination with antibiotics for the

treatment of infectious diseases. In addition, our studies are suggesting the importance of the substituent's topology and moiety for the biological potency. In future studies, it will be necessary to design and analyze systematically more compounds and understand the structure-activity relationships in order to develop potent resistance modifiers.

6. NEW FINDINGS

1. **Bioactive compounds from *Cleistochlamys kirkii***
   - Chamanetin (**CK2**) and dichamanetin (**CK4**) with a benzyl moiety at C-8 showed potent antibacterial effect against both *Staphylococcus aureus* ATCC 25923 reference and methicillin and ofloxacin resistant *S. aureus* 272123 strains. Moreover, dichamanetin proved to be the most active compound isolated from *C. kirkii* on these strains and has higher lipophilic character compared to chamanetin due to its extra benzyl group at C-6. Dichamanetin had synergistic activity with tetracycline and ciprofloxacin on *S. aureus* reference strain. Furthermore, dichamanetin showed synergism in combination with tetracycline on the methicillin and ofloxacin resistant *S. aureus* strain.
   - Chamanetin and acetylmelodorinol (**CK5**) had potent efflux pump modulatory effects in *S. aureus* reference strain. Polycarpol (**CK1**) was the most effective efflux pump inhibitor on *Escherichia coli* AG100 inhibiting the AcrAB-TolC transporter system. All bioactive compounds of *C. kirkii* except isochamanetin (CK3) were able to inhibit effectively the bacterial quorum sensing system.
2. **Fluorinated β-diketo phosphorus ylides**
   - 1,1,1-trifluoro-3-oxo-1-methoxy-3-(triphenylphosphoranylidene)propane-2-one (**PY2**), 4,4,4-trifluoro-3-oxo-2-(triphenylphosphoranylidene)butanal (**PY4**), and 1,1,1-trifluoro-3-(triphenylphosphoranylidene)pentane-2,4-dione (**PY5**) inhibited directly the AcrAB-TolC efflux transporter in *E. coli* AG100 strain compared to the AcrAB-TolC pump mutant AG100 A strain.
3. **Selenocompounds**
   - Methylketone selenoester (**EDA9**) had antibacterial activity against the reference *S. aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 strains.
   - Methylketone selenoester potentiated the effect of tetracycline and ciprofloxacin on the AcrAB-TolC expressing *E. coli* AG100 strain.
   - Almost all selenocompounds showed potent anti-biofilm activity against *Salmonella enterica* serovar Typhimurium 14028s strain.

- Cyclic selenoanhydride (**EDA1**), meta-substituted benzene selenodiester (**EDA4**) and methoxycarbonylmethyl selenoester (**EDA7**) were the most active inhibitors of the AcrAB-TolC system in *E. coli* AG100 strain.

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9. PUBLICATIONS RELATED TO THE THESIS

I. **Kincses A**, Szabó ÁM, Saijo R, Watanabe G, Kawase M, Molnár J, Spengler G. Fluorinated β-diketo phosphorus ylides are novel efflux pump inhibitors in bacteria. *In Vivo*. **30**: 813-817, 2017.
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