Close

From:	liar-anticancer <editor@iiar-submissions.com>	**Sent:**	Tue 23/08/16 9:21 AM
To:	Gabriella Spengler <spengler.gabriella@med.u-szeged.hu>	**Priority:**	Normal
Subject:	Revision of manuscript No 3527-K	**Type:**	Embeded HTML/Text

Dear Dr. Gabriella Spengler:

I am pleased to inform you that your manuscript no. 3527-K has been peer-reviewed to secure excellence in scientific quality and presentation.

Please login to In Vivo, www.iiar-submissions.com to review the manuscript file with all the suggestions and corrections introduced by our reviewer(s), as well as those of our philological department. We believe that these alterations have improved the presentation of your work. We would greatly appreciate your attention in inspecting carefully all changes and making appropriate clarifications, additions or revisions as required.

You may use Microsoft Office Word (version 2007 or higher) and go to the "Review" tab, where you may click the "Accept all changes" option or manually accept changes one by one by clicking the tick box. If a change is not acceptable please click "X" box and explain. Don't forget to check the "Check_Sheet" file for the reviewers' comments.

Following your inspection, please upload your complete revised manuscript on our online platform. We would appreciate receiving your revised manuscript within 5 days.

With my best wishes,
John G. Delinasios
Managing Editor

IIAR journals

Fluorinated Beta-diketo Phosphorus Ylides Are Novel Efflux Pump Inhibitors in Bacteria

Running title: Phosphorus Ylides as Bacterial Efflux Pump Inhibitors

Experimental study

Annamária Kincses[1], Ágnes Míra Szabó[1], Ryosuke Saijo[2], Genki Watanabe[2], Masami Kawase[2], Joseph Molnár[1] and Gabriella Spengler[1]

[1]*Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Szeged 6720, Hungary*

[2]*Faculty of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan*

Correspondence to: Gabriella Spengler, Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Dóm tér 10, H-6720 Szeged, Hungary. Tel: Tel:+3662545115, Fax:+3662545113, e-mail: spengler.gabriella@med.u-szeged.hu

Abstract. *Background:* One of the most important resistance mechanisms in bacteria is the increased expression of multidrug efflux pumps. To combat efflux related resistance, the development of new efflux pump inhibitors is essential. *Materials and Methods:* Ten phosphorus ylides were compared based on their MDR-reverting activity in multidrug efflux pump system consisting of the subunits acrA and acrB or acridine resistance proteins A and B and the multidrug efflux pump subunit TolC or outer membrane factor TolC (AcrAB-TolC) of *Escherichia coli* K-12 AG100 strain and its AcrAB-TolC-deleted strain. Efflux inhibition was assessed by real-time fluorimetry and the inhibition of quorum sensing (QS) was also investigated. The relative gene expression of efflux QS genes was determined by real-time reverse transcriptase quantitative polymerase chain reaction. *Results:* The most potent derivative was $Ph_3P=C(COC_2F_5)CHO$ and its effect was more pronounced on the AcrAB-TolC-expressing *E. coli* strain, furthermore the most active compounds, $Ph_3P=C(COCF_3)OMe$, $Ph_3P=C(COC_2F_5)CHO$ and $Ph_3P=C(COCF_3)COMe$, reduced the expression of efflux pump and QS genes. *Conclusion:* Phosphorus ylides might be valuable EPI compounds to reverse efflux related MDR in bacteria.

Key Words: Phosphorus ylides, multidrug resistance, bacterial AcrAB-TolC, efflux pump, quorum sensing.

Multidrug resistance (MDR) is a serious problem for the treatment of various diseases, such as bacterial and fungal infections and cancer, due to the reduction or lack response of microorganisms as well as cancer cells to applied chemotherapeutic agents (1,2).

One of the major mechanisms of MDR is the overexpression of efflux pumps (EPs), which reduces the accumulation of toxic agents. In bacteria the resistance nodulation division (RND) transporters have a great impact on MDR phenotype. The major cause for the MDR phenotype is due to overexpresseion of efflux pumps that are part of the RND family of transporters, for example the AcrAB-TolC system (1). These efflux pumps have the ability to recognize and extrude a large variety of unrelated antibiotics from the periplasmic space of the cell envelope, or 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 (3).

The quorum-sensing system (QS) in bacteria is a regulatory system that controls gene expression depending on the density of the bacterial cell population. A transcriptional regulator (LuxR homolog), signal synthase (LuxI homologue) and autoinducer (acyl homoserine lactone) are crucial for the QS in most Gram-negative bacteria. SdiA, an *E. coli* LuxR homolog, has a great impact on the colonization of *E. coli* (4, 5). SdiA is a homolog of QS regulators that detects *N*-acylhomoserine lactone (AHL) signals from other bacteria. SdiA represses the expression of virulence factors by interacting with unknown stationary-phase signals in *E. coli* O157:H7, and enhances multidrug resistance by activating MDR efflux pumps in *E. coli* (6).

Organic compounds of phosphorus ylides (P-ylides) are a fascinating class of compounds (7). The biological activity of P-ylides related to their ATP Binding Cassette Subfamily B Member 1 (ABCB1)-modulating activity in mouse lymphoma cells has already been described (8), however, additional information is needed to describe their valuable biological activities in other aspects.

In this article, we report the MDR-modulating activities of P-ylides in bacteria related to the inhibition of efflux activity and QS.

Materials and Methods

Compounds. The synthesis of P-ylides was described previously (8) and the structures of the P-ylides (compounds **1**-**10**) screened for their MDR-modulating activities are shown in **Table I**. The compounds were dissolved in dimethyl sulfoxide (DMSO) for the experiments.

Acridine orange (AO), ethidium bromide (EB), and LB broth were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Modified LB agar (LB*) contained 5 g yeast extract 10 g, trypton, 10 g NaCl, 1 g K_2HPO_4, 0.3 g $MgSO_4.7H_2O$ and 36 mg FeNaEDTA in 1.0 l of distilled water. Mueller Hinton broth was purchased from Scharlau Chemie S.A. (Barcelona, Spain).

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 strain (*E. coli* K-12 AG100A). The strains were kindly provided by Professor Dr. Hiroshi Nikaido (Department of Molecular and Cell Biology and Chemistry, University of California, Berkeley, CA, USA).

Strains used for QS tests: *Chromobacterium violaceum* 026 (CV026) as sensor strain; *Sphingomonas* spp. EZF 10-17 (*Sphingomonadaceae*) isolated from a grapevine crown gall tumor (used as AHL producer), this strain induced pigment production by CV026 and proved to be efficient for the study of QS interactions; *Enterobacter cloaceae* 31298 (clinical wound isolate, used as AHL producer).

Determination of minimum inhibitory concentrations. The minimum inhibitory concentrations (MICs) of P-ylides were tested according to Clinical and Laboratory Standard Institute guidelines (9).

Real-time accumulation assay by Roche LightCycler real-time thermocycler. The effect of the studied compounds on the real-time accumulation of EB was assessed by an automated EB method (10), using a LightCycler real-time thermocycler (LightCycler 1.5; Roche, Indianapolis, IN, USA). Briefly, an aliquot of an overnight culture of the strain in LB medium was transferred to fresh LB medium and incubated until it reached an optical density (OD) of 0.6 at 600 nm. It was then washed with phosphate-buffered saline (PBS; pH 7.4) and centrifuged at 13 000 × *g* for 3 min, the pellets re-suspended in PBS (pH 7.4) and the OD adjusted to 0.6 at 600 nm. The compounds were added individually at different concentrations (in double concentrated form) in 5 µl volumes of their stock solutions to 45 µl of EB solution of 2 mg/ l in PBS. Then, 10 µl of the EB solution containing the compound were transferred into standard glass capillary tubes of 20 µl maximum volume (Roche) and 10 µl of bacterial suspension (OD of 0.6 at 600 nm) was added to the capillaries. The capillaries containing the samples were placed into a carousel (Roche) and the fluorescence was monitored at the FL-2 channel every minute on a real-time basis.

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

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

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

Assay for QS inhibition. LB* was used for these experiments. The sensor strain *C. violaceum* 026 (CV026) and the AHL producer strains EZF 10-17 *Sphingomonas* spp. (*Sphingomonadaceae*) or *E. cloaceae* 31298 were inoculated as parallel lines and incubated at room temperature (20°C) for 24-48 h. QS inhibition was monitored by agar diffusion method. Filter paper discs (7.0 mm in diameter) were impregnated with 10 µl of stock solutions of the compounds in DMSO (10 mg/ml in DMSO). The discs were placed between the parallel lines of sensor and AHL producer strains on the surface of the nutrient agar. The plates were incubated at room temperature for a further 24-48 h and the interactions between the strains and compounds were evaluated for the reduction in size of the zone of pigment production and the zone of growth inhibition of the affected strains, in millimeters. AO was applied as positive control (11).

Expression analyses of genes by real-time reverse transcriptase quantitative polymerase chain (RT-qPCR) reaction. Bacteria were cultured in LB broth and were incubated overnight at 37°C with shaking. On the day of RNA isolation, the bacterial

suspensions (OD of 0.6 at 600 nm) were transferred to 10 ml tubes in 3 ml aliquots and 50 µg/ml of compounds were added to the tubes which were the incubated at 37ºC. At intervals of 4 and 18 h of culture, the tubes were centrifuged at 12,000 × *g* for 2 min. Pellets were suspended in 100 µl TE buffer containing 1 mg/ml lysozyme by vigorous vortexing and were incubated at 37°C for 10 min. The total RNA was isolated in an RNase-free environment using NucleoSpin RNA kit (Macherey Nagel, Germany) according to the manufacturer's instructions. Purified RNA was stored in RNase-free water in nuclease-free collection tubes and was maintained at −20ºC until quantification was performed. The concentration of the extracted RNA templates was assessed by spectrophotometry at 260 nm.

Expression of the genes of the acridine resistance protein A (*acrA)*, acridine resistance protein B (*acrB)*, multiple antibiotic resistance protein R (*marR)* and quorum-sensing transcriptional activator (*sdiA)* genes was studied by reverse transcription of total RNA. 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 real-time one-step RT-qPCR was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA) strictly adhering to the manufacturer recommendations of the SensiFAST^{TM} SYBR No-ROX One-Step Kit (Bioline GmbH, Luckenwalde, Germany). Briefly, each well of 96-well microtiter plates contained, in a final volume of 20 µl, 10 µl of the 2x SensiFAST^{TM} SYBR No-ROX One-Step Mix, 0.2 µl Reverse Transcriptase, 0.4 µl RiboSafe RNase Inhibitor, 5.4 µl Diethylpyrocarbonate (DEPC)-treated water, 500 nM of each primer and approximately 20 ng of total RNA in RNAase-free water. Thermal cycling was

initiated with a denaturation step of 5 min at 95°C, followed by 40 cycles each of 10 s at 95°C, 30 s at 57°C and 20 s at 72°C.

The forward and reverse primers used for assessment of the activity of the transporter genes *acrA*, *acrB*, the regulator *marR* and the QS regulator *sdiA* are shown in Table II.

Compounds **1-10** did not have any antibacterial effect on the AcrAB-TolC-expressing *E. coli* AG100 (MDR) strain nor its AcrAB-TolC-deleted progeny *E. coli* AG100A (EP-deleted strain) (MIC above 100 μg/ml), except for **6**, which had a mild, non-significant effect on the EP-deleted strain (MIC=50 μg/ml).

The activity of the compounds was compared based on the relative final fluorescence index (RFI) of the real-time accumulation curves (**Table III**).

In the case of real-time EB accumulation by Light Cycler thermocycler, the amount of EB accumulated by cells is higher if the difference between $RF_{treated}$ and $RF_{untreated}$ is greater, therefore, the degree of inhibition of the EP system by the compound becomes greater.

The majority of the P-ylides were found to inhibit the AcrAB-TolC system of *E. coli* except $Ph_3P=C(COCF_3)COPh$ (**3**), $Ph_3P=C(COC_2F_5)COPh$ (**7**) and $Ph_3P=C(COC_3F_7)COPh$ (**8**), with had little or no effect on the intracellular EB accumulation in the *E. coli* AG100 and the AG100A strains. Among the P-ylide series, $Ph_3P=C(COCF_3)OMe$ (**2**), $Ph_3P=C(COCF_3)CHO$ (**4**) and $Ph_3P=C(COCF_3)COMe$ (**5**) exhibited strong AcrAB-TolC pump-inhibiting properties compared to the AcrAB-TolC pump-deficient mutant strain. The most potent derivative was **4** and its effect was more pronounced on the MDR *E. coli* strain compared to the pump-deleted *E. coli* strain. This activity suggests that the

compound may interfere with the proton motive force because AcrB utilizes proton motive force as energy for its transport function.

The P-ylides were not able to inhibit the QS in the applied systems compared to the positive control AO (data not shown).

Regarding the effect of P-ylides on the relative expression of efflux pump and QS genes in *E. coli* AG100, the most effective compounds **2**, **4**, and **5** were selected for gene-expression studies. In the assay, the gene of the multidrug efflux pump subunit AcrB, the gene of the periplasmic AcrA subunit, the component of the *E. coli mar* locus (multiple antibiotic resistance), and the gene of the LuxR homologue SdiA were investigated. As shown in **Figure 1A**, compound **2** 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. Compound **4** also significantly up-regulated the secondary RND transporter gene *acrB* (approximately 2-fold increase) after 4 h and 18 h exposures as well. Surprisingly, there was up-regulation of *sdiA* expression after 18 h compared to the expression level after 4 h implicating the ability of compound **2** to influence the QS gene *sdiA*, however, this increase was not significant (**Figure 1B**). Compound **5** up-regulated the expression levels of *acrA* and *acrB* after 4 h, although after 18 h, the up-regulation of these genes was not significant (less than 2-fold increase) as presented by Figure 1C.

Discussion

Some phenothiazines and hydantoins are known to be EPIs against both bacteria and cancer cells (13). However, P-ylides **3**, **7** and **8** have been shown to have activity against the EPs of cancer cells (8), but not to have activity against the EP of bacteria. It is important to note that ABC transporters are primary efflux pumps deriving their

energy from the hydrolysis of ATP, however, the AcrAB-TolC system is a three-component proton motive force-dependent multidrug efflux system. The most effective compounds in the present bacterial system were compounds **2**, **4**, and **5**, which inhibi ted the AcrAB-TolC system and influenced the expression of the genes *acrA* and *acrB*, which are constituents of the AcrAB-TolC system. In addition, although the compounds are not QS inhibitors, compound **4** did increase the expression of *sdiA* after 18 h exposure.

It can be concluded that in the $COCF_3$ series (compounds **1**-**6),** the MDR-reverting activity in the MDR *E. coli* strain was intensified in the following order: CHO (**4**) > OMe (**2**), COMe (**5**) >> CO_2Et (**6**), COPh (**3**), CN (**1**).

Thus, some structurally related trifluoroacetylated P-ylides differ in their MDR-reversal activities between cancer cells and bacterial strains, indicating that the compounds differently act as inhibitors of primary (ABCB1) and secondary (AcrB) efflux pumps because these pumps differ in their energy source for driving the pump (ATP and PMF, respectively).

The present study demonstrated that trifluoroacetylated P-ylides may be attractive lead EPIs for further development as a MDR-reversing agents, however, their mode of action should be elucidated by structure–activity relationship studies.

Acknowledgements

This research was supported by the Szeged Foundation for Cancer Research, the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4. A/2-11-1-2012-0001 ‘National Excellence Program’. This article was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

References

1 Tanwar J, Das S, Fatima Z and Hameed S: Multidrug resistance: an emerging crisis. Interdiscip Perspect Infect Dis 541340. doi: 10.1155/2014/541340, 2014.

2 Nikaido H: Multidrug resistance in bacteria. Annu Rev Biochem *78*: 119-146, 2009.

3 Nikaido H and Pagès JM: Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. FEMS Microbiol Rev *36*: 340-363, 2012.

4 Kanamaru K, Kanamaru K, Tatsuno I, Tobe T and Sasakawa C: SdiA, an *Escherichia coli* homologue of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157:H7. Mol Microbiol *38*: 805-816, 2000.

5 Tavío MM, Aquili VD, Poveda JB, Antunes NT, Sánchez-Céspedes J and Vila J: Quorum-sensing regulator sdiA and marA overexpression is involved in i*n vitro*-selected multidrug resistance of *Escherichia coli*. J Antimicrob Chemother *65*: 1178-1186, 2010.

6 Lee J, Maeda T, Hong SH and Wood TK: Reconfiguring the quorum-sensing regulator SdiA of *Escherichia coli* to control biofilm formation *via* indole and *N*-acylhomoserine lactones. Appl Environ Microbiol *75*: 1703-1716, 2009.

7 El-Batta A, Jiang C, Zhao W, Anness R, Cooksy AL and Bergdahl M: Wittig reactions in water media employing stabilized ylides with aldehydes. Synthesis of alpha,beta-unsaturated esters from mixing aldehydes, alpha-bromoesters, and Ph_3P in aqueous $NaHCO_3$. J Org Chem *72*: 5244-5259, 2007.

8 Spengler G, Ocsovszki I, Tönki ÁS, Saijo R, Watanabe G, Kawase M and Molnár J: Fluorinated β-Diketo Phosphorus Ylides Are Novel Inhibitors of the ABCB1 Efflux Pump of Cancer Cells. Anticancer Res *35*: 5915-5919, 2015.

9 Clinical and Laboratory Standard Institute guidelines. http://clsi.org/

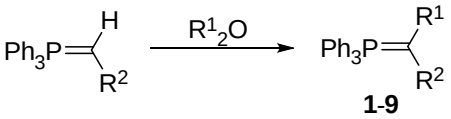
10 Viveiros M, Martins A, Paixão L, Rodrigues L, Martins M, Couto I, Fähnrich E, Kern WV and Amaral L: Demonstration of intrinsic efflux activity of *Escherichia coli* K-12 AG100 by an automated ethidium bromide method. Int J Antimicrob Agents *31*: 458-462, 2008.

11 Varga GZ, Szabo MA, Schelz Zs, Szegedi E, Amaral L and Molnár J: Quorum sensing inhibition by phenothiazines and related compounds. Lett Drug Design Discovery *8*: 133-137, 2011.

12 Viveiros M, Dupont M, Rodrigues L, Couto I, Davin-Regli A, Martins M, Pagès JM and Amaral L:. Antibiotic stress, genetic response and altered permeability of *E. coli*. PLoS One *2*: e365, 2007.

13 Amaral L, Spengler G, Martins A, Armada A, Handzlik J, Kiec-Kononowicz K and Molnar J: Inhibitors of bacterial efflux pumps that also inhibit efflux pumps of cancer cells. Anticancer Res *32*: 2947-2957, 2012.

Table I. Structures of P-ylides (compounds **1**-**10**)



1-**9**

Compound	R^1	R^2
1	$COCF_3$	CN
2	$COCF_3$	OMe
3	$COCF_3$	COPh
4	$COCF_3$	CHO
5	$COCF_3$	COMe
6	$COCF_3$	CO_2Et
7	COC_2F_5	COPh
8	COC_3F_7	COPh
9	$COCH_3$	CN
10	H	CN

Table II. Forward and reverse primers used for assessment of the activity of the transporter genes *acrA* and *acrB*, the regulator *marR* and the quorum-sensing regulator *sdiA* of *Escherichia coli* AG100.

Gene	Full name	Primer sequence (5'-3')	Amplicon size (bp)	Reference
acrA	Acridine resistance protein A	CTTAGCCCTAACAGGATGTG TTGAAATTACGCTTCAGGAT	189	(12)
acrB	Acridine resistance protein B	CGTACACAGAAAGTGCTCAA CGCTTCAACTTTGTTTTCTT	183	(12)
marR	Multiple antibiotic resistance protein R	AGCGATCTGTTCAATGAAAT TTCAGTTCAACCGGAGTAAT	170	(12)
sdiA	Quorum-sensing transcriptional activator	CTGATGGCTCTGATGCGTTTA TCTGGTGGAAATTGACCGTATT	163	This study
gapdh	Glyceraldehyde-3-phospate dehydrogenase	ACTTACGAGCAGATCAAAGC AGTTTCACGAAGTTGTCGTT	170	(12)

Table III. Relative final fluorescence index (RFI) for the affect of P-ylides (compounds **1**-**10**) on the AcrAB-TolC-expressing *Escherichia coli* AG100 and pump-deleted *E. coli* AG100A strains at 50 μg/ml

Compound	RFI[a]	
	AG100	AG100A
1	0.36	0.46
2	0.64	0.49
3	0.04	0.12
4	0.73	0.46
5	0.42	0.27
6	0.25	0.49
7	0.02	0
8	0	0.19
9	0.29	0.32
10	0.42	0.44

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

[a] RFI was calculated according to the formula:

where $RF_{treated}$ is the relative fluorescence at the last point (30 min) of the ethidium bromide (EB) retention curve in the presence an P-ylide and $RF_{untreated}$ is the relative fluorescence at the last point (30 min) of the EB retention curve of the untreated solvent control dimethyl sulfoxide (DMSO).

Figure 1. Relative gene expression levels of the genes of the acridine resistance protein A (*acrA)*, acridine resistance protein B (*acrB)*, multiple antibiotic resistance protein R (*marR)* and quorum-sensing transcriptional activator (*sdiA)* in the presence of compounds **2** (A), **4** (B)**,** and **5** (C) after 4 and 18 h exposure in LB. The line denotes a threshold value, which was set at a two-fold increase in transcripts.