# Fluorinated β-Diketo Phosphorus Ylides Are Novel Inhibitors of the ABCB1 Efflux Pump of Cancer Cells

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**Abstract.** *Efflux pump inhibitors are attractive compounds that reverse multidrug resistance (MDR) in cancer cells. In the present study, 10 phosphorus ylides (P-ylides) were compared based on their MDR-reverting activity in human ATP-binding cassette sub-family B member 1 (ABCB1; P-glycoprotein) gene-transfected L5178Y mouse T-lymphoma cells. Among them, three P-ylides, $Ph_3P=C(COCF_3)COPh$, $Ph_3P=C(COC_2F_5)COPh$ and $Ph_3P=C(COC_3F_7)COPh$ were identified as selectively modulating the ABCB1 pump. These compounds, with low cytotoxicity against mouse T-lymphoma cells, exhibited more potency than the positive control ABCB1 inhibitor verapamil.*

Multidrug resistance (MDR) is a serious problem for the treatment of various diseases such as cancer due to appearance of reduced or missing response of cancer cells to applied chemotherapeutic agents (1-4).

One of the major mechanisms of MDR is the overexpression of efflux pumps, that reduces the accumulation of toxic agents Multidrug pumps fall into one of five distinct families of membrane proteins and the most important MDR transporters of cancer cells belong to the ATP-binding cassette (ABC) transporters. The basic unit of the ABC family of transporters contains four core domains, and is the only multidrug pump family that are primary active transporters, namely, transport is powered by the direct hydrolysis of ATP by the transporter itself (5).

One of the most accepted strategies for overcoming MDR mediated by P-glycoprotein (ABCB1) is based on the development of MDR reversal agents that possess efflux pump inhibitor (EPI) properties (5). A number of MDR reversal agents of synthetic or natural origin have been reported and some of them have reached the stage of clinical trials. However, none of them has been approved for clinical use because of the undesirable side-effects (6, 7). Therefore, it is important to continue research in this area in order to discover new inhibitors of primary efflux pumps.

Organic compounds of phosphorous ylides (P-ylides) comprise a very interesting class of compounds with a long history in organic chemistry (8, 9). P-Ylides react with aldehydes or ketones to give substituted alkenes in a transformation called the Wittig reaction (10) named after George Wittig who was awarded the Nobel prize for this work in 1979. Little has been reported on P-ylides biological activities, besides their versatility and diversity in chemical behavior.

In this context, our interest has focused on the potential EPI activity of P-ylides such as (dicarbonylmethylene) triphenylphosphoranes, beta-diketo phosphorus ylides, which are very stable because of the strong electron-withdrawing effect of the two carbonyl groups (11, 12). In order to identify specific and selective MDR reversal agents, we screened 10 P-ylides (compounds **1-10**, Table I) for activity against ABCB1-mediated MDR in mouse T-lymphoma cells.

In this article, we report the MDR-modulating activities of P-ylides and the identification of selective MDR modulators of the ABCB1 pump in cancer cells.

## Materials and Methods

*General procedure for the preparation of α-acyl-α-perfluoroacyl-methylenetriphenyl-phosphoranes (**1-9**).* These compounds were prepared by employing the method reported by Hamper, with slight modifications (13). To a suspension of a phosphonium salt (11.0 mmol) in tetrahydofuran (THF) (25 ml) was added triethylamine (48.4 mmol) at 0˚C, and the mixture was stirred for 30 min. To the mixture perfluorinated anhydride (12.1 mmol) was added dropwise, and the whole was stirred at room temperature for the time indicated in Table

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*Key Words:* Multidrug resistance, phosphorus ylide, trifluoroacetyl, ABCB1, P-glycoprotein, mouse T-lymphoma.

I. The precipitate was filtered off, washed three times with cold THF, and the filtrate was evaporated. The residue was triturated with water, and the insoluble material was collected by filtration, washed with water, and dried in vacuo to give the product.

*Compound identification*. All melting points (mp) were determined using a Yanagimoto hot-stage melting point apparatus (Yanaco New Science Inc., Kyoto, Japan) and are uncorrected. $^{1}$H-nuclear magnetic resonance (NMR) spectra were measured on Bruker AVANCE500 spectrometer (Bruker Co., MA, USA) with tetramethylsilane (Me4Si) as an internal reference. $^{13}$C-NMR spectra were obtained on a Bruker AVANCE500 spectrometer (at 126 MHz). Both $^{1}$H- and $^{13}$C-NMR spectral data are reported in parts per million (δ) relative to $Me_4Si$. Infrared (IR) spectra were recorded on a JASCO FT/IR-4100 spectrometer (JASCO Co., Tokyo, Japan). Low- and high-resolution mass spectrometry (MS) was carried out with a JEOL JMS-GC mate II spectrometer (JEOL Ltd, Tokyo, Japan) with a direct inlet system at 70 eV and a Bruker micrOTOF-Q mass spectrometer (Bruker Co., MA, USA) with methanol as the solvent. Elemental analyses were carried out on a Yanaco CHN Corder MT-5 (Yanaco Co., Tokyo, Japan) at the Integrated Center for Science, Ehime University. Standard work-up meant that the organic layers were finally dried over $Na_2SO_4$, filtered, and concentrated *in vacuo* below 45˚C using a rotary evaporator. 2-(Triphenylphosphoranylidene)acetonitrile (compound **10**) was obtained from Wako Pure Chem. Ind. Ltd., Osaka, Japan.

*Characterization of compounds*. 4,4,4-Trifluoro-3-oxo-2-(triphenylphosphoranylidene)butanenitrile (**1**), mp 190˚C (CHCl3/hexane) (mp 191-192.5˚C; 13); 1,1,1-trifluoro-3-oxo-1-methoxy-3-(triphenylphosphoranylidene)propane-2-one (**2**). 43% yield. mp 160-161˚C ($CHCl_3$/hexane). IR (KBr): 3054, 2928, 2822, 1579, 1438, 1239, 1176, 1159, 1096, 975, 753, 720, 693, 539, 506 cm$^{-1}$. $^{1}$H-NMR (500 MHz, $CDCl_3$) δ=3.15 (s, 3H, OC*H*$_3$), 7.51 (td, *J*=7.9, 3.2 Hz, 6H, Ar*H*), 7.62 (td, *J*=7.6, 1.8 Hz, 3H, Ar*H*), 7.67 (dd, *J*=12.7, 7.3 Hz, 6H, Ar*H*) ppm. $^{13}$C-NMR (126 MHz, $CDCl_3$) δ=65.4, 104.0 (d, $^{1}J_{C\text{-}P}$=125.4 Hz, C=P), 119.7 (qd, $^{1}J_{C\text{-}F}$=288.9 Hz, $^{3}J_{C\text{-}P}$=19.3 Hz, $CF_3$), 123.1 (d, $^{1}J_{C\text{-}P}$=89.0 Hz, ArC), 129.1 (d, $^{2}J_{C\text{-}P}$=12.7 Hz, ArC), 132.9 (d, $^{3}J_{C\text{-}P}$=3.4 Hz, ArC), 133.7 (d, $^{4}J_{C\text{-}P}$=10.2 Hz, ArC), 167.3 (dq, $^{2}J_{C\text{-}F}$=30.8 Hz, $^{2}J_{C\text{-}P}$=32.7 Hz, *C*=O) ppm. MS (EI) m/z: 402 ($M^+$, 15.8), 262 (100). *Anal*. Calcd for $C_{22}H_{18}F_3O_2P$: C, 65.67; H, 4.51. Found: C, 65.69; H, 4.41. 4,4,4-Trifluoro-1-phenyl-2-(triphenylphosphoranylidene)butane-1,3-dione (**3**), mp 179-181˚C (MeOH) (mp 182-183˚C; 14); 4,4,4-trifluoro-3-oxo-2-(triphenylphosphoranylidene)butanal (**4**), mp 147-149˚C ($CHCl_3$/hexane) (mp 147˚C; 15); 1,1,1-trifluoro-3-(triphenylphosphoranylidene)-pentane-2,4-dione (**5**), mp 138-140˚C ($CHCl_3$/hexane) (14). In the preparation of compound **5**, the reaction solvent was MeCN instead of THF; ethyl 4,4,4-trifluoro-3-oxo-2-(triphenyl phosphoranylidene)butanoate (**6**), mp 119-120˚C (MeOH) (mp 125-127˚C; 13); 4,4,5,5,5-pentafluoro-1-phenyl-2-(triphenylphosphoranylidene) pentane-1,3-dione (**7**). mp 171-173˚C (MeOH) (16); 4,4,5,5,6,6,6-heptafluoro-1-phenyl-2-(triphenylphosphoranylidene)hexane-1,3-dione (**8**), mp 147-149˚C (MeOH) (mp 143˚C; 10); 3-oxo-2-(triphenyl- phosphoranylidene) butanenitrile (**9**), mp 206-207˚C (CHCl3/hexane) (mp 205˚C; 17).

*Cell culture*. L5178Y mouse T-lymphoma cells (ECACC Cat. No. 87111908; U.S. FDA, Silver Spring, MD, USA) were transfected with pHa *ABCB1*/A retrovirus (18). The ABCB1-expressing cell line was selected by culturing the infected cells with 60 ng/ml colchicine to maintain the expression of the MDR phenotype. L5178Y (parental) mouse T-cell lymphoma cells and the human *ABCB1*-transfected subline (MDR) were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cell lines were incubated in a humidified atmosphere (5% $CO_2$, 95% air) at 37˚C.

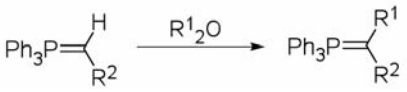
*Cytotoxicity assay*. Each compound was dissolved in 99.5% dimethyl sulfoxide (DMSO) (Sigma, Munich, Germany). In each protocol, DMSO was always tested as solvent control and no activity was observed. The cytotoxic effects of the compounds were tested at a range of increasing concentrations (0.26-133 μM) using the L5178Y mouse T-lymphoma cell line as an experimental model. The cells were distributed into 96-well flat-bottom microtiter plates at a density of $1\times10^5$ cells/ml in a final volume of 100 μl of medium per well. The different concentrations of each compound were added into duplicate wells in 100 μl. The plates were incubated at 37˚C for 24 h; at the end of the incubation period, 20 μl of (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) solution (from a 5 mg/ml stock) was added to each well and cells incubated for 4 h. Then, 100 μl of sodium dodecyl sulfate (10%) solution was measured into each well and the plates were further incubated overnight at 37˚C. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref 630 nm) with a Dynatech MRX vertical beam ELISA reader (Dynatech Laboratories, Inc. MA, USA). The percentage of inhibition of cell growth was determined according to Equation 1.

$$100 - \left[ \frac{OD_{sample} - OD_{medium\ control}}{OD_{cell\ control} - OD_{medium\ control}} \right] \times 100 \quad \text{(Eq. 1)}$$

*Rhodamine-123 accumulation assay*. The parental and MDR mouse T-lymphoma cells were adjusted to a density of $2\times10^6$ cells/ml, and re-suspended in serum-free McCoy's 5A medium and distributed in 500 μl aliquots. The test compounds were added at 10 μg/ml; verapamil (positive control) at 10 μg/ml and DMSO at 2% as solvent control. The samples were incubated for 10 min at room temperature, and then 10 μl (5.2 μM final concentration) of rhodamine-123 were added to the samples, followed by incubation for 20 min at 37˚C. Finally, the samples were washed twice, resuspended in 500 μl phosphate-buffered saline (PBS) and the fluorescence of the cell population was analyzed with a Partec CyFlow flow cytometer (Partec, Munster, Germany). The histograms were evaluated regarding the mean fluorescence intensity, the standard deviation and the peak channel of 20,000 individual cells belonging to the total and the gated populations. The fluorescence activity ratio (FAR) was calculated on the basis of the measured fluorescence values of treated/untreated resistant cell line (FL-1MDR) and the sensitive parental cell line (FL-1PAR), according to Equation 2.

$$FAR = \frac{\left( \frac{FL\text{-}1MDR_{treated}}{FL\text{-}1MDR_{untreated}} \right)}{\left( \frac{FL\text{-}1PAR_{treated}}{FL\text{-}1PAR_{untreated}} \right)} \quad \text{(Eq. 2)}$$

Table I. *Structures and calculated octanol/water partition coefficients (log P) of the studied P-ylides (**1-10**).*



| Compound | R1 | R2 | log P[a] |
|---|---|---|---|
| **1** | $COCF_3$ | CN | 4.77 |
| **2** | $COCF_3$ | OMe | 4.96 |
| **3** | $COCF_3$ | COPh | 6.14 |
| **4** | $COCF_3$ | CHO | 4.81 |
| **5** | $COCF_3$ | COMe | 4.53 |
| **6** | $COCF_3$ | $CO_2Et$ | 5.23 |
| **7** | $COC_2F_5$ | COPh | 6.78 |
| **8** | $COC_3F_7$ | COPh | 7.42 |
| **9** | COCH3 | CN | 4.22 |
| **10**[b] | H | CN | 4.61 |

[a]The log p-values were determined using a commercial software package, Molinspiration Cheminformatics, http://wwwmolinspirationcom. The calculated log P value of verapamil is 4.55. [b]This compound is commercially available.

Table II. *Cytotoxic activity of P-ylides (compounds **1-10**) on parental (PAR) and ABCB1-gene transfected multidrug-resistant (MDR) cancer cells.*

| Compound | $IC_{50}$[a] (μg/ml) | | Selectivity index[b] |
|---|---|---|---|
| | PAR cells | MDR cells | |
| **1** | 24.76±3.11 | 49.48±2.69 | 0.50 |
| **2** | 50.27±3.91 | 69.76±4.74 | 0.72 |
| **3** | 32.51±4.94 | 37.01±1.89 | 0.88 |
| **4** | 34.22±4.03 | 50.20±2.92 | 0.68 |
| **5** | 69.21±6.21 | 84.04±5.36 | 0.82 |
| **6** | 24.84±2.56 | 36.66±2.37 | 0.68 |
| **7** | 36.12±2.62 | 39.35±3.57 | 0.92 |
| **8** | 20.42±2.65 | 42.36±5.67 | 0.48 |
| **9** | >100 | >100 | 1 |
| **10** | 96.13±5.82 | >100 | <0.99 |

$IC_{50}$: Half maximal inhibitorry concentration. [a]Values for $IC_{50}$ are the average±SD of three independent experiments. [b]Selectivity index=$IC_{50}$ (PAR cells)/$IC_{50}$ (MDR cells).

## Results

We screened our in-house library of P-ylides and related compounds, most of them previously synthesized in our laboratory (19). To our knowledge, this is the first report on the identification of P-ylides as novel MDR-reversal agents. The differently fluorinated compounds **1-9** were easily prepared in moderate-to-good yields by the reaction of acyl ylides with perfluorinated anhydrides (9, 10). The structures of the P-ylides (compounds **1-10**) screened for their MDR-modulating activities are shown in Table I.

The cytotoxic activity of P-ylides **1-10** was evaluated using MTT assay in two tumor cell lines, parental L5178Y mouse T-lymphoma cells and human *ABCB1*-gene transfected L5178Y/MDR mouse lymphoma cells. The half-maximal inhibitory concentrations ($IC_{50}$) are presented in Table II and selectivity indices were calculated based upon the comparison of the sensitivity ($IC_{50}$) of the parental to the MDR cell lines. All compounds had a relatively low cytotoxic activity. The determination of the selective index did not show any significant discrimination between the parental (chemosensitive) and MDR (chemoresistant by overexpression of ABCB1) cancer cell lines. This shows that the compounds are equally active against both the sensitive parental and resistant MDR cancer cells.

The rhodamine-123 (a substrate of ABCB1) exclusion assay was used to assess the potential ABCB1-mediated MDR-reversing activity of compounds **1-10**. In this assay, the FAR was evaluated for the accumulation ratio of rhodamine-123 in MDR and parental cells (Table III). The

Table III. *Multidrug resistance (MDR)-reversal activities of P-ylides (compounds **1-10**) on MDR cancer cells.*

| Compound | Conc. (μg/ml) | FSC[a] | SSC[a] | FL-1[a] | FAR[a] |
|---|---|---|---|---|---|
| Verapamil | 10 | 2386 | 817 | 33.4 | 16.6 |
| **1** | 10 | 2380 | 807 | 25.1 | 12.5 |
| **2** | 10 | 2410 | 830 | 1.78 | 0.89 |
| **3** | 10 | 2387 | 795 | 57.6 | 28.7 |
| **4** | 10 | 2433 | 826 | 2.74 | 1.36 |
| **5** | 10 | 2443 | 851 | 1.53 | 0.76 |
| **6** | 10 | 2363 | 893 | 2.74 | 1.36 |
| **7** | 10 | 2453 | 938 | 71.7 | 35.7 |
| **8** | 10 | 2698 | 1019 | 41.2 | 20.5 |
| **9** | 10 | 2514 | 878 | 1.93 | 0.96 |
| **10** | 10 | 2577 | 895 | 1.25 | 0.62 |
| DMSO | 2.00% | 2526 | 872 | 0.83 | 0.41 |

[a]FSC: Forward scatter count of cells in the sample (cell size ratio); SSC: side scatter count of cells in the samples; FL-1: mean fluorescence intensity of the cells; FAR: fluorescence activity ratio. The inhibition of ATP-binding cassette sub-family B member 1 transporter is evident when FAR >1.

mean percentage fluorescence intensity was calculated for the treated MDR cells compared to the untreated cells. The A FAR was then calculated using Equation 2 on the basis of the measured fluorescence values. When the FAR value is greater than 1, a reversal of MDR has taken place (14). MDR is due to an increase in the efflux of a compound from the cell. Consequently, a reversal of MDR means that the accumulation of rhodamine-123 by MDR cells should be

similar to the accumulation by the chemosensitive parental cells. The data in Table III reveal that compounds **1**, **3**, **7**, and **8** displayed a remarkable inhibition of MDR at the non-toxic concentration of 10 μg/ml. These cells overexpress the ABCB1 protein that is responsible for drug efflux.

Firstly, the active compound **1** was tested to define the importance of the trifluoromethyl ketone moiety. Thus, removal of the trifluoroacetyl group of **1**, which results in **10**, abolished the potency of the compound. The replacement of the trifluoroacetyl group of **1** with acetyl group, resulting in the corresponding non-fluorinated analog (**9**), also significantly diminished potency. This indicates the possibility that the trifluoromethyl ketone moiety is necessary for potency.

Secondly, the replacement of the cyano residue of **1** with other substituents, such as methoxy (**2**), formyl (**4**), acetyl (**5**) and ethoxycarbonyl (**6**), resulted in diminished potency. However, a benzoyl group instead of the cyano group of **1**, resulting in **3**, boosed the potency by twofold. In the benzoyl series, **3**, **7** and **8**, the potency decreased in the order $COC_2F_5$ (**7**) > $COCF_3$ (3) > $COC_3F_7$ (**8**). These compounds (**3**, **7** and **8**) demonstrated higher potency than the reference drug verapamil.

A number of studies revealed the high lipophilicity of various MDR-reversal agents (21). The calculated octanol/water partition coefficients (log p-values) of a number of MDR-reversal 1,4-dihydropyridine derivatives were in the region of 5.1-7.5 (22). The calculated log p-values of **1-10** and verapamil are presented in Table I. Compounds **3**, **7** and **8**, displaying significant capabilities in reversing MDR, have log P values in the region of 6-7.5. On the other hand, the MDR-reversal activities are low for the other ylides with low log P values in the region of 4.2-5.5.

## Discussion

As a result of screening in order to obtain possible lead structures bearing a trifluoroacetyl group, compounds **3**, **7** and **8** were found to be the most potent modulators and to be more potent than verapamil.

It is important to note that ABC transporters are primary efflux pumps deriving their energy from the hydrolysis of ATP. The MDR modulators are believed to bind to the transmembrane domains of ABCB1 (P-glycoprotein) which leads to inhibition of ABC transporters due to the induced conformational changes (23). The functionally-active conformation of ABCB1 depends on the integrity of the membrane bilayer in which ABCB1 is embedded. Among the possible mechanisms of MDR reversion by P-ylides, we evaluated the non-specific reactions with cell membrane lipids, because of a high degree of lipophilicity exhibited by the active P-ylides.

The substitution in the $COCF_3$ series (compounds **1-6**) intensified their MDR-reverting activity in the following order: COPh (**3**) > CN (**1**) >> CHO (**4**), CO2Et (**6**) > OMe (**2**) > COMe (**5**). These data show that the decrease of lipophilic properties of P-ylides markedly lowered their effect on the MDR inhibition. The lipophilic nature of P-ylides enables them to easily penetrate and overcome the cell membrane.

## Conclusion

This study identified a novel class of P-glycoprotein-associated MDR-reversal agents, P-ylides **3**, **7** and **8**. These compounds demonstrate high potency against ABCB1 which far exceeds that of the reference drug verapamil. The present study demonstrated that trifluoroacetylated P-ylides may be attractive lead compounds for further development as MDR-reversing agents. In addition, the ease of synthesis and the small size of these compounds make them potential candidates for structure–activity relationships as EPIs in multidrug efflux systems.

## 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.

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*Received July 29, 2015*
*Revised September 15, 2015*
*Accepted September 25, 2015*