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Short communication

Synthesis and biological evaluation of 4-methylideneisoxazolidin-5-ones – A new class of highly cytotoxic α-methylidene-γ-lactones

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Abstract

Two 4-methylideneisoxazolidin-5-ones (**4a**,**b**), which are α-methylidene-γ-lactones containing a nitrogen atom in the lactone ring, were synthesized. Their cytotoxic properties were evaluated against promyelocytic leukemia HL-60 cells. Both **4a** and **4b** exhibited relatively high cytotoxic activity with an IC_{50} of 4.1 and 5.4 μM, respectively. Caspase-3 activity assay revealed that both isoxazolidinones (**4**) were able to induce apoptosis process in time- and concentration-dependent manner. Using multiplex PCR analysis, it was observed that **4** caused distinct inhibition of *BCL-2* gene expression. Expression of *BAX*, a pro-apoptotic gene remained unchanged. It was also found that **4a**,**b** did not induce the expression of *MDR1* and *MRP1* genes, related to multidrug resistance. In addition, cytotoxicity data obtained for drug-sensitive and drug-resistant HL-60 ADR cells revealed that the investigated compounds were poor substrates for transport by MRP1 efflux pump, suggesting that they might be useful for treating drug-resistant tumors. Furthermore, antimicrobial properties of **4a**,**b** were evaluated. They showed significant activity against fungi *Candida albicans*, but only a weak activity against all tested Gram-positive and Gram-negative bacterial strains.

Keywords: Methylideneisoxazolidinones; Cytotoxicity; Apoptosis; Multidrug resistance; Antimicrobial activity

1. Introduction

Sesquiterpene lactones (SQLs) are a class of naturally occurring plant terpenoids, formed by condensation of three isoprene units and subsequent enzyme-mediated cyclizations and oxidative transformations which produce a *cis*- or *trans*-fused lactones. These compounds are known for their different biological activities, such as anti-inflammatory, phytotoxic, antimicrobial and antiprotozoal properties, as well as cytotoxicity against tumor cells [1–6]. Kupchan et al. [7] reported that cytotoxic and antineoplastic activity of SQLs was associated mainly with the presence of α-methylidene-γ-butyrolactone moiety in their structure. Many SQLs with high cytostatic and cytotoxic activity against various cancer cells, bearing such structural motif have been identified [1,7–11]. It is now well established that α-methylidene-γ-lactones exert their biological activities acting as alkylating agents. These compounds react as Michael-type acceptors with bionucleophiles, especially with sulfhydryl groups of cysteine. The precise mechanism of action of SQLs as inhibitors of cell growth is still unclear. Several experimental data indicate that antitumor activity of these compounds is strongly related to their inhibitory effect on many thiol-containing

Abbreviations: AFC, 7-amido-4-trifluoro-coumarin; *MDR1*, gene encoding glycoprotein P; *MRP1*, gene encoding multidrug resistance protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; SQLs, sesquiterpene lactones.

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enzymes, involved in the synthesis and processing of proteins, RNA and DNA [9,12,13]. It has been also observed that SQLs mediate their cytotoxic effect by triggering apoptosis in many types of cell lines [11,14,15]. Another postulated molecular mechanism of anticancer activity of SQLs is by influencing cell signal transduction via transcription factor NF-κB and mitogen-activated protein kinases [2,3,16–18]. It was recently found that a sesquiterpene lactone, parthenolide, can selectively kill primitive leukemia cells without affecting normal stem and progenitor hematopoietic cells [19]. These data indicate that SQLs and related compounds may represent a novel and promising class of antileukemic agents.

In our previous reports we have described several approaches to the synthesis of α-methylidene-γ- and δ-lactones and -lactams. Many of the obtained compounds exerted relatively high cytotoxic activity against cancer cells in vitro [20–22]. Recently, we have published a preliminary report on the synthesis and cytotoxic activity of 4-methylideneisoxazolidin-5-ones, which are α-methylidene-γ-lactones containing a nitrogen atom in the lactone ring [23]. Now we present a full account on the synthesis and biological evaluation of two selected 4-methylideneisoxazolidin-5-ones, one with an aliphatic and the other with an aromatic substituent in position 3. The extended biological studies of these compounds included their cytotoxic, pro-apoptotic and antimicrobial activity. Their influence on the expression of two genes, *MDR1* and *MRP1*, which are important factors in regulating bioavailability of many therapeutics was also investigated.

2. Results and discussion

2.1. Chemistry

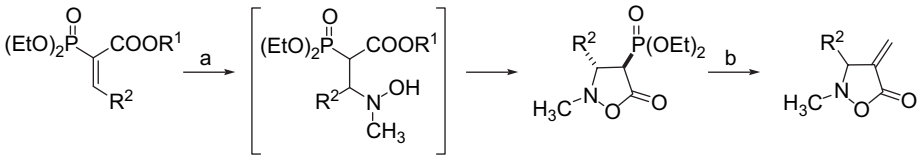
Target 4-methylideneisoxazolidin-5-ones **4a**,**b** were synthesized in a reaction sequence as shown in Scheme 1. A Michael addition of *N*-methylhydroxylamine hydrochloride to easily available ethyl (*E*)-2-diethoxyphosphoryl-4-methyl-2-heptenoate (**1a**) [24] or dicyclohexylammonium 2-diethoxyphosphoryl-3-(4-methylphenyl)propenoate (**1b**) [25] gave adducts **2a** or **2b**, respectively. These adducts were not isolated and lactonized spontaneously to *trans*-4-diethoxyphosphorylisoxazolidin-5-ones **3a**,**b**. Isooxazolidinone **3b** was formed as a mixture of diastereoisomers in 1:1 ratio, due to the additional stereogenic center in R^2 substituent. A Horner–Wadsworth–Emmons olefination of formaldehyde using isoxazolidinones **3a**,**b**, in the presence of K_2CO_3 as a base, gave, after purification by column chromatography on silica gel, the expected 4-methylideneisoxazolidin-5-ones **4a**,**b**. Both intermediate 4-diethoxyisoxazolidinones **3a**,**b** and target 4-methylideneisoxazolidinones **4a**,**b** were fully characterized by IR, ^1H and ^{13}C NMR spectroscopy.

2.2. Biological assays

2.2.1. Cytotoxicity studies

The cytotoxicity of methylideneisoxazolidinones (**4a**,**b**) was assayed against HL-60 leukemia cells. *cis*-Platin was used as a reference compound. Cells were exposed to a broad range of drug concentrations (10^{-7} to 10^{-3} M) for 72 h and cell viability was analyzed by dye-exclusion assay. IC_{50} values are presented in Table 1. Both **4a** and **4b** exhibited relatively high cytotoxicity with the IC_{50} values of about 5 μM or less. The slightly higher cytotoxic activity was observed for **4a** with an IC_{50} of 4.1 ± 0.7 μM. Cytotoxic effectiveness of the new 4-methylideneisoxazolidin-5-ones was comparable to that of the well-known sesquiterpene lactones, parthenolide and helenalin [9,10,14,19] but was lower than *cis*-platin, a well-known therapeutic agent used for the treatment of leukemia. Although a molecular mechanism of the cytotoxic action of these new compounds has not yet been investigated, we may assume that it is similar to that of SQLs with methylidenebutyrolactone ring. However, a better solubility of 4-methylideneisoxazolidin-5-ones in water as compared with SQLs and their less complicated structure may appear as important features in the design of potential therapeutics.

Effect of isoxazolidinones on viability of multidrug-resistant cells was also studied. For this purpose cytotoxicities of **4a**,**b** and adriamycin against maternal HL-60 cells and multidrug-resistant subline HL-60 ADR (adriamycin-resistant, MRP1-overexpressing) were tested and compared. As shown in Table 1, drug-resistant HL-60 ADR cells were much less sensitive to adriamycin in comparison to maternal HL-60 cells. Interestingly, **4a**,**b** were almost equally effective against HL-60 and HL-60 ADR cells. Although, antitumor activity of these compounds was much lower than that of adriamycin, but only against the drug susceptible HL-60 cells. In contrast, they were only slightly less effective than adriamycin towards the drug-resistant HL-60 ADR cells. It indicates that



1a,b **2a,b** **3a,b** **4a,b**

a, R^1= Et, R^2= 

b, R^1= $NH_2(C_6H_{11})_2$, R^2= 4-MeC_6H_4

Scheme 1. Reagents and conditions: (a) $CH_3NHOH \cdot HCl$, CH_2Cl_2, r.t. 12 h; (b) K_2CO_3, 36% formalin, THF, 0 °C to r.t. 45 min.

Table 1
Cytotoxic activity of methylideneisoxazolidinones **4a**,**b**, adriamycin and *cis*-platin to parental HL-60 cells and adriamycin-resistant, MRP1-overexpressing, HL-60 ADR cells

Compound	Cytotoxicity (μM)[a]	
	HL-60	HL-60 ADR
4a	4.1 ± 0.7	2.5 ± 0.3
4b	5.4 ± 0.9	6.0 ± 0.5
Adriamycin	0.013 ± 0.001	2.0 ± 0.2*
cis-Platin	0.7 ± 0.2	–[b]

*Statistically significant difference between the HL-60 and HL-60 ADR cells as determined by *t*-test, $p < 0.001$.
Data are presented as average calculated from 2–4 independent experiments ± SD, each performed in triplicate.
[a] IC_{50} – Concentration of a test compound required to reduce the fraction of survival cells to 50% of that observed in the control, non-treated cells.
[b] Not determined.

isoxazolidinones are poor substrates (if any) for transport by MRP pump, suggesting that they might be useful for treating drug-resistant tumors.

2.2.2. Caspase-3 activity and expression of BCL-2 and BAX genes

Compounds **4a**,**b** were tested for their ability to induce the activity of caspase-3 which is one of the major proteases involved in the process of programmed cell death – apoptosis [26]. Caspases, the key effectors of apoptosis, are a family of cysteine proteases that, once activated, cleave peptide bonds formed by a carboxyl group of the aspartic acid residues. Proteolysis of numerous cellular protein substrates leads to the next steps of programmed cell death. It was postulated earlier that many sesquiterpene lactones bearing methylidenebutyrolactone motif can mediate their cytotoxic effect by triggering apoptosis [11,14,15]. Although induction of apoptosis is rather a frequent phenomenon with growth-inhibiting substances, it still remains important to determine the dynamics of the cell-death induction. In our study HL-60 cells were treated with the tested compounds in two concentrations, $1 \times IC_{50}$ and $10 \times IC_{50}$. The time course of the induction of caspase-3 activity, expressed as a concentration of fluorescent aminofluorocoumarin (AFC) released from the substrate, is shown in Fig. 1. Induction of the caspase-3 activity occurred more rapidly in the cells exposed to **4b** than to **4a**. It is interesting to note that both methylideneisoxazolidinones tested were able to induce the apoptosis process in time- and concentration-dependent manner. Caspase-3 induction in HL-60 cells exposed to isoxazolidinones prompted us to study their effect on some pro- and anti-apoptotic gene expression using the semiquantitative multiplex RT-PCR method. As in our previous study [27] we have determined expression of *BCL-2* gene, a known anti-apoptotic gene. The ratio of *BCL-2* to reference *β-actin* amplicons dropped down about two times in HL-60 cells treated with **4a** and about three times after treating with **4b** (Fig. 2A and B). The obtained data suggest that both analyzed compounds inhibit *BCL-2* gene expression. However, expression of *BAX*, a pro-apoptotic gene, remained unchanged

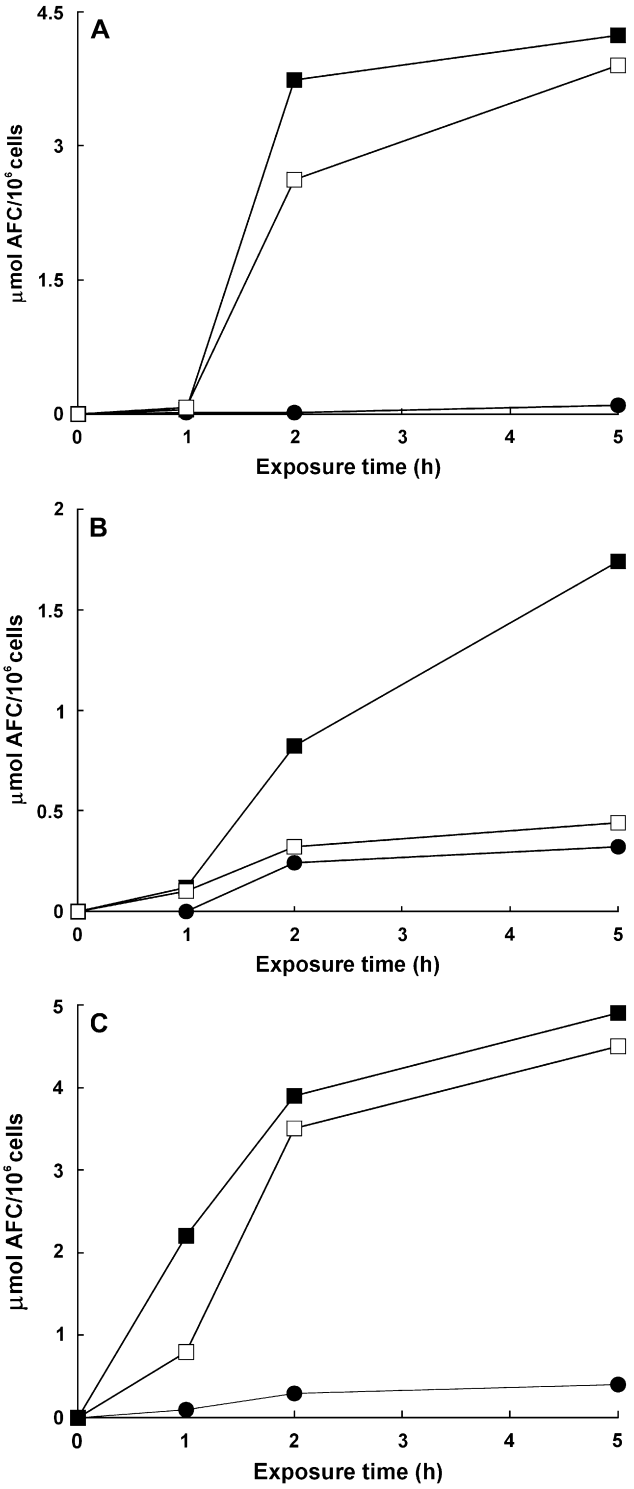


Fig. 1. Time course of caspase-3 activity induction by **4b** (A), **4a** (B) and *cis*-platin used as a reference (C) in HL-60 leukemia cells. Cells were treated with tested compounds at the concentration $1 \times IC_{50}$ (white square) and $10 \times IC_{50}$ (black square). Control, non-treated cells (black circle). After 1, 2 and 5 h of incubation, the cells were lysed and quantified for caspase-3 activity, which is proportional to the amidofluorocoumarin (AFC) released from the labeled substrate. Enzyme activity is expressed as the concentration of AFC released by 10^6 cells. Points on the curves are means of four determinations. SD values were excluded for clarity and did not exceed 15% of the mean value for each point.

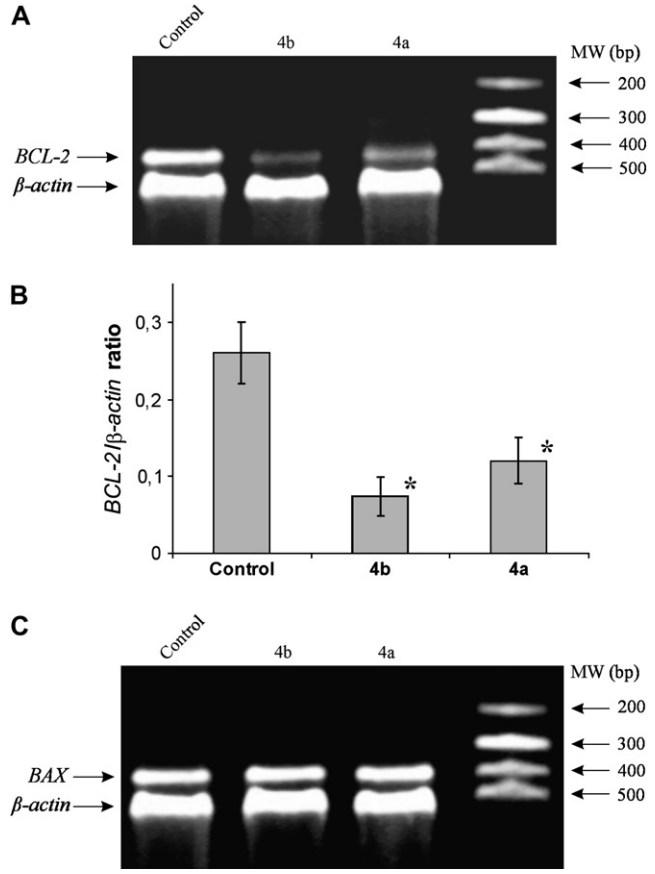


Fig. 2. Multiplex PCR for *BCL-2* (433 bp) and reference *β-actin* (530 bp) genes (A), *BAX* (438 bp) and *β-actin* genes (C) in HL-60 cells treated with **4a** and **4b** at concentration equal to IC_{50} for 72 h. The ratio of the *BCL-2* amplicons to reference *β-actin* genes (B). Mean ± SD, $n = 4$. *Differences statistically significant, $p < 0.05$.

(Fig. 2C). Consequently, the calculated *BCL-2*/*BAX* ratio in drug exposed cells was clearly lower.

2.2.3. The effect of isoxazolidinones on MDR1 and MRP1 gene expression

Subsequently, we examined whether the tested isoxazolidinones could affect expression of *MDR1* and *MRP1* genes. Products of these genes are multidrug-resistant protein-1 (P-glycoprotein; P-gp) and multidrug-resistant-associated protein-1 (MRP1), which belong to the family of membrane transporters with ATP-binding cassette. Both these efflux pumps are potent resistance factors for chemotherapeutic agents. It is widely accepted that their overexpression in malignant cells plays a major role in decreasing the intracellular concentration of anticancer drugs, a phenomenon known as multidrug resistance (MDR) [28]. A number of drugs have been shown to induce P-gp and MRP1 at the transcriptional and protein level [29,30]. In human myeloid leukemia cells it has been demonstrated that induction of *MRP1* overexpression was an early event in the process of acquired multidrug resistance and occurred before that for the *MDR1* gene [31]. In this study we have found that **4a** and **4b** did not have any effect on *MRP1* gene expression. Expression of *MDR1* gene has not been also changed (as illustrated in Fig. 3).

2.2.4. Antimicrobial activity studies

The antimicrobial effect of **4a**,**b**, measured as the minimal inhibitory concentration (MIC), was determined for the following bacterial strains: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and fungi: *Candida albicans*, using the standard microdilution susceptibility test. The growth of Gram-positive bacterial strains *S. aureus* and *S. epidermidis* was inhibited by **4a** at a concentration of 150 μg/mL and by **4b** at a concentration of 300 μg/mL. Minimal inhibitory concentration of both tested compounds for another Gram-positive cocci *E. faecalis* was ≥ 300 μg/mL. Compound **4a** was active against Gram-negative *E. coli* (75 μg/mL), while **4b** was still inactive at a concentration as high as 300 μg/mL. Neither **4a** nor **4b** was effective as a growth inhibitor of Gram-negative *P. aeruginosa* (MICs > 300 μg/mL). The growth of the pathogenic and drug-resistant fungi, *C. albicans*, was fully inhibited by both tested compounds at relatively low concentrations of 37.5 μg/mL.

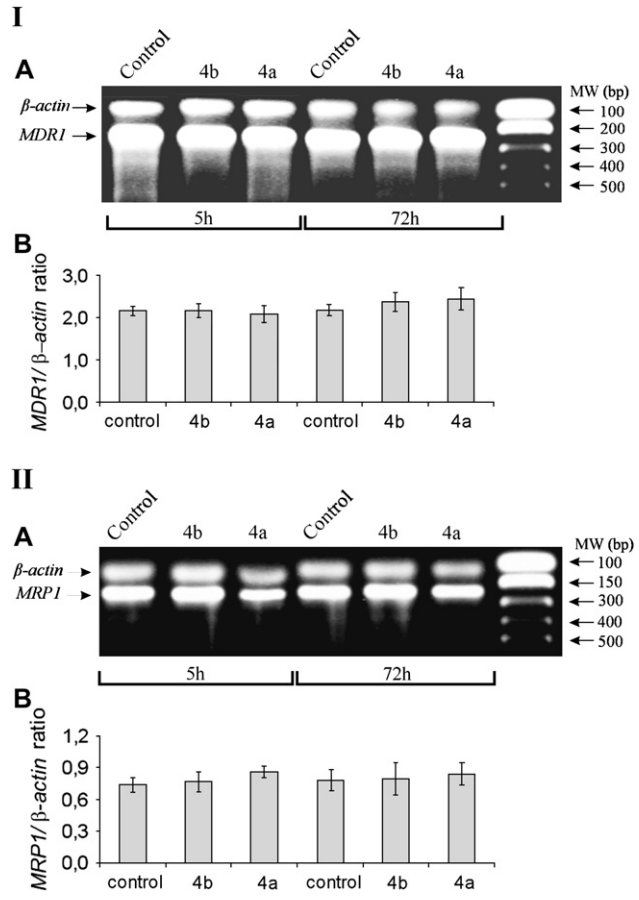


Fig. 3. Multiplex PCR for *MDR1* (287 bp) and reference *β-actin* (123 bp) genes (IA) and *MRP1* (274 bp) and reference *β-actin* (123 bp) genes (IIB) in HL-60 cells treated with **4a** and **4b** at concentration equal to IC_{50} for 5 and 72 h. *MDR1*/*β-actin* ratio (IB) and *MRP1*/*β-actin* ratio (IIB) calculated by densitometric analysis.

3. Conclusion

The novel methylideneisoxazolidinones are promising compounds with high antileukemic and antifungal activity. They do not transcriptionally activate expression of MDR1 and MRP1 cell membrane efflux pumps related to multidrug resistance. Tested isoxazolidinones induce apoptosis in cancer cells. It seems that these compounds are not removed from cells by membrane transporters.

4. Experimental part

4.1. Chemistry

All reactions requiring anhydrous and oxygen-free conditions were conducted in an argon atmosphere. Organic solvents and reagents were purified by the appropriate standard procedures. IR spectra were recorded on a Specord M80 spectrometer. ^{1}H NMR (250 MHz), ^{13}C NMR (62.9 MHz), and ^{31}P NMR (101 MHz) spectra were recorded on a Bruker DPX-250 spectrometer with TMS as an internal standard and 85% H_3PO_4 as an external standard. Elemental analyses were performed on a Perkin–Elmer PE 2400 analyzer. Column chromatography was performed on Fluka® silica gel 60 (230–400 mesh). TLC was performed on Fluka® silica gel plates (5–17 μm, F_{254}). All other reagents and solvents were of analytical grade.

4.1.1. Synthesis of trans-4-diethoxyphosphoryl-2-methyl-3-(1-methylbutyl)isoxazolidin-5-one, ***3a***

To a suspension of MeNHOH · HCl (0.266 g, 3.183 mmol) in anhydrous CH_2Cl_2 (10 mL), ethyl 2-(diethoxyphosphoryl)-4-methyl-2-heptenoate **1a** (0.650 g, 2.122 mmol) and anhydrous triethylamine (0.444 mL, 3.183 mmol) were added. The mixture was stirred at room temperature for 12 h, followed by addition of anhydrous $ZnCl_2$ (0.434 g, 3.183 mmol). Stirring was continued for another 8 h and the reaction mixture was quenched with water (40 mL). The mixture was extracted with CH_2Cl_2 (3 × 15 mL). The combined organic extracts were dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography on silica gel (eluent: $CHCl_3$/EtOAc = 9/1), to give **3a** as a mixture of diastereoisomers in a 1:1 ratio (0.163 g, 25%), oil; IR (film, cm^{-1}): 1772, 1160, 1052; ^{1}H NMR ($CDCl_3$): $\delta = 0.91$ (t, 6H, $^3J_{HH} = 7.0$ Hz, 2 × CH_3(CH$_2$)$_2$CH), 0.94 (d, 3H, $^3J_{HH} = 6.7$ Hz, CH_3CH), 0.96 (d, 3H, $^3J_{HH} = 6.7$ Hz, CH_3CH), 1.09–1.33 (m, 8H, 2 × CH$_3$CH_2CH_2CH), 1.38 (t, 6H, $^3J_{HH} = 7.0$ Hz, 2 × CH_3CH$_2$OP), 1.39 (t, 6H, $^3J_{HH} = 7.0$ Hz, 2 × CH_3CH$_2$OP), 1.61–1.80 (m, 2H, 2 × CH$_3$CH), 3.02 (s, 3H, CH_3N), 3.03 (s, 3H, CH_3N), 3.13 (dd, 1H, $^2J_{PH} = 25.5$ Hz, $^3J_{HH} = 4.7$ Hz, PCHCH), 3.14 (dd, 1H, $^2J_{PH} = 25.7$ Hz, $^3J_{HH} = 5.0$ Hz, PCHCH), 3.47 (ddd, 1H, $^3J_{PH} = 19.2$ Hz, $^3J_{HH} = 5.0$ Hz, $^3J_{HH} = 5.0$ Hz, PCHCH), 3.49 (ddd, 1H, $^3J_{PH} = 19.2$ Hz, $^3J_{HH} = 5.0$ Hz, $^3J_{HH} = 4.7$ Hz, PCHCH), 4.08–4.36 (m, 8H, 2 × (CH$_3$CH_2O)$_2$); ^{13}C NMR ($CDCl_3$): $\delta = 13.95$ (s, CH_3(CH$_2$)$_2$CH), 14.02 (s, CH_3(CH$_2$)$_2$CH), 14.08 (s, CH_3CH), 14.75 (s, CH_3CH), 16.22 (d, $^3J_{CP} = 5.9$ Hz, CH$_3$CH$_2$O), 16.23 (d, $^3J_{CP} = 5.9$ Hz, CH$_3$CH$_2$O), 16.30 (d, $^3J_{CP} = 5.9$ Hz, 2 × CH$_3$CH$_2$O), 20.09 (s, CH$_3$CH$_2$CH$_2$CH), 20.13 (s, CH$_3$CH$_2$CH$_2$CH), 33.87 (s, CH$_3$CH$_2$CH$_2$CH), 34.71 (s, CH$_3$CH$_2$CH$_2$CH), 37.56 (d, $^3J_{CP} = 8.0$ Hz, CH$_3$CH), 37.69 (d, $^3J_{CP} = 8.0$ Hz, CH$_3$CH), 43.41 (d, $^1J_{CP} = 142.1$ Hz, PCHCH), 44.42 (d, $^1J_{CP} = 142.2$ Hz, PCHCH), 48.33 (s, CH$_3$N), 48.63 (s, CH$_3$N), 62.80 (d, $^2J_{CP} = 6.8$ Hz, CH$_3$CH$_2$O), 62.85 (d, $^2J_{CP} = 6.7$ Hz, CH$_3$CH$_2$O), 64.14 (d, $^2J_{CP} = 6.8$ Hz, CH$_3$CH$_2$O), 64.19 (d, $^2J_{CP} = 6.7$ Hz, CH$_3$CH$_2$O), 71.23 (d, $^2J_{CP} = 2.0$ Hz, PCHCH), 71.59 (d, $^2J_{CP} = 2.1$ Hz, PCHCH), 171.37 (d, $^2J_{CP} = 3.0$ Hz, C=O), 171.50 (d, $^2J_{CP} = 2.9$ Hz, C=O); ^{31}P NMR ($CDCl_3$): $\delta = 19.14$, 19.22; Anal. Calcd for $C_{13}H_{26}NO_5P$: C, 50.81; H, 8.53; N, 4.56; P, 10.08. Found: C, 50.70; H, 8.58; N, 4.45; P, 10.22.

4.1.2. Synthesis of trans-4-diethoxyphosphoryl-2-methyl-3-(4-methylphenyl)isoxazolidin-5-one, ***3b***

To a suspension of MeNHOH · HCl (0.261 g, 3.13 mmol) in anhydrous CH_2Cl_2 (10 mL), dicyclohexylammonium (*E*)-2-(diethoxyphosphoryl)-3-(4-methylphenyl)acrylate **1b** (1.000 g, 2.085 mmol) was added. The mixture was stirred at room temperature for 12 h. After that time the reaction mixture was filtrated and quenched with saturated $NaHCO_3$ (20 mL). The mixture was extracted with CH_2Cl_2 (3 × 15 mL). The combined organic extracts were dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography (eluent: EtOAc/hexane = 1/1), on silica gel to give pure **3b** (0.307 g, 45%), white prisms (from Et_2O); mp 79–81 °C; IR (film, cm^{-1}): 1780, 1260, 1060; ^{1}H NMR ($CDCl_3$): $\delta = 1.09$ (t, 3H, $^3J_{HH} = 7.0$ Hz, CH_3CH$_2$O), 1.24 (t, 3H, $^3J_{HH} = 7.0$ Hz, CH_3CH$_2$O), 2.36 (s, 3H, CH_3C$_6$H$_4$), 2.78 (s, 3H, CH_3N), 3.56 (dd, 1H, $^2J_{PH} = 21.5$ Hz, $^3J_{HH} = 12.0$ Hz, PCHCH), 3.86–4.02 (m, 2H, CH$_3$CH_2O), 4.08–4.25 (m, 2H, CH$_3$CH_2O), 4.32 (dd, 1H, $^3J_{PH} = 12.0$ Hz, $^3J_{HH} = 12.0$ Hz, PCHCH), 7.17–7.34 (m, 4H, C$_6H_4$); ^{13}C NMR ($CDCl_3$): $\delta = 15.89$ (d, $^3J_{CP} = 6.3$ Hz, CH$_3$CH$_2$O), 16.16 (d, $^3J_{CP} = 6.2$ Hz, CH$_3$CH$_2$O), 21.11 (s, CH$_3$C$_6$H$_4$), 43.87 (s, CH$_3$N), 50.11 (d, $^1J_{CP} = 152.3$ Hz, PCHCH), 62.60 (d, $^2J_{CP} = 6.8$ Hz, CH$_3$CH$_2$O), 63.80 (d, $^2J_{CP} = 6.3$ Hz, CH$_3$CH$_2$O), 74.58 (s, PCHCH), 128.10 (s, 2C, C$_6$H$_4$), 129.40 (s, 2C, C$_6$H$_4$), 131.78 (s, C$_6$H$_4$), 139.20 (s, C$_6$H$_4$), 168.11 (s, C=O); ^{31}P NMR ($CDCl_3$): $\delta = 16.79$; Anal. Calcd for $C_{15}H_{22}NO_5P$: C, 55.04; H, 6.77; N, 4.28; P, 9.46. Found: C, 55.18; H, 6.61; N, 4.45; P, 9.60.

4.1.3. Synthesis of 2-methyl-4-methylidene-3-(1-methylbutyl) isoxazolidin-5-one, ***4a***

To a solution of 4-diethoxyphosphoryl-2-methyl-3-(1-methylbutyl)isoxazolidin-5-one **3a** (0.150 g, 0.488 mmol) in THF (5 mL), aqueous 36% formaldehyde (0.263 mL, 3.416 mmol) was added. The mixture was cooled to 0–5 °C with simultaneous slow addition of the solution of potassium carbonate (0.202 g, 1.46 mmol) in H_2O (2 mL). The solution was stirred for 45 min at room temperature (monitored by TLC) then extracted with Et_2O (3 × 15 mL). The organic layer was washed with brine and dried over $MgSO_4$, filtrated and

concentrated in vacuo. The remaining oil was purified by column chromatography on silica gel (eluent $CHCl_3$), to give pure **4a** (0.071 g, 80%), as a mixture of diastereoisomers in a 57:43 ratio, oil; IR (film, cm^{-1}): 1764, 1664, 1112; ^1H NMR ($CDCl_3$): $\delta = 0.91$ (t, 6H, $^3J_{HH} = 7.0$ Hz, $2 \times CH_3(CH_2)_2CH$), 0.93 (d, 3H, $^3J_{HH} = 6.8$ Hz, CH_3CH), 0.94 (d, 3H, $^3J_{HH} = 6.8$ Hz, CH_3CH), 1.14–1.49 (m, 8H, $2 \times CH_3CH_2CH_2CH$), 1.66–1.80 (m, 2H, $2 \times CH_3CH$), 2.86 (s, 6H, $2 \times CH_3N$), 3.51 (ddd, 1H, $^3J_{HH} = 5.50$ Hz, $^4J_{HH} = 2.0$ Hz, $^4J_{HH} = 2.0$ Hz, CH_2CCH), 3.58 (ddd, 1H, $^3J_{HH} = 4.50$ Hz, $^4J_{HH} = 2.0$ Hz, $^4J_{HH} = 2.0$ Hz, CH_2CCH), 5.70 (d, 1H, $^2J_{HH} = 2.0$ Hz, $CHHCCH$), 5.71 (d, 1H, $^2J_{HH} = 2.0$ Hz, $CHHCCH$), 6.40 (d, 1H, $^2J_{HH} = 2.0$ Hz, $CHHCCH$), 6.41 (d, 1H, $^2J_{HH} = 2.0$ Hz, $CHHCCH$); ^{13}C NMR ($CDCl_3$): $\delta = 14.10$ (s, $2 \times CH_3(CH_2)_2CH$), 14.99 (s, $2 \times CH_3CH$), 20.17 (s, $2 \times CH_3CH_2CH_2CH$), 33.88 (s, $CH_3CH_2CH_2CH$), 34.71 (s, $CH_3CH_2CH_2CH$), 37.48 (s, CH_3CH), 38.45 (s, CH_3CH), 48.96 (s, CH_3N), 49.29 (s, CH_3N), 73.71 (s, CH_2CCH), 74.25 (s, CH_2CCH), 124.23 (s, CH_2CCH), 124.71 (s, CH_2CCH), 135.84 (s, CH_2CCH), 136.70 (s, CH_2CCH), 170.11 (s, C=O), 170.23 (s, C=O); Anal. Calcd for $C_{10}H_{17}NO_2$: C, 65.54; H, 9.35; N, 7.64. Found: C, 65.42; H, 9.29; N, 7.48.

4.1.4. Synthesis of 2-methyl-4-methylidene-3-(4-methylphenyl)isoxazolidin-5-one, 4b

To a solution of 4-diethoxyphosphoryl-2-methyl-3-(4-methylphenyl)isoxazolidin-5-one **3b** (0.200 g, 0.611 mmol) in THF (5 mL), aqueous 36% formaldehyde (0.33 mL, 4.277 mmol) was added. The mixture was cooled to 0–5 °C with simultaneous slow addition of the solution of potassium carbonate (0.253 g, 1.83 mmol) in H_2O (2 mL). The mixture was stirred for 45 min at room temperature (monitored by TLC) then extracted with Et_2O (3×15 mL). The organic layer was washed with brine and dried over $MgSO_4$, filtrated and concentrated in vacuo. The remaining oil was purified by column chromatography on silica gel (eluent $CHCl_3$), to give pure **4b** (0.066 g, 53%), white prisms (from EtOAc/hexane); mp 43–45 °C; IR (film, cm^{-1}): 1768, 1672; ^1H NMR ($CDCl_3$): $\delta = 2.38$ (s, 3H, $CH_3C_6H_4$), 2.89 (s, 3H, CH_3N), 4.50 (bs, 1H, CH_2CCH), 5.28 (d, 1H, $^2J_{HH} = 3.0$ Hz, $CHHCCH$), 6.27 (d, 1H, $^2J_{HH} = 3.0$ Hz, $CHHCCH$), 7.19–7.27 (m, 4H, C_6H_4); ^{13}C NMR ($CDCl_3$): $\delta = 21.17$ (s, $CH_3C_6H_4$), 45.24 (s, CH_3N), 76.17 (s, CH_2CCH), 122.60 (s, CH_2CCH), 128.56 (s, 2C, C_6H_4), 129.68 (s, 2C, C_6H_4), 132.76 (s, C_6H_4), 139.10 (s, C_6H_4), 140.15 (s, CH_2CCH), 167.31 (s, C=O); Anal. Calcd for $C_{12}H_{13}NO_2$: C, 70.92; H, 6.45; N, 6.89. Found: C, 70.73; H, 6.48; N, 6.62.

4.2. Biological assays

4.2.1. Cell culture

Human promyelocytic leukemia HL-60 and drug-resistant HL-60 ADR (adriamycin-resistant, MRP1-overexpressing) cells were used. HL-60 cell line was obtained from the Institute of Immunology and Experimental Therapy (Wrocław, Poland) and the adriamycin-resistant subline HL-60 ADR was kindly provided by Prof. Grzegorz Bartosz, University of Łódź (Poland). Cells were cultured in RPMI 1640 medium (Cambrex, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Cytogen, Germany) and antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin) at 37 °C in 5% CO_2–95% air atmosphere.

4.2.2. Cytotoxicity assay

Exponentially growing cells were seeded at 3×10^5/well on 24-well plate (Nunc, Roskilde, Denmark). Cells were then exposed to the tested compounds for 72 h. Stock solutions of analyzed compounds were freshly prepared in DMSO and diluted with complete culture medium to obtain the concentration range from 10^{-7} to 10^{-3} M. DMSO concentration never exceeded 0.1% and had no influence on cell growth. Following the incubation, the number of viable cells was counted under microscope in Bürker hemocytometer, using the trypan-blue exclusion assay [32]. IC_{50} values (the concentration of the tested compound required to reduce the cell survival fraction to 50% of the control) were calculated from the concentration–response curves and used as a measure of cellular sensitivity to a given treatment.

4.2.3. Caspase-3 activity studies

HL-60 cells treated with the **4a**,**b** or *cis*-platin (in two concentrations: $1 \times IC_{50}$ and $10 \times IC_{50}$) were used for the determination of caspase-3 activity by the fluorometric immunosorbent enzyme assay (Roche, Germany). After 1, 2 and 5 h of incubation with the tested compound, the cells were spun down, washed twice with cold 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl, and lysed with recommended lysis buffer containing dithiothreitol. Cellular lysates were used directly for determination of the enzyme activity. Caspase-3 activity assay, based on the capture of caspase-3 from cellular lysates by a monoclonal antibody, was performed according to the manufacturer's protocol. Caspase-3 activity is proportional to the concentration of fluorochrome (amino-fluorocoumarin, AFC) released from the substrate [acetyl-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC)]. Generated free AFC was determined fluorometrically at $\lambda = 505$ nm (Victor-2 multifunction counter, Wallac, Finland). Enzyme activity was expressed as the concentration of AFC released by 10^6 cells.

4.2.4. RNA extraction and cDNA synthesis

Total RNA was isolated from HL-60 cells (about 10^5) treated for 5 and 72 h with tested compounds at a dose equal to its IC_{50} value by Total RNA Prep Plus Minicolumn Kit (A&A Biotechnology, Poland). The concentration of isolated RNA was determined by absorbance measurement at 260 nm.

cDNA was synthesized using RevertAid™ cDNA Synthesis Kit (Fermentas, Lithuania). Reaction mixture (total volume of 20 μL) containing 1 μL of total RNA (3 μg), 1 μL of oligo (dT) 18 primer (0.5 μg) and 8 μL of deionized, nuclease free water was prepared. The mixture was spinned down and incubated at 70 °C for 5 min, then chilled on ice. The following components were added: 4 μL of 5× concentrated reaction

buffer, 1 μL of ribonuclease inhibitor (20 U), 4 μL of 10 mM dNTPs mix. The mixture was incubated at 37° C for 5 min and 1 μL of RevertAid M-MulV reverse transcriptase (200 U) was added. The mixture was incubated at 42 °C for 60 min. The reaction was stopped by heating at 70 °C for 10 min.

4.2.5. BCL-2, BAX, MDR1 and MRP1 gene expression

The expression of human *BCL-2*, *BAX*, *MDR1*, *MRP1* and housekeeping *β-actin* genes were analyzed by multiplex polymerase chain reaction (MPCR) with the use of sets of gene specific primers. *BCL-2*: reverse: 5′CGA CTT CGC CGA GAT GTC CAG CCA G′3, forward: 5′ACT TGT GGC TCA GAT AGG CAC CCA G′3; *BAX*: reverse: 5′GCC CAT CTT CTT CCA GAT GGT GAG C′3, forward: 5′CAG CTC TGA GCA GAT CAT GAA GAC A′3; *MDR1*: reverse: 5′GCT TTC TGT CTT GGG CTT GT′3, forward: 5′CTC ATC GTT TGT CTA CAGTTC GT′3; *MRP1*: reverse: 5′GTC TTA CTC ATT GCA GG′3, forward: 5′CTT CTG CAC ATT CAT GGT C′3; *β-actin* (123 bp): reverse: 5′CAT TAA CGA GAA GCT GTG CT′3, forward: 5′GAA GGA AGG CTG GAA GAG T′3; *β-actin* (530 bp): reverse: 5′CTC CTT AAT GTC ACG CAC GAT TTC′3, forward: 5′GTG GGG CGC CCC AGG CAC CA′3.

In the experiments ''hot start'' DNA polymerase (Qiagen, Germany) was used. PCR mixture contained 1.5 mM $MgCl_2$, dNTPs mix, 0.5 U *Taq* Polymerase, reaction buffer and 0.5 μM of each primer. DNA was amplified in 35 cycles using the following parameters: denaturation (94 °C; 30 s), annealing (57 °C; 30 s) and extension (72 °C; 30 s).

4.2.6. Semiquantitative analysis of gene expression

The amplicons obtained in the MPCR were separated by gel electrophoresis with ethidium bromide staining. After archivization gels were analyzed in a densitometer (MiniBis Bio-Imaging System, Jerusalem, Israel). Differences in gene expression were determined by normalizing their expression to expression of the housekeeping *β-actin* gene (123 or 530 bp). Quantification of the investigated gene expression was done on the basis of optical density of the detected bands and the ratio of the products of the investigated gene to the housekeeping gene products was calculated.

4.2.7. Antimicrobial susceptibility testing

The group of microorganisms chosen for the study consisted of Gram-positive bacteria: *S. aureus* ATCC29213, *S. epidermidis* ATCC 12228, *E. faecalis* ATCC29212, Gram-negative bacteria: *E. coli* NCTC 8196, *P. aeruginosa* NCTC 6749 and yeast: *C. albicans* ATCC 10231.

The susceptibility of microorganisms to the tested compounds **4a**,**b** was determined by the standard CLSI (Clinical Laboratory Standards Institute/National Committee for Clinical Laboratory Standards, M7-A5, 2000). Sterile stock solutions of each compound at a concentration of 60 mg/mL were prepared in DMSO. The dilutions used in the antimicrobial tests were 0.29–300 μg/mL, prepared for bacteria in Mueller–Hinton broth (Difco, USA) and for yeast in RPMI 1640 medium supplemented with L-glutamine (Biomed, Poland). To specify the minimal inhibitory concentrations (MIC) for bacterial strains, turbidometric (OD_{600}) studies were carried out using the multifunction counter Victor-2.

Anti-yeast activity was tested by MTT-reduction assay. To each well of a microplate containing *C. albicans*, incubated for 24 h at 37 °C with serial dilutions of **4a** or **4b**, 50 μL of MTT solution (0.3% in PBS) was added and plates were incubated for 2 h at 37 °C. Then plates were centrifuged (3000 rpm, 10 min), emptied and filled with 150 μL of DMSO and 25 μL of glycine buffer (0.1 M, pH 10.2). For complete dissolving of purple formazan crystals the plates were incubated for 15 min at room temperature with gentle agitation. The colour intensity was determined using a microplate reader (Victor-2) at $\lambda = 550$ nm. In parallel experiments the influence of DMSO (used as a solvent for tested compounds) on microorganism viability was investigated.

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