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Original article

Synthesis of structural analogues of hexadecylphosphocholine and their antineoplastic, antimicrobial and amoebicidal activity

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ABSTRACT

Twelve derivatives of hexadecylphosphocholine (miltefosine) were synthesized to determine how the position and length of the alkyl chain within the molecule influence their biological activities. The prepared alkylphosphocholines have the same molecular formula as miltefosine. Activity of the compounds was studied against a spectrum of tumour cells, two species of protozoans, bacteria and yeast. Antitumour efficacy of some alkylphosphocholines measured up on MCF-7, A2780, HUT-78 and THP-1 cell lines was higher than that of miltefosine. The compounds showed antiprotozoal activity against *Acanthamoeba lugdunensis* and *Acanthamoeba quina*. Some of them also possess fungicidal activity against *Candida albicans* equal to miltefosine. No antibacterial activity was observed against *Staphylococcus aureus* and *Escherichia coli*. A difference in position of a long hydrocarbon chain within the structure with maximum efficacy was observed for antitumour, antiprotozoal and antifungal activity.

1. Introduction

Synthetic alkylphosphocholines (APCs) have the capacity to inhibit the growth of cancer cells selectively [1,2]; their cellular uptake is efficient, and they are capable to target some of the important signalling pathways in tumour growth [3]. Some of them were already tested successfully in clinical trials [4–9]. The leading compound miltefosine (hexadecylphosphocholine, **HPC**) is used for topical application in patients with skin metastases of breast cancer [2]. These compounds exhibit inhibitory capacity against tumour cells derived from different malignant localisations, such as breast [10] and ovarian [11,12] cancer. HPC have a co-stimulatory effect on granulopoiesis and thrombocytopoiesis [9], which is in contrast with bone-marrow suppression typical for most chemotherapeutical regimens.

Besides antineoplastic activity alkylphosphocholines possess also antiprotozoal properties. They show a trophocidal action against a wide spectrum of protozoans, including *Leishmania* [13], *Trypanosoma* [14], *Entamoeba* [15], *Acanthamoeba* [16], *Trichomonas* [17], and *Naegleria* [18]. Miltefosine is a registered drug for the treatment of visceral leishmaniasis [19]. *Acanthamoeba* spp. is a free-living amoeba which causes granulomatous amoebic encephalitis (GAE) and amoebic keratitis (AK) [20]. GAE is characterised by high mortality and optimal therapy has not been established yet [21]. Most cases of amoebic keratitis are related to the use of contact lenses. AK is treated with combinations of different drugs: nevertheless, an optimal therapy is also needed to be found [22].

Alkylphosphocholines are known to have antifungal activity as well. Susceptible species are *Candida*, *Cryptococcus*, *Aspergillus*, *Fusarium* and *Scedosporium*. APCs act as fungicidal agents [23].

In previous studies authors investigated antineoplastic or antileishmanial activity of broad spectrum of alkylphosphocholines,

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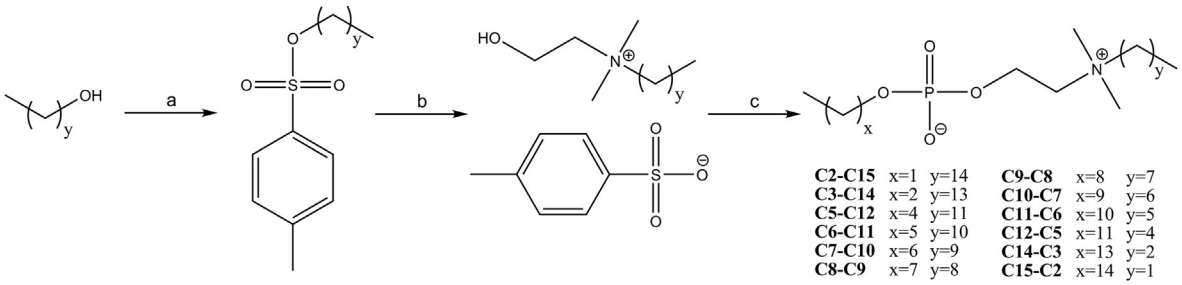

Scheme 1. Synthesis of alkylphosphocholines. Reagents and conditions: a) 4-methylbenzenesulphonyl chloride, pyridine, 20 °C; b) 2-(*N*,*N*-dimethylamino)ethanol, CH_3CN, reflux; c) i: $CH_3(CH_2)_xOH$, $POCl_3$, Et_3N, $CHCl_3$, 25 °C; ii: pyridine, H_2O, 25 °C.

which included molecules with cycles in the structure alone [24] or in combination with unsaturated bonds [13,25], or ether bond in the alkyl chain [26]. Heterocyclic substitutions on aminium nitrogen were also tested [26,27]. The presented compounds have structural analogies with the known alkylphosphocholines and are isomers of miltefosine. We evaluated their cytotoxicity and antiprotozoal and antimicrobial activity in order to study structure–activity relationship. The aim of the study was to discover influence of isomers upon the biological activity, i. e. the influence of the position of the alkyl chain within the molecule on the antineoplastic, antimicrobial and amoebicidal activity.

2. Results and discussion

2.1. Chemistry

A series of twelve alkylphosphocholines, structural derivatives of hexadecylphosphocholine, was synthesized according to Scheme 1 using a procedure modified by Lukáč et al. [27]. This series was prepared to determine the effect of the position and length of the alkyl chain linked to the phosphate or aminium moiety on the biological activity. The synthesized APCs have the same molecular formula as miltefosine. The APCs were prepared by phosphorylation of primary alcohols with phosphorus oxychloride and subsequent reaction with derivatives of choline 4-methylbenzenesulphonate. The reaction mixture was then hydrolysed by addition of water to get the desired alkylphosphocholine. The products were purified by column chromatography and crystallisation. Choline 4-methylbenzenesulphonate derivatives were synthesized by a reaction of 2-(*N*,*N*-dimethylamino)ethanol and alkyl 4-methylbenzenesulphonate in acetonitrile. Alkyl 4-methylbenzenesulphonates were prepared by tosylation of primary alcohols with 4-methylbenzenesulphonyl chloride. The purity of the APCs was determined using ^{31}P NMR spectroscopy, where a single signal of the phosphate group was observed, and by mass spectrometry. Three compounds, **C7**–**C10**, **C8**–**C9**, and **C9-8**, were previously prepared by Menger and Peresypkin [28] by a method using 2-chloro-2-oxo-1,3,2-dioxophospholane as a starting material. Compound **C10**–**C7** is a patented structure of Zentaris GmbH [29].

Several attempts were made to prepare a compound with an inverted hexadecylphosphocholine structure, with the hexadecyl chain linked to the aminium nitrogen and the methyl group attached to the phosphate moiety. Different synthetic approaches were used: the synthetic procedure mentioned above and the procedure which includes the hydrolysis of methyldichlorophosphate and the reaction of the resulting methylphosphonic acid with a choline 4-methylbenzenesulphonate derivative catalysed by 2,4,6-triisopropylbenzenesulphonyl chloride [26]. None of the methods, even the one used by Peresypkin and Menger [30], which should afford higher yields, was successful.

2.2. Antineoplastic activity

Alkylphosphocholines **C2**–**C15** to **C15**–**C2** and **HPC** (miltefosine) showed different inhibitory effect on cell growth in the tumour cell and normal cell cultures. IC50 values (Table 1), reflecting the cytotoxicity of the compounds against MCF-7 breast cancer cells, A2780 ovarian cancer cells, THP-1 and HUT-78 leukaemia cells, were determined on the basis of their dose–effect relationship, in the 95% confidence interval. IC50 is the concentration that inhibited 50% of the population growth; low IC50 values indicate high cytotoxicities.

Normal human peripheral blood cells, mainly a lymphocyte

Table 1
Values of IC50, minimal trophocidal concentration (MTC) and minimal inhibitory concentration (MIC) of prepared compounds. Figures marked with * represent values outside the 95% confidence interval. Figures in bold correspond to the most active compounds.

Compound	IC50 [μM]				MTC [μM]		MIC [μM]		
	MCF-7	A2780	HUT-78	THP-1	*A. lugdunensis*	*A. quina*	*S. aureus*	*E. coli*	*C. albicans*
C2–C15	**14.44**	7.44	35.23	30.54	**62.5**	**62.5**	>1000	>1000	21.14
C3–C14	20.90	9.26	**29.06**	**29.40**	125	125	>1000	>1000	86.30
C5–C12	25.75	18.32	68.02	71.22	125	125	>1000	>1000	352.27
C6–C11	64.50	25.72	96.05	31.22	**62.5**	125	>1000	>1000	359.53
C7–C10	26.21	4.70	436.90*	646.60*	125	125	>1000	>1000	352.23
C8–C9	19.25	**4.32**	279.80*	543.85*	125	125	>1000	>1000	367.14
C9–C8	33.96	7.31	146.10*	170.47	250	250	>1000	>1000	734.29
C10–C7	62.25	35.05	52.93	101.40	250	250	>1000	>1000	179.77
C11–C6	111.37	19.15	82.59	92.05	125	250	>1000	>1000	187.54
C12–C5	66.07	24.82	55.19	93.25	125	125	>1000	>1000	183.57
C14–C3	50.62	28.58	51.37	127.07	125	125	>1000	>1000	11.71
C15–C2	43.34	10.06	62.74	96.80	125	125	>1000	>1000	**2.93**
HPC	35.68	8.87	67.24	81.28	250	125	11.5	>1000	2.87

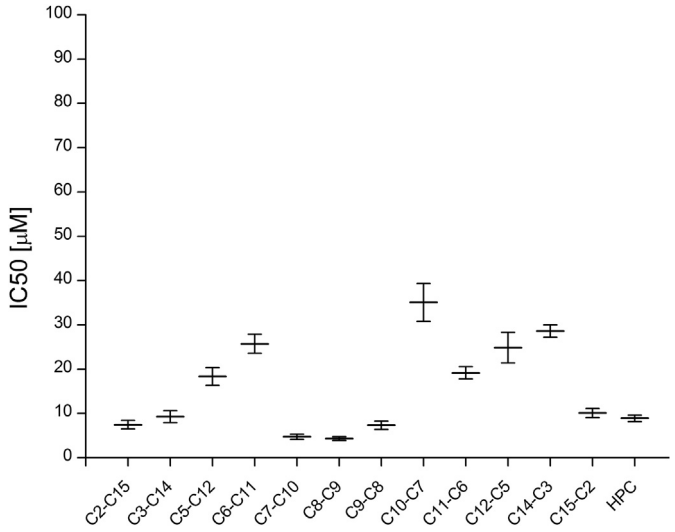

Fig. 1. Inhibitory effect of alkylphosphocholines on the proliferation of A2780 ovarian carcinoma cells.

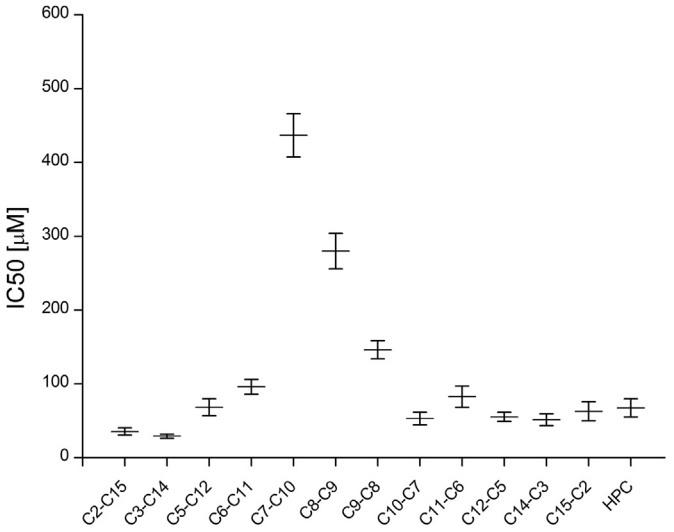

Fig. 3. Activity of alkylphosphocholines activity against HUT-78 leukaemia cell line. IC_{50} values corresponding to **C7**–**C10**, **C8**–**C9** and **C9**–**C8** were approximated by the Graph Pad Prism software outside the 95% confidence interval; one can consider those values as extrapolated.

population, showed very good survival after 24-h treatment with the examined compounds, the viability of control cells was well maintained without other stimuli, and in the treated cells the inhibition was very weak. With these peripheral blood cells, a maximum of 12% inhibition of growth was detected at a concentration of 500 μM in **C3**–**C14**, followed by 10% inhibition in **C2**–**C15**. No cytotoxicity was measured after 24 h in **C7**–**C10**, **C8**–**C9**, and **C9**–**C8**, and this was the reason why for neither of the compounds the IC50 values could be computed using statistic programs in the range of concentrations used.

The synthesized alkylphosphocholines reached the best inhibitory effect against A2780 cell line (Fig. 1). All the IC50 values are below 35.05 μM, activities of the most of the compounds do not differ significantly from that of miltefosine (multiple comparison test, $p < 0.05$), except **C6**–**C11**, **C10**–**C7**, **C12**–**C5** and **C14**–**C3**. Nevertheless, IC50 values of these four compounds are lower in A2780 cells in comparison with the other three cell lines.

In MCF-7 cell population, **C2**–**C15**, **C3**–**C14** and **C8**–**C9** showed the highest activity, followed by **C5**–**C12** and **C7**–**C10**; these five compounds were more efficient than miltefosine (Fig. 2). The new alkylphosphocholines **C2**–**C15**, **C3**–**C14** and **C8**–**C9** have significantly better activity than miltefosine (one-way ANOVA, multiple comparison test, $p < 0.05$), while **C6**–**C11**, **C10**–**C7**, **C11**–**C6**, and **C12**–**C5** show a significantly lower activity in MCF-7 cells than the reference **HPC**.

In HUT-78 cells, the most active are **C2**–**C15** and **C3**–**C14** (Fig. 3); except **C7**–**C10** and **C8**–**C9** having very high IC50 values, the activities of the other ten compounds of the series do not differ significantly from that of miltefosine. In THP-1 leukaemia population, there are three compounds with low activities (significantly lower than miltefosine); while the most efficient are **C2**–**C15**, **C3**–**C14** and **C6**–**C11** (Fig. 4).

The IC50 values of three compounds in HUT-78 and THP-1 T-lymphocyte and monocyte leukaemia differ in an order of

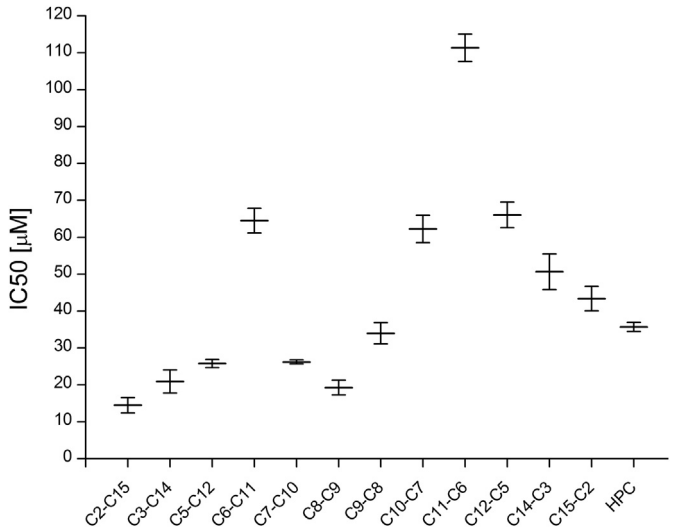

Fig. 2. Inhibitory effect of alkylphosphocholines on the proliferation of MCF-7 breast carcinoma cells.

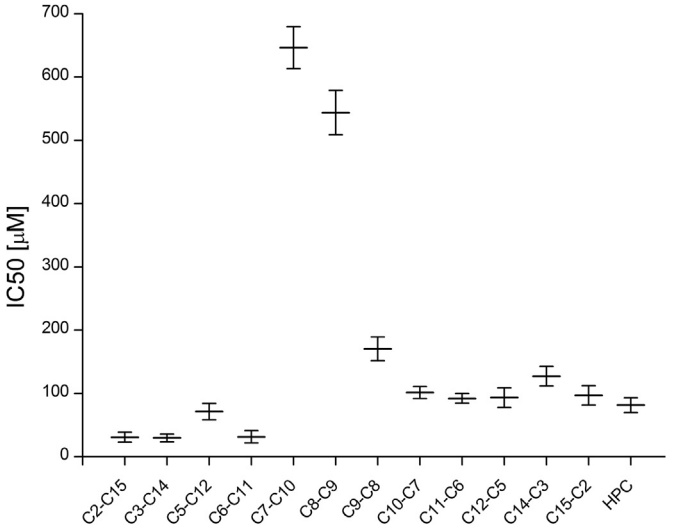

Fig. 4. Activity of alkylphosphocholines activity against THP-1 undifferentiated monocyte leukaemia cell line.

magnitude from the IC50 pattern corresponding to the breast and ovarian cancer cells. In the case of compounds **C7**–**C10**, **C8**–**C9** and **C9**–**C8**, the statistics cannot provide values in 95% confidence interval. The values depicted on the graphs (Figs. 3 and 4) are computed by extrapolation and they are very close to the highest concentration actually used. Therefore it can be concluded that these compounds do not exhibit any toxicity against leukaemia cells.

Previously tested alkylphosphocholines, such as miltefosine, perifosine, erucylphosphocholine and erufosine, and alkylphospholipid edelfosine act on cancer cells by altering the cell membrane, they are capable of interfering with the cell cycle in highly proliferative tumours [3]. They can induce apoptosis by activation of caspases 3 and 8 and by cleavage of cell death substrates, such as poly-ADP-ribose polymerase (PARP) [31].

In the present study, the results put forward the fact that the synthesized compounds were more active against highly proliferative cells in comparison with lymphocytes and malignant cells with lower turnover rate. Also, they are more active against cells which grew attached (A2780 and MCF-7), suggesting that the compounds interfere with adhesion and migration of the malignant cells. Alkylphosphocholines are capable of decreasing the proportion of cells in the S phase and increase the proportion in the G2/M phases of the cell cycle [12], and this could lead to a selective inhibition of highly proliferative cells, in this case the solid tumours versus tumour cells with slower turnover and normal haematopoietic cells.

Selectivity of the synthesized alkylphosphocholines is confirmed by their low inhibitory capacity against normal human lymphocytes. These results are in concordance with the biologic properties of other similar compounds with therapeutic potential [9,31].

MCF-7 and A2780 cells derived from female genital tumours are strongly inhibited by the alkylphosphocholines, but **C2**–**C15** influence is probably not dependent on oestrogen receptors. MCF-7 cells express [10] the oestrogen receptor alpha, while A2780 ovarian carcinoma cells do not express this receptor [32]. Alkylphosphocholines were proven to be effective against breast cancer cells *in vitro*, but they exhibit toxicity against cell lines without discriminate oestrogen receptor positive and negative [33] cell lines and they interfere with the PI3K and Akt [24] cell signalling pathways. The Wnt/β-catenin signalling is also affected [34] by alkylphosphocholines and this leads to the triggering of apoptosis.

The haematopoietic cells, both normal and tumour, are mildly affected by compounds **C2**–**C15** to **C15**–**C2** and **HPC**: tumour lymphoblasts or monocytes are weakly inhibited. The compounds have an insignificant effect on normal lymphocytes, which means they probably do not interfere with the main growth factors, cytokines and chemokines influencing the viability and proliferation of haematopoietic cells. The selectivity against cells derived from blood versus solid tumours was reported for some alkylphosphocholine analogues [35], being the premise of a good therapeutic potential. The behaviour of the prepared compounds is very similar. For example, erufosine affects the leukaemia cells selectively while it is harmless against normal human lymphocytes [36]. A similar pattern was observed in our experiment. On the other hand, erucylphosphohomocholine exhibits cytotoxicity in acute leukaemia with highly proliferative cells [37], by interfering with JNK-ERK pathways. The studied leukaemia cells had a much lower proliferation rate in comparison with acute leukaemia and these cells were of an inferior differentiation stage. The tested compounds displayed a moderate toxicity against these cell lines.

A possible explanation of distinctive antiproliferative effect of the synthesized compounds on carcinoma cells is their effect on the Wnt/β-catenin signalling pathway. In adherent epithelial carcinoma cells β-catenin is localised in cytoplasm and on the cell membrane and its downregulation enhances the apoptosis and inhibits cell proliferation [38]. Very high levels of β-catenin are constitutively expressed in the leukaemia cells and although inhibition of the signalling leads to the decrease of cell proliferation, the apoptotic process is regulated in a different way. Normal human lymphocytes do not contain detectable levels of β-catenin. Phosphorylation is involved in pathways regulated by β-catenin. Therefore the position of the phosphate moiety in the structure of the alkylphosphocholines probably influences the biologic outcome and it could play an important role in the selectivity of the compounds.

2.3. Antiprotozoal activity

The antiprotozoal activity of the prepared compounds was tested on strains of *Acanthamoeba lugdunensis* and *Acanthamoeba quina*, which are responsible for developing amoebic keratitis. Values of minimal trophocidal concentration are summarised in Table 1.

The activities of the synthesized compounds on both strains were comparable to the activity of the reference compound, miltefosine (Fig. 5). In case of *A. lugdunensis*, the compounds achieved a better antiprotozoal effect than the standard except **C9**–**C8** and **C10**–**C7**, which displayed the same activity as miltefosine. Antiprotozoal activities of the compounds against *A. quina* reached the same value as miltefosine, only compounds **C9**–**C8**, **C10**–**C7** and **C11**–**C6** were less effective. The most active compound in both cases was **C2**–**C15**, which was four times more effective against *A. lugdunensis* and twice as active as miltefosine against *A. quina*. Higher antiprotozoal activity of **C2**–**C15** is probably due to its closer structural similarity to quaternary aminium compounds (QUATs). QUATs are known to have better antiprotozoal effects than alkylphosphocholines, on the other hand they are also more toxic to healthy human cells [27,39]. Saran et al. observed by NMR spectroscopy that negatively charged phosphate group of miltefosine interacts with positive aminium nitrogen of the polar head group of the phospholipid in the membrane and vice versa [40]. We supposed that compound **C2**–**C15** has better solubilisation properties than alkylphosphocholine with inverted structure. This compound can better interact with membrane phospholipids of *Acanthamoeba* cells than **C15**–**C2**. Its aminium cation, which is closer to the long alkyl chain, can interact more easily with the phosphate anion of

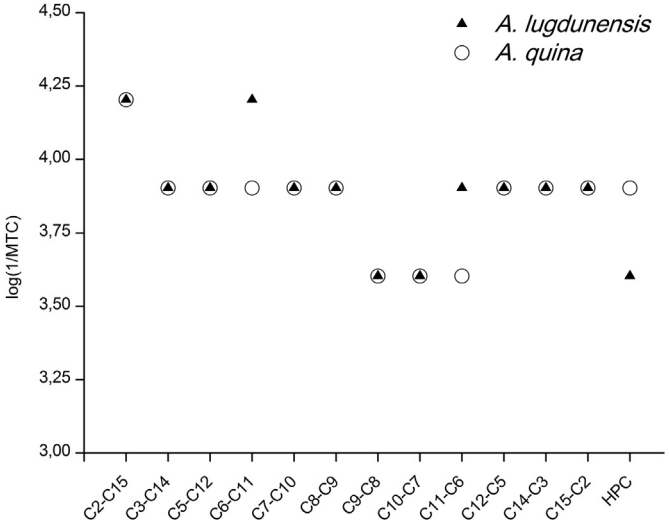

Fig. 5. Antiprotozoal activities of alkylphosphocholines against *Acanthamoeba* spp.

the membrane phospholipid and the phosphate anion of **C2**–**C15** is closer to the aminium cation of the choline part of the phospholipid. In this orientation the long alkyl chain is also incorporated in the cell membrane. It means that the zwitterion of **C2**–**C15** has a better orientation for interaction with a membrane phospholipid than the zwitterion of **C15**–**C2**.

2.4. Antimicrobial activity

Antimicrobial activity was measured against gram-positive bacteria *Staphylococcus aureus*, gram-negative bacteria *Escherichia coli* and yeast *Candida albicans* (Table 1). The prepared APCs showed very weak antibacterial activity against *E. coli*. All compounds had MIC out of range of tested concentrations. These findings correspond to results obtained by Obando et al. with different series of alkylphosphocholines, who reported no activity against gram-negative bacteria [41]. Similar results were observed for *S. aureus*. The only active compound was miltefosine with MIC value of 11.5 μM which is twofold lower than the value measured by Obando et al. for methicillin-resistant strain of *S. aureus* [41].

The prepared compounds showed moderate activity against *C. albicans* although none of them was more effective than miltefosine. MIC of miltefosine was 2.87 μM, which is similar to the value measured by Lukáč et al. [39], Obando et al. [41] or Ravu et al. [42]. Comparable activity was observed in the case of **C15**–**C2** with MIC of 2.93 μM. The antifungal activity of the tested compounds decreased with the shortening of the hydrocarbon chain attached to the phosphate moiety (Fig. 6) with minimum activity for compound **C9**–**C8**. The activity began to smoothly increase again with extension of the alkyl chain on the aminium side. But even the **C2**–**C15** with the longest alkyl chain attached to nitrogen did not achieve the MIC of miltefosine and its activity was ten times lower than the activity of **C15**–**C2**, the compound with an inverted structure. It could be assumed that antifungal activity depends on long hydrocarbon chain attached to the phosphate moiety. However there is limitation in length of the chain. Exceeding the number of carbon atoms over 18 decreases the activity [43].

2.5. Structure–activity relationship

A repeating pattern can be observed in the plots of activities of the prepared compounds against tumour cell lines (Figs. 1–4). The activity of the compounds depends on the presence of long alkyl chain in the molecule. The shortening of the chain causes a decrease of the activity with minimum activity for compound containing 11 carbons in the chain. In case of solid tumours, high activity was also observed for compounds possessing two approximately equal long chains on nitrogen and on the phosphate moiety, **C8**–**C9** and **C7**–**C10** (Figs. 1 and 2). Whereas for activity against leukemic cells the long alkyl chain was essential and could not be substituted by two shorter chains (Figs. 3 and 4). The observed behaviour of activity of the series for leukemic cell lines copies a pattern similar to the cut-off effect [44]. Based on this, one can assume that the lengthening of the chain on the phosphate site or on the aminium nitrogen site could increase antitumour activity. Comparison of two compounds with invert structure (e. g. **C2**–**C15** and **C15**–**C2**) favours the compound with the long alkyl chain linked to nitrogen for all four tested tumour cell lines. Above mentioned higher similarity with quaternary aminium salts could be possible explanation. Long chain aminium salts are more efficient than alkylphosphocholines against tumour cells [27]. An advantage of the synthesized compounds vs. QUATs is their low toxicity for normal cells.

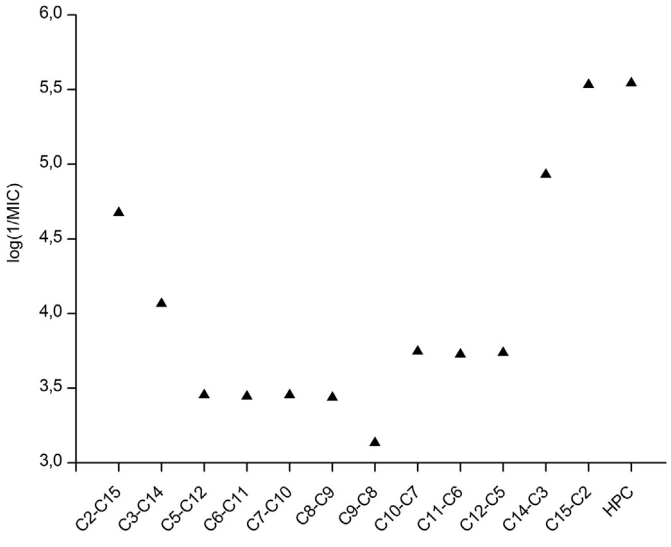

Fig. 6. Activities of the prepared alkylphosphocholines against yeast *Candida albicans*.

The antiprotozoal activity also depends on the position of the long alkyl chain (nitrogen or phosphate). Compounds with two shorter chains showed insufficient activity in comparison with compounds possessing one long chain. With shortening of the alkyl chain the critical micelle concentration (c_k) of the compounds increases. The study of aggregation properties of compounds described here in this paper, namely the c_k, is the subject of our next paper which will be published elsewhere. Preliminary data show that critical micelle concentration is in a range between c_k of miltefosine, 1.25×10^{-6} mol dm^{-3} [45], and c_k of derivative with two eight-carbon long chains, 1.00×10^{-3} mol dm^{-3} [30]. Solubility of all compounds in water was satisfactory for testing of biological activities. Compounds with approximately equally long alkyl chain didn't show better antiprotozoal activity in comparison with miltefosine, except of **C8**–**C9** in case of *A. lugdunensis*. Study made by Lukáč et al. [46] suggests that exchange of one of the hydrocarbon chains for perflourinated one can increase the activity in case of alkylphosphonatocholines with eight carbon chain linked to phosphonate and nitrogen. However this activity is still similar to analogical alkylphosphocholines from our series. The most active compound possesses long alkyl chain linked to aminium moiety. The optimal length of the alkyl chain of the alkylphosphocholine is also important for antiprotozoal activity. The compound with similar polar head, however, with a chain containing 20 carbon atoms (C2–C20) was less active than **C2**–**C15**. Its minimal trophocidal concentration is over 400 μM for *A. lugdunensis* [43]. This can be explained by a relatively poor solubility of this compound in water under normal conditions.

Better antifungal properties were observed for compounds with long alkyl chain linked to the phosphate moiety. The lipophilicity of alkylphosphocholine plays important role. Obando et al. [41] observed that alkylphosphocholines that have hexadecyl or octadecyl alkyl chain connected to phosphate possess the best anticandidal activities. Further increase in alkyl chain up to 20 carbons causes drop of anticandidal activity [43]. These observation is another example of cut-off effect described in detail by Balgavý and Devínsky [44]. However this observation is different to our findings in case of antiprotozoal and antitumour activity, where opposite analogues with long alkyl chains in the aminium moiety were the most active.

3. Conclusions

In this study, twelve compounds of the alkylphosphocholine group were synthesized. The main aim of the study was to find out

how the different position and length of alkyl chains linked to phosphate or aminium moiety affect the biological activity. The total sum of carbons in both chains is 17 which is equal to the number of relevant carbon atoms in the reference compound, miltefosine.

The studied series of alkylphosphocholines displayed a very promising antitumour potential in solid tumours, and two of its representatives, **C2**–**C15** and **C3**–**C14**, are the most active compounds against all four tested tumour cell lines. Nine compounds of the series are highly selective; i. e., their cytotoxic effect is significantly higher in the studied tumour cells versus normal human lymphocytes. The structures having a longer chain linked to the phosphate moiety displayed a better cytotoxic effect and they show similarities with the activity of miltefosine in solid tumour and leukaemia cells.

The prepared compounds also possess antiprotozoal activity against pathogenic strains of amoebae *A. lugdunensis* and *A. quina*. The most active compound against both strains is **C2**–**C15**. The antibacterial efficiency was measured against *E. coli* and *S. aureus*. None of the synthesized alkylphosphocholines showed a significant antibacterial activity. Yeast *C. albicans* was used for tests of antifungal properties. Best activity was observed in the case of compound **C15**–**C2**.

4. Experimental

4.1. Material and methods

Chemicals for synthesis were obtained from commercial suppliers: CentralChem, Slovakia – acetone, acetonitrile, diethyl ether, chloroform, propan-1-ol, propan-2-ol, pyridine, tetrahydrofurane, triethylamine; Fluka, Germany – decan-1-ol, dodecan-1-ol, ethyl 4-methylbenzenesulphonate, 4-methylbenzenesulphonyl chloride, phosphorus oxychloride, methyl 4-methylbenzenesulphonate, *N,N*-dimethyl-2-aminoethanol, nonan-1-ol, undecan-1-ol; Lachema, Czech republic – octan-1-ol, tetradecan-1-ol; Merck, Germany – Amberlite MB-3, pentadecan-1-ol, pentan-1-ol; Reachim, Russia – heptan-1-ol, hexan-1-ol. Chemicals were used as supplied. Solvents for synthesis were purified and dried before use according to Perrin and Armarego [47]. Choline salts were dried at 61 °C at diminished pressure prior to use.

^{1}H, ^{13}C and ^{31}P NMR spectra were measured on a Varian MERCURY plus spectrometer working at frequency 300, 75 and 121.5 MHz respectively. ^{13}C and ^{31}P NMR spectra were decoupled against protons. Spectra were measured in $CDCl_3$ or DMSO-*d6*. TMS was used as the internal standard for ^{1}H and ^{13}C NMR spectra and 85% H_3PO_4 was used as the external standard for ^{31}P NMR spectra. Mass spectra were recorded on an LTQ Orbitrap XL hybrid FTMS spectrometer (Thermo Fisher Scientific) using electrospray Ion Max-ESI in positive mode. Elementary analysis was carried out on FLESCH 2000 (Thermo Fischer Scientific).

Cell cultures were performed in a fully equipped cell culture laboratory, with class II laminar hoods (Lamil Plus and Heto Holten), sterile humidified incubators with 5% CO_2 level and constant 37 °C temperature (from Uniequip and Thermo Electron Corporation), centrifuges with swing-out rotors, for cell cultures (Hettich 320R), −80 °C ultra freezer (Heto Ultra Freeze), −190 °C liquid nitrogen tank (Cryosystem 2000 from MVE), incubator with a shaker (Heidolph Titramax 1000) and other laboratory devices. Colorimetric and fluorimetric measurements were assessed using Synergy 2 multiplate reader (BioTek). MCF-7 (breast tumour), A2780 (ovarian carcinoma), THP-1 and HUT-78 (leukaemia) cell lines were acquired from European Collection of Cell Cultures (ECCAC). Normal human lymphocytes were isolated from whole blood collected by venipuncture from a healthy 42-years old male donor, after his written informed consent. *A. lugdunensis* and *A. quina* were clinical isolates of free-living amoeba isolated from corneas of patients with *Acanthamoeba* keratitis [48,49]. Cultures used for antimicrobial tests were *S. aureus* ATCC 29/58, *E. coli* ATCC 377/79 and *C. albicans* ATCC 8186.

4.2. Chemistry

4.2.1. General procedure for preparation of alkyl 4-methylbenzenesulphonates

4-Methylbenzenesulphonyl chloride (55 mmol) was added slowly to a mixture of a primary alcohol (50 mmol) and 20 ml of pyridine at 10 °C. The reaction mixture was stirred for 3 h at 20 °C. After that 120 ml of 25% hydrochloric acid was slowly added. The reaction mixture was then extracted with chloroform, organic layer dried with Na_2SO_4 and evaporated to yield alkyl 4-methylbenzenesulphonate as colourless oily liquid or white solid, which was used without further purification. Ethyl 4-methylbenzenesulphonate was purchased from Fluka.

4.2.1.1. Propyl 4-methylbenzenesulphonate. Yield 97.0%; ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 3H, J = 7.5 Hz); 1.61–1.73 (m, 2H); 2.45 (s, 3H); 3.98 (t, 2H, J = 6.6 Hz); 7.35 (d, 2H, J = 7.9 Hz); 7.78 (d, 2H, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$) δ 10.0; 21.6; 22.3; 72.2; 127.9; 129.8; 133.2; 144.7.

4.2.1.2. Pentyl 4-methylbenzenesulphonate. Yield 93.3%; ^{1}H NMR ($CDCl_3$) δ 0.82–0.93 (m, 3H); 1.18–1.33 (m, 4H); 1.59–1.68 (m, 2H); 2.44 (s, 3H); 4.01 (t, 2H, J = 6.5 Hz); 7.34 (d, 2H, J = 7.9 Hz); 7.78 (d, 2H, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$) δ 13.5; 21.3; 21.7; 27.1; 28.2; 70.4; 127.5; 129.5; 132.8; 144.4.

4.2.1.3. Hexyl 4-methylbenzenesulphonate. Yield 86.2%; ^{1}H NMR ($CDCl_3$) δ 0.84 (t, 3H, J = 6.9 Hz); 1.17–1.34 (m, 6H); 1.58–1.67 (m, 2H); 2.44 (s, 3H); 4.01 (t, 2H, J = 6.6 Hz); 7.34 (d, 2H, J = 7.9 Hz); 7.78 (d, 2H, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$) δ 13.9; 21.6; 22.4; 25.0; 28.8; 31.1; 70.7; 127.9; 129.8; 133.2; 144.7.

4.2.1.4. Heptyl 4-methylbenzenesulphonate. Yield 95.4%; ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 3H, J = 6.9 Hz); 1.19–1.31 (m, 8H); 1.33–1.69 (m, 2H); 2.44 (s, 3H); 4.01 (t, 2H, J = 6.5 Hz); 7.34 (d, 2H, J = 7.9 Hz); 7.78 (d, 2H, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$) δ 14.0; 21.6; 22.5; 25.3; 28.6; 28.8; 31.6; 70.7; 127.9; 129.8; 133.2; 144.7.

4.2.1.5. Octyl 4-methylbenzenesulphonate. Yield 76.0%; ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 3H, J = 6.9 Hz); 1.22–1.27 (m, 10H); 1.60–1.65 (m, 2H); 2.45 (s, 3H); 4.02 (t, 2H, J = 6.6 Hz); 7.34 (d, 2H, J = 8.1 Hz); 7.78 (d, 2H, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1; 21.6; 22.6; 25.3; 28.8; 28.9; 29.1; 31.7; 70.7; 127.9; 129.8; 133.2; 144.7.

4.2.1.6. Nonyl 4-methylbenzenesulphonate. Yield 81.4%; ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, J = 6.9 Hz); 1.22–1.31 (m, 12H); 1.58–1.65 (m, 2H); 2.44 (s, 3H); 4.01 (t, 2H, J = 6.5 Hz); 7.34 (m, 2H, J = 8.8 Hz); 7.78 (d, 2H, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1; 21.6; 22.6; 25.3; 29.3; 29.4; 29.6; 31.8; 31.9; 70.7; 127.8; 129.8; 133.2; 144.6.

4.2.1.7. Decyl 4-methylbenzenesulphonate. Yield 85.1%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.6 Hz); 1.08–1.18 (m, 14H); 1.27–1.35 (m, 2H); 2.44 (s, 3H); 4.01 (t, 2H, J = 6.6 Hz); 7.33 (d, 2H, J = 8.0 Hz); 7.78 (d, 2H, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1; 21.6; 22.6; 25.3; 28.8; 28.9; 29.3; 29.4; 31.9; 70.7; 127.9; 129.8; 133.2; 144.6.

4.2.1.8. Undecyl 4-methylbenzenesulphonate. Yield 88.7%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.8 Hz); 1.22–1.30 (m, 16H); 1.58–1.71 (m, 2H); 2.45 (s, 3H); 4.01 (t, 2H, J = 6.5 Hz); 7.35 (d, 2H, J = 7.9 Hz); 7.78

(d, 2H, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1; 21.6; 22.7; 25.3; 28.8; 28.9; 29.3; 29.4; 29.5; 29.6; 31.9; 70.7; 127.9; 129.8; 133.3; 144.6.

4.2.1.9. Dodecyl 4-methylbenzenesulphonate. Yield 71.7%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.7 Hz); 1.08–1.18 (m, 18H); 1.27–1.35 (m, 2H); 2.44 (s, 3H); 4.01 (t, 2H, J = 6.6 Hz); 7.33 (d, 2H, J = 7.9 Hz); 7.78 (d, 2H, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1; 21.6; 22.6; 25.3; 28.8; 28.9; 29.3; 29.4; 29.5; 31.9; 70.7; 127.9; 129.8; 133.2; 144.6.

4.2.1.10. Tetradecyl 4-methylbenzenesulphonate. Yield 80.6%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.7 Hz); 1.08–1.18 (m, 22H); 1.27–1.35 (m, 2H); 2.44 (s, 3H); 4.01 (t, 2H, J = 6.6 Hz); 7.33 (d, 2H, J = 7.9 Hz); 7.78 (d, 2H, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1; 21.6; 22.6; 25.3; 28.8; 28.9; 29.3; 29.4; 29.5; 29.6; 31.9; 70.7; 127.9; 129.8; 133.2; 144.6.

4.2.1.11. Pentadecyl 4-methylbenzenesulphonate. Yield 59.5%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.8 Hz); 1.21–1.30 (m, 24H); 1.54–1.60 (m, 2H); 2.45 (s, 3H); 4.01 (t, 2H, J = 6.6 Hz); 7.34 (d, 2H, J = 8.2 Hz); 7.78 (d, 2H, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1; 21.7; 22.7; 25.3; 25.7; 28.8; 28.9; 29.4; 29.5; 29.6; 29.7; 31.9; 32.8; 70.7; 127.9; 129.8; 133.2; 144.6.

4.2.2. General procedure for preparation of choline salts

A solution of alkyl 4-methylbenzenesulphonate (40 mmol) in acetonitrile (20 ml) was added to a solution of 2-(*N,N*-dimethylamino)ethanol (40 mmol) in acetonitrile (20 ml). The reaction mixture was refluxed for 4 h. The solvent was evaporated in vacuum and the crude solid was left for crystallisation from acetone yielding a white, hygroscopic solid as the product. The product was kept in vacuum desiccator over P_4O_{10}.

4.2.2.1. N-ethyl-N,N-dimethyl-2-hydroxyethane-1-aminium 4-methylbenzenesulphonate. Yield 77.9%; ^{1}H NMR (DMSO-*d6*) δ 1.23 (t, 3H, J = 7.2 Hz); 2.29 (s, 3H); 3.02 (s, 6H); 3.51–3.64 (m, 2H); 3.77–3.88 (m, 2H); 5.23–5.33 (m, 2H); 7.11 (d, 2H, J = 7.8 Hz); 7.48 (d, 2H, J = 8.2 Hz); ^{13}C NMR (DMSO-*d6*) δ 7.9; 20.8; 50.1; 54.9; 59.6; 64.0; 125.4; 128.0; 137.6; 145.7. HRMS calcd. for $C_6H_{16}ON^+$ = 118.1226; found m/z: $[M]^+$ 118.1224.

4.2.2.2. N-(2-hydroxyethyl)-N,N-dimethylpropane-1-aminium 4-methylbenzenesulphonate. Yield 67.1%; ^{1}H NMR (DMSO-*d6*) δ 0.88 (t, 3H, J = 7.3 Hz); 1.65–1.73 (m, 2H); 2.29 (s, 3H); 3.06 (s, 6H); 3.26–3.34 (m, 4H); 3.80–3.81 (m, 2H); 5.46 (t, 1H, J = 5.1 Hz); 7.12 (d, 2H, J = 7.9 Hz); 7.49 (d, 2H, J = 8.2 Hz); ^{13}C NMR (DMSO-*d6*) δ 10.4; 15.3; 20.7; 50.7; 54.8; 64.5; 65.3; 125.4; 128.0; 137.6; 145.5. HRMS calcd. for $C_7H_{18}ON^+$ = 132.1383; found m/z: $[M]^+$ 132.1381.

4.2.2.3. N-(2-hydroxyethyl)-N,N-dimethylpentane-1-aminium 4-methylbenzenesulphonate. Yield 78.4%; ^{1}H NMR (DMSO-*d6*) δ 0.89 (t, 3H, J = 7.3 Hz); 1.20–1.37 (m, 4H); 1.64–1.70 (m, 2H); 2.29 (s, 3H); 3.07 (s, 6H); 3.29–3.37 (m, 4H); 3.81 (m, 2H); 5.47 (t, 1H, J = 5.3 Hz); 7.12 (d, 2H, J = 8.4 Hz); 7.48 (d, 2H, J = 7.9 Hz); ^{13}C NMR (DMSO-*d6*) δ 13.6; 20.7; 21.4; 21.5; 27.8; 50.7; 54.8; 63.9; 64.4; 125.4; 128.0; 137.5; 145.5. HRMS calcd. for $C_9H_{22}ON^+$ = 160.1696; found m/z: $[M]^+$ 160.1693.

4.2.2.4. N-(2-hydroxyetyl)-N,N-dimethylhexane-1-aminium 4-methylbenzenesulphonate. Yield 71.7%; ^{1}H NMR (DMSO-*d6*) δ 0.87 (t, 3H, J = 6.7 Hz); 1.22–1.30 (m, 6H); 1.63–1.68 (m, 2H); 2.29 (s, 3H); 3.04 (s, 6H); 3.26–3.32 (m, 2H); 3.34–3.43 (m, 2H); 3.81 (m, 2H); 5.27 (t, 1H, J = 5.0 Hz); 7.11 (d, 2H, J = 7.9); 7.47 (m, 2H); ^{13}C NMR (DMSO-*d6*) δ 13.7; 20.7; 21.6; 21.8; 25.3; 30.6; 50.7; 54.8; 64.0; 64.5; 125.4; 128.0; 137.6; 145.6. HRMS calcd. for $C_{10}H_{24}ON^+$ = 174.1852; found m/z: $[M]^+$ 174.1849.

4.2.2.5. N-(2-hydroxyethyl)-N,N-dimethylheptane-1-aminium 4-methylbenzenesulphonate. Yield 82.6%; ^{1}H NMR (DMSO-*d6*) δ 0.87 (t, 3H, J = 6.6 Hz); 1.27 (m, 8H); 1.64–1.69 (m, 2H); 2.29 (s, 3H); 3.06 (s, 6H); 3.30–3.40 (m, 4H); 3.80 (m, 2H); 5.47 (t, 1H, J = 5.1 Hz); 7.12 (d, 2H, J = 7.9 Hz); 7.48 (d, 2H, J = 8.4 Hz); ^{13}C NMR (DMSO-*d6*) δ 13.8; 20.7; 21.7; 21.9; 25.6; 28.1; 30.9; 50.7; 54.8; 63.9; 64.4; 125.4; 128.0; 137.5; 145.6. HRMS calcd. for $C_{11}H_{26}ON^+$ = 188.2009; found m/z: $[M]^+$ 188.2007.

4.2.2.6. N-(2-hydroxyethyl)-N,N-dimethyloctane-1-aminium 4-methylbenzenesulphonate. Yield 68.1%; ^{1}H NMR (DMSO-*d6*) δ 0.87 (t, 3H, J = 6.8 Hz); 1.27 (m, 10H); 1.63–1.68 (m, 2H); 2.29 (s, 3H); 3.04 (s, 6H); 3.26–3.38 (m, 4H); 3.81 (m, 2H); 5.26 (t, 1H, J = 4.8 Hz); 7.11 (d, 2H, J = 7.8 Hz); 7.47 (d, 2H, J = 8.1 Hz); ^{13}C NMR (DMSO-*d6*) δ 14.0; 20.8; 21.8; 22.1; 25.8; 28.5; 31.2; 50.8; 54.9; 64.1; 64.6; 125.5; 128.0; 137.6; 145.8. HRMS calcd. for $C_{12}H_{28}ON^+$ = 202.2165; found m/z: $[M]^+$ 202.2163.

4.2.2.7. N-(2-hydroxyethyl)-N,N-dimethylnonane-1-aminium 4-methylbenzenesulphonate. Yield 76.0%; ^{1}H NMR (DMSO-*d6*) δ 0.86 (t, 3H, J = 6.6 Hz); 1.26 (m, 12H); 1.63–1.67 (m, 2H); 2.29 (s, 3H); 3.04 (s, 6H); 3.26–3.31 (m, 2H); 3.34–3.37 (m, 2H); 3.80 (m, 2H); 5.27 (t, 1H, J = 5.0 Hz); 7.11 (d, 2H, J = 7.9); 7.47 (d, 2H, 8.2 Hz); ^{13}C NMR (DMSO-*d6*) δ 13.9; 20.7; 21.7; 22.0; 25.7; 28.4; 28.5; 28.7; 31.1; 50.7; 54.9; 64.0; 64.5; 125.4; 128.0; 137.5; 145.6. HRMS calcd. for $C_{13}H_{30}ON^+$ = 216.2322; found m/z: $[M]^+$ 216.2321.

4.2.2.8. N-(2-hydroxyethyl)-N,N-dimethyldecane-1-aminium 4-methylbenzenesulphonate. Yield 79.3%; ^{1}H NMR (DMSO-*d6*) δ 0.88 (t, 3H, J = 6.6 Hz); 1.17–1.50 (m, 18H); 1.60–1.69 (m, 2H); 2.34 (s, 3H); 3.27 (s, 6H); 3.36–3.45 (m, 2H); 3.64–3.74 (m, 2H); 4.09–4.20 (m, 2H); 4.78 (t, 1H, J = 5.7 Hz); 7.15 (d, 2H, J = 8.0 Hz); 7.74 (d, 2H, J = 8.1 Hz); ^{13}C NMR (DMSO-*d6*) δ 14.1; 21.3; 22.7; 22.8; 26.2; 29.1; 29.3; 29.4; 29.5; 29.6; 31.9; 51.8; 56.4; 65.8; 66.0; 125.8; 128.7; 139.5; 143.1. HRMS calcd. for $C_{14}H_{32}ON^+$ = 230.2478; found m/z: $[M]^+$ 230.2477.

4.2.2.9. N-(2-hydroxyethyl)-N,N-dimethylundecane-1-aminium 4-methylbenzenesulphonate. Yield 56.0%; ^{1}H NMR (DMSO-*d6*) δ 0.86 (t, 3H, J = 6.7 Hz); 1.25 (m, 16H); 1.63–1.67 (m, 2H); 2.29 (s, 3H); 3.06 (s, 6H); 3.29–3.39 (m, 4H); 3.80 (m, 2H); 5.48 (t, 1H, J = 5.1 Hz); 7.12 (d, 2H, J = 7.9 Hz); 7.48 (d, 2H, J = 7.9 Hz); ^{13}C NMR (DMSO-*d6*) δ 13.9; 20.7; 21.7; 22.0; 25.7; 28.4; 28.6; 28.7; 28.9; 31.2; 50.7; 54.8; 63.9; 64.4; 125.4; 128.0; 137.5; 145.6. HRMS calcd. for $C_{15}H_{34}ON^+$ = 244.2635; found m/z: $[M]^+$ 244.2633.

4.2.2.10. N-(2-hydroxyethyl)-N,N-dimethyldodecane-1-aminium 4-methylbenzenesulphonate. Yield 92.2%; ^{1}H NMR (DMSO-*d6*) δ 0.86 (t, 3H, J = 6.8 Hz); 1.25 (m, 18H); 1.65 (m, 2H); 2.28 (s, 3H); 3.03 (s, 6H); 3.26–3.36 (m, 4H); 3.80 (m, 2H); 5.26 (m, 1H); 7.10 (d, 2H, J = 7.6 Hz); 7.47 (d, 2H, J = 8.2 Hz); ^{13}C NMR (DMSO-*d6*) δ 13.9; 20.7; 21.7; 22.0; 25.7; 28.4; 28.6; 28.7; 28.8; 28.9; 31.2; 50.7; 54.8; 64.0; 64.5; 125.4; 127.9; 137.5; 145.7. HRMS calcd. for $C_{16}H_{36}ON^+$ = 258.2791; found m/z: $[M]^+$ 258.2787.

4.2.2.11. N-(2-hydroxyethyl)-N,N-dimethyltetradecane-1-aminium 4-methylbenzenesulphonate. Yield 84.4%; ^{1}H NMR (DMSO-*d6*) δ 0.88 (t, 3H, J = 6.7 Hz); 1.17–1.52 (m, 22H); 1.60–1.69 (m, 2H); 2.34 (s, 3H); 3.26 (s, 6H); 3.36–3.45 (m, 2H); 3.65–3.74 (m, 2H); 4.09–4.20 (m, 2H); 5.21 (t, 1H, J = 5.7 Hz); 7.15 (d, 2H, J = 8.0 Hz); 7.74 (d, 2H, J = 8.0 Hz); ^{13}C NMR (DMSO-*d6*) δ 14.1; 21.3; 22.7; 22.8; 26.2; 29.1; 29.3; 29.4; 29.5; 29.6; 29.7; 31.9; 51.8; 56.4; 65.8; 66.0; 125.8; 128.7; 139.6; 143.0. HRMS calcd. for $C_{18}H_{40}ON^+$ = 286.3104; found m/z: $[M]^+$ 286.3099.

4.2.2.12. N-(2-hydroxyethyl)-N,N-dimethylpentadecane-1-aminium 4-methylbenzenesulphonate. Yield 85.9%; ^{1}H NMR (DMSO-*d6*) δ 0.86 (t, 3H, *J* = 6.8 Hz); 1.24 (m, 24H); 1.63–1.67 (m, 2H); 2.29 (s, 3H); 3.04 (s, 6H); 3.26–3.30 (m, 2H); 3.33–3.37 (m, 2H); 3.80 (m, 2H); 5.25 (t, 1H, *J* = 5.7 Hz); 7.11 (d, 2H, *J* = 8.4 Hz); 7.47 (d, 2H, *J* = 8.1 Hz); ^{13}C NMR (DMSO-*d6*) δ 13.9; 20.8; 21.7; 22.0; 25.8; 28.5; 28.7; 28.8; 28.9; 29.0; 31.3; 50.8; 54.9; 64.0; 64.6; 125.5; 128.0; 137.5; 145.8. HRMS calcd. for $C_{19}H_{42}ON^+$ = 300.3261; found *m*/*z*: $[M]^+$ 300.3256.

4.2.3. General procedure for preparation of alkylphosphocholines

To a stirred solution of phosphorus oxychloride (10 mmol) and (triethyl)amine (20 mmol) in chloroform (10 ml) was added dropwise a solution of an alcohol (9 mmol) in chloroform (20 ml) at 0 °C. The reaction mixture was stirred for 2 h at room temperature. The prepared intermediate was used immediately and without purification. Pyridine (15 ml) was added dropwise at 0 °C followed by addition of a choline 4-methylbenzenesulphonate derivative (12.5 mmol). The reaction mixture was stirred at room temperature overnight. After that it was hydrolysed by addition of water (1.5 ml) and stirred for one hour at room temperature. Solvents were evaporated at vacuum and the crude solid was diluted in tetrahydrofuran and water (5:1). Exchange resin Amberlite MB3 was added to the stirred solution until the resin stopped changing colour. The resin was filtered off and solvents were evaporated in vacuum. The product was purified by chromatography using silica gel with the liquid phase containing $CHCl_3$:CH_3OH:H_2O (65:25:3). After purification, solvents were evaporated in vacuum, the product was dried by azeotropic distillation using propan-2-ol, dissolved in chloroform and precipitated with acetone. The product was filtered off as white hygroscopic solid and was stored in desiccator over phosphorus pentoxide.

*4.2.3.1. [2-(N,N-dimethyl-N-pentadecylaminio)ethyl]ethylphosphate (**C2**–**C15**).* Yield 7.0%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, *J* = 6.8 Hz); 1.21–1.33 (m, 27H); 1.71 (m, 2H); 3.33 (s, 6H); 3.41–3.47 (m, 2H); 3.79 (m, 2H); 3.92 (q, 2H, J = 7.0 Hz); 4.31 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 16.7; 22.7; 22.9; 26.3; 29.3; 29.4; 29.5; 29.6; 29.7; 31.9; 51.8; 58.9; 61.2; 64.2; 65.8; ^{31}P NMR ($CDCl_3$) δ −0.29; HRMS calcd. for $C_{21}H_{46}O_4NPNa$ = 430.3057; found *m*/*z*: $[M+Na]^+$ 430.3053; Anal. Calcd. for $C_{21}H_{46}NO_4P \times 0.75\ H_2O$: C, 59.90; H, 11.37; N, 3.33; found: C, 59.89; H, 11.28; N, 3.05.

*4.2.3.2. [2-(N,N-dimethyl-N-tetradecylaminio)ethyl]propylphosphate (**C3**–**C14**).* Yield 5.7%; ^{1}H NMR ($CDCl_3$) δ 0.86–0.93 (m, 6H); 1.25–1.32 (m, 22H); 1.56–1.71 (m, 4H); 3.33 (s, 6H); 3.41–3.47 (m, 2H); 3.76–3.83 (m, 4H); 4.30 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 10.4; 14.1; 22.7; 22.9; 24.1; 24.2; 26.3; 29.3; 29.4; 29.5; 29.6; 29.7; 31.9; 51.8; 58.8; 64.2; 65.7; 67.1; ^{31}P NMR ($CDCl_3$) δ −0.18; HRMS calcd. for $C_{21}H_{46}O_4NPNa$ = 430.3057; found *m*/*z*: $[M+Na]^+$ 430.3056; Anal. Calcd. for $C_{21}H_{46}NO_4P \times 1.6\ H_2O$: C, 57.80; H, 11.36; N, 3.21; found: C, 57.72; H, 10.88; N, 3.01.

*4.2.3.3. [2-(N-dodecyl-N,N-dimethylaminio)ethyl]pentylphosphate (**C5**–**C12**).* Yield 7.0%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 6H, *J* = 6.89 Hz); 1.25–1.33 (m, 22H); 1.56–1.72 (m, 4H); 3.34 (s, 6H); 3.43–3.49 (m, 2H); 3.81–3.88 (m, 4H); 4.33 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.5; 22.7; 22.9; 26.4; 28.0; 29.3; 29.4; 29.5; 29.6; 30.6; 30.7; 31.9; 51.8; 60.0; 64.2; 65.8; ^{31}P NMR ($CDCl_3$) δ −0.48; HRMS calcd. for $C_{21}H_{46}O_4NPNa$ = 430.3057; found *m*/*z*: $[M+Na]^+$ 430.3053; Anal. Calcd. for $C_{21}H_{46}NO_4P \times 0.75\ H_2O$: C, 59.90; H, 11.37; N, 3.33; found: C, 60.12; H, 11.32; N, 3.14.

*4.2.3.4. [2-(N,N-dimethyl-N-undecylaminio)ethyl]hexylphosphate (**C6**–**C11**).* Yield 13.0%; ^{1}H NMR ($CDCl_3$) δ 0.85–0.90 (m, 6H); 1.25–1.33 (m, 22H); 1.57–1.71 (m, 4H); 3.34 (s, 6H); 3.42–3.48 (m, 2H); 3.79–3.86 (m, 4H); 4.29 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.7; 22.9; 25.6; 26.3; 29.3; 29.4; 29.5; 29.6; 31.0; 31.1; 31.7; 31.9; 51.8; 58.9; 64.2; 65.5; 65.7; ^{31}P NMR ($CDCl_3$) δ −0.11; HRMS calcd. for $C_{21}H_{46}O_4NPNa$ = 430.3057; found *m*/*z*: $[M+Na]^+$ 430.3053; Anal. Calcd. for $C_{21}H_{46}NO_4P \times 0.5\ H_2O$: C, 60.55; H, 11.37; N, 3.36; found: C, 60.58; H, 11.40; N, 3.09.

*4.2.3.5. [2-(N-decyl-N,N-dimethylaminio)ethyl]heptylphosphate (**C7**–**C10**).* Yield 7.5%; ^{1}H NMR ($CDCl_3$) δ 0.85–0.90 (m, 6H); 1.26–1.32 (m, 22H); 1.57–1.71 (m, 4H); 3.33 (s, 6H); 3.44 (m, 2H); 3.79–3.85 (m, 4H); 4.30 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 21.9; 22.7; 22.9; 25.9; 26.4; 29.2; 29.3; 29.5; 31.0; 31.9; 51.8; 52.4; 58.9; 64.2; 65.7; ^{31}P NMR ($CDCl_3$) δ −0.17; HRMS calcd. for $C_{21}H_{46}O_4NPNa$ = 430.3057; found *m*/*z*: $[M+Na]^+$ 430.3056; Anal. Calcd. for $C_{21}H_{46}NO_4P \times 0.8\ H_2O$: C, 59.77; H, 11.37; N, 3.32; found: C, 59.67; H, 11.15; N, 3.06.

*4.2.3.6. [2-(N,N-dimethyl-N-nonylaminio)ethyl]octylphosphate (**C8**–**C9**).* Yield 10.2%; ^{1}H NMR ($CDCl_3$) δ 0.85–0.90 (m, 6H); 1.26–1.34 (m, 22H); 1.55–1.62 (m, 2H); 1.71 (m, 2H); 3.35 (s, 6H); 3.44–3.49 (m, 2H); 3.80–3.87 (m, 4H); 4.31 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.6; 22.7; 22.9; 25.9; 26.4; 29.2; 29.3; 29.4; 29.5; 31.0; 31.1; 31.8; 31.9; 51.8; 58.9; 64.2; 65.6; 65.7; ^{31}P NMR ($CDCl_3$) δ −0.20; HRMS calcd. for $C_{21}H_{46}O_4NPNa$ = 430.3057; found *m*/*z*: $[M+Na]^+$ 430.3053; Anal. Calcd. for $C_{21}H_{46}NO_4P \times 0.9\ H_2O$: C, 59.77; H, 11.37; N, 3.32; found: C, 59.68; H, 10.92; N, 3.15.

*4.2.3.7. [2-(N,N-dimethyl-N-octylaminio)ethyl]nonylphosphate (**C9**–**C8**).* Yield 17.5%; ^{1}H NMR ($CDCl_3$) δ 0.85–0.90 (m, 6H); 1.25–1.32 (m, 22H); 1.55–1.64 (m, 2H); 1.70–1.71 (m, 2H); 3.35 (s, 6H); 3.43–3.49 (m, 2H); 3.79–3.86 (m, 4H); 4.29 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.1; 22.6; 22.7; 22.9; 26.0; 26.4; 29.1; 29.3; 29.4; 29.5; 31.1; 31.2; 31.7; 31.9; 51.8; 58.9; 64.2; 65.5; 65.7; ^{31}P NMR ($CDCl_3$) δ −0.06; HRMS calcd. for $C_{21}H_{46}O_4NPNa$ = 430.3057; found *m*/*z*: $[M+Na]^+$ 430.3056; Anal. Calcd. for $C_{21}H_{46}NO_4P \times 1.25\ H_2O$: C, 58.65; H, 11.37; N, 3.26; found: C, 58.74; H, 11.08; N, 3.13.

*4.2.3.8. [2-(N-heptyl-N,N-dimethylaminio)ethyl]decylphosphate (**C10**–**C7**).* Yield 23.0%; ^{1}H NMR ($CDCl_3$) δ 0.86–0.90 (m, 6H); 1.25–1.34 (m, 22H); 1.56–1.60 (m, 2H); 1.71 (m, 2H); 3.32 (s, 6H); 3.43–3.48 (m, 2H); 3.77–3.83 (m, 4H); 4.27 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.0; 14.1; 22.5; 22.7; 22.9; 25.9; 26.3; 29.0; 29.4; 29.6; 29.7; 31.1; 31.2; 31.9; 51.8; 58.8; 64.0; 65.5; 65.6; ^{31}P NMR ($CDCl_3$) δ −0.34; HRMS calcd. for $C_{21}H_{46}O_4NPNa$ = 430.3057; found *m*/*z*: $[M+Na]^+$ 430.3053; Anal. Calcd. for $C_{21}H_{46}NO_4P \times 0.6\ H_2O$: C, 60.29; H, 11.37; N, 3.35; found: C, 60.29; H, 11.33; N, 3.25.

*4.2.3.9. [2-(N-hexyl-N,N-dimethylaminio)ethyl]undecylphosphate (**C11**–**C6**).* Yield 14.7%; ^{1}H NMR ($CDCl_3$) δ 0.86–0.91 (m, 6H); 1.25–1.33 (m, 22H); 1.54–1.61 (m, 2H); 1.71 (m, 2H); 3.35 (s, 6H); 3.45–3.50 (m, 2H); 3.78–3.85 (m, 4H); 4.28 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 13.9; 14.1; 22.5; 22.7; 22.8; 25.9; 26.0; 29.4; 29.5; 29.7; 31.2; 31.4; 31.9; 51.8; 58.9; 64.1; 65.5; 65.6; ^{31}P NMR ($CDCl_3$) δ −0.04; HRMS calcd. for $C_{21}H_{46}O_4NPNa$ = 430.3057; found *m*/*z*: $[M+Na]^+$ 430.3053; Anal. Calcd. for $C_{21}H_{46}NO_4P \times 1\ H_2O$: C, 59.27; H, 11.37; N, 3.29; found: C, 59.31; H, 11.25; N, 3.11.

*4.2.3.10. [2-(N,N-dimethyl-N-pentylaminio)ethyl]dodecylphosphate (**C12**–**C5**).* Yield 19.3%; ^{1}H NMR ($CDCl_3$) δ 0.86–0.94 (m, 6H); 1.25–1.37 (m, 22H); 1.54–1.64 (m, 2H); 1.72 (m, 2H); 3.35 (s, 6H); 3.45–3.51 (m, 2H); 3.79–3.86 (m, 4H); 4.29 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 13.8; 14.1; 22.3; 22.5; 22.7; 25.9; 28.4; 29.4; 29.5; 29.7; 31.1; 31.2; 51.8; 58.8; 64.1; 65.5; 65.6; ^{31}P NMR ($CDCl_3$) δ 0.001; HRMS calcd. for $C_{21}H_{46}O_4NPNa$ = 430.3057; found *m*/*z*: $[M+Na]^+$

430.3053; Anal. Calcd. for $C_{21}H_{46}NO_4P \times 0.75\ H_2O$: C, 59.90; H, 11.37; N, 3.33; found: C, 59.94; H, 11.34; N, 3.21.

*4.2.3.11. [2-(N,N-dimethyl-N-propylaminio)ethyl]tetradecylphosphate (**C14–C3**).* Yield 16.5%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, $J = 6.6$ Hz); 1.02 (t, 3H, $J = 7.2$); 1.25 (m, 22H); 1.54–1.61 (m, 2H); 1.78–1.81 (m, 2H); 3.36 (m, 6H); 3.46–3.51 (m, 2H); 3.78–3.84 (m, 4H); 4.28 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 10.7; 14.1; 16.3; 22.7; 25.9; 29.4; 29.5; 29.7; 31.1; 31.2; 31.9; 51.7; 58.8; 58.9; 64.0; 65.4; 65.5; 66.8; ^{31}P NMR ($CDCl_3$) δ −0.09; HRMS calcd. for $C_{21}H_{46}O_4NPNa = 430.3057$; found *m/z*: $[M+Na]^+$ 430.3055; Anal. Calcd. for $C_{21}H_{46}NO_4P \times 0.75\ H_2O$: C, 59.90; H, 11.37; N, 3.33; found: C, 59.96; H, 11.23; N, 3.17.

*4.2.3.12. [2-(N-ethyl-N,N-dimethylaminio)ethyl]pentadecylphosphate (**C15–C2**).* Yield 18.9%; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, $J = 6.8$ Hz); 1.25–1.41 (m, 27H); 1.56–1.60 (m, 2H); 3.33 (s, 6H); 3.66–3.68 (m, 2H); 3.77–3.84 (m, 4H); 4.28 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 8.5; 14.1; 22.7; 26.0; 29.4; 29.5; 29.7; 31.1; 31.9; 51.1; 58.8; 58.9; 60.8; 63.5; 65.5; 65.6; ^{31}P NMR ($CDCl_3$) δ −0.19; HRMS calcd. for $C_{21}H_{46}O_4NPNa = 430.3057$; found *m/z*: $[M+Na]^+$ 430.3054; Anal. Calcd. for $C_{21}H_{46}NO_4P \times 0.8\ H_2O$: C, 59.77; H, 11.37; N, 3.32; found: C, 59.82; H, 11.33; N, 3.10.

4.3. Cytotoxicity assay

The biological model used for the *in vitro* study was a selection of tumour cell lines and normal human peripheral blood cells as a reference. Two cell lines derived from solid human tumours were used: MCF-7 human breast adenocarcinoma and A2780 ovarian carcinoma cell lines, both adherent immortalized cell lines. Two leukaemia cell lines were also used, namely the THP-1 and HUT-78 cells.

The THP-1 human leukemic monocyte cell line grew in suspension, in cell RPMI-1640 culture media enriched with foetal bovine serum (FCS), glutamine, penicillin-streptomycin, non-essential amino acid solution (NEA), 2-sulfanylethanol and sodium pyruvate. Cells were thawed, carefully; they grew in culture flasks, in a sterile incubator at 37 °C, 5% CO_2, in humidified atmosphere. For cells growing in suspension passages were made by centrifugation of the cell culture flask content and redistribution in subcultures. After expansion and two passages cells were placed on 96-well microplates, in 95 μl of media, at a concentration of 20 000 cells per well. Plates were kept in incubator and after approximately 20 h they were treated with the solution of the compounds.

HUT-78 is a T-cell lymphoma human malignant cell line: the cells have lymphoblast morphology. They were cultivated and prepared for experiments similarly with THP-1 cells.

MCF-7 cells were grown in MEM cell culture media, supplemented with NEA, FCS and glutamine. The passages were made enzymatically with a Trypsine-EDTA solution. The appropriate cell culture media for the A2780 cell line is RPMI-1640 supplemented with FCS and glutamine. The two adherent cell lines were seeded onto a microplate at a concentration of 20 000 cells per well, in 190 μl cell culture media, and after about 20 h they were treated with 10 μl of solution of the studied compounds.

Separation of lymphocytes from whole blood was made using a method improved by our laboratory [50], by density gradient centrifugation, using Histopaque solution. The lymphocyte ring separated between plasma and the separation solution was harvested using a pipette. Cells were washed twice in Hank's balanced salt solution.

The lymphocytes were preserved in an ultra freezer at −70 °C and they were defrosted gently in order to preserve their viability, according to the protocol of Hemelen et al. Cryopreservation does not alter the lymphocyte viability and preserves the surface markers as well, if handled carefully [51]. The lymphocytes were seeded on 96-well plates, at a density of 30 000 cells in 95 μl RPMI-1640 media having the supplements described for the THP-1 cells line. Lymphocytes do not proliferate under these conditions, differently from the leukaemia cells, consequently more cells were needed to obtain a cell density similar to the malignant populations after 24 h.

The compounds were solubilised directly in the RPMI-1640 culture media. For each compound 7 concentrations were prepared, in the range from 20 mM to 10 μM. Cells were treated by adding 10 μl solution of the alkylphosphocholine into each well containing 190 μl media in adherent cells, or 5 μl in 95 μl cellular suspension. The final concentration of the alkylphosphocholines varied between 1 mM and 1 μM. Untreated cells were used as a reference.

After 24-h treatment, cytotoxicity assessment was performed on the cells. The microplates were stained with MTS dye (CellTiter 96 Aqueous Proliferation Assay from Promega), when cell lines were in suspension, or with MTT dye (Thiazolyl Blue Tetrazolium Bromide from Sigma Aldrich) for adherent cells, according to a method described earlier [52]. The two methods are convergent; they are based on the same principle: the capacity of living cells mitochondria to transform the dye into coloured formazan form. The only difference between the two methods is the solubility of the crystals yielded by the viable cells. In adherent cell lines, the MTT is a convenient method, because the supernatants can be easily removed and then solubilise the formazan products in dimethyl sulfoxide, while the MTS formazan form is water-soluble and requires no further processing. The microplates were measured at 492 nm/520 nm using the multiplate reader and the extinctions were analysed with Gen5 software. The IC50 values for each compound were calculated with the Graph Prism5 biostatistics software, using the best-fit values of the log inhibitor versus normalized response sigmoidal curve. Significance of values was examined using the column statistics, one-way ANOVA test and Bonferroni post-test.

4.4. Antiprotozoal assay

The antiprotozoal assay was carried out according to the method modified by Mrva et al. [22]. Amoebas used for testing were *A. lugdunensis* and *A. quina*. From the 2-day monoxenic cultures on agar plates, the trophozoites were axenized by inoculation into the Bacto-Casitone/Serum medium (BCS) with penicillin and ampicillin. After 72 h the active trophozoites were transferred into peptone-yeast extract-glucose medium (PYG) with penicillin and ampicillin. After 5 passages the trophozoites were transferred into a PYG medium without antibiotics and consecutively cultivated in this medium. Cytotoxicity measurements were performed in sterile 96-well microtiter plates. Each well was seeded with 100 μl (2×10^5 cells ml^{-1}) of a trophozoite suspension. Then, 100 μl of a freshly prepared medium containing APC at 6 concentrations was added to all wells except untreated control wells, which received 100 μl of a pure medium. The APCs were tested at final concentrations of 500, 250, 125, 62.5, 31.25, and 15.6 μM. The reduction of trophozoites was recorded after 1, 24 and 48 h by counting the surviving cells in a Bürker-Türk haemocytometer. Viability of trophozoites was determined by trypan blue exclusion; 100% eradication was confirmed by transferring 50 μl of the suspension to a PYG medium and recording the amoeba growth for 14 days. The lowest concentration of the APC supporting 100% eradication of the trophozoites was defined as the minimal trophocidal concentration (MTC). The cultivations and the cytotoxicity measurements were

carried out at 37 °C for *A. quina* as well as *A. lugdunensis*. The experiments were repeated 4 times for each concentration.

4.5. Antimicrobial assay

The antimicrobial activity was measured against Gram-positive bacteria *S. aureus* ATCC 29/58, Gram-negative bacteria *E. coli* ATCC 377/79 and yeast *C. albicans* ATCC 8186. Testing was performed by a method used previously by Lukáč et al. [53]. Solutions of studied compounds were prepared in water. Stock solution had the concentration of 1000 μg/ml. Cultures of bacteria and yeast grew 24 h in blood agar and in the Sabouraud agar respectively. From these cultures suspensions of microorganisms were prepared with concentration of 5×10^7 CFU/ml of bacteria and 5×10^5 CFU/ml of *Candida*. The suspensions were prepared in physiological solution (pH 7.2). Concentrations of microorganisms were determined spectrophotometrically and the suspensions were adjusted to absorbance $A = 0.35$ at $\lambda = 540$ nm. The microorganism suspension (5 μl) was added to solutions containing the tested compound (100 μl) and to double concentrated peptone broth medium (8%) for bacteria or Sabouraud medium (12%) for *Candida* (100 μl). For a testing assay the solutions were serially diluted by half (concentrations of the solutions were: 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 μg/ml) and used. Tests were performed in 96-well microtiter plates. The microorganisms were incubated for 24 h at 37 °C. Then from each well, 5 μl of tested suspension was taken and cultured on blood agar (bacteria) or on Sabouraud agar (yeast). The Petri dishes were incubated for 24 h at 37 °C. The lowest concentration of APC that prevented colony formation was defined as the minimal inhibition concentration (MIC).

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