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Synthesis and biological activity of dialkylphosphocholines

Miloš Lukáč [a,b,*], Martin Mrva [c], Eva Fischer-Fodor [d], Ivan Lacko [a], Marián Bukovský [e], Natalia Miklášová [a], František Ondriska [f], Ferdinand Devínsky [a]

[a] Department of Chemical Theory of Drugs, Faculty of Pharmacy, Comenius University, Kalinčiakova 8, 832 32 Bratislava, Slovakia
[b] NMR laboratory, Faculty of Pharmacy, Comenius University, Odbojárov 10, 832 32 Bratislava, Slovakia
[c] Department of Zoology, Faculty of Natural Sciences, Comenius University, Mlynská Dolina B-1, 842 15 Bratislava, Slovakia
[d] Tumor Biology Laboratory, Research Department, 'I. Chiricuta' Cancer Institute, 34-36 Republici St., 400015 Cluj Napoca, Romania
[e] Department of Cell and Molecular Biology of Drugs, Faculty of Pharmacy, Comenius University, Kalinčiakova 8, 832 32 Bratislava, Slovakia
[f] HPL (Ltd) Department of Parasitology, Microbiological Laboratory, Istrijská 20, 841 07 Bratislava, Slovakia

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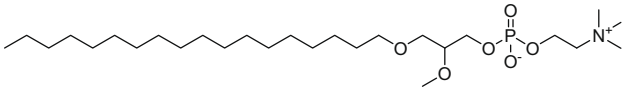
ABSTRACT

A series of dialkylphosphocholines were prepared and evaluated for their biological activity. The antiprotozoal activity was determined against *Acanthamoeba lugdunensis*. Compound **15** exhibited excellent trophocidal activity. None of the tested dialkylphosphocholines exhibited better fungicidal activity against *Candida albicans* than miltefosine. The antineoplastic activity was determined against HeLa. The most cytotoxic was compound **10**, which was more active against tumor cells as against normal cells.

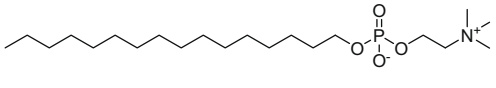
Synthetic alkyletherphospholipids (AEPs) constitute a heterogeneous group of unnatural lipids, which have a potential antineoplastic activity.[1–10] 1-*O*-Octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (edelfosine, **Et-18-OCH$_3$**) and hexadecylphosphocholine (miltefosine, **HPC**) are the most important members of this family (Fig. 1). **Et-18-OCH$_3$** is a prototype of alkyllysophospholipids (ALPs) and **HPC** represents the alkylphosphocholines (APCs). **Et-18-OCH$_3$** shows selective apoptosis in tumor cells, whereas normal cells are sparsed.[11] Gajate et al.[12–15] proved that **Et-18-OCH$_3$** induces selectively apoptosis in leukemic cells due to its preferential incorporation into cancer cells. This process leads to the intracellular activation of the death receptor Fas/CD95 by 'its recruitment together with downstream signaling molecules into cluster of lipid rafts'.[15] Examples of clinical relevant anticancer APCs include the prototypic compound **HPC** and the novel drug octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate (perifosine).[16,17] Miltex®, which is a 6% solution of **HPC**, is used as a palliative drug for treatment of cutaneous metastases from breast cancer.[16]

The effect of AEPs is not related only to antineoplastic activity but they have shown also activity against fungi and protists. **HPC** and other APCs have a broad-spectrum fungicidal activity.[10,18–20] Obando et al.[18] tested the set of 14 AEPs. The highest antifungal activity exhibited octadecylphosphocholine.[18] **Et-18-OCH$_3$** possessed good activity against *Candida albicans* but *Cryptococcus neoformans* was not so sensitive. They observed that the ALPs appear to be less active against fungi than the APCs.[18] Irlbacholine, another type of APCs, it is the bolaamphiphile (bis[2-(trimethylammonio)ethyl]docosane-1,22-diyl bis(phosphate)). Irlbacholine, as a plant metabolite isolated from *Irlbachia alata* and *Anthocleista djalonensis*, exhibited excellent antifungal activity against pathogenic fungi.[21] Other analogues of irlbacholine with shorter or longer alkyl chain were less active.[22]

AEPs posses also a wide range of antiprotozoal activity. They exhibit antiprotozoal activity on *Leishmania* spp.,[23–28] *Trypanosoma*



Edelfosine



Miltefosine (**HPC**)

Figure 1. Alkyletherphospholipids.

* Corresponding author. Tel.: +42 1250117322; fax: +42 1250117357.
E-mail address: lukac@fpharm.uniba.sk (M. Lukáč).

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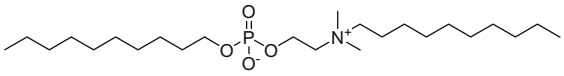
spp.,[29] *Acanthamoeba* spp.,[10,30–36] *Entamoeba histolytica*,[37] *Trichomonas vaginalis*,[38] *Balamuthia mandrillaris*, and *Naegleria fowleri*.[32] **HPC** (Impavido®) was registered as the first oral drug for treatment of visceral leishmaniasis in India and Germany and for treatment of cutaneous leishmaniasis in Colombia.[24] Free-living amoebae of the genus *Acanthamoeba* are the opportunistic pathogens. They are causative agents of *Acanthamoeba* keratitis (AK), and they have also been associated with infections that occur mainly in the immunocompromised host, like, granulomatous amoebic encephalitis (GAE), cutaneous lesions, pneumonitis, and sinusitis.[39–41] There are no efficient and handily treatments for AK or GAE.[42] The most significant reason for the unsuccessful therapy seems to be the existence of a double-wall cyst stage that is highly resistant to the available treatments, causing reinfections.[43] AK is often treated with a combination of polyhexamethylene biguanide or chlorhexidine and propamidine isethionate. However, the therapy with these substances failed in many cases.[41] **HPC** shows a good activity against *A. polyphaga*, *A. lenticulata*, and *A. castellanii*,[30,31] while **Et-18-OCH$_3$**, as a representant of ALPs, possesses very low activity against *A. castellanii* and *A. polyphaga*.[31]

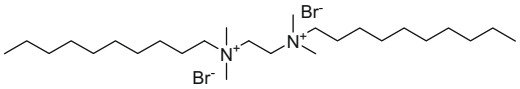
Alkyl$_{(x)}$ 2-[alkyl$_{(y)}$(dimethyl)ammonio]ethyl phosphates with branched or unbranched alkyl chains represent phosphate-quaternary ammonium heterogemini surfactants (HSs).[44,45] Those are special type of APCs, where one methyl group of choline is changed to a long alkyl chain. They can also be named as dialkylphosphocholines (DAPCs). HSs possess interesting physicochemical properties.[44–49] Depending on the length of alkyl chains, they form micelles, vesicles, tubules, or coacervates in water.[44]

This Letter describes the synthesis of a series of DAPCs, alkyl$_{(x)}$ 2-[alkyl$_{(y)}$(dimethyl)ammonio]alkyl$_{(z)}$ phosphates. Two sets of surfactants are presented. The first series contains compounds **1**–**10**, which have 22 carbon atoms alkyl chains bound to both, ammonium and phosphate moieties while the second series (compounds **5**, **11**–**14)** presents as well different linker unit between ammonium and phosphate groups (2, 3, 4, 6, 10 carbon atoms). Compounds **15** and **16** were prepared for comparison of their biological activities to DAPCs versus biscationic surfactant (Fig. 2).

The synthetic strategy for the preparation of the DAPCs is depicted in Schemes 1 and 2. The phosphocholine analogues were prepared according to the literature procedures.[10,25] The strategy

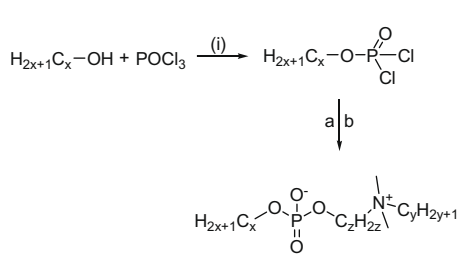


15



16

Figure 2. Structure of DAPC **15** and biscationic surfactant **16**.



Scheme 1. Reagents: (i) $CHCl_3$, triethylamine; (a) (ii) pyridine, choline *p*-toluenesulfonate; (iii) H_2O; (b) (ii) H_2O; (iii) pyridine; (iv) 2,4,6-triisopropylbenzenesulfonyl chloride, choline bromide. Subscripts *x*, *y*, and *z* are described in Table 1.

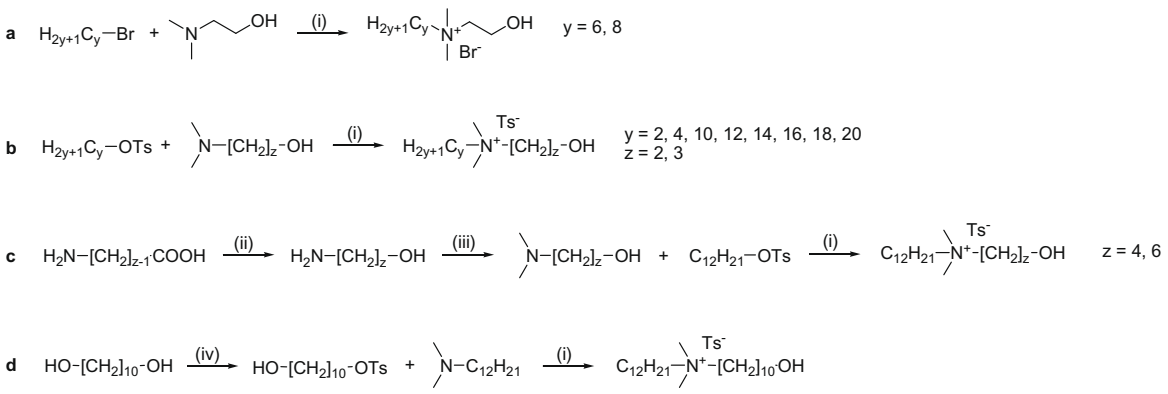
involves phosphorylation of the alcohols using $POCl_3$ and subsequent attachment of the desired choline analogue. Compounds **1**–**6** and **9**–**15** were prepared by reaction of alkyl dichloridophosphate with choline *p*-toluenesulfonate and subsequently hydrolyzed (Scheme 1a). The compounds **7** and **8** were prepared using another strategy (Scheme 1b). Alkyl dichloridophosphate was firstly hydrolyzed by water and transformed to dipyridinium hexadecyl phosphate using pyridine. This salt was then bounded to choline bromide in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride. The choline analogues were prepared by quaternization of tertiary *N*,*N*-dimethylaminoalcohol with bromoalkanes (Scheme 2a) or alkyl *p*-toluenesulfonates (Scheme 2b) while *N*-ethyl-10-hydroxy-*N*,*N*-dimethyldecan-1-ammonium *p*-toluenesulfonate was prepared by other method. Decane-1,10-diol was firstly monotosylated[50] and subsequently was quaternized with dodecyldimethylamine (Scheme 2d). *N*-Ethyl-4-hydroxy-*N*,*N*-dimethylbutan-1-ammonium *p*-toluenesulfonate and *N*-ethyl-6-hydroxy-*N*,*N*-dimethylhexan-1-ammonium *p*-toluenesulfonate were prepared by reduction of desired ω-aminoacids with borane–tetrahydrofurane complex,[51] followed by Eschweiler–Clarke methylation[52] of ω-aminoalcohols (Scheme 2c). Compounds **16**[53] (Fig. 2) and **HPC**[54] were prepared according to the literature procedure.

The final products were purified by crystallization or by silica gel column chromatography and analyzed by ^{1}H, ^{13}C, ^{31}P NMR and IR spectroscopies.[55]

The antiprotozoal activity of these products against *Acanthamoeba lugdunensis* was determined using a previously described assay.[10] *A. lugdunensis* was isolated from a patient suffering from amoebic keratitis.[56] Minimal trophocidal concentration (MTC) of **HPC** was 400 μM. **HPC** (a standard APC) exhibited relatively weak activity against this strain of *Acanthamoeba*. Other species of *Acanthamoeba: A. polyphaga*, *A. lenticulata*, *A. castellanii* seem to be more sensitive to **HPC**.[30,33] DAPCs **1**–**10** were less active than **HPC**. Compounds **11** and **12** (Table 1) exhibited better activity than **5**, which has only two carbon atoms linker chain. The MTC of **11** and **12** was four times, respectively, two times lower than MTC of **HPC**. The most active compound was **15**, which has an excellent value of MTC = 25 μM, while compound **5** shows a poor activity. The difference between compounds **5** and **15** consists two carbon atoms in one alkyl chain. DAPC **15** was approximately 10 times more active than biscationic surfactant **16**.

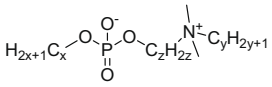
The antimycotic activity of investigated compounds was tested against *C. albicans* according to the procedure described previously.[10] **HPC** exhibited very good fungicidal activity its MFC (minimal fungicidal concentration) was 4 μM. All compounds exhibited MFC values higher than **HPC**. Compounds **1** and **10** were less active than other compounds of the series (**2**–**9**). The length of linker units is important for the activity of DAPCs of the second series. DAPCs with shorter linker chains, **5**, **11**, and **12**, were more active than the other ones with longer linker chains. The activity of DAPC **15** is comparable with the activity of biscationic surfactant **16**.

The cytotoxic activity of these compounds against Hfl-1 (human lung fibroblast cells), normal cells, which have a rapid cellular turnover and against HeLa (human cervix carcinoma cells) was determined using a previously described assay[57] with small modifications.[58] The cytotoxicity was expressed as the half maximum inhibitory concentration (IC_{50}). The most cytotoxic was compound **10**, which was more active against tumor cells as against normal cells. Figures 3 and 4 illustrate the untreated and treated HeLa cells with compound **10**. Standard compound **HPC** exhibited also higher toxicity against tumor cells. The other six tested compounds (**3**, **5**, **6**, **9**, **11**, and **12**) shown higher toxicity against normal cell lines, while aggressive tumor cell line seems to be somehow more resistant. The investigation demonstrated that APCs (**HPC**, **10**) possess a better antineoplastic activity than DAPCs (**3**, **5**, **6**, **9**, **11**, and **12**).



Scheme 2. Reagents: (i) CH_3CN; (ii) $NaBH_4$, I_2, THF; (iii) HCHO, HCOOH; (iv) *p*-toulenesulfonyl chloride, triethylamine.

Table 1
Structural characterization of dialkylphosphocholines and their antiprotozoal, antifungal, and cytotoxic activity



No.	Number of carbon atoms			*A. lugdunensis*	*C. albicans*	Cytotoxic activity	
	x	*y*	*z*	MTC (μM)	MFC (μM)	Hfl-1 IC_{50} (μM)	HeLa IC_{50} (μM)
1	2	20	2	>400	1238.4	nd	nd
2	4	18	2	>400	9.7	nd	nd
3	6	16	2	>400	19.3	110.4	169.8
4	8	14	2	>400	9.7	nd	nd
5	10	12	2	>400	19.3	43.8	118.4
6	12	10	2	>400	9.7	58.8	130.8
7	14	8	2	>400	19.3	nd	nd
8	16	6	2	>400	77.4	nd	nd
9	18	4	2	>400	19.3	113.4	161.1
10	20	2	2	>400	619.2	27.2	**20.3**
11	10	12	3	100	9.4	57.7	111.8
12	10	12	4	200	18.6	94.6	148.3
13	10	12	6	>400	34.8	nd	nd
14	10	12	10	>400	8104.6	nd	nd
15	10	10	2	**25**	20.5	nd	nd
16	–	–	–	200	17.5	nd	nd
HPC	–	–	–	400	**4.0**	203.4	128.9

No. = number of compounds.
Values in bold character correspond to the most active compounds.
nd = not determined.

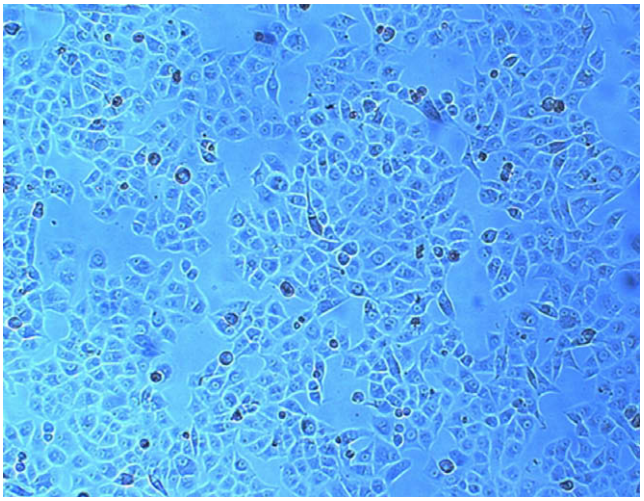


Figure 3. Untreated HeLa cells.

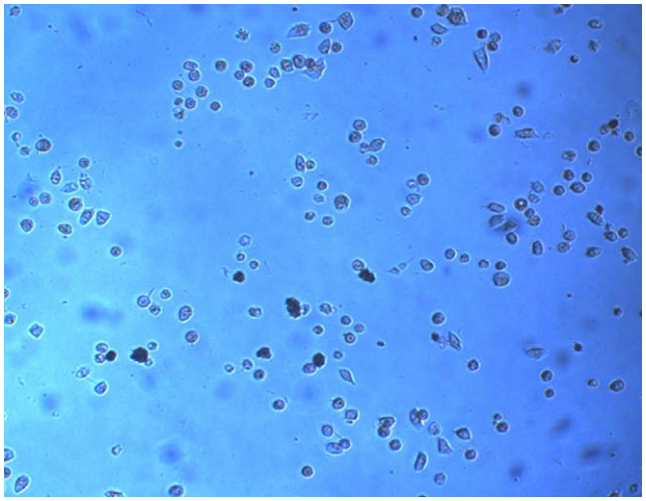


Figure 4. HeLa cells treated with **10**.

In conclusion, we have described synthesis of 15 DAPCs. Their biological activity was compared with **HPC** and biscationic surfactant **16**. The antiprotozoal activity was determined against *A. lugdunensis*. Compound **15** exhibited excellent trophocidal activity. The antifungal activity was determined against *C. albicans*. None of the tested DAPCs exhibited better activity than **HPC**. The cytotoxicity was determined against Hfl-1 and HeLa. The most cytotoxic was compound **10**, which was more active against tumor cells as against normal cells.

Acknowledgments

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55. *Characterization of compound* **5**: ^{1}H NMR ($CDCl_3$, TMS) δ 0.84–0.92 (m, 6H), 1.18–1.41 (m, 32H), 1.55–1.78 (m, 4H), 2.18 (s, 2H), 3.35 (s, 6H), 3.41–3.50 (m, 2H), 3.76–3.89 (m, 4H), 4.27–4.35 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1, 22.7, 22.9, 26.0, 26.3, 29.3, 29.4, 29.5, 29.6, 29.7, 31.0, 31.1, 31.9, 51.9, 58.8, 64.2, 65.5, 65.6, 65.9; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ 0.76; IR ν_{max}/cm^{-1} 3427, 2919, 2851, 1635, 1469, 1242, 1100, 1067, 822.
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58. HeLa cells were cultivated using Dulbecco's Modified Eagle's medium. For the IC_{50} measurements, cells were seeded on 96-well cell culture plates in a concentration of 10,000 cells per well, in 200 μl of cell culture media. Compounds were dissolved in Ca- and Mg-free phosphate buffered saline (PBS) solution in stock concentrations (10 mg/ml). In order to obtain the IC_{50} values, the experiments for all compounds were performed for a concentration series (from 1 to 200 μg/ml).