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Relationship between aggregation properties and antimicrobial activities of alkylphosphocholines with branched alkyl chains

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ABSTRACT

Synthesis of five alkylphosphocholines with branched alkyl chains (Isophol-PCs) with different length of alkyl chains was described. Isophol$_8$-PC and Isophol$_{12}$-PC represent new compounds. The physico-chemical properties of Isophol-PCs were determined, critical micelle concentration and types of formed aggregates in aqueous solutions were investigated. The biological activities of Isophol-PCs have been studied for the first time in the present study. Antimicrobial activities of alkylphosphocholines were studied against bacteria (*Staphylococcus aureus*, *Escherichia coli*), yeast (*Candida albicans*) and pathogenic free-living amoebae (*Acanthamoeba lugdunensis* and *Acanthamoeba quina*). *A. lugdunensis* and *A. quina* are relatively insusceptible to action of miltefosine (standard compound of alkylphosphocholines) and therefore they are good models for studies of amoebicidal action of the investigated compounds. Relationship between structure, physico-chemical and biological activities of Isophol-PCs was discussed. *S. aureus* and *C. albicans* were sensitive to action of Isophol$_{16}$-PC, Isophol$_{20}$-PC. *E. coli* was not sensitive to action of all studied alkylphosphocholines in the concentrations equal to, or less than 10 mM. Among all the synthesized compounds, Isophol$_{16}$-PC had the highest level of activity against both strains of *Acanthamoeba*. The minimum trophocidal concentrations of Isophol$_{16}$-PC against *A. lugdunensis* and *A. quina* are about four times lower than the minimum trophocidal concentrations of miltefosine against booth strains.

1. Introduction

Free-living amoebae of the genus *Acanthamoeba* are known as the causative agents of a painful, progressive, and vision-threatening eye disease *Acanthamoeba* keratitis (AK) in immunocompetent individuals, and granulomatous amoebic encephalitis (GAE) and disseminating infections in immunodeficient patients. The treatment of GAE and disseminated infections is to date only rarely successful, and it is much dependent on early diagnosis. AK was treated with a series of drugs with inconsistent effects. Propamidine isethionate (Brolene®), polyhexamethylene biguanide, and chlorhexidine are used in the treatment, however, their application is difficult to handle as it must be frequent and long-lasting. Simple and easily manageable treatment is not available, and cases of failure of therapy and a resistance to those drugs were also reported (Khan, 2009; Schuster and Visvesvara, 2004a,b).

Yeasts of the genus *Candida* cause candidosis, an emerging mycosis with high frequency, and many health complications. The superficial candidosis, including cutaneous, mucosal, mucocutaneous, onychial, and granulomatous candidosis, are typical with chronic course and recurrence (Farah et al., 2000; López-Martínez, 2010). They are treated by topical application of various antimycotics, e.g., imidazoles, allylamines, nystatin. The systemic candidosis is usually treated with flucytosine and azoles (Pinto et al., 2008). In cases of poor clinical response or resistance observed mostly with fluconazole and itraconazole, amphotericin B is administered intravenously. Due to its nephrotoxicity, the dosage must be adjusted case-by-case (López-Martínez, 2010).

The rise of the resistance of pathogenic bacteria to antibiotics worldwide has sustained the development of new antibacterial drugs (Llull et al., 2007). Although *Escherichia coli* is a pre-dominant species among the facultative anaerobic normal

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flora of the intestine, some strains are capable of causing diseases which can disseminate throughout the body implicating in urinary tract infections, gastro-intestinal infections, and sepsis/meningitis (Chen and Frankel, 2005). *Staphylococcus aureus* is an important human pathogen known for causing a wide variety of infections ranging from skin and soft tissue infections (Ortega-Loayza et al., 2010) to life threatening disseminated diseases (François et al., 2010). Most of the isolates are susceptible to clindamycin, trimethoprim/sulphamethoxazole, doxycycline, gentamycin, vancomycin, chloramphenicol, rifampicin, linezolid (Maltezou and Giamarellou, 2006). However, the capacity of *S. aureus* to develop antimicrobial resistance (Hiramatsu et al., 2001) prompts the permanent development of new drugs.

Alkylphosphocholines (APCs) are groups of compounds with a wide spectrum of biological properties. Hexadecylphosphocholine (HPC) is the main representative of this type of compounds. It was for the first time synthesized in 1958 (Hirt and Brechtold, 1958). However, its biological activities were discovered later. It possesses antineoplastic (Houlihan et al., 1995), antibacterial (Llull et al., 2007), antimycotic (Widmer et al., 2006), antiprotozoal (Croft et al., 2003) and antivirotic (Cugh et al., 2008) activities. Currently, antileishmanial activity of HPC and other APCs has been studied the most (Aguiar et al., 2010; Calogeropoulou et al., 2008; Griewank et al., 2010; Hornillos et al., 2008; Papanastasiou et al., 2010). HPC is conventionally used for the treatment of leishmaniasis (Van Griensven et al., 2010). HPC was registered as the first oral drug for treatment of visceral leishmaniasis (Impavido®) in India and Germany and for treatment of cutaneous leishmaniasis in Colombia (Seifert et al., 2007). The HPC is active against more parasites than merely *Leishmania*, it exhibits also antiprotozoal activity against *Acanthamoeba*, *Balamuthia*, *Naegleria* (Schuster et al., 2006), *Trichomonas* (Blaha et al., 2006), *Entamoeba* (Seifert et al., 2001) or *Trypanosoma* (Saraiva et al., 2009). Trophocidal activities of HPC and other APCs against *Acanthamoeba* spp. were for the first time described by Walochnik et al. (2002). Subsequently, several investigations of amoebicidal activities of APCs were published (Lukáč et al., 2009a,b, 2010c; McBride et al., 2005, 2007; Mrva et al., 2011). HPC was used in successful treatment of disseminated *Acanthamoeba* sp. infection (Aichelburg et al., 2008). Its anti-*Acanthamoeba* efficacy was also tested in an organotypic skin equivalent (Walochnik et al., 2009), and for a topical treatment of experimental *Acanthamoeba* keratitis in Syrian hamsters (Polat et al., 2011).

HPC and other APCs are zwitterionic surfactants. HPC contains hydrophobic and hydrophilic moieties. The hydrophobic part is represented by an alkyl chain and the hydrophilic part is the phosphocholine group. The phosphocholine group contains two charges, one positive (trimethylammonium cation) and one negative (phosphate anion), which are connected with the alkyl chain. Positive and negative charges are compensated in the molecule and therefore some physicochemical properties of HPC and APCs are more similar to nonionic surfactants than to ionic surfactants, e.g., the critical micelle concentration (cmc) of HPC is 12.5 μM (Lukáč et al., 2010b) it is similar to the value of cmc for Brij 56 (cmc = 51 μM) (Kabir-ud-Din et al., 2009) but is much lower than the cmc of cetyltrimethylammonium bromide (cmc = 850 μM) (Lukáč et al., 2010b). The knowledge of physicochemical properties of amphiphilic compounds is important for explaining their biological activities or medical and pharmaceutical properties (Christiansen et al., 2010; Colomer et al., 2011; Lukáč et al., 2011; Weng et al., 2011; Zidan et al., 2011).

The aim of this study was the synthesis of APCs with branched alkyl chains (Isophol-PCs), the study of their physicochemical properties, and the evaluation of their potential efficacies against bacteria, yeasts and amoebae.

2. Materials and methods

2.1. Materials

All chemicals used for the synthesis were purchased from commercial suppliers. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Varian Gemini 2000 spectrometer operating at 300, 75.5, and 121.5 MHz, respectively, with ^{13}C and ^{31}P spectra being recorded with proton-decoupling. The spectra were measured in $CDCl_3$ relative to the internal standard TMS for ^{1}H and ^{13}C NMR spectra and to the external standard 85% H_3PO_4 for ^{31}P NMR spectra. Infrared spectra were recorded on a FT-IR Impact 400 D spectrophotometer as potassium bromide discs. Blood agar base No. 2, Sabourauds agar, Nutrient broth No. 2, Sabouraud medium, glucose, peptone, yeast extract was purchased from Imuna Pharm a.s., Slovakia. Bacto-Casitone was obtained from *E. coli*, Slovakia.

2.2. Synthesis of APCs

The different APCs with branched alkyl chains (Isophol-PCs) were prepared from the respective Guerbet alcohols (Isophols) according to synthetic routes described in Lukáč et al. (2009a). Solution of the alcohol (9 mmol) in chloroform (20 ml) was added dropwise at 0 °C to a stirred solution of phosphorus oxychloride (10 mmol) and triethylamine (20 mmol) in chloroform (10 ml). The resulting mixture was stirred at room temperature (r.t.) for 2 h. This intermediate was used immediately without any purification. Pyridine (15 ml) was added dropwise at $t = 0$ °C to the resulting solution, followed by the addition of choline tosylate (12.5 mmol). The reaction mixture was stirred at r.t. overnight. After cooling, the mixture was hydrolyzed by addition of H_2O (1.5 ml) and stirred for 1 h at r.t. The solvents were evaporated in vacuum and the resulting crude solid was dissolved in a mixture of tetrahydrofuran–water (5:1 V/V). To the stirred solution, exchange resin Amberlite MB-3 was added sequentially until the color of the resin ceased to change. Then, the resin was filtered off and the solvents were evaporated in vacuum. The resulting crude solid was purified by crystallization from a mixture of chloroform and acetone or chloroform and diethyl ether ($Isophol_8$-PC, $Isophol_{12}$-PC) or by flash chromatography using $CHCl_3$/MeOH/H_2O (60/25/4, V/V/V) as a liquid phase ($Isophol_{16}$-PC, $Isophol_{20}$-PC, $Isophol_{24}$-PC). APCs were dried *in vacuo* over P_4O_{10}.

2-ethylhexyl 2-(trimethylammonio)ethyl phosphate × H_2O ($Isophol_8$-PC): Yield 19.2%; ^{1}H NMR ($CDCl_3$) δ: 0.86 (t, 6H, $J = 6.5$ Hz), 1.18–1.41 (m, 8H), 1.41–1.53 (m, 1H), 3.40 (s, 9H), 3.61–3.75 (m, 2H), 3.78–3.85 (m, 2H), 3.96 (s, 2H), 4.21–4.31 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 10.9, 14.1, 23.1, 23.3, 29.0, 30.0, 40.3, 40.4, 54.3, 59.1, 59.2, 66.3, 67.7, 67.8; ^{31}P NMR ($CDCl_3$) δ: −0.26; IR ($_{max}$/cm^{-1} 3413, 2930, 2872, 1664, 1489, 1463, 1245, 1085, 1064, 971.

2-butyloctyl 2-(trimethylammonio)ethyl phosphate × H_2O ($Isophol_{12}$-PC): Yield 26.9%; ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, 6H, $J = 6.5$ Hz), 1.15–1.40 (m, 16H), 1.46–1.59 (m, 1H), 3.40 (s, 9H), 3.61–3.72 (m, 2H), 3.75–3.83 (m, 2H), 4.01 (s, 2H), 4.22–4.30 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 14.1, 14.2, 22.7, 23.1, 26.7, 28.9, 29.8, 30.6, 30.9, 31.9, 38.9, 39.0, 54.3, 59.1, 66.2, 68.2, 68.3; ^{31}P NMR ($CDCl_3$) δ: −1.59; IR υ_{max}/cm^{-1} 3419, 2928, 2858, 1659, 1489, 1465, 1244, 1085, 971.

2-hexyldecyl 2-(trimethylammonio)ethyl phosphate × H_2O ($Isophol_{16}$-PC): Yield 12.5%; ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, 6H, $J = 6.6$ Hz), 1.17–1.48 (m, 24H), 1.48–1.60 (m, 1H), 3.40 (s, 9H), 3.61–3.72 (m, 2H), 3.75–4.00 (m, 4H), 4.25–4.35 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 14.1, 22.7, 26.7, 26.8, 29.4, 29.7, 29.8, 30.1, 30.9, 31.9, 38.9, 39.1, 54.3, 59.1, 66.3, 68.3, 68.4; ^{31}P NMR ($CDCl_3$) δ: −0.31; IR υ_{max}/cm^{-1} 3420, 2926, 2855, 1638, 1488, 1465, 1243, 1086, 970.

2-octyldodecyl 2-(trimethylammonio)ethyl phosphate × 0,5 H_2O ($Isophol_{20}$-PC): Yield 5.9%; ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, 6H, $J = 6.6$ Hz),

1.16–1.45 (m, 32H), 1.50–1.62 (m, 1H), 3.36–3.68 (m, 10H), 3.72–3.89 (m, 2H), 3.92–4.02 (m, 2H), 4.28–4.50 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 14.1, 22.7, 26.7, 29.4, 29.6, 29.7, 29.8, 30.1, 30.7, 31.9, 38.8, 38.9, 54.4, 59.7, 66.1, 69.1; ^{31}P NMR ($CDCl_3$) δ: −0.91; IR υ_{max}/cm^{-1} 3425, 2924, 2854, 1633, 1466, 1241, 1088, 1043, 971.

2-decyltetradecyl 2-(trimethylammonio)ethyl × 0,5 H_2O (Isophol$_{24}$-PC): Yield 15.6%; ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, 6H, J = 6.7 Hz), 1.15–1.45 (m, 40H), 1.50–1.62 (m, 1H), 3.44 (s, 9H), 3.71–3.78 (m, 2H), 3.88–4.03 (m, 2H), 4.29–4.47 (m, 2H), 5.44–6.08 (m, 1H); ^{13}C NMR ($CDCl_3$) δ: 14.1, 22.7, 26.7, 29.4, 29.6, 29.7, 30.2, 30.7, 31.9, 38.8, 38.9, 54.4, 59.6, 66.2, 69.0; ^{31}P NMR ($CDCl_3$) δ: −0.57; IR υ_{max}/cm^{-1} 3399, 2923, 2853, 1643, 1466, 1238, 1087, 1050, 969.

2.3. Equilibrium surface tension

The critical micelle concentration values of the surfactants were determined from the surface tension isotherm according to the described procedure (Lukáč et al., 2010b). The solvent surface tension values were measured by the Wilhelmy plate technique using a Kruss 100 MK2 tensiometer. Deionized water was used in the preparation of all samples. The temperature of the measurements was kept at 25 ± 0.1 °C. Measurements of equilibrium surface tension were taken repeatedly (every 6 min) until the change in surface tension was less than 0.05 mN m^{-1}. The critical micelle concentration (cmc) and surface tension at the cmc (γ_{cmc}) were determined from the break point of the surface tension vs. log c curve. From the surface tension data, the adsorbed amount of surfactant Γ is calculated utilizing the Gibbs adsorption isotherm.

$$\Gamma_{cmc} = \frac{-[d\gamma/d \log c]_T}{(2.303iRT)} \quad (1)$$

where γ is the surface tension (mN m^{-1}), c is the surfactant concentration, i is the prefactor (i = 1), T is the absolute temperature and R the gas constant. Surface excess could be determined from the slope below the cmc in the surface tension vs. log c plots. Surface area at the surface saturation per head group (A_{cmc}) is obtained from the equation

$$A_{cmc} = \frac{10^{16}}{N_A \Gamma_{cmc}} \quad (2)$$

where N_A is the Avogadro constant.

2.4. Determination of critical micelle concentration by ^{13}C NMR and ^{31}P NMR spectroscopy

The measurements of cmcs by ^{13}C NMR and ^{31}P NMR spectroscopy were performed according to a modified procedure (Babu et al., 2005; Desando and Reeves, 1986; Mirgorod et al., 2010; Plückthun and Dennis, 1981). Stock solutions used for the determination of cmc were prepared by mixing a calculated amount of a surfactant with 50 μl of D_2O and 200 μl of H_2O. Dilutions of the stock solutions were performed with deionized water. The spectra were measured at 25 ± 0.1 °C. TMS or 85% H_3PO_4 were used as the external standards. The chemical shifts of the carbon atom signal of a methyl group of the longer alkyl chain or the signal of the phosphate group were recorded. The changes of the chemical shifts depending on the concentration of the surfactant were monitored. The cmc values were determined from the break point of the chemical shift vs. reciprocal concentration curve.

2.5. Measurement of aggregates of APCs in water by ^{31}P NMR

Measurement of aggregation of APCs in water by ^{31}P NMR spectroscopy was performed according to described procedure (Lukáč et al., 2010c). The samples were prepared in an NMR tube. 100 μl of deionized water were added to 50 mg of APCs. The mixtures were homogenized by several cycles of heating to about 50 °C and cooling to −18 °C. ^{31}P NMR spectra were measured on the prepared samples at 25 °C, 37 °C and 75 °C.

2.6. In vitro antibacterial and anticandidal activity assay

The antimicrobial activity was tested against Gram-negative bacteria *Escherichia coli* ATCC 11229, Gram-positive bacteria *Staphylococcus aureus* ATCC 6538, and yeasts *Candida albicans* ATCC 8186 and *Candida albicans* ATCC 4553. The minimum inhibitory concentrations (MIC) of Isophol-PCs were determined by the previously described method (Lukáč et al., 2010a).

2.7. In vitro amoebicidal activity assay

The cytotoxic activity of five Isophol-PCs was tested on two clinical isolates of free-living amoebae, i.e., *Acanthamoeba lugdunensis* AcaVNAK02, and *Acanthamoeba quina* AcaVNAK03, isolated from the corneas of two patients with AK (Ondriska et al., 2006). Both isolates are representatives of group II according to the classification of Pussard and Pons (Visvesvara and Schuster, 2008). The species identification was performed according to the identification key of Page (Page, 1991) based mainly on cyst morphology and temperature tolerance (Ondriska et al., 2006). The molecular classification into the genotype T4 for both strains was revealed (Nagyova et al., 2010).

The experiment was carried out using the modifications of previously described methods (Mrva et al., 2011; Walochnik et al., 2002). Briefly, 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 that received 100 μl of a pure medium. Each APC was 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 hemocytometer. Viability of trophozoites was determined by trypan blue exclusion; 100% eradication was confirmed by transferring 50 μl of the suspension to a PYG medium, then recording the amoeba growth for 14 days. The lowest concentration of APCs supporting 100% eradication of the trophozoites was defined as the minimal trophocidal concentration (MTC). The EC_{50} is defined in this study as the effective concentration of APC that reduces the survival of amoebae by 50%. EC_{50} values after 24 h of incubation were calculated by linear regression analysis. Statistical evaluation was done with the STATISTICA Ver. 7 program package (StatSoft CR Ltd., Prague, Czech Republic). The experiments were repeated 8 times for each concentration. The cultivations and the cytotoxicity measurements were carried out at 37 °C.

3. Results

3.1. Synthesis of APCs

Prepared APCs were synthesized according to Scheme 1, connection of alkyldichloridophosphate with choline tosylate. Five compounds were prepared. They were obtained as an amorphous

Table 1
Isophol-PCs properties obtained by measurement of surface tension and NMR spectroscopy.

Compound	Surface tension			NMR spectroscopy	
	cmc (mol dm^{-3})	γ_{cmc} (mN m^{1})	A_{cmc} (Å)	^{13}C NMR (mol(dm^{-3})	^{31}P NMR (mol dm^{-3})
Isophol$_8$-PC	–	–	–	$(2.3 \pm 0.3) \times 10^{-1}$	$(2.4 \pm 0.2) \times 10^{-1}$
Isophol$_{12}$-PC	$(3.3 \pm 0.1) \times 10^{-3}$	29.6 ± 0.1	54 ± 2	–	$(7.4 \pm 0.2) \times 10^{-3}$
Isophol$_{16}$-PC	$(1.2 \pm 0.1) \times 10^{-4}$	26.1 ± 0.1	64 ± 1	–	–
Isophol$_{20}$-PC	$(3.3 \pm 0.3) \times 10^{-6}$	26.2 ± 0.2	85 ± 6	–	–
Isophol$_{24}$-PC	$<1 \times 10^{-6}$	–	–	–	–
HPC[a]	$(1.25 \pm 0.05) \times 10^{-5}$	38.3 ± 0.1	57 ± 3	–	–

[a] Lukáč et al. (2010b).

powder (Isophol$_{16}$-PC, Isophol$_{20}$-PC, Isophol$_{24}$-PC) or crystalline compounds (Isophol$_8$-PC, Isophol$_{12}$-PC). The purities of the compounds were confirmed by ^{31}P NMR spectroscopy, one signal of phosphate group in the spectra was observed. Amount of crystalline water was determined by ^{1}H NMR spectroscopy. Isophol-PCs were obtained as hemihydrates or monohydrates.

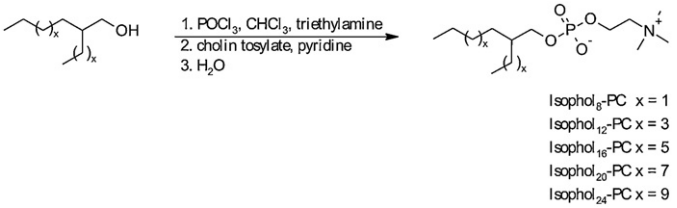
3.2. Physicochemical properties of APCs

The cmc of Isophol$_{12}$-PC, Isophol$_{16}$-PC and Isophol$_{20}$-PC were determined from the plots of surface tension vs. log concentration of APCs in water solutions (Fig. 1a). Data obtained from the plots are shown in Table 1. The cmc of Isophol$_{24}$-PC was very low and it was impossible to determine it by measurement of surface tension. We assumed that the value was lower than 1 μM. The value of γ_{cmc} of Isophol$_{12}$-PC is higher than the values of Isophol$_{16}$-PC and Isophol$_{20}$-PC. The surface areas at the surface saturation per head group of APCs (A_{cmc}) (Table 1) were determined from the premicellar linear region of the surface tension vs. log concentration curve. The values increase from 54 to 85 with increasing length of alkyl chains. The cmc of Isophol$_8$-PC were determined by ^{13}C NMR spectroscopy (Fig. 1b); the cmc was 0.23 M, and confirmed by ^{31}P NMR spectroscopy (Fig. 1c), the cmc was estimated at 0.24 M. The ^{31}P NMR spectroscopy was also used in the determination of the cmc of Isophol$_{12}$-PC (Fig. 1d). The cmc value was 7.4 μM.

Types of aggregates formed in water solutions were determined by ^{31}P NMR spectroscopy. The spectra are depicted in Fig. 2. Three types of spectra were measured. In the spectrum of Isophol$_{24}$-PC, an anisotropic signal was observed (Fig. 2), Isophol$_{20}$-PC was characterized by a superposition of two signals, one signal was anisotropic and the other one was isotropic (Fig. 2). The spectra of the other three compounds contained only isotropic signals (Fig. 2). The shapes of signals of all investigated compounds were similar in the range of temperatures from 25 °C to 75 °C.

3.3. Biological activities of APCs

APCs were more active against *Candida* than the bacteria. The compounds had no effect against *E. coli* up to the concentration of 10 mM. The *S. aureus* strain was sensitive to Isophol$_{16}$-PC and Isophol$_{20}$-PC, with MICs 0.15 mM and 0.31 mM, respectively (Table 2). The best activity against *Candida* was exhibited by the reference compound, miltefosine. The strain ATCC 8186 was



Scheme 1. Synthesis of Isophol-PCs.

Table 2
The values of antimicrobial activities of Isophol-PCs against bacteria and yeasts.

Compound	MIC (mM)			
	S. aureus	*E. coli*	*C. albicans* ATCC 8186	*C. albicans* ATCC 4553
Isophol$_8$-PC	>10	>10	>10	>10
Isophol$_{12}$-PC	>10	>10	>10	>10
Isophol$_{16}$-PC	0.15	>10	0.15	0.08
Isophol$_{20}$-PC	0.31	>10	0.04	0.15
Isophol$_{24}$-PC	>10	>10	>10	>10
HPC	>10	>10	0.005	0.02

about four times more sensitive against HPC (MIC = 5 μM) than the strain ATCC 4553 (MIC = 20 μM). The inhibitory activities were also observed in the cases of compounds Isophol$_{16}$-PC and Isophol$_{20}$-PC but the MICs were higher than the MICs of HPC. They were in the range 40–150 μM. Other APCs were less effective with MICs more than 10 mM (Table 2).

All studied APCs inhibited the growth of *A. lugdunensis* and *A. quina*. Isophol$_{16}$-PC had the highest level of activities against both tested strains. Its MTCs after 24 h of exposure were 125 μM for *A. lugdunensis* and 62.5 μM for *A. quina*, respectively. After 48 h, twofold decrease of the MTC value of Isophol$_{16}$-PC against *A. lugdunensis* was recorded, although in the second strain MTC remained unchanged. For other APCs the MTC values were more than 500 μM for both strains of amoebae. Therefore, their activities were expressed as EC_{50}. The values of EC_{50} are lower than or equal to 1 mM in the case of all compounds, only *A. lugdunensis* was very resistant against Isophol$_{24}$-PC and we estimated that the value was more than 100 times higher than 1 mM. The MTC values of the reference compound were 500 μM and 250 μM for *A. lugdunensis* and *A. quina*, respectively (Table 3).

The time dependence of activities of Isophol-PCs against strains of *Acanthamoeba* showed that all compounds possessed inhibition activities against trophozoite growth. Isophol$_8$-PC and Isophol$_{12}$-PC reached maximum activities after 1 h or 24 h, and subsequently the amount of trophozoites increased after 48 h (Fig. 3). Only the concentrations 500 μM for Isophol$_8$-PC and Isophol$_{12}$PC, and 250 μM for Isophol$_{12}$-PC possessed the trophostatic activities against *Acanthamoeba quina* in all time ranges investigated. *A. lugdunensis* was less susceptible to the action of Isophol$_8$-PC and Isophol$_{12}$-PC, and no investigated

Table 3
The values of MTC and EC_{50} of Isophol-PCs against amoebae.

Compound	*Acanthamoeba lugdunensis*		*Acanthamoeba quina*	
	MTC (μM)	EC_{50} (μM)	MTC (μM)	EC_{50} (μM)
Isophol$_8$-PC	>500	999	>500	921
Isophol$_{12}$-PC	>500	556	>500	246
Isophol$_{16}$-PC	125	46	62.5	29
Isophol$_{20}$-PC	>500	907	>500	202
Isophol$_{24}$-PC	>500	1.6×10^5	>500	530
HPC	500	48	250	26

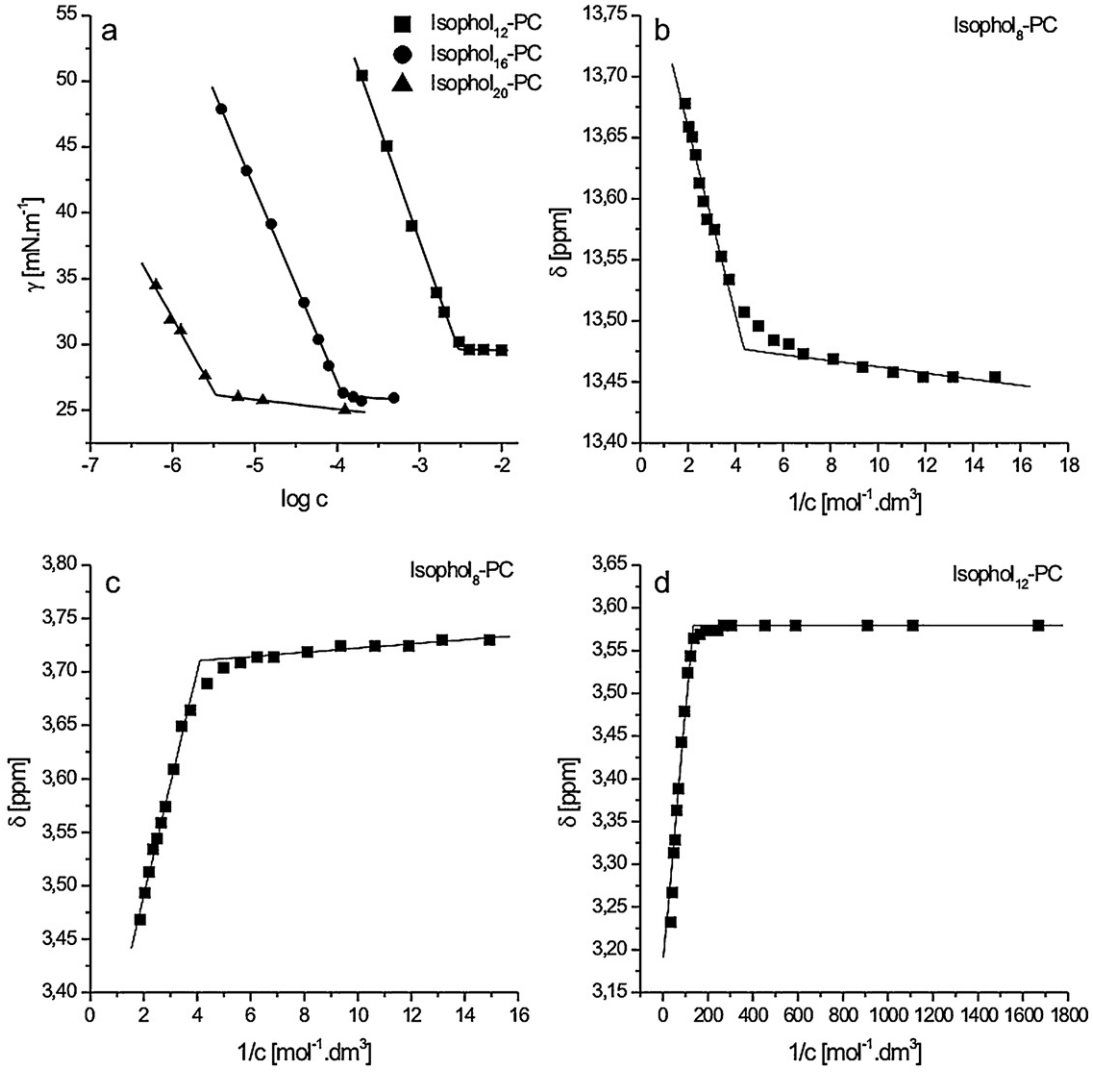


Fig. 1. Plots used in determination of physicochemical properties of Isophol-PCs; (a) plots of surface tension vs. log concentration of Isophol$_{12}$-PC, Isophol$_{16}$-PC and Isophol$_{20}$-PC; (b) plots of the chemical shift of the methyl group carbon atom in the longer alkyl chain (^{13}C NMR spectroscopy) vs. reciprocal concentration of Isophol$_8$-PC; (c) and (d) plots of the chemical shift of the phosphate group (^{31}P NMR spectroscopy) vs. reciprocal concentration of Isophol$_8$-PC and Isophol$_{12}$-PC, respectively.

concentration acted trophostatically after 48 h incubation. Isophol$_{16}$-PC had inhibition activities on growth of both strains of *Acanthamoeba* up to the concentration of 31.5 μM after 48 h of incubation. Interesting amoebicidal activities were possessed by Isophol$_{20}$-PC. Although it was not trophocidal, its inhibition effect on the growth of *Acanthamoeba* strains was more intensive than the inhibition effect of Isophol$_{16}$-PC at the lowest concentrations tested (Fig. 3). It was more apparent in the case of its action against *A. quina*, where all investigated concentrations of Isophol$_{20}$-PC caused inhibition of amoeba growth after 48 h of incubation and trophozoites were not observed in the cultures. All living cells were in the stage of pseudocysts. The time-dependent action of Isophol$_{24}$-PC against *Acanthamoeba* was very similar to that of Isophol$_8$-PC.

4. Discussion

4.1. Synthesis of APCs

APCs were prepared by the known method which coupled alkyldichloridophosphates with choline tosylates. The yields of Isophol-PCs were from 5.9% to 26.9%. The lower yields could be caused by the using of Isophols without purifications and drying, (only 2-ethylhexanol was distilled before application) or not entirely dry choline tosylate for synthesis. However, the yields are comparable with some other results published for synthesis of APCs, alkylphospholipids or dialkylphosphocholines (Koufaki et al., 1996; Peresypkin et al., 2007; Ukawa et al., 1989). The compounds Isophol$_{16}$-PC, Isophol$_{20}$-PC and Isophol$_{24}$-PC were first prepared by Kang et al. (2005) who used a different method for synthesis. Their synthesis of APCs from 2-chloro-2-oxo-1,3,2-dioxaphospholane led to higher yields (e.g. 41.8% for Isophol$_{24}$-PC).

4.2. Physicochemical properties of APCs

The cmc of Isophol-PCs are in Table 1. We obtained similar cmcs for Isophol$_{16}$-PC and Isophol$_{20}$-PC as published Kang et al. (2005). The cmcs of Isophol$_8$-PC and Isophol$_{12}$-PC were confirmed by two methods. The cmc of Isophol$_{24}$-PC was not obtained but we estimated that it is lower than 1 μM. The postmicellar curve until concentration 1 μM for this compound was obtained by measurement of the surface tension.

The values of cmcs are decreasing with lengthening of the alkyl chain. The plots of log cmc vs. length of alkyl chain (Fig. 4)

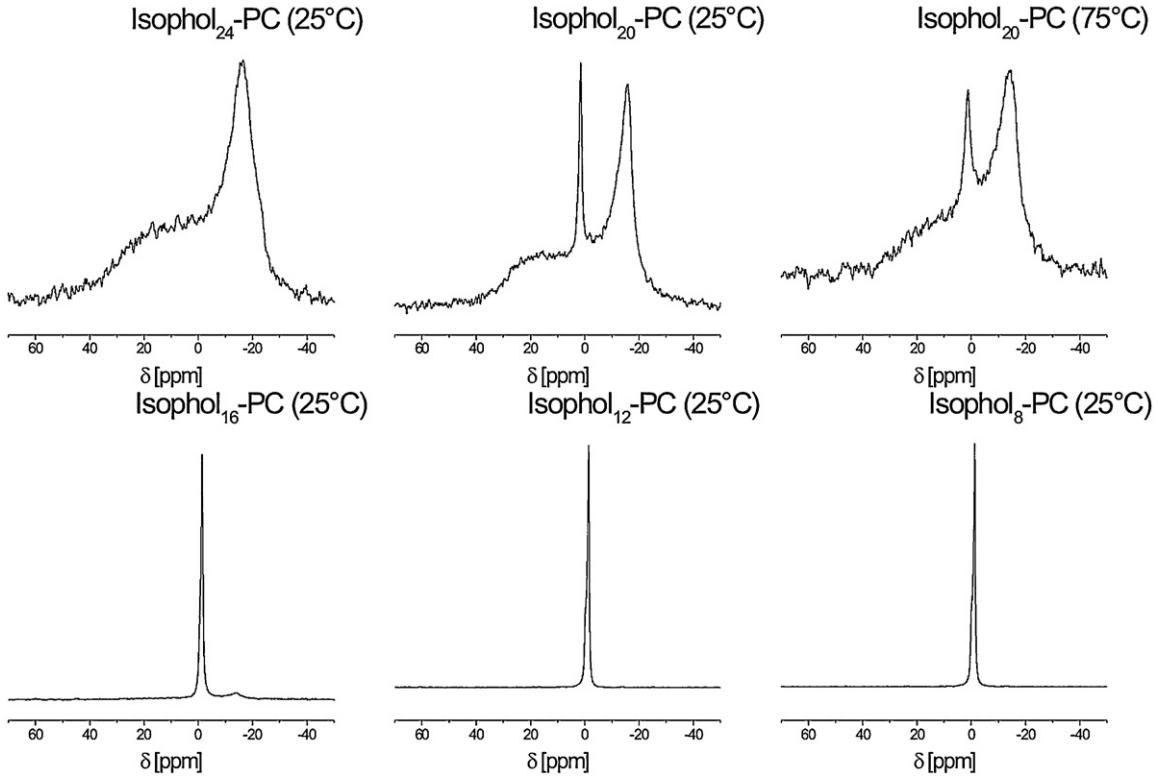


Fig. 2. ^{31}P NMR spectra of Isophol-PCs.

show that the decrease of log cmcs depending on the alkyl chain length (N) is linear and it can be described by the equation log cmc = $-0.40\,N + 2.5$ ($R^2 = 0.9974$). A similar dependence for APCs with straight alkyl chains was observed by Yaseen et al. (2005). The influence of the alkyl chain length on cmcs was described by the equation log cmc = $-0.47\,N + 2.6$. It means that the decrease of cmcs is more intensive in the case of APCs with straight alkyl chains; the cmcs of these compounds fall slightly faster depending on the number of carbon atoms in the hydrophobic parts of the molecules than Isophol-PCs. The equations also show that the cmcs of APCs with straight alkyl chains are lower than the cmcs of Isophol-PCs with the same number of carbon atoms in the alkyl chains. For example, cmc of HPC is 13 μM and cmc of Isophol$_{16}$-PC is 120 μM (Table 1).

The equation which expresses the dependence of log cmc vs. length of alkyl chain enables estimating the cmc of Isophol$_{24}$-PC which could not be measured by surface tension. The extrapolated cmc is 7.9×10^{-8} M. It supports our assumption that the cmc is lower than 1 μM.

The architecture of amphiphilic compounds is important for the formation of aggregates in solutions. ^{31}P NMR spectrum of Isophol$_{24}$-PC shows that this compound forms double-layer aggregates. Isophol$_{24}$-PC can form unilamelar or multilamelar vesicles in aqueous solution. The same assumption was made by Kang et al. (2005). The superposition of two signals and their shape in the ^{31}P NMR spectrum of Isophol$_{20}$-PC indicate that the sample may contain micelles and lamellar phases. The lipophilicity of the other three compounds is lower than Isophol$_{20}$-PC or Isophol$_{24}$-PC and therefore one can expect the presence of micelles in aqueous solutions. The presence of one isotropic signal in ^{31}P NMR spectra of Isophol$_8$-PC, Isophol$_{12}$-PC and Isophol$_{16}$-PC indicates the occurrence of such type of aggregates. The relationship between structure of Isophol-PCs (length of alkyl chains) and types of aggregates formed shows that the aggregates change from micelles to vesicles with increasing lipophilicity of the molecules.

4.3. Biological activities of Isophol-PCs

Gram positive bacteria, *S. aureus*, and yeast species *C. albicans* were sensitive to the action of Isophol$_{16}$-PC, Isophol$_{20}$-PC. *C. albicans* was also susceptible to HPC. Gram negative bacteria, *E. coli* were not sensitive to any of the studied APCs at concentrations equal to or less than 10 mM. Similar results were obtained by Obando et al. (2007). The APCs and some etherphospholipids they studied did not possess antibacterial activity against *E. coli* up to concentrations of 350 μM. Different was the situation with *S. aureus*. In our case, Isophol$_{16}$-PC and Isophol$_{20}$-PC were efficient against gram positive bacteria but HPC was not. It is surprising because the cmc of HPC lies between the values for Isophol$_{16}$-PC and Isophol$_{20}$-PC and the activity of HPC against *S. aureus* (MIC = 44 μM) has been as described by Obando et al. (2007). Other compounds with lower or higher lipophilicity were inactive up to the concentration 10 mM.

C. albicans, other yeasts and various fungi are very sensitive to antifungal action of HPC (Widmer et al., 2006). Our observations are in accordance with the previously published data (Obando et al., 2007; Widmer et al., 2006), as the MIC for the *C. albicans* ATCC 8186 was 5 μM, and for the second strain *C. albicans* ATCC 4553 it reached 20 μM. The Isophol-PCs with cmcs about one order of magnitude higher or lower than HPC (Isophol$_{16}$-PC and Isophol$_{20}$-PC) possessed anticandidal activities but they did not reach the level of action of HPC. On the other hand, Obando et al. (2007) reported that for octadecylphosphocholine (OPC) a better activity was measured against *C. albicans* than for HPC. The cmc of Isophol$_{20}$-PC (cmc = 3.3 μM) is between the cmcs of HPC (cmc = 13 μM (Lukáč et al., 2010b)) and OPC (cmc = 0.35 μM, (Yaseen et al., 2005)), however, its antimicrobial activity is lower than the activity of HPC against both tested strains of *C. albicans*. Several authors (Lu et al., 1999; Lukáč et al., 2009a,b; Obando et al., 2007) have studied the activity

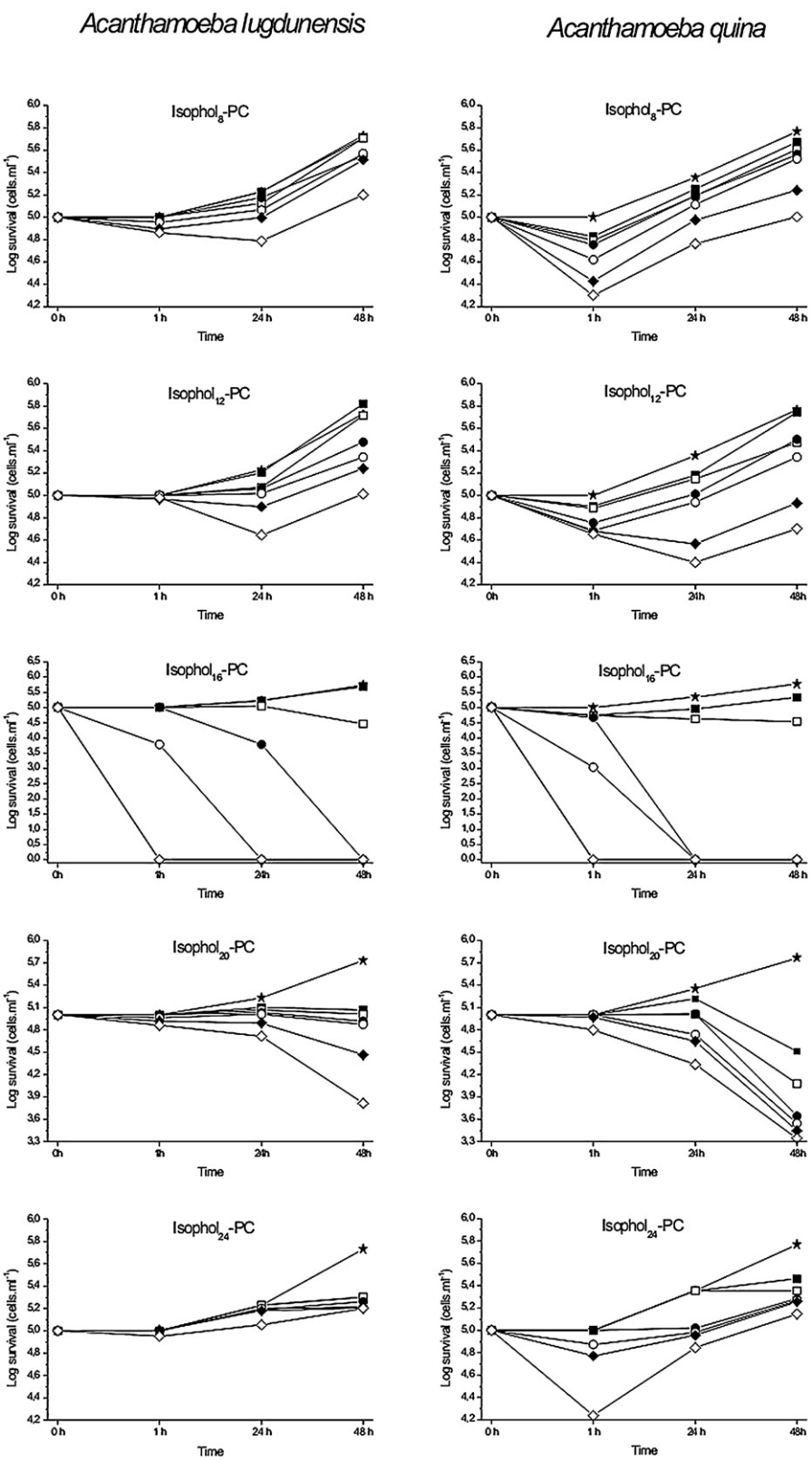


Fig. 3. Antiprotozoal activities of Isophol-PCs against trophozoites of *Acanthamoeba lugdunensis* and *Acanthamoeba quina*. Concentration of 15.6 μM (■), 31.25 μM (□), 62.5 μM (●), 125 μM (○), 250 μM (♦), 500 μM (◊) were used. *, control. The lines are guides to the eye.

of APCs against *C. albicans*. Therefore, we can suppose that the activity of APCs against this yeast could be sensitive to modification of the structure of the standard APC, HPC. The most antimicrobial active compounds were HPC and their analogues with small modifications, diethylamino analogues of HPC (Lukáč et al., 2009a), 16-chlorohexadecylphosphocholine (Lu et al., 1999), OPC, elaidylphosphocholine or octadecyl 2-(trimethylphosphonio)ethyl phosphate (Obando et al., 2007). Other modifications that were made in the structure of HPC (dodecylphosphocholine or HPC analogues with heterocyclic rings in choline moiety) led to

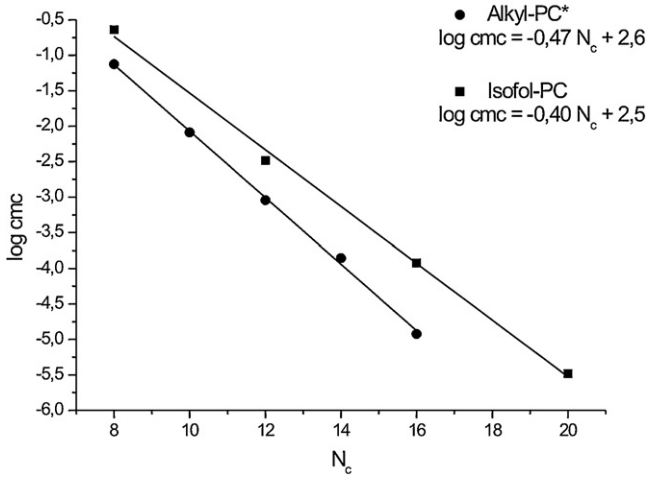


Fig. 4. Plot of log cmc vs. number of carbon atoms, N_c, increase in the alkyl chain of Isophol-PCs (■) and APCs (●).
*The values of the APCs were adopted from Yaseen et al. (2005).

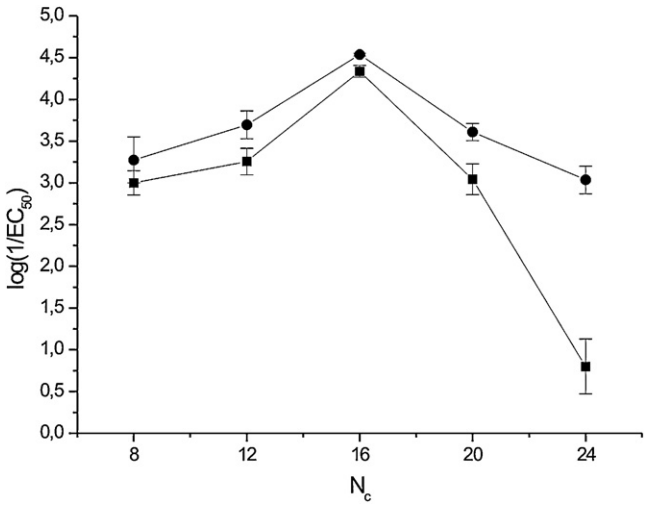


Fig. 5. Plot of log reciprocal EC_{50} vs. number of carbon atoms, N_c, increase in the alkyl chain of Isophol-PCs. ■, *A. lugdunensis*; ●, *A. quina*. The lines are guides to the eye.

decrease of activities (Lu et al., 1999; Lukáč et al., 2009a,b; Obando et al., 2007).

The sensitivity of different strains of *Acanthamoeba* to APCs, in particular HPC, is variable. HPC had higher levels of activities against some strains of *A. polyphaga*, *A. castellanii*, and *A. lenticulata* (McBride et al., 2005, 2007; Walochnik et al., 2002), however, other strains, *A. lugdunensis* and *Acanthamoeba* sp., were significantly less sensitive to HPC (Mrva et al., 2011).

$Isophol_{16}$-PC had the highest level of activity against both strains of *Acanthamoeba*. It is not surprising that this compound possessed the highest activity. Its cmc is about one order of magnitude higher than cmc of HPC. Therefore its solubilization properties of the plasma membrane of *Acanthamoeba* are the best among the studied Isophol-PCs. $Isophol_{12}$-PC has a very high cmc and $Isophol_{20}$-PC has higher lipophilicity and forms vesicles apart from micelles. Walochnik et al. (2002) also observed lower activities for the compounds with higher lipophilicity than HPC (APCs with alkyl chains longer than 16 carbon atoms). McBride et al. (2007) reported similar observations. However, OPC possessed better activity against *A. castellanii* than HPC, nevertheless, other APCs showed lower levels of activities on both investigated strains, *A. polyphaga* and *A. castellanii* (McBride et al., 2007). A modification in the hydrophilic part of the HPC molecule or changing the structure to dialkylphosphocholines with a cmc similar to HPC could lead to an improvement in activity (Lukáč et al., 2009a,b, 2010c). This means that the activity of APCs against *Acanthamoeba* is not as restricted to the structure of HPC as it is in the case of its activity against *Candida*.

The activities of Isophol-PCs are better seen in the EC_{50} expression. The activity is increased with the extension of the alkyl chains in the hydrophobic parts of the molecules until it reaches the maximum ($Isophol_{16}$-PC) and then it falls (Fig. 5). This relationship of cytotoxic activity to the length of the alkyl chain has a bilinear form and is named the cut-off effect (Balgavý and Devínsky, 1996). The cut-off effect observed in the case of Isophol-PCs supports our hypothesis that the APCs can act mainly as solubilizers of cytoplasmic membranes. This non-specific mode of action is typical for amphiphilic compounds (Balgavý and Devínsky, 1996). Devínsky et al. (1985, 1990, 1992) observed a correlation between the cmc of surfactants and MIC as a function of the alkyl chain length. It showed that only amphiphilic compounds with cmcs within a certain range have the best antimicrobial activities. The cmc values interval was 4×10^{-3} to 1×10^{-4} mol dm^{-3} for amine oxides tested against *Staphylococcus aureus*, *Escherichai coli* and *Candida albicans*. The amine oxides (AOs) can be considered as zwitterionic surfactants (Rosen, 2004) and are in the same group of surfactants as alkylphosphocholines. The similarity between AOs and APCs is not only in the structure but it can be observed also in their biological activities. The most effective APCs against *Acanthamoeba*, $Isophol_{16}$-PC has a cmc 1.2×10^{-4} mol dm^{-3} and the value is in the range of cmcs described for amine oxides (Devínsky et al., 1985).

Encystment is a very common reaction of the *Acanthamoeba* spp. trophozoites to the long-lasting presence of unfavorable conditions (Kilvington et al., 2008; Klieščiková et al., 2011a,b; Lonnen et al., 2010; Schuster and Visvesvara, 2004a; Visvesvara and Schuster, 2008). Recently it was found that in the case of rapid onset of acute stress caused by low concentrations of organic solvents, trophozoites form a pseudocyst stage which probably increases their resistance to these compounds (Klieščiková et al., 2011a,b). In this sense, the effect of $Isophol_{20}$-PC against *A. quina* was interesting. In the whole range of concentrations of $Isophol_{20}$-PC after 48 h of incubation, all living amoebae were observed in the stage of rounded cells without any projections or acanthopodia, very similar to pseudocysts. For *A. lugdunensis*, a similar effect was observed only in the case of the highest concentrations (500 μM, 250 μM) of the same compound and the same time of incubation. Other Isophol-PCs did not induce the formation of rounded cells in the present strains of *Acanthamoeba* spp. The morphology of the observed rounded stage is very similar to pseudocysts reported by Klieščiková et al. (2011a,b). Also their formation was induced in a similar manner, by low concentrations of toxic organic compounds. Therefore, we assume the formation of pseudocysts after action of $Isophol_{20}$-PC on trophozoites.

5. Conclusions

Zwitterionic amphiphilic compounds show a potent antimicrobial effect. The knowledge of physicochemical properties of alkylphosphocholines can be helpful for understanding of their biological activities. We synthesized five Isophol-PCs with different length of branched alkyl chains. We investigated their biological activities against bacteria, yeast and amoebae and we determined some of their physicochemical properties like critical micelle concentration or types of aggregates, which Isophol-PCs formed in water solutions. The relationship between biological activities and physicochemical properties show that the most potent compound was $Isophol_{16}$-PC with cmc = 1.2×10^{-4} mol dm^{-3} characteristic with formation of micelles in water solutions. The compounds with

higher cmcs than that of Isophol$_{16}$-PC or the compounds, which formed lamellar phases in water dispersions, were less active.

The most active compound, Isophol$_{16}$-PC, may be a promising candidate for the treatment of *Acanthamoeba* infections. Its trophocidal activities against studied *Acanthamoeba* spp. are better in comparison with HPC and chemical structures and physicochemical properties of both compounds are very similar. We also think that its toxicity may be comparable with toxicity of HPC. Therefore, Isophol$_{16}$-PC may be suitable for topical administration because the HPC shows lower toxicity against human keratinocytes than chlorhexidine, which is a standard drug used for treatment of *Acanthamoeba* keratitis (Walochnik et al., 2009).

Acknowledgments

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References

Aguiar, M.G., Pereira, A.M.M., Fernandes, A.P., Ferreira, L.A.M., 2010. Reductions in skin and systemic parasite burdens as a combined effect of topical paromomycin and oral miltefosine treatment of mice experimentally infected with *Leismania (Leishmania) amazonensis*. Antimicrob. Agents Chemother. 54, 4699–4704.

Aichelburg, A.C., Walochnik, J., Assadian, O., Prosch, H., Steuer, A., Perneczky, G., Visvesvara, G.S., Aspöck, H., Vetter, N., 2008. Successful treatment of disseminated *Acanthamoeba* sp. infection with miltefosine. Emerg. Infect. Dis. 14, 1743–1746.

Babu, P., Chopra, D., Guru Row, T.N., Maitra, U., 2005. Micellar aggregates and hydrogels from phosphonobile salts. Org. Biomol. Chem. 3, 3695–3700.

Balgavý, P., Devínsky, F., 1996. Cut-off effects in biological activities of surfactants. Adv. Colloid Interface Sci. 66, 23–63.

Blaha, C., Duchêne, M., Aspöck, H., Walochnik, J., 2006. In vitro activity of hexadecylphosphocholine (miltefosine) against metronidazole-resistant and -susceptible strains of *Trichomonas vaginalis*. J. Antimicrob. Chemother. 57, 273–278.

Calogeropoulou, T., Angelou, P., Detsi, A., Fragiadaki, I., Scoulica, E., 2008. Design and synthesis of potent antileishmanial cycloalkylidene-substituted ether phospholipid derivatives. J. Med. Chem. 51, 897–908.

Frankel, H.D., Chen, G., 2005. Enteropathogenic *Escherichia coli*: unravelling pathogenesis. FEMS Microbiol. Rev. 29, 83–98.

Christiansen, A., Backensfeld, T., Weitschies, W., 2010. Effects of non-ionic surfactants on *in vitro* triglyceride digestion and their susceptibility to digestion by pancreatic enzymes. Eur. J. Pharm. Sci. 41, 376–382.

Colomer, A., Pinazo, A., Manresa, M.A., Vinardell, M.P., Mitjans, M., Infante, M.R., Perez, L., 2011. Cationic surfactants derived from lysine: effects of their structure and charge type on antimicrobial and hemolytic activities. J. Med. Chem. 54, 989–1002.

Croft, S.L., Seifert, K., Duchêne, M., 2003. Antiprotozoal activities of phospholipid analogues. Mol. Biochem. Parasit. 126, 165–172.

Cugh, P., Bradel-Tretheway, B., Monteiro-Filho, C.M.R., Planalles, V., Maggirwar, S.B., Dewhurst, S., Kim, B., 2008. Akt inhibitors as an HIV-I infected macrophage-specific anti-viral therapy. Retrovirology 5 (art. no. 11).

Desando, M.A., Reeves, L.W., 1986. The effects of high temperatures (29–123 °C) on critical micelle concentrations in solutions of potassium *n*-octanoate in deuterium oxide: a nuclear magnetic resonance study. Can. J. Chem. 64, 1823–1828.

Devínsky, F., Lacko, I., Mlynarčík, D., Račanský, V., Krasnec, Ľ., 1985. Tenside Deterg. 22, 10–15.

Devínsky, F., Kopecká-Leitmanová, A., Šeršeň, F., Balgavý, P., 1990. Cut-off effect in antibacterial activity and in membrane perturbation efficiency of the homologous series of *NN*-dimethylalkylamine ocides. J. Pharm. Pharmacol. 42, 790–794.

Devínsky, F., Lacko, I., Mlynarčík, D., 1992. Aggregation properties as a measure of lipophilicity in QSAR studies of antimicrobially active amphiphiles. In: Kuchař, M., Rejholec, V. (Eds.), QSAR in Design of Bioactive Compounds. Prous Int. Pub., Barcelona, pp. 233–247.

Farah, C.S., Ashman, R.B., Challacombe, S.J., 2000. Oral candidosis. Clin. Dermatol. 18, 553–562.

François, P., Scherl, A., Hochstrasser, D., Schrenzel, J., 2010. Proteomic approaches to study *Staphylococcus aureus* pathogenesis. J. Proteomics 73, 701–708.

Griewank, K., Gazeau, C., Eichhorn, A., Von Stebut, E., 2010. Miltefosine efficiently eliminates *Leishmania major* amastigotes from infected murine dendritic cells without altering their immune functions. Antimicrob. Agents Chemother. 54, 652–659.

Hiramatsu, K., Cui, L., Kuroda, M., Ito, T., 2001. The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. Trends Microbiol. 9, 486–493.

Hirt, R., Brechtold, R., 1958. Zur Synthese der Phosphatide. 2. Eine neue Synthese der Lecithine. Pharm. Acta Helv. 33, 349–356.

Hornillos, V., Carrillo, E., Rivas, L., Amat-Guerri, F., Acuña, A.U., 2008. Synthesis of BODIPY-labeled alkylphosphocholines with leishmanicidal activity, as fluorescent analogues of miltefosine. Bioorg. Med. Chem. Lett. 18, 6336–6339.

Houlihan, W.H., Lohmeyer, M., Workman, P., Cheon, S.H., 1995. Phospholipid antitumor agents. Med. Res. Rev. 15, 157–223.

Kabir-ud-Din, M.S., Sheikh, S., Dar, A.A., 2009. Interaction of a cationic gemini surfactant with conventional surfactants in the mixed micelle and monolayer formation in aqueous medium. J. Colloid Interface Sci. 333, 605–612.

Kang, E.C., Kataoka, S., Kato, K., 2005. Synthesis and properties of alkyl phosphorylcholine amphiphiles with a linear and an asymmetrically branched alkyl chain. Bull. Chem. Soc. Jpn. 78, 1558–1564.

Khan, N.A., 2009. Acanthamoeba, Biology and Pathogenesis. Caister Academic Press, Norfolk, UK.

Kilvington, S., Heaselgrave, W., Lally, M.J., Ambrus, K., Powell, H., 2008. Encystment of *Acanthamoeba* during incubation in multipurpose contact lens disinfectant solutions and experimental formulations. Eye Contact Lens 34, 133–139.

Klieščiková, J., Kulda, J., Nohýnková, E., 2011a. Propylene glycol and contact-lens solutions containing this diol induce pseudocyst formation in acanthamoebae. Exp. Parasitol. 127, 326–328.

Klieščiková, J., Kulda, J., Nohýnková, E., 2011b. Stress-induced pseudocyst formation—a newly identified mechanism of protection against organic solvents in acanthamoebae of the T4 genotype. Protist 162, 58–59.

Koufaki, M., Polychroniou, V., Calogeropoulou, T., Tsotinis, A., Drees, M., Fiebig, H.H., LeClerc, S., Hendriks, H.R., Makriyannis, A., 1996. Alkyl and alkoxyethyl antineoplastic phospholipids. J. Med. Chem. 39, 2609–2614.

Llull, D., Rivas, L., García, E., 2007. In vitro bactericidal activity of the antiprotozoal drug miltefosine against *Streptococcus pneumoniae* and other pathogenic streptococci. Antimicrob. Agents Chemother. 51, 1844–1848.

Lonnen, J., Heaselgrave, W., Nomachi, M., Mori, O., Santodomingo-Rubido, J., 2010. Disinfection efficacy and encystment rate of soft contact lens multipurpose solutions against *Acanthamoeba*. Eye Contact Lens 36, 1–7.

López-Martínez, R., 2010. Candidosis, a new challenge. Clin. Dermatol. 28, 178–184.

Lu, Q., Ubillas, R.P., Zhou, Y., Dubenko, L.G., Dener, J.M., Litvak, J., Phuan, P.W., Flores, M., Ye, Z.J., Gerber, R.E., Truong, T., Bierer, D.E., 1999. Synthetic analogues of irlbacholine: a novel antifungal plant metabolite isolated from *Irlbachia alata*. J. Nat. Prod. 62, 824–828.

Lukáč, M., Mojžiš, J., Mojžišová, G., Mrva, M., Ondriska, F., Valentová, J., Lacko, I., Bukovský, M., Devínsky, F., Karlovská, J., 2009a. Dialkylamino and nitrogen heterocyclic analogues of hexadecylphosphocholine and cetyltrimetylammonium bromide: effect of phosphate group and environment of the ammonium cation on their biological activity. Eur. J. Med. Chem. 44, 4970–4977.

Lukáč, M., Mrva, M., Fischer-Fodor, E., Lacko, I., Bukovský, M., Miklášová, N., Ondriska, F., Devínsky, F., 2009b. Synthesis and biological activity of dialkylphosphocholines. Bioorg. Med. Chem. Lett. 19, 6346–6349.

Lukáč, M., Lacko, I., Bukovský, M., Kyselová, Z., Karlovská, J., Horváth, B., Devínsky, F., 2010a. Synthesis and antimicrobial activity of a series of optically active quaternary ammonium salts derived from phenylalanine. Cent. Eur. J. Chem. 8, 194–201.

Lukáč, M., Pisárčik, M., Lacko, I., Devínsky, F., 2010b. Surface-active properties of nitrogen heterocyclic and dialkylamino derivates of hexadecylphosphocholine and cetyltrimethylammonium bromide. J. Colloid Interface Sci. 347, 233–240.

Lukáč, M., Timko, L., Mrva, M., Ondriska, F., Karlovská, J., Valentová, J., Lacko, I., 2010c. Synthesis, aggregation properties, and antiprotozoal activity of heterocyclic heterogemini surfactants. Heteroatom. Chem. 21, 203–209.

Lukáč, M., Prokipčák, I., Lacko, I., Devínsky, F., 2011. Solubilisation of griseofulvin and rutin in aqueous micellar solutions of gemini and heterogemini surfactants and their mixtures. Eur. J. Pharm. Sci. 44, 194–199.

Maltezou, H.C., Giamarellou, H., 2006. Community-acquired methicillin-resistant *Staphylococcus aureus* infections. Int. J. Antimicrob. Agents 27, 87–96.

McBride, J., Ingram, P.R., Henriquez, F.L., Roberts, C.W., 2005. Development of colorimetric microtiter plate assay for assessment of antimicrobials against *Acanthamoeba*. J. Clin. Microbiol. 43, 629–634.

McBride, J., Mullen, A.B., Carter, C., Roberts, C.W., 2007. Differential cytotoxicity of phospholipid analogues to pathogenic *Acanthamoeba* species and mammalian cells. J. Antimicrob. Chemother. 60, 521–525.

Mirgorod, Y.A., Postnikov, E.B., Borshch, N.A., 2010. ^{13}C NMR investigation of the structure of alkylammonium chloride micells in aqueous solutions. J. Struct. Chem. 51, 1111–1118.

Mrva, M., Garajová, M., Lukáč, M., Ondriska, F., 2011. Weak cytotoxic activity of miltefosine against clinical isolates of *Acanthamoeba* spp. J. Parasitol. 97, 538–540.

Nagyova, V., Nagy, A., Timko, J., 2010. Morphological, physiological and molecular biological characterisation of isolates from first cases of *Acanthamoeba* keratitis in Slovakia. Parasitol. Res. 106, 861–872.

Obando, D., Widmer, F., Wright, L.C., Sorrell, T.C., Jolliffe, K.A., 2007. Synthesis, antifungal and antimicrobial activity of alkylphospholipids. Bioorg. Med. Chem. 15, 5158–5165.

Ondriska, F., Mrva, M., Lichvár, M., Žiak, P., Murgašová, Z., Bieliková, A., Gablasová, K., Nohýnková, E., 2006. In: Furková, K. (Ed.), Akantamébová keratitída–novoobjavená humánna parazitóza na Slovensku. Novinky v pediatrii III, Bratislava, pp. 46–48.

Ortega-Loayza, A.G., Diamantis, S.A., Gilligan, P., Morrell, D.S., 2010. Characterization of *Staphylococcus aureus* cutaneous infections in a pediatric dermatology tertiary health care outpatient facility. J. Am. Acad. Dermatol. 62, 804–811.

Page, F.C., 1991. Nackte rhizopoda. In: Page, F.C., Siemensma, F.J. (Eds.), Nackte Rhizopoda und Heliozoea. G. Fischer Verlag, Stuttgart, New York, pp. 1–170.

Papanastasiou, I., Prousis, K.C., Georgikopoulou, K., Pavlidis, T., Scoulica, E., Kolocouris, N., Calogeropoulou, T., 2010. Design and synthesis of new adamantyl-substituted antileishmanial ether phospholipids. Bioorg. Med. Chem. Lett. 20, 5484–5487.

Peresypkin, A., Clavel, C., Menger, F.M., 2007. Ambidextrous 'hybrid' fluorinated zwitterionic geminis: self-assembly in both organic and aqueous media. Mendeleev. Commun. 17, 82–84.

Pinto, P.M., de Cássia Botelho Weikert-Oliveira, R., Pereira Lyon, J., Cury, V.F., Arantes, R.R., Koga-Ito, C.Y., Resende, M.A., 2008. In vitro antifungal susceptibility of clinical isolates of *Candida* spp. obtained from patients with different predisposing factors to candidosis. Microbiol. Res. 163, 579–585.

Plückthun, A., Dennis, E.A., 1981. ^{31}P nuclear magnetic resonance study on the incorporation of monomeric phospholipids into nonionic detergent micelles. J. Phys. Chem. 85, 678–683.

Polat, Z.A., Obwaller, A., Vural, A., Walochnik, J., 2011. Efficacy of miltefosine for topical treatment of Acanthamoeba keratitis in Syrian hmasters. Parasitol. Res., doi:10.1007/s00436-011-2515-0, in press.

Rosen, M.J., 2004. Surfactants and Interfacial Phenomena, 3rd ed. John Wiley & Sons Inc., New Jersey.

Saraiva, V.B., Wengert, M., Gomes-Quintana, E., Heise, N., Caruso-Neves, C., 2009. Na^+-ATPase and protein kinase C are targets to 1-O-hexadecylphosphocoline (miltefosine) in *Trypanosoma cruzi*. Arch. Biochem. Biophys. 481, 65–71.

Schuster, F.L., Visvesvara, G.S., 2004a. Free-living amoeba as opportunistic and non-opportunistic pathogens of human and animals. Int. J. Parasitol. 34, 1001–1027.

Schuster, F.L., Visvesvara, G.S., 2004b. Opportunistic amoebae: challenges in prophylaxis and treatment. Drug Resist. Updat. 7, 41–51.

Schuster, F.L., Guglielmo, B.J., Visvesvara, G.S., 2006. In-vitro activity of miltefosine and Voriconazole on clinical isolates of free-living amebas: *Balamuthia mandrillaris*, *Acanthamoeba* spp., and *Naegleria fowleri*. J. Eukaryot. Microbiol. 53, 121–126.

Seifert, K., Duchêne, M., Wernsdorfer, W.H., Kollaritsch, H., Scheiner, O., Wiedermann, G., Hottkowitz, T., Eibl, H., 2001. Effects of miltefosine and other alkylphosphocholines on human intestinal parasite *Entamoeba histolytica*. Antimicrob. Agents Chemother. 45, 1505–1510.

Seifert, K., Lemke, A., Croft, S.L., Kayser, O., 2007. Antileishmanial structure–activity relationships of synthetic phospholipids: in vitro and in vivo activities of selected derivatives. Antimicrob. Agents Chemother. 51, 4525–4528.

Ukawa, K., Imamiya, E., Yamamoto, H., Mizuno, K., Tasaka, A., Terashita, Z., Okutani, T., Nomura, H., Kasukabe, T., Hozumi, M., Kudo, I., Inoue, K., 1989. Synthesis and antitumor activity of new alkylphospholipids containing modifications of the phosphocholine moiety. Chem. Pharm. Bull. 37, 1249–1255.

Van Griensven, J., Balasegaram, M., Meheus, F., Alvar, J., De Lynen, L., Boelaert, M., 2010. Combination therapy for visceral leishmaniasis. Lancet Infect. Dis. 10, 184–194.

Visvesvara, G.S., Schuster, F.L., 2008. Opportunistic free living Amebae, Part I. Clin. Microbiol. Newslett. 30, 151–158.

Walochnik, J., Duchêne, M., Seifert, K., Obwaller, A., Hottkowitz, T., Wiedermann, G., Eibl, H., Aspöck, H., 2002. Cytotoxic activities of alkylphosphocholines against clinical isolates of *Acanthamoeba* spp. Antimicrob. Agents Chemother. 46, 695–701.

Walochnik, J., Obwaller, A., Gruber, F., Mildner, M., Tschachler, E., Suchomel, M., Duchêne, M., Auer, H., 2009. Anti-*Acanthamoeba* efficacy and toxicity of miltefosine in an organotypic skin equivalent. J. Antimicrob. Chemother. 64, 539–545.

Weng, Y., Guo, X., Gregory, R.L., Xie, D., 2011. Preparation and evaluation of an antibacterial dental cement containing quaternary ammonium salts. J. Appl. Polym. Sci. 122, 2542–2551.

Widmer, F., Wright, L.C., Obando, D., Handke, R., Ganendren, R., Ellis, D.H., Sorrell, T.C., 2006. Hexadecylphosphocholine (miltefosine) has a broad-spectrum fungicidal activity and is efficacious in a mouse model of cryptococcosis. Antimicrob. Agents Chemother. 50, 414–421.

Yaseen, M., Lu, J.R., Webster, J.R.P., Penfold, J., 2005. Adsorption of single chain Zwitterionic phosphocholine surfactants: effects of length of alkyl chain and head group linker. Biophys. Chem. 117, 263–273.

Zidan, A.S., Rahman, Z., Khan, M.A., 2011. Product and process understanding of a novel pediatric anti-HIV tenofovir niosomes with a high-pressure homogenizer. Eur. J. Pharm. Sci. 44, 93–102.