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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),