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Original article

Dialkylamino and nitrogen heterocyclic analogues of hexadecylphosphocholine and cetyltrimetylammonium bromide: Effect of phosphate group and environment of the ammonium cation on their biological activity

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ABSTRACT

A series of dialkylamino and nitrogen heterocyclic analogues of hexadecylphosphocholine and cetyltrimethylammonium bromide have been synthesized. The prepared compounds exhibit significant cytotoxic, antifungal and antiprotozoal activities. Alkylphosphocholines possess higher antifungal activity against *Candida albicans* in comparison with quaternary ammonium compounds. However, quaternary ammonium compounds exhibit significant higher activity against human tumor cells and *Acanthamoeba lugdunensis* compared to alkylphosphocholines. In addition, their haemolytic toxicity has been investigated. The relationship between structure and biological activity of the tested compounds is discussed.

1. Introduction

Alkylphosphocholines (APCs) represent a class of potential agents for the clinical treatment of cancer [1–3]. The prototype of the APCs is hexadecylphosphocholine (miltefosine, **HPC**) which was initially developed as an anticancer agent [4]. The cytotoxic effect of HPC has been demonstrated in a wide range of tumors. Miltex®, 6% solution of **HPC**, is used as an effective palliative drug for treatment of cutaneous metastases from breast cancer [5]. Piperidine analogues of **HPC** revealed potentiation in cytotoxic activity on some cell lines [6,7]. The effect of APCs did not relate only to antineoplastic activity but they showed activity against fungi and protists. Obando et al. [8] studied antifungal activity of 14 APCs and alkylglycerophosphocholines (AGPCs). APCs exhibited higher antifungal activity than AGPCs [8]. Up to now, no efficient and easily manageable treatment is available for amoebic keratitis (AK) and granulomatous amoebic encephalitis. The most important reason for unsuccessful therapy seems to be the existence of a double-wall cyst stage that is highly resistant to the available treatments, causing reinfections [9]. Therefore, the susceptibility of several *Acanthamoeba* spp. to APCs was investigated by Walochnik et al. [10]. They studied the influence of the alkyl chain of APCs on antiprotozoal activity. In this study, **HPC** exhibited the highest cytotoxicity against trophozoites resulting in complete cell death at 20–40 μM, and also displayed significant cysticidal activity. The **HPC** is active against more parasites than merely *Acanthamoeba*, it exhibits also antiprotozoal activity on *Leishmania* [11], *Plasmodium* [12], *Balamuthia*, *Negleria* [13], *Trichomonas* [14], *Entamoeba* [15] or

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Trypanosoma [16]. **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 [17]. Some heterocyclic analogues of APCs exhibit higher antileishmanial activity than **HPC**. Hexadecyl [2-(*N*-methylpiperidinio)ethyl] phosphate (**HPPip**) is one of the most active APCs, its half maximal effective concentration (EC_{50}) was 0.3 μM [17].

Quaternary ammonium compounds (QUATs) are widely used as disinfectants in medicine, agriculture and industry. As more resistant organisms continue to emerge in the society, identification of additional antimicrobial agents becomes increasingly more important [18,19]. Their antifungal activity is well known and QUATs show high activity against fungi and yeast [20–23]. Cytotoxicity of surfactants has been extensively studied. Many types of QUATs exhibit antineoplastic activity or inhibit enzymes important for tumorigenesis [24–26]. Antiprotozoal activity of QUATs against *Acanthamoeba* spp. is mainly studied in connection with the disinfection of contact lenses. Benzalkonium chloride or polyquaternium 1 represents QUATs, which are used in commercial contact lens solutions [27].

The aim of the study was to prepare the nitrogen heterocyclic and dialkylamino analogues of **HPC** and cetyltrimethylammonium bromide (**CTAB**) and to evaluate their potential efficacies against human tumor cells, *Candida albicans* and *Acanthamoeba lugdunensis*. Moreover, the haemolytic properties of APCs and QUATs were studied on human red blood cells. Effect of phosphate group and ammonium cation environment of prepared compounds on biological activity was investigated.

2. Experimental

2.1. Materials and methods

All chemicals used for synthesis were purchased from commercial suppliers. Sabouraud agar and Sabouraud medium were purchased from Imuna Pharm a.s., Slovakia. Bacto-Casitone was obtained from *E. coli* s.r.o., Slovakia. *C. albicans* CCM 8186 was obtained from the Czech Collection of Microorganisms. *A. lugdunensis* was isolated from a patient suffering from amoebic ceratitis [28]. Jurkat (human T-cell acute lymphoblastic leukemia), HeLa (human cervical adenocarcinoma) MCF-7 (human breast adenocarcinoma, estrogen receptor-positive), MDA-MB-231 (human breast adenocarcinoma, estrogen receptor-negative) and A-549 cell lines (human lung adenocarcinoma) were kindly provided by Dr. M. Hajdúch (Olomouc, Czech Republic). CCRF-CEM cell line (human T-cell acute lymphoblastic leukemia) was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were routinely maintained in RPMI 1640 medium with L-Glutamine and 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Jurkat, HeLa and CCR-CEM) or Dulbecco's modified Eagle's medium with Glutamax-I (MCF-7, MDA-MB-231, and A-549) supplemented with 10% fetal calf serum, penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}) (all from Invitrogen, USA), in humidified air with 5% CO_2 at 37 °C. Before each cytotoxicity assay, cell viability was determined by trypan blue exclusion method and found greater than 95%. Infrared spectra were recorded on a FT-IR Impact 400 D spectrophotometer as potassium bromide discs. ^1H, ^{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 NMR spectra being recorded using proton-decoupling. The spectra were measured in $CDCl_3$ and DMSO-d_6 relative to internal standard TMS for ^1H and ^{13}C NMR spectra and to external standard 85% H_3PO_4 for ^{31}P NMR spectra. The purity of the prepared compounds was examined by TLC plate technique (DC-Alufolien Kiesegel 60 F_{256}, Merck).

2.2. Chemistry

2.2.1. General procedure for the quaternization of aminoalcohols with methyl p-toluenesulfonate

Aminoalcohol (22 mmol), prepared by hydroxyethylation [29], was added to the solution of methyl *p*-toluenesulfonate (20 mmol) in acetonitrile (20 ml). The resulting mixture was refluxed for 4 h. After cooling down, the acetonitrile was evaporated in vacuum. The resulting mixture was crystallized from acetone. The quaternary salts were obtained as white, hygroscopic solids.

Choline *p*-toluenesulfonate **2a**: yield 94%; ^1H NMR (DMSO-d_6, TMS) δ 2.29 (s, 3H), 3.09 (s, 9H), 3.34–3.44 (m, 2H), 3.78–3.88 (m, 2H), 5.27 (t, 1H, $J = 4.9$ Hz), 7.04 (s, 2H, $J = 8.0$ Hz), 7.48 (d, 2H, $J = 8.0$ Hz).

N,*N*-Diethyl-2-hydroxy-*N*-methylethanammonium *p*-toluenesulfonate **2b**: yield 87%; ^1H NMR (DMSO-d_6, TMS) δ 1.20 (t, 6H, $J = 7.2$ Hz), 2.28 (s, 3H), 2.95 (s, 3H), 3.27–3.40 (m, 6H), 3.75–3.83 (m, 2H), 5.26 (t, 1H, $J = 5.1$ Hz), 7.10 (d, 2H, $J = 7.9$ Hz), 7.46 (d, 2H, $J = 8.2$ Hz).

N-(2-hydroxyethyl)-*N*-methylpyrrolidinium *p*-toluenesulfonate **2e**: yield 89%; ^1H NMR (DMSO-d_6, TMS) δ 2.61–2.53 (m, 4H), 2.83 (s, 3H), 3.03 (s, 3H), 3.56–3.40 (m, 6H), 3.87–3.79 (m, 2H), 5.27 (t, 1H, $J = 4.8$ Hz), 7.10 (d, 2H, $J = 8.2$ Hz), 7.45 (d, 2H, $J = 7.9$ Hz).

N-(2-hydroxyethyl)-*N*-methylpiperidinium *p*-toluenesulfonate **2f**: yield 86%; ^1H NMR (DMSO-d_6, TMS) δ 1.58–1.48 (m, 2H), 1.83–1.74 (m, 4H), 2.28 (s, 3H), 3.06 (s, 3H), 3.45–3.27 (m, 6H), 3.88–3.80 (m, 2H), 5.26 (t, 1H, $J = 4.8$ Hz), 7.10 (d, 2H, $J = 8.1$ Hz), 7.45 (d, 2H, $J = 8.0$ Hz).

N-(2-hydroxyethyl)-*N*-methylazepanium *p*-toluenesulfonate **2g**: yield 89%; ^1H NMR (DMSO-d_6, TMS) δ 1.51–1.63 (m, 4H), 1.75–1.87 (m, 4H), 2.28 (s, 3H), 3.05 (s, 3H), 3.35–3.41 (m, 4H), 3.49–3.60 (m, 2H), 3.79–3.88 (m, 2H), 5.29 (t, 1H, $J = 4.8$ Hz), 7.10 (d, 2H, $J = 7.9$ Hz), 7.46 (d, 2H, $J = 8.0$ Hz).

N-(2-hydroxyethyl)-*N*-methylmorpholinium *p*-toluenesulfonate **2i**: yield 84%; ^1H NMR (DMSO-d_6, TMS) δ 2.29 (s, 3H), 3.20 (s, 3H), 3.38–3.61 (m, 10H), 3.83–3.96 (m, 2H), 5.29–5.40 (m, 1H), 7.10 (d, 2H, $J = 8.1$ Hz), 7.47 (d, 2H, $J = 8.1$ Hz).

2.2.2. General procedure for the preparation of APCs from choline p-toluenesulfonates

Solution of hexadecanol (9 mmol) in chloroform (20 ml) was added (dropwise at 0 °C) to a stirred solution of phosphorous 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 further without any subsequent additional purification. Pyridine (15 ml) was added dropwise at 0 °C to the resulting solution followed by the addition of choline *p*-toluenesulfonate or its diethylamino or heterocyclic analogue (12.5 mmol). The reaction mixture was stirred at r.t. over night. 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 tetrahydrofurane and water (5:1, V/V). To the stirred solution, exchange resin MB3 was added sequentially until the color of the resin stopped changing. Then the resin was filtered off and the solvents were evaporated in vacuum. The remaining mixture was dried with propan-2-ol. The resulting crude solid was purified by flash chromatography using CH_2Cl_2/MeOH/25% NH_3 (60/50/5, V/V/V) as a liquid phase. After chromatography the solvents were evaporated *in vacuo*. The residue was dissolved in chloroform and precipitated with acetone or petroleum ether. APC was filtered off and dried in vacuum dessicator.

Hexadecyl [2-(*N*,*N*,*N*-trimethylammonio)ethyl] phosphate × 1.5 H_2O (**HPC**): yield 17.2%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.7$ Hz), 1.22–1.47 (m, 26H), 1.54–1.64 (m, 2H), 2.37 (s, 3H), 3.41 (s, 3H), 3.78–3.86 (m, 4H), 4.25–4.34 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1, 22.7, 25.9, 29.4, 29.5, 29.7, 31.0, 31.1, 31.9, 54.5, 59.2, 65.6, 66.4; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ −0.78; IR υ_{max}/cm^{-1} 3425, 2923, 2852, 1644, 1468, 1250, 1088, 967, 720.

Hexadecyl [2-(*N*,*N*-Diethyl-*N*-methylammonio)ethyl] phosphate × 2.5 H_2O (**HPDEt**): yield 16.7%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.7$ Hz), 1.18–1.41 (m, 32H), 1.55–1.67 (m, 2H), 1.82 (s, 5H), 3.28 (s, 3H), 3.53–3.86 (m, 4H), 3.70–3.77 (m, 2H), 3.85 (q, 2H, $J = 6.5$ Hz), 4.28–4.37 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) 8.1, 14.1, 22.7, 25.9, 29.4, 29.5, 29.7, 31.0, 31.9, 48.4, 56.9, 58.5, 61.4, 65.5; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ −0.63; IR υ_{max}/cm^{-1} 3440, 2922, 2852, 1645, 1467, 1247, 1084, 966, 766, 722.

Hexadecyl [2-(*N*-Methylpyrrolidinio)ethyl] phosphate × 1.5 H_2O (**HPPyr**): yield 23.3%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.6$ Hz), 1.19–1.36 (m, 26H), 1.55–1.65 (m, 2H), 2.13–2.34 (m, 7H), 3.31 (s, 3H), 3.77–3.90 (m, 8H), 4.29–4.38 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1, 22.7, 25.9, 29.4, 29.5, 29.7, 31.0, 31.1, 31.9, 48.3, 58.5, 60.8, 60.9, 64.6, 65.6, 65.7; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ −0.86; IR υ_{max}/cm^{-1} 3399, 2919, 2851, 1653, 1467, 1249, 1081, 1000, 967, 768, 722.

Hexadecyl [2-(*N*-Methylpiperidinio)ethyl] phosphate × 2.5 H_2O (**HPPip**): yield 10.4%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.6$ Hz), 1.20–1.38 (m, 26H), 1.55–1.66 (m, 2H), 1.67–1.77 (m, 2H), 1.84–1.96 (m, 4H), 2.31 (s, 5H), 3.38 (s, 3H), 3.50–3.60 (m, 2H), 3.64–3.75 (m, 2H), 3.80–3.90 (m, 4H), 4.28–4.38 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1, 20.2, 21.0, 22.7, 25.9, 29.4, 29.5, 29.7, 31.0, 31.1, 31.9, 48.8, 58.4, 62.2, 63.9, 65.5, 65.6; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ −0.92; IR υ_{max}/cm^{-1} 3441, 2919, 2851, 1650, 1467, 1250, 1073, 971, 770, 722.

Hexadecyl [2-(*N*-Methylazepanio)ethyl] phosphate × 1.5 H_2O (**HPAzep**): yield 16.5%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.7$ Hz), 1.22–1.48 (m, 26H), 1.56–1.67 (m, 2H), 1.69–1.76 (m, 4H), 1.87–1.96 (m, 4H), 2.12 (s, 3H), 3.39 (s, 3H), 3.48–3.58 (m, 2H), 3.74–3.89 (m, 6H), 4.30–4.38 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1, 21.9, 22.7, 26.0, 27.9, 29.4, 29.5, 29.7, 31.0, 31.1, 31.9, 51.8, 58.8, 65.2, 65.3, 65.5; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ −0.84; IR υ_{max}/cm^{-1} 3388, 2921, 2851, 1648, 1467, 1264, 1082, 1002, 962, 766, 722.

Hexadecyl [2-(*N*-Methylmorpholinio)ethyl] phosphate × 1.5 H_2O (**HPMorph**): yield 10.8%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.6$ Hz), 1.22–1.46 (m, 26H), 1.54–1.63 (m, 2H), 2.75 (s, 3H), 3.53 (s, 3H), 3.68–3.84 (m, 6H), 3.99–4.08 (m, 6H), 4.30–4.39 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1, 22.7, 25.9, 29.4, 29.5, 29.7, 31.0, 31.1, 31.9, 48.3, 58.4, 60.8, 60.9, 64.6, 65.6, 65.7; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ −0.56; IR υ_{max}/cm^{-1} 3435, 2922, 2851, 1646, 1468, 1251, 1095, 1079, 962, 755, 722.

2.2.3. General procedure for the quaternization of N-methylazocane or N-*methyl*-N,N-*dipropylamine with 2-bromoethanol*

N-Methyl-*N*,*N*-dipropylamine or *N*-methylazocane (0.01 mol), prepared by Eschweiler-Clarke methylation [30] of dipropylamine or azocane, was quaternized with 2-bromoethanol (0.012 mol) in acetonitrile. The mixture was refluxed for 4 h. After cooling, the acetonitrile was evaporated in vacuum. The resulting mixture was triturated with ether and crystallized from acetone. White hygroscopic solid was obtained after crystallization.

N-(2-hydroxyethyl)-*N*-methyl-*N*-propylpropan-1-ammonium bromide (**2c**): yield 68%; ^{1}H NMR (DMSO-d_6, TMS) δ 1.05 (t, 6H, $J = 7.0$ Hz), 1.72–2.08 (m, 4H), 3.33 (s, 3H), 3.41–3.57 (m, 4H), 3.68–3.78 (m, 4H), 4.09–4.20 (m, 2H), 5.10 (t, 1H, $J = 4.9$ Hz).

N-(2-hydroxyethyl)-*N*-methylazocanium bromide (**2h**): yield 92%; ^{1}H NMR (DMSO-d_6, TMS) δ 1.45–1.71 (m, 6H), 1.79–1.98 (m, 4H), 3.05 (s, 3H), 3.06–3.56 (m, 6H), 3.79–3.87 (m, 2H), 5.26 (t, 1H, $J = 4.9$ Hz).

2.2.4. General procedure for the preparation of APCs from choline bromides

Hexadecanol (2 mmol) was treated with phosphorous oxychloride (2.1 mmol) and triethylamine (3 mmol) in THF. The solution was hydrolyzed with 1.5 ml H_2O for 1 h and evaporated *in vacuo*. A pyridinium salt was prepared by treatment with pyridine (16 ml) for 2 h at 50 °C. Pyridinium hexadecyl phosphate was coupled with **2c** or **2h** (3 mmol) in the presence of a condensing agent 2,4,6-triisopropylbenzenesulfonyl chloride. APC was purified by the procedure described in the previous method for preparation of APC from choline *p*-toluenesulfonates.

Hexadecyl 2-[*N*-Methyl-*N*,*N*-dipropylammonio]ethyl phosphate × H_2O (**HPDPr**): yield 13.6%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.7$ Hz), 1.03 (t, 6H, $J = 7.3$ Hz), 1.19–1.37 (m, 26H), 1.55–1.66 (m, 2H), 1.68–1.83 (m, 4H), 2.33 (s, 2H), 3.32 (s, 3H), 3.36–3.47 (m, 4H), 3.74–3.80 (m, 2H), 3.84 (q, 2H, $J = 6.7$ Hz), 4.28–4.36 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ 10.7, 14.1, 16.0, 22.7, 25.9, 29.4, 29.5, 29.7, 31.0, 31.1, 31.9, 49.6, 58.5, 62.4, 63.6, 65.5, 65.6; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ −0.48; IR υ_{max}/cm^{-1} 3443, 2923, 2854, 1646, 1467, 1247, 1095, 1075, 960, 761, 722.

Hexadecyl 2-(*N*-Methylazocanio)ethyl phosphate × 2 H_2O (**HPAzoc**): yield 20.4%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.7$ Hz), 1.22–1.38 (m, 26H), 1.55–1.79 (m, 8H), 1.90–2.01 (m, 4H), 2.08 (s, 4H), 3.37 (s, 3H), 3.54–3.73 (m, 4H), 3.80–3.90 (m, 4H), 4.28–4.37 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1, 22.0, 22.7, 24.5, 25.9, 26.2, 29.4, 29.5, 29.7, 31.0, 31.1, 31.9, 50.8, 58.7, 60.9, 63.8, 65.5, 65.6; ^{31}P NMR ($CDCl_3$, H_3PO_4) δ −0.64; IR υ_{max}/cm^{-1} 3394, 2922, 2851, 1648, 1467, 1229, 1080, 1000, 962, 751, 722.

2.2.5. General method for the preparation of QUATs

N-Methylated heterocycles or dialkylamines (0.01 mol) prepared by Eschweiler–Clarke methylation [30], and 1-bromohexadecane (0.011 mol) were added to 30 ml of dry acetonitrile, and then heated under reflux for 12 h. After having distilled the solvent off in vacuum the drying of the product was completed by azeotropic distillation with toluene or benzene. The quaternary ammonium salt was precipitated by diethyl ether. The precipitate was then filtered off and the crude product was recrystallized several times from a mixture of acetone/methanol.

N,*N*-Dietyl-*N*-methylhexadecylammonium bromide (**CMDEt**): yield 82%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.6$ Hz), 1.2–1.54 (m, 32H), 1.63–1.78 (m, 2H), 3.28 (s, 3H), 3.36–3.45 (m, 2H), 3.63 (q, 4H, $J = 7.2$ Hz); ^{13}C NMR ($CDCl_3$, TMS) δ 8.3, 14.1, 22.4, 22.7, 26.4, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 47.9, 56.6, 60.5; IR υ_{max}/cm^{-1} 2919, 2849, 1469, 720.

N-Methyl-*N*,*N*-dipropylhexadecylammonium bromide (**CMDPr**): yield 74%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.6$ Hz), 1.06 (t, 6H, $J = 7.2$ Hz) 1.2–1.48 (m, 26H), 1.64–1.87 (m, 6H), 3.35 (s, 3H), 3.41–3.53 (m, 6H); ^{13}C NMR ($CDCl_3$, TMS) δ 10.8, 14.1, 16.1, 22.5, 22.7, 26.4, 28.7, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 49.0, 61.7, 63.1; IR υ_{max}/cm^{-1} 2917, 2851, 1471, 718.

N,*N*-Dibutyl-*N*-methylhexadecylammonium bromide (**CMDBu**): yield 66%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.6$ Hz), 1.01 (t, 6H, $J = 7.3$ Hz), 1.21–1.53 (m, 30H), 1.64–1.79 (m, 6H), 3.38 (s, 3H), 3.43–3.57 (m, 6H); ^{13}C NMR ($CDCl_3$, TMS) δ 13.7, 14.1, 19.7, 22.5, 22.7, 24.4, 26.3, 29.2, 29.4, 29.5, 29.6, 29.7, 31.9, 48.9, 61.3, 61.4; IR υ_{max}/cm^{-1} 2920, 2851, 1468, 721.

N-Hexadecyl-*N*-methylpyrrolidinium bromide (**CMPyr**): yield 76%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.7$ Hz), 1.20–1.46 (m, 26H), 1.71–1.83 (m, 2H), 2.25–2.37 (m, 4H), 3.32 (s, 3H), 3.62–3.70 (m, 2H), 3.78–3.94 (m, 4H); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1, 21.7, 22.7, 24.1, 26.4, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 48.6, 64.1, 64.4; IR υ_{max}/cm^{-1} 2918, 2851, 1467, 721.

N-Hexadecyl-*N*-methylpiperidinium bromide (**CMPip**): yield 69%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.7$ Hz), 1.21–1.47 (m,

26H), 1.66–2.02 (m, 8H), 3.37 (s, 3H), 3.60–3.71 (m, 4H), 3.79–3.88 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1, 20.2, 20.7, 22.1, 22.7, 26.4, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 48.5, 60.7, 62.6; IR υ_{max}/cm^{-1} 2918, 2850, 1467, 721.

N-Hexadecyl-*N*-methylazepanium bromide (**CMAzep**) yield 75%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.7$ Hz), 1.20–1.46 (m, 26H), 1.68–1.89 (m, 6H), 1.89–2.09 (m, 4H), 3.40 (s, 3H), 3.58–3.69 (m, 4H), 3.71–3.81 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1, 22.1, 22.7, 23.0, 26.4, 27.4, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 51.2, 64.7, 64.9; IR υ_{max}/cm^{-1} 2920, 2851, 1467, 721.

N-Hexadecyl-*N*-methylazocanium bromide (**CMAzoc**): yield 84%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.7$ Hz), 1.22–1.47 (m, 26H), 1.59–1.85 (m, 8H), 1.94–2.03 (m, 4H), 3.36 (s, 3H), 3.45–3.55 (m, 2H), 3.58–3.66 (m, 2H), 3.72–3.83 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1, 22.0, 22.7, 22.8, 24.4, 26.3, 26.4, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 50.2, 59.6, 63.5; IR υ_{max}/cm^{-1} 2919, 2851, 1468, 721.

N-Hexadecyl-*N*-methylmorpholinium bromide (**CMMorph**): yield 58%; ^{1}H NMR ($CDCl_3$, TMS) δ 0.88 (t, 3H, $J = 6.6$ Hz), 1.19–1.50 (m, 26H), 1.65–2.05 (m, 4H), 3.37 (s, 3H), 3.60–3.75 (m, 4H), 3.78–3.90 (m, 2H); ^{13}C NMR ($CDCl_3$, TMS) δ 14.1, 22.0, 22.7, 26.3, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 47.3, 59.7, 60.7, 65.4; IR υ_{max}/cm^{-1} 2920, 2851, 1473, 1120, 901, 720.

2.3. Cytotoxicity assay

The cytotoxic effects of compounds were determined using colorimetric microculture assay with the MTT (3[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) end-point [31]. Briefly, 3×10^3 cells ml^{-1} (A-549, MCF-7, MDA-MB-231), 5×10^3 cells ml^{-1} (HeLa) or 1×10^4 cells ml^{-1} (Jurkat and CCRF-CEM) cells were plated per well in 96-well polystyrene microplates (Sarstedt, Germany) in the culture medium containing tested chemicals at final concentrations of 1×10^{-4} to 1×10^{-8} mol l^{-1}. After 72 h of incubation, 10 μL of MTT (5 mg ml^{-1}) were added in each well. After an additional 4 h, during which insoluble formazan was produced, 100 μl of 10% sodium dodecylsulphate were added in each well and another 12 h were allowed for the dissolution of formazan. Absorbance was measured at 540 nm using the automated MRX microplate reader (Dynatech Laboratories, UK). The blank-corrected absorbance of the control wells was taken as 100% and the results were expressed as a percentage of the control. All experiments were performed in triplicate. IC_{50} values (concentrations of tested agents that inhibited growth of cell cultures to 50% of the untreated control) were determined by GraphPad Prism for Windows version 5.01.

2.4. Fungicidal susceptibility testing of APCs and QUATs

A cell suspension culture of *C. albicans* was prepared from 24-h cultures of yeast in the Sabouraud agar. The concentration of culture was 5×10^5 cells ml^{-1}. The suspensions were prepared in physiological solution (pH 7.2). The concentration of microorganisms was measured spectrophotometrically and the suspensions were concentrated 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 Sabouraud medium (12%) (100 μl). The cultivation was performed in 96-well microtiter plate. The solution of the studied compounds was prepared in water. The microorganisms were incubated for 24 h at 37 °C. Then from each well, 5 μl of tested suspension was taken and cultured on the Sabouraud agar. The Petri dishes were incubated for 24 h at 37 °C. The lowest concentration of the APC and the QUATs supporting no colony formation was defined as the minimal fungicidal concentration (MFC).

2.5. Trophocidal susceptibility testing of APCs and QUATs

These procedures were carried out using the modified methods described by [10]. The cultures of amoeba were grown axenically in 100 ml Erlenmeyr flasks. Axenic culture was obtained from the monoxenic culture of *A. lugdunensis*. Trophozoites were harvested from 2-day monoxenic plate cultures and transferred to Bacto-Casitone/Serum (BCS) medium with penicillin and ampicillin. Actively growing trophozoites were harvested by centrifugation at 500*g* for 7 min and subsequently they were incubated at 37 °C for 3 days. Thereafter, trophozoites were transferred to a BCS medium without antibiotics and cultivated at 37 °C for 3 days. The cultivation in the BCS medium was repeated 5-times, then the trophozoites were transferred to peptone–yeast extract–glucose (PYG) medium and afterwards cultivated in this medium. Experiments were carried out in 96-well microtiter plates at 37 °C under sterile conditions. Each well was seeded with 100 μl (2×10^5 cells ml^{-1}) of a trophozoite suspension. Then 100 μl of test substance solution in PYG medium was added and mixed with the suspension of trophozoites. APCs were tested at six concentrations (25, 50, 100, 200, 400, 800 μM) and QUATs were tested at 5 concentrations (100, 50, 25, 12.5, 7.25 μM). Viability was determined by trypan blue exclusion. One hundred percent eradication was confirmed by transferring 50 μl of the suspension to a PYG medium and by recording the amoeba growth for 14 days. The lowest concentration of the APCs and the QUATs supporting for 100% eradication of trophozoites was defined as the minimal trophocidal concentration (MTC).

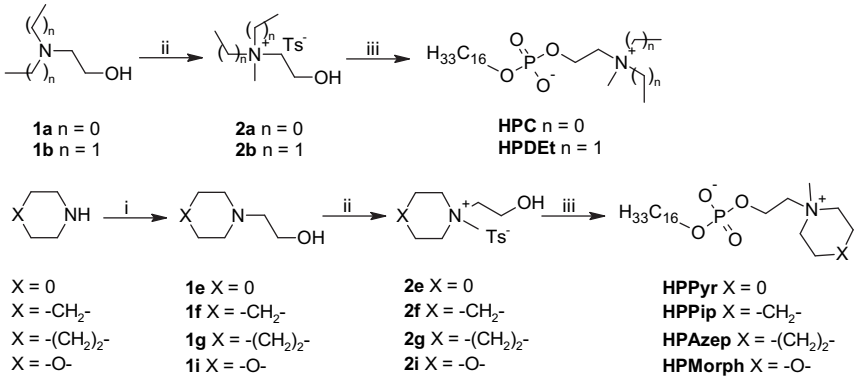
2.6. Haemolytic activity assay of APCs and QUATs

Human blood was collected in 10 ml tubes containing sodium citrate as anticoagulant. The blood was transferred to a centrifuge tube and the cells were washed three times with calcium- and magnesium-free phosphate-buffer saline (PBS). Cells were collected by centrifugation at 2000*g* for 10 min. The cells were then suspended in the buffer (PBS – 145 mM NaCl, 5 mM KCl, 4 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 10 mM glucose, buffer was adjusted to pH = 7.4 [32]). The prepared cell culture could be stored at 4 °C for 48 h. The cell density of the stock suspension of the erythrocytes was 6.7–6.8×10^8 cells ml^{-1}. One volume of the cell suspension was pipetted to 9 volumes of buffer containing different concentrations of the tested compounds. Final erythrocyte concentrations were 6.7–6.8×10^7 cells ml^{-1}. The mixtures were incubated at 37 °C for 1 h with gentle shaking and then centrifugated at 2000*g* for 10 min. The degree of haemolysis was determined by comparing the absorbance (542 nm) of appropriate dilutions of the supernatant with that of control samples haemolysed in 500 μM solution of **CTAB** in PBS.

3. Results and discussion

3.1. Chemistry

The synthetic strategy for the preparation of the APCs is depicted in Schemes 1 and 2. Representative phosphate analogues were prepared to determine the importance of the phosphocholine moiety for bioactivity according to modified procedures [6,33,34]. The strategy involves phosphorylation of the hexadecanol using $POCl_3$ and subsequent attachment of the desired choline analogue. **HPC**, **HPDEt**, **HPPyr**, **HPPip**, **HPAzep** and **HPMorph** were prepared by reaction of hexadecyl dichloridophosphate with choline *p*-toluenesulfonate and subsequently hydrolyzed. **HPDPr** and **HPAzoc** were prepared by another strategy. Hexadecyl dichloridophosphate



Scheme 1. Synthesis of ALPs – **HPC**, **HPDEt**, **HPPyr**, **HPPip**, **HPAzep**, **HPMorph**. Reagents and conditions: i 2-chloroethanol, K_2CO_3, benzene, reflux; ii methyl *p*-toluenesulfonate, CH_3CN, reflux; iii $POCl_3$, hexadecanol, Et_3N, $CHCl_3$, pyridine.

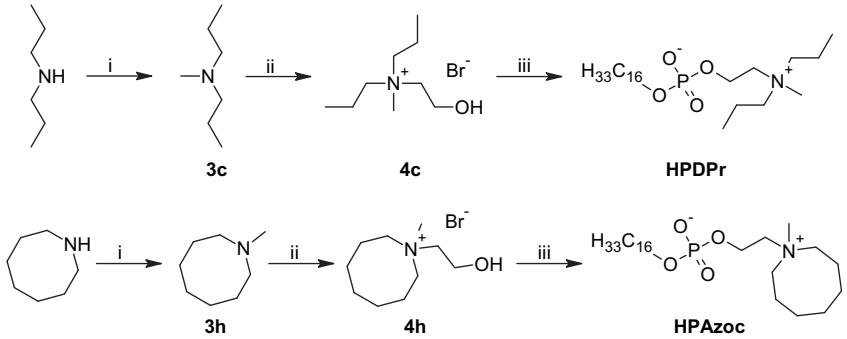
was firstly hydrolyzed by water and transformed to dipyridinium hexadecyl phosphate using pyridine. This salt was then connected with choline bromide in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride. The choline analogues were obtained with two synthetic procedures: 1. Tertiary 2-aminoethanoles **1e**, **1f**, **1g** and **1i** were prepared by the reaction of secondary amine with 2-chloroethanol and potassium carbonate in the presence of a catalytic amount of KI. The appropriate cholines were obtained by quaternization of **1a**, **1b**, **1e**, **1f**, **1g** and **1i** with methyl *p*-toluenesulfonate in acetonitrile. The synthesis of choline *p*-toluenesulfonate analogues **2a**, **2b**, **2e**, **2f**, **2g**, and **2i** is depicted in Scheme 1. *2*. Dipropylamine and azocane were methylated by Eschweiler–Clarke reaction, resulting in tertiary amines **3c** and **3h**. The choline bromides **4c** and **4h** were obtained by quaternization of prepared tertiary amines with 2-bromoethanol. Dibutyl analogue of choline bromide or *p*-toluenesulfonate is very hygroscopic and it is very hard to obtain in solid form (*N*,*N*-dibutyl-2-hydroxy-*N*-methylethanammonium bromide started to crystallize from the acetone at −18 °C after half a year). The prepared APCs crystallized as hydrates. The amount of bounded water was estimated by ^{1}H NMR spectroscopy. The position of signal depends on the concentration of measured substances in $CDCl_3$.

QUATs **CMDEt**, **CMDPr**, **CMDBu**, **CMPyr**, **CMPip**, **CMAzep**, **CMAzoc** and **CMMorph** were prepared by Menschutkin reaction, tertiary amines **3b**, **3c**, **3d**, **3e**, **3f**, **3g**, **3h** and **3i** were quaternized with 1-bromohexadecane in dry acetonitrile. The QUATs were prepared by the procedure as was previously described by Devínsky et al. [35]. The secondary amines were methylated by Eschweiler–Clarke reaction (Scheme 3).

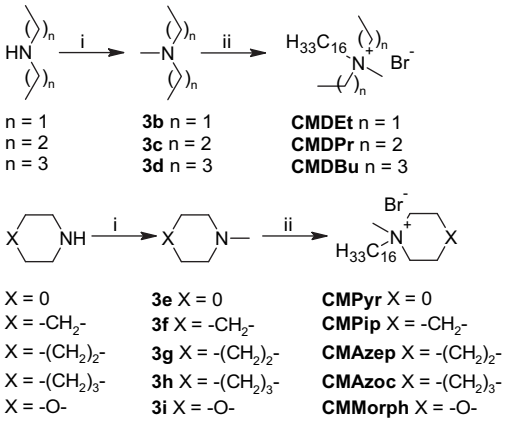
3.2. Antineoplastic activity of APCs and QUATs

HPC was considered as a standard compound of APCs, its cytotoxicity is very well known. **HPC** and other APCs exhibited the best activity against human acute T-lymphoblastic leukemia (Table 1). The APCs with smaller heterocyclic rings or shorter alkyl chains of dialkylamino analogues were more active than the compounds with the biggest heterocyclic ring (**HPAzoc**) or dipropylamino analogue (**HPDPr**). Insertion of oxygen to heterocyclic ring increased the antileukemic activity. **HPMorph** ($IC_{50} = 7.7$ μM) was two times active against Jurkat in comparison with its analogue without oxygen (**HPPip**, $IC_{50} = 14$ μM). The antileukemic activity of the APCs was approximately four times higher than the antineoplastic activity compared to other studied carcinoma cell lines. **HPMorph** again exhibited the highest activity against the remaining investigated cancer cell lines. The least active compound was **HPPip**. Its IC_{50}s against HeLa, MDA-MB-231, MCF-7 and A-549 were higher than 50 μM. This observation is startling. Koufaki et al. [6] tested the cytotoxicity of these two APCs against six human tumor xenografts. **HPPip** was the most active compound from the studied APCs and **HPMorph** exhibited only moderate activity. **HPPip** was very active against small cell and non-small cell lungs carcinoma [6] but we observed that it was inactive against lungs carcinoma A-549 ($IC_{50} > 100$ μM) in our case. Increasing of heterocyclic rings or prolongation of alkyl chains on ammonium cation decreased the cytotoxicity of the APCs against HeLa, MDA-MB-231 and MCF-7 only slightly.

CTAB was considered as a standard compound of QUATs. Its cytotoxic activity is lower on comparison with other QUATs.



Scheme 2. Synthesis of ALPs – **HPDPr**, **HPAzoc**. Reagents and conditions: i 35% HCHO, 85% HCOOH, reflux; ii 2-bromoethanol, CH_3CN, reflux; iii hexadecanol, $POCl_3$, Et_3N, $CHCl_3$, 2.4.6-triisopropylbenzenesulfonyl chloride, pyridine.



Scheme 3. Synthesis of QACs. Reagents and conditions: i 35% HCHO, 85% HCOOH, reflux; ii 1-bromohexadecane, CH_3CN, reflux.

Elongation of alkyl chains in dialkylamino groups increased the activity in the case of cell lines: Jurkat, CCRF-CEM, Hela and MDA-MB-231. On the other hand, the tumor cell lines MCF-7 and A-549 were not so sensitive on elongation of the alkyl chains of the dialkylamino groups. The activity was affected only slightly. The substitution of the dimethyl amino group to nitrogen heterocycle (pyrrolidine, piperidine, azepine and azocane) in the molecule of CTAB led to increased cytotoxic effects. Only **CMAzoc** exhibited lower activity than **CTAB** in the case of tumor cell lines A-549 and MCF-7. Insertion of oxygen to the heterocyclic ring decreased antineoplastic activity. **CMMorph** was the least active QUATs. This observation is very interesting when we compare the activity of the APCs and the QUATs. Morpholine analogue of **HPC** is the most cytotoxic active compounds from the APCs but on the other hand morpholine analogue of **CTAB** was the least active compound from the QUATs. The QUATs were about 10-times more active than the APCs. Disadvantage of the QUATs is in their high cytotoxicity not only on tumor cells but on health human cells. Cationic surfactants induce apoptosis in normal and cancer cells. Benzethonium chloride as a model QUAT increases DNA fragmentation and also accelerated condensation of nuclei in rat thymocytes more than in Jurkat cells [36]. Synthetic APCs are anticancer agents that in contrast to most anticancer drugs do not target DNA [37]. Instead, **HPC** decreases the destructive cytotoxic effect of epirubicine and cyclophosphamide on mouse spermatogenic and hematopoietic cells. For example, **HPC** significantly reduces of aberration of chromosomes (clastogenicity) without changes in proliferative activity of bone marrow cells [38]. Both the APCs and the QUATs inserted into the plasma membrane and subsequently induce a broad range of biological effects, ultimately leading to cell death [36,37].

3.3. Antifungal activity of APCs and QUATs

The MFC of **HPC** against *C. albicans* was 4 μM; this value corresponds to the same value published by Widmer at al. [39]. **HPC** and **HPDEt** presented the best activity against *C. albicans* (Table 1). Expansion of the length of head group alkyl chains from 1–2 carbon atoms to 3 carbon atoms (**HPDPr**) significantly decreased the antifungal activity. **HPDPr** is the least active compound from the studied APCs. Heterocyclic analogues were less active as **HPC**. For the heterocyclic APC compound series, expansion of the heterocyclic ring or insertion of another heteroatom (morpholine) had no effect on antifungal activity.

CTAB as a standard compound of QUATs exhibited lower fungicidal activity (MFC = 21 μM) in comparison with **HPC**. Ahlström et al. observed complete elimination of *Candida* cells by **CTAB** at concentration 100 μM [40]. The higher concentration of *Candida* cells in the tested culture is a possible reason of higher MFC value in the case of fungicidal determinations provided by Ahlström et al. [40]. Dialkylamino analogues of **CTAB** with an even number of carbon atoms in the head group alkyl chains were more effective than **CTAB**, however, **CMDPr** had similar activity as the standard compound. Heterocyclic analogues showed the same activity as **CMDEt** and **CMDBu**, only **CMMorph** was the least active from all QUATs.

The reason of higher antifungal activity of APCs in the comparison with QUATs is probably in a different mode of action. Widmer et al. [39] suggested that the effect of HPC was due to selective lysophospholipase-transacylase of phospholipase B1 (PLB 1) inhibition rather than a nonspecific detergent-like action which is typical for the QUATs [41]. However, the inhibition of PLB 1 is not sufficient for the fungicidal activity of the APCs and they should also have other biochemical targets which are responsible for antifungal effect of APCs [8].

3.4. Antiprotozoal activity of APCs and QUATs

HPC was weakly active against *Acanthamoeba lugdunensis* (Table 1). Its MTC was 400 μM. Walochnik et al. [10] studied three

Table 1
Antineoplastic, fungicidal, trophocidal and haemolytic activities of APCs and QUATs.

Compound	IC_{50} (μM)						MFC (μM)	MTC (μM)	EC_{50} (μM)
	Jurkat	CCRF-CEM	HeLa	MDA-MB-231	MCF-7	A-549	*C. albicans*	*A. lugdunensis*	Haemolytic activity
HPC	10.0	13.5	41.6	37.6	39.2	44.7	4	400	30
HPDEt	19.6	9.0	49.4	34.4	53.7	59.4	4	200	30
HPDPr	28.6	19.2	55.0	40.6	50.6	55.0	16	400	30
HPPyr	10.0	9.3	46.2	37.9	39.3	53.2	8	200	30
HPPip	14.0	11.2	57.2	51.7	54.8	>100	8	200	25
HPAzep	18.2	9.8	53.1	49.8	51.5	55.1	8	50	25
HPAzoc	38.6	43.8	55.0	45.6	53.3	55.0	8	50	30
HPMorph	7.7	8.25	21.7	11.4	22.4	42.0	8	200	30
CTAB	1.98	3.25	4.10	4.50	0.75	0.76	21	25	45
CMDEt	0.8	2.10	1.38	4.90	0.65	0.62	10	25	40
CMDPr	0.85	1.96	1.68	4.27	0.80	0.75	19	25	30
CMDBu	0.76	0.91	0.90	2.55	0.68	0.64	9	25	25
CMPyr	0.82	1.35	2.29	3.80	0.62	0.53	10	12.5	35
CMPip	0.72	0.92	1.47	3.86	0.59	0.63	10	12.5	35
CMAzep	0.80	1.00	1.82	2.84	0.72	0.67	9	12.5	35
CMAzoc	0.78	1.00	1.43	4.12	0.94	0.85	9	12.5	30
CMMorph	3.57	4.16	5.61	5.75	0.89	0.87	24	50	45

species of *Acanthamoeba*: *A. castellanii*, *A. polyphaga*, *A. lenticulata* and they observed lower values of the MTC (MTC = 20–40 μM). The low values of the MTC were also obtained by McBride et al. [42]. They studied the influence of **HPC** on two strains of *Acanthamoeba*: *A. polyphaga* (MTC = 31.25 μM), *A. castellanii* (MTC = 31.25 μM). In our case, **HPC** was about 10-time less active against *A. lugdunensis* [43] in comparison with its activity against *A. castellanii*, *A. polyphaga* and *A. lenticulata* which was observed by other authors. This significant discrepancy can be most likely explained with testing the compound on different species of the genus *Acanthamoeba*. Dialkylamino analogues of APCs were low active compounds similar to **HPC**. The same activity was shown by the heterocyclic analogues **HPPyr**, **HPPip** and **HPMorph**. The expansion of heterocyclic ring to 7–8 atoms caused an increase of the trophocidal activity. **HPAzep** and **HPAzoc** were about 4-times more active than other heterocyclic analogues and they were about 8-times more effective than **HPC**.

QUATs exhibited excellent activity against *A. lugdunensis*. We have not found any investigation of antiprotozoal activity of **CTAB** against *Acanthamoeba* recently, but the MTC of these QUATs is comparable with the MTC of benzalkonium chloride, a commercially available disinfectant [27]. **CTAB** and dialkylamino analogues had the same values of MTC. In the series of the investigated compounds, the activity did not depend on the alkyl chains length of the head group. Heterocyclic compounds showed a 2-times better activity than **CTAB** and dialkylamino analogues, only the **CMMorph** exhibited the least activity of all.

3.5. Haemolytic activity of APCs and QUATs

The investigated APCs and QUATs are relatively haemolytic toxic compounds (Table 1) because their toxicity depended on alkyl chain length [8,44]. The haemolytic activity of the APCs did not depend on head groups. Very low changes of this activity were observed in dependence on structural modifications.

A different situation occurred in the case of QUATs. **CTAB** and **CMMorph** exhibited the lowest haemolytic activity. The alkyl chains length of dialkylamino analogues played an important role in the haemolytic activity. The elongation of alkyl chains led to a gradual increase of haemolytic activity. The haemolytic activity of heterocyclic QUATs did not depend on ring size but they had a higher activity than **CTAB**.

4. Conclusion

16 compounds were prepared; 8 APCs and 8 QUATs. **CTAB** was obtained as a commercially available substance. We investigated the influence of a polar head group of the APCs and the QUATs on cytotoxic, fungicidal, trophocidal and haemolytic activities. In this study, we focussed on modifying the dimethylamino group of **HPC** and **CTAB** as standard compounds for APCs and QUATs. We prepared two series of the APCs and the QUATs: dialkylamino and nitrogen heterocyclic analogues.

The cytotoxic activity of APCs and QUATS were examined on six human cancer cells lines – Jurkat, CCRF-CEM, HeLa, MDA-MB-231, MCF-7 and A-549. The QUATs exhibited better cytotoxic activity in comparison with the APCs. The most active compound was dibutylamino analogue of **CTAB**. **CMDBu** exhibited the best activity on tree cell lines, one leukemia (CCRF-CEM) and two carcinoma (HeLa, MDA-MB-231). The APCs were about 10-times less active than the QUATs. Generally, **HPMorph** exhibited the best cytotoxic activity on all studied cancer cell lines. Generally, the best fungicidal activity against *C. albicans* was exhibited by the APCs. The most active compounds were **HPC** and **HPDEt** with MFC = 4 μM. A different situation was observed in the case of antiprotozoal action of the APCs and the QUATs against *A. lugdunensis*. Higher trophocidal activity appertained to the QUATs. The APCs exhibited very low trophocidal activity, only **HPAzep** and **HPAzoc** achieved activity comparable with the activity of the QUATs. Haemolytic activity of the tested compounds was similar. Haemolysis of erythrocytes caused by the APCs and the QUATs only slightly depended on the structure of the polar head group. The APCs were a little bit more toxic than the QUATs.

Acknowledgements

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