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Increased lipid peroxidation, apoptosis and selective cytotoxicity in colon cancer cell line LoVo and its doxorubicin-resistant subline LoVo/Dx in the presence of newly synthesized phenothiazine derivatives

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ABSTRACT

Cancer cells often develop the resistance to pro-apoptotic signaling that makes them invulnerable to conventional treatment. Therapeutic strategies that make cancer cells enter the path of apoptosis are desirable due to the avoidance of inflammatory reaction that usually accompanies necrosis. In the present study phenothiazines (fluphenazine and four recently synthesized derivatives) were investigated in order to identify compounds with a potent anticancer activity. Since phenothiazines are known as multidrug resistance modulators the sensitive human colorectal adenocarcinoma cell line (LoVo) and its doxorubicin-resistant, ABCB1 overexpressing, subline (LoVo/Dx) have been employed as a model system. In studied cancer cells cytotoxic effect of the phenothiazine derivatives was accompanied by apoptosis and autophagy induction as well as by the increase of cellular lipid peroxidation and intracellular reactive oxygen species generation. Molecular modelling revealed that reactivity of phenothazines (manifested by their low energy gap) but not lipophilicity was positively correlated with their anticancer potency, pro-oxidant properties and apoptosis induction ability. Additionally, some of the studied compounds turned out to be more potent cytotoxic and pro-apoptotic agents in doxorubicin-resistant (LoVo/Dx) cells than in sensitive ones (LoVo). The hypothesis was assumed that studied phenothiazine derivatives induced apoptotic cell death by increasing the production of reactive oxygen species.

1. Introduction

The major mode of programmed cell death is apoptosis, essential for the maintenance of homeostasis in normal tissues. Cancer cells often develop the resistance to pro-apoptotic signaling that makes them invulnerable to conventional treatment [1]. Therapeutic strategies that aim to make cancer cells enter the path of apoptosis are desirable because apoptosis, unlike necrosis, is not accompanied by a strong inflammatory reaction. Apoptosis can be initiated by many different factors including the disturbance in cellular redox balance (reviewed in [2]). The imbalance between the intensity of the oxidative processes resulting in the formation of reactive oxygen species (ROS), and the anti-oxidant activity of the cells causes oxidative stress. Oxidative stress elicits diverse types of cellular responses, such as temporary reduction of growth and proliferation, and eventually cell death [3]. It has been shown that elevated ROS concentration can cause apoptosis by increasing the level of cellular lipid peroxidation and, in consequence, by augmenting permeability of mitochondrial membrane [4]. The disruption of mitochondrial membrane leads to cytochrome c release and to the activation of executive caspases. The ability of ROS to induce apoptosis results in the possibility of eliminating tumor cells by "introducing" them, by means of pharmacological agents, into the state of increased oxidative stress [3,4]. The increased production of ROS was reported after exposure of cancer cells to various cytostatic drugs (*e.g.*, doxorubicin, mitomycin C, and cisplatin) [5].

Phenothiazines are tricyclic compounds in which structure two

Abbreviations: ABu-TPR, S(+-10-[3-(1-ethyl-2-hydroxyethylamino)-2-hydroxypropyl]-2-trifluoromethylphenothiazine hydrochloride; APh-FLU, 10-{3-[4-(4-acetylphenylpiperazin-1-yl]-2-hydroxypropyl}-2-trifluoromethylphenothiazine dihydrochloride; DCF-, DA 2′,7′-dichlorodihydrofluorescein diacetate; FLU, fluphenazine; HBA, hydrogen bond acceptors; HBD, hydrogen bond donors; HEE-FLU, 10-{3-[4-(2-hydroxyethoxyethylpiperazin-1-yl]-2-hydroxypropyl}-2-trifluoromethylphenothiazine dihydrochloride; MAE-TPR, 10-[3-(N-2-hydroxyethyl-*N*-methylamino-2-hydroxypropyl]-2-trifluoromethylphenothiazine hydrochloride; MDA, malondialdehyde; NAC, N-acetylcysteine; PHDs, phenothiazine derivatives; QSAR, structure-activity relationship; ROS, reactive oxygen species

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benzene rings are joined by atoms of nitrogen and sulfur thus forming a 1,4-thiazine middle ring. They constitute the oldest group of neuroleptic drugs. The therapeutic effect of phenothiazines is attributed to the blockade of dopamine receptors, although the interaction with other receptors and channels is also probable [6]. Many intracellular proteins, *e.g.*, calmodulin [7], protein kinase C [8], and troponin C [9] can be inhibited by these drugs. Being cationic, amphiphilic compounds phenothiazines are likely to interact with phospholipid membranes (reviewed in [10]). Many activities of phenothiazines in cancer cells have been reported (reviewed in [11–13]). Induction of apoptosis by phenothiazines was observed in several leukemic cell lines [14], neuroblastoma and glioma cells [15], oral squamous cancer cells [16], colon adenocarcinoma cells [17], as well as in cervical and endometrial cancer cell lines [18]. Phenothiazines were often reported to induce apoptosis in the caspase-dependent way. Their ability to activate of caspase 3 [15,19–21] as well as caspase 8 and 9 [20,22] was demonstrated, indicating the activation of both extrinsic and intrinsic apoptotic pathways. Additionally, in human lung cancer cells co-treated by DNA-damaging drugs and phenothiazines a protracted cell-cycle arrest occurred followed by a form of apoptosis associated with increased endogenous oxidative stress and intense vacuolation suggesting that lysosomal dysfunction might be a major component of cell death in the presence of phenothiazines [22] Phenothiazines are also known as potent modulators of multidrug resistance of cancer cells, able to restore sensitivity to anticancer drugs in resistant cells (reviewed in [23]). Their ability to increase the accumulation of cytotoxic drugs in resistant cells has been known for more than 35 years [24]. Phenothiazines are potent inhibitors of ABCB1 transporter [25,26]. Although phenothiazines were demonstrated to be effective multidrug resistance modulators in numerous *in vitro* studies [27–29], the outcome of clinical trials was negative. The main reason for negative results were the concentrations of phenotiazines achieved in plasma that were too low to reach the desired effect (reviewed in [30]).

In the present study new phenothiazine derivatives (PHDs) were investigated in order to identify compounds with a potent anticancer activity. On the basis of previously identified structure-activity relationships of phenothiazine analogues, the modifications were introduced into the structure of FLU in order to reduce its serious side effects in the central nervous system and to increase its pro-apoptotic activity in cancer cells [31]. A piperazine phenothiazine – fluphenazine and four recently synthesized PHDs have been included to the study. Since phenothiazines are known to be multidrug resistance modulators the pair of sensitive (LoVo) and drug-resistant (LoVo/Dx) human colorectal adenocarcinoma cell lines has been employed as a model system. Our previous studies demonstrated the expression of several ABC transporters (ABCB1, ABCC1, ABCG2) in both cell lines [32]. The overexpression of ABCB1 (P-glycoprotein) in LoVo/Dx cells as compared to LoVo was identified to be a factor responsible for the resistance to doxorubicin in the former cell line. Several PHDs able to differentially suppress growth in doxorubicin-resistant subline LoVo/Dx as compared to the parental LoVo cell line were identified. The putative mechanisms of cancer cell death were investigated and PHDs were demonstrated to induce both apoptosis and autophagy processes. Additionally membrane lipid peroxidation and intracellular ROS generation were enhanced in the presence of PHDs. Methods of molecular modelling were employed to find out the physicochemical as well as structural parameters of PHDs molecules crucial for the their biological activity.

2. Materials and methods

2.1. Compounds

Fluphenazine (FLU) was purchased from Sigma-Aldrich. All compounds used in the experiments were dissolved in DMSO. The structures of derivatives used in the experiments are presented in Fig.1. MAE-TPR 10-[3-(N-2-hydroxyethyl-*N*-methylamino)-2-hydroxypropyl]-2-trifluoromethylphenothiazine hydrochloride; and HEE-FLU 10-{3-[4-(2-hydroxyethoxyethyl)piperazin-1-yl]-2-hydroxypropyl}-2-trifluoromethylphenothiazine dihydrochloride were synthesized as described previously [31]. The synthesis of ABu-TPR S(+)-10-[3-(1-ethyl-2-hydroxyethylamino)-2-hydroxypropyl]-2-trifluoromethylphenothiazine hydrochloride was described by Zyta et al. [33].

APh-FLU 10-{3-[4-(4-acetylphenyl)piperazin-1-yl]-2-hydroxypropyl}-2-trifluoromethylphenothiazine dihydrochloride was synthesized as follows. A solution of 0.00124 mol of 10-(2,3-epoxypropyl)-2-trifluoromethylphenothiazine [33] and 0.00124 mol of (4-acetylphenyl)piperazine in 10 ml of ethanol was refluxed under stirring for 6 h. Then the solvent was evaporated under reduced pressure and the residue was purified through flash chromatography. In the next step the obtained product was transformed to the corresponding salt by means of ethanol saturated with hydrogen chloride gas.

Analytical data for free base:

Formula: $C_{28}H_{28}F_3N_3O_2S$; m.w.: 527.60; m.p.:138-140 °C; 48.7% yield; CC [ethyl acetate, Rf = 0.72]. $_1$H NMR: 2.50–2.68 (m, 9H, CH_3 and $CH_2N(CH_2)_2$), 3.25–3.39 (m, 4H, $N(CH_2)_2$), 4.03–4.14 (m, 3H, $N_{10}-CH_2CH$), 5.84 (brs, 1H, $CHOH_{ex}$), 6.78–7.24 (m, 9H, 7PhtH and 2ArH), 7.80–7.94 (m, 2H, ArH).

2.2. Molecular modelling

Geometry optimization for molecules of investigated compounds was made by the DFT/B3LYP/6-31 + G* model, using the calculating package of SPARTAN 14 (Wavefunction, Inc.). The geometry optimization was carried out in two steps. First, an equilibrium conformer with the lowest energy for the molecule was found using the MM/MMFF model, later the structure optimization of this conformer was performed.

The values of following descriptors were calculated: absolute hardness (η), absolute electronegativity (χ), chemical potential of electrons (μ) and electrophilicity index (ω). These descriptors were determined on the basis of the calculated energy value for HOMO (ε_{HOMO}) and LUMO orbitals (ε_{LUMO}) using the following equations [34] [35]:

$$\chi = -\mu = -\frac{\varepsilon_{LUMO} + \varepsilon_{HOMO}}{2} \quad (1)$$

$$\eta = \frac{\varepsilon_{LUMO} - \varepsilon_{HOMO}}{2} \quad (2)$$

$$\varpi = \frac{\mu^2}{2\eta} \quad (3)$$

2.3. Cell culture

The human colorectal adenocarcinoma cell line LoVo and its doxorubicin-resistant subline LoVo/Dx [36] were obtained from Institute of Immunology and Experimental Therapy (Polish Academy of Science, Wroclaw, Poland). Both LoVo and LoVo/Dx cells were grown in F12 medium (Cytogen) supplemented with 10% fetal bovine serum (Gibco), 1% antibiotic antimycotic solution (Sigma-Aldrich) and 1% glutamine (Prolabo). The cells were cultured at 37 °C in humidified 5% CO_2 atmosphere and passaged twice a week. After each passage, doxorubicin in concentration of 100 ng/ml was added to the medium in order to maintain a drug resistance of LoVo/Dx cells. Experimental procedures were carried out in log-phase of cell growth. Cells were detached from the culture flasks by the use of non-enzymatic cell dissociation solution (Sigma-Aldrich). The density of cells for each experiment was determined with EVE Automatic Cell Counter (NanoEnTek).