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Phenothiazine Conjugate with Mitochondria-Directed Cationic Compound F16. Synthesis and Cytotoxic Action against Human Breast Carcinoma †

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Abstract: The development of new drugs or drug candidates based on the phenothiazine system (10H-dibenzo-[b,e]-1,4-thiazine) is a promising approach in view of the diverse biological activity of this tricyclic system, which is present in traditional drugs (chlorpromazine. thioridazine, trifluoperazine, trifluopromazine) with antipsychotropic, antihistamine and antimuscarinic activities. In practical medicine, these drugs are used as antagonists of dopamine and other neurotransmitter receptors for the treatment of schizophrenia and bipolar disorders. Ongoing studies on the synthesis and biological screening of various phenothiazine derivatives in recent years have revealed other important biological effects of these compounds, among which their antitumor effects are of great interest. This work reports the synthesis of a novel N-substituted phenothiazine analog bearing a mitochondria-directed cationic group (E)-4-(1*H*-indol-3-ylvinyl)-pyridinium (F16) linked to the nitrogen atom of the phenothiazine core by a butane bridge. The lipophilic cationic F16 fragment was used as a means to enhance transmembrane transport and selective delivery of the hybrid molecule into the mitochondria of cancer cells. In tests on the BT474 breast cancer cell line, the phenothiazine-F16 hybrid demonstrated significant cytotoxic activity. The cytotoxic effect of the compound was noticeable at a concentration of 5 μM and further increased dose-dependently, leading to complete tumor cell death at a concentration of 50 μM (IC_{50} 3.3 μM). The F16-derivative of phenotzine showed marked mitochondrial targeting. In experiments on isolated rat liver mitochondria, the tested agent already at a concentration of 5 μM significantly decreased the membrane potential of succinate-energized organelles. Increasing the concentration of phenothiazine hybrid to 20 μM resulted in complete dissipation of the potential. The obtained result of antitumor activity against BT-474 cell culture and significant effect on the reduction of mitochondrial membrane potential allows us to consider phenothiazine-F16 hybrid as a new promising antitumor drug.

Keywords: phenothiazine; conjugates; mitochondria; delocalized lypophilic cations; F16; anti-cancer activity

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1. Introduction

Modern antitumor pharmacotherapy uses a wide range of drugs. However, almost all drugs used today, due to low selectivity of cytotoxic action, give severe side effects and lead to the development of multidrug resistance in tumors. New generation drugs with tumor marker molecules (e.g., tumor-specific receptors) as a target have high selectivity and low toxicity, but they do not solve the problem of resistance of malignant cells to

therapy. Therefore, the discovery of new multi-targeted chemotherapeutic agents directed simultaneously at several targets, with lower systemic toxicity and a novel mechanism of cytotoxic action is of utmost importance. Currently, combining two or more pharmacophores into a single molecule is considered as one of the promising approaches to the development of antitumor drugs. Generally, such hybrid molecules tend to act in different ways simultaneously on multiple targets. The success of molecular hybridization in cancer chemotherapy is quite evident, judging from the number of hybrid compounds that have successfully completed clinical trials and/or entered the drug market in the last decade [1,2]. In this context, hybridization of various pharmacophores with compounds of phenothiazine structure is of great interest.

The development of new drugs or drug candidates based on the phenothiazine system (10H-dibenzo-[b,e]-1,4-thiazine) is a promising approach because of the diverse biological activity of this tricyclic system, which is present in traditional drugs with antipsychotropic, antihistamine, and antimuscarinic effects. In practical medicine, these drugs are used as antagonists of dopamine and other neurotransmitter receptors for the treatment of schizophrenia and bipolar disorders [3]. Ongoing studies in recent years on the synthesis and biological screening of various phenothiazine molecular hybrids have revealed other interesting pharmacological properties of these hybrid compounds such as antibacterial, antifungal, antimalarial, and most importantly, antitumor effects [4]. It has been shown that phenothiazine derivatives can initiate apoptosis, block angiogenesis and inhibit multidrug resistance of cancer cells [5–7].

Hybridization of cytotoxic molecules with lipophilic cationic mitochondria-targeting molecules, among which triphenylphosphonium cation, dequalinium salt, rhodacyanine MCT-077, rhodamines and (E)-4-(1H-indole-3-ylvinyl)-N-methylpyridinium iodide (F-16) are currently considered as a successful approach to enhance the efficacy and specificity of antitumor drug action. These cationic molecules with small molecular weight may facilitate the transport of functional compounds across cell membranes and enhance their selective targeting to the mitochondria of tumor cells. The mitochondrial targeting of such cationic hybrid compounds is due to the high transmembrane potential of mitochondria (negative intrinsically) compared to the membrane potential of cells and other intracellular organelles [8–13].

Thus, the directed introduction of lipophilic cationic fragments into cytotoxic molecules is a very important task in obtaining promising antitumor agents. However, to date, hybrid phenothiazine derivatives conjugated with lipophilic cationic groups have been described in only two papers [14,15]. This communication presents the results of the synthesis of a novel N-substituted phenothiazine analog bearing a mitochondria-directed cationic group (E)-4-(1*H*-indol-3-ylvinyl)-pyridinium (F16) linked to the nitrogen atom of the phenothiazine core by an alkyl bridge. The compound exhibited high cytotoxic activity in vitro (IC_{50} 3.3 μM) against human breast carcinoma BT-474. In experiments on isolated rat liver mitochondria, its significant effect on the reduction of mitochondrial membrane potential was revealed.

2. Materials and Methods

2.1. Chemistry

Reagents and solvents: phenothiazine, gramine, 1,4-dibromobutane, NaH, pyridinecarbaldehyde, tributylphosphine, CH_3CN and DMF were obtained from Acros Organics (Geel, Belgium) and used without further purification. (E)-4-(1H-indol-3-ylvinyl)pyridine, the neutral precursor of F16, was prepared according to the method of [16]. IR spectra (thin film) were obtained on a Vertex 70v spectrometer (Bruker, Karlsruhe, Germany). ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ or in MeOD with Me_4Si as internal standard on an AVANCE-500 instrument (500.13 (^1H), 125.78 MHz (^{13}C)) or on an AVANCE-400 instrument (400.13 (^1H), 100.62 MHz) (^{13}C)) (Bruker). The mass spectrum was recorded on a high-resolution quadrupole mass spectrometer Bruker maXis Impact. Elemental analysis

was performed on an 1106 analyzer (Carlo Erba, Milan, Italy). TLC was performed on Sorbfil plates (Sorbpolymer, Krasnodar, Russia).

2.1.1. Synthesis of N-(4-Bromobutyl) phenothiazine 3

Phenothiazine (1.1 g, 10.4 mmol) was stirred with a suspension of sodium hydride (60% dispersion in mineral oil, 3.0 eq.) in dry DMF (20 mL) at 0 °C for 1 h. Then 1,4-dibromobutane (2.0 eq.) was added dropwise to the reaction mixture and the suspension was stirred at 0 °C for 4 h. The reaction mixture was poured into water (100 mL), extracted with CH_2Cl_2 and dried over $MgSO_4$. The residue after evaporation of the solvent was purified by column chromatography (SiO_2, hexane). Compound **3** was obtained as a colorless viscous oil (1.2 g, 70% yield). The data of ^1H and ^{13}C NMR spectra corresponded to the literature data [17].

2.1.2. Synthesis of 4-(10H-Phenothiazin-10-yl)butyl-N-(E)-4-(1H-indol-3-yl-vinyl)pyridinium Bromide 4

A mixture of phenothiazine derivative **3** (0.3 g, 1 mmol) and (E)-4-(1H-indol-3-yl-vinyl)-pyridine (0.2 g, 1 mmol) was stirred in DMF or CH_3CN (11 mL) at 85 °C under argon atmosphere for 16 hours. The mixture was then cooled to room temperature, the solvent was evaporated under reduced pressure and the dry residue was chromatographed on a SiO_2 column using CH_2Cl_2/MeOH 30:1→10:1. Compound **4** was obtained as a brown powder. The yield was 0.34 g (71%), IR (film) ν_{max} 3400 (NH) cm^{-1}; m.p. 148–150 °C (EtOH); ^1H-NMR (500 MHz, MeOD/$CDCl_3$): δ 1.76–1.77 (4H, m, H-2′, H-3′), 3.89–3.91 (2H, m, H-1′), 4.21–4.23 (2H, m, H-4′), 6.85–6.99 (5H, m, Ph in phenothiosine, H-10′ or H-11′), 7.09–7.14 (4H, m, Ph in phenothiosine), 7.23–7.25 (2H, m, H-16′, H-17′), 7.44–7.47 (1H, m, C-15′ or H-18′), 7.60 (2H, d, J = 6.5 Hz, H-6′, H-8′), 7.79–7.81 (1H, m, H-12′), 7.95–7.98 (2H, m, H-15′ or H-18′, H-10′ or H-11′), 8.15 (2H, d, J = 6.5 Hz, H-5′, H-9′) ppm; ^{13}C-NMR (125 MHz, MeOD/$CDCl_3$): δ 23.6 (C-2′), 28.9 (C-3′), 46.8 (C-1′), 60.4 (C-4′), 113.5 (C-15′ or C-18′), 115.3 (C-13′), 117.0 (C-1, C-9), 117.5 (C-10′ or C-11′), 121.3 (C-15′ or C-18′), 122.7 (C-16′ or C-17′), 122.8 (C-6′, C-8′), 123.8 (C-3, C-7), 124.3 (C-16′ or C-17′), 126.2 (C-14′ or C-19′), 126.6 (C in phenothiosine), 128.4 (C-4, C-6), 128.5 (C-2, C-8), 133.5 (C-12′), 138.4 (C-10′ or C-11′), 139.1 (C-14′ or C-19′), 143.2 (C-5′, C-9′), 146.1 (C in phenothiosine), 156.3 (C-7′) ppm; Anal. Calcd for $C_{31}H_{28}BrN_3S$: C 67.08; H 5.16. Found: C 67.14; H 5.09; MS (HRMS): calcd for $C_{31}H_{28}N_3S$ [M–Br] 474.1991; found: 474.1998.

2.2. Biology

2.2.1. Cell Line BT-474 and Culture Condition

Human breast ductal carcinoma BT474 line (Accession Number—HTB-20) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in DMEM media supplemented with 10% fetal bovine serum, 100 μg/mL gentamycin, and 2 mM of glutamine at 37 °C in a 5% CO_2 atmosphere.

2.2.2. Effect of Compound 4 on the Viability of BT-474 Human Breast Adenocarcinoma Tumor Cells

For cytotoxicity assay, BT-474 cells were seeded into 96-well Nunc cell culture plates (Thermo Scientific) at a concentration of 1×10^5 cells/mL (1×10^4 cells in 100 μL of culture medium per well). Addition of compound **4** was carried out from a freshly prepared solution of compound **1** (5–10 mM) in Hanks' solution at concentrations of (0.01–100 μM). The final concentration of DMSO in freshly prepared conjugate solutions did not exceed 1% and was not toxic to cells. All treatments were performed 24 h after cell seeding. For each concentration, experiments were performed in three repetitions. Incubation of cells with compound **4** lasted 48 h. Cytotoxicity was determined by crystal violet (CV) assay. For this purpose, after incubation the medium was removed from the plates, stained with 0.2% alcoholic solution of crystal violet (CV) (50 μL in each well) and incubated with the

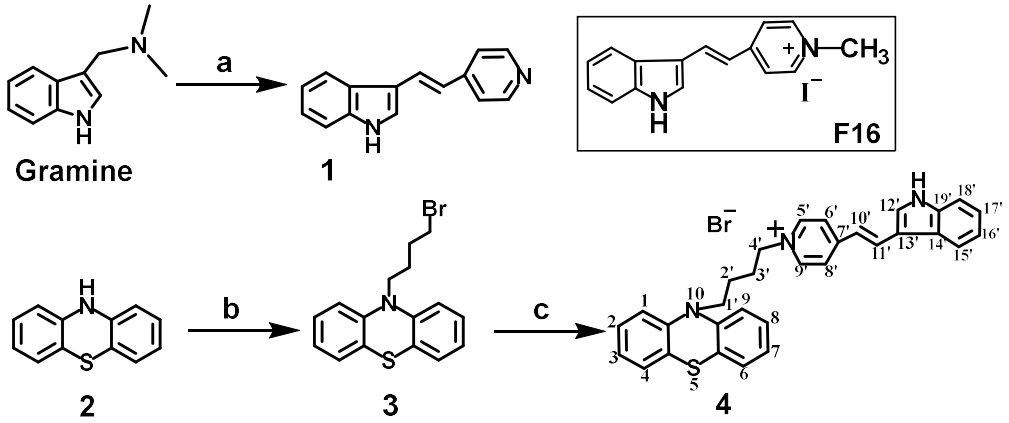
dye for 15 min at room temperature. The medium was then removed from the plates, rinsed several times with tap water and left to dry for 24 h. Inhibition of cell population growth (IC_{50}) was determined based on dose-dependent curves. After 24 h, 100 μL of SDS (sodium dodecyl sulfate) was added to each well to elute the bound dye. The cytotoxicity of the compounds was determined by the ratio of optical densities (at 620 nm) minus the measured background absorbance in treated and untreated cultures 48 h after addition of the compounds on a Tecan plate reader. The optical density value was directly proportional to the number of viable cells. The number of viable cells was estimated by trypan blue exclusion assay after trypsinization of the cell culture. The concentration value causing 50% inhibition of cell population growth (IC_{50}) was determined from dose-dependent curves.

2.2.3. Effect of Compound **4** on the Viability of BT-474 Human Breast Adenocarcinoma Tumor Cells

Mitochondrial potential (Δψ) was assessed with a safranin O fluorescent probe (λ_{ex} = 520 nm; λ_{em} = 580 nm) using a Varioskan LUX plate spectrofluorimeter (Thermo Fisher Scientific, Waltham, MA, USA). Rat liver mitochondria (0.5 mg/mL) were incubated in medium containing 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 1 μM rotenone, 10 mM EGTA, 10 μM safranin O and 10 mM Hepes/KOH, pH 7.4. The increase in safranin O florescence indicated a decrease in membrane potential (Δψ). The protonophore uncoupler 2,4-dinitrophenol (DNP) was used to maximize the decrease in membrane potential.

3. Results and Discussion

The delocalized lipophilic cationic compound F16 was first described in [18,19]. The authors showed that this compound selectively accumulates in the mitochondrial matrix of various tumor cells, causing cell cycle arrest, interruption of the mitochondrial respiratory chain and a decrease in intracellular ATP levels. The neutral precursor F16 (E)-4-(1*H*-indol-3-ylvinyl)-N-methylpyridine **1** was synthesized by us in 64% yield by interaction of gramine and pyridine carboxaldehyde, in the presence of tributylphosphine according to the method of [16]. Commercially available phenothiazine **2** was transformed into a bromalkyl derivative **3** by interaction with sodium hydride in dry DMF with sequential addition of a two-mole excess of 1,4-dibromobutane to the solution. Then, interaction of bromide **3** with (E)-4-(1*H*-indol-3-ylvinyl)pyridine by boiling in CH_3CN or in DMF and followed by purification of the resulting product by column chromatography on SiO_2 afforded the target compound **4** in 71% yield (Scheme 1).



Scheme 1. Synthesis of conjugate **4**: (**a**) pyridnecarboxaldehyde, N-butyl phosphorus, CH_3CN, 60 °C, 24 h; (**b**) 1,4-dibromobutane, NaH, 0 °C, 1 h; (**c**) **1**, DMF or CH_3CN, 85 °C, 16 h.

Compound **4** is a brown powder, the structure of which was proved by elemental analysis, mass spectrometry and ^1H and ^{13}C NMR spectroscopy. In the ^1H NMR spectra,

the presence of (E)-4-(1*H*-indol-3-ylvinyl)pyridinium moiety is evidenced by two characteristic doublets for the pyridine ring at 7.60 and 8.15 ppm with *J* = 6.5 Hz and six multiplets characteristic of the indole and phenyl fragments at 6.85–6.99, 7.09–7.14, 7.23–7.25, 7.44–7.47, 7.79–7.81 and 7.95–7.98 ppm. The ^{13}C NMR spectra show signals for the (E)-4-(1*H*-indol-3-ylvinyl)pyridinium and phenothiazine carbons in the 113.5–156.3 ppm range. The signals of the (C-2′), (C-3′), (C-1′), and (C-4′) carbon atoms resonated at 23.6, 28.9, 46.8 and 60.4 ppm, respectively.

The effect of compound **4** on the viability of human breast adenocarcinoma BT-474 cells was studied in a crystal violet test. The graph (Figure 1) shows that the cytotoxic effect of compound **4** appeared at a concentration of 5 μM and then increased in a dose-dependent manner, leading to the complete death of tumor cells at a concentration of 50 μM.

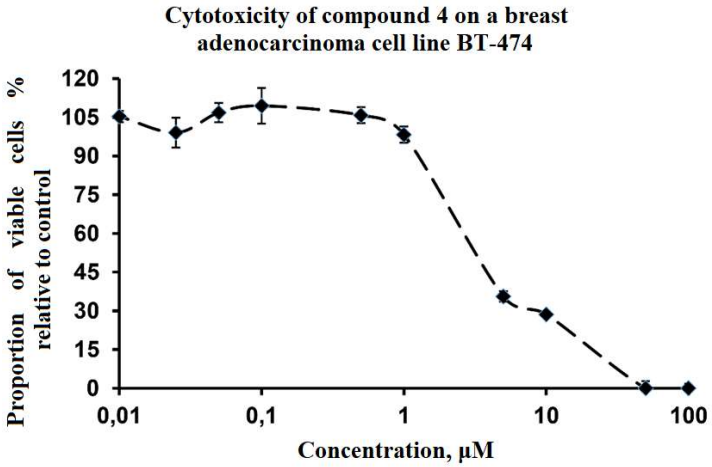


Figure 1. Effect of compound **4** on the viability of BT-474 cells. Means ± SEM are shown (n = 4).

Compound **4** showed a significant mitochondrial targeting. In experiments on isolated rat liver mitochondria, the tested agent, already at a concentration of 5 μM, significantly reduced the membrane potential of organelles energized by succinate. An increase in the concentration of compound **1** to 20 μM led to an almost complete decrease in the potential (Figure 2).

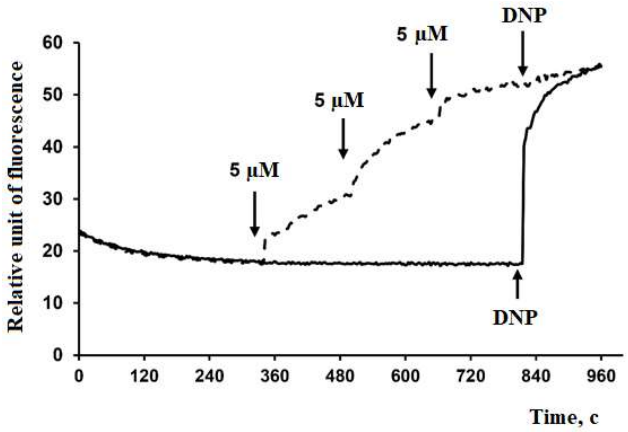


Figure 2. Effect of sequential addition of compound 4 (5 μM, 5 μM, 10 μM) on the membrane potential of rat liver mitochondria energized by succinate. The solid line (control) was obtained in the absence of the test compound. DNP–2,4-dinitrophenol.

Thus, it can be supposed that the cytotoxic effect of the phenothiazine derivative is due to the induction of mitochondrial dysfunction, leading to the activation of various types of cell death.

4. Conclusions

A new hybrid compound of phenothiazine structure was obtained by binding phenothiazine fragment to lipophilic delocalised molecule F16. The hybrid exhibited high cytotoxic activity (IC_{50} 3.3 μM) against human breast carcinoma BT-474.

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