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RESEARCH ARTICLE

Synthesis and anticancer and lipophilic properties of 10-dialkylaminobutynyl derivatives of 1,8- and 2,7-diazaphenothiazines

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Abstract

New derivatives of two isomeric types of azaphenothiazines, 1,8- and 2,7-diazaphenothiazine, containing the triple bond substituents and additionally tertiary cyclic and acyclic amine groups, were synthesized and tested for their anticancer activity. The compounds exhibited differential inhibitory activities. Better results were obtained when the acetylenic group was transformed *via* the Mannich reaction to the dialkylaminobutynyl groups. The most active was 2,7-diazaphenothiazine with the *N*-methylpiperazine-2-butynyl substituent against the human ductal breast epithelial tumor cell line T47D, more potent than cisplatin. The 2,7-diazaphenothiazine system turned out to be more active than isomeric 1,8-diaza one. For the most active compound, the expression of *TP53, CDKN1A, BCL-2* and *BAX* genes was detected by the RT-QPCR method. The gene expression ratio BACL-2/BAX suggests the mitochondrial apoptosis in T47D cells. The synthesis makes possible to obtain many new bioactive phenothiazines with the dialkylaminoalkynyl substituents inserting various tertiary cyclic and acyclic amine moieties to the substituents.

Keywords

Antiproliferative activity, BACL-2/BAX ratio, dialkylaminoalkynyl substituents, dipyridothiazines, phenothiazines

History

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Introduction

Classical tricyclic phenothiazines, representing the dibenzo-1,4-thiazine ring system, attract considerable attention because of their significant biological activities and interesting chemical features. Those with aminoalkyl substituents at the nitrogen atom are valuable drugs exhibiting neuroleptic, antihistaminic, antitussive and antiemetic activities[1]. The molecular scaffold of phenothiazine was very prolific for the structure modification by introduction of new substituents and substitution of one or two benzene rings with homoaromatic and heteroaromatic rings. The substitution with an azine ring leads to formation of azaphenothiazine. Depending on the aromatic rings, new phenothiazines and azaphenothiazines contain not only the tricyclic ring system, but also tetra- and pentacyclic ones with up to four additional nitrogen atoms in the aromatic rings[2–4]. Such structure modifications changed potency and type of biological properties exhibiting very promising anticancer, antibacterial, antifungal, anti-inflammatory activities, reversal of multidrug resistance[4–9] and a potential benefit in treatment of Alzheimer's, Creutzfeldt-Jakob's and AIDS-associated diseases[10–12].

Both classical and modified phenothiazines exert anticancer and cancer chemopreventive activity, and potential as an adjuvant to chemo- and radiotherapy. These compounds affect cancer cells mainly through cell death and cell-cycle arrest. Their ability to induce apoptosis is well recognized; however, the mechanism behind this property is not clearly understood. Various studies demonstrated that phenothiazines inhibit calmodulin, tubulin and protein kinase C action, P-glycoprotein transport function and recently induce lysosomal dysfunction[13–15].

Most of the classical bioactive phenothiazines contain flexible pharmacophoric dialkylaminopropyl substituents at the thiazine nitrogen atom. As aromatic compounds with the alkynyl groups exhibit wide variety of activities such as anticancer[16–23], anti-inflammatory[24], antimicrobial[25,26], antiviral[27,28], antioxidant[29], it was interesting to synthesize and study phenothiazines with more rigid dialkylamino substituents, containing a triple bond linker. We found only one paper in the phenothiazines studies with the dialkylaminoalkynyl substituents exhibiting promising anticancer and multidrug resistance reverting activity[30].

Lipophilicity has been considered for a long time as a vital molecular property in the drug design. This parameter plays a crucial role in the transport of compounds through a biological system and may also influence the formation of a complex with a receptor or a biomacromolecule at the site of action and is modeled by partition of a solute between *n*-octanol and water as log*P*. The lipophilicity of bioactive compounds can be correlated with many physicochemical, pharmacokinetic and pharmacodynamic drug properties, including biological activity, solubility, permeability, distribution, metabolism, target protein and plasma protein binding[31–35]. It may also influence the formation of a complex between a compound and a receptor or a biomacromolecule at the site of action. It was found for anticancer compounds

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that lipophilicity can be regarded as one of the main properties correlating with cytotoxicity[36–38].

The introduction of two pyridine rings instead of the benzene ones in the phenothiazine scaffold leads to various diazaphenothiazines of the dipyrido[1,4]thiazine structure. We synthesized 1,8- and 2,7-diazaphenothiazines with varied alkyl, aryl, heteroaryl, dialkylaminoalkyl, amidoalkyl, sulfonamidoalkyl and ''half-mustard'' substituents. Some of those compounds exhibited very promising anticancer, immunosuppressant and antioxidant activities, and low toxicity[39–42].

In this article, two series of new azaphenothiazines, 1,8- and 2,7-diazaphenothiazines, with more rigid substituents containing a triple bond were synthesized. For those compounds, the anticancer action on the selected tumor cell lines and lipophilic property were investigated. For the most active compound, the expression of *TP53*, *CDKN1A*, *BCL-2* and *BAX* genes was detected using the RT-QPCR method.

Methods

Chemistry

Melting points were determined in open capillary tubes on a Boetius melting point apparatus and are uncorrected. The ^{1}H NMR spectra were recorded on a Bruker Fourier 300 and Bruker DRX spectrometers at 300 and 600 MHz in deuteriochloroform with tetramethylsilane as the internal standard. Fast Atom Bombardment mass spectra (FAB MS, in glycerol) were run on a Finnigan MAT 95 spectrometer at 70 eV. The thin layer chromatography was performed on aluminum oxide 60 F_{254} neutral (type E) (Merck 1.05581) with $CHCl_3$-EtOH (10:1 v/v) as eluents.

10H-1,8-diazaphenothiazine (**1A**), *10H-2,7-diazaphenothiazine* (**1B**), *10-propargyl-1,8-diazaphenothiazines* (**2A**) and *10-propargyl-2,7-diazaphenothiazines* (**2B**) were obtained according to the reported procedures in the literature[42–44].

General procedure for synthesis of compounds (3–9)a,B

A mixture of 10-propargyl-diazaphenothiazine **2A** or **2B** (0.5 mmol), paraformaldehyde (0.5 mmol), amine (0.7 mmol) and cuprous chloride (catalitic amount) in peroxide-free, dry dioxane (10 ml) was heated with continuous stirring at 70–80 °C for 3 h. After cooling 20 ml water was added and mixture was extracted with chloroform, dried with Na_2SO_4, and evaporated *in vacuo*. The dry residue was dissolved in $CHCl_3$ and purified by column chromatography (aluminum oxide, $CHCl_3$) to give:

*10-[4-(*N*-ethyl-*N*-methyl)amino-but-2-ynyl]-1,8-diazaphenothiazine (**3A**).* (0.116 g, 75%); An oil. ^{1}H NMR ($CDCl_3$) δ: 1.03 (t, $J = 7.2$ Hz, 3H, CH_3), 2.26 (s, 3H, N–CH_3), 2.44 (q, $J = 7.2$ Hz, 2H, N–CH_2), 3.35 (s, 2H, CH_2), 4.79 (s, 2H, CH_2), 6.81 (dd, $J = 7.5$ Hz, $J = 4.8$ Hz, 1H, H_3), 6.95 (d, $J = 4.9$ Hz, 1H, H_6), 7.24 (dd, $J = 7.5$ Hz, $J = 1.5$ Hz, 1H, H_4), 8.07 (dd, $J = 5.1$ Hz, $J = 1.5$ Hz, 1H, H_2), 8.11 (d, $J = 4.9$ Hz, 1H, H_7), 8.39 (s, 1H, H_9). FAB MS *m/z*: 311 (M + 1, 20), 252 (M + 1–C_3H_5N, 100), 201 (M + 1–$C_7H_{12}N$, 35). Anal. Calcd for: $C_{17}H_{18}N_4S$ C 65.78, H 5.84, N 18.05. Found: C 65.58, H 5.79, N 17.98.

*10-[4-(*N*-ethyl-*N*-methyl)amino-but-2-ynyl)-2,7-diazaphenothiazine (**3B**).* (0.124 g, 80%); An oil. ^{1}H NMR ($CDCl_3$) δ: 1.08 (t, $J = 7.2$ Hz, 3H, CH_3), 2.31 (s, 3H, N–CH_3), 2.49 (q, $J = 7.2$ Hz, 2H, N–CH_2),3.43 (s, 2H, CH_2), 4.54 (s, 2H, CH_2), 6.99 (d, $J =$ 5.5 Hz, 1H, H-9), 7.04 (d, $J = 5.5$ Hz, 1H, H-4), 8.14 (s, 1H, H-1), 8.17 (d, $J = 5.5$ Hz, 1H, H-3), 8.32 (d, $J = 5.5$ Hz, 1H, H-8), 8.40 (s, 1H, H-6). FAB MS *m/z*: 311 (M + 1, 100), 252 (M + 1–C_3H_5N, 18), 201 (M + 1–$C_7H_{12}N$, 55). Anal. Calcd for: $C_{17}H_{18}N_4S$ C 65.78, H 5.84, N 18.05. Found: C 65.52, H 5.77, N 17.91.

*10-[4-(*N,N*-diethyl)amino-but-2-ynyl]-1,8-diazaphenothiazine (**4A**).* (0.134 g, 82%); An oil. ^{1}H NMR ($CDCl_3$) δ: 1.04 (t, $J = 7.2$ Hz, 6H, $2CH_3$), 2.50 (q, $J = 7.2$ Hz, 4H, 2 N–CH_2), 3.43 (s, 2H, CH_2), 4.78 (s, 2H, CH_2), 6.82 (dd, $J = 7.5$ Hz, $J = 4.8$ Hz, 1H, H_3), 6.94 (d, $J = 4.9$ Hz, 1H, H_6), 7.23 (dd, $J = 7.5$ Hz, $J = 1.5$ Hz, 1H, H_4), 8.07 (dd, $J = 5.1$ Hz, $J = 1.5$ Hz, 1H, H_2), 8.11 (d, $J = 4.9$ Hz, 1H, H_7), 8.38 (s, 1H, H_9). FAB MS *m/z*: 325 (M + 1, 15), 252 (M + 1–$C_4H_{10}N$, 100), 201 (M + 1–$C_8H_{14}N$, 20). Anal. Calcd for: $C_{18}H_{20}N_4S$ C 66.63, H 6.21, N 17.27. Found: C 66.42, H 6.25, N 17.02.

*10-[4-(*N,N*-diethyl)amino-but-2-ynyl]-2,7-diazaphenothiazine (**4B**).* (0.126 g, 78%); An oil. ^{1}H NMR ($CDCl_3$) δ: 1.10 (t, $J =$ 6.0 Hz, 6H, 2 CH_3), 2.55 (q, 5.32 $J = 6.0$ Hz, 4H, 2 CH_2), 3.52 (s, 2H, CH_2), 4.50 (s, 2H, CH_2), 6.98 (d, $J = 5.5$ Hz, 1H, H-9), 7.40 (d, $J = 5.5$ Hz, 1H, H-4), 8.12 (s, 1H, H-1), 8.17 (d, $J = 5.5$ Hz, 1H, H-3), 8.32 (d, $J = 5.5$ Hz, 1H, H-8), 8.39 (s, 1H, H-6). FAB MS *m/z*: 325 (M + 1, 100), 201 (M + 1–$C_8H_{14}N$, 30). Anal. Calcd for: $C_{18}H_{20}N_4S$ C 66.63, H 6.21, N 17.27. Found: C 66.40, H 6.20, N 17.00.

*10-(4-Pyrrolidin-1-yl-but-2-ynyl)-1,8-diazaphenothiazine (**5A**).* (0.129 g, 80%); An oil. ^{1}H NMR ($CDCl_3$) δ: 1.76 (m, 4H, $2CH_2$), 2.60 (m, 4H, $2CH_2$),3.41 (s, 2H, CH_2), 4.78 (s, 2H, CH_2), 6.80 (dd, $J = 7.5$ Hz, $J = 4.8$ Hz, 1H, H_3), 6.94 (d, $J = 4.9$ Hz, 1H, H_6), 7.22 (dd, $J = 7.5$ Hz, $J = 1.5$ Hz, 1H, H_4), 8.05 (dd, $J = 5.1$ Hz, $J = 1.5$ Hz, 1H, H_2), 8.10 (d, $J = 4.9$ Hz, 1H, H_7), 8.37 (s, 1H, H_9). FAB MS *m/z*: 323 (M + 1, 30), 200 (M-$C_8H_{12}N$, 100). Anal. Calcd for: $C_{18}H_{18}N_4S$ C, 67.05, H 5.63, N 17.38. Found: C, 66.91, H 5.65, N 17.17.

*10-(4-Pyrrolidin-1-yl-but-2-ynyl)-2,7-diazaphenothiazine (**5B**).* (0.119 g, 74%); An oil. ^{1}H NMR ($CDCl_3$) δ: 1.68 (m, 4H, $2CH_2$), 2.66 (m, 4H, $2CH_2$), 3.39 (s, 2H, CH_2) 4.57 (s, 2H, CH_2), 6.96 (d, $J = 5.5$ Hz, 1H, H-9), 7.43 (d, $J = 5.5$ Hz, 1H, H-4), 8.12 (s, 1H, H-1), 8.19 (d, $J = 5.5$ Hz, 1H, H-3), 8.30 (d, $J = 5.5$ Hz, 1H, H-8), 8.38 (s, 1H, H-6). FAB MS *m/z*: 323 (M + 1, 20), 201 (M + 1–$C_8H_{12}N$, 20). Anal. Calcd for: $C_{18}H_{18}N_4S$ C, 67.05, H 5.63, N 17.38. Found: C, 66.87, H 5.59, N 17.11.

*10-(4-Piperidin-1-yl-but-2-ynyl)- 1,8-diazaphenothiazine (**6A**).* (0.126 g, 75%); An oil. ^{1}H NMR ($CDCl_3$) δ: 1.34 (m, 2H, CH_2), 1.57 (m, 4H, $2CH_2$), 2.44 (m, 4H, $2CH_2$), 3.26 (s, 2H, CH_2), 4.79 (s, 2H, CH_2), 6.79 (dd, $J = 7.5$ Hz, $J = 4.8$ Hz, 1H, H_3), 6.94 (d, $J = 4.9$ Hz, 1H, H_6), 7.22 (dd, $J = 7.5$ Hz, $J = 1.5$ Hz, 1H, H_4), 8.06 (dd, $J = 5.1$ Hz, $J = 1.5$ Hz, 1H, H_2), 8.09 (d, $J = 4.9$ Hz, 1H, H_7), 8.37 (s, 1H, H_9). FAB MS *m/z*: 337 (M + 1, 30), 201 (M + 1–$C_9H_{14}N$, 100). Anal. Calcd for: $C_{19}H_{20}N_4S$ C, 67.83, H 5.99, N 16.65. Found: C, 67.59, H 5.92, N 16.40.

*10-(4-Piperidin-1-yl-but-2-ynyl)-2,7-diazaphenothiazine (**6B**).* (0.121 g, 72%); An oil. ^{1}H NMR ($CDCl_3$) δ:1.45 (m, 2H, CH_2), 1.70 (m, 4H, 2 CH_2), 2.55 (m, 4H, $2CH_2$),3.40 (s, 2H, CH_2), 4.51 (s, 2H, CH_2), 6.99 (d, $J = 5.5$ Hz, 1H, H-9), 7.44 (d, $J = 5.5$ Hz, 1H, H-4), 8.10 (s, 1H, H-1), 8.15 (d, $J = 5.5$ Hz, 1H, H-3), 8.31 (d, $J = 5.5$ Hz, 1H, H-8), 8.33 (s, 1H, H-6). FAB MS *m/z*: 337 (M + 1, 100), 201 (M + 1–$C_9H_{14}N$, 50). Anal. Calcd for: $C_{19}H_{20}N_4S$ C, 67.83, H 5.99, N 16.65. Found: C, 67.55, H 5.90, N 16.45.

*10-(4-Morpholin-4-yl-but-2-ynyl)-1,8-diazaphenothiazine (**7A**).* (0.113 g, 67%); An oil. ^{1}H NMR ($CDCl_3$) δ: 2.54 (m, 4H, $2CH_2$), 3.29 (s, 2H, CH_2), 3.61 (m, 4H, $2CH_2$), 4.79 (s, 2H, CH_2),

6.81(dd, $J = 7.5$ Hz, $J = 4.8$ Hz, 1H, H_3), 6.95 (d, $J = 4.9$ Hz, 1H, H_6), 7.23 (dd, $J = 7.5$ Hz, $J = 1.5$ Hz, 1H, H_4), 8.06 (dd, $J = 5.1$ Hz, $J = 1.5$ Hz, 1H, H_2), 8.10 (d, $J = 4.9$ Hz, 1H, H_7), 8.36 (s, 1H, H_9). FAB MS *m/z*: 339 (M + 1, 35), 200 (M-$C_8H_{12}NO$, 100). Anal. Calcd for: $C_{18}H_{18}N_4SO$ C 63.88, H 5.36, N 16.56. Found: C 63.72, H 5.41, N 16.39.

*10-(4-Morpholin-4-yl-but-2-ynyl)-2,7-diazaphenothiazine (**7B**).* (0.117 g, 69%); An oil. ^{1}H NMR ($CDCl_3$) δ: 2.52 (m, 4H, 2 CH_2), 3.35 (s, 2H, CH_2), 3.70 (m, 4H, 2CH_2), 4.55 (s, 2H, CH_2), 6.90 (d, $J = 5.5$ Hz, 1H, H-9), 7.41 (d, $J = 5.5$ Hz, 1H, H-4), 8.12 (s, 1H, H-1), 8.15 (d, $J = 5.5$ Hz, 1H, H-3), 8.32 (d, $J = 5.5$ Hz, 1H, H-8), 8.40 (s, 1H, H-6). FAB MS *m/z*: 339 (M + 1, 40), 201 (M + 1–$C_8H_{12}NO$, 100). Anal. Calcd for: $C_{18}H_{18}N_4SO$ C 63.88, H 5.36, N 16.56. Found: C 63.69, H 5.31, N 16.44.

*10-[4-(4-Methylpiperazin-1-yl)but-2-ynyl]-1,8-diazaphenothiazine (**8A**).* (0.121 g, 69%); An oil. ^{1}H NMR ($CDCl_3$) δ: 1.25 (s, 3H, N-CH_3), 2.55 (m, 8H, 4CH_2), 3.42 (s, 2H, CH_2), 4.79 (s, 2H, CH_2), 6.81(dd, $J = 7.5$ Hz, $J = 4.8$ Hz, 1H, H_3), 6.95 (d, $J = 4.9$ Hz, 1H, H_6), 7.22 (dd, $J = 7.5$ Hz, $J = 1.5$ Hz, 1H, H_4), 8.05 (dd, $J = 5.1$ Hz, $J = 1.5$ Hz, 1H, H_2), 8.09 (d, $J = 4.9$ Hz, 1H, H_7), 8.35 (s, 1H, H_9). FAB MS *m/z*: 351 (M + 1, 100), 201 (M + 1–$C_9H_{15}N_2$, 35). Anal. Calcd for: $C_{19}H_{21}N_5S$ C 64.93, H 6.02, N 19.93. Found: C 64.75, H 5.91 N 19.69.

*10-[4-(4-Methylpiperazin-1-yl)but-2-ynyl]-2,7-diazaphenothiazine (**8B**).* (0.125 g, 71%); An oil. ^{1}H NMR ($CDCl_3$) δ:1.25 (s, 3H, N-CH_3), 2.55 (m, 8H, 4 CH_2), 3.40 (s, 2H, CH_2), 4.52 (s, 2H, CH_2), 6.99 (d, $J = 5.5$ Hz, 1H, H-9), 7.44 (d, $J = 5.5$ Hz, 1H, H-4), 8.10 (s, 1H, H-1), 8.15 (d, $J = 5.5$ Hz, 1H, H-3), 8.31 (d, $J = 5.5$ Hz, 1H, H-8), 8.33 (s, 1H, H-6). FAB MS *m/z*: 351 (M + 1, 100), 201 (M + 1–$C_9H_{15}N_2$, 40). Anal. Calcd for: $C_{19}H_{21}N_5S$ C 64.93, H 6.02, N 19.93. Found: C 64.72, H 5.90 N 19.73.

*10-[4-(4-Phenylpiperazin-1-yl)but-2-ynyl]-1,8-diazaphenothiazine (**9A**).* (0.171 g, 83%); An oil. ^{1}H NMR ($CDCl_3$) δ: 2.73 (m, 4H, 2CH_2), 3.22 (m, 4H, 2 CH_2), 3.41 (s, 2H, CH_2), 4.79 (s, 2H, CH_2), 6.82 (dd, $J = 7.5$ Hz, $J = 4.8$ Hz, 1H, H_3), 6.95 (d, $J = 4.9$ Hz, 1H, H_6), 7.23 (m, 6H, $H_{Ar} + H_4$), 8.10 (dd, $J = 5.1$ Hz, $J = 1.5$ Hz, 1H, H_2), 8.11 (d, $J = 4.9$ Hz, 1H, H_7), 8.39 (s, 1H, H_9). FAB MS *m/z*: 414 (M + 1, 100), 201 (M + 1–$C_{13}H_{17}N_2$, 20). Anal. Calcd for: $C_{24}H_{23}N_5S$ C 69.71, H 5.61, N 16.94. Found: C 69.55, H 5.54, N 16.79.

*10-[4-(4-Phenylpiperazin-1-yl)but-2-ynyl]-2,7-diazaphenothiazine (**9B**).* (0.167 g, 81%); An oil. ^{1}H NMR ($CDCl_3$) δ: 2.73 (m, 4H, 2 CH_2), 3.25 (m, 4H, 2 CH_2), 3.42 (s, 2H, CH_2), 4.55 (s, 2H, CH_2), 6.95 (d, $J = 5.5$ Hz, 1H, H-9), 7.10 (d, $J = 5.5$ Hz, 1H,H-4), 7.23–7.32 (m, 5H, H_{Ar}), 8.10 (s, 1H, H-1), 8.13 (d, $J = 5.5$ Hz, 1H, H-3), 8.31 (d, $J = 5.5$ Hz, 1H, H-8), 8.33 (s, 1H, H-6). FAB MS *m/z*: 414 (M + 1, 100), 201 (M + 1–$C_{13}H_{17}N_2$, 30). Anal. Calcd for: $C_{24}H_{23}N_5S$ C 69.71, H 5.61, N 16.94. Found: C 69.59, H 5.52, N 16.81.

Anticancer effects in vitro

Cell culture

Compounds were evaluated for their anticancer activity using three cultured cell lines: SNB-19 (human glioblastoma, DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), C32 (human amelanotic melanoma, ATCC—American Type Culture Collection, Manassas, VA, USA) and T47D (human ductal breast epithelial tumor cell line, ATCC, Manassas, VA, USA). The cultured cells were kept at 37 °C and 5% CO_2. The cells were seeded (1×10^4 cells/well/100 µl DMEM supplemented with 10% FCS and streptomycin and penicillin) using 96-well plates (Corning).

Cell proliferation and viability

In recent years tetrazolium salts have been described, which can be used for the measurement of cell proliferation and viability. The tetrazolium salts are cleaved to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture. The formazan dye produced by metabolically active cells is quantified by a scanning ELISA reader by measuring the absorbance of the dye solution at appropriate wavelengths ($\lambda = 420$–480 nm with a reference wavelength $\lambda = 600$ nm).

WST-1 assay

In this study, the WST-1 assay (Roche Diagnostics, Mannheim, Germany) was used to evaluate the antiproliferative effect of the compounds on the number of cancer cells in cultures. After exposure to tested compounds (at concentrations between 0 and 100 µg/ml) for 72 h, cells were incubated with WST-1 (10 µl) for 1 h. The absorbance of the samples against a background control was read at 450 nm using with a reference wavelength $\lambda = 600$ nm a microplate reader. Results are expressed as means of at least two independent experiments performed in triplicate.

The RT-QPCR method

Genes trancriptional activity (*TP53, CDKN1A, BCL-2, BAX*) was evaluated using real-time RT-QPCR method with OPTICON TM DNA Engine (MJ Research, Watertown, MA) and QuantTect® SYBR® Green RT-PCR Kit (Qiagen, Valencia, CA). Cells were exposed to compound **8B** in 0.5 µg/ml concentration for 24 h. The RNA extraction was made by using QuickRNA™ Kit MiniPrep (ZYMO RESEARCH). Total RNA integrity was analyzed in 1.2% agarose electrophoresis with added ethidium bromide compound. The quantity and purity of the extracted total RNA were determined using spectrophotometric analysis with HP845 (Hewlett Packard, Waldbronn, Germany) spectrophotometer. The statistical analysis was performed using the Statistica 8.0 software (StatSoft, Tulsa, OK). All values were expressed as means ± SE.

Lipophilicity determination

Thin-layer chromatography was performed on 10 cm × 10 cm reversed-phase thin-layer chromatography (RP TLC) plates precoated with silica gel RP-18F_{254S} (Merck, Darmstadt, Germany) with the mobile phase of acetone and aqueous TRIS (*tris*(hydroxymethyl)aminomethane) buffer pH 7.4 (ionic strength 0.2 M) to meet physiological conditions. The concentration of acetone in the mobile phase ranged from 50% to 85% (v/v) in 5% increments. Diazaphenothiazines **(3–9)A** and **(3–9)B**, and the standards **I–V** (benzamide log$P = 0.64$[45], acetanilide log$P = 1.21$[46], acetophenone log$P = 1.58$[46], 4-bromoacetophenone log$P = 2.43$[45], benzophenone log$P = 3.18$[46]) were dissolved in ethanol (2.0 mg/ml) and 2 µl of these solutions were spotted on the plates 10 mm from the bottom edges. Before development of the plates, chromatographic chambers were saturated with the mobile phase for 0.5 h. After development of the plates and drying in a stream of air the chromatograms were observed under UV light at $\lambda = 254$ nm. At least three chromatograms were developed for each solute–solvent combination and R_F values were averaged. The R_M values

calculated from experimental R_F values using the equation $R_M = \log(1/R_F - 1)$ were linearly dependent on the concentration of acetone.

The R_{M0} values were obtained by extrapolation to zero acetone concentration by using the equation $R_M = R_{M0} + bC$, where C is the concentration (%, *v*/*v*) of acetone in the mobile phase.

The correlation between the known log*P* values and the experimental R_{M0} values for standards **I–V** gave the calibration equation:

$$\log P_{TLC} = 0.9795R_{M0} + 0.2302 \ (r = 0.9938,\ s = 0.2156,\ F = 349.97,\ p = 0.0002)$$

This equation was used for transformation of the R_{M0} values into the log*P* values for diazaphenothiazines.

Results and discussion

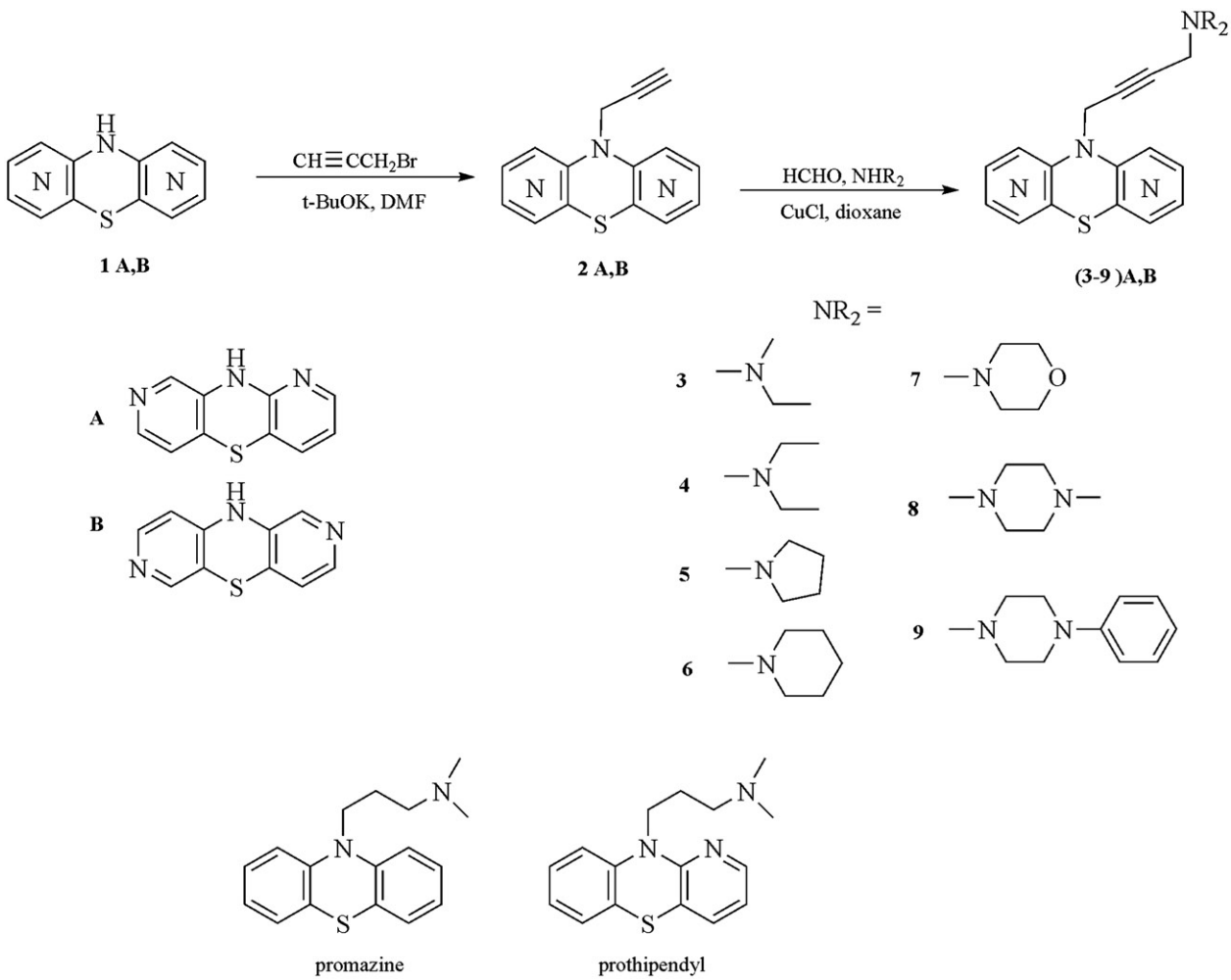
Chemistry

The parent substrates 10*H*-1,8-diazaphenothiazine **1A** and 10*H*-2,7-diazaphenothiazine **1B** were transformed with propargyl bromide into the prop-2-ynyl derivatives **2A** and **2B**, and further using the Mannich reaction (with formaldehyde and selected secondary acyclic and cyclic amines) into the 4-dialkylaminobut-2-ynyl derivatives of 1,8- and 2,7-diazaphenothiazines **(3–9)A** and **(3–9)B** in good yields (67–83%, Scheme 1). The structures of the new compounds were characterized with the use of ^{1}H NMR and FAB MS spectra and elemental analyses. Two compounds (**2A,B**) possess the prop-2-ynyl group, 14 compounds possess the dialkylaminobut-2-ynyl groups with the acyclic ((**3,4**)**A,B**) and 5-membered and 6-membered cyclic ((**5–9**)**A,B**) amine moieties.

Anticancer activity

The activity of two series of compounds **(2–9)A** and **(2–9)B** was investigated *in vitro* using cultured glioblastoma SNB-19, melanoma C-32 and ductal carcinoma T47D cell lines and cisplatin as a reference. To compare the influence of the nitrogen atoms on the anticancer activity classical monoazaphenothiazine drug, prothipendyl, (10-dimethylaminopropyl-1-azaphenothiazine) was also tested.

The tested compounds exhibited different activities against the cell lines (Table 1). The T47D cell line was the most resistant for the tested compounds. The derivatives **2A** and **2B** with the alkynyl group, prop-2-ynyl, (very active in some compounds[17,20,21]) were found to be very weak active or inactive (IC_{50} >50 μg/ml). The transformation of the prop-2-ynyl group into the aminobut-2-ynyl groups led to the compounds with different activities. Most of the dialkylaminobutynyl compounds were more active than the prop-2-ynyl derivatives (Table 1). The location of the nitrogen atoms in the tricyclic ring system also influenced the anticancer activity as the 2,7-diazaphenothiazine series was more active than the 1,8-diaza one. The most active was compound **8B** with the *N*-methylpiperazine-2-butynyl group (containing tertiary cyclic amine moiety with two nitrogen atoms) against the T47D cell line. In this case, the activity was even stronger than for cisplatin. The isomeric compound **8A**,



Scheme 1. Synthesis of 10-dialkylaminobutynyl 1,8- and 2,7-diazaphenothiazines **(3–9)AB** and the structures of promazine and prothipendyl.

containing the same substituent, was found unexpectedly inactive. It seems that the kind of the amine group does not influence significantly the tested activity, but rather combination of the effects of the diazaphenothiazine system (the place of the nitrogen atoms) and the substituent. Prothipendyl, containing only one pyridine ring, was less active than the most active diazaphenothiazines against all examined cell lines.

Apoptosis assay

The cellular stress can change the expression of the *TP53* gene encoding the P53 protein, which is known as the ''guardian of the genome''. This protein influences cell cycle arrest by changing the expression of *CDKN1A* gene encoding the P21 protein. The P53 protein also can stimulate the cell to changes in gene expression *BCL-2* and *BAX* involved in mitochondrial pathway apoptosis[47–50]. Efforts were made to understand the mechanism of anticancer activity for the most active compound **8B** with the *N*-methylpiperazine-2-butynyl group. The compound effect on the transcriptional activity of the *TP53* and *CDKN1A* genes was evaluated after cells incubation by 24 h. The results of analysis of *TP53, CDKN1A, BCL-2, BAX* genes in T-47D, SNB-19 and C-32 cells after 24 h of treatment are collected in Table 2. mRNA copy number of *TP53* gene did not shown any significant changes between T47D samples. In SNB-19 cells, compound **8B** generated a significant increase in the expression of *TP53* but in C-32 cells a reduction of the number of copies mRNA was observed. An increase in the number of *CDKN1A* copies in T47D and SNB-19 suggests possibility of participating in cell cycle arrest. Analysis of the gene expression ratio *BCL-2/BAX* in T47 cells showed activation of the mitochondrial apoptosis. Transcriptional activity of these genes in the SNB-19 and C-32 cells suggests a different way of cell death and protective activation.

Lipophilicity determination

For lipophilicity determination of diazaphenothiazines **(2–9)A,B**, a very convenient RP TLC method was used[51–54]. The obtained parameter R_{M0}, representing relative lipophilicity (Table 3), was transformed in the parameter log*P* with the use of the calibration curve based on five standards of known log*P* values. The tested compounds turned out to be low lipophilic with the log*P* values of 0.92–2.644 at pH = 7.4. Phenothiazines are known as one of the most lipophilic drugs with[55,56] the log*P* values up to 5.9. In comparison with classical phenothiazines, the investigated azaphenothiazines are much less lipophilic mainly due to the presence of additional nitrogen atoms in the ring system. Such a lipophilic property is considered as optimal for achieving appropriate physicochemical characteristics. The latest reports suggested that the compounds with the lipophilic parameter log*P* < 4 stand a much higher chance of success in achievement of biological target. The optimum region of the lipophilicity lies within a narrow range[34,35] between 1 and 3. It is worth noting that anticancer and immunosuppressive 1,8-diazaphenothiazines with the alkyl, dialkylaminoalkyl, acetylaminopropyl, methanesulfonylaminopropyl and chloroethylureidopropyl substituents exhibited the log*P* values[57] in the range of 0.78–2.60.

The correlation of the anticancer activities (the IC_{50} values) of the aminobutynyl derivatives **(3–9)A** and **(3–9)B** with lipophilic properties expressed as log*P* was very weak to moderate with the coefficient $r = 0.141–0.625$ (Table 4). It means that lipophilicity is only one of the parameters influencing the activity.

Conclusion

New derivatives of two isomeric types of azaphenothiazines, 1,8-diaza- and 2,7-diazaphenothiazine, containing triple bond substituents and additionally tertiary cyclic and acyclic amine groups, were synthesized and tested for their anticancer activity. Our investigations show that the introduction of the slightly acidic acetylenic group into the diazaphenothiazine system did not lead to potent anticancer compounds. Better results were obtained when the acetylenic group was transformed via the Mannich reaction to the dialkylaminobutynyl groups. The most active was the *N*-methylpiperazine-2-butynyl derivative **8B** against the ductal carcinoma T47D cell line, more potent than cisplatin. The 2,7-diazaphenothiazine system turned out to be more active than the

Table 1. Anticancer activities IC_{50} (μg/ml) of 10-substituted 1,8-diazaphenothiazines **(2–9)a** and 2,7-diazaphenothiazines **(2–9)B**.

	Anticancer activity IC_{50} (μg/ml)		
No.	SNB-19	C-32	T47D
2A	>50	>50	>50
3A	>50	26.6 ± 1.1	>50
4A	>50	26.1 ± 6.1	>50
5A	36.1 ± 7.7	37.0 ± 2.3	>50
6A	31.9 ± 9.9	35.4 ± 7.4	45.0 ± 4.3
7A	>50	>50	>50
8A	>50	>50	>50
9A	33.1 ± 2.9	27.5 ± 2.8	45.8 ± 4.9
2B	>50	>50	>50
3B	36.2 ± 3.3	29.2 ± 2.7	>50
4B	39.0 ± 3.5	25.0 ± 7.8	40.5 ± 9.7
5B	36.9 ± 1.9	35.1 ± 1.8	34.1 ± 3.2
6B	35.6 ± 3.1	24.8 ± 1.7	41.1 ± 4.9
7B	42.4 ± 5.1	40.6 ± 3.9	>50
8B	21.2 ± 2.3	35.0 ± 2.6	9.6 ± 2.2
9B	35.1 ± 4.6	36.1 ± 4.1	46.4 ± 4.4
Prothipendyl	36.6 ± 9.1	28.1 ± 6.8	32.3 ± 6.2
Cisplatin	7.7 ± 1.5	7.8 ± 1.3	46.9 ± 2.7

Table 2. The influence of compound **8B** on expression of genes encoding *TP53*, *CDKN1A*, *BCL-2*, *BAX* in glioblastoma SNB-19, melanoma C-32 and ductal carcinoma T47D cells.

		number of mRNA copies/μg total RNA		
Gene		SNB-19	C-32	T47D
TP53	Control	1218 ± 284	944 ± 171	100745 ± 30542
	8B	20209 ± 697	475 ± 129	110117 ± 18230
CDKN1A	Control	3184 ± 301	1304 ± 246	17642 ± 2327
	8B	5850 ± 422	1206 ± 351	31258 ± 1265
BCL-2	Control	2795 ± 410	13413 ± 3549	1080 ± 34
	8B	6949 ± 668	17203 ± 964	298 ± 11
BAX	Control	29678 ± 5198	2452 ± 563	26727 ± 4060
	8B	55650 ± 10542	1528 ± 429	20850 ± 5979
BCL-2/BAX	Control	0.09	5.47	0.02
	8B	0.12	11.26	0.04

Table 3. The R_{M0}, $\log P_{TLC}$ values and *b* (slope) and *r* (correlation coefficient) of the equation $R_M = R_{M0} + bC$ for diazaphenothiazines **(2–9)A,B**.

No	$-b$	R_{M0}	*r*	$\log P_{TLC}$	No	$-b$	R_{M0}	*r*	$\log P_{TLC}$
2A	0.0293	1.997	0.994	2.186[52]	**2B**	0.024	1.454	0.984	1.654
3A	0.016	0.704	0.989	0.920	**3B**	0.017	0.753	0.991	0.968
4A	0.016	0.746	0.996	0.961	**4B**	0.018	0.824	0.994	1.037
5A	0.022	1.023	0.988	1.232	**5B**	0.019	1.317	0.989	1.520
6A	0.021	1.143	0.989	1.350	**6B**	0.025	1.325	0.985	1.528
7A	0.019	1.313	0.996	1.516	**7B**	0.020	1.431	0.989	1.632
8A	0.029	2.027	0.997	2.215	**8B**	0.025	2.047	0.993	2.235
9A	0.033	2.261	0.987	2.447	**9B**	0.032	2.464	0.981	2.644

Table 4. The correlation between the anticancer activities IC_{50} and the log*P* values for diazaphenothiazines **(2–9)A,B**.

No	Cell line	Equation	*r*
(3–9)A	SNB-19	$IC_{50} = -109.0\log P^2 + 353.9\log P - 50.9$	0.141
(3–9)B	SNB-19	$IC_{50} = -4.462\log P^2 + 8.889\log P + 33.70$	0.552
(3–9)A	C-32	$IC_{50} = -114.1\log P^2 + 535.5\log P - 415.6$	0.539
(3–9)B	C-32	$IC_{50} = -5.736\log P^2 + 25.84\log P + 6.931$	0.625
(3–9)A	T47D	$IC_{50} = 227.4\log P^2 - 822.6\log P + 783.6$	0.531
(3–9)B	T47D	$IC_{50} = 22.87\log P^2 - 97.18\log P + 134.2$	0.615

1,8-diaza one. The replacement of the well-known flexible dialkylaminoalkyl substituents in the phenothiazine system with more rigid substituents containing a triple bond and a tertiary acyclic or cyclic amine groups also led to anticancer compounds. Analysis of the gene expression ratio *BCL-2/BAX* in T47 cells showed activation of the mitochondrial apoptosis. The correlation between anticancer activities and lipophilicity was moderate. As the Mannich reaction enables to insert various tertiary cyclic and acyclic amine moieties to the aminoalkynyl substituents, we think there is a way to obtain many new bioactive phenothiazines with the aminoalkynyl substituents.

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Declaration of interest

The authors have declared no conflict of interest.

Part CXLIII in the series of Azinyl Sulfides.

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