10H-1,9-diazaphenothiazine and its 10-derivatives: synthesis, characterisation and biological evaluation as potential anticancer agents

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ABSTRACT

10H-1,9-diazaphenothiazine was obtained in the sulphurisation reaction of diphenylamine with elemental sulphur and transformed into new 10-substituted derivatives, containing alkyl and dialkylaminoalkyl groups at the thiazine nitrogen atom. The 1,9-diazaphenothiazine ring system was identified with advanced 1H and 13C NMR techniques (COSY, NOESY, HSQC and HMBC) and confirmed by X-ray diffraction analysis of the methyl derivative. The compounds exhibited significant anticancer activities against the human glioblastoma SNB-19, melanoma C-32 and breast cancer MDA-MB-231 cell lines. The most active 1,9-diazaphenothiazines were the derivatives with the propynyl and N,N-diethylaminoethyl groups being more potent than cisplatin. For those two compounds, the expression of H3, TP53, CDKN1A, BCL-2 and BAX genes was detected by the RT-QPCR method. The proteome profiling study showed the most probable compound action on SNB-19 cells through the intrinsic mitochondrial pathway of apoptosis. The 1,9-diazaphenothiazine system seems to be more potent than known isomeric ones (1,6-diaza-, 1,8-diaza-, 2,7-diaza- and 3,6-diazaphenothiazine).

INTRODUCTION

In the last decades, cancer has been one of the main causes of death worldwide affecting millions of people per year. The main forms of a curative treatment for tumours are surgery, radiation, chemotherapy and biotherapy. The goal of chemotherapeutic agents is to cure the tumour, to prolong survival and to reduce the tumour burden to alleviate symptoms. In recent years, a lot of effort has been applied to the synthesis of potential anticancer drugs with better selectivity and minor or no side effects.

Synthetical and natural bioactive compounds with heterocyclic ring systems play an important role for the development of novel scaffolds in medicinal chemistry. One of the most active heterocyclic rings is a 1,4-thiazine ring, containing the nitrogen and sulphur atoms. This ring fused with two benzene rings forms a dibenzothiazine system, present in one of most valuable drugs – phenothiazines. Classical phenothiazines with the dialkylaminoalkyl groups at the nitrogen atom (and additional simple group at the carbon atom in position 2) are still been recognised as neuroleptic, antihistaminic, antitussive and antiemetic drugs. Recently, many papers were published revealing new activities for these compounds, for example, thioridazine, one of the most known phenothiazines, exhibits promising properties for multidrug-resistant tuberculosis treatment and lung cancer therapy through targeting lung cancer stem cells, due to its efficacy and safety.
On the other hand, the phenothiazine structure has been modified mainly by introduction of new substituents at the thiazine nitrogen atom and by replacement of one or two benzene rings with various azine rings (leading to azaphenothiazines). Recent numerous original reports, reviews and chapters in monographs describe new promising biological activity of both classical and modified phenothiazines such as anticancer, anti-plasmdm, antiviral, anti-inflammatory and antibacterial activities, reversal of multi-drug resistance.

The modifications of the phenothiazine structure with one or two pyridine rings lead to pyridobenzothiazines and dipryidothiazines, respectively. Of the four isomeric pyridobenzothiazines (being 1-aza-, 2-aza-, 3-aza- and 4-azaphenothiazines), the 10-dialkylaminoalkyl derivatives of the 1-aza series (known as prothi- pendyl, isopropylendyp, oxypropylendyp and pipazethate) are still used in the various therapies such as antipsychotic, anti-histaminic, anti-secretory, and antiemetic drugs.

Dipryidothiazines are less known and out of possible isomeric types only 6 types have been synthesised: 1,6-diaza-, 1,8-diaza-, 2,7-diaza-, 3,6-diaza- and 3,7-diazaphenothiazines. Initially, only 1,6-diazaphenothiazines were examined for their biological activities: neuroleptic and antihista- minic. Recently, some derivatives of four types of dipryidothiazines (1,6-diaza-, 1,8-diaza-, 2,7-diaza- and 3,6-diazaphenothiazines) were found to potent antitumor activities, stronger than cisplatins, against various types of cancer cells in mice.

The aim of this paper is elaboration of efficient synthesis of 10H-1,9-diazaphenothiazine, transformation this compound into 10H-1,9-diazaphenothiazine and drying with Na2SO4. The obtained product was purified by column chromatography (aluminium oxide, CHCl3) to give 10H-1,9-diazaphenothiazine (2) (0.083 g, 42%).

In an autoclave. A mixture of 2,2'-dipyridylamine (1) (0.171 g, 1 mmol), elemental sulphur (0.064 g, 2 mmol) and small crystal of iodine was added to the clean quartz reactor vessel. The mixture was heated at 230 °C during 30 min. The obtained brown oil was purified by column chromatography (aluminium oxide, CHCl3) to give 10H-1,9-diazaphenothiazine (2) (0.034 g, 17%).

**Materials and methods**

**Chemistry**

Melting points were determined in open capillary tubes on a Boetius melting point apparatus and are uncorrected. The 1H NMR, COSY, ROESY, HSQC, HMBC spectra were recorded on an Ascend™ 600 spectrometers at 600 MHz in deuteriochloroform with tetramethylsilane as the internal standard. The 13C NMR spectrum was recorded at 75 MHz. Electron impact mass spectra (EI MS), fast atom bombardment mass spectra (FAB MS, in glycerol), chemical ionisation (CI MS) were run on a Finnigan MAT 95 spectrometer at 70 eV and HR MS was run on a Bruker Impact II. The thin layer chromatography was performed on silica gel 60 F254 (Merck 1.05735) with CHCl3-EtOH (10:1 v/v) and on aluminium oxide 60 F254 neutral (type E) (Merck 1.05581) with CHCl3-EtOH (10:1 v/v) as eluents.

**Synthesis of 10H-1,9-diazaphenothiazine (2) through sulphurisation of 2,2’-dipyridylamine (1) with elemental sulphur**

In open air conditions. A mixture of 2,2’-dipyridylamine (1) (0.171 g, 1 mmol), elemental sulphur (0.064 g, 2 mmol) and small crystal of iodine was heated at 250 °C for 1 h. After cooling the reaction mixture was extracted with CHCl3 (3 × 10 ml). The obtained product was purified by column chromatography (aluminium oxide, CHCl3) to give 10H-1,9-diazaphenothiazine (2) (0.056 g, 28%); mp 156–157 °C.

1H NMR (CDCl3) δ: 6.74 (dd, J = 7.8 Hz, J = 4.8 Hz, 2H, H2, H3), 7.16 (dd, J = 7.8 Hz, J = 1.8 Hz, 2H, H4, H5), 7.96 (dd, J = 4.8 Hz, J = 1.8 Hz, 2H, H6, H7), 8.33 (s, 1H, NH). 13C NMR (CDCl3): 112.86 (C4a, C5a), 118.16 (C3, C7), 133.83 (C4, C6), 145.55 (C2, C8), 154.26 (C9a, C10a). EI MS m/z: 201 (M, 100), HR MS (El) m/z calc. for [C10H7N3S + H] 202.0439. Found 202.0455.

In a microwave reactor. A mixture of 2,2’-dipyridylamine (1) (0.171 g, 1 mmol), elemental sulphur (0.064 g, 2 mmol) and small crystal of iodine in dioxane (5 ml) was added to the clean autoclave. The mixture was heated at 230 °C, under pressure of 17 bar, during 2 h. The obtained brown oil was evaporated in vacuo. The dry residue was dissolved in CHCl3 and purified by column chromatography (aluminium oxide, CHCl3) to give 10H-1,9-diazaphenothiazine (2) (0.034 g, 17%).

**Synthesis of 10-substituted 1,9-diazaphenothiazines (3–5)**

To a solution of 10H-1,9-diazaphenothiazine (2) (0.50 g, 0.25 mmol) in dry DMF (5 ml) NaH (0.012 g, 0.5 mmol, 60% NaH in mineral oil was washed out with hexane) was added. The reaction mixture was stirred at room temperature for 1 h and then alkyl or aryl halides (methyl iodide, allyl bromide, benzyl chloride, 0.75 mmol) were added and the stirring was continued for 24 h. The mixture was poured into water (15 ml), extracted with CHCl3 (3 × 10 ml) and dried using Na2SO4. The obtained product was purified by column chromatography (aluminium oxide, CHCl3) to give the following:

- 10-Methyl-1,9-diazaphenothiazine (3) (0.041 g, 75%); mp 122–123 °C.
- 10-Butyl-1,9-diazaphenothiazine (4) (0.045 g, 75%); an yellow oil.
- 10-Benzyl-1,9-diazaphenothiazine (5) (0.046 g, 63%); an beige oil.
Synthesis of 10-propargyl-1,9-diazaphenothiazines (6)

To a suspension of 10H-1,9-diazaphenothiazine (2) (0.100 g, 0.5 mmol) in dry DMF (10 ml) was added 80 mg (0.72 mmol) potassium tert-butoxide. The mixture was stirred at room temperature for 1 h. Then to the solution was added drop-wise a solution of propargyl bromide (0.080 g, 0.64 mmol) in dry toluene. The solution stirred at room temperature 24 h and poured into water (20 ml), extracted with methylene chloride (20 ml), dried with Na2SO4, evaporated to the beige oil. The residue was purified by column chromatography (silica gel, CHCl3) to yield 10-propargyl-1,9-diazaphenothiazine (6) (0.085 g, 71%); mp 119–120 °C.

1H NMR δ: 2.17 (s, 1H, CH), 5.07 (s, 2H, CH2), 6.84 (dd, J = 7.5 Hz, J = 5.1 Hz, 2H, H3, H6), 7.28 (dd, J = 7.18 Hz, J = 1.8 Hz, 2H, H2, H5), 8.12 (dd, J = 4.8 Hz, J = 1.18 Hz, 2H, H3, H6). EI MS: 239 (M+, 90), 200 (M-CH2CH3, 100). HR MS (EI) m/z calc. for [C13H9N3S+H] 240.0595. Found 240.0599.

Synthesis of 10-substituted-1,9-diazaphenothiazines (7–12)

To a solution of 10H-1,9-diazaphenothiazine (2) (0.100 g, 0.5 mmol) in dry dioxane (10 ml) NaOH (0.200 g, 5 mmol) was added. The mixture was refluxed for 2 h and hydrochlorides of dialkylaminoalkyl chloride (3-dimethylamino propyl), 2-diethylaminoethyl, 3-dimethylamino-2-methylpropyl) and hydrochlorides of cycloalkylamino alkyl chloride [1-(2-chloroethyl)-pyrrolidine, 1-(2-chloroethyl) piperidin, 2-(2-chloroethyl)-1-methylpiperidin, 1.5 mmol] were added. The reaction mixture was refluxed for 48 h. After cooling dioxane was evaporated in vacuo and residue was dissolved in CHCl3 (10 ml). The extracts were washed with water, dried with anhydrous sodium sulphate and evaporated in vacuo. The obtained product was purified by column chromatography (alumina oxide, CHCl3) to give the following: 10-(3’-Dimethylamino)propyl)-1,9-diazaphenothiazine (7) (0.100 g, 71%); an oil.

1H NMR δ: 1.97 (m, 2H, CH2), 2.24 (s, 6H, 2CH3), 2.44 (t, J = 7.5 Hz, 2H, NCH2), 4.22 (t, J = 7.5 Hz, 2H, NCH2), 6.84 (dd, J = 7.5 Hz, J = 5.1 Hz, 2H, H3, H6), 7.51 (dd, J = 7.18 Hz, J = 1.8 Hz, 2H, H2, H5), 8.33 (dd, J = 4.8 Hz, J = 1.18 Hz, 2H, H3, H6). Cl MS m/z: 287 (M+1, 100), HR MS (EI) m/z calc. for [C13H11N3S+H] 287.1330. Found 287.1326.

10-(2’-Diethylamino)ethyl)-1,9-diazaphenothiazine (8) (0.105 g, 69%); an oil.

1H NMR δ: 1.08 (t, J = 7.2 Hz, 6H, 2CH3), 2.64 (q, J = 7.2 Hz, 4H, 2CH2), 2.80 (t, J = 7.2 Hz, 2H, NCH2), 4.03 (t, J = 7.2 Hz, 2H, NCH2), 6.85 (dd, J = 7.5 Hz, J = 5.1 Hz, 2H, H3, H6), 7.51 (dd, J = 7.18 Hz, J = 1.8 Hz, 2H, H2, H5), 8.31 (dd, J = 4.8 Hz, J = 1.18 Hz, 2H, H3, H6). Cl MS m/z: 301 (M+1, 20), 228 (M+1-NC3H7O, 100), 200 (M+1-C2H5NC3H7O10, 25). HR MS (EI) m/z calc. for [C13H13N3S+H] 301.1486. Found 301.1476.

10-(3’-Diethylamino-2’-methylpropyl)-1,9-diazaphenothiazine (9) (0.115 g, 80%); an oil.

1H NMR (CDCl3) δ: 0.92 (d, J = 6.5 Hz, 3H, CH3), 2.39 (m, 9H, 2CH3, NCH2CH3), 4.15 (m, 2H, NCH2), 6.85 (dd, J = 7.5 Hz, J = 5.1 Hz, 2H, H3, H6), 7.51 (dd, J = 7.18 Hz, J = 1.8 Hz, 2H, H2, H5), 8.33 (dd, J = 4.8 Hz, J = 1.18 Hz, 2H, H3, H6). Cl MS m/z: 301 (M+1, 100), 228 (M+1-NC3H7O, 100), 200 (M+1-C2H5NC3H7O10, 25). HR MS (EI) m/z calc. for [C13H13N3S+H] 301.1487. Found 301.1494.

10-(2’-Pyrrrolidinyl)ethyl)-1,9-diazaphenothiazine (10) (0.110 g, 75%); an oil.

1H NMR (CDCl3) δ: 1.25 (s, 3H, CH3), 2.83 (m, 4H, 2CH2), 2.99 (t, J = 7.5 Hz, 2H, CH2), 4.44 (t, J = 7.5 Hz, 2H, NCH2), 6.85 (dd, J = 7.5 Hz, J = 5.1 Hz, 2H, H3, H6), 7.51 (dd, J = 7.18 Hz, J = 1.8 Hz, 2H, H2, H5), 8.33 (dd, J = 4.8 Hz, J = 1.18 Hz, 2H, H3, H6). Cl MS m/z: 299
cells is quantified by a scanning ELISA reader that measures the absorbance of the dye solution at appropriate wavelengths. The examined cells were exposed to the tested compounds for 72 h at various concentrations between 0.1 and 100 μg/ml (prepared initially at a concentration of 1 mg/ml in DMSO). The control was performed in order to check that DMSO has no effect on the cells at the concentration used. The cells were incubated with WST-1 (10 μl) for one hour and the absorbance of the samples was measured against a background control at 450 nm using a microplate reader with a reference wavelength of 600 nm. The results are expressed as the means of at least two independent experiments performed in triplicate. The antiproliferative activity of the tested compound was compared to cisplatin. The IC50 values (a concentration of a compound that is required for 50% inhibition) were calculated from the dose–response relationship with respect to control.

The RT-QPCR method

Genes transcriptional activity (H3, p53, CDKN1a, BCL-2, BAX) was evaluated by the real-time RT-QPCR method with OPTICON TM DNA Engine (MJ Research, Watertown, MA) and QuantTech® SYBR® Green RT-PCR Kit (Qiagen, Valencia, CA). Cells were exposed to compounds 5 and 8 at a concentration of 0.5 μg/ml for 24 h. The RNA extraction was made by using Quick-RNA™ Kit MiniPrep (ZYMOS RESEARCH). Total RNA integrity was analysed in 1.2% agarose electrophoresis with added ethidium bromide compound. The quantity and purity of extracted total RNA were determined by spectrophotometric analysis with HP845 (Hewlett Packard, USA) spectrophotometer. The statistical analysis was performed using the Statistica 8.0 software (StatSoft, Tulsa, OK). All values were expressed as means ± SE.

Apoptosis antibody detection array

The Proteome Profiler Human Apoptosis Array (R&D Systems) kit simultaneously detects the relative expression level of 35 apoptosis-related proteins. In short, the SNB-19 cells were treated with compounds 5 and 9 at a concentration of 0.5 μg/ml. All immunodetection steps were performed in accordance with the manufacturer’s instruction. The blots were detected using an enhanced chemiluminescence system using Li-COR C-Digit Blot Scanner.

Annexin-V apoptosis detection assay

Apoptosis was analysed via Annexin-V-FLUOS Staining kit (Roche, Germany) according to the manufacturer’s instruction. Briefly, after 24 h incubation of the SNB-19 cells with compounds 5 and 8 (0.5 μg/ml), the samples were collected, washed with phosphate-buffered solution and treated with annexin and propidium iodide. The stained cells were analysed by a flow cytometry (LSR II Becton Dickinson flow cytometer). Viable, early apoptotic, late apoptotic and necrotic cells were determined by staining Annexin V-PI-, Annexin V+/PI-, Annexin V+/PI+ Annexin V+/PI+, respectively.

Results and discussion

Synthesis

Synthesis of known dipryridothiazines is dependent on the location of the nitrogen atoms in the formed products. Synthesis from amnonitrotdipyridinyl (2,2', 2,4' and 4,4') sulphones or from two appropriate disubstituted pyridines (proceeding through a sulphone formation step without isolation) runs as the Smiles rearrangement to dipyridinylamine (usually not isolated) followed by cyclisation to form the 1,4-thiazine ring. In this way, four dipryrido-thiazines were obtained (1,6-diaza-24,41,45 1,8-diaza-23,46, 2,7-diaza-47,48 and 3,6-diaza-pentathiazines25,49). Direct cyclisation as the Ullmann-type process to 2,6-diaza-, 2,8-diaza- and 4,6-diaza-pheno-thiazines has not been observed40. Symmetrical 1,9-diaza- and 3,7-diaza-pentathiazines were obtained by sulphurisation of 2,2'- and 4,4'-dipyridinylamine with elemental sulphur at higher temperature. The synthesis of 10H-1,9-diazaphenothiazine (2) was mentioned by Rath40 as a procedure of preparation being similar to the process used for converting diphenylene to phenothiazine (without any details of the reaction conditions, and the product isolation and characterisation). The synthesis of isomeric 10H-3,7-diazaphenothiazine in the reaction of sulphurisation of 4,4'-dipyridinylamine was performed at 280–290 °C giving the product in very yield (9%)51.

Our sulphurisation of 2,2'-dipyridinylamine (1) with elemental sulphur at 250 °C for 1 h in open air conditions (without a solvent) gave 10H-1,9-diazaphenothiazine (2) in 28% yield. Sulphurisation in an autoclave using dioxane as a solvent was less efficient than in open air giving the desired product in 17% yield. Attempted sulphurisations in other solvents (ethylene glycol in open air conditions and water, chloroform, DMF and monomethyl ether of diethylene glycol in an autoclave) were not satisfactory. The best result (42%) was achieved when the reaction (without a solvent) was carried in a microwave reactor.

10H-1,9-diazaphenothiazine (2) was transformed into 10-substituted derivatives 3–12 in alkylation reaction with selected alkyl and dialkylaminoalkyl halides (Scheme 1).

Spectroscopic analysis

The Rath product was not characterised at all40. Our spectroscopic study revealed a molecular formula of C12H14N2S from HR MS spectrum and only 3 pyridine proton signals of an AMX system in the 1H NMR spectrum which pointed at a symmetrical structure of the sulphurisation product. To find the location of the azine nitrogen atoms in the dipryridothiazine system, the sulphurisation product was methylated with methyl iodide in dry DMF in the presence of sodium hydride. The simple 1H NMR spectrum revealed four signals: the methyl group and three pyridine protons confirming that the methyl group is attached to the thiazine nitrogen atom.

To find the pyridine nitrogen atoms 2D NMR ROESY spectrum was recorded. An irradiation of the methyl protons at 3.45 ppm did not show any proximity of the methyl group to the protons confirming the pyridine nitrogen atoms to be in positions 1 and 9. The full assignment of the proton and carbon signals came from other 2D NMR spectra showing 1H-1H (COSY) and 13C-1H connectivities (HSQC and HMBC, in Supplementary Material). Those last two spectra showed the C-H relationship through one bond (JC,H Connectivity) and three bonds (2JC,H Connectivity).

The all 1H-1H and 1H-13C relationships were included in Supplementary Material. This spectroscopic analysis confirmed the methylated product to be 10-methyl-1,9-diazaphenothiazine (10-methyl-dipirido[3,2-b;2',3'-e]1,4 thiazine) (3).
The X-ray diffraction study confirmed the product structure concluded from the $^1$H NMR spectra as 10-methyl-1,9-diazaphenothiazine and revealed a spatial arrangement in the molecule in a solid state (Figure 1).

In all known studies of N-methyl dipryridothiazine crystals, the tricyclic ring systems are planar or folded depending on the methyl group location\textsuperscript{15,47,52}. The ring system in compound (3) is also folded along the S–N axis with the butterfly angle of $36.22(4)^\circ$ between two pyridine ring planes. The central thiazine ring is in boat conformation with the angle between two halves (SCCS) of $41.28(6)^\circ$. The methyl group is located in equatorial position with the S5⋯N10–C11 angle of 170.1(1)$^\circ$.

The bond angles on heteroatoms in the central ring C1a–N10–C10a and C4a–S5–C5a are 118.74(12)$^\circ$ and 98.19(7)$^\circ$, respectively. The thiazine nitrogen atom (N10) showed pyramidal configuration as a sum of three bond angles around this atom is $353.12^\circ$. The N10–C bond lengths in the thiazine ring are 1.406–1.409 Å, being significantly longer than the azine N–C bonds (1.331–1.349 Å). The N10–CH$_3$ bond length is the longest at 1.463(2) Å due to the sp$^3$ hybridisation of the carbon atom. The molecule interacts with the neighbouring four molecules with four non-classical C–H⋯N hydrogen bonds (the molecule is an

![Scheme 1. Synthesis of 10H-1,9-diazaphenothiazine (2) and its 10-derivatives (3–12).](image)

![Figure 1. Ortep drawing of 10-methyl-1,9-diazaphenothiazine (3), showing the atom labelling.](image)
acceptor of two and a donor of two others H-bonds) forming 2D layers perpendicular to the c crystallographic axis. Consequently, a layer-type structure was observed.

**Anticancer activity**

As it was mentioned in Introduction, some 1,6-diaza-, 1,8-diaza-, 2,7-diaza- and 3,6-diazaphenothiazines substituted at the thiazine nitrogen atom, exhibited very strong anticancer action against various cancer cell lines. The most active compounds possessed the propargyl and dialkylaminoalkyl substituents, and in some cases even the hydrogen atom. Therefore, 10H-1,9-diazaphenothiazine (2) and its 10-derivatives 3–12 with various alkyl and dialkylaminoalkyl substituents were screened for their anticancer activity against in vitro using cultured glioblastoma SNB-19, melanoma C-32 and breast cancer MDA-MB-231 cell lines and cisplatin as a reference drug. Besides of the dimethylaminomethyl-propyl derivative 9 all the compounds turned out to be very active at least one cancer cell line (Table 1). This type of dipyridothiazine appeared the most potent comparing with other anti-cancer ability against melanoma C-32 with IC50 = 3.83 μM, more potent than cisplatin, IC50 = 13.2 μM) and inactive against other lines. General, an introduction of alkyl and dialkylaminoalkyl groups in position 10 enhanced the activity. Compounds 5–8 and 11 with the propargyl, benzyl, dimethylaminopropyl, diethylaminomethyl and morpholinylethyl groups were highly active against glioblastoma SNB-19 with IC50 = 0.33–3.85 μM. Compounds 6–8 and 10 (having the pyrrolidinylethyl group) was very active against breast cancer MDA-MB-231 with IC50 = 2.13–9.61 μM. Compounds 3–5 (possessing the methyl, allyl and propargyl groups) exerted strong activity against melanoma C-32 with IC50 = 3.37–4.02 μM. The most active compounds were more potent than cisplatin.

**Apoptosis assay**

Compounds 5 and 8 were selected as the most promising 1,9-diazaphenothiazines to study the mechanism of anticancer action using the RT-QPCR method. This method analysed the gene transcriptional activities of proliferation marker (H3) cell cycle regulator (TP53 and CDKN1A) and intracellular apoptosis pathway (BACL-2 and BAX). The obtained results on three cancer cell lines are collected in Table 2.

The growth, division and eventual death of the cells in the body are processes that are controlled by hundreds of genes working together. The gene encoding the histone H3 is involved in the cell cycle progression and is considered as an indicator of proliferation in molecular studies. It plays an important role in regulation of the expression of the genetic information encoded in DNA. Both compounds reduced considerably the number of mRNA copies in all cancer lines what can be a result of a modification of the chromatin structure.

Tumour protein p53 is TP53 gene product, one of the most known tumour suppressor genes, which is involved in anticancer action by various mechanisms. The p53 protein is activated by a variety of cell stresses, such as DNA damage, oncogene activation, spindle damage and hypoxia. Activated p53 transactivates a number of target genes, many of which are involved in DNA repair, cell cycle arrest and apoptosis. Compounds 8 and 5

**Table 1. The anticancer activity of 1,9-diazaphenothiazines.**

| No | SNB-19 | C-32 | MDA-MB-231 |
|----|--------|------|------------|
| 2  | >200   | 3.83 | >200       |
| 3  | >200   | 3.72 | 29.5       |
| 4  | 172.2  | 4.02 | 19.1       |
| 5  | 3.85   | 3.37 | 14.1       |
| 6  | 0.79   | 28.2 | 7.36       |
| 7  | 0.38   | 50   | 9.61       |
| 8  | 0.33   | 14.4 | 2.13       |
| 9  | >200   | >200 | >200       |
| 10 | >200   | >200 | >200       |
| 11 | 3.62   | 21.5 | 14.2       |
| 12 | >200   | >200 | 29.8       |

**Table 2. The influence of compounds 5 and 8 on expression of genes encoding H3, TP53, CDKN1A, BCL-2, BAX.**

| Gene     | SNB-19         | C-32          | MDA-MB-231     |
|----------|----------------|---------------|----------------|
| **H3**   |                |               |                |
| Control  | 17,909 ± 1812  | 995,904 ± 181,187 | 183,104 ± 32,058 |
| 5        | 3717 ± 408     | 18,538 ± 584  | 6374 ± 609     |
| 8        | 4448 ± 420     | 366,840 ± 33,506 | 44,374 ± 2912  |
| **TP53** |                |               |                |
| Control  | 835,734 ± 53,484 | 682,740 ± 84,734    | 299,833 ± 4213  |
| 5        | 1,398,619 ± 245,646 | 500,868 ± 23,049   | 110,292 ± 28,461 |
| 8        | 503,813 ± 66,078 | 636,089 ± 33,506 | 194,374 ± 54,249 |
| **CDKN1A** |               |               |                |
| Control  | 242,104 ± 131,105 | 1,752,117 ± 374,944 | 17,642 ± 2504  |
| 5        | 442,447 ± 43,883 | 1,724,825 ± 162,708 | 100,681 ± 10,814 |
| 8        | 348,748 ± 50,516 | 2,244,254 ± 156,054 | 83,652 ± 4606  |
| **BCL-2** |                |               |                |
| Control  | 15,315 ± 1085  | 68,431 ± 12,161 | 121,241 ± 13,879 |
| 5        | 16,710 ± 622   | 23,277 ± 6412  | 29,376 ± 3256  |
| 8        | 12,765 ± 217   | 74,397 ± 7567  | 88,717 ± 11,951 |
| **BAX**  |                |               |                |
| Control  | 918,016 ± 47,034 | 2,431,793 ± 574,891 | 154,191 ± 11,428 |
| 5        | 1,090,855 ± 133,864 | 490,262 ± 29,522   | 121,389 ± 12,855 |
| 8        | 1,198,420 ± 338,142 | 1,855,193 ± 488,197 | 161,425 ± 27,675 |
| **BAX/BCL-2** | 59.9         | 35.5          | 1.21           |
| 5        | 65.3          | 21.1          | 4.13           |
| 8        | 93.9          | 24.9          | 2.04           |
decreased mRNA copies in 3 or 2 cancer lines, respectively (only an increase in mRNA copies was observed in C-32 line).

The cell cycle inhibitor CDKN1A (p21) tightly controlled by the p53 protein is a protein playing multiple roles not only in the DNA damage response, but also in many cellular processes during unperturbed cell growth. The main and well-known function of protein p21 is to arrest cell cycle progression by inhibiting the activity of cyclin-dependent kinases. This protein is also involved in the regulation of transcription, apoptosis, DNA repair, as well as cell motility. As a biomarker of the cell response to different toxic stimuli, p21 expression and functions were analysed58,59. Compound 8 induced increase in the expression of CDKN1A in all cancer lines but compound 5 only in breast cancer lines.

P53 protein is also responsible for keeping the right balance between expression of a proapoptotic BAX gene and an antiapoptotic BCL-2 gene. These proteins have special significance since they can determine if the cell commits to apoptosis or aborts the process. It is thought that the main mechanism of action of the BCL-2 family of proteins is the regulation of cytochrome c release from the mitochondria via alteration of mitochondrial membrane permeability60. In general, compounds 5 and 8 reduced (with some exception) the mRNA expression of BCL-2. In contrast to this, the compounds enhanced or reduced the expression of BAX. The ratio of BAX/BCL-2 can determine whether cells will die via apoptosis or be protected from it. In comparison with the control, the BAX/BCL-2 ratio was found to be greater for both compounds in relation to SNB-19 and MDA-MB-231 cell lines.

In summary, the analysis of the gene expression revealed that compounds 5 and 8 selectively reduced expression of H3 and TP53, and enhanced expression of CDKN1A in examined cell lines. The gene expression ratio of BAX/BCL-2 indicated the induction of mitochondrial apoptosis in two cancer cell lines (SNB-19 and MDA-MB-231). In melanoma C-32 cell line, the transcriptional gene activity suggests a different way of cell death.

Annexin V Apoptosis Detection Assay showed the populations corresponding to viable, necrotic, early and late apoptotic cells. When the SNB-19 cells were treated with compounds 5 and 8, there was slight increase in early and late apoptotic cell populations and slight decrease in viable cell populations (Supplementary Material).

To further understand the mechanism of action of compounds 5 and 8, we performed a determination of apoptosis-related proteins using Proteome Profiler Human Apoptosis Array. We identified 12 expressed proteins in the response to the compounds in the SNB-19 cells (Supplementary Material). Proteins such as phospho-p53 (S15, phosphorylation at ser15), phospho-p53 (S46) and phospho-p53 (S392) play an important role in cell proliferation as DNA damage response, induction of apoptosis and growth suppression61 and all three proteins were found in the protein array. We found proteins which implicated in apoptosis: BAX, pro-caspase-3, cytochrome c and SMAC/Diablo. The last protein is a proapoptogenic mitochondrial protein which interacts and antagonises inhibitors of apoptosis proteins (IAPs) thus allowing the activation of caspases and apoptosis62. BAX accelerates programmed cell death by binding to mitochondrial membrane (MOMP) releasing cytochrome c, promoting activation of caspase-3 and triggering apoptosis63. It seems that compounds 5 and 8 induce BAX to form a channel in MOMP and release cytochrome c to activate caspases 9 and 3 (promoted also by SMAC/Diablo) thus initiating apoptosis through the intrinsic mitochondrial pathway. However, further studies are required to confirm the precise mechanism of this anticancer action.

**Conclusion**

We report here efficient synthesis of 10H-1,9-diazaphenothiazine in the sulphurisation reaction of diphenylamine with elemental sulphur and its transformation into new 10-substituted derivatives, containing the alkyl and dialkylaminoalkyl groups at the thiazine nitrogen atom. The 1,9-diazaphenothiazine ring system was identified with advanced 1H and 13C NMR techniques and confirmed by single-crystal X-ray crystallography of the methyl derivative. X-ray diffraction analysis revealed nonplanar tricyclic ring system with the substituent at the thiazine nitrogen atom in an equatorial location. The compounds exhibited significant anticancer activities against the human glioblastoma SNB-19, melanoma C-32 and breast cancer MDA-MB231 cell lines. The most active 1,9-diazaphenothiazines were the derivatives with the propynyl and N,N-diethylaminoethyl groups being more potent than cisplatin. The expression of H3, TP53, CDKN1A, BCL-2 and BAX genes for those two compounds was detected by the RT-QPCR method. The analysis of the gene expression revealed that both compounds inhibited the proliferation in all cells (H3) and activated of mitochondrial events of apoptosis (BAX/BCL-2) in two cancer cell lines (SNB-19 and MDA-MB-231). The proteome profiling study showed the most probable compound action on SNB-19 cells through the intrinsic mitochondrial pathway of apoptosis. The 1,9-diazaphenothiazine system seems to be more potent than known isomeric ones (1,6-diaza-, 1,8-diaza-, 2,7-diaza- and 3,6-diazaphenothiazine).

**Disclosure statement**

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Part CLIV in the series of Azinyl Sulphides.

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