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RESEARCH ARTICLE

Synthesis and cytotoxicity studies of bifunctional hybrids of nitrogen mustards with potential enzymes inhibitors based on melamine framework

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Abstract

The new class of hybrid anticancer drugs were obtained by selective functionalization of the triazine scaffold. These were prepared by rearrangement of mono-, bis- and/or tris-(1,3,5-triazin-2-yl)-1,4-diazabicyclo[2.2.2]octanium chlorides leading to formation of 2-chloroethylamino fragments attached to 1,3,5-triazine *via* one, two or three piperazine rings respectively. Their inhibitory effect was found strongly dependent on the structure of substituents in triazine ring. The anti-proliferative activity of the hybrids evaluated *in vitro* by using mammalian tumour cells estimated as IC_{50} was in the range 0.62–139,78 μM. Both cytotoxicity and alkylating activity depended on the substituents of triazine ring, however, also the mono-functional analogues of nitrogen mustards, which are unable to form liaisons between two DNA strands, induced apoptosis and necrosis in the tested cells.

Keywords: Proliferation inhibitors, apoptosis, hybrid anticancer drugs, nitrogen mustard, triazine scaffold, alkylating agent

Introduction

The recent therapeutic approach in which drug candidates are designed to possess diverse pharmacological properties and act on multiple targets has stimulated development of the hybrid drugs. Such dual action drugs with two dissimilar drug molecules combined together by direct linking of the two molecular entities are already known and found to be useful tools in the therapy of complex diseases such as cardiovascular and inflammatory diseases and might be used as antibacterial agents useful in the treatment of drug resistant pathogens[1].

In several cases the collected data provide a conclusive molecular mechanism for the independent modes of action of both drug-like fragments, amplification of effects of fragments or even the extraordinary biological effects not attributed to any of the individual partner of the hybrid construct that make the emergence of drug resistance less likely[2]. Since cancer is a genetic disease with several mutations altering the replication system of the cell, the strategy based on the construction of hybrid anticancer drugs possessing different toxicity profiles seems to be very promising.

In the last decade, several new highly potent cytostatic agents, derived from 1,3,5-triazine, have been presented that are active as inhibitors of enzymes involved in the cell proliferation cycle (see Figure 1).

A melamine derivative, ZSTK474 [2-(2-difluoromethylbenzimidazol-1-yl)-4,6-dimorpholino-1,3,5-triazine] (**1**) has been found, which inhibits the growth of tumour cells with its molecular target identified as phosphatidylinositol 3-kinases (PI3K) with strong antitumour activity against human cancer xenografts without toxic effects on critical organs[3]. For melamine **2** it has been suggested that its antimetastatic and

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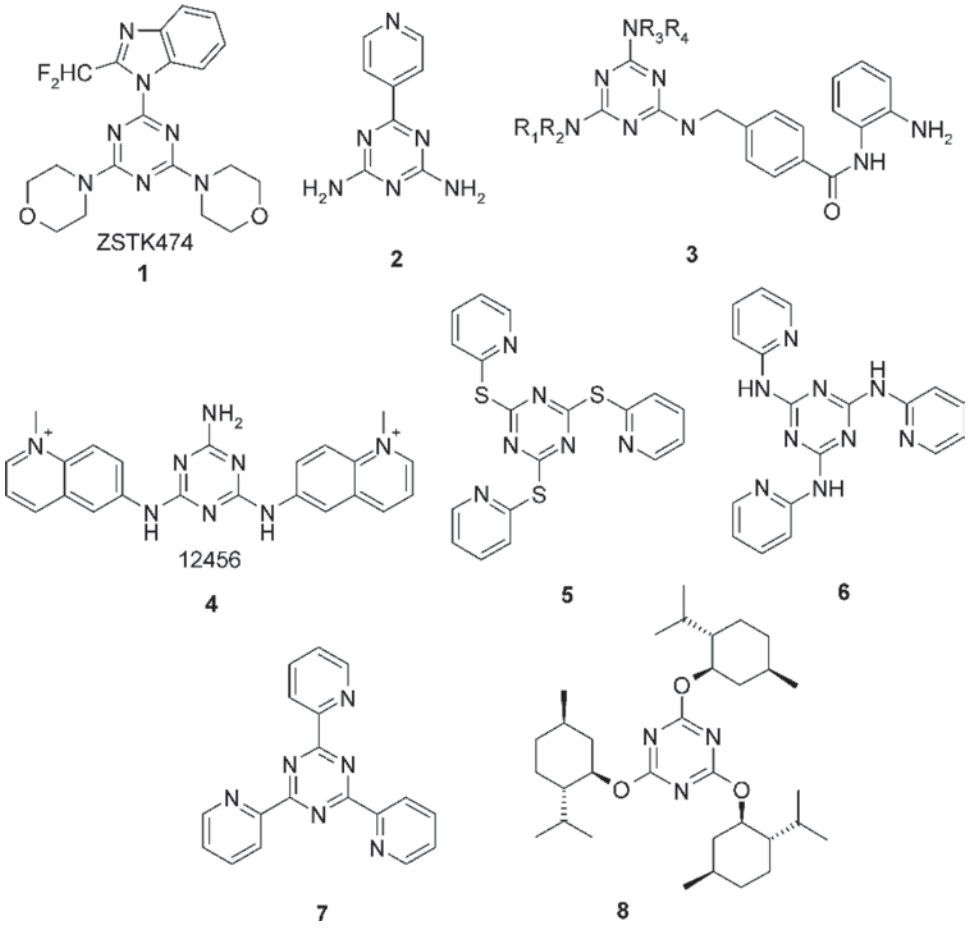


Figure 1. Structures of triazine based antitumour agents and enzyme inhibitors.

antitumour activity is due in part to inhibition of angiogenesis, rather than direct anti-proliferative action on tumour cells[4].

Melamine derivatives **3** were also found to be some of the best histone deacetylase inhibitors with anti-proliferative activities with IC_{50} values below the micromolar range. Some of these compounds can also significantly reduce tumour growth in human tumour xenograft models in mice. In general, derivatives of the triazine series show better profiles than their pyridine, pyrimidine, and purine analogues and moreover, these compounds are selective to the cancer cell line tested (HCT116) with a toxicity index that in some cases is over 100-times higher than that of a normal cell line (HMEC[5]). Melamine derivative **4** were also found to be the potent G-quadruplex ligand, which is shown to induce both telomere shortening and apoptosis in the human A549 cell line as a function of its concentration and time exposure. Several other melamine analogues were also considered as potential antitumour agents that block telomere replication stabilizing the telomeric G-rich single stranded DNA overhang into G-quadruplex and consistently inducing telomere erosion, which results in senescence after prolonged contact. They are also able to induce apoptotic or non-apoptotic cell death, alteration of cell cycle progression, and depression of telomerase activity[6].

A series of thiopyridine **5** and aminopyridine **6** substituted triazine derivatives has been found inducing G2/M arrest and apoptosis with a possible involvement of p53[7]. Triazine **7** has been found to be efficient topoisomeraze inhibitors[8] and terpene substituted triazine **8** is active as a cytostatic, but with an unknown molecular target[9]. Additionally, as a selective inhibitor, 1,3,5-triazine based molecules have been found to act on many different targets which include: HIV-1 reverse transcriptase[10], estrogen receptor beta[11], glutathione S-transferase[12], *M. tuberculosis* dihydrofolate reductase[13], photosynthetic reaction centre[14], guanosine-5′-triphosphate binding site[15], ATP competitive inhibitor of mammalian target of rapamycin[16] and urate oxidase[17]. Considering enzyme inhibition as an intrinsic property of the complete melamine structure, still remains vast potential of decoration of triazine scaffold by introducing "address" fragment increasing selectivity, components facilitating transport through the cell membranes, and last but not least, components of classic anticancer drugs with well-documented therapeutic competence. Therefore recent approaches involve development of hybrid cancer drugs[18] or application of 1,3,5-triazine as inductor of new specific molecular targets[19]. In these studies it has been attempted to introduce into 1,3,5-triazine scaffold

one, two or three fragments bearing 2-chloroethylamine moiety characteristic for nitrogen mustards[20,21] and to confirm anti-proliferative activity of the obtained hybrids.

Materials and methods

General Information

Thin layer chromatographies (TLC) were carried out on SiO_2 (Merck; 60 Å F254) and spots located with: UV light (254 and 366 nm) and 1% ethanolic 4-(4´-nitrobenzyl)-pyridine (NBP). Melting points were determined on a Büchi apparatus, model 510. IR spectra were recorded as KBr pellets or film on a Infracord 137 E spectrometer. ^{1}H-NMR, ^{13}C-NMR, spectra were recorded on a Bruker Avance DPX 250 (250 MHz) spectrometer. Chemical shifts (ppm) are relative to TMS used as an internal standard. The multiplicity were marked as s = singlet, d = dublet, t = triplet, q = quartet, qu = quintet, m = multiplet. Triazines **9**a-d were obtained from cyanuric chloride according to standard procedure described[22].

2,4-Bis-methoxy-6-[4-(2-chloroethyl)-piperazin-1-yl]-[1,3,5] triazine (12a).

General procedure: 1,4-Diazabicyclo[2.2.2]octane (**10**) (DABCO) (1.12 g, 10 mmol) was added to a vigorously stirred solution of 2-chloro-4,6-dimethoxy-1,3,5-triazine (9a) (1.76 g, 10 mmol) in dichloromethane (20 mL), cooled to 5°C. The mixture was stirred at 5°C for 0.5 h and then under reflux condition for 1 h. Progress of reaction was monitored by TLC (R_f = 0 for salt **11a,** R_f = 0.15 for **12a,** DCM, 1% solution of (NBP) for visualization of spots). The organic layer was concentrated under evaporated reduced pressure. 2,4-Bis-methoxy-6-[4-(2-chloroethyl)-piperazin-1-yl]-[1,3,5]triazine (**12a**) was obtained (2.81 g, yield 98%), m.p. = 88–90°C.

^{1}H-NMR ($CDCl_3$): 2.55 (t, 4H, J = 5.2 Hz); 2.77 (t, 2H, J = 7.5 Hz); 3.61 (t, 2H, J = 7.5 Hz); 3.87 (t, 4H, J = 5.2 Hz); 3.95 (s, 6H). ^{13}C-NMR ($CDCl_3$): 40.58; 43.13; 52.50; 54.20; 55.60; 59.41; 166.23; 172.06. IR (film/NaCl): 2952; 2840; 2808; 1584; 1536; 1472; 1368; 1308; 1280; 1255; 1190; 1135; 1040; 990. Anal. Calcd for $C_{11}H_{18}ClN_5O_2$: C, 45.92; H, 6.31; N, 24.34. Found: C, 45.81; H, 6.33; N, 24.39.

2,4-Bis-benzyloxy-6-[4-(2-chloroethyl)-piperazin-1-yl]-[1,3,5] triazine (12b)

Starting materials: **9b** (1.64 g, 5 mmol), DABCO (1.12 g, 5 mmol). *Product*: 2,4-bis-benzyloxy-6-[4-(2-chloroethyl)-piperazin-1-yl]-[1,3,5]triazine (**12b**) (2.09 g, 95%), oil.

^{1}H-NMR ($CDCl_3$): 2.54 (t, 4H, J = 5.0 Hz); 2.76 (t, 2H, J = 7.5 Hz); 3.61 (t, 2H, J = 7.5 Hz); 3.88 (t, 4H, J = 5.0 Hz); 5.38 (s, 4H); 7.26–7.45 (m, 10H). ^{13}C-NMR ($CDCl_3$): 40.60; 43.23; 52.53; 59.44; 68.80; 127.94; 128.06; 128.25; 135.97; 166.35; 171.61. IR (film/NaCl): 3050; 2960; 2815; 2255; 1830; 1695; 1580; 1525; 1495; 1445; 1420; 1345; 1305; 1270; 1155; 1105; 1040; 995. Anal. Calcd for $C_{23}H_{26}ClN_5O_2$: C, 62.79; H, 5.96; N, 15.92. Found: C, 62.29; H, 5.71; N, 15.98.

2-[4-(2-Chloroethyl)-piperazin-1-yl]-4,6-bis-(2,2,2-trifluoroethoxy)-[1,3,5]triazine (12c)

Starting materials: 2-chloro-4,6-bis-(2,2,2-trifluoroethoxy)-[1,3,5]triazine (**9c**) (1.56 g, 5 mmol), DABCO (1.12 g, 5 mmol). *Product*: 2-[4-(2-chloroethyl)-piperazin-1-yl]-4,6-bis-(2,2,2-trifluoroethoxy)-[1,3,5]triazine (**12c**) (2.03 g, 96%), oil.

^{1}H-NMR ($CDCl_3$): 2.67 (t, 4H, J = 5.5 Hz); 2.88 (t, 2H, J = 8.0 Hz); 3.66 (t, 2H, J = 8.0 Hz); 3.94 (t, 4H, J = 5.5 Hz); 4.75 (qw, 4H, J = 7.5 Hz). ^{13}C-NMR ($CDCl_3$): 36.31; 40.08; 43.15; 52.26; 59.13; 63.30; 165.95; 170.67. IR (film/NaCl): 2980; 2940; 2870; 2820; 2250; 1720; 1670; 1590; 1525; 1420; 1375; 1300; 1270; 1160; 1130; 1070; 990; 950. Anal. Calcd for $C_{13}H_{16}ClF_6N_5O_2$: C, 36.85; H, 3.81; N, 16.53. Found: C, 36.71; H, 3.75; N, 16.42.

2-[4-(2-Chloroethyl)-piperazin-1-yl]-4,6-diphenoxy-[1,3,5] triazine (12d)

Starting materials: 2-chloro-4,6-diphenoxy-1,3,5-triazine (**9d**) (1.50 g, 5 mmol), DABCO (1.12 g, 5 mmol). *Product*: 2-[4-(2-chloroethyl)-piperazin-1-yl]-4,6-diphenoxy-[1,3,5]triazine (**12d**) (1.73 g, 84%), m.p. = 138–140°C.

^{1}H-NMR ($CDCl_3$): 2.48 (t, 4H, J= 7.5 Hz); 2.73 (t, 2H, J = 5.0 Hz); 3.57 (t, 2H, J = 5.0 Hz); 3.72 (t, 4H, J = 7.5 Hz); 7.12–7.37 (m, 10H). ^{13}C-NMR ($CDCl_3$): 40.53; 43.20; 52.48; 59.39; 121.58; 125.90; 129.04; 151.92; 166.42; 172.13. IR (film/NaCl): 2970; 2920; 2805; 1740; 1670; 1595; 1575; 1530; 1490; 1445; 1390; 1375; 1310; 1280; 1260; 1210; 1160; 1125; 1070; 1020; 995. Anal. Calcd for $C_{21}H_{22}ClN_5O_2$: C, 61.24; H, 5.38; N, 17.00. Found: C, 61.04; H, 5.41; N, 16.95.

2,4-Bis-[4-(2-chloroethyl)-piperazin-1-yl]-6-methoxy-[1,3,5] triazine (12e)

Starting materials: 2,4-dichloro-6-methoxy-1,3,5-triazine (**9e**, DCMT) (0.90 g, 5 mmol), DABCO (2.24 g, 10 mmol). *Product*: 2,4-bis-[4-(2-chloroethyl)-piperazin-1-yl]-6-methoxy-[1,3,5]triazine (**12e**) (2.02 g, 80%), m.p. = 278–281°C.

^{1}H-NMR ($CDCl_3$): 2.53 (t, 8H, J = 5.1 Hz); 2.76 (t, 4H, J = 8.0 Hz); 3.63 (t, 4H, J = 8.0 Hz); 3.84 (t, 8H, J = 5.1 Hz); 3.87 (s, 3H). ^{13}C-NMR ($CDCl_3$): 38.99; 41.23; 51.10; 53.01; 57.97; 160.68; 164.01; 169.66. IR (film/NaCl): 3000; 2950; 2860; 2810; 2770; 2230; 1675; 1590; 1580; 1525; 1490; 1380; 1350; 1300; 1245; 1190; 1150; 1120; 1090; 1045; 995. Anal. Calcd for $C_{16}H_{27}Cl_2N_7O$: C, 48.81; H, 6.99; N, 23.44. Found: C, 48.54; H, 6.75; N, 23.40.

2,4,6-Tris-[4-(2-chloroethyl)-piperazin-1-yl]-[1,3,5]triazine (12f)

Starting materials: cyanuric chloride (0.92 g, 5 mmol), DABCO (3.36 g, 15 mmol). *Product*: 2,4,6-Tris-[4-(2-chloroethyl)-piperazin-1-yl]-[1,3,5]triazine (**12f**) (2.11 g, 81%), oil.

^{1}H-NMR ($CDCl_3$): 2.35–2.95 (m, 22H); 3.59-3.77 (m, 14H). ^{13}C-NMR ($CDCl_3$): 40.47; 42.69; 52.57; 59.26; 164.69. IR (film/NaCl): 3020; 2930; 2820; 2350; 1725; 1670; 1575; 1540; 1525; 1475; 1440; 1375; 1310; 1260;

1220; 1180; 1140; 1090; 1010; 950. Anal. Calcd for $C_{21}H_{36}Cl_3N_9$: C, 49.39; H, 7.16; N, 23.56. Found: C, 49.31; H, 7.11; N, 23.49.

Cytotoxicity determined by SRB method

The following established *in vitro* human cancer cell lines were applied: SW707 (colorectal adenocarcinoma), Jurkat (leukemia), A549 (lung cancer), LNCaP (prostate cancer) and T47D (breast cancer). All lines were obtained from the American Type Culture Collection (Rockville, Maryland, USA) and maintained at the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

Twenty-four hours before addition of the tested agents, the cells were plated in 96-well plates (Sarstedt, USA) at a density of 104 cells per well in 100 μL of culture medium. The cells were cultured in the opti-MEM medium supplemented with 2 mM glutamine (Gibco, Warsaw, Poland), streptomycin (50 μg/mL), penicillin (50 U/mL) (both antibiotics from Polfa, Tarchomin, Poland) and 5% fetal calf serum (Gibco, Grand Island, USA). The cell cultures were maintained at 37°C in humid atmosphere saturated with 5% CO_2.

Solutions of compounds were prepared by dilution of 1 mg of **12a,b,f** in DMSO (100 μL) and diluted subsequently to 1000 μL with culture media or **12c,e** in DMSO (1000 mL). Compound **12f** was sonicated for 1 minute to enhance dilution. Compounds were tested in final concentration of: 100, 10, 1 and 01 μg/mL for 12a,c,e or 10, 1, 0.1 and 0.01 μg/mL for 12b,d,f in culture media.

SRB assay

The details of this technique were described by Skehan[23]. The cytotoxicity assay was performed after 72-hour exposure of the cultured cells to varying concentrations (0.1–100 μg/mL) of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% TCA (trichloroacetic acid, Aldrich-Chemie, Germany) on the top of the culture medium in each well. The plates were incubated at 4°C for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B (SRB, Sigma, Germany) dissolved in 1% acetic acid (POCh, Gliwice, Poland) for 30 minutes. Unbound dye was removed by rinsing (4×) with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base (POCh, Gliwice, Poland) for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate reader Multiskan RC photometer (Labsystems, Helsinki, Finland). Each compound in given concentration was tested in triplicates in each experiment, which was repeated 3–5 times.

The results of cytotoxic activity *in vitro* were expressed as an IC_{50} (μg/mL), i.e. the concentration of compound, which inhibits the proliferation of 50% of tumour cells as compared to the control untreated cells.

MCF-7 cultures

Stock cultures of breast MCF-7 cancer cells (purchased from the American Type Culture Collection, Rockville, MD) were maintained in continuously exponential growth by weekly passage in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% FBS(Sigma), 50 μg/mL streptomycin, 100 U/mL penicillin at 37°C in a humid atmosphere containing 5% CO_2. Cells were cultivated in Costar flasks and subconfluent detached with 0.05% trypsin and 0.02% EDTA in a calcium-free phosphate buffered saline. The study was carried out using cells from passages 3 to 7, growing as monolayer in 6-well plates (Nunc) (5×10^5 cells per well and preincubated 24 hours without phenol red.

Determination of apoptotic index and cell vialibility

The compounds were dissolved in sterile water and used at concentrations of 1, 10, 50, 100 and 150 μM. Microscopic observations of cell monolayers were performed with a Nikon optiphot microscope. Wright-Giemsa staining was performed using the Fisher Leuko Stat Kit. Adherent MCF-7 cells grown in 6-well plates were stained after induction of apoptosis with a dye mixture (10 μM acridine orange and 10 μM ethidium bromide, prepared in phosphate buffered saline). At the end of each experimental time point, all of the media was removed and cells were harvested by incubation with 0.05% trypsin and 0.02% EDTA for 1 min and washed with the medium. Then, 250 μL of cell suspension was mixed with 10 μL of the dye mix and 200 cells per sample were examined by fluorescence microscopy, according to the following criteria:

- viable cells with normal nuclei (a fine reticular pattern stained green in the nucleus and red-orange granules in the cytoplasm);
- viable cells with apoptotic nuclei (green chromatin which is highly condensed or fragmented and uniformly stained by the acridine orange);
- non-viable cells with normal nuclei (bright orange chromatin with organised structure);
- non-viable cells with apoptotic nuclei (bright orange chromatin witch is highly condensed or fragmented).

Antitumour activity investigated compounds expressed as percentage of non-viable MCF-7 (summarized both apoptotic and necrotic) mammal tumour cells was shown in Table 1.

Determination of alkylating properties (Preussmann test)

The tested compounds (0.01 mmol) were dissolved in 2-metoxyethylether (200 μL) and solution of NBP in 2-metoxyethylether (5%, 200 μL) were added. The samples were heated at 100 ± 0.5°C for 1 h and then quickly cooled to 20°C. 2-Metoxyethylether (500 μL) and piperidine (100 μL) were added to the samples to give a total volume of 1 mL. The final concentration of the tested compounds was 10 μM. After 90 s, the absorbance was measured at λ = 560 nm in a quartz cell (1 cm). 2-Metoxyethylether was used as a reference solvent.

Ethidium bromide (EtBr) assay

Each well of 96-well plate was loaded with Tris buffer containing ethidium bromide (0.1 M Tris, 1 M NaCl, pH 8, 0.5 mM EtBr final concentration, 100 μL). To each well was added 15 μg plasmid. pBR322 as water solution (0.05 μg/μL). Then, to each well was added chlorambucil (CHL) or compound 12a–f (1 μL of a 1 mM solution in water, 10 μM final concentrations). After incubation at 25°C for 30 min, the fluorescence of each well was read on a Multilabel Reader Victor 3V (ex.: 355 nm, em.: 615 nm) in duplicate experiments with two control wells (no drug = 100% fluorescence, no DNA = 0% fluorescence). Fluorescence readings are reported as % fluorescence relative to the controls.

Statistical analysis

In all experiments, the mean values for three independent assays ± standard deviations (S.D) were calculated. The results were submitted to statistical analysis using the Student's test. Differences were considered significant when $p < 0.05$. Mean values, the standard deviations and the number of measurements in the group are presented in the figures.

Results and discussion

As exemplified above, the appropriate modification of 1,3,5-triazine ring system could lead to numerous novel and diverse target-specific inhibiors of enzymes. Thus, the diversity of action mode of triazine based inhibitors and presence of three reactive centres which could be used for the selective functionalization of the triazine scaffold have paved the way to a new class of hybrid anticancer drugs with a lowered toxicity and broader antitumour spectrum[24]. Therefore, while designing the structure of new hybrid anticancer drugs[25] we attempted to attach one, two, or three chloroethylamine groups to surround triazine substituted respectively with one (Scheme 1), two or three piperazine rings (Scheme 2).

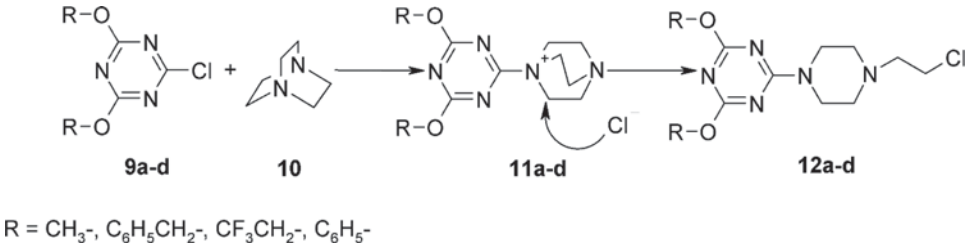
In order to prepare such constructs we made use of the observation[26,27] that some N-triazinylammonium chlorides **11a**-f easy accessible from a broad range of triazines **9a–d** and 1,4-diazabicyclo[2.2.2]octane (DABCO) (**10**) rearranged with a formation of **12a**–f bearing 2-chloroethylamino fragment. Most N-triazinylammonium salts **11** were obtained in almost quantitative yield.

The rearrangement of **11a–f** to **12a–f** proceeds at room temperature relatively slowly and is accelerated in non-aqueous media and at elevated temperatures.

The anti-proliferative activity of the hybrids **12a–f** has been evaluated *in vitro* by using six tumour cell lines. The colorimetric tests were performed in 96-well culture plate and the activity of the cytotoxic drugs presented as IC_{50} values were determined after 72 h of drug exposures (Table 1).

Inhibitory effects of 12a–f was found strongly dependent on the structure of substituents in triazine ring. Unexpectedly, for five tumour cell lines the crosslinking ability, which obligatory required the presence of two or tri chloroethylamino group were found not crucial for activity. Inhibitory effects of **12b,d**, bearing only single chloroethylamino group, expressed as IC_{50} in the range 0.62–3.45 μg/mL on the growth of human tumour cell lines has been found much stronger than bi- and trifunctional analogues **12e,f** (IC_{50} in the range 4.50–6.22 μg/mL or totally negative). This suggests that cell proliferation may be inhibited by mono-alkylation involving chloroethylamino functionality or by the other mechanisms involving, unknown as yet, inhibitory effect of triazine structure.

In the further studies inhibition of proliferation, apoptotic index and cell vialibility was determined *in vitro*

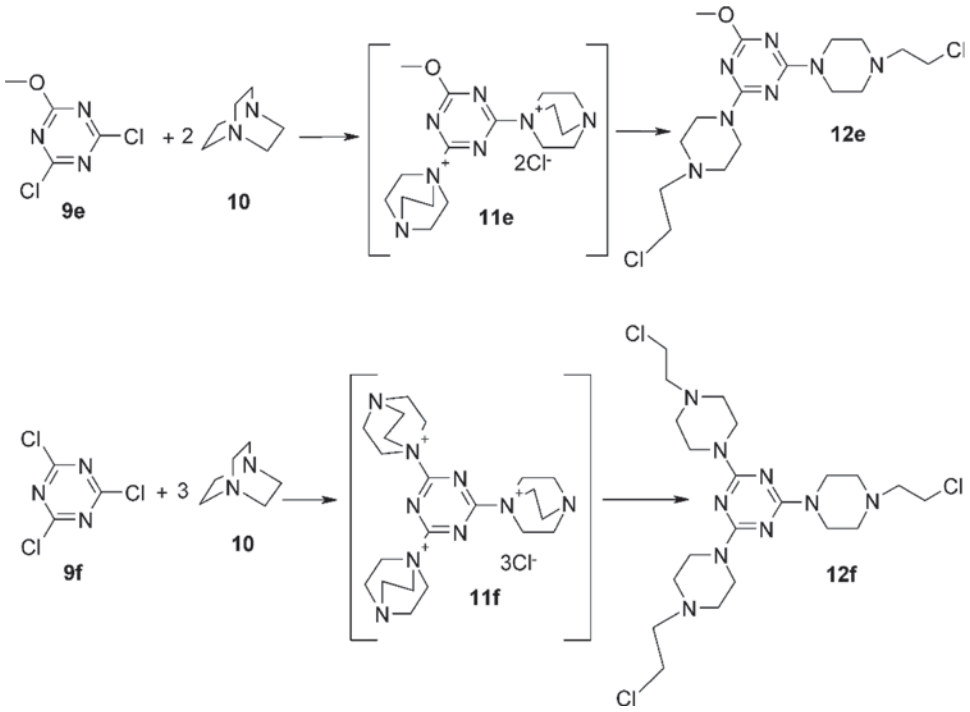


Scheme 1. Formation of triazines 12a-d bearing one 2-chloroethylamine fragment.

Table 1. Inhibitory effects of **12a**–f on the growth of human tumour cell lines expressed as IC_{50} in μg/mL.

	cell line/IC_{50} [μg/mL] mean ± SD				
12	breast cancer T47D	prostate cancer LNCaP	colorectal cancer SW707	lung cancer A549	lymphoblastic leukemia Jurkat
a	27.86 ± 0.03	15.78 ± 6.68	32.38 ± 0.89	34.66 ± 1.55	31.29 ± 0.84
b	1.40 ± 0.33	0.99 ± 0.52	3.45 ± 0.28	2.06 ± 0.66	0.62 ± 0.15
c	96.84 ± 4.47	18.27 ± 14.32	96.84 ± 5.47	61.04 ± 20.31	25.60 ± 2.43
d	2.60 ± 0.99	1.47 ± 0.95	2.93 ± 0.81	2.67 ± 1.33	2.93 ± 0.36
e	36.22 ± 15.85%*	6.22 ± 3.34	25.35 ± 10.82%*	31.58 ± 10.43%*	4.50 ± 1.98
f	negative**	negative**	negative**	negative**	9.01 ± 0.86

*inhibition of cell proliferation *in vitro* expressed as percent of proliferation inhibition at concentration of 12e 10 μg/mL.
no inhibition to the concentration of **12e 10 μg/mL.



Scheme 2. Synthesis 1,3,5-triazine derivatives bearing two 2-chloroethylamino residues **12e** and three 2-chloroethylamino residues **12f**.

on breast MCF-7 cancer cells. The obtained results were compared with the anti-proliferative effect of chlorambucil and summarized in Table 2.

All the compounds **12a–f** showed cytotoxic and anti-proliferative effect. The concentration which inhibits 50% of colony formation was in the range 18.70–139.78 μM. It is worthy to notice that in the case MCF-7 cancer the activity increased with the amount of 2-chloroethylamino fragments. The analogue **12f** with $IC_{50} = 18.70$ μM has three such fragments and is the most active against the investigated cells. It is more active than chlorambucil with $IC_{50} = 29.14$ μM, the therapeutics with alkylating properties belonging to the first class of cytostatics used for cancer therapy[28].

Our other aim was to determine the *in vitro* alkylating activity of the novel mustards **12a–f**. For this purpose an *in vitro* Preussmann test[29] was used. All of the tested compounds demonstrate their alkylating activity toward NBP molecule. Alkylating activity results are presented in Table 3.

Only compound **12c** has a low alkylating activity (+) and is less active than chlorambucil, the other ones are more active and can be included in the group of high (++) alkylating activity.

One can see a correlation between the alkylating activity of the tested compounds and their cytotoxicity on the breast cancer MCF-7 cell line. Both cytotoxicity and alkylating activity increase with the amount of 2-chloroethylamino fragments.

The analyzed triazines **12a–f**, analogously to standard chlorambucil (CHL), inhibited tumour cell growth by exerting direct anti-proliferative affects with the cytotoxic (apoptosis/necrosis) consequences. The investigations showed that all of new compounds induced concentration-dependent apoptosis of MCF-7 cells. The percentage of apoptotic and necrotic MCF-7 cells after treatment with 50 μM solutions of CHL and compounds **12a–f** is shown in Figure 2.

Table 2. Viability of MCF-7 cells treated for 24 h with different concentrations of chlorambucil (CHL) and compounds **12a–f**.

Conc.	Non-viable cells (% of control ± 2)						
[μM]	CHL	12a	12b	12c	12d	12e	12f
0	1a	3	2	3	4	2	2
1	11	6	9	8	23	25	23
10	31	20	24	27	46	31	52
50	48	24	39	46	57	52	62
100	96	42	51	52	63	69	65
150	100	48	61	75	74	72	73
IC_{50}	29.14	139.78	92.02	67.47	32.13	43.95	18.70

[a]Mean values ± SD from three independent experiments done in duplicate are presented.

Table 3. Alkylating activity of compounds **12a–f** on comparison to chlorambucil; NBP test results.

Compound	Molar extinction coefficient (ε)	Absorbance (A)[a] 560 nm	Alkylation activity[b]
12a	103.6	0.1036	++
12b	119.5	0.1195	++
12c	88.3	0.0883	+
12d	119.3	0.1193	++
12e	130.2	0.1302	++
12f	148.9	0.1488	++
Chlorambucil	92.7	0.0927	+

[a]Average data from three determinations.
[b]According to Preussmann: (−) $A < 0.05$, (+) $A = 0.05$–0.1, (++) $A = 0.1$-0.5, (+++) $A > 0.5$.

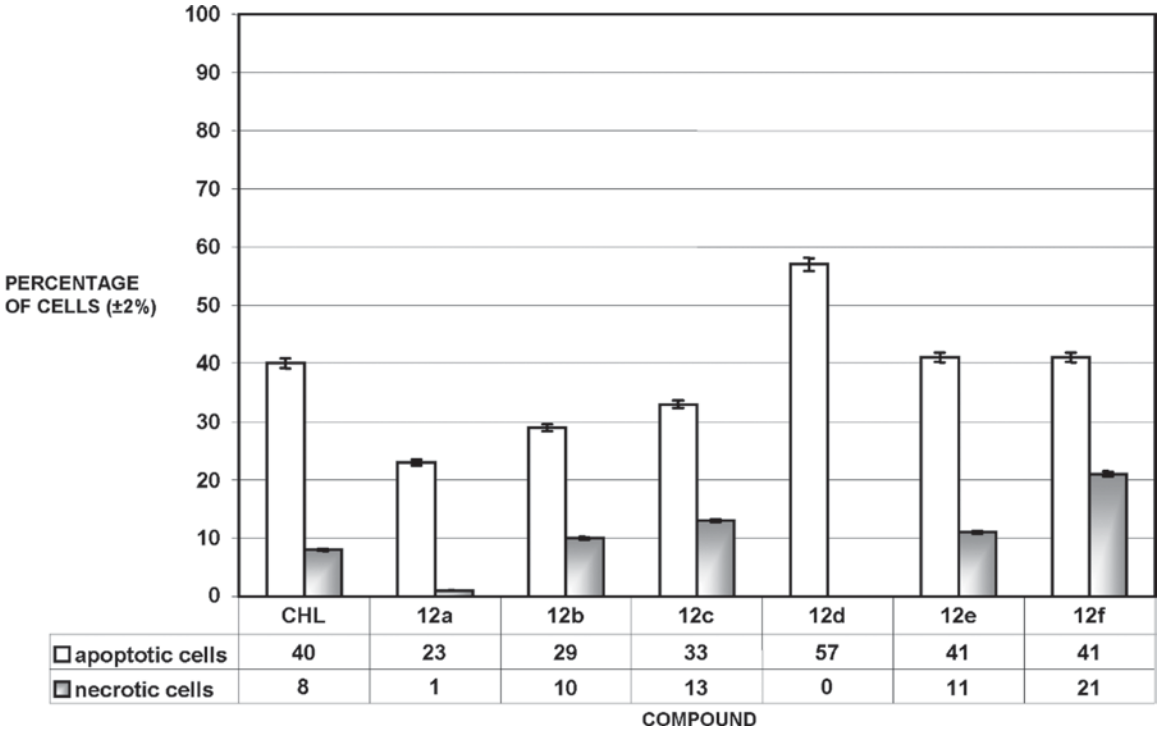


Figure 2. Percentage of apoptotic and necrotic MCF-7 cells after treatment with 50 μM solutions of chlorambucil (CHL) and compounds 12a–12f. 100% = {apoptotic(%) + necrotic(%) + viable cells}.

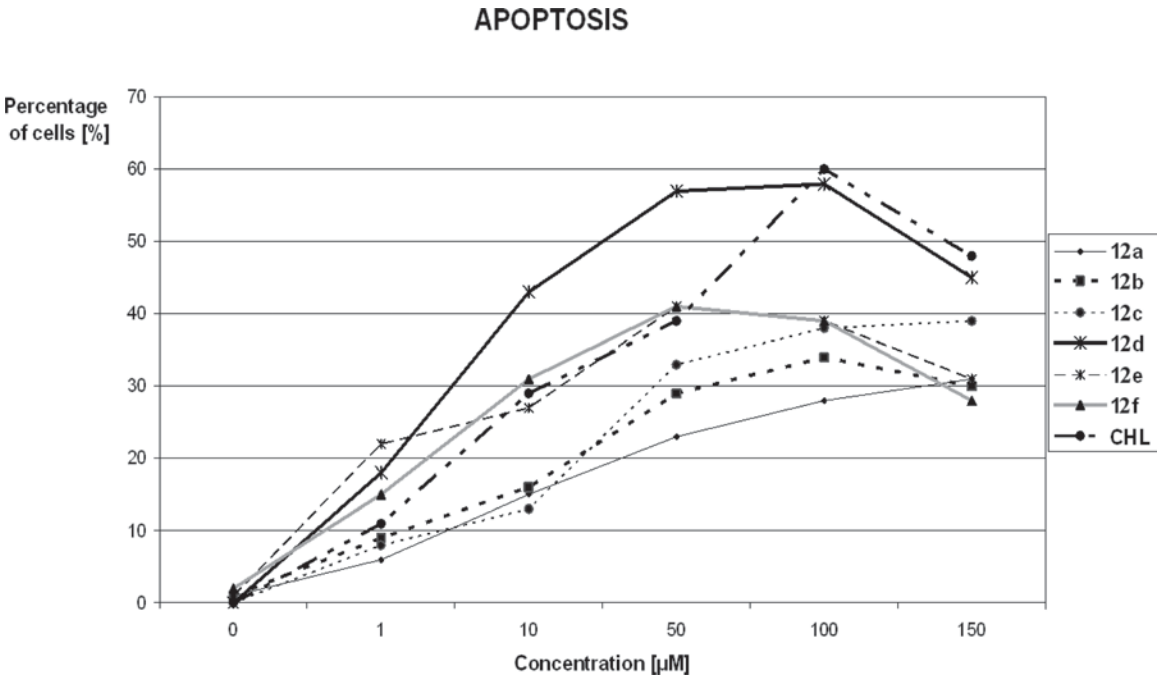


Figure 3. The relationship of apoptotic cells amount from increasing concentration of chlorambucil (CHL) and compounds 12a–f.

Apoptosis is the main way of the cell death at this concentration for all **12a–f**.

Interestingly, the apoptotic cell death was mainly observed for every of them in the range of concentration from 1 to 100 μM, as we can see in Figure 3. These results also show that necrosis is predominant cell death for all of compounds at concentration 150 μM. The ethidinium displacement assay showed that triazines **12a–e** can bind to DNA although relatively weaker than chlorambucil (Table 4).

Conclusions

New dual action drugs combining together by direct linking melamine based potential inhibitors of enzymes with mustard alkylating groups were obtained. The method

Table 4. DNA binding effect of compounds **12a–f**.

Compound	Decrease of fluorescence [%]
CHL	61.73
12a	96.74
12b	98.75
12c	87.50
12d	85.00
12e	92.35
12f	55.62

opened access to mono-, di- and trifunctional derivatives. In case of five tumour cell lines (breast cancer T47D, prostate cancer LNCaP, colorectal cancer SW707, lung cancer A549 and Jurkat lymphoblastic leukemia) the strongest inhibition of proliferation was observed for triazines **12b,d** substituted with single chloroethylamino fragment only. These exclude formation of bifunctional lesions and cross-link of nucleobases within the DNA duplex, but could be attributed to the additional inhibitory activity of triazine scaffold.

However, the experiments on the breast cancer MCF-7 cell line shown that in this case bi- trifunctional analogues **12e,f** of nitrogen mustards, which are capable to form liaisons between two DNA strands, induced apoptosis and necrosis in the tested cells more effectively than mono-functional **12a–d**. The data from ethidium displacement assay suggests that DNA binding may be implicated in the cytotoxicity of the compounds, but there also might be other possible targets and more complex mechanism of action of triazine NM derivatives, so it strongly suggests the need for further studies.

Declaration of interest

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