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Synthesis, physicochemical characterization and preliminary pharmacological *in vitro* evaluation of two novel cytotoxic benzophenone-linked 3,3-dimethyltriazenes

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The synthesis, physicochemical characterization and preliminary pharmacological evaluation of the cytotoxic effects of two novel substances, 1-(4-benzoylphenyl)-3,3-dimethyltriazene and 1-(2-benzoylphenyl)-3,3-dimethyltriazene is presented. The cytotoxicity of the novel benzophenone-linked triazenes and of ten other 1-phenyl-3,3-dimethyl triazene derivatives as well as of the referent alkylating drug melphalan was assessed using the MTT-dye reduction assay. A panel of human tumor cell lines was used: the chronic lymphoid leukemia SKW-3, the acute promyelocyte leukemia HL-60 and its multidrug-resistant subline HL-60/Dox. Both novel compounds showed strong cytotoxic activity, comparable to that of the referent alkylating agent melphalan, whereas the ten ring-substituted 1-phenyl-3,3-dimethyl triazenes proved to be far less active *in vitro*. DNA-fragmentation analysis indicated that after 24 h treatment the novel benzophenone-linked triazenes induced programmed cell death in HL-60 cells.

1. Introduction

Since the discovery of the antineoplastic activity of 1-aryl-3,3-dimethyl triazenes in the 1950's there has been considerable interest in the development of triazene analogues. New substances with higher efficacy and lower organ toxicity and mutagenicity than that the various ring-substituted 1-phenyl analogues have been synthesized (Clarke et al. 1955; Foster 1998; Connors et al. 1976; Gescher et al. 1981). This effort resulted in the consequent discovery of dacarbazine (DTIC) and later of the imidazotetrazinone compound temozolomide, which is a triazene prodrug sharing the same major active metabolite as dacarbazine (Foster 1998; Kohn et al. 1994; Stupp et al. 2001). These drugs currently constitute a distinct class of alkylating antineoplastic agents, characterized by a mono-alkylating mode of DNA modification, low myelosuppressive potential, complex decomposition patterns, and several putative active metabolites. Generally, there is no cross-resistance with the conventional alkylating drugs (Foster 1998; Stupp et al. 2001). Dacarbazine (DTIC) is used in the treatment of lymphomas and malignant melanoma, and temozolomide was shown to be effective for the treatment of certain central nervous system neoplasms (Foster 1998; Stupp et al. 2001).

Recently, an intriguing novel strategy proposed by Metheson et al. known also as the "combi-targeting concept" led to the development of a "chymeric" compound, which due to the presence of a triazene linked to a 4-anilinoquinazoline backbone exerts a double cytotoxic mode of action: DNA-damaging and epidermal growth factor receptor – targeting (Matheson et al. 2003, 2001). The strong cytotoxic effects observed in a panel of human tumour cell lines demonstrated that such chymeric compounds, sharing the properties of a triazene and another tumour inhibiting agent, might represent a tandem approach towards the development of antineoplastic drugs with high potency (Matheson et al. 2003, 2001).

Recent advances provide evidence for the cytotoxic potential of diverse naturally occurring and synthetic benzophenones by alternative mechanisms such as farnesyltransferase or tubulin-polimerization inhibition, induction of apoptosis etc. (Liou et al. 2004; Diaz-Carballo et al. 1994; Mitsch et al. 2003; Matsumoto et al. 2003). In view of these findings, we aimed at combining the properties of a triazene and a benzophenone pharmacophore in a single molecule via the introduction of a benzoyl substituent in the phenyl ring of 1-phenyl-3,3-dimethyl core structure. In this report, we present the synthesis, physicochemical characterization and preliminary cytotoxic evaluation of 1-(4-benzoylphenyl)-3,3-dimethyltriazene and 1-(2-benzoylphenyl)-3,3-dimethyltriazene. The cytotoxic effects of the two novel compounds were investigated in comparison to ten other novel or previously described ring-substituted 1-phenyl-3,3-dimethyl triazene derivatives (see Table).

2. Investigations, results and discussion

2.1. Chemistry

Different substituted aromatic amines were transformed to diazonium salts in water. Consequently, the salts reacted with dimethylamine water solution, according to the scheme.

The products were 1-aryl-3,3-dimethyl triazenes (Threadgill and Stevens 1983). Their structure was confirmed by mass-spectral analysis. The non ring-substituted compound 1-phenyl-3,3-dimethyltriazene had a base-peak m/z 77 (100%) – a phenyl cation-radical, which was a result of the elimination of the other part of the molecule, namely 3,3-dimethyltriazene fragment and m/z 149 (30%) – 1-phenyl-3,3-dimethyltriazene cation-radical of the investigated molecule.

When there was one benzoyl group in the aromatic nucleus, the base-peak of the p-isomer was a cation-radical with m/z 181 and the base-peak of the o-isomer was a cation-radical with m/z 152. The difference between the two isomers is due to their distinct fragmentation pathways. The X-ray crystal structure analysis of 1-(4-benzoylphenyl)-3,3-dimethyltriazene and 1-(2-benzoylphenyl)-3,3-dimethyltriazene confirmed their structure (Fig. 1 and Fig. 2).

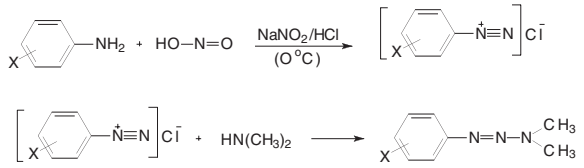
When there was one acetyl group in the aromatic nucleus of the triazene molecule the base-peak of the respective compounds was a cation-radical with m/z 91, while the molecule cation-radical was m/z 191. Common in the process of fragmentation is the formation of fragments with different peaks intensity which can be discriminated by the position of the acetyl group in the aromatic nucleus.

When there was a hydroxymethyl group in the aromatic nucleus, the base-peak of the p- and m-isomers was a cation-radical with m/z 89, and the base-peak of the o-isomer was a cation-radical with m/z 77. The molecule cation-radical of all three isomers was m/z 179. These three compounds form fragments with different peak intensities which can be discriminated by the position of the hydroxymethyl group in the aromatic nucleus.

When there was a nitro group in the aromatic nucleus, the base peak of the p- and m-isomers was the same cation-radical with m/z 122, the base-peak of the o-isomer was a cation-radical with m/z 150. The difference between these three isomers is due to their distinct fragmentation pathways.

The base-peak of 1-(p-tolyl)-3,3-dimethyltriazene was a cation-radical with m/z 106 and the molecule cation-radical was m/z 163.

Scheme



X = o- and p-benzoyl; m- and p-acetyl; o-, m- and p-hydroxymethyl; o-, m- and p-nitro; p-methyl.

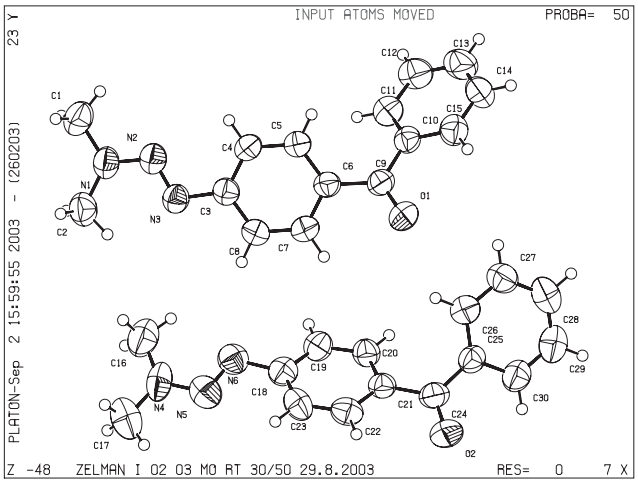


Fig. 1: The solid state structure of 1-(4-benzoylphenyl)-3,3-dimethyltriazene

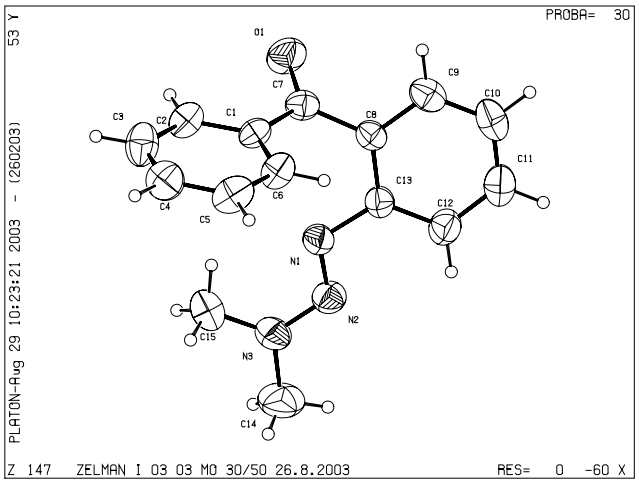
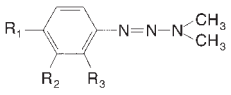
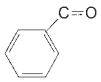


Fig. 2: The solid state structure of 1-(2-benzoylphenyl)-3,3-dimethyltriazene



Compd.	R^1	R^2	R^3
1		H	H
2	H	H	
3	H	H	H
4	H	CH_3CO	H
5	CH_3CO	H	H
6	CH_2OH	H	H
7	H	CH_2OH	H
8	H	H	CH_2OH
9	CH_3	H	H
10	NO_2	H	H
11	H	NO_2	H
12	H	H	NO_2

2.2. Pharmacology

The IC_{50} values of the cytotoxicity determination are summarized in the Table. The newly synthesized benzophenone-linked triazenes exerted concentration-dependent cytotoxic effects in the panel of cell lines investigated (Fig. 3).With respect to the relative potencies shown, they were more effective than the other investigated triazenes. Both compounds caused similar cytotoxicity against SKW-3 lymphoid cells with IC_{50} values of 19.03 and

Table: Relative potency of the investigated 1-aryl-3,3-dimethyltriazenes and melphalan, as expressed by the IC_{50} values against SKW-3, HL-60 and HL-60/Dox

Compd.	IC_{50} value (μM)		
	SKW-3	HL-60	HL-60/Dox
1	19.03	61.98	64.29
2	18.79	20.13	22.44
3	>200	>200	>200
4	73.74	>200	>200
5	>200	>200	>200
6	>200	>200	>200
7	>200	>200	>200
8	>200	>200	>200
9	163.42	>200	>200
10	143.65	121.47	>200
11	162.61	>200	>200
12	>200	>200	>200
Melphalan	22.01	25.41	51.07

18.79 μM, respectively. These results are comparable to the effect of the reference drug melphalan (IC_{50} value of 22 μM). Among the other triazene compounds, only the derivatives bearing 3-acetyl, 3- and 4- nitro or 4-methyl substituents caused 50% tumour cell growth inhibition within the investigated concentration range (25–200 μM), whereas the rest exerted only marginal effects.

The results obtained using the myeloid HL-60 cells showed that 1-(2-benzoylphenyl)-3,3-dimethyltriazene caused the most potent cytotoxic effect, with IC_{50} value of 20.13 μM, lower than that of the referent drug melphalan. The other newly synthesized compounds and 1-(4-benzoylphenyl)-3,3-dimethyltriazene caused 50% cell growth inhibition at approximately 3-fold higher concentrations, although the maximum efficencies of the benzophenone derivatives were similar. All invested triazenes but 4-nitrophenyl-3,3-dimethyltriazene exerted either marginal effects or a lack of cytotoxicity and thus failed to induce 50% inhibition of the malignant cell proliferation.

Both benzophenone-linked triazenes proved to escape the MRP-1 mediated multidrug resistance in HL-60/Dox cells with equal relative potency as in the sensitive subline. Interestingly, melphalan induced 50% inhibition of the malignant cell proliferation in HL-60/Dox cells at approximately twice the concentrations than in HL-60. This resistance pattern could be due to the action of MRP-1 transporter, expressed in HL-60/Dox, which causes driving out of the cells of glutathione-conjugates of diverse substrates. This mechanism might explain the multidrug resistance in these line (Bagrij et al. 2001). Conventional alkylating agents like the bis-(β-chloroethyl)amine derivative melphalan are known to form glutathione adducts, which are the substrate for MRP-1 (Kearns et al. 1998; Bagrij et al. 2001).

The other triazene derivatives exerted only marginal effects against HL-60/Dox cells and actually none of them caused 50% reduction of the viable cell number within the concentration range being investigated.

The 1-aryl-3,3-dimethyltriazenes undergo a complex transformation via a spontaneous hydrolytic decomposition or CytP450 mediated metabolism (*in vivo*) to produce aryl- or alkyl diazonium intermediates, respectively that are highly electrophilic and react with biologic macromolecules (Foster 1998; Connors et al. 1976; Gescher et al. 1981; Stupp et al. 2001). Concerning dacarbazine, it has been assumed that its cytotoxicity is due to the biotransformation product methyltriazenoimidazolecarboxamide

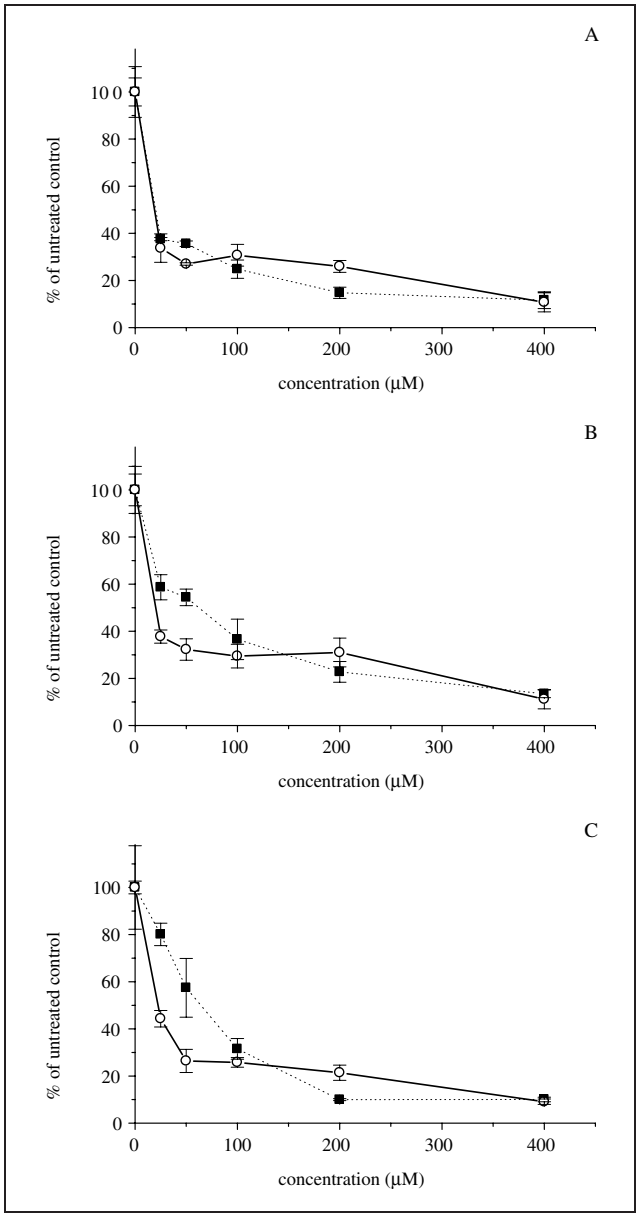


Fig. 3: Concentration-response curves of 1-(4-benzoylphenyl)-3,3-dimethyltriazene (■) and 1-(2-benzoylphenyl)-3,3-dimethyltriazene (O) on SKW-3 (A), HL-60 (B) and HL-60/Dox (C) cells as assessed by the MTT-dye reduction assay after 72 h treatment. Each data point represents the arithmetic mean ± sd for at least 8 independent experiments

(MTIC) which further decomposes to produce methyl diazonium, the latter being a potent DNA monoalkylator. The spontaneous decomposition of dacarbazine to the corresponding carboxamido-imidazolylhydrazine most probably does not contribute to the antineoplastic effects of this drug (Foster 1998; Connors et al. 1976; Stupp et al. 2001). The diazo product obtained is highly reactive, but it undergoes a spontaneous cyclization to the non-toxic azahypoxanthine (Foster 1998).

In contrast to dacarbazine, 1-phenyl-3,3-dimethyltriazenes most probably exert their effects through formation of both degradation products and metabolites, the relative importance of either activation pathway being largely dependent on the chemical stability of the derivatives (Connors et al. 1976). Thus, the greater the ability of a given triazene to hydrolize spontaneously yielding aryldiazonium products is, the higher would be its cytotoxicity *in vitro* in the absence of CytP450 expression in the malignant cell

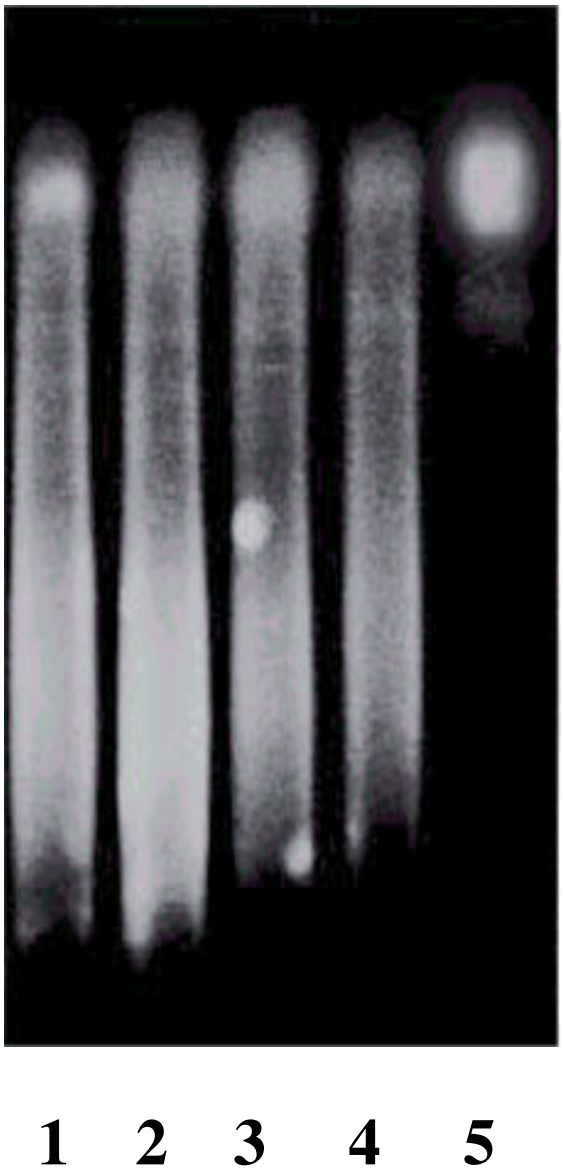


Fig. 4: DNA-fragmentation analysis. DNA extracted from the cytosolic fraction of HL-60 cells following treatment with 1-(4-benzoylphenyl)-3,3-dimethyltriazene (50 μM – lane 1 and 100 μM – lane 2) and 1-(2-benzoylphenyl)-3,3-dimethyltriazene (50 μM – lane 3 and 100 μM – lane 4) and untreated controls (lane 5) was analyzed by 0.8% agarose gel-electrophoresis, ethidium bromide staining and UV-transillumination

lines (Connors et al. 1976). The observed profound cytotoxic effects of the newly synthesized 1-(4-benzoylphenyl)-3,3-dimethyltriazene and 1-(2-benzoylphenyl)-3,3-dimethyltriazene could be due to the formation of reactive benzoylphenyl diazonium ions with DNA and peptide arylating properties (Foster 1998). Considering the profound cytotoxic effects of some aminobenzophenones and other substituted benzophenone derivatives, the contribution of the benzophenone core structure to the effects of these novel triazene-based compounds could not be excluded (Liou et al. 2004).

The electropherogram (Fig. 4) depicts the cytosolic DNA of HL-60 cells following 24 h treatment with either 1-(4-benzoylphenyl)-3,3-dimethyltriazene or 1-(2-benzoylphenyl)-3,3-dimethyltriazene. A typical laddering phenomenon is demonstrated, which is characteristic for the mono- and oligonucleosomal DNA-fragmentation associated with the programmed cell death (Schwartzman and Cidlowski 1993). It is well established that for a variety of antineoplastic agents the primary specific effect, which they induce is the induction of apoptosis, which appears to be a common mechanism by which chemotherapeutic agents destroy tumour cells (O'Connor and Kohn 1992). As our results indicate, the induction of programmed cell death at least partly mediates the cytotoxic potential of the tested triazene compounds.

In summary our data indicate that 1-(4-benzoylphenyl)-3,3-dimethyltriazene and 1-(2-benzoylphenyl)-3,3-dimethyltriazene exert high cytotoxic and apoptosis-inducing activity in micromolecular concentrations. Further pharmacological and toxicological evaluation is needed to assess their potential clinical application.

3. Experimental

3.1. Chemistry

Melting points were measured on a Boetius hot plate microscope (Germany) and were uncorrected. IR spectra (nujol) were recorded on an IR-spectrometer FTIR-8101M Shimadzu. ^{1}H NMR spectra were recorded at ambient temperature on a Bruker 250 WM (250 MHz) spectrometer in $CDCl_3$. Chemical shifts are given in ppm (δ) relative to TMS used as an internal standard. Mass spectra were recorded on a Jeol JMS D 300 double focusing mass spectrometer coupled to a JMA 2000 data system. The compounds were introduced by direct inlet probe, heated from 50 to 400 °C at a rate of 100 °C/min. The ionization current was 300 mA, the accelerating voltage 3 kV and the chamber temperature 150 °C. TLC was performed on precoated plates Kieselgel 60 F_{254} Merck (Germany) with layer thickness 0.25 mm and UV detection (254 nm). Yields of TLC-homogeneous isolated products are given. Elemental analysis was performed at the Faculty of Inorganic Chemistry, Eberhard-Karls-University of Tuebingen. Analyses indicated by the symbols of the elements were within ± 0.4% of the theoretical values.

3.1.1. 1-(4-Benzoylphenyl)-3,3-dimethyltriazene (**1**)

4-Aminobenzophenone (5.91 g, 30 mmol) was suspended in water (50 ml) and 32% hydrochloric acid (16.8 ml) was added. The resulting suspension was cooled to 5–0 °C in an ice bath and a solution of sodium nitrite (2.17 g, 31.2 mmol) in water (6 ml) was added in such a rate that the temperature did not rise above 10 °C. The resulting suspension of the diazonium salt was poured (drop by drop) for 5–10 min into 40% aqueous solution of dimethylamine (60 ml) with vigorous stirring. The mixture was immediately extracted with chloroform (3 × 40 ml). The combined extracts were washed with water (4 × 40 ml), dried over sodium sulphate, filtered and the solvent was evaporated under reduced pressure. The residue was recrystallized from diisopropyl ether, resulting in red-yellowish crystals, m.p. 56–57 °C. Yield 5.81 g (77%). IR ($CHCl_3$) (cm^{-1}): 3019, 3011, 2908, 1647, 1598, 1579, 1477, 1447, 1419, 1402, 1385, 1349, 1305, 1280, 1165, 1148, 1090, 939, 914, 857, 701, 653. ^{1}H NMR ($CDCl_3$): 3.30–3.48 (br. d, 6 H), 7.25–7.83 (m, 9 H – arom.). MS (m/z, %): 253 (17), 209 (38), 181 (100), 152 (73), 105 (56), 77 (37), 51 (7).
$C_{15}H_{15}N_3O$ (253)

3.1.2. 1-(2-Benzoylphenyl)-3,3-dimethyltriazene (**2**)

2-Aminobenzophenone (5.91 g, 30 mmol) was suspended in water (40 ml) and 32% hydrochloric acid (16.8 ml) was added. The resulting suspension was cooled to 5–0 °C in an ice bath and a solution of sodium nitrite (2.18 g, 31.2 mmol) in water (6 ml) was added in such a rate that the temperature did not rise above 10 °C. The resulting suspension of the diazonium salt was poured (drop by drop) for about 30 min into 40% aqueous solution of dimethylamine (60 ml) with vigorous stirring. The mixture was immediately extracted with chloroform (2 × 50 ml). The combined extracts were washed with water (4 × 40 ml), dried with sodium sulphate, filtered and the solvent evaporated under reduced pressure. The residue was recrystallized from diisopropyl ether, resulting in orange-red coloured crystals, m.p. 75–77 °C. Yield 4.13 g (54%). IR ($CHCl_3$) (cm^{-1}): 3066, 3025, 3012, 2907, 1660, 1598, 1500, 1473, 1449, 1404, 1393, 1291, 1247, 937, 830, 702, 688, 654, 631. ^{1}H NMR ($CDCl_3$): 2.55–2.95 (br. s, 3 H), 3.15–3.45 (br. s, 3 H), 7.20–7.74 (m, 9 H arom.). MS (m/z, %): 253 (7), 209 (34), 181 (74), 152 (100), 127 (5), 105 (13), 77 (26), 51 (6).
$C_{15}H_{15}N_3O$ (253)

3.1.3. 1-Phenyl-3,3-dimethyltriazene (**3**)

Aniline (1.87 g, 20 mmol) was suspended in water (12–15 ml) and 32% hydrochloric acid (11.2 ml) was added. The resulting solution was cooled to

5–0 °C in an ice bath and a solution of sodium nitrite (1.48 g, 21.4 mmol) in water (5 ml) was added at such a rate that the temperature did not rise above 10 °C. The diazo-solution, kept at 0 °C, was added dropwise to a cooled and stirred mixture of dimethylamine (40% aqueous solution, 30 ml) and 30% aqueous solution of sodium carbonate (5.94 g Na_2CO_3 in 50 ml H_2O). The resulting oil was stirred for 30 min followed by extraction with diisopropyl ether. The combined extracts were washed with water (4 × 30 ml), dried (KOH), filtered, and the solvent was evaporated under reduced pressure. The residue distilled as a pale yellow liquid. Yield 2.25 g (75%), b.p. 57–58 °C/10^{-2} mbar. IR (film) (cm^{-1}): 3065, 2925, 2833, 1734, 1684, 1653, 1594, 1476, 1461, 1445, 1318, 1286, 1207, 1160, 1087, 917, 764, 694, 651, 568. ^{1}H NMR ($CDCl_3$): 3.06–3.61 (s, 6 H), 7.05–7.50 (m, 5 H). MS (m/z, %): 149 (30), 105 (66), 77 (100), 51 (13).
$C_8H_{11}N_3$ (149)

3.1.4. 1-(3-Acetylphenyl)-3,3-dimethyltriazene (**4**)

3′-Aminoacetophenone (4.07 g, 30 mmol) was suspended in water (24 ml) and 32% hydrochloric acid (16.8 ml) was added. The resulting slurry was cooled to 5–0 °C in an ice bath and a solution of sodium nitrite (2.17 g, 31.2 mmol) in water (6 ml) was added in such a rate that the temperature did not rise above 10 °C. The resulting solution of the diazonium salt was poured (drop by drop) for 5–10 min into 40% aqueous solution of dimethylamine (60 ml) under vigorous stirring. The mixture was immediately extracted with chloroform (3 × 30 ml). The combined extracts were washed with water (4 × 30 ml), dried with sodium sulphate, filtered and the solvent was evaporated under reduced pressure. The residue distilled as a pale yellow-green liquid, b.p. 101–103 °C/10^{-2} mbar. Yield 4.38 g (76%). IR (film) (cm^{-1}): 3353, 3067, 3003, 2908, 1688, 1587, 1476, 1447, 1403, 989, 957, 908, 890, 852, 797, 691, 644, 601. ^{1}H NMR ($CDCl_3$): 1.75 (s, 3 H); 2.59–2.60 (s, 6 H), 7.25–8.00 (m, 4 H – arom.). MS (m/z, %): 191 (22), 147 (53), 119 (91), 91 (100), 77 (21), 43 (38).
$C_{10}H_{13}N_3O$ (191)

3.1.5. 1-(4-Acetylphenyl)-3,3-dimethyltriazene (**5**)

4′-Aminoacetophenone (2.02 g, 15 mmol) was suspended in water (12 ml) and 32% hydrochloric acid (8.4 ml) was added. The resulting slurry was cooled to 5–0 °C in an ice bath and a solution of sodium nitrite (1.08 g, 15.6 mmol) in water (3 ml) was added at such a rate that the temperature did not rise above 10 °C. The resulting solution of the diazonium salt was poured (drop by drop) for 5 min into 40% aqueous solution of dimethylamine (30 ml) under vigorous stirring. The mixture was immediately extracted with chloroform (2 × 30 ml). The combined extracts were washed with water (4 × 30 ml), dried with sodium sulphate, filtered and the solvent was evaporated under reduced pressure. The residue was recrystallized from diisopropyl ether, the resulting product was long, pale buff needles. Yield: 2.52 g (88%), m.p. 90–92 °C. IR ($CHCl_3$) (cm^{-1}): 3011, 2909, 1672, 1598, 1572, 1478, 1445, 1418, 958, 916, 850, 830, 638, 608. ^{1}H NMR ($CDCl_3$): 2.55 (s, 3 H), 3.35 (br. s, 6 H), 7.45 (d, 2 H), 7.90 (d, 2 H). MS (m/z, %): 191 (29), 147 (50), 119 (92), 91 (100), 77 (19), 43 (54).
$C_{10}H_{13}N_3O$ (191)

3.1.6. 1-(4-Hydroxymethylphenyl)-3,3-dimethyltriazene (**6**)

4-Aminobenzylalcohol (3.70 g, 30 mmol) was dissolved in water (40 ml) and 32% hydrochloric acid (16.8 ml) was added. The resulting solution was cooled to 5–0 °C in an ice bath and a solution of sodium nitrite (2.17 g, 31.2 mmol) in water (6 ml) was added in such a rate that the temperature did not rise above 10 °C. The resulting suspension of the diazonium salt was poured (drop by drop) for about 15 min into 40% aqueous solution of dimethylamine (60 ml) with vigorous stirring. The mixture was immediately extracted with chloroform (2 × 60 ml). The combined extracts were washed with water (4 × 50 ml), dried with sodium sulphate, filtered and the solvent was evaporated under reduced pressure. After a few days the residue crystallized and was recrystallized from diisopropyl ether, the resulting product was yellow crystals, m.p. 63–65 °C. Yield 4.74 g (88%). $C_9H_{13}N_3O$ (179) (C, H, N) IR ($CHCl_3$) (cm^{-1}): 3605, 3010, 2987, 1608, 1504, 1479, 1449, 1403, 1340, 1205, 1086, 945, 1010, 945, 846. ^{1}H NMR ($CDCl_3$): 1.82 (s, 2 H), 3.33 (s, 6 H), 4.63 (s, 1 H), 7.25–7.43 (m, 4 H – arom.). MS (m/z, %): 179 (36), 162 (2), 135 (61), 107 (94), 89 (100), 77 (43), 51 (8).

3.1.7. 1-(3-Hydroxymethylphenyl)-3,3-dimethyltriazene (**7**)

3-Aminobenzylalcohol (3.69 g, 30 mmol) was dissolved in water (40 ml) and 32% hydrochloric acid (16.8 ml) was added. The resulting solution was cooled to 5–0 °C in an ice bath and a solution of sodium nitrite (2.17 g, 31.2 mmol) in water (6 ml) was added in such a rate that the temperature did not rise above 10 °C. The resulting solution of the diazonium salt was poured (drop by drop) for about 15–20 min into 40% aqueous solution of dimethylamine (60 ml) with vigorous stirring. The mixture was immediately extracted with chloroform (2 × 60 ml). The combined extracts were washed with water (4 × 50 ml), dried with sodium sulphate, filtered and the solvent was evaporated under reduced pressure. The residue distilled as a pale yellow liquid. B.p. 117–118 °C/3.4 × 10^{-1} mbar. Yield 3.70 g (69%). IR (film) (cm^{-1}): 3368, 3027, 2907, 1734, 1699, 1607, 1589, 1478, 1390, 1339, 1238, 1163, 1139, 1092, 871, 795, 698, 657, 572. ^{1}H NMR ($CDCl_3$): 1.85 (s, 2 H), 3.35 (s, 6 H), 4.65 (s, 1 H), 7.08–7.42 (m, 4 H – arom.). MS (m/z, %): 179 (22), 135 (42), 107 (74), 89 (100), 77 (50), 51 (10).
$C_9H_{13}N_3O$ (179)

3.1.8. 1-(2-Hydroxymethylphenyl)-3,3-dimethyltriazene (**8**)

2-Aminobenzylalcohol (3.70 g, 30 mmol) was dissolved in water (40 ml) and 32% hydrochloric acid (16.8 ml) was added. The resulting solution was cooled to 5–0 °C in an ice bath and a solution of sodium nitrite (2.18 g, 31.2 mmol) in water (6 ml) was added in such a rate that the temperature did not rise above 10 °C. The resulting solution of the diazonium salt was poured (drop by drop) for about 15–20 min into 40% aqueous solution of dimethylamine (60 ml) under vigorous stirring. The mixture was immediately extracted with chloroform (2 × 60 ml). The combined extracts were washed with water (4 × 50 ml), dried with sodium sulphate, filtered and the solvent was evaporated under reduced pressure. The residue distilled as a pale yellow liquid. B.p. 107–108 °C/2.2 × 10^{-1} mbar. Yield 3.53 g (66%). IR (film) (cm^{-1}): 3409, 3067, 2908, 1684, 1597, 1582, 1478, 1446, 1404, 1340, 1315, 1208, 1154, 1084, 870, 762, 650, 603, 589. ^{1}H NMR ($CDCl_3$): 3.11 (br. s, 6 H), 4.01 (s, 2 H), 5.09 (s, 1 H), 7.07–7.84 (m, 4 H – arom.). MS (m/z, %): 179 (18), 135 (55), 107 (55), 79 (39), 77 (100), 51 (11).
$C_9H_{13}N_3O$ (179)

3.1.9. 1-(p-Tolyl)-3,3-dimethyltriazene (**9**)

p-Toluidine (4-aminotoluene) (4.52 g, 31.5 mmol) was suspended in water (26 ml) and 32% hydrochloric acid (25 ml) was added. The resulting solution was cooled to 5–0 °C in an ice bath and a solution of sodium nitrite (2.27 g, 32.81 mmol) in water (6 ml) was added at such a rate that the temperature did not rise above 10 °C. The diazo-solution, kept at 0 °C, was added dropwise to a cooled and stirred mixture of dimethylamine (40% aqueous solution, 63 ml) and 30% aqueous solution of sodium carbonate (9.29 g Na_2CO_3 in 80 ml H_2O). The resulting suspension was stirred for 30 min and was extracted with diisopropyl ether. The combined extracts were washed with water (4 × 30 ml), dried (Na_2SO_4), filtered, and the solvent was evaporated under reduced pressure. The residue crystallized. The crude product was 5.3 g (75%). IR (film) (cm^{-1}): 3448, 3364, 3024, 2922, 2864, 1734, 1700, 1684, 1623, 1517, 1506, 1402, 1317, 1282, 1213, 1083, 915, 822, 713, 559. ^{1}H NMR ($CDCl_3$): 2.30–2.40 (s, 3 H), 3.20–3.30 (s, 6 H), 7.15–7.25 (d, 2 H), 7.32–7.36 (d, 2 H). MS (m/z, %): 163 (13), 119 (17), 107 (70), 106 (100), 91 (59), 77 (13), 65 (13), 51 (6), 39 (6).
$C_9H_{13}N_3$ (163)

3.1.10. 1-(4-Nitrophenyl)-3,3-dimethyltriazene (**10**)

4-Nitroaniline (4.14 g, 30 mmol) was suspended in water (40 ml) and 32% hydrochloric acid (16.8 ml) was added. The resulting suspension was cooled to 5–0 °C in an ice bath and a solution of sodium nitrite (2.18 g, 31.2 mmol) in water (6 ml) was added in such a rate that the temperature did not rise above 10 °C. The resulting suspension of the diazonium salt, kept at 0 °C, was added (drop by drop) for about 15–25 min into 40% aqueous solution of dimethylamine (60 ml) with vigorous stirring. The mixture was immediately extracted with chloroform (2 × 60 ml). The combined extracts were washed with water (4 × 70 ml), dried with sodium sulphate, filtered and the solvent was evaporated under reduced pressure. The resulting product consisted of orange coloured crystals. Yield 3.82 g (75%), m.p. 142–143 °C. IR ($CHCl_3$) (cm^{-1}): 3027, 3008, 2911, 1603, 1591, 1513, 1479, 1443, 1334, 1307, 1206, 1162, 1094, 856, 697, 666, 580. ^{1}H NMR ($CDCl_3$): 3.26 (s, 3 H), 3.38 (s, 3 H), 7.25–7.51 (d, 2 H – arom.), 8.14–8.20 (d, 2 H – arom.). MS (m/z, %): 194 (33), 150 (86), 122 (100), 106 (6), 92 (30), 75 (41), 64 (8), 50 (13), 44 (12).
$C_8H_{10}N_4O_2$ (194)

3.1.11. 1-(3-Nitrophenyl)-3,3-dimethyltriazene (**11**)

3-Nitroaniline (4.15 g, 30 mmol) was suspended in water (40 ml) and 32% hydrochloric acid (16.8 ml) was added. The resulting solution was cooled to 5–0 °C in an ice bath and a solution of sodium nitrite (2.18 g, 31.2 mmol) in water (6 ml) was added in such a rate that the temperature did not rise above 10 °C. The resulting solution of the diazonium salt, kept at 0 °C, was added (drop by drop) for about 15–25 min into 40% aqueous solution of dimethylamine (60 ml) with vigorous stirring. The mixture was immediately extracted with chloroform (2 × 60 ml). The combined extracts were washed with water (4 × 70 ml), dried with sodium sulphate, filtered and the solvent was evaporated under reduced pressure. The resulting product was dark-brown coloured. It was recrystallized from diisopropyl ether. A black crystal was formed around the magnetic stirrer. Yield 2.57 g (44%), m.p. 99–101 °C. IR ($CHCl_3$) (cm^{-1}): 3020, 2910, 2865, 1734, 1701, 1685, 1654, 1528, 1449, 1403, 1387, 1346, 1304, 1273, 1204, 1096, 1075, 999, 899, 819, 801, 701, 696, 679, 587. ^{1}H NMR ($CDCl_3$): 3.23 (s, 3 H), 3.55 (s, 3 H), 7.25–8.25 (m,

4 H – arom.). MS (m/z, %): 194 (22), 150 (82), 122 (100), 106 (2), 92 (16), 75 (30), 64 (7), 50 (6), 45 (18).
$C_8H_{10}N_4O_2$ (194)

3.1.12. 1-(2-Nitrophenyl)-3,3-dimethyltriazene (**12**)

2-Nitroaniline (4.14 g, 30 mmol) was suspended in water (40 ml) and 32% hydrochloric acid (16.8 ml) was added. The resulting suspension was cooled to 5–0 °C in an ice bath and a solution of sodium nitrite (2.18 g, 31.2 mmol) in water (6 ml) was added in such a rate that the temperature did not rise above 10 °C. The resulting solution/suspension of the diazonium salt, kept at 0 °C, was added (drop by drop) for about 15–20 min into 40% aqueous solution of dimethylamine (60 ml) with vigorous stirring. The mixture was immediately extracted with chloroform (2×60 ml). The combined extracts were washed with water (4×70 ml), dried with sodium sulphate, filtered and the solvent was evaporated under reduced pressure. The resulting product was dark liquid. It was distilled as a red liquid, b.p. 99–102 °C/ 3.4×10^{-1} mbar. Yield 3.45 g (60%). IR (film) (cm^{-1}): 3503, 3384, 3074, 2919, 1734, 1700, 1684, 1669, 1624, 1601, 1576, 1527, 1472, 1443, 1403, 1387, 1352, 1313, 1260, 1214, 1159, 1141, 1101, 1081, 949, 858, 822, 774, 750, 686, 635, 598. ^{1}H NMR ($CDCl_3$): 3.20 (s, 3 H), 3.53 (s, 3 H), 6.70–8.11 (m, 4 H – arom.). MS (m/z, %): 194 (28), 150 (100), 138 (6), 92 (19), 76 (7), 64 (14), 51 (13), 39 (10).
$C_8H_{10}N_4O_2$ (194)

4.2. Pharmacology

4.2.1. Cell lines and culture conditions

The cytotoxic activity of the tested compounds was evaluated in a panel of three leukemic cell lines of human origin, namely the chronic lymphoid leukemia SKW-3, the acute promyelocytic leukemia HL-60 and its resistant to anthracyclines variant HL-60/Dox. The human tumour cell lines exploited herein were supplied from the German Collection of Microorganisms and Cell Cultures. They were maintained as suspension – type cultures in a controlled environment (RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine, at 37 °C in a 'Heraeus' incubator with 5% CO_2 humidified atmosphere). HL-60 were cultured in doxorubicine containing medium; in order to avoid a synergistic interaction the HL-60/Dox cells were cultured in doxorubicine-free environment for 72 h prior to drug treatment. In order to maintain the cells in log phase cellular suspension aliquots were re-fed with fresh RPMI-1640 medium two or three times per week. The stock solution of the tested compound was prepared in DMSO and was consequently diluted in RPMI-1640. At the final dilutions obtained, the concentration of the solvent never exceeded 0.5%.

4.2.2. MTT dye-reduction assay

The cytotoxic activity of the tested compounds was assessed *in vitro*, using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) – dye reduction assay as described by Mosman (1983), with some modifications (Konstantinov et al. 1999). Briefly, 100 µl aliquotes of exponentionally growing cells (at a density of $2–3.5 \times 10^5$/ml) were seeded in 96-well microplates and after a 24 h adaptation period they were exposed for 72 h to different concentrations of the test compounds. After incubation with the test compounds, 10 µl aliquots per well of the MTT stock (10 mg/ml in PBS) were added and the plates were further incubated for 4 h at 37 °C. The formazan crystals formed were dissolved by addition of 100 µl/well 5% solution of formic acid in 2-propanol (Merck). The samples absorption was measured using an ELISA reader (Uniscan Titertec, Helsinki, Finland) at wavelength of 580 nm. The survival fractions were calculated as percentage of the untreated control, the latter being set at 100%.

4.2.3. DNA Fragmentation analysis

DNA isolation and electrophoretic analysis were performed as previously described (Konstantinov et al. 1999). In brief, exponentially growing HL-60 cells were treated with 1-(4-benzoylphenyl)-3,3-dimethyltriazene, 1-(2-benzoylphenyl)-3,3-dimethyl-triazene (at 50 or 100 µM) or with 1% DMSO (untreated control). After the incubation period $0.5–1.0 \times 10^6$ treated and untreated cells were washed in PBS. Cell pellets obtained were resuspended in 0.25 ml PBS and lysed with 0.5 ml lysis buffer (0.5% Triton X-100, 20 mM Tris HCl, 1 mM EDTA, pH = 7.4). After centrifugation (13000 rpm for 20 min) the supernatants were transferred into fresh tubes. In order to allow the precipitation of the polar and water soluble DNA, NaCl solution (0.187 ml, 6 M) and 2-propanol (0.937 ml) were added to each sample. After mixing, samples were kept at –20 °C overnight and were spun at 13000 rpm for 20 min. In addition, DNA was washed with 1 ml 70% ethanol, DNA pellets were air dried and dissolved in 20 µl distilled water. Samples were analysed using electrophoresis followed by ethidium bromide staining. DNA ladder was visualized and photographed, using an UV-transilluminator with CCD camera (UVPBioDoc-It^{TM} System, a1-Biotech GmbH, Martinsried, Germany).

4.2.4. Statistics

The data processing exploited MS Excel and OriginPlot software for PC. Differences between groups were assessed using the Student's t-test; $p \leq 0.05$ was considered significant.

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