Triazene salts: Design, synthesis, ctDNA interaction, lipophilicity determination, DFT calculation, and antiproliferative activity against human cancer cell lines

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Synthesis, characterization and investigation of antiproliferative activity of nine triazene salts against human cancer cell lines (MV-4-11, MCF-7, JURKAT, HT-29, Hep-G2, Hela, Du-145 and DAUDI), and normal human mammary epithelial cell line (MCF7-10A) is presented. The structures of novel compounds were determined using 1H and 13C NMR, and GC-APCI-MS analyses. Among the derivatives, compound 2c, 2d, 2e and 2f has very strong activity against bipherenotypic B myelomonocytic leukemia MV4-11, with IC50 values from 5.42 to 7.69 μg/ml. The cytotoxic activity of compounds 2c-2f against normal human mammary gland epithelial cells MCF-10A is 6–11 times lower than against cancer cell lines. Our results also show that compounds 2c and 2f have very strong activity against DAUDI and HT-28 with IC50 4.91 μg/ml and 5.59 μg/ml respectively. Their lipophilicity was determined using reversed-phase ultraperformance liquid chromatography and correlated with antiproliferative activity. Our UV–Vis spectroscopic results indicate also that triazene salts tends to interact with negatively charged DNA phosphate chain. To support the experiment, theoretical calculations of the 1H NMR shifts were carried out within the Density Functional Theory.

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1. Introduction

One of the most aggravating diseases in the present world is cancer. Each year more than ten million people are diagnosed with some type of cancer, and more than half of them can die of it. In many countries, cancer diseases occupy the second place immediately after cardiovascular diseases (Boyle and Levin, 2008). Although public awareness about the treatment and prevention of cancer is still growing, and although new anticancer drugs are still being developed, cancer remains the major health problem in the around the world (Ferlay et al., 2013). Many of the current anticancer drugs have very low selectivity, relatively high side effects, limited bioavailability and oral absorption or rapid metabolism (Zawilska et al., 2013). For this reason, many prodrug groups have been developed that are activated in the cancer cells. Such a group of prodrug alkylating agents are triazenes which are successfully used for the fight against many tumors, such as leukemia, lymphoma, melanoma, and sarcoma (Yahalom et al., 1983; Smith et al., 1990). Some triazenes have also been used as a prodrug candidate for melanocyte-directed enzyme prodrug therapy (MDEPT) (Monteiro et al., 2013).

Approved by the Food and Drug Administration (FDA) for medical use Dacarbazine (1) (5-(3,3-dimethyltriazene)imidazol-4-carboxamide, DTIC) and Temozolomide (2) (8-carbamoyl-3-methylimidazol[5,1-d]-1,2,3,5-tetrazin-4(3H)-one, TMZ) are the only triazines used in the treatment of cancer (Meer et al., 1986; O'Reilly et al., 1993). DTIC requires activation by the cytochrome P450, resulting in the production of a very reactive methylidiazonium...
cations that can react with DNA O6-methylguanine, while Temozolomide does not require enzymatic activation and is hydrolysed to the active form already under physiological conditions (Meer et al., 1986; Friedman et al., 2000). However, many types of cancer cells have a mechanism to repair this type of damage by expressing a protein O6-alkylguanine DNA alkyltransferase thereby reducing the effectiveness of the drugs used (Happold et al., 2012; Kanugula and Pegg, 2003; Friedman et al., 2000).

These results encouraged us to continue our investigation on the synthesis and molecular properties of anticancer agents with diverse mechanisms of action (Łączkowski et al., 2014; Cytarska et al. 2015; Łączkowski et al., 2016, 2018). Our research began with the design and synthesis of nine novel triazene salts and evaluation of their antiproliferative activity against human cancer cell lines (biphenotypic B myelomonocytic leukemia MV4-11, human breast carcinoma MCF-7, human leukemic T-cell lymphoblast JURKAT, human colon adenocarcinoma HT-29, human hepatocellular carcinoma HepG2, human cervical carcinoma HeLa, human prostate carcinoma Du-145, Burkitt lymphoma DAUDI, and normal human DNA damage repair cells). For a better understanding of the mechanism of action we also performed interaction of triazenes with ctDNA using UV–Visible absorption spectroscopic (Sohrabi et al., 2018; Moosavi-Movahedi et al., 2004; Marouzi et al., 2017; Omidvar et al., 2013; Rashidipour et al., 2016; Friedman et al., 2000).

2. Experimental

2.1. Materials and methods

All experiments were carried out under air atmosphere unless stated otherwise. Reagents were generally the best quality commercial-grade products and were used without further purification. 1H NMR (700 and 400 MHz) and 13C NMR (100 MHz) spectra were recorded on a Bruker Avance III multinuclear instrument. MS spectra were recorded on triple quadrupole mass spectrometer detector LCMS-8040 (Shimadzu, Japan). Melting points were determined in open glass capillaries and are uncorrected. Analytical TLC was performed using Macherey-Nagel Polygram Sil G/UV 254 0.2 mm plates. Bis(2-chloro-ethyl)amine hydrochloride, and appropriate anilines were commercial materials (Alrich). 2.1.7. 1-(2-Chloroethyl)-3-(4-nitrophenyl)-4,5-dihydro-1H-1,2,3-triazol-3-ium chloride (2f)

Yield: 1.30 g, 37%, (dichloromethane/methanol, 95:5, Rf = 0.16) mp 107–108 °C decamp. 1H NMR (DMSO d6, 700 MHz), δ (ppm): 4.20 (t, 2H, CH2, J = 5.5 Hz); 4.67 (t, 2H, CH2, J = 14 Hz); 4.91 (t, 2H, CH2, J = 12 Hz); 7.44 (d, 2H, 2CH, J = 8 Hz); 5.76 (d, 2H, 2CH, J = 8 Hz). 13C NMR (DMSO d6, 100 MHz), δ (ppm): 21.08 (CH3); 40.83 (CH2); 52.66 (CH2); 54.37 (CH2); 54.79 (CH2); 118.51 (2CAr); 130.96 (2CAr); 134.30 (C); 139.43 (C); GC-APCI-MS (m/z, %): 188 [(M+–2Cl), 100].

2.1.7. 1-(2-Chloroethyl)-3-(3-trifluoromethyl)phenyl)-4,5-dihydro-1H-1,2,3-triazol-3-ium chloride (2g)

Yield: 1.10 g, 48%, (dichloromethane/methanol, 95:5, Rf = 0.18) mp 131–133 °C decamp. 1H NMR (DMSO d6, 200 MHz), δ (ppm): 4.20 (t, 2H, CH2, J = 5 Hz); 4.67 (t, 2H, CH2, J = 5.5 Hz); 4.75 (t, 2H, CH2, J = 14 Hz); 4.95 (t, 2H, CH2, J = 13 Hz); 7.88 (m, 2H, 2CH); 7.96 (m, 2H, 2CH). 13C NMR (DMSO d6, 100 MHz), δ (ppm): 40.67 (CH2); 52.46 (CH2); 55.51 (CH2); 55.27 (CH2); 115.37 (q, C, JCF = 4 Hz); 120.70 (C); 125.60 (C, JCF = 4 Hz); 131.06 (C, JCF = 33 Hz); 131.98 (2C); 137.49 (C). GC-APCI-MS (m/z, %): 242 [(M+–2Cl), 100].

2.1.7. 1-(2-Chloroethyl)-3-(4-(ethoxycarbonyl)phenyl)-4,5-dihydro-1H-1,2,3-triazol-3-ium chloride (2h)

Yield: 1.60 g, 28%, (dichloromethane/methanol, 95:5, Rf = 0.16) mp 116–117 °C decamp. 1H NMR (DMSO d6, 700 MHz), δ (ppm): 2.64 (s, 3H, CH3); 4.19 (t, 2H, CH2, J = 5 Hz); 4.63 (t, 2H, CH2, J = 5 Hz); 4.73 (t, 2H, CH2, J = 14 Hz); 4.92 (t, 2H, CH2, J = 13 Hz); 7.75 (d, 2H, 2CH, J = 9 Hz); 8.17 (d, 2H, 2CH, J = 9 Hz). 13C NMR (DMSO d6, 100 MHz), δ (ppm): 27.31 (CH3); 40.64 (CH2); 52.22 (CH2); 55.43 (CH2); 55.38 (CH2); 118.47 (2CAr); 130.61 (2CAr); 136.58 (C); 139.89 (C); 197.43 (CO). GC-APCI-MS (m/z, %): 216 [(M+–2Cl), 100].

2.1.7. 1-(2-Chloroethyl)-3-phenyl-4,5-dihydro-1H-1,2,3-triazol-3-ium chloride (2b)

Yield: 1.67 g, 62%, (dichloromethane/methanol, 80:20, Rf = 0.20) mp 89–91 °C decamp. 1H NMR (DMSO d6, 700 MHz), δ (ppm): 4.21 (t, 2H, CH2, J = 5.5 Hz); 4.62 (t, 2H, CH2, J = 5 Hz); 4.74 (t, 2H, CH2, J = 14 Hz); 4.95 (t, 2H, CH2, J = 13 Hz); 7.52 (t, 1H, CH, J = 7 Hz); 7.63 (m, 2H, 2CH); 7.67 (m, 2H, 2CH). 13C NMR (DMSO d6, 100 MHz), δ (ppm): 40.78 (CH2); 52.48 (CH2); 54.94 (CH2); 54.66 (CH2); 118.55 (2CAr); 129.38 (C); 130.56 (2CAr); 136.71 (C). GC-APCI-MS (m/z, %): 174 [(M+–2Cl), 100].

2.1.2. 1-(2-Chloroethyl)-3-phenyl-4,5-dihydro-1H-1,2,3-triazol-3-ium chloride (2a)

Yield: 2.80 g, 80%, (dichloromethane/methanol, 95:5, Rf = 0.15) mp 82–84 °C decamp. 1H NMR (DMSO d6, 200 MHz), δ (ppm): 1.19 (s, 3H, CH3); 3.86 (t, 2H, CH2, J = 5 Hz); 4.89 (t, 2H, CH2, J = 14 Hz); 7.52 (t, 1H, CH, J = 7 Hz); 7.62 (m, 2H, 2CH); 7.65 (m, 2H, 2CH). 13C NMR (DMSO d6, 100 MHz), δ (ppm): 21.08 (CH3); 40.83 (CH2); 52.66 (CH2); 54.37 (CH2); 54.79 (CH2); 118.51 (2CAr); 130.96 (2CAr); 134.30 (C); 139.43 (C); GC-APCI-MS (m/z, %): 188 [(M+–2Cl), 100].
CH₂, J = 13 Hz); 4.96 (t, 2H, CH₂, J = 13 Hz); 7.89 (d, 2H, 2CH, J = 9 Hz); 8.48 (d, 2H, 2CH, J = 9 Hz). ¹³C NMR (DMF-d₇, 100 MHz), δ (ppm): 40.57 (CH₂); 52.15 (CH₃); 55.68 (CH₂); 56.13 (CH₂); 119.30 (2CAr); 127.59 (2CAr); 134.21 (2CAr); 146.61 (C). GC-APCI-MS (m/z): 219 [(M⁺-2CI)⁻], 100.

2.1.8. 1-(2-Chloroethyl)-3-(4-sodium sulfate/phenyl)-4,5-dihydro-1H-1,2,3-triazol-3-ium chloride (2h)

Yield: 2.00 g, 52%, (dichloromethane/methanol, 80:20, Rₛ = 0.10); mp 91–93°C. ¹H NMR (DMF-d₇, 400 MHz), δ (ppm): 4.16 (t, 2H, CH₂, J = 6 Hz); 4.58 (t, 2H, CH₂, J = 5.5 Hz); 4.65 (t, 2H, CH₂, J = 14 Hz); 4.88 (t, 2H, CH₂, J = 15 Hz); 7.58 (d, 2H, 2CH, J = 9 Hz); 7.89 (d, 2H, 2CH, J = 9 Hz). ¹³C NMR (DMF-d₇), δ (ppm): 40.66 (CH₂); 52.31 (CH₂); 54.78 (CH₂); 54.97 (CH₃); 117.84 (2CAr); 127.59 (2CAr); 136.43 (C); 148.59 (C). GC-APCI-MS (m/z): 276 [(M⁺-2CI)⁻], 100.

2.1.9. 1-(2-Chloroethyl)-3-(4-fluorophenyl)-4,5-dihydro-1H-1,2,3-triazol-3-ium chloride (2i)

Yield: 1.30 g, 44%, (dichloromethane/methanol, 80:20, Rₛ = 0.11); mp 91–93°C. ¹H NMR (DMF-d₇, 400 MHz), δ (ppm): 4.16 (t, 2H, CH₂, J = 6 Hz); 4.56 (t, 2H, CH₂, J = 5.5 Hz); 4.67 (t, 2H, CH₂, J = 13 Hz); 4.90 (t, 2H, CH₂, J = 14 Hz); 7.50 (m, 2H, 2CH₂); 7.71 (m, 2H, 2CH₂). ¹³C NMR (DMF-d₇, 100 MHz), δ (ppm): 40.78 (CH₂); 52.88 (CH₂); 54.82 (CH₂); 54.72 (CH₂); 117.52 (2CAr); 121.27 (2CAr); 133.33 (2CAr); 148.59 (C). GC-APCI-MS (m/z): 219 [(M⁺-2CI⁻)], 100.

2.2. Biological activity

2.2.1. Cells

Human cancer cell lines Du-145, HeLa, HepG2, HT-29, MCF-7, MV-4-11 and normal human mammary gland epithelial cells MCF-10A were obtained from American Type Culture Collection (Rockville, Maryland, USA). DAUDI and Jurkat cell lines were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. All cell lines were maintained in liquid nitrogen at the Cell Culture Collection of Institute of Immunology and Experimental Therapy (Wrocław, Poland). The Du-145 and MCF-7 cell lines were grown in Eagle’s medium (IIEF, Wrocław, Poland) with addition of 10% fetal bovine serum (Sigma-Aldrich, Steinheim, Germany). MCF-7 cell line was supplemented with MEM Non-Essential Amino Acids Solution, 2.0 mM L-glutamine, and 4.0 mM L-glutamine (both Sigma-Aldrich, Steinheim, Germany). HepG-2, HT-29, MCF-7, MCF-10A cell lines. The results were presented as an IC₅₀ values (inhibitory concentration 50) – the dose (µM/mL) of tested compounds that inhibits cell proliferation at 50%. Each concentration of examined agents was tested in triplicate in a single experiment, which was repeated at least 3 times (Rubinstein et al., 1990; Bramson et al., 1995). The activity of examined agents was compared to the activity of reference compound – cis-platin (Accord Healthcare Polska, Warszawa, Poland). The control of 99.8% ethanol that was the solvent of the tested agents was also performed.

2.2.2. SRB cytotoxic test

Cells were attached to the bottom of plastic wells by fixing them with cold 50% TCA (trichloroacetic acid, POCH, Gliwice, Poland) on 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 cellular material fixed with TCA was stained with 0.14% sulforhodamine B (SRB, Sigma-Aldrich, Germany) and dissolved in 1% acetic acid (POCH, Gliwice, Poland) for 30 min. Unbound dye was removed by rinsing (4X) in 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for determination of the optical density (λ = 540 nm) in a computer-interfaced, 96-well Synergy H4 (BioTek Instruments USA) photometer microtiter plate reader (Sidoryk et al., 2012).

2.2.5. MTT cytotoxic test

20 µl of MTT solution (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, stock solution: 5 mg/ml) was added to each well and incubated for 4 h. After the incubation time was complete, 80 µl of the lysis mixture was added to each well (lysis mixture: 225 ml dimethylformamide, 67.5 g sodium dodecyl sulfate and 275 ml of distilled water). The optical densities of the samples were read after 24 h on a Synergy H4 (BioTek Instruments USA) photometer microtiter plate reader at 570 nm (Sidoryk et al., 2012). All of chemicals were obtained from Sigma-Aldrich, Germany.

2.3. Determination of lipophilicity by RP UPLC

The studies were performed on the UPLC-MS/MS system equipped with solvent delivery two pumps LC-30AD combined with gradient systems, degasser model DGU-20A5, an autosampler model SIL-30AC, a column oven model CTO-20AC, UV detector model SPD-M20A and triple quadrupole mass spectrometer

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detector LCMS-8040 (Shimadzu, Japan). Kinetex C18 (150 × 4.6 mm; 2.6 μm) column was purchased from Phenomenex Co. The methanol concentration, expressed in volumetric ratio v/v, ranged from 0.65 to 0.95 in constant steps of 0.05. Tested compounds were dissolved in methanol (10 μg/ml). The flow rate of the mobile phase was 0.5 ml/min. All analyses were carried out at 25 °C, and detection wavelength of 254 nm was chosen.

2.4. Spectroscopy

The UV absorption spectra were recorded on T60U spectrophotometer (PG Instruments) equipped with quartz cells of 1 cm path length; the pH value of the solutions were determined with CP-501 pH-meter (Elmetron). ctDNA, ethidium bromide dye (EB) and Tris were obtained from the Sigma-Aldrich Company. Tris-HCl buffer solution (concentration 10 mM) was prepared by dissolving solid substance in doubly distilled water and acidify by HCl to pH 7.4. The stock solution of ctDNA was prepared by dissolving solid substance in Tris-HCl solution. All solutions were stored at 4 °C. The concentrations of ctDNA and EB were determined by absorption spectroscopy using the molar extinction coefficient of 6600 M⁻¹ cm⁻¹ at 260 nm and 5800 M⁻¹ cm⁻¹ at 480 nm, respectively. The solutions of ctDNA had a ratio of UV absorbance at 260 and 280 nm larger than 1.8, which indicated that ctDNA was sufficiently free from protein. The stock solutions of substances in doubly distilled water and acidify by HCl to pH 7.4. The stock solutions of substances were obtained from the Sigma-Aldrich Company. Tris-HCl buffer solution (concentration 10 mM) was prepared by dissolving solid substance in ethanol and Tris-HCl solution. All solutions were stored at 4 °C. The concentrations of ctDNA and EB were determined by absorption spectroscopy using the molar extinction coefficient of 6600 M⁻¹ cm⁻¹ at 260 nm and 5800 M⁻¹ cm⁻¹ at 480 nm, respectively. The solutions of ctDNA had a ratio of UV absorbance at 260 and 280 nm larger than 1.8, which indicated that ctDNA was sufficiently free from protein. The stock solutions of substances of 2a-2i series at concentration 100 mM were prepared by dissolving solid substance in ethanol and Tris-HCl solution (1:10) (Charak et al., 2012).

2.5. Quantum mechanical calculations

Theoretical evaluation of NMR proton chemical shifts was carried out for all investigated compounds employing Density Functional Theory (DFT) approximation. As a first step, optimization of investigated systems geometrical parameters was carried out at the B3LYP/6-311G** level of theory and followed by frequency calculations to confirm that the resulting structures correspond to real minima on the potential energy surface. Single starting point per system was used. Next, chemical shifts were calculated with respect to tetramethylsilane (TMS) and compared to experimental data recorded in DMSO. Based on results of our earlier theoretical study of NMR shifts in similar compounds (Baranowska-Ła˛czkowska et al., 2018) we used M06 and B3LYP functionals, combining them with the aug-pcS-1 basis set of Jensen (Jensen, 2008). London Atomic Orbitals (LAOs) (London, 1937) were employed to ensure gauge-origin independent results. Solvent effects were not included in the calculations, as the proton shifts of investigated compounds are not expected to be strongly solvent-dependent. All calculations were carried out using the Gaussian 09 package (Frisch et al., 2009). The aug-pcS-1 basis set was taken from the EMSL Basis Set Library (Feller, 1996; Schuchardt et al., 2007).

3. Results and discussion

3.1. Chemistry

The target triazene salts containing chloroethyl group were obtained in two steps, one-flask synthesis. In the first step, para- or meta-substituted benzenediazonium chlorides were prepared by diazotization reaction of appropriate anilines 1a-1i in the presence of sodium nitrite in 6 M hydrochloric acid (Scheme 1). In the next step, a series of triazene 2a-2i was synthesized by reaction between different substituted benzenediazonium chlorides and bis(2-chloroethyl)amine hydrochloride, followed by addition of sodium hydrogen carbonate, with good yield (37–91%) and chemical purity. All of the synthesized derivatives were characterized by spectroscopic methods ¹H NMR (700 MHz) and ¹³C NMR (100 MHz), and GC-APCI-MS analyses. ¹H NMR and ¹³C NMR spectra of triazenes 2a-2i showed four characteristic triplets at δ (4.16–4.96 ppm) and peaks at δ (40.57–56.13 ppm) due to the four methylene groups, which indicates the conversion of substrates to the expected products with the simultaneous creation of internal triazene salts 2a-2i. The mass spectra of all compounds showed (M+2Cl) ion in the positive-ion mode which is fully consistent with the assigned structures.

3.2. Calculations

To further confirm the structure of resulting triazene salts 2a-2i, theoretical evaluation of NMR proton chemical shifts was carried out for all investigated compounds employing Density Functional Theory (DFT) approximation. Resulting theoretical chemical shifts are presented in (Table 1) together with the corresponding experimental data. Values of the root mean square error (rmse) calculated with respect to experimental data are also printed. Complete set of geometrical parameters of investigated systems can be found in Supplementary Material. We note here that all calculations have been carried out employing frozen structures, and thus protons being chemically equivalent due to rotations around single bonds, and leading to single experimental signal, in our calculations have different chemical environment and appear at different chemical shifts.

For the purpose of comparison with experimental data average theoretical chemical shifts are thus calculated from chemical shifts of protons which would be chemically equivalent. Next
The assignments made based on the B3LYP results agree very well with those made on the M06 values, with the former function changes up to 0.9 ppm. Small in contrary, the signals of aromatic protons are much more sensitive to the change of the nearby substituent, and their position changes up to 0.9 ppm.

### 3.3. Lipophilicity determination

The ability to penetrate the drug through biological membranes is the decisive parameter responsible for its activity. Parameter describing this property is lipophilicity that is defined as the partition coefficient between the the aqueous phase and the non-aqueous phase usually 1-octanol and is expressed as log P (Arnott et al., 2012). One of the best methods to determine concentration of a compound in various solvents needed to determine lipophilicity is reversed-phase high performance liquid chromatography (RP-HPLC) (Marciniec et al., 2016). Therefore, for the determination of relative lipophilicity of triazene derivatives 2a-2i we used reversed-phase ultra-performance liquid chromatography (RP UPLC), based upon sub 3-µm porous particles. Chromatographic capacity factors (k) were calculated: k = (t_R/t_M) 

#### Table 1

| Triazene 2a-2i | RMSE |
|---------------|------|
| 2a            |      |
| H16           | 4.50 |
| H19           | 4.33 |
| H20           | 4.77 |
| H21           | 4.76 |
| H22           | 3.91 |
| H23           | 4.64 |
| H24           | 3.68 |
| H25           | 3.80 |
| H26           | 8.10 |
| H27           | 7.08 |
| H28           | 8.28 |
| H29           | 8.93 |
| H30           | 2.79 |
| H31           | 2.79 |
| H32           | 2.69 |
| δB3LYP        | 0.31 |
| δM06          | 0.33 |
| δexp          | 0.24 |
| 2b            |      |
| H15           | 4.48 |
| H16           | 4.71 |
| H17           | 4.73 |
| H18           | 3.83 |
| H19           | 4.77 |
| H20           | 3.99 |
| H21           | 4.63 |
| H22           | 4.19 |
| H23           | 7.75 |
| H24           | 8.17 |
| H25           | 8.17 |
| H26           | 2.64 |
| H27           |      |
| H28           | 0.32 |
| H29           | 0.34 |
| H30           | 0.38 |
| H31           | 0.36 |
| H32           | 0.38 |
| δB3LYP        | 0.36 |
| δM06          | 0.36 |
| δexp          | 0.36 |
| 2c            |      |
| H15           | 4.44 |
| H16           | 4.72 |
| H17           | 4.73 |
| H18           | 3.83 |
| H19           | 4.57 |
| H20           | 4.36 |
| H21           | 3.76 |
| H22           | 8.67 |
| H23           | 7.91 |
| H24           | 7.71 |
| H25           | 6.22 |
| H26           | 7.77 |
| H27           | 7.27 |
| H28           | 2.36 |
| H29           | 2.36 |
| H30           | 2.36 |
| H31           | 2.36 |
| H32           | 2.36 |
| δB3LYP        | 0.28 |
| δM06          | 0.28 |
| δexp          | 0.28 |
| 2d            |      |
| H15           | 4.55 |
| H16           | 4.37 |
| H17           | 4.75 |
| H18           | 4.74 |
| H19           | 3.94 |
| H20           | 4.66 |
| H21           | 3.69 |
| H22           | 3.79 |
| H23           | 8.50 |
| H24           | 8.26 |
| H25           | 7.98 |
| H26           | 7.13 |
| δB3LYP        | 0.30 |
| δM06          | 0.30 |
| δexp          | 0.30 |
| 2e            |      |
| H15           | 4.47 |
| H16           | 4.31 |
| H17           | 4.70 |
| H18           | 4.70 |
| H19           | 3.87 |
| H20           | 4.59 |
| H21           | 3.67 |
| H22           | 3.77 |
| H23           | 7.88 |
| H24           | 8.68 |
| H25           | 8.72 |
| H26           | 7.77 |
| δB3LYP        | 0.36 |
| δM06          | 0.36 |
| δexp          | 0.36 |
| 2f            |      |
| H15           | 4.68 |
| H16           | 4.88 |
| H17           | 4.58 |
| H18           | 4.17 |
| H19           | 7.67 |
| H20           | 7.71 |
| H21           | 7.77 |
| δB3LYP        | 0.30 |
| δM06          | 0.30 |
| δexp          | 0.30 |
| 2g            |      |
| H15           | 4.50 |
| H16           | 4.32 |
| H17           | 4.73 |
| H18           | 4.79 |
| H19           | 3.90 |
| H20           | 4.62 |
| H21           | 3.67 |
| H22           | 3.80 |
| H23           | 7.95 |
| H24           | 7.94 |
| H25           | 8.44 |
| H26           | 1.20 |
| H27           | 1.84 |
| H28           | 4.01 |
| H29           | 4.49 |
| δB3LYP        | 0.28 |
| δM06          | 0.28 |
| δexp          | 0.28 |
| 2h            |      |
| H15           | 4.48 |
| H16           | 4.28 |
| H17           | 4.77 |
| H18           | 4.79 |
| H19           | 3.98 |
| H20           | 4.68 |
| H21           | 3.63 |
| H22           | 3.80 |
| H23           | 7.97 |
| H24           | 7.24 |
| H25           | 8.12 |
| H26           | 8.57 |
| H27           | 1.09 |
| H28           | 1.81 |
| H29           | 4.38 |
| δB3LYP        | 0.28 |
| δM06          | 0.28 |
| δexp          | 0.28 |
| 2i            |      |
| H15           | 4.67 |
| H16           | 4.91 |
| H17           | 4.57 |
| H18           | 4.18 |
| H19           | 7.44 |
| H20           | 7.56 |
| H21           | 7.56 |
| δB3LYP        | 0.32 |
| δM06          | 0.32 |
| δexp          | 0.32 |

* Opposite assignment is made based on the M06/aug-pc-1 results. See text for details.

The ability to penetrate the drug through biological membranes is the decisive parameter responsible for its activity. Parameter describing this property is lipophilicity that is defined as the partition coefficient between the the aqueous phase and the non-aqueous phase usually 1-octanol and is expressed as log P (Arnott et al., 2012). One of the best methods to determine concentration of a compound in various solvents needed to determine lipophilicity is reversed-phase high performance liquid chromatography (RP-HPLC) (Marciniec et al., 2016). Therefore, for the determination of relative lipophilicity of triazene derivatives 2a-2i we used reversed-phase ultra-performance liquid chromatography (RP UPLC), based upon sub 3-µm porous particles. Chromatographic capacity factors (k) were calculated: k = (t_R/t_M) 

Thus, the eluent with correlation coefficient (R^2 = 0.967–0.998) value. The analysis shows that the value of the log k_w is in the range from −3.503 to −0.914. From all tested compounds, the lowest log k_w values, in the range from −3.503 to −2.675, were observed for compounds 2c, 2d, 2e and 2f containing methyl, trifluoromethyl, chloride and carboxyethyl substituents respectively. The compounds 2a, 2b, 2g and 2i containing acetyl, hydrogen, nitro, and fluoro substituents, showed average values of log k_w, in the range from −2.002 to −1.478. The highest value of the log k_w was found for compounds 2h containing sodium sulfonate group (log k_w, RMSE)
Antiproliferative activity of triazene nitrogen mustards 2a-2i against cancer cell lines and normal human mammary epithelial cells MCF-10A.

| Triazene salts  | λ1 (nm) | λ2 (nm) |
|-----------------|---------|---------|
| 2a              | 335     | 240     |
| 2b              | 331     | 236     |
| 2c              | 340     | 241     |
| 2d              | 326     | 234     |
| 2f              | 337     | 223     |
| 2g              | 333     | 235     |
| 2h              | 340     | 221     |
| 2i              | 330     | 221     |

Table 3

Antiproliferative activity of triazene nitrogen mustards 2a-2i against cancer cell lines and normal human mammary epithelial cells MCF-10A.

| IC50 ± SD [μg/ml] |
|-------------------|-------------------|
|                  | MV–4-11 | JURKAT | HT–29 | Hep-G2 | HeLa | Du-145 | DAUDI | MCF-10A |
| 2a                | 13.42 ± 5.937  | 45.07 ± 1.654  | 66.20 ± 13.428  | 49.32 ± 17.381  | 38.45 ± 12.341  | 39.42 ± 9.768  | 88.95 ± 9.309  | 16.07 ± 26.485 |
| 2b                | 44.39 ± 4.369  | 50.44 ± 8.079  | 68.54 ± 6.356  | 48.88 ± 18.718  | 37.93 ± 17.106  | 39.41 ± 11.465  | 38.38 ± 8.824  | 40.19 ± 4.286  |
| 2c                | 5.42 ± 2.923   | 19.75 ± 3.017  | 13.14 ± 0.503  | 16.25 ± 3.734  | 23.81 ± 1.051  | 18.11 ± 7.036  | 36.44 ± 5.287  | 4.91 ± 1.527  |
| 2d                | 3.59 ± 3.033   | 16.47 ± 4.144  | 24.29 ± 1.221  | 31.74 ± 5.453  | 14.92 ± 1.079  | 22.70 ± 2.975  | 30.95 ± 3.146  | 17.63 ± 2.288  |
| 2e                | 7.39 ± 6.191   | 15.09 ± 2.773  | 13.64 ± 3.002  | 31.94 ± 8.143  | 14.91 ± 4.658  | 12.53 ± 2.234  | 30.10 ± 10.156  | 13.64 ± 3.656  |
| 2f                | 6.16 ± 1.763   | 20.87 ± 2.194  | 35.98 ± 7.111  | 5.59 ± 0.632  | 27.79 ± 10.104  | 23.51 ± 3.316  | 22.78 ± 10.062  | 61.89 ± 24.810  |
| 2g                | 14.05 ± 7.746  | 23.88 ± 7.482  | 49.11 ± 14.143  | 47.64 ± 3.418  | 32.07 ± 1.399  | 28.97 ± 5.351  | 34.14 ± 1.350  | 61.13 ± 60.483  |
| 2h                | 50.8 ± 36.576  | 5.55 ± 14.192  | 7.95 ± 11.711  | 13.65 ± 12.306  | 27.2 ± 17.459  | 6.65 ± 24.065  | 11.95 ± 15.547  | 89.97 ± 18.485  |
| 2i                | 51.38 ± 11.917  | 61.40 ± 21.808  | 39.53 ± 10.080  | 45.17 ± 4.094  | 70.17 ± 13.874  | 51.26 ± 14.724  | 35.09 ± 7.588  | 75.60 ± 17.762  | 15.66 ± 4.010  |
| cis-platin        | 0.76 ± 0.184   | 1.73 ± 0.443  | 0.24 ± 0.056  | 3.63 ± 0.715  | 0.68 ± 0.147  | 0.37 ± 0.137  | 0.59 ± 0.086  | 1.07 ± 0.208  | 4.65 ± 1.171  |

* Average proliferation inhibition at 100 μg/ml.
For all compounds with increasing concentration of the triazene with constant DNA concentration, the hyperchromic effect was observed (Fig. 1). The absorption of DNA-triazene complexes at 258 nm showed a decrease in absorbance compared to the sum of the individual components, which clearly shows that the test compounds interact with DNA. With increasing concentration of the DNA at a constant triazene concentration, the hypsochromic shifts were observed relative to the sum of the absorbances of the individual components (Fig. 2). Both in experiments with constant DNA concentration and with a constant concentration of

![Fig. 1. The absorption spectrum of the solution containing 100 mM of DNA and increasing amounts of 2a.](image1)

![Fig. 2. The absorption spectrum of the solutions containing 30 µM of 2g and increasing amounts of DNA.](image2)

| Triazene | Binding constant $K_b$ [M$^{-1}$] | Binding constant $K_o$ |
|----------|----------------------------------|------------------------|
| 2a       | 55,330                           | $5.53 \times 10^4$ M$^{-1}$ |
| 2b       | 19,758                           | $1.98 \times 10^4$ M$^{-1}$ |
| 2c       | 23,248                           | $2.32 \times 10^4$ M$^{-1}$ |
| 2d       | 19,503                           | $1.95 \times 10^4$ M$^{-1}$ |
| 2e       | 24,968                           | $2.50 \times 10^4$ M$^{-1}$ |
| 2f       | 58,260                           | $5.83 \times 10^4$ M$^{-1}$ |
| 2g       | 26,037                           | $2.60 \times 10^4$ M$^{-1}$ |
| 2h       | 23,427                           | $2.34 \times 10^4$ M$^{-1}$ |
| 2i       | 19,254                           | $1.92 \times 10^4$ M$^{-1}$ |
compounds, the incubation time does not play a key role in the formation of DNA linkage, suggesting a fast bonding to DNA.

In the next step of our research we calculated the intrinsic binding constant $K_b$ between triazene salts $2a$-$2i$ and DNA (Table 5) using the equation: $[\text{DNA}] / ([e_a - e_b]) = [\text{DNA}] / ([e_b - e_f]) + 1 / K_b ([e_b - e_f])$, where $[\text{DNA}]$ is the concentration of DNA in base pairs, while $e_a$, $e_b$ and $e_f$ are the apparent, free and bound complex extinction coefficients, respectively (Pakravan et al., 2015). Plot of $[\text{DNA}] / ([e_a - e_b] 	imes 10^8$ vs. $[\text{DNA}]$ for triazene $2g$ can be found in Supplementary Material.

As we can see that the largest binding constant $K_b$ equals $5.83 \times 10^4 \text{ M}^{-1}$ ($2f$) and $5.53 \times 10^4 \text{ M}^{-1}$ ($2b$) have compounds having the COOEt and COCH$_3$ groups, which is probably caused by the formation of an additional hydrogen bond between the carbonyl group of these compounds and phosphate chain of DNA. The compounds containing NO$_2$ and Cl groups are characterized by two times smaller binding constant $K_b$ equals $2.60 \times 10^3 \text{ M}^{-1}$ and $2.50 \times 10^3 \text{ M}^{-1}$, respectively. An interesting observation is also that compounds containing strong electron-withdrawing CF$_3$ and F groups are characterized by the smallest binding constant $K_b = 1.95 \times 10^3 \text{ M}^{-1}$ and $1.92 \times 10^3 \text{ M}^{-1}$, respectively. This is probably due to the weakening of the formation of hydrogen bonds by these molecules.

The next experiment with the competitive replacement of ethidium bromide dye (EB) from its complex with DNA by the tested compounds showed no changes in absorbance spectra, suggesting non-intercalative mode of binding between triazenes and DNA (Fig. 3).

In conclusion, our research suggests that cationic triazene species interact fast with the negatively charged DNA phosphate chain outside of the helix.

4. Conclusion

In summary, we have developed an efficient method for the synthesis of triazene salts and confirmed their structure by spectroscopic methods and theoretical calculations. As a result of our research, we have identified new leading structures with very high activity against some types of cancer cells with IC$_{50}$ values from 4.91 to 7.69 µg/ml, and with cytotoxic activity against normal human mammary gland epithelial cells MCF-10A from 6 to 11 times lower than against cancer cell lines. We have also demonstrated a good correlation between determined lipophilicity and the antiproliferative activity of obtained compounds. Our UV–Vis spectroscopic results indicate also that triazene salts tend to interact with negatively charged DNA phosphate chain. Additional calculations show that compounds $2f$ and $2b$ containing COOEt and COCH$_3$ substituents bind more strongly to DNA than other compounds, their $K_b$ values are $5.83 \times 10^4 \text{ M}^{-1}$ and $5.53 \times 10^4 \text{ M}^{-1}$, respectively. Moreover, the calculated binding constant $K_b$ values indicates that the resulting derivatives could also interact with DNA in an in vivo situation, however, to confirm this further studies are required.

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2018.11.012.

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