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Expedient synthesis and anticancer evaluation of dual-action 9-anilinoacridine methyl triazene chimeras

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Abstract

The efficient synthesis of molecular hybrids including a DNA intercalating 9-anilinoacridine (9-AnA) core and a methyl triazene DNA methylating moiety is described. Nucleophilic aromatic substitution (S_NAr) and electrophilic aromatic substitution (EAS) reactions using readily accessible starting materials provide a quick entry to novel bifunctional anticancer molecules. The chimeras were evaluated for their anticancer activity. Chimera **7b** presented the highest antitumor activity at low micromolar IC_{50} values in antiproliferative assays performed with various cancer cell lines. In comparison, compound **7b** outperformed DNA intercalating drugs like Amsacrine and AHMA. Mechanistic studies of chimera **7b** suggest a dual mechanism of action: methylation of the DNA repairing protein MGMT associated with the triazene structural portion and Topo II inhibition by intercalation of the acridine core.

Keywords: Anilinoacridine · Triazene · Molecular chimera · S_NAr · EAS · Anticancer

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1. Introduction:

The discovery of small molecule multi-target drugs with combined pharmacological activities has emerged as a widely used approach in medicinal chemistry (Lomberdino et al. 2004; Zhou et al. 2017). DNA intercalators, comprising a wide range of structurally diverse biologically active compounds mostly with anticancer and antimalarial applications (Martínez et al. 2005; Almaqwashi et al. 2016), have been proposed as advantageous components for successful dual-drug structural hybrids - molecular chimeras.

During the last thirty years, the DNA intercalating 9-anilinoacridine (9-AnAs) has been frequently used as structural core for hybrid drug candidates. This scaffold presents a planar structural fragment with hydrogen bond donor and hydrogen bond acceptor groups that intercalates DNA and binds to minor grooves stabilizing the DNA-drug complex (Gellerman et al. 2012), apparently reducing the risk of secondary cancers (Godzieba et al. 2019). In addition, its DNA topoisomerase II (Topo II) poisoning ability enhances the antiproliferative effects (Gamage et al. 1994; Auparakkitanon et al. 2000). One of the oldest drugs of this family is the antineoplastic Amsacrine that is still used in

clinical treatment of acute lymphoblastic leukemia (Fig.1) (Nitiss et al. 2009; Horstmann et al. 2005). Apparently, this drug suffers from fast deactivation by glutathione associated drug resistance. An improved version of Amsacrine, (9-acridinylamino)-5-hydroxymethylaniline (AHMA) was designed to avoid metabolic oxidation. Compared to Amsacrine, AHMA, has longer half-life in human plasma accompanied by longer duration of its drug action (Rastogi et al. 2002). AHMA was also shown to display better antitumor activity *in vivo* probably due to the substituent (NH_2 and CH_2OH, Fig. 1) arrangement in the meta-positions of the aniline ring, which prevents undesired oxidation processes (Chang et al. 2003; Afzal et al. 2016).

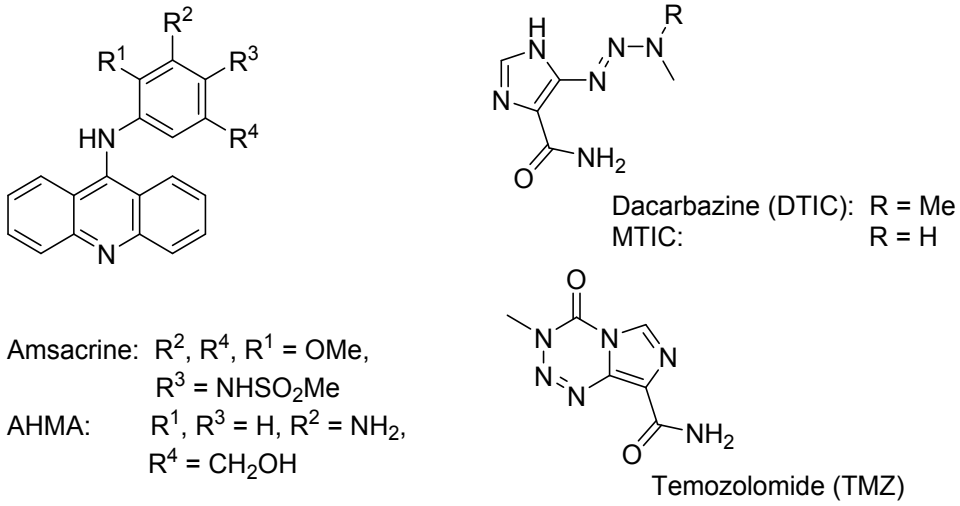


FIGURE 1 Structures of anticancer DNA intercalators, Topoisomerase II inhibitors and DNA methylating agents.

DNA methylating agents like Dacarbazine (DTIC) and Temozolomide (TMZ), bearing a triazene moiety (Fig. 1), are members of a different class of anticancer drugs applied to the treatment of melanoma (DTIC) and glioblastoma (TMZ) (Su et al. 1999; Scarborough et al. 1996; Thomas et al. 2013). So far, DTIC and TMZ are the only triazene-based approved drugs currently in use in clinical chemotherapy (Gerson et al. 2018).

The mechanism of DTIC and TMZ antitumor activity involves the methylation of nucleotides, through *in vivo* generation of highly reactive diazomethane (the actual alkylating agent). Both TMZ and DTIC are prodrugs of the active 5-(3-methyltriazen-1-yl) imidazole-4-carboximide (MTIC)

intermediate (Meer et al. 1986; Jhang et al. 2012). Unlike DTIC, which requires metabolic activation by cytochrome P450, otherwise remaining dormant, TMZ spontaneously converts to MTIC under physiologic conditions (Kolar et al. 1980; Marchesi et al. 2007; Monteiro et al. 2013).

In general, the administration of drug combinations with differing mechanisms of action is a favorable therapeutic treatment to augment the effectiveness of the treatment (Mort et al. 2014; Sonpavde et al. 2016; Raja et al. 2013). Yet, combination chemotherapy by co-administration of two or more drugs, does not appear to prevent the development of drug resistance in cancer cells and, in addition, it is associated with increased toxicity (Szakacs et al. 2006; Pritchard et al. 2012; Shirota et al. 2001**)**. A useful approach to the design of unexplored promising anticancer candidates is the structural combination of bioactive fragments of existing pharmaceutical agents with proven effectiveness. This drug design strategy has led to the development of a sizable number anticancer structural hybrids (chimeras) destined to preclinical and clinical development (Huang et al. 2014**;** Naito et al. 2019; Oliveira et al. 2015). Thus and thus, we decided to design, synthesize and evaluate a family of chimeras including 9-AnAs and methyl triazene moieties to investigate whether a single molecule bearing two anticancer agents operating under two distinct mechanisms (Topo II inhibitors: 9-AnAs and DNA methylating agents: DTIC or TMZ) works better than each one of them alone.

In the past we developed the highly efficient one-pot derivatization of 9-anilonoacridines (9-AAs) at the amino group by simple S_NAr reaction using easily accessible haloaryl starting materials (Gellerman et al. 2010; Gellerman et al. 2011). Such a "reverse" approach (Fig. 2) to synthesize 9-AnAs complements the "classical" electrophilic aromatic substitution (EAS) approach to 9AnA derivatives, in which 9-Cl acridine reacts with substituted anilines (Redko et al. 2012).

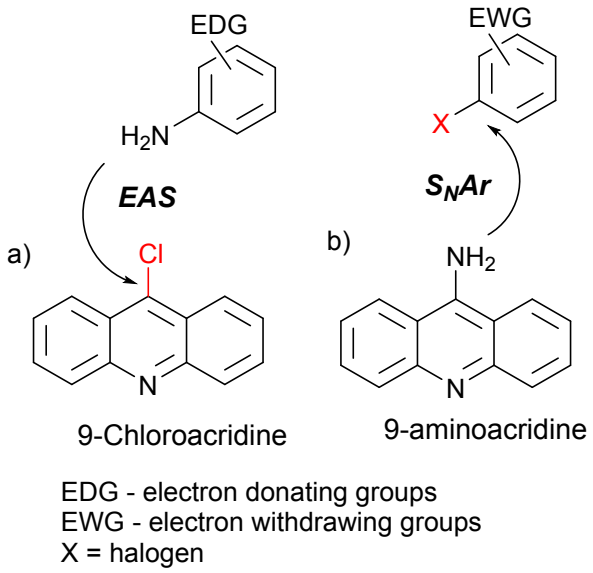


FIGURE 2 (a) "Classical" – EAS and (b) "reverse" – S_NAr approaches for the synthesis of substituted 9-AnAs

Here, we report on the synthesis of chimeras built from 9-AnAs and methyl triazene structural building blocks. Our synthetic strategy is based on our previous experience with the facile derivatization of 9-AnA through aromatic substitution reactions. A total of nine chimeras were prepared by this approach. Preliminary antiproliferative assays against H1299 (NSCLC) and WM-266-4 (human metastatic melanoma) cancer cell lines helped us to identify a lead compound with enhanced antitumor activity compared to the parent control drugs: Amsacrine and AHMA. Chimera **7b** stands out among our synthesized hybrids, exhibiting remarkable cell growth inhibition with IC_{50} = 2.9 and 0.8 μM against the above-mentioned cancer cell lines, respectively. Expectedly, the chemostability of the synthesized chimeras varied according to the type of triazene used as substituent on the aniline ring of 9-AnA. Here we demonstrate that chimera **7b** can bestow its antiproliferative effect by two independent mechanisms: DNA alkylation associated with methyl triazene structural portion and Topo II inhibition affiliated with the 9-acridine core. The findings reported in this work expand the scope of 9-anilinoacridine (9-AnA) based methyl triazene chimeras as efficient anticancer candidates.

2. Materials and methods

2.1 General information

Amsacrin, DTIC, and TMZ were purchased from Tzamal D-Chem Laboratories Ltd. Petah-Tikva, Israel if not indicated otherwise. AHMA was synthesized according a reported literature procedure (Su et al. 2006). All solvents were purchased from Bio-Lab Ltd. Jerusalem, Israel or Gas Technologies Ltd. Kefar Saba, Israel. All reactions were performed in a round bottom flask and monitored by TLC performed on aluminium plates (0.25 mm, E. Merck) precoated with silica gel (Merck 60 F-254). Developed TLC plates were visualized under a short-wavelength UV lamp. Yields refer to spectroscopically (1H, 13C NMR) homogeneous material obtained after column chromatography performed on silica gel (Silica flash P60) supplied by Silicycle. ^{1}H and ^{13}C NMR were recorded in $CDCl_3$ and DMSO-d6 on a Bruker Avance III 400 MHz spectrometer. Chemical shifts (δ) are quoted in ppm, relative to SiMe4 (δ = 0.0) as an internal standard. LC/MS analyses were performed using an Agilent Technologies 1260 Infinity (LC) 6120 quadruple (MS), column Agilent SB-C18, 1.8 mm, 2.1×50 mm, column temperature 50 °C, eluent water- acetonitrile (CH_3CN) + 0.1% formic acid. FT-IR spectra were recorded as KBr pellets on a Shimadzu FT-IR-8400S spectrometer. High-resolution mass spectra (HRMS) were measured using an Agilent QTOF mass-spectrometer in electrospray ionization (ESI) positive ion mode. Melting points were recorded on a standard melting point apparatus.

2.2 Reagents and cell lines

All the cell lines were cultured in an RPMI medium supplemented with 2 mM glutamine, 10% fetal bovine serum and penicillin streptomycin (100 IU/ml of each). The cell culture growth medium and all additives were purchased from Biological Industries, Bet-Ha'emek, Israel. All cell cultures were grown in an incubator at 37 °C in an environment containing 6% CO_2. The cytotoxicity of the materials was determined by measuring the mitochondrial enzyme activity, using a commercial XTT assay kit (Biological Industries, Bet-Ha'emek, Israel). All samples contained DMSO at final concentration below 0.05%. All cancer cell lines were kindly provided by Prof. Albert Pinhasov (Ariel University, Israel).

2.3 Liquid chromatography

Purification of the compounds was done *via* normal phase chromatography by using silica gel (Silica flash @ P60) supplied by Merck. The column was kept at room temperature and eluted with either petroleum ether, ethyl acetate or dichloromethane, neat or combined.

2.4 Liquid Chromatography Mass Spectrometry (LCMS)

Electron spray mass spectra (ESI-MS) were obtained using an Autoflex III smart-beam (MALDI, Bruker), Q-TOF micro (Waters) or an LCQ Fleet^{TM} ion trap mass spectrometer (Finnigan/Thermo). HPLC/LC-MS analyses were made using Agilent infinity 1260 connected to Agilent quadruple LC-MS 6120 series equipped with ZORBAX SB-C18, 2.1 x 50 mm, 1.8 µm column. In all cases, the eluent was A (0.1% formic acid (FA) in H_2O) and B (0.1% FA in ACN) and the elution gradient profile was: 100% A for first 3 min, followed by 5 min (from min 3 to min 8) during which it reached 100% B, followed by 5 min (from min 8 to min 13) of 100% B, followed by two min (from min 13 to min 15) with a linear gradient back to 100% A, followed by 2 min (from min 15 to min 17) of 100% A. The UV detector was set at 254 nm. The column temperature was kept at 50 °C. The flow rate was of 0.3 ml/min. The MS fragmentor was tuned on 30 V or 70 V on positive or negative mode.

2.5 Chemostability studies

Stock solutions were prepared by dissolving 5 mg of the tested compound in 500 µL of DMSO. Aqueous stability was determined at pH 4.6 and 7.4. Aliquots (100 µL) of stock solution were diluted to a total of 2.5 mL with the desired buffer and then incubated at 37 °C. During the incubation period 250 µL portions were drawn at different time intervals, filtered and analyzed by LC–MS.

2.6 Cytotoxicity test (XTT)

The cytotoxicity of the synthesized chimeras was determined by measuring the mitochondrial enzyme activity, using a commercial XTT assay kit. All samples contained DMSO at final concentration below 0.05%. Cells were cultured in micro wells at 5-10 × 10^4 cells/mL and incubated for 48 h and 72 h. After the first incubation period, the cultures were washed and then provided with fresh medium containing different concentrations of the tested substances. At the end of the second incubation, XTT reagent was added and the cells were re-incubated for additional 3 h. During that time, the absorbencies in the wells were measured with a TECAN Infinite M200 ELISA reader at both 480 and 680 nm. The difference between these measurements was used for calculating the % growth inhibition (GI) in test wells compared to two controls: cells that were exposed to the medium and solvent, and

those which were exposed to a solvent-free medium. All the tests were done in tetra-plicate; each experiment was conducted twice.

2.7 Western blot

HCT-116 cells (1 × 106 cells/well), were treated with DTIC, TMZ and Chimera **7b** (100 μM) or DMSO (Sigma Aldrich) for 48 h and were harvested using RIPA lysis buffer and protease inhibitor cocktail (Sigma Aldrich). Equal amounts of protein were separated using 10% SDS- polyacrylamide gel and blotted onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS), containing 1% Tween20 for 2 h at room temperature, probed with the appropriate primary antibody over night at 4 °C and then with the appropriate fluorescently labeled secondary antibody (Li-Cor Biosciences). Membranes were scanned and analyzed using Licor-FC Infrared Imaging System (Li-Cor Biosciences). Primary antibodies used: MGMT (Millipore) and tubulin (Cell Signaling).

2.8 Human topoisomerase IIa kDNA decatenation assay

Topo II activity was measured by the ATP-dependent decatenation of kinetoplast DNA (kDNA). The assay was performed according to manufacturer's (Inspiralis Limited, UK) protocol modified as follows. The reaction mixture contained kDNA (200 ng), 3 units of Topo II and assay buffer with ATP. AHMA and molecular chimera **7b** were diluted in DMSO and added at the indicated concentrations to the reaction mixture to a final volume of 15 ml. The same amount of DMSO was added to control cultures without the drugs. The samples were incubated for 25 min at 37 °C and the reaction was stopped by adding 1 ml of 10% SDS. Samples were electrophoresed in 1% agarose gel in TAE buffer at 4 V/cm for 2 h. The gel was stained with ethidium bromide and photographed under a UV trans-illuminator.

2.9 Chemistry

2.9.1 General procedure for the synthesis of the dimethyl triazene derivative of nitroaniline (1).

A suspension of nitroaniline (0.3 mole, 1 equiv.) in diluted hydrochloric acid in water having a ratio of HCl:H_2O (4:6) was diazotized at 0 °C with sodium nitrite (1.2 equiv.) in water and the resulting solution was stirred for 45 min and to give a clear solution. The diazonium salt solution was treated with 40% aqueous dimethylamine. At this stage, the triazene usually precipitated after reaching a pH

between 7 to 8. The reaction mixture was quenched with a 1% solution of citric acid and extracted with DCM. The organic layer was washed with brine, dried over Na_2SO_4, filtered and the solvents were removed under reduced pressure to give a crude product which was then purified by silica gel chromatography using EtOAc:petroleum ether (1:9), to give pure triazenes **1a-1i** (Scheme 1) with 58 to94% yield.

(*E*)-3,3-dimethyl-1-(2-nitrophenyl) triaz-1-ene (1a). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 363 mg (86%) R_f = 0.78, yellow liquid; MS-ESI, (m/z) = 195 (M+1); ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.64 (dd, *J* = 8.0, 1.34 Hz, 1H-a) 7.52 (dd, *J* = 8.2, 1.34 Hz, 1 H-b), 7.44 (, ddd, *J* = 8.3, 7.1, 1.5 Hz, 1 H-c), 7.16 (, ddd, *J* = 8.2, 7.1, 1.5 Hz, 1 H-d), 3.53 (s,3 H, CH_3-e), 3.19 (s,3 H, CH_3-f). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 144.9, 143.6, 132.1, 130.5, 124.6, 119.5, 36.1 (Me), 29.2 (Me).

(*E*)-3,3-dimethyl-1-(4-methyl-2-nitrophenyl) triaz-1-ene (1b). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 358 mg (87%), *Rf* = 0.73, yellow liquid, MS-ESI, (m/z) = 209 (M+1, 100%), ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.44 (d, *J* = 0.98 Hz, 1H -a), 7.40 (d, *J* = 8.3 Hz, 1H -b), 7.25 (dd, *J* = 0.61, 1.96 Hz, 1H-c), 3.50 (s., 3H, CH_3-d), 3.17 (s., 3H, CH_3-e), 2.35 (s, 3H CH_3-f); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 144.8, 141.5, 135.1, 133.1, 123.8, 119.5, 43.2, 36.2 (Me), 20.7 (Me).

(*E*)-1-(4-methoxy-2-nitrophenyl)-3,3-dimethyltriaz-1-ene (1c). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 0.364 g (91%), *Rf* = 0.66, orange liquid, MS-ESI, (m/z) = 225 (M+1, 98%), ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.46 (d, *J* = 9.1 Hz, 1H-a), 7.17 (d, *J* = 2.8 Hz, 1H-b), 7.03 (dd, *J* = 2.8, 9.1 Hz, 1H-c), 3.82 (s, 3H, OCH_3-d), 3.50 (s., 3H, CH_3- e), 3.17 (s., 3H,CH_3-f); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 156.7, 145.2, 137.6, 120.7, 119.4, 107.8, 56.0 (OMe), 40.2 (Me), 36.3 (Me).

(*E*)-3,3-dimethyl-1-(3-nitrophenyl) triaz-1-ene (1d). The compound was purified by column chromatography [ethyl acetate: petroleum ether (1:9), v/v]; yield: 347 mg (82%), *Rf* = 0.82, yellow solid, MP:100-101 ^{o}C, MS-ESI, (m/z) = 195 (M+1, 56%), 196 (M+2, 35%); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.25 (t, *J* = 2.2 Hz, 1H-a), 7.94 (ddd, *J* = 0.98, 2.2, 8.1 Hz, 1H-b), 7.64 - 7.74 (m, 1H-c), 7.44 (t, *J* = 8.1 Hz, 1H-d), 3.56 (s., 3H,CH_3-e), 3.24 (s., 3H,CH_3-f); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 152.2, 149.1, 129.5, 127.0, 119.5, 114.9, 36.1 (Me), 32.5 (Me).

(*E*)-1-(4-bromo-3-nitrophenyl)-3,3-dimethyltriaz-1-ene (1e). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 220 mg (58 %), *Rf* = 0.80, light yellow solid, MP: 80-81 °C, MS-ESI, (m/z) = 272.9 (M+1, 43%), 274.9 (M+3, 42%) ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.24 (d, *J* = 2.7 Hz, 1H-a), 7.78 (dd, *J* = 2.7, 8.7 Hz, 1H-b), 7.69 (d, *J* = 8.7 Hz, 1H-c), 3.61 (s, 3H,CH_3-d), 3.30 (s, 3H,CH_3-e); ^{13}C NMR (101 MHz, CDCl3) δ (ppm): 149.2, 147.9, 133.7, 126.5, 119.8, 112.9, 43.6 (Me), 36.8 (Me).

Methyl (*E*)-4-(3,3-dimethyltriaz-1-en-1-yl)-3-nitrobenzoate (1f). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 293 mg (76%), *Rf* = 0.76, yellow solid, MP: 96-97 °C, MS-ESI, (m/z) = 253 (M+1, 68%), 254 (M+2, 29%). ^{1}H NMR (400 MHz, CDCl3) δ (ppm): 8.29 (d, *J* = 1.7 Hz, 1H-a), 8.08 (dd, *J* = 1.7, 8.6 Hz, 1H-b), 7.60 (d, *J* = 8.6 Hz, 1H-c), 3.92 (s, 3H,OCH_3-d), 3.60 (s, 3H,CH_3-e), 3.25 (s, 3H,CH_3-f). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 165.4, 147.0, 137.2, 133.0, 126.3, 125.4, 119.3, 58.5, 43.8 (Me), 36.7(Me).

(*E*)-3,3-dimethyl-1-(4-nitrophenyl) triaz-1-ene (1g). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 375 mg (89 %), *Rf* = 0.84, yellow solid, MP: 144-145 °C, MS-ESI, (m/z) = 195(M+1, 66%), 196(M+2, 30%); ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.01 - 8.29 (dd, *J* = 9.2Hz, 2H-a), 7.41 - 7.55 (dd, *J* = 9.2Hz, 2H-b), 3.59 (s., 3H,CH_3-c), 3.27 (s., 3H,CH_3-d). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 156.1, 144.7, 124.9, 120.7, 43.7 (Me), 36.4(Me).

(*E*)-1-(2-bromo-5-nitrophenyl)-3,3-dimethyltriaz-1-ene (1h). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 288 mg (74%), *Rf* = 0.80, light yellow solid, MP: 112-113 °C, MS-ESI, (m/z) = 272.9 (M+1, 48%), 274.9 (M+3, 47%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.90 (d, *J* = 2.3 Hz, 1H-a), 7.63 (d, *J* = 8.7 Hz, 1H-b), 7.47 (dd, *J* = 2.3, 8.7 Hz, 1H-c), 3.57 (s.,3H,CH_3-d), 3.24 (s., 3H,CH_3-e); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 151.2, 150.8, 135.1, 125.5, 116.9, 109.0, 38.3 (Me), 32.1(Me).

(*E*)-1-(4-methoxy-3-nitrophenyl)-3,3-dimethyltriaz-1-ene (1i). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 364 mg (91 %), *Rf* = 0.68, yellow solid, MP: 146-148 °C, MS-ESI, (m/z) = 225 (M+1, 100%), ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.22 (d, *J* = 2.8 Hz, 1H-a), 8.03 (dd, *J* = 2.8, 9.05 Hz, 1H-b), 6.96 (d, *J* = 9.05 Hz, 1H-c), 3.99

(s, 3H,OCH3-d), 3.57 (s., 3H,CH_3-e), 3.29 (s., 3H,CH_3-f); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 158.0, 141.9, 140.7, 121.7, 114.0, 111.1, 56.7, 43.5 (Me), 36.3(Me).

2.9.2 General Procedure for the S_NAr synthesis of compounds 2a and 2b.

A solution of an aryl halide of triazene (1 equiv.) in dry DMF (6 ml) and Cs_2CO_3 (0.5 equiv.) was added slowly to a stirred solution of 9-aminoacridine (1 equiv.), under positive nitrogen pressure at room temperature. The reaction mixture heated to 90 °C, and stirred for 12 h. The reaction was monitored by TLC and LCMS. After reaching its completion, the reaction mixture was diluted with water and extracted with EtOAc (DMF was removed carefully by successive water washings to the organic layer). The organic layer was washed with brine, dried over Na_2SO_4 (anh.), filtered and concentrated under reduced pressure to give a crude product which was then purified by silica gel chromatography using EtOAc:CH_2Cl_2 (2:8), resulting in a pure compound, generally as a brown solid.

(*E*)-N-(4-(3,3-dimethyltriaz-1-en-1-yl)-2-nitrophenyl) acridin-9-amine (2a). The compound was purified by column chromatography [EtOAc:CH_2Cl_2 (2:8) v/v]; yield: 97 mg (68%), *Rf* = 0.43, brown solid, MP: 212-214 °C; FT-IR (KBr, ν, cm^{-1}): 2958, 1582,1501,1430,1380; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.99 (d, *J* = 2.3 Hz, 1H-a), 7.81 - 7.90 (dd, 2H-b), 7.50 - 7.63 (m, 4H-c), 7.37 - 7.42 (dd, 2H-d), 6.94 - 7.06 (m, 2H-e), 6.84 (d, *J* = 8.7 Hz, 1H-f), 3.49 (s,3H, CH_3-g), 3.19 (s, 3H,CH_3-f); ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm): 151.5, 146.2, 144.3, 140.9, 139.9, 138.3, 131.9, 128.6, 121.5, 120.5, 117.5, 116.9, 115.7, 35.9 (Me), 33.7 (Me); HRMS-ESI(+) calculated for $C_{21}H_{19}N_6O_2$, 387.1491; found 387.1856 (98%) $[M+H]^+$

(*E*)-N-(2-(3,3-dimethyltriaz-1-en-1-yl)-4-nitrophenyl) acridin-9-amine (2b). the compound was purified by column chromatography [EtOAc:CH_2Cl_2 (2:8) v/v]; yield: 88 mg, (62%), *Rf* = 0.48, brown solid, MP: 204-206 °C; FT-IR (KBr, ν, cm^{-1}): 3010, 1576,1522,1410,1364; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.23 (dd, *J* = 1.4, 8.0 Hz, 1H-a), 7.92 - 8.12 (m, 2H-b), 7.78 - 7.92 (m, 2H-c), 7.44 - 7.57 (m, 2H-d), 7.32 - 7.43 (m, 2H-e), 7.06 (d, *J* = 8.4 Hz, 1H-f), 6.96 (t, *J* = 7.4 Hz, 2H-g), 3.11 - 3.41 (s, 6H,2CH_3-h); ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm): 154.1, 152.8, 140.9, 137.7, 133.4, 131.7, 126.9, 126.0, 121.7, 117.9, 117.5, 116.3, 112.3, 31.1(Me), 29.8(Me) ; HRMS-ESI(+) calculated for $C_{21}H_{19}N_6O_2$, 387.1491; found 387.1574 (96%) $[M+H]^+$

2.9.3 General procedure for the EAS synthesis of dimethyltraizene 9-AnA derivatives 4a, 4b, 4c and 4d.

A catalytic amount of 10% Pd/C was added to a suspension of nitroaniline dimethyl triazene, (Scheme 1) (0.2 g, 1 equiv.) in ethanol (4 mL) under hydrogen atmosphere (1atm) and the whole reaction mixture was stirred for 4 h room temperature. The reaction was monitored by TLC and LCMS. After completion,the crude of the reaction was filtered through celite and the solvent was removed under reduced pressure. After complete evaporation of the solvent, water was added and the product was extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4 (anh.) and give a crude product after solvent removal. The crude product was subsequently treated as follows: The crude amine triazene (1 equiv.) was dissolved in absolute ethanol under a nitrogen atmosphere at 0 ^oC. Then, a solution of 9-Cl-acridine (1 equiv.) and *N,N*-diisopropylethylamine (DIPEA) (1.5 equiv.) was added. The reaction was stirred at room temperature for 12 h and monitored by TLC. After completion, the reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in EtOAc and washed with distilled water. The aqueous layer was washed three times with 15 mL aliquots of EtOAc. The collected organic fractions were dried over Na_2SO_4 (anh.) and the solvent was removed under reduced pressure to yield the crude product, which was then purified by silica gel chromatography using EtOAc:CH_2Cl_2 (2:8 to 4:6) to afford the pure compound as a brown solid.

(*E*)-N-(4-(3,3-dimethyltriaz-1-en-1-yl) phenyl) acridin-9-amine (4a). The compound was purified by column chromatography [EtOAc:CH_2Cl_2 (5:5) v/v]; yield: 113 mg (64%), *Rf* = 0.64, brown solid, MP: 234-236 ^oC; FT-IR (KBr, ν, cm^{-1}): 2932, 1488,1437,1395,1361; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.03 (d, *J* = 8.56 Hz, 2H-a), 7.91 (s., 2H-b), 7.50 - 7.67 (m, 2H-c), 7.33 - 7.44 (dd, *J* = 8.8Hz, 2H-d), 7.24 (brs, 3H-e), 6.79 - 6.93 (dd, *J* = 8.8 Hz, 2H-f), 3.32 (s, 6H-2CH_3-g); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 146.2, 133.6, 130.7, 127.5, 125.0, 123.7, 122.1, 121.7, 119.7, 119.0, 116.5, 29.9(Me), 29.4 (Me); HRMS-ESI(+) calculated for $C_{21}H_{20}N_5$, 342.1640; found 342.1712 (100%) $[M+H]^+$

(*E*)-N-(3-(3,3-dimethyltriaz-1-en-1-yl) phenyl) acridin-9-amine (4b). The compound was purified by column chromatography [EtOAc:CH_2Cl_2 (5:5) v/v]; yield:132 mg (62%), Rf = 0.63, brown solid, MP: 136-137 ^oC; FT-IR (KBr, ν, cm^{-1}): 2945, 1470,1424,1400,1355; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.02 (d, *J* = 8.6 Hz, 2H-a), 7.94 (d, *J* = 8.5 Hz, 2H-b), 7.57 (t, *J* = 7.5 Hz, 2H-c), 7.26 (s, 1H-d), 7.15 - 7.23 (m, 3H-e), 7.09 - 7.15 (m, 1H-f), 7.07 (s, 1H-g), 6.61 - 6.72 (m, 1H-h), 3.29 (s,

6H,2CH_3-i); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 162.2, 147.2, 130.5, 129.7, 129.5, 126.8, 126.7, 124.7, 123.9, 120.2, 114.8, 114.4, 110.0, 29.7(Me), 29.6 (Me); HRMS-ESI(+) calculated for $C_{21}H_{20}N_5$, 342.1640; found 342.1712 (100%) $[M+H]^+$

(*E*)-N-(4-bromo-3-(3,3-dimethyltriaz-1-en-1-yl) phenyl) acridin-9-amine (4c). The compound was purified by column chromatography [EtOAc:CH_2Cl_2 (5:5) v/v]; yield: 96 mg (62%), Rf = 0.46, brown solid, MP: 160-161 ^oC; FT-IR (KBr, ν, cm^{-1}): 2952, 1463,1502,1429,1348; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.23 (dd, *J* = 1.47, 8.0 Hz, 1H-a), 7.92 (br., s., 2H-b), 7.72 (ddd, *J* = 1.5, 6.9, 8.4 Hz, 1H-c), 7.38 - 7.62 (m, 5H-d), 7.25 (ddd, *J* = 1.0, 6.9, 8.0 Hz, 1H-e), 7.06 (s., 2H-f), 6.87 (d, *J* = 2.1 Hz, 1H-g), 6.51 (dd, *J* = 2.51, 8.4 Hz, 1H-h), 3.44 (br. s., 3H-CH_3-i), 3.17 (brs, 3H,CH_3-j) ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm): 148.5, 140.9, 133.6, 133.4, 131.7, 126.8, 126.7, 126.0, 121.0, 120.5, 117.3, 110.8, 107.9, 36.1 (Me), 31.3 (Me); HRMS-ESI(+) calculated for $C_{21}H_{19}BrN_5$ Exact Mass: 420.0746; found 419.0765, 421.0787 (47% : 53%) $[M+H]^+$.

(*E*)-N-(3-(3,3-dimethyltriaz-1-en-1-yl)-4-methoxyphenyl) acridin-9-amine (4d). The compound was purified by column chromatography [EtOAc:CH_2Cl_2 (5:5) v/v]; yield: 123 mg (74%,) Rf = 0.46 , brownish-black solid, MP: 246-247 ^oC, FT-IR (KBr, ν, cm^{-1}): 3032, 1603, 1443, 1321; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.90 (br., s., 2H-a), 7.50 (br., s., 3H-b), 7.40 (br., s., 2H-c), 6.85 - 7.15 (m, 2H-d), 6.96 (d, *J* = 8.6Hz, 1H-e), 6.72 (br., s, 1H-f), 6.52 (dd, *J* = 2.57, 8.6Hz, 1H-g) , 3.79 (s, 3H-OCH_3-f), 3.10 - 3.40 (brs, 6H,2CH_3- g); ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm): 148.1, 140.8, 140.1, 138.3, 133.4, 131.0, 130.3, 125.8, 122.3, 120.5, 115.4, 114.5, 107.8, 56.3, 31.1(Me), 28.2 (Me); HRMS-ESI(+) calculated for $C_{22}H_{22}N_5O$, 372.1746; found 372.1823 (100%) $[M+H]^+$.

2.9.4 General procedure for the synthesis of the methyl triazene derivatives of nitroaniline (5).

A stirred suspension of a given nitroaniline (0.3 g, 1 equiv.) in 25 mL of hydrochloric acid diluted in water in a ratio of HCl(c):H_2O (4:6) was diazotized at 0^{o}C with sodium nitrite (0.178 g, 1.2 equiv.) in water. The reaction was stirred for 45 min to a clear solution. The diazonium salt solution was treated with 40% aqueous methylamine, whereupon a triazene precipitate usually appeared (pH 7-8). The reaction mixture was quickly extracted with $CHCl_3$ (2 × 10 mL). The combined organic layers were washed with $NaHCO_3$ (4 × 10 mL), brine, dried over Na_2SO_4 (anh.) and the solvents were removed under reduced pressure to afford a red-orange oil that was used without further purification. DIEA

(1.130 mL) was added to a stirred solution of the above mentioned red-orange oil in CH_2Cl_2 (15 mL) at 0 °C followed by careful addition of $ClCO_2Me$ (0.187 mL, 1.1 equiv.) for **5a, 5c**, and PhCOCl (0.297 mL, 1.1 equiv.) was used for **5b**. After stirring for 6 h, the reaction mixture was carefully washed with dilute HCl several times until the aqueous layer reached a pH of 5. The organic layer was washed with brine, dried over Na_2SO_4 (anh.) and the solvents removed under reduced pressure to afford the crude product, which was purified by silica gel chromatography using EtOAc:petroleum ether (2:8) to give pure products.

Methyl (*E*)-1-methyl-3-(2-nitrophenyl) triaz-2-ene-1-carboxylate (5a). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 393 mg (76 %), *Rf* = 0.84, yellow liquid; MS-ESI, (m/z) = 239 (M+1, 26%), 261 (M+23, 62%)*;* ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.79 - 7.87 (m, 1H-a), 7.57 - 7.63 (m, 1H-b), 7.53 (dd, *J* = 1.28, 8.01 Hz, 1H-c), 7.40 - 7.46 (m, 1H-d), 3.98 (s, 3H-OCH_3-e), 3.46 (s, 3H,CH_3-f); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 154.5, 145.9, 142.3, 133.2, 128.6, 124.3, 120.5, 54.5, 31.0 (Me) .

(*E*)-(1-methyl-3-(2-nitrophenyl) triaz-2-en-1-yl) (phenyl) methanone (5b). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: .445 mg (72 %) *Rf* = 0.84, yellow solid, MP: 136 - 137 °C, MS-ESI, (m/z) = 284 (M+, 57%), 285 (M+1, 43%), 307 (M+23%); ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.81 (dd, *J* = 1.34, 8.07 Hz, 1H-a), 7.65 - 7.71 (m, 2H-b), 7.38 - 7.55 (m, 5H-c), 7.24 (dd, *J* = 1.34, 8.07 Hz, 1H-d), 3.63 (s, 3H-CH_3-e); ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 171.9, 146.3, 141.7, 133.9, 132.9, 131.4, 129.9, 128.7, 127.9, 124.2, 119.9, 29.3 (Me).

Methyl (*E*)-1-methyl-3-(4-nitrophenyl) triaz-2-ene-1-carboxylate (5c). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 456 mg (88.2%), *Rf* = 0.86, yellow solid, MP: 96 - 97 °C, MS-ESI, (m/z) = 239 (M+1, 29%), 260.9 (M+23, 60%), 150 (M-88, 18%); ^{1}H NMR (400 MHz, $CDCl_3$)) δ (ppm): 8.15 - 8.39 (d, *J* = 9.05 Hz, 2H), 7.67 - 7.80 (d, *J* = 9.05 Hz, 2H), 4.00 (s, 3H), 3.51 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 154.4, 153.2, 147.3, 124.7, 122.7, 54.4, 30.8 (Me).

2.9.5 General procedure for the synthesis of methyl triazene derivatives of aniline (6).

A given derivative of nitro aniline **5** (0.2 g, 1 equiv.) was added to a suspension of methyl triazene and catalytic amount of 10% Pd/C in ethanol (4 mL). The reaction was carried out under hydrogen

(1atm) for 4 h at room temp. The reaction was monitored by TLC and LCMS. After completion, the crude product was filtered through a Celite® bed and the solvent was removed under reduced pressure. After complete evaporation of the solvent, water was added, and reaction mixture extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4 (anh.) and concentrated under reduced pressure to give a crude product purified by column chromatography.

Methyl (*E*)-3-(2-aminophenyl)-1-methyltriaz-2-ene-1-carboxylate (6a). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 150 mg (86%), *Rf* = 0.54, yellow liquid; MS-ESI, (m/z) = 209 (M+1, 100%); ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.51 - 7.59 (m, 1H-a), 7.13 (ddd, *J* = 1.47, 7.15, 8.13 Hz, 1H-b), 6.69 - 6.80 (m, 2H-c), 5.03 (brs, 2H-d), 3.97 (s, 3H-f), 3.46 (s, 3H-CH_3-g); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 155.1, 141.8, 133.3, 130.1, 124.7, 117.8, 116.9, 54. (CO_2Me), 1, 29.7(Me).

(*E*)-(3-(2-aminophenyl)-1-methyltriaz-2-en-1-yl) (phenyl)methanone (6b). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 164 mg (92%), *Rf* = 0.74, yellow liquid, MS-ESI, (m/z) = 255 (M+1, 95%); ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.57 - 7.68 (m, 2H-a), 7.39 - 7.54 (m, 3H-b), 7.24 - 7.31 (m, 1H-c), 7.01 - 7.12 (m, 1H-d), 6.62 - 6.70 (m, 2H-e), 4.52 (brs, 2H-f), 3.61 (s, 3H-CH_3-g); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 172.0, 142.3, 134.9, 133.0, 130.7, 130.4, 129.3, 127.9, 122.8, 117.9, 116.7, 28.1(Me).

Methyl (*E*)-3-(4-aminophenyl)-1-methyltriaz-2-ene-1-carboxylate (6c). The compound was purified by column chromatography [ethyl acetate:petroleum ether (1:9), v/v]; yield: 164 mg (94.2%), *Rf* = 0.68, yellow solid, MP: 112 - 113 °C, MS-ESI, (m/z) = 209 (M+1, 98%); ^{1}H NMR (400 MHz, $CDCl_3$); δ (ppm): 7.40 - 7.70 (d, *J* = 8.68 Hz, 2H-a), 6.51 - 6.72 (d, *J* = 8.93 Hz, 2H-b), 6.24 - 6.88 (m, 2H-c), 3.95 (s, 3H-d), 3.43 (s, 3H-CH_3-e); ^{13}C NMR (101 MHz, $CDCl_3$)) δ (ppm): 155.2, 147.5, 140.7, 123.7, 114.9, 53.8(CO_2Me), 29.8(Me).

2.9.6 General procedure for the synthesis of methyl triazene 9-AnA derivatives (7).

Sodium hydride (1.2 equiv.) was added in small portions during aperiod of 15 min to the stirred solution of a given amine (**6**) (0.1 g, 1 equiv.), in dry DMF (6 mL) at 0 °C. Then, 9-chloroacridine was slowly added to the reaction mixture under positive nitrogen pressure. After stirring at 0 °C for 45 min the reaction mixture was kept at room temperature for 11 h. The reaction was monitored by TLC

and LCMS. After completion, the crude product was diluted with a saturated solution of NH_4Cl and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4 (anh.), filtered and the solvent removed under reduced pressure to give a product, which was later purified by column chromatography.

Methyl (*E*)-3-(2-(acridin-9-ylamino) phenyl)-1-methyltriaz-2-ene-1-carboxylate (7a). The compound was purified by column chromatography [EtOAc:CH_2Cl_2 (5:5) v/v]; yield: 136 mg, (74 %,) *Rf* = 0.53, brown solid, MP: 132 - 133 ^oC; FT-IR (KBr, ν, cm^{-1}): 3005, 1703, 1657, 1420, 1374; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.23 (br. s., 2H-a), 8.12 (d, *J* = 8.68 Hz, 2H-b), 7.70 - 7.82 (m, 3H-c), 7.44 (t, *J* = 7.40 Hz, 2H-d), 6.92 - 7.05 (m, 2H-e), 6.54 (d, *J* = 8.07 Hz, 1H-f), 3.83 (s, 3H-g), 3.57 (s, 3H-CH_3-h); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 154.8, 134.4, 131.0, 130.7, 130.6, 129.8, 129.6, 125.3, 124.2, 124.0, 122.9, 121.8, 120.1, 116.2, 54.3 (CO_2Me), 29.8 (Me); HRMS-ESI (+) calculated for $C_{22}H_{20}N_5O_2$, 386.1539; found 386.1636 (100%) $[M+H]^+$.

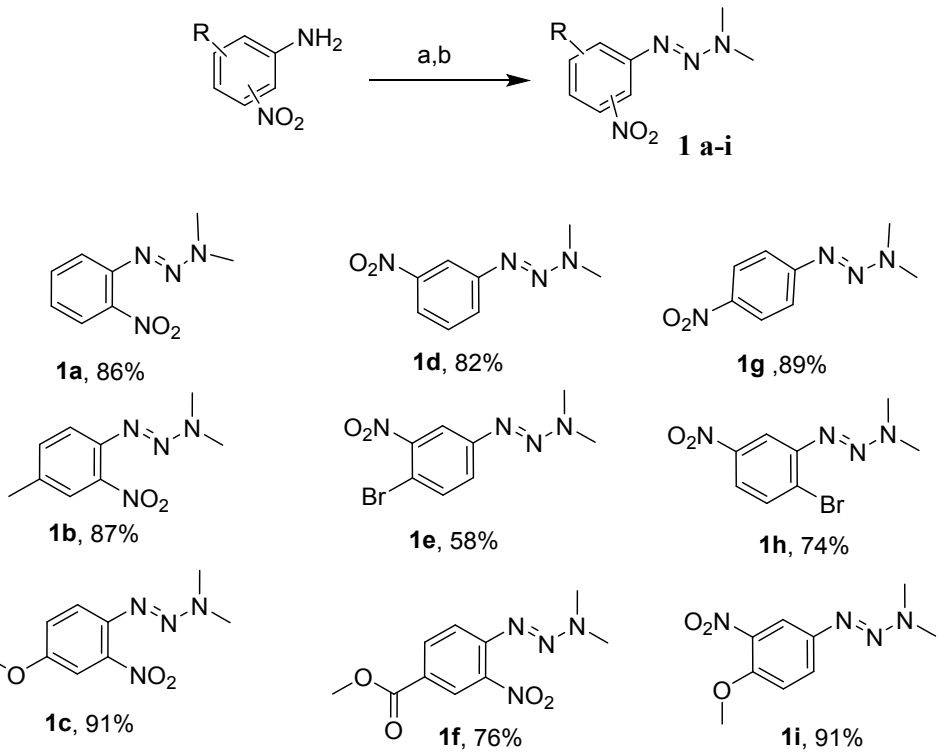
(*E*)-(3-(2-(acridin-9-ylamino) phenyl)-1-methyltriaz-2-en-1-yl)(phenyl) methanone (7b). The compound was purified by column chromatography [EtOAc:CH_2Cl_2 (5:5) v/v]; yield: 114 mg (67 %,) *Rf* = 0.57, brown solid, MP: 228 - 229 ^oC; FT-IR (KBr, ν, cm^{-1}): 2990, 1660, 1531, 1433, 1302; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.21 (d, *J* = 10.64 Hz, 1H-a), 8.09 - 8.17 (m, 2H-b), 7.87 (d, *J* = 8.68 Hz, 1H-c), 7.75 (t, *J* = 7.34 Hz, 1H-d), 7.56 - 7.63 (m, 2H-e), 7.43 - 7.56 (m, 4H-f), 7.34 - 7.41 (m, 2H-g), 7.06 - 7.16 (m, 1H-h), 6.96 - 7.05 (m, 2H-i), 6.80 - 6.96 (m, 2H-k), 3.66 (s, 3H-CH_3-l); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 172.1 (C=O), 147.2, 147.0, 146.3, 140.7, 134.5, 134.4, 131.4, 130.8, 130.6, 130.1, 129.6, 129.2, 128.4, 127.9, 125.1, 124.4, 117.8; 29.8 (Me); HRMS-ESI(+) calculated for $C_{27}H_{22}N_5O$, 432.1746; found 432.1815 (100%) $[M+H]^+$.

Methyl (*E*)-3-(4-(acridin-9-ylamino) phenyl)-1-methyltriaz-2-ene-1-carboxylate (7c). The compound was purified by column chromatography [EtOAc:CH_2Cl_2 (5:5) v/v]; yield: 144 mg, (78 %,) *Rf* = 0.54, brown solid, MP: 138 - 139 ^oC; FT-IR (KBr, ν, cm^{-1}): 2897, 1690, 1608, 1510, 1409; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.92 - 8.16 (d, *J* = 8.44 Hz, 2H-a), 7.43 - 7.64 (m, 5H-b), 7.21 (brs, 3H-c), 6.78 - 6.94 (d, *J* = 8.68 Hz, 2H-d), 3.96 (s, 3H-e), 3.46 (s, 3H-CH_3-f); ^{13}C NMR (101 MHz, $CDCl_3$)) δ (ppm): 155.1, 143.3, 139.3, 130.9, 126.0, 125.8, 124.5, 123.9, 123.7, 119.9, 119.1, 118.0, 54.0 (CO_2Me), 31.4 (Me); HRMS-ESI (+) calculated for $C_{22}H_{20}N_5O_2$, 386.1539; found 386.1636 (100%) $[M+H]^+$.

3. Results and discussion

3.1 Synthesis of dimethyl triazene nitroaryls 1a - 1i.

To demonstrate our synthetic approach, we initially synthesized dimethyl triazene nitroaryl fragments **1a - 1i** mimicking the dacarbazine bioactive core structure (Scheme 1). These building blocks are decorated with representative electron withdrawing (EWG) and electron donating (EDG) groups designed to examine their impact on the course of the acridine derivatization reaction.



Reagents and conditions: a) $NaNO_2$/HCl, 0 ^{o}C, 45 min; b) aq. 40% sol. dimethylamine.

SCHEME 1 Synthesis of the starting dimethyl triazene nitroaryls **1a-1i**

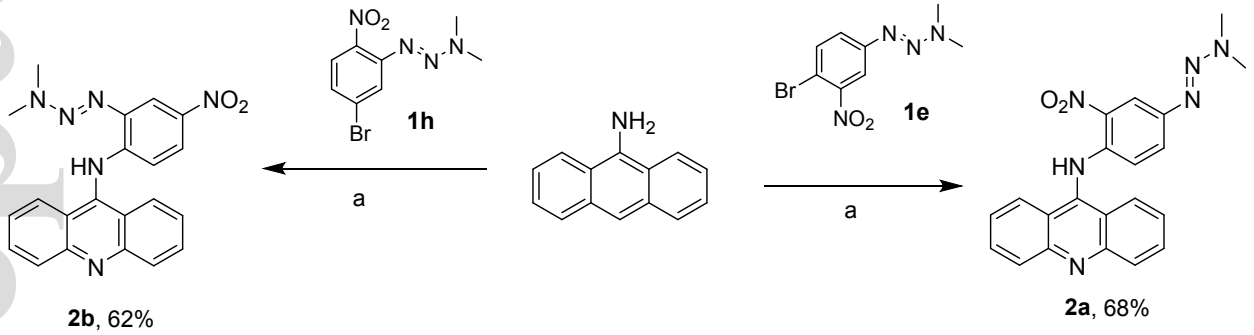
The synthesis of theses derivatives involves two consecutive steps. Diazotization of a starting nitroaniline by sodium nitrite in diluted HCl at 0 °C followed by the condensation of the corresponding diazonium salt with 40% aqueous dimethyl amine, without isolation of the

intermediate. This process led us to the desired dimethyl triazene derivatives **1a - 1i** of the corresponding nitroaryls with H, Me, OMe, CO_2Me and Br substituents at the phenyl ring.

Initially, we followed a literature procedure where neutralization of diazotation reaction mixture using sodium carbonate is followed by the addition 1.2 equiv. of 1 M solution of dimethyl amine in THF (White et al.. 1973; Diana et al. 2011). Unfortunately, the products were obtained in low yields below 20% probably due to the decomposition of the diazonium salts during the neutralization process. We modified the original procedure by skipping the neutralization step and directly adding excess 40% dimethyl amine (aq. sol.) reaching pH 8. This procedure afforded the corresponding triazene compounds **1a - 1i** in moderate to high yields (58 - 91%) (Scheme 1).

3.2 Synthesis of dimethyl triazene anilinoacridine chimeras 2a - 2b by S_NAr.

Previously, we reported on the S_NAr reaction of electrophilic haloaryls bearing one or two strongly electron-withdrawing groups to obtain antiproliferative 9-AnA derivatives (also dubbed: "reverse" approach) (Gellerman et al. 2010; Gellerman et al. 2011). Here, we employed a similar synthetic strategy to obtain two new molecular chimeras. Treatment of arylbromides **1e** and **1h** with 9-AnA in the presence of 1 equiv. of Cs_2CO_3 in DMF at 90 °C for 12 h afforded the dimethyl triazene derivatives of 9-AnA **2a** and **2b** in good yields (68% and 62%, respectively, Scheme 2).



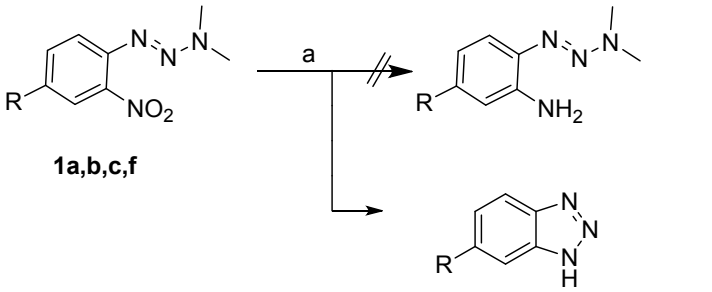
Reagents and conditions: a) Cs_2CO_3, DMF, 90 °C, 12 h.

SCHEME 2 Synthesis of dimethyltraizene 9-AnA derivatives **2a, 2b** by S_NAr reaction

Chimeras **2a** and **2b** are unlikely to be obtained using the "classical" EAS approach (Fig. 2) due to low reactivity of the putative amine group on the required anilines substituted with strong electron withdrawing nitro groups.

3.3 Synthesis of dimethyltriazene 9-AnA chimeras 4a - 4d by EAS.

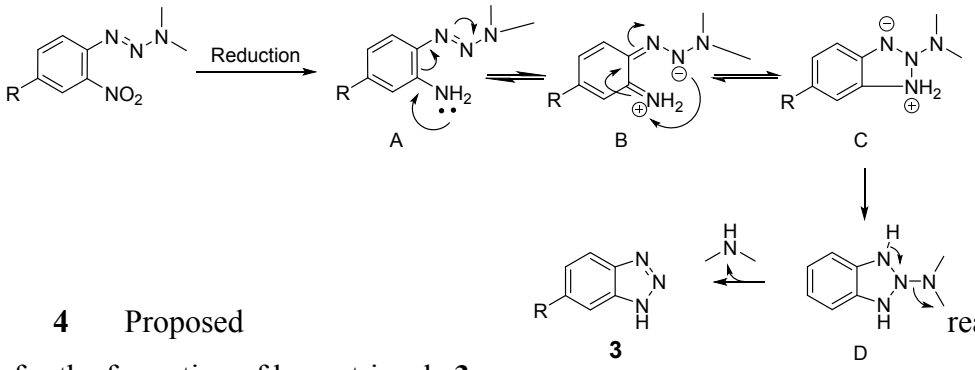
In order to further extend the scope of the library of 9-AnA dimethyl triazene hybrids, we employed the "classical" electrophilic aromatic substitution (EAS) approach, where 9- chloroacridine reacts with a nucleophilic aniline (Blaziak et al. 2016). The reduction of the nitro group in nitroaryl triazenes **1a - 1i** was planned to provide the required anilino triazene nucleophiles. However, during the reduction of **1a - 1i**, we noticed that the formation of anilino triazene derivatives from nitroaryls we observed that only the reduction of *meta*- and *para*- nitro triazenes led to the desired products. In contrast to the reduction of *ortho*-nitro triazenes resulted in a different product (Scheme 3). Based on experimental and spectral data (reaction conditions, ^{1}H, ^{13}C NMR, and MS see supportive information), we concluded that this product is the corresponding benzotriazole **3** (Scheme 3), which forms in 100% conversion from nitro triazenes **1a**, **b**, **c**, and **f** regardless of other substituents in the ring, namely electron-donating or withdrawing groups.



SCHEME 3 First attempt towards the synthesis of *o*-dimethyltriazene aniline building blocks

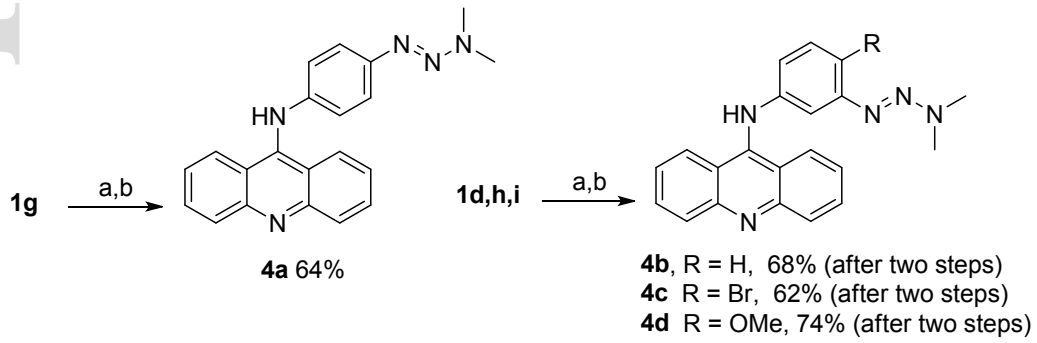
This observation has been previously documented by reports describing the triazene conversion to benzotriazole *via* an intramolecular cyclization (Kumar et al. 2011; Zhou et al. 2011). A possible

mechanism for the benzotriazole formation following the reduction of corresponding *ortho*-nitrotriazenes is depicted in Scheme 4. Initially, the *ortho*-nitrotriazene compound is reduced to an *ortho*-aminotriazene in the presence of Pd/C under a hydrogen atmosphere (1 atm) (A). Then, the amine lone pair of electrons resonates through the phenyl ring to form zwitterion B, which rapidly rearomatizes *via* intramolecular cyclization to intermediate C followed by proton transfer to the 2-triazole amine D. Finally, the formation of the aromatic benzotriazole **3** provides the driving force for the elimination of dimethyl amine.



SCHEME 4 Proposed reaction mechanism for the formation of benzotriazole **3**

We decided not to pursue the *ortho* derivatives by this approach and move forward towards the *meta* and *para* 9-AnA dimethyl triazenes **4a**, **b**, **c**, and **d**. To that end, triazene nitroaryls **1d, g, h,** and **i** were reduced using hydrogen and 10% Pd/C in EtOH. Filtration of the catalyst followed by EAS reaction with 9-Cl acridine finally led to the desired products with good yields (62-74% after two steps without isolation of intermediates, Scheme 5).

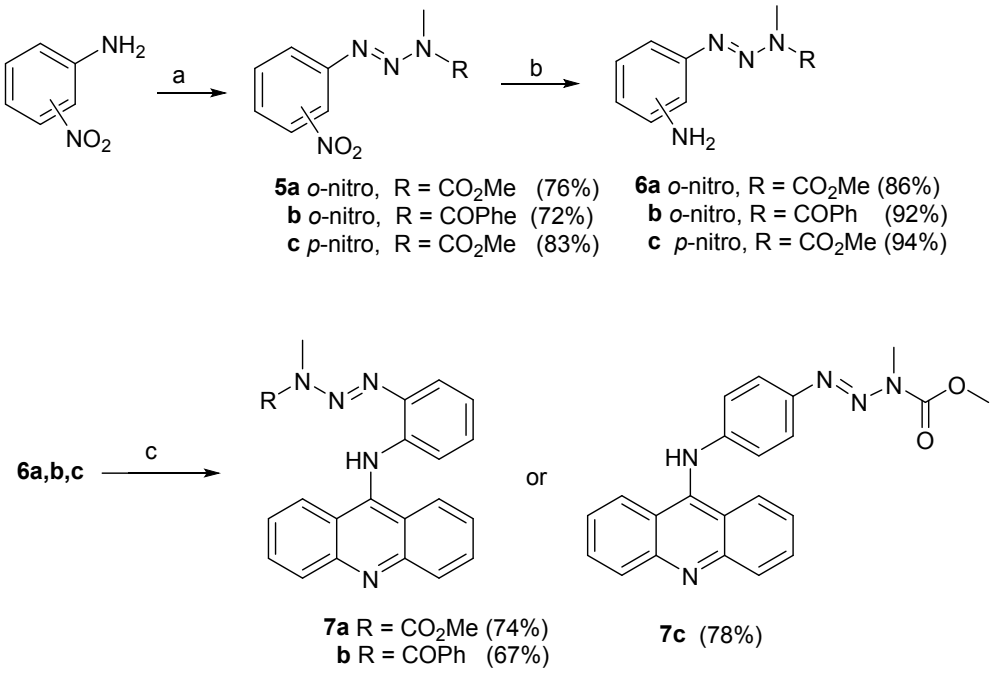


Reagents and conditions: a) 10% Pd/C, H_2 (1 atm), EtOH, r.t., 3h; b) 9-Cl acridine, DIPEA, EtOH ,r.t.,12h.

SCHEME 5 Synthesis of dimethyltriazene 9-AnA derivatives **4a - 4d** by EAS

3.4 Synthesis of protected monomethyl triazene chimeras 7a - 7c by EAS.

Temozolomide (TMZ) is an anticancer drug, which contains an embedded monomethyl triazene structural tether. The therapeutic activation of TMZ involves the spontaneous decomposition of a cyclic urea core, forming the unstable MTIC (Fig. 1), which under physiologic conditions releases diazomethane (a very reactive DNA methylating agent). Therefore, we presumed that the protection of mono methyl amine intermediates using carbamate or amide biodegradable groups (Beckerab et al. 2018) could result in molecular candidates better fit to release DNA methylating agents, similarly but with slower kinetics than TMZ (Fang et al. 2012). In addition, we realized that the *N*-protection of monomethyl triazene in the corresponding *ortho*-nitroaryls could help to overcome the formation of the un-reactive benzotriazole upon reduction of NO_2 to NH_2 as mentioned above. The *ortho*- and *para*-monomethyl triazene nitroaryls **5a**, **b,** and **c** bearing methyl carbamate and benzamide protections, respectively (Scheme 6), were prepared and subjected to catalytic hydrogenation (H_2, 10% Pd/C) directly leading to the protected *ortho* triazene anilines **6a** and **6b** and the *para* triazene aniline **6c** ready to be coupled with 9-Cl acridine. Finally, **6a - c** were reacted with 9-Cl acridine using NaH in DMF. Following this approach we were able to generate the desired 9-AnA monomethyl triazene chimeras **7a - c** in 74%, 67% and 78% yield, respectively.



Reagents and conditions: a) i. $NaNO_2$/HCl, 0 °C, 30 min., ii. aq.methylamine, iii. $ClCO_2Me$, DIPEA, DCM, 0 °C to r.t, 3h for **5a, 5c**; PhCOCl, DIPEA, DCM, 0 °C to r.t, 3h for **5b**. b) 10% Pd/C, H_2 (1 atm), EtOH, r.t., 3 h; c) 9-Cl acridine, NaH, DMF, 0 °C to r.t, 12 h.

SCHEME 6 Synthesis of protected monomethyl triazene 9-AnA chimeras **7a - 7c** by EAS

3.5 Chemostability of triazene chimeras in acetate and PBS buffers.

Chemostability studies of the three chimeras **7a**, **7b** and **7c** as well as the dimethyl triazene 9-AnAs **2a – b** and **4a - d** hybrids were performed at two pH values: 4.6 and 7.4. The results were compared to the chemostabilities of TMZ and DTIC under the same conditions. The two pHs were chosen to mimic the relatively acidic milieu present in some common cancer intracellular fluids (pH 4.6) and the slightly basic physiological environment (pH 7.4), respectively. For this study, samples of all compounds were incubated at 37 ^{o}C. Aliquots were taken at selected time intervals and were analyzed by LC-MS (Fig. 3). In PBS buffer (pH 7.4, Fig. 3A) the dimethyl triazene 9-AnAs **2a – b** and **4a - d** exhibited similar stability ($t_{1/2}$ = 10 - 15 h) as DTIC ($t_{1/2}$ = 10.5 h), while monomethyl triazene counterparts **7a - c** ($t_{1/2}$ = 4.8 - 10.6 h) and TMZ ($t_{1/2}$ = 5.5 h) decomposed faster. However, at the acidic pH (Fig. 3B) a reversed stability pattern, revealed that the protected monomethyl triazenes **7a - c** are more stable than

the dimethyl triazenes **2** and **4**. Our results are consistent with the reported stability of the control drugs TMZ and DTIC in various hydrolytic media (Mirzaei et al. **2015**). The results of these experiments also indicate very little difference in stability between methyl carbamate and benzamide degradable groups in **7a**, **7b** and **7c**. From the data we can conclude that the stability of both series of chimeras is in a comparable range as that established by TMZ and DTIC and therefore the herein described chimeras appear to be promising candidates to display potent anticancer activities.

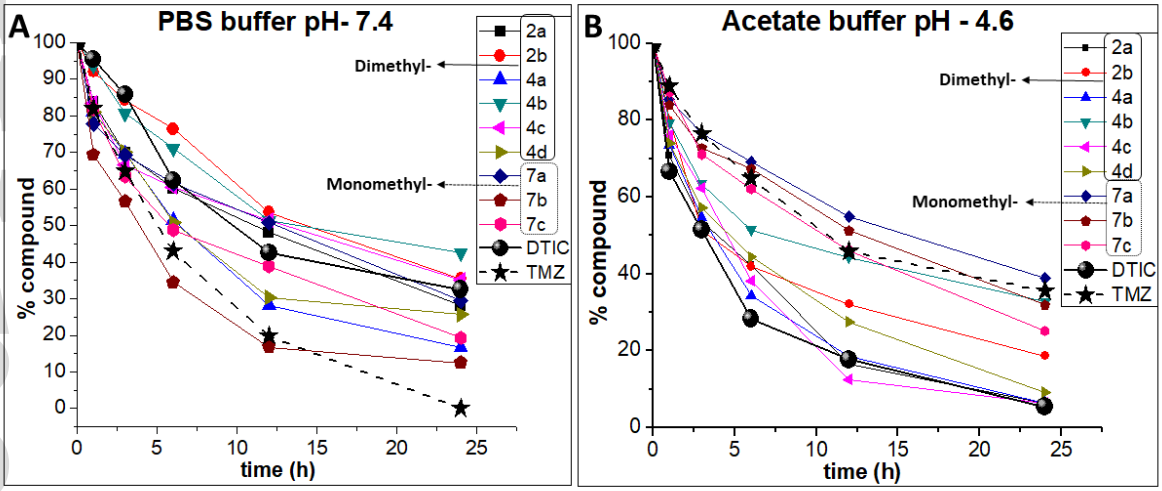


FIGURE 3 Chemostability of dimethyl triazene chimeras **2a**, **b**, **4a - 4d,** and monomethyl chimeras **7a - 7c** in PBS (pH 7.4) and acetate (pH 4.6) buffers

3.6 Cell cytotoxicity of triazene chimeras

A preliminary anticancer study to estimate the potential antitumor activity of all compounds was performed. The chimeras were subjected to a cytotoxicity assay against three cancer cell lines H1299 (NSCLC), WM266.4 (human metastatic melanoma) and HCT116 (colorectal carcinoma). HCT116 cell line expresses elevated levels of MGMT, the enzyme O(6)-methylguanine-DNA methyltransferase that repairs alkylating damage, such as that induced by temozolomide (see supporting info, Fig. S1) (Yusein-Myashkova et al. 2016; Zheng et al. 2008; van Nifterick et al.

2010). The cells were exposed to increasing doses of the compounds, and after 48 h or 72 h of incubation, metabolic activities of the cells were measured using the XTT assay. Antiproliferative tests of compounds **2, 4** and **7** identified them as bioactive chimeras against all cancer cell lines in the micromolar range. These compounds exhibited increased cytotoxic effects compared to Amsacrine and AHMA (Table 1). In general, protected monomethyl triazenes **7** were more active that dimethyl triazene derivatives **2** and **4**, probably because they do not require activation by cytochrome P450 of the dimethyltriazene tether, which occurs only *in vivo*. Among triazenes **7**, compound **7b** is of special interest. This chimera exhibited the highest anticancer activity against H1299 and WM266.4 cancer cell lines (over ten times more active than Amsacrine and AHMA) with IC_{50} values of 2.9 μM and 0.8 μM after 72 h of incubation, respectively. HCT116 cell line was, in general, the most resistant to anticancer treatment. Hybrid **7b** also showed the highest cytotoxic effect on this cell line compared to all other tested candidates and control drugs. Such elevated activity could be the result of a dual action mechanism only forged ahead by a chimera, acting both as a DNA intercalating and alkylating agent. Additional testing to support this idea was performed, see below. The fact that **7b**, as well as all other tested compounds, is less effective on HTC116 can be attributed to its MGMT associated DNA repairing activity. Notably, TMZ was not active in all *in vitro* experiments at the tested concentrations (IC_{50} > 100) (Le Calve et al. 2010). The results pinpoint **7b** as a potent anticancer chimeric candidate to be considered for further development.

TABLE 1 IC_{50} (half maximal inhibitory concentration) values (μM) for **2a - b**, **4a - d**, **7a - c**, AHMA and Amsacrine in H1299, WM266.4 and HTC116 cell lines for 48 h and 72 h incubation. IC_{50} values were calculated using three-parameter non-linear regression. IC_{50} values marked with * were calculated using four-parameter non-linear regression. N/A means IC_{50} values and shifts could not be reliably calculated

Comp. / Cell line	2a	2b	4a	4b	4c	4d	7a	7b	7c	Amsacrine	AHMA	TMZ
H1299-48h	27.7 (+0.12)	28.6 (+0.95)	22.2 (+0.18)	31.4* (+0.41)	27.6 (+0.09)	N/A	24.6 (+0.84)	5.3 (+0.23)	25.2 (+1.02)	25.8 (+2.31)	36.9* (+0.77)	>100

H1299-72h	17.1 (+0.26)	18.2 (+0.18)	12.3 (+0.35)	21.3 (+0.46)	20.5 (+1.89)	33.2 (+1.17)	14.4 (+0.76)	**2.9 (+0.43)**	5.6 (+0.62)	16.5 (+0.03)	17.7 (+0.80)	>100
WM264.4-48h	35.1 (+0.33)	32.3 (+1.52)	46.8 (+0.74)	28.6 (+0.68)	29.9 (+0.73)	13.5 (+0.86)	27.7 (+0.12)	3.6 (+0.48)	13.5 (+0.54)	20.2 (+1.46)	24.3 (+2.30)	>100
WM264.4-72h	28.3 (+0.49)	19.8 (+0.78)	35.1 (+0.81)	18.5 (+1.37)	17.0 (+0.11)	N/A	9.4 (+1.19)	**0.8 (+0.37)**	12.8 (+0.94)	11.5 (+0.98)	15.8 (+1.23)	>100
HCT116-48h	N/A	48.4 (+3.44)	57.1 (+2.32)	N/A	N/A	45.3 (+1.97)	N/A	30.8 (+3.56)	46.0 (+3.69)	36.8 (+0.92)	41.2 (+1.73)	>100
HCT116-72h	N/A	41.1 (+2.35)	38.7 (+3.63)	51.3 (+4.49)	48.0 (+2.92)	37.1 (+1.28)	46.9 (+2.76)	**15.4 (+1.68)**	37.5 (+1.05)	31.9 (+1.33)	32.2 (+2.41)	>100

3.7 Western blot analysis of 7b

Based on the preliminary antiproliferative studies we decided to take a closer look at hybrid **7b** to confirm its anticancer activity and assess its mode of action. Western blot analysis to define O(6)-Methylguanine DNA methyltransferase (MGMT) protein levels in the HTC116 cell line was performed in the presence of **7b**. MGMT is a DNA repairing enzyme that removes the mutagenic alkyl adducts introduced at the O(6) position of guanine and the O(4) position of thymine introduced by various exogenous and endogenous agents (Kaina et al. 2007). Unlike other DNA repairing pathways, human MGMT works as a single protein to remove the guanine O(6) bound alkyl groups by the nucleophilic action of the thiol group on cysteine145 located in its active site (Hegi et al. 2008). Since the amount of MGMT determines repair level of DNA alkylated adducts, the MGMT expression level provides important information about cancer susceptibility and the chances of success of potential drugs based on alkylating mechanisms of action (Christmann et al. 2011). Temozolomide (TMZ) and Dacarbazine (DTIC) belong to a class of drugs known as alkylating agents. The efficacy of treatment with these agents may be limited by inherent or developed resistance, particularly, but not exclusively, due to the expression of MGMT in a significant proportion of tumors, specifically in glioblastoma (Fan et al. 2013). In order to study the effect of chimera **7b** on the level of MGMT we treated HCT116 cells with 100 μM of TMZ, DTIC and chimera

7b for 48 h. Lysates from compound-treated cells were subjected to Western blot analysis for MGMT protein detection. Compared with the two controls, TMZ and DTIC, we found that chimera **7b** was more effective in reducing the expression of MGMT by 50% (Fig. 4). This study indicates that chimera **7b** promotes DNA methylation with certainty. In order to demonstrate that **7b** may rely on a dual action mechanism to exert its enhanced antitumor activity we also investigated its ability to inhibit the enzymatic activity of Topo II.

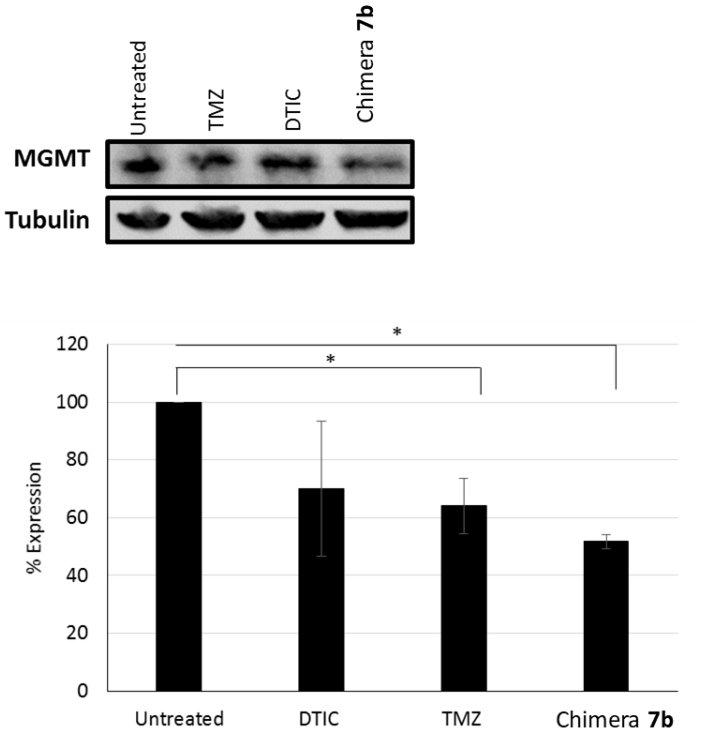


FIGURE 4 HCT-116 cells were treated with DTIC, TMZ and Chimera **7b** in concentration of 100 µM for 48 h. Protein samples were prepared and resolved by SDS-PAGE. MGMT and tubulin (loading control) antibodies were used to detect protein levels. The graph represent the quantification of the protein level as evaluated by image studio software. Values represent a mean of at least 3 experiments. *P-value < 0.05. DTIC - Dacarbazine, TMZ - Temozolomide

3.8 Chimera 7b inhibits enzymatic activity of Topo II

AHMA is an anticancer agent that reversibly binds at the minor groove of DNA and stabilizes the Topo II DNA complex interfering with its enzymatic activity (Delgado et al. **2018**). Chimera **7b** is a structural combination of AHMA and a triazene moiety. The modification of the AHMA scaffold may affect its original DNA-intercalation and DNA-Topo II stabilization effectiveness. The Topo II inhibitory activities of AHMA and chimera **7b** were evaluated employing the kDNA decatenation enzymatic assay. In this assay, catenated DNA (kDNA) remains at the top of the electrophoretic gel, whereas the smaller decatenated DNA, minicircles, produced from enzymatic activity of Topo II runs to the bottom of the gel. The inhibitory activity of the two compounds were assessed at 25 μM and 100 μM. At the higher concentration both compounds showed no development of minicircles, corroborating that AHMA and **7b** strongly inhibit Topo II. At the lower concentration, AHMA was not capable to fully repress the formation of minicircles while **7b** showed no visible signs of them (Fig. 5). The results of the kDNA decatenation enzymatic assay indicates that chimera **7b** is a stronger inhibitor of Topo II than AHMA.

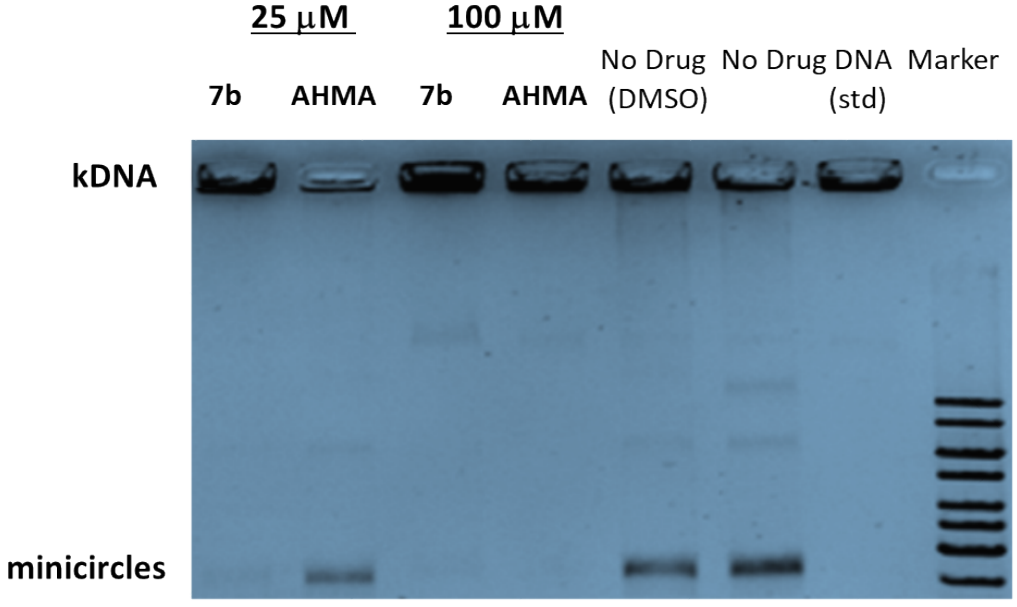


FIGURE 5 Topo II enzymatic inhibition by **7b** and AHMA. Chimera **7b** or AHMA were added to a cell-free lysate containing Topo II and DNA. After incubation the products were separated by agarose

electrophoresis. The upper bands are catenated DNA (due to enzyme inhibition) while the lower bands (minicircles) are decatenated DNA

4. Conclusions

In summary, we developed molecular chimeras toward dual-action anticancer agents. These chimeras consist of a DNA intercalating core tethered to a methyl triazene DNA methylating moiety. An efficient preparation method of aromatic triazene synthetic fragments, followed by their EAS coupling with 9-chloroacridine or S_NAr with 9-aminoacridine, afforded novel molecular chimeras in good yields. Chemostability studies indicated that the chimeras are stable enough to perform as anticancer agents. At physiological pH dimethyl triazenes are more stable than the monomethyl counterparts protected by amide or carbamate degradable groups. In acidic media, the dimethyl triazene derivatives were found to be the less stable bunch. An *in vitro* assay revealed benzamide monomethyl triazene **7b**, which efficiently inhibits growth of MGMT deficient H1299 and WM266.4 cancer cell lines after 72 h incubation with IC_{50} values of 2.9 μM and 0.8 μM, correspondingly, as a promising dual-action candidate to further studies. Positive results from MGMT western blot and Topo II inhibition studies indicate that chimera **7b** may exert its antiproliferative effect either by DNA alkylation or via DNA intercalation. The two inhibitory mechanisms may work independently or, most likely, in a coexistent synchronized fashion. Chimera **7b** was identified as a potent dual-action anticancer candidate for preclinical studies.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Conflict of interest -The author declares no conflict of interests.

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