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Synthesis and primary cytotoxicity evaluation of new diaryltriazenes

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A series of triazenes derived from 5-(4-aminophenyl)-2,4-dihydro–4-substituted-3*H*-1,2,4-triazole-3-thiones **1a-c**, aminoglutethimide or *para*-aminobenzoic acid have been synthesized for *in vitro* anticancer properties against three cell lines. The selected compounds; 1,3-bis[4(4-methyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thioxo-5-yl)phenyl]-3*H*-triazene **2a,** 1,3-bis[4(4-ethyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thioxo-5-yl)phenyl]-3*H*-triazene **2b,** 1,3-bis[4(4-allyl-2,4- dihydro- 3*H*-1,2,4- triazole- 3- thioxo- 5- yl) phenyl]- 3*H*– triazene **2c** and 1-[(4-carboxy)phenyl]-3-[4(4-allyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thioxo-5-yl)phenyl]-3*H*triazene **4** show significant activity but not 1,3-bis[4-(3-ethyl-2,6–dioxo–3-piperidnyl)phenyl]-3*H*-triazene **3**. **2a-c** and **4** that pass the criteria for activity in this assay have been scheduled automatically for evaluation against the full panel of 60 human tumour cell lines from leukemia, melanoma, lung, colon, kidney, ovary, breast, prostate and central nervous system cancer at a minimum of five concentrations at 10-fold dilutions. Sulphorhodamine B (SRB) protein assay has been used to estimate cell viability or growth. Compounds **2a-c** and **4** show variable antitumor activity against most of the tested sub-panel tumor cell lines. The $\log_{10}GI_{50}$ values of these compounds are comparable to values of dacarbazine and mitozolamide based anticancer agents.

Keywords: Diaryltriazenes, anticancer screening

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The rapid spread of cancer has sparked an intense worldwide search for new compounds which may be use in designing antitumor drugs. Arylakyltriazenes have been a subject of study by medicinal chemists and biologists for many years. Dacarbazine; [5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide] which is used in the treatment of malignant melanoma is the first generation of arylalkyltriazenes[1-3]. The development of second generation triazene, prompted by the dose-limiting side effects of dacarbazine, has led to the clinical trial of 4-(3,3-dimethyltriazen-1-yl) benzoic acid[4-8]. A number of cyclic arylmonoalkyltriazenes, 8-carbamoyl-3-alkylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3*H*)-one, have been designed as potential therapeutic alternatives to dacarbazine. Their antitumor activity observed in several murine tumor models has been reported to be comparable with or superior to that of dacarbazine[9-12].

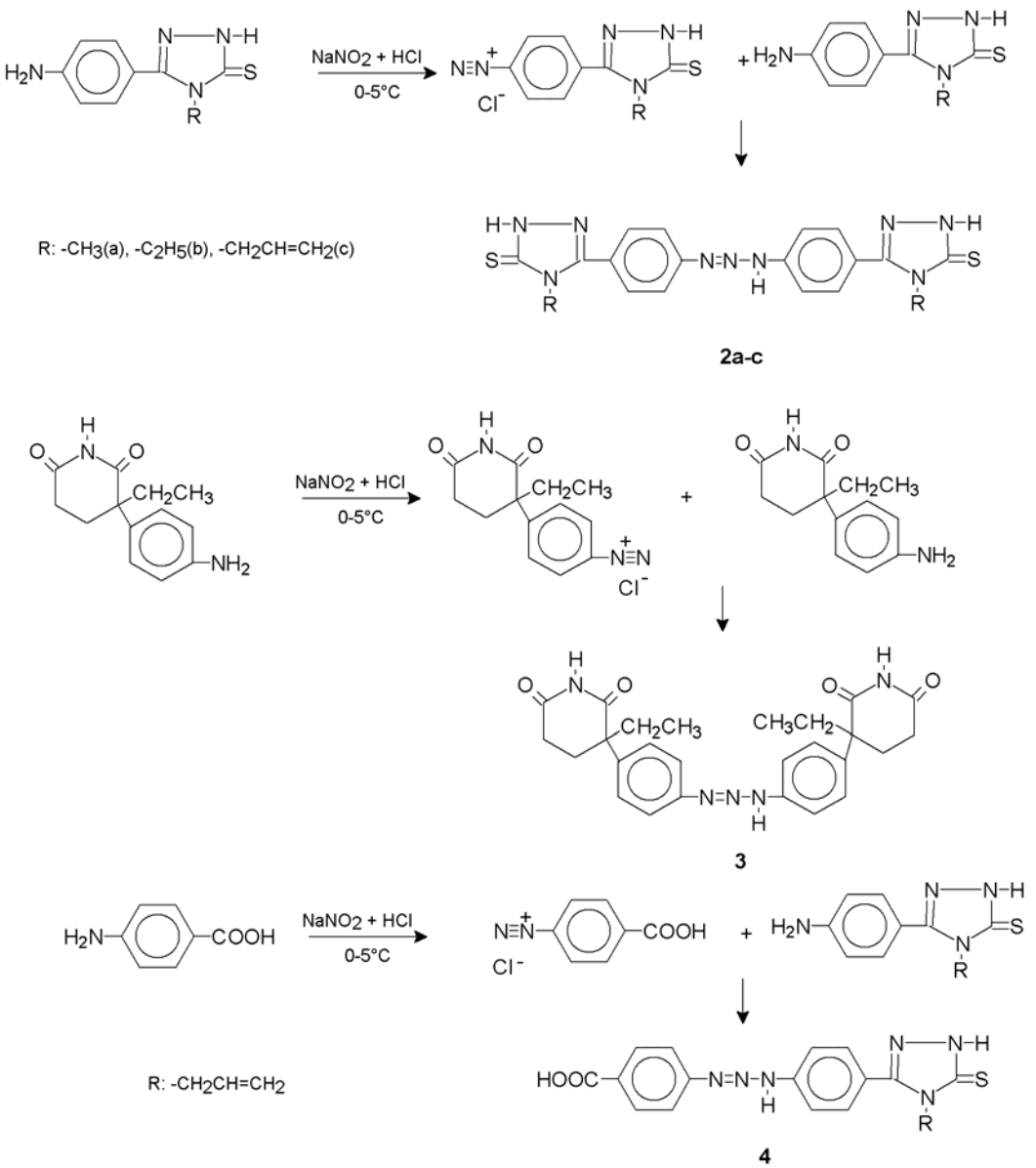
The search for more effective anticancer agents has focused to a large extent on the design of molecules capable of recognizing and binding to target DNA base sequences. Structural and biophysical studies of the antitryponosomal agent, berenil [bis(4-amidinophenyl)-1,3-triazene], have shown that the molecule binds in a DNA duplex minor groove with a preference for Adenine/Timine-rich base tracts[13-17]. The drug design strategies outlined have proved remarkably successful in developing agents. Herein is now described the synthesis, structure properties and cytotoxicity evaluation of a new series of diaryltriazenes derivatives (**Scheme I**). Compounds **2a-c, 3** and **4** were evaluated for their antitumor activity following the NCI screening program[18-21].

Results and Discussion

Chemistry

5-(4-Aminophenyl)-2,4-dihydro–4-substituted-3*H*-1,2,4-triazole-3-thiones **1a-c** were prepared as described previously[22]. **1a-c,** aminoglutetimide, *para*-aminobenzoic acid containing an aromatic primary amine function, were converted to a diazonium cation under nitrite and acid condition and the latter coupled with the unprotonated amino group of second molecule to give compounds **2a-c, 3** and **4** respectively.

The chemical structures of these compounds were confirmed by UV, IR, ^{1}H NMR and MS data in experimental section. In the IR spectra, all of these



Scheme I — The synthesis of target compounds

compounds **2a-c, 3, 4** showed a strong band between 3401-3499 $cm^{-1,}$ which is a characteristic of NH band. Imide and carboxyl C=O stretching bands were observed at 1699 and 1684 cm^{-1} respectively. 1,2,4-Triazolin-3-thiones showed C=S stretching bands in the 1335-1363 cm^{-1} region.

^{1}H NMR spectrum of diaryltriazenes showed no signals which were attributable to the amine at δ 3-5 (Ref. 22). 1,2,4-triazole-3-thione derivatives may exist in thiole and thione tautomeric forms[23]. The presence of a peak due to the NH function of the triazoline ring between δ 13.40-14.10 supported the thione structure[22,23]. NH protons of triazene function group of **2a, 2c** resonated between δ 12.80-13.10,12.87 respectively as a broad singlet or lower field with protons of triazoline NH protons as a broad singlet. The two NH protons of the triazene and triazole ring were seen together as broad singlet between δ 13.40-13.90 for compound **2b** and NH protons of triazoline ring were observed between δ 13.90-14.10 for compound **4**. The signals of the carboxyl protons of **4** were observed between δ 12.80-13.10 with triazene NH proton as a broad singlet. Compound **3** showed a singlet at δ 10.90 and 12.60 corresponding to proton of piperidindion NH,and triazene NH proton respectively.

ESI mass spectra of the compounds confirmed their molecular weights and showed characteristic fragment ions, loss of a hydrogen radical and the further elimination of 28 (N_2), M^+-29 (Ref. 24). In addition, ESI mass spectra of the synthesized compounds exhibited the expected fragmentation pattern of triazoline and aminoglutetimide[25].

Cytotoxicity Evaluation

Compounds **2a-c, 3** and **4** were evaluated for their antitumor activity following the NCI screening program[18,19]. They were evaluated in the three-cell line, NCI-H460 (Lung), MCF7 (Breast), SF-268 (CNS), at 10^{-4} M concentration primary anticancer assay. Compounds were added at a single concentration (10^{-4} M) and the culture was incubated for 48 hr. End point determinations were made with a protein binding dye, sulphorhodamine B (SRB). Results for each compound were reported as the percent of growth of the treated cells when compared to the untreated control cells. All the compounds which reduced the growth of any one of the cell lines to 32% or less (negative numbers indicate cell kill) were passed on for evaluation in the full panel of 60 human tumour cell lines (**Table I**). Compounds **2a-c** and **4** were considered active. The cytotoxic and/or growth inhibitory effects of the these compounds were tested *in vitro* against the full panel of 60 human tumour cell lines organised into subpanels representing leukemia(I) lines CCRF-CEM, HL60(TB), K-562, MOLT-4, RPMI-8226, SR; Non-small cell lung cancer (II) lines A549/ATCC, EKVX, HOP-62, HOP-92, NCI-H226, NCI-H23, NCI-H322M, NCI-H522; colon cancer (III) lines COLO205, HCT-116, HCT-15, HT29, KM12, SW-620; CNC cancer (IV) lines SF-268, SF-295, SNB-19, SNB-75, U251; melanoma (V) lines LOX IMVI, MALME-3M, M14, SK-MEL-2, SK-MEL-28, SK-MEL-5, UACC-257; ovarian cancer (VI) lines IGROV1, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, SK-OV-3; renal cancer (VII) lines 786-0, A498, ACHN, CAKI-1, RXP-393, SN-12C, TK-10, UO-31; prostate cancer (VIII) lines PC-3, DU-145; breast (IX) lines MCF7, NCI/ADR-RES, MDA-MB-231/ATCC, HS 578T, MDA-MB-435, MDA-N, BT-549, T-47D at 10-fold dilutions of five concentrations ranging from 10^{-4} to 10^{-8} M. The percentage growth was evaluated spectrophotometrically versus controls not treated with the test agents. A 48 hr continuous drug exposure protocol was followed and a SRB protein assay was used to estimate cell viability or growth. For each compound, calculations have been made for the concentration of drug causing the 50% growth inhibition (GI_{50}), total growth inhibition (TGI), and 50% cell kill (LC_{50}). The $\log_{10}GI_{50}$ and $\log_{10}TGI$ were then determined, defined as the mean of the log10's of the individual GI_{50} and TGI values. Negative values indicated the most sensitive cell lines. Compounds having values –4 and <-4 were declared to be active. Compounds **2a-c** and **4** showed variable antitumor activities against most of the tested sub-panel tumor cell lines. Assay results were reported as both dose-response curves (**Figure 1**) and pictographically as mean graphs (**Figure 2**).

Compounds **2a, 2b** and **2c** were higly active in the *in vitro* screen on a Leukemia cell lines (CCRF-CEM, HL60-(TB), K-562, MOLT-4, RPMI-8226 and SR) that they were reached lower than –5 of $\log_{10}GI_{50}$ level. Compound **2a** was higly active against non small cell lung cancer cell lines (HOP-92, $\log_{10}GI_{50}$ value –5.54; NCI-H226, $\log_{10}GI_{50}$ value –5.27; NCI-H23, $\log_{10}GI_{50}$ value –5.11; NCI-H522, $\log_{10}GI_{50}$ value –5.39), CNS cancer cell line (U-251, $\log_{10}GI_{50}$ value –4.88), melanoma cell lines (M14, $\log_{10}GI_{50}$ value –5.05; SK-MEL-5, $\log_{10}GI_{50}$ value –5.52), ovarian cancer cell lines (OVCAR-8, $\log_{10}GI_{50}$ value –5.34), renal cancer cell lines (ACHN, $\log_{10}GI_{50}$ value –5.70; UO31 $\log_{10}GI_{50}$ value –5.55) and prostate cancer cell line (DU-145, $\log_{10}GI_{50}$ value –4.89). On these cell lines the $\log_{10}GI_{50}$ values of dacarbazine and mitozolamide used as anticancer agent was found between –4 and -4.7. In addition, the compounds **2b** and **2c** were the most active on all cancer cell lines. Out of these the $\log_{10}GI_{50}$ values were lower than –5, except CNC cancer cell line (SNB 19), ovarian cancer cell lines (OVCAR-5, SK-OV-3), renal cancer cell lines (A498, RXF 393, TK-10). Especially, they demostrated the most marked effects on Breast cell line; T-47D -6.06 for **2b** and -5.95 for **2c** respectively.

Table I — The 3-cell line (Lung; NCI-H460, Breast; MCF7, CNS; SF-268), one dose (10^{-4} M) primary anticancer assay results

Compd	Concentration	Growth percentage			Activity
		Lung NCI-H460	Breast MCF7	CNS SF-268	
2a	10^{-4} M	31	30	-5	Active
2b	10^{-4} M	34	9	-59	Active
2c	10^{-4} M	0	16	-69	Active
3	10^{-4} M	44	44	52	Inactive
4	10^{-4} M	33	29	15	Active

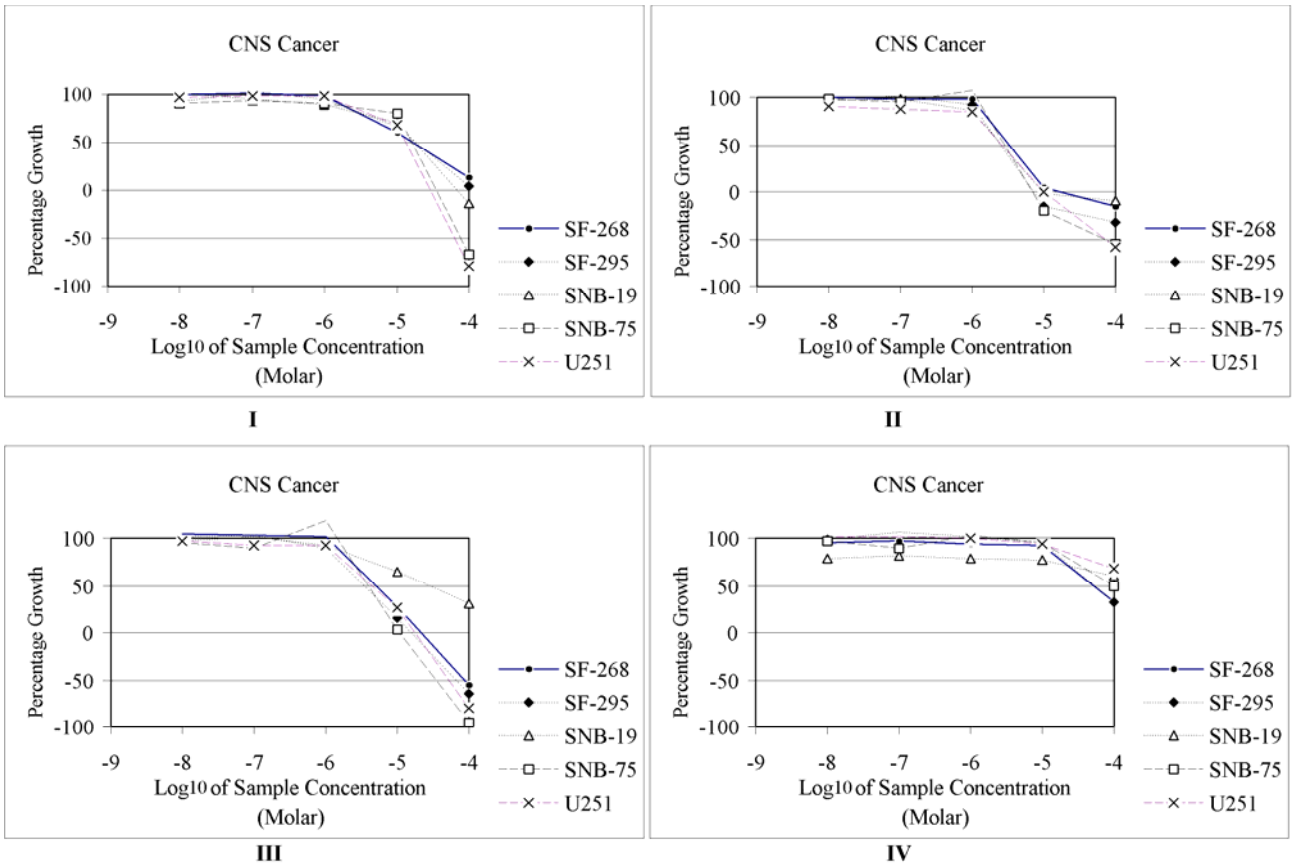


Figure 1 — Dose-response curves of compound **2a (I)**, compound **2b (II)**, compound **2c (III)** and compound **4 (IV)**. The cell line curves are grouped by subpanel. Horizontal lines are provided at the PG (Percentage Growth) values of +50.0 and – 50.0. The concentrations to points where the curves cross this lines are the GI_{50}, TGI and LC_{50} respectively.

When the results of compounds **2a, 2b** and **2c** were compared with dacarbazine and mitozolamide, ıt was speculated that the cytotoxicities of these compounds were comparable to those of anticancer agents. The compound **4** showed only the marked effects on Breast cancer cell lines (BT-549, $\log_{10}GI_{50}$ value -4.57; T-47D, $\log_{10}GI_{50}$ value –4.62) and both were lower than that of dacarbazine (-4) and mitozolamide (-4). The dose-response curves in **Figure 1** was created by plotting the percentage growths (PG) against the $\log_{10}$ of the corresponding concentration for every cell line. The activity of the tested compounds could be tentatively correlated to the structure variations and modifications. The decreased activity of compound **4** compare to compounds **2a-c** might be related to the hydrophilic nature of carboxyl group. As can be seen in **Figure 1**, the most active compounds among diaryltriazenes were **2a-c** derived from 1,2,4-triazole-3-thiones. In conclusion, these preliminary results are promising and some of these compounds may be potential candidates for new anticancer agents.

Experimental Section

Melting points were determined with a Buchi (B-530) apparatus and are uncorrected. Elemental analyses were performed with Leco CHNS-932 elemental analyzer (Turkish Scientific and Technical Institue). UV-Vis spectra were recorded on a Shimadzu 2100S UV-Vis spectrophotometer. IR spectra were recorded on a Perkin Elmer 1600 spectrometer as KBr pellets. ^{1}H NMR spectra were recorded on Brucker AC 200 (Münster, Germany) at 200 MHz using DMSO-d_6 (E.Merck, Darmstadt, Germany) as the solvent. ESI (Electron Spray Ionisation) were recorded at Westfalische-Willhelms Universitat Münster, Germany. Aminoglutetimide and *para*-aminobenzoic acid were supplied from Novartis and Merck respectively. All organic solvents used were purchased from Merck (Darmstadt, Germany).

General synthesis of 5-(4-aminophenyl)-2,4-dihydro-4-substituted-3H-1,2,4-triazole-3-thiones, 1a-c.

1-[4-(Acetylamino)benzoyl]-4-substituted thiosemicarbazides (0.005 mole) were refluxed in 5 mL of

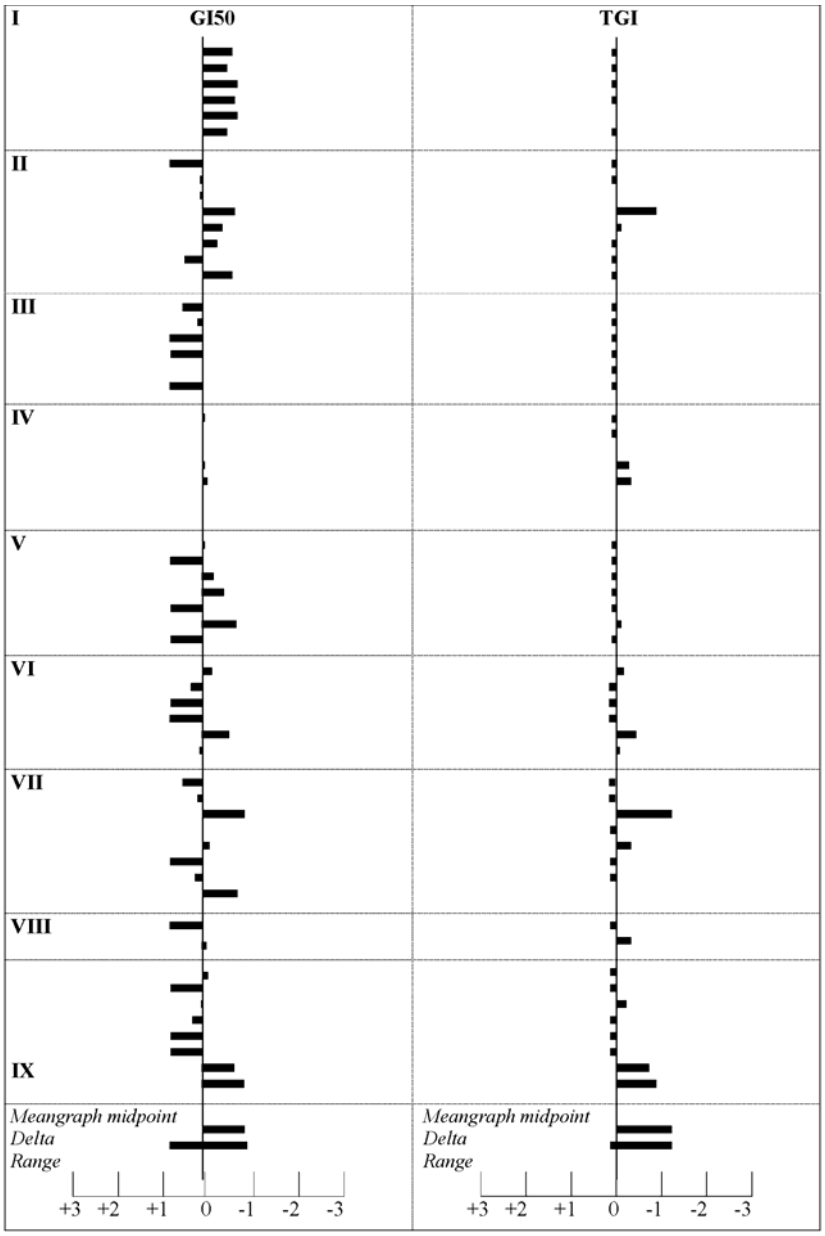


Figure 2 — GI_{50} and TGI-based mean graphs from screening of compound **2a** in the NCI human tumor cell line panel. The tumor cell line subpanels are identified as follows: I (Leukemia); II (lung,non-small-cell); III (colon); IV (CNS); V (melanoma); VI (ovarian cancer); VII(renal); VIII (prostate); IX (breast). The individual cell lines in all subpanels are identified in the Result and Discussion. Bars projecting to the right of the center lines present cell lines which are more sensitive to the compound, whereas bars projecting to the left of the center lines represent those that are less sensitive

NaOH (2*N*) for 4 hr. The solution was allowed to cool and then acidified with HCl (37%). The precipitate formed was filtered and washed with water and purified by recrystallization from ethanol[22].

Synthesis of 1,3 bis [4(4-substituted-2,4-dihydro-3*H*-1,2,4-triazole-3-thioxo-5-yl)phenyl]-3*H*-triazene, 2a-c.

1,3 bis [4-(3–ethyl-2,6–dioxo–3-piperidnyl)-phenyl]3*H*-triazene, 3.

Compounds of **1a-d** (0.002 mole) and amino-glutetimide (0.002 mole) were dissolved 37% HCl (1 mL). The solution was cooled to 0°C and treated portionwise, over 20 min, with sodium nitrite (0.002 mole); the mixture was stirred at 0°C for 15 min, then carefully poured into cooled methanolic solutions of

1a-c and aminoglutetimide (0.002 mole) The mixture was stirred at 0°C for 1 hr and then the coloured precipitate was washed with hot ethanol, methanol and ethylacetate.

2a: UV-Vis (nm): 203, 256, 376; IR (KBr): 3401 (N-H), 3102 (C-H), 1335 cm^{-1} (C=S); ^1H NMR(DMSO-d_6): 3.57 (s, 6H, CH_3), 7.65 (d, 4H, *m*-position to triazoline Ar-H), 7.80 (d, 4H, *o*-position to triazoline Ar-H), 12.80-13.10 (bs, 1H, triazen NH), 13.84 (s, 2H, triazolin NH); ESIMS: *m/z* (%) 423 (M^+,45), 422 (100), 394 (43); m.p. 212-15°C. Yield 66%. Anal. Calcd. for $C_{18}H_{17}N_9S_2.H_2O$: C, 48.96; H, 4.34; N, 28.55. Found: C, 49.65; H, 4.58; N, 27.80%.

2b: UV-Vis (nm): 202, 257, 375; IR (KBr): 3421 (N-H), 3106 (C-H), 1363 cm^{-1} (C=S); ^1H NMR(DMSO-d_6): 1.19 (t, 6H, CH_2CH_3), 4.10 (q, 4H, - CH_2CH_3), 7.65 (d, 4H, *m*-position to triazoline Ar-H), 7.74 (d, 4H, *o*-position to triazoline Ar-H), 13.40-13.90 (bs, 3H, triazene and triazolin NH); ESIMS: *m/z* (%) 451 (M^+, 26), 450 (100), 422 (12); m.p. 210-14°C. Yield 33%. Anal. Calcd. for $C_{20}H_{21}N_9S_2.1.5H_2O$: C, 50.19; H, 5.05; N, 26.34. Found: C, 49.79; H, 4.71; N, 26.03%.

2c: UV-Vis (nm): 202, 257, 376; IR (KBr): 3499 (N-H), 3170 (C-H), 1346 cm^{-1} (C=S). ^1H NMR(DMSO-d_6): 4.74 (d, 4H, CH_2CHCH_2), 4.96 (d, 2H, CH_2CHCHa *J*=17Hz *trans*), 5.14 (d, 2H, CH_2CH=C*Hb* J=10Hz *cis*), 5.81-5.94 (m, 2H, CH_2CH=CH_2), 7.42-7.82 (d, 8H, Ar-H), 12.87 (s, 1H, triazen NH), 13.91 (s, 2H, triazolin NH); ESIMS: *m/z* (%) 475 (M^+, 28), 474 (100), 446 (11); m.p. 198-201°C. Yield 54%. Anal. Calcd. for $C_{22}H_{21}N_9S_2.H_2O$: C, 53.53; H, 4.70; N, 25.54. Found: C, 52.99; H, 3.96; N, 25.30%.

3: UV-Vis (nm): 208, 239, 357; IR (KBr): 3467 (N-H), 3210 (piperidine N-H), 1699 cm^{-1} (C=O); ^1H NMR(DMSO-d_6): 0.71 (t, 6H, CH_2CH_3), 1.80-1.90 (m, 8H, CH_2CH_2), 2.20 (q, 4H, CH_2CH_3), 7.20-7.60 (d, 8H, Ar-H), 10.90 (s, 2H, piperidindion NH), 12.60 (s, 1H,triazen NH); ESIMS: *m/z* (%) 475(M^+,31), 474 (100); m.p. 170°C. Yield 42%. Anal. Calcd. for $C_{26}H_{29}N_5O_4.1.25H_2O$: C, 62.70; H, 6.37; N, 14.06. Found: C, 62.81; H, 6.4; N, 13.79%.

1-[(4-carboxy)phenyl]-3-[4(4-allyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thioxo-5-yl)phenyl]-3*H*-triazene, 4.

para-Aminobenzoic acid (0.002 mole) was dissolved 37% HCl (1 mL). The solution was cooled to 0°C and treated portionwise, over 20 min, with sodium nitrite (0.002 mole); the mixture was stirred at 0°C for 15 min, then carefully poured into cooled methanolic solutions of 5-(4-aminophenyl)-2,4-dihydro-4-allyl-3*H*-1,2,4-triazole-3-thiones **1c** (0.002 mole). The mixture was stirred at 0°C for 1 h and then the coloured precipitate was washed with hot ethanol, and petroleum ether respectively.

4: UV-Vis (nm): 205, 256, 372; IR (KBr): 3401 (NH, OH), 3140 (C-H), 1684 (C=O),1346 cm^{-1} (C=S); ^1H NMR (DMSO-d_6): 4.75 (d, 2H, CH_2CHCH_2), 4.94 (d, 1H, CH_2CHCHa *J*=,17Hz *trans*), 5.19 (d, 1H, CH_2CH=CHb *J*= 10Hz *cis*), 5.81-5.89 (m, 1H, CH_2CH=CH_2), 7.54 (d, 2H, *m*-position to carboxyl Ar-H), 7.64 (d, 2H, *m*-position to triazolin Ar-H), 7.74 (d, 2H, *o*- position to triazolin Ar-H), 7.98 (d, 2H, *o*- position to carboxyl Ar-H), 12.80-13.10 (bs, 2H, triazene NH and carboxyl H), 13.90-14.10 (bs, 1H, triazolin NH); ESIMS: *m/z* (%) 380 (M^+,22), 379 (100), 351 (30); m.p. 160°C. Yield 15%. Anal. Calcd. for $C_{18}H_{16}N_6SO_2.0.5H_2O$: C, 55.52; H, 4.40; N, 21.58. Found: C, 56.15; H, 3.82; N, 20.83%.

Anticancer screening

The anticancer evaluation of the compounds described was undertaken by the National Cancer Institute (NCI), Bethesda, MD (USA). The NCI's *in vitro* anticancer screen consists of 60 human tumor cell lines against which compounds are tested at a minimum of five concentrations at 10-fold dilutions. A 48 hr continuous drug exposure protocol is used, and a sulforhodamine B (SRB) protein assay is used to estimate cell viability or growth[18-21].

The human tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells were inoculated into 96-well microliter plates in 100 μL at plating densities ranging from 5000 to 40000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microliter plates were incubated at 37°C, 5% CO_2, 95% air and 100% relative humidity for 24 hr prior to addition of experimental drugs.

After 24 hr, two plates of each cell line were fixed *in situ* with trichloroacetic acid (TCA), to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs were solubilized in DMSO at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of the drug addition, an aliquot of frozen concentrate was thawed and diluted

to twice the desired final maximum test concentration with complete medium containing 50 μg mL^{-1} gentamicin. Additional four, 10-fold or ½-log serial dilutions were made to provide a total of five drug concentrations plus control. Aliquots of 100 μL of these different drug dilutions were added to the appropriate microliter wells already containing 100 μL of medium, resulting in the required final drug concentrations.

Following drug addition, the plates were incubated for an additional 48 hr at 37°C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 μL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded, and the plates were washed five times with tap water and air dried. SRB solution (100 μL) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at RT. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 μL of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition (GI) was calculated as:

$[(Ti-Tz)/(C-Tz)]*100$ for concentrations for which $Ti \geq Tz$

$[(Ti-Tz)/Tz]*100$ for concentrations for which $Ti < Tz$.

Three dose response parameters were calculated for each experimental agent. GI_{50} was calculated from $[(Ti-Tz)/(C-Tz)]*100=50$, which was the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in TGI was calculated from Ti=Tz. Values were calculated for each of these parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the value for that parameter was expressed as greater or less than the maximum or minimum concentration tested.

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