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Original article

Synthesis and antiproliferative activity of triazenoindazoles and triazenopyrazoles: a comparative study

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Abstract

Several triazenoindazoles and triazenopyrazoles were prepared transforming the appropriate aminoindazoles and aminopyrazoles in the corresponding diazonium salts which were reacted with dimethylamine, diethylamine and pyrrolidine. All the triazenes were tested for their antiproliferative activity against K562, HL60, L1210 and MCF7 cell lines. The biological data showed that the benzocondensation plays a positive role on the antiproliferative activity. The ^{1}H-NMR spectra showed that the rotational barrier around the N_2–N_3 bond in the triazene group can be influenced both by the position of this group in the indazole nucleus and by the substitution pattern in the benzene moiety.

Keywords: Triazenoindazoles; Triazenopyrazoles; Antiproliferative activity; Hindered rotation

1. Introduction

Many chemotherapeutic treatments of tumors are based on the combination of drugs, having a different mechanism of action, among which there is often an alkylating agent [1].

A class of alkylating agents is represented by triazene compounds [1], which continue to be a subject of interest for some research groups [2–4]. These compounds are able to alkylate the DNA molecule, acting mainly on the N-7 or O-6 atoms of guanine [5–8]. Among triazenes, dacarbazine, namely 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide (DTIC), is the best single antineoplastic agent for the treatment of malignant melanoma [9].

The main in vivo mechanism of action proposed for these compounds is based on cytochrome P-450 induced metabolic activation in the liver [10,11], to afford an unstable 3-monoalkyltriazene [12], which evolves in the ultimate alkylating species. Some authors have proposed two other possible mechanisms of action. The first one is based on the reversion of the triazenes into diazo or diazonium precursors, which react with biological macromolecules [13,14], whereas the other mechanism is concerned with the antimetastatic actions of some triazenes among these DM-COOK, the potassium salt of the 4-(3,3-dimethyltriazen–1–yl)benzoic acid (DM-COOH), which was only slightly demethylated by liver microsomes. In this case, the anticancer mechanism is based on the inhibition of neutral proteases on the cell surface of cancer cells [15]. The different mechanisms are compatible and therefore it is possible that triazenes act following more than one mechanism of action.

In the literature, some examples of triazenoheterocycles are reported which are active in vitro in the absence of microsomial activation. In fact, several triazenopyrroles, triazenoindoles and triazenotiofenes resulted active in vitro against leukemia and other tumoral cell lines [4,16,17]. Moreover, despite the accepted view that dacarbazine requires metabolic activation in vivo to exert a cytotoxic activity, it displayed activity in vitro against GM892A cell line (IC_{50} 16 μM) and Raji cell line (IC_{50} 128 μM) [18]. The aryl or heteroaryl group bounded at position 1 of triazenes is considered to be simply a carrier. However, it can influence the antitumor activity, on the basis of its pharmacokinetic properties, as well as the stability of the triazene. In fact, it is reported that triazenopyrazoles and triazenotriazoles are also light sensitive but decompose more slowly than dacarbazine [19]. Moreover, triazenoindoles are significantly more active than triazenopyrroles [16].

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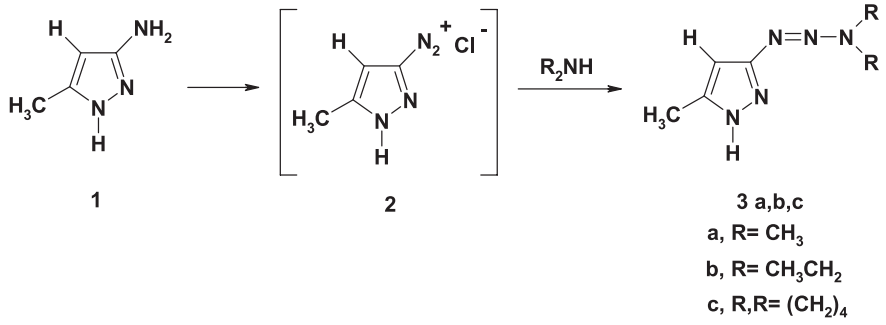
Taking into account the influence exerted by different heterocyclic nuclei, as well as by their substitution pattern, on the pharmacokinetic properties, stability and, as a consequence, activity of 1-heteroaryltriazenes, it was in our interest to synthesize new triazene derivatives containing benzocondensed heterocycles, in order to gain more insight on the role of the benzocondensation in the structure–activity relationships of the alkylating 1-heteroaryltriazenes.

Here we report the synthesis and the cytotoxicity of some triazenoindazoles, which are compared to the related triazenopyrazoles. As the isosteric triazenoindoles and triazenopyrroles were active in vitro without microsomial activation [4,16,17], we tested our compounds under the above condition.

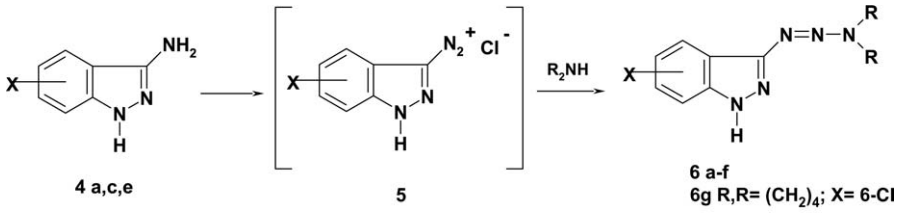
2. Chemistry

Triazenes **3a–c, 6a–g, 9c–f** were prepared as outlined in Schemes 1–3, by reaction of the intermediate diazonium salts **2, 5** and **8** with dimethylamine, diethylamine and pyrrolidine.

The structures of all the new compounds were assigned on the basis of their satisfactory analytical and spectroscopic data. The electron impact MS spectra were indicative of a characteristic fragmentation pathway: the molecular ion loses consecutively the dialkylamino radical and the nitrogen and hydrocyanic molecules. The ^{1}H-NMR spectra showed the signals for the alkyl groups and the indazole or pyrazole nucleus. Moreover, the above spectra indicated that the rota-

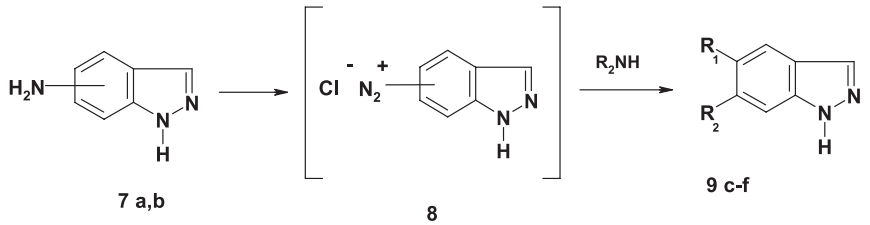


Scheme 1



4,5,6	a	b	c	d	e	f
R	CH_3	C_2H_5	CH_3	C_2H_5	CH_3	C_2H_5
X	H	H	5-Cl	5-Cl	6-Cl	6-Cl

Scheme 2



9	c	d	e	f
R_1	5-X	5-Y	H	H
R_2	H	H	6-X	6-Y

X: —N=N—N(CH3)(CH3)

Y: —N=N—N(C2H5)(C2H5)

Scheme 3

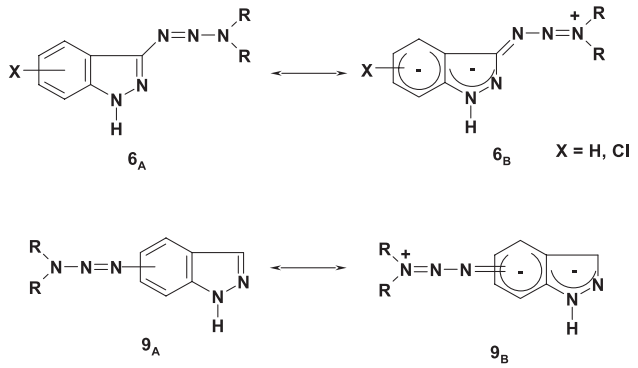


Fig. 1. Mesomeric structures for compounds **6** and **9**.

tional barrier around the N2–N3 bond in the triazene group of the triazenoindazoles can be influenced by the position of this group in the indazole nucleus, as well as by the substitution pattern in the benzene moiety. In fact, the spectrum in $CDCl_3$ or DMSO-d_6 of 3-(3′,3′-dimethyltriazeno)indazole **6a** showed for the methyl groups a very broad signal centered at about 3.4δ, consistent with a partial equivalence of these groups, whereas the positional isomers 5-(or 6-)-(3′,3′-dimethyltriazeno)indazoles **9c,e** produced a sharp singlet at 3.34 and 3.36δ, respectively. The observed difference for the positional isomers is probably due to the diverse contribution from the resonance forms 6_B and 9_B to the 3 or 5-(or 6)substituted structures **6a** and **9c,e**, respectively (Fig. 1).

The 5-(or 6-)chloro-3(3′,3′-dimethyltriazeno)indazoles **6c,e** produced for the two methyl groups two broad singlets centered at 3.20 and 3.57δ, respectively, which present superposition at the bases. The above signals are due to the increased rotational barrier around the N_2–N_3 bond in the compounds **6c,e** than **6a**. The form 6_B is more stabilized for **6c,e** when compared to **6a**, owing to the presence of the withdrawing chlorine atom at the 5 or 6 position (Fig. 1). This last atom is more effective when it is bonded at the 6 position than the 5 one, as the singlets are better separated for **6e** than **6c**.

Despite the behavior of the (3′,3′-dimethyltriazeno) indazoles, the NMR spectra of all the ethyl analogues showed a unique and well split quadruplet for the methylene portion of the ethyl groups, and a unique signal for the remaining methyls, which was a sharp triplet for the 5- or 6-diethyltriazenoindazoles **9d,f** and an unresolved signal more or less broad for the 3-diethyltriazenoindazole **6b** and the 5(or 6)-chloro-3-diethyltriazenoindazoles **6d,f**. In contrast to the diethylderivatives, the 3-(3′,3′-tetrametylene-triazeno)-6-chloroindazole produced in the ^{1}H-NMR spectra two distinct signals at 3.59 and 3.95δ, respectively, for the two N-3 bounded methylene groups. As regards the triazenopyrazoles, ^{1}H-NMR spectra of the dimethyl and tetramethylene derivatives **3a,c** showed a very large signal for the N-bounded methyl and methylene groups, respectively, whereas splitted signals were obtained for the diethyl derivative **3b**.

3. Biological results and discussion

Compounds **3, 6, 9** and dacarbazine were evaluated in vitro for their antiproliferative activity against K562 (human chronic myelogenous leukemia), HL60 (human leukemia), L1210 (murine leukemia) and MCF7 (human adenocarcinoma) cell lines. The percent of growth inhibition at 100 μM is reported in Table 1.

The IC_{50} values, determined for compounds, which exhibited at 100 μM a percent of growth inhibition more than 50%, are reported in Table 2.

Different inhibition profiles were observed for the employed cell lines. The dimethyl and diethyltriazenoindazoles **6a,b** were more active than the corresponding pyrazole derivatives **3a,b** confirming the positive role played by the benzocondensation. The presence of the chlorine atom at the 5 or 6 position of the 3-dimethyltriazenoindazoles **6c,e** lowered the antiproliferative activity showed by the nonsubsti-

Table 1
Percent of growth inhibition found on K562, HL60, L1210 and MCF7 cell lines at 100 μM

Compound	Percent growth inhibition			
	K562	HL60	L1210	MCF7
3a	28.7	33.8	41.7	23.7
3b	34.7	50.0	70.5	31.7
3c	19.4	30.9	41.4	na
6a	73.3	42.8	92.1	33.4
6b	56.1	75.0	100.0	59.1
6c	50.0	35.7	75.7	18.2
6d	100.0	100.0	87.5	91.2
6e	38.2	41.0	37.5	13.5
6f	79.3	94.6	96.5	46.7
6g	42.0	24.0	49.6	na
9c	73.0	59.2	100.0	13.6
9d	39.0	61.1	22.9	21.4
9e	70.3	75.3	99.1	44.7
9f	40.3	61.0	34.2	32.2
Dacarbazine	22.2	33.0	10.2	29.0

na, nonactive.
Values are the mean of at least three independent determinations; variation was less than 15%.

Table 2
IC_{50} values of the most active compounds, found on K562, HL60, L1210, and MCF7 cell lines

Compound	IC_{50} (μM)			
	K562	HL60	L1210	MCF7
3b	>100	>100	91.0	>100
6a	87.1	>100	20.0	>100
6b	71.0	41.7	17.0	90.1
6c	>100	>100	33.0	>100
6d	20.0	11.7	23.0	33.0
6f	44.5	35.5	18.0	>100
9c	75.9	91.3	17.0	>100
9d	>100	81.7	>100	>100
9e	60.0	39.2	20.0	>100
9f	>100	43.8	>100	>100

Values are the mean of at least three independent determinations; variation was less than 15%.

Table 3

Compound	Mp (°C)	Crystallization solvent	Analyses	Formula	Yield (%)
3a	152–155	Ethyl acetate	C, H, N	$C_6H_{11}N_5$	76
3b	86–88	Ethyl acetate/petroleum ether	C, H, N	$C_8H_{15}N_5$	42
3c	209–210	Ethanol	C, H, N	$C_8H_9N_5$	87
6a	200–203	Ethanol	C, H, N	$C_9H_{11}N_5$	85
6b	140–141 [a]	Ethyl acetate	C, H, N	$C_{11}H_{15}N_5$	52
6c	207–209	Ethyl acetate	C, H, N	$C_9H_{10}N_5Cl$	40
6d	137–140	Ethyl ether	C, H, N	$C_{11}H_{14}N_5Cl$	38
6e	221–223	Ethanol	C, H, N	$C_9H_{10}N_5Cl$	30
6f	150–153	Ethanol	C, H, N	$C_9H_{14}N_5Cl$	60
6g	220–221	Ethyl acetate/petroleum ether	C, H, N	$C_{11}H_8N_5Cl$	48
9c	140–141 [b]	Benzene/petroleum ether	C, H, N	$C_9H_{11}N_5$	90
9d	78–80	Ethyl acetate	C, H, N	$C_9H_{11}N_5$	90
9e	140–141	Ethanol/water	C, H, N	$C_{11}H_{15}N_5$	95
9f	–	–	C, H, N	$C_{11}H_{15}N_5$	43

[a] Lit. 139–140 °C (ethanol) [24].
[b] Lit. 144–145 °C [25].

tuted derivative **6a**. The 5 (or 6)-chloro-3-diethyltriazenoindazoles **6d,f** were, in some cases, more effective than the nonsubstituted analogue **6b**. Among compounds **3** and **6**, the ethyl derivatives were more active than the methyl analogues, whereas the tetramethylene substitution decreased the activity for both compounds **3** and **6**. The position of the dialkyltriazeno moiety in the indazole nucleus influenced the activity. In fact, the 5 and 6-substituted dimethyltriazenoindazoles **9c,e** resulted at all slightly more active than 3-substituted isomer **6a**, whereas for the ethyl analogues **9d,f** and **6b** this trend was reversed.

To summarize, among the tested compounds, **6d** resulted to be the most active against all the cell lines. As dacarbazine was practically inactive in the above tests, it would seem that **6d** acts in vitro with a different mechanism [13–15] than that, based on the metabolic activation, generally followed by dacarbazine.

4. Experimental

4.1. Chemistry

All melting points were determined on a Büchi 530 capillary melting point apparatus and are uncorrected; IR spectra were recorded with a Jasco IR-810 spectrophotometer as nujol mull supported on NaCl disks; ^{1}H-NMR spectra were obtained using a Bruker AC-E 250 MHz spectrometer (tetramethylsilane as internal standard). Microanalyses (C, H, N) performed in the laboratories of the Dipartimento di Scienze Farmaceutiche, Università di Catania, were within ±0.4% of the theoretical values.

*4.1.1. Procedure for the preparation of 3-(3′,3′-dialkyltriazeno)indazoles **6a–g***

To a magnetically stirred cold solution (ice bath 0–5 °C) of compound **4a,c,e** [20,21] (3 mmol) in 6 N aqueous hydrochloric acid/acetic acid (1:1) (3 ml) [acetone (1 ml) was added in the case of **4c,e**], a solution of sodium nitrite (0.21 g, 3 mmol) in water (1 ml) was added dropwise. Stirring was continued for 1 h at 0–5 °C and then the reaction mixture was treated at the same temperature with a 40% aqueous dimethylamine solution (90 mmol, 11.4 ml) for compounds **6a,c,e** or with diethylamine (90 mmol, 9.31 ml) for compounds **6d,f**. In the case of **6g** the diazonium salt solution was treated with pyrrolidine (6 mmol, 0.51 ml), and a 40% aqueous solution of sodium hydroxide was added at 0–5 °C to the solution until it become alkaline.

The reaction mixture was stirred overnight at room temperature in the dark. For compounds **6a,c,e,g**, the solid that separated was washed with cold water, filtered off and then crystallized (see Table 3). Instead, as regards compounds **6b,d,f**, the alkaline solution was extracted with dichloromethane (3 × 30 ml), and the combined extracts were dried (Na_2SO_4) and concentrated under reduced pressure. The oily residue was washed with cold water until solidification. The solid was filtered off and then crystallized (see Table 3).

*4.1.2. Procedure for the preparation of 5-(or 6-)-(3′,3′-dialkyltriazeno)indazoles **9c–f***

To a magnetically stirred cold solution (ice bath 0–5 °C) of compounds **7a,b** (3 mmol) in 6 N aqueous hydrochloric acid (2.5 ml) and acetone (2 ml), a solution of sodium nitrite (0.21 g, 3 mmol) in water (1 ml) was added dropwise. Stirring was continued for 1 h at 0–5 °C. To obtain compounds **9c,e**, the solution was treated at the same temperature with a 40% aqueous solution of dimethylamine (30 mmol, 3.8 ml), and the mixture was stirred overnight at room temperature in the dark. The solid that separated from the alkaline medium was washed with cold water, filtered off and then crystallized (see Table 3).

For compounds **9d,f**, the solution was treated at 0–5 °C with diethylamine (30 mmol, 3.1 ml). In the case of **9d**, the mixture was stirred for 15′ affording a solid, which was filtered off, washed with cold water, and then crystallized (see Table 1). Instead, in the case of **9f**, the mixture was stirred overnight at room temperature in the dark and then extracted with dichloromethane (3 × 30 ml). The combined

extracts were dried (Na_2SO_4) and concentrated under reduced pressure to give a brown oily residue, which was treated with a diethyl ether/petroleum ether (40–70 °C) mixture (8:2 V/V) (5 ml). The insoluble material was filtered off, and the solution was chromatographed following the Flash Chromatography procedure [22]: external diameter of the column 4 cm, silica gel (32–63 μm), eluant diethyl ether/petroleum ether (8:2 V/V). Fractions 8–20 (each 50 ml) were collected and evaporated to give **9f** as an oil (see Table 3).

4.1.3. 1H-5-methyl-3-(3′,3′-dialkyltriazeno)pyrazoles ***3a–c***

To a magnetically stirred cold solution (ice bath 0–5 °C) of compound **1** [23] (10 mmol) in 6 N aqueous hydrochloric acid (10 ml), a solution of sodium nitrite (0.7 g, 10 mmol) in water (3 ml) was added dropwise. Stirring was continued for 1 h at 0–5 °C and then the solution was treated at the above temperature with a 40% aqueous dimethylamine solution (200 mmol, 26 ml) for compound **3a**, with diethylamine (200 mmol, 21 ml) for compound **3b**, and with pyrrolidine (20 mmol, 1.7 ml) in the case of **3c**, adding in this last case sodium hydroxide (40%) until the solution became alkaline.

The mixture was stirred overnight at room temperature in the dark, and the solid separated was washed with cold water, filtered off and then crystallized (see Table 3).

4.1.4. 3-(3′,3′-Dimethyltriazeno)indazole ***6a***

MS (*m/e*): 189 (M^+), 145(M^+–$N(CH_3)_2$), 117 (145-N_2), 90 (117-HCN). I.R. (cm^{-1}): 3218 (br, NH), 1616 (N=N). ^{1}H-NMR ($CDCl_3$) (δ): 3.4 (6H, very brs, 2 CH_3), 7.11–8.17 (4H, a set of signal, C_6H_4), 8.86 (1H, br, exchangeable with D_2O, NH).

4.1.5. 3-(3′,3′-Diethyltriazeno)indazole ***6b*** *[24]*

MS (*m/e*): 217 (M^+), 145(M^+–$N(C_2H_5)_2$), 117 (145-N_2), 90 (117-HCN). I.R. (cm^{-1}): 3143(very br, NH), 1618 (N=N). ^{1}H-NMR ($CDCl_3$) (δ): 1.34 (6H, brs, 2 CH_3); 3.88 (4H, q, 2 CH_2 J = 7.14 Hz); 7.10–8.18 (4H, a set of signals, C_6H_4); 9.09 (1H, br, exchangeable with D_2O, NH).

4.1.6. 3-(3′,3′-Dimethyltriazeno)-5-chloroindazole ***6c***

MS (*m/e*): 223 (M^+), 179 (223-N$(CH_3)_2$), 151 (179-N_2), 124 (151-HCN). I.R. (cm^{-1}): 3208–3107(multiple bands, NH), 1622 (N=N). ^{1}H-NMR (DMSO-d_6) (δ): 3.23 (3H, brs, CH_3); 3.57 (3H, brs, CH_3); 7.37 (1H, d, H-7, J = 8.53 Hz); 7.51 (1H, d, H-6, J = 8.53 Hz); 8.08 (1H, s, H-4); 12,90 (1H, s, NH).

4.1.7. 3-(3′,3′-Diethyltriazeno)-5-chloroindazole ***6d***

MS (*m/e*): 251 (M^+), 179 (M^+–$N(C_2H_5)_2$), 151 (179-N_2), 124 (151-HCN). I.R. (cm^{-1}): 3210–3100 (multiple bands, NH), 1623 (N=N). ^{1}H-NMR ($CDCl_3$) (δ): 1.35 (6H, brs, 2 CH_3); 3.88 (4H, q, 2 CH_2, J = 7.25 Hz); 7.28 (1H, d, H_7, J = 8.75 Hz); 7.46 (1H, d, H_6, J = 8.75 Hz); 8.13 (1H, s, H-4); 11,71 (1H, brs, exchangeable with D_2O, NH).

4.1.8. 3-(3′,3′-Dimethyltriazeno)-6-chloroindazole ***6e***

MS (*m/e*): 223 (M^+), 179 (M^+–$N(CH_3)_2$), 151 (179-N_2), 124 (151-HCN). I.R. (cm^{-1}): 3205–3110(multiple bands, NH), 1619 (N=N). ^{1}H-NMR (DMSOd_6) (δ): 3.20 (3H, brs, CH_3); 3.57 (3H, brs, CH_3); 7.11 (1H, d, H-4, J = 8.61 Hz); 7.52 (1H, s, H-7); 8.08 (1H, d, H-5, J = 8.61 Hz); 12,81 (1H, brs, NH).

4.1.9. 3-(3′,3′-Diethyltriazeno)-6-chloroindazole ***6f***

MS (*m/e*): 215 (M^+–$N(C_2H_5)_2$), 151 (179-N_2), 124 (151-HCN). I.R. (cm^{-1}): 3210–3110 (multiple bands, NH). ^{1}H-NMR ($CDCl_3$) (δ): 1.36 (6H, brs, 2 CH_3); 3.92 (4H, q, 2 CH_2, J = 7,32 Hz); 7.11 (1H, d, H-4, J = 8.62 Hz); 7.60 (1H, s, H-7); 8.06 (1H, d, H-5, J = 8.62 Hz); 11.85 (1H, brs, exchangeable with D_2O, NH).

4.1.10. 3-(3′,3′-Tetramethylenetriazeno)-6-chloroindazole ***6g***

I.R. (cm^{-1}): 3200–3111 (multiple bands, NH), 1620 (N=N).^{1}H-NMR (DMSO-d_6) (δ): 2.00 (4H, s, 2 CH_2); 3.59 (2H, brs, N–CH_2–); 3.95 (2H, brs, N–CH_2-); 7.10 (1H, d, H-4 or H-5, J = 8.42 Hz); 7.49 (1H, s, H-7); 8.02 (1H, d, H-5 or H-4, J = 8.42 Hz); 12.75 (1H, s, NH).

4.1.11. 5-(3′,3′-Dimethyltriazeno)indazole ***9c*** *[25]*

MS (*m/e*): 189 (M^+), 145 (M^+–$N(CH_3)_2$), 117 (145-N_2), 90 (117-HCN). I.R. (cm^{-1}): 3200–3100 (multiple bands, NH), 1616 (N=N). ^{1}H-NMR ($CDCl_3$) (δ): 3.35, (6H, s, 2 CH_3); 7.43 (1H, d, H-7, J = 8.33 Hz); 7.64 (1H, d, H-6, J = 8.33 Hz); 7.74 (1H, s, H-4); 8.07 (1H, s, H-3); 10.70 (very br, exchangeable with D_2O, NH).

4.1.12. 5-(3′,3′-Diethyltriazeno)indazole ***9d***

MS (*m/e*): 217 (M^+), 145 (M^+–$N(C_2H_5)_2$), 117 (145-N_2), 90 (117-HCN). I.R. (cm^{-1}): 3210–3100(multiple bands, NH), 1627 (N=N). ^{1}H-NMR ($CDCl_3$) (δ): 1.28 (6H, t, 2 CH_3, J = 7.14 Hz); 3.75 (4H, q, 2 CH_2, J = 7.14 Hz); 7.43 (1H, d, H-7, J = 8.87 Hz); 7.64 (1H, dd, H-6, J = 8.87, 1.92 Hz); 7.72 (1H, d, H-4, J = 1.92 Hz); 8.06 (1H, s, H-3).

4.1.13. 6-(3′,3′-dimethyltriazeno)indazole ***9e***

MS (*m/e*): 189 (M^+), 145 (M^+–$N(CH_3)_2$), 117 (145-N_2), 90 (117-HCN). I.R. (cm^{-1}): 3200–3120 (multiple bands, NH), 1623 (N=N). ^{1}H-NMR ($CDCl_3$) (δ): 3.36 (6H, brs, 2 CH_3); 7.39 (1H, d, H-5, J = 8.83 Hz); 7.47 (1H, s, H-7); 7.68 (1H, d, H-4, J = 8.83 Hz); 8.06 (1H, s, H-3); 9.99 (1H, brs, NH).

4.1.14. 6-(3′,3′-Diethyltriazeno)indazole ***9f***

MS (*m/e*): 217 (M^+), 145 (M^+–$N(CH_3)_2$), 117 (145-N_2), 90 (117-HCN). I.R. (cm^{-1}): 3200–3100 (multiple bands, NH). ^{1}H-NMR ($CDCl_3$) (δ):1.28 (6H, t, 2 CH_3, J = 7.14 Hz); 3.78 (4H, q, 2 CH_2, J = 7.14 Hz); 7.38(1H, dd, H-5, J = 8.74, 1.36 Hz); 7.46 (1H, s, H-7); 7.67 (1H, d, indazole H-4, J = 8.74 Hz); 8.04 (s, 1H, H-3).

*4.1.15. 5-Methyl-3-(3′,3′-dimethyltriazeno) pyrazole **3a***

MS (*m/e*): 153 (M^+), 109 (M^+–$N(CH_3)_2$), 81 (109-N_2), 54 (81-HCN). I.R. (cm^{-1}): 3191–3100 (multiple bands, NH), 1592 (N=N). ^{1}H-NMR (DMSO-d_6) (δ): 2.17 (3H, s, CH_3); 3.21 (6H, brs, 2 CH_3); 5.85 (1H, s, pyrazole H-4); 12.16 (1H, brs, exchangeable with D_2O, NH).

*4.1.16. 5-Methyl-3-(3′,3′-diethyltriazeno) pyrazole **3b***

MS (*m/e*): 181 (M^+), 109 (M^+–$N(C_2H_5)_2$), 81 (109-N_2), 54 (81-HCN). I.R. (cm^{-1}): 3196–3105 (multiple bands, NH), 1593 (N=N). ^{1}H-NMR (DMSO-d_6) (δ): 1.16 (6H, t, 2 CH_3, J = 6.80 Hz); 2.17 (3H, s, CH_3); 3.66 (4H, q, 2 CH_2 J = 6.92 Hz); 5.85 (1H, s, pyrazole H-4); 12.12 (1H, brs, exchangeable with D_2O, NH).

*4.1.17. 5-Methyl-3-(3′,3′-tetramethylenetriazeno) pyrazole **3c***

MS (*m/e*): 179 (M^+), 109 (M^+–$N(CH_2)_4$), 70 (109-N_2). I.R. (cm^{-1}): 3190–3106 multiple bands, NH), 1593 (N=N). ^{1}H-NMR(DMSO-d_6) (δ): 1.93 (4H, s, 2 CH_2); 2.15 (3H, s, CH_3); 3.65 (4H, very brs, N–(CH_2); 5.81 (1H, s, H-4); 12.08 (1H, brs, exchangeable with D_2O, NH).

4.2. Biology

The compounds **3a–c, 6a–g, 9c–f** were tested in vitro for their antiproliferative activity against K562 (human chronic myelogenous leukemia), HL60 (human leukemia), L1210 (murine leukemia) and MCF7 (human breast adenocarcinoma) cell lines. These cell lines were grown at 37 °C in a humidified atmosphere containing 5% CO_2, in RPMI-1640 medium (Sigma), supplemented with 10% fetal calf serum and antibiotics.

The cells were suspended at a density of 2×10^5 cells per ml in growth medium for HL60 cell line and 1×10^5 cells per ml for K562 and L1210 cell lines, transferred to 24-well plate (1 ml per well), cultured with or without test compounds and incubated at 37 °C for 48 h for K562 and HL60 cell lines and for 72 h for L1210 cell line. Numbers of viable cells were determined by counting in a hematocytometer after dye exclusion with trypan blue.

The antiproliferative activity against MCF7 was determined by MTT assay. In this case, cells were suspended at a density of 4×10^5 cells per ml in RPMI-1640 without phenol red, supplemented with 10% fetal calf serum, 0.025% glutamine, and antibiotics, transferred (50 μl per well) to 96-well plate containing 50 μl of described medium, cultured with or without test compounds and incubated at 37 °C for 4 days. The antiproliferative effects of the compounds were estimated in terms of percent of growth inhibition. The activity of those compounds whose growth inhibition value was greater than 15% at screening concentration of 100 μM are reported in Table 2. IC_{50} values (test concentration at which the cell proliferation was inhibited of 50% than the untreated growth control) for compounds that showed at 100 μM a percent of growth inhibition more than 50% are reported in Table 3.

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