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Original article

Copper(II) chloride mediated synthesis and DNA photocleavage activity of 1-aryl/heteroaryl-4-substituted-1,2,4-triazolo[4,3-*a*]quinoxalines

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ABSTRACT

A new class of photonucleases, 1-aryl/heteroaryl-4-substituted-1,2,4-triazolo[4,3-*a*]quinoxalines (**4**) was synthesized in a facile and efficient manner *via* copper(II) chloride mediated oxidative intramolecular cyclization of 2-(arylidenehydrazino)-3-substituted-quinoxalines (**3**). DNA cleavage potency of compounds **4a**–**d** (40 μg each) was quantitatively evaluated on supercoiled plasmid ΦX174 under UV irradiation (312 nm, 15 W) without any additive. Compound **4c** was found to be the most efficient DNA photocleaver which had converted supercoiled DNA (form I) into the relaxed DNA (form II) at 30 μg and the DNA photocleavage activity increases with increase in concentration of **4c**.

1. Introduction

The design of novel photonucleases, which selectively cleave DNA under irradiation with a specific light under mild conditions without any additives such as metals and reducing agents, is very interesting from chemical and biological standpoints and offers a significant potential in medicine especially in post-genome era [1]. Research in this area has revealed that photocleavage is a promising approach for new antitumor and antiviral therapeutic strategies. Several heterocyclic DNA photocleaving molecules such as thiazo- or thiadiazo-naphthalene carboxamides [2] and isoquino [4,5-*bc*]acridines [3] exhibit antitumor activity whereas isoquinolino[5,4-*ab*]phenazine derivatives [4] could be used as topoisomerase I targeted antitumor agents. Other photosensitive DNA cleavers which have been reported include antitumor agents, 3-amino-1,2,4-benzotriazine 1,4-dioxide, (tirapazamine, WIN 59075, SR4233) [5] and chlorobithiazoles derivatives structurally related to bleomycin [6]. In addition, DNA photocleaving molecules find potential use for the design of photofootprinting agents to map the sequence specific sites of DNA binding drugs and proteins or as site-directed photonucleases for accessing structural and genetic information. Wender et al. [7] demonstrated that benzotriazoles when combined with a DNA recognition subunit can upon photoactivation cleave DNA *via* radical mechanism in a potent and selective fashion. The conjugated C=N bond in aromatic heterocycles was reported to generate the photoexcited [8] ($n-\pi^*$) state, which would have radical character and could be able to cleave DNA photochemically [8]. Triostin A and echinomycin, members of the quinoxaline family of antitumor antibiotics, bind to DNA and exhibit high level of cytotoxicity [9]. Prompted by these reports, it seemed worthwhile to synthesize and explore the photochemical DNA cleaving properties of 1,2,4-triazolo[4,3-*a*]quinoxalines as this nucleus exhibits diverse pharmacological activities, *viz.*, antitumor [10], antimicrobial [11,12], and antidepressants [13].

Traditional routes to 1,2,4-triazolo[4,3-*a*]quinoxalines include the reaction of 2-hydrazinoquinoxaline with acid chlorides/acids/acid anhydrides at elevated temperature [14]; ring closure of 2-hydrazino-3-chloroquinoxalines with triethyl orthoalkanoates [14]; pyrolysis of hydrazones [14,15]; cyclodehydration of aroylhydrazine with phosphorus oxychloride [15]; reaction of 2-chloroquinoxaline with mono or diacylhydrazine [14,16]; dehydrogenative cyclization of Schiff's bases with DDQ [17]. Unfortunately, such methods are often limited due to inevitably poor conversions, multistep synthesis, harsh reaction conditions, long

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reaction times at higher reaction temperature, side product formation, sometimes tedious work up procedures, toxic and expensive reagents. Alternatively, this ring system can be obtained by oxidative cyclization of hydrazones with oxidant iodobenzene diacetate [18,19] in order to overcome these limitations. Though IBD itself is an efficient and environmentally benign oxidant, but it is usually synthesized in the laboratory by four step procedure involving harsh reaction conditions, passing of toxic chlorine gas for hours, expensive reagents and cumbersome reaction work ups [20]. Moreover, IBD is used in stoichiometric ratio for oxidation reactions; its molecular weight being very high (MW 322) makes it a cost-ineffective reagent. Therefore, development of an alternative, convenient, economically viable oxidant that requires mild conditions and proceeds to high yields is still in demand.

In recent years, $CuCl_2$ (copper(II) chloride) has emerged as a reagent of choice for various synthetically useful transformations replacing successfully the mercury (II), thallium (III) and lead (IV) compounds, due to its similarity in oxidation reactions, low toxicity, ready availability, ease of handling and inexpensiveness. In this regard, some recent applications include: $CuCl_2$ catalyzed intramolecular C–H oxidation/acylation of formyl-N-arylformamides to indoline-2,3-diones *via* O_2 as terminal oxidant [21], $CuCl_2$-mediated oxidative cyclization of heterocyclically substituted aldimines [22] and regioselective C-allylation of enaminones [23]. Herein, we describe the copper(II) chloride mediated synthesis of fused 1,2,4-triazolo[4,3-*a*]quinoxalines and their photocleavage activities.

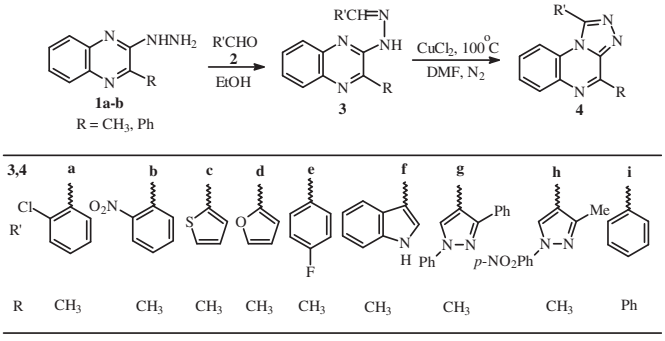
2. Chemistry

2.1. Synthesis

The synthetic pathway of the target compounds (**4a**–**i**) is outlined in Scheme 1. The key intermediate, 2-(arylidenehydrazino)-3-substituted-quinoxalines (**3a**–**i**), was prepared by the condensation of 2-hydrazino-3-methyl/phenylquinoxaline (**1a** or **1b**) with various aromatic/heteroaromatic aldehydes (**2**) in refluxing ethanol. Subsequent oxidative intramolecular cyclization of a variety of **3** with 2 equiv of copper(II) chloride in absolute DMF under nitrogen atmosphere provided the desired 1-aryl/heteroaryl-4-substituted-1,2,4-triazolo[4,3-*a*]quinoxalines (**4**) in excellent yield. The structure of compounds **4** was established by their elemental analysis, mass and a careful comparison of their IR and ^{1}H NMR spectra with those of their corresponding hydrazones **3**.

2.2. Results and discussion

IR spectra of **3** showed characteristic absorption bands due to the stretching vibrations of N–H and C=N at ~3155–3364 and 1597–1642 cm^{-1}, respectively. The ^{1}H NMR spectra of **3** displayed a sharp singlet around δ 8.5–8.9 ppm for azomethine (N=CH) and a broad singlet at δ 9.0–9.4 corresponding to resonance of NH as it was exchangeable with D_2O. IR spectra of **4** revealed the disappearance of NH stretch, thus confirming the oxidation of **3** into **4**. An important characteristic feature in the ^{1}H NMR spectra of **4** was the disappearance of the signals at around δ 8.5–8.9 and 9.0–9.4 corresponding to azomethine (N=CH) and NH, respectively, of its precursor hydrazones **3**, thus indicating the successful triazole ring formation. Also, ^{1}H NMR spectra of **4a**–**h** exhibited a signal at δ 3.1 for CH_3 protons instead of δ 2.5 as shown in the NMR of **3a**–**h**. This downfield shift may be attributed to the lone pair effect of the nitrogen of the triazole ring on the methyl protons at position-3 of quinoxaline ring.



Scheme 1. Synthesis of 1,2,4-triazolo[4,3-*a*]quinoxalines (**4a**–**i**).

3. Biological results and discussion

Out of the nine synthesized compounds, four triazolo[4,3-*a*] quinoxalines **4a**–**d**, bearing 2′-chlorophenyl, 2′-nitrophenyl, 2′-thienyl and 2′-furyl at position-1 were chosen for preliminary DNA photocleavage studies. The choice of these compounds was made due to their high solubility in DMSO and on the observation that these rings play crucial role in cleaving DNA photochemically. It is known that excitation of a haloarene can lead to homolytic cleavage of a carbon–halogen bond, thereby generating a phenyl radical [24], potentially capable of causing single-stranded lesions to DNA. Photoreactivity of nifedipine (NIF), a nitroaromatic drug used in the treatment of myocardial ischemia and hypertension, in *vitro* and in *vivo* has been reported in Ref. [25]. Nitrobenzamido ligands linked to 9-aminoacridine were found to photocleave DNA [26]. α-Terthienyl (2,2′:5,2″-terthiophene), a naturally occurring secondary plant metabolite, is an excellent singlet oxygen sensitizer, causes phototoxicity to a number of insect species and photocleave DNA [27]. In the present study compounds **4c** and **d** were chosen to compare the DNA photocleavage activity of thiophene and furan rings as it has been reported earlier that compounds possessing a thiono or thio group were found to possess significantly enhanced DNA photocleavage compared with oxygen-containing counterparts [28].

The UV–Vis absorption data of compounds **4a**–**d** were shown in Table 1. It was found that compounds **4a**, **b**, **c** and **d** showed maximum absorption wavelength 358, 360, 344, 351 nm, respectively.

3.1. DNA photocleavage

In our initial explorations, DNA cleaving activities of **4a**–**d** toward plasmid ΦX174 DNA under photoirradiative conditions was monitored quantitatively by measuring the conversion of native supercoiled DNA (Form I) *via* densitometry into the photocleave product i.e. relaxed circular DNA (Form II) resulting from nicking of the DNA backbone using Image J Software. These DNAs (Form I & II) though are similar in their molecular weight, charge and size but are very different in their conformations. Because of the tight conformation of supercoiled DNA, it migrates faster through a gel

Table 1
Absorption peaks of **4a**–**d**.

Compd	λ_{abs}/nm (log ε)[a]
4a	255 (3.21), 358 (3.27), 416 (3.25)
4b	255 (3.21), 360 (3.27), 386 (3.12)
4c	261 (3.19), 344 (3.21), 449 (2.64)
4d	257 (3.21), 351 (3.26), 436 (2.86)

[a] In $CHCl_3$.

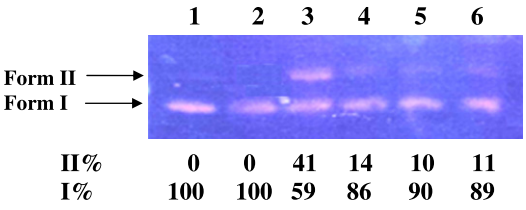


Fig. 1. Ethidium bromide stained agarose gel (1%) showing the DNA photocleavage of 3 μl (60 μg) ΦX174 plasmid (in 0.04 M Tris–borate, 0.114% acetic acid and 50 mM EDTA, pH 8.0, λ_{irr} 312 nm, 15 W, 45 min) by 20 mg/ml (DMSO) stock solution of **4a**, **4b**, **4c** and **4d**. Lane 1: control plasmid DNA only + UV + DMSO, Lane 2: control plasmid DNA + no UV + 40 μg **4c** in DMSO, Lanes 3–6: DNA + UV + 2 μl (40 μg) solution of **4c**, **4a**, **4b** and **4d** in DMSO, respectively.

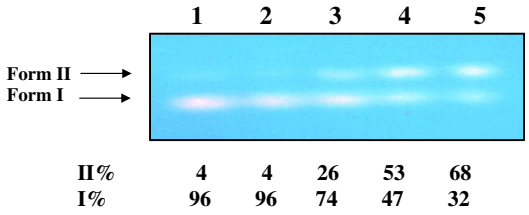


Fig. 3. Effect of concentration of compound **4c** on the photocleavage of DNA. Lane 1: control plasmid only + UV + DMSO, Lane 2: control DNA + 40 μg **4c** in DMSO + no irradiation, Lanes 3, 4, 5: DNA + UV + **4c** at the concentrations of 1.5 μl (30 μg), 4 μl (80 μg) and 6 μl (120 μg) in DMSO, respectively.

than relaxed circular DNA and due to the differences in their migration rate they can be separated easily by gel electrophoresis.

3.2. Results

Fig. 1 shows the gel electrophoresis pattern of 60 μg ΦX174 plasmid after incubation with **4** upon irradiation (λ_{irr} 312 nm, 15 W, 45 min), from which relative amounts of the two DNA forms were evaluated (Fig. 2). Two control experiments conducted under same conditions, (i) whether the DNA (Form I) was incubated with DMSO (2 μl) and UV radiations in absence of compound **4** (lane 1), (ii) or incubation of the mixture of DNA and compound **4c** (40 μg, prepared in DMSO) was left in the dark (lane 2), do not result in DNA damage. However, compounds **4a**–**d** caused single strand nicking of DNA with the conversion of Form I to Form II at concentrations as low as 40 μg when irradiated by light without any further additives (lanes 3–6). It was established through control experiment that in the absence of light (lane 2), no cleavage occurs, confirming that UV light functioned as a trigger to initiate these triazoloquinoxaline derivatives for the DNA strand scission.

On irradiation with UV light, 1-(2′-thienyl)-4-methyl-1,2,4-triazolo[4,3-*a*]quinoxaline (**4c**, lane 3) resulted in most efficient DNA cleavage (41% Form II) than that of **4a**, **4b** and **4d** (Fig. 1, lanes 4–6) of the same series and also compared to control DNA where no DNA damage was observed (lane 1). Therefore, it was decided to study the effect of concentration, varying from 30 μg to 120 μg, on the photocleavage ability of compound **4c** on supercoiled DNA. It is clearly evident from gel electrophoresis (Fig. 3) and bar graph (Fig. 4) representing the % of DNA cleavage that DNA-nicking is directly proportional to concentration of compound **4c**. The original plasmid ΦX174 DNA has about 4% relaxed circular DNA and 96% supercoiled DNA (as in lane 1). In the control experiments, whether the DNA was irradiated under light in the absence of **4c** (lane 1) or the mixture of DNA and the highest conc. of **4c** (40 μg) was left in the dark (lane 2), the amount of the relaxed circular DNA remains about 4%. With increase in **4c** concentration (lanes 3–5) under same assay conditions, nicked DNA (Form II) increases progressively (26% Form II at a concentration as low as 30 μg and 68% form II at concentration of 120 μg) while Form I diminishes compared to control DNA (lane 1) and dark control (lane 2).

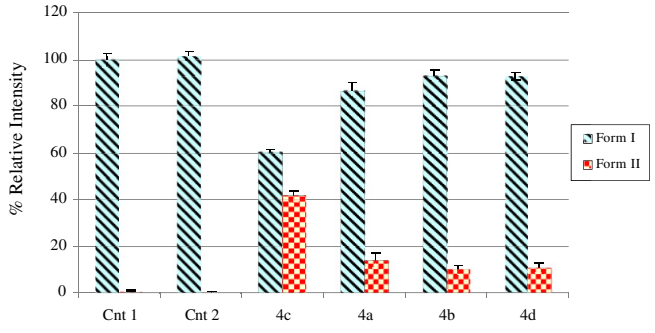


Fig. 2. Quantitative analysis of relative intensities of cleavage bands obtained by densitometry assay of lanes 1–6 shown in Fig. 1 using Image J Software. Reported values reflect the average of three experiments and results are expressed as mean ± S.D.

3.3. Structure–photocleavage efficiency relationship based on molecular orbital calculations

The minimum energy structures for **4a**–**d** have been obtained by performing geometry optimization using molecular mechanics method and mm+ force field, which were used further for single point level calculations. The minimum energy in the most stable conformation, LUMO and HOMO orbital energies for compounds **4a**–**d** were obtained from AM1 force field semi-empirical quantum calculations, using molecular modeling programs HyperChem 7.0 and are listed in Table 2. The relative UV-induced DNA photonicking efficiency of compounds **4a**–**d** (the order is **4c** > **4a** > **4d** > **4b**, Figs. 1 and 2) was compared with their highest occupied molecular orbital and the lowest unoccupied orbital (HOMO–LUMO) gaps. There seems to exist a correlation between relative DNA photocleavage efficiency and the energy gap between LUMO and HOMO (ΔE_{L-H}). For example, the most prominent DNA photocleaver **4c** was found to have the largest HOMO–LUMO gap while the least effective compound **4b** has the minimum HOMO–LUMO gap. This observation is in close agreement with the literature reports describing the relationship between photocleavage ability and HOMO–LUMO gap [29]. Though ΔE_{L-H} for compounds **4a**–**d** is showing variations, but this difference is not significant enough to draw a relationship between structure and DNA photocleavage efficiency of **4a**–**d** to their excited-state properties. The observed fact in the present study that photonicking efficiency of compound

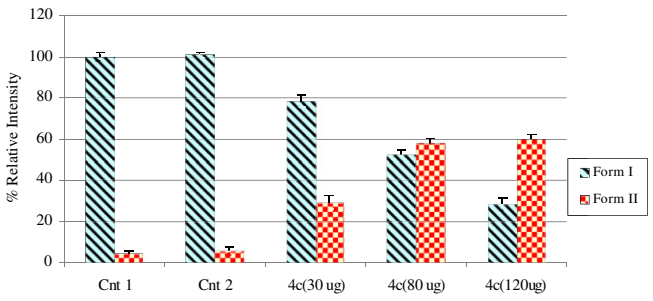


Fig. 4. Scanning densitometry results of effect of concentration of **4c** on the photocleavage of DNA. Reported values reflect the average of three experiments and results are expressed as mean ± S.D.

Table 2
The minimum energy and frontier orbitals energies for **4a**–**d**.

Compd	E^a	LUMO[a]	HOMO[a]	ΔE_{L-H}
4a	−3503.517296	−1.344616	−9.097906	7.75339
4b	−3665.214847	−1.759671	−9.275171	7.51550
4c	−3117.678469	−1.180223	−8.947033	7.76681
4d	−3113.266902	−1.161189	−8.870054	7.708865

[a] *E* (minimum energy of molecule in least strained conformation, kcal/mol) and the frontier orbital energies (ev) were obtained from AM1 force field calculations.

4c with 2′-thienyl moiety was much stronger than its oxygen-containing counterpart **4d**, further supports the earlier observation that the presence of sulfur promotes DNA photocleaving ability more efficiently than its oxo-counterpart [28].

Thus, these compounds of **4** represent promising new class of photonucleases which may be useful as tools for probing DNA structure and for photodynamic chemotherapy. Further experiments aimed to study the mode of action and sequence specific photolytic DNA cleavage are under progress.

4. Conclusion

In summary, the present work provides a general and efficient route to a new class of DNA photocleaving fused triazoloquionoxalines **4** by $CuCl_2$ mediated oxidative cyclization of 2-(arylidenehydrazino)-3-substituted-quinoxalines. The readily available, non-toxic and inexpensive $CuCl_2$ offers a valuable alternative as an oxidizing agent. The data from preliminary studies revealed that 1,2,4-triazolo[4,3-*a*]quinoxalines exhibit promising DNA photocleaving activities and the cleavage efficiency was substrate and concentration dependent.

5. Experimental

Melting points were determined in open capillaries using a melting point apparatus and are uncorrected. The IR spectra were recorded on a Buck Scientific IR M-500 spectrophotometer in KBr discs (ν_{max} in cm^{-1}). ^1H NMR spectra in $CDCl_3$ were recorded on a Bruker instrument at 300 MHz using TMS as an internal standard. Chemical shifts are recorded in δ values and coupling constants *J* in Hz. High resolution mass spectra (HRMS) were recorded on a Kratos MS-50 spectrometer in EI mode. The electronic absorption spectra of compounds **4a**–**d** (dissolved in chloroform to give 10^{-3} M solutions) were recorded on a Smart UV-2202 UV–Vis systronics double beam spectrophotometer (700-200 nm) at room temperature. Data were reported in λ_{max}/nm. Hydrazines **1a**–**b** were prepared according to the literature procedure [14]. 1,3-Diphenylpyrazole-4-carboxaldehyde and 1-(*p*-nitrophenyl)-3-methylpyrazole-4-carboxaldehyde were prepared from the appropriated phenylhydrazones *via* the Vilsmeier–Haack reaction according to the literature procedure [30]. Rest aldehydes (**2**) were commercially available.

5.1. General procedure for the synthesis of 2-(arylidenehydrazino)-3-substituted-quinoxalines (*3a*–*i*)

2-Hydrazino-3-methylquinoxaline (**1a**) (0.6 g, 3.4 mmol) was dissolved in 20 ml ethanol containing three drops of glacial acetic acid and appropriate aldehyde (**2**) (3.4 mmol). The reaction mixture was heated under reflux for 1 h and then mixture was allowed to cool at room temperature. The crude product thus obtained was collected by filtration, dried and recrystallized from ethanol to afford 2-(arylidenehydrazino)-3-substituted-quinoxalines **3**.

2-Benzylidenehydrazino-3-phenylquinoxaline (**3i**) was synthesized by the same method.

5.1.1. 2-(2′-Chlorobenzylidenehydrazino)-3-methylquinoxaline (*3a*)
M.p. 170 °C (Lit. [19] m.p. 169–170 °C); yield 92%.

5.1.2. 2-(2′-Nitrobenzylidenehydrazino)-3-methylquinoxaline (*3b*)
M.p. 184–186 °C (Lit. [19] m.p. 184–186 °C); yield 92%.

5.1.3. 2-(2′-Thiophenemethylidenehydrazino)-3-methylquinoxaline (*3c*)
M.p. 163–164 °C (Lit. [19] m.p. 162–164 °C); yield 80%.

5.1.4. 2-(2′-Furanylmethylidenehydrazino)-3-methylquinoxaline (*3d*)
M.p. 162–163 °C (Lit. [19] m.p. 160–162 °C); yield 78%.

5.1.5. 2-(4′-Fluorobenzylidenehydrazino)-3-methylquinoxaline (*3e*)
M.p. 192–193 °C; yield 82%; IR: 3155 (N–H str), 1597 (C=N); ^1H NMR ($CDCl_3$) δ: 2.59 (s, 3H, CH_3), 7.11–7.13 (m, 1H, quinox-6-H), 7.14–7.19 (t, 2H, J_o = 8.7 Hz, Ph-3′, 5′-H), 7.36–7.40 (t, 1H, J_o = 6.9 Hz, quinox-7-H), 7.66–7.69 (d, 1H, J_o = 7.8 Hz, quinox-5-H), 7.82–7.86 (dd, 2H, J_o = 8.7 Hz, $J_{(m)HF}$ = 5.4 Hz, Ph-2′, 6′-H), 8.11–8.14 (d, 1H, J_o = 8.4 Hz, quinox-8–H), 8.57 (s, 1H, N=C–H), 9.36 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ: 21.21, 114.83, 115.76–116.05 (d, $^2J_{C-F}$ = 21.75 Hz, Ph-3′, 5′-C), 123.05, 128.46, 129.20, 129.74–129.86 (d, $^3J_{C-F}$ = 9.0 Hz, Ph-2′, 6′-C), 131.06, 131.10, 131.50, 133.47, 146.71, 154.86, 162.46–165.79 (d, $^1J_{C-F}$ = 249.75 Hz, Ph-4′-C). HRMS (*m*/*z*): 280.1132 (M^+) ($C_{16}H_{13}N_4F$ (M^+) requires 280.1124). *Anal.* Calcd. for $C_{16}H_{13}N_4F$: C, 68.57; H, 4.64; N, 20.0. Found: C, 68.69; H, 4.53; N, 19.87%.

5.1.6. 2-(1H-Indol-3′-ylmethylidenehydrazino)-3-methylquinoxaline (*3f*)
M.p. 202 °C; yield 87.5%; IR: 3297 (N–H str), 1617 (C=N); ^1H NMR ($CDCl_3$) δ: 2.60 (s, 3H, CH_3), 7.10–7.24 (m, 2H, quinox-6-H, 7-H), 7.35–7.46 (m, 4H, Indole-4′, 5′, 6′, 7′-H), 7.56 (s, 1H, Indole-2′-H), 7.60–7.62 (d, 1H, J_o = 7.8 Hz, quinox-5-H), 7.63–7.66 (d, 1H, J_o = 7.8 Hz, quinox-8-H), 8.55 (s, 1H, N=C–H), 8.84 (s, 1H, Indole-NH), 9.04 (s, 1H, NH). *Anal.* Calcd. for $C_{18}H_{15}N_5$: C, 71.76; H, 4.98; N, 23.25. Found: C, 71.99; H, 5.21; N, 23.65%.

5.1.7. 2-(1′,3′-Diphenyl-1H-pyrazol-4′-ylmethylidenehydrazino)-3-methylquinoxaline (*3g*)
M.p. 204–205 °C; yield 76.9%; IR: 3342 (N–H str), 1620 (C=N); ^1H NMR ($CDCl_3$) δ: 2.55 (s, 3H, CH_3), 7.03–7.06 (m, 1H, quinox-6-H), 7.14–7.16 (m, 1H, quinox-7-H), 7.31–7.39 (m, 3H, Ph-H), 7.49–7.54 (m, 5H, Ph-H), 7.58–7.61 (m, 2H, Ph-H), 7.64–7.66 (d, 1H, J_o = 7.8 Hz, quinox-5-H), 8.01–8.04 (m, 1H, quinox-8-H), 8.49 (s, 1H, pyrazole-5′-H), 8.66 (s, 1H, N=C–H), 9.30 (s, 1H, NH). *Anal.* Calcd. for $C_{25}H_{20}N_6$: C, 74.25; H, 4.95; N, 20.79. Found: C, 74.63; H, 5.21; N, 20.99%.

5.1.8. 2-[3′-Methyl-1′-(p-nitrophenyl)-1H-pyrazol-4′-ylmethylidenehydrazino]-3-methylquinoxaline (*3h*)
M.p. 252–253 °C; yield 79.8%; IR: 3342 (N–H str), 1620 (C=N), 1527 (NO_2), 1350 (NO_2); ^1H NMR ($CDCl_3$) δ: 2.3 (s, 3H, pyrazole-3′-CH_3), 2.43 (s, 3H, CH_3), 7.09–7.12 (m, 1H, quinox-6-H), 7.15–7.18 (m, 1H, quinox-7-H), 7.58–7.61 (m, 1H, quinox-5-H), 7.69–7.72 (d, 2H, J_o = 9.0 Hz, Ph-2′, 6′-H), 8.07–8.10 (m, 1H, quinox-8-H), 8.29–8.31 (d, 2H, J_o = 9.0 Hz, Ph-3′, 5′-H), 8.54 (s, 1H, pyrazole-5′-H), 8.96 (s, 1H, N=C–H), 9.28 (s, 1H, NH). *Anal.* Calcd. for $C_{20}H_{17}N_7O_2$: C, 62.01; H, 4.39; N, 25.32. Found: C, 61.99; H, 4.38; N, 25.55%.

5.1.9. 2-Benzylidenehydrazino-3-phenylquinoxaline (*3i*)
M.p. 118–120 °C (Lit. [14] m.p. 118 °C); yield 73%; IR: 3342 (N–H str), 1636 (C=N); ^1H NMR ($CDCl_3$) δ: 7.14–7.21 (m, 2H, quinox-6-H, 7-H), 7.37–7.55 (m, 10H, Ph-H), 7.61–7.64 (d, 1H, J_o = 7.8 Hz,

quinox-5-H), 8.05–8.07 (d, 1H, J_o = 8.4 Hz, quinox-8-H), 8.87 (s, 1H, =C–H), 9.40 (s, 1H, NH). *Anal.* Calcd. for $C_{21}H_{16}N_4$: C, 77.77; H, 4.93; N, 17.28. Found: C, 77.84; H, 4.96; N, 17.32%.

5.2. General procedure for cyclization of hydrazones (**3a**–**i**) to 1-aryl/heteroaryl-4-methyl-1,2,4-triazolo[4,3-a]quinoxalines (**4a**–**i**)

5.2.1. 1-(2′-Chlorophenyl)-4-methyl-1,2,4-triazolo[4,3-a] quinoxaline (**4a**)

To a solution of hydrazone (**3a**) (0.48 g, 1.6 mmol) in 20 ml absolute DMF at 50 °C was added a solution of $CuCl_2$ (0.43 g, 3.2 mmol) in 10 ml warm absolute DMF. The reaction mixture was stirred at 50 °C for 20 min and then heated at 100 °C for 1 h under nitrogen. After cooling to approximately 30 °C, the mixture was concentrated to 5 ml *in vacuo*. Then a solution of 50 ml water, 25 ml concentrated ammonia and 8.0 g NaCl was added to the residue in order to remove the copper ions as a water-soluble complex. The mixture was stirred at 40 °C for 20 min in presence of air, cooled to room temperature and the precipitated solid was collected by filtration. The solid was treated again with dilute ammonia solution (20 ml). The crude product obtained was washed with water and recrystallized with chloroform to get pure 1-(2′-chlorophenyl)-4-methyl-1,2,4-triazolo[4,3-*a*]quinoxaline (**4a**).

M.p. 197–198 °C (Lit. [19] m.p. 198–200 °C); yield 87%.

All other compounds (**4b**–**i**) were synthesized according to the procedure mentioned above.

5.2.2. 1-(2′-Nitrophenyl)-4-methyl-1,2,4-triazolo[4,3-a]quinoxaline (**4b**)

M.p. 245–247 °C (Lit. [19] m.p. 246–248 °C); yield 91%.

5.2.3. 1-(Thiophen-2′-yl)-4-methyl-1,2,4-triazolo[4,3-a]quinoxaline (**4c**)

M.p. 184 °C (Lit. [19] m.p. 184–186 °C); yield 89%.

5.2.4. 1-(Furan-2′-yl)-4-methyl-1,2,4-triazolo[4,3-a]quinoxaline (**4d**)

M.p. 160–162 °C (Lit. [19] m.p. 162–164 °C); yield 87%.

5.2.5. 1-(4′-Fluorophenyl)-4-methyl-1,2,4-triazolo[4,3-a] quinoxaline (**4e**)

M.p. 232–234 °C; yield 90%; IR: 1605 (C=N); ^{1}H NMR ($CDCl_3$) δ: 3.09 (s, 3H, CH_3), 7.31–7.34 (t, 1H, J_o = 6.9 Hz, quinox-6-H), 7.35–7.40 (t, 2H, J_o = 8.7 Hz, Ph-3′, 5′-H), 7.50–7.53 (d, 1H, J_o = 8.1 Hz, quinox-7-H), 7.59–7.61 (d, 1H, J_o = 8.4 Hz, quinox-5-H), 7.71–7.76 (dd, 2H, J_o = 8.7 Hz, $J_{(m)HF}$ = 5.4 Hz, Ph-2′, 6′-H), 8.06–8.09 (d, 1H, J_o = 8.1 Hz, quinox-8-H); ^{13}C NMR ($CDCl_3$) δ: 21.15, 115.57, 116.40–116.70 (d, $^2J_{C-F}$ = 22.5 Hz, Ph-3′, 5′-C), 124.34, 125.68, 127.70, 128.19, 130.26, 132.13–132.24 (d, $^3J_{C-F}$ = 8.25 Hz, Ph-2′, 6′-C), 136.60, 145.0, 149.09, 152.86, 162.65–165.99 (d, $^1J_{C-F}$ = 250.5 Hz, Ph-4′-C). HRMS (*m*/*z*): 280.1132 (M^+) ($C_{16}H_{13}N_4F$ (M^+) requires 280.1124). *Anal.* Calcd. for $C_{16}H_{11}N_4F$: C, 69.06; H, 3.95; N, 20.14. Found: C, 69.03; H, 3.88; N, 20.18%.

5.2.6. 1-(1H-Indol-3′-yl)-4-methyl-1,2,4-triazolo[4,3-a]quinoxaline (**4f**)

M.p. 188–190 °C; yield 73.6%; IR: 3297 (N–H str), 1616 (C=N); ^{1}H NMR ($CDCl_3$) δ: 3.07 (s, 3H, CH_3), 7.09–7.13 (m, 1H, quinox-6-H), 7.18–7.21 (m, 1H, quinox-7-H), 7.31–7.52 (m, 4H, Indole-4′, 5′, 6′, 7′-H), 7.61 (s, 1H, Indole-2′-H), 7.75–7.77 (d, 1H, J_o = 8.4 Hz, quinox-5-H), 8.01–8.03 (d, 1H, J_o = 8.1 Hz, quinox-8-H), 10.49 (s, 1H, Indole-NH). *Anal.* Calcd. for $C_{18}H_{13}N_5$: C, 72.24; H, 4.34; N, 23.41. Found: C, 72.56; H, 4.72; N, 23.78%.

5.2.7. 1-(1′,3′-Diphenyl-1H-pyrazol-4′-yl)-4-methyl-1,2,4-triazolo [4,3-a]quinoxaline (**4g**)

M.p. 168–170 °C; yield 88%; IR: 1620 (C=N); ^{1}H NMR ($CDCl_3$) δ: 3.1 (s, 3H, CH_3), 7.18–7.26 (m, 2H, quinox-6-H, 7-H), 7.46–7.54 (m, 5H, Ph-H), 7.56–7.59 (m, 3H, Ph-H), 7.68–7.71 (d, 1H, J_o = 8.1 Hz, quinox-5-H), 7.89–8.01 (m, 2H, Ph-H), 8.09–8.12 (d, 1H, J_o = 8.1 Hz, quinox-8-H), 8.41 (s, 1H, pyrazole-5′-H). *Anal.* Calcd. for $C_{25}H_{18}N_6$: C, 74.62; H, 4.47; N, 20.89. Found: C, 74.28; H, 4.66; N, 20.44%.

5.2.8. 1-(3′-Methyl-1′-(p-nitrophenyl)-1H-pyrazol-4′-yl)-4-methyl-1,2,4-triazolo[4,3-a]quinoxaline (**4h**)

M.p. 210–212 °C; yield 87%; IR: 1620 (C=N), 1527 (NO_2), 1350 (NO_2); ^{1}H NMR ($CDCl_3$) δ: 2.4 (s, 3H, pyrazole-3′-CH_3), 3.11 (s, 3H, CH_3), 7.05–7.08 (m, 1H, quinox-6-H), 7.19–7.22 (m, 1H, quinox-7-H), 7.55–7.58 (d, 2H, J_o = 8.1 Hz, Ph-2′, 6′-H), 7.71–7.73 (d, 1H, J_o = 7.8 Hz, quinox-5-H), 8.01–8.04 (d, 1H, J_o = 7.8 Hz, quinox-8-H), 8.25–8.27 (d, 2H, J_o = 8.4 Hz, Ph-3′, 5′-H), 8.49 (s, 1H, pyrazole-5′-H). *Anal.* Calcd. for $C_{20}H_{15}N_7O_2$: C, 62.33; H, 3.89; N, 25.45. Found: C, 62.30; H, 3.82; N, 25.64%.

5.2.9. 1,4-Diphenyl-1,2,4-triazolo[4,3-a]quinoxaline (**4i**)

M.p. 235–237 °C (Lit. [14] m.p. 235 °C); yield 78%; IR: 1632 (C=N); ^{1}H NMR ($CDCl_3$) δ: 7.33–7.38 (t, 1H, J_o = 8.1 Hz, quinox-6-H), 7.54–7.57 (t, 1H, J_o = 8.1 Hz, quinox-7-H), 7.60–7.66 (m, 6H, Ph-H), 7.68–7.71 (d, 1H, J_o = 8.1 Hz, quinox-5-H), 7.75–7.78 (m, 2H, Ph-H), 8.19–8.22 (d, 1H, J_o = 8.4 Hz, quinox-8-H), 8.88–8.91 (m, 2H, Ph-H). *Anal.* Calcd. for $C_{21}H_{14}N_4$: C, 78.26; H, 4.34; N, 17.39. Found: C, 78.27; H, 4.49; N, 17.33%.

6. Biological activity

6.1. Materials and methods

The DMSO, ethylenediaminetetraacetic acid (EDTA) and ethidium bromide (EtBr) were purchased from Sigma–Aldrich, USA. Bacteriophage plasmid, ΦX174, was procured from Invitrogen, USA. Stock solution of compound **4** (20 mg/ml) were prepared in DMSO and stored in brown containers in the refrigerator. All gel electrophoresis experiments [31] were performed in 1 × TAE buffer (0.04 M Tris–borate, 0.114% acetic acid and 50 mM EDTA, pH 8.0). The cleavage efficiency was quantified *via* densitometric analysis using the Image J Software (recommended by NIH, USA).

6.2. Photocleavage of plasmid DNA by **4**

The photoinduced DNA cleaving activities of **4a**, **b**, **c** and **d** were assayed with a supercoiled, covalently closed, circular ΦX174 double-stranded DNA (form I), in presence of UV light, which is a very sensitive molecular biology tool for detection of any changes in DNA. Experiments were carried out under aerobic conditions in six eppendorf microtubes containing 60 μg (3 μl) ΦX174 plasmid DNA and 40 μg of **4** (20 mg/ml, dissolved in DMSO) in 0.04 M Tris–borate, 0.114% acetic acid and 50 mM EDTA at pH 8.0. The solutions were then irradiated at room temperature for 45 min at 312 nm using an ultraviolet lamp (15 W). Following irradiation, the reaction was stopped by removing the UV light and 5 μl of 6× glycerol loading dye (containing 0.25% bromophenol blue in 30% glycerol) in TAE buffer was added and the resulting mixture was loaded onto a 1% agarose gel. Electrophoresis was carried out for 1 h at 110 V in TAE buffer and gel was incubated in 1% ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) solution for 10 min. DNA cleavage was indicated by the formation of relaxed circular DNA (form II). DNA in the gel was visualized by a UV transilluminator and photographs were taken with a digital photocamera. The relative densities of various DNA bands on

ethidium-stain agarose gels were quantified *via* densitometry with Image J Software from which the extent of photocleavage was calculated from the ratio of relaxed circular DNA form II to supercoiled form I DNA.

6.3. Statistical analysis

Results are expressed as mean ± SD. The analysis of variance was used to compare quantitative data populations with normal distributions and equal variance. A value of $P < 0.05$ was considered statistically significant unless otherwise specified. Software GraphPad Prism (version 5.0) was used for data analysis.

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