Triazoloquinoxalines-based DNA intercalators-Topo II inhibitors: design, synthesis, docking, ADMET and anti-proliferative evaluations

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ABSTRACT
Sixteen [1, 2, 4]triazolo[4,3-a]quinoxalines as DNA intercalators-Topo II inhibitors have been prepared and their anticancer actions evaluated towards three cancer cell lines. The new compounds affected on high percentage of MCF-7. Derivatives 7e, 7c and 7b exhibited the highest anticancer activities. Their activities were higher than that of doxorubicin. Molecular docking studies showed that the HBA present in the chromophore, the substituted distal phenyl moiety and the extended linkers enable our derivatives to act as DNA binders. Also, the pyrazoline moiety formed six H-bonds and improved affinities with DNA active site. Finally, 7e, 7c and 7b exhibited the highest DNA affinities and act as traditional intercalators of DNA. The most active derivatives 7e, 7c, 7b, 7g and 6e were subjected to evaluate their Topo II inhibition and DNA binding actions. Derivative 7e exhibited the highest binding affinity. It intercalates DNA at IC50 = 29.06 μM. Moreover, compound 7e potently intercalates DNA at an IC50 value of 31.24 μM. Finally, compound 7e demonstrated the most potent Topo II inhibitor at a value of 0.890 μM. Compound 7c exhibited an equipotent IC50 value (0.940 μM) to that of doxorubicin. Furthermore, derivatives 7b, 7c, 7e and 7g displayed a high ADMET profile.

1. Introduction
DNA is the main aim for hallmark genetic diseases, such as cancer, demonstrates an important role in many diversity of cellular processes. Intercalators reversibly act on the DNA double helix. Many anticancer DNA intercalators are clinically used. Intercalators were transferred to the hydrophobic region between two neighbouring DNA base pairs. There has been a lot of research concentrated on the new prepared compounds’ action when bound to DNA non-covalently. The target actions can lead to cellular death due to disrupting replication and/or transcription. Accordingly, anticancer agents that bind to DNA have potential applications. The binding of the intercalators with DNA may be through insertion between DNA base pairs, minor or major groove binding and/or electrostatic reactions. DNA intercalators have three main structural groups, i) Chromophore (planar polyaromatic rings) that binds to DNA, ii) Cationic species (e.g. protonated amino gp) interact with the phosphate-sugar DNA region, iii) Side chain that can inhibit DNA minor groove. Anticancer drugs binding have three principally different ways. First, the anticancer medicines react with the DNA bonded protein so control transcription factors and polymerases. The second is through interfering with transcriptional activity, where RNA binds to DNA to perform triple helical DNA or DNA-RNA hybrids. The third is by minor groove binders where derivatives non-covalently bind to DNA.

The chromophores are placed between nearby DNA base pairs forming strong non-covalent interactions. These interactions lead to DNA distortion and uncoiling, also interfering with the detection and function of the associated proteins or enzymes leading to the failure of DNA repair systems, transcription processes, and replication of DNA.

In addition, the placing of chromophores between DNA bases results in DNA lengthening and decreasing DNA helical twists. The groove binding ligands may be considered like standard key and lock models. Unlike intercalation, groove binders do not make huge DNA conformational changes. In addition, they are usually semi-circular-shaped ligands that bind to the DNA minor groove.

Intercalators can be classified into two types classical (mono-intercalators) and threading intercalators. The threading intercalation occurs if there are two groove binding side chains. One side chain is directed to the major groove and the other to the minor groove. DNA intercalators as anticancer are already applied or
still under clinical trials (e.g. doxorubicin I\textsuperscript{16}, amsacrine II\textsuperscript{17}, ellipticine III\textsuperscript{9}) (Figure 1).

The imidazoquinoline, imiquimod (Figure 2) is effective in the treatment of skin and breast cancer of different types. Also, its effectiveness in other cancer types of treatment is demonstrated\textsuperscript{18}. EAPB0203 (Figure 2) was recognised to have 45 and 110 fold more active against melanoma A375 cancer cells than imiquimod and fotemustine respectively\textsuperscript{19}. Moreover, it was confirmed to have anticancer activities against leukaemia in different types\textsuperscript{20}. Anastrozole as triazole containing drug was established to have anticancer activity against breast cancer\textsuperscript{20}.

Quinoxaline structure is the scaffold of numerous DNA intercalators\textsuperscript{21–26}. The novel anticancer discovering or developing DNA intercalators is one of the extremely significant objectives in medicinal chemistry\textsuperscript{27}. Quinoxaline derivatives were reported to have high anticancer activities through intercalation of DNA\textsuperscript{28} e.g. echinomycin. It exhibited high activities against a variety of cancers in phases I and II clinical trials\textsuperscript{29}.

Depending on the mentioned facts, and the extension of producing new anti-cancers\textsuperscript{30–36}, especially that intercalators for DNA\textsuperscript{21–24,37–40}, it was reported herein modifications of EAPB0203 through hybridisation with privileged heterocyclic fragments as potent anticancer agents against MCF-7, HepG2 and HCT-116. Inhibition of DNA topoisomerase II, induction of apoptosis, cell cycle arrest, and inhibition of cancer cell proliferation are the main hallmarks applied to estimate potent chemotherapies for their anticancer activities\textsuperscript{41}.

There is a strong relation between apoptosis, inhibition of topoisomerase II and induced cell cycle arrest, in HepG2 Cells (Human Liver Cancer)\textsuperscript{42}. Topoisomerase II expression in MCF-7 has been allied with HER2/neu protein overexpression and cell proliferation\textsuperscript{43}. Moreover, human topoisomerase II catalytic inhibitors, inhibit DNA synthesis resulting in attenuation of cancer cell proliferation and DNA damage in HCT116 cells\textsuperscript{44}. DNA-Topo II binding and docking evaluations of our novel derivatives were carried out.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Approved DNA intercalators derivatives main pharmacophoric groups.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Structures of imiquimod and EAPB0203.}
\end{figure}
According to the main of DNA intercalators-topo II inhibitor pharmacophores, the new derivatives were designed.

1.1. Rationale and structure-based design

Our derivatives were obtained as quinoloxaline chromophores having only a single side chain. Synthesis of our derivatives was performed by fusion of quinoloxaline and triazole rings and joining of chalcones or pyrazole moieties to obtain the main chromophore with one side chain at position-4 as minor groove binder.

The new derivatives represent the key structural requirements to intercalate DNA and also to inhibit the topo II enzyme. The triazoquinoloxaline chromophore is placed between DNA bases. Additionally, all designed derivatives contain basic nitrogen as cationic centres that enhance the selectivity and affinity towards DNA. Lastly, all derivatives have a single side chain to bind with the minor groove enhancing affinities. The selection of various substituents at different positions in the benzene ring was built on their relatively lipophilicity with different electron withdrawing or/and electron donating effects to enable us to investigate the final target SAR.

Overall, the designed derivatives were in vitro evaluated against MCF-7, HCT-166 and HepG2 for their anti-proliferative activities. The results provoked us to carry out further investigations into the mechanism of action of our derivatives. The most potent candidates were assessed for their capability to combine with DNA through DNA/methyl green and Topo II assay. Additionally, in silico studies were done to assess their affinities towards the active site of DNA.

2. Results and discussion

2.1. Chemistry

The reaction sequence for syntheses of our compounds is demonstrated in Schemes 1 and 2. Starting with the heating of benzene-1,2-diamine compounds 1–4 were obtained in agreement with reported methods following the reaction sequence mentioned in Schemes 1 and 2\(^{21,22}\). The heating of compound 4 with 4-aminoacetophenone under reflux afforded the acetyl derivative 5 (Scheme 1).

IR spectrum of 5 showed absorption bands at 3243, 2965, and 1725 cm\(^{-1}\) indicating NH, C-H aliphatic and C=O respectively. \(^1\)H NMR spectrum revealed new signals at \(\delta\) 3.47 and 10.33 (\(\text{D}_2\text{O}\) exchangeable) indicated CH\(_3\) and NH respectively. Heating the ketone derivative 5 with the appropriate aromatic aldehydes afforded the corresponding chalcones (6a–h). On the other hand, cyclisation of the formed chalcones with hydrazine hydrate produced pyrazoles 7a–g (Scheme 2). IR of compound 6f displayed absorption bands at 1660 and 3111 cm\(^{-1}\) indicating the C=O group of \(\alpha,\beta\)-unsaturated ketone and NH. \(^1\)H NMR proved the presence of OCH\(_3\) at \(\delta\) 3.85 ppm. Furthermore, it confirmed the NH group at \(\delta\) 10.67 ppm which disappeared when using \(\text{D}_2\text{O}\).

Also, IR of 7e displayed C=O band disappearance and appearance of 2 NH bands at 3200 cm\(^{-1}\). The \(^1\)H NMR confirmed the presence of CH\(_3\) peak at \(\delta\) 2.28. Also, two \(\text{D}_2\text{O}\) exchangeable singlet peaks appeared at \(\delta\) 10.24 and 10.36 ppm indicating 2NH.

2.2. Docking studies

Molsoft program was used for docking our derivatives and doxorubicin on the binding site of DNA. It used top II complexes with DNA receptors (4G0U)\(^{35}\). The binding energy (\(\Delta G\)) was presented in Table 1. The doxorubicin binding proposed mode showed exothermic energy = -100.31 kcal/mol and formed ten H-bonding interactions. The chromophore was placed in the hydrophobic groove formed by Ala869, Arg945, Asn786, Asn795, Asn867, Asn882, Gln742, Gln789, Gln870, Gly737, Gly868, Luc880, Lys739 and Phe738. It also formed two H-bonds with Asn795, one H-bond with Asn867 and one H-bond with Asn786. The sugar side chain was tilted towards DNA minor groove and formed one H-bond with Leu880 and two H-bonds with Asn882 and three H-bonds with Arg945 (Figure 3).

The new derivative 7e was docked in the same orientation as doxorubicin (\(-97.12\) kcal/mol and eight Hydrogen bonds). The pharmacophore was presented in the same lipophilic channel as in the case of doxorubicin. Two H-bonds were formed with Leu799 and Asn795. The side chain was directed towards DNA minor groove and six H-bonds were formed with Arg945 (Figure 4). Additionally, the expected binding modes of 7c (\(-94.82\) kcal/mol and 8 H-bonding interactions (Figure 5)) and 7b (\(-93.96\) kcal/mol and H-bonding interactions (Figure 6)) have the same orientation and position as that of 7e.

As scheduled, the chromophore HBA, the substituted distal phenyl and the long linkers enable our derivatives to act as DNA binders. Also, the pyrazoline moiety formed six H-bonds improving affinities with DNA active site. Finally, 7e, 7c and 7b exhibited the highest DNA affinities and act as traditional intercalators of DNA.

\[\text{Scheme 1. Target compounds 1–5 synthetic pathways.}\]
2.3. MTT assessment

Assessment of cell multiplication inhibition action of quinoxaline derivatives 5, 6a–h and 7a–g were examined by means of MTT colorimetric assay against MCF-7, HCT-116 and HepG224–26. Doxorubicin was used as a reference. The results were summarised in Table 2. New derivatives have the highest potent effect mainly on MCF-7. Compounds 7e (IC50 = 6.15, 5.75, 3.41 μM), 7c (IC50 = 6.33, 6.22, 4.45 μM) and 7b (IC50 = 7.46, 6.90, 5.88 μM) displayed the greatest anticancer actions against HepG2, HCT116 and MCF-7 cell lines correspondingly and higher than doxorubicin, (IC50 = 7.94, 8.07 and 6.75 μM correspondingly).

With respect to the HepG2 cell line, compound 7g exhibited exceptional anticancer activities (IC50 = 9.51 μM). Compounds 5, 6a, 6c–h, 7d and 7f displayed very good anticancer activities (IC50 from 10.91 to 17.99 μM). Derivative 7a (IC50 = 20.33 μM), demonstrated potent cytotoxic effect. However 6b (IC50 = 35.22 μM) demonstrated moderate cytotoxic action.

HCT-116 cytotoxicity evaluation discovered that compounds 7g and 6c showed significant cytotoxic effects against HCT-116 (IC50 = 8.96 and 9.53 μM respectively. Also, 5, 6a, 6d–h, 7a, 7d and 7f displayed very good anticancer actions (IC50 ranging from 10.16 to 19.44 μM). However 6b (IC50 = 31.22 μM) demonstrated medium cytotoxic effect.

MCF-7 cytotoxicity assessment showed that compounds 7d, 6c, 7g and 6e presented potent anticancer actions (IC50 = 7.56, 8.61, 8.62 and 9.95 μM). Compounds 5, 6a, 6d, 6f, 6g, 6h, 7a and 7f displayed very good anticancer effects (IC50 from 10.11 to 15.36 μM). While, compound 6b with IC50 = 25.82 μM, displayed good cytotoxicity.

Finally, the four most potent derivatives 7b, 7c, 7e and 7g were assessed for their cytotoxicity against VERO normal cell lines. The results discovered that the new derivatives displayed low toxicity against VERO normal cells with IC50 values = 38.77–55.09 μM. The cytotoxicity of these compounds against the cancer cell lines was from 3.41 to 9.51 μM. Derivatives 7b, 7c, 7e and 7g are respectively, 7.88, 10.81, 16.16 and 4.50 fold safer in VERO normal cells compared to breast cancer cell lines (MCF-7, the most sensitive cells).

2.4. In vitro DNA binding evaluation

The extremely potent 6e, 7b, 7c, 7e and 7g were further assessed for their DNA-binding according to the reported procedure using methyl green dye21,22,49. DNA-binding affinities results were presented as IC50 and briefed in Table 3. All results were compared to doxorubicin.

Our new derivatives elicit excellent to very good effects as DNA binders. 7e is the highly potent one. It intercalates nucleic acid at lower IC50 (29.06 μM). Moreover, compound 7e potently intercalates DNA, at an IC50 value of 31.24 μM compared to doxorubicin (31.27 μM). Also 6e, 7b and 7g bind to DNA with high affinities at IC50 values of 38.00, 32.49 and 36.50 μM, respectively.
2.5. In vitro topoisomerase II inhibitory activity

The greatest active compounds 6e, 7b, 7c, 7e and 7g also were evaluated as Topo II enzyme inhibitors agreeing with the stated procedure21,22. All results were compared to the reference drug doxorubicin (Table 3). All derivatives exhibited excellent or very good inhibition activities (IC50 range 0.890 – 1.275 μM) in comparison with doxorubicin (IC50 = 0.94 μM). The obtained results were matched with molecular docking studies, DNA binding and in vitro cytotoxicity activities. Compound 7e was found to be the most potent derivative at IC50 value of 0.890 μM. Also, compound 7c exhibited equipotent IC50 = 0.940 μM to that of doxorubicin, while compounds 6e, 7b and 7g displayed significant Topo II inhibitory activities with IC50 of 1.275, 1.050 and 1.220 μM, correspondingly.

2.6. SAR (structure activity relationship)

The SAR has concentrated on the impact of length and type of linkers, position of the substituents at benzene ring electronic and hydrophobic nature. All derivatives showed variable activity levels with characteristic MCF-7 selectivity. The distal hydrophobic phenyls attached to [1, 2, 4]triazolo[4,3-a]quinazolines chromophore through the novel linkers; prop-2-en-1-one and/or pyrazoline linkers containing (HBA-HBD). These linkers, the substituents lipophilicity and their electronic nature exhibited an essential role in anticancer activity as DNA intercalators. The pyrazoline linker as in 7a–g showed higher activities than prop-2-en-1-one linker as in 6a–h.

The tested derivatives are classified into two groups. The first one is compounded 6a–h. In this group, prop-2-en-1-one linker was used. Compound 6e containing distal phenyl moiety substituted with hydrophobic, electron donating (+I) methyl group exhibited higher anticancer activity than 6d that substituted with hydrophobic, electron withdrawing fluoro group with +I and -1 and 6a that unsubstituted against HepG2 cell lines, while compound 6d displayed higher anticancer activity than 6c and 6a against the two MCF-7 and HCT116 cell lines respectively. Compound 6h substituted with 4-nitro group (−M, −I) exhibited higher activities than 6g that 3-substituted one against the three HepG2, HCT116 and MCF-7 cell lines. This indicated that the 4-position is essential for higher activity. Derivative 6d having 2,6-dichloro (+M, −I) exhibited higher activities than mono substituted one 6b against MCF-7, HCT116 and HepG2. 6f that containing the 4-methoxy (+M, +I) group displayed higher activities than 6b with 4-chloro (+M, −I) one against the three cancer lines.

7a–g derivatives constitute the second group. 6e with 4-methyl substituent showed higher activities than 6d with 4-fluoro group (−M, −I) and 6b with 4-chloro (+M, −I) substituent against the three cancer lines. 7d with 2,6-dichloro substituents resulted in inferior activities to that of 7b with a mono substituent. Compound 6h with a 4-nitro group (−M, −I) exhibited higher activities than 6g with 3-nitro one against the three cancer cell lines. 6a containing unsubstituted phenyl group showed the lowest anticancer activities against the three cell lines. These findings are consistent with the parabolic relationship of the Hansch equation.

2.7. In silico ADMET calculations

Compounds 7b, 7c, 7e and 7g were exposed to a computational study to determine the physicochemical properties according to the rule of Lipinski20. He recommended good absorption of a
Figure 4. DNA-Topo II and 7e expected binding mode.

Figure 5. DNA-Topo II and 7c expected binding mode.
ligand if it at least accomplishes three rules of the following: (1) Hydrogen bond donors are not more than five; (2) Hydrogen bond acceptors are not more than 10; (3) Molecular weight less than 500; (4) Partition coefficient (logP) is not more than 5. In the present study, while doxorubicin missed three rules, only compound 7b exceeds the rule of molecular weight by a small fraction. ADMET prediction was performed online using the algorithm protocol of the pkCSM descriptor (http://biosig.unimelb.edu.au/pkcsm/prediction)\(^5\). Evaluation of the ADMET properties of 7b, 7c, 7e and 7g (Table 4) displayed better absorption (91.581–97.215) compared to doxorubicin (62.3). This preference may be attributed to the high degree of hydrophobicity of our derivatives\(^5\). Moreover, 7b, 7c, 7e and 7c showed good CNS penetration (\(\text{C}_{0}\)1.707 to \(\text{C}_{0}\)2.037), compared to the inability of doxorubicin to cross CNS (\(\text{C}_{0}\)<4.0). On the other hand 7b, 7c, 7e and 7g can inhibit CYP3A4 metabolic enzymes but doxorubicin cannot. Calculation of excretion for our derivatives exhibited lower rates in comparison to doxorubicin. So it showed longer duration of action. Regarding the humans’ maximum tolerated dose, our quinoxaline derivatives 7b, 7c, 7e and 7g showed 0.336, 0.329, 0.332 and 0.299, respectively while 0.081 for doxorubicin. So our directives have a broad therapeutic window. It is also indicated by higher LD\(_{50}\) of our derivatives (2.617–2.660) in comparison to 2.408 for doxorubicin.

3. Conclusion
In summary, new series of DNA intercalators and Topo II inhibitors derived from quinoxalines have been synthesised. Their anti-proliferative activities were estimated against three different types of
cancer. A docking study was carried out to evaluate their DNA-binding activity. Docking data was highly related to that biological testing. MCF-7 was the most affected one by our derivatives influence. Compounds 7e (IC50 = 6.15, 5.75, 3.41 μM), 7c (IC50 = 6.33, 6.22, 4.45 μM) and 7b (IC50 = 7.46, 6.90, 5.88 μM) demonstrated the highest anti-proliferative actions against HepG2, HCT116 and MCF-7 correspondingly. These compounds presented higher activities than that of doxorubicin, (IC50 = 7.94, 8.07 and 6.75 μM correspondingly). Compounds 7g and 6e revealed very high anti-proliferative activities against HepG2, HCT116 and MCF-7 cancers with (IC50 = 9.51, 8.96 and 8.62 μM) and (IC50 = 10.91, 10.16 and 9.95 μM) respectively. The greatest active compounds 7e, 7c, 7b, 7g and 6e were estimated for their DNA-binding and Topo II inhibition activities. Compound 7e displayed the highest binding affinity. This compound potently intercalates DNA at decreased IC50 value (29.06 μM). Finally, compound 7e showed the greatest potency as a Topo II inhibitor at IC50 = 0.890 μM. Docking results concluded that our derivatives 7e, 7c and 7b demonstrated the highest activities as classical DNA intercalators. The pyrazoline moiety formed six H-bonds and increased affinities towards DNA active site. Furthermore, our derivatives 7b, 7c, 7e and 7g displayed wonderful in Silico predicted ADMET profile.

### 4. Experimental

#### 4.1. Chemistry

##### 4.1.1. General

Derivatives 1–4 were prepared according to the reported methods.1,21,22 1H NMR and 13C NMR for all derivatives were done on a Bruker at 400 and 100 MHz using DMSO-d6 solvent and represented on the 0 ppm scale at Cairo university Microanalytical unit. Thin layer chromatography (TLC) was used to monitor the reactions.

#### 4.1.1.1. 1-[(4-[[1, 2, 4]Triazolo[4,3-a]quinoxalin-4-ylamino]phenyl]ethan-1-one (5).

Derivative 4 (4.08 g, 0.02 mol) and 4-aminoacetophenone (3.02 g, 0.02 mol) were heated under reflux in CH3CN (20 ml) with 0.5 ml of TEA for 10 h. The precipitated product was filtered and washed with n-hexane then dried.

Yield, 91%; m.p. 171–72 °C; IRmax (cm−1): 3243 (NH), 3090 (C-H aromatic), 2965 (C-H aliphatic), 1630 (CO of amidic); MS (m/z): 302 (M+), 297 (M+–1), 243 ([M – H2O]+); 1H NMR 8.03 (m, 2H, Phenyl H-3,5), 7.81 (d, 1H, quin H-9), 7.99 (d, 1H, quin H-9), 7.94 (dd, 1H, quin H-7), 7.51 (dd, 1H, quin H-8), 7.74 (d, 1H, quin H-9), 7.94 (dd, 2H, phenyl H-5,3), 8.21 (d, 1H, quin H-6), 8.34 (dd, 2H, phenyl H-6,2), 10.01 (s, 1H, CH triazolo), 10.58 (s, 1H, Ph-NH, D2O exchangeable); 13C NMR, 26.91 (C, CH3), 116.72 (CH, quin C-9), 120.03 (phenyl C-3, 5), 123.22 (quin C-1, 10), 125.83 (quin C-9), 127.60 (quin C-9), 123.19 (quin C-9), 129.66 (phenyl C-2, 6i), 131.61 (phenyl C-4), 136 (quin C-3), 138.78 (triizolo C-3), 143.32 (phenyl C-1), 144.64 (quin C-4), 196.92 (C=O amidic); MS (m/z): 305.02 (M+–2, 6.32%), 304.03 (M+–1, 24.15%), 303 (M+–3, base peak, 100%), 302 (64.34%), 287 (78.78%), 89 (87.43%), 75 (67.97%); Anal. Calcd for C17H13N2O2 (303.33): C, 67.32; H, 4.32; N, 23.09. Found: C, 67.53; H, 4.46; N, 23.24.

#### 4.1.2. General methods for preparation of target derivatives (6a–h)

Ketone 5 (1.0 g, 0.003 mol) and the appropriate aromatic aldehyde (0.0045 mol) were heated under reflux in ethanol (10 ml), ethanolic NaOH (10 ml, 10%) was added dropwise within 15 min. The reaction mixture was stirred for 10 h, the precipitate was filtered, air dried and crystallised from ethyl alcohol to give the corresponding chalcones 6a–h.

#### 4.1.2.1. 1-[(4-[[1, 2, 4]Triazolo[4,3-a]quinoxalin-4-ylamino]phenyl]-3-phenylprop-2-en-1-one (6a).

Yield, 80%; m.p. 275–277 °C; IRmax (cm−1): 3280 (NH), 3101 (C-H aromatic), 1630 (CO of ω,β-unsaturated ketone); 1H NMR 7.46–7.56 (m, 5H, H-2′, 3′, 4′, 5′, 6′), 7.55–7.59 (m, 1H, quin H-7), 7.71–7.74 (m, 1H, quin H-8), 7.79–8.01 (1H, quin H-9), 7.99–8.03 (m, 2H, Phenyl H-3,5),.
4.1.2.2. 1-[[4-[[1, 2, 4]Triazolo[4,3-a]quinoxalin-4-yl]amino]phenyl]-3-(3-nitrophenyl)prop-2-en-1-one (6b). Yield, 85%; m.p. 257$\pm$259°C; IR$_{\text{max}}$ (cm$^{-1}$): 3220 (NH), 3001 (C=H aromatic), 1643 (CO of $\alpha$,$\beta$-unsaturated ketone); 1H NMR 7.53 (dd, 2H, H-4', 5'), 7.72$\pm$7.75 (m, 1H, quin H-7), 7.78$\pm$7.81 (m, 1H, quin H-8), 7.83 (1H, quin H-9), 8.18 (1H, H-6'), 8.24 (dd, 2H, phenyl H-3,5), 8.27 (1H, quin H-6), 8.32 (dd, 2H, phenyl H-2,6), 8.45 (dd, 2H, CO-CH=CH-phenyl), 7.87, 7.91 (m, 1H, H-2'), 10.07 (s, 1H, triazole CH), 10.69 (s, 1H, NH-phenyl); 13C NMR 132.26 (quin C-3), 122.43 (quin C-5), 137.22 (C-5), 148.23 (quin C-4), 150.35 (quin C-3), 131.64 (C-4'), 137.22 (C-5'), 138.77 (triazole C-3), 141.32 (phenyl C-1'), 138.77 (triazole C-3), 134.36 (phenyl C-1), 143.73 (quin C-4), 187.84 (C, C=O amido); MS (m/z): 421.57 (M$^+$, 31.87%), 376.34 (71.52%), 301.94 (59.70%), 274.80 (39.02%), 165.38 (M$,^+$ base peak, 100%), 135.62 (55.11%); Anal. Calc. for C$_{23}$H$_{18}$N$_{2}$O$_{2}$ (421.46): C, 71.25; H, 4.54; N, 16.62. Found: C, 71.52; H, 4.69; N, 16.91.

4.1.2.7. 1-[[4-[[1, 2, 4]Triazolo[4,3-a]quinoxalin-4-yl]amino]phenyl]-3-(3-nitrophenyl)prop-2-en-1-one (6g). Yield, 80%; m.p. 257$\pm$259°C; IR$_{\text{max}}$ (cm$^{-1}$): 3220 (NH), 3001 (C=H aromatic), 1643 (CO of $\alpha$,$\beta$-unsaturated ketone); 1H NMR 7.53 (dd, 2H, H-4', 5'), 7.72$\pm$7.75 (m, 1H, quin H-7), 7.78$\pm$7.81 (m, 1H, quin H-8), 7.83 (1H, quin H-9), 8.18 (1H, H-6'), 8.24 (dd, 2H, phenyl H-3,5), 8.27 (1H, quin H-6), 8.32 (dd, 2H, phenyl H-2,6), 8.45 (dd, 2H, CO-CH=CH-phenyl), 7.87, 7.91 (m, 1H, H-2'), 10.07 (s, 1H, triazole CH), 10.69 (s, 1H, NH-phenyl); 13C NMR 116.79 (quin C-9), 120.13 (p-unsaturated C, C=O amidic); MS (m/z): 392 (M$^+$, 26.63%), 304 (99.81%), 299 (74.16%), 238 (71.41%), 69.01 (base peak, 100%); Anal. Calc. for C$_{23}$H$_{18}$N$_{4}$O$_{2}$ (425.88): C, 67.69; H, 3.79; N, 16.44. Found: C, 68.01; H, 3.85; N, 16.72.

4.1.2.8. 1-[[4-[[1, 2, 4]Triazolo[4,3-a]quinoxalin-4-yl]amino]phenyl]-3-(3-nitrophenyl)prop-2-en-1-one (6h). Yield, 90%; m.p. 257$\pm$259°C; IR$_{\text{max}}$ (cm$^{-1}$): 3120 (NH), 3000 (C=H aromatic), 1633 (CO of $\alpha$,$\beta$-unsaturated ketone); 1H NMR 7.50 (dd, 2H, H-3', 5'), 7.77$\pm$7.80 (m, 1H, quin H-7), 7.81$\pm$7.84 (m, 1H, quin H-8), 7.86 (1H, quin H-9), 8.19 (dd, 2H, phenyl H-3,5), 8.25 (dd, 1H, quin H-6), 8.28 (dd, 2H, phenyl H-2,6), 8.31 (dd, 2H, H-2', 6'), 8.46 (dd, 2H, CO-CH=CH-phenyl), 10.09 (s, 1H, triazole CH), 10.72 (s, 1H, NH phenyl, D$_{2}$O exchangeable); Anal. Calc. for C$_{23}$H$_{16}$F$_{2}$N$_{4}$O$_{2}$ (436.43): C, 66.05; H, 3.70; N, 19.26. Found: C, 66.32; H, 3.89; N, 19.43.

4.1.3. General method for preparation of target derivatives 7a–g. Chalcones 6a–g,h (0.001 mol) and hydrazine hydrate 80% (0.5 g, 0.01 mol) in ethanol (15 ml) were heated under reflux for 6 h, then left at rt for 12 h. The precipitate was washed several times with water, dried and crystallised from ethanol to afford the corresponding pyrazoles 7a–g.

4.1.3.1. N-[[4-[[5-Phenyl-4,5-di-hydro-1H-pyrazol-3-yl]phenyl]amino]phenyl]-[1, 2, 4]triazolo[4,3-a]quinoxalin-4-amine (7a). Yield, 75%; m.p. 150–152°C; IR$_{\text{max}}$ (cm$^{-1}$): 3299 (2NH), 3069 (C=H aromatic), disappearance of the absorption band for CO of chalcone; 1H NMR 2.85–2.87 (m, 2H, pyrazole CH$_{2}$), 4.84 (t, 1H, pyrazole CH), 7.16–8.44 (m, 13H, aromatic protons), 10.04 (s, 1H, triazole CH), 10.24 (s, 1H, NH pyrazole), 10.61 (s, 1H, NH-phenyl, D$_{2}$O exchangeable); MS (m/z): 405 (M$^+$, 23.58%), 392 (19.74%), 311 (75.84%), 262 (base peak, 100%), 180 (56.48%); Anal. Calc. for C$_{23}$H$_{18}$N$_{4}$ (405.47): C, 71.09; H, 4.72; N, 24.18. Found: C, 71.23; H, 4.89; N, 24.39.

4.1.3.2. N-[[4-[[4-(4-Chlorophenyl)-4,5-di-hydro-1H-pyrazol-3-yl]phenyl]amino]phenyl]-[1, 2, 4]triazolo[4,3-a]quinoxalin-4-amine (7b). Yield, 80%; m.p. 160–162°C; IR$_{\text{max}}$ (cm$^{-1}$): 3209 (2NH), 3060 (C=H aromatic), disappearance of the absorption band for CO of chalcone; MS (m/z): 441 (M$^+$ + 2, 3.35%), 439.55 (M$^+$, 9.06%), 319 (44.71%), 289 (68.98%), 287 (base peak, 100%), 66.15 (28.80%); Anal. Calc.
for C_{24}H_{18}ClN_{7} (439.91): C, 71.79; H, 5.11; N, 23.52.

Yield, 85%; m.p. 197 – 205 °C.

4.1.3.5. N-\{4-[5-(3-Nitrophenyl)-4,5-dihydro-1H-pyrazol-3-yl]phenyl\} \(\rightarrow\) \{1, 2, 4\}triazolo[4,3-a]quinoxalin-4-amine (7f). Yield, 80%; m.p. 195 – 197 °C; \(\text{IR}_{\text{max}}\) (cm \(^{-1}\)) = 3259 (2NH), 3087 (C-H aromatic), disappearance of the absorption band for CO of chalcone; \(^1\)H NMR 2.99 – 3.01 (m, 2H, CH\(_2\) pyrazole), 4.29 (t, 1H, pyrazole CH), 7.46 – 8.26 (m, 12H, aromatic proton), 10.06 (s, 1H, triazole CH & NH pyrazole), 10.28 (s, 1H, NH phenyl, D\(_2\)O exchangeable); Anal. Calcd. for C\(_{24}\)H\(_{18}\)N\(_8\)O\(_2\) (450.46): C, 63.99; H, 4.12; N, 24.88. Found: C, 64.34; H, 4.12; N, 25.07.

4.1.3.6. N-\{4-[5-(4-Nitrophenyl)-4,5-dihydro-1H-pyrazol-3-yl]phenyl\} \(\rightarrow\) \{1, 2, 4\}triazolo[4,3-a]quinoxalin-4-amine (7g). Yield, 85%; m.p. 203 – 205 °C; \(\text{IR}_{\text{max}}\) (cm \(^{-1}\)) = 3277 (2NH), 3050 (C-H aromatic), disappearance of the absorption band for CO of chalcone; \(^1\)H NMR 3.05 – 3.07 (m, 2H, CH\(_2\) pyrazole), 4.29 (t, 1H, pyrazole CH), 7.45 – 8.23 (m, 12H, aromatic proton), 10.04 (s, 1H, triazole CH), 10.26 (s, 2H, NH pyrazole & NH-phenyl, D\(_2\)O exchangeable); Anal. Calcd. for C\(_{24}\)H\(_{18}\)N\(_8\)O\(_2\) (450.46): C, 63.99; H, 4.03; N, 24.88. Found: C, 64.34; H, 4.12; N, 25.07.

4.2. Docking studies

Docking experiments were done using molsoft program. Each experiment used DNA-Top II (https://www.rcsb.org/structure/4G0U) downloaded from Protein Databank. The reference ligand used is doxorubicin.

4.3. In vitro anti-proliferative activity

The cytotoxicity assays were performed at Al-Azhar University, Pharmacology & Toxicology Department, Cairo, Egypt. Cancer cells from different cancer cell lines HCT-116, HepG2 and MCF-7, were purchased from ATCC, Manassas, USA and grown on the appropriate growth medium Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin and 10% of heat-inactivated foetal bovine serum in a humidified, 5% (v/v) CO\(_2\) atmosphere at 37 °C.

4.4. In vitro DNA/methyl green assay

Methyl green dye can bind DNA to form coloured DNA/methyl green reversible complex. These complexes at neutral pH are still stable. The methyl green is displaced from DNA upon intercalating agents addition. Colourless carbinol was formed by the addition of H\(_2\)O to the dye, leading to a dramatic decrease in spectrophotometric absorbance. AA value (the difference between DNA/methyl green complex and free carbinol) provides the simplest means for detecting the DNA-binding affinity and relative binding strength. IC\(_{50}\) values were determined using the GraphPadPrism 5.0 software.

4.5. In vitro topoisomerase II inhibitory activity

A mixture of human Topo II (2 \(\mu\)l), substrate super coiled pH\(_{Ht1}\) DNA (0.25 \(\mu\)g), 50 \(\mu\)g/ml test compound (2 \(\mu\)l), and assay buffer (4 \(\mu\)l). The reaction started upon incubation of the mixture for 30 min at 37 °C. The reaction was terminated by the addition of proteinase K (50 \(\mu\)g/ml) and 10% sodium dodecyl sulphate (2 \(\mu\)l) for 15 min at 37 °C, followed by incubation at 37 °C for 15 min. Then, the DNA was run for 1 – 2h on 1% agarose gel in BioRad gel electrophoresis system followed by staining with GelRedTM stain for 2h and destained for 15 min with TAE buffer. The gel was imaged via BioRad’s Gel DocTMEZ system. Both supercoiled and linear strands of DNA were incorporated into the gel as markers for DNA-Topo II intercalators. By using the GraphPad Prism version 5.0, the values of IC\(_{50}\) were calculated. Each reaction was performed in duplicate, and at least three independent determinations of each IC\(_{50}\) were made.

The data is available in a supplementary file.

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