DESIGN, SYNTHESIS, MOLECULAR DOCKING AND ANTI-
PROLIFERATIVE EVALUATION OF NOVEL PYRAZOLO[4,3-
E][1,2,4]TRIAZOLO[4,3-C]PYRIMIDINE DERIVATIVES AS
POTENTIAL DNA INTERCALATORS AND TOPOISOMERASE II
INHIBITORS

Ashraf H. Bayoumi, *Farag F. Sherbiny, Eslam B. Elkaeed, Ahmed A. Gaber

Department of Organic Chemistry, Faculty of Pharmacy (Boys), Al-Azhar University,
Cairo, Egypt.

*Corresponding author: Dr-farag-sherbiny@azhar.edu.eg

ABSTRACT

A series of novel hybrid pyrazolotriazolopyrimidine derivatives was designed and synthesized in synthetically useful yields. All the new synthesized compounds were biologically evaluated in vitro for their cytotoxic activities against a panel of three cancer cell lines namely, HepG-2, MCF-7, and HCT-116. The results of cytotoxic evaluation indicated that compounds 12, and 11 exhibited the most prominent cytotoxic effect against all tested cell lines with IC₅₀ values ranging from 12.41 to 22.18 µM comparable to that of doxorubicin as a control drug (IC₅₀ values of 8.17 and 9.27 µM). Moreover, the most potent compound was further evaluated for its topoisomerase II inhibitory activities and DNA intercalating affinities as potential mechanisms for its anti-proliferative activities. In particular, compound 12 exhibited higher intercalative activity with IC₅₀ value of 30 µM than doxorubicin (31 µM). Interestingly, compound 12 displayed a significant topoisomerase II inhibitory activity with IC₅₀ value of 0.055 µM. Furthermore, molecular docking study was also performed in order to understand the binding mode in the active site and explain the anti-cancer results with prospective target.

Keywords: Anticancer, Pyrazolotriazolopyrimidine derivatives, Topoisomerase II, DNA-intercalator, Molecular docking

GRAPHICAL ABSTRACT

Compound 12 possesses the highest cytotoxic potency against all tested cell lines with IC₅₀ values of 12.41, 14.23, and 15.18 µM respectively, which was additionally estimated for its DNA intercalating affinities with IC₅₀ value of 30.8 µM and for topoisomerase II inhibitory activities with IC₅₀ value of 0.055 µM.
INTRODUCTION

Cancer remains one of the most common causes of death throughout the world and thus the development of potent and more effective anticancer agents represents one of the most important challenges in therapeutics due to the unrivaled pathophysiology of tumors and the predictable emergence of resistance to medication (Thun et al. 2010). Classical methods for cancer treatment including radiotherapy, chemotherapy, and immunotherapy with their own limitations.

Anticancer drugs have been classified into two main target types: the first one is drugs that target DNA, RNA, or proteins. The second target includes other elements involved in the carcinogenesis process, such as the immune system, the endothelium and the extracellular matrix. Most classical chemotherapeutic agents interact with tumour DNA (Espinosa et al. 2003). Compounds that affect DNA include groove binders, alkylating agents, DNA intercalators, and topoisomerase inhibitors (Hurley 2002).

Topoisomerases are important nuclear enzymes, which play a pivotal role in DNA replication, transcription, chromosome segregation, and recombination. There are two fundamental types of topoisomerases; (a) topoisomerase I (Topo I), which is responsible for cleavage, relaxing, and releasing of one strand of the DNA duplex, (b) topoisomerase II (Topo II), which cleaves both strands of the DNA helix simultaneously to remove DNA supercoiling (Wang 2002). These enzymes covalently bind to DNA helix via tyrosine residues in the active site. These linkages are transient and easily reversible, and the covalently bound structure is known as the cleavable complex (Denny 2004). Accordingly, topoisomerases are considered as crucial targets for cancer chemotherapy treatments (Pommier et al. 2010). Topoisomerase inhibitors block the ligation step of the cell-cycle, generating single and double stranded breaks that harm the integrity of the genome (Mlcochova et al. 2018). Introduction of these breaks subsequently leads to apoptosis (Kaina 2003).

Some anticancer drugs targeting Topo II inhibit the enzymatic activity as a primary mode of action and are known as catalytic Topo II inhibitors (Nitiss 2009). Another type of Topo II-targeting drugs, including intercalating drugs, interfere with the enzyme’s cleavage and rejoining activities by trapping the cleavable complex and thereby increasing the time of the transient Topo II-catalyzed DNA breaks. These drugs are referred to as Topo II poisons because they convert the Topo II enzyme into a DNA-damaging agent (Pommier et al. 2010, Nitiss 2009).

These class of drugs act either by topo poisoning via inter-chelation with DNA as doxorubicin (Liu et al. 1989), amsacrine (Sung et al. 2005) and mitoxantrone (Shenkenberg et al. 1986). On the other hand, drugs act as catalytic inhibitors of Top-II as TSC24 (Huang et al. 2010), HY-1 (zhao et al. 2011) and compound I (Islam et al. 2017).

DNA Intercalators and Topo II poisons share three common essential structural features. The first one is a planar polyaromatic system (chromophore) which is sandwiched between DNA base-pairs (Laponogov et al. 2013). The second feature is a cationic species, interacting with the negatively charged phosphate group of DNAs. The cationic center may be an amino or nitrogen containing heterocyclic group, which can be protonated at physiological pH (Lee et al. 2017). The third feature is a flexible side chain that anchors DNA (Bailly et al. 2012) (Figure 1).

On the other hand, pyrazolopyridine moieties have anticancer activities (Schenone et al. 2014). In addition, the discovery of new therapeutic DNA intercalators
for the treatment of cancer are considered one of the most important goals in the field of medicinal chemistry (Varrica et al. 2018). Pyrazolopyrimidine analogs exhibited excellent anticancer activities through DNA intercalation. Pogorelčnik and co-workers optimized the first anti-topoisomerase II pharmacophore belonging to pyrazolo[3,4-d]pyrimidine scaffold performing systematic screening to predict the bioactivity between molecule and drug target. Compound II was a result of this high-throughput screening (HTS) and efficacious candidate in the series of pyrazolo[3,4-d]pyrimidine which showing promising anticancer activities in hepatocellular carcinoma (HepG2) and breast cancer (MCF-7) cell lines with mean IC\textsubscript{50} value of 1.30 μM. besides, its topoisomerase inhibition (Pogorelčnik et al. 2015) (Figure 2).

(Figure 1). DNA intercalators and their basic pharmacophoric features

(Figure 2). Pyrimidine derivatives as topoisomerase II Inhibitors
A series of new pyrazolo[3,4-d]pyrimidine possessing 4-(1H-benzimidazol-2-yl)-phenylamine moiety at C4 position and primary as well as secondary amines at C6 position has been designed and synthesized. Their antitumor activities were evaluated against a panel of 60 human cancer cell lines. Compound III proved to be the most active and efficacious candidate in this series, with mean IC\textsubscript{50} values of 1.30 μM. Further biological evaluation suggested that this compound induce apoptosis and inhibit human topoisomerase (Topo) IIα (Singla et al. 2017). On the other hand, roscovitine, belongs to the family of purine and is used for the treatment of lung cancer with IC\textsubscript{50} value of 2.7 μM (Whittaker et al. 2004).

Therefore, on the basis of previously above-mentioned findings and in resumption of our previous efforts in the design and synthesis of new anticancer agents (Gaber et al. 2018), we report the design, synthesis, DNA intercalating, and docking studies of a new series of pyrazolotriazolopyrimidine derivatives. These derivatives were designed based on the main pharmacophoric features of DNA intercalators.

**Rationale drug design**

As reported previously, the basic influential chemical features of anti-topo II activity are indispensable for anticancer activity. Also, it is well known that triazole moiety is one of the most important chemical features necessary for anticancer activity and thus, anti-topo II activity (Huang et al. 2013) that also its effect is enhanced when fused with other heterocyclic moieties such as quinoxaline nucleus (Ibrahim et al. 2018). Therefore, the essential core of the rationale is to hybridize bioisostere pyrazolo[4,3-c]pyrimidine nucleus with the triazole moiety which display significantly different hydrogen bonding potentials in order to get an efficacious anti-topo II activity. Furthermore, some alkyl moieties, which play as groove-binding side chain, have been hybridized with pyrazolotriazolopyrimidine nucleus (Figure 3).

(Figure 3). Proposed rationale drug design of new potential DNA intercalators

**Results and discussion**

**Chemistry**
The designed compounds were synthesized as outlined in schemes (1, 2). Ethoxymethylene malononitrile, 1 (Ding et al. 2012) was allowed to refluxed with commercially available phenylhydrazine in ethanol to produce 5-amino-1-phenyl-1H-pyrazole-4-carbonitrile 2 (Cheng et al. 1956). Compound 2 was underwent a partial hydrolysis using alcoholic NaOH to produce carboxamide derivative 3 (He et al. 2011). 1,6-Diphenyl pyrazolo[3,4-d]pyrimidine core 4 (Miyashita et al. 1990) was formed from the reaction of compound 3 with methyl benzoate with subsequent chlorination using phosphoryl trichloride to afford compound 5 (Miyashita et al. 1998). The obtained compound 5 was heated with hydrazine hydrate to afford 4-hydrazinyl-1,6-diphenyl-1H-pyrazolo[3,4-d]pyrimidine 6 (Gaber et al. 2018). Cyclocondensation reaction of the hydrazine derivative 6 using potassium hydroxide and carbon disulphide in absolute ethanol followed by treatment with hydrochloric acid which resulted in the key intermediate compound, pyrazolo[4,3-e][1,2,4]triazolo[4,3-c]pyramidine-3-thiol 7 (scheme 1). There is tautomerism between (SH) and neighboring N of triazole moiety, so that the $^1$H-NMR spectrum of compound 7 revealed singlet signal at 14.42 ppm (D$_2$O exchangeable) corresponding to thiole proton and singlet signal at 13.85 ppm (D$_2$O exchangeable) corresponding to (NH) proton.

(Scheme 1). General procedure for synthesis of the key intermediate triazole derivative (1-7). Reagents and conditions of reaction; (a) phenyl hydrazine, absolute ethanol, reflux, 2 h; (b) sodium hydroxide, absolute ethanol, HCl, reflux, 5 h; (c) methyl benzoate, sodium ethoxide, absolute ethanol, HCl, reflux, 14 h; (d) phosphoryl trichloride, reflux, 6 h; (e) hydrazine hydrate 99%, reflux, 8 h; (f) carbon disulphide, absolute ethanol, potassium hydroxide, reflux, 16 h, hydrochloric acid

Furthermore, compounds 8-12 were prepared by the reaction of different appropriate alkyl bromides, namely, 1-bromobutane, 1-bromo-2-methylpropane, 1-bromo-2,2-dimethylpropane, bromocyclopentane and (bromomethyl)cyclobutane with compound 7 in the presence of anhydrous potassium carbonate in DMF. This reaction proceeded smoothly and the desired compounds were obtained in good yields (~ 70%) (scheme 2).
General procedure for synthesis of target compounds (8-12). Reaction of the triazole derivative 7 with different alkyl halides. Reagents and conditions of reaction; (a) 1-Bromobutane, (b) 1-Bromo-2-methylpropane, (c) 1-Bromo-2,2-dimethylpropane, (d) Bromocyclopentane, and (e) (Bromomethyl)cyclobutane in the presence of anhydrous potassium carbonate and DMF.

The structures of all newly synthesized compounds, (8-12) were confirmed by IR, $^1$H-NMR, $^{13}$C-NMR and mass spectra. For example, IR spectrum of compound 10 showed that the presence of new peak 2955 cm$^{-1}$ specific for aliphatic -CH stretching. $^1$H-NMR spectrum of compound 10 revealed that singlet peak at 3.41 ppm corresponding to (S-CH$_2$) protons and sharp singlet peak at 1.05 ppm specific for nine protons of neopentyl group and disappear of signals corresponding to thiol proton at 14.42 ppm.

**Biological evaluation**

**In vitro antiproliferative activities**

The new synthesized compounds have been in vitro evaluated for their anticancer activities against three different cancer cell lines namely, hepatocellular carcinoma (HepG-2), human breast adenocarcinoma (MCF-7) and human colon cancer (HCT-116) cells using neutral red assay Borenfreund et al. 1985). The screened compounds displayed different levels of cytotoxicity ranging from excellent, and weak activities against all tested cell lines.

Data represented in (Table 1) revealed that, compounds 11, and 12 exhibit the highest significant cytotoxic effect against all tested cell lines with IC$_{50}$ values ranging from 12.41 to 22.18 µM compared with doxorubicin as control drug. Compound 12 was the most potent one with IC$_{50}$ values of 12.41, 14.23, and 15.18 µM, respectively compared with other active compounds which can be explained by important chemical
features of cyclobutylmethyl moiety which was used as a linker and required to hold binding site. As a result, the replacement of the cyclobutylmethyl moiety with other bioisostere (cyclopentyl) as compound 11 lead to almost the same potency (IC$_{50}$ values of 18.38, 22.18, and 19.39 µM respectively). However, the substitution with butyl, isobutyl, or neopentyl moiety instead of cyclobutylmethyl moiety (8, 9, 10) might be unfavorable to cytotoxicity, indicating that the size of cyclic moiety of compound 12 was likely required for activity.

(Table 1) In vitro cytotoxicity against hepatocellular carcinoma (HepG-2), human breast adenocarcinoma (MCF-7) and human colon cancer (HCT-116) activity of new synthesized compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM) HepG-2</th>
<th>IC$_{50}$ (µM) MCF-7</th>
<th>IC$_{50}$ (µM) HCT-116</th>
<th>IC$_{50}$ (µM) DNA intercalation</th>
<th>IC$_{50}$ (µM) Topo-II inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>NT$_b$</td>
</tr>
<tr>
<td>8</td>
<td>79.24±3.2</td>
<td>84.15±2.8</td>
<td>72.24±3.2</td>
<td>72.06±3.1</td>
<td>NT$_b$</td>
</tr>
<tr>
<td>9</td>
<td>61.10±2.9</td>
<td>70.52±2.6</td>
<td>65.52±2.6</td>
<td>53.48±2.5</td>
<td>NT$_b$</td>
</tr>
<tr>
<td>10</td>
<td>33.79±1.8</td>
<td>38.06±2.0</td>
<td>35.41±0.6</td>
<td>45.90±2.0</td>
<td>NT$_b$</td>
</tr>
<tr>
<td>11</td>
<td>18.38±1.0</td>
<td>22.18±1.3</td>
<td>19.38±1.0</td>
<td>38.62±1.7</td>
<td>NT$_b$</td>
</tr>
<tr>
<td>12</td>
<td>12.41±0.6</td>
<td>14.23±0.8</td>
<td>15.18±1.3</td>
<td>30.81±1.4</td>
<td>0.055±0.2</td>
</tr>
<tr>
<td>DoX</td>
<td>8.50±0.2</td>
<td>8.17±0.2</td>
<td>9.27±0.2</td>
<td>31.72±1.4</td>
<td>0.01±0.1</td>
</tr>
</tbody>
</table>

Doxorubicin (DoX) was used as positive standard. *Not tested

**DNA intercalation activity**

The synthesized compounds were tested for their DNA-binding affinities using the methyl green dye according to the reported technique (Burres et al. 1992). The DNA intercalative results displayed that the compound 12 is more intercalative than doxorubicin with IC$_{50}$ value of 30.81 µM, whereas compounds 9, 10 and 11 showed moderate DNA-binding activities with IC$_{50}$ values of 53.48, 45.90, and 38.62 µM respectively. Finally, the rest of compounds were shown to possess weak DNA-binding affinities.

**Topoisomerase II inhibitory activity**

The most potent compound 12 was further examined as topo II inhibitors, according to the reported method (Singla et al. 2017). Doxorubicin was used as a positive control. The results of tested compound displayed that compound 12 showed good inhibitory activity with IC$_{50}$ value of 0.055 µM compared with doxorubicin (IC$_{50}$ = 0.01 µM).

**Molecular docking**

In this study, the docking of the most potent compound (12) with the DNA binding site of topoisomerase II (ID:3qX3), was carried out in order to predict and estimate the preferred binding mode (Ibrahim et al. 2018). The binding site of topoisomerase II has been reported, which includes the amino acid and nucleotide residues involved in the binding interactions (Arthur et al. 2019). In particular, the compound 12 inhibits
topoisomerase by intercalating the DNA and thereby inhibiting topoisomerase in the process. The results of the docking study was reported as topoisomerase II binding free energy (ΔG).

The proposed binding mode of reference ligand, doxorubicin, showed affinity value equals -79.58 kcal/mol. Doxorubicin is involved in hydrogen bonding interactions with Arg503, DA12, and DG13 and water mediated interactions with backbone amino groups of Ser480, Asp479 and Mg ion. Furthermore, doxorubicin made additional hydrogen bonding interactions with DT9 and DG10 and is stabilized by aromatic stacking interactions with nucleotide residues, DC8, DT9, DA12, and DG13, and hydrophobic interactions with hydrophobic part of Gln778, Met782 and Leu192 (Figure 4).

(Figure 4) Predicted binding mode of Doxorubicin at the binding site of topoisomerase II. H-bonds (yellow dotted lines), Hydrogen (white), nitrogen (blue), oxygen (red) and sulfur (yellow).

The suggested binding mode of compound 12 shows affinity value of -80.14 kcal/mol. It demonstrated that compound 12 is involved in a water mediated interaction DG13, and aromatic stacking interactions with nucleotide residues, DC8, DT9, DA12, and DG13. In addition, compound 12 was also stabilized by anionic π interaction with key residue, Asp479 and located in the hydrophobic pocket formed by hydrophobic parts of Arg503, and Gln778 and Met782 (Figure 5).
(Figure 5) Predicted binding mode of compound 12 at the binding site of topoisomerase II. H-bonds (yellow dotted lines), Hydrogen (white), nitrogen (blue), oxygen (red) and sulfur (yellow).

Experimental Chemistry

All the chemicals which employed in this study were commercially available with analytical grade and used without any further purification. Solvents were purified and freshly distilled before using according to the standard procedures. The progress of the reaction mixtures was monitored by thin layer chromatography (TLC). The spots on the TLC plates were visualized with a UV lamp (254 nm). Melting points were measured using Thermo Fisher Scientific. IR spectra were recorded Bruker tensor 27, FT-IR Spectrophotometer. All 1H NMR and 13C NMR spectra were recorded on a Bruker 400 and 100 MHz Spectrophotometer. Chemical shifts (δ) are reported in parts per million (ppm) using tetramethylsilane (TMS) as an internal standard. Ultraviolet–visible (UV–vis) absorption spectra were recorded on Perkin–Elmer spectrophotometer at the wavelength of maximum absorption (λmax) in a range of DMSO at same concentrations (1×10⁻⁶ M). The mass spectra were run on a Shimadzu Qp 5050 Ex Spectrometer. The microanalyses for C, H and N were performed on Perkin–Elmer elemental analyzer.

Preparation of 5,7-diphenyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-c]pyrimidine-3-thiol (7)

A mixture of hydrazine derivative 6 (0.3, 0.001 mol), potassium hydroxide (0.056 g, 0.001 mol) and carbon disulfide (0.12 g, 0.002 mol) in absolute ethanol (20 mL) was heated under reflux on a steam bath until the evolution of hydrogen sulfide (16 h). The excess solvent was removed by distillation and the residue was stirred with water, and filtered. The filtrate was acidified with 10 % HCl. The separated precipitate was collected, washed with water and crystallized from butanol to produce triazole derivative 7.

Green crystal; yield 81% (0.28 g); m.p. 230-232 °C, IR (KBr, v cm⁻¹): 3422 (NH), 3101 (Ar–H), 1193 (C=S). 1H-NMR (DMSO-d6, δ, ppm): 7.40 – 8.53 (m, 10H, Ar–H), 8.64 (s, 1H, Ar-H, C3–H pyrazole), 13.85 (s, 1H, NH D2O exchangeable), 14.42 (s, 1H, SH D2O exchangeable); 13C-NMR (DMSO, 100 MHz): 104.88, 123.70, 125.21, 126.11, 126.99, 129.13, 129.79, 133.96, 135.51, 138.56, 152.86, 154.50, 158.40, 167.88. MS (m/z): 344 (M⁺, 28.50%), 77(100%). Anal. Calcd. For C18H12N6S (344): C, 62.78; H, 3.51; N, 24.40. Found: C, 62.82; H, 3.55; N, 24.43.

General procedure for the synthesis of compounds (8-12)

A mixture of compound 7 (0.34 g, 0.001 mol), anhydrous potassium carbonate (1.38 g, 0.001 mol) and appropriate alkyl bromide (0.001mol) namely, 1-bromobutane, 1-bromo-2-methylpropane, 1-bromo-2,2-dimethylpropane, bromocyclopentane and (bromomethyl) cyclobutane in DMF (20 ml) was refluxed on a water-bath for specific time. The reaction mixture was poured onto crushed ice with continuous stirring. The
obtained solid was collected by filtration and recrystallized from ethanol to give a corresponding compound.

3-(Butylthio)-5,7-diphenyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-c]pyrimidine (8)

White solid; reaction time: 0.5 h yield 63% (0.25 g); m.p. 159-161°C; IR (KBr, ν cm⁻¹): 3075 (Ar-H), 2965 (Aliph-H). ¹H-NMR (DMSO-d₆, δ, ppm): 0.95 (t, J = 7.46 Hz, 3H, -SCH₂-CH₂-CH₂-CH₃), 1.44 - 1.52 (m, 2H, -SCH₂-CH₂-CH₂-CH₂-CH₃), 1.81 (quin, J = 7.58 Hz, 2H, -SCH₂-CH₂-CH₂-CH₂-CH₂-CH₃), 3.28 - 3.31 (m, 2H, -SCH₂-CH₂-CH₂-CH₂-CH₃), 7.46 - 8.47 (m, 10H, Ar-H), 8.80 (s, 1H, Ar-H, C₃–H pyrazole); ¹³C-NMR (DMSO, 100 MHz): 13.56, 22.03, 25.05, 31.69, 102.51, 122.14, 127.81, 128.80, 129.95, 130.94, 131.71, 132.46, 133.83, 138.71, 146.44, 146.91, 149.70, 166.18; MS (m/z): 400 (M⁺, 18.46), 279 (100.00). Anal. Calcd. for C₂₂H₂₀N₆S (400): C, 65.98; H, 5.03; N, 20.98. Found: C, 65.94; H, 5.07; N, 20.95

3-(Isobutylthio)-5,7-diphenyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-c]pyrimidine (9)

White solid; reaction time: 0.5 h yield 75% (0.30 g); m.p. 145-147 °C; IR (KBr, ν, cm⁻¹): 3078 (Ar-H), 2957 (Aliph-H). ¹H-NMR (DMSO-d₆, δ, ppm): 1.05 (d, J = 6.85 Hz, 6H, -SCH₂-C(CH₃)₂), 2.03 - 2.14 (m, 1H, -SCH₂-C(CH₃)₂), 3.19 (d, J = 6.60 Hz, 2H, -SCH₂-C(CH₃)₂), 7.46 - 8.43 (m, 10H, Ar-H), 8.76 (s, 1H, Ar-H, C₃–H pyrazole); ¹³C-NMR (DMSO, 100 MHz): 22.05, 28.66, 40.57, 102.58, 122.24, 127.81, 128.80, 129.95, 130.94, 131.72, 132.46, 133.83, 138.71, 146.44, 146.98, 149.79, 166.28; MS (m/z): 400 (M⁺, 27.25), 77 (100.00). Anal. Calcd. for C₂₂H₂₀N₆S (400): C, 65.98; H, 5.03; N, 20.98. Found: C, 65.95; H, 5.00; N, 20.96.

3-(Neopentylthio)-5,7-diphenyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-c]pyrimidine (10)

White solid; reaction time: 1 h yield 68% (0.28 g); m.p. 152-154 °C; IR (KBr, ν, cm⁻¹): 3072 (Ar-H), 2955 (Aliph-H). ¹H-NMR (DMSO-d₆, δ, ppm): 1.05 (s, 9H, -SCH₂-C(CH₃)₃), 3.41 (br. s., 2H, -SCH₂-C(CH₃)₃), 7.44 - 8.44 (m, 10H, Ar-H), 8.79 (s, 1H, Ar-H, C₃–H pyrazole); ¹³C-NMR (DMSO, 100 MHz): 28.85, 32.16, 46.66, 102.83, 122.70, 127.81, 128.80, 129.18, 130.94, 131.72, 132.46, 133.83, 138.71, 146.44, 146.91, 149.14, 166.95; MS (m/z): 414 (M⁺, 27.25), 77 (100.00). Anal. Calcd. for C₂₃H₂₂N₆S (414): C, 66.64; H, 5.35; N, 20.27. Found: C, 66.60; H, 5.33; N, 20.31.

3-(Cyclopentylthio)-5,7-diphenyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-c]pyrimidine (11)

White solid; reaction time: 1.5 h yield 58% (0.24 g); m.p. 135-137 °C; IR (KBr, ν, cm⁻¹): 3080 (Ar-H), 2950 (Aliph-H). ¹H-NMR (DMSO-d₆, δ, ppm): 1.61 - 1.78 (m, 8H), 4.07 (br. s., 1H), 7.46 - 8.45 (m, 10H, Ar-H), 8.76 (s, 1H, Ar-H, C₃–H pyrazole); ¹³C-NMR (DMSO, 100 MHz): 25.55, 35.75, 42.26, 103.03, 122.80, 127.81, 128.80, 129.18, 130.94, 131.71, 132.46, 133.58, 138.71, 146.48, 146.91, 149.18, 166.98; MS (m/z): 412 (M⁺, 8.20), 288(100.00). Anal. Calcd. for C₂₃H₂₂N₆S (412): C, 66.97; H, 4.89; N, 20.37. Found: C, 67.94; H, 4.92; N, 20.41.
3-((Cyclobutylmethyl)thio)-5,7-diphenyl-7H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-c]pyrimidine (12)

White solid; reaction time: 1 h yield 70% (0.29 g); m.p. 139 - 141°C; IR (KBr, ν, cm⁻¹): 3075 (Ar-H), 2954 (Aliph-H). ¹H-NMR (DMSO-d₆, δ, ppm): 1.77 - 1.88 (m, 4H), 2.06 - 2.15 (m, 2H), 2.76 (quin, J = 7.64 Hz, 1H), 3.48 (d, 2H, overlapped with H₂O), 7.45 – 8.45 (m, 10H, Ar-H), 8.79 (s, 1H, Ar-H, C₃–H pyrazole); ¹³C-NMR (DMSO, 100 MHz): 18.80, 27.53, 35.18, 47.25, 103.61, 122.60, 127.86, 128.60, 129.55, 130.94, 131.71, 132.46, 133.58, 138.71, 146.48, 146.91, 149.32, 166.32; MS (m/z): 412 (M⁺, 3.90), 77 (100.00). Anal. Calcd. for C₂₃H₂₀N₆S (412) C, 66.97; H, 4.89; N, 20.37. Found: C, 67.95; H, 4.92; N, 20.35.

Biological Activities Screening

In vitro antiproliferative activities

The current synthesized compounds have been tested for their anti-cancer activities against three different cancer cell lines; hepatocellular carcinoma (HepG-2), human breast adenocarcinoma (MCF-7) and human colon cancer (HCT-116), through neutral red assay protocol (Borenfreund et al. 1985). The Cell lines were cultured on DMEM media (Lonza) supplemented with 200 mM of L-glutamine and 10% of fetal bovine serum (FBS); Gibco-BRL. The tested compounds were dissolved in a mixture of Dimethyl Sulfoxide and DMEM with ratio 4:100 (v/v), respectively. An initial dose of (1 mg/ml) was tested on all cell lines and subsequences by seven more dilutions using value of 50% as dilution factor from the starting dose. Cells were seeded with concentration of (6 × 10⁴ cell/ml) for 24 hours in flat bottom 96 well plates at 5% CO₂ and 37°C until semi confluent cell layer was obtained then, treated with 100 μl of each of serially diluted compounds. After 48 hours, the antitumor activity of the compounds was measured quantitatively by ELISA microplate reader at wave length 540-nm using neutral red assay protocol. Doxorubicin was used as a positive control. The results of cytotoxicity were reported as IC₅₀ values.

DNA intercalation assay (DNA/methyl green assay)

The synthesized compounds were tested for their DNA-binding affinities using the methyl green dye according to the reported procedure (Burres et al. 1992). A mixture of methyl green (20 mg) and Calf thymus DNA (10 mg) (Sigma– Aldrich) were suspended in 0.05 M Tris-HCl buffer (100 ml, pH 7.5) containing 7.5 ml of MgSO₄. This mixture was stirred continuously for 24 h at 37 °C. The tested compounds were dissolved in ethanol and pitted into the wells of a 96-well microtiter tray at a concentration of 10,100 and 1000 μM. The excess solvent was removed from each well under vacuum, with subsequent addition of 200μl of the DNA/methyl green solution. The test samples were incubated for 24 h in a dark at ambient temperature. Then, absorbance of each sample was determined at 642.5-645 nm. Readings were corrected for initial absorbance and normalized as the percentage of the untreated DNA/methyl green absorbance value. In this test, the methyl green dye reversibly binds DNA to form persistent colored complex of DNA/methyl green. This color still stable at neutral pH. When the DNA intercalators were added, the methyl green was displaced from DNA with addition of H₂O molecule to the dye resulting in formation of the colorless carbinol.
leading to a decrease in spectrophotometric absorbance. ΔA value (the difference between DNA/methyl green complex and free cabinol) provides the simplest means for detecting the DNA-binding affinity and relative binding strength. IC<sub>50</sub>'s were determined by linear regression of data plotted on a semi-log scale. The results of DNA-binding affinity are reported as IC<sub>50</sub> values. Doxorubicin, as one of the most powerful DNA intercalators, was used as a positive control.

**Topoisomerase II inhibitory activity**

The most active compound was further examined as topo II inhibitors, according to the reported method (Singla et al. 2017). In general, the reaction started upon incubation of a mixture consisted of human topo II (2 μl), substrate super coiled pHot1 DNA (0.25 μg), 50 μg/ml test compound (2 μl), and assay buffer (4 μl) in 37 °C for 30 min. In order to terminate the reaction, 10% sodium dodecylsulphate (2 μl) and proteinase K (50 μg/ml) were added at 37 °C for 15 min followed by incubation for 15 min at 37 °C. Then, the DNA was run on 1% agarose gel in BioRad gel electrophoresis system for 1–2 h followed by staining with GelRedTM stain for 2 h and destained for 15 min with TAE buffer. The gel was imaged via BioRad’s Gel DocTMEZ system. Both supercoiled and linear strands DNA were incorporated in the gel as markers for DNA-Topo II intercalators. The results were reported IC<sub>50</sub> (50% inhibition concentration) values calculated from the concentration-inhibition response curve. Doxorubicin was also tested using the same procedure as a positive control. The IC<sub>50</sub> values were calculated from the concentration–inhibition response curve.

**Molecular docking**

In the present work, we used AutoDock program (Morris et al. 1998). AutoDock is a suit of automated docking tools, which allows flexible ligand docking and freely available under the GNU general public license (Huey et al. 2007). AutoDock predicts how small molecules, such as substrates or drug candidates bind to a receptor of known 3D structure. AutoDock suit includes two main programs: the AutoGrid, which pre-calculates the grids describing the target protein and the AutoDock, which performs the docking of the ligand to the target protein.

The scoring function used is empirically derived, for empirical binding free energy force field that allows the prediction of binding free energies for docked ligands. AutoDock is based on the United Atom force-filed of AMBER, which uses only polar hydrogens, this helps to reduce the number of atoms that must be modelled explicitly during the docking, thus speeding up the calculations.

**Conclusion**

This research reported the design and synthesis a series of novel pyrazolotriazolopyrimidine derivatives as potential antitumor agents. Thus, the new synthesized compounds were evaluated for in vitro against a panel of three cancer cell lines, HepG-2, MCF-7, and HCT-116. The anticancer activity results displayed that compounds 12, and 11 exhibited the best significant anticancer activity among the newly synthesized compounds against all tested cell lines with IC<sub>50</sub> values ranging from
12.41 to 22.18 µM compared with doxorubicin. Additionally, the most active compound was further evaluated for their topoisomerase II inhibitory activities and DNA intercalating affinities with IC₅₀ values of 0.005 and 30 µM, respectively compared with reference ligand, doxorubicin, with IC₅₀ values of 0.01 and 31 µM. Moreover, the docking studies were also performed to suggest possible explanation of the results obtained from topoisomerase II inhibitory activities. Finally, pyrazolotriazolopyramidine derivatives represent a talented starting point for further study as anticancer agents.

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تصميم و تحضير والأرساء الجزيئي و تقييم النشاط المضاد للسرطان لبعض مشتقات البيرازولوتريبتولايربميينديد الجديدة كمثلاً تحتلها مع الحمض النووي و مثبطات أذرز توبويزيمبراز اثاث

أشرف حسن بومو و *فرج فاروق شيربي و إسلام بسوني القعد و أحمد عادل جابر

قسم الكيمياء العضوية - كلية الصيدلة بني - جامعة الأزهر - مدينة نصر - القاهرة - مصر

Dr-farag-sherbiny@azhar.edu.eg

الملخص

حى حصى و ححضش سسه ييشخبث انبشاصٕحشٚبصٔنٕبٛشٚذٍٚ ونٓذٍٚ انٓدٍٛ انذذٚذة فٗ اَخبخّٛ يفٛذة صُبعٛب يٍ خلال بعض انًشكببث انٕسٛطّ حى إثبث انٓدٕغ انبُبئٛت نهًشكببث ببسخعًبل خٓبص اٜشعت دٌٔ انحًشاء ٔخٓبص انشٍَٛ انُٕٔ٘ ٔ انًغُبطٛسٙ ٔيطٛبف انكخهت ْزا ببلإضبفت إنٗ انخحهٛم انذلٛك نعُبصش انًشكببث انخٗ حبٍٛ َسبت انكشبٌٕ ٔانٓٛذسٔخٍٛ ٔانُخشٔخٍٛ فٗ انًشكببث .لذ حى حمٛٛى خًٛع انًشكبث انذذٚذة بٕٛنٕخٛب فٗ انًخخبش لاَشطخٓب انسبيّ نهخلاٚب انسشطبَّٛ فٗبعض إَاع  انسشطبٌ كسشطبٌ انثذ٘ ٔسشطبٌ انكبذ ٔسشطبٌ انمٕنٌٕ ٔكزنك حى حمٛٛى كفبءة ْزِ انًشكبث اندذٚذة كًخذاخلاث يع انحًض انُٕٔ٘ ٔلذ حى اخخٛبس اَشظ ْزِ انًشخمبث لاخخببسِ كًثبظ لاَضٚى حٕبٕاٚضٔيٛشاص اثُبٌ.

وحى إخشاء الإخخببساث انبٕٛنٕخٛت عهٗ انًشكبث انذذٚذة فٕخذ أٌ نٓب حأثٛش يضبد انسشطبٌ ٔرنك ببنًمبسَت ببنًضبد نهسشطبٌ (دٔكسٕسٔبسٍٛ) كًشخع.اظٓشث َخبئح حمٛٛى انسًبّ نٓزة انًشحبّ انًشحبّ الابشص ضذ خًٛع انخلاٚب انسشطبَّٛ انًخخبشِ بُخبئح حخشأذ بٍٛ 23.52 انٗ 33.2 ٔ 8.28 ٔ 8.38 يٛكشٔيٕل ببنًمبسَّ بمٛى َخبئح ان ذٔكسٕسٔبسٍٛ كعمبس يحكى انخٙ حخشأذ بٍٛ 44.88 انٗ 46.52 يٛكشٔيٕل ببنًمبسَّ بذٔكسٕسٔبسٍٛ ايب انًشكببث الخشٖ.ببنُسبّ نخذاخم ْزِ انًشكبث باشخٛش الابشصضذ خًٛع انخلاٚب انسشطبَّٛ انًخخبشِ بُخبئح حخشأذ بٍٛ 21 لًٛب يخٕسطّ حخشأذ بٍٛ 48.73 انٗ 64.58 يٛكشٔيٕل ايب انًشكبث 8 ٔ 21 ٔ 22 فمذ خبءث َخبئدٓب الم لِٕ ببنًمبسَّ ببنًشكببث الاخشٖ.

وقد أظهرت نتائج تقييم السمء لهذه المركبات على النحو التالي بالنسبة للنشاط المضاد للسرطان فقد كانت أقوى النتائج هي للمركبات ١٢ و ١١ حيث أظهرت الابز ضد جميع الخلايا السرطانية المختبرية بناتج تتجاوز بين ٢٢ ٠.١٨ الى ٢٢ ٠.١٨ ميكرومول بالمقارنة بقيم نتائج الدوكسوريبسين كعقار محاك لتي تتراوح بين ١٠٨ الى ١٢٠ ميكرومول. بينما أظهر المركب ١٠ فيما متوسط تتجاوز بين ٤٣.٧٤ الى ٣٥.٤١ ميكرومول بالمقارنة بدوكسوريبسين أما باقي المركبات فكانت نتائجها أقل قوة بمثابة بالمقارنة بالمركبات الأخرى. بالنسبة لتفاعل هذه الوعاوة مع الحمض النووي فقد جاء في المرتبة الأولى حيث تداخل مع الحمض النووي بصورة أقوى من تداخل دوكسوريبسين بقيمة ٣٠.٨١ ميكرومول اما المركب ٩ و ١٠ و ١١ فقد جادت نتائجها بين ٣٠.٢٦ الى ٤٦.٤٨ ميكرومول مما يجعلها في المرتبة الوسطى بالمقارنة بالمضاد لدوكسوريبسين بينما تأتي في المرتبة الأخيرة المركب ٨ و قد تم استناد أقوى هذه المركبات وهو المركب ١٢ من حيث نشاط مثبط لنشاط الازيم توبويزيمبراز اثاث حيث أعطيت نتيجة قوية وهي ٥٥ ٠.٥٥ ميكرومول وذلك بالمقارنة بدوكسوريبسين. وتم إجراء دراسات المتمم لفهم ارتباط المركبات وعرقلة انزيم التوبويزيمبراز اثاث وتنصير النتائج البيولوجية.

الكلمات المفتاحية: مضاد للسرطان، مشتقات البيرازولوتريبتولايربميينديد، توبويزيمبراز اثاث، مثبطات الحمض النووي، الأرساس الجزيئي