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Molecular modeling study and synthesis of novel pyrrolo[2,3-*d*]pyrimidines and pyrrolotriazolopyrimidines of expected antitumor and radioprotective activities

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Abstract—Novel pyrrolo[2,3-*d*]pyrimidine derivatives **4a**–**e**, **10**, **14**, **15**, pyrazolopyrrolopyrimidine **13**, pyrrolotriazolopyrimidine **5**–**9**, **17** and pyrrolopyrimidotriazine **18** are reported herein. The design of these compounds was based upon the molecular modeling simulation of the fitting values and conformational energy values of the best-fitted conformers to VEGFRTK inhibitor hypothesis. This hypothesis was generated from its corresponding lead compounds using CATALYST software. The structures of these compounds were confirmed by microanalyses, IR, ^{1}H NMR, and mass spectral data. Compounds **6** and **15** showed interesting in vitro antitumor activity compared to doxorubicin as positive control. These results are nearly consistent with the molecular modeling studies. Docking studies were made on compound **15** to predict its binding mode. Moreover, compound **10** exhibited a significant radioprotective activity.

1. Introduction

In current cancer chemotherapy, several agents with DNA-cleavage properties, antimitotics, antimetabolites, inhibitors of topoisomerases, and most recently, signal transduction inhibitors are used as drugs. Protein tyrosine kinases are enzymes that provide a central switch mechanism in cellular signal transduction pathways. As such they are involved in many cellular processes such as cell proliferation, metabolism, survival and apoptosis. Several protein tyrosine kinases are known to be activated in cancer cells and to drive tumor growth, angiogenesis, progression, and metastasis. Blocking tyrosine kinase activity therefore represents a rational approach to cancer therapy.[1]

In 1970, Folkman and Kerbel proposed that inhibition of angiogenesis can prevent tumor growth.[2,3] Subsequently, it was also recognized that metastasis can be affected by angiogenesis. For these reasons, inhibitors of angiogenesis are expected to be valuable drugs for cancer therapy. The cancer cell is characterized by oncogene-derived tumor expression of pro-angiogenic proteins, such as vascular endothelial growth factor (VEGF), placenta-like growth factor, basic fibroblast growth factor (FGF), platelet-derived endothelial growth factor (PDGF), angiopoietin-2 (Ang-2), hepatocyte growth factor, and insulin-like growth factor (ICF).[4] The mentioned pro-angiogenic growth factors bind to specific receptors that possess receptor tyrosine kinase (RTK) activity.

Pyrrolo[2,3-*d*]pyrimidines have aroused recent attention from chemical and biological view points since they have useful properties as antimetabolites in purine biochemical reactions.[5] Several mechanisms are also involved in their cytotoxic activities as being antifolate inhibitors of dihydrofolate reductases,[6,7] tyrosine Kinase c-Src or

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Lck inhibitors,[8,9] Cyclin dependent kinase (CDK) inhibitors[10,11] or adenosine receptor antagonists.[12,13]

On the other hand, sulfonamides have recently been reported to show substantial antitumor activity in vitro and/or in vivo.[14–17]

Regarding radioprotective activity, some heterocyclic compounds[18–24] especially pyrrole and pyrrolopyrimidines[25,26] were proved to exhibit significant radioprotective activity. Also some sulfur-containing compounds are known to have good radioprotective activity.[27–29]

To explore the synergistic effect resulted from combining both sulfonamides and pyrrolopyrimidines, the present work was carried out to design and synthesize a novel series of pyrrolo[2,3-*d*]pyrimidine derivatives bearing sulfonamide moiety, for studying their structure activity relationship and to be evaluated as antitumor and radioprotective agents (Schemes 1–3).

2. Results and discussion

2.1. Molecular modeling

2.1.1. Generation of vascular epithelial growth factor receptor tyrosine kinase (VEGFRTK) inhibitor hypothesis using CATALYST software. The lead compounds **I–VII** which were reported to have selective VEGFRTK inhibitory activity were used to generate common feature hypothesis of VEGFRTK inhibitor (Fig. 1).[8] The generated pharmacophore consisted of five features with constraint dimensions (Figs. 2 and 3).

i. Two hydrogen bond acceptor (HBA) appeared as a vector (green spherical mesh).
ii. Three hydrophobic functions (HY_1, HY_2, HY_3) (blue spherical meshes).

Molecular modeling simulation studies were then conducted by measuring the compare/fit values, separately, between the conformational models of lead vatalanib, compounds **2–18** and ideal selective VEGFRTK inhibitor hypothesis (Fig. 4). The results of the best fitting values, as well as the conformational energy of the best-fitted conformer with this hypothesis are given in Table 1. The results of simulation studies have revealed that compounds **6**, **10**, **14**, **15** and **17** might be promising active hit molecules.

2.1.2. Docking studies

- Regarding Vatalanib, four clusters of docked conformations were obtained, the largest cluster (four poses) was the lowest in mean docked energy (−9.17 kcal/mol) (Table 2), and the best member (in terms of docking score) was selected as the docked solution for Vatalanib, examination of the docking pose shows that Vatalanib forms a hydrogen bond with the amidic NH in Cys919, in addition to extensive non-polar interactions, especially with Val848, Leu840, and Leu1035 (Fig. 5).
- Using the RMSD tolerance of 1 Å, compound **15** gave ten distinct clusters. The best pose of compound **15** showed good mapping to the docked pose of Vatalanib (Fig. 6).

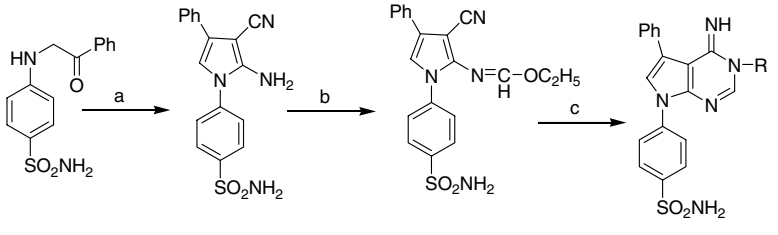
2.2. Chemistry

Pyrrolo[2,3-*d*]pyrimidines are known to have a broad spectrum of biological activities including antitumor activity, also pyrrole and pyrrolo[2,3-*d*] pyrimidines possess antioxidant properties and hence have radioprotective activity.[30]

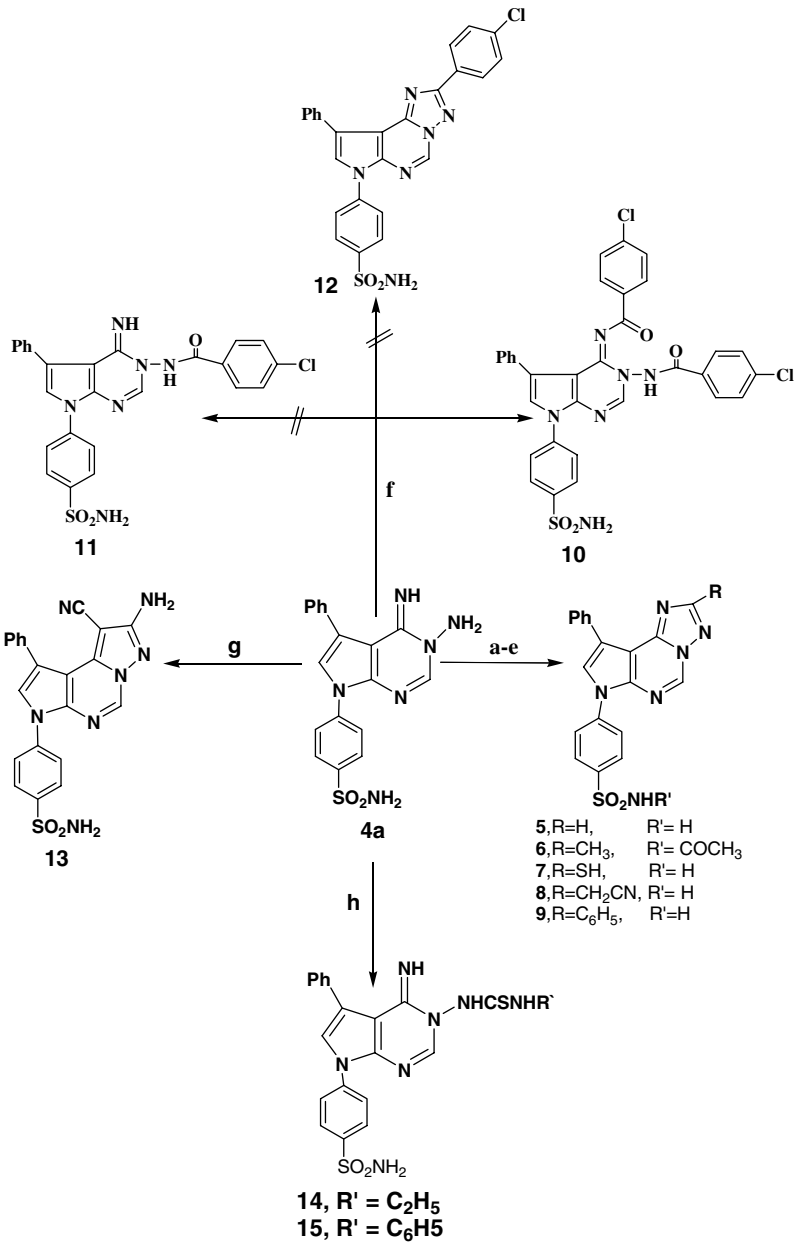
Additionally, sulfonamides have been explored as potential anticancer agents. In the light of these facts and guided by molecular modeling results, the present work was carried out, hoping that the newly synthesized compounds might exhibit significant antitumor and radioprotective activities.

Interaction of sulfanilamide with phenacyl bromide furnished 4-(2-oxo-2-phenyl-ethylamino)-benzenesulfonamide **1**,[31] which was reacted with malononitrile in ethanol in the presence of sodium ethoxide to give the strategic starting material pyrrole derivative **2**[31] (Scheme 1).

The 2-amino-3-cyano-4-phenyl-pyrrole derivative **2** was subjected to reaction with triethylorthoformate under reflux to give the Ethyl *N*-3-cyano-4-phenyl-1-(4-sulfamoylphenyl)-1*H*-pyrrol-2-yl formimidate **3**. The structure of compound **3** was confirmed by its micro-analytical and spectral data, where the IR spectrum showed bands at 2200 cm^{-1} and 1580 cm^{-1} attributed to the presence of (C≡N) and (C=N) groups, respectively. Moreover, the ^1H NMR spectrum of compound **3** revealed a triplet at 1.3 ppm and a quartet at 4.3 ppm attributed to the ethyl group, and a singlet at 10.5 assigned to the proton of (N=CH) group (Scheme 1).



Scheme 1. Compounds: **4a**, R = NH_2; **4b**, R = C_2H_5; **4c**, R = CH_2CH_2OH; **4d**, R = $CH_2CH_2CH_2OH$; **4e**, R = CH_2 Ph. Reagents: a, malononitrile, sodium ethoxide; b, triethylorthoformate; c, appropriate aliphatic amines.



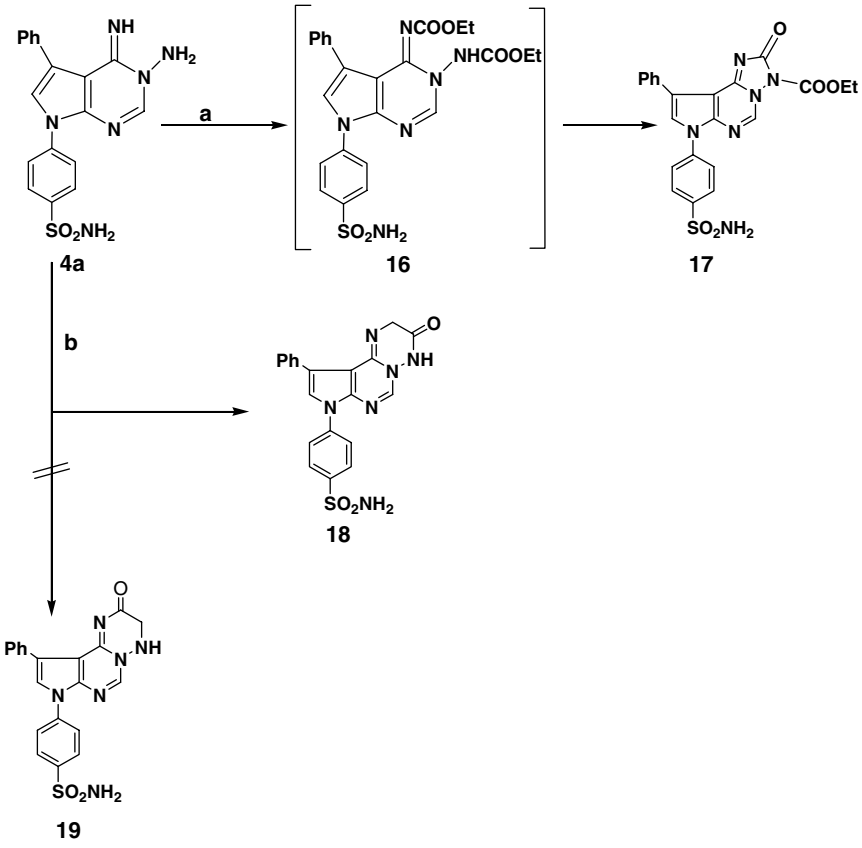
Scheme 2. Reagents: a, formic acid; b, acetic anhydride; c, carbon disulfide; d, ethyl cyanoacetate; e, benzoyl chloride; f, 4-chlorobenzoylchloride g, malononitrile; h, appropiate isothiocyanate.

When the triethylorthoformate derivative **3** was reacted with various aliphatic amines a series of 3,4-dihydropyrrolo[2,3-*d*]pyrimidin derivatives **4a**–**e** were obtained (Scheme 1). The structures of compounds **4a**–**e** were established on the bases of elemental analyses and spectral data. Their IR spectra showed the disappearance of the (C≡N) band. Moreover, ^{1}H NMR spectrum of compound **4b** revealed a triplet at 1.3 ppm and a quartet at 4.0 ppm attributed to the ethyl group. Also, the ^{1}H NMR spectrum of compound **4c** showed multiplet signal at 2.5 ppm assigned to the four protons of the ($2CH_2$) group. Regarding compound **4a**, its mass spectrum exhibited a molecular ion peak *m*/*z* at 380 (M^+, 1.78%) and a base peak at 55.

Compound **4a** was reacted with different one-carbon cyclizing agents to prepare some new pyrrolopyrimidine derivatives containing [1,2,4]triazole moiety which was reported to have a broad spectrum of biological activity including antitumor effects[32–34] (Scheme 2).

Compound **5** was obtained via reaction of compound **4a** with formic acid under reflux. Structure of pyrrolotriazolopyrimidine derivative **5** was confirmed via its mass spectrum, which showed a molecular ion peak *m*/*z* at 390 (M^+, 100% base peak).

Also, reaction of compound **4a** with acetic anhydride gave the pyrrolotriazolopyrimidine derivative **6**. Cyclization at the imino and amino centers took place in



Scheme 3. Reagents: a, ethyl chloroformate; b, ethyl chloroacetate.

addition to acetylation of the amino group of the sulfamoyl moiety (Scheme 2).

IR spectrum of compound **6** showed a band at 1740 cm^{-1} confirming the presence of (C=O) of the acetyl group, also its mass spectrum revealed a molecular ion peak *m/z* at 446 (M^+, 5%) with a base peak at 404.

Carbon disulfide was used also to cyclize compound **4a** under reflux in the presence of pyridine to give compound **7**. Mass spectrum of compound **7** exhibited a molecular ion peak *m/z* at 422 (M^+, 0.34%) with a base peak at 63.

On the other hand compound **4a** reacted under reflux with ethylcyanoacetate in the presence of ethanol containing sodium ethoxide afforded the pyrrolotriazolopyrimidine derivative **8** (Scheme 2).

Microanalytical and spectral data were in accordance with the expected structure, hence its IR spectrum showed band at 2220 cm^{-1} assigned to the presence of (C≡N) group. Mass spectrum of compound **8** showed a molecular ion peak *m/z* at 429 (M^+, 7.71%) with a base peak at 429.

Interaction of compound **4a** with benzoyl chloride afforded the pyrrolotriazolopyrimidine derivative **9** (Scheme 2). This assumed through benzoylation at the amino group followed by cyclization. The structures of compound **9** was confirmed based on its mass spectrum, which revealed a molecular ion peak *m/z* at 466 (M^+, 1.38%) with a base peak at 103.

Also, when compound **4a** was made to reaction with 4-chlorobenzoylchloride, the dibenzoyl derivative **10** (Scheme 2) was isolated, and both the monobenzoyl **11** and the fused system **12** were eliminated from consideration on the basis of elemental analyses and mass spectrum. IR spectrum of compound **10** revealed bands at 1680 cm^{-1} and 1700 cm^{-1}, attributed to the two carbonyl groups. Its mass spectrum showed a molecular ion peak *m/z* at 657 (M^+, 4.55%) with a base peak at 139.

Interaction of compound **4a** with the active methylene compound, malononitrile, afforded the fused tricyclic compound **13** (Scheme 2). IR spectrum of compound **13** showed a band at 2200 cm^{-1} assigned to the presence of (C≡N) group. Its mass spectrum exhibited a molecular ion peak *m/z* at 441 (M^+) with a base peak at 64.

Nucleophilic reaction of compound **4a** on the highly positive carbon of the isothiocyanate RNCS yielded compounds **14** and **15** (Scheme 2). Their structures were confirmed based on their spectral data, where the IR spectrum of compound **14** showed bands at 3460, 3330, and 3150 cm^{-1} assigned to (NH, NH_2) groups, whereas a band at 1250 cm^{-1} due to the introduction of (C=S) group appeared in the IR spectra of both com-

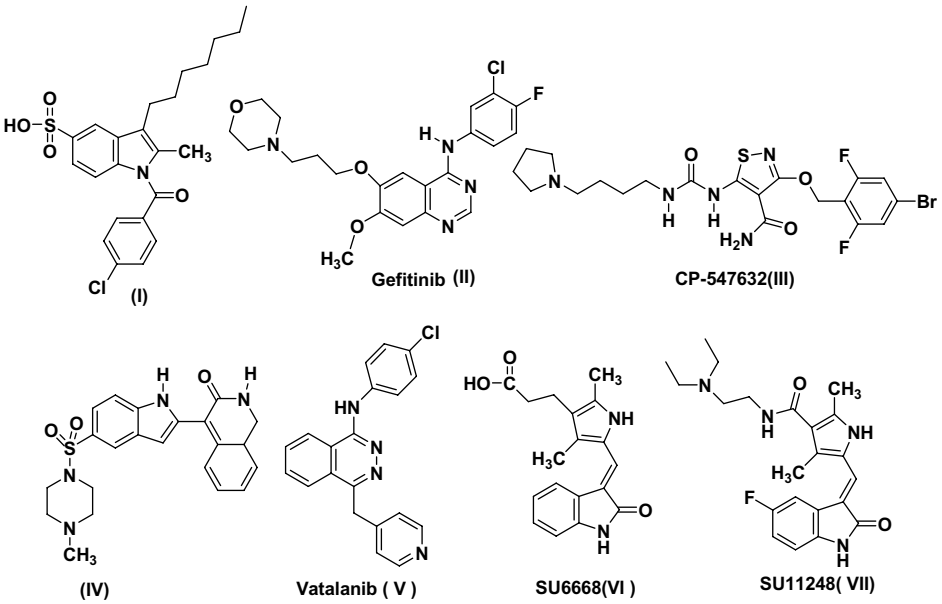


Figure 1. Structures of selective VEGFRTK inhibitory lead compounds **I–VII** used for generation of common feature VEGFRTK inhibitor hypothesis.

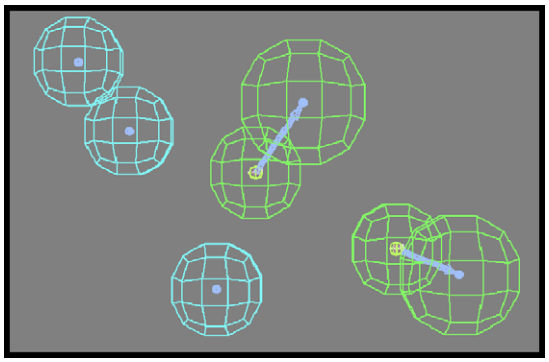


Figure 2. Generated hypothesis for VEGFRTK inhibitors with five features [three HY (pale blue) and two HBA (green)].

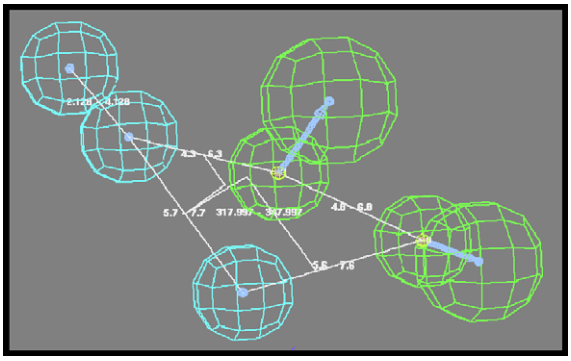


Figure 3. Constraint distances and torsion angel of VEGFRTK hypothesis.

pounds **14** and **15**. Furthermore, ^{1}H NMR spectrum of compound **14** revealed a triplet at 1.2 ppm and a quartet at 4.0 ppm attributed to the ethyl group. The mass spectrum of compound **15** showed a molecular ion peak *m*/*z* at 515 (M^{+}, 3.65%) with a base peak at 77.

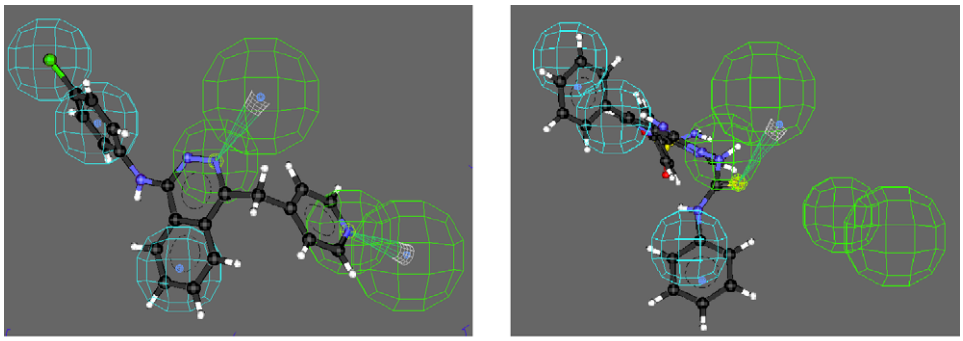
Condensation of compound **4a** with ethyl chloroformate afforded 4-(2-Oxo-9-phenyl-7*H*-pyrrolo[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-7-yl) benzenesulfonamide **17** (Scheme 3). The formation of compound **17** proceeded via initial formation of the intermediate **16** followed by intramolecular cyclization via loss of one mole ethanol. Structure of compound **17** was established on the bases of microanalytical and spectral data. IR spectrum revealed bands at 3360, 3330 cm^{-1} for NH_2 group, 2930, 2895 cm^{-1} for CH aliph., 1710 cm^{-1} for C=O (ester) and at 1680 cm^{-1} for C=O (amide). Moreover, mass spectrum of compound **17** showed a molecular ion peak *m*/*z* at 480 (M^{+}, 6.09%) and a base peak at 227.

Reaction of compound **4a** with ethyl chloroacetate in refluxing sodium ethoxide solution yielded the pyrrolo[2,3-*d*]pyrimidotriazinederivative **18** rather than its isomeric structure **19** (Scheme 3).

Structure of **18** was suggested rather than structure **19** based on assumption that the reaction basic condition allowed it to proceed through formation of sodium salt on the less basic imino nitrogen atom, and elimination of sodium chloride followed by cyclization.[35] In addition, IR spectrum of the isolated product **18** showed a carbonyl band at 1620 cm^{-1}, which was at less frequency than that expected for structure **19**. Also, ^{1}H NMR spectrum of compound **18** revealed singlet at 4.3 for the (CH_2CO) group. Moreover, the mass spectrum showed a molecular ion peak *m*/*z* at 419 (M−1, 2.81) with a base peak at 80.

2.3. Antitumor activity

Doxorubicin, the reference drug used in this study, is one of the most effective antitumor agents used to produce regressions in acute leukemia's, Hodgkin's disease,



Lead **V** (**Vatalanib**) **15,** fit value 2.95, energy = 5.47

Figure 4. Mapping of VEGFRTK inhibitor hypothesis with Vatalanib and compound **15**.

Table 1. Fit and energy values and in vitro cytotoxic activity of some newly synthesized compounds

Compound	Fit value	Energy K/cal^{-1}	Non viable cells (%)					
			Concentration (μmol/mL)					
			200	100	75	50	25	IC_{50}[a] (nM/ml)
2	2.13	7.39	100	100	100	80	40	88.75
5	1.5	0.1	90	60	15	0	0	100
6	2.97	10.00	100	80	20	5	0	75.78
7	1.78	0.46	80	80	20	0	0	90.25
8	1.78	0	90	80	15	0	0	90.25
9	1.73	0.44	0	0	0	0	0	>200
13	0.9	7.78	0	0	0	0	0	>200
15	2.95	5.47	100	95	80	60	50	26.5
Vatalanib	5.00	0.13	—	—	—	—	—	—
Doxorubicin	—	—	100	75	64	55	20	81.5

[a] IC_{50} value which corresponds to the compound concentration causing 50% mortality in net cells.

Table 2. Docked energies and estimated K_i values for Vatalanib and compound **15**

Compound	Docked energy	K_i
Vatalanib	−9.41	+1.37e−06
15	−9.54	+2.25e−06

and other lymphomas. The relationship between survival ratio and drug concentration was plotted to obtain the survival curve of Ehrlich Ascites Carcinoma (EAC) cell line. The response parameter calculated was IC_{50} value (Table 1), which corresponds to the compound concentration causing 50% mortality in net cells.

Compounds **6** and **15** showed significant in vitro antitumor activity compared to the reference drug Doxorubicin. One must mention that compound **15** (IC_{50}, 26.5) possesses thioureido moiety which is known to have antitumor activity.[36]

Also, fusion of pyrrolopyrimidine in a tricyclic structure namely pyrrolotriazolopyrimidine **6** (IC_{50}, 67.5) enhances the antitumor activity which agrees with the literature.

2.4. Radioprotective activity

Radiation exposure significantly increases lipid peroxidation (LPx), such increase seems to be the result of inactivation of scavenger enzymes activities induced by reactive oxygen species (ROS). Oxidative stress occurs in living organisms when the production of ROS exceeds the ability of organisms to prevent their accumulation.[37,38] Such elevation of LPx is accompanied by decline in GSH content and in the activity of related antioxidant enzyme SOD. Additionally LPx can be initiated by hydrogen abstraction from lipid molecules by lipid radiolytic products. This leads to permeability changes, secondary alteration in membrane proteins, and other sequences.[39,40]

2.5. Effect of compound 10 and/or γ-irradiation on lipid peroxidation and antioxidant status

2.5.1. Glutathione level in blood (GSH). As summarized in Table 3, a significant depletion in GSH level was observed in blood of irradiated mice compared to their corresponding controls. While mice exposed to γ-irradiation and treated with compound **10** showed significant increase of GSH content in blood.

2.5.2. Lipid peroxidation content (MDA) in plasma. As shown in Table 3, mice exposed to γ-irradiation showed significant elevation in Malonaldialdehyde (MDA) level in plasma compared to the control values. Treatment with compound **10** prior to irradiation exhibited a higher reduction in MDA levels in blood as compared to irradiated group.

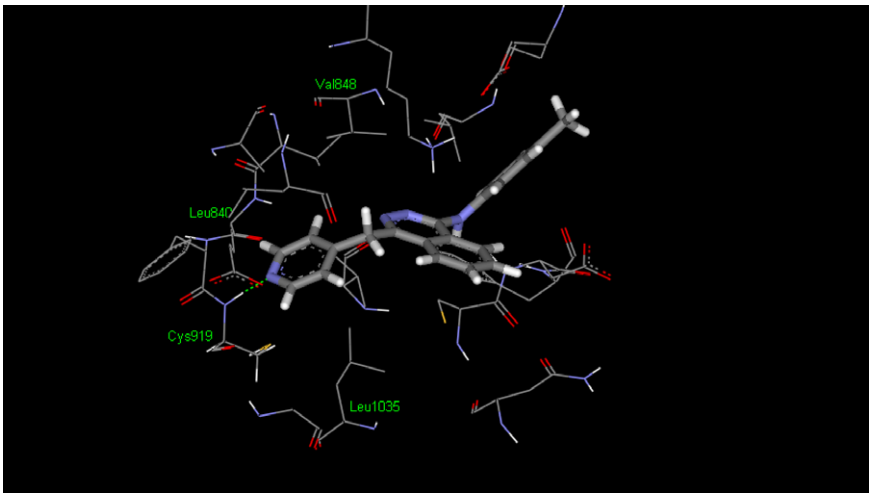


Figure 5. The proposed binding mode of Vatalanib inside the active site of VEGFT kinase resulting from docking. The most important amino acids are shown together with their respective numbers. Vatalanib forms only hydrogen bond with Cys919 through its nitrogen (HB acceptor) in pyridine moiety. The other interactions are hydrophobic bonding with Val848, Leu840, and Leu1035 amino acids.

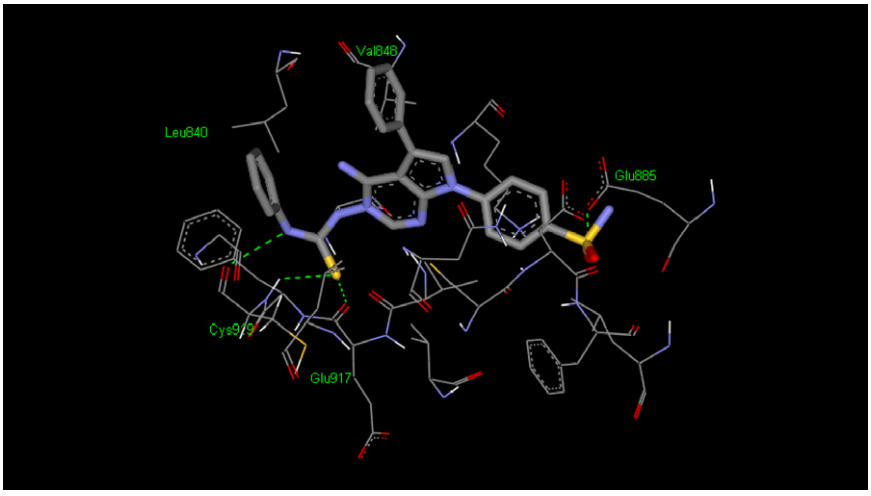


Figure 6. The proposed binding mode of compound **15** inside the active site of VEGFT kinase resulting from docking. The most important amino acids are shown together with their respective numbers. Compound **15** forms three hydrogen bonds with Cys919 and Glu917 through its isothiocyanate moiety and other hydrogen bond with Glu885 through its sulfonamide moiety. The other interactions are hydrophobic bonding with Val848 and Leu840 amino acids.

Table 3. Effect of compound **10** administration on blood glutathione (GSH) content and plasma lipid peroxide concentrations (LPx) of normal and irradiated mice

Groups		GSH mg/dl	LPx μmol/ml
Control	Mean ± SE% of change	78.59 ± 5.45 (100%)	78.05 ± 0.79 (100%)
CMC	Mean ± SE$^{\#}$	75.45 ± 0.28 (96.15%)	80 ± 1.18 (103.9%)
Rad	Mean ± SE$^{\#}$	38.98 ± 1.03*** (49.59%)	101.29 ± 0.11*** (129.78%)
Compound **10**	Mean ± SE$^{\#}$	138.07 ± 13.75** (175.68%)	80.96 ± 2.23 (103.7%)
Compound **10** + Rad	Mean ± SE$^{\#}$	303.51 ± 35.99*** (386.19%)	80.32 ± 1.00 (100.9%)

Each value is the mean of eight mice ±SE.
$^{\#}$ Percentage of change from control group.
** High significance at $P < 0.01$ and very high significance *** at $P < 0.001$.
CMC: Carboxy methyl cellulose.

3. Conclusion

The present data showed that some compounds combining both pyrrole or pyrrolo[2,3-*d*]pyrimidine and benzenesulfonamide moieties exhibited promising in vitro cytotoxic activity against (EAC) cell line. Compounds **6** and **15** showed the highest in vitro cytotoxic activity when compared to other tested compounds and Doxo-

rubicin as a reference drug. Additionally, compound **10** showed significant radioprotective activity. Docking studies revealed that compound **15** showed binding mode similar to vatalanib which was reported to have VEGFRTK inhibitory effects.

4. Experimental protocols

4.1. Catalyst molecular modeling experiments

All molecular modeling work was performed on Silicon Graphic (SGI), Fuel workstation (500 MHz, R 14000 A™ processor, 512 MB memory) using the catalyst package of Molecular Simulation (version 4.8), under an IRIX 6.8 operating system, at the Faculty of Pharmacy, Ain Shams University. A generalized visualizer, confirm, info, HipHop, compare/fit, force field was used throughout.

Training sets, lead compounds **I**–**VII**, were selected. Molecules were built within the catalyst and conformational models for each compound were generated automatically using the poling algorithm. This emphasizes representative coverage over a 20 kcal mol^{-1} energy range above the estimated global energy minimum and the best searching procedure was chosen. The training molecules with their associated conformational models were submitted to catalyst by using default common features hypothesis generation by using HipHop commands. All needed features for VEGFRTK inhibitors were selected from the Dictionary List: HB Acceptor, Hydrophobic (*HY*) and HB donor. By this step, we specified the expected features required for the activity of VEGFRTK inhibitors. Then, a Generate Hypothesis order was given to the computer. Finally, the Process data were collected, and the Process log files were examined which showed that 10 hypotheses were generated.

All the 10 generated hypotheses were analyzed. The assessment of the ideal hypothesis among the generated ones indicated that hypothesis ranked number 2 was the ideal one.

4.2. Docking studies

- The docking was performed using Autodock 3.05.[41]
- The docking was performed on two compounds; Vatalanib and **15**.
- The structures were minimized by Merck Molecular force field (MMFF) to a gradient of 4.5×10^{-5} using Spartan'06. The optimized geometries were exported as Sybyl mol2 file which were exported to Autodock tools. Gasteiger charges were calculated for each molecule and the torsional degrees of freedom were set to the maximum number of rotatable bonds.
- The receptor structure was downloaded from the protein databank (pdb) [www.rcsb.org], pdb entry 1VR2. Water molecules were deleted from the file and hydrogens were added, then Kollman charges were calculated. No further geometry optimization was performed.
- Non-polar hydrogens were merged with the parent atoms for both the ligands and the receptor.
- The grid maps were generated for a box centered on the ATP binding site (extension relative to the original pdb file coordinates: *x*: 27.2–42.2, *y*: 26.0–41.0, *z*: 9.0–24.0), and the grid points spacing was kept to the default value of 0.375 Å.
- The docking itself was performed using the Lamarckian Genetic Algorithm, with a translational increment of 0.5 Å, and both quaternion and torsional angles were incremented by 15 Å each. The generation size was set to 150 individuals per generation, and the rest of the docking parameters were kept to the default values. Ten runs of the Genetic algorithm were performed, thus for each compound, we got 10 docked poses, which were subsequently clustered relative to the original MMFF structure using root mean square deviation (RMSD) tolerance of 1.0 Å. The poses were evaluated using the default empirical scoring function in Autodock.

4.3. Chemistry

Melting points are uncorrected and were determined on a Stuart melting point apparatus (Stuart Scientific, Redhill, UK). Elemental analyses (C, H, N) were performed on Perkin-Elmer 2400 analyzer (Perkin-Elmer, Norwalk, CT, USA) at the Microanalytical Unit of Cairo University. All compounds were within ±0.4% of the theoretical values. IR spectra (KBr) were measured on (Pye Unicam SP1000 IR spectrophotometer Thermoelectron, Egelbach, Germany). ^{1}H NMR spectra were obtained on a Bruker 300 MHz NMR spectrophotometer (Bruker, Munich, Germany) in DMSO-d_6 as a solvent, using tetramethyl-silane (TMS) as internal standard. Mass spectra were run on Varian MAT 311-A70 eV (Varian, Fort Collins, USA).

4.3.1. 4-(2-Oxo-2-phenyl-ethylamino)-benzenesulfonamide (1). A mixture of sulfanilamide (1.72 g, 0.01 mol) with phenacyl bromide (2 g, 0.01 mol) was refluxed in dimethylformamide (30 ml); in the presence of triethylamine (three drops); for 3 h. The solid obtained was filtered and recrystallized from ethanol[31] (88% yield). M.p.: 218–220 °C as reported,[31] IR (KBr, cm^{-1}): 3474, 3259, 3130 (NH_2,NH), 1676 (C=O), 1306, 1195 (SO_2). ^{1}H NMR, (DMSO-d_6): δ 4.80 (d, 2H, CH_2), 6.60–7.70 (m, 11H, Ar–H + NH_2), 8.10 (s, 1H, NH), $C_{14}H_{14}N_2O_3S$ (290).

4.3.2. 4-(2-Amino-3-cyano-4-phenyl-pyrrol-1-yl)benzenesulfonamide (2). A mixture of compound **1** (2.9 g, 0.01 mol) with malononitrile (0.7 g, 0.01 mol) was refluxed in ethanol (30 ml) containing sodium ethoxide (0.5 g) for 3 h. The reaction mixture was acidified with diluted HCl. The solid obtained was recrystallized from ethanol[31] (85% yield), m.p. 200–202 °C as reported,[31] IR (KBr, cm^{-1}): 3400, 3340, 3230 (NH_2), 2200 (C≡N), 1350, 1170 (SO_2), ^{1}H NMR, (DMSO-d_6): 6.20 (br. s,

2H, NH2), 6.90 (s, 1H, pyrrole-H), 7.30–7.90 (m, 11H, Ar–H + SO_2NH_2), MS (*m*/*z*): 338 (M^+, 33.9%), 258 (9.7%), 155 (100%), 139 (2.9%), $C_{17}H_{14}N_4O_2S$ (338).

4.3.3. Ethyl *N*-3-cyano-4-phenyl-1-(4-sulfamoylphenyl)-1*H*-pyrrol-2-yl-formimidate (3). A mixture of compound **2** (3.4 g, 0.01 mol) and triethylorthoformate (20 ml) was refluxed for 5 h. The reaction mixture was cooled and then poured onto ice cold water. The formed residue was recrystallized from dioxane (75% yield), m.p. 120–122 °C, IR (KBr, cm^{-1}): 3350, 3230 (NH_2), 2200 (C≡N), 1580 (C=N), 1340, 1155(SO_2), ^1H NMR (DMSO-d_6) δ: 1.3 (t, 3H, CH_3), 4.3 (q, 2H, CH_2), 5.0 (s, 1H, CH pyrrole), 7.3–8.2 (m, 11H, Ar–H + NH_2), 10.5 (s, 1H, N=CH). Anal. Found: C, 60.60; H, 4.20; N, 14.40%; $C_{20}H_{18}N_4O_3S$ Calcd C, 60.91; H, 4.56; N, 14.21%.

4.3.4. 4-(3-Amino-4-imino-5-phenyl-3,4-dihydropyrrolo[2,3-*d*]pyrimidin-7-yl)benzenesulfonamide(4a),4-(3-ethyl-4-imino-5-phenyl-3,4-dihydropyrrolo[2,3-*d*]pyrimidin-7-yl)benzenesulfonamide(4b), 4-(3-2 hydroxyethyl-4-imino-5-phenyl-3,4-dihydropyrrolo[2,3-*d*]pyrimidin-7-yl)benzenesulfonamide(4c), 4-(3-3 hydroxypropyl-4-imino-5-phenyl-3,4-dihydropyrrolo[2,3-*d*]pyrimidin-7-yl)benzenesulfonamide (4d), and 4-(3-benzyl-4-imino-5-phenyl-3,4-dihydropyrrolo[2,3-*d*]pyrimidin-7-yl)benzenesulfonamide(4e). A mixture of **3** (3.9 g, 0.01 mol) and the appropriate aliphatic amines (0.01 mol) was stirred in ethanol at room temperature for 30 min, the solid formed was filtered, and recrystallized from dioxane, **4a**: (87% yield), m.p. 206–207 °C, IR (KBr, cm^{-1}) 4a: 3450, 3315, and 3150 (NH, NH_2), 1580 (C=N), 1360, 1170 (SO_2), MS (*m*/*z*) **4a**: 381 (M + 1, 1.78%), 55 (100%), 194 (3.56%), 116(6.87%), Anal. Found: C, 56.50; H, 4.60; N, 21.80%; $C_{18}H_{16}N_6O_2S$ Calcd C, 56.84; H, 4.21; N, 22.11%. **4b**: (70% yield), m.p. 237–239 °C. IR (KBr, cm^{-1}): 3460, 3330, 3200 (NH, NH_2), 1600 (C=N), 1360, 1170 (SO_2), ^1H NMR (DMSO-d_6) δ: 1.3 (t, 3H, CH_3), 4.0 (q, 2H, CH_2), 5.0 (s, 2H, NH_2), 7.3–7.7 (m, 11H, Ar–H + NH + CH pyrrole), 8.1 (s, 1H, CH pyrimidine), Anal. Found: C, 61.30; H, 4.50; N, 17.50%; $C_{20}H_{19}N_5O_2S$ Calcd C, 61.07; H, 4.83; N, 17.81%. **4c**: (78% yield), m.p. 260–262 °C, IR (KBr, cm^{-1}): 3400, 3310, 3190 (NH, NH_2), 1590 (C=N), 1360, 1170 (SO_2), ^1H NMR, (DMSO-d_6) δ: 2.5 (m, 4H, $2CH_2$), 6.4 (s, 1H, CH pyrrole), 7.2–8.3 (m, 12H, Ar–H + NH + SO_2NH_2), 9.2 (s, 1H, CH pyrimidine), Anal. Found: C, 58.30; H, 4.50; N, 17.40%; $C_{20}H_{19}N_5O_3S$ Calcd C, 58.68; H, 4.65; N, 17.11%. **4d**: (80% yield), m.p. 220–222 °C, IR (KBr, cm^{-1}): 3450, 3315, and 3150 (NH, NH_2), 1580 (C=N), 1360, 1170 (SO_2). Anal. Found: C, 59.30; H, 4.60; N, 16.30%; $C_{21}H_{21}N_5O_3S$ Calcd C, 59.57; H, 4.96; N, 16.55%. **4e**: (65% yield), m.p. 160–162 °C, IR (KBr, cm^{-1}): 3440, 3325, 3200 (NH, NH_2), 1585 (C=N), 1360, 1170 (SO_2). Anal. Found: C, 65.70; H, 4.30; N, 15.60%; $C_{25}H_{21}N_5O_3S$ Calcd C, 65.93; H, 4.62; N, 15.38%.

4.3.5. 4-(9-phenyl-7*H*-pyrrolo[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-7-yl)benzenesulfonamide (5). A solution of **4a** (3.8 g, 0.01 mol) in formic acid (20 ml) was refluxed for 4 h and the reaction mixture was then concentrated. The separated crystals were recrystallized from dioxane (63% yield), m.p. 260–262 °C, IR (KBr, cm^{-1}): 3300, 3190 (NH_2), 1580 (C=N), 1350, 1160 (SO_2), MS (*m*/*z*): 390 (M^+, 100% base peak), Anal. Found: C, 58.80; H, 3.80; N, 21.70%; $C_{19}H_{14}N_6O_2S$ Calcd C, 58.46; H, 3.59; N, 21.54%.

4.3.6. 4-(2-Methyl-9-phenyl-7*H*-pyrrolo[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-7-yl)benzenesulfonamide (6). A solution of **4a** (3.8 g, 0.01 mol) in acetic anhydride (20 ml) was refluxed for 4 h and the reaction mixture was then concentrated. The separated crystals were recrystallized from dioxane (65% yield), m.p. 268–270 °C, IR (KBr, cm^{-1}): 3230 (NH), 1740 (C=O), 1350, 1160 (SO_2), MS (*m*/*z*): 446 (M^+, 5%), 404 (100% base peak), Anal. Found: C, 59.30; H, 4.20; N, 18.50%; $C_{22}H_{18}N_6O_3S$ Calcd C, 59.19; H, 4.04; N, 18.83%.

4.3.7. 4-(2-Mercapto-9-phenyl-7*H*-pyrrolo[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-7-yl)benzenesulfonamide (7). A solution of **4a** (3.8 g, 0.01 mol) in carbon disulfide (2 ml) was refluxed for 8 h in the presence of pyridine (20 ml), the reaction mixture was then concentrated and poured into ice cold water. The separated solid was filtered off and recrystallized from ethanol (82% yield), m.p. 259–260 °C, IR (KBr, cm^{-1}): 3300, 3230, (NH_2), 1580 (C=N), 1350, 1150 (SO_2).

MS (*m*/*z*): 422 (M^+, 0.34%), 63 (100%). Anal. Found: C, 54.30; H, 3.10; N, 19.60%; $C_{19}H_{14}N_6O_2S_2$Calcd C, 54.03; H, 3.32; N, 19.91%.

4.3.8. 4-(2-Cyanomethyl-9-phenyl-7*H*-pyrrolo[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-7-yl)benzenesulfonamide (8). A mixture of **4a** (3.4 g, 0.01 mol) and ethyl cyanoacetate (1.3 g, 0.01 mol) was refluxed for 8 h in ethanol (30 ml) containing sodium ethoxide (0.23 g, 0.01 mol). The reaction mixture was then acidified with diluted HCl. The separated solid was filtered, and recrystallized from dioxane (76% yield), m.p. 264–266 °C, IR (KBr, cm^{-1}): 3300, 3230 (NH_2), 2220 (C≡N), 1590 (C=N), MS (*m*/*z*): 429 (M^+, 7.71%), 429(100%). Anal. Found: C, 58.50; H, 3.20; N, 22.60%; $C_{21}H_{15}N_7O_2S_2$ Calcd C, 58.74; H, 3.49; N, 22.84%.

4.3.9. 4-(2,9-Diphenyl-7*H*-pyrrolo[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-7-yl)benzenesulfonamide (9). A mixture of **4a** (3.4 g, 0.01 mol) and benzoyl chloride (10 ml) was refluxed for 2 h. The reaction mixture was then concentrated and the formed precipitate was filtered off and recrystallized from dioxane, (70% yield), m.p. 268–270 °C, IR (KBr, cm^{-1}): 3340, 3195 (NH_2), 1585 (C=N), 1350, 1160 (SO_2). MS (*m*/*z*): 466 (M^+, 1.38%), 103 (100%), 77 (15.4%). Anal. Found: C, 64.70; H, 3.60; N, 18.40%; $C_{25}H_{18}N_6O_2S$ Calcd C, 64.38; H, 3.86; N, 18.03%.

4.3.10. 3-(4-Chlorobenzoylamino)-4-(4-chlorobenzoylamino)5-phenyl-7-(4-sulfamoylphenyl)-3,4-dihydropyrrolo[2,3-*d*]pyrimidin (10). A mixture of compound **4a** (3.8 g, 0.01 mol) and 4-chlorobenzoylchloride (10 ml) was refluxed for 3 h, the reaction mixture was then concentrated and the obtained solid was recrystallized from dioxane

(80% yield), m.p. 223–224 °C, IR (KBr, cm^{-1}): 3340, 3240, 3230, (NH, NH_2), 1700, 1680, (2 C=O), 1310, 1150 (SO_2). MS (*m/z*): 657 (M^+, 4.55%), base peak at 139 (100%). Anal. Found: C, 58.50; H, 3.10; N, 12.40%; $C_{32}H_{22}N_6O_4SCl_2$ Calcd C, 58.36; H, 3.34; N, 12.77%.

4.3.11. 4-(8-Amino-9-cyano-1-phenyl-3*H*-pyrazolo[1,5-*c*]pyrrolo[3,2-*e*]pyrimidin-3-yl)benzenesulfonamide (13). A mixture of **4a** (3.8 g, 0.01 mol) and malononitrile (0.7 g, 0.01 mol) was refluxed in ethanol (30 ml) containing sodium ethoxide (0.23 g, 0.01 mol) the reaction mixture was then acidified with diluted HCl. The obtained residue was filtered and recrystallized was from ethanol, (61% yield), m.p. 152–153 °C, IR (KBr, cm^{-1}): 3460, 3330 (NH_2), 2200 (C≡N), 1340, 1160 (SO_2). MS (*m/z*): 441 (M^+, 4.87%), base peak at 64 (100%). Anal. Found: C, 58.60; H, 3.20; N, 22.50%; $C_{21}H_{15}N_7O_2S$ Calcd C, 58.74; H, 3.49; N, 22.84%.

4.3.12. 4-(3-(3-Ethyl thioureido)-4-imino-5-phenyl-3,4-dihydropyrrolo[2,3-*d*]pyrimidin-7-yl)benzenesulfonamide (14), 4-(3-(3-phenyl thioureido)-4-imino-5-phenyl-3,4-dihydropyrrolo[2,3-*d*]pyrimidin-7-yl)benzenesulfonamide (15). A mixture of **4a** (3.8 g, 0.01 mol) and the appropriate isothiocyanate derivatives (RNCS) was refluxed in ethanol for 3 h, the reaction mixture was concentrated, and the separated crystals were recrystallized from ethanol, **14**: (70% yield), m.p. 258–260 °C, IR (KBr, cm^{-1}): 3460, 3330, and 3150 (NH, NH_2), 3100 (CH arom.), 1250 (C=S), 1350, 1170 (SO_2), ^1H NMR (DMSO-d_6): δ 1.20 (t, 3H, CH_3), 4.0 (q, 2H, CH_2), 6.2 (s, 1H, CH pyrrole), 7.3–8.0 (m, 11H, Ar–H + SO_2NH_2), 8.3 (s, 1H, CH pyrimidine), 9.3(s, 3H, +3NH). Anal. Found: C, 53.60; H, 4.20; N, 21.20%; $C_{21}H_{21}N_7O_2S_2$ Calcd C, 53.96; H, 4.49; N, 20.98%.

Compound **15**: (81% yield), m.p. 270–271 °C, IR (KBr, cm^{-1}) 15: 3450, 3320, 3130 (NH, NH_2), 3080 (CH arom.), 1245 (C=S), 1350, 1170 (SO_2), MS (*m/z*): 515 (M^+, 3.65%), 481(61.13%), 77 (100%), 65(53.16%). Anal. Found: C, 58.50; H, 4.40; N, 19.30%; $C_{25}H_{21}N_7O_2S_2$ Calcd C, 58.25; H, 4.08; N, 19.03%.

4.3.13. 4-(2-Oxo-9-phenyl-7*H*-pyrrolo[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-7-yl)benzenesulfonamide (17). A mixture of **4a** (3.8 g, 0.01 mol) and ethyl chloroformate (1.4 g, 0.015 mol) in benzene (20 ml) was refluxed for 5 h. The reaction mixture was then filtered off and recrystallized from ethanol (80% yield), m.p. >300 °C, IR (KBr, cm^{-1}): 3360, 3330 cm^{-1} (NH_2), 2983, 2895 cm^{-1} (CH aliph.), 1710 cm^{-1} (C=O ester), 1680 cm^{-1} (C=O amide), 1350, 1150 (SO_2). MS (*m/z*): 478 (M^+, 6.09%), 227 (100%), 174(56.09%), 134(61.07%), 73 (53.14%). Anal. Found: C, 55.50; H, 4.50; N, 17.30%; $C_{22}H_{18}N_6O_5S$ Calcd C, 55.23; H, 3.76; N, 17.57%.

4.3.14. 4-(2-Oxo-10-phenyl-3,4-dihydro-pyrrolo[2,3-*d*]pyrimido[3,4-e][1,2,4]triazine-8-yl)benzenesulfonamide (18). A mixture of **4a** (3.8 g, 0.01 mol) and ethyl chloroacetate (1.2 g, 0.01 mol) was refluxed in ethanol (30 ml) containing sodium ethoxide (0.23 g, 0.01 mol) for 10 h, the reaction mixture was then cooled and poured into ice cold water, the solid separated was recrystallized from dioxane (80% yield), m.p. >300 °C, IR (KBr, cm^{-1}): 3450, 3315 (NH_2), 1620 (C=O), 1580 (C=N), 1350, 1160 (SO_2), ^1H NMR (DMSO-d_6): δ 4.3 [s, 2H, CH_2CO], 6.7 [s, 1H, CH pyrrole], 7.1–7.9 [m, 11H, Ar–H + SO_2NH_2], 8.3 [s, 1H, CH pyrimidine], 9.2 [s, 1H, NH], MS (*m/z*): 419 (M-1, 2.81%), 80 (100%). Anal. Found: C, 57.30; H, 3.60; N, 19.70%; $C_{20}H_{16}N_6O_3S$ Calcd C, 57.14; H, 3.81; N, 20.00%.

4.4. Biological testing

4.4.1. Antitumor activity. Ehrlich Ascites Carcinoma cells (EAC) were obtained by needle aspiration of ascitic fluid from the preinoculated mice under aseptic conditions. Tumor cells suspension (2.5×10^6 per ml) was prepared in saline. Tested compounds solutions were prepared by dissolving 10 μmol of the tested compounds in a mixture of 7 ml DMSO and 3-ml saline. Some compounds failed to be dissolved in either DMSO or DMF so could not be screened as cytotoxic agents using this method.

In a set of sterile test tubes 200, 100, 75, 50 and 25 μL of each tested compound were mixed with 100 μL of tumor cell suspension, then completed to 1 mL with saline to obtain a solution of 200, 100, 75, 50 and 25 μM, respectively, for each tested compound. The test tubes were incubated at 37 °C for 2 h. Trypan blue exclusion test was carried out to calculate the percentage of non-viable cells after 2 h of incubation.[42] The results of in vitro cytotoxic activity experiments are presented in Table 1.

4.4.2. Radioprotective activity. This study was conducted to evaluate the potency of one of the synthesized compounds **10** as protective agent against γ-irradiation-induced toxicity. Female Swiss albino mice were injected intraperitoneally with suspension of the tested compound in carboxy methylcellulose once every other day for a total of three injections during 7 days. Each injection was given 30 min prior to exposure to a single dose of whole body γ-irradiation at a dose level of 6 Gy. Lipid peroxide level (LPx) and Glutathione content (GSH) were estimated in blood of animals at the end of the experiment.

4.4.2.1. Chemicals and facilities. All chemicals and reagents were of the highest grade commercially available. Facilities including animal house and biochemical equipments have been made available by the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt.

4.4.2.2. Animals. Female Swiss albino mice weighing 25–30 g were used in this study. Mice were housed at a constant temperature (24 ± 2 °C) with alternating 12-h light and dark cycles and fed standard laboratory food and water.

4.4.2.3. Irradiation. Whole body γ-irradiation was performed at the NCRRT, Cairo, Egypt, using Gamma cell-40 (Cesium-137 source). Animals were irradiated at three doses, every other day 30 min after drug injection at dose level of 6 Gy delivered at a dose rate of 0.86 Gy/min.

4.4.2.4. Compound 10 dosing. Tested compound was suspended in carboxy methylcellulose (CMC) and given to mice by intraperitoneal injection (ip) of the maximum tolerated dose (150 mg/kg body weight) once every other day for a total of three injections during 7 days.

4.4.2.5. Experimental design. From the beginning of the experiment, mice were divided into eight groups. Each group consists of 10 animals. All experimental animals were categorized as follows:

1. Control: Animals served as untreated control group.
2. CMC: Animals were treated by ip injection of Carboxymethylcellulose.
3. Rad: Animals were subjected to three doses; every other day of whole body γ-irradiation at a dose level of 6 Gy starting from day 10.
4. Compound 10: Animals were treated by ip injection of suspension of compound **10** in Carboxymethylcellulose.
5. Compound **10** + Rad: Mice injected ip with compound **10** and subjected to whole body γ-irradiation.

4.4.2.6. Sample collection. Animals were fasted for 16 h prior to each sampling. Samples were collected after 1 day post last irradiation dose. Whole blood was collected by heart puncture after light anesthesia using heparinized syringes. One part was used for glutathione (GSH) estimation. The separated plasma from heparinized blood was used for the determination of lipid peroxide as malondialdehyde (MDA).

4.4.2.7. Analytical procedures. Lipid peroxide (Lpx) level in plasma was ascertained by the formation of MDA and measured as described by Yoshioka et al.[43] GSH content was determined according to Beutler et al.[44]

4.4.2.8. Statistical analysis. Student's *t* test[45] was used for the evaluation of the biochemical parameters.

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