New quinoxaline-based VEGFR-2 inhibitors: design, synthesis, and antiproliferative evaluation with *in silico* docking, ADMET, toxicity, and DFT studies†

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A new series of 3-methylquinoxaline-based derivatives having the same essential pharmacophoric features as VEGFR-2 inhibitors have been synthesized and evaluated for their antiproliferative activities against two human cancer cell lines, MCF-7 and HepG-2. Compounds 15b and 17b demonstrated a significant antiproliferative effect with IC_{50} ranging from 2.3 to 5.8 μM. An enzymatic assay was carried out for all the tested candidates against VEGFR-2. Compound 17b was the most potent VEGFR-2 inhibitor (IC_{50} = 2.7 nM). Mechanistic investigation including cell cycle arrest and apoptosis was performed for compound 17b against HepG-2 cells, and the results revealed that 17b induced cell apoptosis and arrested cell cycle in the G2/M phase. Moreover, apoptosis analyses were conducted for compound 17b to evaluate its apoptotic potential. The results showed upregulation in caspase-3 and caspase-9 levels, and improving the Bax/Bcl-2 ratio by more than 10-fold. Docking studies were performed to determine the possible interaction with the VEGFR-2 active site. Further docking studies were carried out for compound 17b against cytochrome P450 to present such compounds as non-inhibitors. *In silico* ADMET, toxicity, and physico-chemical properties revealed that most of the synthesized members have acceptable values of drug-likeness. Finally, DFT studies were carried out to calculate the thermodynamic, molecular orbital and electrostatic potential properties.

1. Introduction

Deregulation of the cell cycle may cause cancer onset, progression, and metastasis. 1,2 Receptor tyrosine kinases (RTKs) play a central role in cellular proliferation. 3 RTK expression is highly organized in normal cells; however, in cancer cells overexpression of some RTKs was observed. 4 Similarly, the vascular endothelial growth factor receptor (VEGFR), an important RTK, plays a remarkable role in angiogenesis. 5 It is composed of three isoforms, VEGFR-1, VEGFR-2 and VEGFR-3. 6 Chiefly, VEGFR-2 is the main mediator of angiogenesis in cancer cells. 7 Signaling of VEGFR-2 is up-regulated at specific phases of cancer to support tumor proliferation and expansion. 8 The main concept to discover novel VEGFR-2 inhibitors is to hinder autophosphorylation and dimerization processes of the receptor. 9 Small molecule inhibitors targeting the kinase domain (KD) leading to blocking signaling pathway hence, suppression of tumor growth. 10 In 2007, sorafenib I turned into the first VEGFR-2 inhibitor 11 to be utilized in the treatment of hepatocellular carcinoma and renal cell carcinoma. 12 During the previous few decades, several VEGFR-2 inhibitors were designed as an adjunctive for cancer therapy. Regorafenib II, 13 lenvatinib III, 14 cabozantinib IV, 15 tivozanib V, 16 and sunitinib VI 17 were marketed for the treatment of different types of cancers (Fig. 1).

VEGFR-2 inhibitors are classified into three classes: (i) ATP competitive inhibitors, binds to the zone which is fitted by adenine ring of ATP *e.g.* sunitinib. 18 (ii) Inhibitors that are not able to bind at adenine binding site but bind beside the hydrophobic pocket *e.g.* sorafenib. 19 (iii) Covalent inhibitors which covalently bind to cysteine amino acid residue at the binding site and hinder binding of ATP *e.g.* vatalanib. 19

Quinoxaline derivatives are a widespread class of the heterocycles receiving the most attention especially in the field of chemotherapy. 20–22 Many drugs incorporating quinoxaline moiety achieved promising results and have been submitted to clinical trials for anticancer therapeutic purposes. 23,24

In this work, some quinoxaline derivatives were synthesized and evaluated for their cytotoxicity and VEGFR-2 inhibitory activity. The most active candidate was assessed for its...
apoptotic effect and cell cycle arrest. Different in silico docking studies were carried out to predict the binding interaction with the prospective target (VEGFR-2) via docking studies. Also, in silico ADMET and toxicity studies were performed to predict the level of drug likeness. Furthermore, DFT studies were carried out to predict the HOMO and LUMO energy as well electrostatic potential map.

1.1 Design concept

Based on the above-mentioned findings and in the extension of our former work targeting anticancer derivatives, especially VEGFR-2 inhibitors we synthesized new quinoxaline derivatives as based on the study of the structure–activity relationships (SAR) of different VEGFR-2 inhibitors. VEGFR-2 inhibitors were found to share basic pharmacophoric features. (i) A head group which is required to be flat hetero aromatic to occupy the hinge region (colored green in Fig. 2). (ii) A hydrophobic spacer to occupy the linker area between the ATP binding domain and the DFG domain of the enzyme (colored purple in Fig. 2). (iii) A hydrogen-bonding (pharmacophore) moiety that is required to achieve hydrogen bond interactions with Asp1044 and Glu883 in the DFG motif (colored red in Fig. 2). (iv) A terminal hydrophobic (tail) moiety which occupies the allosteric hydrophobic back pocket (colored blue in Fig. 2).

The main concept of our design was achieved by bioisosteric alteration of VEGFR-2 inhibitors (sorafenib & sunitinib). Such modifications were done at four positions. Firstly, bioisosteric replacement of pyridine or indole rings by 3-methylquinoxaline-2(1H)-one or 3-methylquinoxaline-2-thiol moieties in the hinge region to occupy the adenine region in the ATP binding pocket. The second strategy was to use N-phenylacetamide moiety in the spacer region instead of the central aryl ring of the lead structures aiming to improve VEGFR-2 binding affinity. Thirdly, we noticed that the conserved hydrogen-bonding moiety between the spacer and the allosteric site residues was done using urea (in case of sorafenib) or amide (in case of sunitinib) moieties. In this regard, we designed our quinoxaline compounds with one or two amides pharmacophoric linking moiety containing HBA–HBD functional groups. The fourth strategy was to replace the terminal hydrophobic tail with other different hydrophobic moieties including aliphatic or substituted phenyl derivatives. The concept of using these hydrophobic moieties was to guarantee different lipophilic and electronic environments, which could result in additional hydrophobic interactions with the receptor.

2. Results and discussion

2.1 Chemistry

The classical method of quinoxaline preparation is to condense phenylenediamine with a dicarbonyl compound. This procedure requires high temperatures, a strong acid catalyst, and long reaction times. Other strategies described for the synthesis of quinoxaline derivatives involve 1,4-addition of 1,2-diamines to diazenylbutenes. There are also several green synthetic methods e.g. one-pot synthesis, microwave-assisted synthesis, recyclable catalysts and reactions in aqueous
In this study and in the light of above findings, we used 3-methylquinoxalin-2(1H)-one as the scaffold to design new antitumor agents. For the synthesis of our target compounds, compounds 5, 6, 10a–d, and 14 were initially synthesized according to the reported procedures as outlined in Schemes 1 and 2.

The final compounds 15a–d, 16, 17a–d and 18 were obtained in good yields following the reported procedures described in Schemes 3 and 4.

### 2.2 Biological evaluation

#### 2.2.1 In vitro anti-proliferative activity

MTT assay protocol was applied for all the tested compounds to evaluate their in vitro antiproliferative activities against MCF-7 and HepG-2. Sorafenib was used as positive control. The results (IC50 values) were summarized in Table 1. Among the tested compounds, 15b and 17b were the most potent antiproliferative candidate. Comparing to sorafenib (IC50 = 3.51 and 2.17 μM against MCF-7 and HepG-2, respectively), compound 17b (the most potent member) showed IC50 value of 2.3 μM and 2.8 μM against MCF-7 and HepG-2, respectively. In addition, compound 15b exhibited IC50 value of 5.8 μM and 4.2 μM against MCF-7 and HepG-2, respectively.

#### 2.2.2 In vitro VEGFR-2 enzyme assay inhibition

All final synthesized compounds were investigated for their VEGFR-2 inhibitory effect using sorafenib as a positive control. The
Scheme 2: General synthetic route of target intermediates 10a–d and 14; reaction conditions: (i) ClCH₂COCl/NaHCO₃/DMF/r.t./1 h, (ii) SOCl₂/DMF/DCE/reflux/4 h, (iii) RNH₂/TEA/CH₃CN/r.t./8 h, (iv) CH₃OH/conc. H₂SO₄/reflux/8 h, (v) NH₂NH₂·H₂O/ethanol/reflux/8 h, (vi) 13/TEA/CH₃CN/r.t./8 h.

Scheme 3: General synthetic route of target final compounds 15a–d and 16; reaction conditions: (i) 10a–d/cat. KI/DMF/WB/6 h, (ii) 14/cat. KI/DMF/WB/6 h.

Scheme 4: General synthetic route of target final compounds 17a–d and 18; reaction conditions: (i) 10a–d/cat. KI/DMF/WB/6 h, (ii) 14/cat. KI/DMF/WB/6 h.
Compounds containing aliphatic hydrophobic tail (3-methylquinoxalin-2(1H)-one) and the corresponding members containing 3-chlorophenyl moiety (15b and 17b with IC<sub>50</sub> values of 3.4 and 2.7 nM, respectively) indicate that aromatic ring containing electron withdrawing group is more preferred biologically than aliphatic moiety. Comparing the IC<sub>50</sub> values of compound 15b and 17b incorporating electron withdrawing group with their corresponding members 15c and 17c incorporating electron donating group, indicate that substitution with electron withdrawing group is more advantageous.

2.2.3 In vitro cytotoxicity against normal hepatic cells. One of the main problems of cancer chemotherapy is the unwanted damage to normal cells caused by the high toxicities of anticancer drugs. To assess the selectivity of the synthesized compounds against cancer cells over normal ones, the cytotoxicity of compound 17b was evaluated in vitro against primary rat hepatocytes using sorafenib as reference. The results revealed that 17b showed cytotoxic activity against cancer HepG-2 cell line (10-fold) more than cytotoxic activity against normal hepatic cells in comparison to sorafenib (11-fold), Table 1. Such results indicate that compound 17b has a significant effect in rapidly proliferating cells but not in normal cells.

2.2.4 Effect of 17b on cell cycle progression. The effect of compound 17b on the cell cycle distribution was evaluated against HepG-2 cells. In this method, HepG-2 cells were treated with 17b (2.3 μM, IC<sub>50</sub> value) and the technique was carried out according to the reported procedure. The results (Table 2 and Fig. 3) revealed that compound 17b arrested cell growth in G2-M phase, accretion of cells at that phase became 30.38% after being 10.81 in control cells. The apoptosis data for compound 17b is described in ESI.

2.2.5 Effects of 17b on the apoptotic markers, caspases, BAX, and Bcl-2. In the current study, western plot technique was utilized for compound 17b (2.3 μM) to investigate its effect on the expression levels of caspases, BAX, and Bcl-2. Our results showed that compound 17b clearly increased the level of caspase-3 by 1.8 fold and caspase-9 by 1.74 fold compared to the control cells (Fig. 4). Also, the results showed that 17b boosted the level of BAX by approximately 4-fold. Moreover, compound 17b markedly downregulated the levels of Bcl-2 by around 3-fold. Finally, compound 17b interestingly boosted the Bax/Bcl-2 ratio by more than 10-fold (Table 3).

2.3 In silico studies

2.3.1 Molecular docking against VEGFR-2. Molecular Operating Environment MOE, package version 2014.09 software was used. The results (IC<sub>50</sub> values) and reported in Table 1. Matching with the cytotoxicity results, compound 17b was the most potent inhibitor with an IC<sub>50</sub> value of 2.7 nM which was more than that of sorafenib (IC<sub>50</sub> value 3.12 nM).

Based on the biological data, we can reach valuable SAR. It was found that the second series compounds incorporating 3-methylquinoxalin-2(1H)-one is more active than corresponding members 15a-d and 16 (incorporating 3-methylquinoxaline-2-thiol). Such results indicate that 3-methylquinoxalin-2(1H)-one moiety is more advantageous than 3-methylquinoxaline-2-thiol moiety. The comparison between compounds containing aliphatic hydrophobic tail (15a and 17a with IC<sub>50</sub> values of 23.1 and 11.2 nM, respectively) and the corresponding members containing 3-chlorophenyl moiety (15b and 17b with IC<sub>50</sub> values of 3.4 and 2.7 nM, respectively) indicate that aromatic ring containing electron withdrawing group is more preferred biologically than aliphatic moiety. Comparing the IC<sub>50</sub> values of compound 15b and 17b incorporating electron withdrawing group with their corresponding members 15c and 17c incorporating electron donating group, indicate that substitution with electron withdrawing group is more advantageous.

### Table 1 In vitro anti-proliferative activities of the synthesized compounds against MCF-7 and HepG-2 cell lines, their VEGFR-2 inhibitory activities on cancer HepG-2, and cytotoxicity for compounds 17b against normal hepatocytes

| Comp. | MCF-7<sup>a</sup> (IC<sub>50</sub>, μM) | HepG-2<sup>a</sup> (IC<sub>50</sub>, μM) | VEGFR-2<sup>a</sup> (IC<sub>50</sub>, nM) | Normal hepatocytes<sup>a</sup> (IC<sub>50</sub>, μM) |
|-------|------------------|------------------|-----------------|------------------|
| 15a   | 62.1 ± 3.2       | 41.2 ± 1.9       | 23.1 ± 0.8      | NT<sup>b</sup>   |
| 15b   | 5.8 ± 0.6        | 4.2 ± 0.3        | 3.4 ± 0.2       | NT<sup>b</sup>   |
| 15c   | 62.2 ± 2.9       | 50.4 ± 2.4       | 27.8 ± 1.2      | NT<sup>b</sup>   |
| 15d   | 61.5 ± 2.3       | 42.8 ± 1.3       | 31.5 ± 1.3      | NT<sup>b</sup>   |
| 16    | 35.8 ± 1.9       | 27.1 ± 1.2       | 18.5 ± 0.8      | NT<sup>b</sup>   |
| 17a   | 29.3 ± 2.1       | 24.5 ± 1.0       | 11.2 ± 0.4      | NT<sup>b</sup>   |
| 17b   | 2.8 ± 0.2        | 2.3 ± 0.2        | 2.7 ± 0.1       | 24.68 ± 1.3      |
| 17c   | 17.9 ± 0.6       | 14.3 ± 0.6       | 13.9 ± 0.5      | NT<sup>b</sup>   |
| 17d   | 35.2 ± 1.6       | 22.4 ± 1.3       | 11.2 ± 0.3      | NT<sup>b</sup>   |
| 18    | 22.3 ± 1.2       | 14.8 ± 0.5       | 11.2 ± 0.2      | NT<sup>b</sup>   |
| Sorafenib | 3.51 ± 1.1   | 2.17 ± 0.1       | 3.12 ± 0.8      | 24.34 ± 1.6      |

<sup>a</sup> IC<sub>50</sub> values are the mean ± S.D. (standard deviations) of three separate experiments. <sup>b</sup> NT: not tested.

### Table 2 Values of different stages of cell cycle progression in HepG-2 after application of the most active compound 17b

| Sample      | % Sub-G1 | % G1     | % S       | % G2/M     |
|-------------|----------|----------|-----------|-----------|
| HepG-2      | 1.56 ± 0.30 | 58.69 ± 2.04 | 28.94 ± 2.39 | 10.81 ± 0.22 |
| 17b/HepG-2  | 1.27 ± 0.17 | 40.03 ± 2.82** | 28.31 ± 1.01 | 30.38 ± 2.93** |

<sup>a</sup> Three independent experiments were applied for each value. **p < 0.01.
Fig. 4  The immunoblotting of the apoptotic markers (normalized to β-actin).  *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 3  Cycle phases after the treatment with compound 17b,  **p < 0.01.
was used for the docking simulations against VEGFR-2 kinase to rationalize the obtained biological results. At the beginning, validation of the docking process was verified and the RMSD value was 0.48 which indicated the validity of the docking process (Fig. 5). The binding pattern of sorafenib to VEGFR-2 active site has been explained in Fig. 6. And the results were matched with the reported data.18,51 The energy scores of the tested candidates and sorafenib were summarized in Table 4.

The mode of interaction of compound 15b against VEGFR-2 active site was the same of sorafenib ($\Delta G = -23.27$ kcal mol$^{-1}$).

The amide group was involved in two hydrogen bonds, where the amide NH formed a hydrogen bond with the carboxylate moiety of Glu883 (2.66 Å) and the carbonyl group formed another hydrogen bond with the NH of Asp1044 (3.0 Å). Additionally, the quinoxaline moiety occupied the hinge region forming two hydrophobic interactions with Leu383 and Phe916. The central phenyl ring formed three hydrophobic interactions with Val897, Val914, and Cys1043. The terminal 3-chlorophenyl

| Sample          | BAX       | Bcl-2     | BAX/Bcl-2 ratio | Caspases-9 | Caspases-3 |
|-----------------|-----------|-----------|-----------------|------------|------------|
| HepG-2          | 1.00 ± 0.31 | 1.00 ± 0.12 | 1.00 ± 0.38     | 1.00 ± 0.10 | 1.00 ± 0.13 |
| 17b/HepG-2      | 4.21 ± 0.60** | 0.32 ± 0.04* | 9.30 ± 2.66*    | 1.74 ± 0.20* | 1.85 ± 0.26* |

Values are given as mean ± SEM of three independent experiments. *p < 0.05 and **p < 0.01 indicate statistically significant differences from the corresponding control (HepG-2) group in unpaired t-tests.

Table 3 Effect of compound 17b on levels of BAX, Bcl-2, active caspases-9, and active caspases-3 protein expression in HepG-2 cells treated for 24 h.

Table 4 The calculated $\Delta G$ (binding free energies) of the synthesized compounds, sorafenib, and co-crystallized ligand against VEGFR-2 ($\Delta G$ in kcal mol$^{-1}$).

| Comp. | $\Delta G$ [kcal mol$^{-1}$] | Comp. | $\Delta G$ [kcal mol$^{-1}$] |
|-------|-----------------|-------|-----------------|
| 15a   | −24.63          | 17b   | −23.97          |
| 15b   | −23.27          | 17c   | −23.62          |
| 15c   | −23.03          | 17d   | −23.67          |
| 15d   | −23.47          | 18    | −24.14          |
| 16    | −22.33          | Sorafenib | −22.15        |
| 17a   | −24.89          |       |                 |

Fig. 5 Validation of the docking process.

Fig. 6 Interaction of sorafenib with the essential amino acids inside VEGFR-2 active site.

Fig. 7 3D representation of 15b with VEGFR-2.

Fig. 8 3D representation of 17b with VEGFR-2.
moiety was involved in three hydrophobic moieties with Ile890, Lue887, and Ile886. In addition, it formed one electrostatic interaction with Asp1044 (Fig. 7).

The docking results of compound 17b ($\Delta G = -23.97 \text{ kcal mol}^{-1}$) are nearly similar to that of sorafenib. In the DFG region, $-\text{NH}$ of the amide moiety in the pharmacophore region formed a hydrogen bond with the carboxylate moiety of Glu883 (1.72 Å). Also, carbonyl group of the amide moiety formed another hydrogen bond with the NH of Asp1044 (2.99 Å). In addition, the terminal hydrophobic (3-chlorophenyl moiety) formed three hydrophobic interactions with Leu887, Ile886, and Ile890. Also, it formed electrostatic interaction with Asp1044. Moreover, the quinoxaline moiety occupied the hinge region forming five hydrophobic interactions with Leu1033, Phe916, Leu838, Phe1045, and Leu1047. The central phenyl group formed four hydrophobic bonds with Val914, Val897, Cys1043, and Phe1047. Such binding pattern may explain the promising biological activity of this member comparing the other candidates (Fig. 8).

The binding modes of compounds 16 and 18 are depicted in ESI† All figures in our docking study were visualized using Discovery Studio Visualizer.

2.3.2 Molecular docking for compound 17b against cytochrome P450. In this work, further molecular docking investigational study was performed for the most active compound against cytochrome P450 3A4 (CYP3A4). This study was carried out to gain further insight into the binding modes of the most active compound into the binding site of CYP3A4 (PDB ID: 4D7D). The co-crystallized ligand (PKT) was used as a reference molecule. The binding free energies ($\Delta G$) were reported in Table 5.

The proposed binding mode of PKT showed binding energy of $-25.73 \text{ kcal mol}^{-1}$. It formed one hydrogen bond with Ser119 and one electrostatic bond with Cys442. In addition, it formed seven hydrophobic interactions with Leu210, Leu211, Ile301, Ala305, and Phe304 (Fig. 9).

The proposed binding mode of 17b was illustrated in Fig. 10 with binding energy $-16.05 \text{ kcal mol}^{-1}$, far less than that of the co-crystallized ligand. In addition, the binding mode of this compound was different from that of the co-crystallized ligand. These results revealed that 17b cannot be CYP3A4 inhibitors and consequently indicates its less liver toxicity.

2.3.3 In silico ADMET studies for compounds 15b and 17b. Blood–brain barrier (BBB) penetration, intestinal absorption, aqueous solubility, CYP2D6 binding, and plasma protein binding properties of compounds 15b and 17b were calculated using Discovery studio 4.0. The BBB penetration levels of the tested compounds were in the low and very low range. Depending on these results, it may be concluded that there are no CNS side effects associated with these compounds. In addition, compounds 15b and 17b showed good levels of intestinal absorption and aqueous solubility. For cytochrome P450 2D6 (CYP2D6) inhibition, both of them were predicted as non-inhibitors. Finally, 15b and 17b were expected to bind plasma protein more than 90% (Table 6). In silico ADMET studies for the rest of the compounds are explained in ESI†.

2.3.4 In silico toxicity studies for compounds 15b and 17b. Toxicity profile of compounds 15b and 17b were predicted according to the built-in models of Discovery studio 4.0 software using seven toxicity parameters.52,53

At first, the carcinogenic potency TD$_{50}$ values (from 9.366 to 142.906 mg per kg body weight per day) of the tested compounds were higher than that of the reference molecule; sorafenib (TD$_{50} = 19.236$ mg per kg body weight per day). In addition, the maximum tolerated dose values (from 0.096 to 0.333 g per kg body weight) of both compounds were higher than sorafenib (0.089 g per kg body weight). Furthermore, the tested compounds showed oral LD$_{50}$ values ranging from 4.703

| Comp. | Binding free energy (kcal mol$^{-1}$) | No. of hydrogen bonds | No. of electrostatic interaction | No. of hydrophobic interaction |
|-------|-------------------------------------|------------------------|---------------------------------|-------------------------------|
| 17b   | $-16.05$                            | 0                      | 0                               | 5                             |
| PKT   | $-25.73$                            | 1                      | 1                               | 7                             |

Table 5. The binding free energies of 17b and PKT against cytochrome P450 (PDB ID: 4D7D).
to 12.496 mg per kg body weight per day which were higher than that of sorafenib (0.823 mg per kg body weight per day). For rat chronic lowest observed adverse effect level (LOAEL), the tested molecules showed higher values (from 0.072 to 0.583 g per kg body weight) than sorafenib (0.005 g per kg body weight). Moreover, the tested compounds were predicted to be mild irritant against eyes and non-irritant against skin. For aerobic body weight) than sorafenib (0.005 g per kg body weight). Moreover, the tested compounds were predicted to be mild irritant against eyes and non-irritant against skin. For aerobic toxicity studies for the synthesized compounds are explained in ESI.†

### 2.3.5 Physico-chemical properties of compounds 15b and 17b

The $A\log P$ values express the degree of lipophility of the chemical compound, where the log $D$ values express the degree of lipophility of the chemical compound taking into account the ionization states of the molecule. An increase in these values indicates an increase in the lipophilic character of the tested compound. It is worthwhile to note that the $A\log P$ and log $D$ values for most compounds in acceptable range for oral and intestinal absorption (1.44–4.74).†

In addition, the molecular polar surface area (MPSA) is another key property linked to drug bioavailability; the passively absorbed molecules with MPSA >140 have low oral bioavailability. Compounds 15b and 17b showed acceptable values of MPSA less than 140. Moreover, molecular volume (MV) descriptor determines transport characteristics of molecules, such as intestinal absorption. The drug diffusivity is inversely proportional to the molecular volume. Molecules with lower MV have higher diffusivity. It was observed that both compounds exhibited low molecular volume values (from 315.21 to 353.63) when compared with sorafenib (MV = 323.1). Finally, Lipinski rule of five was applied for compounds 15b and 17b. It was found that both of them have molecular weight less than 500, hydrogen bond acceptor groups less than 10, and hydrogen bond donor group less than 5. This indicates that these compounds are likely to be orally bioavailable (Table 7). Physico-chemical properties for the rest of the compounds are explained in ESI.†

### 2.3.6 DFT studies for compound 15b and 17b

Discovery studio software was used to carry out density functional theory (DFT) calculations. Different molecular and atomic properties were calculated including (i) total energy of the molecules, (ii) binding energy which describes the interaction energy between all the atoms in the molecule, (iii) the energy of the highest occupied molecular orbital (HOMO), (iv) the energy of the lowest unoccupied molecular orbital (LUMO), (v) gap energy which describes the energy difference between LUMO and HOMO, (v) the magnitude of the dipole moment ($\mu$).

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**Table 6**: ADMET parameters for compounds 15b and 17b

| Comp. | BBB level | Solubility level | Absorption level | CYP2D6 prediction | PPB prediction |
|-------|-----------|-----------------|-----------------|-------------------|--------------|
| 15b   | ++++      | ++              | 0               | F                 | More than 90%|
| 17b   | +++       | ++              | 0               | F                 | More than 90%|
| Sorafenib | +++      | +               | 0               | F                 | More than 90%|

*a* BBB level, 0 = very high, + = high, ++ = medium, +++ = low, ++++ = very low. *b* Solubility level, 0 = very low, + = low, ++ = good, +++ = optimal. *c* Absorption level, 0 = good, + = moderate, ++ = poor, +++ = very poor. *d* CYP2D6, cytochrome P2D6, T = inhibitor, F = non inhibitor. *e* PBB, plasma protein binding (less than 90% or more than 90%).

**Table 7**: Physico-chemical properties of 15b, 17b and sorafenib

| Comp. | $A\log P$ | log $D$ | MPSA | MSA | MV | HBA | HBD | M. WT |
|-------|-----------|--------|------|-----|----|-----|-----|-------|
| 15b   | 4.74      | 4.74   | 109.28 | 444.39 | 339.56 | 6 | 2 | 462.951 |
| 17b   | 3.12      | 3.12   | 90.87 | 430.37 | 328.25 | 7 | 2 | 446.886 |
| Sorafenib | 4.17     | 4.17   | 92.35 | 434.9 | 323.1 | 7 | 3 | 464.825 |

*a* Log of the octanol–water partition coefficient. *b* The octanol–water partition coefficient calculated considering the ionization states of the molecule. *c* Molecular surface area: calculates the total surface area for each molecule using a 2D approximation. *d* Molecular polar surface area: calculates the polar surface area for each molecule using a 2D approximation. *e* Molecular volume: calculates the 3D volume for each molecule using the current 3D coordinates. *f* Hydrogen bond acceptor atoms. *g* Hydrogen bond donor atoms. *h* Molecular weight.

**Table 8**: Thermodynamic parameters of compounds 15b, 17b and sorafenib

| Name | Total energy (kcal mol$^{-1}$) | Binding energy (kcal mol$^{-1}$) | HOMO energy (kcal mol$^{-1}$) | LUMO energy (kcal mol$^{-1}$) | Gap energy (kcal mol$^{-1}$) | $\mu$ |
|------|-------------------------------|--------------------------------|-------------------------------|-------------------------------|-------------------------------|------|
| 15b  | −2139.791                     | −10.576                        | −0.200                        | −0.107                        | 0.093                         | 2.365|
| 17b  | −1817.724                     | −10.717                        | −0.201                        | −0.103                        | 0.098                         | 3.061|
| Sorafenib | −2000.377                    | −9.866                         | −0.200                        | −0.091                        | 0.109                         | 3.088|

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The results (Table 8) revealed that the total energies of compounds 15b, 17b and sorafenib have negative values which are favorable for spontaneous binding and interaction. In addition, both 15b and 17b have dipole moment values very close to that of sorafenib. The improved dipole moment can enhance hydrogen bond and non-bonded interactions in drug receptor complexes which keep an important role to increase binding affinity. Elevated dipole moment indicated the increased binding affinity with target enzyme during VEGFR-2 inhibitory activities. Thermodynamic parameters for the rest of the compounds are explained in ESI.†

2.3.6.1 Molecular orbital analysis for compound 17b. According to the frontier molecular orbital theory, the energies of HOMO and LUMO play an important role in chemical reactivity.59 It was evident that compound 17b have gap energy values very close to that of sorafenib. Fig. 11 showed the spatial distribution of molecular orbitals for compound 17b. Molecular orbital analysis for sorafenib and compound 15b are depicted in ESI.†

2.3.6.2 Electrostatic potential map for compound 17b. Electrostatic interactions are one of the forces guiding the binding of molecules to proteins. The assessment of this interaction through computational approaches makes it possible to evaluate the energy of protein–drug complexes.60 Next to steric complementarity, electrostatics are one of the main driving forces involved in molecular recognition.61 Electrostatics are known to play a key role in protein–DNA,62 protein–protein63 and protein–substrate64 recognitions.

There are many colored patches in MEP surface according to availability of electron cloud. Atoms with high electronegativity and negative charges display red color and can form hydrogen bonding acceptor. While atoms with poor electron and positive charge display blue color and can form hydrogen bonding donor. The atoms with zero charge values display green to yellow color and can form π- and other types of staking interactions. This molecular detail helps to predict how much they are potential to take part in chemical reactions and to realize their mechanism of interactions.64

The most active compound 17b showed MEP map like that of sorafenib to some extent. The quinoxaline moiety showed a red patch at the nitrogen atoms and carbonyl group which can form hydrogen bond with polar amino acids at the hinge region. The two amide groups in each molecule showed red and blue patches which indicate the possibility of hydrogen bond formation. The aromatic moieties in each molecule showed high electron cloud (green to yellow patches) which can favor the π-staking interaction with aromatic amino acid residues (Fig. 12). Molecular electrostatic potential map for sorafenib and 15b are depicted in ESI.†

3. Conclusion

In the presented work ten quinoxaline derivatives (15a–d, 16, 17a–d, and 18) were designed and synthesized. Compound 17b was the most promising candidate against MCF-7, HepG-2, and VEGFR-2 with IC_{50} values of 2.8 μM, 2.3 μM, and 2.7 nM respectively, more than that of sorafenib 3.51 μM, 2.17 μM, and 3.12 nM respectively. Also, compound 17b arrested the cell cycle in the G2/M phase and induced apoptosis in HepG-2 cells. Moreover, the mentioned compound upregulated the level caspase-3, caspase-9 and boosted the Bax/Bcl-2 ratio by more than 10-fold, as compared to the control. Docking studies revealed that most compounds have similar binding pattern with VEGFR-2. In silico ADMET, toxicity, and physico-chemical properties divulged that target compounds exhibited acceptable pharmacokinetic profile, and physicochemical properties. Further docking studies for compound 17b against cytochrome P450 showed the non-inhibitory effect of this compound. DFT calculations including total energy, binding energy, HOMO, LUMO, gap energy, dipole moment, and electrostatic potential were performed. The development of other VEGFR-2 inhibitors involving quinoxaline derivatives is ongoing and will be reported in due course.

4. Experimental

4.1 Chemistry

All the reagents, chemicals, apparatus were described in ESI.† Compounds 3, 4, 5, 6, 8, 9, 10a–d, 12, 13, and 14 were obtained according to the reported procedures.58,65–68

4.1.1 General procedure for preparation of the target compounds 15a–d and 16. A mixture of potassium 3-methylquinoxaline-2-thiolate 5 (0.214 g, 0.001 mol) and the appropriate 4-(2-chloroacetamido)-N-(substituted)benzamide 10a–d (0.001 mol) or 2-chloro-N-(4-(2-(2-hydroxybenzoyl) hydrazine-1-carbonyl)phenyl)acetamide 14 (0.001 mol), anhydrous K_{2}CO_{3} (0.001 mol) and KI (0.001 mol) in DMF (10 ml) was heated on a water bath for 8 h. The reaction mixture was then poured on crushed ice. The precipitates were filtered, dried, and crystallized from methanol to give the corresponding target compounds 15a–d and 16.
4.1.1.1  N-Butyl-4-(2-((3-methylquinolin-2-yl)thio)acetamido)benzamide 15a. Yellow crystal (yield, 65%); mp = 190–192 °C; FT-IR (ν max, cm⁻¹): 3450, 3261, 2910, 1647, 1603, 1524; ¹H NMR (700 MHz, DMSO-d₆) δ 11.97 (s, 1H), 10.76 (s, 1H), 10.67–10.66 (m, 1H), 10.58 (s, 1H), 7.95 (d, J = 1.7 Hz, 2H), 7.77 (d, J = 8.5 Hz, 2H), 7.47 (d, J = 7.0 Hz, 2H), 7.00–6.98 (m, 4H), 6.96 (d, J = 7.1 Hz, 2H), 4.33 (s, 2H), 2.68 (s, 3H); ¹³C NMR (176 MHz, DMSO-d₆) δ 168.28, 167.12, 165.49, 162.78, 159.80, 155.44, 151.97, 142.78, 140.82, 139.36, 134.66, 130.05, 129.06, 128.73, 119.53, 118.91, 117.88, 115.00, 36.26, 35.45, 31.24, 22.19; MS (m/z): exact mass calefd for C₂₃H₂₁N₂O₅S [M⁺]: 487.1. Found: 488.1. Anal. calcxd for C₂₃H₂₁N₂O₅S: C, 61.59; H, 4.34; N, 14.37. Found: C, 62.2; H, 3.9; N, 13.27.

4.1.2 General procedure for preparation of the target compounds 17a-d and 18. A mixture of potassium 3-methylquinoline-2-thiolate 6 (0.198 g, 0.001 mol) and the appropriate 4-(2-chloracetamido)-N-(substituted)benzamide 10a-d (0.001 mol) or 2-chloro-N-[4-(2-hydroxybenzoyl)hydrazine-1-carboxy]phenyl)acetamide 14 (0.001 mol), anhydrous K₂CO₃ (0.001 mol) and KI (0.001 mol) in DMF (10 ml) was heated on a water bath for 8 h. Next, the reaction mixture was poured on crushed ice. The precipitates were filtered, dried, and crystallized from methanol to give the final compounds 17a-d and 18.

4.1.2.1  N-Butyl-4-(2-(3-methyl-2-oxoquinolin-1(2H)-yl)acetamido)benzamide 17a. White crystal (yield, 60%); mp = 279–281 °C; FT-IR (ν max, cm⁻¹): 3429, 3318, 2910, 1641, 1601, 1530; ¹H NMR (700 MHz, DMSO-d₆) δ 10.68 (s, 1H), 8.33 (t, J = 5.6 Hz, 1H), 7.83–7.81 (m, 2H), 7.81–7.78 (m, 1H), 7.65–7.63 (m, 2H), 7.57 (dd, J = 8.6, 7.1, 1.5 Hz, 1H), 7.52 (dd, J = 8.5, 1.2 Hz, 1H), 7.38 (dd, J = 8.2, 7.1, 1.2 Hz, 1H), 7.37 (dd, J = 8.0, 1.5 Hz, 1H), 7.28 (m, 2H), 7.19 (m, 2H), 7.09 (m, 4H), 6.96 (m, 2H), 5.18 (s, 2H), 2.49 (s, 3H), 1.50 (dd, J = 8.6, 6.3, 1.9 Hz, 2H), 1.34–1.31 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H); ¹³C NMR (176 MHz, DMSO-d₆) δ 165.91, 165.67, 157.97, 154.85, 141.43, 133.46, 132.46, 130.19, 130.05, 129.27, 128.56, 123.93, 118.76, 115.19, 45.75, 39.28, 31.76, 21.59, 20.14, 14.21; MS (m/z): exact mass calefd for C₂₁H₁₈N₂O₃ [M⁺]: 392.2. Found: 392.2. Anal. calcxd for C₂₁H₁₈N₂O₃: C, 67.33; H, 6.16; N, 14.28. Found: C, 67.72; H, 5.87; N, 13.38.

4.1.2.2  N-(3-Chlorophenyl)-4-(2-(3-methyl-2-oxoquinolin-1(2H)-yl)acetamido)benzamide 17b. Yellowish white crystal (yield, 70%); mp >300 °C; FT-IR (ν max, cm⁻¹): 3429, 3318, 2910, 1641, 1603, 1524; ¹H NMR (700 MHz, DMSO-d₆) δ 10.80 (s, 1H), 10.31 (s, 1H), 7.98–7.96 (m, 3H), 7.81 (dd, J = 8.0, 1.5 Hz, 1H), 7.74–7.73 (m, 2H), 7.71–7.70 (m, 1H), 7.59–7.57 (m, 1H), 7.54 (dd, J = 8.5, 1.3 Hz, 1H), 7.40–7.37 (m, 2H), 7.16 (dd, J = 8.0, 2.1, 0.9 Hz, 1H), 5.19 (s, 2H), 2.49 (s, 3H); ¹³C NMR (176 MHz, DMSO-d₆) δ 168.55, 165.48, 157.97, 154.85, 142.25, 141.22, 133.47, 133.38, 132.47, 130.78, 130.20, 129.57, 132.93, 123.29, 123.66, 120.11, 119.05, 118.75, 115.21, 45.80, 21.59; MS (m/z): exact mass calefd for C₂₁H₁₈N₂O₃ [M⁺]: 446.1. Found: 447.1. Anal. calcxd for C₂₂H₁₉N₂O₄: C, 64.50; H, 4.29; N, 12.54. Found: C, 64.98; H, 4.05; N, 12.09.

4.1.2.3  N-(4-Hydroxyphenyl)-4-(2-(3-methyl-2-oxoquinolin-1(2H)-yl)acetamido)benzamide 17c. Yellowish white crystal (yield, 70%); mp >300 °C; FT-IR (ν max, cm⁻¹): 3303, 3261, 2958, 1644, 1601, 1513; ¹H NMR (700 MHz, DMSO-d₆) δ 10.75 (s, 1H), 9.93 (s, 1H), 9.25 (s, 1H), 7.94 (d, J = 8.6 Hz, 2H), 7.70 (d, J = 8.5 Hz, 2H),
The used cell lines were obtained from ATCC (American Type Culture Collection) via the holding company for biological products and vaccines (VACSERCA) (Cairo, Egypt).

4.2 In vitro VEGFR-2 assay. Human VEGFR-2 ELISA kit was used was carried out in this test following the reported method illustrated in ESI.†

4.3 Biological testing

4.3.1 In vitro anti-proliferative activity. MTT cytotoxicity assay was utilized and it has been detailed in ESI.† In this test, all the synthesized compounds were evaluated for their anti-proliferative activities against MCF-7 and HepG-2 cell lines. The used cell lines were obtained from ATCC (American Type Culture Collection) via the holding company for biological products and vaccines (VACSERCA) (Cairo, Egypt).

4.2.2 In vitro VEGFR-2 assay. Human VEGFR-2 ELISA kit was used was carried out in this test following the reported method illustrated in ESI.†

4.2.3 Cell cycle and apoptosis analysis. Flow cytometry assay was applied according to the reported methods described in ESI.†

4.2.4 Western blot analysis. Western blot technique was performed for the most promising member against caspase-3, caspase-9, BAX, and Bcl-2 as described in ESI.†

4.3 In silico studies

4.3.1 Docking studies. Docking studies were carried out against VEGFR-2 (PDB ID: 2OH4) and CYP3A4 (PDB ID: 4D7D) using MOE 2014 and the results were visualized using Discovery studio 4.0 according to the procedure reported in ESI.†

4.3.2 ADMET studies. ADMET descriptors were determined using Discovery studio 4.0 as according to the reported method (ESI†).

4.3.3 Toxicity studies. Discovery studio 4.0 software was used to predict the toxicity potential of the synthesized compounds as reported in ESI.†

4.3.4 DFT studies. Discovery studio 4.0 software was used to calculate the DFT parameter as reported in ESI.†

Conflicts of interest

There is no conflict of interest. Many thanks for Dr Mohamed R. Elnagar, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt for his valuable technical assistance.

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