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FULL PAPER



N-Substituted-4-phenylphthalazin-1-amine-derived VEGFR-2 inhibitors: Design, synthesis, molecular docking, and anticancer evaluation studies

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Abstract

In accordance with the significant impetus of the discovery of potent vascular endothelial growth factor receptor 2 (VEGFR-2) inhibitors, herein, we report the design, synthesis, and anticancer evaluation of 12 new N-substituted-4phenylphthalazin-1-amine derivatives against HepG2, HCT-116, and MCF-7 cells as VEGFR-2 inhibitors. The results of the cytotoxicity investigation indicated that HCT-116 and MCF-7 were the most sensitive cell lines to the influence of the newly synthesized derivatives. In particular, compound 7a was found to be the most potent derivative among all the tested compounds against the three cancer cell lines, HepG2, HCT116, and MCF-7, with IC₅₀ = 13.67 ± 1.2 , 5.48 ± 0.4 , and $7.34 \pm 0.6 \mu$ M, respectively, which is nearly equipotent to that of sorafenib ($IC_{50} = 9.18 \pm 0.6$, 5.47 ± 0.3 , and $7.26 \pm 0.3 \mu$ M, respectively). All synthesized derivatives, **4a,b-8a-c**, were evaluated for their inhibitory activities against VEGFR-2. The tested compounds displayed high to low inhibitory activity, with IC₅₀ values ranging from 0.14 ± 0.02 to $9.54 \pm 0.85 \,\mu$ M. Among them, compound **7a** was found to be the most potent derivative that inhibited VEGFR-2 at an IC₅₀ value of $0.14 \pm 0.02 \,\mu$ M, which is nearly 72% of that of the sorafenib IC₅₀ value ($0.10 \pm 0.02 \,\mu$ M). Compounds 7b, 8c, 8b, and 8a exhibited very good activity with IC_{50} values of 0.18 ± 0.02 , 0.21 ± 0.03 , 0.24 ± 0.02 , and $0.35 \pm 0.04 \mu$ M, respectively. Molecular modeling studies were carried out for all compounds against the VEGFR-2 active site. The data obtained from biological testing highly correlated with that obtained from molecular modeling studies. However, these modifications led to new phthalazine derivatives with higher VEGFR-2 inhibitory activities than vatalanib and which are nearly equipotent to sorafenib.

KEYWORDS

4-phenylphthalazin-1-amine, anticancer agents, molecular docking, VEGFR-2 inhibitors

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1 | INTRODUCTION

Extensive studies have been reported concerning the synthesis of several phthalazine derivatives^[1-7] as promising anticancer agents and potent vascular endothelial growth factor receptor 2 (VEGFR-2) inhibitors.^[8-11] Phthalazin-1,4-diones have been reported as potent type II IMP dehydrogenase inhibitors and as effective anti-proliferative agents against different human and murine tumor cells, particularly against hepatocellular carcinoma.^[3] Moreover, 1,4-disubstituted phthalazines have attracted considerable attention as promising and effective anticancer agents.^[3,5] In addition, many triazolo[3,4-*a*]phthalazine derivatives were reported to exhibit promising antitumor activities against MGC-803, EC-9706, HeLa, and MCF-7 human cancer cell lines.^[12]

During the last two decades, there was a growing interest in the synthesis of several phthalazines as promising drug candidates for the treatment of cancer. The latter research efforts have led to the discovery of several leading phthalazines with different cellular and enzymatic targets. For example, AMG 900 I (Figure 1) was synthesized by Amgen as an orally bioavailable, potent, and highly selective pan-Aurora kinase inhibitor that is active against taxane-resistant tumor cell lines.^[6] AMG 900 I was found to be active against an AZD1152-resistant HCT116 variant cell line that harbors an Aurora-B mutation (W221L).^[7] Thereafter, Cee et al.^[13] have discovered two selective and orally bioavailable pyridinyl-pyrimidine phthalazines Aurora kinase inhibitors. Vatalanib (PTK787) II^[14] (Figure 1) inhibits both VEGFR-1 and VEGFR-2 with IC₅₀ values of 380 and 20 nM, respectively. Vatalanib is well absorbed orally and shows an in vivo antitumor activity against a panel of human tumor xenograft models; however, vatalanib is currently in phase III clinical trials for the treatment of colorectal cancer.^[15,16] In addition, many anilinophthalazines have been reported as potent inhibitors of VEGFR-2, such as AAC789 III and IM-023911 IV with IC₅₀ = 20 and 48 nM, respectively (Figure 1).^[8-11,17,18]



FIGURE 1 Structures of the lead anticancer phthalazine derivatives I–IV, sorafenib, and the designed target 4-phenylphthalazinon-1-amine derivatives 4–9a–c

In this context, the 1-substituted-4-phenylphthalazine scaffold, bearing different 4-substituted anilines, in particular, emerged as an interesting scaffold for designing VEGFR inhibitors (Figure 1). The abovementioned facts have aggravated us to design novel *N*-substituted-4-phenylphthalazin-1-amine derivatives in an attempt to obtain more potent anticancer agents. Sorafenib (Nexavar®, **V**) (Figure 1) is a potent VEGFR-2 inhibitor, which has been approved as an antiangiogenic drug.^[19,20]

The common pharmacophoric features shared by various VEGFR-2 inhibitors, for example, vatalanib and sorafenib, revealed four main features, as shown in Figure $1^{[21-23]}$: (i) The core structure of most inhibitors that occupied the catalytic adenosine triphosphate (ATP)-binding domain consists of a flat heteroaromatic ring system that contains at least one N atom. (ii) A central aryl ring (hydrophobic spacer) is present, occupying the linker region between the ATPbinding domain and the DFG domain of the enzyme.^[24] (iii) Also, there exists a hydrophilic linker containing a functional group (e.g., amino or urea) acting as pharmacophore that possesses both H-bond donor and acceptor to bind with Glu883 and/or Asp1044, the two crucial residues in the DFG (Asp-Phe-Gly) motif, which is an essential tripeptide sequence in the active kinase domain. The NH group of the linker moiety usually forms one hydrogen bond with Glu883, whereas the C=O group forms another hydrogen bond with Asp1044. (iv) The hydrophobic interactions are usually achieved when a terminal hydrophobic moiety of the inhibitors occupies the newly formed allosteric hydrophobic pocket, which is exposed when the DFG loop phenylalanine residue flips out of its lipophilic pocket.^[25] Furthermore, the X-ray analysis of the VEGFR-2 receptor confirmed the presence of adequate space available for various substituents around the terminal heteroaromatic ring.^[26,27]

VEGFR-2 is the earliest recognized marker for the development of endothelial cells. VEGFR-2 has been documented as a regulator of tumor angiogenesis; therefore, several VEGFR-2 inhibitor molecules have been developed as effective anticancer agents.^[28,29] VEGFR-2 was reported to be substantially upregulated in HepG2 cells in a dose-dependent manner with the stimulation of the hepatocyte growth factor, which is involved in cell proliferation, invasion, and angiogenesis of hepatocellular carcinoma.^[30-32] Blockade of VEGFR-2 signaling revealed a marked inhibition on both the growth and metastasis of HCC.^[32,33] Also, VEGFR-2 was found to be crucial for cell survival, which regulates endothelial differentiation in both the breast cancer cells (MCF-7)^[32,33] and human colorectal carcinoma (HCT-116).^[34,35] The overexpression of VEGFR-2 receptors in breast cancer cells has been documented as a contributor in resistance of such cancer type to the chemotherapeutic effect of tamoxifen.^[36] VEGFR-2 degraders were documented to impair the in vitro endothelial differentiation and to promote angiogenesis, suggesting a VEGFR-2-mediated mechanism of antiproliferative activity in human colorectal carcinoma.^[34]

In continuation of our efforts to obtain new anticancer agents,^[37-43] the goal of the present work was the synthesis of new agents with the same essential pharmacophoric features of the reported and clinically used VEGFR-2 inhibitors (e.g., vatalanib and sorafenib). The main core of our molecular design rationale comprised bioisosteric modification strategies of VEGFR-2 inhibitors at the four different pharmacophoric positions (Figure 1).

2 | RESULTS AND DISCUSSION

2.1 | Rationale and structure-based design

N-Substituted-4-phenylphthalazin-1-amine derivatives have the essential pharmacophoric features of VEGFR-2 inhibitors^[22,39-44] (Figure 1), which include the following: First, there exists a sixmembered heteroaromatic ring, phthalazine, as a central aryl moiety, substituted with phenyl ring, as a hydrophobic portion, forming the 4-phenylphthalazine scaffold. Second, the target phenyl group at



FIGURE 2 Superimposition of compound 7a and vatalanib inside the binding pocket of vascular endothelial growth factor receptor 2 (1YWN)



FIGURE 3 Superimposition of compound **7a** and sorafenib inside the binding pocket of vascular endothelial growth factor receptor 2 (1YWN)

position-4 is used to replace pyridine and *N*-methylpicolinamide moieties of vatalanib and sorafenib (Figures 2 and 3), respectively. N at position-2 of phthalazine nucleus acts as an H-bond acceptor (HBA), forming an H-bond with the essential amino acid residue Asp1044. The third strategy is using NH linker (as NH of vatalanib) which acts as H-bond donor (HBD), forming an H-bond with the essential amino acid residue Glu883. Fourth, the hydrophobic substituted phenyl tail of the reported ligands vatalanib and sorafenib (Figures 2 and 3) is replaced by other one substituted with distal hydrophobic moieties linked through amide (NHCO), urea (NHCONH), thiourea (NHCSNH), and/or amide (CONH) linkers as in **6**, **7a,b**, **8a-c**, and/or **9a-c** derivatives, respectively.

The used substituted hydrophobic phenyl tail occupied the hydrophobic pocket formed by Asp1044, Cys1043, Ile1042, Val897, His892, Gly891, Ile890, Leu887, Ile886, and Glu883. Furthermore,

these amide, urea, and/or thiourea linkers were used to resemble the urea linker of sorafenib to obtain both linkers of vatalanib and sorafenib in the same compound to increase H-bonding interactions with the active site of VEGFR-2 and consequently the anticancer activities. These linkers increased the length of the structures to enable the distal moieties to occupy new hydrophobic grooves formed by Arg1025, His1024, Ile1023, Cys1022, Lys1021, Arg1020, Leu1017, Ile890, His889, and Ile886 (Figures 2 and 3).

Additionally, the substitution pattern was selected to ensure different electronic and lipophilic environments that could influence the activity of the target compounds. These modifications were performed to carry out further elaboration of the phthalazine scaffolds and to explore a valuable structure-activity relationship (SAR). The designed target phthalazine derivatives were synthesized and evaluated as potential VEGFR-2 inhibitors and antitumor





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agents against three human tumor cell lines, namely, hepatocellular carcinoma (HepG2), breast cancer (Michigan Cancer Foundation-7 [MCF-7]), and human colorectal carcinoma-116 (HCT-116).

2.2 | Chemistry

The synthetic strategy for the preparation of the target compounds (4–9) is depicted in Schemes 1-3. The synthesis was initiated by cyclocondensation of 2-benzoylbenzoic acid (1) with hydrazine hydrate to afford the corresponding 4-phenylphthalazin-1(2*H*)-one (2), which underwent chlorination by reaction with phosphorous oxychloride to afford 1-chloro-4-phenylphthalazine (3).^[3,4] The chloro derivative (3) was refluxed with the appropriate 4-substituted aniline, namely, 4-nitroaniline and/or 4-aminobenzoic acid, to afford the corresponding amino derivatives **4a,b**, respectively (Scheme 1). However, the 4-nitro derivative **4a** was reduced to the corresponding 4-amino derivative **5** by heating with SnCl₂ as a reducing agent in the presence of hydrochloric acid following the reported procedure.^[45] The produced amino derivative **5** was allowed to react with benzoic acid, the appropriate isocyanates, namely, phenyl and cyclohexyl isocyanates, and/or the appropriate isothiocyanates, namely, propyl, butyl, and cyclohexyl isothiocyanates, to afford the corresponding amide derivative **6**, urea **7a,b**, and/or thiourea **8a-c** derivatives, respectively (Scheme 2). Finally, the acid derivative **4b** was reacted with the appropriate amine, namely, aniline, 4-chloroaniline, and/or 4-methylaniline, following the mixed anhydride method to give the corresponding acid amide derivatives **9a-c**, respectively (Scheme 3).

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2.3 | In vitro antiproliferative activity

The antiproliferative activity of the newly synthesized phthalazine derivatives **4a,b-9a-c** was examined against three human tumor cell lines, namely, hepatocellular carcinoma (HepG2), colorectal carcinoma (HCT-116), and breast cancer (MCF-7), using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described by



SCHEME 2 The synthetic route for the preparation of the target compounds 5-8a-c





Mosmann.^[46-48] Sorafenib was used as a reference cytotoxic drug. The results were expressed as growth inhibitory concentration (IC_{50}) values, which represent the compound concentrations required to produce a 50% inhibition of cell growth after 72 h of incubation, calculated from the concentration–inhibition response curve and summarized in Table 1. From the obtained results, it was explicated that most of the prepared compounds displayed an excellent to low growth inhibitory activity against the tested cancer cell lines. Investigations of the cytotoxic activity indicated that HCT-116 and MCF-7 were the most sensitive cell lines to the influence of the new derivatives, respectively. In particular, compound **7a** was found to be the most potent derivative over all the tested

TABLE 1 In vitro cytotoxic activities of the newly synthesized compounds against HepG2, HCT-116, and MCF-7 cell lines and VEGFR-2 kinase assay

	IC ₅₀ (μM) ^a						
Compound	HepG2	HCT116	MCF-7	VEGFR-2			
4a	>100	97.43 ± 5.3	99.09 ± 5.1	9.54 ± 0.85			
4b	>100	95.67 ± 5.3	96.97 ± 5.1	8.99±0.74			
5	>100	93.93 ± 4.9	96.43±5.1	6.76 ± 0.62			
6	79.41 ± 4.8	73.46 ± 4.2	86.55 ± 4.9	3.02 ± 0.07			
7a	13.67 ± 1.2	5.48 ± 0.4	7.34 ± 0.6	0.14 ± 0.02			
7b	19.69 ± 1.8	9.38 ± 0.9	8.41 ± 0.8	0.18 ± 0.02			
8a	45.24 ± 3.3	38.23 ± 2.5	49.06 ± 3.2	0.35 ± 0.04			
8b	41.45 ± 3.2	39.49 ± 2.6	44.83 ± 3.0	0.24 ± 0.02			
8c	29.46 ± 2.6	17.38 ± 1.5	14.16 ± 1.3	0.21 ± 0.03			
9a	82.16 ± 4.8	77.06 ± 4.5	88.16 ± 5.0	3.68 ± 0.36			
9b	52.60 ± 3.7	46.28 ± 3.1	55.35 ± 3.6	1.99 ± 0.06			
9c	49.40 ± 3.6	43.90 ± 3.0	54.21 ± 3.5	1.56 ± 0.05			
Sorafenib	9.18 ± 0.6	5.47 ± 0.3	7.26 ± 0.3	0.10 ± 0.02			

 $^{a}IC_{50}$ values are the mean ± SD of three separate experiments.

compounds against the three HepG2, HCT116, and MCF-7 cancer cell lines with $IC_{50} = 13.67 \pm 1.2$, 5.48 ± 0.4 , and $7.34 \pm 0.6 \mu$ M, respectively, which is nearly equipotent to that of sorafenib ($IC_{50} = 9.18 \pm 0.6$, 5.47 ± 0.3 , and $7.26 \pm 0.3 \mu$ M, respectively).

With respect to the HepG2 hepatocellular carcinoma cell line, compounds **7b** and **8c** displayed very good anticancer activities with $IC_{50} = 19.69 \pm 1.8$ and $29.46 \pm 2.6 \mu$ M, respectively. Compounds **8b**, **8a**, **9c**, and **9b**, with $IC_{50} = 41.45 \pm 3.2$, 45.24 ± 3.3 , 49.40 ± 3.6 , and $52.60 \pm 3.7 \mu$ M, respectively, displayed good cytotoxicity. Compounds **6** and **9a**, with $IC_{50} = 79.41 \pm 4.8$ and $82.16 \pm 4.8 \mu$ M, respectively, exhibited moderate cytotoxicity. However, compounds **4a**, **4b**, and **5** with $IC_{50} > 100 \mu$ M displayed the lowest cytotoxicity.

Cytotoxicity evaluation against colorectal carcinoma (HCT-116) cell line revealed that compounds **7b** and **8c** displayed very good anticancer activities with $IC_{50} = 9.38 \pm 0.9$ and $17.38 \pm 1.5 \,\mu$ M, respectively. Compounds **8a**, **8b**, **9c**, and **9b**, with $IC_{50} = 38.23 \pm 2.5$, 39.49 ± 2.6 , 43.90 ± 3.0 , and $46.28 \pm 3.1 \,\mu$ M, respectively, exhibited good cytotoxicity. Compounds **6** and **9a**, with $IC_{50} = 73.46 \pm 4.2$ and $77.06 \pm 4.5 \,\mu$ M, displayed moderate cytotoxicity. However, compounds **4a**, **4b**, and **5**, with IC_{50} ranging from 93.93 ± 4.9 to $97.43 \pm 5.3 \,\mu$ M, displayed the lowest cytotoxicity.

With respect to the MCF-7 cell line, compounds **7b** and **8c** displayed very good anticancer activities with $IC_{50} = 8.41 \pm 0.8$ and $14.16 \pm 1.3 \,\mu$ M, respectively. Compounds **8b**, **8a**, **9c**, and **9b**, with $IC_{50} = 44.83 \pm 3.0$, 49.06 ± 3.2 , 54.21 ± 3.5 , and $55.35 \pm 3.6 \,\mu$ M, respectively, exhibited good cytotoxicity. Compounds **6** and **9a**, with $IC_{50} = 86.55 \pm 4.9$ and $88.16 \pm 5.0 \,\mu$ M, displayed moderate cytotoxicity. However, compounds **4a**, **4b**, and **5**, with IC_{50} ranging from 96.43 ± 5.1 to $99.09 \pm 5.1 \,\mu$ M, displayed the lowest cytotoxicity.

2.4 | In vitro VEGFR-2 kinase assay

All the synthesized derivatives, **4a,b-8a-c**, were evaluated for their inhibitory activities against VEGFR-2 by using an

antiphosphotyrosine antibody with the Alpha Screen system (PerkinElmer). The results were reported as a 50% inhibition concentration value (IC₅₀), which is calculated from the concentration-inhibition response curve and summarized in Table 2. Sorafenib was used as a positive control in this assay. The tested compounds displayed high to low inhibitory activity with IC₅₀ values ranging from 0.14 ± 0.02 to $9.54\pm0.85\,\mu\text{M}$ (Figure 4). Among them, compound 7a was found to be the most potent derivative that inhibited VEGFR-2 at an IC_{50} value of 0.14 \pm 0.02 $\mu M,$ which is nearly 72% of that of sorafenib IC_{50} value (0.10 ± 0.02 µM). Compounds 7b, 8c, 8b, and 8a exhibited a very good activity with IC_{50} values of 0.18 ± 0.02 , 0.21 ± 0.03 , 0.24 ± 0.02 , and $0.35 \pm 0.04 \,\mu\text{M}$, respectively. Also, compounds 9c and 9b possessed good VEGFR-2 inhibition with IC₅₀ values of 1.56 ± 0.05 and $1.99 \pm 0.06 \,\mu$ M, respectively. Finally, compounds 4a, 4b, 5, 6, and 9a displayed the lowest VEGFR-2 inhibition with IC₅₀ values ranging from 3.02 ± 0.07 to $9.54 \pm 0.85 \,\mu$ M, respectively.

2.5 Structure-activity relationship

The preliminary SAR study focused on the effect of hydrophobic and electronic nature of the substituents used in this study. Also, it focused on the effect of the type, length, and the number of linkers used and the distal moieties. The data obtained revealed that the tested compounds displayed different levels of anticancer activity and possessed a distinctive pattern of selectivity against the HCT116 cell lines. Generally, the 4-phenylphthalazine scaffold, bearing different 4-substituted anilines, was connected with the hydrophobic distal moieties through amide, urea, and/or thiourea linkers containing HBA-HBD. Lipophilicity and electronic nature of the distal moieties exhibited an important role in VEGFR-2 inhibition and, consequently, the anticancer activities.

In molecular docking studies, the presence of the amino and urea linkers, of both vatalanib and sorafenib in the same molecule, like 7a,b, imparts higher VEGFR-2 binding affinity, and consequently higher anticancer activity, compared with either vatalanib (with only NH linker) or sorafenib (with only urea linker). This higher affinity of such derivative may be attributed to the increased length of the molecular structures so as to enable the distal moieties to occupy the



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FIGURE 4 IC₅₀(µM) of the synthesized compounds and sorafenib as vascular endothelial growth factor receptor 2 inhibitors

hydrophobic grooves formed by Arg1025, His1024, Ile1023, Cys1022, Lys1021, Arg1020, Leu1017, Ile890, His889, and Ile886.

From the structure of the synthesized derivatives and the data shown in Table 1, we can divide these tested compounds into three groups. The first group contains amino and (thio)urea linkers as in compounds 7a,b, and 8a-c. Generally, in this group, the urea linker derivatives 7a,b exhibited higher anticancer activities than that containing thiourea linkers 8c, 8b, and 8a, respectively. The hydrophobic electron-donating (+inductive [+I]) aliphatic cyclohexyl moiety in compound 7a showed higher anticancer activities than the hydrophobic electron-withdrawing (-I) phenyl one, 7b. The more hydrophobic electron-donating (+I) aliphatic cyclohexyl moiety connected to thiourea linker in compound 8c displayed higher anticancer activities than the butyl 8b and propyl 8a against the HepG2. HCT116. and MCF-7 cell lines. except for the propyl derivative 8a, which exhibited a higher anticancer activity than the butyl one, 8b, against MCF-7 cell lines.

The second group, 6 and 9a-c, contains shorter amide CONH and NHCO linkers, respectively. Generally, the 4-substituted phenyl distal moieties, as in compounds 9c and 9b, exhibited higher activities than the unsubstituted ones, 6 and 9a, respectively, against the HepG2, HCT116, and MCF-7 cell lines. In this group, the arrangement of CO and NH atoms is very important for the activity. In compound 6, the NHCO linker showed higher activities than the

IADLE Z	The calculated free ene	ergy of binding (AG if	r kcal/mol) for the ligands

Compound	ΔG (kcal/mol)	RMSD (Å)	Compound	ΔG (kcal /mol)	RMSD (Å)
4a	-56.38	0.98	8b	-88.86	1.15
4b	-59.88	1.05	8c	-90.66	0.83
5	-61.67	0.95	9a	-76.50	0.92
6	-77.27	1.02	9b	-81.74	0.96
7a	-99.06	1.19	9c	-82.44	1.16
7b	-94.99	1.04	Vatalanib	-77.51	1.02
8a	-87.24	1.12	Sorafenib	-95.36	1.06

Abbreviation: RMSD, root-mean-square deviation.

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CONH linker in compound 9c, where the two compounds have the same structure but only differ in the arrangement of the amide linkers. The presence of hydrophobic electron-donating (+1) methyl group 9c showed higher activities than that substituted with hydrophobic electron-withdrawing (-1) chloro group 9b.

The third group is the one with no distal moieties, **4a**, **4b**, and **5** derivatives. This group generally contains only the hydrophobic phenyl tail substituted at position-4 with nitro, carboxylic, and/or amino groups, respectively. The hydrophilic electron-donating amino group **5** (–I and +mesomeric effect [+M]) exhibited higher activities than the hydrophilic electron-withdrawing carboxylic group **4b** (–I and –M) and hydrophobic electron-withdrawing nitro group **4a** (–I and –M) against the HCT116, MCF-7, and HepG2 cell lines, respectively. The obtained data were correlated with the Hansch equation for a linear relationship.

The data obtained from VEGFR-2 inhibition assay concluded that the presence of the hydrophobic distal moieties connected to the urea linkers **7a** and/or **7b** exhibited higher VEGFR-2 inhibition activities than that attached to thiourea linkers **8c**, **8b**, and/or **8a**, respectively. The longer urea linkers **7a** and **7b** and thiourea linkers **8c**, **8b**, and **8a** displayed higher activities than CONH **9c**, **9b**, NHCO **6**, and CONH **9a**, respectively. Finally, compounds having distal moieties exhibited higher activities than that containing only hydrophobic phenyl tails, **5**, **4b**, and **4c**, respectively.

2.6 | Docking studies

All modeling experiments in the present work were performed using Molsoft software. Each experiment used VEGFR-2 downloaded from the Brookhaven Protein Data Bank (PDB ID 1YWN).^[49]

All studied ligands have a similar position and orientation inside the recognized binding site of VEGFR-2, which reveals a large space bounded by a membrane-binding domain that serves as an entry channel for the substrate to the active site (Figure 5). The obtained results of the free energy of binding (ΔG) explained that most of these compounds had a good binding affinity toward the receptor and the computed values reflected the overall trend (Table 1).

The proposed binding mode of sorafenib revealed an affinity value of -95.66 kcal/mol and exhibited four H-bonds. The urea linker formed one H-bond with the key amino acid Glu883 (2.01 Å) through its NH group and one H-bond with Asp1044 (2.09 Å) through its carbonyl group. The N-methylpicolinamide moiety was stabilized by the formation of two H-bonds with Cys917, where the pyridine N atom formed one H-bond with the NH of Cys917 (2.51 Å), whereas its NH group formed one H-bond with the carbonyl of Cvs917 (1.95 Å). The N-methylpicolinamide moiety occupied the hydrophobic groove formed by Leu1033, Gly920, Lys918, Cys917, Phe916, Glu915, Leu838, and Ala864. Moreover, the central phenyl ring occupied the hydrophobic pocket formed by Cys1043, Leu1033, Val914. Val897, Lys866, and Val865. Furthermore, the hydrophobic 3-trifluoromethyl-4-chlorophenyl moiety attached to the urea linker occupied the hydrophobic pocket formed by Asp1044, Ile1042, His1024, Leu1017, His892, Gly891, Ile890, Leu887, and Glu883 (Figure 6).

The proposed binding mode of vatalanib revealed an affinity value of -77.51 kcal/mol and exhibited three H-bonds. The NH linker formed one H-bond with the key amino acid Glu883 (1.73 Å) through its carbonyl group. The N-2 of phthalazine nucleus formed one H-bond with the essential amino acid residue Asp1044 (2.74 Å). The pyridine N atom also formed one H-bond with the NH of Cys917 (2.39 Å). The pyridine moiety occupied the hydrophobic groove formed by Leu1033, Cys917, Phe916, Glu915, Ala864, and Leu838. Moreover, the central phthalazine scaffold occupied the hydrophobic pocket formed by Asp1044, Cys1043, Glu915, Val914, Val897,



FIGURE 5 Superimposition of some docked compounds inside the binding pocket of 1YWN



FIGURE 6 The predicted binding mode of sorafenib with 1WYN. H-bonded atoms are indicated by dotted lines

Lys866, Val865, and Ala864. Furthermore, the hydrophobic 4-chlorophenyl moiety attached to the urea linker occupied the hydrophobic pocket formed by Asp1044, Ile1042, Leu1017, His892, Gly891, Ile890, Leu887, Ile886, and Glu883 (Figure 7).

The urea and NH linkers played an important role in the binding affinities toward the VEGFR-2 enzyme, where it was responsible for the higher binding affinities of sorafenib and vatalanib, respectively. These findings encourage us to use different linkers resembling sorafenib and vatalanib in the same compound, hoping to obtain potent VEGFR-2 inhibitors.

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As planned, the proposed binding mode of compound 7a is virtually the same as that of sorafenib and vatalanib, which revealed an affinity value of -99.06 kcal/mol and formed four H-bonds. The NH linker formed one H-bond with the key amino acid Glu883 (1.60 Å)



FIGURE 7 The predicted binding mode of vatalanib with 1WYN. H-bonded atoms are indicated by dotted lines

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through its carbonyl group, whereas the N-2 of phthalazine nucleus formed one H-bond with the essential amino acid residue Asp1044 (2.95 Å). Moreover, the urea linker formed two H-bonds with Asp1044 (2.88 Å) and His1024 (2.91 Å). The 4-phenyl group occupied the hydrophobic groove formed by Leu1033, Cys917, Phe916, Glu915, and Ala864. Moreover, the central phthalazine scaffold occupied the hydrophobic pocket formed by Asp1044, Cys1043, Glu915, Val914, Val897, Lys866, Val865, and Ala864. The hydrophobic phenyl tail occupied the hydrophobic pocket formed by Asp1044, Cys1043, Val897, His892, Gly891, Ile890, Leu887, Ile886, and Glu883. Furthermore, the distal cyclohexyl moiety occupied the hydrophobic groove formed by Asp1021, Arg1020, Leu1017, Ile890, His889, and Ile886 (Figure 8). These interactions may explain the highest anticancer activity of compound **7a**.

The proposed binding mode of compound 7b is virtually the same as that of 7a, which revealed an affinity value of -94.99 kcal/ mol and displayed four H-bonds. The NH linker formed one H-bond with the key amino acid Glu883 (2.63 Å) through its carbonyl group, whereas the N-2 of phthalazine nucleus formed one H-bond with the essential amino acid residue Asp1044 (2.96 Å). Moreover, the urea linker formed two H-bonds with Asp1044 (2.90 Å) and His1024 (2.89 Å). The 4-phenyl group occupied the hydrophobic pocket formed by Leu1033, Cys917, Phe916, Glu915, and Ala864. Moreover, the central phthalazine scaffold occupied the hydrophobic pocket formed by Asp1044, Cys1043, Glu915, Val914, Val897, Lys866, Val865, and Ala864. The hydrophobic phenyl tail occupied the hydrophobic pocket formed by Asp1044, Cys1043, Val897, His892, Gly891, Ile890, Leu887, Ile886, and Glu883. Furthermore, the distal phenyl ring occupied the hydrophobic groove formed by Arg1025, His1024, Ile1023, Cys1022, Lys1021, Arg1020, Leu1017, Ile890, His889, and Ile886

(Figure 9). These interactions of compound **7b** may explain its high anticancer activity.

The proposed binding mode of compound **8c** is virtually the same as that of 7a and 7b, which revealed an affinity value of -90.66 kcal/mol and three H-bonds. The NH linker formed one H-bond with the key amino acid Glu883 (1.70 Å) through its carbonyl group, whereas the N-2 of phthalazine nucleus formed one H-bond with the essential amino acid residue Asp1044 (2.58 Å). Moreover, the thiourea linker formed one H-bond with Asp1044 (2.75 Å). The 4-phenyl group occupied the hydrophobic groove formed by Leu1033, Cys917, Phe916, Glu915, and Ala864. Moreover, the central phthalazine scaffold occupied the hydrophobic pocket formed by Asp1044, Cys1043, Glu915, Val914, Val897, Lys866, Val865, and Ala864. The hydrophobic phenyl tail occupied the hydrophobic pocket formed by Asp1044, Cys1043, Val897, His892, Gly891, Ile890, Leu887, Ile886, and Glut883. Furthermore, the distal cyclohexyl moiety occupied the hydrophobic groove formed by His1024, Cys1022, Lys1021, Arg1020, Leu1017, Ile890, His889, and Ile886 (Figure 10). These interactions of compound 8c may explain its high anticancer activity.

From the obtained docking results (Table 2), we concluded that the NH linker occupied the same groove occupied by NH and urea linkers of vatalanib and sorafenib, and played the same role, which is essential for higher affinity toward VEGFR-2 enzyme. The distal hydrophobic moieties increased hydrophobic interactions and, consequently, affinities toward VEGFR-2 enzyme. The phthalazine enables the new compounds to form a new H-bond through its N atom at position-2 with the amino acid Asp1044. The use of urea, thiourea, and/or amide linkers leads to elongations of the structures, which plays an important role in their VEGFR-2 inhibitory activities. The hydrophobic distal moieties and linkers formed hydrophobic and hydrogen bonding interactions, which increased the affinity toward the VEGFR-2 enzyme. However, these





FIGURE 9 The predicted binding mode of 7b with 1WYN



FIGURE 10 The predicted binding mode of 8c with 1WYN

modifications result in obtaining new phthalazine derivatives with higher VEGFR-2 inhibitory activities than vatalanib to be nearly equipotent to sorafenib.

3 | CONCLUSION

In summary, 12 new *N*-substituted-4-phenylphthalazin-1-amine derivatives have been designed, synthesized, and evaluated for their anticancer activities against three human tumor cell lines, hepatocellular carcinoma (HepG2), colorectal carcinoma (HCT-116), and breast cancer (MCF-7), as VEGFR-2 inhibitors. All the tested compounds showed variable anticancer activities. The molecular modeling was performed to investigate the binding mode of the proposed compounds with the VEGFR-2 active site. The data obtained from biological testing highly correlated with that obtained from molecular modeling studies. Investigations of the cytotoxic activity indicated that HCT-116 and MCF-7 were the most sensitive cell lines to the influence of the new derivatives, respectively. In particular, compound **7a** was found to be the most potent

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derivative over all the tested compounds against the HepG2, HCT116, and MCF-7 cancer cell lines with $IC_{50} = 13.67 \pm 1.2$, 5.48 ± 0.4 , and $7.34 \pm 0.6 \mu$ M, respectively, which is nearly equipotent to that of sorafenib (IC₅₀ = 9.18 ± 0.6 , 5.47 ± 0.3 , and 7.26 ± 0.3 µM, respectively). All synthesized derivatives 4a,b-8a-c were evaluated for their inhibitory activities against VEGFR-2. The tested compounds displayed a high to low inhibitory activity with IC_{50} values ranging from 0.14 ± 0.02 to 9.54 ± 0.85 μ M. Among them, compound 7a was found to be the most potent derivative that inhibited VEGFR-2 at an IC_{50} value of $0.14 \pm 0.02 \,\mu\text{M}$, which is nearly 72% of that of the sorafenib IC₅₀ value (0.10 \pm 0.02 μ M). Compounds 7b, 8c, 8b, and 8a exhibited a very good activity with IC₅₀ values of 0.18 ± 0.02 , 0.21 ± 0.03 , 0.24 ± 0.02 , and $0.35 \pm 0.04 \,\mu$ M, respectively. Also, compounds 9c and 9b possessed good VEGFR-2 inhibition with IC₅₀ values of 1.56 ± 0.05 and $1.99 \pm 0.06 \,\mu$ M, respectively. From the obtained docking results, it was concluded that the NH linker occupied the same groove occupied by NH and urea linkers of vatalanib and sorafenib, respectively, and played the same role, which is essential for higher affinity toward the VEGFR-2 enzyme. The distal hydrophobic moieties increased hydrophobic interactions and, consequently, affinities toward the VEGFR-2 enzyme. The phthalazine enables the new compounds to form a new H-bond through its N atom at position-2 with the amino acid Asp1044. The use of urea, thiourea, and/or amide linkers leads to elongations of the structures and enables the hydrophobic distal moieties to occupy a new hydrophobic groove, which increases the affinity toward the VEGFR-2 enzyme. However, these modifications result in obtaining new phthalazine derivatives with higher VEGFR-2 binding affinities than vatalanib to be nearly equipotent to sorafenib.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

All melting points were determined by the open capillary method on a Gallenkamp melting point apparatus at the Faculty of Pharmacy Al-Azhar University and were uncorrected. The infrared spectra were recorded on a pyeUnicam SP 1000 IR spectrophotometer at the Pharmaceutical Analytical Unit, Faculty of Pharmacy, Al-Azhar University, using the potassium bromide disc technique. The proton magnetic resonance ¹H NMR spectra were recorded on a Jeol 400 MHZ-NMR spectrophotometer at Microanalytical Center, Faculty of Pharmacy, Ain Shams University, and a Jeol 400 MHZ-NMR spectrophotometer at Microanalytical Unit, Faculty of Pharmacy, Cairo University. The ¹³C NMR spectra were recorded on a Bruker 100 MHZ-NMR spectrophotometer at Microanalytical Unit, Faculty of Pharmacy, Cairo University. Tetramethylsilane was used as an internal standard and chemical shifts were measured in the δ scale (ppm). The mass spectra were performed on Direct Probe Controller Inlet part to Single Quadropole mass analyzer in Thermo Scientific GCMS model ISQ LT using Thermo X-Calibur software at the Regional Center for Mycology and Biotechnology, Al-Azhar University. Elemental analyses (C, H, N) were performed on a CHN analyzer at Regional Center for Mycology and Biotechnology, Al-Azhar University. All compounds were within ±0.4 of the theoretical values. The reactions were monitored by thin-layer chromatography (TLC) using TLC sheets precoated with UV fluorescent silica gel Merck 60 F254 plates and were visualized using a UV lamp and different solvents as mobile phases.

4-Phenylphthalazin-1(2*H*)-one (**2**) and 1-chloro-4-phenylph thalazine (**3**) were obtained according to the reported procedures.^[3,4]

The original spectra of the investigated compounds, together with their InChI codes and some biological activity data, are provided as Supporting Information.

4.1.2 | General procedure for the synthesis of compounds 4a,b

Equimolar quantities of 1-chloro-4-phenylphthalazine (**3**) (2.40 g, 0.01 mol) and the appropriate *p*-substituted aniline, namely, 4-nitroaniline and/or 4-aminobenzoic acid (0.01 mol), in acetonitrile (20 ml) were heated under reflux for 6 h. The mixture was left to cool and the separated solid was filtered and crystallized from ethanol to give the target compounds **4a,b**, respectively.

N-(4-Nitrophenyl)-4-phenylphthalazin-1-amine (4a)

Yield, 84%; m.p. 180–182°C; $IR_{\nu max}$ (cm⁻¹): 3,418 (N–H), 3,073 (C–H aromatic), 1,513 (C=N), and 1,334 (NO₂); ¹H NMR (400 MHz, dimethyl sulfoxide [DMSO]-*d*₆): 7.69–7.71 (m, 3H, H-3, H-4, H-5 of C₆H₅), 7.77 (d, 2H, H-3, H-5 of NH–C₆H₄, *J* = 6.22), 8.05 (d, 1H, H-5 of phthalazine, *J* = 8.20), 8.14 (d, 1H, H-6 of phthalazine, *J* = 7.36), 8.18 (d, 2H, H-2, H-6 of C₆H₅, *J* = 6.12), 8.29 (d, 1H, H-7 of phthalazine, *J* = 7.76), 8.33 (d, 2H, H-2, H-6 of NH–C₆H₄, *J* = 6.22), 9.01 (d, 1H, H8 of phthalazine, *J* = 8.28), and 10.33 (s, 1H, NH, D₂O exchangeable); Anal. calcd. for C₂₀H₁₄N₄O₂ (342.11): C, 70.17; H, 4.12; N, 16.37. Found: C, 69.89; H, 4.34; N, 16.53.

4-[(4-Phenylphthalazin-1-yl)amino]benzoic acid (4b)

Yield, 82%; m.p. 245-247°C; $IR_{\nu max}$ (cm⁻¹): 3,363 (NH), 3,039 (C-H aromatic), 1,786 (C=O acid), and 1,541 (C=N); ¹H NMR (400 MHz, DMSO-*d*₆): 7.68-7.72 (m, 3H, H-3, H-4, H-5 of C₆H₅), 7.74 (d, 2H, H-3, H-5 of NH-C₆H₄, *J* = 8.00), 7.97-8.02 (m, 5H, H-2, H-6 of C₆H₅, H-2, H-6 of NH-C₆H₄ and H-5 of phthalazine), 8.13 (t, 1H, H-6 of phthalazine, *J* = 8.60), 8.25 (t, 1H, H-7 of phthalazine, *J* = 8.60), 9.12 (d, 1H, H-8 of phthalazine, *J* = 7.8 Hz), and 10.93 (s, 1H, OH, D₂O exchangeable); MS (*m*/*z*): 342 (M⁺+1, 11.12%), 341 (M⁺, 56.49%), and 340 (M⁺-1, 100%, base peak); Anal. calcd. for C₂₁H₁₅N₃O₂ (341.12): C, 73.89; H, 4.43; N, 12.31. Found: C, 74.15; H, 4.62; N, 12.58.

4.1.3 | Synthesis of N^1 -(4-phenylphthalazin-1-yl)benzene-1,4-diamine (5)

A mixture of N-(4-nitrophenyl)-4-phenylphthalazin-1-amine (4a) (3.42 g, 0.01 mol) and SnCl₂ (19.0 g, 0. 1 mol) was heated under reflux in ethanol (100 ml) for 4 h in the presence of a catalytic amount of HCI (0.02 M). After cooling to room temperature, the reaction mixture was neutralized with aqueous NaOH. The ethanol was evaporated and the aqueous solution was continuously extracted with ether. The ether solution was dried over Na₂SO₄, concentrated, and the precipitated solid was washed with water, filtered, dried, and crystallized from ethanol to give the corresponding target compound (5). Yield, 62%; m.p. 217-219°C; IR_{ymax} (cm⁻¹): 3,291 (NH₂), 3,167 (NH), 3,014 (C-H aromatic), and 1,506 (C=N); ¹H NMR (400 MHz, DMSO-d₆): 4.93 (s, 2H, NH₂, D₂O exchangeable), 6.63 (d, 2H, H-3, H-5 of NH-C₆H₄, J = 8.80), 7.56-7.61 (m, 5H, H-3, H-4, H-5 of C₆H₅ and H-2, H-6 of NH-C₆H₄), 7.62 (d, 2H, H-2,H-6 of C₆H₅, J = 9.60), 7.81 (d, 2H, H-6, H-7 of phthalazine, J = 7.40), 7.92 (d, 1H, H-5 of phthalazine, J = 7.40), 8.59 (d, 1H, H-8 of phthalazine, J = 9.60), and 8.95 (s, 1H, NH, D₂O exchangeable); MS (*m*/*z*): 313 (M⁺+1, 13.43%), 314 (M⁺, 72.98%), 311 (M⁺-1, 100%, base peak), and 77 (6.07%); Anal. calcd. for C₂₀H₁₆N₄ (312.14): C, 76.90; H, 5.16; N, 17.94. Found: C, 76.72; H, 5.33; N, 18.12.

4.1.4 | Synthesis of *N*-{4-[(4-phenylphthalazin-1-yl)-amino]phenyl}benzamide (6)

A mixture of benzoic acid (0.19 g, 0.0016 mol) and triethanolamine (TEA) (0.18 g, 0.0018 mol) in methylene chloride (20 ml) was put in an ice-salt bath while stirring. Next, a solution of ethyl chloroformate (0.19 g, 0.0018 mol) in methylene chloride (10 ml) was added to the reaction mixture dropwise for over 20 min and left for 1 h while stirring at -10° C to -5° C. Then, N¹-(4-phenylphthalazin-1yl)benzene-1,4-diamine (5) (0.46 g, 0.15 mmol) was added to the reaction mixture portionwise. The reaction mixture was stirred for 3 h at ambient temperature. The formed precipitate was filtered and washed with water and ethanol to afford the target compound (6). Yield, 75%; m.p. 247-249°C; IR_{vmax} (cm⁻¹): 3,207 (2NH), 3,010 (C-H aromatic), and 1,656 (C=O amide); ¹H NMR (400 MHz, DMSO-d₆): 7.52 (d, 2H, H-3, H-5 of NH-C₆H₄, J = 7.40), 7.59 (d, 1H, H-4 of C=O-C₆H₅, J = 6.80), 7.64-7.68 (m, 4H, H-2, H-6 of NH-C₆H₄ and H-3, H-5 of C=O-C₆H₄), 7.69-7.72 (m, 3H, H-3, H-4, H-5 of C₆H₅), 7.95 (t, 1H, H-5 of phthalazine, J = 7.20), 8.01 (dd, 4H, H-2, H-6 of C_6H_5 , H-2, H-6 of C=O- C_6H_4 , J = 8.40), 8.16 (t, 1H, H-6 of phthalazine, J = 7.00), 8.23 (t, 1H, H-7 of phthalazine, J = 7.00), 9.27 (s, 1H, NH, D₂O exchangeable), and 11.70 (s, 1H, NH-C=O, D₂O exchangeable); ¹³C NMR (100 MHz, DMSO-d₆): 119, 121.68, 121.83 (2C), 125.88, 126.01, 126.57, 127.67, 128.24 (2C), 128.50, 128.89 (2C), 129.37 (2C), 130.26 (2C), 130.83, 132.17, 134.82, 135.19, 135.98, 138.72, 152.35, 152.80, 154.08 and 166.11; MS (m/z): 417 (M⁺1, 27.43%), 416 (M⁺, 100% base peak), 415 (M⁺-1, 90%), and 77 (10.98%); Anal. calcd. for $C_{27}H_{20}N_4O$ (416.16): C, 77.87; H, 4.84; N, 13.45. Found: C, 78.21; H, 4.97; N, 13.62.

4.1.5 | General procedure for the synthesis of compounds 7a,b

A mixture of the N^1 -(4-phenylphthalazin-1-yl)benzene-1,4-diamine **5** (0.31 g, 0.001 mol) and the appropriate isocyanate, namely, cyclo-hexylisocyanate and/or phenylisocyanate (0.001 mol), was refluxed in absolute ethanol (25 ml) for 3 h. The solution was cooled and the obtained solid was filtered and recrystallized from ethanol to produce the corresponding urea derivatives **7a,b**, respectively.

1-*Cyclohexyl*-3-{4-[(4-*phenylphthalazin*-1-*yl*)*amino*]*phenyl*}*urea* (7*a*) Yield, 83%; m.p. 262–264°C; IR_{νmax} (cm⁻¹): 3,207 (3NH), 3,010 (C-H aromatic), 2,931 (C-H aliphatic), and 1,656 (C=O amide); ¹H NMR (400 MHz, DMSO-*d*₆): 1.06–1.19 (m, 2H, C-4 of cyclohexyl), 1.32–1.58 (m, 4H, C-3 and C-5 of cyclohexyl), 1.64–1.82 (m, 4H, C-2 and C-6 of cyclohexyl), 3.74–3.78 (m, 1H, C-1 of cyclohexyl), 6.03 (s, 1H, NH-cyclohexyl, D₂O exchangeable), 7.39 (d, 2H, H-3, H-5 of NH–C₆H₄, *J* = 8.00), 7.56–7.70 (m, 5H, H-3, H-5 of C₆H₄ and H-2, H-6 of NH–C₆H₄), 7.78 (m, 5H, H-2, H-6 of C₆H₅, H-5, H-6 of phthalazine, NH–C=O, D₂O exchangeable), 8.05 (d, 1H, H-5 of phthalazine, *J* = 7.40), 8.27 (d, 1H, H-7 of phthalazine, *J* = 8.80), 8.64 (d, 1H, H-8 of phthalazine, *J* = 8.20), and 9.14 (s, 1H, NH–C₆H₄, D₂O exchangeable); Anal. calcd. for C₂₇H₂₇N₅O (437.22): C, 74.12; H, 6.22; N, 16.01. Found: C, 74.38; H, 6.39; N, 16.34.

1-Phenyl-3-{4-[(4-phenylphthalazin-1-yl)amino]phenyl}urea (7b)

Yield, 85%; m.p. 253–255°C; $IR_{\nu max}$ (cm⁻¹): 3,489, 3,252, 3,260 (3NH), 3,042 (C–H aromatic), 1,681 (C=O amide), and 1,545 (C=N); ¹H NMR (400 MHz, DMSO-*d*₆): 6.97 (t, 1H, H-4 of NH-C₆H₅, *J* = 8.40), 7.26 (dd, 2H, H-3, H-5 of NH-C₆H₅, *J* = 8.80), 7.47 (m, 4H, H-2, H-6 of NH-C₆H₅ and H-3, H-5 of NH-C₆H₄), 7.53–7.59 (m, 3H, H-3, H-4, H-5 of C₆H₅), 7.65 (d, 2H, H-2, H-6 of NH-C₆H₄, *J* = 7.60), 7.85–7.94 (m, 4H, H-2, H-6 of C₆H₅ and H-6, H-7 of phthalazine), 7.99 (d, 1H, H-5 of phthalazine, *J* = 8.60), 8.64–8.67 (m, 3H, H-8 of phthalazine, NH-C=O-NH, D₂O exchangeable), and 9.20 (s, 1H, NH, D₂O exchangeable); MS (*m*/*z*): 431 (M⁺, 2.25), 430 (M⁺+1, 1.13), and 337 (100%, base peak); Anal. calcd. for C₂₇H₂₁N₅O (431.17): C, 75.16; H, 4.91; N, 16.23. Found: C, 75.34; H, 5.12; N, 16.45.

4.1.6 | General procedure for the synthesis of compounds 8a-c

A mixture of the N^1 -(4-phenylphthalazin-1-yl)benzene-1,4-diamine 5 (0.31 g, 0.001 mol) and the appropriate isothiocyanate, namely, propyl isothiocyanate, butyl isothiocyanate, and/or cyclohexyl isothiocyanate (0.001 mol), was refluxed in absolute ethanol (25 ml) for 3 h. The solution was cooled and the formed solid was filtered and

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recrystallized from ethanol to obtain the corresponding thiourea derivatives **8a-c**, respectively.

1-{4-[(4-Phenylphthalazin-1-yl)amino]phenyl}-3-propylthiourea (8a) Yield, 83%; m.p. 202-204°C; IR_{vmax} (cm⁻¹): 3,194 (3NH), 3,095 (C-H aromatic), 2,993 (C-H aliphatic), and 1,634 (C=O amide); ¹H NMR (400 MHz, DMSO-d₆): 0.87 (t, 3H, CH₃, J = 8), 1.52 (q, 2H, CH₂CH₃, J = 8.00), 3.44 (t, 2H, CH₂N, J = 8.00), 7.36 (d, 2H, H-3, H-5 of NH-C₆H₄, J = 8.40), 7.52-7.56 (m, 3H, H-3, H-4, H-5 of C₆H₅), 7.65-7.68 (m, 3H, H-2, H-6 of NH-C₆H₄ and NH-CH₂, D_2O exchangeable), 7.87-7.94 (m, 4H, H-2, H-6 of C₆H₅ and H-6, H-7 of phthalazine), 7.99 (d, 1H, H-5 of phthalazine, J = 9.00), 8.65 (d, 1H, H-8 of phthalazine, J = 10.80), 9.29 (s, 1H, NH-C=S, D₂O exchangeable), and 9.40 (s, 1H, NH-C₆H₄, D₂O exchangeable); ^{13}C NMR (100 MHz, DMSO-d₆): 11.86, 22.35, 46.12, 118.90 (2C), 121.66, 123.12, 124.49, 126.20, 128.89, 129.01, 130.13 (2C), 132.05 (2C), 132.63 (2C), 133.94, 137.24 (2C), 137.78, 152.29, 153.77, and 180.91; Anal. calcd. for $C_{24}H_{23}N_5S$ (413.17): C, 69.71; H, 5.61; N, 16.94. Found: C, 69.47; H, 5.87; N, 17.12.

1-Butyl-3-{4-[(4-phenylphthalazin-1-yl)amino]phenyl}thiourea (8b)

Yield, 85%; m.p. 216–218°C; IR_{ymax} (cm⁻¹): 3,177 (3NH), 3,050 (C–H aromatic), 2,978 (C–H aliphatic), 1,675 (C=O amide); ¹H NMR (400 MHz, DMSO-*d*₆): 0.89 (t, 3H, CH₃), 1.28 (m, 2H, -CH₂–CH₃), 1.50 (m, 1H, CH₂CH₂CH₃), 3.48 (t, 2H, CH₂CH₂CH₂CH₃), 7.35 (d, 2H, H-3, H-5 of NH–C₆H₄, *J* = 9.60), 7.53–7.60 (m, 4H, H-3, H-4, H-5 of C₆H₅ and NH-CH₂, D₂O exchangeable), 7.65 (d, 4H, H-2,H-6 of NH-C₆H₄,*J* = 8.80), 7.86–7.95 (m, 4H, H-2, H-6 of C₆H₅ and H-6, H-7 of phthalazine), 8.02 (d, 1H, H-5 of phthalazine, *J* = 7.20), 8.65 (d, 1H, H-8 of phthalazine, *J* = 9.20), 9.30 (s, 1H, NH–C=S, D₂O exchangeable), 9.38 (s, 1H, NH, D₂O exchangeable); ¹³C NMR (100 MHz, DMSO-*d*₆): 14.23, 20.10, 31.20, 44.08, 118.90, 121.66 (2C), 123.12, 124.43 (2C), 126.20 (2C), 128.89 (2C), 129.01, 130.13 (2C), 132.04, 132.63, 133.94, 137.25, 137.76, 152.28, 153.76, and 180.85 (C=S); Anal. calcd. for C₂₅H₂₅N₅S (427.18): C, 70.23; H, 5.89; N, 16.38. Found: C, 70.05; H, 5.97; N, 16.59.

1-Cyclohexyl-3-{4-[(4-phenylphthalazin-1-yl)amino]phenyl}thiourea (**8c**)

Yield, 81%; m.p. 230–232°C; $IR_{\nu max}$ (cm⁻¹): 3,143 (3NH), 3,040 (C–H aromatic), 2,965 (C–H aliphatic), and 1,667 (C=O amide); ¹H NMR (400 MHz, DMSO-*d*₆): 1.01–1.08 (m, 2H, C-4 of cyclohexyl), 1.23–1.58 (m, 4H, C-3 and C-5 of cyclohexyl), 1.66–1.82 (m, 4H, C-2 and C-6 of cyclohexyl), 4.11–4.16 (m, 1H, C-1 of cyclohexyl), 7.42 (d, 2H, H-3, H-5 of NH–C₆H₄, *J* = 8.00), 7.50–7.57 (m, 4H, H-3, H-4, H-5 of C₆H₅, NH-cyclohexyl, D₂O exchangeable), 7.67 (d, 2H, H-2, H-6 of NH–C₆H₄, *J* = 8.00), 7.90–7.98 (m, 4H, H-2, H-6 of C₆H₅ and H-6, H-7 of phthalazine), 8.03 (d, 1H, H-5 of phthalazine, *J* = 10.00), 8.12–8.14 (d, 2H, H-6, H-7 of phthalazine), 8.67 (d, 1H, H-8 of phthalazine, *J* = 8.00), 9.28 (s, 1H, NH–C=S, D₂O exchangeable), and 9.58 (s, 1H, NH-C₆H₄, D₂O exchangeable); Anal. calcd. for C₂₇H₂₇N₅S (453.20): C, 71.49; H, 6.00; N, 15.44. Found: C, 71.68; H, 6.13; N, 15.51.

4.1.7 | General procedure for the synthesis of compounds 9a-c

A mixture of 4-[(4-phenylphthalazin-1-yl)amino]benzoic acid (4b) (0.50 g, 1.48 mmol) and TEA (0.18 g, 1.78 mmol) in methylene chloride (20 ml) was put in an ice-salt bath with stirring. Next, a solution of ethyl chloroformate (0.19 g, 1.77 mmol) in methylene chloride (10 ml) was added to reaction mixture dropwise for over 20 min and left for 1 h while stirring at -10 to -5° C. Then, the appropriate amine, namely, aniline, 4-chloroaniline, and/or 4-methylaniline (1.48 mmol), was added to the reaction mixture portionwise. The reaction mixture was stirred for 3 h at ambient temperature. The formed precipitate was filtered and washed with water and ethanol to afford the corresponding compounds **9a-c**, respectively.

N-Phenyl-4-[(4-phenylphthalazin-1-yl)amino]benzamide (9a)

Yield, 83%; m.p. 253–255°C; $IR_{\nu max}$ (cm⁻¹): 3,365 (2NH), 3,095 (C–H aromatic), and 1,671 (C=O amide); ¹H NMR (400 MHz, DMSO-*d*₆): 7.15 (t, 1H, H-4 of NH–C₆H₅, *J* = 10.80), 7.35 (t, 2H, H3, H-5 of NH–C₆H₅, *J* = 10.80), 7.69–7.72 (m, 3H, H-3, H-4, H-5 of C₆H₅), 7.75 (d, 2H, H-3, H-5 of NH–C₆H₄, *J* = 8.00), 7.82 (d, 2H, H-2, H-6 of NH–C₆H₅, *J* = 10.40), 7.99 (d, 2H, H-2, H-6 of C₆H₅, *J* = 7.20), 8.04 (t, 1H, H-5 of phthalazine, *J* = 6.40), 8.13 (d, 2H, H-2, H-6 of NH–C₆H₄, *J* = 10.00), 8.18 (t, 1H, H-6 of phthalazine, *J* = 7.60), 8.31 (t, 1H, H-7 of phthalazine, *J* = 7.60), 9.08 (d, 1H, H-8 of phthalazine, *J* = 8.40), 10.33 (s, 1H, NH–C₆H₄, exchangeable with D₂O), and 10.93 (s, 1H, C=O–NH, exchangeable with D₂O); Anal. calcd. for C₂₇H₂₀N₄O (416.16): C, 77.87; H, 4.84; N, 13.45. Found: C, 78.11; H, 4.97; N, 13.69.

N-(4-Chlorophenyl)-4-[(4-phenylphthalazin-1-yl)amino]benzamide (9b)

Yield, 81%; m.p. 287–289°C; $IR_{\nu max}$ (cm⁻¹): 3,143 (2NH), 3,040 (C–H aromatic), and 1,667 (C=O amide); ¹H NMR (400 MHz, DMSO-*d*₆): 7.32 (d, 2H, H-3, H-5 of 4-Cl–C₆H₄, *J* = 8.00), 7.49 (d, 2H, H3, H-5 of C₆H₅–NH, *J* = 10.40), 7.57–7.64 (m, 3H, H-3, H-4, H-5 of C₆H₅), 7.71 (d, 2H, H-4, H-6 of 4-Cl–C₆H₄, *J* = 6.00), 7.95–8.04 (m, 3H, H-2, H-6 of C₆H₅ and H-5 of phthalazine), 8.08 (dd, 2H, H-2, H-6 of NH–C₆H₄, *J* = 8.40), 8.16 (d, 1H, H-6 of phthalazine, *J* = 10.80), 8.27 (d, 1H, H-7 of phthalazine, *J* = 10.80), 8.71 (d, 1H, H-8 of phthalazine, *J* = 8.40), 9.09 (s, 1H, NH–C₆H₄, D₂O exchangeable), and 9.88 (s, 1H, C=O-NH, D₂O exchangeable); Anal. calcd. for C₂₇H₁₉ClN₄O (450.12): C, 71.92; H, 4.25; N, 12.43. Found: C, 71.69; H, 4.38; N, 12.08.

4-[(4-Phenylphthalazin-1-yl)amino]-N-(p-tolyl)benzamide (9c)

Yield, 85%; m.p. 262–264°C; IR_{ymax} (cm⁻¹): 3,320 (2NH), 3,065 (C–H aromatic), 2,965 (C–H aliphatic), 1,660 (C=O amide); ¹H NMR (400 MHz, DMSO-*d*₆): 2.29 (s, 3H, CH₃), 7.15 (d, 2H, H-3, H-5 of 4-CH₃-C₆H₄, *J* = 10.80), 7.55–7.63 (m, 3H, H-3, H-4, H-5 of C₆H₅), 7.68–7.701 (m, 4H, H-2, H-6 of C₆H₄–CH₃ and H-3, H-5 of NH–C₆H₄), 7.90–8.09 (m, 5H, H-2, H-6 of C₆H₅, H-2, H-6 of NH–C₆H₄ and H-5 of phthalazine), 8.17 (d, 2H, H-6, H-7 of phthalazine, *J* = 10.00), 8.72 (d, 1H, H-8 of phthalazine, *J* = 7.60), 9.62 (s, 1H, NH–C₆H₄, D₂O exchangeable) and 10.07 (s, 1H, C=O–NH, D₂O exchangeable); MS (*m*/*z*): 363 (M⁺, 3.5%), 256 (71.6%), 82 (base

beak, 100%), and 76 (17.84%); Anal. calcd. for $C_{28}H_{22}N_4O$ (430.18): C, 78.12; H, 5.15; N, 13.01. Found: C, 78.40; H, 4.98; N, 13.23.

4.2 | Docking studies

In the present work, all the target compounds were subjected to docking study to explore their binding mode toward the VEGFR-2 enzyme. All modeling experiments were performed using molsoft program, which provides a unique set of tools for the modeling of protein/ligand interactions. It predicts how small flexible molecule such as substrates or drug candidates bind to a protein of known three-dimensional structure represented by grid interaction potentials (http://www.molsoft.com/icm_pro.html). Each experiment used the biological target VEGFR-2 downloaded from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb/explore/explore.do? structureId=1YWN). To qualify the docking results in terms of accuracy of the predicted binding conformations in comparison with the experimental procedure, the reported VEGFR-2 inhibitor drugs vatalanib and sorafenib were used as reference ligands.

4.3 | In vitro cytotoxic activity

The cytotoxicity assays were performed at Pharmacology and Toxicology Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt. Cancer cells from different cancer cell lines, hepatocellular carcinoma (HepG2), colorectal carcinoma (HCT-116), and breast cancer (MCF-7), were purchased from American Type Cell Culture Collection and grown on the appropriate growth medium, Roswell Park Memorial Institute medium-1640, supplemented with 100 mg/ml of streptomycin, 100 units/ml of penicillin, and 10% of heat-inactivated fetal bovine serum, in a humidified, 5% (v/v) CO₂ atmosphere at 37°C. Cytotoxicity assay was carried out by using 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT).

Exponentially growing cells from different cancer cell lines were trypsinized, counted, and seeded at the appropriate densities (2,000-1,000 cells/0.33-cm² well) into 96-well microtiter plates. Cells were then incubated in a humidified atmosphere at 37°C for 24 h. Next, cells were exposed to different concentrations of compounds (0.1, 10, 100, and 1,000 µM) for 72 h. Then, the viability of treated cells was determined using the MTT technique as follows: Cells were incubated with 200 μ l of 5% MTT solution/well (Sigma-Aldrich) and were allowed to metabolize the dye into colored insoluble formazan crystals for 2 h. The remaining MTT solution was discarded from the wells and the formazan crystals were dissolved in 200 µl/well acidified isopropanol for 30 min, covered with aluminum foil, and continuously shaken using a MaxQ 2000 plate shaker (Thermo Fisher Scientific, Inc.) at room temperature. Absorbance was measured at 570 nm using a Stat FaxR 4200 plate reader (Awareness Technology, Inc.). The cell viability was expressed as the percentage of control and the concentration that induces 50% of maximum inhibition of cell proliferation (IC50) was determined using GraphPad Prism software, version 5 (GraphPad Software, Inc.).[46-48]

4.4 | In vitro VEGFR-2 kinase assay

The kinase activity of VEGFR-2 was measured by using an antiphosphotyrosine antibody with the Alpha Screen system (PerkinElmer) according to the manufacturer's instructions.^[50] Enzyme reactions were performed in 50 mM Tris-HCl, pH 7.5, 5 mM MnCl₂, 5 mM MgCl₂, 0.01% Tween-20, and 2 mM dithiothreitol, containing 10 µM ATP, 0.1 µg/ml biotinylated poly-GluTyr (4:1), and 0.1 nM VEGFR-2 (Millipore). Before catalytic initiation with ATP, the tested compounds at final concentrations ranging from 0 to $300 \,\mu$ g/ml and enzyme were incubated for 5 min at room temperature. The reactions were quenched by the addition of $25\,\mu$ l of 100 mM EDTA, 10 μ g/ml Alpha Screen streptavidin donor beads, and 10 µg/ml acceptor beads in 62.5 mM 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid, pH 7.4, 250 mM NaCl, and 0.1% bovine serum albumin. The plate was incubated in the dark overnight and then read by ELISA Reader (PerkinElmer). Wells containing the substrate and the enzyme without compounds were used as the reaction control. Wells containing biotinylated poly-GluTyr (4:1) and enzyme without ATP were used as the basal control. The percent inhibition was calculated by the comparison of compounds treated with control incubations. The concentration of the test compound causing 50% inhibition (IC₅₀) was calculated from the concentration-inhibition response curve (triplicate determinations) and the data were compared with sorafenib (Sigma-Aldrich) as a standard VEGFR-2 inhibitor.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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