Design, Synthesis, and Biological Evaluation of a Novel VEGFR-2 Inhibitor Based on a 1,2,5-Oxadiazole-2-Oxide Scaffold with MAPK Signaling Pathway Inhibition

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Abstract: Over the past few decades, the development of broad-spectrum anticancer agents with anti-angiogenic activity has witnessed considerable progress. In this study, a new series of pyrazolo[3,4-d]pyrimidines based on a phenylfuroxan scaffold were designed, synthesized, and evaluated, in terms of their anticancer activities. NCI-60 cell one-dose screening revealed that compounds 12a–c and 14a had the best MGI%, among the tested compounds. The target fluorinated compound 12b, as the most active one, showed better anticancer activity compared to the reference drug sorafenib, with IC50 values of 11.5, 11.6, and 13 µM against the HepG-2, A2780CP, and MDA-MB-231 cell lines, respectively. Furthermore, compound 12b (IC50 = 0.092 µM) had VEGFR-2-inhibitory activity comparable to that of the standard inhibitor sorafenib (IC50 = 0.049 µM). Furthermore, the ability of compound 12b in modulating MAPK signaling pathways was investigated. It was found to decrease the level of total ERK and its phosphorylated form, as well as leading to the down-regulation of metalloproteinase MMP-9 and the over-expression of p21 and p27, thus leading to subG1 cell-cycle arrest and, thus, the induction of apoptosis. Additionally, compound 12b decreased the rate of wound healing in the absence of serum, in comparison to DMSO-treated cells, providing a significant impact on metastasis inhibition. The quantitative RT-PCR results for E-cadherin and N-cadherin showed lower expression of the neuronal N-cadherin and increased expression of epithelial E-cadherin, indicating the ability of 12b to suppress metastasis. Furthermore, 12b-treated HepG2 cells expressed a low level of anti-apoptotic BCL-2 and over-expressed proapoptotic Bax genes, respectively. Using the DAF-FM DA fluorescence probe, compound 12b produced NO intracellularly as efficiently as the reference drug JS-K. In silico molecular docking studies showed a structural similarity through an overlay of 12b with sorafenib. Interestingly, the drug-likeness properties of compound 12b met the expectations of Pfizer’s rule for the design of new drug candidates. Therefore, this study presents a novel anticancer lead compound that is worthy of further investigation and activity improvement.

Keywords: pyrazolo[3,4-d]pyrimidines; furoxan; VEGFR-2; DAF-FM DA; MAPK; metastasis; apoptosis

1. Introduction

Cancer is a large group of diseases, and is the result of the uncontrollable growth of abnormal cells [1]. In 2018, according to the WHO, the second leading cause of death worldwide was cancer, and it was estimated that 9.6 million people worldwide died from various cancers [1]. About 300,000 new cases are diagnosed every year among children...
only. The total annual economic cost of cancer was estimated to be about $1.16 trillion per year, and this number has been rising [2]. Cancer affects everyone—men, women, and children—alike, and represents an enormous burden for all. Cancer not only affects the diseased organ, but also invades adjoining organs in a process called metastasis, which is a major cause of death from cancer [1]. The interaction of tumor cells with their microenvironment is essential for their development [3]. Angiogenesis is the key regulatory process for tumor growth and metastasis, which begins with splitting or sprouting pre-existing blood vessels to form new ones, in order to deliver the essential nutrients to the tumor, and is a focus of anticancer therapy [3–6]. This process involves the growth, migration, and differentiation of blood vessel endothelial cells, which line their inside wall [7]. Therefore, tumor growth and metastasis may be suppressed by anti-angiogenic agents.

Vascular endothelial growth factor (VEGF) expression, through the expression of the cell-surface kinase VEGFR-2 (KDR/Flk-1), is strongly associated with both angiogenesis and tumor aggressiveness [5,8]. KDR is overexpressed in different types of cancers, such as breast cancer [9,10], cervical cancer [11,12], non-small cell lung cancer (NSCLC) [11,13,14], hepatocellular carcinoma (HCC) [5,11,15], and renal carcinoma [5,11,16]. VEGF overexpression has been observed in solid tumors, which initiates the activation of VEGFR-2 [4]. This, in turn, triggers a downstream signaling cascade, including the Raf/MEK/ERK pathway, ultimately leading to the excessive formation of new blood vessels and accelerated angiogenesis, tumor proliferation, and metastasis [4,5,17,18]. Hence, effective anti-angiogenic agents are those having potential inhibition of VEGFR-2 [4,18].

Sunitinib, sorafenib, and cabozantinib were recently shown to be among the anti-angiogenic drugs that can inhibit VEGFR-2, and which are active against a vast array of cancer types [4]. Their adverse side-effects and influence on patient health are the main cause of concern relating to chemotherapeutic drugs [19]. Therefore, there is an urgent need to develop new classes of VEGFR-2 inhibitors with low toxicity and enriched anti-angiogenic potential.

The chemical fragments pyrazolo[3,4-d]pyrimidine and 1,2,5-oxadiazole-N-oxide may serve as promising scaffolds for the design of a new class of anti-angiogenic agents through improved bioavailability and enhanced biological activity [20–22]. They have been identified based on the combination principle of multiple pharmacophore and bioisosteric replacements of the lead potent multi-target kinase inhibitor sorafenib (Nexavar®) [23,24]. Sorafenib is a small molecule inhibitor of VEGFR-2, VEGFR-3, PDGFR-ß, and c-Kit kinases with downstream targeting of the Raf/MEK/ERK pathway [23,25].

The basic pharmacophoric features of sorafenib are characterized by its hydrophobic tail (allosteric binding site; substituted phenyl), hydrogen bond donor and acceptor activities (binding with Glu:883:A and Asp:1044:A in the DFG domain; urea group), central aryl ring (occupying the linker region; arylxy group), and heteroaromatic ring (ATB-binding domain; substituted pyridine) [26–28]; see Figure 1. Interestingly, the next generation of potent VEGFR-2 inhibitors such as regorafenib [28], cabozantinib [28], nintedanib [28] and lenvatinib [26,28] demonstrate the same basic pharmacophoric features of sorafenib [26–28]: hydrophobic tail (substituted phenyl, piperazinyl, or cyclopropyl), hydrogen bond donor and acceptor activities (urea or amide group), central aryl ring (aryloxy or arylamino group), and heteroaromatic ring (substituted pyridine, quinoline, or indole); see Figure 2. Hence, the design of a new series with these essential structural pharmacophoric features has drawn our interest.
Furthermore, the pyrimidine-based core was selected as the ATP-binding domain isostere. It has recently been reported that numerous pyrazolo[3,4-d]pyrimidine derivatives exhibit potent anti-tumor activities [29–32]. In 2018, Wand et al. reported a potent VEGFR-2 inhibitor having a pyrazolo[3,4-d]pyrimidine scaffold [20]. Therefore, the privileged pyrazolo[3,4-d]pyrimidine nucleus was deemed to be a promising scaffold for developing a new VEGFR-2 inhibitor class.
On the other hand, the nitric oxide (NO)-releasing furoxan moiety was shown to have potent anti-tumor activity in earlier reports [21,22]. It is worth mentioning that NO plays a pivotal role as a signaling free radical molecule, functioning as a vascular smooth muscle relaxant, platelet aggregation inhibitor, neurotransmitter, and immune regulator [33]. The anti-tumor activity of NO has been observed at high levels (more than 300 nM), through the initiation of DNA damage and nitrosative stress [34,35], the phosphorylation of P53, and the increasing of MKP-1 expression, leading to cellular respiration inhibition. High levels of NO also play a crucial role in the reversal of drug resistance through MRP efflux pump inhibition [36,37], hypoxia-induced drug-resistance attenuation [36], hypoxic radio-sensitization [38], transcription factor hypoxia-inducible factor (HIF-1) activation blocking [36,38], inhibition of epithelial–mesenchymal transition (EMT) [39], and metastatic inhibition [39]. Therefore, increasing the concentration of intracellular NO may have a significant impact on tumor growth and metastasis [35,36].

A convenient way to achieve high levels of intracellular NO is through the use of NO donors [40]. Among them, the most stable is the furoxan moiety [41], which releases NO in the presence of plasma, reduced glutathione (GSH), or albumin (i.e., through thiol mechanisms) and, therefore, may exert better biological activity and rarely leads to tolerance, compared to other nitric oxide-releasing motifs [40,41]. In summary, the synergistic effects discussed above have raised hopes for its therapeutic potential and, as such, endowing the pyrazolo[3,4-d]pyrimidine scaffold with a furoxan moiety may have a significant impact on tumor and metastasis suppression [20–22,41].

Enlightened by these findings, we were inspired to design and synthesize a new series of pyrazolo[3,4-d]pyrimidines tethered to a NO-releasing furoxan moiety through a central aryl bridge. In this context, as shown in Figure 3, our program began with the replacement of the pyridine ring with an isosteric pyrazolo[3,4-d]pyrimidine base-scaffold (in order to fit the ATP-binding domain), the replacement of the oxygen atom with the bioisosteric NH as a hydrogen bond donor (HBD), the keeping of the central aryl moiety without modification as a linker, the replacement of the hydrogen-bonding moiety urea with furoxan methoxy/amino to bind the DFG domain and, finally, the incorporating of the phenyl group as a hydrophobic tail to occupy the allosteric binding region. Furthermore, C3 and C6 functionalizations were studied.

Figure 3. Rational design for target compounds.
2. Results and Discussion

2.1. Chemistry

The synthetic process for the target compounds was initiated with a trial to prepare the furoxan moiety from allyl ether precursors 3a–d. As depicted in Scheme 1, the phenolic derivatives 2a–h were prepared from alkylidyne malononitrile, through a multi-step reaction, with reasonable yields [42]. Compounds 2a–d were alkylated, using allyl bromide, to obtain the allyloxy derivatives 3a–d [42–45]. Upon treating compounds 3a–d with sodium nitrite, an electrophilic aromatic substitution reaction took place at the ortho position to an allyloxy group, wherein the carbons were more shielded, thus providing the nitrated derivatives 5a–d, rather than the desired compound 4 [46]. The unaffected chemical shift of allyl protons through the reaction and disappearance of the doublet at 7.00 ppm corresponding to the protons ortho to allyloxy group evidenced the proposed chemical structures. On the other hand, the furoxan moiety was successfully installed through the alkylation of phenolic OH precursors 2a–h using phenyl furoxan methyl mesylate to afford the target compounds 6a–h [21].

The fluorinated pyrazolo[3,4-d]pyrimidine derivatives were synthesized as depicted in Scheme 2, in which the bioisosteric furoxan methylamino group was successfully in-

![Scheme 1. Synthesis of unexpected compounds 5a-d and target compounds 6a-h. (i) Ar-NHNH₂; (ii) R₂COOH; (iii) POCl₃; (iv) p-aminophenol; (v) allyl bromide, EtOH, reflux, 45 min.; (vi) NaNO₂, HOAc, 25–70 ºC, 1 h; (vii) 3-(((methylsulfonyl)oxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide, NaOH, THF, reflux, 2 h.](image-url)
stalled on the central aryl ring. The reaction cascade began with the installation of a trifluoromethyl group at C6 of the pyrazolopyrimidine core 8a–c, through the cyclization of the intermediate 5-amino 4-cyano pyrazole derivatives 7a–c with trifluoroacetic acid in the presence of a catalytic amount of phosphorus oxychloride, thus yielding the intermediate 6-trifluoromethyl derivatives 8a–c [47]. The 4-chlorinated derivatives 9a–c were obtained through the refluxing of 6-trifluoromethyl-pyrazolopyrimidinone intermediates 8a–c in phosphorus oxychloride [48]. The NO-releasing source, phenylfuroxan alkarylamine 11, was obtained with good yield from furoxan mesylate 10, through refluxing with p-phenylenediamine in THF in a ratio of 1:6 [21]. Finally, the target compounds 12a–c were obtained, with reasonable yield, through alkylation of phenylfuroxan alkarylamine 11 with the 4-chloro derivatives 9a–c [42].

Moreover, the C6-substituted derivatives 14a–c were obtained through the series of chemical reactions depicted in Scheme 3. The 6-chloromethyl intermediates 13a–c were synthesized through intermolecular cyclization of the 5-amino-4-cyano intermediates 7a–c, using chloroacetic acid and a catalytic amount of phosphorus oxychloride [48,49]. Next, the target compounds 14a–c were prepared by N-alkylation of phenylfuroxan alkarylamine 11 with the 6-chlormethyl intermediates 13a–c [50]. Finally, the des-furoxan analogs 15a and b

**Scheme 2.** Synthesis of target compounds 12a–c. (i) Ar-NNH$_2$, EtOH, reflux, 15 h; (ii) CF$_3$COOH, POCl$_3$, reflux, 2 h; (iii) POCl$_3$, reflux, 10 h; (iv) p-phenylene diamine, TEA, THF, reflux, 2 h; (v) 11, TEA, NaI, i-PrOH, reflux, 2 h.
were prepared, according to Scheme 4, through the alkylation of \( p \)-phenylenediamine with the 6-trifluoromethyl-4-chlor derivatives 9b and c in a 6:1 ratio [42].

![Scheme 3. Synthesis of C6-appending compounds 14a–c.](image)

15a, and 15b were prepared, according to Scheme 4, through the alkylation of \( p \)-phenylenediamine with the 6-trifluoromethyl-4-chlor derivatives 9b and c in a 6:1 ratio [42].

14a; \( R = H, X = H, 63\% \)
14b; \( R = Me, X = H, 58\% \)
14c; \( R = Me, X = Br, 69\% \)

**Scheme 3.** Synthesis of C6-appending compounds 14a–c. (i) \( \text{ClCH}_2\text{COOH}, \text{POCl}_3, \text{THF}, \text{reflux, 2 h} \); (ii) 11, TEA, NaI, THF, reflux, 2 h.

15a. \( R_1 = H, 73\% \)
15b. \( R_1 = \text{CH}_3, 78\% \)

**Des-NO-Releasing compounds**

![Scheme 4. Synthesis of des-NO-releasing compounds 15a and b.](image)

2.2. Biological Evaluation

2.2.1. In Vitro Anticancer Activity

NCI-60 Cell Line Screening

The structures of the synthesized compounds 6a–h, 12a–c, 11, 13a–c, and 14a–c were submitted to the National Cancer Institute’s Developmental Therapeutics Program (NCI-DTP), and all of them were selected for preliminary in vitro anticancer activity screening against NCI-59 human cancer cell lines at a concentration of 10 \( \mu \)M, using the sulforhodamine B (SRB) assay to determine the growth percentage and cell viability [51]. The
results for a panel of 59 cancer cell lines demonstrating nine types of cancer (leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate, and breast cancers) are depicted in Tables S1 and S2 (from the Supplementary Material File), and the mean % growth inhibition values (GI%) for the tested compounds against the NCI-59 cancer cell line’s full panel are depicted in Figure 4.

As shown in Figure 4, considering the mean growth inhibition % (MGI%) at 10 µM, compounds 6a and f demonstrated no mean growth inhibition percentage, while compounds 6b, d, and e enhanced the growth percentage by 1–5%. Compounds 6c, g, and h demonstrated low anticancer activity, with GI% = 1.58–4.57%. Additionally, compounds 12a–c exhibited significant inhibitory effects in their anticancer activity, with GI% in the range of 23.2–33.17%.

On the other hand, moving the substitution from C4 (compounds 12a–c) to C6, as in compounds 14a and 14b, reduced the growth-inhibitory activity (exhibiting GI% of 27.93 and 8.22, respectively), while compound 14c enhanced the growth by 3.35%.

Moreover, based on the above findings, compound 12b was identified as the most active analog among the tested compounds, displaying a better anticancer profile. As shown in Table S1 (from the Supplementary Material File), compound 12b exhibited growth-inhibitory % activity against the tested panel of up to 77% in leukemia, 64% in non-small cell lung cancer, 62% in colon cancer, 45% in CNS cancer, 72% in melanoma, 40% in ovarian cancer, 69% in renal cancer, 52% in prostate cancer, and 61% in breast cancer cells. Hence, the anticancer profile of compound 12b against the NCI-59 cancer cell lines encouraged us to further explore its mode of action.

Preliminary SARs Study

The methyl substitution (EDG) at C3 and C6 (i.e., compounds 6a–h) had no significant effect on the anticancer activity. Notably, the replacement of EDG at C6 in the tested series (i.e., compounds 6a–h) with the electron-withdrawing group CF₃, and the replacement of the oxygen of the central aryl ring with NH, caused compounds 12a–c to exhibit significant inhibitory effects in their anticancer activity, indicating that the electron-withdrawing substituents at C6 very likely led to enhanced potency. On the other hand, moving the substitution from C4 (compounds 12a–c) to C6, as in compounds 14a and 14b, reduced the growth-inhibitory activity while compound 14c enhanced the growth, indicating that
appending the furoxan moiety at C6 and keeping both the pyrazole C3 and 1-phenyl ring unsubstituted may have a good impact on the activity. Furthermore, to test the hybridization hypothesis, compounds 11 and 13a-c were also tested, and the results displayed a significant improvement in antitumor activity, especially for compound 14a, indicating synergistic efficiency. Consequently, appending the furoxan moiety at C4, utilizing an NH in the central aryl ring at C4, and substituting the C6 with an electron-withdrawing CF$_3$ group can be suggested as vital elements for the growth-inhibitory activity of the furoxan-based pyrazolo[3,4-d]pyrimidine hybrids reported herein. A summary, based on the above discussion, of the preliminary structure–activity relationships (SARs) observed in this study is provided in Figure 5.

![Figure 5. Preliminary SAR of target compounds.](image)

**In Vitro Anti-Proliferative Activities**

The cell growth-inhibitory activities of the most active compound 12b were evaluated against human cancer HepG-2 [52–54], ovarian [55,56], breast [54], and colon [54] cell lines highly expressing the VEGFR-2 protein. The cells were treated with serial dilutions for 48 h, after which the cell viability was determined by standard MTT assay, using sorafenib as a reference drug. The initial screening of the target compounds showed that 12b was the most active compound, at a single 50 µM dose, against liver (HepG2), breast (MDA-MB-231 and MCF-7), colon (HT-29), and ovarian (SKOV-3, A2780, and A2780CP) cancer cell lines. In order to determine the concentration that killed 50% of cells, serial dilutions were used in the treatment, and the determination of percentage of viability identified that 12b was the most active compound. The IC$_{50}$ values are shown in Table 1 and Figure 6. Compared to sorafenib, the target compound 12b showed better antitumor activity, with IC$_{50}$ values of 11.5, 11.6, and 13 µM against HepG-2, A2780CP, and MDA-MB-231 cell lines, respectively, while the IC$_{50}$ for SKOV-3 was comparable to that of the reference drug sorafenib. In addition, the morphological changes in 12b-treated cancer cell lines at concentrations of 6.25 and 12.50 µM are shown in Figure 6. In contrast, the des-NO-releasing compounds 15a and b were tested, but did not inhibit the proliferation of cancer cells at concentrations as high as 50 µM, indicating the significance of the phenylfuroxan moiety in the anticancer activity of 12b.
Colon HT-29: 15 ± 0.24

± represents the standard deviation from triplicates.

Figure 6. Morphological changes in 12b-treated cancer cell lines at concentrations of 6.25 and 12.50 µM.
Table 1. IC\textsubscript{50} values for compound 12b against different types of cancers.

| Cancer   | Cell Line | 12b (µM) | Sorafenib (µM) |
|----------|-----------|----------|----------------|
| Liver    | HepG2     | 11.5 ± 0.5 | 14 ± 0.4 |
| Ovarian  | SKOV-3    | 18.6 ± 0.2 | 16.9 ± 0.2 |
|          | A2780     | 15 ± 0.15 | 10.5 ± 0.5 |
|          | A2780CP   | 11.6 ± 0.1 | 15.2 ± 0.1 |
| Breast   | MDA-MB-231| 13 ± 0.2  | 16.6 ± 0.3 |
|          | MCF-7     | 22.3 ± 0.3 | 5.1 ± 0.4  |
| Colon    | HT-29     | 15 ± 0.24 | 11 ± 0.2   |

± represents the standard deviation from triplicates.

Furthermore, the selectivity index (SI) of compound 12b towards the various cancer cell lines was evaluated by detecting the required concentration to kill 50% of normal human skin fibroblast cells (HSF). The results revealed that compound 12b did not cause any morphological changes in HSF cells at a concentration as high as 50 µM, indicating its safety and selectivity towards cancer cells, rather than normal cells.

2.2.2. VEGFR Kinase Inhibitory Profile of 12b

The fluorinated pyrazolo[3,4-d]pyrimidine derivative based on the 1,2,5-oxadiazole-2-oxide scaffold (12b) was evaluated for its VEGFR\textsubscript{x} (VEGFR-1, VEGFR-2, and VEGFR-3) kinase inhibitory activities, using a VEGFR inhibitory assay with sorafenib as a reference drug. As shown in Figure 7, the results indicated that compound 12b possesses potency that is comparable to that of sorafenib, displaying noticeable selectivity against VEGFR-2 with an IC\textsubscript{50} of 0.09 µM.

![Figure 7. VEGFR\textsubscript{x} kinase selectivity profiles of 12b and the reference drug sorafenib.](image-url)
2.2.3. Flow Cytometric Studies

The mechanism of action of 12b was investigated by evaluating its effectiveness in modulating the cell cycle and induction of apoptosis. As seen in Figure 8A,B, treating HepG2 cells with compound 12b for 48 h caused cell-cycle arrest at subG1 (24 vs. 5.6 in DMSO-treated cells), leading to the induction of apoptotic pathways (Figure 8C,D).

![Figure 8](image)

Figure 8. Flow cytometric studies of 12b in HepG-2 cells: (A) Cell-cycle distribution. (B) histogram for the change in cell count in each phase in comparison to the control cells. Cells were treated with increasing concentrations for 48 h: control (DMSO), 5.5 µM (1/2 IC50), and 11 µM (IC50). (C) Apoptotic effect of 12b through Annexin-V-FITC/PI staining assay. Cells were treated with 12b at the tested concentrations for 24 h. (D) Histograms for induction of apoptosis and showing the decrease in viable cell count. **** = significant results in comparison to DMSO-treated cells (p < 0.0001). The statistical difference was analyzed by One-Way ANOVA by GraphPad Prism 8.0.

2.2.4. Intracellular Measurement of NO

The NO-releasing properties of 12b were evaluated by measuring the level of intracellular NO by flow cytometry, using a cell-permeable and photo-stable NO fluorescent indicator, 3-Amino-4-aminomethyl-2’,7’-difluorofluorescein diacetate (DAF-FM DA), with a detection limit of ~3 nM. DAF-FM diacetate penetrates the cells and is deacetylated by esterase enzyme to yield a non-fluorescent dye, DAF-FM, which, upon reaction with NO, produces a fluorescent benztriazole product [57]. Compound 12b was efficiently able to increase the production of NO in the treated cells (9.9% vs. 0.1% in DMSO-treated cells; Figure 9). Furthermore, compound 12b increased NO production as efficiently as the positive control reference drug JS-k.

2.2.5. Wound-Healing Assay

A wound-healing assay is a simple and economical assay used to investigate the anti-metastatic activity of compounds. Cells were treated with compound 12b in serum-free media for 24 h. After that, the cells were fixed with ice-cold methanol and air-dried, then stained with 0.5% crystal violet. As shown in Figure 10, compound 12b decreased the rate of wound healing in the absence of serum, in comparison to the DMSO-treated cells. These data revealed that 12b is an anticancer compound that possesses anti-metastatic activity.
2.2.5. Wound Healing Assay

A wound healing assay is a simple and economical assay used to investigate the anti-metastatic activity of compounds. Cells were treated with compound 12b in serum-free media for 24 h. After that, the cells were fixed with ice-cold methanol and air-dried, then stained with 0.5% crystal violet. As shown in Figure 10, compound 12b decreased the rate of wound healing in the absence of serum, in comparison to the DMSO-treated cells. These data revealed that 12b is an anticancer compound that possesses anti-metastatic activity.

2.2.6. Apoptosis and Metastatic Proteins

Epithelial cells express specific genes involved in maintaining apical–basal polarity and adherence, such as epithelial E-cadherin. The epithelial cells migrate to distant locations by over-expressing mesenchymal genes, including neuronal N-cadherin and vimentin [58]. Cancer aggressiveness has been positively correlated with the over-expression of N-cadherin.
Therefore, in this study, the partial mechanism of activity of 12b was investigated, by quantitative RT-PCR, with respect to E-cadherin and N-cadherin. The 12b-treated cells showed lower expression of neuronal N-cadherin and increased expression of epithelial E-cadherin, indicating the ability of 12b to suppress metastasis (Figure 11A). Additionally, the ability of 12b was confirmed by detecting the change in expression of both pro-apoptotic (Bax) and anti-apoptotic (BCL-2) genes. Compound 12b-treated HepG2 cells (for 12 h) expressed a low level of BCL-2 and over-expressed Bax (Figure 11A).

Figure 11. Compound 12b inhibited cell migration and induced cell-cycle arrest and apoptosis in treated HepG2 cells. (A) relative fold of change in metastatic and apoptotic genes. (B) western blot analysis for the change in proteins involved in MAPK pathway signaling. Beta-actin was used as a loading control.

The mitogen-activated protein kinase (MAPK) pathway is considered to be a key player in the gene expression of survival, proliferative, and anti-apoptotic proteins. Aberrant dysregulation in the MAPK pathway activity has been linked to the overall poor prognosis in various cancers [59]. Compound 12b decreased the level of total ERK and its phosphorylated form (Figure 11B), indicating its ability to modulate signaling pathways. Inhibition of the MAPK pathway led to the downregulation of metalloproteinase MMP-9 (metastatic protein), the over-expression of cell-cycle inhibitors (p21 and p27), and a slight increase in the expression of p53; see Figure 11B. Additionally, compound 12b was further tested against p38, MKK3, and JNK using ELISA assay protocols [60–62] at a concentration of 11 µM. The results indicated that 12b caused low expression in p38 and MKK3 while JNK was overexpressed; see Figure 12. The overexpression of JNK might have contributed to the NO released from 12b and reactive oxygen species (ROS). In summary, the novel anticancer lead compound 12b is worthy of further investigation and activity improvement.
2.3. In Silico Molecular Modeling

2.3.1. EON Scaffold Hopping

The discovery of new molecular targets can be achieved by whole-molecule replacements, through the use of physically realistic shapes and electrostatic potential similarities that permit the identification of biologically active molecules with significantly different structures, with respect to existing active agents [63]. OpenEye’s EON application [64] can contribute significantly to lead generation and library design, through the creation of electrostatic Tanimoto (ET) grids using full Poisson–Boltzmann (PB) electrostatics and attaching them to each output molecule [65]. EON is also influenced by the pKa state and formal charges, which have major impacts on the electrostatics, as the query and database molecule are adjusted to a neutral pH model [65]. The data manager in the VIDA module can easily read the EON report file [66]. Sorafenib was included in the library in order to validate the EON results. The designed library was tested against sorafenib as a query reference, and the compounds were ranked according to their shape and electrostatic similarity to sorafenib. As shown in Table 2, the top-scoring compounds were those of the C6-appending series 14a–c, which showed the best alignment, followed by the fluorinated derivatives 12a–c and, then, the rest of the designed compounds.

Table 2. EON shape and electrostatic potential similarity of target compounds with respect to the query drug sorafenib.

| CPD Name | EON ET coul a | EON ET pb b | EON Shape Tanimoto c | EON ET combo d | EON Rank |
|----------|---------------|-------------|----------------------|----------------|----------|
| Sorafenib | 1             | 1           | 1                    | 2              | 1        |
| 14a      | 0.153         | 0.126       | 0.106                | 0.232          | 2        |
| 14b      | 0.155         | 0.127       | 0.102                | 0.229          | 3        |
| 14c      | 0.153         | 0.126       | 0.103                | 0.228          | 4        |
| 6d       | 0.067         | 0.057       | 0.086                | 0.143          | 5        |
| 12a      | 0.023         | 0.032       | 0.107                | 0.139          | 6        |
| 6h       | 0.056         | 0.049       | 0.089                | 0.138          | 7        |
| 12b      | 0.012         | 0.019       | 0.117                | 0.136          | 8        |
| 6b       | 0.055         | 0.048       | 0.088                | 0.135          | 9        |

Figure 12. Relative expression of p38, MKK3, and JNK in 12b-treated HepG-2 cells.
2.3.2. Docking Studies

In silico molecular docking studies were performed using the OpenEye Scientific Software, version 2021.spr, in order to explore the binding modes of the target compounds with the VEGFR-2 active site, adopting the crystal structure of VEGFR-2 in complex with sorafenib (PDB ID: 3WZE). The tested compounds were ranked according to their FRED Chemgauss4 scores—the sum of shape, HB, and desolvation energies—with the lowest score indicating the best binding interactions (see Table 3). To validate the docking results and rationalize the predictive protein–ligand interactions of the target compounds within the active site, the positive drug sorafenib was re-docked using the same procedure as the target compounds. The results demonstrated that the docking pose of the co-crystallized ligand (see Figure 13A), showing interactions similar to those existing in the crystal structure, and the hydrophobic interactions were roughly the same.

Table 3. FRED Chemgauss4 scores of target compounds 6a–h, 12a–c, and 14a–c.

| Compound | FRED Chemgauss4 Score |
|----------|------------------------|
| 6e       | -12.8024               |
| 6a       | -11.6995               |
| 6b       | -11.5059               |
| 6g       | -11.4424               |
| 12b      | -11.3747               |
| 6c       | -11.3160               |
| 6h       | -11.1254               |
| 6f       | -11.0437               |
| 14c      | -10.9153               |
| 12a      | -10.7872               |
| 6d       | -10.3713               |
| 14a      | -10.1469               |
| 14b      | -9.9950                |
| 12c      | -9.8026                |
| Sorafenib| -18.5752               |

* Electrostatc Tanimoto using only the coulombic (coul) part of Poisson–Boltzmann (PB) electrostatics. \(^b\) Electrostatic Tanimoto using full PB electrostatics. \(^c\) Shape Tanimoto between the given molecule and the query. \(^d\) Sum of ET-pb and EON shape Tanimoto.
Figure 13. Binding interactions in the active site of VEGFR-2: (A) 3D representation of sorafenib; (B) 3D representation of 12b (grey) showing an overlay with sorafenib (green); (C) 2D diagram of 12b; (D) 2D diagram of sorafenib.
The target compounds interacted with the active site in different ways, as follows:

1. The first set, \(6a-h\), had the same binding pose, in which compounds \(6a, b, d,\) and \(e\) displayed H-bonds between N2 and Cys:919:A (with length ranging from 1.71 to 2.09 Å), as well as between NH and Asp:1046:A (with length ranging from 1.89 to 2.38 Å) in the DFG domain, while compounds \(6c, f, g,\) and \(h\) possessed an additional H-bond, arising from the interaction between furoxan-\(N\)-oxide and Lys:868:A (length ranging from 2.24 to 2.41 Å); Figure S74A (from the Supplementary Material File).

2. The second set, \(14a-c\), with C6 appending and pyrimidine amide, showed H-bonds between the carbonyl group at C4 and Cys:919:A (length ranging from 1.61 to 2.23 Å), as well as H-bonds between NH and Asp:1046:A (bond length 2.04 Å), Glu:885:A (bond length 2.23 Å), and Asp:1046:A (bond length 1.42 Å) in the DFG region, while N5-H and furoxan-2-oxide in compound \(14c\) displayed two additional hydrogen bonds, with Glu:917:A (bond length 2.27 Å) and Lys:868:A (length 2.15 Å); Figure S74B (from the Supplementary Material File).

3. The third set, \(12a-c\), with trifluoromethyl at C6 and appended C4, displayed different modes of interactions, in which compounds \(12a\) and \(12c\) stacked well together (but did not overlie sorafenib), showing two H-bonds coming from furoxan-2-oxide and NH with Lys:919:A (bond lengths 2.18 and 1.95 Å, respectively) and Glu:885:A (bond lengths 2.12 and 1.39 Å, respectively); Figure S74C (from the Supplementary Material File).

Overall, the results recommended extensions or modifications for the abovementioned compounds on the central aryl ring, which projects towards the protein cavity.

The most active compound against NCI-60 cell lines, \(12b\), was selected as the most plausible binding conformation and, thus, we explored its binding mode within the binding pocket. Compound \(12b\) displayed a unique binding mode that could underlie its broad-spectrum anticancer activities. As shown in Figure 13B, in spite of not displaying a strong H-bond, \(12b\) stacked well with sorafenib and tightly fit into the protein pocket without any projection to the protein cavity. The depicted 2D interaction of the protein with \(12b\) (see Figure 13C,D), generated by Discovery Studio Visualizer \cite{67,68}, showed two weak hydrogen bonds, coming from the NH group (HBD) on the furoxan methylamino linker and Glu:885:A (HBA, bond length 4.79 Å), as confirmed by the 2D depiction of the protein–ligand interaction using the OpenEye docking report (Figure S75; from the Supplementary Material), as well as between fluorine and Cys:919:A (HBD). Furthermore, there were hydrophobic interactions with the contacted residues. The central aryl ring, pyrazole, and 1-phenyl formed pi–cation electrostatic, pi–pi T-shaped, and pi–pi stacking hydrophobic interactions, respectively. Additionally, \(12b\) formed Van der Waals and other mixed hydrophobic interactions. The docking report of OpenEye revealed that the furoxan-\(N\)-oxide formed a hydrogen bond (3%) with Lys:868:A (HBD), as shown in Figure S75 (from the Supplementary Material File). In addition, the FRED docking report showed an additional property relating to the guidance of the optimization of compound \(12b\), displaying shape, hydrogen bonding interaction, and protein and ligand desolvation energies, identified through the FRED Chemgauss4 score. Compound \(12b\) had a FRED chemgauss4 score of \(-11.37\) (where that of sorafenib was \(-18.58\)). Thus, either the hydrogen bonds or hydrophobic interactions of \(12b\) were weaker than those of sorafenib. Simultaneously, as a whole molecule, \(12b\) spatially stacked well with the co-crystallized ligand and tightly fit into the pocket of VEGFR-2; this provided a reasonable explanation as to why compound \(12b\) had better anticancer activity among the tested analogs, as confirmed by the NCI-60 cell line screening and its molecular mechanisms.

2.4. ADME/Toxicity Analysis

Prediction of the physicochemical, pharmacokinetic, and drug-likeness properties of compound \(12b\) was achieved using the web-based tool ADMETlab 2.0 \cite{69} which offers a straightforward approach to the comprehensive, accurate, and efficient prediction of ADMET properties using a high-quality database of 250,000 entries covering 53 endpoints and a multi-task graph attention framework \cite{70}. As shown in the radar graph in Figure S76 (from the Supplementary Material File), compound \(12b\) displayed physico-
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were reported as s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), or br (broad) and the J-coupling constants were in Hz. High-resolution mass spectra (HRESI-MS) were acquired on a Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) linked to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and pump). Thin-layer chromatography (TLC) was performed for reaction monitoring on pre-coated silica gel 60 F254 (Merck) sheets and visualized by ultraviolet light (254 nm). Purification of synthesized compounds was achieved via either column chromatography using 150–250 µm silica gel or recrystallization from an appropriate solvent. Solvents and reagents were acquired from commercially available suppliers.

Compounds 2a–h [42], 7a–c [42], 8a [71], 10 [21], and 13a–c [49,72] were prepared as reported.

3.1.1. General Procedure for Preparation of Allyloxy Derivatives (3a–d)

To a stirred solution of phenolic derivatives 2a–d (0.002 mol) in absolute ethanol (15 mL), allyl bromide (1.209 g, 0.010 mol) and sodium hydroxide (0.080 g, 0.002 mol) were added. After 15 min of reflux, another equivalent of sodium hydroxide was added, and the reflux was continued for another 30 min (TLC monitoring). After cooling, the precipitated solid was filtered, washed with ethanol, dried, and recrystallized from acetonitrile to yield the allyloxy derivatives 3a–d [43–45].

N-(4-(allyloxy)phenyl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (3a)

White powder; 65% yield; 1H NMR (400 MHz, DMSO-d6, δ = ppm) δ = 10.11 (s, 1H, NH), 8.47 (s, 2H, 3-H, 6-H), 8.21 (d, J = 8.0 Hz, 2H, H-Ar), 7.69 (d, J = 8.0 Hz, 2H, H-Ar), 7.56 (t, J = 8.0 Hz, H-Ar), 7.36 (t, J = 8.0 Hz, 1H, H-Ar), 7.02 (d, J = 8.0 Hz, 2H, H-Ar), 6.12–6.02 (m, 1H, OCH2CH=CH2), 5.42 (dd, J = 17.8, 2.2 Hz, 1H, OCH2CH=CH2), 5.27 (dd, J = 10.4, 2.2 Hz, 1H, OCH2CH=CH2), 4.58 (d, J = 4.0 Hz, 2H, OCH2). HRESI-MS m/z calcld for [M+H]+: C20H17N3O: 344.1506; found: 344.1497.
N-(4-(allyloxy)phenyl)-6-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (3b)

White powder; 70% yield; 1H NMR (400 MHz, DMSO-d6, δ = ppm) δ = 9.96 (s, 1H, NH), 8.45 (s, 1H, 3-H), 8.20 (d, J = 8.0 Hz, 2H, H-Ar), 7.71 (s, 2H, H-Ar), 7.55 (t, J = 8.0 Hz, 2H, H-Ar), 7.34 (t, J = 8.0 Hz, 1H, H-Ar), 7.01 (d, J = 8.0 Hz, 2H, H-Ar), 6.11–6.02 (m, 1H, OCH2CH=CH2), 5.42 (dd, J = 17.3, 1.7 Hz, 1H, OCH2CH=CH2), 5.27 (dd, J = 10.6, 1.8 Hz, 1H, OCH2CH=CH2), 4.58 (d, J = 5.4 Hz, 2H, OCH2), 2.75 (s, 3H, CH3). HRESI-MS m/z calcd for [M+H]+: C21H15N3O: 358.1662; found: 368.1655.

N-(4-(allyloxy)phenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (3c)

White powder; 78% yield; 1H NMR (400 MHz, DMSO-d6, δ = ppm) δ = 8.73 (s, 1H, NH), 8.36 (s, 1H, 6-H), 8.18 (d, J = 8.0 Hz, 2H, H-Ar), 7.55–7.50 (m, 4H, H-Ar), 7.32 (t, J = 8.0 Hz, 1H, H-Ar), 6.99 (d, J = 8.0 Hz, 2H, H-Ar), 6.11–6.01 (m, 1H, OCH2CH=CH2), 5.41 (dd, J = 17.4, 1.8 Hz, 1H, OCH2CH=CH2), 5.27 (dd, J = 10.6, 1.8 Hz, 1H, OCH2CH=CH2), 4.58 (d, J = 5.4 Hz, 2H, OCH2), 2.75 (s, 3H, CH3). 13C NMR (101 MHz, DMSO-d6) δ 14.77 (3-C, 133.83 (OCH2CH=CH2), 138.93 (C-Ar), 142.41 (C-Ar), 154.09 (C3), 155.27 (C7a), 155.85 (C4), 156.02 (C6).

3.1.2. General Procedure for Preparation of Nitrated Allyloxy Derivatives (5a–d)

To a flask charged with allyloxy derivatives 3a–d (0.015 mol) in glacial acetic acid (3 mL), saturated aqueous NaNO2 (0.045 mol) solution was added dropwise so that the temperature did not exceed 70 °C [46]. After 1 h, the mixture was diluted with water and extracted with EtOAc. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was then purified by column chromatography using hexane/ethyl acetate (8:2) as eluent to yield the nitrated derivatives 5a–d.

N-(4-(allyloxy)-3-nitrophenyl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (5a)

Orange crystals; 55% yield; 1H NMR (400 MHz, DMSO-d6, δ = ppm) δ = 10.50 (s, 1H, NH), 8.42 (s, 1H, 6-H), 8.34 (s, H, 3-H), 8.18 (d, J = 8.0 Hz, 2H, H-Ar), 7.64–7.55 (m, 4H, H-Ar), 7.42–7.35 (m, 2H, H-Ar), 6.12–6.02 (m, 1H, OCH2CH=CH2), 5.45 (dd, J = 17.3, 1.6 Hz, 1H, OCH2CH=CH2), 5.32 (dd, J = 10.5, 1.4 Hz, 1H, OCH2CH=CH2), 4.72 (d, J = 5.2 Hz, 2H, OCH2). 13C NMR (101 MHz, DMSO-d6) δ 69.09 (OCH2), 102.20 (C3a), 110.42 (C-Ar), 118.10 (OCH2CH=CH2), 120.85 (C-Ar), 120.91 (C-Ar), 124.69 (C-Ar), 129.22 (C-Ar), 132.99 (C-Ar), 133.59 (OCH2CH=CH2), 138.62 (C-Ar), 145.08 (C3), 153.14 (C7a), 155.81 (C4), 155.90 (C6). HRESI-MS m/z calcd for [M+H]+: C20H15N6O6: 389.1357; found: 389.1347.

N-(4-(allyloxy)-3-nitrophenyl)-6-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (5b)

Orange crystals; 60% yield; 1H NMR (400 MHz, DMSO-d6, δ = ppm) δ = 10.48 (s, 1H, NH), 8.32 (s, 1H, 3-H), 8.18 (d, J = 8.0 Hz, 2H, H-Ar), 7.58–7.53 (m, 4H, H-Ar), 7.40–7.32 (m, 2H, H-Ar), 7.34 (t, J = 8.0 Hz, 1H, H-Ar), 7.01 (d, J = 8.0 Hz, 2H, H-Ar), 6.11–6.03 (m, 1H, OCH2CH=CH2), 5.45 (dd, J = 17.3, 1.5 Hz, 1H, OCH2CH=CH2), 5.32 (dd, J = 10.5, 1.4 Hz, 1H, OCH2CH=CH2), 4.71 (d, J = 5.3 Hz, 2H, OCH2), 2.39 (s, 3H, CH3). 13C NMR
(101 MHz, DMSO-d$_6$) $\delta$ 25.79 (6-CH$_3$), 69.07 (OCH$_2$), 100.31 (C3a), 110.30 (C-Ar), 118.06 (OCH$_3$CH=CH$_2$), 120.63 (C-Ar), 120.84 (C-Ar), 126.26 (C-Ar), 128.40 (C-Ar), 129.15 (C-Ar), 133.04 (C-Ar), 133.45 (OCH$_2$CH=CH$_2$), 138.81 (C-Ar), 144.88 (C-Ar), 153.98 (C3), 154.26 (C7a), 155.53 (C4), 165.08 (C6); HRESI-MS m/z calcd for [M+H]$^+$: C$_{21}$H$_{18}$N$_6$O$_3$: 403.1513; found: 403.1503.

N-(4-(allyloxy)-3-nitrophenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (5c)

Orange crystals; 58% yield; $^1$H NMR (400 MHz, DMSO-d$_6$, $\delta$ = ppm) $\delta$ = 9.59 (s, 1H, NH), 8.40 (s, 1H, 6-H), 8.20–8.15 (m, 3H, H-Ar), 7.67–7.32 (m, 5H, H-Ar), 6.07–6.04 (m, 1H, OCH$_2$CH=CH$_2$), 5.44 (d, $J$ = 7.4 Hz, 1H, OCH$_2$CH=CH$_2$), 5.31 (d, $J$ = 10.6 Hz, 1H, OCH$_2$CH=CH$_2$), 4.71 (d, $J$ = 5.4 Hz, 2H, OCH$_2$), 2.79 (s, 3H, CH$_3$). HRESI-MS m/z calcd for [M+H]$^+$: C$_{21}$H$_{18}$N$_6$O$_3$: 403.1513; found: 403.1502.

N-(4-(allyloxy)-3-nitrophenyl)-3,6-dimethyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (5d)

Orange crystals; 53% yield; $^1$H NMR (400 MHz, DMSO-d$_6$, $\delta$ = ppm) $\delta$ = 9.59 (s, 1H, NH), 8.28–8.14 (m, 3H, H-Ar), 7.62–7.28 (m, 5H, H-Ar), 6.07–6.02 (m, 1H, OCH$_2$CH=CH$_2$), 5.43 (d, $J$ = 17.3 Hz, 1H, OCH$_2$CH=CH$_2$), 5.30 (d, $J$ = 10.6 Hz, 1H, OCH$_2$CH=CH$_2$), 4.67 (d, $J$ = 4.5 Hz, 2H, OCH$_2$), 2.72 (s, 3H, 3-CH$_3$), 2.44 (s, 3H, 6-CH$_3$). HRESI-MS m/z calcd for [M+H]$^+$: C$_{22}$H$_{20}$N$_6$O$_3$: 417.1670; found: 417.1659.

3.1.3. General Procedure for Preparation of Target Compounds 6a–h

To a flask charged with phenolic derivatives 2a–h (0.002 mol) in THF (15 mL), furoxan mesylate 10 (0.540 g, 0.002 mol) and NaOH (0.080 g, 0.002 mol) were added and the mixture was heated at reflux for 2 h (TLC monitoring) [21]. Then, the mixture was cooled to room temperature, diluted with water, and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was then purified by column chromatography using EtOAc:Hex (1:4) as eluent to provide the target compounds 6a–h.

4-phenyl-3-((4-((1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)methyl)-1,2,5-oxadiazole 2-oxide (6a)

White powder; 62% yield; $^1$H NMR (400 MHz, DMSO-d$_6$, $\delta$ = 10.21 (s, 1H, NH), 8.55 (s, 1H, 3-H), 8.51 (s, 1H, 6-H), 8.22 (d, $J$ = 8.0 Hz, 2H, H-Ar), 7.88–7.86 (m, 2H, H-Ar), 7.76 (d, $J$ = 7.6 Hz, 2H, H-Ar), 7.69–7.62 (m, 3H, H-Ar), 7.57 (t, $J$ = 8.0 Hz, 2H, H-Ar), 7.38 (t, $J$ = 7.4 Hz, 1H, H-Ar), 7.08 (d, $J$ = 8.0 Hz, 2H, H-Ar), 5.25 (s, 2H, OCH$_2$); $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 157.71 (C-Fu), 156.66 (C6), 153.53 (C4), 139.23 (C7a), 134.20 (C3), 133.66 (C-Ar), 132.07 (C-Ar), 129.97 (C-Ar), 129.70 (C-Ar), 128.08 (C-Ar), 126.24 (C-Fu), 121.26 (C-Ar), 115.84 (C-Ar), 113.18 (C3a), 59.38 (OCH$_2$); DEPT135 $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 156.66, 134.19, 132.07, 129.97, 129.70, 128.08, 126.84, 121.26, 115.84, 59.38 (inverted); Anal. Calcd. for C$_{26}$H$_{19}$N$_7$O$_3$ (477.48): C, 65.40; H, 4.01; N, 20.53; Found: C, 65.57; H, 4.24; N, 20.49.

3-((4-((6-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)methyl)-1-phenyl-1,2,5-oxadiazole 2-oxide (6b)

White powder; 66% yield; $^1$H NMR (400 MHz, DMSO-d$_6$, $\delta$ = 10.05 (s, 1H, NH), 8.43 (s, 1H, 3-H), 8.22 (d, $J$ = 8.0 Hz, 2H, H-Ar), 7.91–7.83 (m, 2H, H-Ar), 7.69–7.60 (m, 3H, H-Ar), 7.57 (t, $J$ = 7.4 Hz, 1H, H-Ar), 7.35 (t, $J$ = 7.4 Hz, 1H, H-Ar), 7.07 (d, $J$ = 8.0 Hz, 2H, H-Ar), 5.24 (s, 2H, OCH$_2$), 2.55 (s, 3H, CH$_3$); $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 165.97 (C6), 157.70 (C-Fu), 154.67 (C4), 139.43 (C7a), 134.03 (C3), 132.04 (C-Ar), 129.95 (C-Ar), 129.61 (C-Ar), 128.07 (C-Ar), 126.59 (C-Ar), 126.24 (C-Fu), 121.21 (C-Ar), 115.83
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(C-Ar), 113.16 (C3a), 59.37 (OCH2), 26.81 (6-CH3); Anal. Calcd. for C27H21N7O3 (491.51): C, 65.98; H, 4.31; N, 19.95; Found: C, 65.76; H, 4.59; N, 20.21.

3-(((3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (6c)

White powder; 58% yield; 1H NMR (400 MHz, DMSO-d6) δ 8.95 (s, 1H, NH), 8.39 (s, 1H, 6-H), 8.18 (d, J = 8.0 Hz, 2H, H-Ar), 7.85 (d, J = 5.0 Hz, 2H, H-Ar), 7.67–7.55 (m, 7H, H-Ar), 7.34 (s, 1H, H-Ar), 7.07 (d, J = 8.0 Hz, 2H, H-Ar), 5.26 (s, 2H, OCH2), 2.77 (s, 3H, CH3); 13C NMR (101 MHz, DMSO-d6) δ 157.70 (C-Fu), 156.13 (C6), 155.99 (C4), 154.43 (C7a), 143.01 (C3), 139.10 (C-Ar), 133.07 (C-Ar), 132.08 (C-Ar), 129.97 (C-Ar), 129.61 (C-Ar), 128.07 (C-Ar), 126.44 (C-Ar), 126.24 (C-Fu), 126.17 (C-Ar), 121.06 (C-Ar), 115.51 (C-Ar), 113.18 (C-Ar), 101.65 (C3a), 59.33 (OCH2), 15.28 (3-CH3); Anal. Calcd. for C27H21N7O3 (491.51): C, 65.98; H, 4.31; N, 19.95; Found: C, 65.81; H, 4.56; N, 20.14.

3-(((3,6-dimethyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (6d)

White powder; 75% yield; 1H NMR (400 MHz, DMSO-d6) δ 8.61 (s, 1H, NH), 8.20 (d, J = 8.0 Hz, 2H, H-Ar), 7.87 (d, J = 8.0 Hz, 2H, H-Ar), 7.67–7.65 (m, 5H, H-Ar), 7.53 (t, J = 7.6 Hz, 2H, H-Ar), 7.31 (t, J = 7.1 Hz, 1H, H-Ar), 7.05 (d, J = 8.0 Hz, 2H, H-Ar), 5.25 (s, 2H, OCH2), 2.72 (s, 3H, C3-CH3), 2.48 (s, 3H, C6-CH3); 13C NMR (101 MHz, DMSO-d6) δ 165.72 (C6), 157.70 (C-Fu), 155.69 (C4), 155.67 (C7a), 153.97 (C-Ar), 142.62 (C3), 139.36 (C-Ar), 133.63 (C-Ar), 132.06 (C-Ar), 129.96 (C-Ar), 129.53 (C-Ar), 128.07 (C-Ar), 126.24 (C-Fu), 126.12 (C-Ar), 125.29 (C-Ar), 120.92 (C-Ar), 114.25 (C-Ar), 113.18 (C-Ar), 99.82 (C3a), 59.32 (OCH2), 26.70 (6-CH3), 15.25 (3-CH3); Anal. Calcd. for C28H23N7O3 (505.54): C, 66.52; H, 4.59; N, 19.39; Found: C, 66.34; H, 4.70; N, 19.61.

3-(((1-((4-bromophenyl))-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (6e)

White powder; 71% yield; 1H NMR (400 MHz, DMSO-d6) δ 10.24 (s, 1H, NH), 8.58 (s, 1H, C3-H), 8.51 (s, 1H, C6-H), 8.24 (d, J = 8.0 Hz, 2H, H-Ar), 7.87 (d, J = 8.0 Hz, 2H, H-Ar), 7.78–7.74 (m, 4H, H-Ar), 7.67–7.64 (m, 3H, H-Ar), 7.08 (d, J = 8.8 Hz, 2H, H-Ar), 5.25 (s, 2H, OCH2); Anal. Calcd. for C26H18BrN7O3 (556.38): C, 56.13; H, 3.26; N, 17.62; Found: C, 56.42; H, 3.48; N, 17.80.

3-(((1-((4-bromophenyl))-6-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (6f)

White powder; 72% yield; 1H NMR (400 MHz, DMSO-d6) δ 10.08 (s, 1H, NH), 8.45 (s, 1H, C3-H), 8.23 (d, J = 8.0 Hz, 2H, H-Ar), 7.87 (dd, J = 7.6, 1.6 Hz, 2H, H-Ar), 7.76 (d, J = 8.9 Hz, 4H, H-Ar), 7.70–7.59 (m, 3H, H-Ar), 7.07 (d, J = 8.0 Hz, 2H, H-Ar), 5.24 (s, 2H, OCH2), 2.55 (s, 3H, C6-CH3); 13C NMR (101 MHz, DMSO-d6) δ 166.19 (C6), 157.70 (C-Fu), 154.81 (C4), 138.73 (C7a), 134.50 (C3), 133.89 (C-Ar), 132.55 (C-Ar), 132.06 (C-Ar), 129.97 (C-Ar), 128.09 (C-Ar), 126.25 (C-Fu), 122.73 (C-Ar), 118.84 (C-Ar), 115.86 (C-Ar), 113.18 (C3a), 59.38 (OCH2), 26.83 (6-CH3); Anal. Calcd. for C25H20BrN7O3 (570.41): C, 56.85; H, 3.53; N, 17.19; Found: C, 56.97; H, 3.72; N, 17.42.

3-(((1-((4-bromophenyl))-3-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenoxy)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (6g)

White powder; 68% yield; 1H NMR (400 MHz, DMSO-d6) δ 8.86 (s, 1H, NH), 8.40 (s, 1H, C6-H), 8.20 (d, J = 8.8 Hz, 2H, H-Ar), 7.87 (d, J = 6.4 Hz, 2H, H-Ar), 7.73 (d, J = 8.8 Hz, 2H, H-Ar), 7.69–7.62 (m, 3H, H-Ar), 7.57 (d, J = 8.8 Hz, 2H, H-Ar), 7.06 (d, J = 8.8 Hz, 2H, H-Ar), 5.26 (s, 2H, OCH2), 2.76 (s, 3H, C3-CH3); 13C NMR (101 MHz, DMSO-d6) δ 157.69
3-[[((1-(4-bromophenyl))-3,6-dimethyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl]amino)phenoxy]methyl]-4-phenyl-1,2,5-oxadiazole 2-oxide (6h).

White powder; 66% yield; 1H NMR (400 MHz, DMSO-d6) δ 8.64 (s, 1H, NH), 8.21 (d, J = 8.9 Hz, 2H, H-Ar), 7.90–7.84 (m, 2H, H-Ar), 7.71 (d, J = 8.9 Hz, 2H, H-Ar), 7.68–7.59 (m, 5H, H-Ar), 7.04 (d, J = 8.9 Hz, 2H, H-Ar), 5.25 (s, 2H, OCH2), 2.71 (s, 3H, C3-CH3), 2.47 (s, 3H, C6-CH3); 13C NMR (101 MHz, DMSO-d6) δ 165.95 (C6), 157.70 (C-Fu), 155.84 (C4), 155.69 (C7a), 154.05 (C-Ar), 143.19 (C3), 138.66 (C-Ar), 133.51 (C-Ar), 132.49 (C-Ar), 129.97 (C-Ar), 128.07 (C-Ar), 126.24 (C-Fu), 125.39 (C-Ar), 122.42 (C-Ar), 115.44 (C-Ar), 113.19 (C-Ar), 99.97 (C3a), 59.32 (OCH2), 26.71 (6-CH3), 15.25 (3-CH3); Anal. Calcd. for C28H22BrN2O3 (584.43): C, 57.54; H, 3.79; N, 16.78; Found: C, 57.69; H, 3.95; N, 16.97.

3.1.4. General Procedure for Preparation of Pyrazolopyrimidinone Derivatives 8b,c

To a suspension of 5-amino-4-cyanopyrazole intermediates 7b,c (0.002 mol) in TFA (6 mL), phosphorus oxychloride (0.4 mL) was added instantly and the mixture was heated at reflux for 2 h [47]. Then, the reaction mixture was cooled to room temperature and added dropwise to ice-cooled water. The solid formed was filtered, washed with water, dried, and recrystallized from n-hexane to yield the 4-chlorinated compounds 8b,c in good yields.

1-(4-bromophenyl)-6-((trifluoromethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (8b)

White powder; 72% yield; 1H NMR (400 MHz, DMSO-d6) δ 13.21 (s, 1H, NH), 8.52 (s, 1H, 3-H), 7.99 (d, J = 6.4 Hz, 2H, H-Ar), 7.83 (d, J = 6.4 Hz, 1H, H-Ar); 13C NMR (101 MHz, DMSO-d6) δ 158.85 (C4), 150.95 (C6), 137.50 (C7a), 136.89 (C3), 132.83 (C-Ar), 123.94 (C-Ar), 120.66 (C-Ar), 119.82 (C-Ar), 117.07 (CF3), 108.43 (C3a).

1-(4-bromophenyl)-3-methyl-6-((trifluoromethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (8c)

White powder; 64% yield; 1H NMR (400 MHz, DMSO-d6) δ 13.75 (s, 1H, NH), 7.97 (s, 2H, H-Ar), 7.80 (s, 2H, H-Ar), 2.57 (s, 3H, CH3); 13C NMR (101 MHz, DMSO-d6) δ 159.31 (C4), 151.18 (C6), 146.91 (C7a), 137.47 (C3), 132.76 (C-Ar), 123.59 (C-Ar), 120.18 (C-Ar), 119.71 (C-Ar), 116.96 (CF3), 106.58 (C3a), 13.74 (3-CH3).

3.1.5. General Procedure for Preparation of 4-Chloro Derivatives 9a–c

A flask charged with suspension of pyrazolopyrimidinone derivatives 8a–c (0.002 mol) in POCl3 (16 mL) was heated at reflux for 10 h [48]. Then, the mixture was cooled to room temperature and added dropwise to ice-cooled water. The solid formed was filtered, washed with water, dried, and recrystallized from n-hexane to yield the 4-chlorinated derivatives 9a–c.

4-chloro-3-methyl-1-phenyl-6-((trifluoromethyl)-1H-pyrazolo[3,4-d]pyrimidine (9a)

White powder; 70% yield; 1H NMR (400 MHz, DMSO-d6) δ 8.07 (d, J = 7.7 Hz, 2H, H-Ar), 7.64 (t, J = 7.7 Hz, 2H, H-Ar), 7.47 (t, J = 7.1 Hz, 1H, H-Ar), 2.79 (s, 3H, CH3); 13C NMR (101 MHz, DMSO-d6) δ 156.22 (C6), 153.22 (C4), 144.50 (C7a), 137.70 (C3), 130.08 (C-Ar), 129.85 (C-Ar), 128.16 (C-Ar), 122.10 (C-Ar), 121.96 (CF3), 115.14 (C3a), 14.33 (3-CH3).
1-(4-bromophenyl)-4-chloro-6-(trifluoromethyl)-1H-pyrazolo[3,4-d]pyrimidine (9b)

White powder; 74% yield; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 9.04 (s, 1H, 3-H), 8.09 (d, \(J = 8.9\) Hz, 2H, H-Ar). 37.27 (inverted), 15.20; Anal. Calcd. for C\(_{126.01}\) (129.80 (0.282 g, 0.001 mol), TEA (0.001 mol), and NaI (0.0005 mol) were added, and the mixture was heated under reflux for 2 h (TLC monitoring) [42]. Then, the reaction mixture was cooled to room temperature and extracted with EtOAc, then washed with hot water 3 times or until the disappearance of unreacted \(p\)-phenylenediamine. The organic layer was dried over anhydrous Na\(_2\)SO\(_4\) and concentrated in vacuo. The residue was then purified by column chromatography using EtOAc:Hex (1:1) to give the phenylfuroxan derivative 11 in good yield.

3-(((4-aminophenyl)amino)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (11)

White powder; 89% yield; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 7.86 (dd, \(J = 7.9, 1.4\) Hz, 2H, H-Ar), 7.68–7.54 (m, 3H, H-Ar), 7.69 (d, \(J = 8.6\) Hz, 2H, H-Ar), 6.42 (d, \(J = 8.6\) Hz, 2H, H-Ar). 5.40 (t, \(J = 5.8\) Hz, 1H, NH, exchangeable), 4.42 (s, 2H, NH\(_2\), exchangeable), 4.25 (d, \(J = 5.8\) Hz, 2H, OCH\(_2\)); \(^13\)C NMR (101 MHz, DMSO-\(d_6\)) \(\delta\) 157.83 (C-Fu), 140.82 (C-Ar), 138.66 (C-Ar), 131.70 (C-Ar), 129.73 (C-Ar), 128.19 (C-Ar), 126.70 (C-Ar), 115.80 (C-Ar), 114.95 (C-Ar), 114.61 (C-Ar), 38.42 (NHCH\(_2\)).

3.1.6. General Procedure for Preparation of Phenylfuroxan Derivative

To a flask charged with a solution of furoxan mesylate 10 (0.270 g, 0.001 mol) in THF (10 mL), \(p\)-phenylenediamine (0.642 g, 0.006 mol) and TEA (0.001 mol) were added, and the mixture was heated at reflux for 2 h (TLC monitoring) [21]. Then, the mixture was cooled to room temperature and diluted with water. The isolated solid was recrystallized in isopropanol (10 mL), 3-(((4-aminophenyl)amino)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (12a–c) was isolated in good yield.

3-(((4-aminophenyl)amino)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (12a–c)

To a stirred solution of 6-trifluoromethyl-4-chloro derivatives 9c (0.001 mol) in isopropanol (10 mL), 3-(((4-aminophenyl)amino)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (11) (0.282 g, 0.001 mol), and NaI (0.0005 mol) were added, and the mixture was heated under reflux for 2 h (TLC monitoring) [42]. Then, the reaction mixture was cooled to room temperature and diluted with water. The isolated solid was recrystallized form absolute ethanol to give the target compounds 12a–c in good yields.

3-(((4-(3-methyl-1-phenyl-6-(trifluoromethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenylamino)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (12a)

White powder; 89% yield; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 9.12 (s, 1H, NH), 8.11 (d, \(J = 7.1\) Hz, 2H, H-Ar), 7.85 (d, \(J = 5.7\) Hz, 2H, H-Ar), 7.60 (m, 5H, H-Ar), 7.36 (d, \(J = 7.2\) Hz, 3H, H-Ar), 6.59 (d, \(J = 7.6\) Hz, 2H, H-Ar), 6.38 (s, 1H, NHCH\(_2\)), 4.41 (s, 2H, NHCH\(_2\)), 2.72 (s, 3H, C\(_3\)-CH\(_3\)); \(^13\)C NMR (101 MHz, DMSO-\(d_6\)) \(\delta\) 157.81 (C3a), 153.19 (C7a), 152.84 (C3), 145.57 (C2), 145.36 (C6), 153.83 (C4), 153.19 (C7a), 152.84 (C3), 145.57 (C2), 143.39 (C1), 138.66 (C-Ar), 131.79 (C-Ar), 129.80 (C-Ar), 129.75 (C-Ar), 128.80 (C-Ar), 128.19 (C-Ar), 126.99 (C-Ar), 126.56 (C-Fu), 126.01 (C-Ar), 121.39 (C-Ar), 114.57 (CF\(_3\)), 112.48 (C-Ar), 101.80 (C3a), 37.27 (NHCH\(_2\)), 15.20 (3-CH\(_3\)); DEPT\(^{13}\)C NMR (101 MHz, DMSO-\(d_6\)) \(\delta\) 131.79, 129.80, 129.75, 128.19, 126.99, 126.01, 121.38, 112.48, 37.27 (inverted), 15.20; Anal. Calcd. for C\(_{28}\)H\(_{22}\)F\(_3\)N\(_3\)O\(_2\) (558.53): C, 60.21; H, 3.79; N, 20.06; Found: C, 59.97; H, 3.88; N, 19.89.

3-(((4-(1-(4-bromophenyl)-6-(trifluoromethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenylamino)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (12b)

White powder; 85% yield; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 10.56 (s, 1H, NH), 8.68 (s, 1H, C3-H), 8.13 (d, \(J = 8.3\) Hz, 1H, H-Ar), 8.10–7.99 (m, 1H, H-Ar), 7.83 (dd, \(J = 14.4, 7.5\) Hz, 4H, H-Ar), 7.68–7.57 (m, 3H, H-Ar), 7.53 (d, \(J = 8.2\) Hz, 1H, H-Ar), 7.16 (m, 1H,
7.68–7.63 (m, 2H, H-Ar), 6.83 (s, 1H, NHCH$_2$), 4.44 (s, 2H, NHCH$_2$); Anal. Calcd. for C$_{27}$H$_{37}$Br$_3$F$_3$N$_3$O$_7$ (623.39): C, 52.02; H, 2.91; N, 17.98; Found: C, 52.24; H, 3.14; N, 18.15.

3′-(((4-((1-(4-bromophenyl)-3-methyl-6-(trifluoromethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenyl)amino)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (12c)

White powder; 91% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.13 (s, 1H, NH), 8.08 (d, J = 8.1 Hz, 2H, H-Ar), 7.85 (d, J = 6.1 Hz, 2H, H-Ar), 7.74 (d, J = 8.1 Hz, 2H, H-Ar), 7.62 (d, J = 7.1 Hz, 3H, H-Ar), 7.54 (d, J = 7.8 Hz, 2H, H-Ar), 6.59 (d, J = 7.8 Hz, 2H, H-Ar), 6.38 (s, 1H, NHCH$_2$), 4.40 (d, J = 3.4 Hz, 2H, NHCH$_2$), 2.70 (s, 3H, C$_3$-CH$_3$); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 157.79 (C-Fu), 156.48 (C6), 153.93 (C4), 153.25 (C7a), 152.90 (C3), 145.60 (C6), 143.79 (C-Ar), 137.94 (C-Ar), 132.60 (C-Ar), 131.77 (C-Ar), 127.99 (C-Ar), 127.91 (C-Ar), 126.55 (C-Fu), 126.02 (C-Ar), 122.76 (C-Ar), 121.52 (C-Ar), 119.18 (C-Ar), 118.78 (C-Ar), 114.54 (CF$_3$), 112.46 (C-Ar), 101.94 (C3a), 37.26 (NHCH$_2$), 15.20 (3-CH$_3$); Anal. Calcd. for C$_{28}$H$_{33}$Br$_3$F$_3$N$_3$O$_7$ (637.42): C, 52.76; H, 3.16; N, 17.58; Found: C, 52.93; H, 3.42; N, 17.81.

3.1.8. General Procedure for Preparation of C6 Appending Target Compounds 14a–c

To a stirred solution of 6-chloromethyl derivatives $^{13}$a–c (0.001 mol) in THF (10 mL), phenylfluoroxan derivative $^{11}$ (0.282 g, 0.001 mol), TEA (0.001 mol), and NaI (0.0005 mol) were added, and the reaction mixture was heated under reflux for 2 h (TLC monitoring) [50]. Then, the mixture was cooled to room temperature, diluted with water, and extracted with EtOAc. The organic layer was dried over anhydrous Na$_2$SO$_4$ then concentrated in vacuo to afford the crude product, which was purified by column chromatography using EtOAc:Hex (3:7) as eluent to yield the C6 appended target compounds $^{14}$a–c.

3′-(((4-((4-oxo-1-phenyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)methyl)amino)phenyl)amino)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (14a)

Yellow powder; 63% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.20 (s, 1H, CONH, D$_2$O exchangeable), 8.29 (s, 1H, C3-H), 7.98 (d, J = 7.8 Hz, 2H, H-Ar), 7.81 (d, J = 7.1 Hz, 2H, H-Ar), 7.57–7.47 (m, 5H, H-Ar), 7.36 (t, J = 7.4 Hz, 1H, H-Ar), 6.54 (d, J = 8.7 Hz, 2H, H-Ar), 6.42 (d, J = 8.7 Hz, 2H, H-Ar), 5.55–5.53 (two s, 2H, two NH, D$_2$O exchangeable), 4.26–4.25 (two s, 4H, two CH$_2$)$_2$; $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 160.99 (C4), 158.20 (C6), 157.76 (C-Fu), 152.49 (C7a), 140.56 (C3), 139.54 (C-Ar), 138.78 (C-Ar), 136.41 (C-Ar), 131.65 (C-Ar), 129.66 (C-Ar), 129.62 (C-Ar), 128.14 (C-Ar), 127.28 (C-Ar), 126.66 (C-Fu), 121.81 (C-Ar), 118.47 (C-Ar), 114.74 (C-Ar), 114.33 (C-Ar), 106.58 (C3a), 46.89 (6-CH$_2$), 38.18 (NHCH$_2$).

3′-(((4-((3-methyl-4-oxo-1-phenyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)methyl)amino)phenyl)amino)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (14b)

Yellow powder; 58% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.11 (s, 1H, CONH, D$_2$O exchangeable), 7.97 (d, J = 7.7 Hz, 2H, H-Ar), 7.81 (d, J = 7.0 Hz, 2H, H-Ar), 7.55–7.42 (m, 5H, H-Ar), 7.31 (t, J = 6.9 Hz, 1H, H-Ar), 6.55 (d, J = 8.0 Hz, 2H, H-Ar), 6.44 (d, J = 8.0 Hz, 2H, H-Ar), 5.56–5.52 (two s, 2H, two NH, D$_2$O exchangeable), 4.24 (s, 4H, two CH$_2$)$_2$, 2.51 (s, 3H, CH$_3$)$_3$; $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 160.99 (C4), 158.95 (C6), 157.72 (C-Fu), 152.87 (C7a), 146.31 (C3), 140.53 (C-Ar), 139.56 (C-Ar), 138.80 (C-Ar), 131.62 (C-Ar), 129.64 (C-Ar), 129.63 (C-Ar), 129.49 (C-Ar), 128.11 (C-Ar), 126.76 (C-Fu), 126.63 (C-Ar), 121.46 (C-Ar), 114.82 (C-Ar), 114.77 (C-Ar), 114.35 (C-Ar), 104.68 (C3a), 46.93 (6-CH$_2$), 38.17 (NHCH$_2$), 13.78 (3-CH$_3$).

3′-(((4-(((4-bromophenyl)-3-methyl-4-oxo-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)methyl)amino)phenyl)amino)methyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (14c)

Yellow powder; 69% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.17 (s, 1H, CONH, D$_2$O exchangeable), 7.96 (d, J = 8.8 Hz, 2H, H-Ar), 7.79 (d, J = 7.2 Hz, 2H, H-Ar), 7.64...
3.1.9. General Procedure for Preparation of Des-NO-Releasing Compounds 15a,b

To a stirred solution of 6-trifluoromethyl-4-chloro derivatives 9b,c (0.001 mol) in isopropanol (10 mL), p-phenylenediamine (0.108 g, 0.001 mol), TEA (0.001 mol), and NaI (0.0005 mol) were added and the reaction mixture was heated under reflux for 2 h (TLC monitoring) [42]. Then, the mixture was cooled to room temperature and diluted with water. The formed precipitate was filtered and recrystallized form absolute ethanol to give the des-furoxan derivatives 15a,b in good yields.

N1-(1-(4-bromophenyl)-6-(trifluoromethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)benzene-1,4-diamine (15a)

White powder; 73% yield; 1H NMR (400 MHz, DMSO-d6) δ 10.46 (d, J = 6.6 Hz, 1H, NH), 8.64 (s, 1H, C3-H), 8.11 (d, J = 8.6 Hz, 1H, H-Ar), 8.02 (d, J = 8.5 Hz, 1H, H-Ar), 7.75 (dd, J = 12.8, 8.3 Hz, 2H, H-Ar), 7.43 (d, J = 8.4 Hz, 1H, H-Ar), 7.08 (d, J = 8.1 Hz, 1H, H-Ar), 6.77–6.61 (m, 2H, H-Ar), 5.47 (s, 1H, NH2), 5.14 (s, 1H, NH2); 13C NMR (101 MHz, DMSO-d6) δ 159.48 (C6), 154.83 (C4), 153.26 (C7a), 146.55 (C3), 138.07 (C-Ar), 134.94 (C-Ar), 132.72 (C-Ar), 128.65 (C-Ar), 123.59 (C-Ar), 123.13 (C-Ar), 114.89 (CF3), 114.25 (C-Ar), 103.21 (C3a).

N1-(1-(4-bromophenyl)-3-methyl-6-(trifluoromethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)benzene-1,4-diamine (15b)

White powder; 78% yield; 1H NMR (400 MHz, DMSO-d6) δ 9.39–8.97 (s, 1H, NH), 8.09 (d, J = 8.6 Hz, 2H, H-Ar), 7.75 (d, J = 8.6 Hz, 2H, H-Ar), 7.24 (d, J = 8.2 Hz, 2H, H-Ar), 6.62 (d, J = 8.2 Hz, 2H, H-Ar), 5.18 (s, 2H, NH2), 2.93–2.54 (s, 3H, CH3); 13C NMR (101 MHz, DMSO-d6) δ 154.02 (C6), 153.34 (C4), 153.30 (C7a), 147.27 (C3), 143.88 (C-Ar), 137.97 (C-Ar), 132.65 (C-Ar), 126.44 (C-Ar), 126.20 (C-Ar), 122.89 (C-Ar), 121.54 (C-Ar), 119.21 (C-Ar), 114.02 (CF3), 101.84 (C3a), 15.22 (3-CH3).

3.2. Biological Evaluation

3.2.1. NCI-60 Cell Line Screening

The in vitro NCI-60 cell line screening assay was conducted at the National Cancer Institute (NCI), Bethesda, USA against 59 cancer cell lines representing 9 types of cancers, as reported earlier [51,73]. Briefly, the human cancer cell lines were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine and inoculated into 96-well microtiter plates in 100 µL (5000 to 40,000 cells/well). Then, the plates were incubated at 37 °C, 5% CO2, 95% air, and 100% relative humidity for 24 h. After that, two plates of each cell line were fixed in situ with TCA, to represent cell population at time zero (Tz). The tested compounds were dissolved in DMSO at 400-fold and added so that the final concentration became 10 µM. The plates were then incubated for an additional 48 h followed by the addition of cold TCA to terminate the assay in case of adherent cells. Then, the cells were fixed in situ by cold 50% (v/v) TCA (50 µL; final concentration, 10% TCA) and incubated for 1 h at 4 °C. After discarding the supernatant, the plates were washed 5 times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µL) at 0.4% (w/v) in 1% HOAc was added to each well, and plates were incubated for 10 min. at rt. Then, washing with 1% acetic acid was conducted 5 times to remove the unbound dye followed by air drying. After that, 10 mM trizma base was added to solubilize the bound
stain, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. On the other hand, the methodology for suspension cells was the same except that 50 µL of 80% TCA (final concentration, 16% TCA) was gently added to terminate the assay by fixing settled cells at the bottom of the wells. Percentage growth inhibition (GI%) was calculated as follows:

\[
\text{Growth percentage} = \frac{T_i (\text{test growth in the presence of compound at } 10 \mu M) - T_z (\text{time zero})}{T_z (\text{control growth}) - T_z (\text{time zero})} \times 100
\]

for \( T_i \geq T_z \);

\[
\text{Growth percentage} = \frac{T_i (\text{test growth in the presence of compound at } 10 \mu M) - T_z (\text{time zero})}{T_z (\text{time zero})} \times 100
\]

for \( T_i < T_z \).

where values of 100, 40, 0, −40, and −100 mean no GI, 60% GI, no net G, 40% lethality, and all cells are dead, respectively.

Data are expressed as a one-dose mean graph of growth percentages including the mean, delta, and range.

3.2.2. VEGFRx Kinase Assay

The kinase assay was performed according to BPS Bioscience® kinase assay kit protocols [17,74–77]. Briefly, 5x Kinase Buffer 1, ATP and Poly-(Glu,Tyr 4:1) (10 mg/mL) in case of FLT1 (Cat. No. 78019)/50x PTK substrate in case of KDR (Cat. No. 40325)/MBP (5 mg/mL) in case of FLT3 (Cat. No. 79797) were thawed and the master mixture (25 µL per well: N wells x (5x Kinase Buffer 1 + ATP (500 µM) + substrate + water)) was prepared. The master mixture (25 µL) was added to every well then inhibitor solution of each well, labeled as “Test Inhibitor”, was added. For the “Positive Control” and “Blank”, the same solution without inhibitor (Inhibitor buffer) was added. To the wells designated as “Blank”, 1x Kinase Buffer 1 was added. FLT1/VEGFR2/FLT3 enzyme was thawed on ice and diluted with 1x Kinase Buffer 1 then added to the wells designated “Positive Control” and “Test Inhibitor Control”. The plate was incubated at 30 °C for 45 min then ADP-Glo reagent/Kinase-Glo Max reagent was added to each well. The plate was covered with aluminum foil and incubated at room temperature for another 45 min. The luminescence was measured immediately using the Tecan–spark® reader. A “Blank” value was subtracted from all readings. All samples and controls were tested in duplicate, and IC \( _{50} \)s were represented as ±SD. Sorafenib was used as a reference drug.

3.2.3. Cell Lines and Reagents

All cell lines were purchased from Nawah Scientific Company (Almokattam Mall, Cairo, Egypt) except A2780 and A2780CP, which were a kind gift from Prof. Jan Brabek (BIOCEV, Vestec u Prahy, Czech Republic). Cells were grown in DMEM medium (BioWhitaker™, Walkersville, MD, USA) or RMPI-1640 supplemented with bovine serum albumin (10%, Life Science Group L, Bedford, UK, Cat No: S-001B-BR) and with 100 IU/mL penicillin/streptomycin (100 µg/mL) (Lonza, Walkersville, MD, USA, 17-602E). Doxorubicin (D1515), sorafenib (SML2653), and JS-K (J4137) were obtained from Sigma-Aldrich, solubilized in DMSO, and kept at −20 °C as a stock solution. DAF-FM™ diacetate was purchased from Invitrogen (Cat. No. D-23844, Waltham, MA, USA). The tested compounds were prepared in dimethyl sulfoxide (10 mM stock) (DMSO Cat. No. 20385.02, Serva, Heidelberg, Germany) and stored at −20 °C.

The Initial Screening and Cell Viability by MTT Assay

According to the procedures of Skehan et al. (with minor modifications) [78,79], the cancer or normal cells were seeded in a 96-well plate (100 µL/well). After overnight incubation at 37 °C and 5% CO₂, the cells were incubated with 50 µM of each tested compound or DMSO (0.5% v/v). After 48 h of incubation, MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide) 5 mg/mL Phosphate-Buffered Saline (PBS) was added,
and the plate was incubated for 4 h. After that, acidified sodium dodecyl sulfate (SDS) solution (10% SDS containing 0.01N HCl in 1× PBS) was used to solubilize formazan crystals. The absorbance was measured after 14 h of incubation at λ570–630 nm by a Biotek plate reader (Gen5™ ). To determine the IC50 for the most active compound 12b, the cells were treated with serial dilutions of the 12b compound for 48 h, then the viability was detected using MTT as mentioned above.

Selectivity of 12b towards Cancer Cells

According to the procedures of Bézivin et al. (with minor modifications) [80–82], the selectivity of compound 12b against cancer cell lines was evaluated by seeding human skin fibroblast cells (HSF) at 5 × 10^4 cells/mL in a 96-well plate and incubating them overnight at 37 °C and 5% CO2. On the second day, serial dilution of the compound 12b was added. After 48 h of incubation, the viability of HSF cells was determined by MTT assay as mentioned above and the selectivity index (SI) was calculated according to Equation (1):

Equation (1) Calculation of selectivity index.

\[
SI = \frac{IC_{50\ normal\ cells}}{IC_{50\ cancer\ cell}}
\]

Cell-Cycle Analysis

According to Gray et al. [83], HepG2 cells were seeded in a 6-well plate (2 mL/well) and incubated at 37 °C and 5% CO2 for 12 h. Cell synchronization at the G1 phase was performed by incubating the cells with serum-free medium for 12 h, then cells were treated with 5.5 or 11 µM of 12b. After 48 h of incubation, cells were washed twice with ice-cold 1× PBS and subjected to trypsinization. The detached cells were centrifuged at 500 × g for 5 min at 4 °C. Then, the cell pellet was resuspended in ice-cold 1× PBS and fixed with absolute ethanol. After 2 h of incubation at −20 °C, the fixed cells were washed with ice-cold 1× PBS. The cell pellet was resuspended in propidium (PI)/RNase (BD Biosciences, BDB550825, Franklin Lakes, NJ, USA) and incubated for 30 min at room temperature (RT) in the dark, then the DNA content in each phase was measured by flow cytometry using BD FACS Calibur [84].

Apoptosis Analysis

Briefly, HepG2 cells (10^6 cells/mL) were treated either with 12b (5.5 or 11 µM) or with DMSO for 24 h, and then were collected after trypsinization and washed twice with 1× PBS. The induction of apoptosis was analyzed by staining cells with PE-Annexin-V [85] in the presence of PI and analyzed using the BD FACS Calibur Flow cytometer.

Detection of Nitric Oxide Level by DAF-FM Diacetate

HepG2 cells were collected, and the number was adjusted at 10^6 cells/test, and then they were stained with 5 µM DAF-FM™ diacetate (Invitrogen, Cat. No. D-23844) for 60 min at 37 °C and 5% CO2. After that, the stained cells were washed to remove the excess probe, and then were re-incubated for another 30 min. Following that, the stained cells were treated either with 12b (5.5 or 11 µM) or JS-K as a positive nitric oxide donor or DMSO as a negative control for another 1 h. The fluorescence was detected by flow cytometry using the FITC detection system in a BD flow Cytometer [86–88].

RT-PCR for Gene Expression

HepG2 cells were treated either with DMSO, 5.5 or 11 µM of 12b for 12 h, and then the total RNA was extracted by TRIzol™ Reagent (Invitrogen, Cat. No. 15596018). The RNA concentration and purity were evaluated using a Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer. Four µg RNA was reverse transcribed to cDNA using the RevertAid First Strand cDNA Synthesis kit. The level of N-cadherin,
**E-cadherin, BCL-2, Bax, and GAPDH** was detected using the SensiFAST<sup>™</sup> SYBR<sup>®</sup> High-ROX Kit with the StepOne<sup>™</sup> Real-Time PCR System (Applied Biosystems<sup>™</sup>). The expression level was firstly normalized to the level of GAPDH, then calculated as $2^{-(\Delta \Delta Ct)}$, where $\Delta Ct_{12b} = (Ct - Ct_{GAPDH})$; $\Delta Ct_{DMSO} = (Ct_{DMSO} - Ct_{GAPDH})$; $\Delta Ct_1 = (\Delta Ct_{12b} - \Delta Ct_{DMSO})$. Ct is the cycle threshold. Primers were synthesized by Eurofins Scientific and are listed in Table 4 [58,89].

**Table 4.** Primer sequence for the genes used in RT-PCR.

| Gene    | Primer Sequence 5′-3′ |
|---------|----------------------|
| Bax     | GTT TCA TCC AGG ATC GAG CAG CAT CTT CTT CCA CCA GAT GGT GA |
| BCL-2   | CAG AGA CAG CCA GGA GAA ATC A TCG CCC TGT GGA TGA CTG A |
| E-cadherin | GAG TGC CAA CTG GAC CA T TCA GTA AGT CAC CCA CCT CTA AGG CCA TC |
| N-cadherin | GAG ATC CTA CTG GAC GGT TCG TCT TGG CGA ATG ATC TTA GGA |
| GAPDH   | ACC ACA GTC CAT GCC ATC AC TCC ACC ACC CTG TTG CTG TA |

**Western Blot Analysis**

The cells were seeded (2.5 × 10< sup>5</sup> cells/mL) and treated as described above. After 48 h of incubation with compound 12b, cells were lysed by RIPA buffer containing 1x protease and phosphatase inhibitor cocktail (Thermo Scientific<sup>™</sup>, Waltham, MA, USA). The supernatant was collected after centrifugation at 4 °C and the Pierce BCA Protein Assay Kit (Thermo Scientific<sup>™</sup>, 23225, Waltham, MA, USA) was used for protein concentration detection. Proteins were then separated using SDS-PAGE at 100 V for 45 min. After that, the proteins were transferred to a nitrocellulose membrane. The membrane was subjected to a suitable primary antibody overnight. Then, an appropriate HRP-conjugated secondary antibody was used. GAPDH was used as a loading control. The membrane was developed using WesternBright<sup>®</sup> ECL (Advansta Inc., K-12045-D20, San Jose, CA, USA), and the signal was detected using ChemiDoc XRS+ (1708265, Bio-Rad Laboratories, Hercules, CA, USA). All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and are listed in Table 5 [59].

**Table 5.** Antibodies used in the assay.

| Antibody          | Dilution | Cat. No. |
|-------------------|----------|----------|
| β-actin           | 1:5000   | 4970     |
| P27               | 1:4000   | 2552     |
| P21               | 1:2000   | 2947     |
| pERK              | 1:2000   | 4370     |
| ERK               | 1:2000   | 4695     |
| MMP9              | 1:2000   | 13667    |
| P53               | 1:5000   | 9282     |
| Anti-Rabbit IgG-HRP-linked | 1:5000 | 7074 |

**ELISA Assay**

The p38, MKK3, and JNK assays were performed according to the manufacturer’s ELISA assay protocols [60–62]. Briefly, a 96-well plate was seeded with a cell density of 10,000–40,000 cells/well. Then, 50 µL of compound 12b or control (DMSO) was added to appropriate wells in triplicate. Following this, 50 µL of the antibody cocktail (in case of p38; Cat. No. ab221012, Abcam (Waltham, MA, USA) and MKK3; Cat. No. PEL-MKK3-S189-T, RayBiotech, Peachtree Corners, GA, USA) was added to each well. The plate was then
sealed and incubated for 1–2.5 h at room temperature with gentle shaking. Each well was washed with 1X Wash Buffer PT (4 × 350 µL), and then the plate was inverted and tapped gently against clean paper towels to remove excess liquid. In case of JNK (Cat. No. ab176662, Abcam, Waltham, MA, USA), this washing step was applied before and after adding 1X rabbit anti-phospho-MKK3 antibody, then 100 µL of HRP-conjugated anti-rabbit IgG solution was added and the plate was incubated for 1 h at room temperature with gentle shaking followed by washing (4 × 350 µL). After that, 100 µL of TMB Substrate was added to each well and incubated for 15–30 min in the dark with gentle shaking. Finally, 100 µL of Stop Solution was added to each well and the plate was shaken for 1 min to mix well. The OD was recorded at 450 nm.

3.3. ADME/Toxicity Analysis

The web-based tool ADMETlab 2.0 was used for prediction of the physicochemical, pharmacokinetic, and drug-likeness properties of compound 12b, with respect to the reference drug sorafenib. ADMETlab 2.0 offers a straightforward approach for comprehensive, accurate, and efficient prediction of ADMET properties, using a high-quality database of 250,000 entries covering 53 endpoints, and a multi-task graph attention framework [69,70].

3.4. Docking Studies

Molecular modeling studies were performed using the OpenEye® scientific software (2021.spr) [19,42,90–93]. A library of pyrazolopyrimidines based on 1,2,5-oxadiaole-2-oxide were minimized using the MMFF94 minimization force field. OMEGA® application was used for the generation of multi-conformers and the whole library was aligned against the reference drug sorafenib, according to shape and electrostatic potential, using the EON module. The VEGFR-2 (PDB code: 3WEZ) receptor was prepared using the Spruce® module, after which the FRED® application was used for docking and generating FRED Chemgauss4 scores. Then, the visualization tool, Vida® application, was employed to display the potential binding interactions of the target compounds to the receptor active site. The Discovery Studio Visualization software was used to generate a 2D depiction of the protein–ligand interaction [67,68]. The docking report was generated using the Docking-Report application through the OpenEye Applications 2021.1.1 command line.

4. Conclusions

In this paper, the design, synthesis, and biological evaluation of a new series of pyrazolo[3,4-d]pyrimidines based on a phenylfuroxan scaffold were detailed. One-dose screening of the NCI-60 cell revealed that compounds 12a–c and 14a had the best MGI% among the tested compounds. The target compound 12b, as the most active one, showed better anticancer activity, with IC50 values of 11.5, 11.6, and 13 µM against the HepG-2, A2780CP, and MDA-MB-231 cell lines, respectively, than the reference anticancer drug sorafenib. Furthermore, compound 12b showed VEGFR-2-inhibitory activity comparable to that of sorafenib. The mechanistic study revealed that compound 12b decreased the level of total ERK and its phosphorylated form and led to the down-regulation of the metastatic protein metalloproteinase MMP-9 and the over-expression of the cell-cycle inhibitors p21 and p27. Cell-cycle analysis demonstrated that compound 12b can arrest cells in the subG1 phase and, thus, induce apoptosis. Compound 12b was found to inhibit wound healing in the absence of serum, in comparison to DMSO-treated cells, as shown by a wound-healing assay, while quantitative RT-PCR for E-cadherin and N-cadherin indicated lower expression of neuronal N-cadherin and increased expression of epithelial E-cadherin, demonstrating the ability of compound 12b to suppress metastasis. Furthermore, 12b-treated HepG2 cells expressed a low level of anti-apoptotic BCL-2 and over-expressed pro-apoptotic Bax genes. Intracellular NO measurement indicated that the NO released from 12b was similar to that from the diazeniumdiolate JS-K reference drug, using DAF-FM DA as a fluorescence probe. The docking results indicated that the most active derivative, 12b, overlaid well with sorafenib, while the anticancer activity of the less active analogs could be enhanced through
Supplementary Materials: The following supplementary materials are available online at https://www.mdpi.com/article/10.3390/ph15020246/s1, Figure S1: 1H NMR of 3a, Figure S2: 1H NMR of 3b, Figure S3: 1H NMR of 3c, Figure S4: 13C NMR of 3c, Figure S5: 1H NMR of 3d, Figure S6: 13C NMR of 3d, Figure S7: 1H NMR of 5a, Figure S8: 13C NMR of 5a, Figure S9: 1H NMR of 5b, Figure S10: 13C NMR of 5b, Figure S11: 1H NMR of 5c, Figure S12: 13C NMR of 5d, Figure S13: 1H NMR of 6a, Figure S14: 13C NMR of 6a, Figure S15: DEPT135 13C NMR of 6a, Figure S16: 1H NMR of 6b, Figure S17: 13C NMR of 6b, Figure S18: 1H NMR of 6c, Figure S19: 13C NMR of 6c, Figure S20: 1H NMR of 6d, Figure S21: 13C NMR of 6d, Figure S22: 1H NMR of 6e, Figure S23: 1H NMR of 6f, Figure S24: 13C NMR of 6f, Figure S25: 1H NMR of 6g, Figure S26: 13C NMR of 6g, Figure S27: 1H NMR of 6h, Figure S28: 13C NMR of 6h, Figure S29: 1H NMR of 8b, Figure S30: 13C NMR of 8b, Figure S31: 1H NMR of 8c, Figure S32: 13C NMR of 8c, Figure S33: 1H NMR of 9a, Figure S34: 13C NMR of 9a, Figure S35: 1H NMR of 9b, Figure S36: 1H NMR of 9c, Figure S37: 1H NMR of 11, Figure S38: 1H NMR (D2O) of 11, Figure S39: 13C NMR of 11, Figure S40: 1H NMR of 12a, Figure S41: 13C NMR of 12a, Figure S42: DEPT135 13C NMR of 12a, Figure S43: 1H NMR of 12b, Figure S44: 1H NMR of 12c, Figure S45: 13C NMR of 12c, Figure S46: 1H NMR of 14a, Figure S47: 13C NMR of 14a, Figure S48: 1H NMR of 14b, Figure S49: 13C NMR of 14b, Figure S50: 1H NMR of 14c, Figure S51: 13C NMR of 14c, Figure S52: 1H NMR of 15a, Figure S53: 13C NMR of 15a, Figure S54: 1H NMR of 15b, Figure S55: 13C NMR of 15b, Figure S56: One dose mean graph of 6a, Figure S57: One dose mean graph of 6b, Figure S58: One dose mean graph of 6c, Figure S59: One dose mean graph of 6d, Figure S60: One dose mean graph of 6e, Figure S61: One dose mean graph of 6f, Figure S62: One dose mean graph of 6g, Figure S63: One dose mean graph of 6h, Figure S64: One dose mean graph of 11, Figure S65: One dose mean graph of 12a, Figure S66: One dose mean graph of 12b, Figure S67: One dose mean graph of 12c, Figure S68: One dose mean graph of 13a, Figure S69: One dose mean graph of 13b, Figure S70: One dose mean graph of 13c, Figure S71: One dose mean graph of 14a, Figure S72: One dose mean graph of 14b, Figure S73: One dose mean graph of 14c, Figure S74: Docking poses of some target compounds inside VEGFR-2 active site: (A) Compound 6a; (B) Compound 14a; (C) Compound 12a, Figure S75: FRED docking report of 12b in VEGFR-2, showing fitness in the binding pocket and residues in contact, Figure S76: Radar graph of physicochemical properties of (A) compound 12b and (B) sorafenib, obtained from ADMETlab 2.0, Table S1: NCI-60 cell line growth percentage screening results for target compounds 6a–h and 12a–c, Table S2: NCI-60 cell line growth percentage screening results for compounds 11, 13a–c, and 14a–c, Table S3: ADMET properties of compound 12b, predicted using the web-based ADMETlab 2.0 tool.

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