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The Design and Discovery of Water Soluble 4-Substituted-2,6-dimethylfuro[2,3-*d*]pyrimidines as Multitargeted Receptor Tyrosine Kinase Inhibitors and Microtubule Targeting Antitumor Agents

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Abstract

The design, synthesis and biological evaluations of fourteen 4-substituted 2,6-dimethylfuro[2,3-*d*]pyrimidines are reported. Four compounds (**11–13**, **15**) inhibit vascular endothelial growth factor receptor-2 (VEGFR-2), platelet-derived growth factor receptor β (PDGFR- β), and target tubulin leading to cytotoxicity. Compound **11** has nanomolar potency, comparable to sunitinib and semaxinib, against tumor cell lines overexpressing VEGFR-2 and PDGFR- β . Further, **11** binds at the colchicine site on tubulin, depolymerizes cellular microtubules and inhibits purified tubulin assembly and overcomes both β III-tubulin and P-glycoprotein-mediated drug resistance, and initiates mitotic arrest leading to apoptosis. In vivo, its HCl salt, **21**, reduced tumor size and vascularity in xenograft and allograft murine models and was superior to docetaxel and sunitinib, without overt toxicity. Thus **21** affords potential combination chemotherapy in a single agent.

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Supporting Information Available: Elemental Analyses and High Resolution Mass Spectrum (HRMS) (EI) are available via the internet.

Introduction

Angiogenesis, the formation of new blood vessels, is essential for tumorigenesis and plays an essential role in tumor growth, invasion and metastasis.^{1,2} Solid tumors depend on the newly formed vascular network to provide nutrients and to remove metabolic waste in order to grow beyond a few millimeters in diameter.³

The activation of Receptor Tyrosine Kinases (RTKs) regulates the transduction of signals from the extracellular domain of endothelial cells to the nucleus and represents the most important factor that triggers angiogenesis.⁴

Tubulin binding agents (Figure 1) belong to an important class of antitumor agents and are widely used in the clinic for cancer chemotherapy.⁵⁻⁷ Most microtubule targeting agents can be divided into 3 classes based on their interactions within the taxane, vinca, or colchicine site on tubulin.^{6,7} Drugs that bind within the taxoid site include paclitaxel (Taxol), docetaxel (Taxotere), and the epothilones. Paclitaxel and other taxoids (and the epothilones) bind to β -tubulin in the interior of the microtubule.^{8,9} Unlike the other two classes of antimicrotubule agents, the taxoids stimulate tubulin polymerization and are designated as microtubule stabilizers.^{6,10} They are useful in the treatment of breast, lung, ovarian, head and neck and prostate cancers.^{7,10} The second class of microtubule disrupting agents are the vinca alkaloids and these include vincristine, vinblastine, vindesine and vinorelbine. The vinca alkaloids bind between two $\alpha\beta$ -tubulin dimers at a site that is distinct from the taxane site.⁶ The vinca alkaloids are important in the treatment of leukemias, lymphomas, non-small cell lung cancer and childhood cancers.⁷ A diverse collection of small molecules, including colchicine and the combretastatins, bind to the colchicine site on β -tubulin at its interface with α -tubulin, a site distinct from the vinca site.^{11,12} Similar to the vinca alkaloids, colchicine site agents inhibit tubulin polymerization. Colchicine itself is not used as an antitumor agent but is useful in the treatment of gout and familial Mediterranean fever.¹³ Although there are no clinically approved anticancer agents that bind within the colchicine site, several agents including 2-methoxyestradiol, combretastatin A-4 (CA-4) phosphorylated prodrug combretastatin A-4 phosphate (CA-4P) (fosbretabulin), the combretastatin CA-1P prodrug (OXi4503), BNC105P, ABT-751 and plinabulin (NPT-2358) have been evaluated in clinical trials.^{11,14,15} While CA-4P, CA-1P and others continue to be evaluated in clinical trials,^{11,16} to date no colchicine site agent has advanced to Phase III studies or FDA approval for anticancer indications, demonstrating the need of developing additional colchicine site agents for potential clinical evaluations.¹¹

Tumor angiogenic mechanisms that are vital for tumor growth and metastasis are inhibited by antiangiogenic agents.¹⁷ Antiangiogenic agents that target RTKs, especially the VEGF receptor, have found utility in the treatment of multiple types of cancer,¹⁸ but these agents are usually not cytotoxic and are mainly cytostatic.^{19,20} Combination chemotherapy with antiangiogenic and cytotoxic agents has shown significant promise, and several studies with such combinations are in progress in the clinic.^{19,20} Single agents with both antiangiogenic activities and cytotoxicity could circumvent the pharmacokinetic problems of multiple agents, avoid drug-drug interactions, be used at lower doses to alleviate toxicity, be devoid of overlapping toxicities and delay or prevent tumor cell resistance. In this study, we report

the design and discovery of novel bicyclic furo[2,3-*d*]pyrimidines, some of which possess both RTK and tubulin inhibitory properties along with potent in vivo antitumor activities.

Rationale

The inhibition of RTKs disrupts angiogenesis and provides an approach for the treatment of cancer.^{18, 21} The ATP-binding site of RTKs is an attractive target for small molecule drug design. Structure determination of ATP-RTK complexes have revealed the regions within or close to the binding cleft that are not fully occupied by ATP.^{21, 22} These unoccupied regions show structural diversity between members of the kinase family. The commonality as well as diversity among the ATP-binding sites of kinases has allowed the development of general pharmacophore models for rational drug design.^{22–25} The general model proposed²² consists of an Adenine region which is a hydrophobic binding site for the adenine ring of ATP (Figure 2a) as well as for the heterocyclic scaffold of RTK inhibitors such as quinazolines and pyrimidines. The N1- and N6-amino nitrogen of the adenine ring of ATP are hydrogen bonded to two amino acid residues of the Hinge region. The Sugar binding pocket in the ATP binding site accommodates the sugar moiety of ATP, and the Phosphate binding region binds the triphosphate moiety of ATP. In addition, a Hydrophobic site I extends in the direction of the lone pair of the N7 of ATP and a Hydrophobic site II lies below the Adenine region. Both hydrophobic regions are unoccupied by ATP in the binding site.²²

We had reported previously^{29, 30} that the furo[2,3-*d*]pyrimidine scaffold containing compound **1** (Figure 3) had afforded both VEGFR-2 and PDGFR- β inhibitory activity. In addition, we^{31–33} had also reported that 6-methyl cyclopenta fused pyrimidines **2** demonstrated potent tubulin depolymerization activity along with potent in vitro and in vivo antitumor activity. The remarkable recent success and clinical trials^{19, 20, 34, 35} of RTK inhibitors in general and particularly of VEGFR-2 and/or PDGFR- β inhibitors in combination with antitubulin agents such as paclitaxel prompted our design and development of single agents with both VEGFR2 and/or PDGFR- β inhibition and antitubulin activity.

As an initial study, we elected to structurally engineer tubulin inhibitory activity into our existing RTK inhibitor **1** (Figure 3). Transposing the 5-substitution of the RTK inhibitor **1** on to the 4-NH moiety of **1** and placing a 6-CH₃ group on **1** afforded a hybrid furo[2,3-*d*]pyrimidine scaffold (Figure 3) capable of antitubulin activity structurally akin to **2** (Figure 3) with the ability to access the hydrophobic region 1 on the general pharmacophore for RTKs in Figure 2.

Thus the hybrid molecules **3–15** with the furo[2,3-*d*]pyrimidine scaffold (Figure 4) were designed with potentially both attributes of RTK (VEGFR-2 and/or PDGFR- β) inhibition and antitubulin activity in single molecules. The 2-substitution of **3–15** was designed as a methyl group, which is not present in most other 6–6 or 6–5 pyrimidine fused ring system RTK inhibitors reported³⁶ such as gefitinib,³⁷ erlotinib,³⁸ vandetanib,³⁹ lapatinib,⁴⁰ afatinib,⁴¹ canertinib,⁴² and 6-[4-[(4-ethylpiperazin-1-yl)methyl]phenyl]-*N*-[(1*R*)-1-phenylethyl]-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (AEE788)⁴³ and analogs of linifanib⁴³ (Figure 5).

We³³ have shown that for similarly fused cyclopenta[*d*]pyrimidines, the 2-methyl group affords the most potent activity against tubulin and tumor cells. On the basis of molecular modeling (see below), there is sufficient space in the ATP binding site of RTKs to accommodate a 2-methyl moiety that provides additional hydrophobic binding with the ATP site, compared with 2-desmethyl analogs. Compounds **3–15** also have a 6-methyl substitution, which has a small size and can be accommodated in the phosphate or sugar binding site (Figure 2) of multiple RTKs. Thus compounds **3–15** were designed to fit into the ATP binding site of RTKs exemplified by **11** in Figures 2b and 2c.

Similar to several known ATP competitive RTK inhibitors (Figure 5), gefitinib,³⁷ erlotinib,³⁸ vandetanib,³⁹ lapatinib,⁴⁰ among others,⁴⁴ **3–10** (Figure 4) have a 4-anilino substitution. Different ring anilino substitutions at the 4-position of the pyrimidine A-ring were expected to be involved in RTK binding at the Hydrophobic Region I and to influence inhibition selectivity as well as antitumor activity. Palmer *et al.*⁴⁵ reported that small lipophilic electron-withdrawing groups at the 3-position of the anilino moiety in tricyclic ring systems are beneficial. Bold *et al.*⁴⁶ also reported that small lipophilic electron-withdrawing groups at the 4-position of the anilino moiety are beneficial for VEGFR-2 and PDGFR- β inhibition. Thus, anilines with electron-withdrawing groups at either the 3- or 4-position were incorporated into compounds **6–8**. For comparison, compounds **3** and **10** with a phenyl group and a 4'-methoxy phenyl substitution, respectively, were also included as 4-substitution on the pyrimidine A ring. Compound **4** with a naphthalene substitution was designed to determine the bulk tolerance in Hydrophobic region I among different RTKs. Similar to erlotinib,³⁸ a 3-acetylenyl substitution was included in compound **5**. The indole ring rather than a substituted phenyl ring has been reported to provide potent and selective RTK inhibition.^{25a} Thus in compound **9**, an indole ring was incorporated as the 4-substitution on the pyrimidine A-ring via a nitrogen linker.

Compared to **3–10**, in compounds **11–15**, an additional alkyl group with increasing bulk was designed on the aniline-nitrogen. Based on the general binding mode and molecular modeling (see below), the ATP site has sufficient space to accommodate a methyl group in **11**, while the larger alkyl groups in **12–15** may not be tolerated due to steric hindrance with the hinge region in the ATP site. However, these larger alkyl groups could be accommodated in an alternate vertical binding mode indicated from molecular modeling (see below). The methyl group on the aniline nitrogen in **11** was introduced to restrict free rotation of the 4-position C-N bond as well as the 1'-position C-N bond (Figure 2a) and thus restrict the conformation of the anilino ring. A methyl group on the aniline-nitrogen was therefore expected to force the phenyl ring into the Hydrophobic site I (Figures 2b and 2c) and increase RTK inhibition. We were aware that the replacement of the NH-hydrogen with a methyl group would preclude its hydrogen bond donor ability in the hinge region and might result in a loss of RTK inhibitory activity. In addition to conformational restriction, we^{31–33} recently reported that methylation of the anilino nitrogen in similar 6–5 ring systems afforded potent antitubulin activity. In light of the extensive literature^{19, 20a, 20b, 34, 35} on clinical combination chemotherapy protocols that combine RTK inhibitors (bevacizumab, apatinib, gefitinib, sorafenib, erlotanib, lapatinib, pazopanib, inatinib, crizotinib, sunitinib) with antitubulin agents (paclitaxel, docetaxel, vinorelbine,

ixabepilone and others), it was of paramount importance to determine if N-alkylation of **10**, particularly the N-methyl analog **11**, would possess both anti-RTK and antitubulin activity thus affording dual activity and the potential of combination chemotherapy in a single agent.

Molecular Modeling and Computational Studies

Docking studies were performed for proposed compounds **3** – **15** in the colchicine binding site of tubulin (PDB: 1SA0⁴⁷, 3.58Å) and in VEGFR-2 (PDB: 1YWN²⁷, 1.71Å) and in a homology model of PDGFR-β²⁸ using Molecular Operating Environment (MOE 2010.10)²⁶ (validated by re-docking the crystal structure ligands) using previously reported methods.^{28, 31} Docking studies of the lead and standard compounds (semaxanib for VEGFR-2 and sunitinib for PDGFR-β) were performed using methods previously reported.²⁸

Multiple low-energy conformations (within 1 kcal/mol of the best pose) were obtained on docking **3** – **15** in the colchicine site of tubulin. The docked conformation of **11** (Figure 6, score: –5.95 kcal/mol) was selected as a working model for compounds **3** – **15** based on their similarity to the crystal structure of the bound conformation of DAMA-colchicine (Figure 6, purple) in tubulin. In addition we elected to use **11** to depict the molecular modeling since it turned out to be the most potent dual acting agent of the target compounds (see below). The 4'-OMe phenyl group of **11** overlaps with the triOMe containing A-ring of DAMA-colchicine and interacts with Leuβ246, Alaβ248, Leuβ253, Alaβ314, Ileβ376 and Valβ316 (Figure 6). The 4'-OMe of **11** overlaps with the 3'-OMe group in the A ring of DAMA colchicine. The 4'-OMe of **11** forms a hydrogen bond with Cysβ241 as is observed with the 3'-OMe group of DAMA-colchicine. The N-Me group of **11** occupies a region in space in proximity to the C5 and C6-positions in the B-ring of DAMA colchicine and is involved in hydrophobic interactions with Lysβ252, Alaβ248 and Leuβ246. The furo[2,3-*d*]pyrimidine scaffold of **11** partly overlaps with the C-ring of DAMA-colchicine and forms hydrophobic interactions with Leuβ253, Asnβ256 and Lysβ250. These results provide molecular modeling support for the potential tubulin inhibitory activity for proposed compound **11** and its analogs (**3**–**10** and **12**–**15**) and are in keeping with results from recently published molecular modeling studies at the colchicine site of tubulin.⁴⁸

The key binding interactions seen with **11** were also retained for the best docked poses of analogs **3** – **10** in tubulin. In the docking studies of **12**–**15** in the colchicine site, homologation at the aniline nitrogen was expected to increase hydrophobic interactions with the side chain atoms of Leuβ248 and Metβ259 in the binding pocket and thus maintain potency against tubulin like **11**.

Figure 7a shows the docked pose of **11** (selected as a working example representative of **3** – **15**) in a vertical binding mode in the ATP binding site of a crystal structure of VEGFR-2.²⁷ The furo[2,3-*d*]pyrimidine scaffold of **11** is placed in the adenine binding pocket of the ATP binding site of VEGFR-2. The N1 nitrogen of **11** forms a hydrogen bond with the backbone NH of Cys917 in the Hinge region. Additionally, the scaffold is stabilized by hydrophobic interactions with Leu838 and Leu1033. The 4-N-methyl anilino moiety interacts with the side chain carbon atoms of Ile1042 and Cys1043. The 4'-OMe phenyl side chain of **11** is

oriented towards Hydrophobic pocket I and interacts with Leu838, Gly839 and Val846. The docked score of **11** in the vertical binding mode was -5.42 kcal/mol, similar to that seen with the best docked pose of the standard compound semaxanib (-5.02 kcal/mol) using identical docking parameters.

Figure 7b shows an alternate docked pose of **11** in the horizontal binding mode in the binding site of VEGFR-2. In this lower scoring (-3.50 kcal/mol) pose, the furo[2,3-*d*]pyrimidine scaffold of **11** is oriented like ATP⁴⁹ in the pocket. Compound **11** forms hydrophobic interactions with Leu838, Val846, Ala864, Phe916 and Cys917 (not labeled). The 4-*N*-methyl moiety of **11** is oriented towards the Hinge region and interacts with the side chain carbon atoms of Phe916 and Glu915 (not labeled). The 4'-OMe phenyl side chain binds in Hydrophobic pocket I and interacts with Ile847, Ala864, Val865, Val897, Val912, Val914, Ile1042 and Cys1043. The 6-Me moiety of **11** is oriented towards the Sugar site and forms hydrophobic interactions with the side chain carbon atoms of Leu838. Both these modeling results (Figures 7a and 7b) support the rationale that the designed analogs should possess VEGFR-2 inhibitory potential. Modeling of the N-H analogs (**3** – **10**) indicate that these prefer the horizontal, ATP-like binding mode (Figure 7b) whereas the N-alkylated analogs (**11** – **15**) prefer the vertical mode as depicted for **11** in Figure 7a.

Figure 8a shows the best docked pose of **11** in the putative ATP binding pocket of a PDGFR- β homology model.²⁸ The bound pose of the furo[2,3-*d*]pyrimidine scaffold of **11** occupies the adenine binding pocket of the ATP binding site and is in the vertical binding mode like that in Figure 7a for the docked pose of **11** in VEGFR-2. The N1 nitrogen of **11** forms a hydrogen bond with the backbone NH of Tyr683 in the Hinge region. Additionally, the scaffold is stabilized by hydrophobic interactions with Leu838 and Leu1033. The 4-*N*-methyl aniline moiety interacts with the side chain carbon atoms of Ile1042 and Cys1043. The 4'-OMe phenyl side chain of **11** is oriented towards Hydrophobic pocket I and interacts with Leu838, Gly839 and Val846.

The docked score of **11** in the vertical binding mode was -6.66 kcal/mol, similar to the score of the standard compound sunitinib (-6.38 kcal/mol).

Figure 8b shows the lower scoring (-5.06 kcal/mol) alternate docked pose of **11** (horizontal binding mode) in the homology model of PDGFR- β binding site.²⁸ In this pose, the furo[2,3-*d*]pyrimidine scaffold of **11** interacts with Leu604, Val614, Tyr683, Cys684 and Gly687 (not labeled). The 4-*N*-methyl moiety is oriented towards the Hinge region and forms hydrophobic interactions with Tyr683 and Cys684. The 4'-OMe phenyl side chain binds in Hydrophobic pocket I and interacts with Thr681, Glu682, Tyr683, Leu833, Cys834 and Phe845. The 6-Me moiety of **11** is oriented towards the Sugar site and forms hydrophobic interactions with the side chain carbon atoms of Val614 and Ala848.

Thus, molecular modeling studies in a VEGFR-2 crystal structure (PDB: 1YWN²⁷) and a PDGFR- β homology model²⁸ show that target compounds exemplified by **11** can exist in a docked form in either a horizontal or a vertical pose. This is similar to the vertical and horizontal bound conformations observed for other furo[2,3-*d*]pyrimidines in crystal structures with RTKs.⁵⁰ The energy difference between the best docked horizontal pose of

11 was within 2 kcal/mol of the lowest energy vertical docked pose in both VEGFR-2 crystal structure and the PDGFR- β homology model. The horizontal pose conformationally orients the phenyl ring of the aniline group towards the 5-position of the furo[2,3-*d*]pyrimidine as demonstrated by a ^1H NMR study (see below). The vertical pose, however, orients the methyl group of the aniline nitrogen over the 5-position of the furo[2,3-*d*]pyrimidine of **11**.

As expected, the preference for the binding mode(s) adopted by **3–15** was dependent on the substitution at the aniline nitrogen. Compound **3** (with an N-H) bound predominantly in the horizontal ATP-like binding mode in both VEGFR-2 and PDGFR- β , while both binding modes were seen for the bound poses of compound **11** which has a 4-N-methyl group with a preference for the vertical mode. Further homologation of the 4-N alkyl substitution in **12–15** led to predominant docked binding in the vertical binding mode. This can be explained by two reasons: (a) in the horizontal binding mode, where the 4-N substitution is oriented towards the Hinge region, increase in the size of the substitution leads to steric clashes with the amino acids; and (b) in the vertical binding modes where the N-alkyl substitution is oriented towards Sugar site (Figure 7a, VEGFR-2) or towards the Hydrophobic pocket I (Figure 8a, PDGFR- β), increasing the size of the alkyl substitution was predicted to promote hydrophobic interactions with amino acids in the binding pocket (for example, with Ile1042 and Cys1043 in VEGFR-2 and Val614 in PDGFR- β).

Thus, molecular modeling studies of compounds **3 – 15** (exemplified by **11**) in tubulin, VEGFR-2 and PDGFR- β suggested that these compounds have the potential for both kinase and tubulin inhibitory activity.

Chemistry

The synthesis of the key intermediate, compound **20**, is shown in Scheme 1. A three step reaction, starting from diethyl propargyl malonate **16** was successfully employed in the synthesis of 4-chloro-2,6-dimethylfuro[2,3-*d*]pyrimidine **20** (Scheme 1). The condensation of diethyl propargyl malonate **16** and acetamidine hydrochloride **17** was attempted as a route to pyrimidine **18**. The use of anhydrous MeOH and sodium metal were essential for the cyclization of the pyrimidine ring. Intramolecular cyclization of **18** to the furo[2,3-*d*]pyrimidine **19** was carried out under H_2SO_4 (conc.) at r.t, and provides high yields (87%) of **19**. This procedure is also used to scale up the reaction for the synthesis of gram quantities of **19**, as an intermediate, for the bulk synthesis of **11** and **21** required for preclinical and in vivo studies. During scale up, conversion of **18** to **19** required cooling the reaction to r.t. in an ice water bath.

Chlorination of **19** with POCl_3 afforded the 4-chloro-2,6-dimethylfuro[2,3-*d*]pyrimidine **20**. This key intermediate was reacted with the appropriate anilines (Scheme 2), in *i*PrOH or *n*BuOH at reflux in the presence of a catalytic amount of HCl, to give target compounds **3–11**. Compounds **12–15** were synthesized *via* alkylation of **10** with the appropriate iodoalkane in the presence of NaH (Scheme 2).

Treatment of **11** with HCl (g) in anhydrous ether (Scheme 3) afforded the HCl salt **21** in 96% yield. Several different conditions were attempted to obtain **21**. In the initial attempt, 100 mg of **11** was dissolved in anhydrous ether to obtain a clear solution. After HCl (g) was bubbled through the solution, a white precipitate formed but instantly re-dissolved. A similar phenomenon was observed when the amount of **11** was increased up to 300 mg. An alternate method to obtain **21** involved *i*PrOH as the solvent and conc. HCl as the acid source. However, no precipitate was observed under these conditions. A literature search⁵¹ revealed that HCl (g) reacts with diethyl ether to form an oxonium salt, which can dissolve a small amount but not large amounts of organic salts. Thus, the amount of **11** was increased to 2 g. Using diethyl ether and HCl (g), **21** was obtained in 96% yield. Although the ¹H NMR spectrum did not show a distinguishable peak for the newly formed 1-N⁺H proton, **11** and **21** have distinct melting points (108 °C for **11** and 273 °C for **21**). In addition, elemental analysis confirmed the stoichiometry of the salt as exactly one molecule of HCl per molecule of **21** with an additional 0.3 molecules of H₂O. Salt **21** has excellent water solubility (>1 g/mL) and was prepared as an aqueous solution for in vivo evaluations.

Biological evaluations and discussion

RTK inhibitory activity of compounds **3–15** and **21** was evaluated using human tumor cells known to express high levels of EGFR, VEGFR-1, VEGFR-2 or PDGFR- β using a phosphotyrosine ELISA cytotblot and are listed in Table 1. Compounds known to inhibit a particular RTK were used as positive controls for these assays. The effect of compounds on cell proliferation was measured in A431 cancer cells known to overexpress EGFR. Finally, the effects of selected compounds on blood vessel formation was assessed using the chicken embryo chorioallantoic membrane (CAM) assay, a standard test for angiogenesis and the results are presented in Table 1. In the CAM assay, purified angiogenic growth factors were placed locally on a vascularized membrane of a developing chicken embryo together with compounds of interest. Digitized images of the vasculature were taken 48 h after growth factor administration and the number of vessels per unit area evaluated as a measure of vascular density.

In the cellular assays using cells with high expression of EGFR, all the compounds showed lower potency than the standard erlotinib. Compounds **5** and **7** were the most potent and were about 25-fold less potent than erlotinib. An *i*Pr substitution on the N4-aniline moiety in **14** affords the least potent analog. Against VEGFR-2 expressing cells, all the target compounds showed good to excellent potency comparable to the standard compounds, sunitinib and semaxanib. Among them **11** and **21** were the most active compounds. An N4-alkyl substitution affords the most potent compounds (**11 – 15**). In the PDGFR- β expressing cells, all the tested compounds showed better potency than the standard sunitinib. Compound **5** was the most potent compound in the series and had an IC₅₀ of 16.2 nM. Compounds **11** and **21** were equipotent and were the second most potent compounds in the series against PDGFR- β expressing cells. They were about 4-fold more potent than sunitinib. In the A431 cytotoxicity assay, all of the target compounds were about 10- to 50-fold less potent than cisplatin.

In the CAM angiogenesis inhibitory assay, **11** and **21** were again the most potent compounds of the series showing single-digit μM inhibition of angiogenesis, which is about 2-fold less potent than sunitinib and 9-fold more potent than erlotinib.

Although the anilino nitrogen in the N-methyl compounds **11** and **21** cannot form hydrogen bonds as hydrogen bond donors with RTK at the hinge region, both compounds showed activities in cells expressing various RTKs, comparable to and even slightly better than the other NH analogs. These results validate our design rationale for RTK inhibitory activity of the proposed hybrid compounds.

A ^1H NMR study, in DMSO-d_6 , was carried out to explore the conformation of **10** and **11** (Figure 9). In compound **10**, the σ bonds ($\text{C}_1\text{-N}$ and N-C_4) connecting the phenyl ring and the pyrimidine ring are both freely rotatable, while these bonds were restricted in **11**, where the additional methyl group was introduced on the N4-position. According to the ^1H NMR spectrum, the furo[2,3-*d*]pyrimidine 5-H proton in **11** is more shielded than in **10**, which suggested a nearby diamagnetic anisotropic cone. Due to the bulk of the N-methyl group, the conformation of **11** is restricted and the phenyl ring in **11** has to position itself on top of the 5-H proton (Figure 9), which accounts for the observed shielding effect.

^1H NMR studies of **11** in a DMSO-d_6 solution indicates that the compound exists in a conformation similar to that seen in the horizontal docked poses (Figures 6, 7b and 8b) and not in the higher scoring vertical docked pose predicted for VEGFR-2 and PDGFR- β . Clearly, steric hindrance of the 4-N-substitution with the Hinge region in the docked pose forces the alternate vertical orientation which adopts the higher energy conformation in order for the OMe-phenyl ring to interact with Hydrophobic pocket I. In the docked pose in the tubulin colchicine site (Figure 7a), **11** approximates the conformation suggested by the ^1H NMR and predicted by the energy minimized conformation of **11** by molecular modeling (MOE 2010.10²⁶). The ^1H NMR conformation for **10** is also predicted by the lowest energy conformation for **10** obtained by molecular modeling (MOE 2010.10²⁶).

Compound **11** was initially evaluated in the NCI 60 cell line panel and showed potent GI_{50} s against most of the NCI 60 cancer cell lines (Table 2). A COMPARE analysis⁵² (Table 3) of the NCI 60 cell line data showed tubulin inhibitor vincristine sulfate to have the closest Pearson's correlation coefficient with **11**. Other compounds, such as vinblastine sulfate and maytansine, also tubulin binding agents, were ranked as the next closest correlations. This clearly suggested antitubulin activity and warranted the further evaluation of **11** and the other analogs as potential tubulin binding agents.

Based on the results of the COMPARE correlation, the effects of **11** and **21** on cellular microtubules were evaluated. A-10 cells were treated with a range of concentrations of **11**, **21** and related compounds. Both **11** and **21** caused dose-dependent loss of cellular microtubules, similar to the effects of CA-4 (Figure 5). Using these cells an EC_{50} (concentration required to cause 50% loss of cellular microtubules) (Table 4) was calculated for **11** and other analogs. The EC_{50} value for **11** was 103 nM, whereas CA-4 had an EC_{50} of 7 nM,³³ confirming the COMPARE analysis results and validating our initial design rationale with respect to antitubulin inhibitory activity for the proposed hybrid compounds

(**11** – **13**, **15** and **21**). To our knowledge, **11** and **21** are the first furo[2,3-*d*]pyrimidines reported to have potent dual RTK inhibition and microtubule disrupting activity. Structure–activity analyses of the other members of this series identified that the size of the N-alkyl group determines the microtubule depolymerization potency (Table 4). Compound **11** (EC₅₀ = 103 nM) with an N-methyl group is the most potent tubulin depolymerizer in the series. The ethyl substituted **12** (EC₅₀ = 1010 nM) is about 10-fold less potent than **11**. Larger N-substitutions, including propyl, isopropyl and *n*-butyl groups, resulted in a loss of microtubule depolymerizing activity (Table 4). In addition, an N-H, without an alkyl group (**10**) is also inactive.

Studies were conducted to determine if **11** and **21** could interact directly with purified tubulin and inhibit its polymerization. At a concentration of 10 μM, both **11** and **21** almost completely inhibited the polymerization of tubulin, consistent with the effects seen in cells (Figure 11). These biochemical studies provide support for a direct interaction of the compounds with tubulin. Further studies were conducted to determine the IC₅₀ for inhibition of tubulin polymerization of **11** as compared to CA-4. In this assay, **11** inhibited tubulin assembly about as well as CA-4 (Table 5). The data in Table 5 also shows that **11** binds at the colchicine site on tubulin, since it inhibited [³H]colchicine binding to the protein, although not as potently as CA-4.

Multidrug resistance (MDR) is a major limitation of cancer chemotherapy, and MDR tumors are resistant to several microtubule disrupting agents. Overexpression of P-glycoprotein (Pgp) represents one of the major mechanisms of tumor resistance. Pgp expression has been reported in the clinical setting in a number of tumor types, particularly after patients have received chemotherapy.⁵³ In addition, Pgp expression has also been reported to be a prognostic indicator in certain cancers and is associated with poor response to chemotherapy.^{53, 54} Due to the overwhelming lack of success of Pgp inhibitors in the clinic,⁵⁵ new microtubule targeting agents that are able to overcome Pgp-mediated multidrug resistance would have advantages over the taxoids and vinca alkaloids.^{32, 53, 54} Such agents will fill an unmet need in the clinic for patients that develop resistance due to Pgp expression. The expression of the βIII-isotype of tubulin is additionally involved in resistance to taxoids and vinca alkaloids in multiple tumor types including non-small cell lung,^{36, 56–59} breast,⁶⁰ and ovarian cancers.^{61,62} Stengel *et al.*⁶³ showed that colchicine site agents circumvent βIII-tubulin mediated resistance, which attests to the critical importance of developing new agents that bind to the colchicine site as an alternative to the taxoids for the treatment of refractory cancers.

The ability of this series of compounds to circumvent βIII-tubulin mediated drug resistance expression was assessed in an isogenic HeLa cell line pair.⁶⁴ Each of the compounds were essentially equally potent in the parental and βIII-tubulin expressing cell line and **11** was found to be slightly more potent in the βIII-tubulin expressing cell line leading to a relative resistance (Rr) value of 0.82 (Table 6). Rr was determined by dividing the IC₅₀ of the expressing cell line by the IC₅₀ of the parental cell line. The rest of the compounds in the series including **21** had comparable effects with Rr values ranging from 1–1.4. Paclitaxel, on the other hand, had a Rr value of 8.3 showing that compounds **11** and **21** and their analogs can overcome this mechanism of drug resistance.

Pgp-mediated drug resistance was evaluated using an SK-OV-3 isogenic cell line pair (Table 6).⁶⁴ In this cell line pair the resistance index (Rr) of paclitaxel, a well-known Pgp substrate, is greater than 800 while a Rr value of 1.7 was obtained with **11**, and the others in the series were also able to overcome drug resistance mediated by the expression of Pgp with Rr values of 1.2–2, consistent with the Rr values obtained with other colchicine site agents including CA-4 and 2-methoxyestradiol (2ME2) of 1.5–2.6.^{64, 65} These data suggest that **11** and **21** are not substrates of Pgp and could overcome clinical tumor resistance mediated by Pgp with distinct advantages over other antitubulin agents such as paclitaxel.

The ability of **11** and related compounds to inhibit cancer cell proliferation was evaluated using the sulforhodamine B assay. The IC₅₀ values were calculated from the linear portions of log dose response curves. The ability of the compounds to overcome drug resistance mediated by the expression of β III tubulin or P-glycoprotein was determined by calculating Rr values in two pairs of isogenic cell lines. Paclitaxel was used as a positive control n = 3.

The effect of **21** on multiple breast cancer cell lines including MCF-7 (human ER positive breast cancer cells), MDA-MB-231 and MDA-MB-468 (human triple negative breast cancer cells), MDA-MB-435 cells, whose origins are unclear since they express both mammary and melanoma markers,⁶⁶ and 4T1 (murine triple negative breast cancer cells) was examined because use of a range of cells provides a better idea of how active **21** would be in the treatment of this disease. The data show that **21** was active against MDA-MB-435 and MDA-MB-468 cells, had intermediate activity against the 4T1 cells and had lower potency against MDA-MB-231, MCF-7 and normal HUVEC endothelial cells (Table 7). Importantly, these data show that **21** was as active in the MCF-7 TAX taxoid resistant cells as in MCF-7 cells, suggesting a lack of cross-resistance between **21** and taxoids (Table 7). Additionally, the MDA-MB-231 and MDA-MB 435 cells were much more sensitive to **21** as compared to the EGFR-1 kinase inhibitor erlotinib.

A typical characteristic of microtubule targeting agents is their ability to cause mitotic arrest in cancer cell lines resulting in cell death. The effects of **11** and **21** on the cell cycle distribution were evaluated by flow cytometry. HeLa cells treated with vehicle show a normal cell cycle distribution with most of the cell population in the first peak (G₁) with 2N DNA content, as well as a population in the second peak (G₂/M) with a 4N DNA content (Figure 7A). Cells in S phase with intermediate DNA content were observed between the 2 peaks. Consistent with the effects of CA-4 (Figure 7B), 40 and 50 nM **11** initiated mitotic accumulation as evidenced by the accumulation of cells in the G₂/M phase of the cell cycle (Figure 7C, D). Compound **21** also initiated dose-dependent mitotic accumulation (Figure 7E, F). Similar results were observed in MDA-MB-435 cells (data not shown). The ability of **11** and **21** to cause apoptosis was analyzed by Western blotting, probing for the presence of cleaved PARP. Treatment of MDA-MB-435 cells with 75 nM **11** for 18 h led to a slight increase in cleaved PARP (Fig 7G). A 36 h treatment with **11**, however, led to much higher levels of cleaved PARP, comparable to those seen with a 50 nM treatment of CA-4 (Figure 7G). Similar effects were seen in lysates from cells treated with **21**. These data show that **11** and **21**, like other microtubule targeting agents, cause mitotic arrest of cancer cells and that this culminates in apoptosis.

Poor water solubility is an additional problem associated with several of the currently used antitubulin agents, particularly the taxoids. It is necessary to formulate such drugs in Cremophor or polysorbate, which can cause hypersensitivity reactions and require long administration times.⁶⁷ Thus the development of water soluble microtubule targeted agents is highly desired, and has attracted enormous research effort. The successful conversion of **11** to the water soluble salt form **21** (Scheme 3), and the ability of **21** to inhibit tumor cells in culture warranted the testing of **21** for in vivo efficacy.

The in vivo antitumor activity and toxicity of **21** were determined in a murine xenograft and allograft models. First, the maximal tolerated dose of **21** was determined in mice. This dose of **21** was used in the murine xenograft model to compare its antitumor efficacy to the FDA approved antimicrotubule agent docetaxel and to the VEGFR-2/PDGFR- β (and Flt-3) kinase inhibitor sunitinib. Compound **21** significantly decreased the growth of primary tumors in this model, both compared to carrier-treated mice and as compared to mice treated with docetaxel or sunitinib (Figure 8A). Further, **21** decreased tumor vascular density (as measured by CD31/PECAM-1 immunohistochemical staining) as compared to carrier treated mice, but not as much as the anti-angiogenic agent sunitinib (Figure 8B). Finally, all of the treated animals gained weight during the study, indicating a lack of gross systemic toxicity (Figure 8C).

We next sought to determine the in vivo efficacy of **21** in a relevant triple negative breast cancer model where metastasis can be assessed (Figure 9). Because the MDA-MB-231 human triple negative model requires 10 million cells, Matrigel and immune deficient animals to grow in vivo and because the MDA-MB-468 line only metastasizes to the liver (unlike human tumors), the mouse 4T1 triple negative orthotopic allograft model, where 750 cells were implanted into immune proficient BALB/c mice was used. Compound **21** was as active as docetaxel at reducing primary tumor growth in the 4T1 model (Figure 9A). Additionally, **21** reduced the size (Figure 9D; metastases at arrows) but not the number (Figure 9C) of resulting lung metastases as compared to docetaxel (Figures 9C and 9D). Finally, treatment with **21** resulted in less weight loss than docetaxel, indicating less overall toxicity to the animals (Figure 9B).

Summary

A series of 14 furo[2,3-*d*]pyrimidine compounds were designed and evaluated as multitargeted RTK inhibitors and as possible antimitotic agents. Three of these compounds (**11** – **13**) were discovered to possess dual acting RTK and tubulin inhibitory activities and antitumor activity. Among them, compound **11**, the most potent analog, showed excellent dual RTK (VEGFR-2 and PDGFR- β) and microtubule inhibitory activity. Compound **11** showed antiproliferative activity against the NCI-60 panel of cancer cells at low nanomolar levels and was active in paclitaxel resistant tumor cell lines with β III tubulin and those that overexpress Pgp. In addition **11** showed potent inhibition of tumor cells expressing VEGFR-2 and PDFGR- β , comparable to sunitinib. Compound **11** also causes cell cycle arrest in the G₂/M phase and apoptosis. Compound **11** has additional advantages over clinical antimitotic agents, such as paclitaxel, in that it is easily synthesized and is readily converted to the water soluble salt form. Microtubule depolymerization through binding at

the colchicine site was determined to be the primary mechanism of antitubulin action for **11**. Compounds **11** and its HCl salt **21** are the first in a class of furo[2,3-*d*]pyrimidines that exhibit potent dual antiangiogenic (via RTK inhibition) and antitubulin activities. Compound **21** provided excellent antitumor activity in vivo in two murine models of cancer, reducing tumor size, vascularity and metastases with activity superior to docetaxel and sunitinib, without overt toxicity. Compound **21** is a lead analog for further preclinical development and for the synthesis of single agents as dual RTK and tubulin inhibitors for possible clinical development.

Experimental section

All evaporations were carried out in vacuo with a rotary evaporator. Analytical samples were dried in vacuo (0.2 mmHg) in a CHEM-DRY drying apparatus over P₂O₅ at 55 °C. Melting points were determined on a MEL-TEMP II melting point apparatus with FLUKE 51 K/J electronic thermometer and are uncorrected. Nuclear magnetic resonance spectra for proton (¹H NMR) were recorded on a Bruker WH-300 (300 MHz) or a Bruker 400MHz/52 MM (400 MHz) spectrometer. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as internal standard: s) singlet, d) doublet, t) triplet, q) quartet, m) multiplet, br) broad singlet. The relative integrals of peak areas agreed with those expected for the assigned structures. High-resolution mass spectra (HRMS), using Electron Impact (EI), were recorded on a VG AUTOSPEC (Fisons Instruments) micromass (EBE Geometry) double focusing mass spectrometer. Thin-layer chromatography (TLC) was performed on Whatman Sil G/UV254 silica gel plates with fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Column chromatography was performed on a 230–400 mesh silica gel (Fisher, Somerville, NJ) column. Elemental analyses were performed by Atlantic Microlab, Inc. Norcross, GA. Element compositions were within ±0.4% of the calculated values. Fractional moles of water or organic solvents found in some analytical samples could not be prevented despite 24–48 h of drying in vacuo and were confirmed where possible by their presence in the ¹H NMR spectra. All solvents and chemicals were purchased from Aldrich Chemical Co. or Fisher Scientific and were used as received. Purities of the final compounds **3–9** and **21** were > 95% by elemental analysis and **10** by HPLC.

2-Methyl-6-hydroxy-5-prop-2-yn-1-ylpyrimidin-4(3*H*)-one (**18**)

A mixture of diethyl prop-2-yn-1-ylmalonate **16** (11.9 g, 60 mmol), sodium metal (1.38 g, 60 mmol) and acetamidine hydrochloride **17** (5.68 g, 60 mmol) was heated to reflux in MeOH (100 mL) for 24 h. The suspension was then cooled in an ice-bath to room temperature. The precipitate formed was collected by filtration and dissolved in 40 mL of water. This solution was adjusted to pH 3–4 with 1 N HCl whereupon a thick precipitate formed. The mixture was filtered and the filter cake was washed with a small amount of water followed by acetone and dried over P₂O₅ to afford 4.1 g (42%) of **18** as a white solid; mp >300 °C; *R*_f 0.11 (CHCl₃/MeOH 6:1); ¹H NMR (DMSO-*d*₆) δ 2.23 (s, 3 H), 3.05 (s, 2 H), 3.32 (s, 1 H), 11.92 (s, 2 H).

2,6-Dimethylfuro[2,3-*d*]pyrimidin-4(3*H*)-one (19)

To a 25 mL round flask were added **18** (1.64 g, 10 mmol) and concentrated sulfuric acid (15 mL). The resulting solution was stirred overnight and poured into 100 mL distilled water and extracted by 30 mL chloroform 3 times. The organic layers were pooled and concentrated to afford **19** (1.36 g, 83%) as a yellow powder; mp >300 °C; *R_f* 0.35 (CHCl₃/MeOH 6:1); ¹H NMR (DMSO-*d*₆) δ 2.42 (s, 3 H, CH₃), 2.44 (s, 3 H, CH₃), 6.63 (s, 1 H, CH), 12.50 (s, 1 H, 3-NH exch).

4-Chloro-2,6-dimethylfuro[2,3-*d*]pyrimidine (20)

To a 50 mL flask were added **19** (1.64 g, 1 mmol) and 10 mL POCl₃. The resulting mixture was refluxed for 2 h, and the solvent was removed under reduced pressure to afford a dark residue. The crude mixture was purified by silica gel column chromatography using hexane: EtOAc = 20:1 as the eluent. Fractions containing the product (TLC) were combined and evaporated to afford 1.55 g (85%) **20** as a yellow solid; mp 47.6–48.1 °C; *R_f* 0.26 (Hexane/EtOAc 15:1); ¹H NMR (DMSO-*d*₆) δ 2.48 (s, 3 H), 2.63 (s, 3 H), 6.77 (s, 1 H).

General procedure for the synthesis of 3–11

To a 100-mL round-bottomed flask, flushed with nitrogen, were added **20** (127 mg, 0.7 mmol), the appropriate aniline (1.05 mmol), BuOH (20 mL), and 2–3 drops of conc. HCl. The reaction mixture was heated at reflux with stirring for 2 h until the starting material **20** disappeared (TLC). The reaction solution was allowed to cool to room temperature; the solvent was removed under reduced pressure to dryness, and the residue was purified by column chromatography on silica gel with EtOAc/Hexane (1:10) as the eluent. Fractions containing the product (TLC) were combined and evaporated to afford target compounds.

2,6-Dimethyl-*N*-phenylfuro[2,3-*d*]pyrimidin-4-amine (3)

Using the general procedure described above, compound **3** was obtained as a yellow powder (137 mg, 82%): mp 157.9–159.1 °C; *R_f* 0.16 (EtOAc/Hexane, 1:3); ¹H NMR (CDCl₃) δ 2.32 (s, 3 H), 2.60 (s, 3 H), 5.64 (s, 1 H), 6.88 (s, 1 H, *exch*), 7.24–7.39 (m, 5 H), Anal. C₁₄H₁₃N₃O·0.1H₂O.

2,6-Dimethyl-*N*-(naphthalen-1-yl)furo[2,3-*d*]pyrimidin-4-amine (4)

Using the general procedure described above, compound **4** was obtained as a pale yellow powder (214 mg, 74%): mp 253.6–254.7 °C; *R_f* 0.1 (EtOAc/Hexane, 1:3); ¹H NMR (CDCl₃) δ 2.15 (s, 3 H), 2.60 (s, 3 H), 4.84 (s, 1 H), 7.52 (s, 1 H, *exch*), 7.51–8.07 (m, 7 H), Anal. C₁₈H₁₅N₃O·0.2H₂O.

***N*-(3-ethynylphenyl)-2,6-dimethylfuro[2,3-*d*]pyrimidin-4-amine (5)**

Using the general procedure described above, compound **3** was obtained as a brown solid (213 mg, 81%): mp 108.6–109.7 °C; *R_f* 0.15 (EtOAc/Hexane, 1:3); ¹H NMR (DMSO-*d*₆) δ 2.36 (s, 3 H), 2.50 (s, 3 H), 4.16 (s, 1 H), 6.74 (s, 1 H), 7.13 (d, 1 H, *J* = 7.7 Hz), 7.36 (t, 1 H, *J* = 7.7 Hz), 7.89 (d, 1 H, *J* = 7.7 Hz), 7.98 (s, 1 H), 9.56 (s, 1 H, *exch*), Anal. C₁₆H₁₃N₃O·0.4H₂O.

2,6-Dimethyl-*N*-[4-(trifluoromethyl)phenyl]furo[2,3-*d*]pyrimidin-4-amine (6)

Using the general procedure described above, compound **6** was obtained as a yellow powder (174 mg, 57%): mp 144.9–146.7 °C; R_f 0.16 (EtOAc/Hexane, 1:3); ^1H NMR (DMSO-*d*₆) δ 2.45 (s, 3 H), 2.50 (s, 3 H), 6.82 (s, 1 H), 7.68 (d, 2 H, $J = 7.7$ Hz), 8.08 (d, 1 H, $J = 7.7$ Hz), 9.85 (s, 1 H, *exch*), Anal. C₁₅H₁₂FN₃O.

***N*-(3-chloro-4-fluorophenyl)-2,6-dimethylfuro[2,3-*d*]pyrimidin-4-amine (7)**

Using the general procedure described above, compound **7** was obtained as a pale yellow powder (154 mg, 53%): mp 191.6–193.1 °C; R_f 0.13 (EtOAc/Hexane, 1:3); ^1H NMR (DMSO-*d*₆) δ 2.40 (s, 3 H), 2.46 (s, 3 H), 6.70 (s, 1 H), 7.40 (t, 1 H, $J = 7.6$ Hz), 7.77 (d, 1 H, $J = 7.6$ Hz), 8.19 (d, 1 H, $J = 7.6$ Hz), 9.64 (s, 1 H, *exch*), Anal. C₁₄H₁₁FCIN₃O.

***N*-(4-chlorophenyl)-2,6-dimethylfuro[2,3-*d*]pyrimidin-4-amine (8)**

Using the general procedure described above, compound **8** was obtained as a yellow powder (172 mg, 63%): mp 156.6–157.2 °C; R_f 0.13 (EtOAc/Hexane, 1:3); ^1H NMR (DMSO-*d*₆) δ 2.43 (s, 3 H), 2.50 (s, 3 H), 6.77 (s, 1 H), 7.39 (d, 2 H, $J = 8.4$ Hz), 7.87 (d, 2 H, $J = 8.4$ Hz), 9.74 (s, 1 H, *exch*), Anal. C₁₄H₁₂ClN₃O.

***N*-(1*H*-indol-4-yl)-2,6-dimethylfuro[2,3-*d*]pyrimidin-4-amine (9)**

Using the general procedure described above, compound **9** was obtained as a brown powder (122 mg, 63%): mp 241.0–242.2 °C; R_f 0.16 (EtOAc/Hexane, 1:1); ^1H NMR (CDCl₃) δ 2.20 (s, 3 H), 2.61 (s, 3 H), 5.24 (s, 1 H), 6.49 (t, 1 H), 7.11 (m, 4 H, 1 H *exch*), 7.34 (d, 1 H), 8.41 (s, 1 H, *exch*), Anal. C₁₆H₁₄N₄O·0.2H₂O.

***N*-(4-methoxyphenyl)-2,6-dimethylfuro[2,3-*d*]pyrimidin-4-amine (10)**

Using the general procedure described above, compound **10** was obtained as a white powder (110 mg, 82%): mp 135.2–136.7 °C; R_f 0.28 (EtOAc/Hexane, 1:3); ^1H NMR (DMSO-*d*₆) δ 2.34 (s, 3 H, CH₃), 2.39 (s, 3 H, CH₃), 3.74 (s, 3 H, OCH₃), 6.52 (s, 1 H, CH), 6.92 (d, 2 H, $J = 8.8$ Hz, C₆H₄), 7.61 (d, 2 H, $J = 8.8$ Hz, C₆H₄), 9.34 (s, 1 H, 4-NH *exch*); HRMS C₁₅H₁₆N₃O₂.

***N*-(4-methoxyphenyl)-*N*,2,6-trimethylfuro[2,3-*d*]pyrimidin-4-amine (11)**

Using the general procedure described, above compound **11** was obtained as a white powder (106 mg, 75%): mp 108–109 °C; R_f 0.36 (Hexane/EtOAc 3:1); ^1H NMR (DMSO-*d*₆) δ 2.14 (s, 3 H, CH₃), 2.45 (s, 3 H, CH₃), 3.43 (s, 3 H, NCH₃), 3.81 (s, 3 H, OCH₃), 4.55 (s, 1 H, CH), 7.04 (d, 2 H, $J = 9.2$ Hz, C₆H₄), 7.25 (d, 2 H, $J = 9.2$ Hz, C₆H₄); HRMS C₁₆H₁₈N₃O₂; Anal. C₁₆H₁₇N₃O₂.

General procedure for the synthesis of 12–15

To a stirred suspension of **10** (1 mmol) in 2 ml DMF was added NaH (1.1 mmol) in portions at 0 °C. The resulted mixture was stirred at ambient temperature until there was no further gas release. To the above mixture was added the appropriate alkyl iodide (1 mmol) at ambient temperature. The resulted mixture was stirred at ambient temperature for 4 h and

then poured into 10 ml H₂O to afford a white precipitate, which was collected through filtration and purified by column chromatography to afford the desired compounds **12–15**.

***N*-ethyl-*N*-(4-methoxyphenyl)-2,6-dimethylfuro[2,3-*d*]pyrimidin-4-amine (**12**)**

The above mentioned general procedure was applied to afford **12** as a colorless crystal (246 mg, 83%): mp 87.6–88.7 °C; *R_f* = 0.30 (Hexane/EtOAc 3:1); ¹H NMR (DMSO-*d*₆) 1.13–1.16 (t, 3 H, *J* = 5.6 Hz, NCH₂CH₃), 2.15 (s, 3 H, CH₃), 2.47 (s, 3 H, CH₃), 3.84 (s, 3 H, OCH₃), 3.98–4.01 (q, 2 H, NCH₂CH₃), 4.47 (s, 1 H, 5-CH), 7.07–7.09 (d, 2 H, *J* = 6.8 Hz, C₆H₄), 7.24–7.26 (d, 2 H, *J* = 6.8 Hz, C₆H₄); Anal. C₁₇H₁₉N₃O₂.

***N*-(4-methoxyphenyl)-2,6-dimethyl-*N*-propylfuro[2,3-*d*]pyrimidin-4-amine (**13**)**

The above mentioned general procedure was applied to afford **13** as a light yellow solid (248 mg, 80%): mp 87.2–87.9 °C; *R_f* = 0.30 (Hexane/EtOAc 3:1); ¹H NMR (DMSO-*d*₆) 0.87–0.90 (t, 3 H, *J* = 6.0 Hz, NCH₂CH₂CH₃), 1.57–1.62 (m, 2 H, NCH₂CH₂CH₃), 2.15 (s, 3 H, CH₃), 2.46 (s, 3 H, CH₃), 3.84 (s, 3 H, OCH₃), 3.91–3.94 (t, 2 H, NCH₂CH₂CH₃), 4.46 (s, 1 H, 5-CH), 7.06–7.08 (d, 2 H, *J* = 7.2 Hz, C₆H₄), 7.24–7.26 (d, 2 H, *J* = 7.2 Hz, C₆H₄); Anal. C₁₈H₂₁N₃O₂.

***N*-butyl-*N*-(4-methoxyphenyl)-2,6-dimethylfuro[2,3-*d*]pyrimidin-4-amine (**14**)**

The above mentioned general procedure was applied to afford **14** as an orange crystal (230 mg, 74%): mp 65.2–67.1 °C; *R_f* = 0.30 (Hexane/EtOAc 3:1); ¹H NMR (DMSO-*d*₆) 0.89–0.91 (t, 3 H, *J* = 5.6 Hz, NCH₂CH₂CH₂CH₃), 1.28–1.36 (m, 2 H, NCH₂CH₂CH₂CH₃), 1.52–1.58 (m, 2 H, NCH₂CH₂CH₂CH₃), 2.15 (s, 3 H, CH₃), 2.46 (s, 3 H, CH₃), 3.84 (s, 3 H, OCH₃), 3.97–3.98 (t, 2 H, NCH₂CH₂CH₃), 4.46 (s, 1 H, 5-CH), 7.06–7.08 (d, 2 H, *J* = 7.2 Hz, C₆H₄), 7.24–7.26 (d, 2 H, *J* = 7.2 Hz, C₆H₄); Anal. C₁₉H₂₃N₃O₂.

***N*-(4-methoxyphenyl)-2,6-dimethyl-*N*-(propan-2-yl)furo[2,3-*d*]pyrimidin-4-amine (**15**)**

The above mentioned general procedure was applied to afford **15** as an orange crystal (256 mg, 79%): mp 131.1–132.7 °C; *R_f* = 0.30 (Hexane/EtOAc 3:1); ¹H NMR (DMSO-*d*₆) 1.09–1.11 (d, 6 H, *J* = 6.4 Hz, 2 CH₃), 2.12 (s, 3 H, CH₃), 2.47 (s, 3 H, CH₃), 3.84 (s, 3 H, OCH₃), 4.20 (s, 1 H, 5-CH), 5.37–5.45 (m, 1 H, NCH), 7.08–7.10 (d, 2 H, *J* = 8.8 Hz, C₆H₄), 7.19–7.21 (d, 2 H, *J* = 8.8 Hz, C₆H₄); Anal. C₁₈H₂₁N₃O₂.

***N*-(4-methoxyphenyl)-*N*,2,6-trimethylfuro[2,3-*d*]pyrimidin-4-amine hydrochloride (**21**)**

To a 50 mL flask were added **11** (2.0 g, 7.07 mmol) and anhydrous ether (20 mL). The resulting mixture was stirred to afford a clear solution. Anhydrous HCl gas was bubbled into the solution until there was no further precipitation. The white precipitate was collected through filtration then dried over P₂O₅ to afford 2.16 mg (96%) of **21** as a colorless crystal; mp 287.3–287.7 °C; *R_f* 0.01 (CH₃Cl/MeOH 6:1); ¹H NMR (DMSO-*d*₆) δ 2.14 (s, 3 H, CH₃), 2.46 (s, 3 H, CH₃), 3.44 (s, 3 H, NCH₃), 3.80 (s, 3 H, OCH₃), 4.55 (s, 1 H, CH), 7.04–7.06 (d, 2 H, *J* = 8.0 Hz, 2 CH), 7.26–7.28 (d, 2 H, *J* = 8.0 Hz, 2 CH); Anal. C₁₆H₁₈ClN₃O₂·0.3H₂O.

Molecular modeling

The X-ray crystal structures of tubulin co-crystallized with *N*-deacetyl-*N*-(2-mercaptoacetyl) colchicine (DAMA colchicine), a close structural analogue of colchicine (PDB: 1SA0⁴⁷, 3.58 Å resolution) and VEGFR-2 co-crystallized with a furo[2,3-*d*]pyrimidine inhibitor (PDB: 1YWN,²⁷ 1.71 Å resolution) were obtained from the protein database. The preparation and validation of the PDGFR-β homology model has been previously reported.²⁸

Preparation of receptor and ligands for docking

The crystal structures of tubulin and VEGFR-2 and the homology model for PDGFR-β were imported into MOE 2010.10.²⁶ After addition of hydrogens, the protein was then “prepared” using the LigX function in MOE 2010.10. LigX is a graphical interface and collection of procedures for conducting interactive ligand modification and energy minimization in the active site of a flexible receptor. The procedure was performed with the default settings.

Ligands were built using the molecule builder function in MOE and were energy minimized to its local minima using the MMF94X forcefield to a constant (0.05 kcal/mol). Ligands were docked into the active site of the prepared protein using the docking suite as implemented in MOE 2010.10. The docking was restricted to the active site pocket residues using the Alpha triangle placement method. Refinement of the docked poses was carried out using the Forcefield refinement scheme and scored using the Affinity dG scoring system. Around 30 poses were returned for each compound at the end of each docking run. The docked poses were manually examined in the binding pocket to ensure quality of docking and to confirm absence of steric clashes with the amino acid residues of the binding pocket.

Docking Procedure

Colchicine site on tubulin

Docking studies were performed using the docking suite of Molecular Operating Environment software (MOE 2010.10).²⁶ The A and B subunits of the protein, along with the crystallized ligand, were retained, while the C, D, and E subunits, GTP, GDP, and Mg ions were deleted. After addition of hydrogen atoms, the protein was then “prepared” using the LigX function in MOE as described above.

To validate the utility of MOE 2010.10 for docking ligands into the active site, DAMA colchicine, the native ligand in the crystal structure (PDB: 1SA0),⁴⁷ was built using the molecule builder, energy minimized, and docked into the active site using the above parameters. The best docked pose of DAMA colchicine displayed an rmsd of 0.9451 Å compared with the crystal structure pose of DAMA colchicine. MOE 2010.10 was thus validated for our docking studies. Docking studies were performed for **3–15** and the standard compounds in MOE using the same settings. Poses from the docking experiment were visualized using MOE and CCP4mg.⁶⁸

VEGFR-2 docking

Docking studies were performed using the docking suite of MOE 2010.10. After addition of hydrogen atoms, the protein was then “prepared” using the LigX function in MOE as described above.

To validate the utility of MOE 2010.10 for docking ligands into the active site, the native ligand in the crystal structure (PDB: 1YWN²⁷) was built using the molecule builder, energy minimized using MMFF94x forcefield to a gradient of 0.05 kcal/mol and docked into the active site using the above parameters. The best docked pose of the native ligand displayed an RMSD of 0.6821 Å compared to the crystal structure pose. MOE 2010.10 was thus validated for our docking process.

Docking studies were performed for **3–15** and the standard compounds in MOE using the same settings. Poses from the docking experiment were visualized using MOE and CCP4mg.⁶⁸

Methods

Antibodies

The PY-HRP antibody was from BD Transduction Laboratories (Franklin Lakes, NJ). Antibodies against EGFR, PDGFR- β , and VEGFR-2 were purchased from Cell Signaling Technology (Danvers, MA).

Phosphotyrosine ELISA

Cells used were tumor cell lines naturally expressing high levels of EGFR (A431), VEGFR-2 (U251), and PDGFR- β (SH-SY5Y). Expression levels at the RNA level were derived from the NCI Developmental Therapeutics Program (NCI-DTP) web site public molecular target information. Briefly, cells at 60–75% confluence were placed in serum-free medium for 18 hr to reduce the background of phosphorylation. Cells were always >98% viable by Trypan blue exclusion. Cells were then pretreated for 60 min with a dose-response relation of 10,000–0.17 nM compound followed in $\frac{1}{3}$ Log increments by 100 ng/mL EGF, VEGF, or PDGF-BB for 10 min. The reaction was stopped and cells were permeabilized by quickly removing the media from the cells and adding ice-cold Tris-buffered saline (TBS) containing 0.05% Triton X-100, protease inhibitor cocktail and tyrosine phosphatase inhibitor cocktail. The TBS solution was then removed and cells fixed to the plate for 30 min at 60 °C and further incubation in 70% ethanol for an additional 30 min. Cells were further exposed to block (TBS with 1% BSA) for 1 h, washed, and then a horseradish peroxidase (HRP)-conjugated phosphotyrosine (PY) antibody was added overnight. The antibody was removed, cells were washed again in TBS, exposed to an enhanced luminol ELISA substrate (Pierce Chemical EMD, Rockford, IL) and light emission was measured using a UV products (Upland, CA) BioChemi digital darkroom. Data were graphed as a percent of cells receiving growth factor alone and IC₅₀ values were calculated from two to three separate experiments ($n = 8–24$) using non-linear regression dose-response relation analysis.

Chorioallantoic membrane assay of angiogenesis

The CAM assay is a standard assay for testing antiangiogenic agents. The CAM assay used in these studies was modified from a procedure by Sheu⁶⁹ and Brooks⁷⁰ and as published previously.⁷¹ Briefly, fertile leghorn chicken eggs (CBT Farms, Chestertown, MD) were allowed to grow until 10 days of incubation. The proangiogenic factors, human VEGF-165 and bFGF (100 ng each) were then added at saturation to a 6 mm microbial testing disk (BBL, Cockeysville, MD) and placed onto the CAM by breaking a small hole in the superior surface of the egg. Antiangiogenic compounds were added 8 hr after the VEGF/bFGF at saturation to the same microbial testing disk and embryos allowed to incubate for an additional 40 h. After 48 h, the CAMs were perfused with 2% paraformaldehyde/3% glutaraldehyde containing 0.025% Triton X-100 for 20 sec, excised around the area of treatment, fixed again in 2% paraformaldehyde/3% glutaraldehyde for 30 min, placed on Petri dishes, and a digitized image was taken using a dissecting microscope (Wild M400; Bannockburn, IL) at 7.5X and SPOT enhanced digital imaging system (Diagnostic Instruments, Sterling Heights, MI). A grid was then added to the digital CAM images and the average number of vessels within 5–7 grids counted as a measure of vascularity. Sunitinib and semaxanib were used as a positive controls for antiangiogenic activity. Data were graphed as a percent of CAMs receiving bFGF/VEGF only and IC₅₀ values calculated from two to three separate experiments ($n = 5-11$) using non-linear regression dose-response relation analysis.

Indirect Immunofluorescence

A-10 cells were used to evaluate the effects of the compounds on cellular microtubules using indirect immunofluorescence. Cells were treated for 18 h with compounds and microtubules visualized with an antibody towards β -tubulin (Sigma-Aldrich, St. Louis, MO) Ec₅₀ values were calculated as previously described and represent an average of a minimum of three independent experiments.⁷²

Sulforhodamine B (SRB) Assay

The antiproliferative activity and evaluation of activity in cellular resistance models of all compounds was evaluated using the SRB assay as previously described.⁶⁴ The IC₅₀'s represent an average of 3 independent experiments using triplicates plus or minus the standard deviation.

Cell Cycle Analysis

HeLa cells were plated in 60 mm dishes and allowed to adhere for 24 h. Drug was then added and cells harvested 24 h later. 50 nM CA-4 was used as a positive control. Once cells were harvested they were stained with Krishan's reagent and analyzed for DNA content using a BD LSRII flow cytometer.

In Vitro Tubulin Polymerization

The effects of the compounds on tubulin polymerization were measured using purified porcine brain tubulin (Cytoskeleton Inc.). Briefly, 2.2 mg/mL of purified porcine brain tubulin was incubated with tubulin polymerization buffer (80 mM Na-PIPES, pH 6.9, 1 mM

EGTA, 1 mM MgCl₂, 10 mM GTP and 10% glycerol) and 10 μM of each corresponding drug. The polymerization of tubulin was monitored turbidimetrically by measuring the absorbance at 340 nm at 37°C in a SpectraMax 96-well plate spectrophotometer.

Apoptosis detection

MDA-MB-435 cells were treated with vehicle, 75 nM **11**, 120 nM **21**, or 50 nM CA-4 for 18 or 36 h. Cells were lysed on ice with cell extraction buffer (Life Technologies, FNN0011) containing PMSF and protease inhibitors (Sigma-Aldrich, P2714) added immediately before use. Lysates were kept on ice for 15 min and then centrifuged at 13,000 rpm, 4°C for 10 min. The Bradford protein assay was used to determine protein content and samples containing equal protein content were loaded and resolved using SDS-PAGE. Western blotting was used to detect caspase 3 dependent cleaved PARP (Cell Signaling). Actin was used as a loading control. A Geliance 600 Imaging System was used to image the ECL reaction.

Maximal tolerated dose in mice

To determine maximal tolerated dose (MTD) of compounds and drugs, a dose finding study was performed using BALBc/J mice (Jackson Laboratories, Bar Harbor, ME). Drugs were first dissolved in 50 mg/ml DMSO and frozen in aliquots at -80°C. Solutol-15 (BASF, Ludwigshafen, Germany) was melted at 60°C for 5–10 min then mixed in a ratio of 1 part DMSO/drug to 1.8 parts solutol-15 to 7.2 parts sterile dextrose 5% in water (D₅W). This solvent mixture was used for all drugs. Starting at 10 mg/kg and 15 mg/kg body weight (n = 2 mice per treatment), mice were weighed and doses increased in 10 mg/kg increments every other day until weight loss was observed. At this point the maximal tolerated dose was estimated to be the approximate dose of first weight loss. The MTD of docetaxel was found to be 35 mg/kg, sunitinib 30 mg/kg and compound **21** 50 mg/kg.

MDA-MB-435 flank xenograft model

MDA-MB-435 human BLBC (500,000) in media were implanted into the lateral flank of 8 wk old female NCr athymic nu/nu nude mice (Charles River, Wilmington, DE). Tumor sizes (length, width, depth) were measured twice weekly by Vernier calipers and volumes calculated by the ellipsoid formula $\text{volume} = 0.52(\text{length} \times \text{width} \times \text{depth})$. When tumor volumes reached 75–100 mm³, treatment with drugs at their MTD (above) was begun and animal weights and tumor volumes measured twice weekly. At the experiment end, animals were humanely euthanized using the AALAC approved method of carbon dioxide asphyxiation. Tumors were removed, fixed in neutral buffered formalin, paraffin embedded, sectioned and sections stained against CD31/PECAM-1 using an antibody from Abcam (ab28364) and staining done using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Vessel density was assessed by counting the number of CD31-positive vessels in a 200× microscope field in a blinded fashion and graphed as a percent vessel density of carrier treated animals.

4T1 triple negative breast orthotopic allograft model

4T1-Luc2GFP dual luciferase/GFP tagged cells were purchased from Caliper Life Sciences (Hopkinton, MA) and maintained in Dulbecco's modification of minimal essential media (DMEM) containing 10% Cosmic Calf Serum (Hyclone, Logan, UT). 750 cells (verified by fluorescence imaging to be >98% GFP positive and counted three times on a TC10 automated cell counter (BioRad, Hercules, CA)) in 100 μ L PBS with 1 mM EDTA were implanted subcutaneously into the left fat pad #4 of 8 wk old female BALBc/J mice using a tuberculin syringe. The MTD of drugs were delivered to animals twice weekly on Tuesday and Friday starting three days after implantation. Tumor sizes (length, width, depth) were measured three times weekly by Vernier calipers and volumes calculated by the ellipsoid formula $\text{volume} = 0.52 (\text{length} \times \text{width} \times \text{depth})$. Animal weights were also taken twice weekly. At day 33 post implantation, the experiment was ended due to moribund animals in the untreated group. At the experiment end, animals were humanely euthanized using the AALAC approved method of carbon dioxide asphyxiation. Tumors and lungs were removed and fresh lungs imaged using a LumaScope fluorescent imaging system (Bulldog Bio, Portsmouth, NH) at 25 $\times$ magnification with the number of metastases per lung counted by hand from captured images.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CA-4	combretastatin A-4
VEGFR-2	vascular endothelial growth factor receptor-2
RTKs	Receptor Tyrosine Kinases
2ME2	2-methoxyestradiol
PDGFR-β	platelet-derived growth factor receptor β
CAM	chorioallantoic membrane

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Figure 1.
Structures of microtubule targeting agents.

Figure 2.

- Binding of ATP with RTKs. Representative amino acids from the four binding pockets (Hinge Region, Hydrophobic Pocket I, Hydrophobic Pocket II and the Phosphate Site) in the ATP site from VEGFR-2 and PDGFR- β and potential interactions with ATP are shown. The potential interaction map for ATP was generated using MOE 2010.10²⁶ by superimposition of the docked poses of ATP in VEGFR-2 (PDB: 1YWN²⁷) and a homology model²⁸ of PDGFR- β .
- Proposed horizontal binding mode 1 of 11 with RTKs similar to ATP binding mode.
- Proposed alternate vertical binding mode of 11 with RTKs.

Figure 3.
Design of antitubulin and RTK inhibitory activities in single agents.

Figure 4.
The structures of furo[2,3-*d*]pyrimidine RTK inhibitors 3–15.

Figure 5.
Structures of kinase inhibitors that lack a 2-methyl group.

Figure 6.

Docking of compound 11 in the colchicine site of tubulin: Superimposition of the docked poses of 11 (pink) and DAMA colchicine (purple) in the colchicine site of tubulin at the interface of the α -subunit (magenta) and β -subunit (blue) of tubulin; Pocket amino acids are depicted in green as sticks. PDB: 1SA0.⁴⁷

Figure 7.

Compound 21 induces cell cycle arrest in G₂/M phase and apoptosis similar to CA-4. HeLa cells were treated with vehicle (A), 25 nM CA-4 (B), 40 nM 11 (C), 50 nM 11 (D), 75 nM 21 (E), or 100 nM 21 (F) for 18 h, stained with Krishan's reagent and cell cycle distribution determined by flow cytometry. Whole cell protein lysates were prepared from MDA-MB-435 cells treated for 18 or 36 h with vehicle, 50 nM CA-4, 75 nM 11, or 120 nM 21, and Western blotting performed to determine levels of cleaved PARP (G). Actin was used as a loading control.

a. Docking of 11 in the ATP binding site of VEGFR-2 and PDGFR- β : Docked pose of 11 (pink) in the vertical binding mode in the ATP binding pocket of VEGFR-2 (PDB: 1YWN²⁷). Pocket amino acids are depicted in green as sticks. Amino acids in the pocket that form strongest interactions with 11 are depicted in green as cylinders.

b. Docked poses of 11 (pink) in the ATP-like horizontal binding mode in the putative ATP binding pocket of VEGFR-2 (PDB: 1YWN²⁷). Pocket amino acids are depicted in green as sticks. Amino acids in the pocket that form strongest interactions with 11 are depicted as green cylinders.

A. MDA-MB-435 xenograft growth **B. tumor vascularity** **C. animal weight**

Figure 8.

Treatment with 21 decreased primary tumor growth and tumor vascular density in MDA-MB-435 flank xenograft model. 500,000 MDA-MB-435 cells were implanted into the lateral flank of NCr athymic nu/nu mice and treated with carrier, docetaxel, sunitinib and 21 twice weekly at their MTD starting 2 wk after tumor implantation until the end of the experiment. Data is representative of 6–8 animals. (A) Tumor volume was calculated with the ellipsoid formula: volume = 0.52 (length × width × depth) and graphically represented as days after implantation. (B) Tumor vascular density was examined at the end of the experiment by staining tumor sections with CD31/PECAM-1 antibodies and a Vectastain ABC kit. Vascular density was determined by counting CD31-positive vessels and graphed as percent vessel density of carrier treated animals. (C) Graphical representation of percent change in animal weight as determined by measuring animal weight at the beginning and end of the experiment.

a. Docked poses of 11 (pink) in the vertical binding mode in the putative ATP binding pocket of PDGFR- β homology model.²⁸ Pocket amino acids are depicted in green as sticks. Amino acids in the pocket that form strongest interactions with 11 are depicted as green cylinders.

b. Docked poses of 11 (pink) in the ATP-like horizontal binding mode in the putative ATP binding pocket of a PDGFR- β homology model. Pocket amino acids are depicted in green as sticks. Amino acids in the pocket that form strongest interactions with 11 are depicted as green cylinders.

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Figure 9.

The conformations and ¹H NMRs of compound **10** (A) and **11** (B).

Compound 21 decreased primary tumor growth and lung metastases in 4T1 orthotopic breast model. 750 4T1-Luciferase/GFP tagged cells were implanted orthotopically into BALB/c mice and treated with carrier or docetaxel and 21 at their MTD, docetaxel once weekly and 21 twice weekly starting at day 7 and continuing to day 32. Number of animals in each group = 7–10. (A) Tumor volume was determined by measuring tumor length, width, and depth with Vernier calipers and by using the ellipsoid formula: volume = 0.52 (length × width × depth) twice weekly. (B) Animal weights were recorded before each treatment and graphed as percent weight change. (C) After 32 days, animals were euthanized and lungs were excised and immediately imaged at 25× using LumaScope fluorescent imaging system. The number of metastases per lung were counted by hand and graphically represented. (D) Three representative images of areas of the lung with metastases (arrows).

Figure 10.

Compounds 11 and 21 inhibit the polymerization of purified porcine brain tubulin. Compounds were incubated at 10 μ M with 2.2 mg/mL purified porcine brain tubulin in the presence of 10% glycerol, and GTP. CA-4 was used as a positive control.

Figure 11.

Compound 11 and 21 cause a loss of cellular microtubules. A-10 cells were treated with vehicle (DMSO), 50 nM CA-4, 100 nM 11, or 1 μ M 21 for 18 h. Cells were then fixed and microtubules visualized by indirect immunofluorescence techniques.

Reagents and conditions: (a) Na/MeOH/reflux, 24 h; (b) H₂SO₄ (conc.), rt, overnight; (c) POCl₃, reflux, 2 h

Scheme 1.

The synthesis of 4-chloro-2,6-dimethylfuro[2,3-*d*]pyrimidine **20**.

Reagents and conditions: (a) the appropriate aniline, ⁱPrOH or ⁿBuOH, 1 drop of HCl (conc.), reflux; (b) NaH, the appropriate iodoalkane.

Scheme 2.

The synthesis of *N*-aryl-2,6-dimethylfuro[2,3-*d*]pyrimidin-4-amines 3–15.

Reagents and conditions: (a) ether, HCl (g).

Scheme 3.

The synthesis of hydrochloric acid salt **21**.

Table 1

RTK inhibitory activities of compounds **3** – **15** and **21**.

Compound	EGFR kinase inhibition (nM)	VEGFR-2 kinase inhibition (nM)	PDGFR- β kinase inhibition (nM)	A431 Cytotoxicity (nM)	CAM angiogenesis inhibition (μ M)
3	113.2 $\pm$ 30.2	50.3 $\pm$ 10.2	n.d.	48.2 $\pm$ 5.9	n.d.
4	145.2 $\pm$ 20.8	32.2 $\pm$ 5.3	n.d.	49.1 $\pm$ 8.0	n.d.
5	30.0 $\pm$ 4.9	76.3 $\pm$ 10.1	16.2 $\pm$ 2.5	22.6 $\pm$ 3.0	18.2 $\pm$ 1.8
6	122.1 $\pm$ 3.4	66.2 $\pm$ 8.1	66.1 $\pm$ 7.2	55.5 $\pm$ 9.0	n.d.
7	30.2 $\pm$ 4.3	58.2 $\pm$ 7.1	n.d.	18.0 $\pm$ 3.0	n.d.
8	102.3 $\pm$ 19.1	70.2 $\pm$ 8.0	n.d.	60.0 $\pm$ 10.1	n.d.
9	90.2 $\pm$ 10.2	102.1 $\pm$ 17.2	28.3 $\pm$ 4.0	50.0 $\pm$ 6.7	22.6 $\pm$ 3.2
10	42.2 $\pm$ 7.1	43.1 $\pm$ 5.0	63.5 $\pm$ 22.8	n.d.	n.d.
11	68.2 $\pm$ 6.2	19.1 $\pm$ 3.0	22.8 $\pm$ 4.9	20.8 $\pm$ 4.2	3.9 $\pm$ 0.4
12	70.7 $\pm$ 14.3	10.6 $\pm$ 2.3	27.1 $\pm$ 4.0	50.1 $\pm$ 8.1	19.2 $\pm$ 3.0
13	73.3 $\pm$ 9.2	8.5 $\pm$ 0.9	29.9 $\pm$ 4.6	42.3 $\pm$ 6.3	16.7 $\pm$ 2.1
14	340.1 $\pm$ 28.3	34.4 $\pm$ 7.1	56.5 $\pm$ 6.2	120.7 $\pm$ 20.1	32.6 $\pm$ 1.9
15	84.4 $\pm$ 10.3	15.6 $\pm$ 3.0	23.1 $\pm$ 3.7	50.2 $\pm$ 13.1	18.9 $\pm$ 2.3
21	82.3 $\pm$ 10.2	20.2 $\pm$ 3.8	21.9 $\pm$ 3.0	55.8 $\pm$ 8.0	3.7 $\pm$ 0.5
semaxanib	n.d.	12.9	n.d.	n.d.	60.0 $\pm$ 10.1
DOX	n.d.	n.d.	n.d.	1.3 $5 \pm$ 0.03	0.04 $\pm$ 0.0031
cisplatin	n.d.	n.d.	n.d.	10.6	18.2 $\pm$ 2.1
sumitinib	172.1 $\pm$ 19.4	18.9 $\pm$ 2.7	83.1 $\pm$ 10.1	n.d.	1.3 $\pm$ 0.07
erlotinib	1.2 $\pm$ 0.2	124.7 $\pm$ 18.2	n.d.	n.d.	29.1 $\pm$ 1.9

IC₅₀ values of compounds in cell lines with high expression levels of the indicated kinase, A431 cytotoxicity and inhibition of the CAM formation

Table 2

Tumor cell inhibitory activity (GI₅₀, nM) of **11** (NCI 60 cell line panel).

Panel/Cell line	GI ₅₀ (nM)	Panel/ Cell line	GI ₅₀ (nM)	Panel/ Cell line	GI ₅₀ (nM)	Panel/ Cell line	GI ₅₀ (nM)
NSCLC		Renal Cancer		Ovarian cancer		Prostate Cancer	
A549/ATCC	37.6	786 – 0	43.6	OVCAR-3	29.1	DU-145	26.2
EKVX	64.8	A498	19.5	OVCAR-4	60.4	Breast Cancer	
HOP-62	32.2	ACHN	55.9	OVCAR-5	55.8	MCF7	42.2
NCI-H226	84.1	CAKI-1	16.3	OVCAR-8	36.6	MDA-MB-231/ATCC	44.3
NCI-H23	40.7	SN 12C	56.7	SK-OV-3	25.2	HS 578T	15.3
NCI-H460	33.1	UO-31	75.2	Melanoma		BT-549	48.0
NCI-H522	<1	Colon Cancer		LOX IMVI	54.9	MDA-MB-468	34.9
CNS Cancer		COLO 205	20	MALME-3M	42.3	Leukemia	
SF-268	33.6	HCC-2998	28.5	M14	23.3	CCRF-CEM	25.3
SF-295	13.8	HCT-116	34	SK-MEL-2	33.9	HL-60(TB)	17.3
SF-539	20.0	HCT-15	25.3	SK-MEL-28	37.7	K-562	13.2
SNB-19	33.3	HT29	32.5	SK-MEL-5	22.9	MOLT-4	67.9
SNB-75	53.2	KM12	21.1	UACC-62	14.6	RPMI-8226	42.8
U251	30.8	SW-620	29.8			SR	32.0

Table 3NCI COMPARE analysis results for **11**.

Rank	Vector	Correlation	Cell line
1	vincristine sulfate S67574 –3M TGI 2 days AVGDATA	0.600	49
2	maytansine S 153858 –4M TGI 2 days AVGDATA	0.494	49
3	vinblastine sulfate S49842 –5.6M TGI 2 days AVGDATA	0.458	49

Table 4Microtubule depolymerizing activities of **10** – **15**.

Compound	EC ₅₀ for microtubule depolymerization
CA-4	7 nM
10	> 40 μ M
11	103 nM
12	1.1 μ M
13	13.5 μ M
14	> 40 μ M
15	>40 μ M
21	570 nM

Table 5Effects of **11** on tubulin polymerization and colchicine displacement.

Compound	Inhibition tubulin assembly IC ₅₀ (μ M) $\pm$ SD	Inhibition of colchicine binding % inhibition $\pm$ SD	
		1 μ M	5 μ M
CA-4	1.0 $\pm$ 0.09	88 $\pm$ 2	99 $\pm$ 0.2
11	1.9 $\pm$ 0.1	63 $\pm$ 4	86 $\pm$ 2

Table 6

Antiproliferative activity ($IC_{50} \pm SD$, nM) of **11** and related compounds.

Compound	MDA-MB-435	HeLa	HeLa WT β III	Rr	SK-OV-3	SK-OV-3 MDR-1/M-6-6	Rr
11	17.1 $\pm$ 1.5	33.1 $\pm$ 1.4	27.2 $\pm$ 1.6	0.82	36.7 $\pm$ 2.3	63.3 $\pm$ 4.8	1.72
12	136.6 $\pm$ 1.8	167.1 $\pm$ 9.9	205.6 $\pm$ 16.5	1.2	228.2 $\pm$ 14.3	270.0 $\pm$ 11.2	1.2
13	1,400 $\pm$ 300	1,240 $\pm$ 70	1,500 $\pm$ 100	1.2	1,600 $\pm$ 200	3,400 $\pm$ 600	2.1
14	> 10 μ M	> 10 μ M	> 10 μ M	n.d.	> 10 μ M	> 10 μ M	n.d.
15	3,000 $\pm$ 400	2,900 $\pm$ 200	4,070 $\pm$ 40	1.4	3,800 $\pm$ 200	7,400 $\pm$ 300	2
21	47.0 $\pm$ 1.3	40.6 $\pm$ 0.8	44.7 $\pm$ 1.1	1.1	50.4 $\pm$ 2.1	62.1 $\pm$ 1.2	1.2
Paclitaxel	1.93 $\pm$ 0.1	1.21 $\pm$ 0.1	10 $\pm$ 0.9	8.3	3.0 $\pm$ 0.1	2,655 $\pm$ 399	885

Table 7

IC₅₀ nM ± SD of Compound **21** and Control Drugs in Breast Cancer and Endothelial Cells.^a

	MDA-MB-435	MDA-MB-231	MDA-MB-468	MCF-7	MCF-7 TAX	4T-1	HUVEC
21	53 ± 4.4	147.6 ± 2.9	7.1 ± 0.9	137.7 ± 2.8	129.8 ± 16.2	97.9 ± 0.12	172.2 ± 1.4
Sunitinib	9.7 ± 0.8	22.3 ± 1.9	6.1 ± 0.7	27.1 ± 4.0	29.3 ± 3.0	16.0 ± 2.1	11.8 ± 1.2
Erlotinib	622.1 ± 100.5	438.5 ± 53.8	235.2 ± 26.3	1.2 ± 0.04	n.d.	n.d.	165.2 ± 1.4
Lapatinib	29.7 ± 4.0	20.0 ± 3.6	83.2 ± 6.7	64.9 ± 10.3	n.d.	10.37 ± 1.8	n.d.
CA-4	52.2	228 ± 2.9	63.7 ± 7.1	24.9 ± 1.4	n.d.	69.6 ± 8.0	17.7 ± 3.6
Docetaxel	2.7 ± 0.32	16.0 ± 1.9	69.1 ± 9.2	59.3 ± 6.4	> 1000	63.4 ± 1.9	56.7 ± 2.9

^aIC₅₀ in nM ± SD from 2–3 experiments.