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Discovery of Biarylaminquinazolines as Novel Tubulin Polymerization Inhibitors

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Abstract

Cell cycle experiments with our previously reported 4-biphenylaminoquinazoline (**1–3**) multityrosine kinase inhibitors revealed an activity profile resembling that of known tubulin

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Supporting Information

Structure of *N*-(4-methoxyphenyl)-*N*,2-dimethylquinazolin-4-amine; kinase inhibition data; and ¹H NMR, ¹³C NMR, and MS spectra of the tested compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes

The authors declare no competing financial interest.

polymerization inhibitors. Novel 4-biarylaminoquinazoline analogues of compound **2** were synthesized and evaluated as inhibitors of several tyrosine kinases and of tubulin. Although compounds **1–3** acted as dual inhibitors, the heterobiaryl analogues possessed only anti-tubulin properties and targeted the colchicine site. Furthermore, molecular modeling studies allowed the rationalization of the pharmacodynamic properties of the compounds.

INTRODUCTION

The discovery of the anticancer drugs erlotinib¹ and gefitinib² in the early 2000s prompted intensive research on 4-anilinoquinazoline compounds, leading to the development of new attractive compounds such as lapatinib,³ vandetanib,⁴ and afatinib.⁵ Several published patents and articles showed the feasibility of the anilinoquinazoline scaffold for the development of tyrosine kinase (TK) inhibitors (TKIs).^{6,7} The main biomolecular target of this class of compounds remains epidermal growth factor receptor (EGFR), although some compounds do not show high selectivity for it. For example, lapatinib is a dual EGFR/Her-2 inhibitor, whereas vandetanib inhibits the kinase activities of both EGFR and VEGFR-2. In this regard, we have recently reported that the functionalization of the quinazoline scaffold with both a fused dioxxygenated ring at the 6 and 7 positions and a 3-biphenylamino function at the 4 position leads to multi-TKIs.⁸ In particular, compound **2** (Figure 1) was found to inhibit the kinase activities of EGFR, FGFR-1, PDGFR β , Abl1, and Src at submicromolar concentrations and also showed EC₅₀'s against several cancer cell lines in the low nanomolar range.⁹ For example, in A431 cells, **2** was about 10 times more cytotoxic than analogues **1** and **3**. Further investigations on the mechanism of action of the biphenylaminoquinazolines indicated that they could have other molecular targets than the TKs. In particular, the effects of compounds **1–3** on cell cycle progression (vide post) were quite similar to known tubulin inhibitors, suggesting an interaction with cytoskeletal components.

Interestingly, in 2008, Sirisoma et al. reported some *N*⁴-methyl-*N*⁴-phenyl-4-aminoquinazolines (e.g., *N*-(4-methoxyphenyl)-*N*,2-dimethylquinazolin-4-amine; see Figure S1, Supporting Information) endowed with potent cytotoxic effects, which were discovered through a cell-based anticancer screening apoptosis platform.¹⁰ The authors proposed and validated tubulin as the main target of these compounds. In fact, although the structural similarity to other substituted anilinoquinazolines, such as erlotinib or gefitinib, might suggest EGFR as being a plausible target, the *N*⁴-methyl and the presence of a substituent at the 2 position of *N*-(4-methoxyphenyl)-*N*,2-dimethylquinazolin-4-amine impaired the ability of the compounds to act also as kinase inhibitors.¹¹

Combining the results of cell cycle analysis with Sirisoma's finding, we were even more convinced that, besides the anti-TK activity, the previously described 4-anilinoquinazolines probably inhibited tubulin polymerization. Here, we demonstrate that biphenylaminoquinazolines possess anti-tubulin effects as well as TKI properties. Thus, they are promising examples of dual inhibitors. Moreover, in order to establish the molecular determinants required to achieve dual TK/tubulin inhibition or to be selective anti-tubulin compounds, we synthesized and evaluated several heterobiaryl analogues of compound **2** in

which one of the two benzene rings of the biphenylamino moiety was replaced by a 5- or 6-atom heterocycle. Finally, all of the synthesized compounds were evaluated by a molecular modeling approach, with the aim of rationalizing the structure–activity relationship.

RESULTS AND DISCUSSION

To investigate the structural determinants required for the inhibition of kinases and tubulin polymerization, we planned the synthesis of some novel analogues of compound **2**, which was the most cytotoxic (among the three biphenylaminoquinazolines previously discovered) against several cell lines (Figure 2).⁸

Compounds **1–3** and **10** were prepared as previously described.⁸ Compounds **4–8** were synthesized starting from 4-chloro-7,8-dihydro[1,4]-dioxino[2,3-*g*]quinazoline (**11**) (Scheme 1).

Chloroquinazoline intermediate **11** was condensed with the appropriate heterobiaryl amines through two different microwave-assisted protocols according to the type of the nucleus bearing the amine function. Final products **4–6**, in which the nucleus bearing the amine was a benzene, were obtained as hydrochlorides using *i*-PrOH as solvent, whereas compounds **7** and **8**, in which the nucleus bearing the amino group was a heterocycle, were obtained as free bases using NaH in THF. In fact, because of the low nucleophilicity of the amine function, when 2-amino-4-phenylpyrimidine or 2-amino-4-phenylthiazole was mixed with chloroquinazoline in *i*-PrOH and irradiated, no products were formed. Consequently, for these two heteroarylamines, a stronger alkaline medium was required.

For the synthesis of compound **9**, the 3-(2-pyrrolyl)aniline synthon was prepared starting from 3-bromoaniline (**12**), protected as ethyl carbamate **13**, which was condensed with *N*-*tert*-boc-2-pyrroleboronic acid via Suzuki coupling.

Intermediate **14** was deprotected at both amine functions in alkaline medium to give the desired derivative **15**, which was finally condensed with chloroquinazoline **11** in *i*-PrOH to afford compound **9** as the hydrochloride (Scheme 2).

All of the newly synthesized compounds, together with **1–3**, were evaluated against a panel of isolated tyrosine kinases and against tubulin (Table 1). Compounds **1–3** inhibited both tubulin polymerization and the tested TKs. Moreover, the biphenylaminoquinazolines were found to bind at the colchicine site, as demonstrated by their ability to inhibit the binding of [³H]colchicine to tubulin. Conversely, heterobiaryl analogues **4–9** were almost inactive at the tested concentrations (IC₅₀'s > 10 μM) against the TKs, whereas they maintained varying anti-tubulin activity. Compound **10** (bearing the biphenylamino moiety but not bearing the fused dioxygenated ring on the quinazoline core) was inactive against all of the tested targets. Interestingly, the inhibitory activity against tubulin polymerization of compounds **1–3**, **5**, **6**, and **9**, especially that of compounds **1** and **5**, was comparable with that of combretastatin A-4 (C A-4).

In view of the data acquired on isolated targets, compounds **1–3**, **5**, **6**, and **9** were also tested on human ovarian carcinoma cell line OVCAR-8 and its cognate P-glycoprotein (P-gp)-overexpressing cell line, NCI/ADR-RES (Table 2).

All compounds, except the paclitaxel control, had essentially identical antiproliferative activities in both the parental and P-gp-overexpressing multidrug-resistant cells. Moreover, the activity of the compounds against the OVCAR-8 cell line may indicate a hormone-independent mechanism of action for the tested compounds.¹² In the cell-based assays, only compounds **1** and **5** exerted effects similar to that of C A-4. Contrary to what was previously reported for other cell lines, compound **1** was more active than compound **2** in inhibiting the proliferation of both the OVCAR-8 and NCI/ADR-RES cell lines.

Because of the above results, compounds **1–3**, **5**, **6**, and **9** were also subjected to analysis for their effects on cell cycle distribution. All compounds were added to the cells at a concentration of 1.0 μ M. In the OVCAR-8 cell line, these experiments revealed a profile of activity typically reported for inhibitors of tubulin polymerization (Figure 3),¹³ further confirming the previous findings. In particular, **1** and **5**, the two most active compounds against tubulin and as cytotoxic agents, significantly increased the proportion of cells arrested at G2/M as compared to that of control cells ($P < 0.05$).

The 4-anilinoquinazoline moiety represents a widely studied scaffold in the field of TKIs,⁷ and a large number of examples are available for both the type I and II TKIs.¹⁴ However, in the field of tubulin polymerization inhibitors, the 4-anilinoquinazoline core is not commonly used, and *N*-(4-methoxyphenyl)-*N*,2-dimethylquinazolin-4-amine represents, to our knowledge, the most active published derivative.¹⁰ Because this chemotype is quite unexplored as a class of tubulin inhibitors, we initiated a molecular modeling study with the goal of rationalizing the pivotal pharmacophore features required to achieve dual inhibition of tubulin polymerization and TKs. Some attempts to draw a common SAR were previously published,^{10,11} but these were aimed mainly at determining differences between the requirements of the two targets.

Thus, the binding mode of **1–9** was evaluated by docking studies. As a consequence of the structural similarity with *N*-(4-methoxyphenyl)-*N*,2-dimethylquinazolin-4-amine and on the basis of their inhibition of colchicine binding, we modeled them into the colchicine site of tubulin. From among the available crystal structures with colchicine site inhibitors,¹⁵ we selected 1SA0 (tubulin with DAMA-colchicine)¹⁶ and 3N2G (tubulin with ethyl-5-amino-2-methyl-1,2-dihydro-3-phenylpyrido[3,4-*b*]-pyrazin-7-yl-carbamate).¹⁷ 3N2G showed a larger binding site than 1SA0 because the Leu255 β residue moves its side chain far from the binding site, thereby opening a small subpocket deeply buried within the tubulin β -subunit (Figure 4). We docked compounds **1–9** into both structures, and we observed two very similar binding modes for each compound (data not shown). The poses obtained with the 3N2G structure had the best docking score.

The cell cycle results strongly indicated that the cytotoxic effects of the reported derivatives result from interactions of the compounds with tubulin. In further support of our hypothesis, we superimposed the selected binding poses of the 4-biphenylamino-quinazolines with the

Nguyen seven-point pharmacophore model (Figure 4).¹⁸ Not all of the model requirements for polar interactions were met, but the hydrophobic requirements were fully satisfied. Hence, our modeling data strongly support the view that derivatives **1–9** are tubulin polymerization inhibitors, in agreement with the biological results presented above.

The specific amino acid residue interactions of derivative **2** (tubulin assembly IC_{50} = 4.0 μ M) are shown in Figure 5.

The binding mode analysis was carried out by splitting the structure of **2** into three different portions: the terminal phenyl ring, the aniline ring, and the quinazoline moiety. The terminal phenyl ring bound in the subpocket opened by the shift of the Leu255 β side chain in 3N2G. In this cleft, the phenyl ring was strongly stabilized by hydrophobic contacts with Tyr202 β , Asn167 β , Val238 β , Leu242 β , and Leu252 β . The aniline ring was well-stabilized by a series of hydrophobic contacts with Leu255 β , Ala316 β , and Ile387 β . Lastly, the quinazoline moiety established nonpolar interactions with Leu248 β , Asn258 β , Met259 β , Lys352 β , and Val181 α . Molecular dynamics carried out for **2** indicated that the described interactions were stable (data not shown).

For the essentially inactive derivatives **7** and **8**, AutoDock provided some binding poses similar to those found for the active compounds. As a consequence, we suggest that a stable/lasting binding was also related to the logP of the bridge cycle. The mainly hydrophobic features of the colchicine site were matched better by phenyl than by pyrimidine (**7**) or thiazole (**8**). To confirm this hypothesis, we are preparing additional compounds. In contrast, with derivative **10** (characterized by two methoxy functions in the quinazoline moiety instead of a fused dioxxygenated ring) we did not observe any binding pose consistent with either reference compound.

Similar modeling experiments were also performed for the studied kinases (EGFR, FGFR1, Abl1, and Src; Table 1). We previously performed docking analyses with compounds **1–3** and **10**,⁸ from which it was concluded that more reliable binding conformations were obtained using kinase crystal structures with the DFG-out conformation. The in/out movement of the DFG triplet (aspartic–phenylalanine–glycine) largely affects the morphology of the ATP binding site. The kinases in the DFG-out form have, as an important feature, a closed conformation of the activation loop, and this prevents ATP binding and exposes an additional hydrophobic site.¹⁹ Using derivative **2** as reference, we noted three major contacts (Figure 6): (i) the quinazoline nitrogen N1 forms an H-bond with the hinge region; (ii) the terminal phenyl moiety forms an “edge-to-face” phenyl–phenyl interaction with the phenylalanine of the DFG triplet; and (iii) the aniline ring lies in a lipophilic cleft formed by hydrophobic residues. (EGFR: Val716, Ala743, Leu844, and Thr854; FGFR: Val492, Lys514, and Phe489; Abl1: Ala269, Lys271, and Ala380; Src: Lys295, Val281, and Thr338).

Among the AutoDock proposed binding conformations of **4–9**, we observed a cluster of poses similar to the one selected for active compounds, but this cluster never scored as one of the best or the most populated. In spite of this, we observed a very poor correlation between docking score and experimental biological activity. In an attempt to readily

rationalize their inactivity, we hypothesize that for compound **7** the loss of kinase inhibition was due to the polarization induced by the pyrimidine ring. In contrast, in the case of compounds **4–6** and **9**, we hypothesize that the heterocycles impair the T-shaped interaction, probably because of the different partial charge distributions. Indeed, it was reported that heterocycles tend to form a “face-to-face” arene–arene interaction in contrast to the “edge-to-face” interaction that is preferred by benzene rings.²⁰

Summarizing, the 4-anilinoquinoline scaffold can be used for the development of both TKIs and tubulin polymerization inhibitors, with the opportunity to modulate these biological activities through the selection of appropriate substituents.

Merging the results of the computational analysis with the biological data, we can define the requirements for dual activity (Figure 7):

1. The carbon atom linker between the oxygen atoms may be varied from one carbon to three, but the dioxygenated function must be included in a cycle;
2. The substitution of the aniline benzene (ring A) with any other heteroaryl is not allowed;
3. The terminal cycle (ring B) represents the switching force between the two activities: with a heterocycle, we observed selective anti-tubulin activity, whereas with a phenyl ring, we obtained dual anti-tubulin/anti-TK activity.

CONCLUSIONS

The previously reported biphenylaminoquinazoline derivatives (compounds **1–3**), initially developed as multi-TKIs, were also found to inhibit tubulin polymerization. By examining the inhibition of the binding of [³H]colchicine to tubulin, we demonstrated that the quinazolines bound in the colchicine site of tubulin. In order to establish a structure–activity relationship, several heteroaryl analogues were synthesized and evaluated as TKIs and as tubulin polymerization inhibitors. The newly synthesized compounds were all found to be almost inactive against the isolated kinases, whereas some derivatives maintained inhibitory activity on tubulin polymerization. Consequently, we found that it is possible to obtain both dual TK/tubulin polymerization inhibitors and selective tubulin inhibitors bearing the same quinazoline scaffold. Molecular modeling studies allowed us to rationalize our structure–activity relationship findings.

Specifically, we found that (1) the 3-phenylaniline moiety was essential for the dual TK/tubulin polymerization inhibition, but substituents could further enhance hydrophobic interactions; (2) generally, the introduction of an heteroatom in the biphenyl moiety was detrimental for anti-TK activity; (3) the substitution of the ring A with an heteroaryl (compounds **7** and **8**) caused the abrogation of both anti-TK and anti-tubulin activities; (4) the introduction of a heteroatom in ring B did not affect anti-tubulin activity; and (5) the fused dioxygenated ring was essential for anti-tubulin activity (compare compounds **1–3** with **10**), as previously reported for inhibition of multiple tyrosine kinases.⁸

The functionalization of the biphenyl moiety with lipophilic substituents (halogen atoms or methyl or methoxy function) is in progress, with the aim to investigate more thoroughly the pharmacophore features of dual TK/tubulin inhibitors. We are also planning the synthesis of heteroaryl analogues of compound **1**, because it showed higher inhibitory activity against the OVCAR-8 and NCI/ADR-RES cell lines than reference compound **2**. Moreover, because the novel compounds did not show anti-TK activities, we will also synthesize the *N*⁴-methyl analogues of the compounds described here.

EXPERIMENTAL SECTION

Chemistry

All commercial chemicals and solvents used were analytical grade and were used without further purification. Analytical thin-layer chromatography (TLC) was performed on precoated silica gel plates (Merck 60-F-254, 0.25 mm). Preparative column chromatography was performed using silica gel 60 (0.063–0.100 mm; Merck), eluting with CHCl₃. Melting points were determined on a Gallenkamp MFB-595-010 M melting point apparatus and are uncorrected. The ¹H NMR spectra were recorded on a Bruker 300-AMX spectrometer with TMS as an internal standard. Coupling constants are given in hertz (Hz), and the relative area peaks were in agreement with all assignments. Elemental analyses were characterized on a PerkinElmer 2400 analyzer. Mass spectra were recorded on an Applied Biosystem Mariner System 5220 with direct injection of the sample. Microwave-assisted reactions were performed on a CEM Discover monomode reactor with the temperature monitored by a built-in infrared sensor and automatic control of power; all reactions were performed in closed devices with pressure control. Compounds **1–3** and **10** were prepared as previously described.⁸ Starting product **11** was synthesized as previously reported.²¹ All of the amine reagents were commercial products except for 3-(2-pyrrolyl)aniline, which was synthesized as described below. The purity for all of the tested compounds was determined by elemental analyses and was found to be equal to or greater than 95%.

General Procedure for Aminoquinazolines 4–9

Method A. A mixture of **11** (1.0 mmol) and 3-(2-heteroaryl)aniline (1.0 mmol) in *i*-PrOH (3 mL) was microwave-irradiated at 80 °C (power set point, 60 W; ramp time, 1 min; hold time, 15 min). After cooling, the resulting precipitate was collected by filtration to give **4–6** as hydrochlorides. **Method B.** To a solution of 2-amino-4-phenylheteroaryl derivatives (1.0 mmol) in anhydrous THF (6 mL) was added NaH (4.5 mmol), and the mixture was stirred at room temperature for 30 min. Compound **11** (1.0 mmol) was then added, and the mixture was microwave-irradiated at 80 °C (power set point, 60 W; ramp time, 1 min; hold time, 15 min). After cooling, the mixture was diluted with water (100 mL) and extracted with EtOAc (3 × 100 mL). The organic phase was evaporated under reduced pressure, and the residue was crystallized from *i*-PrOH to give **7** or **8**.

(7,8-Dihydro[1,4]dioxino[2,3-*g*]quinazolin-4-yl)-[3'-(furan-2''-yl)phenyl]amine Hydrochloride (**4**)

From 3-(2-furyl)aniline with method A: yield 52%. mp 75 °C. ¹H NMR (300 MHz, DMSO-*d*₆): δ 11.02 (broad s, 1H, *NH*), 8.83 (s, 1H, 2-H), 8.33 (s, 1H, 5-H or 10-H), 8.05 (d, *J* = 1.7

Hz, 1H, 5''-H), 7.81 (s, 1H, 2'-H), 7.71-7.62 (m, 2H, 4'-H and 6'-H), 7.53 (t, $J = 7.7$ Hz, 1H, 5'-H), 7.32 (s, 1H, 5-H or 10-H), 7.00 (d, $J = 3.4$ Hz, 1H, 3''-H), 6.64 (dd, $J = 3.4, 1.7$ Hz, 1H, 4''-H), 4.55–4.43 (m, 4H, $\text{OCH}_2\text{CH}_2\text{O}$). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 158.36, 152.26, 151.22, 149.41, 144.93, 143.16, 137.41, 134.76, 130.74, 129.27, 123.33, 121.23, 119.22, 112.10, 110.53, 108.01, 106.40, 105.44, 64.97, 64.08. Anal. Calcd for $\text{C}_{20}\text{H}_{15}\text{N}_3\text{O}_3\cdot\text{HCl}$: C, 62.91; H, 4.22; Cl, 9.29; N, 11.01. Found: C, 62.94; H, 4.26; Cl, 9.20; N, 11.04. HRMS (ESI-TOF) for $\text{C}_{20}\text{H}_{16}\text{N}_3\text{O}_3[\text{M} + \text{H}]^+$: calcd, 346.1186; found, 346.1135.

(7,8-Dihydro[1,4]dioxino[2,3-g]quinazolin-4-yl)-[3'-(thien-2''-yl)phenyl]amine Hydrochloride (5)

From 3-(2-thienyl)aniline with method A: yield 70%. mp > 300 °C. ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 10.96 (broad s, 1H, NH), 8.82 (s, 1H, 2-H), 8.33 (s, 1H, 5-H or 10-H), 8.01 (d, $J = 1.7$ Hz, 1H, 2'-H), 7.71 (dd, $J = 8.0, 1.7$ Hz, 1H, 4'-H or 6'-H), 7.63 (m, 1H, 4'-H or 6'-H), 7.61 (dd, $J = 5.1, 1.2$ Hz, 1H, 5''-H), 7.55 (dd, $J = 3.6, 1.2$ Hz, 1H, 3''-H), 7.51 (t, $J = 8.0$ Hz, 1H, 5'-H), 7.32 (s, 1H, 5-H or 10-H), 7.18 (dd, $J = 5.1, 3.6$ Hz, 1H, 4''-H), 4.55–4.44 (m, 4H, $\text{OCH}_2\text{CH}_2\text{O}$). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 158.37, 151.21, 149.34, 144.92, 142.52, 137.56, 134.78, 134.19, 129.41, 128.49, 126.06, 124.05, 123.44, 123.07, 121.23, 110.63, 108.07, 105.43, 64.98, 64.08. Anal. Calcd for $\text{C}_{20}\text{H}_{16}\text{ClN}_3\text{O}_2\text{S}$: C, 60.37; H, 4.05; Cl, 8.91; N, 10.56; S, 8.06. Found: C, 60.35; H, 4.06; Cl, 8.96; N, 10.55; S, 8.09. HRMS (ESI-TOF) for $\text{C}_{20}\text{H}_{16}\text{N}_3\text{O}_2\text{S} [\text{M} + \text{H}]^+$: calcd, 362.0958; found, 362.0896.

(7,8-Dihydro[1,4]dioxino[2,3-g]quinazolin-4-yl)-[3'-(pyridin-yl-2''-yl)phenyl]amine Hydrochloride (6)

From 3-(2-pyridinyl)-aniline with method A: yield 56%. mp > 300 °C. ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 11.01 (broad s, 1H, NH), 8.82 (s, 1H, 2-H), 8.70 (d, $J = 3.2$, 1H, 6''-H), 8.45 (s, 1H, 2'-H), 8.34 (s, 1H, 5-H or 10-H), 8.05–7.82 (m, 4H, 4'-H, 5'-H, 6'-H and 3''-H), 7.60 (t, $J = 7.6$ Hz, 1H, 4''-H), 7.41 (dd, $J = 7.6, 3.2$ Hz, 1H, 5''-H), 7.30 (s, 1H, 5-H or 10-H), 4.55–4.42 (m, 4H, $\text{OCH}_2\text{CH}_2\text{O}$). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 158.49, 154.50, 151.32, 149.11, 148.56, 145.00, 138.55, 138.11, 137.35, 134.17, 129.16, 125.53, 124.54, 123.29, 122.93, 120.96, 110.87, 107.97, 105.03, 65.01, 64.08. Anal. Calcd for $\text{C}_{21}\text{H}_{17}\text{ClN}_4\text{O}_2$: C, 64.21; H, 4.36; Cl, 9.02; N, 14.26. Found: C, 64.21; H, 4.30; Cl, 9.07; N, 14.27. HRMS (ESI-TOF) for $\text{C}_{21}\text{H}_{17}\text{N}_4\text{O}_2[\text{M} + \text{H}]^+$: calcd, 357.1346; found, 357.1318.

(7,8-Dihydro[1,4]dioxino[2,3-g]quinazolin-4-yl)-(4'-phenyl-pyrimidin-2''-yl)amine (7)

From 2-amino-4-phenylpyrimidine with method B: Yield 5%. mp > 300 °C. ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 8.95 (d, $J = 5.4$ Hz, 1H, 6'-H), 8.87 (s, 1H, 2-H), 8.46 (s, 1H, 5-H or 10-H), 8.32–8.26 (m, 2H, 2''-H and 6''-H), 8.04 (d, $J = 5.4$ Hz, 1H, 5'-H), 7.67–7.58 (m, 3H, 3''-H, 4''-H and 5''-H), 7.44 (s, 1H, 5-H or 10-H), 4.57–4.45 (m, 4H, $\text{OCH}_2\text{CH}_2\text{O}$). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 164.06, 158.96, 158.92, 149.69, 143.89, 136.19, 131.09, 128.90, 127.09, 112.33, 111.26, 109.75, 64.52, 64.07. Anal. Calcd for $\text{C}_{20}\text{H}_{16}\text{ClN}_5\text{O}_2$: C, 60.99; H, 4.09; Cl, 9.00; N, 17.78. Found: C, 70.01; H, 4.07; Cl, 9.03; N, 17.76. HRMS (ESI-TOF) for $\text{C}_{20}\text{H}_{16}\text{N}_5\text{O}_2[\text{M} + \text{H}]^+$: calcd, 358.1299; found, 358.1204.

(7,8-Dihydro[1,4]dioxino[2,3-g]quinazolin-4-yl)-(4'-phenylthiazol-2''-yl)amine (8)

From 2-amino-4-phenylthiazole with method B: yield 10%. mp 278 °C. ¹H NMR (300 MHz, DMSO-*d*₆): δ 12.14 (broad s, 1H, NH). 8.68 (s, 1H, 2-H), 8.37 (s, 1H, 5-H or 10-H), 7.99 (d, *J* = 7.7 Hz, 2H, 2''-H and 6''-H), 7.69 (s, 1H, 5-H or 10-H), 7.46 (t, *J* = 7.7 Hz, 2H, 3''-H and 5''-H), 7.35 (t, *J* = 7.7 Hz, 1H, 4''-H), 7.28 (s, 1H, 5'-H), 4.47–4.38 (m, 4H, OCH₂CH₂O). ¹³C NMR (75 MHz, DMSO-*d*₆): δ 151.32, 151.16, 149.54, 143.96, 128.60, 127.60, 125.68, 125.56, 122.15, 112.58, 112.55, 109.74, 109.38, 108.91, 108.69, 64.42, 63.99. Anal. Calcd for C₁₉H₁₅ClN₄O₂S: C, 57.21; H, 3.79; Cl, 8.89; N, 14.05; S, 8.04. Found: C, 57.24; H, 3.81; Cl, 8.90; N, 14.04; S, 8.06. HRMS (ESI-TOF) for C₁₉H₁₅N₄O₂S [M + H]⁺: calcd, 363.0910; found, 363.0861

(7,8-Dihydro[1,4]dioxino[2,3-g]quinazolin-4-yl)-[3'-(pyrrol-2''-yl)phenyl]amine Hydrochloride (9)

From 3-(2-pyrrolyl)aniline (**15**) with method A: yield 67%. mp 270 °C. ¹H NMR (300 MHz, DMSO-*d*₆): δ 11.38 (broad s, 1H, NH). 11.10 (broad s, 1H, NH); 8.81 (s, 1H, 2-H), 8.34 (s, 1H, 5-H or 10-H), 7.89 (broad s, 1H, 2'-H), 7.58 (dt, *J* = 7.7, 2.0 Hz, 1H, 4'-H or 6'-H), 7.46 (t, *J* = 7.7 Hz, 1H, 5'-H), 7.46–7.41 (m, 1H, 4'-H or 6'-H), 7.33 (s, 1H, 5-H or 10-H), 6.88 (dd, *J* = 4.1, 2.7 Hz, 1H, 5''-H), 6.54 (dd, *J* = 5.1, 4.1 Hz, 1H, 4''-H), 6.14 (dd, *J* = 5.1, 2.7 Hz, 1H, 3''-H), 4.55–4.43 (m, 4H, OCH₂CH₂O). ¹³C NMR (75 MHz, DMSO-*d*₆): δ 158.49, 151.25, 149.41, 144.97, 137.15, 134.68, 133.61, 130.38, 129.07, 121.77, 121.24, 119.83, 119.71, 110.51, 109.15, 107.92, 106.02, 105.48, 65.02, 64.13. Anal. Calcd for C₂₀H₁₇ClN₄O₂: C, 63.08; H, 4.50; Cl, 9.31; N, 14.71. Found: C, 63.06; H, 4.52; Cl, 9.29; N, 14.71. HRMS (ESI-TOF) for C₂₀H₁₇N₄O₂[M + H]⁺: calcd, 345.1346; found, 345.1364

Ethyl 3-Bromophenylcarbamate (13)

To a solution of 3-bromoaniline (**12**) (0.4 g, 2.0 mmol) in THF (20 mL) and TEA (1.0 mL, 8.0 mmol) was added ethyl chloroformate (0.8 mL, 8.0 mmol) dropwise. The mixture was heated at reflux for 1 h. After cooling, the precipitate was removed by filtration, and the solution was evaporated under reduced pressure. The solid was suspended in H₂O (100 mL) and extracted with EtOAc (3 × 50 mL). The organic phase was evaporated under reduced pressure to give **13** (0.5 g, yield 84%) as an oil. ¹H NMR (300 MHz, DMSO-*d*₆): δ 9.82 (broad s, 1H, NH), 7.74 (t, *J* = 1.3 Hz, 1H, 2-H), 7.41 (dt, *J* = 8.1, 1.3 Hz, 1H, 4-H or 6-H), 7.23 (t, *J* = 8.1 Hz, 1H, 5-H), 7.16 (dt, *J* = 8.1, 1.3 Hz, 1H, 4-H or 6-H), 4.13 (q, *J* = 7.0 Hz, 2H, CH₂CH₃), 1.24 (t, *J* = 7.0 Hz, 3H, CH₂CH₃). Anal. Calcd for C₉H₁₀BrNO₂: C, 44.29; H, 4.13; Br, 32.74; N, 5.74. Found: C, 44.31; H, 4.15; Br, 32.70; N, 5.78.

Ethyl [3-(1'-tert-Butyloxycarbonylpyrrol-2'-yl)phenyl]-carbamate (14)

To a mixture of ethyl 3-bromophenylcarbamate (**13**) (0.4 g, 1.6 mmol) and *N*-Boc-2-pyrroloboronic acid (0.4 g, 2.0 mmol) in ethylene glycol dimethyl ether (25 mL) were added a solution of Na₂CO₃ (0.8 g, 7.2 mmol) in H₂O (2.0 mL) and Pd(PPh₃)₄ (100 mg). The mixture was heated at reflux for 4 h. After cooling, the mixture was poured into H₂O (100 mL) and extracted with CH₂Cl₂ (3 × 100 mL). The organic phase was evaporated under reduced pressure and purified by column chromatography to give **14** (0.35 g, yield 64%). mp 126 °C. ¹H NMR (300 MHz, DMSO-*d*₆): δ 9.66 (broad s, 1H, NH), 7.44–7.40 (m, 2H, 2-H

and 4-H or 6-H), 7.33 (dd, $J = 3.6, 1.7$ Hz, 1H, 3'-H or 5'-H), 7.26 (t, $J = 7.6$ Hz, 1H, 5-H), 6.93 (d, $J = 7.6$ Hz, 1H, 4-H or 6-H), 4.12 (q, $J = 7.3$ Hz, 2H, CH_2CH_3), 1.29 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.24 (t, $J = 7.3$ Hz, 3H, CH_2CH_3). Anal. Calcd for $\text{C}_{18}\text{H}_{22}\text{N}_2\text{O}_4$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.40; H, 6.74; N, 8.48.

3-(2-Pyrrolyl)aniline (15)

A solution of ethyl [3-(1'-*tert*-butyloxycarbonylpyrrol-2'-yl)phenyl]carbamate (**14**) (0.33 g, 1.0 mmol) in 5% KOH(aq) (100 mL) was heated at reflux for 4 h. After cooling, the mixture was poured into H_2O (100 mL) and extracted with CH_2Cl_2 (3×50 mL). The organic phase was evaporated under reduced pressure to give **14** (0.15 g, yield 96%). mp 89 °C. ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 11.08 (broad s, 1H, NH), 6.98 (t, $J = 7.7$ Hz, 1H, 5-H), 6.82–6.74 (m, 3H, 2-H and 4-H or 6-H and 3'-H or 5'-H), 6.38 (dt, $J = 7.7, 1.5$ Hz, 1H, 4-H or 6-H), 6.33–6.29 (m, 1H, 3'-H or 5'-H), 6.06 (dd, $J = 5.0, 2.6$ Hz, 1H, 4'-H), 4.98 (broad s, 2H, NH_2). Anal. Calcd for $\text{C}_{10}\text{H}_{10}\text{N}_2$: C, 75.92; H, 6.37; N, 17.71. Found: C, 75.95; H, 6.35; N, 17.74.

Biology. Cell Lines and Culture Conditions

Human cell lines, ovarian carcinoma cells OVCAR-8 and NCI/ADR-RES, used in the studies were generously provided by the National Cancer Institute Drug Screen, Frederick, MD. Cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere for 96 h. Cell protein was measured using sulforhodamine B.

In Vitro Kinase Assays

Novel compounds were tested for in vitro kinase activity as previously described⁸ using purified EGFR (Invitrogen), FGFR-1, Src, and Abl1 (the latter three from Sigma). Kinase reactions were performed in precooled microcentrifuge tubes in a 25 μL final volume of kinase buffer containing 200 ng/mL active kinase, 0.5 mg/mL myelin basic protein (Sigma), 50 μM unlabeled ATP (Sigma), and 1 $\mu\text{Ci/mL}$ [γ - ^{32}P]ATP (PerkinElmer Italia). Compounds were added at concentrations ranging from 10 μM to 10 nM. Negative controls were prepared replacing the substrate solution with water, whereas positive controls were setup by replacing the tested compound with water. The reactions were carried out at 30 °C for 30 min and stopped by addition of loading buffer containing 0.25 mM β -mercaptoethanol. Reactions were then subjected to electrophoresis on a 12% SDS-PAGE gel. Gels were dried, and phosphorylated myelin basic protein was identified by autoradiography.

Tubulin Studies

Electrophoretically homogeneous bovine brain tubulin was purified as described previously in detail.²² The tubulin assembly assay was performed with 10 μM (1.0 mg/mL) tubulin and 0.4 mM GTP in 0.8 M monosodium glutamate (pH of 2 M stock solution adjusted to 6.6 with HCl). Reaction mixtures contained varying compound concentrations in 4% (v/v) dimethyl sulfoxide, and control reaction mixtures contained only the solvent. Reaction mixtures were preincubated without GTP at 30 °C for 15 min and chilled on ice. GTP was added, and the reaction mixtures were placed at 0 °C in cuvettes in Beckman DU7400 and

DU7500 recording spectrophotometers equipped with electronic temperature controllers. After baselines at 350 nm were established, the reaction temperature was rapidly increased to 30 °C. The IC₅₀ was defined as the compound concentration that inhibited the extent of assembly, measured turbidimetrically, by 50% after 20 min at 30 °C. Reaction mixture volume was 0.25 mL.²³

Inhibition of [³H]colchicine binding to tubulin was performed as described previously²⁴ using reaction conditions that strongly stabilize the colchicine binding activity of tubulin.²⁵ Reaction mixtures (0.1 mL) contained 1.0 μM (0.1 mg/mL) tubulin, 5.0 μM [³H]colchicine, 5.0 μM potential inhibitor, 5% (v/v) dimethyl sulfoxide, and the stabilizing components described previously.²⁵ Incubation was for 10 min at 37 °C, a time selected so that the control reaction without inhibitor was 40–60% complete.

Fluorescence-Activated Cell Sorting Analysis

OVCAR-8 cells were grown at 37 °C in a humidified incubator in the presence of 5% CO₂. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin G and 100 μg/mL streptomycin (all provided by Gibco). Cells were cultured for 24 h in a drug-free medium or supplemented with different compounds (1 μM). Cells were harvested, washed twice with PBS, and fixed in 70% cold ethanol (30 min at –20 °C). Cells were then washed once in citrate phosphate buffer (0.2 N Na₂HPO₄ and 0.1 M citric acid, 24:1) followed by PBS and were lastly incubated in an RNase solution (100 μg/mL in PBS). After 30 min at 37 °C, the cells were incubated in a propidium iodide solution (100 μg/mL in PBS, Sigma) at room temperature for a further 30 min. Samples were analyzed on a BD FACS Calibur flow cytometer, collecting 10 000 events. Results of cell cycle analysis were examined using WinMDI 2.9.

Statistical Analysis

Biological results are reported as means ± standard error. Statistical analysis was performed by using one-way analysis of variance. A *P* value of less than 0.05 was considered to be statistically significant.

Molecular Modeling

All molecular modeling studies were performed on a MacPro dual 2.66 GHz Xeon running Ubuntu 12. The tubulin structure was downloaded from the Protein Data Bank (<http://www.rcsb.org/>; PDB IDs: 1SA0¹⁶ and 3G2N¹⁷). Hydrogen atoms were added to the protein using Molecular Operating Environment (MOE) 2007.09,²⁶ structures were minimized, and all heavy atoms were kept fixed until an rmsd gradient of 0.05 kcal mol^{–1} Å^{–1} was reached. Ligand structures were built with MOE and minimized using the MMFF94x force field until an rmsd gradient of 0.05 kcal mol^{–1} Å^{–1} was reached. The docking simulations were performed using AutoDock²⁷ using a 80:80:80 grid box and a spacing of 0.375 Å, with the grid center obtained by the average of cocrystallized inhibitor coordinates. Molecular dynamics was performed with the AMBER 9 package²⁸ using the settings previously reported.²⁹

The kinase structures were downloaded from the Protein Data Bank (<http://www.rcsb.org/>; PDB IDs: EGFR, 1XKK;³ FGFR1, 3C4F;³⁰ Abl1, 2QOH;³¹ and SRC, 3ENF³²). Docking studies were carried out by AutoDock using already reported settings.⁸ The images in the article were created with PyMol.³³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

C A-4	combretastatin A-4
compds	compounds
DFG	Asp–Phe–Gly
MW	microwave
<i>i</i>-PrOH	isopropanol
TEA	triethylamine
THF	tetrahydrofuran
TK	tyrosine kinase

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Figure 1.
Previously reported quinazoline-based multi-TK inhibitors.

Figure 2.
Newly synthesized quinazoline compounds.

Figure 3.

Effect of quinazoline compounds on cell cycle progression. Data are presented as the mean $\pm$ SEM of three independent experiments.

Figure 4.

Tubulin structures are shown as ribbons: green/brown for the β -subunits and yellow/red for the α -subunits of 1SA0 and 3N2G, respectively. The binding mode of **2** (cyan) is shown. Global view of the Nguyen pharmacophore model: the red, pink, and yellow balls are H-bond acceptors; the magenta ball is an H-bond donor; the blue and skyblue balls are hydrophobic points; and the green ball is a planar group. DAMA-colchicine (purple) is also shown. Residue Leu255 is shown as a stick (green and brown for 1SA0 and 3N2G, respectively).

Figure 5. Binding mode of compound **2** in the colchicine site. Residues forming the hydrophobic interactions are shown using a color code: in green are residues interacting with the phenyl moiety; in magenta are those interacting with the aniline ring; and in white are those interacting with the dioxoquinazoline moiety.

Figure 6.

Binding mode of derivative **2** in the ATP binding site of: EGFR (green), FGFR (cyan), Src (magenta), and Abl (yellow). Residues of the hinge region are shown. The Phe residue of the DFG triplet is also shown. In all cases, the biphenyl moiety is stabilized in a lipophilic cleft (EGFR: Val716, Ala743, Leu844, and Thr854; FGFR: Val492, Lys514, and Phe489; Abl: Ala269, Lys271 and Ala380; Src: Lys295, Val281, and Thr338).

Figure 7.
Structure-activity relationship for biaryl aminoquinazolines.

Scheme 1. Synthesis of Compounds 4-8^a

^aReagents and conditions: (a) method A: 3-(2-furyl)aniline, 3-(2-thienyl)aniline, or 3-(2-pyridinyl)aniline, *i*-PrOH, MW, 80 °C, 15 min; method B: 2-amino-4-phenylpyrimidine or 2-amino-4-phenylthiazole, NaH, THF, MW, 80 °C, 15 min. See Figure 2 for Ar specification.

Scheme 2. Synthesis of Compound 9^a

^aReagents and conditions: (a) ClCOOEt, TEA, THF, reflux, 1 h; (b) *N*-boc-2-pyrroloboronic acid, Na₂CO₃(aq), ethylene glycol dimethyl ether, Pd(PPh₃)₄, reflux, 4 h; (c) KOH(aq), reflux, 4 h; (d) compound **11**, *i*-PrOH, MW, 80 °C, 15 min.

Table 1
In Vitro Evaluation of Quinazoline Compounds

compd	IC ₅₀ (μM) ^a					colchicine binding inhibition (%) ^c
	EGFR	FGFR1	Abl1	Src	tubulin ^b	
1	0.002 ± 0.001	0.063 ± 0.015	0.051 ± 0.010	0.032 ± 0.008	1.1 ± 0.03	72 ± 0.4
2	0.042 ± 0.005	0.178 ± 0.030	0.024 ± 0.010	0.642 ± 0.105	4.0 ± 0.2	41 ± 7
3	0.027 ± 0.012	0.063 ± 0.025	0.056 ± 0.016	0.645 ± 0.085	2.3 ± 0.02	34 ± 1
4	>10 ^e	>10 ^e	>10 ^e	>10 ^e	12 ± 0.4	nd ^d
5	>10 ^e	>10 ^e	>10 ^e	>10 ^e	1.2 ± 0.06	75 ± 0.8
6	>10 ^e	>10 ^e	>10 ^e	>10 ^e	3.2 ± 0.1	25 ± 4
7	>10 ^e	>10 ^e	>10 ^e	>10 ^e	>20	nd ^d
8	>10 ^e	>10 ^e	>10 ^e	>10 ^e	>20	nd ^d
9	>10 ^e	>10 ^e	>10 ^e	>10 ^e	3.0 ± 0.2	33 ± 7
10	>10 ^e	>10 ^e	>10 ^e	>10 ^e	>20	nd ^d
vandetanib	0.190 ± 0.085	>10	0.151 ± 0.024	0.044 ± 0.016	>20	nd ^d
CA-4	>10	>10	>10	>10	1.1 ± 0.1	99 ± 0.7
paclitaxel	>10	>10	>10	>10	nd ^f	nd ^f

^aValues are reported as the mean ± SD of at least three independent experiments.

^bInhibition of tubulin polymerization with tubulin at 10 μM.

^cPercentage of inhibition of [³H]colchicine binding. Tubulin was 1 μM. Both [³H]colchicine and the inhibitor were 5 μM.

^dnd, not determined because tubulin polymerization's IC₅₀ was greater than 10 μM.

^eThe percentage of inhibition at 10 μM is reported in the Supporting Information.

^fPaclitaxel stimulates tubulin assembly.

Table 2
Cytotoxic Activity of Quinazoline Compounds

compd	EC ₅₀ (nM) $\pm$ SD ^a	
	OVCAR-8	NCI/ADR-RES ^b
1	30 $\pm$ 0 ^c	15 $\pm$ 7
2	500 $\pm$ 0	350 $\pm$ 80
3	550 $\pm$ 70	300 $\pm$ 100
5	10 $\pm$ 0	4.0 $\pm$ 1
6	150 $\pm$ 70	250 $\pm$ 70
9	4000 $\pm$ 1000	3600 $\pm$ 200
paclitaxel	5.5 $\pm$ 0.7	5000 $\pm$ 0
C A-4	18 $\pm$ 4	13 $\pm$ 4

^a Data were obtained in at least two independent experiments.

^b A cell line isogenic with OVCAR-8 except that it overexpresses P-gp.

^c A standard deviation of 0 means that the same value was obtained in all experiments.