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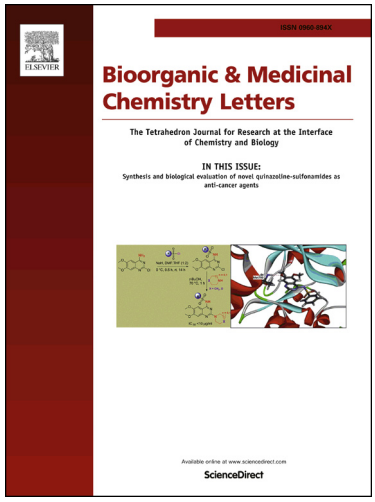

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PII: S0960-894X(17)30429-8
DOI: http://dx.doi.org/10.1016/j.bmcl.2017.04.053
Reference: BMCL 24906

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 18 November 2016
Revised Date: 13 April 2017
Accepted Date: 15 April 2017

Please cite this article as: Fischer, T., Krüger, T., Najjar, A., Totzke, F., Schächtele, C., Sippl, W., Ritter, C., Hilgeroth, A., Discovery of novel substituted benzo-anellated 4-benzylamino pyrrolopyrimidines as dual EGFR and VEGFR2 inhibitors, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: http://dx.doi.org/10.1016/j.bmcl.2017.04.053

Discovery of novel substituted benzo-anellated 4-benzylamino pyrrolopyrimidines as dual EGFR and VEGFR2 inhibitors

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Abstract – The quinazoline scaffold is the main part of many marketed EGFR inhibitors. Resistance developments against those inhibitors enforced the search for novel structural lead compounds. We developed novel benzo-anellated 4-benzylamine pyrrolopyrimidines with varied substitution patterns at both the molecular scaffold and the attached residue in the 4-position. The structure-dependent affinities towards EGFR are discussed and first nanomolar derivatives have been identified. Docking studies were carried out for EGFR in order to explore the potential binding mode of the novel inhibitors. As the receptor tyrosine kinase VEGFR2 recently gained an increasing interest as an upregulated signalling kinase in many solid tumors and in tumor metastasis we determined the affinity of our compounds to inhibit VEGFR2. So we identified novel dually acting EGFR and VEGFR2 inhibitors for which first anticancer screening data are reported. Those data indicate a stronger antiproliferative effect of a VEGFR2 inhibition compared to the EGFR inhibition.

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Keywords: structure-dependent activity, lead structure, compound evaluation, anticancer screening

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The understanding of cell signaling processes in the last decades led to the identification of novel target structures for a more selective anticancer therapy.[1,2] Early identified protein kinases have been cyclin dependent kinases (cdks) which were found deregulated in cancer cells to cause enhanced proliferation rates.[3,4] Then receptor tyrosine kinases (RTKs) gained certain interest because of their central role in various cellular pathways of regulating cell growth and apoptosis.[5-7] Such RTKs can be found either overexpressed in cancer cells or overactivated by the production of endogenous ligands.[6,8,9] After binding to the extracellular region of such a RTK the membrane-located receptor half undergoes a dimerization with a second neighboured receptor half to give a functional unit which proceeds in an intracellular phosphorylation of amino acid residues.[5,6] After such an autophosphorylation cell signaling proteins dock at the phosphorylated RTK binding site and are phosphorylated to mediate further cellular processes.[6,10]

One central target structure for anticancer therapies became the epidermal growth factor receptor (EGFR) tyrosine kinase.[11,12] EGFR can be activated by various ligands and plays a role in enhanced cell proliferation, escape from apoptosis that ensures a cancer cell survival and, finally, in aggressive invasive tumor growth.[11,12] So EGFR became a central target structure for the development of inhibitors as potential anticancer drugs.[13] Early inhibitors have been small-molecule inhibitors which bind to the ATP-binding site so that the intracellular phosphorylation of the EGFR tyrosine residues is blocked.[14,15] Meanwhile, resistances against such inhibitors have been described which result from amino acid

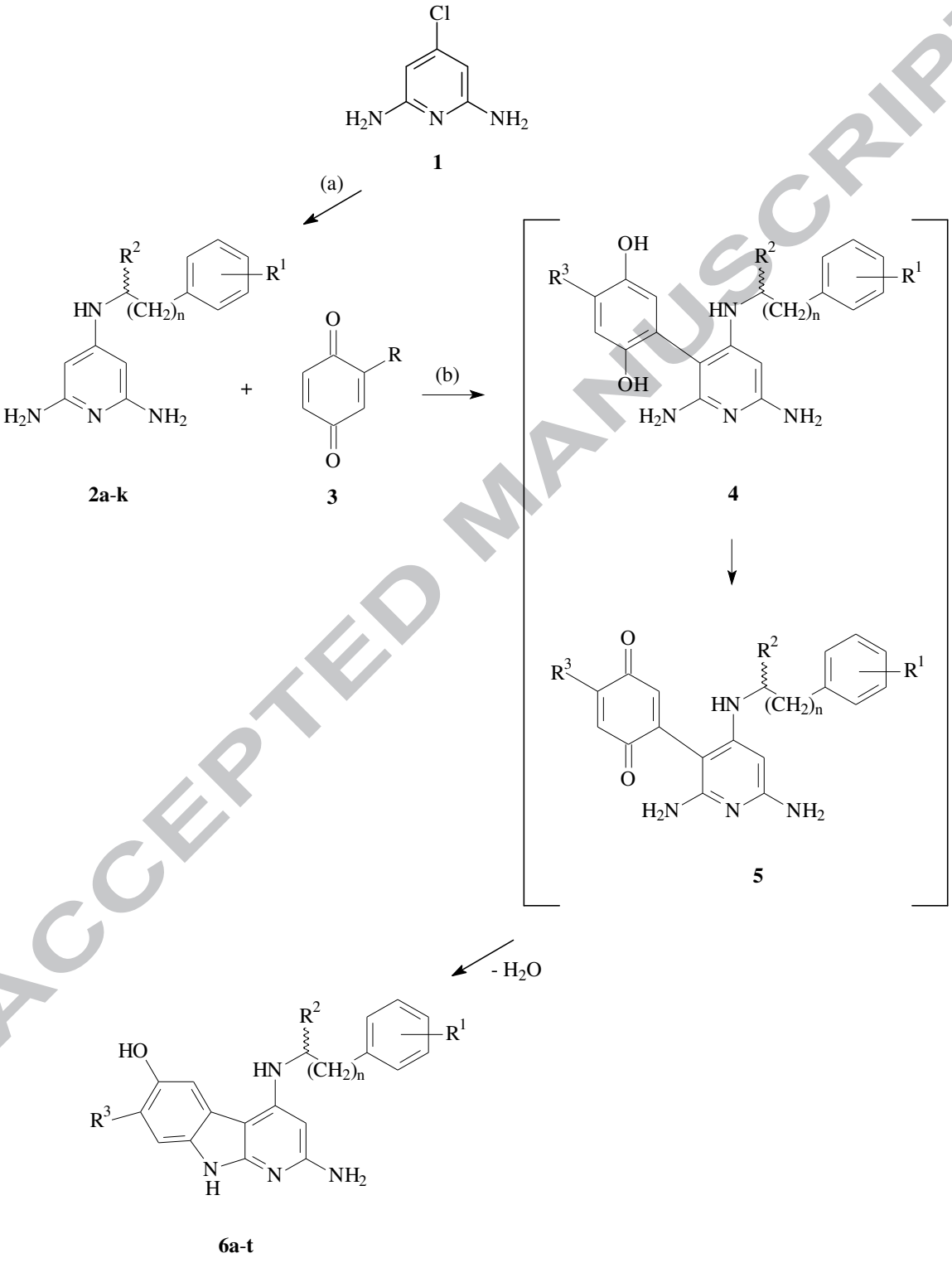
mutations within the encoding EGFR gene.[16,17] While single muations like L858R have been tolerated by the inhibitors, certain mutations in the exon 20 gene region led to inhibitor resistances, mainly the T790M mutation or a combined L858R_T790M mutation.[18,19] So prominent marketed inhibitors are affected from such mutations like erlotinib which reversibly binds to EGFR contrasting novel inhibitors like afatinib which bind to EGFR in an irreversible manner.[19,20]

Erlonitnib owns a quinazoline scaffold which can be found in several marketed EGFR inhibitors.[21] Due to the increasing resistance developments against the established EGFR inhibitors there is a strong demand for novel lead structures.[22] While the pyrimidine part of the quinazoline core and the attached 4-anilino residue were maintained in all recent alternative structures the attached phenyl residue has been exchanged with a five-ring heterocycle.[23,24] Merely micromolar activites have been reported for all those alternative structures as EGFR inhibitors without a selectivity of kinase inhibition.

We synthesized a series of novel benzo-anellated pyrrolopyrimidines as potential EGFR inhibitors with a varied 4-benzylamino function. We evaluated the EGFR inhibiting properties of our novel compounds and additionally investigated their potential to inhibit the vascular endothelian growth factor (VEGFR) 2 that supports the invasive tumor growth by enforcing tumor-angiogenesis.[25] Moreover, recent findings of VEGFR2 in many solid tumors made it a most interesting target structure for anticancer therapy.[26-28] So we identified dual inhibitors of both EGFR and VEGFR2 which have been evaluated in first cellular anticancer studies.

Our target structures **6a**-**t** resulted from the reaction of the 4-benzylamino varied compounds **2a**-**k** with the respective *p*-benzoquinone **3** in a solution mixture of dried ethanol and acetic acid (4/1) under reflux conditions.[29] The 4-substituted starting compounds **2a**-**k** were yielded from the reaction of the 2,6-diamino-4-chloropyrimidine **1** with the respective varied benzylamine at a temperature of 135 °C after 2-4 h.[30] The suggested mechanism for the

target compound formation started with a primary Michael addition of both starting compounds to give the 5-substituted pyrimidine intermediate **4**.



Scheme 1. Reagents and conditions: (a) varied benzylamine, 135 °C, 2-4 h, yields: 48-80%; (b) reflux, 3-5 h, ethanol, acetic acid, yields: 2-22%.

Then the hydroquinone substructure was oxidized by the remaining excess of the original benzoquinone **3** to give the quinone substructure in compound **5**. Finally, a cycloaddition reaction took place under elimination of water to result in the benzo-anellated derivative **6** given in an average yield of 14%.

The protein kinase inhibitory activity has been determined in an ATP competition assay for different inhibitor concentrations.[31] The reduced phosphorylation of a respective kinase substrate under inhibitor application has been determined with scintillation measurements counting the radioactive incorporated phosphorus.

Table 1. Protein kinase inhibiting data of target compounds **6a**-**t**.

					K_i [µM]	
	n	R^1	R^2	R^3	EGFR	VEGFR2
6a	0	-	H	H	0.70	2.76
6b	0	-	H	Me	0.64	4.83
6c	0	-	H	MeO	0.61	13.41
6d	0	-	H	Br	0.37	n.a.[a]
6e	0	-	H	Cl	0.27	n.a.[a]
6f	0	4-Me	H	H	0.09	3.26
6g	0	4-Me	H	Me	5.67	n.a.[a]
6h	0	4-OMe	H	H	0.12	n.d.[b]
6i	0	4-OMe	H	Me	5.95	n.d.[b]

6j	0	4-Cl	H	H	0.08	3.24
6k	0	4-Cl	H	Me	0.11	n.a.[a]
6l	0	3-OMe	H	H	1.26	3.99
6m	0	2-OMe	H	H	1.87	0.85
6n	0	2-OMe	H	Me	4.03	7.24
6o	0	3-Cl	H	H	0.29	n.d.[b]
6p	0	3-Cl	H	Me	0.90	n.d.[b]
6q	0	3,4-OMe	H	H	3.68	11.07
6r	1	-	H	H	0.64	4.47
6s	0	-	*(R)*Me	H	0.91	1.93
6t	0	-	*(S)*Me	H	0.52	4.15

[a] not active.

[b] not determinable.

We found a submicromolar affinity towards EGFR for the 4-benzylamine substituted compound **6a**. Then we introduced a methyl function into the 7-position of the molecular scaffold in derivative **6b**. The affinity was almost similar to that of the unsubstituted scaffold in compound **6a**. A methoxy function instead of the methyl group in derivative **6c** also resulted in an almost unchanged affinity. If halogen atoms were placed into the 7-position we found improvements in the affinity. The bromo substitution in compound **6d** led to an almost twofold increase in affinity and the chloro substitution in derivative **6e** resulted in the best affinity of those 4-benzylamine substituted compounds with a further molecular scaffold substitution.

We then introduced a methyl function into the 4-position of the benzylamino residue in compound **6f**. We found a main increase in affinity reaching nanomolar ranges of 90 nM

similar to the reversibly binding erlotinib for which 45 nM have been determined. If that 4-methly benzylamino substitution was combined with a 7-methyl function at the molecular scaffold in compound **6g** the affinity was mainly lowered and was also almost tenfold lower than that of the only 7-methyl substituted compound **6b**. If the 4-methyl benzylamino function was replaced with a methoxy function in derivative **6h** the affinity was similar. For the combination of the 4-methoxy benzylamino substitution with the additional 7-methyl group at the molecular scaffold in derivative **6i** we found the same tendency of a reduced affinity if compared to the 4-methyl benzylamino substituted derivatives **6f** with the nanomolar affinity and the additionally 7-methyl substituted compound **6g** with main decreases in the EGFR affinity.

Next we investigated the influence of a 4-chloro substitution in the benzylamino residue in derivative **6j**. The compound showed the best affinity of 80 nM for the only 4-benzylamine substituted compounds. The introduction of a methyl function into the 7-position of the molecular scaffold in compound **6k** led to an almost similar affinity with only a slight reduction.

We then varied the substituent position within the benzylamino residue. A methoxy function in the 3-position of the benzylamino residue in compound **6l** led to a tenfold lower affinity towards EGFR if compared to the 4-positioned residue in derivative **6h**. If placed in the 2-position of the benzylamine residue in derivative **6m** the affinity was further lowered. The combination with the additional 7-methyl function in compound **6n** was not favourable with a mainly lowered affinity.

When the 4-chloro substituent in the benzylamine residue of derivative **6j** moved to the 3-position the affinity was found decreased in derivative **6o**. If combined with the 7-methyl functions in compound **6p** a further affinity reduction was observed. We also investigated one disubstituted benzylamine compound. The 3,4-dimethoxy benzylamine derivative **6q** showed

a mainly decreased affinity if compared to the both monomethoxy substituted derivatives **6h** and **6l**, respectively.

Next we varied the benzyl residue first with a phenylethyl residue. The elongation of the 4-amino substituent in compound **6r** was not of favour with an almost similar activity than that of the benzylamine derivative **6a**. The additional (*R*) methyl function in compound **6s** was less favourable, whereas slight improvements in the affinity resulted for the (*S*) methyl function in compound **6t**.

If we summarize the results of our substituent-dependent affinities towards EGFR so far, it can be stated that additional molecular scaffold substitutions were not favourable if combined with substituted benzylamine residues. Best nanomolar affinities resulted for the benzylamine derivatives substituted in the 4-position. Both 3- and 2-substituted benzylamine compounds showed lowered EGFR affinities for the respective substituents. A benzylamine disubstitution is not favourable and changes in the 4-aminoalkyl chain partly influenced the EGFR affinity with slight decreases or increases, respectively.

The docking results with EGFR[32] showed that the benzo-anellated pyrrolo[2,3-*b*]pyridine scaffold is mimicking the adenine ring of ATP and represents the hinge-binding motifs. They also showed that potent inhibitors (e.g. **6f** and **6h**) are able to fom two hydrogen bonds to the hinge region residues: one to the *N*H of Met793 and one to the CO of Gln791. In case of **6f** and **6h** the 4-methyl and 4-methoxy substituted benzyl group is interacting with the hydrophobic pocket nearby Leu788 (Figure 1A). If the 4-methoxy or methyl substitution on the benzylamino ring is combined with a methyl substitution on position 7 a dramatic loss of activity is observed. The docking of **6i** to EGFR showed that the 7-methyl group is facing into the solvent region which is unfavourable (Figure 1B). In case of the compounds without a 4-methoxy/methyl benzylamino group (e.g. **6b**) the pyrrolo[2,3-b]pyridine scaffold and the 7-methyl substitutent are more buried which might explain the similar activities of **6a** and **6b.**

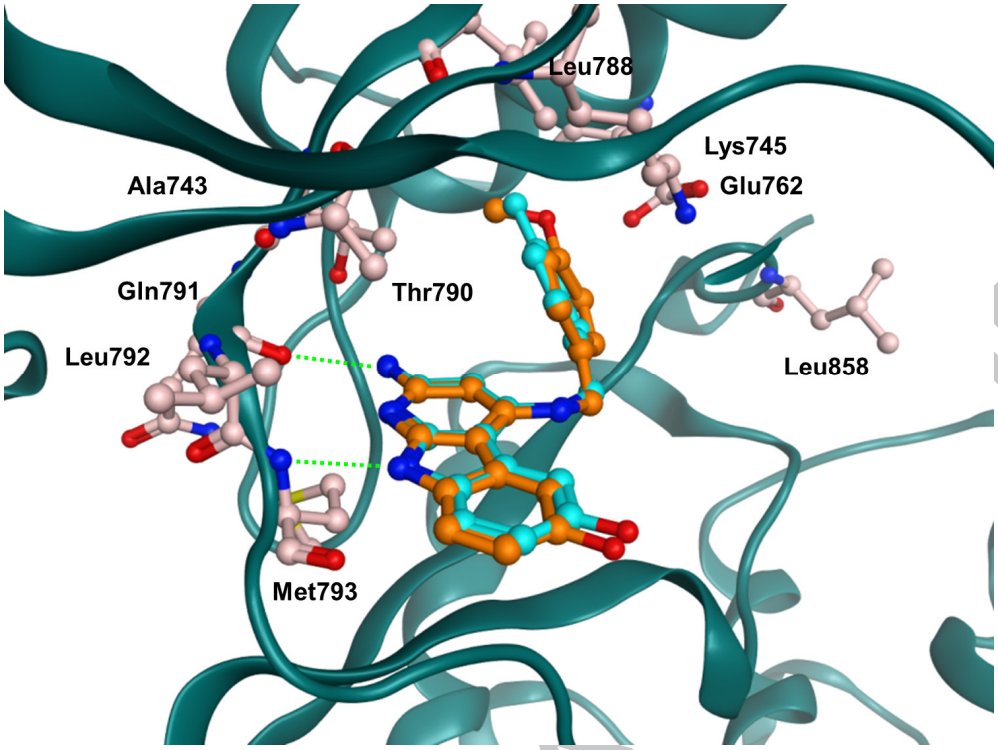


Figure 1A. Interaction of the most active EGFR inhibitors **6f** (cyan carbon atoms) and **6h** (orange carbon atoms) at the active site of EGFR. The substituted benzyl ring is completely buried in a hydrophobic pocket between Thr790 and Leu788. Hydrogen bonds are shown as dashed lines. Only relevant amino acids are displayed.

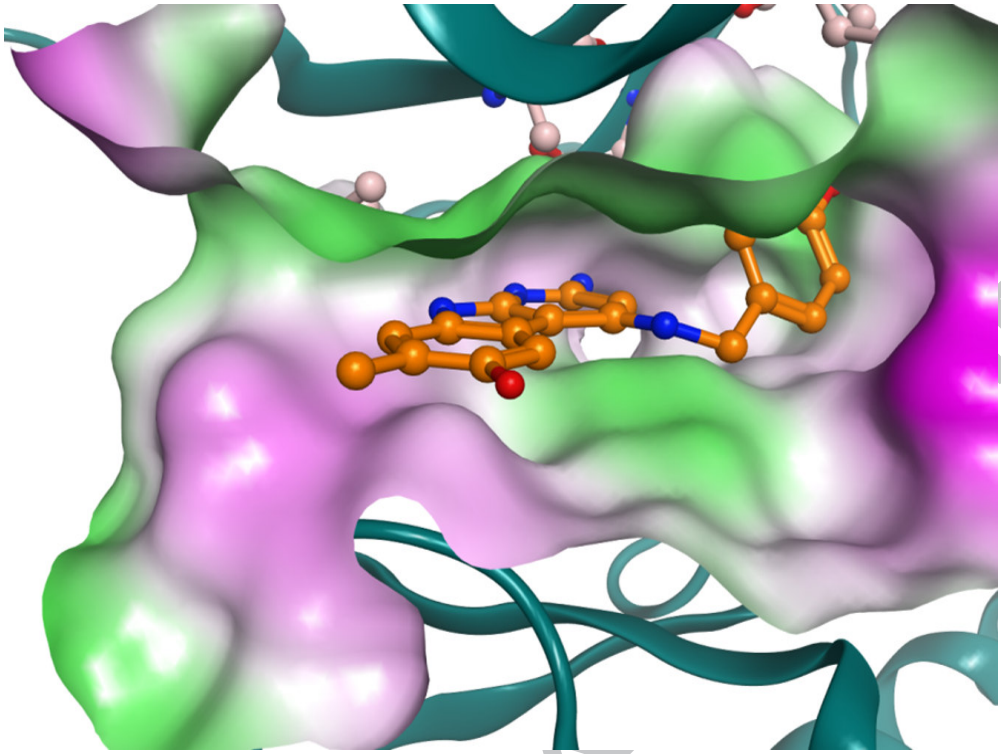


Figure 1B. Docking result for the less active inhibitor **6i** (orange carbon atoms) at EGFR. The 7-methyl group is facing into the polar solvent region resulting in unfavourable interactions. The molecular surface of EGFR is displayed and colored according to the hydropbhobicty (polar region = magenta, hydrophobic region = green).

With first identified nanomolar EGFR inhibitors of various substitution patterns we decided to determine the VEGFR2 affinities of our compounds to estimate their potential for further anticancer studies of cell proliferation. VEGFR2 not only supports the EGFR activity in tumor growth with activity in angiogenesis, but is also found in many solid tumors like bladder cancer, breast cancer, lung cancer and prostate cancer according to recent studies.[28,33] The role of VEGFR2 in such solid tumors is under discussion and indications have been found that VEGFR2 is envolved in metastasis of such tumors. So VEGFR2 became an interesting target structure either for combined therapies to address both EGFR and VEGFR2 or to address VEGFR2 alone.

Within our first compound series **6a**-**6e** with a 7-substitution of the molecular scaffold and an unsubstituted 4-benzylamine residue we found best affinities towards VEGFR2 for the 7-unsubstituted derivative **6a** with a lower micromolar affinity. The 7-methyl function caused a decreased compound affinity and the 7-halogen substituted compounds **6d** and **6e** were not active as VEGFR2 inhibitors. The 7-methoxy function in compound **6c** was also unfavourable. The 4-methyl function in the benzylamine compound **6f** decreased the affinity if compared to the unsubstituted derivative **6a** and the additional methyl function in 7-position of the molecular scaffold led to a loss of VEGFR2 affinity. Similar results were found for the 4-chlorobenzylamine function in derivative **6j** with decreases in the VEGFR2 affinity if compared to the unsubstituted compound **6a** and a loss of affinity for the additionally 7-methly substituted derivative **6k**.

A methoxy function in the 3-position of the benzylamine residue in compound **6l** led to a lower micromolar affinity towards VEGFR2. If moved to the 2-position of the benzylamine in compound **6m** the affinity mainly increased reaching submicromolar ranges with a K_i value of 0.85 µM. An additional methyl function in the 7-position of the molecular scaffold in derivative **6n** was unfavourable with main decreases in the VEGFR2 affinity.

A dimethoxy substitution of the benzylamine residue in compound **6q** was also unfavourable if compared to the monomethoxy substitution in compound **6l**.

Within the series of 4-aminoalkyl side chain varied derivatives **6r**-**t** we found best affinities for the (*R*) methyl substituted compound **6s** being more active than the only benzylamine substituted compound **6a**. The two other derivatives **6r** and **t** showed similar reduced affinities.

Next we carried out a first selectivity screening of protein kinase inhibition for compound **6g** which was not active as VEGFR2 inhibitor and showed a sufficient solubility in the used protein kinase inhibition assays even at higher concentrations > 100 µM. We tested against protein kinases of all the protein kinase families. We determined no activity against MARK1

and DAPK1 from the cAMK familiy and against MEK1 and MEKK2 of the STE family with IC_{50} values > 100 µM. The compound was not active as PKCα and as DMPK inhibitor, again with IC_{50} values > 100 µM. Both kinases belong to the AGC kinase family again. Similar IC_{50} values were determined for the inhibition of CK1α and Wee1 of the CK1 family and for ALK1, MLK4 and, finally, for IRAK of the TKL familiy. We found no activity against c-Abl and JAK2 of the TK family with IC_{50} values > 100 µM. Finally, we determined the activity against CDK1 and CDK2 of the CMGC family and found a residual activity with values of 61 µM for CDK1 and of 48 µM for CDK2.

High expression rates of EGFR and its activating ligands as tumor-inducing agents have been recently reported to occur mainly in breast cancer and non-small lung cancer cells.[11] EGFR was found as tumor-relevant growth factor in breast cancer, lung cancer and bladder cancer.[27-29] So we selected relevant cancer cells lines of breast cancer and non-small lung cancer to initially estimate the potential of our compounds to act as antiproliferative agents in anticancer tumor growth. In these studies 10 µM of the selected inhibitors were used and the growth inhibition of the relevant tumor cells was determined in the sulforhodamine assay at the National Cancer Institute of Health (NCI).[34,35]

Table 2. Anticancer cell screening data of NCI selected compounds.

	Growth inhibition [%]		
	6e	**6j**	**6m**
Breast cancer			
MDA-MB-231/ATCC	34	65	73
MDA-MB-468	28	71	55
BT-549	21	24	37
HS 578T	16	33	41
Non-small lung cancer			
HOP-92	19	57	66
HOP-62	20	31	63
NCI-H460	5	25	82

[a] not determined.

The NCI selected one submicromolar EGFR inhibitor which showed no VEGFR2 affinity (**6e**), one nanomolar EGFR inhibitor with a VEGFR2 affinity in low micromolar range (**6j**) and, finally, one low micromolar active EGFR inhibitor with a submicromolar VEGFR2 affinity (**6m**) for first anticancer screening studies. The determined compound growth inhibition activities in respective tumor cells are shown in table 2.

Comparing all the anticancer screening cell line results for the selected compounds we found the lowest activity for the only EGFR-inhibiting compound **6e** with best results in the relevant breast cancer cell lines ranging from 16 to 34%. The activity was similar to that of

gefitinib for which a 20% growth inhibition has been reported in the breast cancer cell line HS 578T at a concentration of 4 μM. Our nanomolar active EGFR inhibitor **6j** with a low micromolar VEGFR2 affinity showed increased activities in the EGFR-relevant breast cancer cell and all non-small lung cancer cell lines. Surprisingly, our submicromolar VEGFR2 inhibitor **6m** with only a low micromolar EGFR affinity showed best growth inhibition rates for three of the breast cancer and for all of the non-small lung cancer cell lines.

Novel lead compounds as EGFR inhibitors are demanded alternatively to the established quinazoline derivatives. We discovered benzo-anellated pyrrolopyrimidines as novel class of receptor tyrosine kinase EGFR inhibitors and identified first nanomolar active compounds which showed a favourable 4-substitution of the 4-benzylamino residue. Furthermore, we determined the VEGFR2 affinities of our compounds and identified first dual inhibitors of EGFR and VEGFR2. Our dual inhibitors were initially screened at the NCI for anticancer cell growth and proved to be effective in selected EGFR- and VEGFR2-cancer relevant cells. The most active VEGFR2 inhibitor showed the best anticancer growth as far as investigated. The results reveal the importance of VEGFR2 inhibition for antiproliferative cancer cell growth in respective VEGFR2-relevant tumors. With first kinase inhibition data of selectivity we found perspective lead compounds for further drug development studies.

Acknowledgement

Financial support of the work was given from the Deutsche Forschungsgemeinschaft (DFG) within the project HI 687/10-1 to Andreas Hilgeroth and to Christoph Ritter within the DFG projects RI 1196/4-1 and, finally, to Tim Fischer as fellowship of the graduate support program of the country saxony anhalt.

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[29] General procedure for the formation of the 4-benzylamino substituted benzo-anellated pyrrolopyrimidines **6**: 2-5 mmol of the respective 4-substituted diaminopyrimidine **2** were dissolved in a mixture of dried ethanol (20 mL) and acetic acid (5 mL). Then 1.8 equiv. of the respective quinone **3** were added and the solution was refluxed for 3-5 h. The reaction procedure was followed with tlc and after finishing the solvent was removed in vacuum. The raw product was purified by column chromatography using silica gel and an eluent mixture of chloroform and methanol in a relation of 95 to 5 that changed to 90 to 10 during column separation. The unified product fractions were evaporated and the remaining solid was recrystallized from dried chloroform. *2-Amino-4-(benzylamino)-9H-pyrimido[4,5-b]indole-6-ol* ***6a***. Yield 0.168 g (22%); grey solid; mp 253-265 °C; ^{1}H NMR (dmso-d_6) δ 5.32 (s, 2H, CH_2), 5.94 (br, 2H, 2-NH_2), 6.51 (br, 1H, NH), 6.58 (dd, *J* = 8.3, 2.3 Hz, 1H, 7-H), 7.01 (d, *J* = 8.3 Hz, 1H, 8-H), 7.15-7.26 (m, 5H, phenylic H), 7.40 (d, *J* = 2.3 Hz, 1H, 5-H), 8.73 (br, 1H, OH), 11.92 (br 1H, 9-H); MS (ESI, cation), *m/z* = 306 [M+H$^+$]. IR (KBr): 3460, 3342, 3065, 3034, 2929, 1641, 1620, 1496, 1460, 1595, 1567, 1452, 1415, 1360 cm^{-1}. Elemental Anal. calcd. (%) for $C_{17}H_{15}N_5O$: C, 66.87; H, 4.95; N, 22.94. Found: C, 66.83; H, 5.25; N, 22.87.

[30] General procedure for the formation of the varied 4-benzylamino substituted diaminopyrimidine **2**: 10 mmol of the 2,6-Diamino-4-chloropyrimidine **1** were

mixed with 30 mmol of the benzylamine and heated to 135 °C for 2-4 h. The reaction was controlled by tlc and stopped when no more of the starting compound **1** could be detected by tlc. After cooling to room temperature the raw product was purified using column chromatography on silica gel and an eluent mixture of ethylacetate and methanol (95/5) that switched to a relation of 90/10. The resulting products of the collected and evaporated fractions were recrystallized from mixtures of methanol and diethylether. *N^4-benzylpyrimidine-2,4,6-triamine* ***2a***. Yield 1.56 g (73%); white solid; mp 144-146 °C; ^{1}H NMR (dmso-d_6) δ 4.27 (s, 2H, CH_2), 4.89 (s, 1H, 5-H), 5.38 (br, 2H, 2-NH_2), 5.59 (br, 2H, 6-NH_2), 7.11 (m, 1H, 4´-H), 7.17-7.21 (m, 4H, 2´-, 3´-, 5´-, 6´-H), 7.32 (br, 1H, NH); MS (ESI, cation), *m/z* = 216 [M+H^+]; Elemental Anal. calcd. (%) for $C_{11}H_{13}N_5$: C, 61.38; H, 6.09; N, 32.53. Found: C, 61.05; H, 5.85; N, 32.33.

[31] The measuring of protein kinase activity was performed in 96-well FlashPlates™ in a 50 µL reaction volume. The reaction mixture consisted of 20 µL of assay buffer solution, 5 µL of an ATP solution in water, 5 µL of the used test compound in a 10% dmso solution and finally a premixture of each 10 µL of used substrate and enzyme solutions. The assay buffer solution contained 70 mM of HEPES-NAOH pH 7.5, each 3 mM of magnesium chloride and manganese(II) chloride, 3 µM of sodium orthovanadate, 1.2 mM of DTT, 50 µg/mL of PEG_{20000} and finally 15 µM of [γ-^{33}P]-ATP making approximately 7 x 10^5 cpm per well. The final kinase concentration had been 20 ng/50 µL for EGFR and VEGFR2. The used substrate was $Poly(Glu,Tyr)_{4:1}$ in a concentration of 125 ng/50 µL.The reaction mixtures were incubated at 30 °C for 60 min. The reaction was stopped with 50 µL of a 2% (v/v) solution of phosphoric acid. Then the plates were aspirated and washed twice with 200 µL of water or a 0.9% solution of sodium chloride. The incorporation of ^{33}Pi was determined with a microplate scintillation counter. Ten different inhibitor concentrations were measured

in a range of 3 nM to 100 µM. The residual activity (%) and the IC_{50} values were finally calculated. From the IC_{50} values the affinity constants K_i were determined using the equation: $IC_{50} = 1/2\ [E_t] + K_i \times (1 + [S] / K_m)$.

[32] The 3D structure of the kinase EGFR was taken from the Protein databank. We took from the available X-ray structures the ones which were co-crystallized with an inhibitor structurally most similar to the benzo-anellated pyrrolo[2,3-b]pyridines under study (PDB ID 2ITY). The protein structures were prepared as follows: water molecules and bound small molecules were deleted, hydrogen atoms were added and the resulting structures were minimized using the MMF94 force field and the conjugate gradient method until a gradient of 0.01 kcal/mol was reached. We used the docking program GOLD 5.2 (Cambridge Crystallographic Data Centre, Cambridge UK) to dock all molecules into the ATP binding site of EGFR. For all docking runs the program default settings were used. The binding site was defined on the gatekeeper residue with a radius of 15 Å. Goldscore was chosen as fitness function. For each molecule 30 docking runs were performed.

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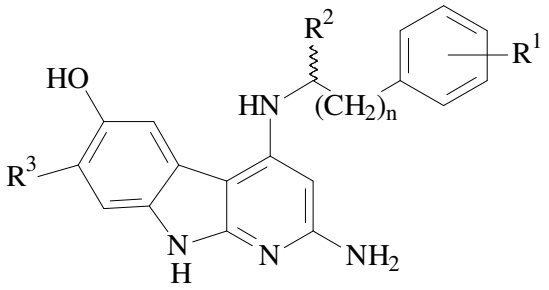

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