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Discovery of 4-anilino α -carbolines as novel Brk inhibitors

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ABSTRACT

Dysregulation of cell signalling processes caused by an enhanced activity of protein kinases mainly contributes to cancer progression. Protein kinase inhibitors have been established as promising drugs that inhibit such overactive protein kinases in cancer cells. The formation of metastases, which makes a therapy difficult, remains a great challenge for cancer treatment. Recently, breast tumor kinase (Brk) was discovered as novel and interesting target for a cancer therapy because Brk participates in both cell dysregulation and metastasis. We discovered 4-anilino substituted α -carboline compounds as a novel class of highly active Brk inhibitors. In the current work, structure–activity relationships are discussed including docking results obtained for 4-anilino α -carbolines. A first profiling of selective kinase inhibition and a proof of concept for the antiproliferative effects is demonstrated. These results qualify the compounds as a promising class of novel antitumor agents.

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The understanding of cell signalling processes in the last decades led to the identification of many protein kinases which were found dysregulated in the various types of cancer.^{1–4} Being either overexpressed or overactivated such protein kinases cause increased cancer cell proliferation or antiapoptotic effects resulting in progressive tumor growth. Protein kinase inhibitors have been established as effective therapeutics for several forms of cancer.^{5–} ⁷ However, they partly cause side effects because they do not only inhibit protein kinases in cancer cells but also the protein kinases found in normal cells.^{8–10}

Therefore, it would be helpful to identify protein kinases in a cancer cell that mainly contribute to the dysregulation to start a personalized cancer treatment. Breast tumor kinase (Brk) was identified as a non-receptor protein kinase. The cDNA of Brk was originally cloned in a screen of tyrosine kinases which were found expressed in a metastatic breast tumor.¹¹ Brk occurred in a majority of breast tumors.^{11,12} However, normal tissues show only low and undetectable amounts of Brk.^{12,13} Thus, Brk became an ideal target protein for a potential tumor therapy because selective targeting of Brk promises no critical side effects for normal cells.

Meanwhile, several cellular substrates of Brk have been identified including RNA-binding proteins, transcription factors and cell signalling molecules.^{14–20} Thus, Brk is discussed to increase oncogenic signal events by phosphorylation of members of the ErbB family.²¹ Additionally to a potential role in cancer progression Brk is discussed to support the survival of detached cancer cells by preventing an autophagic cell death.²² As a consequence Brk may play an important role in tumor metastasis because of its contribution to cellular survival.

β-Carbolines have recently been published to act as protein kinase inhibitors.²³ We have been interested to develop related α-carbolines and to investigate their inhibitory effects for different kinases. We discovered novel 4-anilino α-carbolines in screening studies as novel class of Brk inhibitors. A first profiling of selectivity towards other protein kinases is presented and structure–activity relationships are discussed. First nanomolar inhibitors have been investigated in first breast cancer cell studies. They demonstrate a successful antiproliferative activity as a result of a Brk inhibition.

The synthesis started from benzotriazole **1** which was treated with 2-bromopyridine in toluene under reflux conditions to give the pyridin-2-yl benzotriazole **2**. Compound **2** was heated in polyphosphoric acid to cyclize to the α -carboline scaffold **3** (Scheme 1).²⁴

The 4-position of compound **3** was activated for the following 4-chlorination reaction by *N*-oxide formation in boiling acetic acid using hydrogen peroxide solution. The 4-chlorination reaction succeeded with phosphorus(V) oxychloride at room temperature. Then the different aniline substituents were introduced with an excess of the respective aniline in a *N*-methylpyrrolidone solution under reflux heating.







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Scheme 1. Reagents and conditions for the preparation of compounds: (a) toluene, reflux, 18 h, 97%; (b) polyphosphoric acid, 160–175 °C, 3 h, 47%; (c) hydrogen peroxide, acetic acid, reflux, 6.5 h, 73%; (d) phosporus oxychloride, dimethyl formamide, rt, 24 h, 71%; (e) aniline, *N*-methylpyrrolidone, reflux, 6–36 h, 28–85%.

We first introduced substituents into the 2-position of the aniline residue. Compound $4a^{25}$ with a 2-methyl aniline function showed no inhibitory activity towards Brk. We then replaced the 2-methyl group with an annelated 2,3-phenyl residue in compound **4b**. We found a Brk inhibition with an IC₅₀ value of 43.5 nM, which strongly indicated that the substitution of the 3-position of the aniline residue would be more promising compared to the sole 2-position (Table 1).

The bulky and hydrophobic trifluoromethyl substituent instead of the phenyl residue in compound **4c** led to a slight decrease in activity with an IC₅₀ value of 59.4 nM. A lipophilic thioether function at the 3-position was found unfavourable because the respective compound **4d** showed no Brk-inhibitory activity. We then replaced the thioether function with a chloro substituent with less space demand and the potential to undergo halogen bonding. The resulting 3-chloro compound **4e** showed a strong increase in inhibitory activity with an IC₅₀ value of 4.75 nM. Thus, a first highly active compound was identified among the 4-anilino α -carbolines.

Subsequently, docking studies were performed for the most active Brk inhibitors to analyze the potential binding mode. The compounds were docked into the ATP-binding pocket of a Brk homology model.²⁶ Docking results suggest a common binding mode for the 4-anilino α -carbolines, where the *N*H- and *N*-atoms

Table 1			
Brk inhibition	of target	compounds	4a-r

of the α -carboline scaffold participate in two hydrogen bond interactions with the hinge region, namely with backbone atoms of Met267. Additionally, the α -carboline and phenyl ring of these inhibitors are making a number of hydrophobic interactions with residues in the ATP binding site and the adjacent gatekeeper pocket. Besides the above mentioned interactions, the 3-chloro group of inhibitor **4e** is suggested to interact with the carbonyl oxygen of lle262, acting as halogen bond donor. The putative binding mode of compound **4e** is shown in Figure 1.

The introduction of a 3-hydroxy function which is also able to make a hydrogen bond to Ile262 led to best activities of the respective compound **4f** with an IC₅₀ value of 3.15 nM. We then produced various 3-alkoxy aniline derivatives **4g**–**i** to evaluate a combination of favourable hydrogen bonding and observed hydrophobic substituent effects on Brk inhibition.

The methylated 3-hydroxy derivative **4g** was inactive similar to the 3-thioether compound **4d**. The elongation of the methyl side chain by one methylene group led to an IC₅₀ value of 155 nM for compound **4h**. This increased activity was as a result of the partly increased substituent lipophilicity. A benzylation of the 3-hydroxy function in compound **4i** further increased the activity with a resulting IC₅₀ value of 40.7 nM. This meant an activity similar to that of the 2,3-phenyl ring annelated compound **4b**.

When the most favourable 3-hydroxy group was replaced with a nitro function in derivative **4j** the activity maintained high with a resulting IC_{50} value of 5.65 nM.

We then moved the aniline mono-substitution from the favourable 3- to the 4-position of the aniline residue. The corresponding 4-methoxy derivative **4k** proved to be active with an IC₅₀ value of 190 nM. However, the 3-methoxy aniline compound **4g** has been found inactive as Brk inhibitor. The 4-chloroaniline derivative **4l** was found to be inactive. So it could be concluded that a sole 4-substituent is unfavourable compared to the related 3-substituted inhibitors.

The best activities among the series of monosubstituted aniline compounds were obtained for the 3-substituted derivatives with potential hydrogen/halogen bonding properties as suggested by the docking studies. However, lipophilic substituents also result in compounds with promising nanomolar activities.

The high sensitivity of the aniline substitution for the degree of Brk inhibition encouraged us to investigate combined substituent effects in the following disubstituted derivatives.

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Compound	\mathbb{R}^1	R ²	R ³	\mathbb{R}^4	IC ₅₀ value ^{a,b} (nM)		
4a	Me	Н	Н	Н	n.a. ^c		
4b	Н	-CH2-CH=CH-C	$H_2 - R^3$	Н	43.5		
4c	Н	CF ₃	Н	Н	59.4		
4d	Н	SMe	Н	Н	n.a. ^c		
4e	Н	Cl	Н	Н	4.75		
4f	Н	OH	Н	Н	3.15		
4g	Н	OMe	Н	Н	n.a. ^c		
4h	Н	OEt	Н	Н	155		
4i	Н	OBn	Н	Н	40.7		
4j	Н	NO ₂	Н	Н	5.65		
4k	Н	Н	OMe	Н	190		
41	Н	Н	Cl	Н	n.a. ^c		
4m	Н	Cl	Me	Н	75.6		
4n	Н	Cl	F	Н	69.5		
40	Н	Cl	OBn	Н	64.5		
4p	Н	Cl	Н	Cl	53.4		
4q	Н	OMe	Br	Н	4.41		
4r	Н	OEt	Br	Н	154		

 $^{\rm a}~$ IC_{50} value determination follow the below described protocols. $^{\rm 28}$

^b Standard errors are typically below 20%. In many cases standard errors below 10% are found.

^c n.a. = not active.



Figure 1. Docking solution of compound **4e** (cyan sticks) for Brk. The protein is shown as pale green ribbon and important residues of the ATP binding site are displayed. Hydrogen and halogen bonds between the inhibitor and the kinase are displayed as dashed lines.

First we combined the favourable 3-chloro substitution of the aniline residue with varying 4-substitutions. An additional lipophilic 4-methyl function in derivative **4m** decreased the compound activity to an IC₅₀ value of 75.6 nM. Similar results were found for the 4-fluoro derivative **4n** and the slightly more favourable 4-benzyloxy substitution in derivative **4o**. We also prepared one additionally 5-substituted compound with a 3,5-dichloro substitution of the aniline residue in derivative **4p**. Also this disubstitution was less favourable than the sole 3-chloro function with a resulting IC₅₀ value of 53.4 nM for the respective compound **4p**.

Then the combination of a 3-alkoxy compound with a 4-bromo substitution was investigated. The 3-methoxy anilino compound **4g** was found to be inactive. However, the additional bromo function in compound **4q** led to highest activities with an IC₅₀ value of 4.41 nM. If the 4-bromo substitution was combined with the 3-ethoxy function the activity remained unchanged compared to the sole 3-ethoxy function with an IC₅₀ value of 154 nM for derivative **4r**.

Thus, we identified highly potent Brk inhibitors and decided to determine the activity of the best 3-hydroxy aniline compound 4f towards kinases from all kinase families of the human kinome. With determined $IC_{50} > 10 \,\mu\text{M}$ we observed no activity against the kinases PIM2 of the PIM kinase family, NEK1 and 2 of the NIMA family, CAMK4, the isoforms MAPKAPK3 and 5 and MARK 1, 3 and 5 of the CAMK family. No activity was observed against the isoforms AKT1-3 and against DMPK of the AGC family, VRK1 of the CK1 family, against the isoforms MST3 and 4 and PAK1-3 of the STE family, against ERK1 and 2, GSK-3β, JNK1 and 3 and the HIPK isoforms 1-3 of the CMGC family. No activity resulted for JAK3 of the TK family and with IC_{50} values of $3.04\,\mu M$ and of $2.2\,\mu M$ against Src of the TK family and against ALK1 of the TKL family, respectively, the activity nearly reached the 10 µM which means activities of a factor of thousand higher than that of 3.2 nM as the determined Brk inhibitory activity of compound 4f.

This first protein kinase activity study including more than 30 kinases from all kinase families profiles the inhibitors of being

Table 2

Antiproliferative activity of compound class-representing derivatives **4f** and **4n** in various breast tumor cancer cell lines

Compound	GI ₅₀ value ^a (μM)			
	MCF7	HS-578/T	BT-549	
4f 4n	0.99 8.12	1.02 10.2	1.58 >100	

 $^{\rm a}\,$ GI_{50} value determination was carried out using the sulfur rhodamine assay of recent protocols. 29,30

selective and thus they prove to be promising anticancer drug candidates. We then determined the antiproliferative activity of two selected compounds in various breast cancer cell lines. Beside the involvement in signalling pathways Brk is discussed to play a role in tumor progression by the phosphorylation of tumor-relevant receptor tyrosine kinases of the ErbB family. It was suspected that an inhibition of Brk might reduce such a receptor-mediated proliferative effect in breast tumor cells where Brk has been found overexpressed. We selected our most active Brk inhibitor **4f** and determined the growth inhibition (GI) of three breast tumor cell lines under inhibitor application as shown in Table 2.

The highest antiproliferative activity was observed in the MCF7 cell line with a GI_{50} value of 0.99 μ M.²⁷ The activity in the BT-549 cell line was only less increased with a GI_{50} value of 1.58 μ M. Compound **4f** was more active compared to lapatinib that is used as protein kinase inhibitor in breast cancer therapy with a reported GI_{50} value of 2.0 μ M in the MCF7 cell line.

We then investigated the disubstituted 3-chloro and 4-fluoro substituted anilino derivative **4n** with reduced Brk inhibition of 69.5 nM as determined IC₅₀ value if compared to compound **4f**. The antiproliferative activity of derivative **4n** was found reduced in the MCF7 cell line with a GI₅₀ value of 8.12 μ M compared to that of derivative **4f** which means a similar range in the antiproliferative activity reduction as in the Brk inhibition compared to compound **4f**. A same tendency of both reduction of the antiproliferative activity and of the Brk inhibition was found in the HS-578/T cell line with an IC₅₀ value of 10.2 μ M. However, the activity of derivative **4n** in the third breast cancer cell line BT-549 was only residual. The best Brk inhibition that obviously correlates with the antiproliferative activity in the BT-549 cell line. Thus, we present a first proof of concept for a Brk inhibition that obviously correlates with the antiproliferative activity in relevant breast cancer cells.

In conclusion, the presented series of 4-anilino α -carbolines turned out as a highly promising class of anticancer agents. The Brk inhibition depends on the kind and positioning of the aniline substituents, which lead to nanomolar as well as to inactive inhibitors. The observed protein kinase inhibition profile documented a first selectivity of Brk inhibition. The correlation of Brk inhibition and mediated antiproliferative activity better than that of the reported lapatinib qualifies the new compound class for further preclinical studies.

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- Spectroscopical data of 4-anilino α -carboline compound class representing compound **4a**: Yield 0.206 g (61%); mp 215–218 °C; IR (ATR ν = 3422 (NH), 3252 (Arly-CH), 1692 (NH), 1593 (C=C) cm⁻¹; ¹H NMR (DMSO- d_6) δ = 11.52 (s, 1H, 9-NH), 8.15 (d, J = 7.9 Hz, 1H, 5-H), 8.00 (s, 1H, aniline NH), 7.94 (d, $\begin{array}{l} 7.31 \\ 7$ 273 (100, M⁺). Elem. Anal. Calcd (%) for C₁₈H₁₅N₃: C, 79.10; H, 5.53; N 15.37. Found: C, 78.88; H, 5.56; 15.48.
- Computational methods: Since there is no crystal structure available for Brk, the homology modeling methodology was used to predict the 3D structure. The sequence of human protein kinase Brk (residues 13-450) was retrieved from the UniProtKB repository (entry number Q13882). Following the NCBI Basic Local Alignment Search Tool (BLAST) guery for the search of template structure in PDB, it was identified that members of the Src kinase family share the highest sequence similarity (>50%) with Brk. Crystal structure of c-Src in the

active conformation (PDB code: 1Y57_A, resolution 1.91 Å) was chosen as a template as it shows the highest sequence identity with Brk (around 53%). The homology model was generated using MODELLER-9v11. The sequence alignment between Brk and c-Src was made using align2d in MODELLER. Hydrogen atoms and partial charges were assigned and the protein structure was subjected for energy minimization in implicit solvent with RMSD deviation of maximum 0.3 Å using the OPLS-AA 2005 forcefield (Maestro 9.3, Schrödinger Inc.). The stereochemical analysis of Ramachandran plot with the PROCHECK program confirmed that this model is reasonable, showing 88.4% of the residues were in the favoured region, 11.3% were in the allowed region and only 0.3% was in the disfavoured region. All ligands were prepared using the LigPrep utility of the Schrödinger suite. MMFF force field was used for energy minimization. Possible ionization states of each ligand were generated at pH 7.4 by using the Ionizer module. Options to generate tautomers, stereoisomers and up to 10 low energy ring conformations were set on. Ligands were docked into the ATP-binding pocket of Brk using GOLD version 5.1. The center of the Brk binding site was set at Leu319 with a radius of 14 Å. Goldscore was chosen as fitness functions and rescoring with Chemscore was applied, since this protocol was validated on the available Src X-ray structures

- 27. Compared to the nanomolar Brk inhibition activity the cellular micromolar activity may be plausible by the differences of in vitro activity of a direct enzyme inhibition and the resulting cellular effects on proliferation which are a result of following cell regulating effects so that excellent in vitro activities consequently lead to lowered activities in a whole cellular system of growth regulation.
- The measuring of protein kinase activity was performed in 96-well 28 FlashPlates™ in a 50 µL reaction volume. The reaction mixture consisted of 20 µL of assay buffer solution, 5 µL of ATP solution in water, 5 µL of 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, each 3 mM of magnesium chloride and manganese(II) chloride, 3 µM of sodium orthovanadate, 1.2 mM of DTT, 50 µg/mL of PEG₂₀₀₀₀ and finally 1 μ M of [γ -³³P]-ATP making approximately 1.2 \times 10⁶ cpm per well. The $K_{\rm m}$ for ATP to Brk was determined with 4.08 μ M. The final Brk concentration has been 6.1 nM. The used substrate was Poly(Glu,Tyr)4:1 in an amount of 125 ng/50 µL. The reaction mixtures were incubated at 30 °C for 60 min. The reaction was stopped with 50 μ L of a 2% (v/v) solution of phosphoric acid. Then the plates were aspirated and washed twice with 200 µL of water or 0.9% solution of sodium chloride. The incorporation of ³³Pi was determined with a microplate scintillation counter. Ten different inhibitor concentrations were measured in a range of 3 nM-100 µM. The residual activity and the IC₅₀ values were finally calculated.
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