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Novel inhibitors of breast cancer relevant kinases Brk and HER2†

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Novel 4-anilino pyrido[2,3-*b*]indoles have been discovered as inhibitors of the breast cancer relevant protein kinase Brk. Within this first series favourable aniline substituents have been characterized. Combinations with substituents of the molecular scaffold have been further investigated and led to additional nanomolar Brk inhibitors. Due to the reported role of Brk in breast cancer progression *via* HER2 activation we determined the inhibition profile of our novel Brk inhibitors to additionally inhibit HER2. These studies characterized the first dually acting Brk and HER2 inhibitor and the first exclusive HER2 inhibitors.

Therapeutic treatment options in breast cancer have long been limited to those types of breast cancer which are mediated by addressing the estrogen receptor (ER).[1,2] Different from that a causative dysregulation of breast cancer cells by protein kinase activities may be limited by the use of protein kinase inhibitors.[3,4] Such protein kinase inhibitors have been established in the therapy of cancer with strong indications to each special type of cancer.[5,6]

Breast cancer cell dysregulation is known to be mediated by receptor tyrosine kinase (RTK) types with HER2 from the ErbB family playing an important role.[7,8] Monoclonal antibodies have been developed as hopeful inhibitors of such RTKs as they block the RTK activation by a competing binding with the original ligand of the RTK.[9,10] In breast cancer therapy trastuzumab has been introduced as a monoclonal antibody for such HER2-positive types of cancer.[11,12] The third group of breast cancer which is both ER and HER2-negative can hardly be treated with present therapeutics.[13]

So strong efforts have been made to identify novel target structures with relevance to breast cancer. Breast tumor kinase (Brk) was found in various breast tumors after its encoding cDNA has been cloned from a metastatic tumor.[14] Brk belongs to the non-receptor tyrosine kinases with occurrence in the cytoplasma. The role of Brk in various cell signalling processes is presently investigated.[15] Brk has been found to be overexpressed in breast tumor cells and has been discovered to potentiate the role of HER2 in tumor progression.[14,16,17] With a limited occurrence of high levels of Brk exclusively in tumor cells Brk is discussed as a promising target for future cancer therapies because of expected low side effects for normal cells under a Brk inhibition.[14–16] Furthermore, Brk is promising for the treatment of HER2 negative tumors which are known to be aggressive. Their metastatic properties may be enforced by Brk because Brk has been reported to prevent the autophagic death of detached cells and thus may favour metastasis progression.[18,19]

Recently, pyrido[3,4-*b*]indoles have been demonstrated to act as moderate protein kinase inhibitors.[20] We have been interested to study closely related pyrido[2,3-*b*]indoles. In our studies we identified novel 4-anilino pyrido[2,3-*b*]indoles as inhibitors of Brk. Structure–activity relationships of the Brk inhibition will be discussed. Beside aromatic substituents we varied the molecular scaffold substitutions to gain insight into favourable inhibition properties of the novel compound class. Additionally, we present HER2 inhibition data to select compounds for further breast cancer cell studies and to estimate the anticancer potential of the Brk inhibition alone and beside a HER2 inhibition in later studies.

The pyrido[2,3-*b*]indole **3** resulted from a polyphosphoric acid catalyzed cyclization reaction of the pyridin-2-yl benzotriazole **2** which was given by the reaction of benzotriazole **1** and 2-bromopyridine carried out in toluene under reflux.[21] The 4-chloro substitution in compound **4** was introduced with phosphorus oxychloride into the prepared N-oxide of the pyrido[2,3-*b*]indole after treatment with hydrogen peroxide in acetic acid (Scheme 1).[22]

The 4-chloropyrido[2,3-*b*]indole **4** was derivatized with the various aniline compounds in *N*-methylpyrrolidone (NMP) under reflux conditions. The first yielded target structures were evaluated as Brk inhibitors (Table 1).

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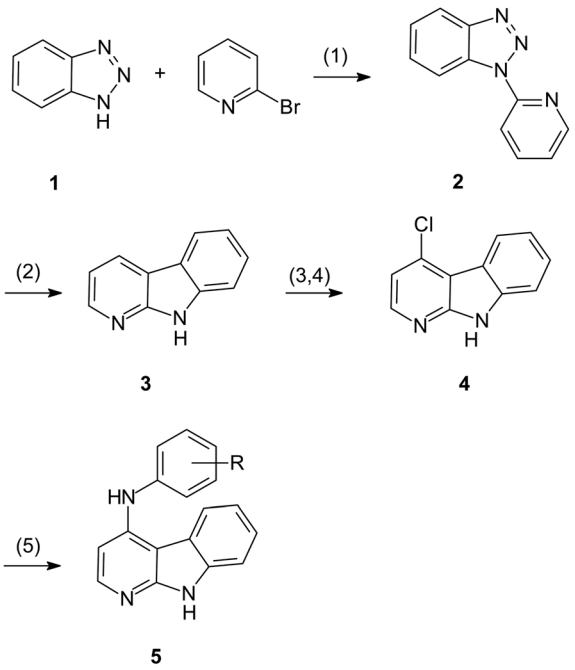


Scheme 1 Molecular scaffold formation and derivatization reaction to aniline target structure series **5**.

Table 1 Inhibitory properties towards Brk and HER2 determined as IC_{50} values of our only aniline substituted target compounds. **5a–d**, **10a–d**, **11a and b**, **12a–c**, **14a–d** and **16a–d**

		IC_{50} value[a]	
Compound	R	Brk (nM)	HER2 (nM)
5a	2-Me	n.a.[b]	978 ± 116
5b	3-OH	3.2 ± 0.26	1300 ± 169
5c	3-Cl	4.7 ± 0.06	66 ± 1.6
5d	4-Cl	n.a.[b]	310 ± 60
10a	OH	n.a.[b]	298 ± 88
10b	OMe	5.7 ± 0.06	1240 ± 311
10c	OEt	410 ± 14	54 800 ± 3564
10d	OBn	55 ± 17	28 300 ± 3960
11a	OMe	4.8 ± 0.55	390 ± 22
11b	OEt	26 ± 0.85	576 ± 71
12a	OMe	9.2 ± 0.04	689 ± 25
12b	OEt	479 ± 4.2	6530 ± 168
12c	OH	n.a.[b]	376 ± 49
14a	OH	n.a.[b]	29 ± 5.2
14b	OMe	186 ± 13	91 200 ± 16 416
14c	Cl	13 ± 0.42	1150 ± 84
14d	NO_2	4.1 ± 0.05	8830 ± 420
16a	OH	n.a.[b]	13 ± 1.3
16b	NO_2	21 ± 0.75	978 ± 115
16c	NH_2	7.6 ± 0.85	1640 ± 52
16d	Cl	13 ± 0.42	1150 ± 84

[a] Mean of two determinations. [b] Not active, IC_{50} value >1 000 000 nM.

The 2-methyl aniline derivative **5a** showed no inhibition of Brk, whereas the 3-hydroxy compound **5b** was highly active as Brk inhibitor with an IC_{50} value of 3.2 nM as discovered in previous screening studies.[23,24] Thus, it seemed that a 2-substitution of the aniline residue is less favourable because of possible steric interactions or a hindered rotability of the aniline residue. Moreover, we failed to synthesize a 2-chloro aniline compound for comparison which may also be plausible because of such steric interactions. Additionally, the reactivity of the aniline nitrogen might be reduced for the electrophilic substitution reaction with the 4-chloropyrido[2,3-*b*]indole **4**. Docking studies were performed for the studied 4-anilino pyrido[2,3-*b*]indole derivatives. Compounds were docked into the ATP-binding pocket of a recently generated homology model of Brk.[25] The derived docking results suggest a common binding mode for these types of inhibitors, where the NH- and N-atoms of the pyrido[2,3-*b*]indole participate in two H-bond interactions with hinge region residues (backbone atoms of Met267). Additionally, the pyrido[2,3-*b*]indole and phenyl rings are making hydrophobic interactions with residues in the ATP binding site and the adjacent gatekeeper pocket. The typical binding mode for compounds of this series is shown for the highly active Brk inhibitor **5b** in Fig. 1. Except the above-mentioned interactions, the 3-hydroxy group of **5b** makes an additional H-bond with Glu235 of Brk. The observations support the favourable effect of the 3-substitution for inhibition of Brk. In comparison, the 2-methyl aniline derivative **5a** or the 4-chloro derivative **5d** lacks such an interaction which could explain the loss of activity of these compounds.

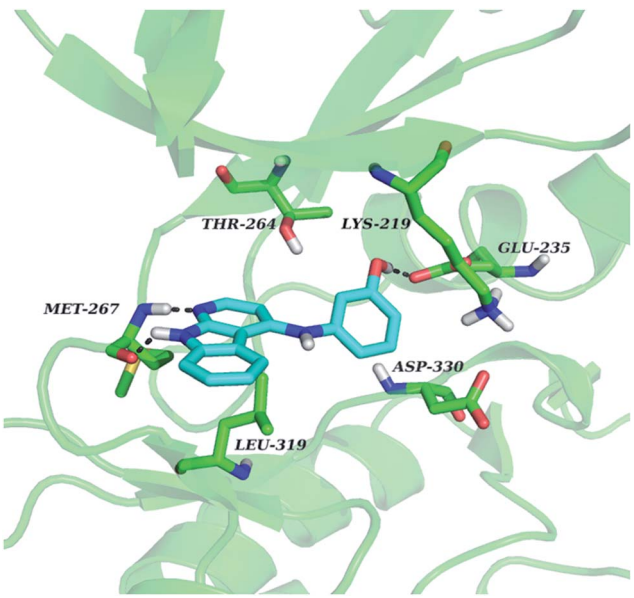


Fig. 1 Top-ranked docking pose of compound **5b** in the binding site of Brk. The protein backbone is represented as green ribbon. Interacting amino acids are displayed as green sticks. The inhibitor is shown with cyan sticks. Hydrogen bonds between inhibitor and Brk are displayed as dashed lines.

As the 3-hydroxy function may serve as both hydrogen bond donator and acceptor function we replaced it with a chloro function known to possibly act as hydrogen bond acceptor or in halogen bonding actions. The corresponding 3-chloro derivative **5c** was nearly as active as Brk inhibitor than the 3-hydroxy compound with a resulting IC_{50} value of 4.7 nM. We then moved the chloro substituent from the favourable 3- to the 4-position of the aniline residue. The resulting compound **5d** was found to be inactive similar to the 2-methyl aniline derivative **5a**. So we could state from the results of this first series of novel Brk inhibitors that the 3-position of the aniline residue is exclusively favourable for both hydrogen bond acceptor and donator functions.

We then substituted the molecular scaffold **4** in the 6-position with various sulfonamide functions. Such functional groups may serve as hydrogen bond acceptor functions favour the enhancement of the hydrophilic compound properties with respect to a better compound solubility. The docking solution for the 3-hydroxy aniline **5b** suggests that the phenyl ring of the pyrido[2,3-*b*]indole system is exposed to the solvent-accessible region of the ATP-binding pocket in Brk. So a hydrophilic substituent may be of favour in this position. Thus, we decided to test several compounds with different hydrophilic substituents at the 6-position of the pyrido[2,3-*b*]indole.

The following series of sulfonamide compounds was prepared by the reaction of the 4-chloropyrido[2,3-*b*]indole **4** with chlorosulfonic acid at room temperature to the 6-chlorosulfonic compound **6**. The 6-chlorosulfonic substituent then reacted with the various amine compounds morpholine, piperazine and *N*-hydroxyethylpiperazine at room temperature in THF to derivatives **7–9** (Scheme 2).

First we combined the morpholine sulfonamide compound **7** and a 3-hydroxy aniline residue in derivative **10a** by heating compound **7** and the aniline in NMP. Surprisingly, compound **10a** was inactive as a Brk inhibitor. So we wondered whether the 3-hydroxy aniline hydrogen bond donator function might be favourable in this substitution combination. We replaced the 3-hydroxy with a 3-methoxy function with an exclusive hydrogen bond acceptor function similar to the 3-chloro function. In the resulting compound **10b** the biological activity of Brk inhibition was restored with an IC_{50} of 5.7 nM. We then increased the alkyl ether residue with one methylene function in derivative **10c**. The compound was found to have a reduced activity with an IC_{50} value of 410 nM. However, a more lipophilic substituent in the 3-benzyloxy aniline compound **10d** improved the reduced activity with a determined IC_{50} value of 55 nM. So we concluded that if substituted at the 6-position of the molecular scaffold a hydrogen bond acceptor function with a partially lipophilic character may be more favourable than the hydrophilic 3-hydroxy function in the aniline residue.

A replacement of the morpholino with a piperazino residue in derivative **8** was carried out because the morpholino residue may easily be protonated under physiological conditions and thus increase the compound solubility. We prepared the 3-methoxy and the 3-ethoxy aniline derivatives **11a** and **11b**, respectively, of compound **8**.

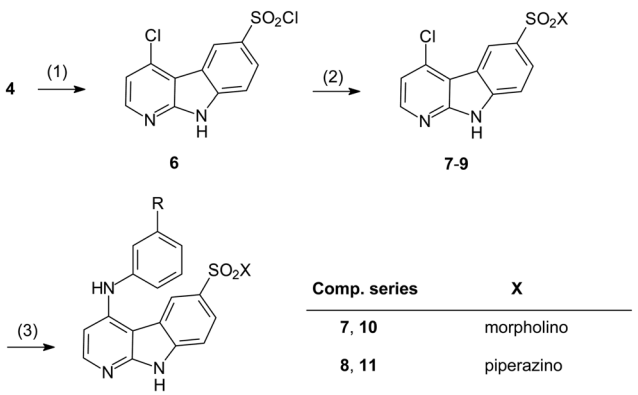
Similar to the 3-methoxy aniline compound **10c** the activity of **11a** was excellent with an IC_{50} value of 4.8 nM. The 3-ethoxy compound **11b** resulted in a partly reduced activity with an IC_{50} value of 26 nM.

Finally, we prepared the 3-methoxy and 3-ethoxy aniline derivatives **12a** and **12b** of the hydroxyethylpiperazine scaffold compound **9**. Similar to both discussed sulfonamide compounds the 3-methoxy aniline compound **12a** showed the highest activity with an IC_{50} value of 9.2 nM. The 3-ethoxy aniline **12b** compound showed a reduced activity with an IC_{50} value of 479 nM. For comparison we prepared the 3-hydroxy aniline derivative **12c** which was found as inactive as the corresponding morpholino sulfonamide derivative **10a**.

So we found consistent structure–activity relationships for our 6-sulfonamide compound series with excellent activities for the 3-methoxy aniline substitution, reduced activity with increased lipophilic alkoxy functions and a loss of activity for the 3-hydroxy aniline function.

Interestingly, the docking of the Brk-inactive compounds containing a 3-hydroxy aniline moiety and a H-bond acceptor function at position 6 of the pyrido[2,3-*b*]indole system, such as **10a**, **12c** and **16a**, showed an inverted docking pose. Instead of interaction with Glu235 the 3-hydroxy aniline interacts with residues at the entrance of the binding pocket (Fig. S2, ESI†). The sulfo or carboxy group at position 6 is found to be near Lys219. In this docking pose the hydrogen bonds to the hinge region are lost. In addition, we carried out a conformational analysis for the inactive derivatives and observed an intramolecular H-bond between the 3-hydroxy group and the H-bond acceptor at position 6 (sulfonyl or carbonyl oxygen, Fig. S3, ESI†). This more closed conformation that is stabilized by an intramolecular H-bond is not able to fit into the binding pocket and might explain the lack of activity of these derivatives.

We then replaced the 6-sulfonamide function with a bromo function. Such a bromo function may show reduced hydrogen bond acceptor properties compared to the more hydrophilic sulfonamide function. The 6-bromo 4-chloropyrido[2,3-*a*]indole



Scheme 2 6-Sulfonamide formation and final derivatization reaction to aniline target structure series **10–12**.

scaffold **13** resulted from the bromination reaction of the unsubstituted 4-chloro compound **4** in acetic acid (Scheme 3). The following 4-anilino derivatives were given by the described procedure in NMP.

The 3-hydroxy aniline compound **14a** was inactive similar to all the sulfonamide derivatives of the previous series. Then we tested the 3-methoxy aniline derivative **14b**. We found an activity with an IC_{50} value of the Brk inhibition of 186 nM. As longer and more lipophilic alkoxy substituents were unfavourable in the sulfonamide series we replaced the 3-methoxy with a 3-chloro function which resulted in a high activity in the scaffold-unsubstituted first compound series **5**. The 3-chloro derivative **14c** showed a mainly improved activity with an IC_{50} value of 13 nM. Then we replaced this chloro substitutent with a nitro function which is a more polar function able to undergo hydrogen bonding as acceptor function. The resulting 3-nitro derivative **14d** showed best lower nanomolar activities with an IC_{50} value of 4.1 nM.

In our next series we replaced the 6-bromo function with an acetyl function with stronger hydrogen bond acceptor properties. The resulting 6-acetyl 4-chloropyrido[2,3-*b*] compound **15** was given by the aluminium chloride catalyzed reaction with acetyl chloride in dichloromethane under reflux conditions (Scheme 3). The final 4-chloro derivatizations again followed the described procedure in NMP.

We first prepared the 3-hydroxy aniline derivative **16a** which was found inactive as a Brk inhibitor like the other 6-substituted compounds. The replacement of the unfavourable 3-hydroxy with the 3-nitro function similar to the 6-bromo series resulted in compound **16b** which showed a significantly increased activity with an IC_{50} value of 21 nM. We wondered whether a further reduction of this nitro function to an amino function might be of favour. The reduction reaction was carried out in hydrochloric acid using tin-II-chloride and resulted in the 3-amino aniline compound **16c** with an IC_{50} value of 7.6 nM. A final aniline substituent change with a 3-chloro aniline yielded compound **16d** with a yet high inhibitory activity of 13 nM similar to the corresponding 6-bromo compound.

Similar to the sulfonamide series the 3-hydroxy aniline function is unfavourable in our further 6-bromo and 6-acetyl substituted series. The alkoxy function, however, was less favourable in those series whereas introduced 3-chloro and 3-nitro functions mainly increased the activity.

Due to the discovered role of Brk inhibitors to potentiate the HER2 activity in HER2-positive breast cancer and thus strengthen a breast cancer cell proliferation[17] we have been interested to determine the HER2 inhibitory properties of our novel inhibitor class (Table 1).

Within our first series of only aniline varied compounds **5a–d** the 3-hydroxy derivative **5b** showed the poorest HER2 inhibitory activity with an IC_{50} value of 1300 nM similar to the 2-methyl substituted compound **5a**. The 3-chloro aniline derivative **5c** showed mainly improved activity with an IC_{50} value of 66 nM and thus was identified as the first dually acting nanomolar inhibitor of both Brk and HER2. The 4-chloro aniline substitution also resulted in an improved activity of HER2 inhibition with an IC_{50} value of 310 nM. This derivative has been not active as a Brk inhibitor.

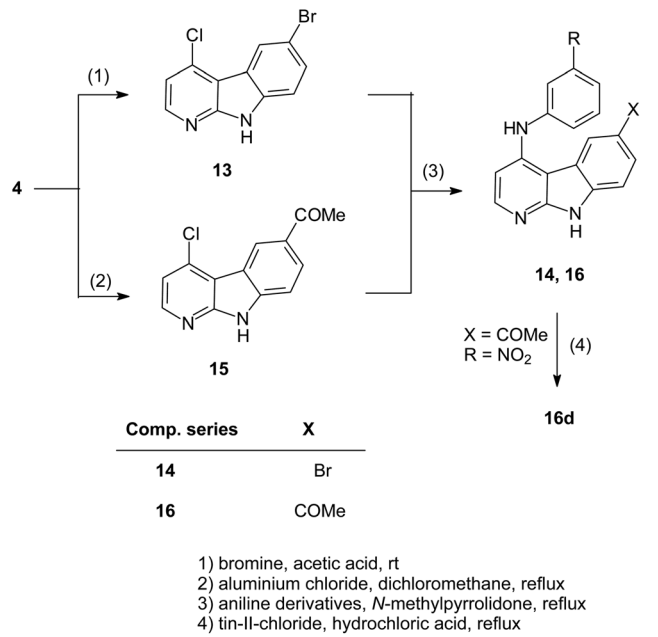
In our series of 6-sulfonamide compounds **10–12** we found best activities of HER2 inhibition for the 3-hydroxy aniline derivatives **10a** and **12c**, respectively. With IC_{50} values between 298 and 376 nM in similar ranges the corresponding compounds were more active than the only 3-hydroxy aniline derivative **5b** so that the sulfonamide substitution is of favour to potentiate the compound activity towards HER2 contrasting to the activity to inhibit Brk as the 3-hydroxy aniline derivatives exclusively inhibited HER2 and no longer Brk.

The 3-methoxy derivatives **10b**, **11a** and **12a** were the most active compounds from the series of 3-alkoxy 6-sulfonamide derivatives with best activities for the piperazino substitution in compound **11a** with an IC_{50} value of 390 nM. An elongation of the 3-alkyl ether side chain led to decreases in activity and resulting IC_{50} values in partly higher micromolar ranges.

Within the series of our bromo substituted compounds **14a–d** we found best activities for the 3-hydroxy aniline substitution in derivative **14a** with a nanomolar IC_{50} value of 29 nM. This compound showed no activity towards Brk and thus was an exclusive HER2 inhibitor. From the other 6-bromo compounds only the 3-chloro aniline substitution was favourable with an IC_{50} value of 1150 nM. The other substitutions led to main decreases in activity.

We finally characterized the inhibition of HER2 of our 6-acetyl compound series **16a–16d**. Within this series we identified the most active HER2 inhibitor **16a** with the 3-hydroxy aniline substitution which was the most favourable aniline substitution in all 6-substituted series. However, all other 6-acetyl derivatives showed moderate activities in lower micromolar ranges.

Thus, we identified exclusive nanomolar HER2 inhibitors with best activities of 29 nM and of 13 nM for the 3-hydroxy



Scheme 3 6-Bromo and -acetyl formation and final derivatization reaction to aniline target structure series **14** and **16**.

aniline substitution in the 6-bromo and the 6-acetyl derivatives **14a** and **16a**, respectively. The only 3-chloro aniline substituted derivative **5c** was the first dually acting inhibitor of both Brk and HER2.

It can be summarized that pyrido[2,3-*b*]indoles with respective 4-aniline and 6-substitutions are highly potent inhibitors of Brk and/or HER2 each depending from the substitution patterns. So they are perspective therapeutic agents to defend both HER2-positive as well as the aggressive HER2 negative type of breast cancer with the combined or exclusive inhibition of the respective causative protein kinases as will be shown in future studies.[26] This would mean a great progress for breast cancer treatment.

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23 In the previous screening studies we collected first kinase panel data for compound **5b** which demonstrated the first selectivity for the low nanomolar inhibition of Brk. Various kinases belonging to different kinase families of the human kinome were tested. We found no activity in the inhibition of JAK3 of the TK family,no inhibition of ERK1 and 2 and of JNK1 and 3 of the CMGC family, no inhibition of PAK1–3 isoforms of the STE family and of VRK1 and AKT1–3 of the AGC family. Compared to the low nanomolar inhibition of compound **5b** the inhibition of HER2-related EGFR with an IC_{50} value of 270 nM was poor. Similar poor inhibition data were obtained for ALK1 and 2 of the TKL family with IC_{50} values of each 220 nM and for AURORA A and C of the AURORA family with IC_{50} values of 250 nM and of 259 nM, respectively.
24 The effectiveness of the nanomolar Brk inhibitor **5b** in the cellular phosphorylation of the downstream Brk effector substrate STAT3 was demonstrated in a western blot analysis and is shown in Fig. S1 in the ESI.† At concentrations >100 nM the phosphorylation of STAT3 is mainly blocked by the Brk inhibitor revealing the corresponding cellular effects in relation to the nanomolar Brk *in vitro* inhibition.
25 Computational methods: since there is no crystal structure available for Brk, the homology modelling 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) query 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. The 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 the 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% were 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 c-Src X-ray structures.

26 First antiproliferative data were obtained for compound **5b** with a growth inhibition of 50% (GI_{50}) for MCF7 breast cancer cells at a concentration of 0.89 μM and for the MDA-MB-468 breast cancer cell line at a concentration of 1.00 μM. The HER2 inhibitor **14a** reduced the growth of MCF 7 cells with a GI_{50} value of 0.44 μM and of the MDA-MB-468 cell line with a GI_{50} value of 1.35 μM. The growth inhibition was determined in the sulfur rhodamine assay following recent protocols.[27,28]
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