Aromatic Interactions with Phenylalanine 691 and Cysteine 828: A Concept for FMS-like Tyrosine Kinase-3 Inhibition. Application to the Discovery of a New Class of Potential Antileukemia Agents

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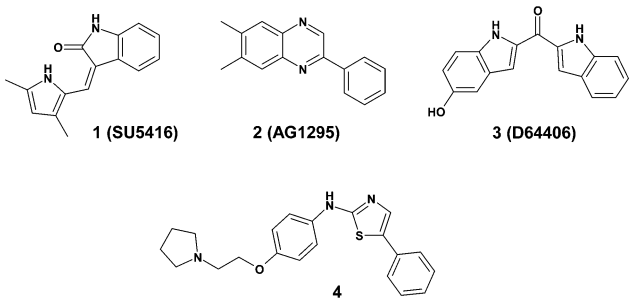
Abstract: FLT3 kinase inhibitors are currently under investigation as a new treatment for acute myeloid leukemia. We report here a molecular concept invoking interactions between an aromatic ring and the side chains of Phe691 and Cys828, two residues of the ATP pocket, to obtain potent and specific inhibitors of this kinase. The hypothesis has been validated by the successful design of a new inhibitor prototype showing promising antiproliferative activity in cellular assays.

Inhibition of the tyrosine kinase activity of the FLT3 (FMS-like tyrosine kinase-3) receptor is a therapeutic concept of current interest in antileukemia drug research.[1−4] This new approach to the treatment of acute myeloid leukemia (AML) has emerged following evidence that constitutive activation of the FLT3 receptor plays an important role in the development of this aggressive hematological malignancy for which no effective cure exists at the moment.[5−7]

The first generation of FLT3 kinase inhibitors was obtained by screening compounds well established as inhibitors of other protein kinases.[8] Several compounds of this type, exemplified by the staurosporine derivative PKC412, have entered clinical trials in AML patients.[9] Medicinal chemistry efforts in the field are now focusing on identifying follow-up clinical candidates with improved properties, in particular with regard to specificity. In this respect, to support our own efforts in this direction, we initiated molecular modeling studies aimed at understanding the structural determinants of inhibition of the FLT3 kinase by small molecules interacting with its ATP binding site. We present here a concept for FLT3 kinase inhibition resulting from this work. Its validation by a first successful application to the design of the prototype of a new class of potent and specific FLT3 inhibitors is also reported.

To gain insight into the structural determinants of FLT3 kinase inhibition, we constructed by homology a model of the three-dimensional atomic structure of the kinase domain of the FLT3 receptor and used it to perform docking studies of compounds reported as inhibitors of this kinase in the literature.[10,11] In particular, we docked **1** (SU5416), **2** (AG1295), and **3** (D64406) into the ATP binding site of the model. **1**−**3** were prominent FLT3 kinase inhibitors in the literature at the time we initiated our work.[12−15] The compounds were reported to inhibit the autophosphorylation of the FLT3 receptor in the submicromolar range in cellular assays. As described below, the resulting binding modes of **1**−**3** in the ATP pocket of the homology model suggested that a specific structural feature common to these molecules was conferring them high inhibitory activity against the FLT3 kinase.

Chart 1



The majority of known kinase inhibitors possess chemical moieties interacting with three crucial parts of the ATP binding site that we term the adenine region and hydrophobic regions I and II (Figure 1).[16]

According to our binding models (Figure 2), **1**−**3** conform to this rule in their interactions with the FLT3 kinase. The central portions of the inhibitors[17] occupy the adenine region where they form a hydrophobic sandwich with residues Ala642 and Leu818. In the same region, they are engaged in the typical hydrogen bonds with the backbone of the hinge segment, precisely at residues Cys694 and Glu692.[18] The inhibitors also contain a moiety[19] occupying hydrophobic region II, the lipophilic slot opened to solvent constituted by residues Leu616 and Gly697 in the FLT3 kinase.

However, what most attracted our attention is the way, according to the models, **1**−**3** interact with hydrophobic region I, the back pocket of the ATP site formed by residues Met665, Val675, Leu689, Phe691, and Cys828. The three inhibitors have in common a phenyl ring (shown in green in Figure 2) that, based on interatomic distance criteria, can establish a S−H/π interaction with the side chain of Cys828 and at the same time make an aromatic−aromatic edge to face C−H/π interaction with the side chain of Phe691.[20−22] The residues corresponding to positions Phe691 and Cys828 in the sequence of the FLT3 kinase domain are not conserved in the protein kinase family.[23]

These residues are located at the entrance of the hydrophobic region I pocket. For this reason the residue corresponding to Phe691 has been named the gate keeper in the literature, and

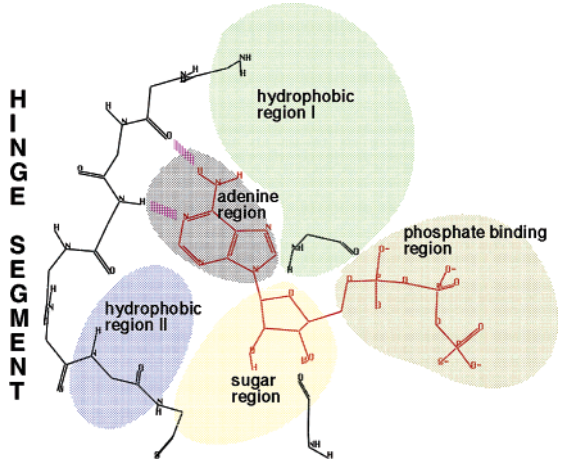


Figure 1. Schematic representation of the ATP binding site of protein kinases (active conformation).

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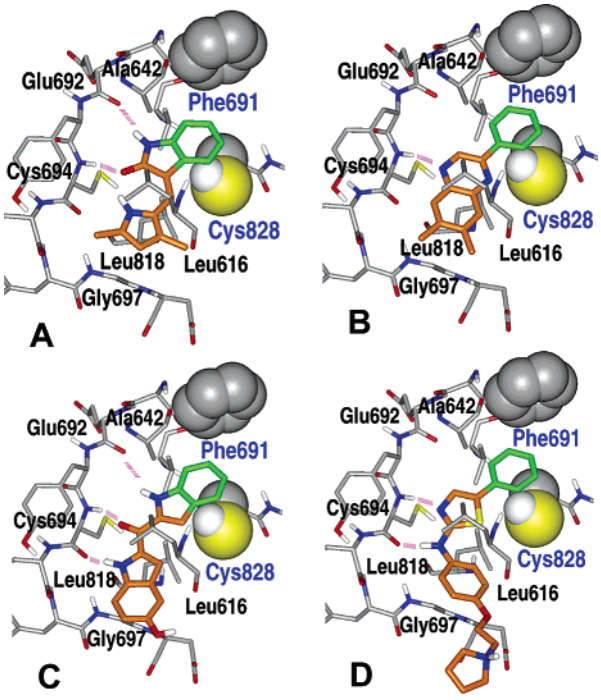


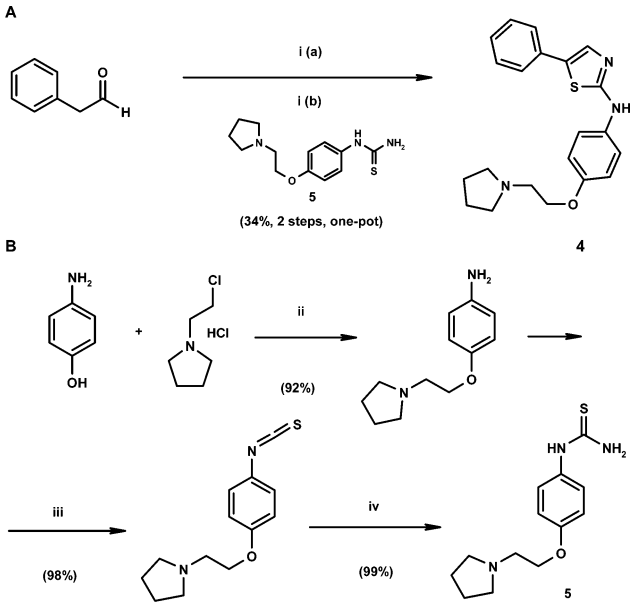
Figure 2. Binding models of reference compounds **1**−**3** (A−C) and designed prototype **4** (D) in the ATP site of the FLT3 kinase. Hydrogen bonds to the hinge segment are indicated by magenta lines. The phenyl ring interacting with Phe691 and Cys828 is shown in green.

the role it plays in determining the selectivity of kinase inhibitors is well established.[24,25] In the case of the FLT3 kinase, the concomitant presence at these positions of two amino acids able to form specific and particularly stable interactions with aromatic rings is remarkable. On the basis of the above analysis, we postulated that a sufficient condition for a molecule to potently inhibit the FLT3 kinase was to possess an aryl ring able to make a S−H/π interaction with Cys828 and an aromatic−aromatic edge to face interaction with Phe691 and this in addition to possessing two chemical moieties conforming to the more generic hydrophobic region II and adenine region pharmacophores.

To test this hypothesis, we designed by interactive molecular modeling a prototype compound (**4** shown in Figure 2D) with the three postulated important structural features. In this molecule, the 2-aminothiazole moiety was chosen to interact with the adenine region. Once this moiety was anchored in the binding site by formation of hydrogen bonds with the backbone of Cys694, we realized that the 5-position of the thiazole ring was very well suited to attach a phenyl ring targeting Cys828 and Phe691 to form the desired aromatic interactions. Another phenyl ring attached to the 2-amino position was adequate to fill the hydrophobic region II slot by formation of a hydrophobic sandwich with Leu616 and Gly697. The design was completed by attachment of a solubilizing cyclopentylamino side chain extending into the solvent from the para position of the latter phenyl ring.

5-Aryl-*N*-aryl-2-aminothiazoles can be prepared by reacting thioureas with 2-aryl-2-bromoacetaldehydes. A drawback associated with such a synthetic approach is the purification and handling of the α-bromoaldehydes that appear prone to undergo degradation. Thus, we have developed an efficient, two-step, one-pot protocol that involves the in situ preparation of the 2-aryl-2-bromoacetaldehydes followed by cyclocondensation with the thiourea (Scheme 1A). The bromination is accomplished by treatment of the aldehyde with trimethylsilyl-

Scheme 1[a]



[a] (i) (a) $(CH_3)_3SiBr$, DMSO, CH_3CN, 0 °C, room temp; (b) **5**, reflux, 34%; (ii) NaOH, DMF, 2 h, 75 °C, 92%; (iii) $CSCl_2$, $CHCl_3$, saturated $NaHCO_3$, 1 h, room temp, 98%; (iv) 2 M NH_3 in MeOH, 1 h, 60 °C, 99%.

bromide and dimethyl sulfoxide in acetonitrile. Thiazole **4** was prepared according to this procedure and obtained in 34% overall yield (Scheme 1A). The corresponding thiourea **5** was synthesized in three steps starting from *p*-aminophenol, as shown in Scheme 1B. O-Alkylation of *p*-aminophenol with 1-(2-chloroethyl)pyrrolidine hydrochloride is performed in DMF and in the presence of finely powdered NaOH. Reaction of the resulting aniline with thiophosgene affords the corresponding isothiocyanate, which is then converted into the desired thiourea upon treatment with ammonia in methanol (Scheme 1B). It is noteworthy that the synthesis of thiazole derivatives devoid of a basic moiety (the cyclopentylamino group for thiazole **4**) requires a further step, namely, the addition of diisopropylethylamine (three-step, one-pot protocol).[26] As observed by HPLC and mass spectrometry, it appears that the tertiary amine facilitates the dehydration of a reaction intermediate to provide the final product.

The designed prototype **4** was tested in biochemical assays measuring its capacity to inhibit the catalytic activity of the FLT3 receptor kinase domain and that of various other recombinant kinases. The resulting experimental data are reported in Table 1. As can be seen, **4** turned out to inhibit the FLT3 kinase with an IC_{50} of 50 nM while most of the other kinases were not significantly inhibited at a concentration as high as 10 μM. Thus, to our satisfaction, potent and selective inhibition of the FLT3 kinase was achieved, thereby validating the design concept.

Two other kinases in Table 1, c-Kit and KDR, were inhibited in the submicromolar range by **4**, although less potently than FLT3. Interestingly, both kinases also present a cysteine residue at the position corresponding to Cys828 in FLT3. Along the same line, CDK1, the only kinase in Table 1 sharing a phenylalanine gate keeper residue with FLT3, is significantly inhibited by **4** (IC_{50} = 2.1 μM). These observations give support to our hypothesis that aromatic interactions with Cys828 and Phe691 provide significant binding affinity for the ATP site of the FLT3 kinase. From a comparison of the data reported in Table 1, it is in principle difficult to make a precise quantitative assessment of the contribution of these interactions to the overall

Table 1. IC_{50} Values of Compound **4** in Enzymatic Assays and Nature of Gate Keeper Residues

kinase	IC_{50},[a] μM	gate keeper 1[b]	gate keeper 2[c]
FLT3	0.050	Phe	Cys
c-Src	2.8	Thr	Ala
c-Abl	1.2	Thr	Ala
c-Kit	0.26	Thr	Cys
c-Met	>10	Leu	Ala
KDR	0.91	Val	Cys
Tie-2	8.0	Ile	Ala
EGF-R	>10	Thr	Thr
IGF1-R	>10	Met	Gly
EphB4	>10	Thr	Ser
JAK2	>10	Met	Gly
CDK1	2.1	Phe	Ala
PKA	>10	Met	Thr
PKB	>10	Met	Thr
FGFR-1	>10	Val	Ala
Ins-R	>10	Met	Gly
c-Raf-1	>10	Thr	Gly
Axl	>10	Leu	Ala
Ret	>10	Val	Ser

[a] All IC_{50} values represent averages of at least three experimental determinations. The reference compound PKC412 has an IC_{50} of 0.50 μM in the FLT3 inhibition assay used in this study. [b] Residue corresponding to Phe691 in the FLT3 kinase. [c] Residue corresponding to Cys828 in the FLT3 kinase.

binding affinity because **4** interacts with other parts of the ATP site (the adenine region and hydrophobic region II) where amino acid differences also exist within the protein kinase family.[27] However, some of the kinases differ, in terms of the residues in close contact with the inhibitor, only by the nature of one of the gate keeper residues. Comparing the data in such cases can provide a rough estimate of these contributions. Thus, all the residues in close contact with **4** but corresponding to Cys828 in FLT3 are identical in c-Kit and c-Src. This residue is also a cysteine in c-Kit, while it is an alanine in c-Src. Comparison of the inhibition data for these two kinases, assuming that the difference in activity is mainly due to the cysteine−alanine change, suggests that the proposed S−H/π interaction contributes approximately 1 order of magnitude to potency. From a similar comparison for FLT3 and c-Kit, which only differ by the nature of the gate keeper residue Phe691, it can be concluded that the modeled aromatic−aromatic edge to face interaction provides a 5-fold improvement in potency compared to the corresponding interaction between a threonine side chain and a phenyl ring.

Encouraged by the potent inhibition of the FLT3 kinase displayed by **4** at the enzymatic level, the compound was tested in cellular assays measuring its ability to inhibit the proliferation of cells whose growth is driven by a constitutively activated FLT3 receptor. These included BaF3 cell lines transfected with FLT3 constructs bearing two forms of activating mutations, the ITD (internal tandem duplication) and D835/Y mutations, as well as the MV4:11 cell line derived from AML patients carrying the ITD mutation. Consistent with the high potency observed in the enzymatic assay, **4** was able to block, in a dose-dependent manner, the proliferation of these cell lines at low concentrations. This is testified by IC_{50} values of 0.24, 0.76, and 0.052 μM obtained respectively in the BaF3-ITD, BaF3-D835/Y, and MV4:11 assays.[28,29] Hence, with **4** we had an entry in a new class of FLT3 kinase inhibitors active at the cellular level. To gain additional evidence that **4** is a specific inhibitor of the FLT3 kinase interacting with its gate keeper residue, the activity of the compound in inhibiting the proliferation of BaF3 cells expressing FLT3-ITD with Phe691 mutated to isoleucine was measured. An IC_{50} of 4.1 μM was obtained. The loss of 1 order of magnitude in potency compared to the inhibition observed with the BaF3-ITD cells having the wild-type gate keeper residue (IC_{50} = 0.24 μM) clearly supports the existence of the postulated aromatic−aromatic interaction with Phe691.

In conclusion, modeling of the binding modes of reported FLT3 kinase inhibitors led us to propose that aromatic interactions with residues Phe681 and Cys828 of the ATP pocket can be exploited to obtain potent and specific inhibitors of this kinase. The discovery of **4** on the basis of this concept is very encouraging for its further use in the search for new classes of FLT3 kinase inhibitors as potential antileukemia agents.

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Supporting Information Available: Experimental procedures and analytical data for all intermediate and final compounds and description of biochemical and cellular assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) At the time we initiated this work, no X-ray crystal structure of the FLT3 kinase was available. We constructed a homology model of the FLT3 kinase using the coordinates of the Lck kinase in complex with AMP−PNP (PDB code 1QPC). This template was chosen because it was the highest resolution structure of a tyrosine kinase adopting an active conformation (conformational state appropriate for docking the type of inhibitor discussed here) available at that time. In the meantime, the crystal structure of the FLT3 kinase in an autoinhibited conformation was reported.[11] In this structure, the ATP binding site is obstructed by residue Phe830 of the activation loop ("DFG out" conformation), which in principle precludes its use for docking the inhibitors of our study. However, we could derive a model of the ATP binding site corresponding to an active conformation from this structure by changing the conformation of the DGF motif to a "DFG in" conformation. Docking the inhibitors using this model gives the same results as with the homology model. Details of protein model construction and inhibitor docking are given in the Supporting Information.
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(17) The lactam ring in **1**, the pyrazine ring in **2**, and the bis-pyrrolyl methanone moiety in **3**.
(18) The three inhibitors accept a hydrogen bond from the backbone NH group of Cys694. **1** and **3** donate a hydrogen bond to the backbone carbonyl group of Glu692. **3** gives in addition a hydrogen bond to the backbone carbonyl group of Cys694.
(19) The pyrrole ring in **1**, the phenyl ring fused to the pyrazine ring in **2**, and the phenol ring in **3**.
(20) For a detailed description of these two types of intermolecular interactions see ref 21 and references therein. By S−H/π interaction, it is meant an interaction between the thiol group of Cys828 and a phenyl ring of the inhibitor in which the side chain sulfur atom is positioned 3.5−4.0 Å below the plane of the phenyl ring with the thiol proton pointing towards its center. Such an interaction, also termed aromatic−thiol π hydrogen bond, has been calculated to provide 2.6 kcal/mol of stabilization energy (gas phase) in ref 22. Aromatic−aromatic edge to face interactions are more common and are very well reviewed in ref 21.
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(27) For instance, **4** is expected to have a different interaction energy in the hydrophobic region II for CDK1 compared to FLT3 because CDK1 has a deletion in this region; the residue corresponding to Gly697 does not exist in CDK1.
(28) The reference compound PKC412 has IC_{50} values of 0.040, 0.006, and 0.024 μM in the BaF3-ITD, BaF3-D835/Y, and MV4:11 assays, respectively.
(29) As control, **4** was also tested on the wild-type BaF3 cell line. No significant inhibition of the proliferation of these cells, whose growth is independent of FLT3 signaling, was obtained at concentrations up to 3 μM.

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