

Crystal Structure of the MAP3K TAO2 Kinase Domain Bound by an Inhibitor Staurosporine

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Abstract Mitogen-activated protein kinase (MAPK) signal transduction pathways are ubiquitous in eukaryotic cells, which transfer signals from the cell surface to the nucleus, controlling multiple cellular programs. MAPKs are activated by MAPK kinases [MAP2Ks or MAP/extracellular signal-regulated kinase (ERK) kinases (MEK)], which in turn are activated by MAPK kinase kinases (MAP3Ks). TAO2 is a MAP3K level kinase that activates the MAP2Ks MEK3 and MEK6 to activate p38 MAPKs. Because p38 MAPKs are key regulators of expression of inflammatory cytokines, they appear to be involved in human diseases such as asthma and autoimmunity. As an upstream activator of p38s, TAO2 represents a potential drug target. Here we report the crystal structure of active TAO2 kinase domain in complex with staurosporine, a broad-range protein kinase inhibitor that inhibits TAO2 with an IC_{50} of 3 μ M. The structure reveals that staurosporine occupies the position where the adenosine of ATP binds in TAO2, and the binding of the inhibitor mimics many features of ATP binding. Both polar and nonpolar interactions contribute to the enzyme-inhibitor recognition. Staurosporine induces conformational changes in TAO2 residues that surround the inhibitor molecule, but causes very limited global changes in the kinase. The structure provides atomic details for TAO2-staurosporine interactions, and explains the relatively low potency of staurosporine against TAO2. The structure presented here should aid in the design of inhibitors specific to TAO2 and related kinases.

Key words TAO2; MAP3K; inhibitor; staurosporine; crystal structure

Mitogen-activated protein kinase (MAPK) signaling modules are one of the most widespread signaling systems in eukaryotes that transfer signals from the cell surface to the nucleus, controlling multiple cellular programs such as embryogenesis, cell differentiation, cell proliferation and cell death. Each MAPK pathway consists of a central three-tiered protein kinase in which MAPKs are activated by dual phosphorylation on a motif of Thr-X-Tyr catalyzed by a family of dual specificity kinases known as MAPK kinases (MAP2Ks) or MAP/extracellular signal-regulated kinase (ERK) kinases (MEKs). MAP2Ks in turn are activated by a protein kinase superfamily referred to as MAPK kinase kinases (MAP3Ks) or MEK kinases (MEKKs).

Finally, activated MAPKs phosphorylate various substrates in cytoplasm and nucleus to change the cellular program and regulate gene expression patterns [1–3]. Among the 12 or more homologous mammalian MAPKs that have been identified, three, ERKs, p38s and c-Jun N-terminal kinases (JNKs), have been well-studied. ERKs are activated by mitogenic stimuli, such as hormones, growth factors and phorbol esters, thus associated with proliferative processes. By contrast, p38s and JNKs are more potently activated in response to physical and chemical stresses such as osmotic shock, ultraviolet radiation, oxidative stress and inflammatory cytokines, and are linked both to reparative and apoptotic responses [1,3].

MAP3Ks represent the entry level of the MAPK signaling modules, and link MAP2K/MAPK components to a wide variety of upstream activators such as MAPK kinase kinase kinases (MAP4Ks), adaptor proteins and small

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guanosine triphosphate-binding proteins [4]. TAO2 is a MAP3K level kinase, which was identified from rats by isolating mammalian cDNAs encoding proteins related to Ste20p, a yeast MAP4K that regulates the MAPK cascade in the pheromone-induced mating pathway of *Saccharomyces cerevisiae* [5,6]. The TAO2 human homolog, prostate-derived Ste20-like kinase, was identified in a screen for RNA overexpressed in human prostate carcinoma and shares over 90% sequence identity with rat TAO2 [7]. TAO2 phosphorylates and thus activates the MAP2Ks MEK3 and MEK6 [6,8], which are direct activators of p38 MAPKs (p38 α , p38 β , p38 γ and p38 δ). Because p38 MAPKs are key regulators of the expression of inflammatory cytokines such as tumor necrosis factor- α and interleukin-1, their pathways are thought to be involved in human diseases such as asthma, arthritis, and other inflammatory or immunoresponsive diseases [9]. As a result, p38 MAPKs are the target of most extensive activities in MAPK inhibitor development, and the testing of selective small-molecule inhibitors of p38 α has progressed into animal and clinical trials [10]. TAO2, as an upstream activator of p38, represents a potential drug target.

There are three members in the TAO family, TAO1 [5], TAO2 and TAO3 [11,12], and TAO2 is the best studied one. We have recently determined the crystal structure of TAO2 kinase domain (1–320), solved in an active form [13]. Here we report the crystal structure of TAO2 kinase domain in complex with an inhibitor staurosporine (Fig. 1), which is the microbial alkaloid from *Streptomyces* sp. and a potent broad-range small-molecule inhibitor for a number of serine/threonine protein kinases. In an *in vitro* kinase assay, we determined that staurosporine inhibits

the activity of TAO2 kinase domain towards myelin basic protein with an IC₅₀ of 3 μ M (data not shown). As observed in other protein kinases such as protein kinase A (PKA) [14], cyclin-dependent kinase 2 (CDK2) [15] and lymphocyte-specific kinase (Lck) [16], staurosporine is bound to the ATP binding site of TAO2. Both polar and nonpolar interactions contribute to the formation of the kinase-inhibitor complex. The TAO2-staurosporine structure provides the structural basis for the kinase-inhibitor interactions, which should help in the design of inhibitors specific to TAO family kinases.

Materials and Methods

Cloning, expression and purification

The details of the production and purification of TAO2 kinase domain protein have been described elsewhere [13]. Briefly, rat TAO2 kinase domain (1–320) was cloned into *Baculovirus* and expressed in insect cells. The TAO2 kinase domain protein was first purified with Ni²⁺-nitrilotriacetic acid-agarose (Qiagen, Valencia, USA), and followed by a MonoQ5/5 column (Pharmacia, Piscataway, USA). Fractions of the MonoQ5/5 column were concentrated and dissolved into buffer containing 30 mM HEPES, pH 7.5, 0.1 M NaCl, 1 mM dithiothreitol and 1 mM EDTA before crystallization trials.

Crystallization, complex formation and data collection

The TAO2 kinase domain crystallized in a buffer containing 0.1 M imidazole, pH 7.8–8.2, 18%–20% polyethylene glycol 1000 and 0.2 M Ca(OAc)₂, as described previously [13]. To make the TAO2(1–320)-staurosporine complex, the preformed TAO2 kinase domain crystals were soaked for 2–6 h with staurosporine (Calbiochem, San Diego, USA) dissolved in dimethylsulfoxide at a final concentration of 0.5 mM. Then the crystals were cryoprotected stepwise with 10%, 20% and 30% polyethylene glycol 200 before flash freezing with propane and liquid nitrogen. The crystal diffraction data to 2.6 Å were collected at 100 K from a single crystal with a RAXIS IV image plate detector equipped with a Rigaku RU300 generator and Osmotic mirrors (Rigaku, The Woodlands, USA).

Structure determination and refinement

The structure of the TAO2(1–320)-staurosporine complex was determined with molecular replacement using active TAO2(1–320) as the search model (Protein Data Bank code 1U5Q). The calculated difference Fourier map

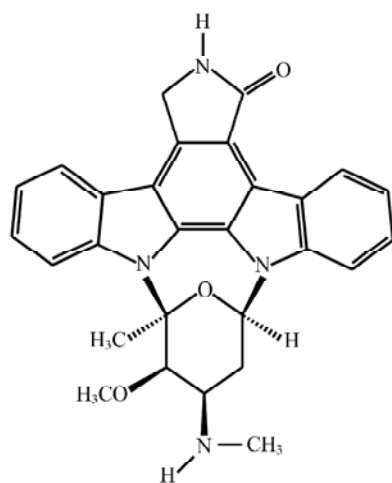


Fig. 1 Chemical structure of staurosporine

using this model reveals clear density for the bound staurosporine. After several cycles of refinement of the TAO2(1-320) model in crystallography and NMR system (CNS) [17] combined with manual rebuilding in O (a crystallographic model building program) [18], the staurosporine model was built into the density. This was followed by further refinement in CNS and O until convergence was reached. The crystal data and refinement statistics of TAO2(1-320)-staurosporine are summarized in **Table 1**.

Table 1 Crystal data and refinement statistics of TAO2(1-320)-staurosporine

Parameter	Value
Space group	P6 ₃ 22
Unit cell dimension (Å)	<i>a</i> = <i>b</i> =186.0, <i>c</i> =94.6
Wavelength (Å)	1.5418
Resolution (Å)	2.6
Number of total observation	802911
Number of unique reflection	30207
Completeness (%) (last shell)	97.9 (92.3)
Intensity <i>I</i> / σ (last shell)	16.3 (1.9)
<i>R</i> _{merge} (last shell) ^a	0.051 (0.458)
<i>R</i> _{work} / <i>R</i> _{free} ^{b,c}	0.21/0.27
Non-H protein atom	5050
Hetero group	2 staurosporine
Number of water molecules	293
No. of reflections in refinement	28004
R.m.s.d. in bond length (Å)	0.012
R.m.s.d. in bond angle (°)	1.65
Average protein B value (Å ²)	42.9
Average ligand B value (Å ²)	26.5
Ramachandran plot statistics (%)	
Residues in most favored region	86.0
Residues in additionally allowed region	12.4
Residues in generously allowed region	1.6
Residues in non-allowed region	0

^a $R_{\text{merge}} = \sum_{\text{hkl}} [(\sum_j |I_j - \langle I \rangle|) / \sum_j I_j]$; ^b $R_{\text{work}} = \sum_{\text{hkl}} |F_o - F_c| / \sum_{\text{hkl}} |F_o|$, where *F*_o and *F*_c are the observed and calculated structure factors, respectively; ^c *R*_{free} is the *R* factor calculated for a randomly selected 5% of the reflections that were omitted from the refinement. R.m.s.d., root-mean-square-deviation.

Results and Discussion

Overall structure of TAO2(1-320)-staurosporine

The staurosporine-soaked TAO2 kinase domain crystals

belong to P6₃22 space group with cell dimensions *a*=*b*=186.0 Å and *c*=94.6 Å. There are two TAO2(1-320)-staurosporine complexes in the asymmetric unit that are not related by non-crystallographic symmetry, but are almost identical in conformation. The electron density is clear for the bound staurosporine molecule [**Fig. 2(A)**]. Also, electron density is good throughout the TAO2 molecule, except for the first 11 amino acids and the His₆-tag at the N-terminus, for which there is no density. Residues 63–65 (in the loop connecting strand 3 and helix C) and residues 302–312 (in the loop connecting helices J and K) are partially disordered. The final model is comprised of 618 of the total 640 residues in the two TAO2(1-320) monomers plus two molecules of staurosporine and 293 water molecules. The TAO2(1-320)-staurosporine model has been refined with reasonable stereochemistry to free *R* factor and *R* factor of 27.0% and 21.0%, respectively (**Table 1**).

The inhibitor-bound TAO2 kinase domain was constitutively phosphorylated on serine 181 at the activation loop, and adopts an active conformation as its apo form [13]. TAO2(1-320) in the complex possesses the typical protein kinase two-domain architecture. The N-terminal domain is composed of an antiparallel five-stranded β -sheet and helix C plus two small helices at an N-terminal extension to the kinase core (labeled helices A and B) [**Fig. 2(A)**]. The C-terminal domain also possesses a standard structure, including six major helices, two β -ribbons, the catalytic loop, and the activation loop. In the truncated form (1–320) that was crystallized, TAO2 possesses two additional helices, J and K, which are in the C-terminal extension to the kinase core (277–320). As observed in other serine/threonine protein kinases such as PKA [14] and CDK2 [15], staurosporine binds in the ATP binding site of TAO2 between the domain interface, occupying a position where the adenosine moiety of ATP binds in the enzyme [**Fig. 2 (A)**].

Interactions between TAO2(1-320) and staurosporine

Staurosporine is a natural microbial alkaloid that was first characterized in 1986 and shown to be a potent inhibitor of protein kinase C [19]. It was subsequently found that staurosporine is a potent and nonspecific inhibitor of a number of protein kinases with IC₅₀ values in the 1–100 nM range, such as PKA and CDK2 [20]. The surface of the staurosporine nucleus is highly hydrophobic (**Fig. 1**). This is complemented by the large hydrophobic surface of the ATP-binding cleft of TAO2, so that staurosporine bound to TAO2 makes extensive favorable van der Waal contacts with residues surrounding staurosporine: Ile34

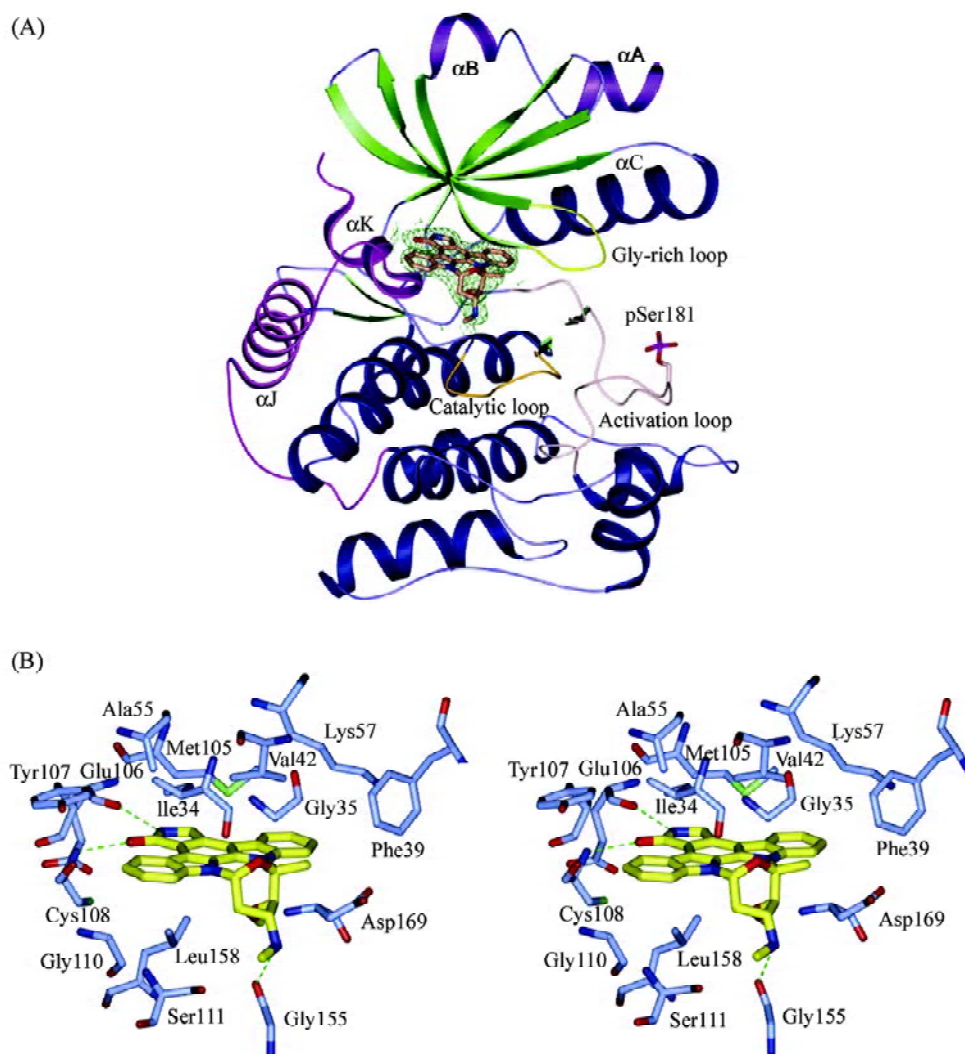


Fig. 2 Crystal structure of TAO2 kinase domain in complex with staurosporine

(A) Overview of the TAO2(1-320)-staurosporine complex in a ribbon diagram with the bound staurosporine molecule shown in stick representation. Also shown is the $F_o - F_c$ difference Fourier map for staurosporine, contoured at 3.5σ . (B) Stereo view of the interactions between TAO2(1-320) (colored blue) and staurosporine (yellow). Hydrogen bonds are denoted by dotted lines. Residues that form van der Waals contacts with staurosporine (approximately 4 Å distance) are shown in stick representation.

(*Leu49* in PKA); Gly35 (*Gly50*); Phe39 (*Phe54*); Val42 (*Val57*); Ala55 (*Ala70*); Lys57 (*Lys72*); Ile89 (*Val104*); Met105 (*Met120*); Tyr107 (*Tyr122*); Gly110 (*Gly126*); Ser111 (*Glu127*); Leu158 (*Leu173*) and Asp169 (*Asp184*) [Fig. 2(B)]. Thus the nonpolar interactions between TAO2 (1-320) and staurosporine are very similar to those observed in PKA [14] and other protein kinases. In particular, Val42 from β_2 and Leu158 from β_7 , two highly conserved residues in protein kinases, sandwich the bulky indole carbazole rings of staurosporine [Fig. 2(B)]. The positions of most residues that interact with staurosporine are altered to some extent with respect to their positions in the uncomplexed structure.

The polar interactions between TAO2 and staurosporine involve three hydrogen bonds which are mimics of those observed in the TAO2(1-320)-MgATP complex [13]. These are the two bonds between N19, O30 of the lactam moiety of staurosporine and backbone carbonyl oxygen of Glu106 and amide nitrogen of Cys108, respectively, and one bond between N31 from the methylamino group of the glycosyl portion of staurosporine and the main-chain carbonyl oxygen of Gly155 [Fig. 2(B)].

Staurosporine-induced conformational changes

The TAO2(1-320)-staurosporine complex was formed through diffusion of 0.5 mM staurosporine into preformed

crystals of the active TAO2 kinase domain. Although the magnitude is relatively small [the structure of TAO2(1-320)-staurosporine superimposed with that of TAO2(1-320) alone at 0.4 Å root-mean-square-deviation], staurosporine induces many conformational changes in TAO2(1-320), most of which are directly linked to the inhibitor binding, indicating that the inhibitor binding proceeds through an “induced fit” mechanism [Fig. 3(A, B)]. Although changes in the main chain positions are rather limited, many residues that form direct contacts with staurosporine change their side chain conformations in binding the inhibitor molecule. These include Phe39, Ile89, Met105, Tyr107, Leu158, Asp169 and Lys314, among which Met105, Tyr107 and Lys314 move away from the inhibitor-binding site whereas Phe39 and Asp169 slightly approach the inhibitor [Fig. 3(B)]. The main loci of the main chain conformational changes are the Gly-rich loop (from His36 to Ala41), the hinge region in the domain interface (from Tyr107 to Ser111), the β_7 - β_8 reverse turn (from Ile157 to Gly163), the helix K and the linker connecting helices J and K [Fig. 3(A)]. The slight changes in the Gly-rich loop seem to be coupled with the movement of Phe39, and the closest contact between staurosporine and the Gly-rich loop of TAO2 occurs between the glycosidic oxygen of staurosporine (O29) and the C α atom of Gly35 (3.3 Å), as observed in other kinases such as PKA [14], CDK2 [15] and Lck [16]. The movements in the hinge region, the β_7 - β_8 reverse turn and the

helix K are to make room for the bulky indole carbazole rings of staurosporine. As a result, the loop connecting helices J and K becomes more flexible and is less well-ordered in the inhibitor-bound TAO2(1-320) structure. Staurosporine induces no changes in the domain orientation of TAO2, which is in contrast to PKA where staurosporine causes opening of the N-terminal domain relative to the C-terminal domain [14]. Also, no changes were observed in the catalytic loop and the DFG motif (a highly conserved motif involved in binding Mg²⁺) [Fig. 3(A)].

Conformational changes at the C-terminal extension

The full length TAO2 protein possesses 1235 residues in its polypeptide chain, and the kinase domain is located at its N-terminus [6]. In the truncated form with residues 1–320 that was crystallized, TAO2 possesses 44 residues as a C-terminal extension, which forms two helices, J and K. Helix K, which adopts a predominantly 3/10 conformation and spans the gap between the two domains of the kinase near the hinge region, is participating in ATP binding; Lys314 from helix K forms a hydrogen bond with the 2' hydroxyl of the ribose [13]. In the complex of TAO2(1-320)-staurosporine, a similar interaction is not possible due to lack of a homologous binding partner in the inhibitor molecule. So the charged tip of Lys314 turns away from the voluminous and hydrophobic indole carbazole ring I of staurosporine, whereas the aliphatic part of the residue

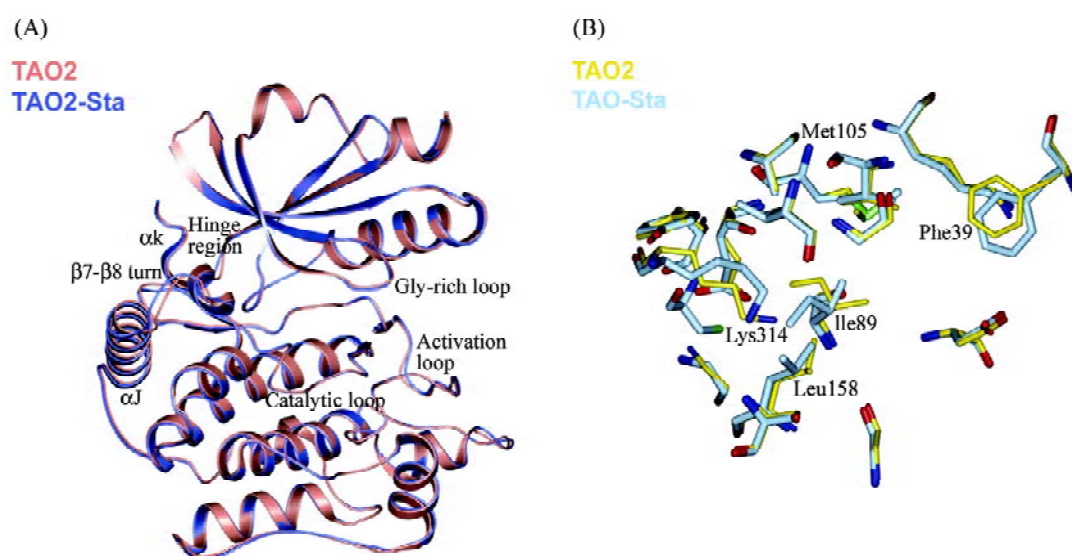


Fig. 3 Staurosporine-induced conformational changes in TAO2(1-320)

(A) Overlay of TAO2(1-320) alone (colored pink) with TAO2(1-320)-staurosporine (blue), showing the conformational changes induced by staurosporine. (B) Superposition of the two structures in the inhibitor-binding pocket. TAO2(1-320) alone is colored yellow, and TAO2(1-320) in complex with staurosporine, blue. Several residues undergo “induced fit” rearrangements in binding the inhibitor, especially in their side chain conformations. TAO2, TAO2(1-320); TAO2-Sta, TAO2(1-320)-staurosporine.

makes van der Waals contacts with the inhibitor [Figs. 3 (B) and 4]. In PKA, a structurally similar position to Lys314 of TAO2 is occupied by Phe327, which is from the C-terminal tail that crosses the two structural domains of the kinase [21]. Phe327 was also displaced in the PKA-staurosporine-PKI (protein kinase inhibitor) complex to make room for the inhibitor, and makes direct contacts with the inhibitor through its hydrophobic side chain [14].

Comparison with TAO2(1-320)-MgATP structure

The staurosporine molecule occupies a site in TAO2 that overlaps with that of the adenosine moiety of ATP [13]. The lactam ring of staurosporine mimics the amino pyrimidine ring of adenine in its hydrogen bonding interactions with the backbone carbonyl group of Glu106 and the amide NH group of Cys108 in the linker region [Fig. 2 (B)]. The indolylcarbazole ring system of the inhibitor is coplanar with the adenine moiety, and the sugar moiety overlaps the ribose of ATP, such that the methylamino nitrogen of staurosporine is located closely to the 3' hydroxyl of ATP (Fig. 4). As a result, the hydrogen bond to the 3' hydroxyl of the ribose of ATP is also mimicked in staurosporine, to the methylamino nitrogen. The unique interaction in ATP recognition of TAO2, a hydrogen bond formed between Lys314 of helix K and the 2' hydroxyl of the ribose, is missing in the TAO2(1-320)-staurosporine complex. Another difference between the two complex

structures is the conformational changes observed in Phe39, which resides at the tip of the Gly-rich loop. In TAO2(1-320)-MgATP, Phe39 rotates away to make room for the triphosphate chain of ATP, whereas in TAO2(1-320)-staurosporine, the phenyl ring of Phe39 moves slightly inward towards staurosporine, making van der Waals contacts with C4 and C35 of the inhibitor (Fig. 4), and blocking the solvent accessibility of the bound staurosporine in this part of the cleft. In addition, Met105 in the hinge region, Lys57 in $\beta 3$ and its ligand partner Glu76 in helix C undergoes different conformational changes in TAO2(1-320) bound MgATP or staurosporine. In TAO2(1-320)-staurosporine, the side chain of Met105 adopts a different rotamer conformation than that in TAO2(1-320) alone, to avoid steric conflict with both the lactam ring and the indole carbazole ring III of the inhibitor (Fig. 4). In TAO2(1-320)-MgATP, the conformation of this residue remains unchanged. The ion pair between Lys57 and Glu76 is a hallmark of active protein kinases, and in the TAO2(1-320)-MgATP complex, Lys57 of TAO2 moves towards the triphosphate chain of ATP and forms direct contact with the β -phosphate [13]. In staurosporine, a similar structure to the triphosphate of ATP is lacking, and Lys57 and Glu76 remain nearly unchanged in the inhibitor-bound TAO2(1-320) (Fig. 4).

Comparison with other protein kinases

In TAO2(1-320), the position and conformation of staurosporine in the ATP-binding cleft are highly similar to those observed in PKA [14] or CDK2 [15]. However, there are several differences among these structures in inhibitor binding and the conformational changes induced. First, a total of three hydrogen bonds were observed in the TAO2(1-320)-staurosporine structure compared to four in both the PKA and CDK2 complexes. The fourth bond between the methylamine group of staurosporine and the side chain of Glu127 in PKA or Asp86 in CDK2 is absent in TAO2, due to an amino acid replacement in this position (Ser111 in TAO2) [Fig. 2(B)]. This should partially explain the much less potent inhibition of staurosporine against TAO2 compared to both PKA and CDK2. Second, conformational changes occur in the C-terminal extension of both TAO2 and PKA, to make room for the inhibitor. In CDK2, a similar structure is not present, leaving this region of the ATP-binding cleft accessible to solvent [22]. Third, in PKA, binding of staurosporine in the ternary complex staurosporine-PKA-PKI(5-24) [14] causes a 13° domain opening relative to 5'-adenylylimido-diphosphate (AMPPNP)-PKA-PKI(5-24) [23]. In contrast, in both TAO2 and CDK2, staurosporine induces no significant

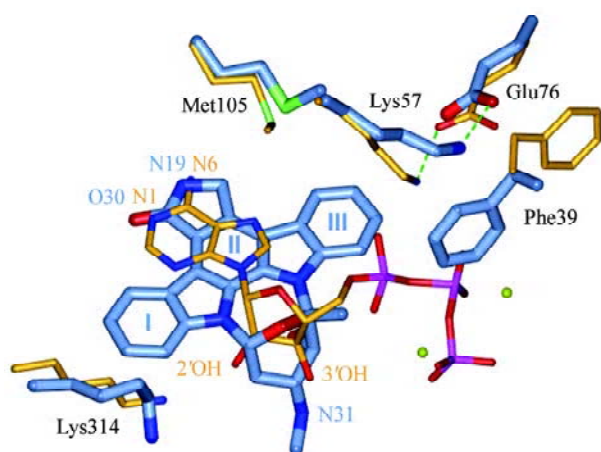


Fig. 4 Comparison of TAO2(1-320)-staurosporine (colored blue) with TAO2(1-320)-MgATP (yellow), highlighting the similarities and dissimilarities in the binding mode of the two ligands

The superposition was carried out against the ATP-binding site of TAO2(1-320). Also shown are Met105, Lys57, Glu76, Phe39 and Lys314, colored blue for TAO2(1-320)-staurosporine and yellow for TAO2(1-320)-MgATP, which adopt different conformations in the two complex structures.

changes in domain orientation.

Insights for the design of inhibitors specific to TAOs

Initial interest in protein kinases as drug targets was stimulated by the findings that many viral oncogenes encode structurally modified cellular protein kinases with constitutive enzyme activity [22]. These findings are suggestive of the potential involvement of proto-oncogene-encoded protein kinases in human proliferative disorders. Thus, specific protein kinase inhibitors could block the disease pathologies resulting from aberrant protein kinase activity. In fact, after G protein-coupled receptors, protein kinases have become the second most important class of targets for drugs in the past 20 years. One of the successful cases is imatinib mesylate (Gleevec; Novartis Pharmaceuticals Inc., Cambridge, USA), which specifically inhibits the inactive form of Bcr-Abl tyrosine kinase, thereby exerting its treatment effects on chronic myeloid leukemia disease [24]. Because p38 α MAPK regulates the production of cytokines such as tumor necrosis factor- α and interleukin-1, its inhibitors might inhibit not only the production of these pro-inflammatory cytokines, but also their actions, interrupting the vicious cycle that often occurs in inflammatory and immunoresponsive diseases. The most extensive activity in MAPK inhibitor development is on p38 α [10]. It is believed that TAO2, an upstream regulator of p38, represents a potential drug target for the treatment of p38 MAPK-associated diseases such as arthritis, autoimmunity, and other diseases yet to be identified. Due to its broad spectrum of protein kinase inhibitory effects, staurosporine proved to be too toxic to use directly as a therapeutic agent, exhibiting a maximal tolerated dose in the order of 10 nM [25]. Nevertheless, staurosporine could serve as a template for the design of inhibitors specific to TAO2. In fact, structure-based drug design has become an integral part of modern drug discovery. In particular, the efforts in designing specific TAO2 inhibitors should take advantage of the unique interaction in TAO2, observed between Lys314 of helix K and the 2' hydroxyl of the ribose of ATP, as a lysine at this position is present only in TAO family kinases [13].

In summary, the crystal structure of the active MAP3K TAO2 kinase domain in complex with staurosporine has been determined at 2.6 Å resolution. The inhibitor targets the ATP binding site of TAO2, and the binding mimics many features of MgATP recognition by the enzyme. Conformational changes occur locally to the inhibitor-binding pocket, whereas global changes are rather limited. The structure presented here thus provides a structural basis for the kinase-inhibitor interactions, and should be helpful

in the design of inhibitors specific to TAO2 and related kinases.

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