Structural basis for UCN-01 (7-hydroxystaurosporine) specificity and PDK1 (3-phosphoinositide-dependent protein kinase-1) inhibition

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PDK1 (3-phosphoinositide-dependent protein kinase-1) is a member of the AGC (cAMP-dependent, cGMP-dependent, protein kinase C) family of protein kinases, and has a key role in insulin and growth-factor signalling through phosphorylation and subsequent activation of a number of other AGC kinase family members, such as protein kinase B. The staurosporine derivative UCN-01 (7-hydroxystaurosporine) has been reported to be a potent inhibitor for PDK1, and is currently undergoing clinical trials for the treatment of cancer. Here, we report the crystal structures of staurosporine and UCN-01 in complex with the kinase domain of PDK1. We show that, although staurosporine and UCN-01 interact with the PDK1 active site in an overall similar manner, the UCN-01 7-hydroxy group, which is not present in staurosporine, generates direct and water-mediated hydrogen bonds with active-site residues. Inhibition data from UCN-01 tested against a panel of 29 different kinases show a different pattern of inhibition compared with staurosporine. We discuss how these differences in inhibition could be attributed to specific interactions with the additional 7-hydroxy group, as well as the size of the 7-hydroxy-group-binding pocket. This information could lead to opportunities for structure-based optimization of PDK1 inhibitors.

Key words: 7-hydroxystaurosporine (UCN-01), kinase alignment, kinase inhibitor specificity, 3-phosphoinositide-dependent protein kinase-1 (PDK1).

INTRODUCTION

Insulin and growth-factor signalling is mediated by the activation of a lipid kinase, phosphoinositide 3-kinase (PI3K), which produces the second-messenger molecule PtdIns(3,4,5)P_3 [1]. Upon generation of PtdIns(3,4,5)P_3, PDK1 (3-phosphoinositide-dependent protein kinase-1) and protein kinase B (PKB; also known as Akt) are co-localized at the plasma membrane through interaction of their pleckstrin homology (PH) domains with PtdIns(3,4,5)P_3 [2,3]. PDK1 activates PKB by phosphorylation of its T-loop (Thr^{308} in PKBα) [4,5]. PDK1 also activates other protein kinases related to PKB, including isoforms of p70 ribosomal S6 kinase (S6K) [6], serum- and glucocorticoid-responsive kinases (SGKs) [7] and p90 ribosomal S6 kinase (RSK) [8]. These kinases lack PH domains and do not bind PtdIns(3,4,5)P_3, and are thought to be activated by a different mechanism, in which the substrates require a priming phosphorylation in a conserved hydrophobic motif (HM) at their C-terminus (for a review, see [9]). This phosphorylation creates a docking motif that specifically interacts with a pocket on the N-terminal lobe of the PDK1 kinase domain [termed PDK1-interacting fragment (PIF) pocket] [10,11], bringing PDK1 together with its substrate and enabling PDK1 to phosphorylate these kinases in their T-loop, thereby activating them. A significant number of human cancers possess elevated PtdIns (3,4,5)P_3 levels due to mutations in a number of genes that regulate the production and degradation of this 3-phosphoinositide. One of the most frequently found mutations occurs in the PtdIns(3,4,5)P_3 3-phosphatase (PTEN), resulting in constitutive activation of PKB and S6K, which are thought to be major contributors to the proliferation and the survival of such tumour cells [12]. Thus inhibitors of PDK1 have the potential to act as anticancer agents, since they would be expected to suppress activation of S6K and PKB and to inhibit cell growth and induce apoptosis of cancer cells that possess elevated levels of PtdIns(3,4,5)P_3.

PDK1 consists of an N-terminal kinase domain and a C-terminal PH domain [13]. The structure of the PDK1 kinase domain has recently been solved, and leads to a definition of the residues lining the ATP-binding site and an understanding of the PDK1 activation mechanism [11]. The PIF-binding pocket could be identified, together with a specific pocket for the phosphorylated serine/threonine residue on the HM of substrate kinases. Staurosporine, a natural-product, ATP-competitive inhibitor, inhibits many kinases in the low nanomolar range [14], and therefore displays a high cytotoxicity [15]. UCN-01 (7-hydroxystaurosporine) is a derivative with an additional hydroxy group on the lactam ring (Figure 1). It was originally described as a protein kinase C (PKC)-selective inhibitor isolated from *Streptomyces* sp. cultures [16], although further studies showed it to be more non-specific [14,17]. UCN-01 potently inhibits the growth and induces apoptosis of many cancer cells, and these effects are thought to be unrelated to PKC inhibition [18,19]. Owing to its antitumour activity, UCN-01 is currently undergoing clinical trials, with positive effects being reported in the Phase I studies (for a review, see [19]). Relatively recent reports have suggested that cell-cycle-checkpoint kinase-1

Abbreviations used: CDK2, cyclin-dependent kinase 2; CHK1, cell-cycle-checkpoint kinase-1; HM, hydrophobic motif; MAPKAP-K2, mitogen-activated-protein-kinase-activated protein kinase 2; PDK1, 3-phosphoinositide-dependent protein kinase-1; PH, pleckstrin homology; PIF, PDK1-interacting fragment; (PK)A/B/C, protein kinases A, B and C respectively; SGK, serum- and glucocorticoid-responsive kinase; S6K, p70 ribosomal S6 kinase; UCN-01, 7-hydroxystaurosporine.

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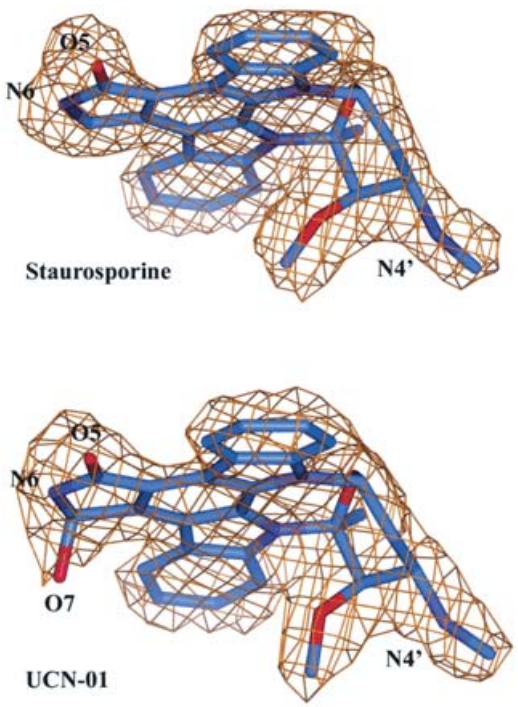

Figure 1 Staurosporine and UCN-01 electron density

The staurosporine and UCN-01 molecules are shown in a stick representation with carbon atoms in light blue, oxygen atoms in red and nitrogen atoms in dark blue. Hydrogen-bonding atoms (Table 2) are labelled as described in [49]. The unbiased $|F_o|-|F_c|, \Phi_{calc}$ maps are shown in orange, and are contoured at 2.5σ.

Table 1 Details of data collection and structure refinement

Values shown in parentheses are for the highest-resolution shell. Crystals were cryogenically cooled to 100 K. All measured data were included in structure refinement. RMSD, root-mean-square deviation.

Dataset	Inhibitor . . .	Staurosporine	UCN-01
Space group		$P3_221$	$P3_221$
Cell dimensions (Å)		$a = 124.17$ $b = 124.17$ $c = 47.31$	$a = 123.39$ $b = 123.39$ $c = 47.12$
Resolution range (Å)		25-2.30 (2.38-2.30)	25-2.50 (2.59-2.50)
No. of observed reflections		31730 (3091)	68515 (6290)
No. of unique reflections		18018 (1794)	14395 (1430)
Redundancy		1.8 (1.7)	4.8 (4.4)
$I/\sigma I$		7.5 (1.8)	4.5 (2.6)
Completeness (%)		95.8 (95.8)	100.0 (99.9)
R_{merge}		0.096 (0.505)	0.167 (0.688)
R_{cryst}, R_{free}		0.218, 0.255	0.189, 0.257
RMSD from ideal geometry			
bonds (Å)		0.007	0.009
angles (°)		1.7	1.8
B-factor RMSD (Å^2) (bonded, main chain)		1.5	1.4
⟨B⟩ protein (Å^2)		31.5	27.3
⟨B⟩ inhibitor (Å^2)		18.5	17.4

(CHK1) [20] and PDK1 [21] may be key targets of UCN-01 in inhibiting the growth of cancer cells, since both kinases are inhibited by UCN-01 in the low nanomolar range.

Here, we report the structures of the PDK1 kinase domain in complex with staurosporine and UCN-01, demonstrating the presence of a pocket that accommodates the 7-hydroxy group of UCN-01. Specificity tests against a panel of 29 kinases show that, although both staurosporine and UCN-01 are relatively non-specific inhibitors, the fingerprint analysis of UCN-01 inhibition with a panel of protein kinases is significantly different from that of staurosporine. We also perform an analysis of the residues predicted to line the UCN-01 hydroxy pocket on a number of protein kinases, and propose a general model that could account for the different sensitivity of protein kinases for staurosporine and UCN-01.

MATERIALS AND METHODS

Expression, purification and crystallization

Human PDK1 (residues 51–359) was expressed and purified from baculovirus-infected SF21 insect cells, as described previously [11], with the following differences: after elution of the His_6-tagged protein from the Ni^{2+}-nitrilotriacetate–agarose beads with 200 mM imidazole, the protein was dialysed against 250 mM NaCl/25 mM Tris/HCl, pH 7.5/1 mM dithiothreitol for 3 h at 4 °C. Proteolysis with glutathione S-transferase–tagged PreScission protease was performed overnight at 4 °C.

For crystals of the PDK1–UCN-01 complex, 100 μl of PDK1 at a concentration of 6.6 mg/ml was mixed with 30 μl of UCN-01 [5.3 mM in 50 % (v/v) ethanol] and incubated on ice for 2 h. The protein was crystallized using sitting-drop vapour diffusion. Protein solution (1.25 μl) was mixed with 0.25 μl of cobaltous chloride hexahydrate (0.1 M) and 1 μl of the mother liquor, consisting of 0.1 M Tris/HCl, pH 8.5 and 2.1 M ammonium sulphate. Hexagonal, rod-shaped crystals grew at 20 °C and reached a maximum size of $0.05 \times 0.05 \times 0.3$ mm after 7 days. After soaking for 3 s in a cryoprotection solution [2.1 M ammonium sulphate/0.1 M Tris/HCl (pH 7.2)/25 % (v/v) glycerol], crystals were frozen in a stream of cold nitrogen.

PDK1 in complex with staurosporine was crystallized using the hanging-drop technique. Drops consisted of 1 μl of PDK1 (at a concentration of 7.6 mg/ml), 1 μl of mother liquor [2.1 M ammonium sulphate/0.1 M Tris/HCl (pH 7.2)] and 0.25 μl of staurosporine (10 mM in DMSO). Hexagonal shaped crystals suitable for data collection appeared after 6 weeks at 20 °C. Crystals were soaked in solution containing 1.7 M ammonium sulphate, 0.1 M Tris/HCl, pH 7.2 and 15 % glycerol, and frozen in a stream of cold nitrogen.

Data collection, structure solution and refinement

Data on the PDK1–staurosporine and PDK1–UCN-01 complexes were collected at the European Synchrotron Radiation Facility (Grenoble, France) beam-line ID14-EH4. The temperature of the crystals was maintained at 100 K using a nitrogen cryostream. Data were processed using the HKL package [22] with final statistics shown in Table 1. The structures were solved by rigid-body refinement with the program CNS [23], using the previously published PDK1 structure (PDB code 1H1W) [11] as a starting model, which resulted in an initial R-factor of 0.306 (R_{free} 0.284) for the PDK1–staurosporine complex and 0.299 (R_{free} 0.311) for the PDK1–UCN-01 complex. Model building with Oxygen software [24] and iterative refinement in CNS, including solvent molecules and the T-loop phosphorylation site, resulted in final R-factors as shown in Table 1. The ATP-binding site showed well-defined density in the unbiased $|F_o|-|F_c|, \Phi_{calc}$ maps for all atoms of staurosporine and UCN-01, including the 7-hydroxy group (Figure 1). CNS topologies and coordinates for

the inhibitors were generated with PRODRG [25]. No electron density could be observed for residues 51–72 (N-terminus), residues 231–239 (T-loop) or residue 359 (C-terminus) in the PDK1–UCN-01 complex. Residues 51–71 (N-terminus) and 233–238 (T-loop) were disordered in the PDK1–staurosporine complex.

Determination of inhibition and mutagenesis

Protein kinase assays: PDK1 was assayed for 10 min at 30 °C in a 50 μl assay mixture consisting of 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1 % 2-mercaptoethanol, also containing 100 μM PDKtide substrate peptide (KTFCGTPEYLAPEVRRE-PRILSEEEQEMFRDFDYIADWC), 10 mM magnesium acetate and 100 μM [γ-^{32}P]ATP (200 c.p.m./pmol), as described previously [10]. Other protein kinases employed (shown below in Table 3) were assayed as described previously [17,26].

The Thr^{222} → Ala mutant of PDK1 was generated by site-directed mutagenesis using a QuikChange kit (Stratagene) following instructions provided by the manufacturer, and was expressed and purified as described previously [11].

RESULTS AND DISCUSSION

Structures of the staurosporine and UCN-01 complexes

PDK1 (residues 51–359) was co-crystallized with staurosporine and UCN-01, and synchrotron diffraction data on a thin hexagonal needle were collected to 2.3 Å (1Å = 0.1 nm) and 2.5 Å resolution respectively. In the unbiased $|F_o|-|F_c|, \Phi_{calc}$ maps, well-defined ($> 3.0\sigma$) density could be observed in the ATP-binding site of the kinase, covering all staurosporine/UCN-01 atoms including the 7-hydroxy group (Figure 1). After initial rounds of protein model building and inclusion of water molecules, the inhibitor molecules were built and refined with full occupancy to average B-factors of 18.5 Å^2 (staurosporine) and 17.3 Å^2 (UCN-01). Further refinement resulted in a final PDK1–staurosporine model with R = 0.218 (R_{free} 0.255), and a final PDK1–UCN-01 model with R = 0.184 (R_{free} 0.257), both with good stereochemistry (Table 1).

The staurosporine molecule is located in the ATP-binding site (which lies between the N-terminal and C-terminal lobes of kinases [27,28]), at the same position described for the inhibitor in complex with the closely related (38 % sequence identity) protein kinase A (PKA [29]; PDB code 1STC) (Figure 2). Hydrophobic residues on both sides of the ATP-binding cleft 'sandwich' the heterocyclic moiety of staurosporine, namely Leu^{88}, Val^{96}, Ala^{109} and Leu^{98} (N-terminal lobe), and Thr^{222} and Leu^{212} of the C-terminal lobe (Figures 2 and 3A). Similarly to the PKA–staurosporine complex, the lactam group mimics the interactions of the adenine base in ATP with the protein backbone, where two conserved hydrogen bonds are formed between the lactam-nitrogen N6 in staurosporine (nomenclature as described in [30]) and the backbone-oxygen of Ser^{160}, and the lactam-oxygen at the C5 position and the backbone-nitrogen of Ala^{162} (Table 2). An additional hydrogen bond is mimicked in the staurosporine sugar moiety, where the methylamino group contacts oxygen Oδ2 of Glu^{166}, similar to the hydrogen bond with the ribose in the PDK1–ATP complex [11], and also the backbone carbonyl of Glu^{209} (Table 2).

The UCN-01 molecule occupies the same position in the ATP-binding site as staurosporine (maximum atomic shift of 0.35 Å) (Figures 2 and 3B). The same hydrophobic interactions are made to the heterocyclic moiety in the PDK1–staurosporine complex. Hydrogen-bonding interactions to the heterocycle and the sugar moiety are also conserved, with similar geometry (Table 2). However, the 7-hydroxy group of UCN-01 forms several novel hydrogen bonds (Figure 3B and Table 2). It hydrogen bonds directly to Oγ1 of Thr^{222} (Figure 3B and Table 2). In addition, an ordered water molecule (B-factor 24.0 Å^2) is found in a position where it contacts the 7-hydroxy group (distance = 3.0 Å; Table 2) and the oxygen Oε1 of Gln^{220} (distance = 2.5 Å), the side chain of which is shifted towards the ligand (1.5 Å for Cδ, rotation of 82° around φ_1) compared with the PDK1–staurosporine complex. However, the water molecule is buried in a predominantly hydrophobic pocket, lined by Val^{143}, Leu^{212} and Cγ2 of Thr^{222} (Figure 3B). Val^{143} also changes its position compared with the PDK1–staurosporine complex (Figure 3), by moving further towards the back of the pocket (shift of 0.7 Å for the Cα carbon, and a rotation of 100° around φ_1) and displacing an ordered water molecule present in the PDK1–staurosporine complex (Figure 3A), which is also observed in other kinase–staurosporine complexes, such as CHK1 [30,31] (Figure 3C). These changes result in more space to accommodate the bulky 7-hydroxy group on UCN-01 (6 Å^3 increase in ligand volume, calculated with VOIDOO [32]).

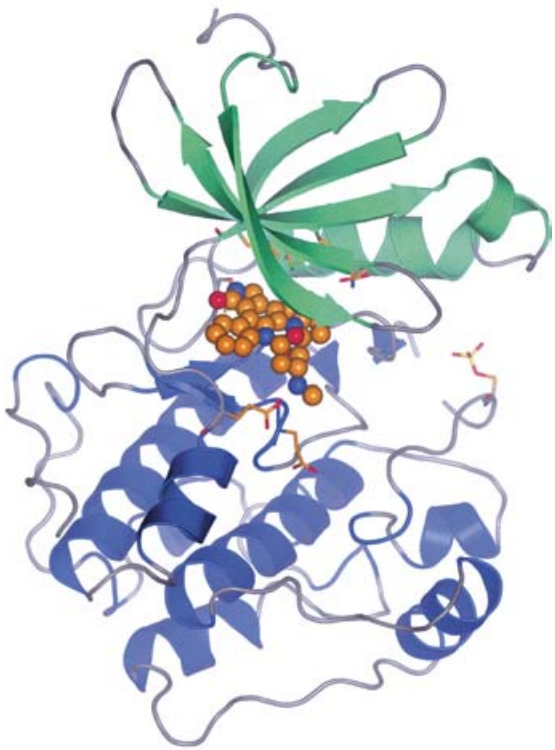

Figure 2 Overview of PDK1-kinase domain bound to staurosporine

The two lobes [in green (N-terminal lobe) and blue (C-terminal lobe)] of the kinase enclose the inhibitor molecule (shown by the orange spheres). The phosphorylated T-loop (shown as a stick representation with a yellow phosphorous atom and red oxygen atoms) lacks residues, due to disorder. Side chains interacting with the inhibitor molecule are drawn as a stick representation with green carbon atoms (refer to Figure 3).

Comparison with the CHK1–UCN-01 complex

High-resolution data for the CHK1 kinase bound to staurosporine and UCN-01 is available (PDB codes 1NVR, 1NVQ; [30]). In CHK1, Ser^{147}, the equivalent of Thr^{222} in PDK1, also hydrogen bonds the UCN-01 7-hydroxyl directly (Figure 3C). Val^{68} in

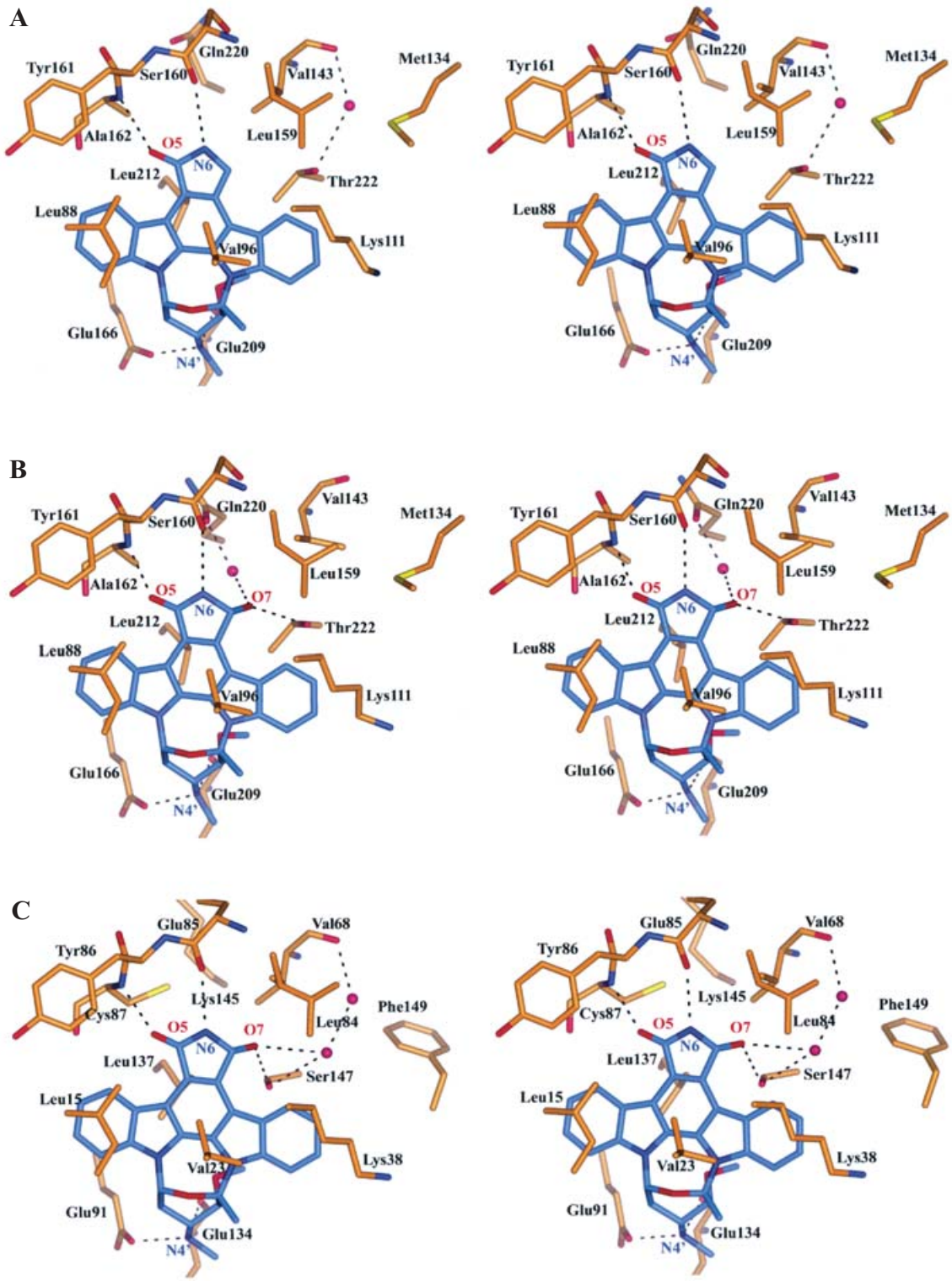

Figure 3 Details of the inhibitor binding sites

Ligand carbon atoms are coloured in light blue and protein carbon atoms are in orange. Nitrogen atoms are coloured in dark blue; oxygen atoms are coloured in red. Water molecules are shown as purple spheres. Hydrogen bonds are indicated by black dotted lines. Stereo-images are shown of (**A**) staurosporine bound to PDK1; (**B**) UCN-01 bound to PDK1; and (**C**) UCN-01 bound to CHK1.

CHK1 (corresponding to Val^{134} in PDK1) is not flipped, as observed for the PDK1–UCN-01 complex, and the buried water molecule generates a water-mediated network of hydrogen bonds to UCN-01. In CHK1, a second water molecule at a different position (shifted 5.2 Å compared with the PDK1–UCN-01 complex) hydrogen bonds O7 of UCN-01 (Figure 3C). CHK1

Table 2 Hydrogen bonding between inhibitors and PDK1

Hydrogen bonds between PDK1 and UCN-01/staurosporine (STO) were calculated with the WHAT IF program [36] using the HB2 algorithm [37]. This algorithm gives a 0 (no hydrogen bond) to 1 (optimal hydrogen bond) score to reflect hydrogen-bond geometry (HB2 column). Donor–acceptor distances are also listed (D-A).

Atom of inhibitor	Atom of protein/H_2O	UCN-01 D-A (Å)	UCN-01 HB2	STO D-A (Å)	STO HB2	Comment
O5	N-Ala^{162}	2.8	0.76	3.0	0.81	Conserved
N6	O-Ser^{162}	2.9	0.80	3.1	0.67	Conserved
N4′	O-Glu^{209}	3.2	0.68	3.1	0.39	Conserved
N4′	Oε2-Glu^{166}	2.6	0.63	2.5	0.45	Conserved
O7	Oγ1-Thr^{222}	3.0	0.56			7-Hydroxy group
O7	H_2O	3.0	0.89			Water mediated to (Oε1-Gln^{220})

appears to have a more extended hydrophilic cavity, as there are two additional buried water molecules present also in the CHK1–staurosporine complex. The corresponding residue to Gln^{220} in PDK1 is a lysine (Lys^{145}) in CHK1, which does not interact with the ligand, but points away from it.

UCN-01 inhibition and specificity

PDK1 inhibition by UCN-01 and staurosporine was measured using kinase assays with ^{32}P-labelled ATP. PDK1 is inhibited by UCN-01 with an IC_{50} value of 5 nM, and by staurosporine with an IC_{50} of 6.5 nM. As a measure for overall specificity of UCN-01 and staurosporine, the effect of these inhibitors was tested against a panel of 29 protein kinases, as described previously [17,26]. The results are shown in Table 3 as percentages of control activity. These data confirm further that UCN-01 and staurosporine are aspecific inhibitors. UCN-01 at a concentration of 1 μM reduces the activity of nine kinases in the panel to less than 10 %, and that of ten others to below 60 % of control activity. Staurosporine at 1 μM will inhibit 12 kinases to less than 10 % control activity, and another 10 to below 60 %. Interestingly, however, several of the protein kinases were differentially inhibited by staurosporine and UCN-01 (Table 3). In an attempt to understand these differences, the panel of kinases was divided into four distinct classes: (a) similar inhibition; (b) stronger inhibition by staurosporine than by UCN-01; (c) stronger inhibition by UCN-01 than by staurosporine; and (d) no inhibition (Table 3). As the additional 7-hydroxy group is the only difference between the ligand molecules (Figure 1), and staurosporine and UCN-01 occupy the same position with similar interactions in the binding site (Figure 3), the residues contacting the extra hydroxy group were identified for PDK1 and extracted from a sequence alignment of all protein kinases used in the panel (Table 3). A structure-based sequence alignment of known kinase structures was obtained from [33], and used to validate the sequence-based sequence alignment (Table 3). The nature of the side chains lining the hydroxy-group-binding pocket could provide a partial explanation for the relative difference between UCN-01 and staurosporine inhibition. Two trends can be observed. For the kinases that are inhibited by UCN-01, there appears to be a preference for a side chain capable of hydrogen bonding the 7-hydroxy group in the hydroxy-group-binding pocket. This is in agreement with the presence of a serine/threonine residue that hydrogen bonds the 7-hydroxy group in the structures of PDK1 (Thr^{222}) and CHK1 (Ser^{147}) bound to UCN-01. Five out of eleven kinases that are hit equally by staurosporine and UCN-01 (group a) appear to have a potential hydrogen-bonding residue at the position equivalent to Thr^{222} in PDK1 (Table 3), and two (PKB and PKC) have a threonine at the position equivalent to Val^{143} in PDK1, which could potentially form a similar hydrogen-bond interaction with the 7-hydroxy group of UCN-01 (Figure 3). The kinases that are more potently inhibited by UCN-01 than by staurosporine (group c) contain a threonine at the Thr^{222}-equivalent position (Table 3). However, mutation of Thr^{222} to alanine in PDK1 did not have a significant effect on either UCN-01 or staurosporine inhibition (results not shown). This shows that Thr^{222} is not the only determinant of binding, suggesting that more factors need to be taken into account, such as the flexibility of the side chains lining the pocket.

Five out of nine kinases that are inhibited more potently by staurosporine than by UCN-01 (group b) lack a potential hydrogen-bonding partner in the 7-hydroxy-group-binding pocket (Table 3). Two of the kinases possessing a threonine at the position corresponding to Thr^{222} [PKA and MAPKAP-K2 (mitogen-activated-protein-kinase-activated protein kinase 2)] in this group have a bulkier methionine at the PDK1 Leu^{159}-equivalent position. PKA contains a possible hydrogen-bonding partner for UCN-01 (Thr^{183}), but Met^{134} in the centre of the hydroxy-group-binding pocket may leave no space for the extra hydroxy group (Table 3). Modelling an extra O7-hydroxy group to staurosporine in the structure of the PKA–staurosporine complex (PDB code 1STC [29]) generates steric clashes, possibly explaining why PKA is not hit by UCN-01. Therefore a second trend that may contribute to specificity is the size of the residues lining the hydroxy-group-binding pocket. If the predicted total volume of the residues (calculated with the BL– set of side chain volumes [34]) at the positions indicated in Table 3 is set against the activity in the presence of UCN-01, a correlation coefficient (r) of 0.6 is obtained. This suggests that, despite inaccuracies in this approach, such as the absence of structural information on precise side chain conformation and water molecules, a weak correlation between predicted hydroxy-group-binding pocket volume and UCN-01 inhibition exists. This size dependency may also have a role for the protein kinases hit by neither staurosporine nor UCN-01. The sequence alignment shows that the Val^{143}- and Thr^{222}-equivalent residues are replaced by bulkier leucine or isoleucine residues in several of the mitogen-activated protein kinase families (Table 3). To investigate the effect of these bulkier side chains on the hydroxy-group-binding pocket, we, starting from the PDK1 crystal structure, replaced residues Val^{143} with isoleucine and Thr^{222} with leucine in standard side chain rotamers (in O [24]), which indeed resulted in van der Waals clashes with C7 of staurosporine (shortest distances: 2.8 Å for Leu^{222}; 3.5 Å for Ile^{143}), and may therefore explain the lack of susceptibility towards UCN-01 in the mitogen-activated protein kinase families. CDK2 (cyclin-dependent kinase 2) is inhibited by both staurosporine and UCN-01 similarly; however,

Table 3 Comparison of inhibition by UCN-01 versus staurosporine and hydroxy-group-binding pocket-lining residues

The indicated protein kinases were assayed at 0.1 mM ATP, as described previously [17,26], in the absence or presence of 1 μM staurosporine (STO) or UCN-01. Results in the two columns labelled 'STO' and 'UCN-01' are presented as the percentage of kinase activity compared with that in control incubations, and the activity results displayed in these two columns are an average of triplicate determinations. For further details concerning the categorization of the groups (**a**)–(**d**), see the text. Numbered residues are referred to in the text. Abbreviations for kinases not otherwise defined in the abbreviations footnote: AMPK, AMP-activated protein kinase; CK1/CK2, (protein formerly known as) casein kinase-1 or -2 respectively; CSK, C-terminal Src kinase; DYRK, dual-specificity tyrosine-phosphorylated and -regulated kinase; GSK3β, glycogen synthase kinase 3β; JNK, c-Jun N-terminal kinase; MAPK2/ERK2, mitogen-activated protein kinase-2/extracellular-signal-regulated kinase 2; MKK1, mitogen-activated protein kinase kinase-1; NEK6, [NIMA (never in mitosis, gene A)]-related kinase-6; PHK, phosphorylase kinase; PRAK, p38-regulated/activated protein kinase; ROCKII, Rho-dependent protein kinase-II;. Residues lining the hydroxy-group-binding pocket are shown in the rightmost five columns, as derived from a multiple sequence alignment with T-Coffee [38]. Kinases with indicated PDB codes (in parentheses) were aligned using 'The Protein Kinase Resource' [33].

Group	Kinase	STO	UCN-01	Residues lining the hydroxy-group-binding pocket				
(**a**) Both STO and UCN-01 inhibit								
	PDK1	5 ± 1	0 ± 1	Met^{134}	Val^{143}	Leu^{159}	Gln^{220}	Thr^{222}
	CHK1 (1NVQ [30])	3 ± 1	1 ± 0	Asn^{59}	Val^{68}	Leu^{84}	Lys^{145}	Ser^{147}
	PKCα	8 ± 2	1 ± 0	Leu	Thr	Met	Lys	Ala
	AMPK	0 ± 0	1 ± 1	Leu	Ile	Met	Lys	Ala
	PHOS.KINASE (1PHK [39])	2 ± 3	1 ± 2	Leu	Ile	Phe	Lys	Thr
	Lck (1QPJ [40])	0 ± 0	3 ± 1	Met	Val	Thr	Lys	Ala
	CDK2/cyclin A (1AQ1 [31])	12 ± 12	8 ± 0	Leu	Val	Phe^{80}	Lys	Ala
	PKBδPH (1O6K [41])	8 ± 2	9 ± 1	Leu	Thr	Met	Lys	Thr
	ROCK-II	9 ± 5	13 ± 2	Met	Val	Met	Lys	Ala
	S6K1	24 ± 8	21 ± 4	Leu	Val	Leu	Lys	Thr
	GSK3β (1I09 [42])	29 ± 6	25 ± 5	Met	Val	Leu	Lys	Cys
(**b**) STO inhibits more strongly than UCN-01								
	MSK1	1 ± 0	11 ± 0	Leu	Val	Leu	Val	Thr^{406}
	DYRK1α	2 ± 2	15 ± 2	Leu	Met	Phe	Lys	Val
	PKA (1STC [29])	4 ± 1	27 ± 2	Leu	Val	Met^{134}	Gln	Thr^{183}
	MKK1	5 ± 8	53 ± 1	Leu	Val	Met	Lys	Cys
	MAPKAP-K2	23 ± 1	60 ± 1	His	Val	Met	Lys	Thr
	CSK (1BYG [43])	25 ± 8	58 ± 3	Met	Val	Thr	Lys	Ser
	SAPK3/p38γ (1CM8 [44])	37 ± 0	94 ± 8	Leu	Ile	Met	Lys	Leu
	SAPK4/p38δ	40 ± 5	100 ± 7	Leu	Ile	Met	Lys	Leu
	PRAK	48 ± 1	89 ± 4	His	Val	Met	Lys	Cys
(**c**) UCN-01 inhibits more strongly than STO								
	MAPKAP-K1a	18 ± 10	1 ± 1	Leu	Val	Leu	Lys	Thr^{341}
	SGK1	51 ± 4	22 ± 4	Leu	Val	Leu	Val	Thr^{407}
(**d**) Neither UCN-01 nor STO inhibits								
	MAPK2/ERK2 (1ERK [45])	100 ± 4	107 ± 5	Leu	Ile	Gln	Lys	Cys
	JNK/SAPK1c	91 ± 3	112 ± 6	Met	Ile	Met	Lys	Leu
	SAPK2α/p38 (1P38 [46])	76 ± 4	107 ± 5	Leu	Ile	Thr	Lys	Leu
	SAPK2β/p38β2	84 ± 11	106 ± 4	Leu	Ile	Thr	Arg	Leu
	CK2 (1F0Q [47])	95 ± 4	102 ± 11	Leu	Val	Phe	Arg	Ile
	CK1 (1CKI [48])	95 ± 11	96 ± 0	Tyr	Pro	Met	Tyr	Ile
	NEK6	109 ± 2	80 ± 7	Leu	Ile	Leu	Lys	Gly

this kinase lacks a hydrogen-bonding partner for the 7-hydroxy group and contains a bulky phenylalanine (Phe^{80}) at the Leu^{159}-equivalent position. In a superimposition of CDK2–staurosporine structure [31] with PDK1–UCN-01 (root-mean-square deviation = 1.3 Å on Cα atoms), staurosporine is seen to be shifted by 1.2 Å out of the potential hydroxy-group-binding pocket due to the presence of the bulky Phe^{80}. Interaction of the 7-hydroxy group on UCN-01 was described to be water-mediated in CDK2 due to the lack of hydrogen-bonding residues [35]. This particular example highlights the limitations of the approach described above. Other examples where none of the described effects account for the observed behaviour are the tyrosine kinases Lck and Csk (C-terminal Src kinase), as well as AMPK (AMP-activated protein kinase) and MSK1 (mitogen- and stress-activated kinase 1) (Table 3). Both MSK1 and SGK1 show the same sequence in their hydroxy-group-binding pocket, with Thr^{406} or Thr^{407} respectively as potential hydrogen-bonding partners, but both were placed in different groups. MSK1 activity is abolished by 1 μM staurosporine, but shows residual activity (11 %) with UCN-01. SGK1 activity is 25 % that of control activity with 1 μM UCN-01, but is twice as high with staurosporine.

Conclusions

UCN-01 was subjected to a specificity analysis against an in-house panel of 29 protein kinases. Contrary to the suggestions of some previous reports, the data show that UCN-01 is not a specific inhibitor, since it inhibited more than half of the tested kinases at significant levels. A direct comparison with staurosporine, however, showed a different pattern of inhibition, and was the subject of further analysis. We have reported the crystal structures of PDK1 in complex with the inhibitors staurosporine and UCN-01. Both inhibitors appear to bind to PDK1 in a similar fashion compared with the CHK1–UCN-01 [30] or PKA–staurosporine [29] complexes, with additional hydrogen-bonding interactions at the UCN-01 7-hydroxy group. This moiety is hydrogen-bonded directly to Thr^{222} and indirectly via an ordered water molecule to Gln^{220}. A different water-mediated hydrogen-bonding network

is also observed in other UCN-01 complexes known to date [30,35], and might serve as a starting point for further structure-based optimization. The residues around the 7-hydroxy group (the hydroxy-group-binding pocket) were aligned with known kinase structures and kinase sequences. It is apparent that spatial effects in the identified pocket have a key role in determining UCN-01 inhibition, as does the presence of hydrogen-bonding partners for the additional hydroxy group.

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