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Structure-based design, synthesis, and study of pyrazolo[1,5-a][1,3,5]triazine derivatives as potent inhibitors of protein kinase CK2

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Abstract—The structure-based design, synthesis, and anticancer activity of novel inhibitors of protein kinase CK2 are described. Using pyrazolo[1,5-a][1,3,5]triazine as the core scaffold, a structure-guided series of modifications provided pM inhibitors with μM-level cytotoxic activity in cell-based assays with prostate and colon cancer cell lines.

CK2 is a highly conserved and ubiquitous serine/threonine protein kinase that only until recently has been considered as a possible target in cancer chemotherapy.[1] It has been shown to be elevated in a wide variety of cancers that have been examined[2] and has been shown to be correlated with the tumors' aggressive growth. Targeted over-expression of CK2 in transgenic mice results in mammary tumorigenesis and CK2 has been demonstrated to increase a cell's oncogenic potential by sensitizing a cell to transformation by other oncogenic proteins.[3] In addition, the recent interest stems in part from new data concerning its involvement in cell proliferation, protection from apoptosis, and its involvement in angiogenesis, in part, through its regulatory function on HIF-1.[4] The modulation of CK2 activity has been demonstrated to cause a decrease in cellular proliferation as well as an increase in the level of apoptosis observed in cancer cells. This has been accomplished through the use of small molecules, dominant negative over-expression of kinase inactive mutants,[5] anti-sense methods,[1] and small interfering RNA molecules (siRNA).[6] These methods have been applied to tumor bearing mice and in the best case, demonstrated complete eradication of the PC3 human prostate cancer tumor.[7] Clearly, modulation of CK2 protein levels or kinase activity results in an interesting and promising pre-clinical result.

The currently available CK2 inhibitors[8] lack the potency, physiochemical, and pharmacological properties required to be successful in a clinical setting. The need for new classes of CK2 inhibitors satisfying these challenges is clear. Using a structure-based design approach, we have developed a series of pyrazolo[1,5-a][1,3,5]triazine derivatives as potent inhibitors of CK2 that also demonstrate cell-based anti-proliferative activity in multiple cancer cell lines.

The medicinal chemistry effort to find small molecule inhibitors of CK2 started with lead identification utilizing both a protein structure specific library screen and de novo rational design techniques. Initial screening of our pre-selected library was accomplished using the human recombinant CK2α catalytic subunit. N^2,N^4-Diphenyl-pyrazolo[1,5-a][1,3,5]triazine-2,4-diamine **1** (Fig. 1) was found to be a potent inhibitor of CK2.

The active site of human and that of corn CK2 are virtually identical with only minor conservative amino acid substitutions. Corn CK2 (cCK2) protein readily co-crys-

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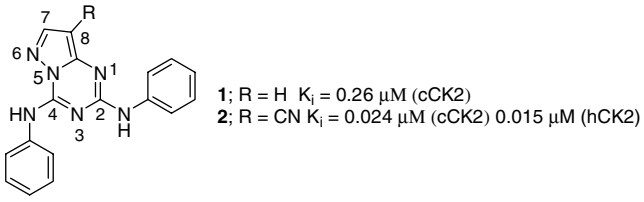


Figure 1. Pyrazolo[1,5-a][1,3,5]triazine inhibitors of CK2.

tallized within our laboratory and the resulting structural coordinates were therefore used in structure-based drug design of CK2 inhibitors. A 1.9 Å resolution co-crystal structure of **1** with cCK2 was solved in which the pyrazolo-triazine core occupied the adenine binding site and was sandwiched between Ile66 and Met163. Two crucial hydrogen bonds between the inhibitor and the backbone of Val116 in the proteins, hinge region anchored the inhibitor in the ATP binding site (Fig. 2.1). The two phenyl groups of the inhibitor adopted a folded conformation due to intra-molecular stacking interactions, in which the C4-NHPh made hydrophobic interactions with the side chains of Arg43, and the surface amino acids Tyr115 and Asn118 (Fig. 2.2). The C2-NHPh extended toward the substrate binding crevice between the phosphate binding loop (Gly46 and Arg47) and the catalytic loop (Fig. 2.3).

It was recognized that three regions of the cCK2 binding site were left largely untouched by the inhibitor: (1) a deeply buried, hydrophobic pocket lined by Val95 and Phe113; (2) a large hydrophobic back pocket composed of residues Val53 and Ile174; and (3) a pair of solvent shielded polar residues Lys68 and Asp175 (Fig. 2.4). Therefore, our initial efforts were focused on these regions. Indeed, as expected, addition of a nitrile group at the C8 position of **1** provided compound **2** with an improvement of potency by ~10-fold (Fig. 1). The nitrile group interacts with hydrophobic residues Val95 and Phe113, which is confirmed by later solved co-crystal structures. A focused library of C2 substitutions was prepared next to explore the large back pocket.

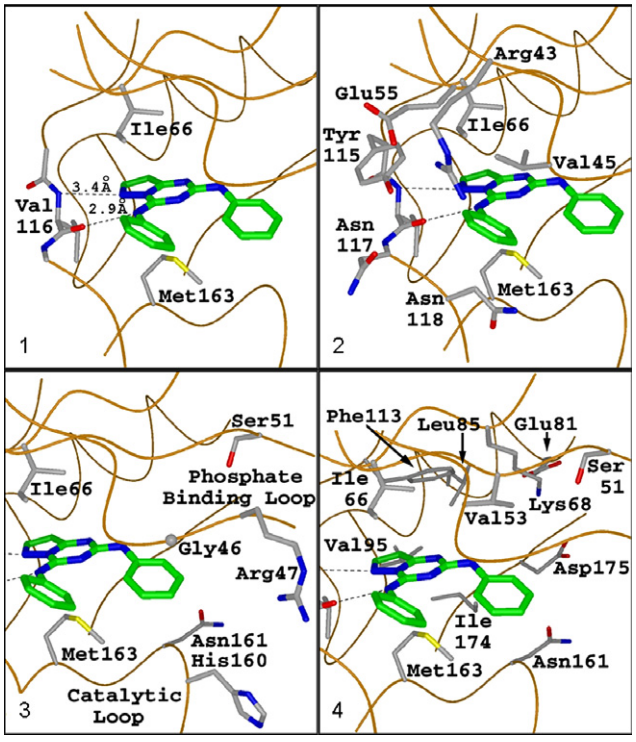
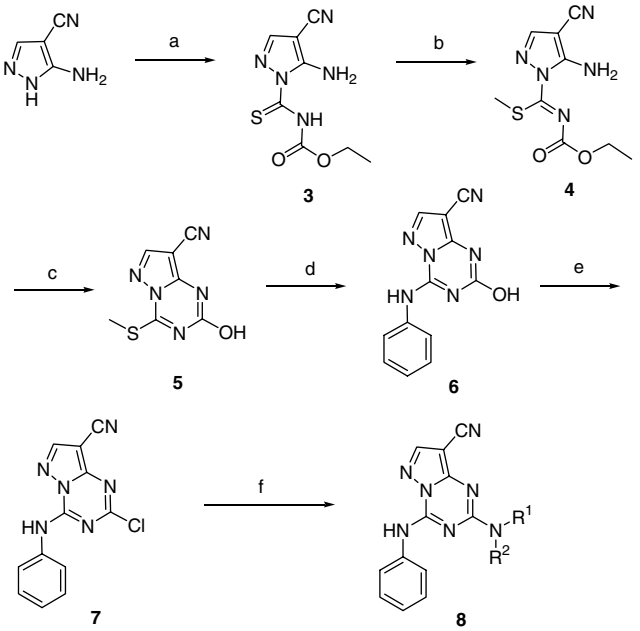


Figure 2. cCK2α active site complex with inhibitor **1** (PDB code: 2PVH). Four panels highlight active site residues that can potentially interact with the pyrazole-triazine core or substitutions at the C2, C4 or C8 positions. All cCK2α residue numbers are derived from those of the highly homologous human CK2α.

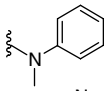
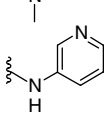
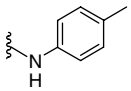
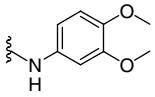
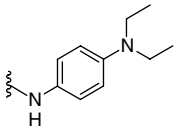
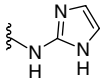
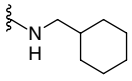
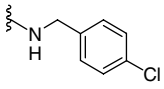
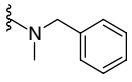
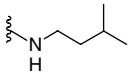
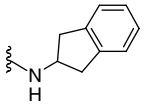
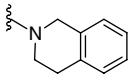
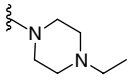
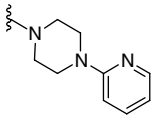
According to Scheme 1, 5-amino-1*H*-pyrazole-4-carbonitrile was first reacted with ethoxycarbonyl-isothiocyanate to give the corresponding pyrazolo-thiourea derivative **3** at room temperature. Direct cyclization failed to yield the desired pyrazolo-triazine. However, once the thiol was methylated, it was cyclized smoothly onto the pyrazole in the presence of DBU in DMF. C4 substitution with amines was carried out at 90 °C to give **6**, followed by chlorination in refluxing phosphorus oxychloride to provide **7**. The chlorine could be displaced easily with a variety of amines in NMP at 50 °C to provide **8**.

Using compound **7** as the building block, a focused library of 184 compounds with C2 substitutions was synthesized in 96-deep well plates with 2 μmol/compound/well. Without further purification, these library compounds were screened against CK2 at 50 and 250 nM concentrations. Compounds in the library that showed >80% inhibition at 50 nM were re-synthesized and characterized. The data are summarized in Table 1. In general, these compounds show potent enzymatic inhibition with the best K_i values in the low nM range.



Scheme 1. Reagents and conditions: (a) $(CO_2Et)NCS$, EtOAc/benzene, rt; (b) MeI, NEt_3, THF; (c) DBU, DMF; (d) Aniline, 90 °C; (e) $POCl_3$, reflux; (f) HNR^1R^2, *i*-Pr_2NEt, NMP, 50 °C.

Table 1. Inhibition data of C2 substituted pyrazolo-triazines

8	–NR^{1}R^2	hCK2[a] K_i (μM)
a		0.242
b		0.168
c		0.005
d		0.009
e		0.023
f		0.045
g		0.008
h		0.005
i		0.055
j		0.013
k		0.012
l		0.014
m		0.126
n		0.011
o		0.024
p		0.007

[a] All K_i values reported in this paper were determined using human CK2α (hCK2), except for **1** and **8a** which were obtained from corn CK2α.

Co-crystal structures of compounds **8g**, **8h** and **8o** in complex with cCK2 were solved (Fig. 3). Interestingly, these three inhibitors all adopted the folded conformation and occupied the same regions as observed in our initial co-crystal structure (Fig. 2). Apparently, intramolecular hydrophobic stacking interaction between C2 substitutions and C4 aniline dominates the conformation of the C2 substitutions. Although these compounds failed to reach the back pocket as we hoped, the interaction of appropriately positioned C2-substitutions (**8g**, **8h**, and **8o**) with adjacent protein residues such as His160, Asp120, Phe121, Val45, and Met163 resulted in significant enhancement of their inhibitory potency as compared to the parent compound **2**.

Clearly, in order to reach the back pocket, the C2-substitution needs to adopt an extended conformation, which could be achieved by interrupting the intra-molecular stacking interactions between C2 and C4 substitutions, or designing an appropriately substituted C2-group which would be induced-fit into the back pocket. When C4-aniline was replaced with a flexible group, a loss of potency was observed for compounds **9a**, **9b**, and **9c** (Table 2). The co-crystal structure of **9a** in complex with cCK2 revealed that without a preference for intra-molecular stacking interactions, C2 aniline indeed adopted the extended conformation, which fit into the back pocket as expected (Fig. 3). Next, compound **9d** was synthesized with an acetamide on the C2-phenyl ring designed to interact with the salt bridge between Lys68 and Asp175. As predicted by modeling, the increase in potency was profound with **9d** being ~20-fold more active than **9b**. Compound **9e**, which has aromatic anilines on both C2 and C4 positions, was synthesized, and another ~20-fold enhancement in potency was achieved (Table 2).

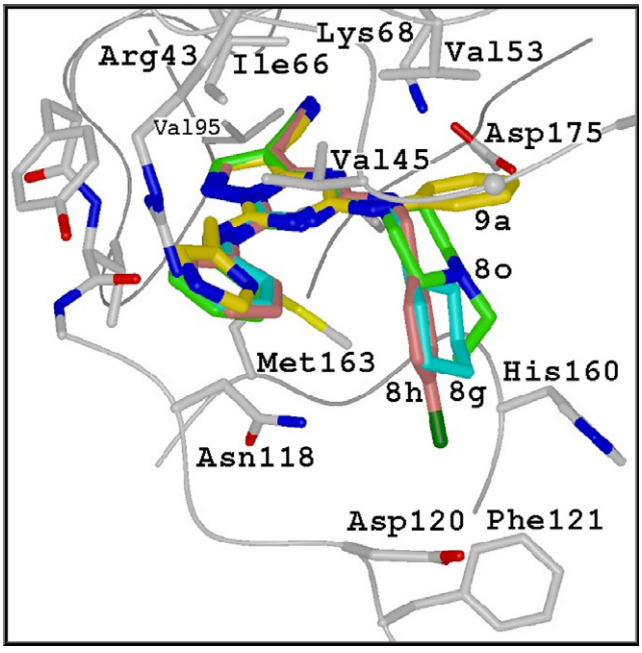
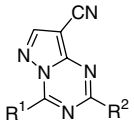
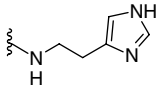
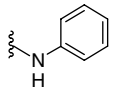
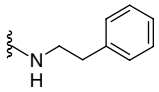
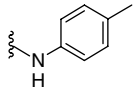
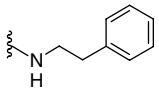
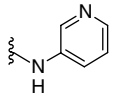
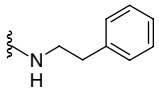
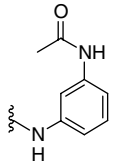
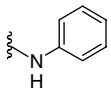
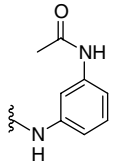


Figure 3. cCK2α binary complex with **8h** and inhibitors **8g**, **8o**, and **9a** superposed using their respective protein Cα atoms. (PDB codes: 2PVJ, 2PVK, 2PVL and 2PVM.)

Table 2. Effect of C4 substitutions on the binding site of C2 substitutions



9

9	R^1	R^2	hCK2 K_i (μM)
a			0.36
b			0.137
c			3.4
d			0.008
e			0.00035

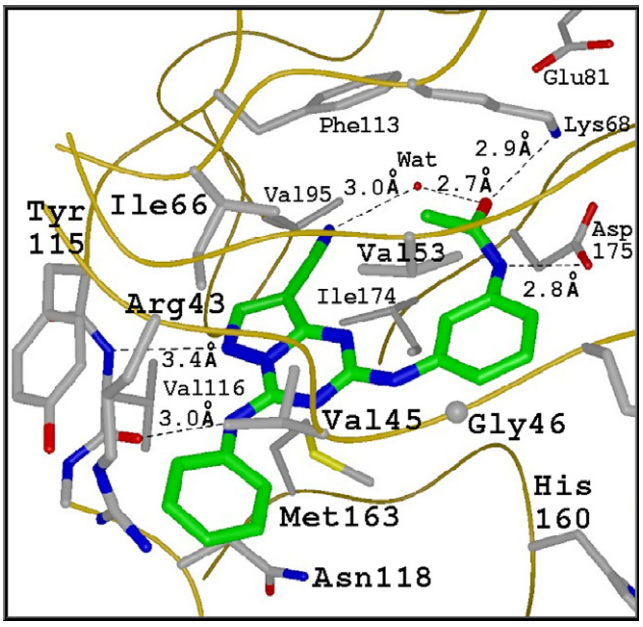
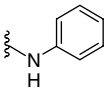
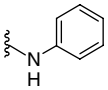


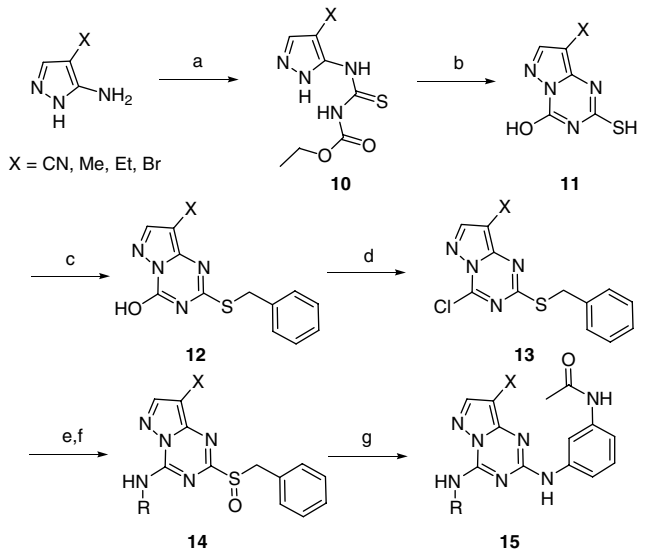
Figure 4. cCK2α with inhibitor **9e** (PDB code: 2PVN) bound to the active site in an extended conformation. Compound **9e** accesses a buried pocket with both hydrophobic and hydrophilic interactions.

The 2.0 Å co-crystal structure for **9e** in complex with cCK2 revealed that, in addition to the normal interactions between inhibitor and protein observed previously, the C2 phenyl group indeed adopted an extended conformation and occupied the back pocket, making hydrophobic interactions with Val53 and Ile174. More importantly, the acetamide group on the phenyl ring adopted a cis conformation, forming three strong hydrogen bonds with Asp175, Lys68, and a buried water molecule (Fig. 4). Thus, all these highly favorable hydrophobic and hydrogen bond interactions between ligand and protein made **9e** one of the most potent CK2 inhibitors ever reported with $K_i = 0.35$ nM.

Further modifications at C4 and C8 positions of the pyrazolo-triazine core were carried out as illustrated in Table 3. Preparation of these pyrazolo-triazines starts with the reaction between pyrazoles and ethoxycarbonyl-isothiocyanate as shown in Scheme 2. As discussed earlier in Scheme 1 and by others,[9] at room temperature, isothiocyanate reacted readily with the ring nitrogen of 3-aminopyrazole when X is an electron withdrawing group, such as CN or Br. In contrast, when X is an electron donating group, isothiocyanate reacted with both the ring nitrogen and the 3-amino group of the pyrazole. However, by heating in EtOAc, 3-thiourea derivatives **10** were the only products obtained due to its thermal stability. Cyclization of **10** in the presence of base afforded the pyrazolo-triazine derivatives **11**, followed by benzylation and chlorination to give **12** and **13**. Displacement of C4-chloro on **13** with primary amines followed by mCPBA oxidation yielded various C4-NHR derivatives **14**. The final products **15** were obtained by substitution of C2-sulfoxide with the amine at high temperature.

Table 3. Inhibitory activity of C4 and C8 substituted pyrazolo-triazines

Compound	–X	–HNR	hCK2 K_i (μM)	HCT116 IC_{50} (μM)
9e	CN		0.00035	0.99
15a	CN		0.00026	2.3
15b	CN		0.0019	5.5
15c	Me		0.035	43
15d	Me		0.011	7.6
15e	Et		2	ND
15f	Br		0.017	18



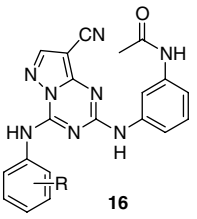
Scheme 2. Reagents and conditions: (a) (CO_2Et)NCS, EtOAc, reflux; (b) NH_4OH, MeOH; (c) BnBr, *i*-Pr_2NEt, NMP; (d) *N*,*N*-dimethylaniline, $POCl_3$, reflux; (e) RNH_2, NMP, rt; (f) mCPBA, CH_2Cl_2; (g) *N*-(3-amino-phenyl)-acetamide, NMP, 160 °C.

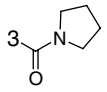
Structure activity relationships of compounds (**15**) clearly indicated that a small cyclopropyl group at the C4 position (**15a**) was at least as active as the phenyl group (**9a**). However, small substitutions at the C8 position, such as methyl (**15d**), ethyl (**15e**) or bromo (**15f**), resulted in significant decreases in inhibitory potency, probably because of their size and/or electronic properties (Table 3).

As shown in Table 3, in an MTT cell-based assay, compound **9e** showed sub-μM inhibition against the HCT116 cancer cell line. Therefore, a series of compounds **16** with a variety of substitutions on the C4-aromatic ring were synthesized (Table 4). All of the compounds are very potent inhibitors of human CK2 with $K_i < 1$ nM (many of them are more potent than **9e**). Compounds **16a**, **16f**, and **16g** strongly inhibit cell growth when tested against HCT116 and PC3 cancer cell lines. It should be noted that, there is still a big discrepancy between their enzymatic potency and cell growth inhibition, probably due to their low aqueous solubility and poor cell membrane permeability. Further optimization of this series of compounds is ongoing to address these problems.

In summary, we have described the design and synthesis of pyrazolo[1,5-a][1,3,5]triazines as novel CK2 inhibitors. Using X-ray crystal structures of cCK2, protein structure-based design enables us to identify the most potent CK2 inhibitors with $K_i < 1$ nM. These compounds also show strong inhibition against cancer cell growth. Future work will be focused on the improvement of their biophysical properties to yield drug-like pre-clinical candidates for in-vivo animal studies.

Table 4. Cell activity of selected pyrazolo-triazines



16	R	hCK2 K_i (nM)	HCT116 IC_{50} (μM)	PC3 IC_{50} (μM)
a	4-*N*-Methylpiperazine	0.21	1.06	1.4
b	3-NMe_2	0.55	2.5	ND
c	4-NEt_2	0.5	2.2	ND
d	3-COOH	0.095	NI	ND
e	3-$CONH(CH_2)_2NMe_2$	0.19	39	ND
f		0.70	1.4	0.78
g	3-$CONEt_2$	0.37	0.76	0.61
h	3-OEt	0.19	1.48	1.2
i	3-$OCHF_2$	0.19	2.1	1.7

NI, no inhibition; ND, not determined.

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