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Structure-based design and synthesis of novel macrocyclic pyrazolo[1,5-*a*] [1,3,5]triazine compounds as potent inhibitors of protein kinase CK2 and their anticancer activities

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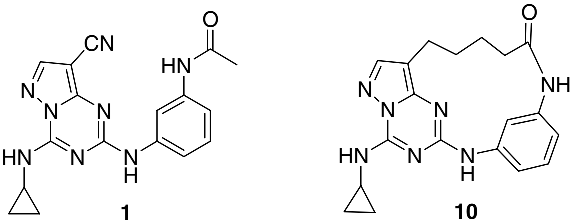
Abstract—A series of macrocyclic derivatives has been designed and synthesized based on the X-ray co-crystal structures of pyrazolo[1,5-*a*] [1,3,5]triazines with corn CK2 (cCK2) protein. Bioassays demonstrated that these macrocyclic pyrazolo[1,5-*a*] [1,3,5]triazine compounds are potent CK2 inhibitors with K_i around 1.0 nM and strongly inhibit cancer cell growth with IC_{50} as low as ~100 nM.

Protein kinase CK2 is a highly conserved and pleiotropic serine/threonine kinase that plays key roles in cell growth, proliferation, and survival. It is localized in both the cytoplasm and the nucleus of normal cells, but is particularly more abundant in the nucleus compartment of cancer cells.[1] It is comprised of a heterotetrameric structure of $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ or $\alpha'_2\beta_2$ where the α or α′ subunits are catalytic, while the β subunit provides the anchor for assembly of the scaffold and interacts with CK2's various protein substrates. The α and α′ subunits are highly homologous for a large part of the amino acid sequence, only differing significantly in the extreme C terminus where homology is essentially non-existent.[2] CK2 has been observed to be frequently up-regulated in a wide variety of human cancers examined.[3–5] Targeted overexpression of CK2 catalytic subunits results in tumoriogenesis in animal and cellular models, and CK2 has been demonstrated to increase a cell's oncogenic potential by sensitizing a cell to transformation by other oncogenic proteins.[6] Down-regulation of CK2 activity has been demonstrated to decrease cellular proliferation as well as to induce apoptosis in cancer cells.[7] Various approaches have been employed to study the cancer related bio-functions of CK2, such as, the use of small molecule inhibitors, dominant negative overexpression of kinase inactive mutants,[8] anti-sense CK2α oligodeoxynucleotide,[7] as well as small interfering RNA molecules (siRNA).[9] Recent studies by Ahmed and co-workers[10] demonstrated that antisense CK2α caused cancer cell death in vitro as well as in a PC3 xenograft model due to induction of apoptosis. Complete eradication of the tumor was observed with higher concentrations of the antisense agents. The effect on normal and non-cancer cells however was minimal under the same conditions. Clearly, data presented by these studies support the rationale of inhibiting CK2 as a potential approach for cancer therapy. Specific,



1

CK2 K_i = 0.26 nM
IC_{50}= 2.3 μM (HCT116)
IC_{50}= 5.1 μM (PC3)

10

CK2 K_i = 24 nM
IC_{50}= 0.29 μM (HCT116)
IC_{50}= 0.88 μM (PC3)

Figure 1. Pyrazolo[1,5-*a*] [1,3,5]triazine and macrocyclic derivative.

Keywords: CK2; Kinase; Anticancer.

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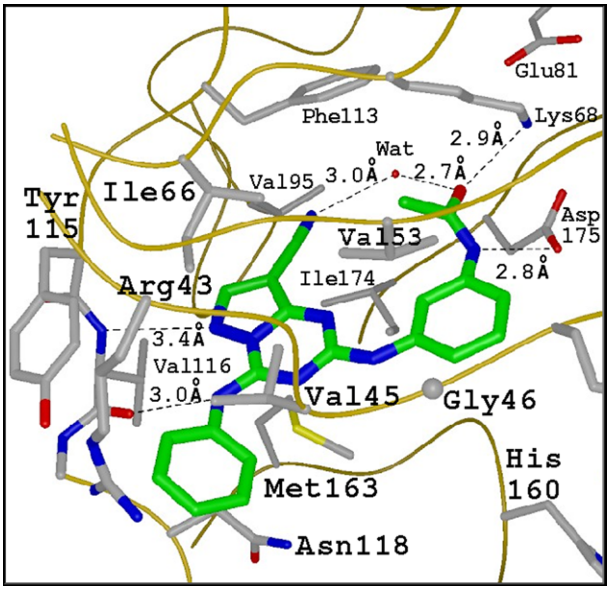
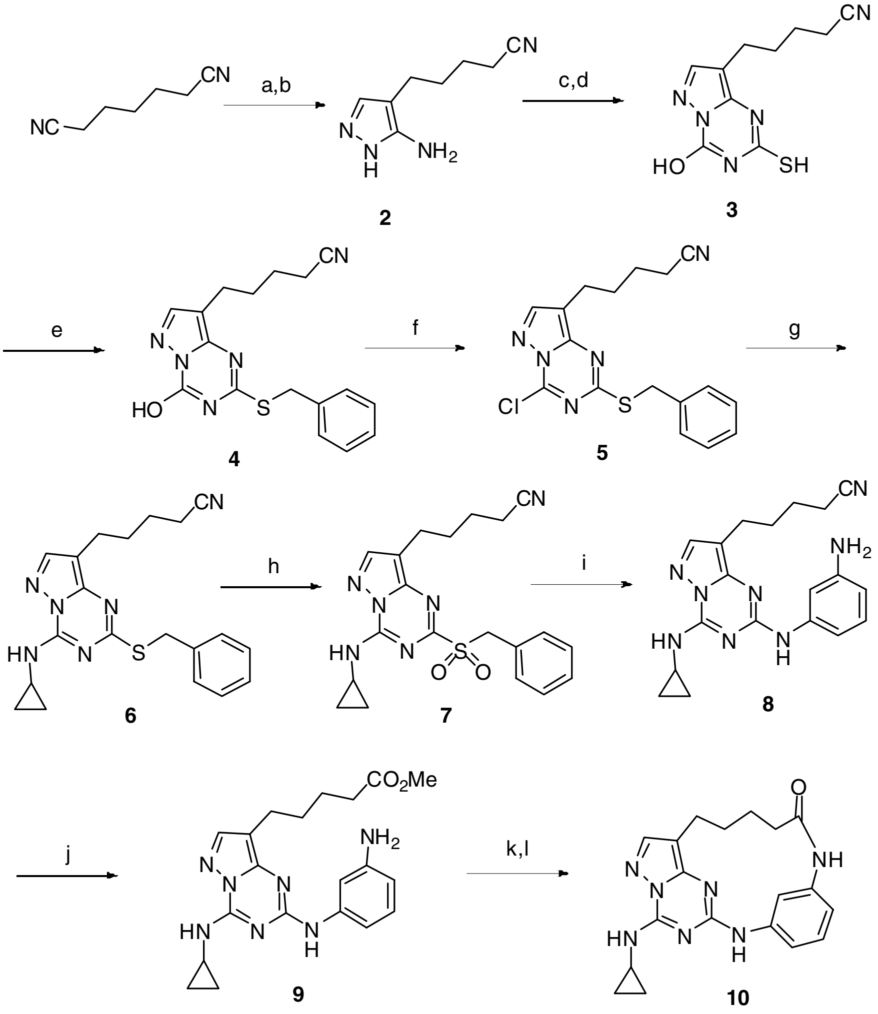


Figure 2. cCK2α with pyrazolo[1,5-*a*] [1,3,5]triazine inhibitor binding to the active site (PDB code: 2PVN).

potent, and cell-permeable inhibitors of CK2 have been developed in the past,[11] with K_i values in the submicromolar range (the best being 20 nM).[11b] These compounds have also shown a remarkable pro-apoptotic effect by down-regulating CK2 activity when tested against multiple cancer cell lines. Therefore, development of cell-permeable CK2 inhibitors should lead to potential anti-cancer drugs.

Recently, we described pyrazolo[1,5-*a*] [1,3,5]triazines **1** (Fig. 1) as potent inhibitors of protein kinase CK2 ($K_i < 1$ nM).[12] However, these compounds exhibited only relative weak cellular activity against prostate and colon cancer cell lines with IC_{50} at low μM levels. The nature of a near planar molecular structure for this class of compounds as observed in the X-ray co-crystal structure (Fig. 2) presumably produces poor membrane permeability and thus poor cellular activity.

As we discussed in the preceding paper,[12] the cis-acetamide group provided two strong hydrogen bond interactions with protein residues Asp175 and Lys68, which induced the C2-phenyl group to adopt an extended con-



Scheme 1. Reagents and conditions: (a) NaH, HCO_2Et, Et_2O, reflux; (b) $NH_2NH_2{\cdot}HCl$, EtOH/H_2O, reflux; (c) $(CO_2Et)NCS$, EtOAc, reflux; (d) NH_4OH, EtOAc, rt; (e) BnBr, *i*-Pr_2NEt, NMP; (f) *N*,*N*-dimethylaniline, $POCl_3$, reflux; (g) cyclopropylamine, NMP, rt; (h) mCPBA, CH_2Cl_2; (i) benzene-1,3-diamine, acetic acid, 70 °C; (j) (1) HCl (g), MeOH; (2) HCl, MeOH, reflux; (k) NaOH, MeOH, reflux; (l) HATU, *i*-Pr_2NEt, NMP, sonicate.

formation and bury into the hydrophobic back pocket. It was observed that the methyl group of cis-acetamide was only about 4 Å away from the nitrogen atom of C8-nitrile group. Therefore, an appropriately placed spatial alkyl chain would provide a linkage between C8 and the acetamide to form a macrocyclic system.

By design, macrocyclic pyrazolotriazines could be beneficial in several aspects. First, an alkyl chain would fit into the deep hydrophobic cavity composed of hydrophobic residues, such as Val95, Phe113, Val53, and Ile174, to provide maximal interactions between ligand and protein. Second, the conformationally constrained cis-acetamide could still interact with Asp175 and Lys68 to form two strong hydrogen bonds for enhanced potency as well as significantly increased metabolic stability of the acetamide, facilitating in vivo animal studies. Third and most importantly, an alkyl side chain in the macrocyclic system would form a bulge out from the molecule and change the planar characteristics of non-cyclized pyrazolotriazines to improve their cell membrane permeability.

In direct comparison, compound **10** was synthesized by following the procedure described in Scheme 1. 1,5-Dicyanopentane was treated with NaH followed by ethylformate to give an intermediate, 2-formylheptanedinitrile, which was then cyclized with hydrazine, providing 4-substituted amino pyrazole **2**. Using a similar procedure as described earlier,[12] pyrazolotriazine **3** was obtained by reacting **2** with thioisocyanate in refluxing EtOAc, followed by cyclization in the presence of base. Benzylation and chlorination afforded compounds **4** and **5**. The C4-chloro group was then displaced with cyclopropylamine to provide compound **6**. Sulfone derivative **7** was obtained by treatment of **6** with 2.5 equivalents of mCPBA, which was then substituted with benzene-1,3-diamine in acetic acid to give **8**. A mixture of imine and ester was initially obtained when **8** was treated with HCl gas in methanol at room temperature overnight. However, after brief reflux in methanol, the methyl ester **9** was the only product isolated. The ester was then saponified. The intra-molecular cyclization was readily achieved by treatment with the coupling re-

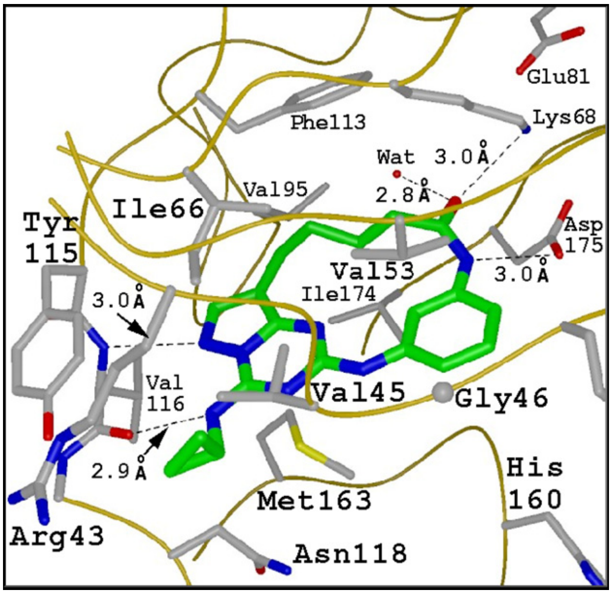
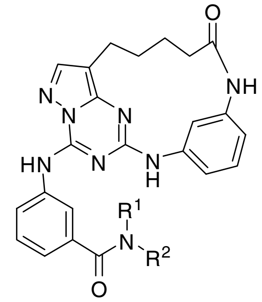


Figure 3. cCK2α active site complex with **10**, at 2.0 Å resolution (PDB code: 3BE9).

Table 1. Cell activity of macrocyclic pyrazolotriazines



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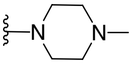
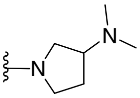
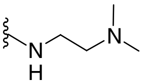
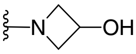
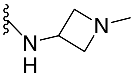
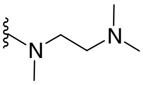
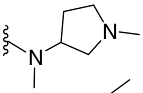
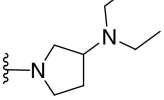
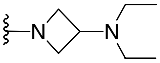
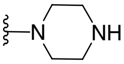
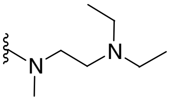
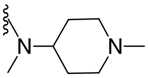
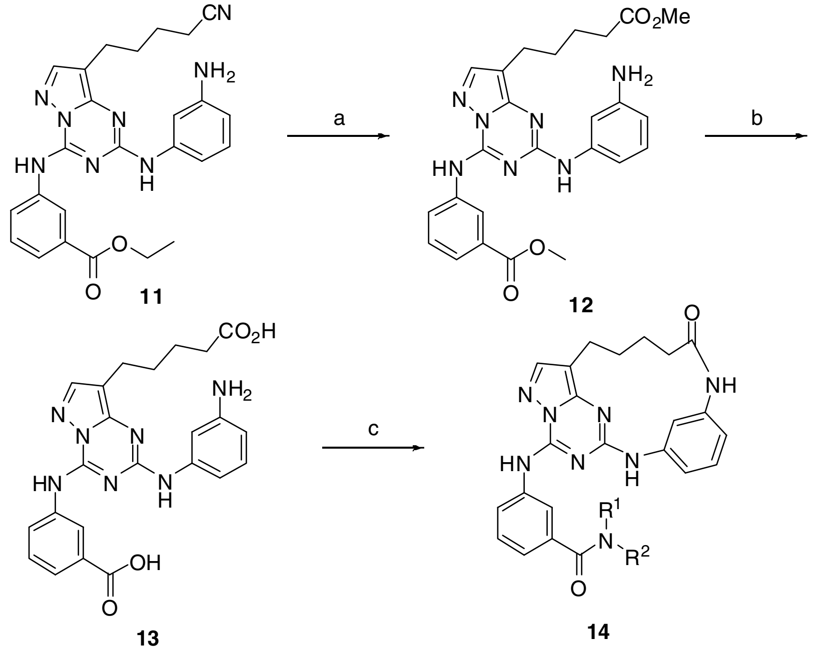
Compound	NR^1R^2	K_i (nM)	HCT116 IC_{50} (μM)	PC3 IC_{50} (μM)
14a		1.3	0.25	0.5
14b		1.4	0.19	0.23
14c		5.2	0.24	0.38
14d		3.6	0.33	0.60
14e		7.4	0.27	0.33
14f		4.0	0.13	0.21
14g		2.0	0.083	0.12
14h		2.2	0.13	0.14
14i		3.4	0.30	0.62
14j		0.8	0.44	ND
14k		2.4	0.15	ND
14l		1.4	0.18	ND

ND, not determined.



Scheme 2. Reagents and conditions: (a) (1) HCl (g), MeOH; (2) HCl, MeOH, reflux; (b) NaOH, MeOH, reflux; (c) (1) HATU, *i*-Pr_2NEt, NMP, sonicate; (2) HNR^1R^2, HATU, NMP.

agent, HATU, in the presence of base in NMP to provide macrocyclic pyrazolotriazine **10.**

The 2.0 Å co-crystal structure of compound **10** in complex with cCK2α revealed that the alkyl linker of the macrocyclic derivative fits quite elegantly into the hydrophobic cavity at the back of the deep pocket, making all the hydrophobic interactions with protein as predicted by modeling. The amide group still made two hydrogen bonds with the salt bridge of cCK2α (Asp175 and Lys68) without interruption by the four carbon chain linkage (Fig. 3).

MTT cell based assay[13] demonstrated that compound **10** was about 10-fold more potent than compound **1**, even though it lost ~100-fold enzyme inhibitory potency (Fig. 1). Clearly, the significantly improved cellular activity of **10** is most likely the result of enhanced membrane permeability imparted by the less planar, more three-dimensional structure imparted by the macrocyclic lactam ring system.

Encouraged by the improvement of cellular activity, a series of macrocyclic pyrazolotriazine derivatives (Table 1) was designed and synthesized. Based on the available co-crystal structures, meta-position of the aniline points to the solvent exposed region. Therefore, incorporation of meta-amide groups might enhance their enzyme inhibitory potency and increase solubility.

Preparation of this series of compounds is illustrated in Scheme 2. Compound **11** was obtained by following a similar procedure as described in Scheme 1. The nitrile group was then converted to the ester. Upon saponification, the di-acid compound **13** was obtained. The cyclization and amide formation were carried out in a one-pot two-coupling procedure, in which 1.5 equivalents of coupling reagent HATU was added in a dilute solution of **13** to form the macrocyclic system followed by additional 1.0 equivalent of HATU and excess of corresponding amines to give desired compounds **14**.

Bioassays clearly demonstrated that replacing the C4-cyclopropyl group with a substituted phenyl group regained enzyme inhibitory potency. At the meantime, the substituent groups of the meta-amide greatly increased the aqueous solubility, which, in turn, greatly enhanced cellular activity of these macrocyclic pyrazolotriazines against both human prostate (PC3) and colon cancer (HCT116) cell lines (Table 1).

In summary, we have described the design and synthesis of a novel class of macrocyclic pyrazolo[1,5-*a*] [1,3,5]triazines as potent CK2 protein kinase inhibitors. These compounds strongly inhibit cell growth in vitro in both human prostate and colon cancer cell lines. Compounds with potent CK2 and cancer cell inhibitory activity are currently being evaluated in animal xenograft models for their anti-tumor activities in vivo.

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13. MTT cell-based assay was based on the formation and the subsequent colorimetric detection of insoluble purple formazan crystals by the metabolically active cells when tetrazolium salt (MTT reagent) was available in the culture media.