Biochemical and three-dimensional-structural study of the specific inhibition of protein kinase CK2 by [5-oxo-5,6-dihydroindolo-(1,2-a)quinazolin-7-yl]acetic acid (IQA)

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IQA {[5-oxo-5,6-dihydro-indolo(1,2-a)quinazolin-7-yl]acetic acid} is a novel ATP/GTP site-directed inhibitor of CK2 ('casein kinase 2'), a pleiotropic and constitutively active protein kinase whose activity is abnormally high in transformed cells. The K_i value of IQA (0.17 μ M) is lower than those of other CK2 inhibitors reported so far. Tested at 10 μ M concentration in the presence of 100 μ M ATP, IQA almost suppresses CK2 activity *in vitro*, whereas it is ineffective or weakly effective on a panel of 44 protein kinases and on phosphoinositide 3-kinase. In comparison, other CK2 inhibitors, notably apigenin and quercetin, are more promiscuous. The *in vivo* efficacy of IQA has been assessed by using the fact that treatment of Jurkat cells with IQA inhibits endogenous CK2 in a dose-dependent manner. IQA has been co-crystallized with maize CK2 α , which is > 70 % identical with its human homologue, and the structure of the complex has been determined at 1.68 Å (1 Å = 0.1 nm) resolution. The inhibitor lies in the same plane occupied by the purine moiety of ATP

with its more hydrophobic side facing the hinge region. Major contributions to the interaction are provided by hydrophobic forces and non-polar interactions involving the aromatic portion of the inhibitor and the hydrophobic residues surrounding the ATP-binding pocket, with special reference to the side chains of V53 (Val⁵³), I66, M163 and I174. Consequently, mutants of human CK2 α in which either V66 (the homologue of maize CK2 α I66) or I174 is replaced by alanine are considerably less sensitive to IQA inhibition when compared with wild-type. These results provide new tools for deciphering the enigmatic role of CK2 in living cells and may pave the way for the development of drugs depending on CK2 activity.

Key words: dual-specificity tyrosine-phosphorylated and -regulated kinase 1a (DYRK1a), protein kinase inhibitor, protein phosphorylation, 4,5,6,7-tetrabromobenzotriazole (TBB).

INTRODUCTION

CK2 (an acronym derived from the misnomer 'casein kinase 2') is an extremely conserved pleiotropic protein kinase [1,2] with a growing list of more than 300 substrates, the majority of which are proteins implicated in signal transduction, gene expression and transcription-related functions [2,3]. In contrast with many protein kinases that are switched on in response to specific stimuli, CK2 is constitutively active either with or without its non-catalytic β subunits, by virtue of two independent activation mechanisms. First, the basal activity of the isolated catalytic subunits (α and/or α' , whose sequences are highly similar but encoded by distinct genes) is conferred by intra-molecular interactions between the N-terminal segment and the activation loop that is maintained in its open conformation [4]. Secondly, whenever the activity is abolished by disrupting these interactions, it can be restored to normal values on association with the β subunits even though the latter are not making any contact with the activation loop [5]. Such a dual activation mechanism maintains catalytic activity

both in the presence and absence of the β subunit. Constitutive activity may reflect both the extreme pleiotropy of CK2 and its purported role as a promoter of cell survival 'at all costs' [6]. This in turn probably accounts for the anti-apoptotic potential of CK2 [7,8] and for a number of reports showing invariably elevated levels of CK2 in abnormally proliferating cells and in a variety of tumours (as reviewed by Guerra and Issinger [9]). Moreover, in different experimental models, it co-operates with certain oncogenes to produce a profound enhancement of the tumour phenotype (reviewed by Tawfic et al. [10]).

From a practical point of view, constitutive activity hampers the understanding of the biological roles of CK2, as there is no physiological device for turning catalytic centre activity on or off within living cells. This limitation, in conjunction with the lethal consequences of deleting both genes of CK2 catalytic subunits [11], makes the use of potent and highly specific inhibitors the first-choice approach left to gain information about the cell-specific contributions of this pleiotropic and enigmatic kinase. CK2 inhibitors would also provide valuable leads for

Abbreviations used: CK2, 'casein kinase 2'; DRB, 5,6-dichloro-ribofuranosylbenzimidazole; DYRK, dual-specificity tyrosine-phosphorylated and -regulated kinase; G-CK, Golgi CK; HS1, haematopoietic cell-specific protein 1; IQA, [5-oxo-5,6-dihydroindolo-(1,2-a)quinazolin-7-yl]acetic acid; PI3K, phosphoinositide 3-kinase; TBB, 4,5,6,7-tetrabromobenzotriazole; for brevity the one-letter system for amino acids has been used, V53, for example, means Val⁵³.

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Atomic co-ordinates of the CK2 α -IQA complex have been deposited in the Protein Data Bank with the accession code 1om1.

the development of drugs with anti-neoplastic potential, once the mechanism by which CK2 contributes to cell transformation has been clarified.

Several ATP site-directed ligands have been reported to act as more or less selective inhibitors of CK2, and some are marketed specifically for this purpose. Among these are: DRB (5,6-dichloro-ribofuranosylbenzimidazole) [12], the hydroxy-anthraquinone emodin [13] and some flavonoids, notably quercetin and apigenin [14]. DRB, however, displays a rather high K_i value (approx. 5 μ M) and it does not discriminate between CK2 and CK1 [15], whereas the specificity of quercetin is quite broad [16] and emodin is known to inhibit some receptor tyrosine kinases almost as efficiently as CK2 [17]. Moreover, with the exception of quercetin, the selectivity of these compounds towards a substantial number of different kinases has not been thoroughly investigated. This makes it difficult to interpret their effects on living cells [18]. Improvements in both efficiency and specificity were obtained by modifying the scaffold of DRB [15]. In particular, a brominated derivative TBB (4,5,6,7-tetrabromobenzotriazole) in which the imidazole ring has been replaced by a triazole one, and in which the sugar moiety has been suppressed [19], displays a remarkable selectivity towards CK2 among a panel of 30 kinases tested, and a K_i value slightly below 1 μ M [20]. Therefore, TBB has been successfully used to inhibit CK2 in intact cells and to probe its role in apoptosis [8]. The crystal structure of maize CK2 α subunit (> 70 % identical with its human homologue) in complex with TBB [21] as well as with emodin [22], and three emodin-related scaffolds [23] has been solved, revealing a similar mode of binding mostly stabilized by van der Waals interactions within a hydrophobic pocket, partially overlapping the ATP-binding site. In the present study, we describe a novel CK2 inhibitor IQA {[5-oxo-5,6-dihydro-indolo-(1,2-a)quinazolin-7-yl]acetic acid} structurally unrelated to any of those described previously. Also, we show that in terms of selectivity and efficiency IQA is superior to TBB both *in vitro* and *in vivo* and is far superior to emodin and apigenin both *in vitro* and *in vivo*. This makes IQA the first-choice CK2 inhibitor available to date, and the determination of its structure in complex with CK2 α also provides valuable hints for the rational design of more powerful compounds with pharmacological potential.

EXPERIMENTAL

Inhibitors

IQA, formerly referred to as CGP029482, staurosporine and its derivative CG44171A were provided by Novartis Pharma Inc. (Basel, Switzerland). Solutions of IQA in DMSO were freshly prepared before each experiment, since the compound slowly undergoes chemical modifications with a concomitant decrease in its inhibitory power. Emodin, apigenin and quercetin were purchased from Sigma (St. Louis, MO, U.S.A.).

Source and purification of protein kinases

Native CK1 and CK2 were purified from rat liver [24]. G-CK (Golgi CK), purified from lactating mammary gland of rats [25], was a gift from Dr A. M. Brunati (University of Padova, Padova, Italy). Protein tyrosine kinases Lyn [26], c-Fgr [27] and Syk (also termed TPK-IIB) were purified from rat spleen. Human recombinant α and β subunits of CK2 were expressed in *Escherichia coli* and the holoenzyme was reconstituted and purified as described previously [28]. The CK2 α mutant V66A was obtained by the method described in [29] using the oligonucleotide 5'-AAAAAGTTGCTGTAAAAT-3'. The S51G and

I174A mutants were obtained using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). Each mutant was generated using two synthetic oligonucleotide primers, each complementary to opposite strands of human α cDNA inserted in pT7-7 vector, namely 5'-GGCCGAGGT-AAATACGGTGAAGTATTTGAAGCC-3' and 5'-GCACAGAA-AGCTACGACTAGCAGACTGGGGTTTGGC-3' for S51G and I174A respectively. Mutants were confirmed by sequencing and used to transform competent *E. coli* BL21(DE3) cells. Expression and purification of α V66A, α S51G and α I174A were performed as described in [28]. Catalytic α subunits were dialysed against 25 mM Tris/HCl (pH 7.5) containing 50 % (v/v) glycerol and were stored at -20°C . *Saccharomyces cerevisiae* pD261 was kindly provided by Dr S. Facchin (University of Padova, Padova, Italy). Sources of all other protein kinases are either described or referred to in [16].

Phosphorylation experiments

All protein kinase activities were linear with respect to time and enzyme concentration in each incubation. Phosphorylation conditions and evaluation of the phosphate incorporated were as described in [20] for CK2, CK1, G-CK, pD261 and the tyrosine kinases Lyn, c-Fgr and Syk. All other specificity assays were performed using an automated multichannel pipette system (Multi-drop 384; Thermo Life Sciences, Colchester, U.K.) at room temperature (25°C) in a total assay volume of 25 μ l. In detail, to plates containing 0.5 μ l of compounds, DMSO controls (without inhibitors) or acid blanks, 15 μ l of an enzyme mixture containing the enzyme and the peptide/protein substrate in buffer was added. Compounds were preincubated in the presence of the enzyme and the peptide/protein substrate for 5 min before initiation of the reaction by adding 10 μ l of ATP (final concentration of 100 μ M for all enzymes). Assays were performed for 40 min at room temperature before termination by the addition of 5 μ l of orthophosphoric acid. After mixing, 10 μ l of the reaction mixture was spotted on to P30 filtermats, which were then washed in phosphoric acid. The filters were subjected to washes of 1×3 min, followed by 3×8 min.

Cell culturing and treatment

The human leukaemia Jurkat T-cell line was maintained in RPMI 1640 (Dutch modification; Sigma), supplemented with 10 % (v/v) foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin, in an atmosphere containing 5 % CO_2 .

Before treatment, cells were washed, resuspended at a density of approx. 10^6 cells/ml in a medium containing 1 % foetal calf serum, and then incubated at 37°C in the presence of the compound at the indicated concentration for different periods of time, as described in the Figure legends. Control cells were treated with equal amounts of the solvent.

At the end of the incubations, cells were centrifuged, washed and lysed by the addition of ice-cold hypo-osmotic buffer consisting of 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, protease inhibitor cocktail (Roche, Basel, Switzerland), 10 mM NaF and 1 μ M okadaic acid. Cytosol and the nuclear extract were prepared as described in [8]. Immunoprecipitation of HS1 (haematopoietic cell-specific protein 1) and Western-blot analysis were performed for the cytosolic fraction using an anti-HS1 total protein antiserum as described in [8]. The assay of CK2 activity in lysates of cells pretreated with increasing concentrations of IQA was performed as described previously [8].

Table 1 Data collection and final model statistics

Number in parentheses refer to the highest resolution bin.

Parameter	Value
Data collection	
Maximum resolution	1.68 (1.77) Å
Independent reflection	41 163 (5404)
Multiplicity	2.9 (2.6)
$\langle I/\sigma(I) \rangle$	5.6 (1.6)
R_{merge}	0.069 (0.069)
Completeness	97.8 (97.8)%
Final model	
Reflection used in refinement	40 448
Protein atoms (+ inhibitor)	2750
Solvent molecules	252
R/R_{free}	20.5/22.6
R.m.s. on bond distance	0.006 Å
R.m.s. on bond angle	1.5°

Preparation of crystals and data collection

The catalytic α subunit of *Zea mays* CK2 was expressed in *E. coli* and purified as described elsewhere [30]. Crystals of the IQA inhibitor–enzyme complex were obtained by a co-crystallization technique, using the sitting drop vapour-diffusion method as described previously [23]. The protein–inhibitor complex was prepared by preincubating an 8 mg/ml protein stock solution for 2 h with a 100 mM inhibitor solution (in 100 % (v/v) DMSO). An adequate volume of inhibitor solution was used to have an inhibitor/protein molar ratio of 3:1 and not to exceed a maximum of 5 % DMSO concentration in the final stock solution.

In approx. 1 week, crystals grew from wells where 2 μ l drops of preincubated stock solution were mixed with 4 μ l of water and 2 μ l of precipitant solution [20 % (w/v) poly(ethylene glycol) 4000, 0.2 M sodium acetate and 0.1 M Tris, pH 8], and equilibrated against a 500 μ l reservoir of the same precipitating agent. Crystals grew at a temperature of 283 K and data were collected at a temperature of 100 K. Crystals were not cryo-protected before mounting.

A complete data set for the IQA complex was measured from a single crystal at the macromolecule diffraction beam-line of the synchrotron ELETTRA (Trieste, Italy) on a MarCCD detector, at a wavelength of 1.2 Å. The completeness of the data set was 97.8 % at a maximum resolution of 1.68 Å (Table 1). The crystal belonged to the space group C2 and the cell parameters were $a = 141.52$ Å, $b = 60.27$ Å, $c = 44.35$ Å and $\beta = 102.63^\circ$, with one molecule in the asymmetric unit, in analogy with previously published CK2 α –inhibitor complex structures. Matthews coefficient V_M was 2.35 Å³ Da⁻¹ for a 47.8 % solvent fraction.

Structure determination and refinement

Data were indexed and then scaled using the programs MOSFLM [31] and SCALA respectively from the CCP4 software package [32]. A rigid-body transformation was applied to the model of a high-resolution enzyme–inhibitor complex [23], deprived of the inhibitor. The entire refinement procedure was performed using the CNS software package [33]. Both $|F_o - F_c|$ and $|2F_o - F_c|$ maps (where F_o and F_c are the observed and calculated structure factors respectively) highlighted the presence of the inhibitor in the active site since the beginning of the refinement.

The definition files for the inhibitor were created by Hic-Up [34] and fixed for the CNS software for the whole refinement

Compound	structure	name	IC ₅₀ (μM)
CGP029482		(5-oxo-5,6-dihydro-indolo[1,2-a]quinazolin-7-yl)-acetic acid (IQA)	0.39
CGP06028		3-(4-{2-[3-(1,1,2,2-tetrafluoro-ethoxy)-phenylamino]-pyrimidin-4-yl}-pyridin-2-ylamino)-propan-1-ol	3.60
CGP060476		N*1*-[4-{2-(3-chloro-phenylamino)-pyrimidin-4-yl}-pyridin-2-yl]-ethane-1,2-diamine	9.60
CGP062644		(3-chloro-phenyl)-(4-{2-[2-(1H-imidazol-4-yl)-ethylamino]-pyridin-4-yl}-pyrimidin-2-yl)-amine	3.20
TBB		4,5,6,7-tetrabromo-benzotriazole	0.50
emodin		6-methyl-1,3,8-trihydroxy-anthraquinone	0.89

Figure 1 Structure and IC₅₀ values of four Novartis compounds selected as CK2 inhibitors by virtual screening

For the sake of comparison, TBB and emodin are also shown. CK2 activity was assayed using a specific peptide substrate as described elsewhere [20], either in the absence or in the presence of increasing concentrations (0.1–40 μ M) of each compound. The results shown are the means from three independent experiments with S.D. never exceeding 15 %.

procedure that was performed by alternating automated cycles and manual inspection steps using the graphic program QUANTA (version 98.1111). During the final steps of the refinement, water molecules were added, and the stereochemistry was checked with the program PROCHECK [35].

RESULTS AND DISCUSSION

IQA is a novel selective inhibitor of CK2

CGP029482 was identified as a CK2 inhibitor by virtual screening of the Novartis Compound Collection. In the ATP-binding site of a homology model of human CK2 [36], 400 000 molecules of this collection have been docked systematically. From the list of best-scored compounds that were subsequently tested in a CK2-inhibition assay, four appeared to be the most promising, based on their relatively low IC₅₀ values (Figure 1).

In particular, CGP029482, the conventional name of IQA, displayed the lowest K_i value among a series of widely employed inhibitors of CK2 (Table 2). Even more remarkable, however, is its selectivity, as judged from the residual activity of a panel of 44 protein kinases tested in the presence of 100 μ M ATP and 10 μ M inhibitors (Table 3). The only kinase whose activity is 90 % inhibited by IQA is CK2, whereas all the others are either not affected or inhibited by < 50 %. The IC₅₀ values of those kinases that were significantly inhibited by 10 μ M IQA, namely cAMP-dependent protein kinase, glycogen synthase kinase 3 β , Lck and DYRK1a (dual-specificity tyrosine-phosphorylated and -regulated kinase 1a) were also determined. As shown in Table 4,

Table 2 *K_i* values of IQA and other compounds used in the present study as inhibitors of CK2

K_m values were determined either in the absence or in the presence of increasing concentrations of inhibitor from Lineweaver–Burk double-reciprocal plots of the data. Inhibition constants were then determined by linear-regression analysis of plots of *K_m*/*V_{max}* versus inhibitor concentration.

Inhibitor	<i>K_i</i> (μM)
IQA	0.17
DRB	4.50
TBB	0.40
Emodin	1.85
Apigenin	0.74
Quercetin	1.18

Table 3 Specificity spectrum of CK2 inhibitors

Residual activity determined in the presence of 10 μM inhibitor is expressed as a percentage of the control without inhibitor. IQA was also tested at 10 μM on the following protein kinases: Abl, Her-1, Her-2, KDR, Flt-1, Flt-3, Flt-4, Tek, IGF-1R (insulin-like-growth-factor-1 receptor), Ins-R, c-Raf-1, c-Kit, c-Src, c-Met, PDGFR-β (platelet-derived-growth-factor receptor β) and FGFR-1 (fibroblast-growth-factor receptor 1). All of these displayed residual activity 80 %.

Protein kinase*	IQA	Apigenin	Emodin
MKK1	85	98	111
MAPK2/ERK2	92	83	88
JNK/SAPK1c	100	92	101
SAPK2a/p38	101	101	92
SAPK2b/p38β2	92	87	101
SAPK3/p38γ	96	102	92
SAPK4/p38δ	100	89	100
MAPKAP-K1a	91	86	103
MAPKAP-K2	70	91	82
MSK1	97	59	90
PRAK	76	93	81
PKA	66	87	94
PKCα	107	100	71
PDK1	93	86	90
PKBα	84	82	82
SGK	85	41	55
S6K1	84	65	84
GSK3β	66	54	68
ROCK-II	94	88	90
AMPK	92	57	89
CHK1	90	67	89
Phosphorylase kinase	79	70	104
CK2	10	57	51
CDK2/cyclin A	91	18	75
CK1	77	106	79
G-CK	98	96	89
PiD261	100	103	90
Lck	52	45	79
Lyn	93	88	90
c-Fgr	91	48	99
Syk	87	95	96
CSK	82	77	109
DYRK1a	50	20	29

* AMPK, AMP-activated protein kinase; CDK, cyclin-dependent kinase; CHK, checkpoint kinase; CSK, c-Src kinase; ERK, extracellular-signal-regulated kinase; GSK3, glycogen synthase kinase-3; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAP, MAPK-activated protein kinase; MKK, MAPK kinase (also called MEK); MSK, mitogen- and stress-activated protein kinase; PDK, 3-phosphoinositide-dependent kinase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B (also called Akt); PKC, protein kinase C; PRAK, p38-regulated/activated kinase; ROCK, Rho-dependent protein kinase; S6K, ribosomal protein S6 kinase; SAPK, stress-activated protein kinase; SGK, serum- and glucocorticoid-induced kinase.

they are 1–2 orders of magnitude higher than the *IC*₅₀ value of CK2. Table 4 also shows that IQA is not effective on PI3K (phosphoinositide 3-kinase), which is not strictly a protein kinase,

Table 4 *IC*₅₀ values of IQA-sensitive protein kinases and PI3K

Protein kinases whose activities were inhibited > 30 % by 10 μM IQA (Table 3) and the lipid kinase PI3K (γ isoform) were assayed in the absence and in the presence of increasing concentrations of IQA. For protein kinases, conditions were the same as in Table 3. The assay of recombinant PI3K γ, expressed in SF21 insect cells and purified on nickel agarose, was performed as described in [41]. No significant level of inhibition was observed by increasing the IQA concentration beyond 200 μM. Similar results were obtained with PI3K α and β whose activities were not affected by IQA up to 20 μM concentration.

Kinase	<i>IC</i> ₅₀ (μM)
CK2	0.39
PKA	16
GSK3β	14
Lck	11
DYRK1a	8
PI3K γ	> 200

Figure 2 Dose-dependent inhibition of CK2 and DYRK1a by IQA and TBB

(A) Inhibition by IQA. (B) Inhibition by TBB. CK2 and DYRK1a were assayed on their specific peptide substrates as described in the Experimental section, either in the absence or in the presence of increasing concentrations of the inhibitors, as indicated. Stimulation of DYRK1a by 5–100 nM concentration of either IQA or TBB was consistently observed.

but has been reported to share with CK2 inhibitability by quercetin [16] and apigenin [37]. Note that, at 10 μM concentration apigenin and emodin are significantly less effective compared with IQA on CK2, and the former inhibits 11 kinases with similar or greater potency than CK2 (Table 3).

TBB has a selectivity comparable with that of IQA [20]; however, DYRK1a, a kinase not included in previous screenings, is readily inhibited by TBB, with an *IC*₅₀ value < 1 μM (Table 2), whereas it is much less sensitive than CK2 to IQA (Table 4 and Figure 2). Taken together, these results indicate that IQA is the

Figure 3 Inhibition of endogenous CK2 by treating Jurkat cells with IQA

(A) The upshift of HS1, symptomatic of CK2-dependent phosphorylation [20], was analysed by Western-blot analysis with anti-HS1 antibodies. HS1 was immunoprecipitated from Jurkat cells treated for 2 h with 2 μ M okadaic acid and with the indicated CK2 inhibitors at 40 μ M. (B) CK2 activity in lysates of cells preincubated for 2 h with increasing concentrations of IQA (as indicated) was assayed as described elsewhere [8].

most effective *in vitro* inhibitor of CK2 reported to date, in terms of both efficacy and selectivity.

IQA inhibits CK2 in living cells

To establish whether IQA is cell permeable and usable for *in vivo* experiments, the method previously applied to TBB [8] was exploited. This method takes advantage of the behaviour of HS1, which on intracellular phosphorylation by endogenous CK2 specifically undergoes an SDS/polyacrylamide gel up-shift. Treatment of Jurkat cells with 40 μ M IQA decreases the up-shift of HS1 as effectively as TBB does (Figure 3A). Consistent with this observation, endogenous CK2 in the lysate of Jurkat cells treated with IQA is drastically inhibited when compared with CK2 from control cells (Figure 3B). As described elsewhere [8], inhibition of CK2-mediated phosphorylation on addition of either TBB or emodin makes HS1 more prone to caspase-dependent degradation, an event that in turn correlates with increased apoptosis as judged from a number of criteria, including release of cytochrome *c* from mitochondria. IQA promotes the same functional effects with the advantage that, whereas treatment of both isolated mitochondria and cultured cells with 5 μ M TBB depolarizes the mitochondrial membrane by opening the pore, these side-effects are not observed on treatment with up to 50 μ M IQA (M. Ruzzene, G. Di Maira and L.A. Pinna, unpublished work).

Structural basis for IQA inhibition

Inhibition of CK2 by IQA is competitive with respect to the phosphodonor substrates ATP and GTP, as judged from its effect on K_m but not on K_{cat} values (results not shown). The K_i values of IQA determined using either ATP or GTP as phosphate donor were nearly identical (0.17 μ M and 0.21 μ M for ATP and GTP respectively) consistent with the view that IQA interferes with interactions shared by the two nucleotides and not with those specific to each of them [38].

Table 5 Variable effects of mutating CK2 α V66 and I174 on the inhibitory potency of IQA and other compounds

CK2 holoenzyme was reconstituted *in vitro* by mixing recombinant wild-type or mutated CK2 α , obtained as described in the Experimental section, with equimolar amounts of recombinant β subunit before adding to the phosphorylation reaction. CGP44171A is a staurosporine derivative whose ability to inhibit CK2 has been described elsewhere [40].

	IC ₅₀ (μ M)		
	Wild-type	V66A (fold*)	I174A (fold*)
IQA	0.39	27.0 (62.2)	11.0 (28.5)
Emodin	0.89	38.00 (42.6)	7.3 (8.2)
TBB	0.50	13.0 (26.0)	1.74 (2.8)
Apigenin	1.20	10.0 (8.3)	4.29 (3.5)
Quercetin	0.85	1.10 (1.2)	3.20 (3.7)
Staurosporine	14.22	28.23 (1.9)	7.62 (0.5)
CGP44171A	1.62	1.98 (1.1)	1.49 (0.9)

* Relative to wild-type.

Some additional clues about the mode of binding of IQA were provided by point mutations of residues in the proximity of the ATP/GTP-binding site, notably V66 and I174. Both are unique or nearly unique to CK2, being replaced by either alanine or, in any case, small side chains in the other kinases. The bulky side chains of V66 and I174 contribute to the relative smallness of the hydrophobic pocket where TBB and emodin are situated [21,22]. Consequently, the mutation of either V66 or, to a lesser extent, I174 to alanine significantly decreases the inhibitory efficiency of emodin and TBB [39]. As shown in Table 5, both mutations are even more detrimental to the inhibitory efficiency of IQA. In contrast, the V66A mutation has almost no effect on the inhibitory efficiency of the flavonoid quercetin as well as on those of staurosporine and a staurosporine derivative, CGP44171A, which inhibits CK2 more potently than staurosporine itself [40]. Especially noticeable are the consequences of mutating I174, with an almost 30-fold increase in the IC₅₀ value of IQA as opposed to a much less detrimental effect on emodin, TBB, apigenin and quercetin, whereas the IC₅₀ values of the two compounds of the staurosporine family are actually 'decreased' by the I174 mutation.

These results are in agreement with the knowledge that the hydrophobic cavity in the active site of CK2 is smaller than those found in most of the other kinases, a circumstance that may account for both its specific inhibition by the relatively small TBB molecule, which fits into the pocket almost perfectly [21], and for its exceptionally modest responsiveness to staurosporine [40], whose large molecular mass may hamper accessibility to the active site of CK2. Our results suggest that both V66 and I174 are important for the binding of IQA probably by making non-polar interactions with its hydrophobic moiety.

The concept outlined above has been corroborated by solving the structure of a complex between IQA and maize CK2 α (whose active core is almost identical with that of its mammalian counterparts), disclosing the crucial role of a number of hydrophobic side chains, especially I66 (the maize homologue of V66) and I174.

The structure of the complex, solved at 1.68 Å resolution, has a well-defined electron density, with clear side-chain details for all residues. The inhibitor electron density occupies a flat central region of the active pocket, in analogy with other enzyme-inhibitor complexes. The extent of the electron density assigned to the small ligand is broader than the inhibitor dimension, suggesting a couple of possible orientations inside the cavity.

Figure 4 IQA polar interactions

The main polar interactions (white dotted lines) between the two conformations of IQA (**a**, **b**) and some residues of the CK2 ATP-binding site, i.e. S51, K68, D175 and a water molecule, are shown. Carbon atoms are shown in yellow, nitrogen in blue and oxygen in red.

Although the position of the aromatic rings could be unequivocally assigned, it is the variable orientation of the acetate moiety that may give rise to two possible conformers, as illustrated in Figure 4. The inhibitor position has been refined in two alternative conformations with occupancies of 0.50.

It has been observed that IQA can undergo a slow hydrolysis in the presence of water, with the opening of the lactam ring and a slight decrease in inhibitory efficiency reflected an approx. 3-fold increase in K_i . However, in the resulting molecule, the benzoic aromatic ring lies in a plane tilted by approx. 30° with respect to the rest of the molecule, which is not in agreement with the completely flat electron density observed in our structure. For this reason, we argue that IQA alone is present in the complex, and we have refined our model using only the native chemical structure of IQA.

The inhibitor molecule penetrates into the catalytic pocket of CK2 lying on the same plane occupied by the ATP purine moiety [38]. The hydrophobic side of IQA faces the hinge region of the protein, whereas the polar side is orientated towards K68. For both conformations, the interactions occurring between the protein and the inhibitor are almost entirely determined by van der Waals contacts with several hydrophobic residues of the ATP-binding pocket, notably V45, V53, I66, K68, V95, F113, V116, M163 and I174 (Figure 5). The total buried surface, after inhibitor binding, is approx. $730 \text{ \AA}^2$, a rather large value. Probably, this value accounts completely for the affinity of this inhibitor. In contrast, there are only few polar interactions that involve the carboxylic portion of the acetate moiety of IQA. In one conformation (Figure 4a), the inhibitor carboxy group makes three hydrogen bonds: one with the amine function of K68, one with the backbone nitrogen of D175 and one with a water molecule further connected to NH-W176. Interestingly, the same water molecule, which is present in all the CK2 structures solved to date except for the complex with emodin, is also involved in a polar interaction with the central nitrogen of the triazole ring of TBB [21]. In the other conformation (Figure 4b), the carboxy group makes an internal hydrogen bond with the lactam nitrogen and interacts with the hydroxyl oxygen of S51. In both situations, the main contribution to the strength of the interaction probably comes from the large surface subtracted from water on inhibitor binding, as also suggested by

Figure 5 Penetration of IQA into the ATP-binding pocket

IQA surrounded by hydrophobic residues in the ATP-binding pocket. One conformation of IQA and only the side chains of the encircling residues are shown. Note the proximity of I/V66 and I174 to IQA, whose importance is discussed in the Results and discussion section. Carbon atoms are shown in yellow, nitrogen in blue and oxygen in red.

the inability of directional polar interactions to fix the inhibitor in one single conformation. This conclusion is also supported by the observation that, whereas the mutation of the hydrophobic side chains of V66 and I174 dramatically decreases the inhibitory efficiency of IQA (Table 3), the mutation of S51 to glycine does not significantly increase the K_i value (results not shown). This also supports the notion that the two different forms of IQA found in the complex with CK2, which share the hydrophobic contacts but differ in whether or not they interact with S51 (see Figure 4), bind with similar affinity.

Conclusions

IQA appears to be the first-choice inhibitor of CK2 described to date both in terms of *in vitro* efficiency and selectivity and it is of practical utility in cell cultures. In all these respects, IQA is superior to TBB, i.e. the most potent CK2 inhibitor described previously, and their modes of binding are similar despite their different chemical structure. Both the IQA and TBB complexes are in fact mainly stabilized by non-polar energetic contributions, i.e. hydrophobic forces and van der Waals interactions.

As a matter of fact, IQA is more responsive than TBB to the mutation of the bulky side chains of V66 and I174 (Table 5), suggesting that the interactions of IQA with individual side chains of the hydrophobic pocket are stronger. This could explain, at least in part, why DYRK1a, where V66 is replaced by alanine, is less sensitive to IQA than CK2 (Figure 2), although it is inhibited by TBB as drastically as CK2. It is also noteworthy that IQA is ineffective on PI3K, which, on the contrary, is inhibited by apigenin as effectively as CK2. This rules out the possibility that the apoptotic effect of IQA might be due to inhibition of PI3K rather than of CK2.

In conclusion, the scaffold of IQA looks more amenable than that of TBB to the modifications aimed at improving selectivity. Also important is the finding that treatment of cells with IQA promotes the expected consequences of CK2 blockade without causing mitochondrial depolarization and uncoupling as observed on treatment with TBB (M. Ruzzene, G. Di Maira and L.A. Pinna, unpublished work), suggesting that IQA is more sensitive to mitochondrial functions. This could also represent a crucial advantage for a compound intended to become a lead for developing CK2-directed drugs devoid of undesirable side effects.

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REFERENCES

- Pinna, L. A. (2002) Protein kinase CK2: a challenge to canons. *J. Cell Sci.* **115**, 3873–3878
- Litchfield, D. W. (2003) Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem. J.* **369**, 1–15
- Meggio, F. and Pinna, L. A. (2003) One-thousand-and-one substrates of protein kinase CK2? *FASEB J.* **17**, 349–368
- Sarno, S., Ghisellini, P. and Pinna, L. A. (2002) Unique activation mechanism of protein kinase CK2. The N-terminal segment is essential for constitutive activity of the catalytic subunit but not of the holoenzyme. *J. Biol. Chem.* **277**, 22509–22514
- Niefind, K., Guerra, B., Ermakowa, I. and Issinger, O.-G. (2001) Crystal structure of human protein kinase CK2: insights into basic properties of the CK2 holoenzyme. *EMBO J.* **20**, 5320–5331
- Ahmed, K., Gerber, D. A. and Cochet, C. (2002) Joining the cell survival squad: an emerging role for protein kinase CK2. *Trends Cell Biol.* **12**, 226–230
- Guo, C., Yu, S., Wang, H., Davis, A. T., Green, J. E. and Ahmed, K. (2001) A potential role of nuclear matrix-associated protein kinase CK2 in protection against drug-induced apoptosis in cancer cells. *J. Biol. Chem.* **276**, 5992–5999
- Ruzzene, M., Penzo, D. and Pinna, L. A. (2002) Protein kinase CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) induces apoptosis and caspase-dependent degradation of haematopoietic lineage cell-specific protein 1 (HS1) in Jurkat cells. *Biochem. J.* **364**, 41–47
- Guerra, B. and Issinger, O.-G. (1999) Protein kinase CK2 and its role in cellular proliferation, development and pathology. *Electrophoresis* **20**, 391–408
- Tawfic, S., Yu, S., Wang, H., Faust, R., Davis, A. and Ahmed, K. (2001) Protein kinase CK2 signal in neoplasia. *Histol. Histopathol.* **16**, 573–582
- Padmanabha, R., Chen-Wu, J. L.-P., Hanna, D. E. and Glover, C. V. C. (1990) Isolation, sequencing and disruption of the yeast CKA2 gene: casein kinase II is essential for viability in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**, 4089–4099
- Zandomeni, R., Zandomeni, R. C., Shugar, D. and Weinmann, R. (1986) Casein kinase type II is involved in the inhibition by 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole of specific RNA polymerase II transcription. *J. Biol. Chem.* **261**, 3414–3420
- Yim, H., Lee, Y. H., Lee, C. H. and Lee, S. K. (1999) Emodin, an anthraquinone derivative isolated from the rhizomes of *Rheum palmatum*, selectively inhibits the activity of casein kinase II as a competitive inhibitor. *Planta Med.* **65**, 9–13
- Critchfield, J. W., Coligan, J. E., Folks, T. M. and Butera, S. T. (1997) Casein kinase II as a selective target of HIV-1 transcriptional inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6110–6115
- Meggio, F., Shugar, D. and Pinna, L. A. (1990) Ribofuranosyl-benzimidazole derivatives as inhibitors of casein kinase-2 and casein kinase-1. *Eur. J. Biochem.* **187**, 89–94
- Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95–105
- Jayasuriya, H., Koonchanok, N. M., Geahlen, R. L., McLaughlin, J. L. and Chang, C. J. (1992) Emodin, a protein tyrosine kinase inhibitor from *Polygonum cuspidatum*. *J. Nat. Prod.* **55**, 696–698
- Perkel, J. M. (2002) Researchers are getting specific about protein kinase inhibitors. *The Scientist* **16**, 37–39
- Szyska, R., Grankowski, N., Felczak, K. and Shugar, D. (1995) Halogenated benzimidazoles and benzotriazoles as selective inhibitors of protein kinases CKI and CKII from *Saccharomyces cerevisiae* and other sources. *Biochem. Biophys. Res. Commun.* **208**, 418–424
- Sarno, S., Reddy, H., Meggio, F., Ruzzene, M., Davies, S. P., Donella Deana, A., Shugar, D. and Pinna, L. A. (2001) Selectivity of 4,5,6,7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 ('casein kinase-2'). *FEBS Lett.* **496**, 44–48
- Battistutta, R., De Moliner, E., Sarno, S., Zanotti, G. and Pinna, L. A. (2001) Structural features underlying selective inhibition of protein kinase CK2 by ATP site-directed tetrabromo-2-benzotriazole. *Protein Sci.* **10**, 2200–2206
- Battistutta, R., Sarno, S., De Moliner, E., Papinutto, E., Zanotti, G. and Pinna, L. A. (2000) The replacement of ATP by the competitive inhibitor emodin induces conformational modifications in the catalytic site of protein kinase CK2. *J. Biol. Chem.* **275**, 29618–29622
- De Moliner, E., Moro, S., Sarno, S., Zagotto, G., Zanotti, G., Pinna, L. A. and Battistutta, R. (2003) Inhibition of protein kinase CK2 by anthraquinone-related compounds. A structural insight. *J. Biol. Chem.* **278**, 1831–1836
- Meggio, F., Donella Deana, A. and Pinna, L. A. (1981) Endogenous phosphate acceptor proteins for rat liver cytosolic casein kinases. *J. Biol. Chem.* **256**, 11958–11961
- Brunati, A. M., Contri, A., Muenchbach, M., James, P., Marin, O. and Pinna, L. A. (2000) GRP94 (endoplasmic reticulum chaperone) co-purifies with and is phosphorylated by Golgi apparatus casein kinase. *FEBS Lett.* **471**, 151–155
- Donella Deana, A., James, P., Staudenmann, W., Cesaro, L., Marin, O., Brunati, A. M., Ruzzene, M. and Pinna, L. A. (1996) Isolation from spleen of a 57-kDa protein substrate of the tyrosine kinase Lyn. Identification as a protein disulfide-isomerase and localisation of the phosphorylation sites. *Eur. J. Biochem.* **235**, 18–25
- Brunati, A. M., James, P., Donella Deana, A., Matoskova, B., Robbins, K. C. and Pinna, L. A. (1993) Isolation and identification of two protooncogene products related to c-fgr and c-fyn in a protein tyrosine fraction of rat spleen (TPK-III). *Eur. J. Biochem.* **216**, 323–327
- Sarno, S., Vaglio, P., Meggio, F., Issinger, O.-G. and Pinna, L. A. (1996) Protein kinase CK2 mutants defective in substrate recognition. *J. Biol. Chem.* **271**, 10595–10601
- Boldyreff, B., Meggio, F., Pinna, L. A. and Issinger, O.-G. (1992) Casein kinase-2 structure–function relationship: creation of a set of mutants of the β subunit that variably surrogate the wildtype β subunit function. *Biochem. Biophys. Res. Commun.* **188**, 228–234
- Guerra, B., Niefind, K., Pinna, L. A., Schomburg, D. and Issinger, O.-G. (1998) Expression, purification and crystallization of the catalytic subunit of protein kinase CK2 from *Z. mays*. *Acta Crystallogr. D* **54**, 143–145
- Leslie, A. G. W. (1991) In *Crystallographic Computing* (Moras, D., Podjarny, A. D. and Thierry, J. P., eds.), pp. 27–38, Oxford University Press, Oxford
- Collaborative Computational Project Number 4 (1994) *Acta Crystallogr. D* **50**, 760–763
- Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S. et al. (1998) Crystallography and NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. D* **54**, 905–921
- Kleywegt, G. J. and Jones, T. A. (1998) Databases in protein crystallography. *Acta Crystallogr. D* **54**, 1119–1131

- 35 Laskowski, R. A., MacArthur, M. W., Moss, D. S. and Thornton, J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structure. *J. Appl. Crystallogr.* **26**, 283–291
- 36 Vangrevelinghe, E., Zimmermann, K., Schoepfer, J., Portmann, R., Fabbro, D. and Furet, P. (2003) Discovery of a potent and selective protein kinase CK2 inhibitor by high-throughput docking. *J. Med. Chem.* **46**, 2656–2662
- 37 Agullo, G., Gamet-Payraastre, L., Manenti, S., Viala, C., Remesy, C., Chap, H. and Payraastre, B. (1997) Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. *Biochem. Pharmacol.* **53**, 1649–1657
- 38 Niefind, K., Putter, M., Guerra, B., Issinger, O.-G. and Schomburg, D. (1999) GTP plus water mimic ATP in the active site of protein kinase CK2. *Nat. Struct. Biol.* **6**, 1100–1103
- 39 Sarno, S., Moro, S., Meggio, F., Zagotto, G., Dal Ben, D., Ghisellini, P., Battistutta, R., Zanotti, G. and Pinna, L. A. (2002) Toward the rational design of protein kinase CK2 inhibitors. *Pharmacol. Ther.* **93**, 159–168
- 40 Meggio, F., Donella Deana, A., Ruzzene, M., Brunati, A. M., Cesaro, L., Guerra, B., Meyer, T., Mett, H., Fabbro, D., Furet, P. et al. (1995) Different susceptibility of protein kinases to staurosporine inhibition. Kinetic studies and molecular bases for the resistance of protein kinase CK2. *Eur. J. Biochem.* **234**, 317–322
- 41 Gray, A., Olsson, H., Batty, I. H., Priganica, L. and Peter Downes, C. (2003) Nonradioactive methods for the assay of phosphoinositide 3-kinases and phosphoinositide phosphatases and selective detection of signaling lipids in cell and tissue extracts. *Anal. Biochem.* **313**, 234–245

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