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Structural bases of protein kinase CK2 inhibition

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Abstract. Protein kinase CK2 is involved in many fundamental aspects of normal cell life, but it is also able to establish favourable conditions for tumorigenesis. CK2 is elevated in various cancers, it is a potent suppressor of apoptosis, it strongly promotes cell survival, it strengthens the multi-drug resistant phenotype and can be considered a valuable drug target for cancer therapy. In this review, the structural bases of CK2 inhibition deduced from the analysis of crystal structures of CK2α-inhibitor complexes are presented and discussed. The best ATP-competitive inhibitors show an adequate hydrophobic character, an excellent shape complementarity with the unique active site of CK2, and the ability to establish polar interactions with both the hinge region and the positive electrostatic area near the conserved water W1 and the Lys68-Glu81 salt bridge. The state of the art of non-ATP-competitive inhibitors is also presented. (Part of a Multi-author Review)

Keywords. Protein kinase, CK2, inhibitors, cancer, conserved water molecules, positive electrostatic area, ATP-binding site, CK2 pharmacophore.

Inhibition of protein kinases

Since the identification of the first protein kinase inhibitors, around 20 years ago, interest in the development of these compounds has grown enormously, both in basic research and pharmaceutical industry [1]. This is mainly due to the involvement of the superfamily of protein kinases in many key functions of cell life, including cell cycle regulation, development, proliferation, signal transmission and apoptosis. Indeed, protein phosphorylation is the most common post-translational modification in signal transduction, and the amount of cellular proteins that are phosphorylated on at least one residue has been estimated around 30% [2–4]. Small-molecule inhibitors of protein kinases are essential tools for studying signal transduction. In principle, genetic techniques, such as RNAi (RNA interference) and knockout animals, could offer an alternative to small-molecule inhibitors to study kinase function. In any event, these techniques often result in phenotypes different from those induced by small-molecule inhibitors, targeting signalling pathways in different ways. Hence, parallel studies using both genetic and chemical approaches seem preferable [5].

Abnormalities in the natural role of this class of enzymes are often associated with human diseases, especially cancer and tumour pathologies, whose treatment was restricted to cytotoxic and hormonal agents until few years ago [6, 7]. In general, protein kinases are in an inactive state (inactive conformation), and the catalytic activity is triggered only by specific extra- or intra-cellular stimuli, often resulting in the phosphorylation of key residues by means of tightly controlled mechanisms. If there is a failure in this regulation, i.e. for overexpression, upregulation, mutations or other genetic and epigenetic alterations, the kinase can display an uncontrolled activity that often is associated with oncogenic behaviours. Protein kinases are enzymes (they catalyze the reversible phosphorylation of proteins in specific residues such as Ser/Thr or Tyr), and this makes them a really attractive target for the development of drugs; their pathological effects can be decreased or abolished by molecules able to bind to the catalytic site, therefore acting as co-substrate competitive inhibitors. Indeed, although kinases are encoded by less than 2% of the

human genome, the so-called human kinome [7], nowadays they correspond to more than 20% of the druggable genome, i.e. of human genes whose products are likely to represent useful therapeutic targets [8]. Therefore, small molecule inhibitors of protein kinases are not only widely used to dissect signalling pathways, but also represent a new important class of potential drugs in a variety of human diseases. It has been estimated that the protein kinase family is the second most important drug target, after G-protein-coupled receptors, with kinase inhibitors as a target of around 30% of drug discovery projects in pharmaceutical companies [9, 10].

ATP-competitive inhibitors

More than 500 different protein kinases are encoded by the human genome. This, at least in principle, sets serious hurdles in the development of molecules that are able to specifically target the active site of a single protein without affecting closely related kinases. This is due to the conservation of the structural features within and nearby the ATP-binding cleft among the members of this family of enzyme. A couple of other obvious drawbacks of the ATP-competitive inhibitors are the presence of different ATP-binding proteins, with more or less similar active sites, and the high intracellular concentration of ATP, ~1–10 mM, which competes with the potential inhibitor in the binding to the target kinase. The latter statement is not fully correct for those inhibitors that bind to the inactive conformation of the kinases (e.g. Imatinib for Abl kinase), which face weaker competition from cellular ATP, and this may enhance their activity *in vivo*.

The intracellular concentration of ATP is much larger than its K_m (for many protein kinases the K_m^{ATP} values are in the low- to mid-micromolar range), with the consequence that inhibitors, to be effective, must be present at intracellular concentrations that significantly exceed their *in vitro*-determined K_i values, as indicated by the Cheng-Prusoff equation $IC_{50} = K_i (1+[ATP]/K_m^{ATP})$ [11], with $IC_{50}/K_i \cong 100-1000$. On the basis of this equation, the potency of an inhibitor in cells depends critically on the K_M^{ATP} of its target. An inhibitor that has similar K_i values against multiple kinases will inhibit more potently in cells those kinases that have a higher K_M^{ATP} [5]. Protein kinase CK2, the subject of this review, has a relatively low K_M^{ATP}, ~14 μM, and therefore can be more deeply affected by this problem compared to other kinases with higher K_M^{ATP}.

Another problem in the development of successful kinase inhibitors with druglike properties is the necessity of the experimental screening of large starting libraries of candidate compounds, followed by a substantial synthetic effort and the preparation of more focused libraries based on initially identified hit compounds.

Despite all these difficulties, which make the development of ATP-competitive kinase inhibitors a demanding task, with the involvement of people with additional competences to those of medicinal chemistry, e.g. structural biology, enzymology, biochemistry, pharmacology and others, many protein kinase inhibitors are now in clinical trials (probably more than a hundred) or available in the market, the majority as anti-tumour drugs [9,12].

The selectivity issue

As mentioned, selectivity is one of the major issues of ATP-competitive inhibition. Structural studies on complexes between kinases and ATP site-directed inhibitors have indicated that, although there is a significant structural conservation in the main features of the active site, small but crucial differences among diverse kinases are present. This variability is significant enough to account for substrate selectivity (even if not complete), and in fact, most available protein kinase inhibitors are ATP site-directed. Indeed, a complete selectivity against a specific target seems not to be a mandatory requirement for kinase inhibitors that aim to become useful drugs. A study of a panel of 113 kinases revealed that none of the ATP-competitive inhibitors in clinical trials or clinically used at the time was totally selective [13]. A more recent analysis of 38 kinase inhibitors tested against an enlarged panel of 317 distinctive human kinases (~55% of the human kinome) confirmed those results [14]. In particular, it showed that even the more selective inhibitor, lapatinib (Tykerb, an EGFR/ERBB2 inhibitor), binds to three distinct kinases with a $K_d < 3$ μM, while staurosporine, as expected being the most promiscuous kinase inhibitor, binds to as many as 253 kinases (with a $K_d < 3$ μM). An interesting observation of this study is that, whereas compounds originally described as Tyr-kinase inhibitors effectively bind this class more frequently than Ser/Thr-kinases, many of those originally developed as Ser/Thr kinase inhibitors bind Tyr-kinases more frequently. This can be explained by the fact that Tyr kinases have a deeper catalytic cleft than Ser/Thr kinases (which comprise about 80% of all protein kinases). This is also the reason why, whereas there are several examples of Ser/Thr kinases that can phosphorylate Tyr residues to some extent, the converse (a Tyr kinase that can phosphorylate Ser or Thr residues) appears to be rarer. As shown below, the Ser/Thr kinase CK2 is one of the kinases that shows this dual specificity.

The lack of absolute selectivity holds also for imatinib mesylate (Gleevec® or STI571), the first and probably

the most known kinase inhibitor on the market, approved by the FDA on 10 May 2001. Originally, imatinib was approved for the treatment of chronic myelogenous leukaemia (CML), targeting the intrinsically active Tyr-kinase BCR-ABL, an aberrant fusion protein generated by a translocation involving chromosomes 8 and 22. Nowadays, this drug is used not only in the treatment of certain types of leukaemia but also in the treatment of other blood cell cancers, in gastrointestinal stromal tumours (GISTs) and in dermatofibrosarcoma protuberans [15, 16]. Indeed, since the beginning of development, imatinib was seen to be a potent inhibitor, both *in vitro* and in cellular systems, of three different Tyr-kinases, Abl, c-Kit and PDGFR (platelet-derived growth factor receptor) [17].

The concept of 'one drug – many targets', i. e. that non-specific drugs are sometimes useful and even desirable, has recently attracted increasing attention and has been recognized not only for kinase inhibitors but also for other classes of drugs [18]. For instance, amiodarone, the most effective anti-arrhythmic compound, cyclooxygenase-2-inhibitors, propranolol, a β-sympatholytic agent, oestrogens and papaverine, a smooth-muscle relaxant agent, were all recognized as 'dirty' or 'promiscuous' drugs. However, it remains possible, of course, that the lack of specificity could represent a serious drawback owing to the possibility of giving rise to unwanted side effects; it can also be a problem when inhibitors are used to analyse the biological function of a given kinase. Indeed, the biological consequences of multi-kinase activity are still poorly defined [14].

Of course, these arguments are also relevant for the ATP-competitive inhibitors of CK2, as exemplified by the discussion on the selectivity of inhibitors TBB, TBI and DMAT presented below.

Non ATP-competitive inhibitors

Alongside the development of ATP-competitive inhibitors, there is a growing interest in compounds that do not target the active site but are still able to downregulate kinase activity. This is true also for protein kinase CK2, for which some interesting non-ATP-competitive inhibitors have been recently proposed. In general, these inhibitors are expected to show fewer off-target side effects and a higher degree of specificity, mainly because residues outside the ATP-binding pocket tend to be less conserved. Since these inhibitors do not compete with cellular ATP, they can normally be used at concentrations closer to their biochemical K_i, unlike ATP-competitive ones. The competition with endogenous substrates, generally present at a concentration much lower than that of the intracellular ATP, is generally relatively unimportant.

Some of them are small-molecule ATP non-competitive allosteric inhibitors, able to alter the kinase conformation and prevent binding of the substrate or co-substrate. Others are small molecules or, more frequently, peptide-based inhibitors, able to directly compete with the binding of the specific protein substrate [19, 20]. As shown below, examples of inhibitors belonging to both these classes are available for CK2. Bisubstrate analogs, able to bind simultaneously to both the ATP binding- and the substrate-binding sites, have also been proposed as potent and highly specific kinase inhibitors [21–23]. At least 'on paper', agents able to selectively target key protein-protein interactions would appear to serve as ideal inhibitors of cell signalling as well as potential therapeutics. In some cases, non-ATP-competitive inhibitors can be used to selectively inhibit the activity of a kinase against only a subset of its targets.

In general, peptide-based inhibitors show some inconveniences, notably, low affinity for the target and hence low inhibitory potency; intracellular instability and difficult uptake; and general bioavailability problems with respect to classical druglike small organic compounds. Recent advances (delivery technologies, new strategies to transform these otherwise modest binding consensus sequences into high-affinity ligands) have led to a renewed interest in the development of such compounds able to disrupt intracellular protein-protein interactions. In several of these instances, unprecedented affinity (<nM) and selectivity (>1000-fold versus closely related protein targets) have been achieved [24]. For instance, cyclization of peptides improves stability against proteases while affording conformational constraints that may enhance inhibitory potency; globally modified peptides, for example with the addition of long alkyl chain, can have enhanced active site affinity via coordination to ancillary binding pockets. In any event, the conversion of consensus recognition sequences into small molecules with druglike properties (peptidomimetics) is time-consuming and requires a combination of detailed structural information of the target protein and an intensive synthetic effort.

General properties of protein kinase CK2

In this and the following sections, an overview of the properties of protein kinase CK2, relevant for the discussion concerning its inhibition, will be presented. Most of these topics are discussed in more detail in the other reviews of this monographic issue of CMLS ('Protein kinase CK2 in health and disease').

CK2, one of the first protein kinases ever discovered [25], is a eukaryotic acidophilic Ser/Thr protein kinase. This ubiquitous enzyme is among the most highly conserved molecules throughout evolution and is present in every cell, both in the cytosol and the nucleus, at a strictly regulated level depending on the cell type. The catalytic α subunit belongs to the so-called 'other' kinase group; among the nearest relatives are the cyclin-dependent kinases, belonging to the CMGC group. Two catalytic (α, with three possible isoforms) and two regulatory (β) subunits compose the tetrameric holo-enzyme $\alpha_2\beta_2$. CK2 is considered a quite anomalous protein kinase for the following peculiar properties: i) it is highly pleiotropic, ii) it can use both ATP and GTP as co-substrate, iii) the target serine or threonine must be surrounded by acidic residues (with the minimal consensus sequence Ser/Thr-X-X-Asp/Glu) and iv) the CK2α catalytic subunit is intrinsically active [26].

More than 300 substrates are known for CK2 [27]. Although most of these substrates are phosphorylated on serine or threonine residues, and consequently CK2 is traditionally classified as a protein Ser/Thr kinase, some examples of tyrosine phosphorylation have also been documented [28, 29]. In this respect, evidence was presented that both the α and α′ catalytic subunits of CK2 can undergo an intermolecular autocatalytic event on a tyrosine placed in the activation loop, very likely Tyr182 [30]. Very recently, strong evidence was provided that CK2 also exhibits tyrosine kinase activity in mammalian cells and therefore can be considered a dual-specificity kinase [31].

Constitutive activity and regulatory mechanisms

Mechanisms regulating the catalytic activity of most protein kinases that have been recognized to date include the control by additional subunits or domains and phosphorylation and dephosphorylation events [32, 33]. In the regulation of most, but not all, protein kinases, also a (auto)phosphorylation process takes place in the activation loop, often at a residue corresponding to the threonine-197 in cAPK (for instance in cdc2 and MAPK). None of the aforementioned regulations appears to be appropriate for CK2. Indeed, the regulatory mechanism of this kinase is still a matter of debate, although it was discovered more than 50 years ago [25,34] and has been the subject of extensive investigation.

The catalytic subunit of this enzyme is intrinsically active, even in absence of the regulatory subunit; once the tetrameric holo-enzyme is formed, its activity can both increase and decrease, depending on the substrate involved. Hence CK2 is a representative of the so-called constitutively active kinases, whose catalytic activity is controlled by neither by second messengers nor phosphorylation events nor association with its regulatory subunits [35, 36]. It was also suggested that CK2 might be regulated by inactivation [26].

It has been proved that the dimeric β-subunit can indeed interact with several partners other than CK2α [35, 37], suggesting that the former can also play the role of a docking platform for substrates and effectors [38, 39]. Evidence of possible distinctive roles for the isolated catalytic and regulatory subunits have led to the hypothesis that a balance in the expression of the two subunits, together with their different specific localization in the cell compartments, can contribute to the regulation of CK2 functions [40]. Other regulatory mechanisms can be those triggered by interactions with particular proteins that are not direct substrates for CK2 but, having a specific localization within the cells, can recruit CK2 in specific sites [41]. It has also been hypothesized that high-order interactions between CK2 tetramers can play a regulatory role [42–46].

It is of some interest to note that, so far, there are no natural mutations known to affect the constitutive activity of protein kinase CK2.

Biological function: Protein kinase CK2 as drug target

The catalytic subunit of CK2 has been demonstrated essential for cell viability in *Saccharomyces cerevisiae* [47], and the regulatory one as well in *Caenorhabditis elegans* [48, 49] and in mice [49]. CK2 is involved in many cellular processes such as cell cycle regulation [50], circadian rhythms, gene expression, cell growth and differentiation, embryogenesis and apoptosis [51–54]. It has been proposed that CK2 has also a crucial role in the transduction of survival signals, protecting the cell against stress events. CK2 is localized in both the cytoplasm and the nucleus of normal cells, but it is particularly more abundant in the nuclear compartment of cancer cells [51]. Abnormal levels of the enzyme detected in several types of cancer, including prostate, mammary gland, lung, kidney, and head and neck, have suggested the involvement of CK2 in tumorogenesis [41, 55, 56]. Targeted overexpression of CK2α results in tumoriogenesis in animal and cellular models, and CK2 has been demonstrated to increase cell oncogenic potential by sensitizing a cell to transformation by other oncogenic proteins [57]. Many of these data, in particular the demonstration of the anti-apoptotic potential of this kinase [57–60], have been acquired by using selective and potent cell-permeable ATP-competitive CK2 inhibitors [58,61–65]. Downregulation of CK2 activity has been demonstrated to decrease cellular proliferation as well as to induce apoptosis in cancer cells [66]. This has been accomplished not only through the use of small-molecule

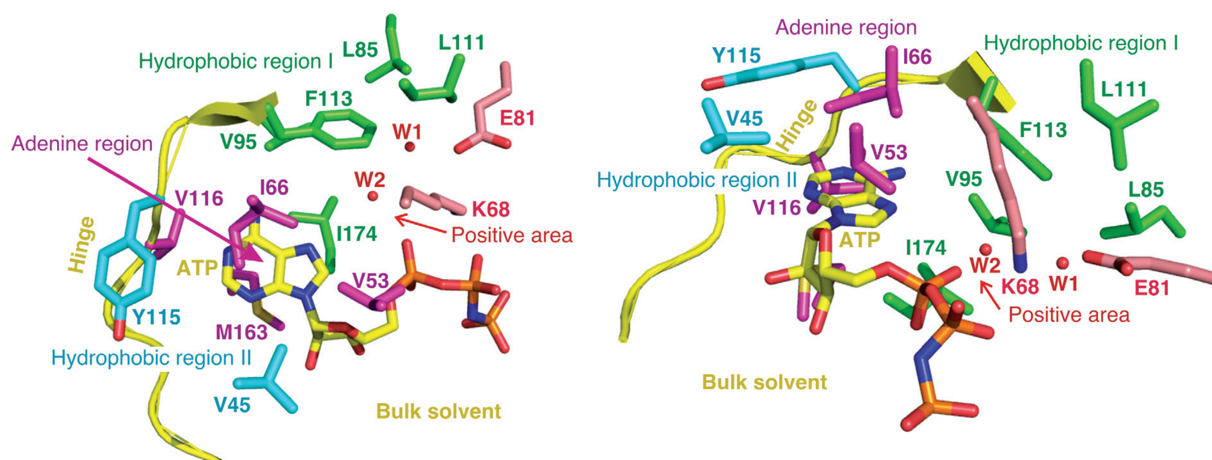

Figure 1. Two views of the CK2α ATP-binding site showing the main structural features. Left panel, from the top of the ATP molecule (yellow carbon atoms); right panel, side view, from the phosphates chain. The three hydrophobic regions common to the 'kinase pharmacophore' are shown in green (hydrophobic region I, in the deepest part of the cavity), in cyan (hydrophobic region II, at the entrance of the cavity) and in magenta (adenine region, occupied by the ATP adenine moiety). Other important areas of the CK2α pharmacophore are the hinge region and the area with a positive electrostatic potential identified near the salt bridge between Lys68 and Glu81, where the two conserved water molecules W1 and W2 are located. The last two regions are the main polar anchoring points for ATP and inhibitors.

ATP-competitive inhibitors, but also of dominant-negative overexpression of kinase inactive mutants, antisense methods and small interfering RNA molecules (siRNA) [60, 67]. These methods have been applied to tumour-bearing mice and, in the best case, demonstrated complete eradication of the PC3 human prostate cancer tumour.

In summary, CK2 can be considered a valuable drug target for cancer therapy essentially on the basis of the following arguments: a) at protein level, CK2 is elevated in various cancers; b) it is a potent suppressor of apoptosis and strongly promotes the survival of the cell; c) it strengthens the multi-drug-resistant phenotype [68]; and d) for the previous reasons, it establishes favourable conditions for tumorigenesis [55]. Clearly, the effects seen upon modulation of CK2 protein levels or kinase activity are an interesting and promising pre-clinical result, and support the rationale of inhibiting CK2 as a potential approach for cancer therapy. Since it hass been seen that viruses are able to exploit cellular CK2 to phosphorylate proteins essential for their life cycle [27] and that the active site of human CK2 shows significant differences in comparison with that of some pathogenic parasites (as in *Plasmodium* and *Leishmania*), CK2 inhibitors have also an anti-viral and anti-infectious potential interest.

Structural properties of protein kinase CK2α

Main structural characteristics of protein kinases

Protein kinase catalytic domains are structurally well conserved throughout evolution, from prokaryotes to mammals, while their amino acid sequences show a much lower degree of similarity [7, 69]. Residues essential for the function of the enzymes are conserved; in particular, the residues fully conserved in all the protein kinases are the following five, indicated with CK2α numbering: Lys68, which stabilizes the α- and β-phosphates of ATP; Glu81, in the αC-helix, salt-bridged to Lys68, which contributes to the stabilization of ATP; Asp156, in the so-called catalytic loop, the catalytic base that favours the deprotonation of the substrate phosphate acceptor, either Ser, Thr or Tyr; Asn161, again part of the catalytic loop, which keeps the γ-phosphate in the correct orientation and interacts with the secondary divalent cation; Asp175, which coordinates the primary divalent cation and is the first of the conserved DFG triplet (DWG in CK2α), at the beginning of the 'activation segment', involved in the movement of the segment itself [70]. Between the N- and the C-terminal lobes, which characterize the protein kinase fold, an essentially hydrophobic deep cleft is formed, where the co-substrate (ATP or, less frequently, GTP) can bind. According to the pharmacophore model, the ATP-binding site of protein kinases can be divided into five regions, three hydrophobic (adenine region and hydrophobic regions I and II) (Fig. 1 for CK2α), and two hydrophilic (sugar pocket and phosphate-binding region) [71, 72].

A segment known as the hinge region, where the ATP adenine moiety (and many inhibitors) can be anchored by means of a couple of hydrogen bonds, connects the two lobes. In order to be active, the conformation of a kinase must ensure the access of

both the co-substrate and the protein substrate to the catalytic site, and the correct location and orientation of the catalytic and binding residues; in particular, the proper orientation of ATP is crucial for catalysis. The binding of the ATP phosphate tail is favoured by two metal ions, mainly magnesium but also manganese to a lesser extent. Two important segments are the Gly-rich loop, or G-loop or p-loop, and the structurally conserved helix αC; the p-loop contributes to strengthen the anchoring of ATP, while the orientation of the αC helix is essential for the activity of the enzyme. In kinases that have inactive and active forms, as in the case of cAPK and CDK2, helix αC has been found with substantially different orientations in the two arrangements [73]. In a transition from an inactive to an active conformation, usually there is a rearrangement of the relative orientation of the two lobes for the proper positioning of essential residues of the catalytic site, and a simultaneous rearrangement in the correct orientation of the αC-helix. Another important structural change occurring for the activation is represented by the displacement of the activation segment, a long loop in the C-terminal domain, that moves away from the close position of the inactive form to an open one, allowing the binding of the substrate and co-substrate to the enzyme [33]. This movement can be triggered by a phosphorylation event, as in the case of cAPK when Thr197 is phosphorylated, sometimes coupled with an interaction with a regulatory subunit, as in the case of CDK2 with cyclin. The activation segment displacement occurs through a rotation around a short stretch of three amino acids at the beginning of the loop; this tripeptide, Asp-Phe-Gly, is highly conserved in kinases but not in CK2, where the phenilalanine is substituted by a tryptophan at position 176. The C-terminal end of the activation segment, the p+1 loop, contributes to substrate recognition and binding.
It must be noted, however, that not all protein kinases show a dramatic transition from an active to an inactive form as exhibited by CDK2 and cAPK. As already mentioned, CK2 is one of the few examples being found solely in the active form.

Key structural properties of CK2α

CK2α bears most of the sequence and structural features common to all protein kinases briefly reported above. Regarding the sequence, two notable exceptions are: a) in the p-loop (residues 46–51 in CK2), the third glycine in the consensus sequence GXGXøG is missing (ø is usually a tyrosine, as in CK2, or a phenylalanine, as in PIM kinases, discussed below); b) at the beginning of the activation segment a tryptophan (residue 176 in CK2) substitutes a phenylalanine in the otherwise conserved DFG triplet. Another distinctive feature of CK2α is the presence of a basic cluster at the beginning of the αC-helix (residues 74–80), where 6 out of 7 consecutive amino acids are basic. The presence of this basic cluster, also known as the substrate recognition site, is related to the propensity to phosphorylate highly acidic substrates. Other basic amino acids present in the p+1 loop have been found relevant for substrate recognition [74].
The determination of the 3D structure of CK2α (from maize) has revealed the basis for the intrinsic activity of the enzyme [75], showing that CK2α carries all the structural properties responsible for the active state of cAPK, taken as the prototype of an active Ser/Thr kinase [76]. In CK2α, many of the 30 N-terminal residues make hydrophobic and polar interactions with other important regions of the protein, located both in the N- and C-terminal domains, stabilizing the active conformation. Of particular interest are the interactions with the activation segment, which is constrained in an open active state. These interactions are unique among the available protein kinase structures, and indeed they occur between residues distinctive of CK2α. The presence of the characteristic Trp176 contributes to wedge the activation segment in the open active conformation. The active conformation of the enzyme is granted also by the proper relative orientation of the N- and C-terminal lobes and by the correct orientation of the αC helix.
In vivo, human CK2 is found mainly, although not exclusively, in a tetrameric assembly. The three-dimensional structure of the human $\alpha_2\beta_2$ holoenzyme, with the α subunits carrying a C-terminal deletion, has been determined at 3.1 Å resolution [77]. The β_2 dimer binds two α subunits in such a way that they do not interact with each other, and, at least within the limit of the relatively low resolution, the catalytic subunit is poorly affected by its interaction with the regulatory one.

Structural aspects of CK2 inhibition

The oncogenic potential of CK2 and its involvement in virally mediated pathologies has led to an increasing number of studies aimed to the discovery of selective inhibitors. These molecules could constitute not only useful tools for the clarification of the protein function *in vivo*, but also lead compounds for drug development. An important CK2 feature that influences the inhibitor design process is constitutive activity, with the consequence that only the active conformation can be targeted. The strategy to induce and stabilize an inactive conformation, successful in the case of Imatinib, is not feasible. Further, constitutive activity,

together with the long half-life, cellular localization and high expression of endogenous CK2 in cells, complicates the use of non-pharmacological approaches, such as RNAi or antisense strategies, to examine the cellular function of CK2 [78].

On the other hand, the inability (or high difficulty) of CK2α to adopt an inactive conformation has some important advantages. If a compound targets the active rather than the inactive conformation, mutations leading to drug resistance through the induction of structural changes that do not affect the enzymatic activity (as in the case of Abl) are less likely to occur [17]. This type of molecular resistance seen in Abl is due to mutants of the so-called gatekeeper residue Thr315, whose side chain contributes to controlling the size of a hydrophobic back pocket, the hydrophobic region I of the protein kinase pharmacophore [71]. This pocket accommodates moieties of ATP-competitive inhibitors, such as Imatinib and some potent CK2 inhibitors (as shown below), but no functional groups of ATP itself. A study of mutations introduced in the corresponding residue of oncogenic Tyr kinases, such as EGFR, βPDGFR, Src and FGFR1, revealed that, with one important exception, all of the inhibitor classes tested were prone to a conserved mechanism of molecular resistance formation [79]. Replacement of Thr315 by larger residues, able to hinder the access to the deepest part of the back pocket, reducing its size, interferes with inhibitor binding, while leaving the kinase activity unaffected. This mechanism of resistance can hardly occur in protein kinases that possess a larger, hydrophobic residue, such as methionine, leucine and phenylalanine, in the equivalent position (about 75 % of all protein kinases). This is the case of CK2α, which at the gatekeeper position carries a phenylalanine (Phe113). Ser/Thr kinases that possess a threonine gatekeeper (for example, Raf and p38α) are often sensitive to tyrosine kinase inhibitors, and vice versa.

The catalytic site of CK2 displays some unique properties that can be exploited in the design of inhibitors with a high degree of specificity, as indicated by the ability to utilize both ATP and GTP and by the low sensitivity to staurosporine inhibition (IC_{50} of 19.5 μM versus values in the low nanomolar range for other kinases [80]). Actually, as described below, fairly specific, potent and cell-permeable inhibitors of CK2 have been successfully developed in recent years [63, 64, 81–83].

Nowadays, probably more than 300 co-crystal structures of inhibitors have been solved, involving more than 40 individual protein kinases, several of them not publicly available in the PDB, as part of medicinal chemistry programs activated in pharmaceutical companies. Besides the tightly regulated PKA and CDK2, the constitutively active CK2 is one of the kinases with the largest number of known structures in complex with inhibitors. The close structural similarities between maize and human CK2α (with a sequence identity of more than 77 %), supported in the past the choice to work with the maize enzyme, with a higher tendency to produce good diffracting crystals, in structural inhibition studies. Now, thanks to the work of Niefind and co-workers, a truncated form of the human enzyme is also amenable to these kinds of studies [84, 85]. As we will see, to what extent the maize and human enzymes are actually structurally similar still remains to be fully clarified. Nevertheless, the value of the studies performed on the maize enzyme is unquestionable, as demonstrated by the following sections of this review. Indeed, the majority of the CK2 inhibitors available today, some with a K_i below 0.1 nM, have been developed mainly through structure-based drug design approaches that exploit maize CK2α complexes.

Common structural properties of CK2α-inhibitor complexes

Like the majority of the known kinase inhibitors, those crystallized in complex with CK2α are ATP-competitive and, therefore, bind to the catalytic site of the enzyme in the position normally occupied by an ATP (or GTP) molecule. In general, the overall structure of the protein is only marginally affected by the binding of a small molecule, either a cosubstrate or an inhibitor. The C-terminal lobe is poorly influenced by the formation of the complex, while the N-terminal lobe shows areas with a higher degree of flexibility. Near the active site, few side chains, notably that of His160, and the p-loop have shown a significant flexibility. Other zones showing some plasticity are the hinge region and that spanning from residue 102 to residue 108, comprising the external loop between the β strands β4 and β5. The nature and relevance of the flexibility of these regions will be analysed below. The position and orientation of helix αC in the N-terminal domain is particularly well conserved. As reported above, the correct position of this helix is essential to the active state of a protein kinase. The overall rigidity of key elements of the enzyme is in accordance with the notion of the constitutive activity of CK2, whose active conformation has strong structural bases (see the review by Karsten Niefind in this same issue).

The stretch of residues whose conformation is less characterized is that spanning from Leu70 to Lys76, just before and at the beginning of helix αC. The electron density of these residues is always worse defined than that of the remaining part of the protein, especially in the side chains, indicating an intrinsic flexibility of this zone. In spite of that, the orientation

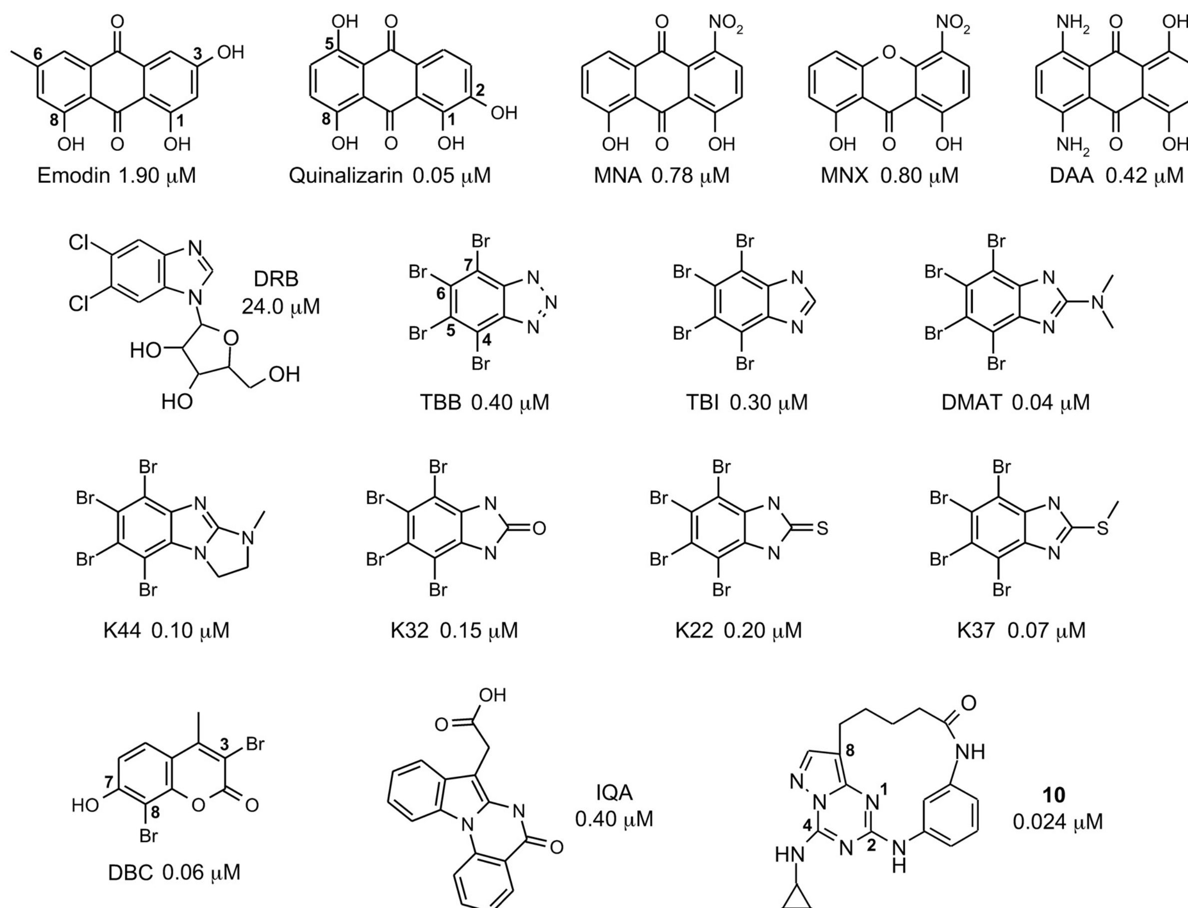

Figure 2. Chemical formulae of the main inhibitors discussed in this review, with the value of the inhibition K_i reported. For some of inhibitors, numbers indicate the most important positions discussed in the text.

of the αC-helix is not affected, as reported above. As the relatively mobile 70–76 segment is involved in substrate recognition, its flexibility probably reflects this function.

Anthraquinones and xanthenones inhibitors

Because of their structural similarities with adenine, anthraquinones are able to interact with nucleotide-binding enzymes such as dehydrogenases, ATPases and kinases. Anthraquinones and xanthenones extracted from natural sources have several potential therapeutic applications, for instance as antiviral, antimicrobial or anti-cancer drugs. On the other hand, the possibility to intercalate DNA due to their extended planar structure makes these compounds potentially cytotoxic. In any case, the study of these compounds as CK2 inhibitors was useful for the understanding of some relevant feature of the ATP-binding site of this kinase.

Emodin

Emodin (1,3,8-trihydroxy-6-methyl-antraquinone) (Fig. 2) was the first ATP-competitive inhibitor crystallized in complex with CK2α, in particular with the maize enzyme [86]. Recently, the complex with a truncated form of the human enzyme was also determined (Fig. 3) [87]. Emodin is a natural compound extracted from the rhizomes of *Rheum palmatum* and is used in Oriental medicine for supposed anti-inflammatory and anti-cancer activity [88]. It is able to inhibit CK2 with an inhibitory constant K_i of 2.0 μM [88,89] and shows modest selectivity. Indeed, this compound was initially reported to be a tyrosine kinase inhibitor, although with a lower efficiency. As seen above, it is not uncommon for Tyr-kinase inhibitors also to target Ser/Thr kinases, while the converse is more unusual. Emodin binds deeper than ATP or GTP to the ATP-binding site of CK2, reaching the hydrophobic region I, which shows some variability among protein kinases [90], and this may be responsible for the selectivity, albeit limited, of this inhibitor. Emodin is entrapped into the active site

mainly by hydrophobic contacts involving residues Val45, Val53, Ile66, Leu85, Val95, Leu111, Phe113, Val116, Met163 and Ile174. The binding of emodin causes alterations in the conformation of some residues near the active site of the maize enzyme, in particular of Asn118, His160 and the p-loop. The last one is shifted towards the interior of the cavity, entrapping emodin inside. The orientation of Asn118 side chain contributes to the closure of the cavity, as well as that of the imidazole ring of His160, which forms an H-bond with the carbonyl oxygen of Arg47. A similar arrangement of the protein matrix is also found in the complexes of maize CK2α with other anthraquinone derivatives, quinalizarin, and, to a lesser extent, MNX (see below for details).

The complex of emodin with human CK2α shows some differences, both in the orientation of the inhibitor and in the conformation of the protein in the hinge region and the p-loop. As in the maize enzyme, in the human one there is also movement of Asn118 towards the ATP-binding cleft, but in the latter case the conformational change is larger. Until now, such orientation of the hinge region has been seen only in two other human structures (in complex with DRB, see below, and with the mutant V66A/M163L), but not in maize. Further, unlike in the maize complex, in the human structure the p-loop and His160 do not deviate from the typical conformations seen in many CK2α structures. Whether there really is a difference in the conformational plasticity of the two enzymes, and if yes to what extent, and its relevance for inhibitor binding and design, requires deeper scrutiny.

The two complexes also differ in the orientation of emodin (while the general position inside the binding pocket is very similar), with the 3-hydroxyl group or the 6-methyl group in the deeper part of the cavity for the human and the maize enzyme, respectively. It is possible that the different orientation of emodin in the maize complex is due to a technical artefact caused by the relatively low resolution (2.63 Å) and by the presence of a pseudo-twofold symmetry axis in the emodin molecule. To clarify whether this is true, a more comprehensive analysis based on further experimental data is necessary.

MNA, MNX and DAA

Several other anthraquinones and related xanthenones were tested *in vitro* as potential CK2 inhibitors, allowing the identification of some interesting compounds with lower IC_{50} (or K_i) compared to emodin [89]. The crystal structures in complex with maize CK2α are available for the inhibitors MNA (a mono-nitro derivative, 1,8-dihydroxy-4-nitro-anthraquinone; K_i 0.78 μM), MNX (a mono-nitro xanthenone, 1,8-dihydroxy-4-nitro-xanthen-9-one; K_i 0.80 μM) and DAA, (a di-amino derivative, 1,4-diamino-5,8-dihydroxy-anthraquinone; K_i 0.42 μM) (Fig. 2) [91]. Among these three inhibitors, MNX binding causes the greatest variation in the N-terminal domain of the enzyme, in particular at the level of the backbone from residues 72 to 75 and of the p-loop (residues 45–51). The latter ‘collapses’ into the cosubstrate binding cavity, in a manner similar to that displayed by emodin and quinalizarin complexes. In the case of MNX, this displacement is not coupled with a rotation of the His160 and Asn118 side chains. MNA and MNX are fully superimposable and orient the nitro group towards the hinge region, so that the hydroxyl groups can make contact with Lys68 (Fig. 3). The importance of the nitro function is outlined by the observation that, if missing, as in 1,8-dihydroxy-anthraquinone and 1,8-dihydroxy-xanthen-9-one, the inhibitory constants K_i rise to values higher than 40 μM. The effect of the NO_2 group is related to its electron-withdrawing property, which increases the dissociation constant of the phenolic hydroxyl group in *para* (as confirmed by theoretical calculations), which becomes more acid and can better interact with the positive area identified near Lys68 (see below).

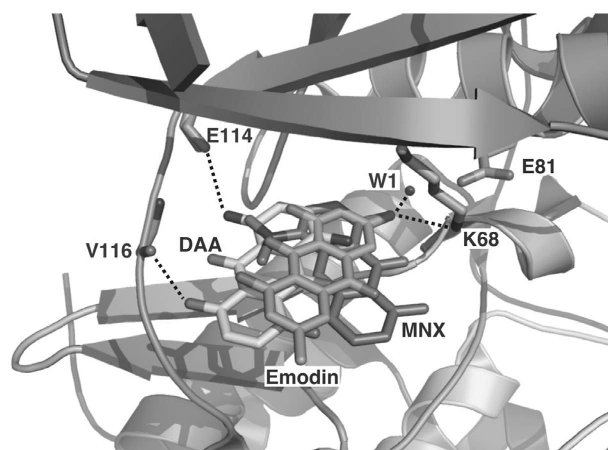

Figure 3. Anthraquinones emodin (human complex), MNX and DAA in the CK2 ATP-binding site as determined by the co-crystal structures. DAA is the only anthraquinone that interacts with the hinge region (hydrogen bonds with carbonyls of Val116 and Glu114). Note that, even with the aromatic rings slightly rotated, both emodin and MNX position an acidic hydroxyl group in the positive area (see also Fig. 1), near the conserved water molecule W1 and the salt bridge Lys68-Glu81.

An interesting feature of MNA is that, while it shows a fairly good inhibitory efficiency towards the CK2 tetrameric holo-enzyme (IC_{50}=0.30 μM), unlike the closely related MNX, it is much less effective towards the isolated α-catalytic subunit (IC_{50}=2.5 μM) [92]. One can speculate that the origin of these different behaviours could be ascribed to the fact that the β subunit, upon interaction with the catalytic one, can

make it stiffer, reducing the flexibility of some regions of the N-terminal lobe. As seen in the crystal structure, MNA can adopt two different conformations, and the nitro function is not coplanar with the rest of the molecule as in the case of MNX [91]. Possibly, the binding of MNA could be favoured by the loss of flexibility of the p-loop and, in general, of the N-terminal domain, to a higher extent in comparison with MNX; in other words, in the case of the tetrameric holoenzyme, the catalytic chain can be 'frozen' in a state that is easier targeted by MNA.

DAA binds to the active site in a different way with respect to the other inhibitors with similar scaffolding (quinalizarin included), preferring a position near the hinge region, where it interacts with the backbone carbonyls of Glu114 and Val116 via two hydrogen bonds (Fig. 3). This is due to the lack of an acidic hydroxyl function that in the other inhibitors shifts the position towards the positive area near Lys68.

Quinalizarin

Quinalizarin (1,2,5,8-tetrahydroxy-anthraquinone; Fig. 2) was identified as a potential CK2 inhibitor by means of computer-aided virtual screening [unpublished observations]. Quinalizarin is a powerful, cell-permeable inhibitor of CK2, with the lowest K_i (around 50 nM) among the anthraquinones and xanthenones and, notably, the most selective among CK2 inhibitors analysed so far, with a 'promiscuity score' of 11.1. This score is inversely proportional to the average selectivity of an inhibitor, i.e. the lower the score the higher the selectivity [93]. This inhibitor establishes the following polar interactions with maize CK2α: 2-hydroxyl group with the conserved water molecule 'W1' (a fully conserved water molecule discussed below) and the nearby Lys68; 5-hydroxyl group with the hinge region (carbonyl backbone of Val116) through a water molecule; 8-hydroxyl group with His160 and the backbone carbonyl of Arg47 (the latter two also interact with each other) (Fig. 4). These interactions stabilize the kinase into a close conformation similar to that shown by maize CK2α in complex with emodin. The scaffold of quinalizarin is coplanar and superimposable to that of emodin in the human complex, but the lack of the hydroxyl group in the corresponding position 5 of emodin prevents the formation of the interaction with His160 and the backbone of Arg47 found with quinalizarin. An additional disadvantage of emodin (human complex) compared to quinalizarin is that the 1-hydroxyl group (in a substantially hydrophobic environment near Ile66, Val95, Phe113 and Val116) and the 6-methyl group (exposed to the solvent) have an unfavourable environment, therefore contributing in a negative way to the binding energy. This may explain, at least in part, the higher potency of quinalizarin, where these adverse interactions are absent, compared to emodin. Somewhat surprisingly the mutations of Val66 and Ile174 to alanines have only a marginal effect on quinalizarin inhibition, while causing a 50-fold drop in inhibitory efficacy with emodin, in accordance with what is usually seen for other CK2 inhibitors. This is not straightforward to explain based solely on the crystal structure; probably more general solvation/desolvation effects must be considered, taking into account the more hydrophobic character of emodin compared to quinalizarin.

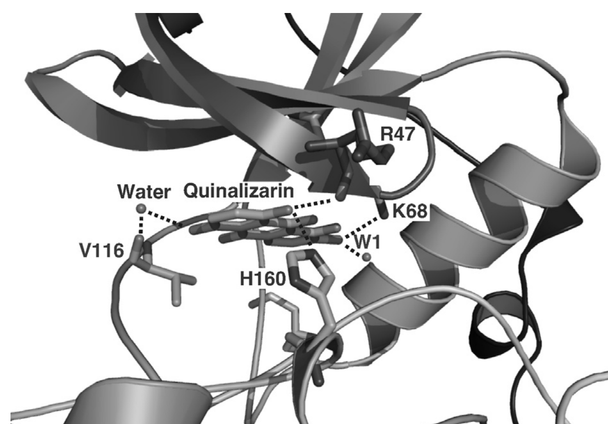

Figure 4. Polar interactions (dotted lines) of quinalizarin with residues and water molecules in the CK2 ATP-binding site. Note the simultaneous interactions with the side chain of His160 and the backbone carbonyl of Arg47 (in the p-loop), that cause the closure of the site, with the 'lowering' of the p-loop from the 'canonical' position.

Indoloquinazoline IQA

A virtual screening analysis of the in-house collection of chemical compounds performed by researchers of Novartis Pharma led to the discovery of a new class of potent and selective inhibitors of CK2, the indoloquinazolinones [94]. The most potent is IQA ([5-oxo-5,6-dihydro-indolo(1,2-a)quinazolin-7-yl]acetic acid; Fig. 2), with a K_i of 0.17 μM, whose structure in complex with maize CK2α was determined at 1.68 Å resolution [95]. The inhibitor was found with an orientation opposite to that in the computationally docked complex. IQA seats inside the catalytic pocket in the same plane occupied by the ATP purine moiety. The hydrophobic side of IQA faces the hinge region of the protein, while the polar side is oriented towards Lys68. IQA is found with two different conformations, differing for a rotation of the acetate function. In one conformation, the carboxyl makes an intra-molecular hydrogen bond with the lactam nitrogen, closing a six-member ring largely favoured in solution, and interacts with the Ser51 hydroxyl. The second conforma-

tion is stabilized by interaction with the CK2α active site; in this case, the carboxylate is involved in three hydrogen bonds, with the amine function of Lys68, the backbone nitrogen of Asp175, and the conserved water molecule W1. For both conformations, the major contribution to binding comes from hydrophobic interactions with non-polar residues, i. e. Val45, Val53, Ile66, Lys68, Val95, Phe113, Val116, Met163 and Ile174. In this respect, it is remarkable that the total buried surface upon inhibitor binding is quite large, about 730 $Å^2$. Mutation of Ser51 with a glycine does not significantly affect the K_i value, excluding that its interaction with IQA plays a significant role.

Tetralogenobenzo derivatives

Tetrabromobenzo-imidazoles/triazoles

The two most widely used CK2α inhibitors belong to this class of compounds, and are TBB (4,5,6,7-tetrabromo-1-benzotriazole) and its derivative DMAT (2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole, also called K25), both commercially available. Initially, they were considered quite selective inhibitors of CK2, but a recent study on an enlarged panel of 80 kinases revealed that DMAT (and also TBI, 4,5,6,7-tetrabromo-benzimidazole, a close TBB relative, also called K17 or TBBz) is a potent inhibitor of some other kinases, above all PIM1, PIM2, PIM3, PKD1, HIPK2 and DYRK1a, while TBB shows a higher selectivity towards CK2, although it also inhibits PIM1 and PIM3 [93]. Notably, the two derivatives K64 (3,4,5,6,7-pentabromo-1*H*-indazole) and K66 (1-carboxymethyl-2-dimethylamino-4,5,6,7-tetrabromo-benzimidazole) display similar efficacy but better selectivity compared to TBB.

A different behaviour between the two tetrabromobenzo-imidazoles DMAT and TBI and the tetrabromobenzo-triazole TBB was also observed in a chemoproteomics study on their effect in cells [96]. While CK2α and CK2α′ were identified as 'bona fide' targets of all three inhibitors in cells, inhibitor-specific cellular effects were observed, indicating that the inhibitors have unique pharmacokinetic properties. In accordance with the lower specificity noted in the *in vitro* study on the panel of 80 kinases, it was shown that, differently from TBB, DMAT and TBI had off-target effects and that resistant CK2 mutants were unable to rescue TBI- and DMAT-induced apoptosis. These two inhibitors were also found to target a non-kinase protein, the detoxifying enzyme Quinone Reductase 2 (QR2), a member of the NAD(P)H dehydrogenase (quinone) family [96]. It is interesting to note that TBB differs from the two inhibitors DMAT and TBI also in the binding mode to CK2α, because of its more acidic pK_a, as shown below.

TBB is a CK2 inhibitor developed starting from the 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (DRB) parent molecule. DRB was found inhibiting CK2 with a modest inhibitory constant (K_i~24 μM), and a rather low specificity [97]. After several modifications, the most relevant being the introduction of four bromine atoms instead of chlorines, the final compound not only showed a lower K_i (0.2–0.6 μM), but also a higher selectivity for CK2 [98–100]. TBB binds to the CK2α active site essentially by means of hydrophobic interactions involving residues Val45, Val53, Ile66, Val95, Phe113, Val116, Met163 and Ile174 [101]. Due to the bulkiness of the four bromine atoms, TBB establishes good van der Waals interactions with the surrounding residues of active site. In the CK2α-TBB complex, His160 side chain adopts the same orientation of the maize CK2α-emodin complex. However, due to the large size of the bromines, in the case of TBB, the p-loop does not interact with His160, but, conversely, it is pushed up, away from the binding cleft, adopting a particular conformation never found in any other structure available so far. The only polar interaction present in the complex is that between nitrogen N1 of TBB and Glu81 side chain, mediated by two water molecules, one of which is the conserved water molecule W1 (Fig. 5). This interaction is possible because of the acidic nature of this inhibitor, as demonstrated by the different binding mode of the closely related compound TBI, which carries an imidazole ring instead of the TBB triazole ring, with a higher pK_a (8.9 vs. 5.0 [102]) (Fig. 5). At neutral pH, TBB is expected to be an anion, with the negative charge on the five-membered ring, while its benzimidazole counterpart is not charged. In the case of TBI, the preferred interaction of the inhibitor is with the hinge region, with the backbone carbonyls of Glu114 and Val116, by means of a couple of halogen bonds involving two bromines [103]. The positive effect of the substitution of chlorines with bromines can possibly be ascribed to different factors: better shape complementarity with the active site (better van der Waals interactions); an increase in the hydrophobic effect favouring the binding; and higher efficacy of the two halogen bonds established with the hinge region [104, 105]. This trend of a higher potency with the increasing dimensions, hydrophobicity and polarizability, respectively, from Cl to Br to I, was confirmed by the higher efficacy of tetraiodobenzo-imidazole derivatives [unpublished observation] and by an analogous trend observed with the tetrahalogeno-isoindole-dione compounds [106].

K22 (4,5,6,7-tetrabromo-1H,3H-benzimidazol-2-thione), DMAT and K32 (4,5,6,7-tetrabromo-1H,3H-benzimidazol-2-one) (Fig. 2) bind to maize CK2α in a

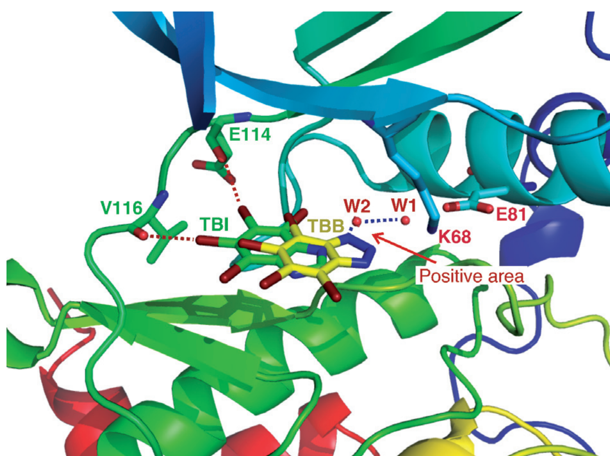

Figure 5. Different binding modes of the two closely related tetrabromobenzo-triazolo TBB and tetrabromobenzo-imidazolo TBI. TBB, due to the more acidic pKa (around 5), is shifted towards the positive area and interacts with water molecule W2. TBI, with a higher pKa (around 9), interacts with the hinge regions establishing two halogen bonds with the backbone of Val116 and Glu114 (red dotted lines).

way similar to that of TBI, using the same two bromines (Br5 and Br6) for the halogen bonds with the backbone carbonyls of Glu114 and Val116 in the hinge region [103, 107]. K44 (N1,N2-ethylene-2-methylamino-4,5,6,7-tetrabromo-benzimidazole; Fig. 2) is rotated about 60° around an axis perpendicular to the plane of the molecule, with the consequence that now the two bromines Br4 and Br5 interact with the hinge region. Br6 and Br7 of K44 establish two additional halogen bonds, with a water molecule and the Asp175 side chain, respectively, for a total of four halogen bonds, one for each bromine atom. K37 (4,5,6,7-tetrabromo-2-(methylsulfanyl)-1*H*-benzimidazole) was found in two orientations, one similar to that of K44, with Br4 and Br5 interacting with the hinge region, and the other similar to that of TBI, K22, K25 and K32, with Br5 and Br6 making the two halogen bonds.

For this class of compounds, a linear relationship between log(K_i) and the variation in the accessible surface area (ΔASA) upon binding to CK2α revealed that the extent of the hydrophobic interactions is ultimately responsible for their rank in potency, while the polar interactions came out to play a major role in orienting the ligands inside the active site [103, 107]. This was confirmed by a study using a Linear Interaction Energy (LIE) model to evaluate free-energy binding values, resulting from three contributions that estimate the van der Waals interactions (parameter coefficient α), the electrostatic contributions (β), and the 'cavity parameter' linked to the energy penalty for forming a solute cavity (γ), a term proportional to the solvent accessible area. An important outcome of this analysis was that the most crucial binding interactions for this class of compounds are due to hydrophobic and van der Waals contributions, with the solvent accessible area term playing a fundamental role [108].

The replacement of the five-membered ring of the tetrabromobenzo derivatives with polar side chains generated a new interesting class of inhibitors, the best one being TBCA (tetrabromocinnamic acid). It has a K_i value of 0.11 μM for CK2, it reduces the viability of Jurkat cells more efficiently than TBB, and, more important, it displays a good selectivity [109, and unpublished observations]. In particular, it does not inhibit DYRK kinases usually targeted by tetrabromobenzimidazole derivatives. Molecular modelling indicates that it might bind to CK2α in a way significantly different from the other members of this class of inhibitors.

DRB

The tetrabromobenzimidazole/triazole derivatives described above were developed starting from the parent molecule DRB [97, 107, 110], most probably the first designed CK2 inhibitor. DRB is able to inhibit CK2 with a modest inhibitory constant (K_i~24 μM), and a rather low specificity [111]. Recently, the crystal structure of human CK2α in complex with DRB was described [112, 113]. One molecule of the inhibitor binds to the active site, in the adenine region but farther away from the hinge region with respect to ATP, with the 6-member ring roughly superposed to the 5-member one of the ATP adenine moiety. Its position is similar to that of DMAT, but slightly rotated towards the entrance of the cavity, where the ribofuranosyl group is pointing. This position, together with a small rearrangement of the protein backbone, does not allow DRB to interact strongly with the hinge region; a weak halogen bond is established between a chlorine atom and the carbonyl of Val116, at a distance of 3.2 Å, within the maximum distance possible for a Cl···O halogen bonds, 3.27 Å, and with a C–Cl···O angle of 168° and a Cl···O–X angle of 114°, close to the optimal values of ~165° and ~120°, respectively [104]. The polar interactions made by the ribofuranosyl group with Asn118 and the p-loop seem to significantly contribute to this not particularly favourable position and orientation of the aromatic portion of DRB. This could explain the observation that deletion of the ribofuranosyl moiety lowers the K_i from 24 μM for DRB to 10 μM for DCB, the dichlorobenzotriazole derivative. As seen above, the introduction of the four bromines instead of the two chlorides was essential for the further improvement of the inhibitors, with K_i values of 0.40 μM for the

benzotriazole TBB and 0.30 μM for the closely related benzimidazole TBI.

An interesting feature of the DRB-CK2α complex is that this is one of the three structures of human CK2α where the hinge region around Phe121 moves towards the interior of the ATP-binding site, occupying the hydrophobic region II, the outer region of the ATP-binding site.

The interest of this structure relies also on the fact that another DRB molecule was found bound to CK2α, in a hydrophobic pocket near the beginning of beta strand β1 and adjacent to the surface made up by beta strands β3, β4 and β5, in a region that is also the interface between the subunits α and β in the tetrameric $\alpha_2\beta_2$ holoenzyme (Figs. 7, 8). Based on kinetic data that indicate that DRB has a mixed inhibitory effect on human CK2α catalytic activity, this 'secondary binding site' is described as allosteric and a site for non-competitive inhibition [112]. The non-ATP-competitive effect is less evident for the maize enzyme compared to the human one, and only a pure ATP-competitive inhibition is observed for the tetrameric holoenzyme, indicating that DRB is not able to disrupt the tetrameric assembly. Quantitative analysis shows that the affinity of DRB for the secondary binding site is lower than that for the ATP-binding one. This secondary binding site looks interesting also for the development of small-molecule antagonists against the CK2α-CK2β interaction. In the structure of the DRB complex, the loop between β4 and β5 was found bent toward the N-lobe β sheet, in a so-called closed conformation. From the comparison of the conformation of the same loop in other structures of the human and maize enzymes, it was inferred that 'the loop provides a further example of a subtle conformational plasticity of CK2α which is evident from human enzyme but not from the maize homolog' [112]. As a matter of fact, the conformational plasticity of this loop is also evident from the maize enzyme, as discussed in three previous papers [83, 91, 107]. It was associated to shrinkage of the crystallographic unit cell (with a shorter 'b' axis and a closer β angle) and attributed to a dehydrating effect of the cryoprotectant (PEG or glycerol) rather than to structural properties of the complex under study. This conclusion was corroborated by the fact that when using silicon oil for cryoprotection, the cell shrinkage effect was no more observed. Taken together, these data seems to indicate that the β4-β5 loop has a similar flexibility in solution in the maize and the human enzyme, and that it can be frozen in an open or closed conformation in the crystal packing, mainly depending on the experimental conditions. It can be tentatively hypothesized that this flexibility plays some role in the recognition process between the α and the β subunits and hence also in the binding of small molecules (for instance DRB) or peptides to the nearby secondary binding site.

Coumarins

Recently, using different virtual screening approaches, the coumarins, natural substances present in several foods derived from plants, were identified as an attractive CK2 inhibitor scaffold. One of the most promising inhibitors, 3,8-dibromo-7-hydroxy-4-methylchromen-2-one (DBC) (Fig. 2), with a K_i of 0.06 μM and good selectivity, has been crystallized in complex with maize CK2α, and the experimental binding mode has been used to derive an LIE model, to rationalize the different free energies of binding and the key interactions of several coumarin derivatives [114]. DBC binds to CK2α in a position similar to that of the anthraquinone MNA and of the xanthenone MNX and, likewise, it does not interact directly with the hinge region. The position of the DBC hydroxyl group is almost identical to the analogous function in MNX, in the area of positive electrostatic potential near Lys68. This OH establishes two hydrogen bonds, one with the amine function of the Lys68 side chain and another with the conserved water molecule W1. This H-bonding network has a crucial role in the recognition process of all phenol-like CK2 inhibitors. The crystal structure adequately explains the qualitative Structure-Activity Relationship (SAR) derived from a panel of more than 60 analysed coumarins. First, the 7-hydroxyl group, even if not sufficient to achieve an IC_{50} in a sub-micromolar range, can be considered an essential feature due to its key interactions with Lys68 and the conserved water W1. To increase the inhibitory potency, an electron-withdrawing substituent should be simultaneously present at the position 8, to increases the 7-hydroxyl acidity, as confirmed by the *in silico* prediction that to achieve a sub-micromolar activity is necessary that the pK_a of the 7-hydroxyl group be lower than 7. The presence of hydrophobic groups at positions 3, 4 and 8, such as bromide or methyl, improve the activity through van der Waals interactions with the CK2 active site.

A quantitative estimate of the free energy of binding was possible using an LIE method that allowed evaluation of the principal contributions to the inhibitors activity. Likewise in the LIE analysis of the tetrabromobenzimidazole derivatives, apolar interactions give the major contribution to the binding energy. In this case, however, the strongest effect results from van der Waals interactions (α=0.029), not from the hydrophobic effect, in agreement with the high steric complementarily between the CK2 active pocket and almost all coumarins studied. The second contribution to the binding comes from the hydro-

phobic effect (γ=0.016), which is the most important factor in discriminating the relative activity of coumarins, as indicated by γ normalized value of 0.854. The electrostatic contribution to the binding energy is modest, as indicated by the low absolute weight of β (–0.003). As seen for other inhibitors which carry a negatively ionisable group (typically an acidic OH), the strong interaction with the conserved water molecule W1 is crucial for the inhibitor orientation but it is not so important for the differences in activity of compounds of the same family.

From the same virtual screening approach that identified coumarins as potential CK2 inhibitors, it also emerged that ellagic acid could be a compound of interest. In the docked position, it lies in a plane similar to that of other polyaromatic inhibitors, but with the important difference that the more extended ring system allows interaction with both the hinge region and the positive area near the salt bridge Lys68-Asp81. Ellagic acid shows good steric and chemical complementarities with the ATP binding site and the right balance of polar and hydrophobic interactions, and most likely these are the reasons for its high potency (K_i = 20 nM) [115]. In addition, ellagic acid also displays a fairly narrow selectivity, is cell permeable and displays cytotoxic effect in ALCL cell lines. The possibility of the presence of the two anchor points seems confirmed by a preliminary low-resolution crystal structure of maize CK2α in complex with ellagic acid [unpublished observations]. A crystal structure of human CK2α in complex with ellagic acid at 2.35 Å has been deposited in the PDB (access code 2ZJW) but is still not publicly available because it is on ‘hold until publication’.

Pyrazolo-triazines

The most recent family of inhibitors whose structures in complex with CK2 are available is based on the pyrazolo-triazine (pyrazolo[1,5-a][1,3,5]triazine) scaffold [116, 117]. These derivatives were developed by Polaris Pharmaceuticals Inc. and are the most potent CK2 inhibitors known up to now, with a K_i as low as 0.095 nM for the best one. In their medicinal chemistry project, they used the structure of human CK2α for the initial screening of a pre-selected library and the X-ray crystal structures of the inhibitors in complex with maize CK2α for the subsequent structure-based drug design optimization process (for a total of seven determined crystal structures). Unfortunately, while the potency achieved by these compounds is quite remarkable, selectivity data are not publicly available at present.

The first inhibitor crystallized in complex with maize CK2α was the N2,N4-diphenyl-pyrazolo[1,5-a][1,3,5]triazine-2,4-diamine compound 1, with a K_i of 0.26 μM. The pyrazolo-triazine core occupies the adenine region and interacts with the backbone of Val116 in the hinge region via two hydrogen bonds. An unfavourable intra-molecular stacking interaction was noted between the two phenyl groups, which adopt a folded conformation and point towards the entrance of the cavity. In addition, there are specific regions of the ATP-binding site that are not touched by compound 1, in particular the hydrophobic region I. Starting from the structure of this complex, several other inhibitors were designed, synthesized, tested and crystallized. The optimization process allows the identification of compound 9e which, with respect to compound 1, carries a nitrile group at the C8 position and an acetamide on the C2-phenyl ring. 9e is one of the most potent CK2 inhibitors ever reported, with a K_i of 0.35 nM. The crystal structure of 9e in complex with maize CK2α revealed that the C2 phenyl group adopts an extended conformation and occupies a back pocket, making hydrophobic interactions with Val53 and Ile174. The intra-molecular stacking seen for compound 1 is avoided because of the key presence of the new acetamide group, which adopts a *cis*-conformation, forming three strong hydrogen bonds with Asp175, Lys68 and a buried water molecule connected to the conserved water W1. It is relevant to note that this high potency is attained also because of the capability of this compound to interact with both the hinge region and the positive electrostatic area near Lys68. The importance of the simultaneous targeting of both these two key regions was anticipated in the concluding remarks of a structural study of CK2 anthraquinone-related inhibitors [91]. SAR analysis on a series of derivatives of 9e revealed that the phenyl group at the C4 position can be substituted by a smaller cyclopropyl group without loss of potency, while substitution of the CN function in position 8 invariably results in a significant decrease in inhibitory potency.

Despite the high potency, in an MTT (tetrazolium salt) cell-based assay, compound 9e showed only relatively weak inhibition (0.99 μM) against the HCT116 colon cancer cell line, 1000-fold higher than the enzymatic potency. This is probably because of the low aqueous solubility and poor cell membrane permeability due to the near planar molecular structure of the compound, as observed in the X-ray crystal structure. In an attempt to obtain druglike pre-clinical candidates for *in vivo* animal studies, a series of macrocyclic pyrazolo-triazines were analysed and the crystal structure of one of them, compound 10 (Fig. 2), in complex with maize CK2α, was determined (Fig. 6). The alkyl linker introduced between the methyl group of the *cis*-acetamide and the nitrogen atom of the nitrile group (at a distance of about 4 Å in compound 9e) fits into

the hydrophobic region I, making important hydrophobic interactions with the protein. The amide group maintains the two hydrogen bonds with Asp175 and Lys68. Even though compound 10 is 100-fold less potent than compound 9e in terms of CK2 inhibition ($K_i = 24$ nM), MTT assay on human prostate and colon cancer cell lines demonstrated that it was about 10-fold more potent than compound 9e as cytotoxic agent. The significantly improved cellular activity was attributed to enhanced membrane permeability due to the overall less planar structure of the inhibitor, induced by the macrocyclic lactam ring system. Compounds of this class with potent CK2 and cancer cell inhibitory activity are currently being evaluated in animal xenograft models for their anti-tumor activities *in vivo*.

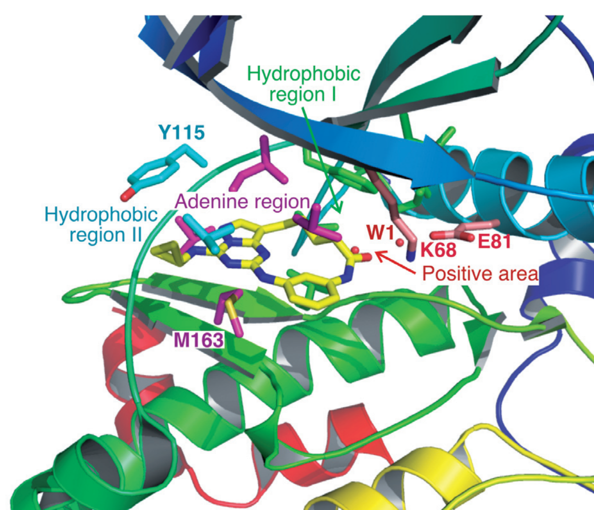

Figure 6. The pyrazolo-triazine compound 10 (yellow carbon atoms) bound to the active site of CK2α. It can interact with all three hydrophobic areas of the binding site (colour-coded as in Figure 1) and makes polar interactions with both the hinge region and the positive area, near the conserved water molecule W1 and the salt bridge Lys68-Glu81.

General principles of CK2 ATP-competitive inhibition

Role of the positive region

From the analysis of the known maize and human CK2α co-crystal structures, it was noted that if a negatively charged moiety is present in a ligand (inhibitor or co-substrate), it tends to cluster in a well-specified zone of the ATP-binding cleft, near the salt bridge Lys68-Glu81. As seen above, this holds for the acidic hydroxyl groups of MNA and MNX as well as for the carboxylic function of IQA, the acidic triazole ring of TBB, the chloride anions present in the DMAT and DRB complexes and for an oxygen of the α-phosphate in ATP and GTP [103]. A quantitative analysis of the electrostatic potential in the CK2α active site revealed the presence of a positively charged region located in the deeply buried area of the cavity, between the hydrophobic region I and the salt bridge formed by the fully conserved Lys68 and Asp81 (Fig. 1), with a mean positive electrostatic potential of 1.5–2.0 kcal/mol. As seen by the systematic analysis of the binding of different classes of CK2 inhibitors, the electrostatic interaction with this area is responsible for the different orientation of the ligands in the active site of CK2. A striking example of this effect is that seen for the different binding modes of the two closely related tetrabromobenzo derivatives TBB and TBI. TBB, with a pK_a~5, binds with the triazole ring inside the positive area, while TBI, with a pK_a~9, is shifted towards the hinge region and forms two halogen bonds with Glu114 and Val116 (Fig. 5), like all the other tetrabromobenzo-imidazole derivatives analysed so far.

Conserved water molecules

In the apo form of CK2α, the positive electrostatic area described in the previous section is occupied by three water molecules. The one in the deepest part of the cavity, called water molecule 1 (W1), is highly conserved in all the known human and maize CK2α crystal structures [103]. It makes hydrogen bonds with the amidic NH of Trp176, with a carboxylic oxygen of Glu81 and with another water molecule (W2) that is present in many structures (Fig. 1). When W2 is absent, its position is invariably occupied by a portion of a ligand, as in the case of MNA, MNX, emodin, IQA and benzamidine, and this suggests that it is directly expelled by the ligand itself, and that this water should be considered a sort of competitor for that position. The third water of the positive area of apoCK2α, W3, is present in only two other structures, namely in the complexes with TBI and K22; in the complexes with DMAT and DRB a chloride ion was found in that position. In the other cases, W3 is usually replaced by atoms of the bound ligand and, most important, by functional groups that can carry a negative charge. In other words, ligands carrying an acidic function have a propensity to cluster in a position corresponding to that of waters W2 and W3, in the region with the positive electrostatic potential at about 3.5 Å from Lys68. Ligands without acidic functions prefer to interact with the hinge region, in particular with the backbone carbonyls of Glu114 and Val116. The scaffold of the macrocyclic pyrazolo-triazines is so extended that it occupies almost entirely the CK2 binding pocket; in this case (see compound 10), W3 is substituted by the lactam carbonyl function that anchors the compound to the positive electrostatic area (Fig. 6).

Role of apolar interactions and small dimension of the CK2α ATP-binding site in binding and selectivity

For many inhibitors described here, the main energetic contribution to the binding appears to be due to apolar forces, namely hydrophobic interactions and van der Waals contacts, involving the hydrophobic surface of the CK2 binding cleft formed by residues Leu85, Val95, Leu111, Phe113, and Ile174 (hydrophobic region I), Val53, Ile66, Val116 and Met163 (adenine region), and Val45 and Tyr115 (hydrophobic region II) (Fig. 1). In particular, for the tetrabromobenzo derivatives, a linear correlation between the log(K_i) and the variation in the accessible surface area (ΔASA) upon binding was identified, indicating that the apolar interactions are ultimately responsible for their rank in potency, as confirmed by a LIE model [103, 107]. Furthermore, the structure-activity analysis of more than 60 different coumarins and the derived LIE model showed that apolar interactions give the largest contribution to the free energy of binding also for this class of compounds [114]. For the pyrazolo-triazine derivatives, the SAR analysis confirmed the important role played by the apolar interactions, involving the extended hydrophobic portions of the inhibitors with hydrophobic region I (alkyl linker), with adenine region (pyrazolo-triazine ring system) and hydrophobic region II (cyclopropyl group) (Fig. 6).

From the analysis of the active sites of different kinases it turned out that the one of CK2α is smaller in size, due to some bulky side chains which reduce the space available to cofactors and inhibitors. The most important of these residues are Ile66 (maize) or Val66 (human) and Ile174 (Fig. 1), which in many protein kinases are replaced with less bulky amino acids, namely alanine versus Ile/Val66, alanine, threonine or leucine versus Ile174. For instance, the active site of CDK2 (belonging to the CMGC group of protein kinases, the nearest to CK2 in the phylogenetic tree of the human kinome) is larger than that of CK2 for the presence of Ala31 instead of Ile66 and Ala144 instead of Ile174. As a consequence, TBB binds in different ways to the two proteins and shows a remarkable selectivity for CK2 [118]. In tetrabromobenzo derivatives the bulkiness of the four halogen atoms appears to be essential for the potency and selectivity of the inhibitors. Inhibition data on maize CK2α mutants confirmed the importance of Ile66 and Ile174; for the single mutants Ile174Ala or Val66Ala and for the double mutant Ile174Ala/Val66Ala, the TBB IC_{50} increases from 0.50 to 1.74, 13.0 and 12.5 μM, respectively [64]. The smaller size of the CK2α active site can also account for the unusually modest sensitivity to the large molecular size promiscuous protein kinase inhibitor staurosporine [89].

Another evidence of the peculiar properties of the CK2α-binding site comes from the observation that PKA is poorly inhibited by emodin, with a 2 orders of magnitude higher K_i. Residues Val66, Ile174 and Phe113 in CK2α are replaced by Ala70, Thr183 and Met120 in PKA, with the latter two closer in space (only 3.80 Å apart). A comparison of the 3D structures indicated that emodin cannot interact in the same way with the two proteins, unless a large rearrangement of residues Thr183 and Met12 of PKA takes place.

CK2 inhibitors often target also PIM kinases with same efficacies, with the remarkable exception of quinalizarin and the tertrabromobenzo derivative K66. The docking of these compounds into the active site of PIM1 (whose 3D structure is known) seems to indicate that the different orientations of a corresponding residue in the p-loop could contribute to the higher inhibitory activity against CK2 [93, and unpublished observations]. In CK2α, Tyr50 is pointing towards the bulk solvent, while in PIM1, the structurally equivalent Phe49 is oriented towards the interior of the active site.

Non-ATP-competitive inhibitors of CK2

As seen before, a second molecule of DRB was found bound to an external hydrophobic binding site ('secondary binding site') near the β4-β5 loop, showing a non-competitive inhibitory effect on CK2 activity (Figs. 7, 8). It is interesting to note that an opposite effect was observed in the case of various peptides reproducing the C-terminal end of the β subunit, encompassing the region that physically interacts with the α subunit in the crystal structure of the tetrameric holoenzyme. These peptides showed a stimulatory effect on the phosphorylation of peptide substrates [119, 120]. In particular, a 23-mer peptide corresponding to the C-terminal sequence 181–203 of the human CK2 regulatory β subunit is able to stimulate the activity of CK2α against a calmodulin-derived peptide, even if to a lesser extend compared to the full-length β subunit. The occurrence of strong interactions between CK2α and the β peptide (181–203) was confirmed by plasmon resonance experiments. The crystal structure of the complex with maize CK2α revealed that the peptide binds in the same hydrophobic pocket of the second DRB molecule, in the secondary binding site (Figs. 7, 8), interacting with the protein through several polar and apolar interactions [121].

More recently, a cyclized 11-mer peptide containing the sequence Arg186-His193 of CK2β, designed on the basis of the structure of the CK2 tetramer, was shown to inhibit the assembly of the holoenzyme (as was previously seen also for the CK2β linear peptide (170–215, [119]) and to affect its substrate preference

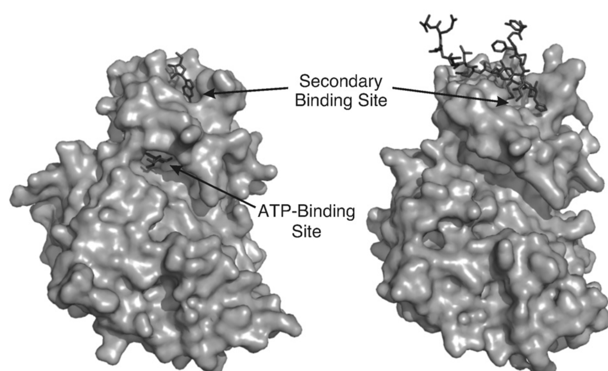

Figure 7. ATP-binding and secondary-binding sites of CK2α. Left panel, DRB occupies both these sites; right panel, β peptide (181–203) bound to the secondary binding site, which is also involved in the α-β subunit interactions in the tetrameric holoenzyme. See also Figure 8.

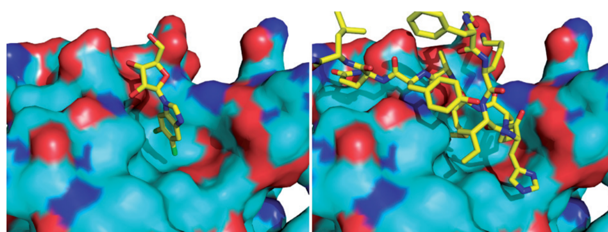

Figure 8. Close-up view of the secondary binding site occupied by DRB (left panel) or by the β peptide (181–203) (right panel). The protein surface is coloured according to the following scheme: carbon atoms in cyan, oxygen atoms in red, nitrogen atoms in blue. See also Figure 7.

[122]. Presumably, this peptide binds in the secondary binding site as the linear β peptide [181–203] and DRB (whose co-crystal structures as available), but the exact mode of binding is not known, nor whether it has a stimulatory or inhibitory activity on CK2α.

In this respect, another interesting peptide is the one derived from the cystic fibrosis transmembrane conductance regulator protein CFTR, corresponding to the 500–518 sequence of the NBD1 domain. Despite this peptide encompassing a region displaying the consensus for CK2 phosphorylation, and hence the possibility that it could behave as a substrate-competitive inhibitor, it was shown to inhibit the CK2α activity via a purely non-competitive mechanism. The inhibitory effect of the peptide is only detectable with the isolated catalytic subunit, not with the heterotetrameric CK2 holoenzyme [123]. Collectively taken, these data suggest that the CFTR peptide binds to an allosteric site of CK2α that is no longer accessible in the tetrameric holoenzyme, as is the case of the secondary binding site.

Very recently, another non-ATP-competitive inhibitor was identified, the podophyllotoxine indolo derivative W16, which was assumed to represent an allosteric inhibitor [124]. It is also able to selectively disrupt the CK2 α/β assembly, and hence it was hypothesised that it binds to the same secondary binding site.

Polyoxometalates are another family of interesting non-classical inhibitory compounds. They are inorganic inhibitors with inhibitory activity in the low nanomolar range (IC_{50}<10 nM for the best ones) that target CK2α neither in the ATP-binding nor in the protein substrate-binding sites, acting as potentially allosteric compounds [125]. They showed a promising selectivity on a panel of 29 protein kinases.

To date, there are no clear structural explanations for the hypothesized allosteric mechanism of these non-ATP-competitive CK2α inhibitors. Four structures are available with different molecules in the secondary binding site, those of human CK2α in complex with DRB, glycerol [112] and the full β subunit (whose binding can have both stimulatory and inhibitory effects), and that of maize CK2α with the β peptide (181–203). None of these shows remarkable conformational changes in the active site that can be clearly correlated to inhibition of the catalytic activity of isolated CK2α. In the case of the β peptide (181–203), the stimulatory effect was tentatively ascribed to the induced oligomerization of CK2α that causes two binding sites to interact to each other.

From this analysis, it is clear that more experimental data are needed to clarify the nature and the structural bases of the hypothesised allostery of the secondary binding site and the reasons why the binding to this site can have both stimulatory and inhibitory effects. This is a quite important issue to address because of the potential relevance of the availability of non-ATP-competitive kinase inhibitors. Furthermore, compounds targeting the secondary binding site should also be able to inhibit CK2 subunit regular assembly and would be valuable tools for the study of the *in vivo* role of the β subunit, especially in the case of those substrates whose phosphorylation is entirely dependant on the β subunit (class III, see [26]).

Inhibitors in clinical trials

Other important tools for studying CK2 function would be compounds able to selectively inhibit the phosphorylation activity of the enzyme by interfering with the binding of protein substrates, i. e. substrate-competitive inhibitors. In this respect, up to now there is only one peptide that has been reported to inhibit CK2 *in vivo* acting as a protein substrate competitor. This CIGB-300 peptide (formerly P15-Tat) was identified from the screening of a random cyclic peptide phage library using a synthetic CK2 phosphoacceptor site of the HPV-16 E7 oncoprotein, and fused to the cell-penetrating peptide derived from the HIV-Tat

protein. This inhibitor exhibited proapoptotic activity and anticancer properties *in vitro*, in tumor animal models and in patients with cervical malignancies [126–128]. Whether the anti-cancer potential of this peptide is mediated by CK2 inhibition or not remains an open question.

Very recently (Investigational New Drug Application submitted in October 2008), Cylene Pharmaceuticals announced that it has initiated a Phase I clinical trial of an orally administered CK2 protein kinase inhibitor, CX-4945, in patients with advanced solid tumors, Castleman's disease or multiple myeloma. CX-4945 is a small molecule reported to inhibit CK2 with IC_{50}=2 nM. Tested against a panel of over 145 protein kinases, it was also able to inhibit DAPK3 (IC_{50}=17 nM), FLT3 (IC_{50}=35 nM), HIPK3 (IC_{50}=45 nM), PIM1 (IC_{50}=46 nM), CDK1/cyclin B (IC_{50}=56 nM) and DYRK2 (IC_{50}=91 nM). While CX-4945 was found to inhibit CDK1 in the molecular screen, it did not inhibit CDK1 activity in the cell. In preclinical studies, it was able to promote tumor regression as a single agent, with broad-spectrum anti-proliferative activity against diverse cancer cell lines (www.lifesciencesworld.com; www.cylenepharma.com).

Concluding remarks

Structural studies of different families of CK2 inhibitors, in particular the tetrabromobenzo derivatives, the anthraquinones and the coumarins, have revealed the main characteristics an inhibitor must hold in order to efficiently target the CK2α active site: appropriate hydrophobicity, excellent shape complementarity with the quite unique and small active site of CK2, and the ability to establish polar interactions with both the two main anchoring points identified in the active site, the hinge region and the positive electrostatic area near the conserved water W1 and the Lys68-Glu81 salt bridge. Indeed, the most potent pyrazolo-triazine derivatives, the best CK2α inhibitors known so far, with a K_i as low as 0.1 nM, carry all these features. Unfortunately, nothing is publicly known about their selectivity, and hence it is not possible to fully estimate their real anti-cancer prospective. Anyway, it must be noted that the practical selectivity of a compound depends on its potency: more potent compounds are more selective because they can be used at a lower dose. This is also true within the protein-kinase family, in which there is a strong correlation between inhibitory potency and selectivity [129]. An important corollary is that there is a minimum threshold of potency without which a molecule cannot be selective, irrespective of any *in vitro* data.

The ATP-binding sites of protein kinases are made by both polar and apolar elements. While the properties of the polar areas are quite conserved among the family, due to the conservation of key residues involved in the catalytic event, the hydrophobic ones have a higher degree of variability. Therefore, while the presence of polar interactions (hydrogen and halogen bonds, salt bridges) increases the inhibitory potency, selectivity is ensured mainly by targeting the hydrophobic portions of the active site. The pyrazolo-triazine compound 10 has a high hydrophobicity and is able to interact simultaneously with all the hydrophobic regions of the CK2-binding site, the adenine region and the hydrophobic regions I and II (Fig. 6). This could really favour high selectivity for the compound. Quinalizarin is a quite small and 'simple' organic compound, an anthraquinone derivative without any torsional degree of freedom, which is clearly an entropic advantage for the binding energy. Quinalizarin is sufficiently hydrophobic and benefits from the strong hydrophobic nature of the CK2 ATP-binding pocket (and the consequent desolvation effect in ligand binding) and from its small dimension. This is at the basis of its quite remarkable selectivity, with a promiscuity score of 11.1. At the same time, it is able to establish a reasonable (for its small size) number of polar interactions via the hydroxyl groups placed in critical positions.

Most of the CK2 inhibitors described here show good cell permeability, an important property for a 'drug-like' compound. Another common positive feature of these inhibitors, excluding the pyrazolo-triazine family, is that they are entropically very constrained, with few or none freely rotatable torsional bonds (Fig. 2), and this makes an important contribution to the free energy of binding.

Efficient lead optimization for potency, selectivity, efficacy and biopharmaceutical properties to generate a useful kinase inhibitor with druglike properties is a demanding task. A great amount of focused, target-driven chemistry is required to find a single potent compound whose selectivity, then, must be extensively characterized *in vitro* and *in vivo*. The scale of this task is such that the best compounds today are developed mainly by the pharmaceutical industry, and the pyrazolo-triazine inhibitors and compound CX-4945 are examples of that.

As underscored above and confirmed by the different degree of selectivity of some CK2 inhibitors, such as DMAT and TBI, when tested in panels of different size, selectivity data based on small panels must be considered with caution. In this respect, to validate CK2-dependent phenotypes, the use of CK2α mutants resistant to individual inhibitors in rescue experiments (proposed by Sarno et al. [64]) seems really useful and

perhaps necessary, as indicated by some recent work where this approach was successfully utilized [58, 62, 96].

Nowadays, much is known about the structural basis of the inhibition of protein kinase CK2, but probably much more remains to be discovered. Some crucial issues that should be addressed are the following: How can the selectivity of the ATP-competitive inhibitors be improved? How dissimilar (or similar) are the human and the maize enzymes really, with particular reference to their plasticity? Are these differences relevant for the optimization of inhibitors? What are the nature and structural properties of the hypothesized allosteric site (or sites)? Can this (or these) be successfully targeted by non-ATP-competitive inhibitors? Is the development and exploitation of specific peptide-based substrate-competitive inhibitors really feasible? How does the β subunit contribute to the regulation of CK2 activity, and how can it be targeted for inhibition?

Even if different types of approaches can contribute to the development of new drugs, pharmacological inhibition still plays an important role because it remains the fundamental mode of intervention. No disease can be treated with a mutation (yet), and no genetic experiment can reliably predict the outcome of targeting a pathway with a small molecule [5]. For this reason, it is crucial to understand how potent and selective kinase inhibitors function in physiologically relevant model systems, even if the specific molecules themselves are not destined to be drugs, as is the case of most of the inhibitors described here. However, they do represent a valuable tool in systematically scrutinizing signalling pathways involving protein kinase CK2.

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