Inhibition of Protein Kinase CK2 by Anthraquinone-related Compounds

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Erika De Moliner‡§, Stefano Moro®, Stefania Sarno‡§, Giuseppe Zagotto‡, Giuseppe Zanotti‡§, Lorenzo A. Pinna¶**, and Roberto Battistutta‡§***
From the ‡Department of Organic Chemistry, and §Department of Pharmaceutical Sciences, University of Padova, Padova 35131, Italy, and ¶Department of Biological Chemistry, University of Padova, Padova 35121, Italy and **Venetian Institute for Molecular Medicine, Padova 35131, Italy

Protein kinases play key roles in signal transduction and therefore are among the most attractive targets for drug design. The pharmacological aptitude of protein kinase inhibitors is highlighted by the observation that various diseases with special reference to cancer are because of the abnormal expression/activity of individual kinases. The resolution of the three-dimensional structure of the target kinase in complex with inhibitors is often the starting point for the rational design of this kind of drugs, some of which are already in advanced clinical trial or even in clinical practice. Here we present and discuss three new crystal structures of ATP site-directed inhibitors in complex with “casein kinase-2” (CK2), a constitutively active protein kinase implicated in a variety of cellular functions and misfunctions. With the help of theoretical calculations, we disclose some key features underlying the inhibitory efficiency of anthraquinone derivatives, outlining three different binding modes into the active site. In particular, we show that a nitro group in a hydroxyanthraquinone scaffold decreases the inhibitory constants Ki because of electron-withdrawing and resonance effects that enhance the polarization of hydroxylic substituents in paraposition.

The crucial role of protein kinases in cell signaling, gene expression, and metabolic regulation is highlighted by the fact that nowadays this family of enzymes is the second most important drug target (1). Actually, abnormal activity of individual protein kinases is often associated with human diseases, especially tumors whose treatment has been so far restricted to cytotoxic and hormonal agents (2). Many kinase inhibitors are currently in clinical trials, mostly as anti-tumor drugs (1, 3), and two of them, Gleevec (STI-571) and rapamycin, are in clinical use for the treatment of a form of leukemia and to prevent tissue rejection after organ transplantation, respectively. One major problem with kinase inhibitors is that the human genome encodes for >500 different protein kinases; therefore, inhibitors designed to target specifically an individual kinase are likely to bind to closely related kinases as well, thus interfering with other cell functions. In addition, the most promising inhibitors are directed to the highly conserved ATP binding site with the consequence that their selectivity is hardly absolute, and they have to compete against high intracellular concentrations of ATP. In this respect, peptide inhibitors directed to the phosphoacceptor substrate binding site may in principle display higher specificity. Their pharmacological utilization, however, is hampered by a number of practical drawbacks, primarily reduced bioavailability.

Many of the chemical scaffolds or building blocks studied as ATP site-directed kinase inhibitors are based on more or less complex heterocyclic molecules (mainly with nitrogen and oxygen as heteroatoms). The most common scaffolds are derivatives of the following: quinazolines; phenylamino-pyrimidines, pyrido-pyrimidines, pyrrolo-pyrimidines, pyrimido-pyrimidines, or pyrazolo-pyrimidines; pyrrolo-pyrimidines; indolin-2-ones; purines; pyridinyl-imidazoles or pyrimidinyl-imidazoles; and phthalazines. Other examples are natural products such as halanol and alkaloids, flavopiridol (belonging to the flavonoid family), and staurosporine and its derivatives (4).

Besides the traditional medicinal chemistry and the relatively new combinatorial approaches (with the employment of high throughput screenings on molecules libraries), the solution of the crystal structure of complexes between an individual kinase and its inhibitors also represents a powerful tool for the discovery of new drugs by a rational drug design approach. In fact, the structural bases for selectivity and potency are now being clarified by means of crystallization of a number of such targets in complex with inhibitors (5). A telling example is that of Cdk2 whose crystal structures in complex with a number of ligands have been exploited to design more potent and selective inhibitors (6–8). It is now a common exercise to run a virtual screen of thousands low molecular weight compounds on the crystal structure of a kinase in complex with an inhibitor with the aim to identify the most promising chemical scaffolds to develop (9). The progress made in the crystallization of protein kinases has corroborated the concept that the ATP-binding domain is an attractive target for drug design. Three successful examples of drug design using a tyrosine kinase as a molecular target are the following: 1) PKI166, a pyrrolo[2,3-d]pyrimidine derivative that inhibits both epidermal growth factor receptor...
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and ErbB2 kinases, 2) the anilino-phthalazine derivative PTK787/ZK222584, a potent and selective inhibitor of the kinase domain receptor and Fli-1 kinases, and 3) the aforementioned STI-571 (10).

CK2 (an acronym derived from the misnomer “casein kinase-2”) is one of the most pleiotropic protein kinases with hundreds of protein substrates involved in a variety of cellular functions with special reference to signaling, nuclear organization, and gene expression (11). An intriguing hallmark of CK2 is the high constitutive activity, which is believed to underlie its pathogenic potential (12). Although there are no known mutations of CK2 associated with neoplasia, CK2 is abnormally elevated in a wide variety of tumors and there are several experimental models where the unscheduled expression of the catalytic subunits of CK2 cooperates with the altered expression of proto-oncogene or tumor suppressors to promote cell transformation and neoplastic growth (13–16). Because of its constitutive activity, CK2 is also exploited by many viruses to phosphorylate proteins essential to their life cycle. This has triggered an increasing interest for CK2 inhibitors that could act as anti-neoplastic and anti-infectious drugs. Although CK2 is essential to viability, it is conceivable that, as pointed out in the case of the MAPK cascade (1), its essential roles in proliferation and differentiation are required only at individual developmental stages, a circumstance that would make applicable a transient pharmacological treatment with CK2 inhibitors.

Our interest has been recently focused on CK2 inhibitors belonging to the anthraquinone (17) and xanthenone families. Anthraquinones have been used for the purification of proteins by affinity techniques taking advantage of their nucleotide-specific ligand capability (18). This enables them to interact with ATP, ADP, and NAD binding sites of enzymes such as dehydrogenases, kinases, and ATPases. Anthraquinone and xanthenone derivatives often obtained from natural sources have several potential therapeutic applications for instance as antiviral, antimicrobial, and anti-cancer drugs (19). A potential drawback of these compounds is that their cyclic planar structure confers them the feature of DNA-intercalators with expectable cytotoxic effects. Even with this limit, the optimization of highly specific and selective inhibitors of this category could be exploited for the elucidation of the still somewhat enigmatic cellular functions of CK2. Another important benefit of the improvement of the inhibition potency and selectivity of anthraquinone and xanthenone derivatives is the rationalization of the effect of different substrates on a common scaffold in order to draw information regarding their effects on the interaction energy involved in target binding. This sort of information is useful for the optimization of the force fields used in drug design and docking studies.

Here we present the crystal structure of three different complexes of maize CK2a (70% identical to its human homologue and almost 100% conserved in the catalytic core) with two anthraquinone derivatives and one related xanthenone. The three inhibitors studied whose chemical formulae and $K_i$ values with CK2 are shown in Fig. 1 are: 1,8-dihydroxy-4-nitro-anthraquinone (MNA), 1,8-dihydroxy-4-nitro-xanthen-9-one (MNX), and 1,4-diamino-5,8-dihydroxy-anthraquinone (DAA). These compounds were selected from a panel of many anthraquinones and xanthenones because of their relatively low $K_i$ values and ability to originate diffracting quality crystals in co-crystallization trials. A relevant issue we also wanted to address was the rationalization of the higher inhibitory efficiency of these molecules as compared with another inhibitor of the anthraquinone family, emodin (also shown in Fig. 1), whose crystal structure in complex with CK2 has previously been solved (17). Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) is extracted from the medicinal herb Rheum palmatum and has been used for a long time in the Orient to cure inflammatory and neoplastic diseases. Our studies will provide hints on how to improve the efficiency and selectivity of emodin-related compounds toward CK2 and possibly other protein kinases.

EXPERIMENTAL PROCEDURES

Crystals Preparation and Data Collection—The catalytic α subunit of Zea mays CK2 was expressed in Escherichia coli and purified according to a method described previously (20). The crystals of the three inhibitor–enzyme complexes with MNA, MNX, and DAA were obtained by co-crystallization with the sitting drop vapor-diffusion technique. The synthesis and characterization of the three inhibitors are described elsewhere.2 The 8-mg/ml protein stock solution was preincubated with 100 mM inhibitor solution (100% Me2SO) in the proper amount to have a transient pharmacological treatment with CK2 inhibitors.



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the β angle are different for MNA crystal with respect to MNX and DAA (see below), which implies a different Matthews coefficient: Vₐₜ is 2.08 Å³ Da⁻¹ for MNX and DAA against a Vₐₜ of 2.40 Å³ Da⁻¹ for MNA. In addition, the solvent content is different: 41% in MNX and DAA compared to 30% as precipitating agent or glycerol.

Structure Determination and Refinement—Data were indexed with MOSFLM (22) and then scaled with SCALA from the CCP4 software package (23). To solve the three new structures, a rigid body transformation was made on the basis of the H-bonds length (longer for an NH acceptor or the hydroxyl or amino groups of the inhibitor H-bonded to the protein) and the hydroxyl or amino groups of the inhibitor H-bonded to the protein was made on the basis of the H-bonds length (longer for an NH acceptor than for an OH one) and on the results of DAA binding simulations (see below).

The definition files for the inhibitors were initially created by Hic-Up (25) corrected with the adequate parameters and used in CNS in the whole refinement procedure that was carried out alternating automated cycles and manual inspection steps using the graphic program QUANTA (26). During the final steps of the refinement, water molecules were added and the stereochemistry was checked with the program Procheck (27). Statistics on data collection and final models are reported in Table I. The final model for the complex with the MNA inhibitor presents an overall crystallographic R-factor of 22.2 (R_m, 24.3) with 177 water molecules and a good stereochemistry with no residues in disallowed regions of the Ramachandran plot. For the complexes with MNX and DAA, the R-factors are 19.8 (R_m, 23.3) and 19.0 (R_m, 21.6), respectively, with 161 and 205 final water molecules and no residues with disallowed stereochemistry.

Computational Methodologies—Calculations were performed on a Silicon Graphics Octane R12000 work station. The ground state geometry of charged and uncharged docked structures was fully optimized using Spartan O2 (version 6.0) software (40). MOE-Dock performs a user-specified number of independent docking runs (50 in our case) and writes the resulting conformations and their energies to a molecular data base file. The resulting docked complexes were subjected to a MMFF94 energy minimization protocol until the root mean square of the conjugate gradient was < 0.1 kcal mol⁻¹ Å⁻¹. The charges for the ligands were imported from the Spartan output files. To model the solvent effects more directly, corrections for the electrostatic interactions were used. The MOE suite utilized includes an implemented version of generalized born/surface area contact function (30) that models the electrostatic contribution to the free energy of solvation in a continuum solvent model. The interaction energy values were calculated as the energy of the complex minus the energy of the ligand minus the energy of protein: ΔE_inter = E(complex) - (E(L) + E(protein)). Apparent pKₐ values of all anthraquinone and xanthone derivatives were theoretically calculated by using ACD/pKa DB (version 6.0) software (40).

Coordinates—Coordinates have been deposited in the Protein Data Bank with the following accession codes: 1M2P (for MNA-CK2 complex); 1M2Q (for MNX-CK2 complex); and 1M2R (for DAA-CK2 complex).

RESULTS AND DISCUSSION

The optimization of a co-crystallization protocol for inhibitors MNA, MNX, and DAA allowed data collections with a maximal resolution higher than that obtained by soaking methods used in the case of emodin and TBB complexes (2.63 and 2.19 Å, respectively) (17, 20). The new protocol consists of a preincubation for 1–2 h of the protein with the inhibitor dissolved in Me₂SO (final Me₂SO concentration ≤ 5%) and then the set up of the crystal trials as described under “Experimental Procedures.” With this new procedure, we could collect data at 2.0-Å resolution for MNA, 1.79-Å resolution for MNX, and 1.70-Å resolution for DAA. It may be interesting to note that the CK2 complexes crystallized so far are not perfectly isomorphous. As outlined in Table I, although the differences in the a and c axis lengths can be considered within the experimental errors, axis b varies from 59.5 ± 1 Å (with ATP or MNA bound and in the apo-form) to 52.2 ± 0.5 Å (in the case of emodin, MNX, and DAA). This change is coupled with an adjustment in the β angle from 103.0° and a decrease of the R-factor to 19.0 (with ATP or MNA bound and in the apo-form) to 18.5 (2.4 Å) and a decrease of the solvent content from 49 to 41%. From the analysis of the final three-dimensional structures, we have noted that these variations reflect two different conformations of the protein loop between β strand 4 and 5 (residues 102–108) (Fig. 2). This segment has a bent conformation with the short b axis and an extended one with the stretched b axis. This is the only evident structural difference between the two diverse groups of cell parameters, and it involves a shrink in the crystal packing roughly along the b axis. One possible explanation is that high PEG concentrations (≥ 30%) as precipitating agent or glycerol as cryoprotectant are responsible of the cell shrinkage. A direct implication of the different inhibitors located at 20 Å far away from the hydrophobic regions of the enzyme is the lability of the inhibitor enzymes, as shown in the crystallographic phases of the MNA-CK2 complex (18), which are more accurate than those of MNX-CK2 and DAA-CK2 complexes (19).
from the 102–108 loop is hardly conceivable.

As expected by analogy with emodin, the three inhibitors bind in the co-substrate binding cavity of CK2 between the N- and C-terminal lobes in the proximity of the Gly-rich loop (Fig. 2). As already noted (20), the C-terminal domain of the kinase is quite rigid, whereas the N-terminal one is more inclined to alterations induced by the presence of different ligands. A noteworthy exception is helix αC that is conformationally very well conserved in all structures solved until now. Among the three complexes described here, the one with MNX shows the greatest variation in the N-terminal domain with special reference to the positions of the backbone between residues 72 and 75 and of the Gly-rich loop (residues 45–51). The latter collapses into the co-substrate binding cavity, but this movement is not accompanied by the rotation of His-160 and Asn-118 side chains as in the case of the emodin complex (17).

The orientation and precise location of MNA and MNX differ from that of emodin, which enters the active site with hydroxyl groups 1 and 8 on the side of the hinge region. In contrast, both MNA and MNX penetrate with the nitro group oriented toward the hinge region, and consequently, the hydroxyl groups make contacts with Lys-68. The structures of MNA and MNX in the active site are fully superimposable (Fig. 3). Emodin and TBB bind to the protein mainly through hydrophobic and van der Waals interactions. In the case of MNX and MNA, additional polar interactions between the inhibitors and the active site of the enzyme contribute both to increase the affinity as indicated by the lower $K_i$ values and to orient the molecules in a different way.

In MNA, the nitro group is not co-planar with the aromatic rings because of the steric hindrance of the adjacent carbonyl (ortho effect). In fact, in our structure, the nitro group is roughly perpendicular to the ring plane. The two hydroxyl groups and the carbonyl of the same side are involved in four hydrogen bonds with the side chains of Asp-175 and Lys-68 and a water molecule (Fig. 4). The latter is also bound to Glu-81 and Trp-176 as in the other CK2 complexes presented here. The nitro group is preferentially oriented toward the opening of the binding pocket (0.67 occupancy), but it is also present in the depth of the cavity (0.33 occupancy); therefore, MNA was refined with a double conformation with final

![Diagram](image-url)

**Fig. 2.** Overview of CK2 three-dimensional folding. The ribbon diagram of CK2 in complex with MNA (in ball-and-stick) bound in the ATP binding site between the N- and the C-terminal lobes is shown (gray). The location of the inhibitor DAA is also shown (ball-and-stick in black). On the top right are shown the two different positions of the loop 102–108 in the case of the MNA complex (gray) and DAA (or MNX) complex (black) that correspond to a long b-cell axis (around 59.5 Å) and a short one (around 52.2 Å), respectively.
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In the case of MNX- and MNA monoanionic derivatives, the neutral and the monoanionic form can fit the CK2 binding cavity whose local pH can be different from that of the external solvent. In addition, the equilibrium between the two conformations of MNX inside the cavity can also be affected by steric contributions because of non-favorable contacts between the hydroxyl group in the inner position and Ile-66 and Val-116.

In the case of DAA where the dissociation constant of hydroxyl groups is not affected by the presence of any nitro function, the binding mode is different (Figs. 3 and 4). DAA binds into the enzyme co-substrate pocket on the side of the hinge region, making two specific H-bonds with backbone carboxyls of Glu-114 and Val-116, which are also responsible for interactions with the adenine moiety of bound ATP (21). This specific binding mode confers to DAA the lowest \( K_a \) value (0.35 \( \mu \)M) among anthraquinone inhibitors tested so far.

In summary, the four anthraquinone derivatives analyzed so far display, despite their common scaffold, three significantly different modes of binding into the active site of CK2: (a) anchoring to the hinge region (DAA); (b) anchoring to Lys-68 and Asp-175 albeit with different orientations (MNA and MNX); and (c) sitting in the middle of the cavity with no strong polar interactions (emodin). These three binding modalities correlate with a gradual increase in the \( K_a \) value from (a) (0.35 \( \mu \)M) to (c) (1.85 \( \mu \)M). Based on this information, a strategy to improve the affinity to CK2 could be to design new molecules able to fill the entire cavity and therefore to interact on both sides, with the hinge region on one hand and Lys-68/Asp-175 on the other. However, a complication arises from the fact that DAA lies on the same plane of the ATP adenosine moiety, i.e. slightly tilted with respect of the other three anthraquinone inhibitors (as shown in Fig. 3). This difficulty could be overcome by replacing the hydroxyl group of the DAA scaffold with more extended flexible chains bearing hydroxylated and/or negatively charged functions.

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ACD/pKa DB software, we theoretically calculated the acid ionization constants of both 1,8-dihydroxy-anthraquinone and 1,8-dihydroxy-xanthen-9-one and of the corresponding 4-nitro-substituted derivatives (Fig. 1). As experimentally demonstrated (42), the first dissociation constant of both 1,8-dihydroxy-anthraquinone and 1,8-dihydroxy-xanthen-9-one is at least three orders of magnitude lower than that of phenol itself (\( pK_a = 10.0 \)). The presence of the nitro group further reduces the first \( pK_a \) value. In the case of the anthraquinone derivative, the \( pK_a \) value is 5.3, whereas for the xanthenone derivative, the value is 4.8 because of the resonance effect that is not possible in anthraquinones. At physiological pH, both nitro derivatives should be present, at least partially, in their monoanionic forms. To better understand the role of the monoanionic species in the CK2 recognition process, a molecular docking study has been performed starting from our CK2 crystallographic coordinates (see “Experimental Procedures”).

In the case of MNX- and MNA-monooanionic derivatives, the energetically most stable and statistically most representative docked conformation is extremely close to the MNX crystallographic structures with the nitro group located only in the inner position and with the negatively charged oxygen at position 1 near to the positive side chain of Lys-68 (see Fig. 5). This result can support the hypothesis that if the monoanionic form is present, it can bind CK2 with high efficiency. In this case, the strong electrostatic interaction between Lys-68 and the monoanionic form seems to play a crucial role in the recognition process. However, considering the neutral form of both MNX and MNA compounds, the modeling procedure sampled two different families of docked conformations: the first one as observed for the monoanionic form with the nitro group located in the inner position and the protonated phenolic oxygen at position 1 close to the positive side chain of Lys-68; and the second one exactly in the opposite configuration with the nitro group located in the external position and the hydroxyl group at position 1 close to the negative side chain of Asp-175. The second one appears to be thermodynamically more stable (\(-9 \) kcal mol\(^{-1}\)) because of the formation of a strong hydrogen bond between the hydroxy group at position 1 of the anthraquinone structure and the negatively charged carboxyl group of Asp-175. This prediction is in good agreement with the crystal structure of the MNA-CK2 complex (see Fig. 5). These data indicate that the neutral and the monoanionic forms bind to the CK2 recognition cavity using a different network of stabilizing interactions, depending on the ionization capabilities of inhibitors. This can generate at least two different configurations inside the binding pocket. Considering the increase of the first \( pK_a \) of MNA due to the ortho effect, we can speculate that MNA is present in solution both as a neutral and monoanionic form and that both can fit the CK2 binding cavity whose local pH can be different from that of the external solvent. In addition, the equilibrium between the two conformations of MNA inside the cavity can also be affected by steric contributions because of non-favorable contacts between the nitro group in the inner location and Ile-66 and Val-116.
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