Journal of
Medicinal
Chemistry

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Volume 49, Number 8 April 20, 2006

Letters

Identification of Ellagic Acid as Potent Inhibitor of Protein Kinase CK2: A Successful Example of a Virtual Screening Application

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Received February 2, 2006

Abstract: Casein kinase 2 (CK2) is a ubiquitous, essential, and highly pleiotropic protein kinase whose abnormally high constitutive activity is suspected to underlie its pathogenic potential in neoplasia and other diseases. Using a virtual screening approach, we have identified the ellagic acid, a naturally occurring tannic acid derivative, as a novel potent CK2 inhibitor. At present, ellagic acid represents the most potent known CK2 inhibitor (K_i = 20 nM).

Casein kinase 2 (CK2) is probably the most pleiotropic protein kinase known, with more than 300 protein substrates already recognized, a feature which might, at least partly, account for its lack of strict control over catalytic activity.[1] Its catalytic subunits (α and/or α′) are in fact constitutively active either with or without the regulatory β-subunits, which appear to play a role in targeting and substrate recruiting, rather than controlling catalytic activity. Although constitutively active CK2 is ubiquitous, essential, and implicated in a wide variety of important cell functions,[2] evidence has been accumulating that its catalytic subunits may behave as oncogenes,[3−6] consistent with the observation that they display an antiapoptotic effect in prostate cancer cell lines.[7] Actually, they are invariably more abundant in tumors as compared to normal tissues and their overexpression is causative of neoplastic growth in animal and cellular models presenting alterations in the expression of cellular oncogenes or tumor suppressor genes.[8] These data, in conjunction with the observation that many viruses exploit CK2 as phosphorylating agent of proteins essential to their life cycle,[1] are raising interest in CK2 as a potential target for antineoplastic and/or antiinfectious drugs.[9]

In the past few years, we have performed an intensive screening program, using both conventional and in silico approaches, with the aim of discovering novel potent and selective CK2 inhibitors.[10,11] In particular, we have recently implemented an in-house molecular database (defined as "MMS-database") in which almost 2000 natural-occurring compounds are collected for specific virtual screening applications. Several families of polyphenolic compounds, including a large class of flavones, flavonols, isoflavones, catechins, anthraquinones, coumarins, and tannic acid derivatives, are represented in our molecular database. Following some recent reports of success in the discovery of new kinase inhibitors by high-throughput docking of large collections of compounds,[12,13] we have performed a virtual screening experiment targeting the ATP binding site of CK2 by browsing the MMS-database. In principle, this strategy could represent a very useful approach to prioritizing compounds for biological screening.[14] In our virtual screening protocol, we have decided to utilize a combination of high-throughput docking protocols in tandem with a *consensus* scoring strategy as recently presented by Miteva and collaborators.[15] In particular, a combination of four docking protocols (MOE-Dock,[16] Glide,[17] Fred,[18] and Gold[19]) and five scoring functions (MOE-Score,[16] GlideScore,[17] GoldScore,[19] ChemScore,[19] and Xscore[20]) has been utilized to appropriately dock and rank all MMS-database candidates. The flowchart of our high-throughput consensus docking is shown in Figure 1 (detailed information about the described virtual screening strategy are described in the Supporting Information). After high-throughput consensus docking, a naturally occurring tannic acid derivative known as ellagic acid, depicted in Figure 2, has been found to sit in the top 5% of the ranked database independently from the nature of the used scoring function.

Considering the unexpected and encouraging virtual screening result, we have prioritized the acquisition and the biochemical characterization of ellagic acid as new potential CK2 inhibitor.

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10.1021/jm060112m CCC: $33.50 © 2006 American Chemical Society
Published on Web 03/16/2006

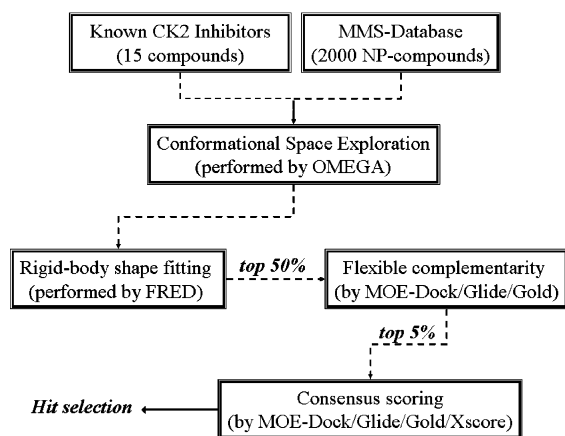

Figure 1. Flowchart of our high-throughput consensus docking.

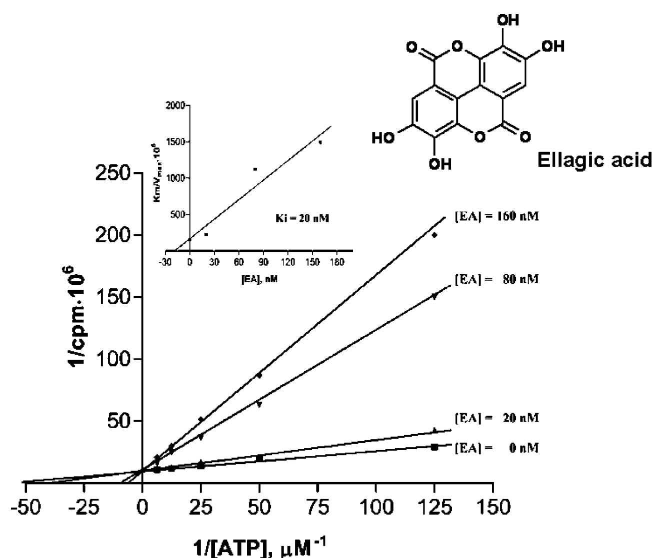

Figure 2. Kinetic analysis of ellagic acid/CK2 complexation consistent with a reversible and competitive mechanism of inhibition. CK2 activity was determined as described in the Experimental Section either in the absence or in the presence of the indicated EA concentrations. The data represent means of triplicate experiments with SEM never exceeding 15%.

As shown in Figure 2, inhibition of CK2 by ellagic acid is competitive with respect to the phosphodonor substrate ATP, and a 20 nM K_i value has been calculated from linear regression analysis of Lineweaver−Burk double reciprocal plots, which is the lowest K_i reported so far of any CK2 inhibitor. Ellagic acid has been previously reported to inhibit the catalytic activity of other kinases such as PKA ($IC_{50} = 2\ \mu M$) or PKC ($IC_{50} = 8\ \mu M$).[21] However, ellagic acid seems to be a much more potent and a quite specific inhibitor of CK2, according to a preliminary selectivity study (Table 1). Further selectivity investigations are ongoing in our laboratories. Moreover, beside ellagic acid, other interesting CK2 inhibitor candidates have been selected from our consensus "docked/scored list", and an intensive validation work is being carried out to determine their activities.

From a molecular point of view, similar to other CK2 inhibitors such as 4,5,6,7-tetrabromo-1-benzotriazole (TBB) and to a number of condensed polyphenolic compounds,[10,11] ellagic acid is ATP-competitive and, as expected, it can occupy the ATP binding region between the N-terminal and the C-terminal lobes of the CK2 enzyme. As shown in Figure 3, our molecular docking investigations have clearly demonstrated that ellagic acid displays a very good steric and chemical complementarity with the ATP binding cavity. Interestingly, the docked conformation shown in Figure 4 has been the best ranked in our high-throughput consensus docking strategy by all docking programs. Specifically, ellagic acid lies essentially in the same plane of all other already known polyaromatic structures but penetrates deeper, reaching the hinge region of the enzyme.[10,11] However, the peculiarity of the ellagic acid binding mode in comparison to those presented by all other CK2 inhibitors is its ability to simultaneously bind the hinge region and the phosphate-binding region of the ATP-binding cleft. At present, this presents a unique binding motif among all known CK2 inhibitors. In fact, in this binding configuration, ellagic acid makes a stabilizing interaction between the hydroxyl group at the 3-position and the backbone carbonyls of Glu114 in the hinge region (Figure 3). Notably, these are the same regions that interact with the adenine moiety of ATP when bound to the active site.[10,11] On the other side, both hydroxyl groups at 7- and 8-positions interact with the carboxylic group of Asp175 (Figure 3) through double hydrogen-bonding interactions. In addition, several hydrophobic interactions (Val53, Val66, Phe113, Met163, Ile174) contribute to strongly stabilize ellagic acid in complex with CK2. In conclusion, the shape and the reduced dimension of the CK2-ATP binding site appear to play a key role in determining the selectivity of CK2 inhibitors.[10,11] The right balance of both polar and hydrophobic interactions and the perfect shape complementarity with the ATP-binding cleft are ultimately responsible for the high potency of ellagic acid.

Our findings disclose a molecular mechanism that predict, at least in part, therapeutic potentials of ellagic acid.[22] When taken as a supplement, the ellagic acid impacts cancer as well as aiding in conventional radiation and chemotherapy.[23,24] Moreover, ellagic acid has been demonstrated to inhibit tumor growth induced by several chemical carcinogens in animal models.[25,26] As shown in Figure 4A, in vitro growth of four ALCL cell lines, either expressing NPM-ALK (KARPAS299, SR786 and SUDHL1) or not (FE-PD), was inhibited at micromolar concentrations in a dose-dependent manner by treatment with ellagic acid.

Cytotoxicity profile measured after 48-h exposure demonstrated that ellagic acid almost completely inhibited the growth of SUDHL1 and FEPD cells at the highest concentrations (85 and 70%, respectively), whereas about 60 and 40% growth inhibition was observed in KARPAS299 and SR786 cells, respectively, under the same experimental conditions. The findings that ellagic acid strongly reduces viability also of FE-PD cells that are NPM-ALK negative together with the absence of a direct inhibition of NPM-ALK observed in vitro on the recombinant enzyme (Table 1) support the view that the cytotoxic effect of ellagic acid in all ALCL cell lines is mediated by CK2. In line with previous reports, we observed that higher concentrations than those used in vitro were required to assess the antiproliferative activity of ellagic acid in living cells. This

Table 1. Inhibition of Protein Kinases by Ellagic Acid Calculated as IC_{50} (μM)[a]

CK2	CK1	PKA	GSK3	DYRK1a	CSK	LYN	SYK	FGR	NPM-ALK	RET	FLT3
0.04	13.0	3.5	7.5	>40	>40	2.9	4.3	9.4	>40	>40	>40

The activity of each protein kinase was determined as described in the Supporting Information. [a] The values of IC_{50} represent the means of at least three independent experiments with SEM never exceeding 15%.

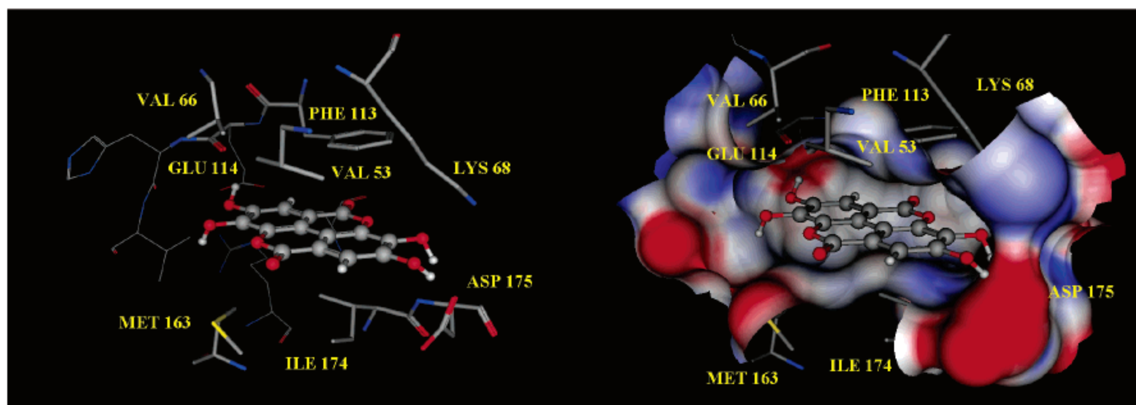

Figure 3. Molecular docking of ellagic acid bound to the active site of the CK2 catalytic subunit. On the left, analysis of the binding mode of ellagic acid in which the interactions with the most crucial amino acids are highlighted. On the right, Connolly's electrostatic charge distribution surface of ATP-binding cleft of CK2 (blue indicates positive surface charge and red indicates negative surface charge).

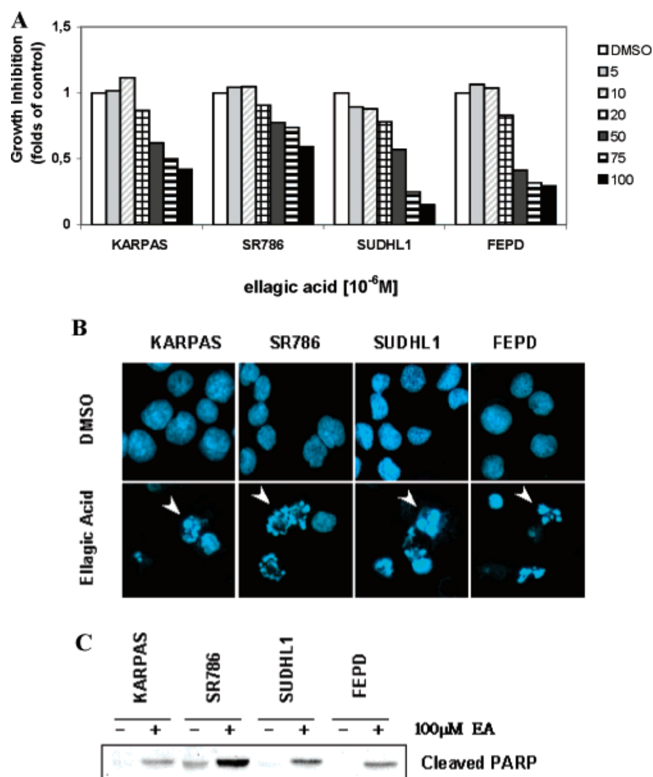

Figure 4. (A) Effect of ellagic acid on cell viability. Karpas299, SUDHL1, SR786, and FE-PD cell lines were cultured in the presence or absence of increasing concentrations of ellagic acid ($5-100 \times 10^{-6}$ M) for 48 h. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Values represent the mean of triplicate cultures. Control cells (DMSO) were assigned viability = 1.0. (B) Log-phase growing ALCL cells (0.5×10^6/mL) were treated for 48 h with ellagic acid (100×10^{-6} M) or left untreated (DMSO) and then processed for immunofluorescence analysis as described in Material and Methods. The cells were stained with 4′-6-diamidino-2-phenylindole (DAPI), and nuclei were observed using a fluorescence microscope. (C). To assay cleavage of the apoptotic hallmark PARP protein, exponentially growing ALCL cells were treated with ellagic acid (+) or left untreated (−) as described above, and then lysed in 0.1% SDS RIPA buffer. PARP protein steady-state was analyzed by western blotting, using a specific antibody recognizing the 89 kDa-cleaved fragment.

may be due to a number of factors, notably slow permeability, instability, and/or competitive mechanisms inside the cells, such as the high concentration of endogenous ATP.[28] However, we cannot exclude that even in the absence of appreciable cytotoxicity the drug is effective in inducing cell cycle arrest in a dose- and time-dependent manner, as previously shown.[28] To better elucidate the possible mechanism of action of ellagic acid in ALCL cell lines, exponentially growing cells were cultured in the presence of the drug for 48 h, and induction of programmed cell death was investigated by assessing molecular events occurring downstream activation of caspase cysteine proteases, which are known to be responsible for execution of apoptosis. To this end, the activity of effector caspases in cells undergoing apoptosis has been shown to promote the activation of enzymes responsible for chromatin condensation and fragmentation, as well as to result in proteolytical inhibition of selected target proteins, such as the DNA-damage repairing enzyme PARP (poly(ADP-ribose)polymerase). Collapse of nuclear integrity and the appearance of apoptotic bodies was observed in all four ALCL cell lines at 48 h posttreatment (Figure 4B, arrowheads), and this was accompanied by increased cleavage of the 116 kDa apoptotic marker protein PARP into its 89 kDa polypeptide (Figure 4C, cleaved PARP). Hence, although we cannot exclude the involvement of caspase-independent effectors, these data indicate that induction of programmed cell death, through activation of pro-apoptotic caspases, takes place in ALCL treated cells and perhaps represents a leading mechanism for the cytotoxicity of ellagic acid in vivo. We are presently synthesizing different focused libraries of ellagic acid analogues with the aim of increasing the potency and selectivity against CK2.

Acknowledgment. The molecular modeling work coordinated by S.M. has been carried out with financial support from the University of Padova, Italy, and the Italian Ministry for University and Research (MIUR), Rome, Italy. S.M. is also very grateful to Chemical Computing Group for the scientific and technical partnership. The biochemical work was financially supported by grants from MIUR (PRIN 2004), AIRC (Italian Association for Cancer Research) and EU (Prokinaseresearch 503467) to L.A.P. and F.M.

Supporting Information Available: Information concerning all experimental details are available free of charge via the Internet at http://pubs.acs.org.

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JM060112M