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Selectivity of 4,5,6,7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 ('casein kinase-2')

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Abstract The specificity of 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB), an ATP/GTP competitive inhibitor of protein kinase casein kinase-2 (CK2), has been examined against a panel of 33 protein kinases, either Ser/Thr- or Tyr-specific. In the presence of 10 μM TBB (and 100 μM ATP) only CK2 was drastically inhibited (>85%) whereas three kinases (phosphorylase kinase, glycogen synthase kinase 3β and cyclin-dependent kinase 2/cyclin A) underwent moderate inhibition, with IC_{50} values one–two orders of magnitude higher than CK2 (IC_{50} = 0.9 μM). TBB also inhibits endogenous CK2 in cultured Jurkat cells. A CK2 mutant in which Val66 has been replaced by alanine is much less susceptible to inhibition by TBB as well as by another ATP competitive inhibitor, emodin. These data show that TBB is a quite selective inhibitor of CK2, that can be used in cell-based assays. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Inhibitor; Protein kinase; Casein kinase; Casein kinase-2; Protein phosphorylation

1. Introduction

Protein kinases make up one of the largest family of enzymes, being committed to the catalysis of protein phosphorylation, which is the most general and frequent mechanism controlling diverse aspects of cell life. About one third of mammalian proteins contain covalently bound phosphate and there are likely to be about 1000 protein kinases encoded by the human genome, considering that in the eukaryotic organism *Caenorhabditis elegans* 2.5% of the genome encodes protein kinases [1]. Given these premises, it is not surprising that protein kinases play a key role in nearly all signal transduction pathways and that altered functions of individual protein kinases underlie numerous pathological conditions, including those due to uncontrolled cell proliferation. Pertinent to this is the observation that more than half of the proto-oncogenes encode protein kinases, the others being either targets or regulators (or both) of protein kinases. A major challenge in this field is therefore to develop highly selective inhibitors of individual protein kinases: these may prove useful for identifying the physiological targets and precise cellular functions of these enzymes and they may give rise to new therapeutic agents applicable in many different disease indications [2,3]. Despite their wide differences in substrate specificity and regulatory mechanism Mg-ATP is a common substrate to all eukaryotic protein kinases, whose ATP binding site consequently is highly conserved [4]. Initially, therefore, the design of ATP site-directed inhibitors was considered unlikely to result in selectivity, due to the assumption that the ATP binding site is almost identical among protein kinases. However empirical observations that some quite specific inhibitors of protein kinases, currently in the early phases of clinical trials (e.g. [5–7]), are competitive with respect to ATP, in conjunction with the solution of crystal structures of protein kinases in complex with inhibitors (e.g. [8–10]), have shown that such a priori concerns were unfounded. The structural rationale for the specificity of inhibitors competitive with respect to ATP is provided by a number of variable and sometimes quite unique features surrounding the otherwise conserved ATP binding site [11]. These provide specific pockets and/or docking sites where the inhibitor binds thereby interfering with the correct binding of ATP [9,10,12].

The most pleiotropic protein kinase known is probably CK2, an acronym derived from the misnomer 'casein kinase-2' used for a long time to indicate a ubiquitous Ser/Thr-specific, acidophilic kinase tested routinely in vitro on casein. CK2 is, however, unrelated physiologically to casein, while it phosphorylates a plethora of cellular proteins (more than 200 are currently known) sharing with casein the consensus sequence S/T-x-x-E/D/Sp/Yp [13–15]. Recently it has been shown that CK2 can also phosphorylate tyrosyl residues [16–18] albeit less efficiently than Ser/Thr ones. The CK2 ho-

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Abbreviations: OA, okadaic acid; TBB, 4,5,6,7-tetrabromo-2-azabenzimidazole or 4,5,6,7-tetrabromobenzotriazole; DRB, 1-(β-D-ribofuranosyl)-5,6-dichlorobenzimidazole; HS1, hematopoietic lineage cell-specific protein 1; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase (also called MEK); ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MAPKAP, MAPK-activated protein kinase; MSK, mitogen- and stress-activated protein kinase; PRAK, p38-regulated/activated kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PDK, 3-phosphoinositide-dependent kinase; PKB, protein kinase B (also called Akt); SGK, serum- and glucocorticoid-induced kinase; p70S6K, p70 ribosomal protein S6 kinase; GSK3, glycogen synthase kinase 3; ROCK, Rho-dependent protein kinase; AMPK, AMP-activated protein kinase; CHK, checkpoint kinase; PHK, phosphorylase kinase; CDK, cyclin-dependent kinase; CK, casein kinase; nCK, native casein kinase; rCK, recombinant casein kinase; G-CK, Golgi casein kinase; CSK, C-terminal Src kinase

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loenzyme is a tetramer composed of two catalytic (α and/or α′) and two 'regulatory' β subunits. The crystal structure of maize CK2α subunit (70% identical to its human homologue) has been solved either alone [19] or in complex with a fragment of the human β subunit, to give a tetramer that may provide some features of the whole holoenzyme [20]. The latter has been crystallized [21] but its structure is not yet available.

High constitutive activity is suspected to underlie the pathogenic potential of CK2, which is exploited by several viruses to perform the phosphorylation of proteins essential to their life cycle (reviewed in [22]), and whose catalytic subunits cooperate with other proto-oncogenes to promote cell transformation in different experimental models [23–26]. This makes CK2 an attractive target in the search for new antineoplastic and antiviral agents. In addition, the availability of small, cell-permeable inhibitors of CK2 could be used to implicate this pleiotropic enzyme in different cellular functions and pathways. Among the compounds which have been reported to inhibit CK2 in a relatively selective manner are emodin [27] and a halogenated derivative of benzotriazole, 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB) [28]. The latter belongs to a class of compounds related to commercially available 1-(β-D-ribofuranosyl)-5,6-dichlorobenzimidazole (DRB), which is marketed as a CK2 inhibitor. Emodin (3-methyl-1,6,8-trihydroxyanthraquinone), however, is also used widely as an inhibitor of receptor tyrosine kinases [29,30] and DRB, which has been tested only on a small number of protein kinases, inhibits the other class of ubiquitous CKs (CK1) almost as efficiently as CK2 [31]. By contrast, TBB discriminates between CK2 and CK1, being much more effective with the former [28,32]. However a systematic analysis of the inhibitory power of TBB toward a wide spectrum of protein kinases was lacking. This gap has been filled now by testing the inhibitory power of TBB on a panel of 33 protein kinases, which includes the classical second messenger-dependent protein kinases implicated in signaling pathways, all the different types of CKs and a number of protein tyrosine kinases. Our data disclose a remarkable selectivity of TBB toward CK2 since no other kinase is inhibited with comparable efficiency. They also provide evidence that TBB can be used to inhibit CK2 in cultured cells and show that the replacement of a conserved Ala present in nearly all the other kinases by a more hydrophobic residue (Val66 in human CK2) is an important feature in conferring inhibitability by this compound.

2. Materials and methods

2.1. Materials

Histone H1 was purchased from Life Technologies (Paisley, UK). Synthetic peptides RRRADDSDDDDD, RRKDLHDDEEDEAMSITA and KKIEKFQSEEQQQ were kindly provided by Dr. O. Marin (Padova, Italy). Synthesis of TBB was performed as in [28].

2.2. Source and purification of protein kinases

Native CK1 (nCK1) and nCK2 were purified from rat liver [33]; Golgi CK (G-CK), purified from rat lactating mammary gland [34], was a gift of Dr. A.M. Brunati (Padova, Italy); protein tyrosine kinases Lyn [35], c-Fgr [36], Syk (also termed TPK-IIB) and C-terminal Src kinase (CSK) [37] were purified from rat spleen. Human recombinant α and β subunits of CK2 were expressed in *Escherichia coli* and the holoenzyme was reconstituted and purified as described in [38]. The generation, expression and purification of the V66A mutant of CK2α subunit are described in [39]. *Saccharomyces cerevisiae* piD261 and *Xenopus laevis* CK1α, kindly provided by Dr. S. Facchin (Padova, Italy) and by Dr. V. Pulgar (Santiago, Chile), respectively, were expressed as His-tagged recombinant proteins in *E. coli* and purified as described in [40,41]. Cyclin-dependent kinase (CDK) 2/cyclin A (human) and CDK1/cyclin B (starfish) were a generous gift from Dr. J. Endicott (Oxford University, Oxford, UK) and Dr. L. Meijer (Roscoff, France), respectively. The source of all the other protein kinases is either described or referenced in [42].

2.3. Protein kinase assays

All protein kinase activities were linear with respect to time in every incubation. Assays were performed as detailed in [42] for all kinases except CK2, CK1 and G-CK which were tested on their specific peptide substrates, RRRADDSDDDDD, RRKDLHDDEEDEAMSITA [43] and KKIEKFQSEEQQQ [44], respectively, piD261, tested on casein [40], and tyrosine kinases Lyn, c-Fgr, Syk and CSK whose assays were performed using angiotensin II as substrate [35]. CDK2/cyclin A was assayed using histone H1 as substrate. The assays were carried out in a final volume of 25 μl containing 50 mM HEPES pH 7.5, 1 mM dithiothreitol, 0.02% Brij 35, 100 mM NaCl, 1 mg/ml histone H1, 10 mM magnesium acetate and 0.1 mM [γ-^{33}P]ATP (500–1000 cpm/pmol) and incubated for 40 min at room temperature. These conditions are suited for reaching maximal velocity with all protein kinases. Assays were stopped by addition of 5 μl of 0.5 M orthophosphoric acid before spotting aliquots onto P30 filtermats (Wallac). Filtermats were washed in 75 mM phosphoric acid (5–10 ml/each filtermat) four times then once in methanol and dried before counting.

2.4. Cell culturing, treatment and lysis

The human leukemia Jurkat T-cell line was maintained in RPMI 1640 supplemented with 10% fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cell treatment was performed by incubation of cell suspension ($\sim 10\times10^6$ cells/ml) for 2 h at 37°C in the presence of 2 μM okadaic acid (OA) with the addition of protein kinase inhibitors where indicated. After incubations, cells were centrifuged and lysed for 15 min in ice-cold buffer consisting of 20 mM Tris–HCl pH 7.4, 2 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol, 1 mM Na_3VO_4, 0.05% Nonidet P-40, protease inhibitor cocktail (Boehringer), 10 mM NaF, 1 μM OA. The lysate was cleared by centrifugation for 10 min at 14 000 rpm (Eppendorf Microfuge 5415C).

2.5. Immunoprecipitation and immunoblotting

The lysate corresponding to $\sim 5\times10^6$ cells was used for HS1 (hematopoietic lineage cell-specific protein 1) immunoprecipitation, performed by incubation at 4°C for 2 h with 2.5 μl of anti-HS1 antiserum [45], followed by addition of protein A-Sepharose. Immunoprecipitates were washed twice with NET buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.05% (v/v) Nonidet P-40, 2 mg/ml bovine serum albumin) and once with 50 mM Tris–HCl pH 7.5, boiled for 3 min and loaded onto 10% SDS–PAGE. Gels were transblotted to Immobilon-P membranes (Millipore) and analyzed by Western blot with the same anti-HS1 antiserum. Development was performed with an enhanced chemiluminescence detection system (Amersham Pharmacia). In the case of phosphatase treatment, before boiling, pellets from immunoprecipitation were incubated for 2 h at 30°C with acidic phosphatase from potato (3 μg) (Boehringer Mannheim) in the presence of 100 mM acetate buffer, pH 5.5.

3. Results

In Table 1 the activity of 33 protein kinases, assayed at 100 μM ATP in the presence of 10 μM TBB, is expressed relative to that measured in the absence of the inhibitor. Only with CK2 inhibition is $>85\%$. A significant, albeit much less pronounced inhibition is also observed with three Ser/Thr protein kinases, besides CK2, namely CDK2/cyclin A (30%), phosphorylase kinase (PHK) (51%) and glycogen synthase kinase 3β (GSK3β) (36%). With the remaining 29 protein kinases (including all the tyrosine protein kinases tested) no appreciable inhibition was observed.

The IC_{50} values for TBB were determined with CK2 and

Table 1
Susceptibility of protein kinases to 10 mM TBB

Protein kinase	Activity (10 μM TBB)	S.D.
MKK1	94	1
MAPK2/ERK2	111	4
JNK1/SAPK1c	93	4
SAPK2a/p38	107	1
SAPK2b/p38b2	103	1
SAPK3/p38g	89	2
SAPK4/p38d	99	2
MAPKAP-K1b	90	7
MAPKAP-K2	108	2
MSK1	111	5
PRAK	87	1
PKA	90	2
PKCα	102	1
PDK1	97	1
PKBα	91	3
SGK	97	6
p70S6K	89	4
GSK3β	64	1
ROCK-II	91	3
AMPK	96	1
CHK1	101	1
PHK	49	1
CDK1/cyclin B	102	3
CDK2/cyclin A	70	5
CK2	13	1
CK1	91	1
G-CK	95	4
piD261	98	2
Lck	98	4
Lyn	99	1
c-Fgr	98	1
Syk	102	2
CSK	101	3

Activity is expressed as a percentage of controls without TBB (in the presence of DMSO alone). ATP was always 100 μM. Source of protein kinases and assay conditions are described in Section 2.
MAPK, mitogen-activated protein kinase; MKK, MAPK kinase (also called MEK); ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MAPKAP, MAPK-activated protein kinase; MSK, mitogen- and stress-activated protein kinase; PRAK, p38-regulated/activated kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PDK, 3-phosphoinositide-dependent kinase; PKB, protein kinase B (also called Akt); SGK, serum- and glucocorticoid-induced kinase; p70S6K, p70 ribosomal protein S6 kinase; ROCK, Rho-dependent protein kinase; AMPK, AMP-activated protein kinase; CHK, checkpoint kinase; CDK, cyclin-dependent kinase.

Table 2
TBB concentration required for 50% inhibition of TBB sensitive protein kinases

Protein kinase	IC_{50} (mM) ± S.D.
nCK2 (rat liver)	0.9 ± 0.4
rCK2 (human)	1.6 ± 0.3
nCK1 (rat liver)	83.0 ± 2.5
rCK1α (*X. laevis*)	80.1 ± 1.9
CDK1/cyclin B	> 100.0
CDK2/cyclin A	15.6 ± 2.1
GSK3β	11.2 ± 1.6
PHK	8.7 ± 0.7

The ATP concentration was 100 μM in all assays. Phosphorylation conditions are described under Section 2.

the other three kinases affected significantly by 10 μM TBB. As shown in Table 2, the IC_{50} values with CDK2, PHK and GSK3β are one–two orders of magnitude higher than those calculated with either nCK2 or recombinant CK2 (rCK2). The K_i values for TBB of CK2 and CK1 were found to be 0.4 and 47 μM, respectively. Similar results were obtained if CK2 activity was monitored using the tyrosyl peptide substrate DEADIYDEEDYDL [17] instead of the usual seryl one (not shown).

Next we wanted to check whether TBB also inhibits CK2 in intact cells. For this purpose, we took advantage of the behavior of HS1 which upon Ser/Thr phosphorylation by CK2 undergoes a shift on SDS–PAGE not observed when the same protein is tyrosine-phosphorylated by Syk and Src family protein kinases [45]. As shown in Fig. 1A, HS1 immunoprecipitated from Jurkat cells treated with OA undergoes an upward shift similar to that observed in vitro upon phosphorylation by CK2 (Fig. 1B). The up-shift of the HS1 immunodetected band is counteracted by treatment of Jurkat cells with 60 μM TBB (compare lanes 3 and 1). The same is observed if the cells are treated with emodin, another inhibitor of CK2 [12,27] albeit less selective than TBB, while 0.1 μM staurosporine, a broad specificity inhibitor of protein kinases, does not prevent the up-shift of the HS1 band. Unlike most protein kinases, which are inhibited by staurosporine with IC_{50} values in the low nanomolar range [46,47], CK2 is unusually resistant to staurosporine, with a IC_{50} of about 19.5 μM [48]. These data corroborate the concept that CK2 is responsible for the up-

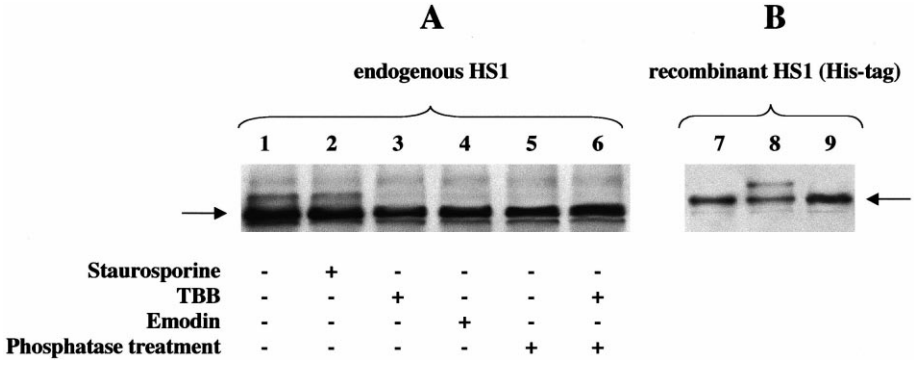

Fig. 1. TBB inhibits CK2-mediated HS1 phosphorylation in Jurkat cells. (A) HS1 was immunoprecipitated from Jurkat cells treated for 2 h with 2 μM OA and the indicated protein kinase inhibitors at the following concentrations: staurosporine, 0.1 μM; TBB, 60 μM; emodin, 60 μM. Samples of lanes 5 and 6 were treated with acidic phosphatase, after HS1 immunoprecipitation, as described under Section 2. (B) Recombinant His-tagged HS1 was analyzed directly (lane 7) or after in vitro phosphorylation by CK2, as described in [41] (lanes 8 and 9). In the case of lane 9, the phosphorylation reaction was followed by thermal inactivation of CK2 and incubation with acidic phosphatase. Analyses were performed by SDS–PAGE and Western blot with anti-HS1 antibodies. The arrows denote the positions of unphosphorylated HS1 (A) and recombinant HS1 expressed with a His tag (B), respectively. The up-shifted immunoreactive band detectable both in vivo (A) and in vitro (B) is due to phosphorylated HS1 as it disappears upon treatment with acidic phosphatase.

shift of HS1. That indeed such an up-shift is due to HS1 phosphorylation was confirmed by showing that it was reversed if the immunoprecipitates were treated with acidic phosphatase (see Fig. 1A, lane 5), a treatment that also reverses the HS1 up-shift induced by incubating recombinant HS1 with CK2 in vitro (Fig. 1B).

Modelling studies suggested that TBB is accommodated into a hydrophobic pocket generated by several non-polar residues [48]. One of these, Val66, attracted our attention for two reasons: firstly this valine is unique to CK2, being replaced by an alanine in nearly all the other protein kinases; secondly this valine is replaced conservatively by isoleucine in maize CK2α (70% identical to its human homologue) where it makes important contacts with another ATP-directed inhibitor, emodin, in the crystal structure of a complex between emodin and CK2α [12]. This prompted us to check whether the replacement of Val66 by alanine would result in a mutant whose catalytic activity is comparable to wild type [39,49] but in which inhibition by either TBB or emodin is impaired. As shown in Fig. 2A, this is indeed the case since the IC_{50} of TBB for the mutant V66A is about one order of magnitude higher than that found with wild type CK2. The same mutation is even more detrimental as far as inhibition by emodin is concerned (Fig. 2B). It should be noted that the K_m value for ATP is not significantly affected by the Val66→Ala mutation. It has to be concluded, therefore, that Val66 plays a relevant role in the binding of diverse classes of inhibitors by CK2.

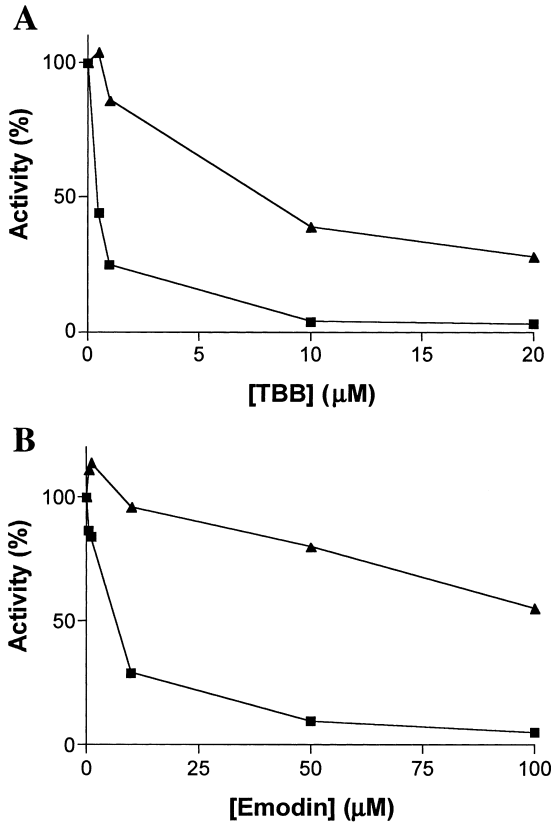

Fig. 2. CK2 susceptibility to inhibition by TBB (A) and emodin (B) is reduced by Val66→Ala mutation. The activity of wild type (squares) and of V66A mutated (triangles) rCK2α subunit was assayed as described under Section 2. The values represent a mean obtained from at least three independent experiments with S.D. not exceeding 14%.

The same mutant is also less sensitive than wild type to GTP vs radiolabeled ATP competition (IC_{50} values 0.35 vs 2.3 mM in the presence of 100 μM ATP). This corroborates the observation that the Val66 to Ala mutation specifically decreases the affinity of CK2 for GTP [49].

4. Discussion

In the light of the data presented it appears that TBB is by far the most selective inhibitor of CK2 among those analyzed, and one of the most specific protein kinase inhibitors described so far. Besides CK2 only three protein kinases out of 33 tested display significant inhibition by TBB; their IC_{50} values, moreover, are one–two orders of magnitude higher than that calculated with CK2: two of these, CDK2 and GSK3β, are kinases belonging to the same protein kinase subfamily (the CMGC group [50]), while the other, PHK, belongs to another subfamily (the CaMK group).

It should be noted however that another CDK, CDK1, closely related to CDK2, is quite refractory to TBB, thus corroborating the notion that closely related protein kinases may exhibit differential sensitivity to a given inhibitor. From these data, it appears that TBB is the first choice inhibitor of CK2 available to date. Interestingly, in our hands, it also discriminates very neatly between the two classes of pleiotropic CKs, as its IC_{50} value with CK1 is almost 100-fold higher than that for CK2. The K_i values, 0.4 μM and 47 μM with CK2 and CK1, respectively, also reflect this difference which is greater than that previously reported [28] for CK1 and CK2 preparations from rat liver both tested using casein as phosphorylatable substrate. In our experiments, specific peptide substrates were used that discriminate between CK1 and CK2 [43] and the data were confirmed using the recombinant enzymes expressed in bacteria (see Table 2). We have also shown that a third class of CK, localized to the Golgi apparatus (G-CK) [51] is fully refractory to TBB. The same applies to the protein kinase piD261, also tested on casein [40], which is required for the normal growth of yeast cells [52], but is also detectable in higher eukaryotes. It can therefore be concluded that the only known CK activity strongly inhibited by TBB is that due to CK2.

TBB is not only very selective, it also is fairly effective, with an IC_{50} value in the low micromolar range. This property in conjunction with cell permeability and lack of evident short term cytotoxicity, make it useful for in vivo experiments, as shown by its inhibition of CK2 in intact platelets [45] and its inhibition of the phosphorylation of a CK2 protein substrate in cultured Jurkat cells (see Fig. 1). This finding corroborates the conclusions of a recent study showing that TBB induces in cultured tobacco cells a number of biological effects which are attributed by the authors to CK2 inhibition [53].

Both the selectivity and efficiency of TBB may be improved further by molecular modifications aimed at rendering its binding to CK2 tighter and more specific. In this respect TBB could become a promising lead for designing new compounds possibly with therapeutical potential, considering that a role for CK2 is implicated in infectious and neoplastic diseases. Toward this aim the solution of the crystal structure of CK2 complexed with TBB and a parallel mutational study of the residues implicated in TBB interactions will be very helpful. Right now, valuable information was provided by the finding that a CK2 mutant in which a hydrophobic residue

that makes contacts with the related inhibitor emodin when the latter is bound to maize CK2α [12] has been replaced by alanine displays reduced susceptibility to inhibition by both emodin and TBB (see Fig. 2). This finding highlights the relevance of this hydrophobic residue, which is conserved in CK2 from different species, but otherwise replaced by alanine in other protein kinases, in conferring specificity to TBB inhibition: it also paves the way towards the generation of CK2 mutants refractory to TBB inhibition, which may be useful for validating the specificity of TBB as an inhibitor of CK2 in vivo.

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