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2-Dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole: a novel powerful and selective inhibitor of protein kinase CK2

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Abstract

Protein kinase CK2 is a highly pleiotropic enzyme whose high constitutive activity is suspected to be instrumental to the enhancement of the tumour phenotype and to the propagation of infectious diseases. Here we describe a novel compound, 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (DMAT), which is superior to the commonly used specific CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) in several respects. DMAT displays the lowest K_i value ever reported for a CK2 inhibitor (40 nM); it is cell permeable and its efficacy on cultured cells, both in terms of endogenous CK2 inhibition and induction of apoptosis, is several fold higher than that of TBB. The selectivity of DMAT assayed on a panel of >30 protein kinases is comparable to that of TBB, with the additional advantage of being ineffective on protein kinase CK1 up to 200 μM. These properties make DMAT the first choice CK2 inhibitor for in vivo studies available to date.

Keywords: Protein kinase CK2; CK2 inhibitors; Polyhalogenated benzimidazoles; TBB; DMAT

CK2 probably is the most pleiotropic protein kinase known with more than 300 protein substrates already identified [1], a feature which might, at least partly, account for its lack of strict control over catalytic activity. 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]. Actually they are invariably more abundant in tumours 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 tumour suppressor genes [7]. These data in conjunction with the observation that many viruses exploit CK2 as phosphorylating agent of proteins essential to their life cycle are rising interest on CK2 as potential target for anti-neoplastic and/or anti-infectious drugs.

Consequently, an increasing number of reports dealing with the development and usage of CK2 inhibitors recently appeared in the literature. Among these inhibitors, 4,5,6,7-tetrabromobenzotriazole (TBB) proved especially successful for in cell and in vivo studies, notably to highlight the anti-apoptotic potential of CK2 [8], the role of CK2 in the repair of chromosomal DNA strand breaks [9], and its implication in the potentiation of Bcr/Abl mediated cell proliferation [10]. While

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the selectivity of TBB is remarkable [11], its efficacy, though higher than those of most other CK2 inhibitors, is not outstanding: in vitro it inhibits purified CK2 with IC_{50} values around 1 μM while the concentration required to induce half-maximal apoptosis of Jurkat cells is around 20 μM.

It is possible that the relatively low efficacy of TBB as well as of other CK2 inhibitors, as opposed to their fairly high specificity, is due to their mode of binding almost exclusively based on apolar contacts with unique hydrophobic side chains, while polar interactions, quite common with potent inhibitors of other kinases, are nearly absent.

In an attempt to increase the binding affinity of the TBB scaffold to the active site of CK2 a number of derivatives have been synthesized [12]. In some of these the triazole ring was replaced by an imidazole one derivatized with substituents which could provide additional interactions with polar side chains in the kinase active site. Here we show that one of these derivatives, 2-dimethylamino-4,5,6,7-tetrabromobenzimidazole (DMAT), displays the lowest K_i value ever reported of a CK2 inhibitor and is several fold more potent than TBB to inhibit CK2 both in vitro and in vivo and to induce apoptosis of Jurkat cells.

Materials and methods

Protein kinases. Native CK1 (a mixture of α, γ, and δ isoforms [13]) and CK2 were purified from rat liver [14]. The source of the other protein kinases was as previously described or referenced [15].

Synthesis and characterization of 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole. All chemicals and solvents used for the synthesis were purchased from Sigma–Aldrich. Melting points (uncorr.) were measured in open capillary tubes on a Gallenkamp-5 melting point apparatus. Ultraviolet absorption spectra were recorded on Kontron Uvikon 940 spectrometer. ^{1}H NMR spectra (in ppm) were measured with Varian Gemini 200 MHz and Varian UNITYplus spectrophotometers at 298 °K in D_6 (DMSO), using tetramethylsilane as the internal standard. Mass spectra (70 eV) were obtained with AMD-604 (Intectra) spectrometer. Flash chromatography was performed on silica gel (Merck) (230–400 mesh). Analytical thin-layer chromatography (TLC) was carried out on precoated silica gel F_{254} (Merck) plates (0.25 mm thickness). Elemental analyses of the new compounds were within ±0.4% of the respective theoretical values.

DMAT was synthesized by using 2,4,5,6,7-pentabromo-1*H*-benzimidazole as a starting compound [12].

The reaction of 2,4,5,6,7-pentabromo-1*H*-benzimidazole with 30% ethanolic dimethylamine at 110–115 °C in steel autoclave provided the corresponding 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (710 mg, 75%). M.P. >330 °C (with decomp.). TLC ($CHCl_3$/MeOH, 90:10): Rf = 0.61. UV (0.1 N HCl/MeOH, 1:1): 237 (39,300), 309 (10,500); (water/MeOH, 1:1): 242 (37,600), 272 (11,800), 316 (10,900); (0.1 N NaOH/MeOH, 1:1): 248 (36,000), 269 (5300), 319 (11,100). ^{1}H-NMR (D_6 (DMSO)): 3.13 (s, 2× CH_3), 11.5 (bs, NH-benzim.). MS: 481 (17), 480 (8), 479 (64), 478 (13), 477 (100), 476 (11), 475 (69), 474 (5), 473 (17). Anal. calcd for $C_9H_7Br_4N_3$ (476.79): C, 22.67; H, 1.48; N, 8.81. Found: C, 22.71; H, 1.65; N, 8.68. Freshly prepared 10 mM solutions of DMAT in DMSO were used for both in vitro and in vivo experiments.

Cell culture, treatment, and viability assay. The human leukemia Jurkat T-cell line was maintained in RPMI-1640, supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For the treatment, cells were suspended at a density of 10^6 cells/ml in a medium containing 1% (v/v) foetal calf serum and then incubated at 37 °C, in the presence of the compounds at the indicated concentrations. Control cells were treated with equal amounts of solvent (DMSO). At the end of incubations, cells were lysed by the addition of hypo-osmotic buffer, as previously described [15].

Cell viability was assessed by means of 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltriazolium bromide (MTT) reagent while caspase activation was followed by Western blot monitoring of PARP degradation as previously described [8].

Phosphorylation assays. Phosphorylation assays for CK1 and CK2 were carried out in the presence of increasing amounts of the inhibitor in a final volume of 25 μl containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$, and 0.02 μM [γ-^{33}P]ATP (500–1000 cpm/pmol), unless otherwise indicated, in the presence of specific peptide substrate as described in [11] and incubated for 10 min at 37 °C. Assays were stopped by addition of 5 μl of 0.5 M orthophosphoric acid before spotting aliquots onto phosphocellulose filters. Filters were washed in 75 μM phosphoric acid (5–10 ml/each) four times then once in methanol and dried before counting. Activity assays of all other protein kinases were performed as described or referenced elsewhere [16,17].

Kinetics. Initial velocities were determined at each of the substrate concentrations tested. K_m values were calculated either in the absence or in the presence of increasing concentrations of inhibitor, from Lineweaver–Burk double-reciprocal plots of the data. Inhibition constants were then calculated by linear regression analysis of K_m/V_{max} versus inhibitor concentration plots.

Results

The structure of the novel inhibitor, 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (DMAT), is shown in Fig. 1A together with that of the parent compound TBB. In Figs. 1B and C the dose-dependent inhibition of CK2 and CK1, respectively, by the two compounds is shown. DMAT inhibits CK2 more potently than TBB, with an IC_{50} value around 0.15 μM. In contrast, DMAT is ineffective on CK1 up to 200 μM concentration, whereas 50% inhibition of CK1 activity is attained with 26 μM TBB.

As shown in Fig. 2, inhibition of CK2 by DMAT is competitive with respect to the phosphodonor substrate ATP: a 40 nM K_i value has been calculated from these experiments, which is the lowest K_i reported so far of any CK2 inhibitor.

TBB is a quite specific inhibitor of CK2 [11] with only one protein kinase, DYRK1a, out of a panel of more than 30 inhibited with comparable efficacy [15]. As detailed elsewhere (manuscript in preparation) this also applies to DMAT: a part from CK2 and DYRK1a both of which are almost 100% inhibited by 10 μM DMA-TBB, all the other kinases of the panel used in [15] still display significant activity under these conditions, with only three (CDK2, SGK, and phosphorylase kinase)

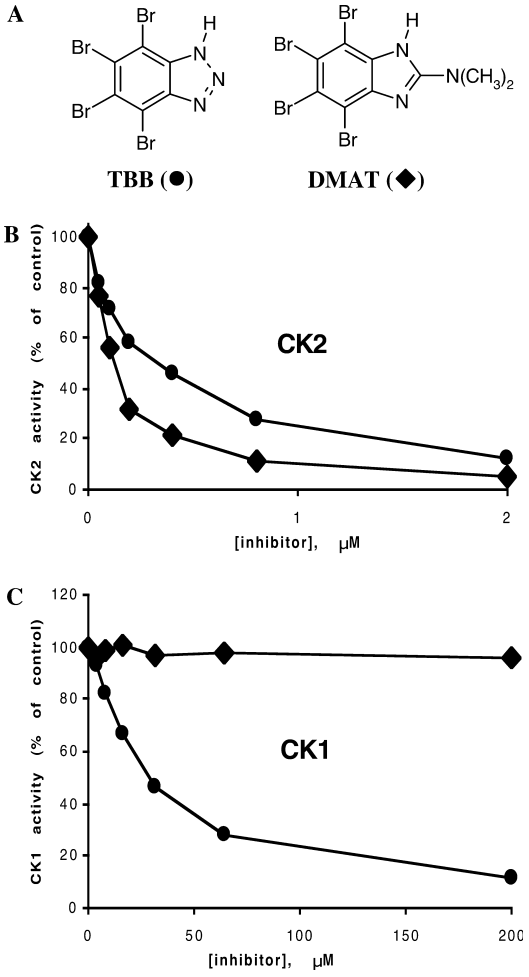

Fig. 1. Dose-dependent inhibition of protein kinases CK1 and CK2 by TBB and DMAT. The molecular structures of TBB and DMAT are shown in (A). Activity assays of protein kinases CK2 (B) and CK1 (C) were performed as described in Materials and methods in the presence of increasing concentrations of either TBB (●) or DMAT (◆). The data represent means of at least three independent experiments with SE never exceeding 10%.

inhibiting more than 50%. Phosphorylase kinase and CDK2 are also partially inhibited by TBB [11]; in contrast, GSK3 which is 40% inhibited by 10 μM TBB [11] is entirely unaffected by the same concentration of DMAT.

Next we wanted to check the inhibitory efficacy of DMAT in vivo. We first showed that by treating Jurkat cells with 5 and 10 μM DMAT endogenous CK2 was drastically inhibited as judged from both direct assay of CK2 with a specific peptide substrate in the cell lysates (Fig. 3) and inhibition of the CK2 promoted up-shift of the endogenous substrate HS1 protein [8] (not shown). By both criteria DMAT proved more powerful than TBB whose concentration required for 80% inhibition of endogenous CK2 is around 50 μM [8].

The superiority of DMAT as CK2 inhibitor in vivo also reflects in its higher efficacy to induce apoptosis

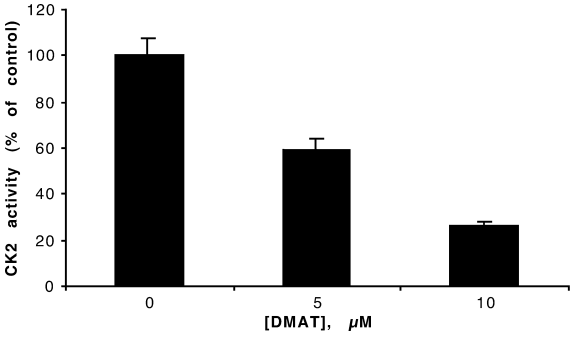

Fig. 3. Inhibition of endogenous CK2 activity by DMAT in Jurkat cells. Jurkat cells were treated for 2 h with the indicated DMAT concentrations, collected by centrifugation, and washed and lysed by addition of hypo-osmotic buffer [15]. CK2 activity in the cell lysates (1–2 μg) was measured toward the synthetic peptide substrate RRRADDSDDDDD (0.1 mM). The activity in the control cells, treated with the solvent DMSO, was normalized to 100%.

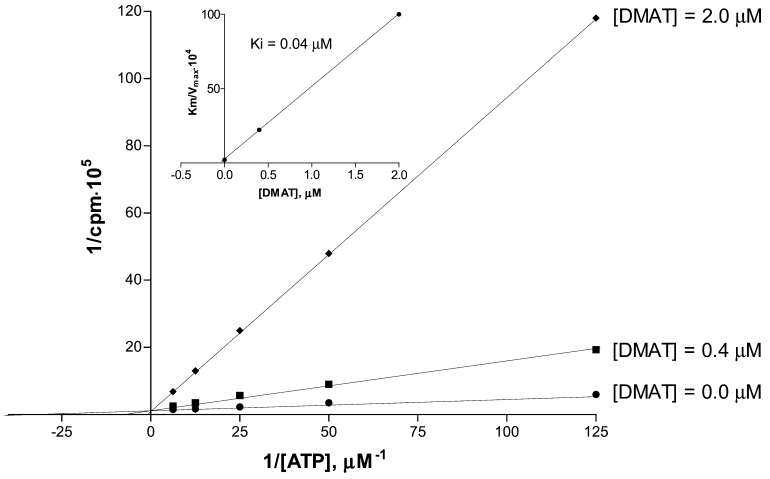

Fig. 2. Lineweaver–Burk inhibition plots of protein kinase CK2 by DMAT. CK2 activity was determined as described in the experimental section either in the absence (●) or in the presence of 0.4 (■) and 2 (◆) μM DMAT. The data represent means of experiments run in triplicate with SE never exceeding 10%.

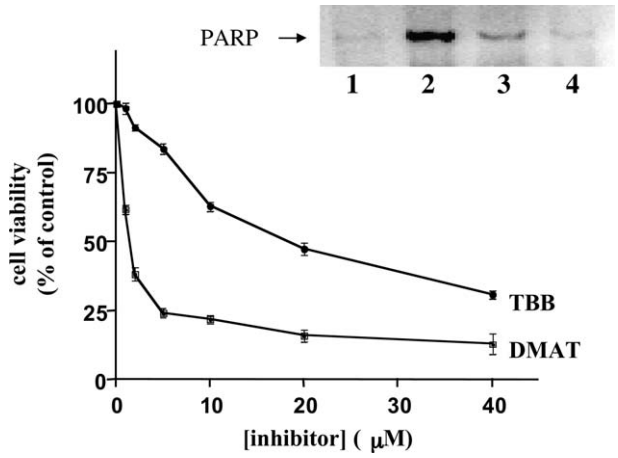

Fig. 4. Dose-dependent effect of DMAT and TBB on cell viability. Jurkat cells were treated for 24 h with the indicated concentrations of the inhibitor. Cell viability data, measured by the method of MTT (see experimental section), represent means of three independent experiments with SE never exceeding 12%. In the inset caspase activation by DMAT (5 and 10 μM in lanes 3 and 4, respectively) is monitored by following the degradation of the caspase substrate PARP with specific antibodies. Lane 2, untreated cells. Lane 1, cells treated with 50 ng/ml antiFas as a positive apoptotic control.

as compared to TBB. As shown in Fig. 4 cell viability is reduced by 50% upon incubation with 2.5 μM DMAT, whereas 20 μM TBB is required to obtain the same effect. As it has been shown of TBB [8] cell death induced by the new compound is also accounted for, at least in part, by apoptosis, based on several criteria, namely the degradation of PARP (see inset of Fig. 4), HS1 protein fragmentation, and release of cytochrome C from mitochondria (not shown).

Discussion

Based on the data presented the new compound DMAT appears to be the first choice inhibitor of protein kinase CK2 described so far for in vitro and, even more, in vivo investigations, due to a number of properties. First, its K_i value for CK2 inhibition is the lowest reported so far of any other inhibitor of this kinase. Second, the selectivity of DMAT is quite remarkable and comparable to that of TBB, with only DYRK1A, out of a panel of >30 protein kinases, inhibited to the same extent as CK2. Third, DMAT is readily cell permeable: it inhibits endogenous CK2 and induces the same cellular effects as TBB at several fold lower concentration. This is an especially notable advantage considering that TBB is effective on cultured cells at 25–50 μM concentration, which can also, at least partially, inhibit endogenous CK1, whereas the same effect is attained with 5–10 μM DMAT, which is fully inactive on CK1 up to 200 μM. Therefore, DMAT will prove especially valuable whenever it is important to discriminate between constitutive "casein kinase" activities due to either CK2 or CK1. Inability to discriminate between CK2 and DYRK1a, on the other hand, is also shared by TBB and other inhibitors of CK2, namely emodin and some emodin derivatives [15]. This finding suggests that CK2 and DYRK1a share similar features at their inhibitor binding sites. Actually a hydrophobic residue (Ile174) which in CK2 has been shown by mutational analysis to participate in apolar interactions with TBB and other inhibitors [15] and which is replaced in the majority of other protein kinases by smaller and/or hydrophilic side chains is instead conservatively replaced by a valine (Val307) in DYRK1a. The only CK2 inhibitor reported so far to be significantly less effective on DYRK1a than it is on CK2 is IQA [15]. It still 50% inhibits DYRK1a activity at 10 μM concentration, however. In addition, IQA is slightly less potent than DMAT and rather unstable in solution, a circumstance which makes somewhat tricky its usage with cultured cells. Potent inhibition of DYRK1a by DMAT, on the other hand, may represent an added value, since specific inhibitors for this class of protein kinases, possibly implicated in neurological disorders [18,19], are presently not available. In perspective, proper derivatizations of the DMAT scaffold could give rise to new more selective inhibitors able to discriminate between DYRK1a and CK2.

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