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Selectivity of 4,5,6,7-tetrabromobenzimidazole as an ATP-competitive potent inhibitor of protein kinase CK2 from various sources☆

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Abstract

Like the previously reported 4,5,6,7-tetrabromobenzotriazole (TBBt), the structurally related 4,5,6,7-tetrabromobenzimidazole (TBBz) is a selective ATP-competitive inhibitor of protein kinase CK2 from such divergent sources as yeast, rat liver, *Neurospora crassa* and *Candida tropicalis*, with K_i values in the range 0.5–1 μM. It is virtually inactive vs. PKA, PKC, and a very weak inhibitor of protein kinase CK1. The corresponding tetrachlorobenzimidazole (TCBz) is a much weaker inhibitor of CK2, like tetrachlorobenzotriazole (TCBt) relative to TBBt. Bearing in mind the similarity of the van der Waals radii of Br (1.95 Å) and CH_3 (2.0 Å), the corresponding much less hydrophobic 4,5,6,7-tetramethylbenzotriazole (TMeBt) was prepared and found to be a very weak inhibitor of CK2, as well as of CK1. An unexpected, and significant, difference between TBBt and TBBz are their inhibitory activities vs. the yeast protein kinase PK60S, which phosphorylates, both in vitro and in intact yeast cells, three of the five pp13 kDa ribosomal surface acidic proteins in yeast cells. TBBt was previously noted to be a more effective inhibitor of PK60S than of yeast CK2; by contrast, TBBz is a relatively feeble inhibitor of PK60S, hence more selective than TBBt vs. CK2 in yeast cells. TMeBt was virtually inactive vs PK60S. Like TBBt, TBBz is an additional lead compound for development of more potent inhibitors of CK2.

Keywords: Protein kinase CK2; Inhibitors; Tetrahalogeno benzimidazoles; Tetrahalogeno benzotriazoles; Tetramethylbenzotriazole

Protein kinases play key roles in signal transduction pathways, and dysfunctions of these enzymes are frequently associated with various pathological states [1]. It is consequently not surprising that major efforts are currently devoted to development of specific inhibitors. Sequencing studies indicate that 2–3% of the genes of the human genome encode protein kinases and estimates suggest that 25% of cellular proteins are phosphorylated. Bearing in mind that ATP is the common, but not necessarily unique, phosphate donor, i.e., that the ATP binding site is highly conserved, it was long considered that ATP-competitive inhibitors would not distinguish between different kinases, an assumption questioned some time ago [2], since it does not take into account subtle structural features in the vicinity of the otherwise conserved ATP binding site. There are now many potent ATP-competitive specific inhibitors, which effectively discriminate a given kinase from many others, and some of these are in preclinical and clinical trials.

Protein kinase CK2 (originally known as casein kinase 2, because it phosphorylates in vitro the non-physiological substrate casein) is the most pleiotropic protein kinase known and is able to phosphorylate in vitro more than 200 cellular proteins, which share with casein the consensus sequence S/T-XX-/D/Sp/Yp [3].

☆ *Abbreviations:* CK1, protein kinase CK1 (casein kinase 1); CK2, protein kinase CK2 (casein kinase 2); PKC, protein kinase C; PKA, cAMP-dependent protein kinase; DRB, 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole; DiBr-RBz, 5,6-dibromo-1-(β-D-ribofuranosyl)benzimidazole; TCBz, 4,5,6,7-tetrachlorobenzimidazole; TBBz, 4,5,6,7-tetrabromobenzimidazole; DiCl-Bt, 5,6-dichloro-benzotriazole; TCBt, 4,5,6,7-tetrachlorobenzotriazole; TBBt, 4,5,6,7-tetrabromobenzotriazole; TMeBt, 4,5,6,7-tetramethylbenzotriazole (see Fig. 1).

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The finding that 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (DRB), see Fig. 1, an inhibitor of eukaryotic mRNA transcription, inhibits in a parallel manner the activity of mammalian CK2, and that inhibition of transcription may be partially reversed by an excess of CK2, pointed to involvement of a phosphorylation step in mRNA transcription, and to DRB as an inhibitor of CK2 [4]. A subsequent study with other halogenated DRB analogs demonstrated that these inhibit both CK1 and CK2, competitively with respect to ATP, with a marked preference for CK2, but not a variety of other kinases [5]. Extension of these studies led to the finding that 5,6-dichloro-benzotriazole (DiCl-Bt, Fig. 1), in which the benzimidazole moiety has been replaced by 2-azabenzimidazole (benzotriazole), is a 10-fold better inhibitor of CK2 than CK1 [6]. This, in turn, led to 4,5,6,7-tetrachlorobenzotriazole (TCBt) and 4,5,6,7-tetrabromobenzotriazole (TBBt, see Fig. 1), which even more effectively discriminated between the two enzymes, with a K_i for TBBt vs. CK2 in the low micromolar range, and two orders of magnitude lower than that for TCBt [7,8]. The remarkable specificity of TBBt was further confirmed by testing against a panel of more than 30 protein kinases [3], and it is presently the most specific, as well as a cell-permeable [3,8], inhibitor of CK2. The crystal structure of TBBt (also referred to as TBB), in a complex with the *Zea mays* CK2α catalytic subunit, has been reported [9].

Bearing in mind that various halogenated benzimidazoles were those initially described as inhibitors of CK2 [4–8], it appeared logical to turn to the 4,5,6,7-tetrahalogeno benzimidazoles, i.e., TBBz and TCBz (Fig. 1), in which the tetrahalogenobenzene moiety is retained intact. Syntheses of TBBz, and its chloro analog TCBz, have been reported [10,11]. We have prepared both by an independent route, and herein describe their inhibitory properties vs. CK2 and CK1 relative to those of TBBt and TCBt, and for comparison vs. several other protein kinases. For reasons described below, the hitherto unknown 4,5,6,7-tetramethylbenzotriazole (TMeBt) was prepared by diazotization of 1,2,3,4-tetramethyl-5,6-diaminobenzene with sodium nitrite, according to the procedure of Benson et al. [12].

Materials and methods

Enzymes

The yeast CK1 (45 kDa) enzyme was purified to apparent homogeneity as elsewhere described [13]. Highly purified CK2 from *Saccharomyces cerevisiae*, *Candida tropicalis*, *Neurospora crassa*, and rat liver was prepared according to Szyszka et al. [14], followed by heparin–Sepharose and α-casein–Sepharose column chromatography, to give preparations with near homogeneity. Rat liver CK1 was isolated according to Meggio et al. [15] and yeast PKA according to Hixson and Krebs [16]. Yeast PKC was prepared by the procedure of Jimenez et al. [17], concentrated by elution from DEAE-cellulose at 90 mM NaCl, and rendered salt-free by dialysis. The yeast protein kinases PK60S and RAP1, which phosphorylate the ribosomal acidic proteins P1/P2, were purified to apparent homogeneity as described by Pilecki et al. [18] and Szyszka et al. [19], respectively.

ATP, casein, and histones H1 and H2A were products of Sigma (St. Louis, MO, USA); and [γ-^{32}P]ATP (spec. act. 167 TBq/mmol) was from ICN Biomedicals (Irvine, CA, USA). CK1 and CK2 activities, in the presence and absence of inhibitors, were routinely assayed as elsewhere reported [20], with casein or recombinant yeast ribosomal P2B protein (rYP2B), and labelled ATP as phosphate donor. Assays of PKA and PKC were performed as described by Hixson and Krebs [16] and Jimenez et al. [17], respectively.

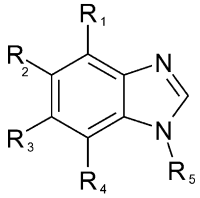
Stock solutions of inhibitors consisted of 2 mg/ml in DMSO; these were appropriately diluted with water or buffer solution to desired concentrations. Possible effects of the presence of small concentrations of DMSO in the enzymatic assays were eliminated by suitable controls.

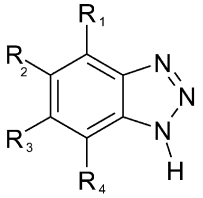
Synthesis of tetrahalogeno benzimidazoles

General. Melting points (uncorr.) were obtained on a Boetius microscope hot stage. Thin layer chromatography (TLC) was run on Merck silica gel F254 glass plates, using the solvent system $CHCl_3$:MeOH (9:1). UV absorption spectra were recorded on a Cary 300 instrument with 10-mm pathlength cuvettes. A Cole–Parmer instrument, with a combination electrode, was employed for pH measurements; and extremes of pH made use of standard solutions of HCl and NaOH. Mass spectra were recorded on a Micro-mass ESI Q-Tof spectrometer upgraded to 8000.

4,5,6,7-Tetrachlorobenzimidazole. A solution of 472 mg (4 mM) of benzimidazole in 100 ml concentrated HCl and 40 ml HNO_3 was heated under reflux for 36 h. After cooling, the precipitate was collected by filtration and crystallized twice from EtOH. Yield, 972 mg (95%), m.p. 320–322 °C, as against 325 °C reported by Buechel [10]. Mass spectroscopy gave *m/z* (MH^+) = 256.893 (theor. for $C_7H_3Cl_4N_2^+$ = 256.905); R_f = 0.73 (benzimidazole 0.43). UV λ_{max} (ε): pH 2: 267 nm (4000), 285 nm (2250), and 296 nm (1900); pH 6: 267 nm (5500), 286 nm (2660), and 296 nm (2370); pH 12: 280 nm (6800), 288 nm (7200).

4,5,6,7-Tetrabromobenzimidazole. To 590 mg (5 mM) of benzimidazole in 15 ml concentrated HNO_3 was added, dropwise, 3 ml of Br_2 and the solution was heated under reflux for 24 h. After cooling, the precipitate was collected by filtration and crystallized twice from EtOH. Yield 1.46 g (67%), m.p. 330–332 °C, cf. to 339 °C reported [11]. Mass spectroscopy gave *m/z* (MH^+) = 434.76 (theor. for $C_7H_3Br_4N_2^+$ = 434.73); R_f = 0.75 (benzimidazole 0.43). UV λ_{max} (ε): pH 2: 266 nm





Compound	R_1	R_2	R_3	R_4	R_5	Compound	R_1	R_2	R_3	R_4
DRB	H	Cl	Cl	H	Rib	DiCl-Bt	H	Cl	Cl	H
DiBr-RBz	H	Br	Br	H	Rib	TCBt	Cl	Cl	Cl	Cl
TCBz	Cl	Cl	Cl	Cl	H	TBBt	Br	Br	Br	Br
TBBz	Br	Br	Br	Br	H	TMeBt	CH_3	CH_3	CH_3	CH_3

Fig. 1. Structures of: (left) halogenated benzimidazole ribosides, halogenated benzimidazoles; (right) benzotriazoles (2-azabenzimidazoles).

(9200), 297 nm (4600); pH 6: 267 nm (10,970), 296 nm (5030); pH 12: 281 nm (10,630).

Stock solutions of the foregoing, stored at 4 °C for up to 4 weeks, fully retained their inhibitory activities and exhibited unchanged mass spectra.

4,5,6,7-Tetramethylbenzotriazole. To a cooled solution of 0.9 g (3.8 mM) 1,2,3,4-tetramethyl-5,6-diaminobenzene hydrochloride [21] in 7 ml water was added a solution of 0.47 g of sodium nitrite in 5 ml water. The mixture turned dark green in color and soon changed to orange. The mixture was kept at room temperature for 12 h, then cooled, and filtered. The residue was washed with water and the product was crystallized from ethanol/water to give 0.46 g TMeBt (68% yield), m.p. 281–282 °C (decomposition). Mass spectroscopy gave *m/z* MH^+ 176.0802 (theor. for $C_{10}H_{14}N_3^+$ 176.2352); $R_f = 0.64$. UV λ_{max} (ε) MeOH: 270 nm (6700), pH 2: 276 nm (6600), pH 7: 275 nm (6400), and pH 12: 281 nm (9060); ^{1}H NMR (d_6-DMSO) 2.24 (s, 6H, CH_3), 2.52 (d, 6H, CH_3); ^{13}C NMR 14.262; 15.674; 114.883; 121.786; 131.031; 132.248; and 142.722.

Results

Initially, protein kinases CK1 and CK2 from yeast and rat liver were tested for inhibition by the eight selected compounds shown in Fig. 1, each at 10 μM, with ATP as phosphate donor. From Table 1, it will be seen that, in agreement with previous results for the corresponding rat and plant enzymes [5,6], DiBr-RBz is appreciably more effective than DRB as an inhibitor of CK2 from yeast and the rat, and much less effective against CK1. The observed lack of inhibition of CK1 from both yeast and mammalian sources by DiCl-Bt, which is a good inhibitor of the CK2 enzymes (Table 1), is also consistent with earlier results for the corresponding plant enzymes [6]. The tetrahalogeno benzotriazoles, TCBt and TBBt, are weak inhibitors of CK1, but much more effective vs. CK2, with TBBt significantly more potent than TCBt, again in agreement with previous findings [7].

Turning to the two new analogs, TCBz and TBBz, the former was only a very weak inhibitor of the CK1 enzymes from both sources, and a moderate inhibitor of CK2. By contrast, TBBz weakly inhibited the CK1 enzymes, but was much more potent vs. CK2 than its tetrachloro congener TCBz. Note, in particular, that TBBz appears to be somewhat more effective vs. CK2 than TBBt (but see below), hitherto considered the most potent inhibitor of this enzyme.

Attention was then directed to the effects of the most potent CK2 inhibitor, TBBz and, for comparison, TBBt and TMeBt. At a concentration of 5 μM, TBBz exhibited little activity vs. the yeast CK1 enzyme, and only moderate inhibition of CK1 from rat liver (Table 2), but was an excellent inhibitor of CK2 from yeast, rat liver, *C. tropicalis*, and *N. crassa*. It is virtually inactive vs. PKA and PKC from yeast, and only moderately inhibits the yeast protein kinases PK60S and RAP1, thus recognizing, typical for CK2, the acidic amino acid sequence EESDDD in YP2B ribosomal protein [22–24]. Turning to TMeBt, it should be recalled that the van der Waals radius of a CH_3 (2.00 Å) is very close to that of a Br (1.95 Å), so that the volume of the tetramethylbenzene moiety of TMeBt is close to that of the tetrabromobenzene moiety of TBBt. However, TMeBt is

Table 1
Inhibition of protein kinases CK1 and CK2 from yeast and rat liver by halogenated benzimidazole ribosides, benzotriazoles, and benzimidazoles

Inhibitor	Activity (%)			
	S. cerevisiae		Rat liver	
	CK1	CK2	CK1	CK2
DRB	95	86	93	83
DiBr-RBz	89	58	91	56
DiCl-Bt	102	52	102	56
TCBt	100	49	100	51
TBBt	89	28	87	12
TmeBt	91	82		
TCBz	96	62	94	67
TBBz	93	16.7	84	19.4

Kinase activity was assayed with 1.5 mg/ml casein and 10 μM ATP in 20 mM Tris–HCl buffer, pH 7.5. Inhibitor concentration was 5 μM. Activities are relative to that in absence of inhibitor.

Table 2
Effect of inhibitors (5 μM) on the activity of various Ser/Thr protein kinases

Protein kinase	Substrate used (mg/ml)	Activity (% of control)		
		TBBz	TBBt	TmeBt
S. cerevisiae CK1	Casein (1.5)	93	89	91
S. cerevisiae CK2	Casein (1.5) or rYP2B (0.1)	16.7	28	82
S. cerevisiae PKA	Histone H2A (0.5)	102.2	108	
S. cerevisiae PKC	Histone H1 (1.0)	100.8	103	
S. cerevisiae PK60S	rYP2B (0.1)	91.2	25	97
S. cerevisiae RAP 1	rYP2B (0.1)	87.5	—	
Rat liver CK1	Casein (1.5)	84	87	
Rat liver CK2	Casein (1.5)	19.4	12	
C. tropicalis CK2	Casein (1.5)	20.3		
N. crassa CK2	Casein (1.5)	18.7		

Kinase activity was assayed with 0.1–1.5 mg/ml of appropriate protein substrate: recombinant yeast ribosomal P2B protein (rYP2B), histone H1, histone H2A, or casein, and 10 μM ATP in 20 mM Tris–HCl buffer, pH 7.5. Activities are relative to that in absence of inhibitor.

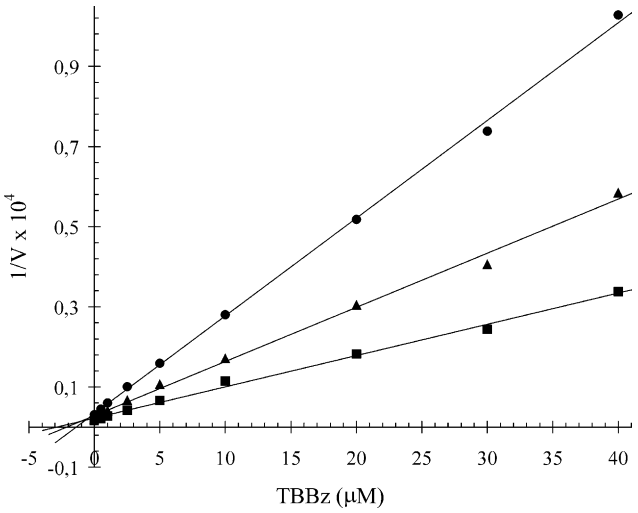


Fig. 2. Dixon plots for inhibition of yeast CK2 by TBBz with ATP concentrations of 10 μM (■), 20 μM (▲), and 40 μM (●).

Table 3
K_i values for inhibition by TBBz and TCBz of protein kinases CK1 and CK2 from various sources

Kinase	K_i (μM)		
	TBBz	TCBz	TBBt[a]
S. cerevisiae CK1	54.4	84.6	120
S. cerevisiae CK2	0.5	18.7	0.58
Rat liver CK1	64.2	73.9	39
Rat liver CK2	0.7	21.0	0.64
C. tropicalis CK2	0.9	18.2	
N. crassa CK2	1.1	23.1	

[a] Data from [7].

much less hydrophobic than TBBt, and this probably accounts for the fact that it is far less effective than TBBt (Table 2). It is also a weak inhibitor of yeast CK1 and virtually inactive vs. PK60S (Table 2).

Inhibition constants, K_i were then determined for the two new halogen analogs vs. CK1 and CK2 from different sources, with the use of Dixon plots, one example of which is shown in Fig. 2. Inhibition of all four CK2 enzymes from widely divergent sources by TCBz and TBBz is competitive with respect to ATP, as previously noted with respect to ATP or GTP for TBBt with the mammalian and yeast enzymes [7]. The calculated K_i values are listed in Table 3.

Discussion

TBBt was previously shown to be a potent and selective ATP-competitive inhibitor of CK2 from yeast, rat liver, Krebs II mouse ascites cells [8], as well as the catalytic CK2α subunit of *Zea mays* [3]. The present results demonstrate that the structurally related TBBz is an equally effective ATP-competitive inhibitor of CK2 from such widely divergent sources as yeast, rat liver, *C. tropicalis*, and *N. crassa*. The potency of TBBz, with K_i values in the range 0.5–1 μM, is similar to that for TBBt, pointing to the key role of the tetrabromobenzene moiety of the two compounds. This is further underlined by the observation that TCBz is a much less effective inhibitor than TBBz (Table 3), as previously noted for TCBt relative to TBBt [8].

The foregoing is readily accounted for by the crystal structure of the complex of TBBt (also referred to as TBB) with the CK2α subunit from *Z. mays* [9]. In this structure, TBBt occupies a hydrophobic pocket in the active site, normally occupied by the adenine moiety of the cosubstrate ATP. In addition, the ring N(1) or, because of symmetry, N(3) of the triazole ring is hydrogen bonded to a water molecule which, in turn, interacts with an additional solvent molecule that is hydrogen bonded to the Oε2 of Glu81.

It should be noted that TBBt may exist in two major tautomeric forms (Fig. 3). In one of these the triazole H

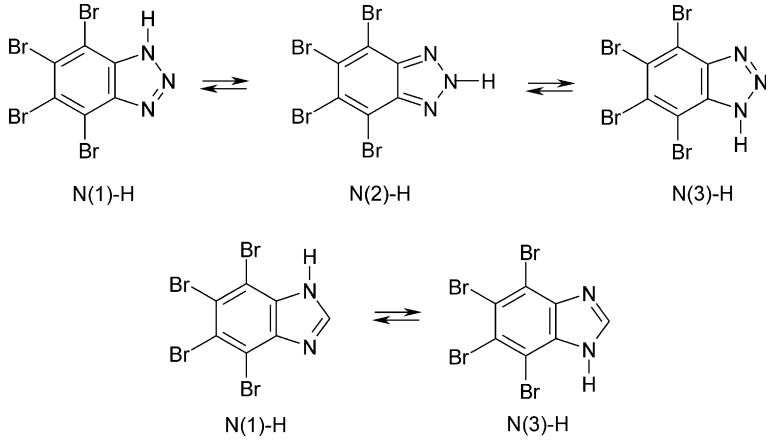


Fig. 3. Prototropic tautomerism of tetrabromo benzotriazoles and benzimidazoles. Note that, because of symmetry, the N(1)–H and N(3)–H tautomers are equivalent.

is located on the ring N(1) or, because of symmetry, on N(3). In the other tautomer the proton is located on the triazole ring N(2). Such prototropic tautomerism of the parent non-halogenated benzotriazole has been extensively investigated, and it is the N(2)–H tautomer which predominates in the gas phase, whereas only the N(1)–H/N(3)–H tautomer is observed in solution and in the solid state [25]. It is, consequently, of interest that, in the crystal structure of CK2α with TBBt, the latter is constrained to bind as the N(2)–H tautomer.

Bearing in mind that the K_i values for TBBt and TBBz are similar, that both are ATP-competitive, and that the corresponding TCBt and TCBz are both much poorer inhibitors, it is clear that TBBz must bind to CK2 as described for TBBt, above [9]. TBBz is therefore an additional lead compound in searches for more effective inhibitors, e.g., by attachment of various substituents at C(2) of the imidazole ring. A further promising approach would involve replacement of the imidazole ring by a pyrazole or pyrrole ring.

One unexpected, and significant, difference between TBBt and TBBz are their inhibitory activities vs. the yeast PK60S, which phosphorylates, both in vitro and in vivo, three of the five pp13 kDa ribosomal surface acidic proteins in yeast cells [8]. No counterpart of this enzyme has hitherto been detected in mammalian cells. It was previously shown that TBBt is a more effective inhibitor of PK60S ($K_i \approx 0.1\,\mu M$) than of yeast CK2 ($K_i \approx 0.6\,\mu M$). In striking contrast, TBBz is a relatively weak inhibitor of PK60S (Table 2), hence more selective vs. CK2 than TBBt, at least in yeast cells.

Acknowledgments

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