Vol. 208, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 8, 1995 Pages 418-424

HALOGENATED BENZIMIDAZOLES AND BENZOTRIAZOLES AS SELECTIVE INHIBITORS OF PROTEIN KINASES CK I AND CK II FROM *SACCHAROMYCES CEREVISIAE* AND OTHER SOURCES**

Ryszard Szyszka,[a] Nikodem Grankowski,[a] Krzysztof Felczak[b] and David Shugar[b,*]

[a]Department of Molecular Biology, Institute of Microbiology, Maria Curie-Skłodowska University, 19 Akademicka St., 20-033 Lublin (Poland)

[b]Institute of Biochemistry & Biophysics, Polish Academy of Sciences, 5a Pawińskiego St., 02-106 Warszawa (Poland)

Received February 1, 1995

Abstract - Several halogeno benzimidazole riboside inhibitors of animal and plant protein kinases CK I and CK II (also known as casein kinases I and II), were found to be effective inhibitors of *Saccharomyces cerevisiae* CK II, but not of the 27-kDa CK I or the 45-kDa CK I. The previously reported 5,6-dichloro-2-azabenzimidazole, which preferentially inhibits plant CK II relative to CK I, discriminates even more effectively between the yeast CK I and CK II enzymes. Two new analogues, tetrahalogeno-2-azabenzimidazoles, are even more potent inhibitors of CK II and much less so of CK I from yeast and animal sources. All inhibitors are competitive with respect to ATP (and GTP with CK II), the two latter with K_i values in the range 0.2 - 0.6 μM for CK II from yeast and mammalian sources.

The ubiquitous protein kinases CK I and CK II (hitherto referred to as casein kinases I and II) recognize Ser/Thr residues in acidic proteins like casein, although the latter is not a physiological substrate. Natural substrates include components of transcription and translation systems, non-histone nuclear proteins, glycogen synthase, topoisomerases, etc. CK I type kinases are monomeric, with molecular masses in the range 20-60 kDa, and utilize ATP as the phosphate donor. The type II enzymes are oligomers with M_r about 130 kDa, usually tetramers with $\alpha\alpha'\beta_2$ or $\alpha_2\beta_2$ form, where α and α' are the catalytic subunits, with either ATP or GTP as phosphate donor (1, 2). The yeast *Saccharomyces cerevisiae* has been shown to contain at least two monomeric CK I type enzymes, one 43 kDa (3, 4), the other 27 kDa (5, 6; but see below); and a type II kinase comprised of four different subunits: 42, 41, 35 and 32 kDa (7, 8) with M_r about 150 kDa.

**Dedicated to the late Professor Eugeniusz Gasior (1930 - 1993), a pioneer in research on yeast CK kinases.

* Corresponding author. Fax: (48)-39-12-16-23; E-mail: SHUGAR@ASP.BIOGEO.UW.EDU.PL.

0006-291X/95 $5.00

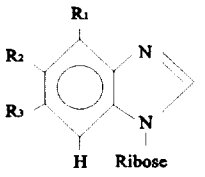
In contrast to many other protein kinases, relatively little attention has been devoted to development of inhibitors of CK kinases (9), notwithstanding the large number of natural substrates for these enzymes *in vitro* (more than 100 for CK II, according to L. A. Pinna). Potent inhibition by polyamines such as heparin (1, 2) has long been a diagnostic tool for CK II, but the high molecular weights of such polyanions, coupled with their high negative charge, limits their applicability to intact cells; while several important exceptions to such behaviour have been noted (9).

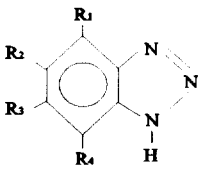
The finding that 5,6-dichloro-1-(ß-D-ribofuranosyl)benzimidazole (DRB, see Scheme 1), a specific inhibitor of eukaryotic mRNA transcription, inhibits in a parallel manner the activity of mammalian CK II, and that inhibition of transcription may be partially reversed by an excess of CK II, pointed to involvement of a phosphorylation step in mRNA transcription, and to DRB as a good inhibitor of CK II (10). A subsequent study with halogenated DRB analogues demonstrated that these specifically inhibit both CK I and CK II, but not a variety of other kinases (11). Several of the analogues exhibited weak discrimination between the two enzymes. Extension of these studies to the corresponding plant enzymes showed that DiCl-2-azaBz (Scheme 1) is a 10-fold better inhibitor of CK II than of CK I (12).

We have now extended these studies to the CK enzymes of *S. cerevisiae*, and describe also two new halogenated 2-azabenzimidazoles (benzotriazoles), both more potent inhibitors and with much better discrimination between CK I and CK II from different sources.

MATERIALS AND METHODS

The two yeast CK I (45 kDa and 27 kDa) enzymes were purified to apparent homogeneity as elsewhere described (4, 5). CK II from yeast, and from rat liver, were prepared according to Szyszka et al. (13), followed by heparin-Sepharose column chromatography. Highly purified CK II from Krebs II mouse ascites tumour cells was obtained by the procedure of Grankowski et al. (14). Rat liver CK I was isolated according to Meggio et al. (15), and yeast cAMP-dependent protein





R_2	R_3	Compound
Cl	Cl	DRB
Br	Br	DiBr-DRB
R_1	Cl	4-Cl-DRB

R_1	R_2	R_3	R_4	Compound
H	Cl	Cl	H	DiCl-2-azaBz
Cl	Cl	Cl	Cl	TetraCl-2-azaBz
Br	Br	Br	Br	TetraBr-2-azaBz

Scheme 1. Structures of halogenated benzimidazole ribosides (left) and halogenated 2-azabenzimidazoles or benzotriazoles (right).

kinase (PKA) according to Hixson et al. (16). Yeast protein kinase C (PKC) was isolated by the procedure of Jimenez et al. (17), followed by elution from DEAE-cellulose at 90 mM NaCl.

Syntheses of the halogeno benzimidazole nucleosides have been elsewhere described (18, 19). We are indebted to Dr. Z. Kazimierczuk for a sample of DiCl-2-azaBz. The 4,5,6,7-tetrachloro- and tetrabromo- benzotriazoles were prepared as described by Büchel (20). All compounds (Scheme 1) were chromatographically homogeneous, and their structures established by various criteria, including NMR spectroscopy. We are particularly indebted to Dr. R. L. Tolman (21) for identification of the 4,5,6-trichloro derivative of DRB (4-Cl-DRB, Scheme 1).

ATP, casein, histones H1 and H2A were products of Sigma (St. Louis, MO., USA); and [γ-^{32}P]ATP (sp. act. 5000 Ci/mmol) was from the Institute of Biochemistry & Biophysics PAN (Warsaw), [γ-^{32}P]GTP was from Amersham (Little Chalfont Buckinghamshire, UK).

CK activities, in the presence and absence of inhibitors, were routinely assayed as elsewhere reported (4, 7), with casein as substrate and labelled ATP (or GTP with CK II) as donor. Stock solutions of inhibitors consisted of 2 mg/ml ethanol, subsequently appropriately diluted with water or buffer solution to desired concentrations. Assays of PKA and PKC were conducted as described by Hixson et al. (16) and Jimenez et al. (17), respectively.

RESULTS AND DISCUSSION

The three yeast enzymes were initially tested for inhibition by the six selected compounds shown in Scheme 1, each at 20 μM, with ATP as donor. The results are listed in Table 1. Somewhat unexpected was the observation that DRB and DiBr-DRB, previously shown to inhibit both CK I and CK II from mammalian and plant (maize seedling) sources (11, 12), were virtually without effect on the two yeast CK I enzymes under conditions where there was significant inhibition of yeast CK II (Fig. 1).

As previously noted for rat liver and plant CK II (11, 12), DiBr-DRB is appreciably more effective than DRB *vs* yeast CK II. By contrast, the apparent lack of inhibition of both yeast CK I enzymes by DiCl-2-azaBz, which is a good inhibitor of yeast CK II (Fig. 1), is consistent with results for the corresponding plant enzymes, where K_i is 10-fold higher for CK I than for CK II (12).

Table 1. Inhibition of the yeast protein kinases CK I (45 kDa), CK I (27 kDa) and CK II by the halogeno benzimidazole ribosides and 2-azabenzimidazoles shown in Scheme 1. 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 20 μM. Activities are relative to that in absence of inhibitor.

Inhibitor	Activity (%)		
	CK I (45 kDa)	CK I (27 kDa)	CK II
DRB	103	102	65
DiBr-DRB	101	99	27
4-Cl-DRB	110	100	37
DiCl-2-azaBz	108	112	31
TetraCl-2-azaBz	99	98	10
TetraBr-2-azaBz	82	74	6

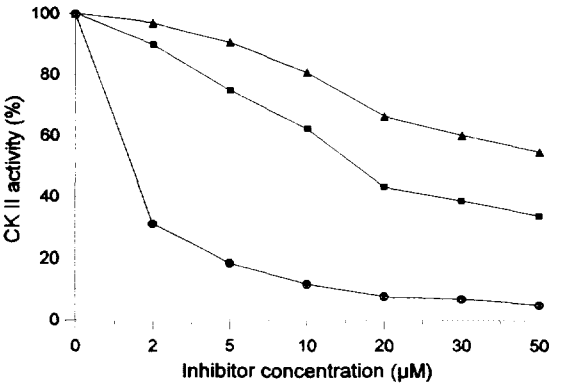


Fig. 1. Inhibition of yeast CK II activity by increasing concentrations of (-▲-▲-▲-) DRB; (-■-■-■-) DiCl-2-azaBz; (-●-●-●-) TetraBr-2-azaBz. Enzyme activity monitored with 1.5 mg casein and 10 μM ATP in 20 mM Tris-HCl buffer, pH 7.5.

With the two new analogues, TetraCl-2-azaBz and TetraBr-2-azaBz, the former was without apparent effect on the CK I enzymes, but was a potent inhibitor of CK II. The tetrabromo congener only weakly inhibited the CK I enzymes (Table 1), but was even more potent *vs* CK II (Fig. 1) than its tetrachloro derivative (Table 1). TetraBr-2-azaBz appears, in fact, to be the most potent inhibitor of protein kinase CK II hitherto reported.

A common feature of all six compounds is their very marked discrimination between the yeast CK I and CK II kinases, as previously noted for the for the corresponding plant enzymes with DiCl-2-azaBz (12).

Attention was then directed to the effects of the most potent inhibitor, TetraBr-2-azaBz, on CK I and CK II kinases from other sources. At a concentration of 10 μM, this compound exhibited little activity *vs* the yeast CK I enzymes, and only moderate inhibition of rat liver CK I (Table 2); but was a good inhibitor of CK II from yeast, rat liver and Krebs II mouse ascites cells. Its specificity is further underlined by the fact that it was virtually inactive *vs* PKA and PKC, in line with the earlier demonstration that two other analogues, DRB and DiBr-DRB, are without effect on a variety of protein kinases other than CK I and CK II (11).

Inhibition constants, K_i were then determined for inhibition of the yeast, and several other, kinases by the two tetrahalogeno analogues, with the aid of Dixon plots, two examples of which are shown in Fig. 2, from which it follows that inhibition is competitive with respect to ATP, as previously noted for the mammalian (11) and plant (12) enzymes.

The calculated K_i values are listed in Table 3, which also includes data for two CK II enzymes with the use of GTP as donor.

In contrast to CK II from mammalian sources, for which the K_m values for ATP and GTP are comparable (1, 2), the K_m for GTP with yeast CK II is inordinately high, 55 μM, as compared

Table II. Effect of TetraBr-2-azaBz (10 μM) on the activity of various Ser/Thr protein kinases

Protein kinase	Activity (% of control)
Yeast CK II	19
Yeast CK I (45 kDa)	93
Yeast CK I (27 kDa)	82
Yeast PKA	108
Yeast PKC	103
Rat liver CK II	21
Rat liver CK I	63
Krebs II mouse ascites CK II	18

to 7.5 μM for ATP (7, 8). Nonetheless, the K_i values are virtually identical with either ATP or GTP as donor, as is also the case for Krebs II mouse ascites CK II (Table 3).

CONCLUDING REMARKS

Several points are worthy of additional comment. The two yeast CK I kinases apparently differ from their mammalian and plant counterparts by their negative response to inhibitors such as DRB and DiBr-DRB, and their feeble inhibition by TetraBr-2-azaBz , the most potent inhibitor of CK II from all sources. It follows that all six compounds are specific inhibitors of yeast CK II. It is also clear from Table 3 that the two new tetrahalogeno congeners, particularly TetraBr-2-azaBz, exhibit excellent discrimination between CK I and CK II from mammalian sources, and may

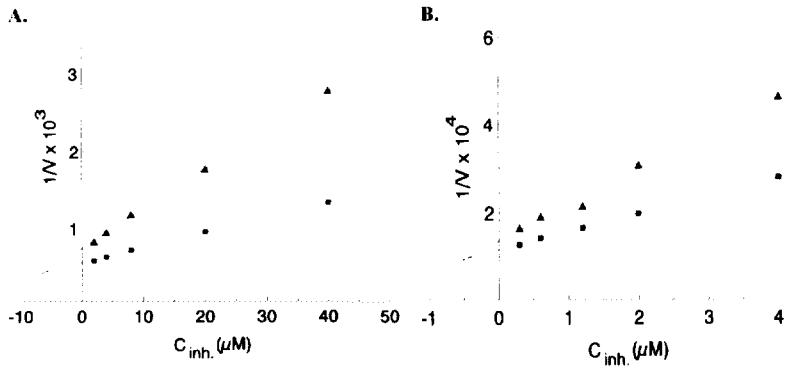


Fig.2. Dixon plots for inhibition of yeast CK II by (A) TetraCl-2-azaBz and (B) TetraBr-2-azaBz, with ATP concentration of 8 μM (-▲-▲-▲-) and 16 μM (-■-■-■-).

Table III. K_i values for inhibition by TetraCl-2-azaBz and TetraBr-2-azaBz of protein kinases CK I and CK II from various sources, with ATP or GTP as donors

Casein kinase	K_i (μM)	
	TetraBr-2- azaBz	TetraCl-2-azaBz
Yeast CK II	0.58 (0.7)*	6.0 (4.7)*
Yeast CK I (45 kDa)	120	260
Yeast CK I (27 kDa)	76	85.2
Rat liver CK II	0.64	4.0
Rat liver CK I	39	64
Krebs II mouse ascites CK II	0.20 (0.30)*	5.3 (3.6)*

*With GTP as donor.

therefore be considered as specific inhibitors of the latter. It will be of interest to determine whether the same applies to the plant enzymes.

There have been sundry reports of the existence of additional CK I-type kinases in yeast, including one apparently involved in DNA repair (22, 23). Vancura et al. (24) refer to "at least three CK I enzymes from yeast" and have reported isolation from yeast of a 56 kDa soluble fragment resulting from proteolysis of the YCK2 gene product, with properties homologous to those of a CK I. The kinase activity of this fragment was inhibited by DiBr-DRB with a K_i of about 30 μM, but was insensitive to the reported selective inhibitor of mammalian CK I, N-(2-aminoethyl)-5-chloro-isoquinoline-8-sulphonamide, also known as CK I-7 (25). The relevance of this to our results awaits an examination of the behaviour of our yeast enzymes towards CK I-7.

Attention should also be drawn to the widely expressed belief that, because of the close sequence homology between the catalytic domains of protein kinases, inhibitors competitive with respect to ATP are unlikely to be specific for a given kinase. Our previous (11) and present findings with benzimidazole and azabenzimidazole inhibitors of CK I and CK II, competitive with respect to ATP (or GTP), do not offer credence to such a belief. We have elsewhere drawn attention to even more striking examples of attainment of very high specificity of suitable designed inhibitors of PKC and a protein tyrosine kinase, notwithstanding that these are competitive with respect to ATP (9). An additional, more recent, example is the development of various purine analogues as inhibitors of cyclin-dependent kinases (26). One of these, 2-(2-hydroxyethylamino)-6-benzylamino-9-methyl-purine (referred to as olomoucine) proved to be a specific inhibitor of several cyclin-dependent kinases, notwithstanding that it is a competitive inhibitor for ATP binding.

ACKNOWLEDGMENT: This investigation was supported by the State Committee for Scientific Research (KBN), Grant No. KBN 662539203p/01.

REFERENCES

1. Tuazon, P. T. and Traugh, J. A. (1991) Ad. Second Messenger Phosphoprotein Res. **23**, 123-164.
2. Pinna, L. A. (1990) Biochim. Biophys. Acta **1054**, 267-284.
3. Lerch, K., Muir, L. W. and Fisher, E. H. (1975) Biochemistry **14**, 2015-2023.
4. Kudlicki, W., Szyszka, R., Palen, E. and Gasior, E. (1980) Biochim.Biophys. Acta **633**, 376-385.
5. Szyszka, R. Kudlicki, W., Grankowski, N. and Gasior, E. (1985) Biochim. Biophys. Acta **838**, 171-174.
6. Donella-Deana, A., Grankowski, N., Kudlicki, W., Szyszka, R., Gasior, E. and Pinna, L. A. (1985) Biochim. Biophys. Acta **829**, 180-189.
7. Kudlicki, W., Szyszka, R. and Gasior, E. (1984) Biochim. Biophys. Acta **784**, 102-107.
8. Padmanabha, R. and Glover, C. V. C. (1987) J. Biol. Chem. **262**, 1829-1835.
9. Shugar, D. (1994) Cellular Mol. Biol. Res., in press.
10. Zandomeni, R.O., Carrera-Zandomeni, M., Shugar, D. and Weinman, R. (1986) J. Biol. Chem. **261**, 3414-3419.
11. Meggio, F., Shugar, D. and Pinna, L. A. (1990) Eur. J. Biochem. **187**, 89-94.
12. Dobrowolska, G., Muszynska, G. and Shugar, D. (1991) Biochim. Biophys. Acta **1080**, 221-226.
13. Szyszka, R., Lopaczynski, W., Galasinski, W., Grankowski, N. and Gasior, E. (1986) Acta Biochim. Polon. **23**, 39-46.
14. Grankowski, N., Boldyreff, B. and Issinger, O.-G. (1991) Eur. J. Biochem. **198**, 25-30.
15. Meggio, F., Donella-Deana, A. and Pinna, L. A. (1981) J. Biol. Chem. **256**, 11958-11961.
16. Hixson, C. S. and Krebs, E. G. (1980) J. Biol. Chem. **255**, 2137-2145.
17. Jimenez, B., Pestana, A. and Fernandez-Renart, M. (1989) Biodchem. J. **260**, 557-561.
18. Kazimierczuk, Z., Stolarski, R., Dudycz, L. and Shugar, D. (1981) Z. Naturforsch. **36c**, 126-134.
19. Kazimierczuk, Z., Stolarski, R. and Shugar, D. (1985) Z. Naturforsch. **40c**, 715-720.
20. Buchel, K. H. (1970) Z. Naturforsch. **25b**, 945-948.
21. Tolman, R. L. & Doss, G. A. (1994) Nucleosides & Nucleotides **13**, 293-295.
22. Gottlieb, T. M. and Jackson, S. P. (1994) TIBS **19**, 500-503.
23. Dhillon, V. and Hoekstra, M. F. (1994) EMBO J. **13**, 2777-2788.
24. Vancura, A., O'Connor, A., Patterson, S. D., Mirza, U., Cheit, B. T. and Kuret, J. (1993) Arch. Biochem. Biophys. **305**,47-53.
25. Hidaka, H., Watanabe, M. and Kobayashi, R. (1991) Methods Enzymol. **201**, 328-339.
26. Vesely, J., Havlicek, L., Strand, M., Blow, J. J., Donella-Deana, A., Pinna, L., Letham, D. S., Kato, J., Detivaud, L., Leclerc, S. and Meier, L. (1994) Eur. J. Biochem. **224**, 771-786.