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## The selectivity of inhibitors of protein kinase CK2. An update.

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Running title: CK2 inhibitors selectivity

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Abbreviations: CK2, casein kinase 2; TBB (TBBt), 4,5,6,7-tetrabromo-1*H*-benzotriazole; TBI (TBBz), 4,5,6,7-tetrabromo-1*H*-benzimidazole; DMAT, 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole.

### Synopsis

CK2 is a very pleiotropic Ser/Thr protein kinase whose abnormally high constitutive activity has been often correlated to pathological conditions with special reference to neoplasia. The two most widely used cell permeable CK2 inhibitors, 4,5,6,7-tetrabromo-1*H*-benzotriazole (TBB) and 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (DMAT), are marketed as quite specific CK2 blockers. Here we show by using a panel of about 80 protein kinases that DMAT and its parent compound 4,5,6,7-tetrabromo-1*H*-benzimidazole (TBI or TBBz) are potent inhibitors of several other kinases, with special reference to PIM1, PIM2, PIM3, PKD1, HIPK2 and DYRK1a. In contrast TBB is significantly more selective toward CK2, although it also inhibits PIM1 and PIM3. In an attempt to improve selectivity toward CK2 a library of 68 TBB/TBI related compounds have been tested for their ability to discriminate among CK2, PIM1, HIPK2 and DYRK1a, ending up with 7 compounds whose efficacy toward CK2 is markedly higher than that toward the second most inhibited kinase. Two of these, 3,4,5,6,7-pentabromo-1*H*-indazole (K64) and 1-carboxymethyl-2-dimethylamino-4,5,6,7-tetrabromo-benzimidazole (K66), display an overall selectivity much higher than TBB and DMAT when tested on an 80 kinases panel and display similar efficacy as inducers of apoptosis.

### Key words

CK2 inhibitors; drug design; selectivity; ATP-mimetics; PIM-1; HIPK2

## Introduction

CK2 (an acronym derived from the misnomer “casein kinase” 2) denotes one of the most pleiotropic Ser/Thr protein kinases, with more than 300 protein substrates already identified [1] and responsible for the generation of a substantial proportion of the eukaryotic phosphoproteome [2]. Such an extreme pleiotropy may, at least partially, account for the observation that CK2 catalytic activity is not controlled by either second messengers, phosphorylation events or association of its catalytic ( $\alpha$  and/or  $\alpha'$ ) with its regulatory  $\beta$ -subunits to give a “constitutively active” heterotetrameric holoenzyme [3,4]. In turn constitutive activity and lack of physiological down-regulatory devices may underlie the pathogenic potential of CK2 whose abnormally elevated levels have been correlated to a number of global diseases, with special reference to neoplasia. Over-representation of CK2 has been documented in a number of cancers including kidney [5], mammary gland [6], lung [7], head and neck [8] and prostate [9] and a number of experimental models and coincidental arguments support the notion that CK2 promotes cell survival through the regulation of oncogenes and plays a global anti-apoptotic role (reviewed by Duncan et al. [10]). Studies aimed at dissecting the multifarious functions of CK2 are hampered by its constitutive activity and limitations in strategies exploited to inhibit the cellular activity of other kinases, notably usage of kinase inactive dominant-negative mutants and RNA interference techniques. These techniques are hindered by long half-life, cellular localization and high expression of endogenous CK2 in cells [10]. Indeed conflicting data about the effectiveness of down-regulation devices (e.g. compare Wang et al. [11] and Seeber et al. [12]) have been reported, suggesting that RNAi or antisense strategies sometimes may be inefficient, requiring continual treatment of cells in order to achieve effective CK2 depletion. These limitations make the pharmacological approach the most promising tool to dissect the cellular functions of CK2 and explain why considerable efforts have been made to develop specific cell permeable CK2 inhibitors (reviewed in Sarno et al. [13]). Some of these inhibitors proved extremely helpful for the identification of CK2 cellular targets and the implication of CK2 in specific cellular functions, as well as in malignancy and defective apoptosis of cancer cells [10,13,14]. In a few cases the concept that the observed pharmacological effect was actually mediated by CK2 inhibition was validated by transfecting the cells with CK2 mutants refractory to inhibitors [15,16]. Generally, however, the implication of CK2 was inferred from the assumption that the compounds used are “specific” inhibitors of this kinase. In this respect the most successful commercially available “specific” CK2 inhibitors are: i) TBB (4,5,6,7-tetrabromo-1*H*-benzotriazole) also termed TBBt to distinguish it from its 4,5,6,7-tetrabromo-1*H*-benzimidazole analogue (TBI or TBBz), also a potent inhibitor of CK2 [17], and, ii) DMAT (2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole). The specificities of TBB and DMAT, though not that of TBI, were assessed on a panel of about 30 protein kinases none of which was inhibited as drastically as CK2 [18,19]. In contrast other commercially available CK2 inhibitors, notably quercetin, apigenin and emodin didn't display a comparable selectivity [20,21]. Recently, however, the panel of protein kinases currently available for running selectivity profiles has been substantially enlarged, to include about 80 kinases representative of nearly all the branches of the kinome (see Fig. 1). This has provided a tool for updating the selectivity of kinase inhibitors and has led to the discovery of “new” targets for “old” inhibitors [22]. This study didn't include any CK2 “specific inhibitor”. On the other hand the solution of the crystal structure of the complex between CK2 and TBI/TBBz [23] has allowed a comparison with the structures of the complexes with TBB [24] and with DMAT [25] leading to the conclusion that despite their structural similarities TBB binds in a quite different manner from TBI and DMAT. Altogether these observations prompted us to profile the selectivity of TBB, DMAT and TBI on the new large panel of protein kinases now available. The results,

presented here, support the view that the selectivity of TBB for CK2 is narrower than those of DMAT and TBI/TBBz which also are powerful inhibitors of kinases belonging to the PIM, HIPK, DYRK and PKD families, and led to the identification of new and more selective inhibitors of CK2.

## Experimental

*Enzymes.* Native CK2 (nCK2) was purified from rat liver [26]. Human recombinant  $\alpha$  and  $\beta$  subunits of CK2 were expressed in *E. coli* and the holoenzyme was reconstituted and purified as previously described [27]. Single and double mutants of CK2 $\alpha$  subunit were generated as reported in [19, 20]. The source of all the other protein kinases used for specificity assays is as previously either described or referenced [22].

*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, 2mM L-glutamine, 100 units/ml penicillin and 100  $\mu$ g/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, then incubated at 37 °C, in the presence of the compounds at the indicated concentrations. Control cells were treated with equal amounts of solvent (0.5 % v/v DMSO). At the end of incubations, cells were lysed by the addition of hypo-osmotic buffer, as previously described [20]. Cell viability was assessed by means of 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltriazolium bromide (MTT) reagent, while apoptosis was evaluated by means of the Cell Detection Elisa kit (Roche), based on the quantification of nucleosomes present in the cytosol of the apoptotic cells, following the manufacturer instructions. 10000 cells were used for each determination.

*Phosphorylation assays.* CK2 activity was tested in a final volume of 25  $\mu$ l containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 12 mM MgCl<sub>2</sub>, 100  $\mu$ M synthetic peptide substrate RRRADDSDDDDD and 0.02 mM [ $\gamma$ -<sup>33</sup>P-ATP] (500-1000 cpm/pmole), unless otherwise indicated, and incubated for 10 minutes at 37 °C. Native CK2 purified from rat liver (0.5-1 pmol) was usually the phosphorylating enzyme, unless otherwise indicated. Assays were stopped by addition of 5  $\mu$ l of 0.5 M orthophosphoric acid before spotting aliquots onto phosphocellulose filters. Filters were washed in 75 mM phosphoric acid (5-10 ml/each) four times then once in methanol and dried before counting.

PIM1 activity was determined by following the same procedure by incubating the kinase in the presence of 50 mM Tris-HCl pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM EGTA, 30  $\mu$ M synthetic peptide substrate RKRRQTSMTD [29] and 100  $\mu$ M [ $\gamma$ -<sup>33</sup>P-ATP]. HIPK2 activity was determined under the same conditions used for PIM1 assays except for ATP which was 20  $\mu$ M and the use of MBP (10  $\mu$ g) as phosphorylatable substrate.

Conditions for the activity assays of all other protein kinases tested in selectivity experiments are described or referenced elsewhere [22].

*Kinetic determination.* Initial velocities were determined at each of the substrate concentration 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. Alternatively inhibition constants were also deduced from the  $IC_{50}/K_i$  Cheng-Prusoff relationship [28] by determining  $IC_{50}$  for each compound at 1  $\mu$ M ATP concentration, i.e. much below  $K_m$  value. The rationale underlying this approach, based on

the assumption that inhibition is competitive with respect to ATP, is illustrated by Burlingham and Widlanski [29].

#### *Molecular modelling.*

Human CK2 $\alpha$  subunit and PIM1 catalytic subunit were retrieved from the PDB (PDB code: 1JWH and 3C4E, respectively) and processed in order to remove the ligands and water molecules. Hydrogen atoms were added to the protein structure using standard geometries with the MOE program. To minimize contacts between hydrogen's, the structures were subjected to Amber94 force field minimization until the rms of conjugate gradient was  $<0.1$  kcal mol $^{-1}$  Å $^{-1}$  keeping the heavy atoms fixed at their crystallographic positions. To strictly validate the model generated and to calibrate the high-throughput docking protocol, a small database of known CK2 and PIM1 inhibitors was built and a set of docking runs was performed. After the calibration, phase flexible ligand-docking steps with three different programs, MOE-Dock, Glide, and Gold were performed essentially as previously described [30].

*Synthesis of new analogues.* All chemicals and solvents used for the syntheses were purchased from Sigma Aldrich. Details for the synthesis of most benzimidazoles and benzotriazoles have been described elsewhere [19,25,31,32]. New derivatives discussed in this paper have been synthesized as follows.

The synthesis of 4,5,6,7-tetrabromo-1*H*-indazole (**1**, **K59**) was realized by the ring closure of the intermediate 3,4,5,6-tetrabromo-2-methyl-N-nitroso-N-acetylaniline [33]. The bromination of **1** was performed using bromine in anhydrous acetic acid, at elevated temperature, yielding the pentabromoderivative **4** (**K64**). Alkylation of **1** with 2-bromoethanol and 2-dimethylaminoethylchloride provided, as expected, two regioisomers each, **2a, b** (**K60, K62**) and **3a, b** (**K61, K63**), respectively. The mixtures of N $^1$ - and N $^2$ -substituted derivatives were separated by flash chromatography and the structures of the derivatives were determined by comparing their  $^1$ H NMR- and UV-spectra to those of the respective known N-substituted indazoles [34,35]. Dissociation constants of **1** (10.6) and **4** (8.40) measured in 50% aqueous ethanol showed the inductive effect of bromine substituents by lowering the respective pK $_a$  value by several units as compared to that of unsubstituted indazole (14.0). A similar effect was observed for TBB (pK $_a$  = 4.95) and 4,5,6,7-tetrabromobenzimidazole (pK $_a$  = 8.95) [17]. Similarly to tetrabromobenzimidazole, the two brominated indazoles **1** and **4** described here are practically neutral molecules at physiological pH (Scheme 1).

The synthesis of 1-substituted acetic acid ethyl ester of 2-dimethylamino-4,5,6,7-tetrabromobenzimidazole **6** (**K67**) was performed by N-alkylation of DMAT (**5**) with bromoacetic acid ethyl ester in acetone in presence of anhydrous K $_2$ CO $_3$  as base. The alkaline hydrolysis of **6** in ethanol-water solution gave the N $^1$ -carboxymethyl derivative of DMAT (**7**, **K66**) (Scheme 2).

The other modified tetrabromobenzimidazoles were obtained from 1-methyl-4,5,6,7-tetrabromobenzimidazole (**8**) as substrate. The heating of **8** with thiourea in 1-propanol gave respective 2-mercapto derivative **9** (**K72**). Alkylation of **9** with bromoacetic acid ethyl ester yielded 2-sulfanylacetic acid ethyl ester **10**, which was substrate for further modifications. Aminolysis of **10** with methylamine gave respective N-methylamide (**11**, **K74**), whereas alkaline hydrolysis yielded 1-methyl-4,5,6,7-tetrabromobenzimidazole-2-sulfanylacetic acid (**12**, **K75**). (Scheme 3).

## Results

### Re-evaluation of TBB, DMAT and TBI selectivity.

TBB (4,5,6,7-tetrabromobenzotriazole) and its 2-dimethylamino derivative DMAT are among the most widely used, commercially available inhibitors of CK2. At variance with other compounds used to inhibit CK2, notably DRB and apigenin, both TBB and DMAT are believed to be very specific based on selectivity profiles run on panels of 30 and 33 protein kinases, respectively [18,19]. In the meantime, however, the size of the panel of kinases available for testing inhibitors' specificity has more than doubled [22] including now-a-days representatives of nearly all subfamilies of the kinome (Fig. 1). This prompted us to re-evaluate the selectivity of TBB and DMAT toward CK2. Since DMAT shares its benzimidazole scaffold with the parent compound TBI (or TBBz) (see Fig. 2) also marketed as a CK2 inhibitor though its selectivity was not profiled before, TBI was also included in our analysis.

The residual activity of a panel of 76 protein kinases tested in the presence of 10  $\mu\text{M}$  TBB, DMAT and TBI and expressed as per cent of their activity in the absence of inhibitors is reported in Table 1 or mentioned in its legend. Even a cursory scrutiny of the data reveals that CK2 is not the only protein kinase drastically affected, several other kinases (values bold typed in Table 1) being more than 90% inhibited by one or more of the three compounds. A more detailed analysis allows the following conclusions to be reached: 1) Although the selectivity spectra of TBB, DMAT and TBI are similar they are not super-imposable, denoting higher similarity between DMAT and TBI (characterized by an imidazole ring) both of which also tend to be more promiscuous than TBB (composed instead of a triazole ring). This point is highlighted by drastic inhibition of PKD1 and CDK2/CyclinA by either DMAT or TBI, in contrast to fairly high residual activity observed in the presence of the same concentration of TBB. 2) There are three subfamilies of protein kinases, none of which was represented in the 30 protein kinases panel originally used to define the TBB specificity [18] whose components tend to be inhibited as drastically as CK2 by all the three compounds at 10  $\mu\text{M}$  concentration, with residual activities below 10%; these are DYRKs (1a, 2 and 3), PIMs (1, 2, 3) and HIPK2. A >90% inhibition of HIPK3 is also observed by DMAT and TBI, while TBB is significantly less effective in this respect.

More detailed information about the relative efficiency of each inhibitor toward protein kinases whose activity is 90% or more inhibited by 10  $\mu\text{M}$  concentration was obtained by determining their  $\text{IC}_{50}$  values at 50  $\mu\text{M}$  ATP. These data are reported in Table 2 and they strengthen the notion that among the three inhibitors the most selective one toward CK2 is TBB with a  $\text{IC}_{50}$  value (0.15  $\mu\text{M}$ ) 3-5 fold lower than those of DYRK2, PIM1 and PIM3, and one order of magnitude or more below those of the other kinases. In contrast TBI appears to be the least selective toward CK2, as it inhibits PIM3, PIM1, PIM2, DYRK2 and PDK1 more efficiently than CK2 itself, and HIPK2 and CDK2 with comparable efficiency. This is partially due also to its relatively high  $\text{IC}_{50}$  value with CK2 (0.60  $\mu\text{M}$ ).

The overall superiority of TBB over DMAT is also highlighted by selectivity profiles performed with 1  $\mu\text{M}$  instead of 10  $\mu\text{M}$  inhibitor concentration (last two columns on the right in Table 1). Under these conditions the number of protein kinases inhibited  $\geq 50\%$  are 3 and 12 with TBB and DMAT, respectively (values underlined in Table 1). Note moreover that 7 out of the 12 PKs  $\geq 50\%$  inhibited by DMAT are inhibited as drastically as CK2 itself.

### DMAT as a powerful inhibitor of kinases other than CK2.

Although the  $\text{IC}_{50}$  value with CK2 is quite low (0.13  $\mu\text{M}$ ) DMAT displays submicromolar  $\text{IC}_{50}$  values with almost all the other kinases in Table 2, with special reference to PKD1, PIM3 and PIM1 whose  $\text{IC}_{50}$  value (148 nM) is comparable to that of the most potent PIM1 inhibitor (BIM-1) reported to date [36].

Similar to CK2 [19] PIM1 is also inhibited by DMAT by a mechanism which is competitive with respect to ATP, as shown by the double reciprocal plot reported in Fig. 3A. However,  $IC_{50}$  determinations at increasing ATP concentration denote weak competition by ATP which, at almost physiological concentration (0.6 mM), causes only a 5.3-fold decrease in DMAT inhibition, as compared to 1  $\mu$ M ATP concentration, while in the same range of ATP concentration the  $IC_{50}$  with CK2 increases 22.1-fold, doubling the value calculated with PIM1 (1.2  $\mu$ M) (Fig. 3B). It is expectable therefore that in the cellular environment (ATP  $\geq$  1 mM) PIM1 will represent the preferred target of DMAT, among all protein kinases probed in this respect.

### Improvement of selectivity toward CK2

The observation that CK2 inhibitors tend to inhibit with comparable efficacy at least three other groups of kinases, notably PIMs, HIPK2 and DYRKs, prompted us to search for more selective compounds in the available library of TBB/TBI derivatives. This presently includes besides several dozens of tetrabromobenzimidazole congeners previously described [19,32] also a number of newly developed compounds with a pyrazole penta-atomic ring replaced for the imidazole one, whose prototype K59, 4,5,6,7-tetrabromoindazole, is shown in Fig. 2 together with its triazole (TBB) and imidazole (TBI) congeners. All the compounds of the library (68 altogether) were tested at 20  $\mu$ M concentration on CK2, PIM1 and HIPK2. The assays were run at ATP concentrations around the  $K_m$  values of the kinases (20  $\mu$ M for CK2 and HIPK2, 100  $\mu$ M for PIM1) and the residual activities are on display in Table S2 (see supplementary data).

Focusing our attention only on compounds highly effective on CK2 (residual activity  $\leq$  15%, bold typed in the table) we observed that most of these are equally or even more potent inhibitors of PIM1, and two actually inhibit HIPK2 even more efficiently than CK2. A few compounds, however, namely K36, K59, K60, K62, K64, K66, K74 and K75, whose structures are shown in Table 3, displayed toward CK2 an inhibitory efficacy significantly higher than those observed with either PIM1 and HIPK2.

The  $K_i$  values of these compounds with respect to CK2, PIM1, HIPK2 and DYRK1a were calculated (Table 3). In all cases CK2 displays the lowest  $K_i$  value as compared to the other three protein kinases. In particular two compounds, K59 and K64 display  $K_i$  values below 100 nM with CK2 while their  $K_i$  values with the second most sensitive protein kinase, which is PIM1 in both cases, are several fold higher. Both K59 and K64 are characterized by a pyrazole ring replaced for the triazole/imidazole rings of TBB and DMAT. K64 also contains a 5<sup>th</sup> bromine atom on the pyrazole ring in addition to the 4 bromine atoms on the benzene ring which are a common denominator of all TBB derivatives described so far. Three other promising compounds belong to the benzimidazole class: these are K66, K74 and K75. In this case the  $K_i$  calculated with CK2 is somewhat higher than 100 nM: this disadvantage however is amply compensated by proportionally higher  $K_i$  values with PIM1, HIPK2 and DYRK1a. We focused further attention on those compounds whose  $K_i$  values with CK2 are 10-fold or more lower than those with the second most inhibited kinase, notably K64, K66, K74 and K75.

We firstly investigated if and to what extent the double mutation of CK2 residues Val-66 and Ile-174 to alanine, previously reported to decrease susceptibility to TBB and DMAT [19,20], also affected inhibition by the new compounds. This is clearly the case, as shown in Table 4; however responsiveness to double mutation is sharply variable: two inhibitors, K74 and K75, are somewhat more sensitive than TBB and DMAT to the double mutation whereas K66 is almost insensitive to it, with a just 6-fold increase in  $IC_{50}$  value. By sharp contrast the pentabrominated inhibitor K64 has lost almost all its efficacy toward the CK2 double



mutant, with a 600-fold raise in  $IC_{50}$ . These differences clearly reflect diverse modes of binding of these compounds, as further indicated by the variable efficacy of the two single mutations, that of Val66 and that of Ile174. In particular the role of Ile174 is crucial for the binding of K64 whereas the binding of K66 relies more on interactions with Val66 as also confirmed by modelling (see Discussion).

The behaviour of K66 attracted our attention because its scaffold is identical to that of DMAT, having in addition a short carboxylic side chain adjacent to the imidazole ring nitrogen (compare structures in Fig. 2). While this carboxylic adduct is still compatible with fairly potent inhibition of CK2 it makes its binding almost independent of Ile174, while more dependent on Val66 (Table 4), and, more important, it drastically reduces the ability to inhibit PIM1 and HIPK2 which are instead dramatically sensitive to DMAT (see Table 2).

Once ascertained that the new CK2 inhibitors considered in Table 3, with special reference to K64, K66, and K75 have lost most of their potency toward those protein kinases, notably PIM1, HIPK2 and DYRK1a which display the tendency of being susceptible to CK2 inhibitors, we wanted to make sure that their overall selectivity was preserved. This analysis was performed on a panel of 78 protein kinases almost completely but not entirely overlapping the one of 70 protein kinases used for the screening presented in Table 1. The data are shown in Fig. 4. For sake of comparison also the data relative to DMAT are reported. The most remarkable outcome is indeed the dramatic gain of selectivity resulting from the derivatization of DMAT with a carboxymethyl side chain to give K66 (Fig 4): while, as noted above, DMAT under these conditions inhibits  $\geq 50\%$  34 kinases out of 70 (i.e. almost 50%), this figure with K66 drops to just 5 kinases out of 78. Only 2 of these, moreover, a part from CK2, are drastically inhibited ( $\geq 80\%$ ) by K66: ERK8, and DYRK2. However, the  $IC_{50}$  values for inhibition of DYRK2 is far above that of CK2 (8  $\mu\text{M}$  as compared to 0.25, see legend of Fig. 4). Consequently the only kinase whose inhibition by K66 is comparable to that of CK2 is ERK8 ( $IC_{50}=0.50 \mu\text{M}$ ) thus placing K66 in the category of “dual kinase inhibitors” able to block with equal efficiency two unrelated kinases. Note in this respect that it would be easy to discriminate between ERK8 and CK2 using the inhibitor BAY439006 which at 10  $\mu\text{M}$  concentration suppresses ERK8 activity without significantly affecting CK2 (27).

The overall selectivity of K64 (Fig. 4) is also good, with 6 protein kinases (including CK2) inhibited  $\geq 50\%$  by 10  $\mu\text{M}$  inhibitor. One of these is again ERK8, but in this case it is inhibited significantly less than CK2. The same applies to PIM2, PIM3, HIPK2 and PKD1, whose  $IC_{50}$  values accordingly are at least one order of magnitude higher than that of CK2.

Due to solubility problems the selectivity of K75 was profiled at 1  $\mu\text{M}$  concentration (Fig. 4). Under these conditions only 3 kinases are  $\geq 50\%$  inhibited: CK2 and PIM3 (both 70% inhibited), and MSK1 (54% inhibited).

K74 turned out to be unstable when kept for >2 hrs in DMSO solution, a circumstance which prevented its robotic profiling on the panel of protein kinases and probably also seriously hampers its usage for in cell experimentation.

### Pro-apoptotic efficacy of K64 and K66

In the light of the data above the first choice novel CK2 inhibitors worthy to be tested on cell cultures were K64 and K66, whose selectivity is comparable. The former is somewhat more potent but the latter has the advantage that its only known target inhibited as drastically as CK2, ERK8, is absent in hematopoietic cells [37], which are those most frequently used for testing the pro-survival and oncogenic potential of CK2 (e.g. [16,38-40]). An added value of K64 is instead its almost complete loss of efficacy toward the CK2 double mutant V66/I174AA (see Table 4), a property which can be exploited to provide the proof of concept

that the cellular effects of a given inhibitor are actually mediated by CK2 rather than by other cellular targets.

As shown in Fig. 5A both K64 and K66 display cytotoxic activity comparable to that of TBB. Interestingly the cytotoxic efficacy of the three compounds roughly correlates to their potency as CK2 inhibitors calculated from their  $K_i$  values (0.048, 0.12 and 0.25 for K64, TBB and K66, respectively). It should be also noted that while in principle the cytotoxic effect of TBB could be accounted for by the inhibition of protein kinases of the PIM and DYRK families having  $IC_{50}$  values close to that of CK2 (Table 2), this doesn't apply to K64 and K66 (see above). As shown in Fig. 5B the cytotoxic effect of K64, similar to that of TBB [40] and of K66 is, at least partially, due to apoptosis as judged from the quantification of nucleosomes present in the cytosol of the apoptotic cells: these in cells treated with K64 5  $\mu$ M are comparable to those induced by etoposide 25  $\mu$ M.

## Discussion

Considering that the human kinome is composed by more than 500 members whose catalytic site is highly conserved and that the most promising cell permeable protein kinase inhibitors are competitive with respect to the universal kinase co-substrate ATP, it is understandable that the development of really specific protein kinase inhibitors is a formidable challenge. While absolute selectivity may be not required for a kinase inhibitor to become a valuable drug and some promiscuity could even be an advantage in terms of therapeutic efficacy, as exemplified by clinical practice with Imatinib/Gleevec [41], lack of specificity is always a serious drawback whenever inhibitors are used to dissect the biological functions of a given protein kinase, where the unwanted blockage of other kinases may generate misleading results. This shortcoming can be attenuated by using more than one inhibitor of the same kinase and can be circumvented by the generation and cell transfection of kinase mutants which are refractory to a given inhibitor. Since however clusters of kinases often tend to be inhibited by the same classes of inhibitors and cell transfection not always is feasible and is likely to alter anyway cell functionality, the availability of pharmacological tools that are as selective as possible, invariably represents a critical advantage for dissecting signaling pathways. This is especially true in the case of protein kinases like CK2 which are constitutively active and cannot be easily up or down-regulated within the cell using physiological effectors.

In the past considerable efforts have been made to develop CK2 specific inhibitors, belonging to different chemical classes (reviewed by Sarno et al. [13,14]). Apart from apigenin, a flavonoid devoid of narrow selectivity [20,42], the two most successful CK2 inhibitors were both derived from the nucleoside DRB (5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole), a rather weak CK2 inhibitor [43], by deleting its sugar moiety and replacing the chlorine and hydrogen atoms of its benzene ring with 4 bromine atoms. These were shown to be critical to encapsulate the inhibitor in a hydrophobic cavity which in CK2 is smaller than in the majority of other protein kinases [24,25]. This feature, which underlies the fairly narrow selectivity of these compounds toward CK2, has been exploited in many recent reports to probe the implication of this kinase in a variety of cellular functions [10]. Despite their structural similarity however TBB and DMAT bind in different ways into the active site of CK2 [25], denoting a role of the negative charge present at neutral pH on the triazole ring of TBB, which is not present in DMAT nor in the other TBB derivatives [17,23]. In these latter the N<sup>2</sup> atom is replaced by a carbon, to give a tetrabromobenzimidazole derivative (TBI or TBBz) devoid of the negative charge at neutral pH [17]. The relevance of this difference to determine kinase selectivity was overlooked in our original studies because the panel of kinases used at that time for selectivity profiles was not large enough to disclose significant differences. It was concluded therefore that, apart from some minor differences, TBB and DMAT exhibited

quite super-imposable inhibition profiles and a similar, quite remarkable selectivity toward CK2, although DMAT is somewhat more potent, especially if used in cell culture [44]. Another advantage of DMAT, as well as of TBI and the other benzimidazole derivatives analysed in this respect [23] is that, owing to its high  $pK_a$  value (8.9 for TBI and 10.6 for DMAT), at physiological pH the N-H of the imidazole ring is not dissociable and therefore these compounds are unable to depolarize mitochondria [19] by shuttling protons across the membrane. The same applies to the pyrazole ring of the indazole derivatives, e.g. K59 ( $pK_a$  10.6) and K64 ( $pK_a$  8.40) (see Materials and Methods). In contrast mitochondria depolarization is a drawback of TBB [20] whose triazole ring exhibits a much lower pH value, allowing proton shuttling under the effect of the pH gradient on the two sides of the mitochondrial membrane.

In this paper, exploiting a panel of kinases more than twice as large as the original one, and including members of nearly all the kinome subfamily (Fig. 1) we have reached the conclusion that the selectivity of TBB is definitely narrower than those of DMAT and TBI. While in fact both DMAT and TBI drastically inhibit several kinases in addition to CK2, notably PIM 1, 2 and 3, DYRK1a, 2 and 3, PKD1, HIPK2 and 3 and CDK/CyclinA, TBB is significantly less effective on these kinases than it is on CK2 as highlighted by comparing both  $IC_{50}$  values (Table 2) and selectivity profiles of TBB and DMAT performed at 1  $\mu$ M inhibitor concentration (see Table 1). On the other hand it should be taken into account the possibility that besides protein kinases also non kinase enzymes interact with CK2 inhibitors: this applies to TBB, but, even more to TBI and DMAT [45].

Incidentally the work presented here provides valuable hints for the development of PIM and HIPK inhibitors more potent and/or selective than those currently available. In the case of PIM1, the product of a proto-oncogene whose dysfunction has been implicated in diverse cancers with special reference to diffuse large cell lymphoma (DLCL) [46], some inhibitors are available [36], whose potency however is comparable to those of TBI and DMAT and whose selectivity has not been explored. Our data moreover disclose the possibility to discriminate among PIM isoforms exploiting their different susceptibility to TBI and DMAT (see Table 2). In contrast inhibitors of HIPK2, a kinase required for normal hematopoiesis and blood vessel formation [47], which plays a critical role in triggering p53-dependent apoptosis in response to the antineoplastic drug cisplatin [48], are not available at all, to the best of our knowledge, a circumstance which makes DMAT and TBI the best tools presently available to downregulate HIPK2.

Once established that TBB and, even more, DMAT and TBI are CK2 inhibitors less selective than it was previously thought, our next aim was the identification of DMAT/TBB derivatives which still are effective on CK2 having lost most of their efficacy toward PIM1, HIPK2 and DYRK1a. This goal turned out to be hard to attain since most compounds tested which are potent inhibitors of CK2 also tend to inhibit PIM1 and/or HIPK2 with similar potency (see Table S2 of supplementary data). A few notable exceptions however were provided by two compounds belonging to the class of indazoles (K59 and K64), having a pyrazole ring replaced for the triazole and imidazole pentatomic rings present in TBB and DMAT, respectively, and three new benzimidazole derivatives (K66, K74 and K75). These are all characterized by the ability to inhibit CK2 more potently than PIM1, HIPK2 and DYRK1a, with  $K_i$  values which in the case of K64 and K66 are many-fold lower than those calculated with the second most inhibited kinase (PIM1 in both cases)

Especially remarkable appears in this respect the behaviour of K66, considering its close structural relatedness with DMAT: while DMAT however inhibits PIM1 with the same potency as CK2, K66 has lost most of its efficacy toward PIM1, as judged from big differences in  $K_i$  and  $IC_{50}$  values.

The structural similarity between DMAT and K66, in conjunction with availability of 3D

structures of both PIM1 and CK2, this latter also in complex with DMAT [25] provide a key to rationalize the preferential binding of K66 to CK2. The structure of DMAT bound into the active site of CK2 together with the derived model in which DMAT has been replaced by K66 are shown in Fig. 6 A, C. Incidentally this accounts also very well for the finding that Ile174 is less important for the binding of K66 (see Table 4). Note in fact that K66, owing to its carboxylic group, is making electrostatic interactions with the positive area close to Lys68 thus displacing the dimethylamino group from its apolar contacts with Ile174 and placing it closer to Val66. In the case of PIM1, DMAT is rotated about 60° with respect the crystallographic data, due to the presence of Phe49. In fact, unlike its CK2 homologue Tyr50, which is exposed toward the solvent, Phe49 is instead oriented toward the hydrophobic cavity forcing the inhibitor to a new pose. As shown in figure 6B the dimethylamino group at position 2 is placed inside a new favourable hydrophobic pocket formed by PIM1 residues Val52, Leu120, Ile185 and Phe49, while in CK2 is simply oriented toward the outside (Figure 6A). These structural observations may explain the similar activity of DMAT in CK2 and PIM1, even in the presence of Val66/Ala65 natural substitution. On the other hand K66 interacts with PIM1 binding cleft in the same way showed for DMAT due to the presence of the dimethylamino group. Under these conditions its carboxyl side chain at position 1 cannot interact with Lys67, homologous to CK2 Lys-68, and thereby is placed in an unfavourable hydrophobic zone (Figure 6D).

Importantly moreover the improved selectivity of K66 as compared to DMAT is not restricted to its ability to discriminate between CK2 and PIM1, but it also reflects in a reduced or abolished inhibitory efficiency toward a large number of kinases deeply affected by DMAT, as outlined by comparing their specificity profiles in Fig. 4: while 34 kinases are  $\geq 50\%$  inhibited by 10  $\mu\text{M}$  DMAT, this figure drops to just 5 with K66, under identical condition. Only one of these latter moreover (ERK8) is inhibited as drastically as CK2 (i.e.  $>80\%$ ), while 12 are those  $>80\%$  inhibited by DMAT.

In an attempt to quantify these differences a “promiscuity score” has been devised, expressing “average” inhibition of all the kinases of the whole panel, by a given concentration of the inhibitor, sufficient to drastically inhibit (about 90%) the kinase considered (in our case CK2) (see legend of Table 5). Such a score, which reflects the promiscuity of the kinase, and is inversely proportional to its “average” selectivity, equals 18.36 and 46.66 with 10  $\mu\text{M}$  K66 and DMAT, respectively. Interestingly a promiscuity score similar to that of K66 has been calculated for K64 (16.8) reinforcing the concept that both K64 and K66 are markedly more selective than the three related commercially available CK2 inhibitors, TBB, DMAT, and TBI, this latter having a score  $>50$  (see Table 5). It should be also noted that while at 10  $\mu\text{M}$  inhibitor the promiscuity scores of TBB and DMAT are similar, if the profiling is performed at 1  $\mu\text{M}$  concentration, where CK2 is about 80% inhibited by either TBB or DMAT (see Table 1), the promiscuity score of DMAT (22.6) doubles that of TBB (11.14) confirming also by this criterion the concept that TBB is more selective than DMAT. Comparing however the selectivity of TBB with those, much higher, of K64 and K66 and considering that these latter are equally effective as TBB as inducers of apoptosis it is predictable that K64 and K66 will replace TBB (not to say about DMAT and TBI) as first choice tool for studying cellular functions of CK2. Since the chemical names of K64 and K66 are exceedingly long (3,4,5,6,7-pentabromo-1*H*-indazole and 4,5,6,7-tetrabromo-2-methylamino-1-carboxymethyl-benzimidazole, respectively) we propose that, by analogy with TBB and DMAT, they are denoted by acronyms PBIN and TMCB.

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### Supplementary Data Available

Detailed description of the synthesis and analytical data (Table S1) for the newly obtained compounds discussed in the present work. Profiling of a library of compounds on CK2, PIM1 and HIPK2 (Table S2). This material is available online.

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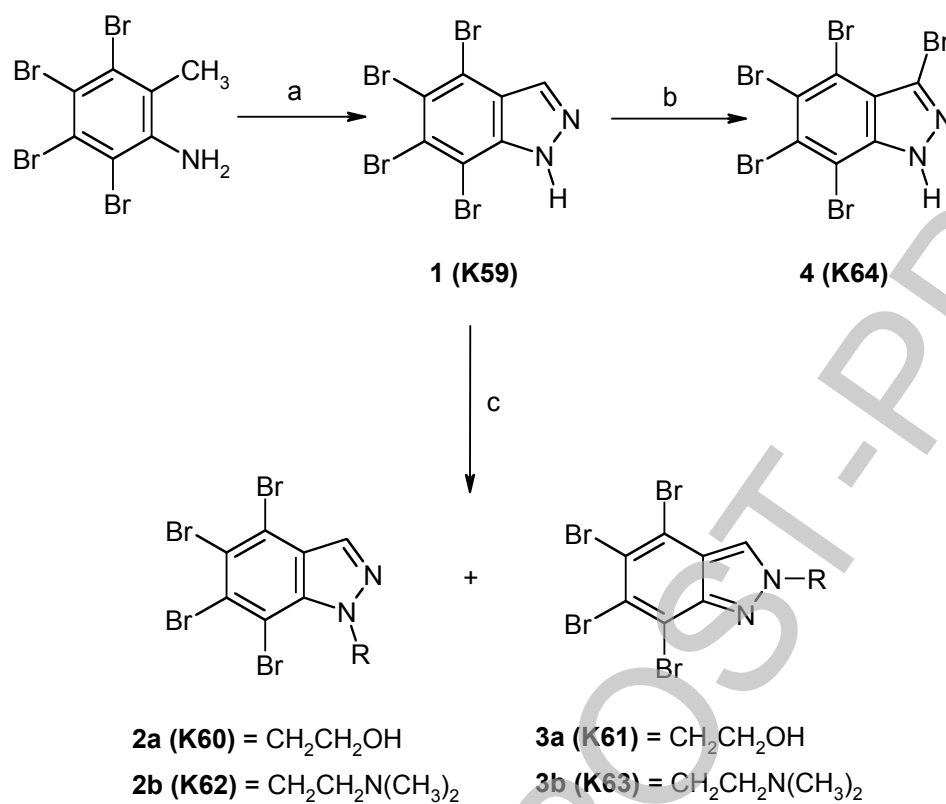
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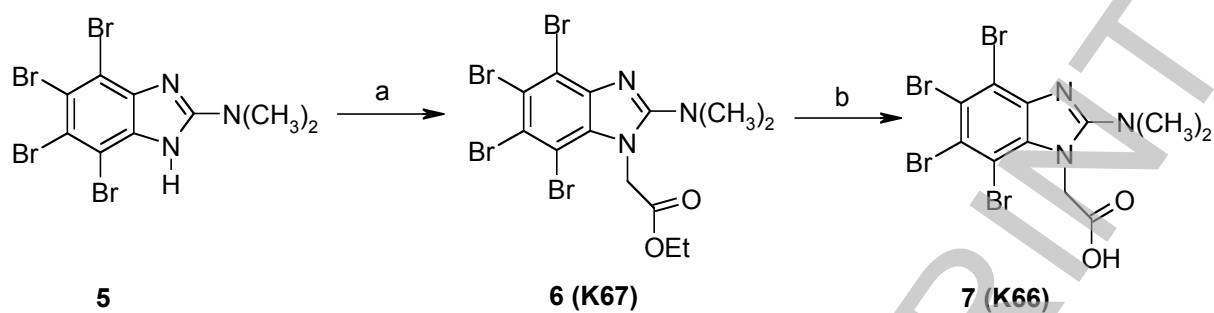
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**Scheme 1.** Synthesis of substituted tetrabromoindazoles and pentabromoindazole.

Reagents: (a) 1)  $\text{Ac}_2\text{O}$ , 2) nitrous gases; (b)  $\text{Br}_2$ ,  $\text{AcOH}$ ; (c)  $\text{K}_2\text{CO}_3$ , 2-butanone, 2- $\text{BrC}_2\text{H}_4\text{OH}$  or  $(\text{CH}_3)_2\text{NCH}_2\text{CH}_2\text{Cl}$ .



**Scheme 2.** Synthesis of 1-substituted derivatives of DMAT.Reagents: (a) 1)  $\text{K}_2\text{CO}_3$ , acetone,  $\text{BrCH}_2\text{COOEt}$ ; b) 1)  $\text{NaOH}$ ,  $\text{EtOH}/\text{H}_2\text{O}$ , 2)  $\text{H}^+$ 

**Scheme 3.** Synthesis of 1-methyl-4,5,6,7-tetrabromobenzimidazole derivatives.

Reagents: (a) thiourea, 1-PrOH; (b)  $K_2CO_3$ , acetone,  $BrCH_2COOEt$ ; (c)  $NH_2CH_3$ , EtOH; (d) 1) NaOH, EtOH/ $H_2O$ , 2)  $H^+$

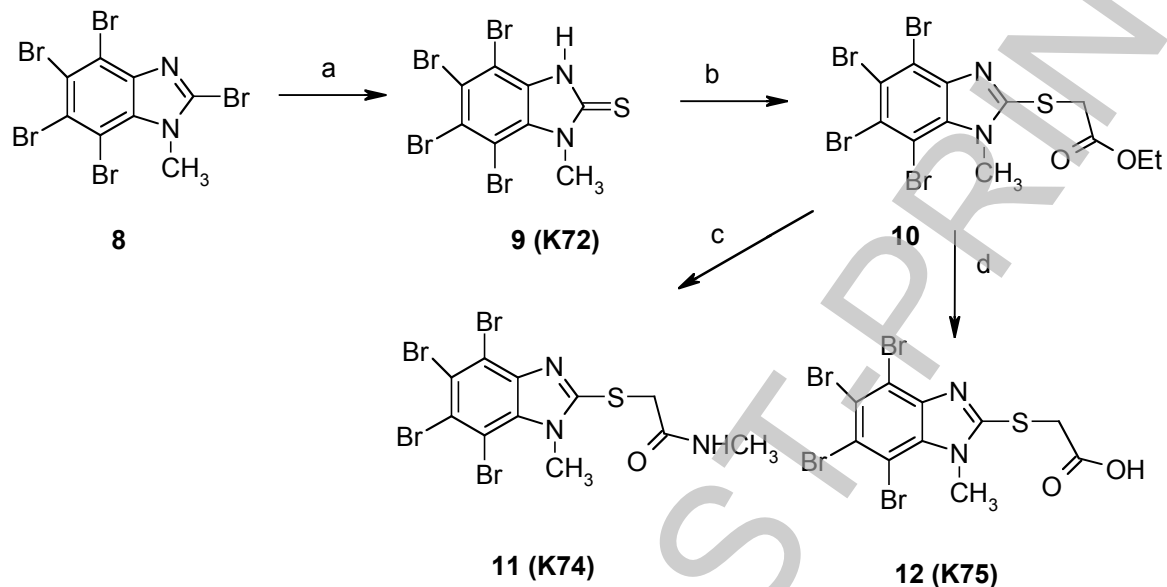


Table 1: Selectivity profiles on large kinase panel of TBB, DMAT and K17.

Activity assays were linear with respect to time and enzyme concentration and were performed as previously described [22]. Residual activity was determined in the presence of the indicated concentrations of inhibitor and expressed as a percentage of the control without inhibitor. Residual activities  $\leq 10\%$  are bold typed, those  $\leq 50\%$  are underlined. Other protein kinases not included in the selectivity panel, namely the isoforms  $\alpha$ ,  $\gamma$  and  $\delta$  of CK1, the tyrosine kinases Lyn, c-Fgr and Syk, and the Golgi casein kinase (G-CK), were tested under conditions specified elsewhere [18] and found to be unaffected ( $<10\%$  inhibited) by 10  $\mu\text{M}$  TBB, DMAT and TBI.

	Kinase	TBB (10 $\mu\text{M}$ )	DMAT (10 $\mu\text{M}$ )	TBI (TBBz) (10 $\mu\text{M}$ )	TBB (1 $\mu\text{M}$ )	DMAT (1 $\mu\text{M}$ )
1	MKK1	41	32	30	95	43
2	ERK1	106	78	78	91	96
3	ERK2	96	71	79	96	85
4	JNK1	33	66	42	91	84
5	JNK2	29	70	62	101	98
6	JNK3	52	85	68	100	95
7	p38 $\alpha$ MAPK	100	79	88	83	81
8	P38 $\beta$ MAPK	96	89	89	98	94
9	p38 $\gamma$ MAPK	24	57	54	105	92
10	p38 $\delta$ MAPK	100	66	67	98	107
11	ERK8	15	13	11	92	<u>27</u>
12	RSK1	63	29	36	102	91
13	RSK2	59	41	59	98	81
14	PKD1	86	76	81	94	100
15	PKB $\alpha$	41	66	53	93	88
16	PKB $\beta$	66	88	81	103	99
17	SGK1	30	24	16	100	73
18	S6K1	26	37	34	106	79
19	PKA	45	76	75	86	80
20	ROCK 2	58	42	31	90	90
21	PRK2	74	59	47	104	88
22	PKC $\alpha$	84	87	92	93	95
23	PKC zeta	37	40	44	79	62
24	PKD1	37	<b>10</b>	<b>5</b>	83	<u>27</u>
25	MSK1	36	29	24	107	90
26	MNK1	31	41	33	90	79
27	MNK2	30	30	33	103	68
28	MAPKAP-K2	64	90	74	96	90
29	MAPKAP-K3	72	94	94	107	99
30	PRAK	51	82	52	84	81
31	CAMKK $\alpha$	71	84	68	116	118
32	CAMKK $\beta$	26	44	48	76	73
33	CAMK1	101	42	25	101	96
34	SmMLCK	28	31	11	95	73
35	PHK	56	35	25	98	83
36	CHK1	105	71	70	85	82

37	CHK2	82	33	16	113	83
38	GSK3 $\beta$	31	35	41	80	73
39	CDK2-Cyclin A	48	18	23	102	94
40	PLK1	25	41	29	88	81
41	PLK1 (Okadaic Acid)	14	39	34	90	78
42	AURORA B	47	17	15	85	67
43	AURORA C	41	28	24	73	76
44	AMPK	71	54	45	100	119
45	MARK3	68	58	47	99	85
46	BRSK2	72	55	24	72	63
47	MELK	47	36	43	79	70
48	CK1	61	80	48	95	100
49	CK2	<b>6</b>	<b>7</b>	<b>10</b>	<u>21</u>	<u>19</u>
50	DYRK1A	<b>4</b>	<b>5</b>	<b>3</b>	51	<u>21</u>
51	DYRK2	<b>3</b>	<b>6</b>	<b>9</b>	68	<u>24</u>
52	DYRK3	<b>4</b>	<b>3</b>	<b>6</b>	76	<u>35</u>
53	NEK2a	118	94	74	101	103
54	NEK6	63	105	79	108	142
55	NEK7	61	96	68	113	107
56	IKK $\beta$	58	35	37	89	60
57	PIM1	<b>2</b>	<b>1</b>	<b>2</b>	<u>27</u>	<u>3</u>
58	PIM2	<b>7</b>	<b>6</b>	<b>2</b>	77	<u>32</u>
59	PIM3	<b>1</b>	<b>1</b>	<b>2</b>	<b>10</b>	<b>5</b>
60	SRPK1	55	70	76	93	98
61	MST2	63	45	35	102	90
62	EFK2	91	84	70	86	78
63	HIPK2	<b>5</b>	<b>2</b>	<b>3</b>	94	<u>20</u>
64	HIPK3	18	<b>6</b>	<b>9</b>	103	<u>40</u>
65	PAK4	71	70	58	95	89
66	PAK5	71	64	72	94	93
67	PAK6	75	96	93	94	103
68	Src	93	93	95	115	105
69	Lck	91	87	79	99	88
70	CSK	84	86	83	101	96

Table 2: IC<sub>50</sub> (μM) of selected protein kinases for TBB, DMAT and TBI (TBBz). Phosphorylation conditions are either described in the Experimental section or referenced elsewhere [22]. ATP concentration was 50 μM. Data with CK2 were obtained using native holoenzyme purified from rat liver. Replacement of native CK2 with recombinant human CK2 holoenzyme (α<sub>2</sub>β<sub>2</sub>) or with the isolated α subunit caused only minor alterations in the IC<sub>50</sub> values.

Kinase	TBI	DMAT	TBB
CK2	0.60	0.13	0.15
PIM1	0.115	0.148	1.04
PIM2	0.18	1.6	4.3
PIM3	0.07	0.097	0.86
HIPK2	0.7	0.37	5.3
HIPK3	1.22	0.59	4.9
DYRK1a	2.1	0.41	4.36
DYRK2	0.34	0.35	0.99
DYRK3	3.7	1.7	5.3
PKD1	0.34	0.18	5.9
CDK2	0.58	0.64	14.0

Table 3.  $K_i$  values ( $\mu\text{M}$ ) of selected compounds for CK2, PIM1, HIPK2 and DYRK1a protein kinases.

Kinetic determinations were performed as described in the Experimental section (with native CK2 holoenzyme) according to the Cheng-Prusoff equation [28].

Kinase	K36	K59	K60	K62	K64	K66	K67	K75
CK2	0.08	0.068	0.71	0.40	0.048	0.25	0.28	0.16
PIM1	0.56	0.55	3.66	4.50	0.738	8.65	4.00	2.55
HIPK2	2.29	1.53	11.33	8.88	1.594	15.25	6.31	12.77
DYRK1a	>25.00	1.00	3.45	2.85	1.64	11.90	>10.00	12.10

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Table 4: IC<sub>50</sub> (μM) of selected indazole and benzimidazole derivatives for wild type and mutated CK2.

Recombinant CK2 holoenzyme was reconstituted in vitro by mixing at 1:1 molar ratio β subunit with wild type or mutated CK2α subunit. The data represent the mean of three independent experiments with SD never exceeding 12%. dm= double mutant in which both Val66 and Ile174 have been replaced by Ala.

Compound	CK2 wt	Val66Ala	Ile174Ala	CK2 dm	Ratio dm/wt
K64	0.052	2.60	>40.00	31.28	601.5
K66	0.50	22.37	1.14	3.32	6.6
K74	0.60	>40.00	>40.00	40.64	67.3
K75	0.31	40.00	>40.00	15.22	49.0
DMAT <sup>a</sup>	0.14	1.78	3.30	5.79	30.47

<sup>a</sup>Data obtained from ref. [19].



Table 5: “Promiscuity score” of selected CK2 inhibitors.

Promiscuity score was calculated from the selectivity profiles (Fig. 4 and Table 1) as the mean of % inhibitions observed with all the kinases tested, excluding the kinase of interest (CK2). Inhibition of CK2 was 93-95% in the presence of 10  $\mu$ M inhibitors, 80% in the presence of 1  $\mu$ M DMAT and TBB. Values of inhibition <0 (i.e. stimulation) were adjusted to 0%.

Inhibitor	Concentration ( $\mu$ M)	Score (%)
K64	10	17.00
K66	10	19.33
DMAT	10	48.60
TBB	10	47.37
DMAT	1	23.10
TBB	1	10.18

## Figure legends

### **Fig. 1: Distribution within the human kinome of protein kinases tested for selectivity against CK2 inhibitors.**

The human kinome [50] is shown and the protein kinases included in the selectivity profiling tested for selectivity are indicated by blue circles. Protein kinases included in the 2001 TBB selectivity test [18] are marked in red. In yellow additional protein kinases tested only in Padova under conditions previously described [18].

### **Fig. 2: Molecular structure of representative benzotriazole, benzimidazole and benzopyrazole derivatives dealt with in this paper.**

### **Fig. 3: Kinetic analysis of PIM-1 inhibition by DMAT.**

Phosphorylation assays were performed as described in the Experimental section in the absence and in the presence of the indicated concentrations of DMAT. The data represent the mean of values obtained in triplicate with SD never exceeding 15%. Lineweaver-Burk double reciprocal plots obtained in the absence and in the presence of the indicated DMAT concentrations are shown in A. The inset refers to the  $K_m/V_{max}$  vs. [I] replot the value of  $K_i$  has been determined from. B:  $IC_{50}$  values for the inhibition of PIM1 and CK2 by DMAT calculated at increasing ATP concentrations.

### **Fig. 4: Comparison between the selectivity profiles of DMAT and some new CK2 inhibitors.**

Activity assays were linear with respect to time and enzyme concentration and were performed as previously described [22]. Residual activity was determined in the presence of 10  $\mu$ M inhibitor except in the case of K75 which was tested at 1  $\mu$ M (see text) and expressed as a percentage of the control without inhibitor. DMAT is also shown for comparison (data drawn from Table 1).  $IC_{50}$  values of K66, determined for ERK8 and DYRK2, were 0.5 and 8, respectively.

### **Fig. 5: Proapoptotic efficacy of new CK2 inhibitors on Jurkat cells.**

A. Jurkat cells were treated for 24 h with increasing concentration of the indicated inhibitors. Cell viability was assessed by the MTT method, assigning 100% value to the control cells, treated with the solvent. Reported values represent the means  $\pm$ S.E.M. from four separate experiments.

B. Jurkat cells were treated with the indicated concentrations of inhibitor for 4h. Apoptosis was evaluated by quantification of nucleosomes present in the cytosol of the apoptotic cells, using the Cell Detection Elisa kit (Roche). Treatment with 25  $\mu$ M etoposide (Eto) was used as a positive control for apoptosis induction. Nucleosome enrichment was calculated from the ratio between the signal in control cells and treated cells.

### **Fig. 6: Molecular Docking of DMAT (A, B) and K66 (C, D) bound to the active site of CK2 (left) and PIM1 (right).**

Molecular docking was performed as described in the Experimental Section. Active LP distribution surface of CK2 and PIM1 binding pockets were represented. Green indicates hydrophobic regions, while in violet are hydrogen bonding or metal-LonePair regions.

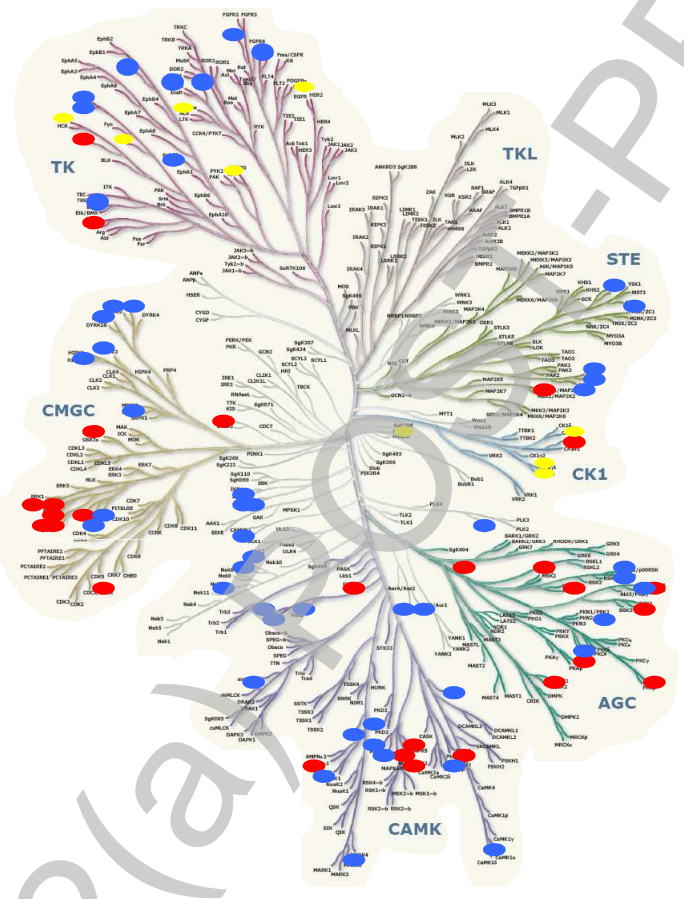


Figure 1

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**Figure 2**

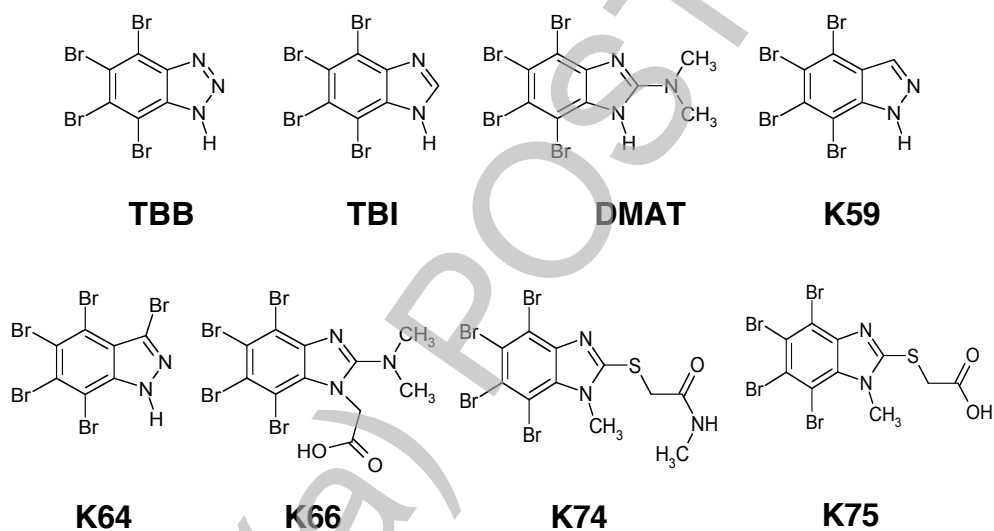


Figure 3

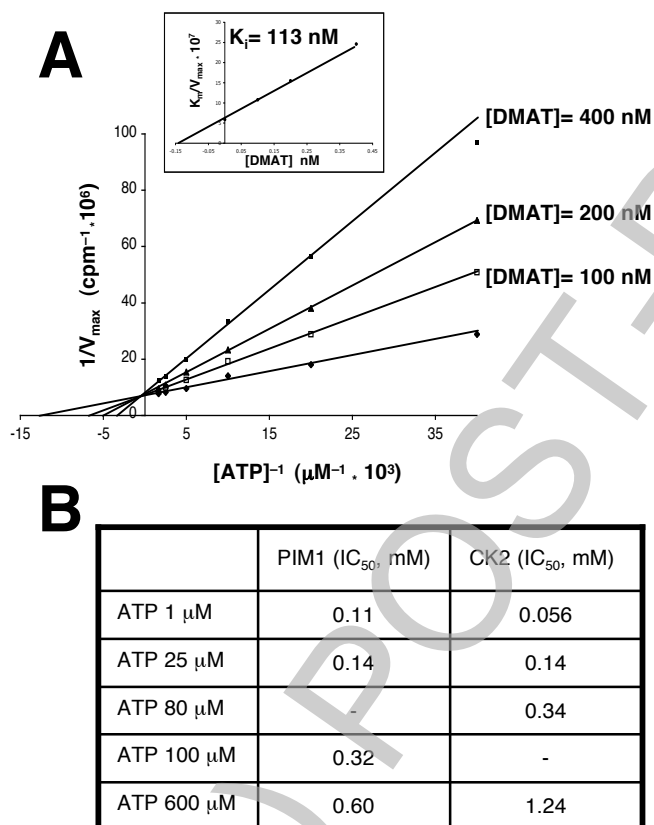


Figure 4

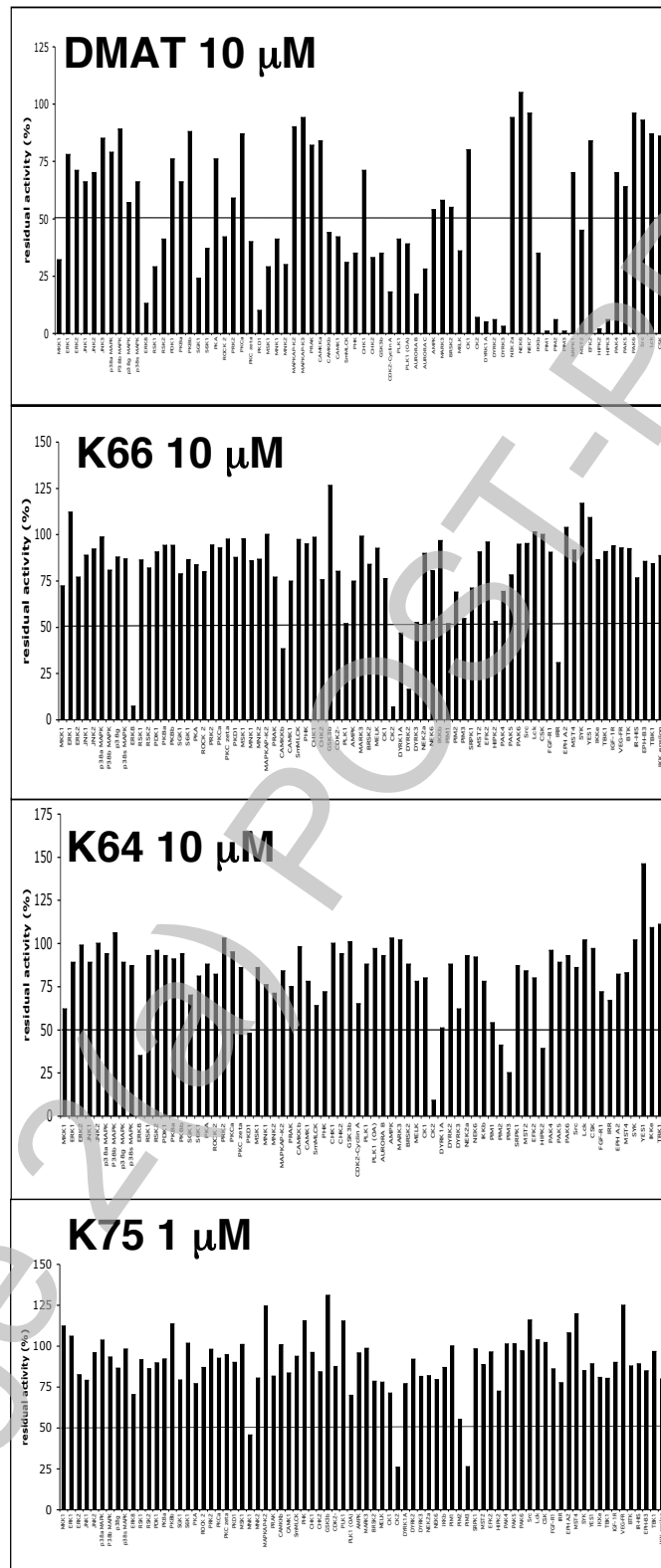
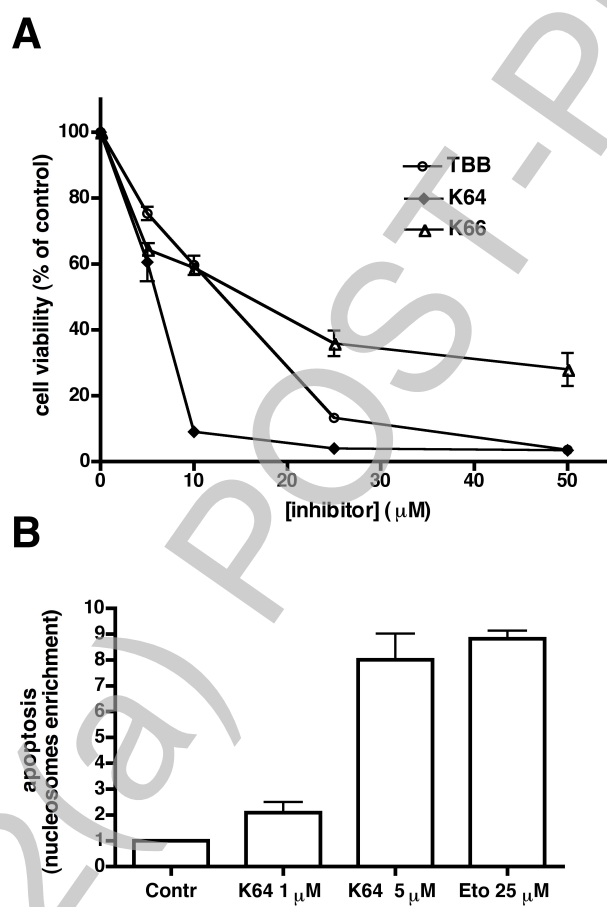
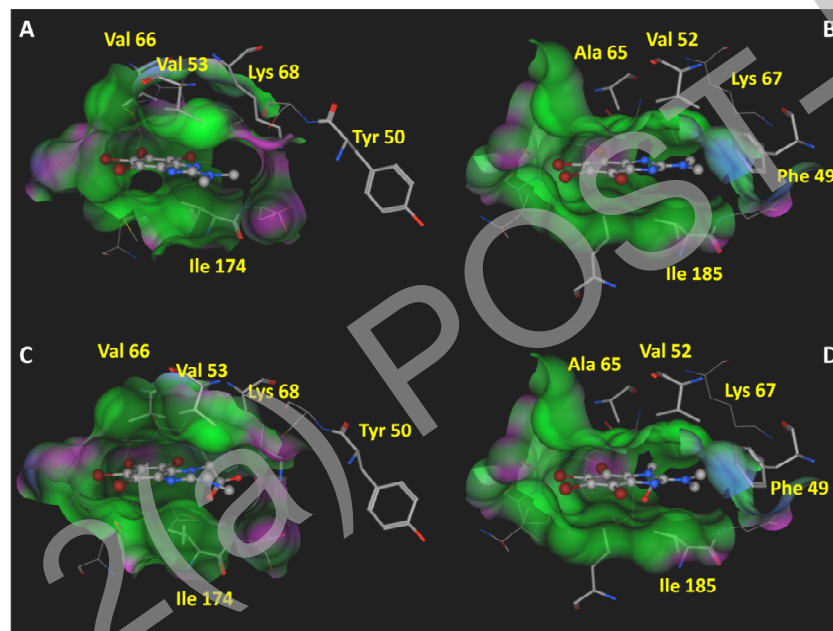


Figure 5



**Figure 6**



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