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Rational drug-design approach supported with thermodynamic studies — a peptide leader for the efficient bi-substrate inhibitor of protein kinase CK2

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Numerous inhibitors of protein kinases act on the basis of competition, targeting the ATP binding site. In this work, we present a procedure of rational design of a bi-substrate inhibitor, complemented with biophysical assays. The inhibitors of this type are commonly engineered by combining ligands carrying an ATP-like part with a peptide or peptide-mimicking fragment that determines specificity. Approach presented in this paper led to generation of a specific system for independent screening for efficient ligands and peptides, by means of thermodynamic measurements, that assessed the ability of the identified ligand and peptide to combine into a bi-substrate inhibitor. The catalytic subunit of human protein kinase CK2 was used as the model target. Peptide sequence was optimized using peptide libraries [KGDE]-[DE]-[ST]-[DE]₃₋₄-NH₂, originated from the consensus CK2 sequence. We identified KESEEE-NH₂ peptide as the most promising one, whose binding affinity is substantially higher than that of the reference RRRDDDSDDD peptide. We assessed its potency to form an efficient bi-substrate inhibitor using tetrabromobenzotriazole (TBBt) as the model ATP-competitive inhibitor. The formation of ternary complex was monitored using Differential Scanning Fluorimetry (DSF), Microscale Thermophoresis (MST) and Isothermal Titration Calorimetry (ITC).

Protein kinases catalyze the transfer of the γ -phosphate from ATP to the acceptor group of a specific amino acid of a target protein¹. Protein kinases are attractive targets in cancer treatment, since the overexpression or mutation of particular kinases has been associated with cancerogenesis²⁻⁴. The majority of available protein kinase inhibitors act on the basis of competition, targeting the ATP binding site. The structure of the latter is highly preserved in various protein kinases, and thus, in spite of their high affinity, the inhibitors of this type may not be selective enough for a particular protein kinase. However, in addition to the ATP-binding site, each protein kinase has a specific binding site for the target protein(s), which enables the recognition of a so-called consensus sequence, identifying residues that preferably flank the phosphorylation acceptor site. This sequence, whose presence is necessary and sufficient to be recognized by a particular kinase, describes the general target specificity of the protein kinase⁵. Short peptides or peptide-mimetics that block the substrate binding site are therefore another group of potentially selective inhibitors of protein kinases⁶⁻⁸. However, due to the nature of kinase interaction with a target protein, the ligands of such type, mimicking that interaction, are rather weak binders.

To overcome all those problems and to enhance both the affinity and the specificity, bi-substrate inhibitors have been designed^{9,10}. Ligands of this type target simultaneously the ATP- and substrate-binding sites, therefore, they should be designed on the basis of the shape and the location of natural substrates⁹. Such inhibitors are usually engineered by coupling ligands targeting ATP-binding site with a peptide fragment that determines the specificity¹¹. This concept has been implemented leading to some moderately efficient inhibitors, consisting of a peptide fragment conjugated with either adenosine¹²⁻¹⁵ or nonhydrolyzable ATP derivatives¹⁶⁻¹⁸. Alternatively, a

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peptide may be conjugated with an ATP-competitive inhibitor^{11,19–22}. This concept of the protein kinase inhibitor design is very promising, since the combination of two substrates required by the target enzyme may display very high specificity²³.

However, there are several pitfalls to avoid in the development of new bi-substrate inhibitors. First of all, the interaction with the target peptides may be associated with conformational changes occurring in the kinase upon ligand binding. This includes global domain movement or local changes in the glycine rich P-loop, C-helix and activation loop^{6,24}. Since the ligand-induced conformational changes in the target protein are still difficult to predict, we decided to support molecular modeling with experimental thermodynamic studies to identify the preferred sequence of the peptide part, and to check experimentally whether possible conformational changes interfere with the binding of the ATP-competitive part of a potential bi-substrate inhibitor of CK2 α .

CK2 is a pleiotropic kinase that regulates many independent processes in cells because of its ability to phosphorylate multiple proteins (until now over 200 substrates of this kinase have been identified)²⁵. Unlike the majority of Ser/Thr protein kinases, which recognize basic phosphoacceptor sites, the consensus sequence of CK2 displays preference towards acidic residues flanking the phosphate donor. Minimal consensus requires the presence of glutamate, aspartate, or occasionally phosphoserine (Sp) or phosphotyrosine (Yp) at the position +3 relative to the phosphoacceptor site. Although a single acidic residue at this position is sufficient for recognition, the presence of multiple acidic amino acids located upstream is strongly preferred. The significance of individual residues at a given position depends on the overall peptide sequence. An acidic residue at position +1 is almost as important as that at +3, while acidic residue at +2 is rather important for peptides in which no other acidic residues are located upstream^{26,27}. Summarizing, the general consensus sequence, most frequently recognized by CK2, is X-[ST]-X-X-[EDSpYp]-X, where X is preferably acidic^{5,26–28}.

Although the contribution of protein kinase CK2 to neoplasia formation remains unclear, CK2 has become a therapeutic target in anticancer treatments²⁹. As for the most protein kinases, the majority of CK2 inhibitors target the ATP-binding site. There are also several reports regarding very promising bi-substrate inhibitors against CK2^{30–33}. Most of them were designed using a common general approach. Enkvist *et al.* developed a highly potent inhibitor ARC-1502 ($K_i = 0.5$ nM), a conjugate of 4,5,6,7-tetrabromo-1H-benzimidazole with peptides containing multiple aspartic acid residues³⁰, and was very successful in further optimization³². More recently, a bi-substrate inhibitor K137-E4 ($IC_{50} = 25$ nM), also derived from tetrabromo-1H-benzimidazole, was reported by Cozza *et al.*, who combined K137 inhibitor (N1-(4,5,6,7-tetrabromo-1H-benzimidazol-2-yl)-propane-1,3-diamine) with Glu₄ tetrapeptide, highly improving both the affinity and selectivity towards CK2³³. In both cases, the design of inhibitors originated from a low-mass competitive inhibitor, to which acidic residues were linked without any broader sequence optimization.

Designing bi-substrate inhibitors by the independent optimization of the part targeting ATP-binding site, and peptides or peptidomimetics targeting substrate binding site may be a good alternative. In the broader view, this approach may be useful for designing generally bivalent inhibitors (i.e. inhibitors targeting two different sites of a protein, not necessarily substrate binding sites³⁴) as there are already known some non-classic CK2 peptide-derived inhibitors that target the interface between the catalytic domain (CK2 α) and the regulatory one (CK2 β), i.e. CAM7117 with $K_d = 150$ nM³⁵. There are also some CK2 β motifs known to interact with other binding partners of CK2³⁶, so peptides or peptidomimetics interfering with these interactions may also be a promising alternative.

Herein, we propose a rational approach based on the independent screening for efficient ligands and peptides, both performed with thermodynamic methods, that score the ability of particular compounds to combine further into an efficient bi-substrate inhibitor. We present the first step of rational design of the bi-substrate inhibitor. We tested three libraries of peptides, originated from the known consensus sequence of CK2, to identify the optimal one with the highest affinity toward the catalytic subunit of human protein kinase CK2. We further verified the possibility of combining the developed peptides with an ATP-competitive ligand to form an efficient bi-substrate inhibitor, using tetrabromobenzotriazole (TBBt) as a model ATP-competitive inhibitor. Finally, we analyzed the inhibitory activity of an ad hoc synthesized bi-substrate ligand.

Results and Discussion

Peptide library screening against CK2. Peptides that efficiently bind to the target protein were selected using pull-down method with immobilized His-tagged catalytic subunit of human CK2 (CK2 α), and further identified using liquid chromatography-mass spectrometry (LC-MS). Three peptide libraries were tested. The first one, [KGDE]-[DE]-[ST]-[DE]₃-NH₂, contained 128 hexapeptides originating directly from the CK2 consensus sequence. The next library, containing 256 heptapeptides, was divided into two separate sub-libraries: [KGDE]-[DE]-[S]-[DE]₄-NH₂ and [KGDE]-[DE]-[T]-[DE]₄-NH₂, each consisting of 128 peptides. This approach was applied to overcome the substantial difference in the efficiency of synthesis of threonine- and serine-containing peptides.

Signals identified in the MS spectra were assigned to a given peptide according to the indicated mass. However, this procedure led to several ambiguities, since a number of different peptides in the library displayed the same mass. LC-MS data was collected independently for the initial [KGDE]-[DE]-[ST]-[DE]₃-NH₂ library (Fig. 1a), and for the eluent released after passing this library through the column with immobilized hCK2 α (Fig. 1b). Although in both runs the same peaks (i.e. M/z) were identified, their relative intensities visibly varied. We assumed that peptides displaying higher affinity to the protein should be enhanced in the second run. Some peptides were excluded from the further analysis due to their unspecific binding to the column's resin (Fig. 1c). Finally, three peaks with substantially increased relative intensity in the eluent fraction were identified.

The first peak of 750 Da mass corresponds to an ensemble of six hexapeptides: EETEED-NH₂, EETEDE-NH₂, DETEEE-NH₂, EETDEE-NH₂, EDTEEE-NH₂ and EESEEE-NH₂, while the next two of 763 and 764 Da mass were identified as KETEEE-NH₂ and EETEEE-NH₂, respectively. The same procedure was performed for the two other libraries leading to the results consistent with those obtained for the first library (See Supplementary Figs S1, S2 and Table 1S).

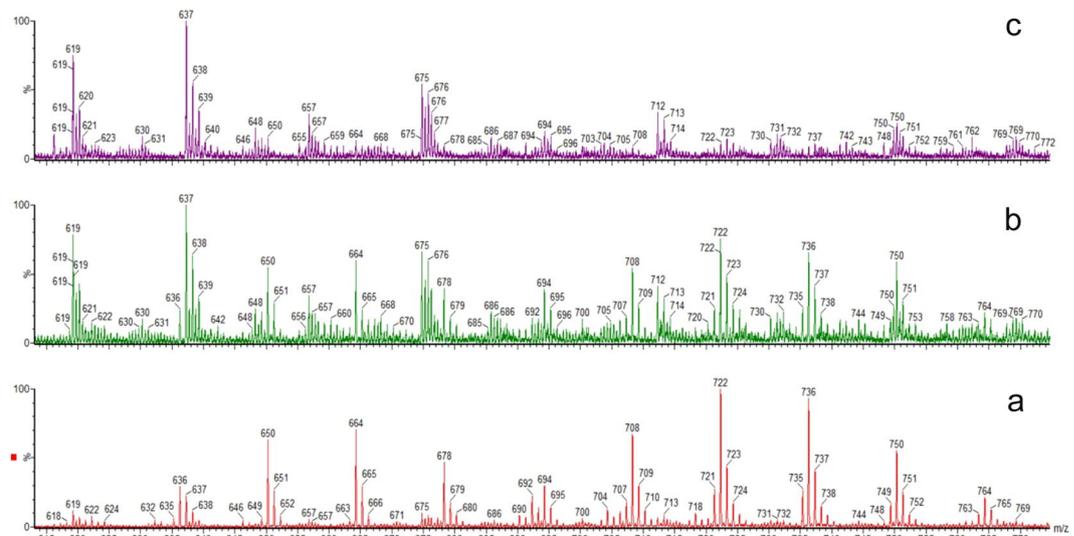


Figure 1. Mass Spectra of [KGDE]-[DE]-[ST]-[DE]₃-NH₂ library pull-down experiment (a) library (b) eluent of the library from immobilized His-tagged CK2 α column (c) eluent of the library from column without CK2 α (control).

Comparison of peptides identified in the pull-down assays pointed to favored peptides exhibiting two features: (1) glutamate residues flanking threonine/serine that undergo phosphorylation by CK2 (at most single aspartate residue) and (2) the N-terminal lysine. Nine hexapeptides (EETEEE-NH₂; KETEEE-NH₂; EESEEE-NH₂; KESEEE-NH₂; EETEED-NH₂; EETEDE-NH₂; DETEEE-NH₂; EETDEE-NH₂; EDTEEE-NH₂) were synthesized in a larger-scale and subjected to thermodynamic studies. DDTDDD-NH₂ peptide was also used to compare the advantage of glutamic acid over the aspartic acid. Binding affinity to hCK2 α for all of the above peptides was assessed semi-quantitatively, using relaxation-filtered NMR spectroscopy.

Transverse relaxation rate (R_2), which reflects the efficiency of the spin-spin exchange mechanism, depends on the hydrodynamic properties of the molecule, in particular on its rotational diffusion in solution. The experiment senses changes in ¹H relaxation rates caused by the presence of hCK2 α , binding to which decreases average rotational diffusion of the solute and thus almost uniformly affects relaxation rates of all ¹H nuclei. In general, the transverse relaxation is much faster for larger molecules, whose nuclear spins experience much more efficient exchange^{37,38}. Dynamic properties of the peptide, when virtually bound to the protein, resemble those of its molecular target. Assuming a fast exchange between free peptide and low-populated protein-bound state, the apparent properties of the free peptide are affected by the contribution of the bound form to the overall nuclear relaxation, which visibly increases the efficiency of spin-spin exchange. Assuming that properties of the protein target do not vary significantly upon ligand binding, the stronger the peptide binding to its molecular target, the faster the transverse relaxation is.

To ensure that the observed effect was not associated with individual relaxation rates of free peptides in the solution, we performed a relaxation-filtered version of the experiment, with the relaxation delay varying in the range of 1 up to 200 ms. We observed substantial intensity variation of particular resonance lines caused by the increase of the delay, which were further quantitatively analyzed according to the model of two-exponential decay. The results of the analysis performed for the EETEED-NH₂ peptide are shown in Fig. 2.

For each peptide we globally analyzed signals determined for the set of the representative ¹H resonances (see methods), assuming that the faster relaxation rate ($R_{2,f}$) corresponds to the relaxation processes averaged over free and bound states, thus revealing the population of molecules interacting with the target protein. The slower one ($R_{2,s}$) describes the relaxation of molecules that did not sense the protein. The higher $R_{2,f}$ (or $R_{2,f}/R_{2,s}$ ratio), the higher population of the peptide is bound, implying its stronger binding to the protein. The estimated relaxation rates are summarized in Table 1 and shown in Fig. 3.

All of the above data indicate that the KESEEE-NH₂ peptide binds subtly stronger than any of the other tested peptides. Remaining peptides that do not carry the aspartic acid at position -2 relative to Ser/Thr bind to the hCK2 α with comparable affinity, regardless the arrangement of further residues. Interestingly, Lys at position -2 improves the affinity for peptide with serine at the acceptor site, while no such effect is observed for peptide with threonine. This may suggest that some tiny differences between binding of serine and threonine substrates take place, therefore allowing CK2 to discriminate them. The observed difference in binding of KESEEE-NH₂ and EESEEE-NH₂ must result from electrostatic interactions rather than from steric boundaries, since DESEEE-NH₂ binds to hCK2 α with lower affinity. So, one may assume that the interaction pattern involving the first residue may depend on its type, and the preferred location of the N-terminal Glu may differ from that of Lys. This variation should be taken into account during the design of a linker to the low-mass ligand. Finally, DDTDDD-NH₂, which corresponds to the C-terminal part of the commercially available reference peptide RRRDDSDDD (used in the biochemical assays), binds visibly weaker to hCK2 α .

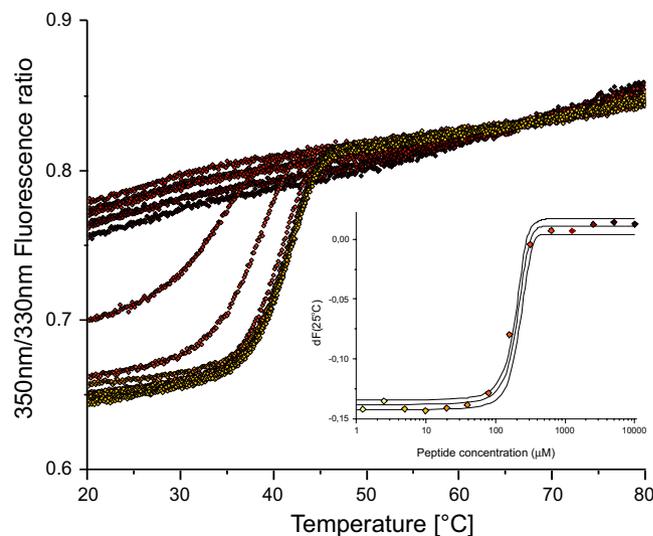


Figure 4. Thermal denaturation profile of KESEEE-NH₂-hCK2 α complex monitored as 350 nm/330 nm fluorescence ratio recorded at the increasing KESEEE-NH₂ peptide concentration (from the lowest – yellow, to the highest – brown). For clarity, all curves were aligned at the high-temperature asymptotes. Nested graph summarizes changes in the fluorescence at 25 °C as a function of KESEEE-NH₂ concentration.

Analogous experiments were performed for three other peptides (See Supplementary Fig. S3). All tested peptides destabilize the protein, and for all of them the decrease of the melting temperature was found dependent on the peptide concentration. For the high-temperature region (i.e. $T > 65^\circ\text{C}$ in Fig. 4) a minor dependence on peptide concentration is indicative of its unspecific binding to the unfolded form of the protein, while at the lower temperature binding of peptides causes a strong decrease of 350 nm/330 nm fluorescence intensity ratio. These changes were used to estimate the binding affinity at 25 °C, assuming the single binding site model. The same procedure was applied to the data collected at 75 °C. Interestingly, dissociation constants estimated at 25 °C and at 75 °C differ visibly (K_d for KESEEE-NH₂ at 75 °C is ~ 1 mM and 0.35 mM at 25 °C), therefore indicating that peptides bind to both folded and unfolded state of hCK2 α . It could be hypothesized that the destabilization of the protein at low peptide concentration may be caused by unfavorable entropy balance upon binding of such hydrophilic ligands.

The general order of peptides derived from nanoDSF is consistent with that obtained in the relaxation-filtered NMR experiments, confirming again that the binding affinity of KESEEE-NH₂ is the highest (Table 2). The thermodynamic parameters of binding of KESEEE-NH₂ peptide by hCK2 α were also determined, with Microscale Thermophoresis, and further compared with the affinity of a commercially available reference peptide, RRRDDSDDDD (Table 2 and Fig. 5).

The peptide at concentration of approximately 1 mM causes hCK2 α unfolding, which is manifested by the increase of fluorescence and distortion of raw MST data. This observation confirms the results obtained with nanoDSF, proving again that the peptide binds to the unfolded protein with K_d of about 1 mM. It should be emphasized that the binding affinity of KESEEE-NH₂ towards hCK2 α ($K_d = 0.39 \pm 0.21$ mM) is significantly higher than that of commercially available RRRDDSDDDD peptide (2.1 ± 0.7 mM, ~ 1 mM³⁹).

Testing the cross-dependency of ligand and peptide binding. To verify the ability of the identified KESEEE-NH₂ peptide to bind with a low-mass ligand and form an efficient bi-substrate inhibitor, the interactions between the peptide, protein and TBBt (model ligand that binds at the ATP-binding site) were monitored using three methods - ITC, MST and nanoDSF. Combination of these techniques allowed for determination of the formal binding constant of peptide and ligand to the free protein, as well as peptide to ligand-protein complexes and ligand to peptide-protein complexes (Fig. 6 Table 2)

Comparison of peptide affinity towards the *apo* form of hCK2 α and the hCK2 α /TBBt complex was performed using nanoDSF and MST. Both methods confirmed that the presence of TBBt does not significantly affect peptide binding. The same applies to the KESEEE-NH₂ interference with the TBBt binding, which effect was studied with ITC and MST. The corresponding values of dissociation constant remain the same within the experimental error. It could be thus concluded that the presence of peptide does not change the TBBt affinity, so both of them can be used as templates for designing a bi-substrate inhibitor.

Molecular modeling of bi-substrate inhibitor. Molecular modeling of the ternary complex of hCK2 α , TBBt, and EESEEE-NH₂ or KESEEE-NH₂ peptide was performed by a combination of modeling by homology with iterative modification of the ligand peptide followed by restrained molecular dynamics. The final structure of both complexes was found stable in terms of 30 ns unrestrained molecular dynamics (Fig. 7). The location of KESEEE-NH₂ is stabilized by electrostatic interactions formed with proximal side-chains of Arg47, Lys49, Lys74, Lys76, Lys77, Lys158, His160, Arg191 and Lys198. All these interactions contribute to the stabilization of protein-peptide, which was estimated with FoldX to 4.2 kcal/mol. The *in silico* determined $k_d = \sim 0.8$ mM is

titrate	nanoDSF				
	hCK2 α				hCK2 α + TBBt
titrant	KESEEE-NH ₂	EESEEE-NH ₂	KETEEE-NH ₂	EETEEE-NH ₂	KESEEE-NH ₂
Kd(mM)	0.35 \pm 0.02	1.58 \pm 0.06	0.95 \pm 0.05	0.96 \pm 0.03	0.45 \pm 0.02
Δ G (kJ/mol)	-19.73 \pm 0.14	-15.99 \pm 0.09	-17.25 \pm 0.13	-17.23 \pm 0.08	-19.10 \pm 0.11
titrate	MST				
	hCK2 α		hCK2 α + KESEEE-NH ₂		hCK2 α + TBBt
titrant	KESEEE-NH ₂	RRRDDDSDDD	TBBt		KESEEE-NH ₂
Kd(mM)	0.39 \pm 0.21	2.1 \pm 0.7	(96 \pm 44) \cdot 10 ⁻⁶	(86 \pm 28) \cdot 10 ⁻⁶	0.36 \pm 0.23
Δ G (kJ/mol)	-19.5 \pm 1.3	-15.3 \pm 0.8	-40.1 \pm 1.1	-40.3 \pm 0.8	-19.7 \pm 1.6
titrate	ITC				
	TBBt		TBBt		
titrant	hCK2 α		hCK2 α + KESEEE		
K _d (μ M)	0.06 \pm 0.03		0.10 \pm 0.07		
Δ G (kJ/mol)	-41.3 \pm 1.1		-40.0 \pm 1.6		
Δ H (kJ/mol)	-25.1 \pm 1.2		-17.7 \pm 1.7		
Δ S (J/mol/K)	47 \pm 8		70 \pm 11		

Table 2. nanoDSF-, MST- and ITC-derived thermodynamic parameters of binding of the peptides and TBBt to hCK2 α , separately and for their ternary complexes.

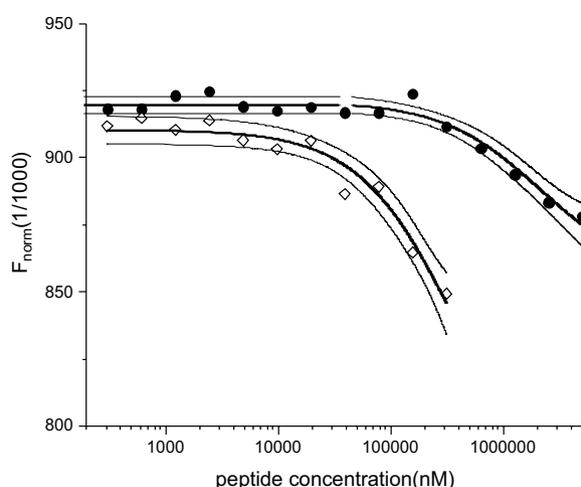


Figure 5. Microscale thermophoresis (MST) data for binding of: KESEEE-NH₂ (diamonds) and commercially available peptide – RRRDDDSDDD (circles) to hCK2 α .

therefore close to the value of 0.3 \pm 0.2 mM determined experimentally with MST. It is worth noting that the side-chain nitrogen of the N-terminal lysine of the peptide points towards TBBt, located at the ATP binding site, thus directing the way for setting up a bi-substrate ligand. The same procedure was applied for the EESEEE-NH₂ peptide. In this case, the side-chain of the N-terminal residue was preferably oriented away from TBBt, therefore disqualifying side-chain of the N-terminal Glu as a potential linker, which could be however linked via the N-terminal amino group. Importantly, the complex with KESEEE-NH₂ remained in the open conformation, while that with EESEEE-NH₂ has switched to the closed one.

Potency of bi-substrate inhibitor against human CK2 α . To confirm the validity of our approach, we synthesized ad hoc a simple, bi-substrate compound, based on the optimized peptide sequence, that was conjugated by an amide bond formed between side chain of the N-terminal lysine and 7-COOH-Br₃Bt. The inhibitory activity of this preliminary bi-substrate inhibitor, IC₅₀ = 0.67 \pm 0.15 μ M, is comparable to that of TBBt (0.62 \pm 0.28 μ M), but higher than that of the leading 7-COOH-Br₃Bt (8.0 \pm 6.3 μ M). Therefore, when compared with the affinity of the low-mass precursor, we obtained over 10-fold enhancement of inhibitory activity for bi-substrate ligand, while coupling of Glu₄ with K137 improved the inhibitory activity only 5-fold³³. This clearly exemplifies the potency of the proposed approach, proving the importance of the optimization of peptide sequence. However, taking into account IC₅₀ values reported for CK2 bi-substrate inhibitors K137-E4 and ARC-1502 (25 nM³³ and 2.7 nM³⁰, respectively), it is clearly understandable that the low-mass ligand as well as the linker must be further optimized.

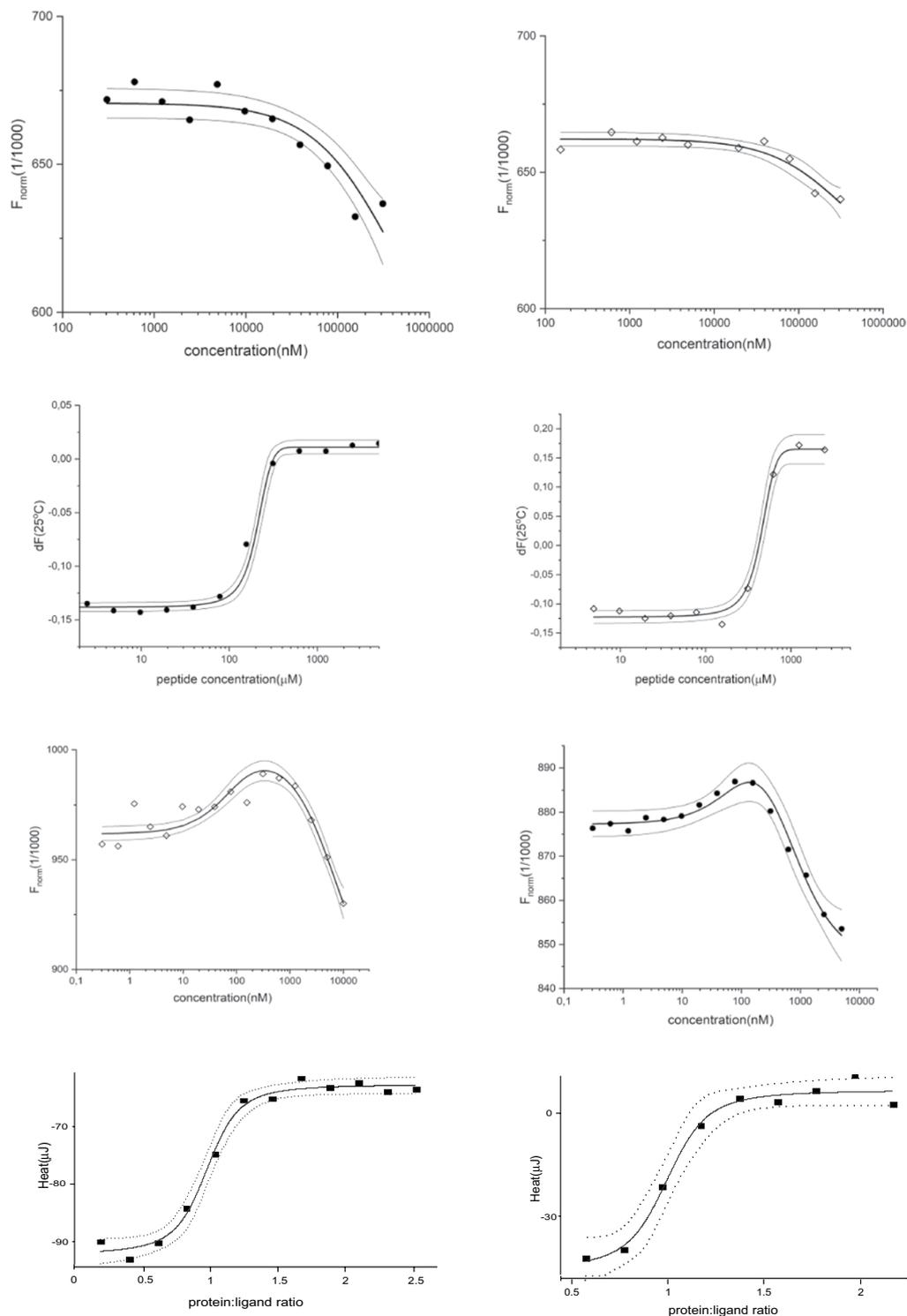


Figure 6. Ternary system analysis. MST data for binding of: (a) KESEEE-NH₂ to hCK2 α (left panel) and to TBBt/hCK2 α complex (right panel) (c) tetrabromobenzotriazole to hCK2 α (left panel) and KESEEE-NH₂/hCK2 α complex (right panel). (b) nanoDSF data for binding of KESEEE-NH₂ to hCK2 α (left panel) and to TBBt/hCK2 α complex (right panel). (d) ITC data for TBBt binding to hCK2 α (left panel) and to KESEEE-NH₂/hCK2 α complex (right panel).

Conclusions

In this work we presented a rationalized approach in CK2 drug design, in which the peptide part of a bi-substrate inhibitor was optimized to obtain an effective bi-substrate inhibitor. Combining experimental thermodynamic methods, we successfully screened three peptide libraries and identified the KESEEE-NH₂ hexapeptide that binds to hCK2 α with affinity higher than any of the studied peptides previously used as substrates for this kinase. We

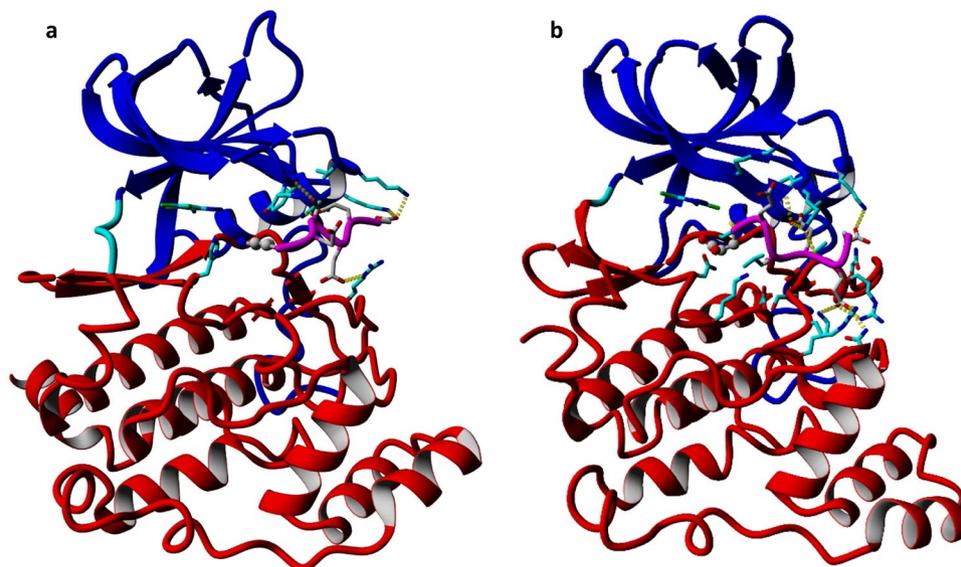


Figure 7. Snapshots of the Molecular Dynamic trajectory performed for the ternary complex of hCK2 α and TBBt with KESEEE-NH₂ (a) and with EESEEE-NH₂ (b). The peptide backbone is denoted in magenta with the N-terminal Lys/Glu residue in ball-and-stick representation.

also proved that the binding of this peptide does not significantly attenuate the binding of an ATP-competitive ligand, which makes the proposed peptide a promising part of an efficient bi-substrate inhibitor. Molecular modeling additionally supports this hypothesis, clearly demonstrating that the linking of halogenated benzotriazole with the N-terminal lysine of the peptide may form an efficient bi-substrate ligand. In summary, the presented experimental approach supports the rational design of specific bi-substrate inhibitors of CK2, which also can be applied to other protein kinases.

Methods

Expression and purification of hCK2 α . The catalytic subunit of human CK2, hCK2 α , was expressed and purified according to the method described previously⁴⁰.

Peptide library synthesis. Three peptide libraries [KGDE]-[DE]-[ST]-[DE]₃-NH₂, [KGDE]-[DE]-[S]-[DE]₄-NH₂ and [KGDE]-[DE]-[T]-[DE]₄-NH₂ were synthesized by standard solid-phase peptide synthesis on TentaGel S RAM resin (Rapp polymere) using a Prelude peptide synthesizer (Protein technologies) with equimolar peptide building blocks. Side chain protecting groups were Boc, tBu and OtBu for Lys, Ser/Thr and Asp/Glu respectively. Cleavage was performed with 95:2.5:2.5% trifluoroacetic acid (TFA):triisopropylsilane:H₂O, and the resulting peptides were precipitated with cold diethyl ether, centrifuged, the pellets were then dissolved in H₂O and finally lyophilized. The compositions of libraries were analyzed by ESI-MS⁴¹. To avoid losing single peptides, the products were purified by dialysis against TBS buffer (25 mM Tris-HCl, 0.15 M NaCl, pH 7) and verified by ESI-MS using LC-MS separation on RP-C18 resin.

Peptide synthesis. 10 Peptides: EETEEE-NH₂; KETEEE-NH₂; EESEEE-NH₂; KESEEE-NH₂; EETEED-NH₂; EETEDE-NH₂; DETEEE-NH₂; EETDEE-NH₂; EDTEEE-NH₂ and DDTDDD-NH₂ were synthesized as described above. The products were purified by HPLC using Empower system (Waters) equipped with RP-amide semi-preparative column (5 μ m particle size, 250 \times 10 mm) (Ascentis). The mobile phase consisted of (A) 0.1% TFA in water and (B) 0.1% TFA with 90% acetonitrile in water. The purity and identity of lyophilized peptides was verified by ESI-MS using LC-MS separation. The final concentration was determined using Direct Detect Infrared Spectrometer (Merck).

Bi-substrate inhibitor synthesis. 4,5,6-tribromo-7-methyl-1H-benzotriazole (7-CH₃-Br₃Bt) (1) was obtained in good yield, according to the previously published procedure⁴². Subsequent oxidation with KMnO₄ led to 7-COOH-Br₃Bt (2) in 22% yield and high purity (see supplementary Fig. S4).

Bi-substrate inhibitor was synthesized and purified the same way as peptides described above, with 7-COOH-Br₃Bt (2) as the first residue in sequence. The purity and identity of lyophilized compounds was verified by ESI-MS using LC-MS separation.

Pull-down assays. Pull-down assays were used to identify peptides that strongly interact with CK2. For this purpose the Pierce™ His Protein Interaction Pull-Down Kit was used. Protein diluted in TBS buffer was incubated with cobalt resin supplemented in Kit (initially equilibrated with TBS buffer) at 4 °C for 1 h with continuous mixing. The resin was separated by centrifugation and the supernatant was removed. The resin was then washed

five times with TBS buffer. Next, TBS buffer containing peptide library was added and incubated overnight at 4 °C with continuous mixing. To separate peptides that efficiently bind to the protein, the resin was centrifuged and washed two times with TBS buffer. Material bound to the column was eluted with 2 M glycine pH 2.5 and further identified by ESI-MS separation on RP-18 resin.

Relaxation-filtered NMR spectroscopy. NMR samples containing tested peptides and hCK2 α were prepared in 25 mM Tris-HCl (pH 7, 0.25 M NaCl) buffer with 10% D₂O. The protein concentration was 25 μ M, and a 100-fold peptide excess (2.5 mM) was used throughout the studies.

The scoring of the peptides' binding affinities towards hCK2 α was done according to the results of ¹H transverse (T_2) relaxation filtering experiment⁴³. All spectra were recorded at 25 °C on Bruker Avance 500 NMR spectrometer (11.7 T) using 18 delays (0, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 100, 150 and 200 ms). The spectra of 7500 Hz spectral width, 16k data points each, were collected using 16 scans with 2.0 s recycling delay preceded by 8 dummy scans.

NMR data were further processed and analyzed with the SpinWorks software (version 4.2.8.0), using cosine-squared window function and Gaussian filter resulting in 0.3 Hz broadening with no solvent filter applied prior to Fourier transformation. We analyzed ¹H transverse relaxation rates of selected nuclei representative for each type of amino acid residues present in the tested peptides, i.e. H γ of Glu (2.3 ppm), H β of Asp (2.8 ppm), H β of Ser (3.8 ppm), H γ (CH₃) of Thr (1.1 ppm) and H ϵ of Lys (2.9 ppm), respectively. Additionally, the resonance signals of amide protons (8.2–8.9 ppm) were also analyzed. We performed the relaxation-filtered experiment, with the relaxation delay varying in the range of 1 to 200 ms. Variation in the intensity of particular resonance signals were analyzed using the model of two-exponential decay (1):

$$y = A_1 \cdot \exp(t * R_{2,f}) + A_2 \cdot \exp(t * R_{2,s}) + \gamma_0 \quad (1)$$

where $R_{2,s}$ and $R_{2,f}$ are the transverse relaxation rates of the free and the fraction of the virtually bound peptide, respectively. For each peptide the global optimization procedure was applied for all identified resonance signals.

Low-volume screening with differential scanning fluorimetry (nanoDSF). The assay was carried out in 25 mM Tris-HCl (pH 7, 0.5 M NaCl) buffer with protein concentration preserved constant at 2.5 μ M, while peptide concentrations varied in the 300 nM–10 mM range. The samples were loaded into nanoDSF Grade Standard Capillaries (NanoTemper Technologies), and analyzed using the Prometheus NT.48 nanoDSF device (NanoTemper Technologies). Thermal unfolding of the protein was monitored using a linear thermal ramp (1 °C/min; 20 °C to 80 °C) with an excitation power of 30%. Binding affinity of peptides was estimated from the ratio of 330 nm and 350 nm estimated at 25 °C and 75 °C for the native and unfolded forms of the protein, respectively. The data was analyzed using the numerical model implemented in Origin 9.0 package (www.originlab.com).

Microscale Thermophoresis (MST). The hCK2 α sample was initially labeled with the commercially available NT-647 dye, using NanoTemper Protein Labeling Kit RED. The concentration of the ligand varied in the range of 100 nM–10 mM, while the concentration of the labeled protein was kept constant at 50 nM. All obtained data were analyzed according to the method described previously⁴⁴, using appropriate model implemented in Origin 9.0 (www.originlab.com). Dissociation constants were estimated globally for a series of three independent pseudo-titration MST experiments, using effect of thermophoresis with temperature jump as the reporter of protein-ligand interaction.

Isothermal titration calorimetry (ITC). All ITC measurements were carried out at 25 °C with the Nano ITC calorimeter (TA Instruments), using 250 rpm stirring and 1000 s delay between succeeding injections to the sample cell of the volume 950 μ l. The 12 injections of 20 μ l volume following first pre-injection of a reduced volume (4 μ l) were analyzed. The sample cell was initially filled with 4–10 μ M TBBt solution, while 20–60 μ M protein solution was placed in the syringe. Ternary complex formation was tested using the same experimental setup with the tested peptide present both in the cell and in the syringe at identical concentration of 500 μ M. For each protein/ligand combination at least two titration experiments were done with different reagent concentrations. The data were further processed using the numerical model implemented in Origin 9.0 package (www.originlab.com), assuming existence of one type of binding sites of the apparent stoichiometry close to 1:1.

Molecular modeling. Initial structure of the ternary complex of hCK2 α with TBBt and KDS tripeptide was adopted from the accessible structures of TBBt bound to the catalytic subunit of maize CK2 (pdb1J91)⁴⁵ and kinase Pim-1 with its substrate peptide (pdb3MA3)²². After replacement of the last three residues by a required sequence KES, the resulting structure was optimized and further relaxed with 5 ns molecular dynamics in the NPT ensemble, in the presence of explicit water molecules, with coordinates of protein backbone and serine of the ligand peptide constrained. In the successive steps, three Glu residues were added one-by-one at the C-terminus of the peptide, and each of these structures were successively subjected to 5 ns constrained MD, in which various distance restraints were tested to facilitate formation of salt-bridges with proximal basic residues of hCK2 α . The final model of the ternary complex hCK2 α /TBBt/KESEEE-NH₂ was further subjected to 50 ns unrestrained MD performed in the NTP ensemble (298 K, 1 atm). The structure of the ternary complex hCK2 α /TBBt/EESEEE-NH₂ was adopted from that with KESEEE-NH₂, and further subjected to 30 ns unrestrained molecular dynamics. All calculations were performed with Yasara Structure using Yasara2 force-field.

In vitro inhibitory activity towards human CK2 α . The ADP-Glo kinase assay (Promega) was used to measure the inhibition effect of the tested compounds. The kinase assay was carried in 96-well plate in volume of 25 μ l in 20 mM Tris-HCl (pH 7.5) buffer, containing 100 ng hCK2 α (1 μ l), 10 μ M CK2 substrate peptide

RRRDDSDDD (Biaffin GmbH & Co KG), 10 μ M ATP, 20 mM MgCl₂ and 2.5 μ l of serially diluted ligand in DMSO. The reaction was initiated with enzyme in and kept going for 20 min at 30 °C. Reaction was stopped by adding 25 μ l of ADP-Glo reagent. After incubation at room temperature for 40 min (to consume the remaining ADP) 50 μ l of kinase detection reagent were added and incubated for additional 30 minutes to produce luminescence signal. Luminescence was measured with The SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices). IC₅₀ values were determined using three independent experiments, 7 inhibitor concentrations in the range of 1 nM–1 mM each, by global fitting of the sigmoidal dose–response equation implemented in Origin 9.0 package (www.originlab.com). Single experiment was analyzed for TBBT to confirm agreement with already published data^{42,46,47}.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on a reasonable request.

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Author Contributions

M.W.S. and J.P. planned all experiments. M.W.S. conducted the experiments. D.P. performed all peptide synthesis. I.Z. contributed to design and performed some NMR experiments. M.W.S. and J.P. analyzed the results. All authors contributed to the final version of the manuscript.

Additional Information

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