

Scientific paper

N-Substituted Indole-2 and 3-Carboxamide Derivatives as Inhibitors of Human Protein Kinase CK2: In Vitro Assay and Molecular Modelling Study

Süreyya Ölgün,^{1,*} Andreas Gratz,² Zühal Kılıç Kurt¹ and Joachim Jose²¹ Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry Tandogan, Ankara, 06100, Turkey² Institut für Pharmazeutische und Medizinische Chemie, Westfälische Wilhelms-Universität Münster, Hittorfstr. 58-62, 48149 Münster, Germany* Corresponding author: E-mail: olgen@ankara.edu.tr
Tel: +90 (312) 203 30 73; Fax: +90 (312) 213 10 81

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Abstract

Protein kinase CK2 (Casein Kinase 2) is involved in cell growth; proliferation and suppression of apoptosis. Hence, it strongly promotes cell survival and can be considered an important target for human cancers. In the present study, a series of *N*-substituted indole-2- and 3-carboxamide derivatives were tested for inhibitions of human recombinant protein kinase CK2 to evaluate their anticancer properties. The inhibition test revealed that the most active compound **4** (1-benzyl-*N*-(2,4-dichlorobenzyl)-1*H*-indole-2-carboxamide) showed an IC₅₀ value of 14.6 μM towards human protein kinase CK2. A molecular docking study of the compounds with CK2 was performed and revealed the binding mode of the most active compound **4**, underlying its inhibitory activity.

Keywords: Casein Kinase-2, Indole derivatives, Inhibitors, Docking

1. Introduction

The acidophilic Ser/Thr protein kinase CK2 (Casein Kinase 2) plays important roles in gene expression, DNA repair, RNA and protein synthesis, apoptosis, transformation and signal transduction from extracellular growth factors.¹ In several mice models, it was shown that CK2 cooperates with other protooncogenes to generate lymphomas and promotes cytotoxic effects.² Overexpression of protein kinase CK2 is an unfavorable prognostic marker in prostate, lung cancers and acute myeloid leukemia and suggests that CK2 may represent an important therapeutic target. Furthermore, many viral proteins are reported as CK2 substrates, indicating that CK2 kinase may play a role in viral infections as well. For this reason, CK2 inhibitors have become an attractive target as anticancer and anti-viral compounds.³ In a number of different cancers such as prostate,⁴ mammary gland,⁵ lung⁶ and others⁷ abnormally high levels of CK2 have been observed. In cancer cells, increased CK2 activity prevents apoptosis⁸ and promotes the transforming potential of oncogenes. It affects the chaperone machinery protecting the “onco-kinome”, enhances the multidrug re-

sistance (MRD) phenotype, counteracts the efficacy of anti-tumor drugs and supports angiogenesis.⁹ CK2 has emerged as an upcoming tumor target in human kinome and diverse strategies have been released to inhibit CK2 functions. The ATP binding site of CK2 is smaller than most of the other protein kinases, which allows for designing selective ATP-competitive inhibitors. Among the many known inhibitors of CK2 today, only **CX-4945** (Fig. 1) has been reached in clinical trials for cancer as an orally bioavailable ATP-competitive inhibitor of protein kinase CK2.¹⁰

A number of different classes of small molecule inhibitors of CK2 have been described.^{11,12} Benzimidazole, benzotriazole and indole derivatives have been thoroughly investigated and led to the development of more potent compounds.¹³ Among them, several 4, 5, 6, 7-tetrabromo-1*H*-benzimidazole (**TBBI**), (IC₅₀ = 0.40 μM), 4, 5, 6, 7-tetrabromo-1*H*-benzotriazole (**TBBt**) (IC₅₀ = 0.30 μM), and 4,5,6,7-tetraiodobenzimidazole (**TIBI**) derivatives were found to be powerful inhibitors of CK2^{14–17}, (Fig. 1). Moreover, some polyhalogeno benzimidazole^{18,19} and 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole compounds exhibited considerable inhibition against CK2.^{9,20}

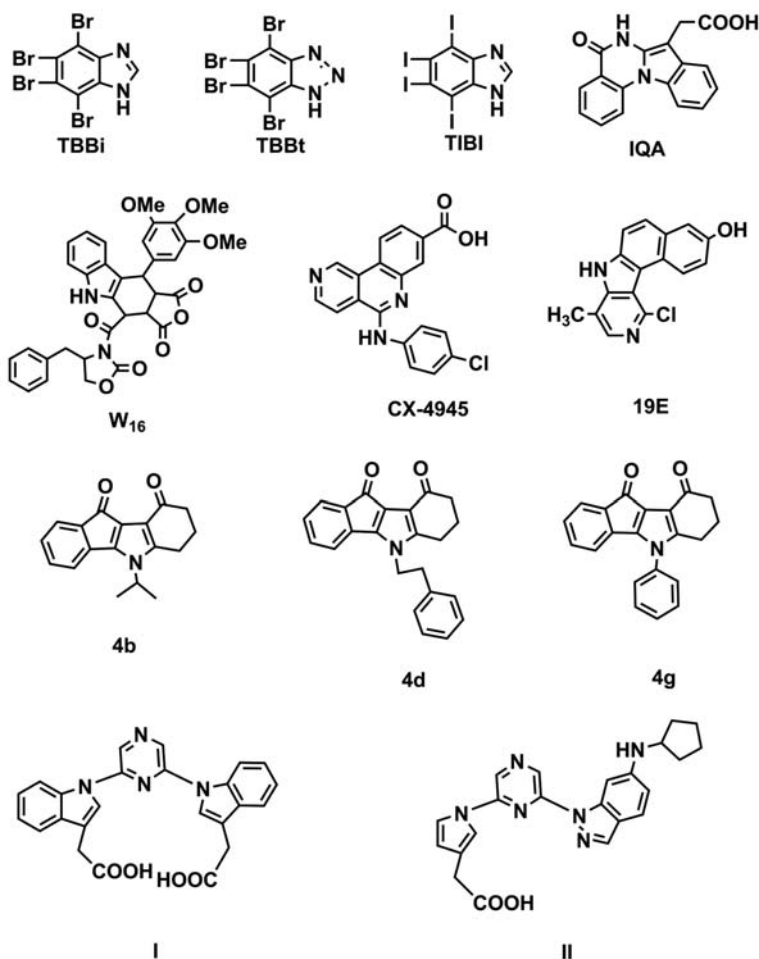


Fig. 1. Indole and benzimidazole derivatives known as inhibitors of CK2

From the group of indole based compounds, a podophyllotoxine indole analog (**W16**) (Fig. 1), was identified as a potent inhibitor, which inhibited CK2 with IC_{50} values of $30 \mu\text{M}$.²¹ In addition, several indazole derivatives were found to inhibit CK2 to a considerable extent.^{22,23} A benzopyridoindole derivative, **19E** (11-chloro-8-methyl-7H-benzo[e]pyrido[4,3-b]-3-ol, Fig. 1), showed a good pharmacologic profile, causing a marked inhibition ($IC_{50} = 0.67 \mu\text{M}$) of CK2 activity associated with cell cycle arrest and apoptosis in human cancer cells.²⁴ More recently, substituted indeno[1,2-b]indole derivatives, **4b**, **4d**, **4g** (Fig. 1), were found to be potent inhibitors of CK2 with IC_{50} of $0.11 \mu\text{M}$, $0.82 \mu\text{M}$ and $1.44 \mu\text{M}$, respectively.²⁵

Three dimensional quantitative structure-activity relationship (QSAR) and molecular docking are useful approaches for drug design. Combining these techniques could offer more insight to understanding the structural features of active site of protein, and thus could more effectively design new potent inhibitors. Recently novel CK2 inhibitors have been identified by these computational methods.^{18,26} 7-substituted indoloquinazoline (IQA, 5-

oxo-5,6-dihydroindolo-(1,2-a)-quinazoline-7-yl-acetic acid, Fig. 1) was obtained as a potent CK2 inhibitor ($IC_{50} = 0.40 \mu\text{M}$) out of a 400.000-compound library by using a virtual screening approach.^{27,28} In addition, N-aryl indole-3-acetic acid derivative (compound I, Fig. 1) and 2,6-disubstituted pyrazine derivative (compound II, Fig. 1), were discovered as new molecules, which exhibited high CK2 inhibitory activity in both enzymatic ($IC_{50} = 9.3 \text{ nM}$) and cell-based ($IC_{50} = 0.9 \text{ nM}$) assays.²⁹

As part of our interest in the area of protein kinase CK2 inhibitors, we previously reported promising 3-(substituted-benzylidene)-1,3-dihydro-indolin-2-one, and thione derivatives as inhibitors of human CK2.³⁰ To investigate more indole derivatives as inhibitors of human CK2, we have tested some N-substituted indole-2- (**1-10**) and 3-carboxamide (**11-20**) derivatives against CK2 enzyme to continue our effort to finding new inhibitors. Moreover, docking analyses were performed to evaluate binding properties of the compounds compared with the potent CK2 inhibitor **19E** and relationships between the biological activities and the binding affinities of the compounds were established.

2. Results and Discussion

2. 1. Experimental

A recently developed assay for the determination of protein kinase CK2 activity based on the quantification of phosphorylated substrate by capillary electrophoresis was used to test the inhibitory activities of the compounds.^{31,32} The inhibition values of all new derivatives which were tested for their antioxidant properties^{33,34} toward protein kinase CK2 are presented in Table 1. Inhibition was tested during the linear range (Fig. 2). Enzymatic reaction was set up in a volume of 2 mL but without the addition of an inhibitor. 200 μ L aliquots were taken from the reaction tube in intervals indicated in the diagram. The CE/UV assay was used to quantify the amount of phosphopeptide formation represented by the area of the pro-

duct peak in each of the 10 aliquots. Inhibition of CK2 holoenzyme was measured after incubation with compound **4** in concentrations ranging from 0.01 to 100 μ M using the CE/UV assay.

The dose-response curve of compound **4** was obtained from three independent CK2 kinase assays. Reduction of CK2 activity was plotted in a semi-logarithmic dose-response curve. The IC_{50} value was obtained by extrapolating the compound concentration at a residual CK2 activity of 50% from a four-parameter logarithmic curve fit (Fig. 3). Although compound **4**, which has 2,4-dichloro benzamide group at second position was found the most active with an IC_{50} value of 14.6 μ M, the corresponding compound **14** at third position did not show any inhibition. Reference compound **19E** inhibits CK2 with an IC_{50} value of 0.67 μ M.

Table 1. Inhibition of human protein kinase CK2 by *N*-substituted indole-2- and 3-carboxamide derivatives

Comp. No	X	R ₁	R ₂	% CK2 Inhibition ^a	LogP ^b
1	H	H	H	17	5.28 (\pm 0.43)
2	H	H	Cl	5	5.83 (\pm 0.50)
3	H	H	F	3	5.32 (\pm 0.46)
4	H	Cl	Cl	30 (IC_{50} = 14.60 μ M)	6.38 (\pm 0.54)
5	H	F	F	-14	5.55 (\pm 0.44)
6	F	H	H	-1	5.32 (\pm 0.46)
7	F	H	Cl	-1	6.02 (\pm 0.46)
8	F	H	F	-1	5.49 (\pm 0.46)
9	F	Cl	Cl	-2	6.59 (\pm 0.51)
10	F	F	F	-12	5.70 (\pm 0.50)
11	H	H	H	-2	5.18 (\pm 0.43)
12	H	H	Cl	n.a	5.66 (\pm 0.45)
13	H	H	F	-2	5.17 (\pm 0.42)
14	H	Cl	Cl	-1	6.30 (\pm 0.50)
15	H	F	F	2	5.41 (\pm 0.44)
16	F	H	H	5	5.23 (\pm 0.42)
17	F	H	Cl	22	5.89 (\pm 0.44)
18	F	H	F	6	5.36 (\pm 0.46)
19	F	Cl	Cl	7	6.45 (\pm 0.49)
20	F	F	F	3	5.50 (\pm 0.51)
19E				IC_{50} = 0.67 μ M ^c	
Emodin				IC_{50} = 0.27 μ M	
TBB				IC_{50} = 1.33 μ M	

^a CK2 activity of a sample including the test compound was calculated as a fraction of the CK2 activity at 10 μ M concentration, measured without test compound but with DMSO (positive control).

^b LogP values calculated from website: www.vcllab.org

^c IC_{50} was taken from PDB data bank.

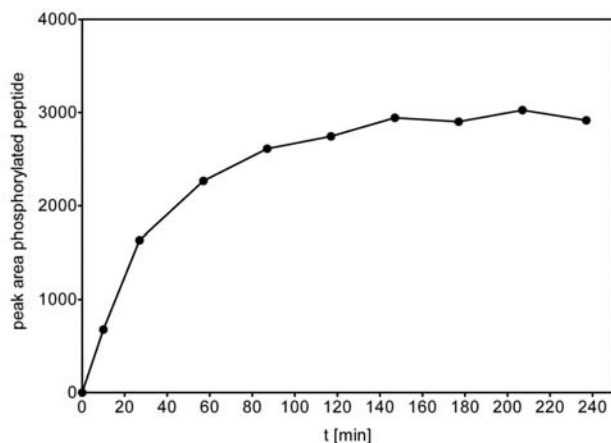


Fig. 2. Kinetics of CK2 reaction

Therefore compound **4** can be considered as moderately active in comparison with **19E**. In addition, the other dichlorinated compounds **9** and **19**, and fluorine containing compounds **10** and **20** did not show any inhibition. The F substitution at para position of *N*-benzyl group caused the activity loss of indole 2-carboxamide derivatives. On the contrary, F substitution at para position of *N*-benzyl group of indole-3-carboxamide derivatives showed better inhibition. Among the indole-2-carboxamide derivatives, compound **1**, which has no substitutions on both aromatic rings showed 17% inhibition of CK2. Compound **17** having F and Cl substitutions at aromatic rings have 22% inhibition of CK2 among the indole-3-carboxamide derivatives. These results indicate that the type of *N*-substitutions and positions of substituents on indole ring are important for activity. The findings are in agreement with the current literature, which point that halogen bonding interactions are responsible for the different conformations of the molecules in the CK2 active site. These interactions also affect many factors that influence inhibitor recognition and binding properties such as specificity surface and hydrophobic effects.^{32,35} The theoretical Log P values of indole-2- and 3-carboxamides were calculated from using the program at the website www.vcclab.org and are given in Table 1.

Although dichlorinated compounds have high Log P values, among them only compound **4** has good inhibition against CK2. This result points out that it is more important where the halogen substitutions are placed than the hydrophobic properties of compounds.

2. 2. Computational

Even if some indole derivatives were studied as ATP-competitive inhibitors, pharmacological inhibition still plays an important role to understand the concept of inhibition design. For this reason, it is important to understand how potent and selective indole derivatives can be

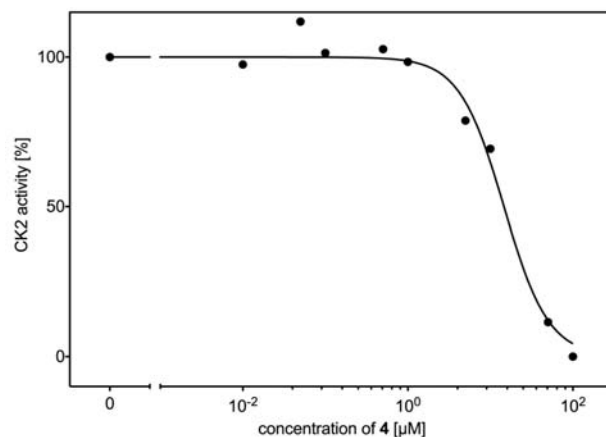


Fig. 3. IC₅₀ determination of compound **4** as a CK2 inhibitor in a dose-dependent manner

designed as CK2 inhibitors. Despite the small number of indole compounds studied and the efficacy of CK2 inhibition could not be clearly explained by structure-activity relationships, the obtained results prompted us to evaluate binding conformation and the degree of fit into the CK2 binding sites of compounds **1–20** by using Autodock 4.2.1 and Vina 1.0.2. The crystallographic structure of human 19E-CK2 complex (entry 3OWL of the Brookhaven protein data bank) was chosen as the starting point to perform molecular docking. The ligand **19E** (Fig. 1) is a potent CK2 inhibitor that docked onto CK2 to validate the docking process. The reliability of the docking protocol was first tested by simulations of the binding mode of CK2 inhibitor **19E** and comparison of the modeled complexes with the available three-dimensional structures derived by X-ray crystallography (3OWL). A root mean square deviation (rmsd) value of 1.641 Å, calculated on the whole structure, was found with no significant conformational rearrangement of the hinge region. The program successfully reproduced the X-ray coordinates of the inhibitor binding conformations (rmsd values ranging from 1.034–2.078 Å between docked and crystallographic conformers). During our work, we also tried the dockings for compound **12** and **13** which have interactions with the protein, but the rmsd values were higher than 2.08 which is outside the error range for interaction between ligand and target. The docking result shows that **19E** occupies in the ATP binding site (Fig. 4) and overlapped with crystallographic **19E**. One hydrogen bond was identified between OH of crystallographic **19E** and NH₂ proton of VAL116. Other hydrogen bond was observed between OH hydrogen of crystallographic **19E** and CO of GLU114. Other gatekeeper amino acids of CK2 binding site are LYS 68, VAL 53, ILE 174 and VAL 66 (Fig. 4).

The docking results of compounds **1–20** suggested that all of the compounds occupied in the ATP binding site of CK2 but with a different orientation than **19E**. Each docked compound was assigned a score according to its

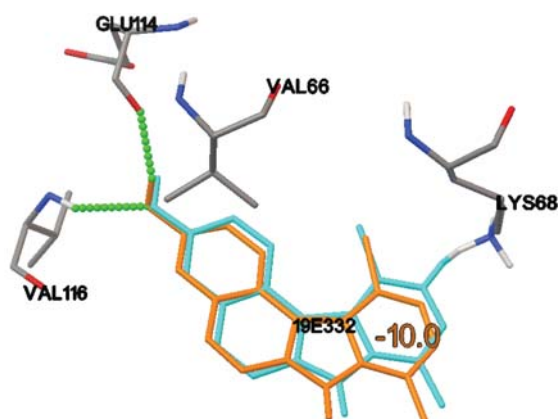


Fig. 4. Comparison of experimentally determined (orange) and docked (cyan) positions of **19E** in the 3OWL active site.

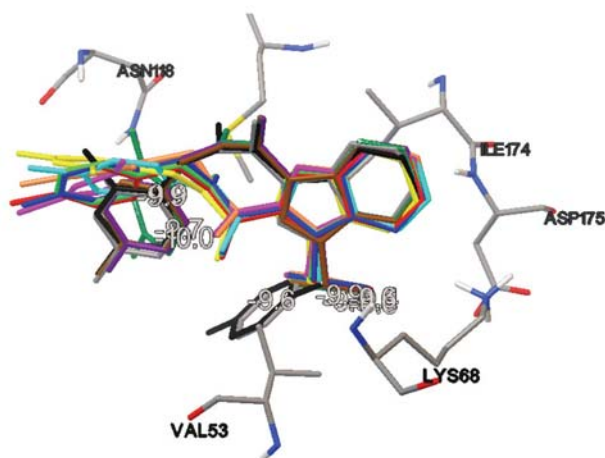


Fig. 5. Overlay of the compounds inside the active site of the CK2.

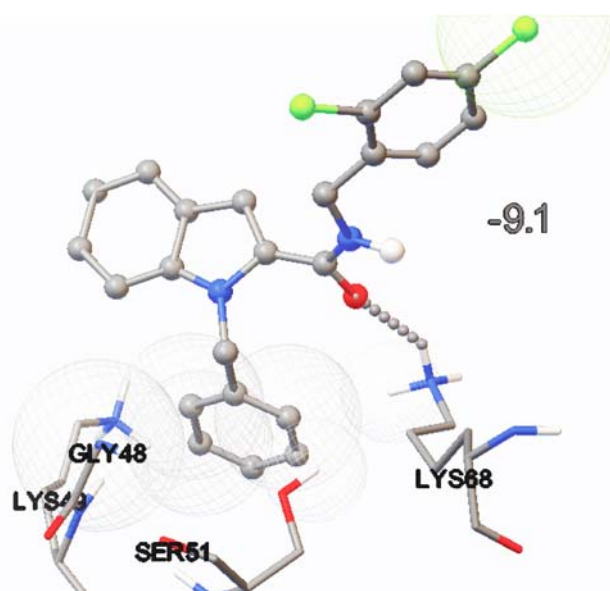


Fig. 6. Interaction of compound **4** with CK2 binding site.

binding mode onto the binding site of enzymes. Compounds showed similar interactions and overlapped in the active site, except compounds **1**, **3**, **4**, **11**, **12**, **13**, **15** and **16** (Fig. 5).

Compound **1** did not show any hydrogen bond between active site amino acids and occupy in the ATP binding site. Compound **17** interacted with ATP binding site and a hydrogen bond was observed between the oxygen of amide carbonyl and NH_2 hydrogen of ASN 118.

The results suggest that ligands with lowest binding affinities have a better chance of interaction with enzymes and have also a good probability of acting as inhibitors. Among the active compounds **1**, **4** and **17**, one hydrogen bond was identified between the amide carbonyl oxygen of compound **4**, and NH_2 hydrogen of LYS 68 (Fig. 6).

3. Conclusion

In this study, a combination of docking studies and biological evaluation allowed us to get into the relationships between biological activity and binding properties of compounds under investigation. Nevertheless, there were no certain relationships between binding properties, structures and activity of compounds. The only conclusion that can be made is that the presence of hydrogen bond acceptor group ($\text{C}=\text{O}$) of the side chain at the indole ring is important for binding to the enzyme active site. Compound **4** showed different binding properties than the reference compound **19E**. Compound **4** has a hydrogen bond between the oxygen of amide carbonyl and NH_2 hydrogen of LYS 68, which is a different interaction than **19E**. This difference is found also with the other indole compounds. In conclusion, it may be necessary to design some compounds with exactly the same binding properties with **19E** to obtain better activity results.

4. Experimental

ATP, Tris and EDTA were bought from Roth (Karlruhe, Germany), MgCl_2 from VWR (Darmstadt, Germany), NaCl and HCl from J. T. Baker (Deventer, The Netherlands), DTT from AppliChem (Darmstadt, Germany), and DMSO from Sigma-Aldrich (Steinheim, Germany) were purchased. CK2 substrate peptide had the amino acid sequence RRRDDDSDDD and was synthesized with its C-terminus conjugated to 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (EDANS). It was synthesized by the Merrifield solid phase procedure.³⁶ Fmoc was used as the N-terminal protecting group,³⁷ and a final purification step was done by RP-HPLC. If not stated otherwise, all solutions were prepared with fresh ultrapure water and have been stored at 4 °C, for short time periods, or at -20 °C, if storage lasted longer than 14 days.

4. 1. Preparation of Recombinant Human CK2 Enzyme

A purification protocol was set up according to the method of Granowski with modifications.³⁸ For this purpose α -subunit (CSNK2A1) and β -subunit (CSNK2B) of protein kinase CK2 were expressed separately in a bacterial expression system (pT7-7/BL21(DE3)).³¹ Freshly transformed bacterial cells were grown overnight at 37 °C until they reached the stationary phase. With the separate overnight culture for each subunit, CSNK2A1 and CSNK2B, 6 L of fresh medium was inoculated and cultivated until an OD500 of 0.6 was reached. Protein expression was induced by adding IPTG to a final concentration of 1 mM and was run at 30 °C for 5–6 h for CSNK2A1 and at 37 °C for 3 h for CSNK2B. Bacterial cells were harvested by centrifugation (6.000 \times g at 4 °C for 10 min) and disrupted by sonification (3 \times 30 s on ice). Cell debris was removed by centrifugation and the bacterial extracts by this strategy for both subunits were combined and subjected to a three-column purification procedures as described previously.³¹ The fractions containing active CK2 holoenzyme were determined by activity measurement. Fractions exhibiting CK2 enzymatic activity were combined and analyzed by SDS PAGE and western blot. They were stored in aliquots at – 80 °C until used for testing.

4. 2. CE-determination of CK2 Inhibition

80 μ L of the CK2-supplemented kinase buffer (kinase buffer + 1 μ g CK2) was either preincubated with 2 μ L test compound, previously dissolved in pure DMSO, or incubated with pure DMSO as a control, for 10 min at 37°C before it was mixed with 120 μ L CE assay buffer (25 mM Tris/HCl, pH 8.5, supplemented with 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 100 μ M ATP and 190 μ M substrate peptide (RRRDDSSDD-[EDANS])). After incubation for 15 min at 37°C, 2 μ mol EDTA were added to stop the enzyme reaction. The samples were stored at 4°C until about 20 nL were injected into a bare fused silica capillary with 50 cm effective and 60 cm total length (50 μ M ID and 375 μ M OD, Beckman Coulter GmbH, Krefeld, Germany). Separations were conducted on a ProteomeLab PA800 System operated by 32 karat 7 software (Beckman Coulter GmbH, Krefeld, Germany) with 2 mol/L aqueous acetic acid as background electrolyte (voltage: 30 kV). Absorption detection was recorded at a wavelength of 214 nm. Before using the capillary for the first time, it was conditioned with 0.1 mol/L NaOH, then rinsed with deionized water and equilibrated with 2 mol/L acetic acid before the sample was injected. This procedure was also performed prior to every measurement. Peak integrations usually had an RSD < 1 %. For determination of IC₅₀ values a concentration-response analysis of the compound in eleven concentrations

ranging from 0.5 nM to 50 μ M was performed. One hundred percent CK2 activity was determined in the absence of the compound but in the presence of DMSO. A control value for no enzyme activity was obtained by adding all components but without the co-substrate ATP. A non-linear regression analysis of the relative inhibition at different compound concentrations was conducted. Therefore a four parameter logistic fit of the data points was calculated by Graphpad Prism using the analysis option “sigmoid curve with variable slope” (GraphPad, La Jolla, CA, USA). The IC₅₀ value represents the concentration at the midpoint (50 %) of CK2 inhibition on a semi logarithmic dose-response plot. The CE assay was finally validated by determining the IC₅₀ values of the known inhibitors of human CK2 Emodin and TBB, which turned out to be 0.27 μ M and 1.33 μ M, respectively, and which were in agreement with the IC₅₀ values that have been determined before with radiometric assays.²⁵

4. 3. Docking Calculations

For this study, X-ray crystal structure of CK2-19E complex (PDB entry 3OWL) was downloaded from Protein Data Bank (PDB). Solvents of molecules were deleted and hydrogen atoms were added to the protein to obtain the docking grid the active site was defined using Autogrid. The grid size was set to 40 \times 40 \times 40 points with grid spacing of 0.375 Å. The grid box was centered on the center of the ligand from the corresponding crystal structure complexes. 2D structures of inhibitors were established by using ChemBioDrawUltra 11.0 and minimized with Hyperchem 8.0.7 (Hypercube Inc, FL, USA) using the semi-empirical the Austin Model-1 (AM-1) method. To be exported to Autodock, the minimized structures were converted into the mol2 file format. The structures of proteins and ligands were combined using the Autodock 4.2.1 and Vina 1.0.2 software package (The Scripps Research Institute, La Jolla CA, USA). The rigid root and rotatable bonds were defined using ADT. The results were ranked by the scoring functions of ligand-fit, and analyzed for relatively lower binding energy, and potential in forming hydrogen bonds with CK2. The ligand was fully optimized inside the binding site during the docking simulations. The conformation with the lowest predicted binding free energy of the most occurring binding modes in CK2 active pockets were evaluated to binding properties and relationships between biological activity-binding affinities of compounds.

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Povzetek

Protein kinaza CK2 (kazein kinaza 2) je vključena v procese celične rasti, proliferacije in zaviranja apoptoze. Tako močno spodbuja preživetje celic in jo lahko štejemo kot pomembno tarčo za zdravljenje raka pri človeku. V tej študiji smo proučevali skupino N-substituiranih derivatov indol-2-karboksamida. Da bi preverili njihove učinke proti raku, smo spojine testirali kot inhibitorje rekombinantne protein kinase CK2. Testi inhibicije so pokazali, da je najučinkovitejša spojina **4** (1-benzil-*N*-(2,4-diklorobenzil)-1*H*-indol-2-karboksamid), z IC₅₀ 14,6 μM proti človeški protein kinazi CK2. Naredili smo tudi študije molekularnega sidranja spojin s CK2 in opredelili vezavni model, ki je pokazal inhibično aktivnost najučinkovitejše spojine **4**.