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Indeno[1,2-*b*]indole derivatives as a novel class of potent human protein kinase CK2 inhibitors

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ABSTRACT

Herein we describe the synthesis and properties of indeno[1,2-*b*]indole derivatives as a novel class of potent inhibitors of the human protein kinase CK2. A set of 19 compounds was obtained using a convenient and straightforward synthesis protocol. The compounds were tested for inhibition of human protein kinase CK2, which was recombinantly expressed in *Escherichia coli*. New inhibitors with IC_{50} in the micro- and sub-micromolar range were identified. Compound **4b** (5-isopropyl-7,8-dihydroindeno[1,2-*b*]indole-9,10(5*H*,6*H*)-dione) inhibited human CK2 with an IC_{50} of 0.11 μM and did not significantly inhibit 22 other human protein kinases, suggesting selectivity towards CK2. ATP-competitive inhibition by compound **4b** was shown and a K_i of 0.06 μM was determined. Our findings indicate that indeno[1,2-*b*]indoles are a promising starting point for further development and optimization of human protein kinase CK2 inhibitors.

1. Introduction

Protein kinases are of high importance in metabolic and regulatory processes. Usually, peptides or proteins are phosphorylated by kinases at the hydroxyl groups of serine, threonine, or tyrosine residues. Protein kinases play major roles in signal transduction and control fundamental cellular functions such as cell–cell-communication, differentiation, migration, the cell cycle and apoptosis.[1]

Human CK2 is a second-messenger- and phosphorylation independent constitutively active S/T protein kinase, with a growing substrate list of more than 400 potential physiological targets reported in the literature up to date.[2] CK2 is a heterotetrameric protein composed of two catalytic α (or α′) subunits and two regulatory β subunits.[3] It shows 'dual cosubstrate specificity', that is, the ability to utilize adenosine triphosphate (ATP) or guanosine triphosphate (GTP) as the cofactor.[4,5] Besides its role in a variety of non-cancer related diseases (such as neurodegenerative disorders, inflammatory processes, angiogenesis-related diseases and viral infections),[6] there is a strong focus on its involvement in cancer. Increased activity of the human protein kinase CK2 has been detected in many different tumors, including those of the prostate, colon, breast and lung,[7] and its impact in cell survival and neoplasia is supported by a growing amount of evidence.[7,8]

In the last three decades numerous inhibitors addressed to the target CK2 have been identified: One of the first reported inhibitors dichlororibobenzimidazole (DRB) showed a rather weak IC_{50} of 15 μM.[9] Tetrabromobenzotriazole (TBB) revealed an IC_{50} of 1.6 μM[10] and the anthraquinone emodin was reported to have an IC_{50} of 0.89 μM.[11] Further inhibitors with IC_{50} in the upper sub-micromolar range include the polybrominated benzimidazoles (IC_{50} from 0.93 to 0.49 μM) described by Andrzejewska et al.[12] and their polyiodinated derivatives, which are around tenfold more potent.[13] The potent and selective indoloquinazoline acetic acid derivative IQA revealed an IC_{50} of 0.08 μM,[14] and for the natural polyphenol ellagic acid an IC_{50} of 0.04 μM was found (Fig. 1).[15] Thus far only one very potent and highly selective inhibitor, the benzonaphthyridine derivative CX-4945 (Fig. 1), with an IC_{50} of 0.001 μM, has reached advanced clinical trials for the treatment of cancer.[16]

Besides the concept of alternative inhibition modes[17] (e.g., CK2β-targeted inhibitors[18] or inhibitors disturbing CK2α/CK2β-subunit interaction[19]), most of the published inhibitors share common structural features: they consist of small and planar heterocyclic scaffolds, able to fit into the nucleotide-binding pocket of CK2α. They serve as ATP/GTP-competitive inhibitors of CK2 and

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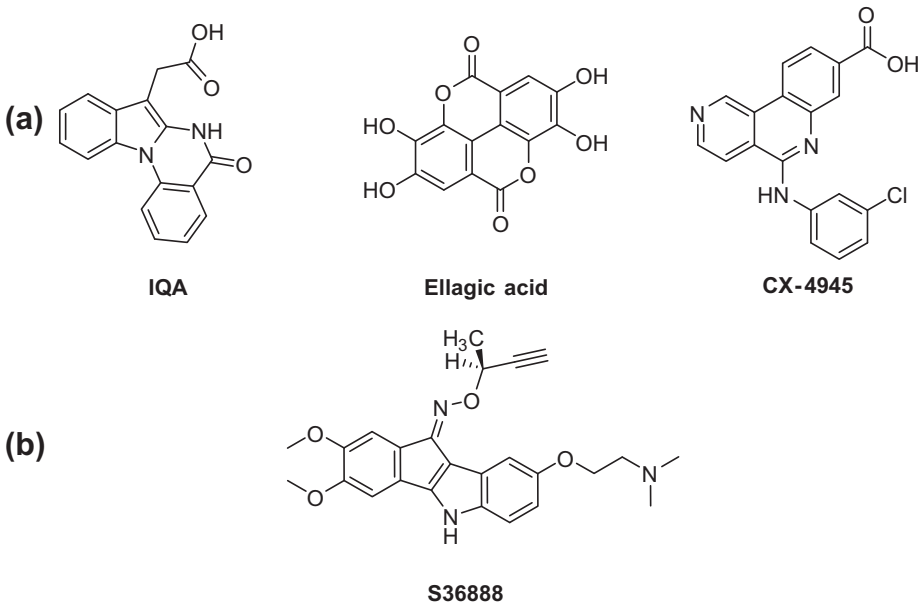


Figure 1. (a) Known CK2 inhibitors IQA, ellagic acid and CX-4945. (b) S36888, a topoisomerase II inhibitor of the indeno[1,2-*b*]indole type.

displace the cofactor.[16,17,20,21] These structural features are also found in indeno[1,2-*b*]indoles such as S36888 (Fig. 1). These compounds have previously been shown to act as DNA-intercalators and inhibitors of topoisomerase II with considerable cytotoxic activity towards cancer cells.[22]

Based on the assumption, that the small and planar heterocyclic indeno[1,2-*b*]indoles could fit into the ATP/GTP-binding pocket of the CK2, we aimed to develop a new class of CK2 inhibitors. Within 19 indeno[1,2-*b*]indoles synthesized, four potent CK2 inhibitors were found. One of them, 5-isopropyl-7,8-dihydroindeno[1,2-*b*]-indole-9,10(5*H*,6*H*)-dione **4b**, inhibited the human CK2 in the sub-micromolar range. As it displayed only low inhibition towards a panel of 22 other human kinases, our results suggest **4b** to be a rather selective inhibitor of CK2. The mode of inhibition was shown to be ATP-competitive and we present evidence of cell permeability.

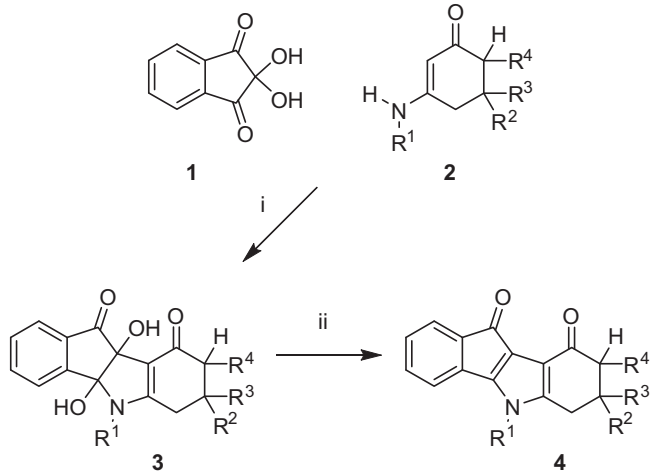
2. Results and discussion

2.1. Synthesis

Compounds of type **4** were synthesized according to a recently published deoxygenation procedure of *vic*-dihydroxyindeno[1,2-*b*]indoles **3** using *N*,*N*,*N*′,*N*′-tetramethylsulfuric acid diamide (tetramethyl-thionylamide, TMTA) as a novel reagent. This resulted in moderate to very good yields of 60–95% (Scheme 1).[23,24] Compounds **3** were prepared by condensation of indanetrione hydrate **1** and 3-amino-cyclohex-2-enones **2** in yields ranging from 40% to 90%. Precursor **1** is commercially available and **2** is readily available by refluxing a solution of cyclohexane-1,3-diones and primary amines in benzene with azeotropic removal of water.[23,24]

For the chiral derivatives **4l** and **4m**, a coupling constant of 10.5 Hz ($^3J_{H7-H8}$) between H-7 and H-8 was observed, showing a 1,2-diaxial coupling of the protons at C-7 and C-8. This results in the conformers with both the methyl and the carbomethoxy groups at C-7 and C-8 in equatorial position on the half-chair, which is in accordance with the crystal structure for the enaminone precursor described by Greenhill et al.[25] with the (*S*)-configuration at C-7 and the (*R*)-configuration at C-8.

The enaminone precursors of **4n** and **4o** were prepared from symmetric 1,3-cyclohexanediones. Condensation of the dione with



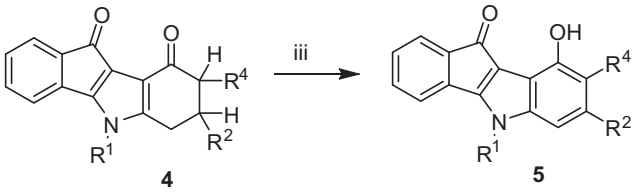
Scheme 1. Preparation of indeno[1,2-*b*]indoles **4**. For R^1, R^2, R^3 and R^4 refer to Table 1. Reagents and conditions: (i) $CHCl_3$ or MeOH, rt, 8–24 h; (ii) DMF, acetic acid, TMTA, rt, 3-12 h.

the amines can occur at both carbonyls with the same probability. Thus, a racemic mixture of both enantiomers of **4n** and **4o** is formed and was used for primary testing. Due to low activity of the racemate in CK2 testing, the isomers were not separated.

For derivative **4n**, 1,2-diaxial coupling constants between H-7 and H-6/H-8 of 10.7 Hz ($^3J_{H6-H7}$ and $^3J_{H7-H8}$) were observed, giving evidence of the equatorial position of the phenyl ring at C-7 for both enantiomers.

Also for **4o**, H-7 shows large couplings with the vicinal protons H-6/H-8 of 10.1 and 11.1 Hz ($^3J_{H6-H7}$ and $^3J_{H7-H8}$) proving the equatorial position of the methyl group at C-7 for both enantiomers.

In order to convert the 7,8-dihydroindeno[1,2-*b*]indole-9,10(5*H*,6*H*)-diones **4** into the aromatic 9-hydroxyindeno[1,2-*b*]indole-10(5*H*)-ones **5** some representative dehydrogenation reactions were explored. The reaction with mercury(II)acetate in boiling acetonitrile according to Iida et al.[26] did not give the desired products. Efforts to aromatize **4** by dehydrogenation in the presence of 10% Pd/C in boiling xylene[27] resulted in the formation of **5**,



4 5

Scheme 2. Preparation of indeno[1,2-*b*]indoles **5**. For R^1, R^2 and R^4 refer to Table 1. Reagents and conditions: (iii) 1,4-dioxane, DDQ, 60–70 °C, 24–72 h.

contaminated by a number of additional compounds such as dimerization products, which made the separation difficult. Finally, a slightly modified version of Rebeks approach to mitomycins by means of DDQ gave the best results.[28,29] The reaction in 1,4-dioxane at 60-70 °C gave **5** in moderate to good yields of 54–74% (Scheme 2). However, the products were strongly dependent on the reaction conditions. In refluxing 1,4-dioxane as well as in refluxing acetonitrile a lot of additional byproducts were formed. A total of 19 indeno[1,2-*b*]indole-derivatives were prepared in yields that ranged from moderate to very good (Table 1).

2.2. Inhibition of human CK2 holoenzyme

The indeno[1,2-*b*]indole derivatives were tested for their inhibitory activity towards human CK2 holoenzyme (Table 1). The synthetic peptide RRRDDDSDDD was used as the substrate, which is reported to be most efficiently phosphorylated by CK2.[30] For initial testing, inhibition was determined relative to the controls at inhibitor concentrations of 10 μM in DMSO as the solvent. The radioactivity of the pure solvent without inhibitor was used as negative control and set to 0% inhibition; Reactions without CK2 were used as positive control and set to 100% inhibition. Compounds with more than 50% inhibition at a concentration of 10 μM were subjected to an IC_{50} determination. For this purpose, inhibition was measured at final concentrations ranging from 0.01 to 30 μM in appropriate intervals. IC_{50} were calculated from the resulting dose–response curves. Each value was determined at least in triplicate in independent experiments. For the known inhibitor emodin we measured an IC_{50} of 0.46 μM in this assay, which is in good agreement with the reported value of 0.89 μM.[11]

From the 19 synthesized indeno[1,2-*b*]indoles, we found four potent compounds with an inhibition of more than 50% at a concentration of 10 μM (**4b**, **4d**, **4f** and **4g**). Two of them (**4b** and **4d**) revealed IC_{50} in the sub-micromolar range (0.11 and 0.82 μM). The CK2 inhibition by compounds **4** was in most cases (**4b**, **4c**, **4j** vs **5b**, **5c**, **5j**) stronger than their dehydrogenated counterparts **5**. An exception was the phenolic indeno[1,2-*b*]indole **5n**, which showed higher inhibition than its analog **4n**. Both derivatives bear the sterically demanding phenyl ring at R^2, which is shifted in-plane in **5n**. Additionally, the lower activity of **4n** could be, at least in part, due to the use of the racemic mixture of both isomers, assuming that only one isomer was active towards CK2. In general for compounds **4**, substitutions at R^2 or R^3, with one or two methyl groups (**4k**, **4o**) or a phenyl ring (**4n**), lowered the inhibitory activity of the compounds. An ester group at R^4 (**4l**, **4m**) had barely any effect on potency. Phenyl or phenylalkyl residues at the indole nitrogen led to a stronger inhibition (**4d**, **4f** and **4g**), but for longer alkyl-linkers (**4e**) inhibitory activity was lost again. *N*-substitution with 2-pyridyl-alkyl and methoxy-substituted phenyl-alkyl residues led to a somewhat lesser activity compared to the phenyl-alkyl derivatives with the same chain-length. Best effects for the cyclohexeneone derivatives **4** were achieved with an isopropyl residue at the indole nitrogen (**4b**), leading to a sevenfold stronger inhibition than the most potent phenylalkyl derivative. Within the phenolic derivatives **5**, *N*-substitution with the isopropyl group also led to the strongest inhibition (48% for **5b**).

2.3. Mode of inhibition

To determine the mode of inhibition, we measured CK2 activity using the non-radiometric capillary electrophoresis (CE) method of Gratz et al.[31] For the strongest inhibitor **4b**, IC_{50} were determined at six different ATP concentrations ranging from approximately 1/10 to 10 times the K_m for ATP. At an ATP concentration at the K_m which was also used for the radiometric measurements, the IC_{50} for **4b** was 0.36 μM, which is in good agreement with the radiometrically determined IC_{50} of 0.11 μM. A linear increase of the IC_{50} with increasing ATP could be observed (Fig. 2), showing the assumed ATP competitivity.[32]

2.4. Determination of K_i

With the demonstrated mode of inhibition, the inhibition constant (K_i) can be calculated from the IC_{50} using the Cheng–Prusoff

Table 1
Synthesized indeno[1,2-*b*]indole derivatives and inhibition of human CK2 holoenzyme

Compound	R^1	R^2	R^3	R^4	Inhibition (%)[a]	IC_{50} (μM)[b]
4a	H	H	H	H	19	n.d.
4b	*iso*-C_3H_7	H	H	H	93.4	0.11
4c	$CH_2C_6H_5$	H	H	H	48	n.d.
4d	$(CH_2)_2C_6H_5$	H	H	H	67	0.82
4e	$(CH_2)_3C_6H_5$	H	H	H	27	n.d.
4f	$CH(CH_3)C_6H_5$	H	H	H	66	4.66
4g	C_6H_5	H	H	H	60	1.44
4h	CH_2-2-C_5H_4N	H	H	H	30	n.d.
4i	$CH_2C_6H_4$-4-OCH_3	H	H	H	41	n.d.
4j	$(CH_2)_2C_6H_3$-3,4-$(OCH_3)_2$	H	H	H	50	n.d.
4k	$CH_2C_6H_5$	CH_3	CH_3	H	10	n.d.
4l	H	CH_3	H	CO_2CH_3	28	n.d.
4m	$CH_2C_6H_5$	CH_3	H	CO_2CH_3	15	n.d.
4n[c]	$CH_2C_6H_5$	C_6H_5	H	H	3	n.d.
4o[c]	$CH_2C_6H_5$	CH_3	H	H	11	n.d.
5b	*iso*-C_3H_7	H	—	H	48	n.d.
5c	$CH_2C_6H_5$	H	—	H	25	n.d.
5j	$(CH_2)_2C_6H_3$-3,4-$(OCH_3)_2$	H	—	H	34	n.d.
5n	$CH_2C_6H_5$	C_6H_5	—	H	31	n.d.

[a] Given is the average percent inhibition at 10 μM.
[b] n.d.: not determined.
[c] A racemic mixture of both enantiomers was used for testing.

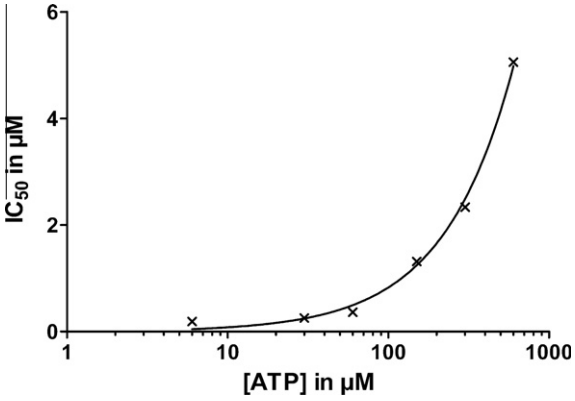


Figure 2. Graphical determination of the inhibition modality. IC_{50} were plotted against the corresponding ATP concentrations. The abscissa was plotted on a logarithmic scale for clarity.

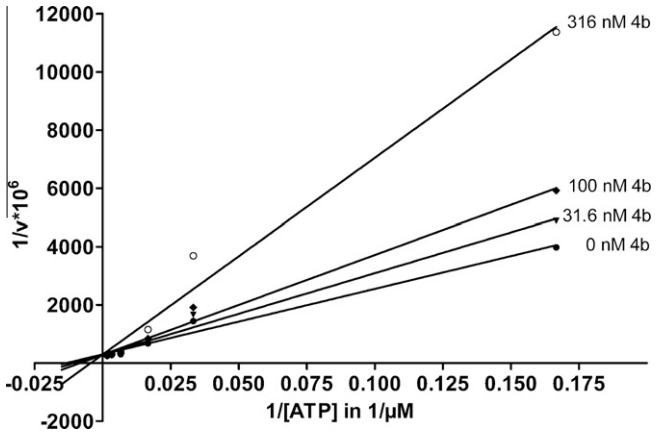


Figure 3. Determination of K_m^{app} for different concentrations of the inhibitor **4b**. Double reciprocal plot of reaction velocity against the cosubstrate concentration.

equation for competitive inhibition.[33] Based on the measurements from the radiometric method, the K_i for the strongest inhibitor **4b** was 0.06 µM.

For comparison, K_i for **4b** was also determined directly by the method described by Copeland.[34] Therefore, reaction velocities (v) at different inhibitor and ATP concentrations were measured with the CE-based CK2 activity assay[31] and were plotted in a Lineweaver–Burk diagram against the varied ATP concentrations (Fig. 3). $1/v_{max}$ is given by the intersections with the ordinate, $-1/K_m^{app}$ are given by the intersections with the abscissa. As already shown in Section 2.3, competitive inhibition is demonstrated by the increasing K_m^{app} with increasing inhibitor concentrations and the unaffected v_{max}.[34]

For the calculation of K_i, K_m^{app} were plotted against the corresponding inhibitor concentrations (Fig. 4). The negative K_i is given by the intersection of the linear regression with the abscissa. For the CE-based measurements, a K_i of 0.15 µM for **4b** was determined, which is in good agreement with the calculated K_i of 0.06 µM from the radiometric measurements.

2.5. Cell permeability

For an evaluation of the cell permeability of **4b**, fragment-based partition coefficients ($C\log P$) and topological polar surface areas (TPSA) were calculated. The data suggest, that **4b** should be able to penetrate cell membranes, with a $C\log P$ of 3.317 and a TPSA of 39.076 Å^2 (calculated by the Molinspiration web services[35]).

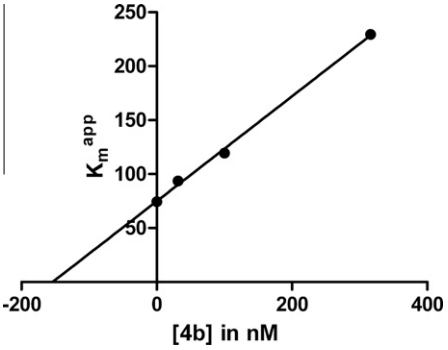


Figure 4. Plot of K_m^{app} against the concentration of **4b**, for the determination of K_i.

Poor absorption and low permeability is predicted for drugs with $C\log P \geqslant 5$ and TPSA $\geqslant 140$ Å^2.[36–38]

To support this prediction, cytotoxicity of **4b** towards a human cancer cell line was evaluated. Therefore, growth inhibition by compound **4b** was measured using the human esophageal cancer cell line KYSE-70. At a concentration of 20 µM, a growth inhibition of 25% (±3%, $n = 3$) was observed. Although the effect was weak, this demonstrates membrane permeability of the compound. The low effects of **4b** towards the cancer cell line could be due to biological conversion of the compounds into inactive derivatives. Alternatively, multidrug resistance efflux transporters as P-glycoprotein (P-gp), which are known to be responsible for the drug resistance of tumors,[39] could have lead to a reduction of intracellular concentrations of the active compound.

2.6. Selectivity profiling

Compound **4b**, which showed the highest inhibition of CK2 (IC_{50} = 0.11 µM), was screened for inhibitory activity against a panel of 22 different human kinases (Table 2). IC_{50} were measured by testing 10 concentrations of each compound in singlicate and calculated from the resulting dose–response curves. The known inhibitor ellagic acid, with a reported IC_{50} towards the CK2 of 0.04 µM,[15] was used as a reference.

Compound **4b** showed selectivity towards CK2 in the panel tested. None of the 22 other kinases were significantly inhibited by **4b** (IC_{50} >35 µM). The inhibition of CK2 by this compound is therefore 300-fold higher than any other kinase tested. Preliminary studies against a panel of 11 kinases suggest that ellagic acid is a strong and selective inhibitor of CK2.[15] Our results suggest that **4b** is a much more selective inhibitor than ellagic acid, which also inhibited eight other kinases with IC_{50} values below 1 µM (ARK5, EGF-R, IGF1R, SRC, VEGF-R2, INS-R, MET and TIE2).

3. Conclusions and outlook

We show that substituted indeno[1,2-*b*]indole derivatives are a novel class of potent inhibitors of the human protein kinase CK2. A set of 19 indeno[1,2-*b*]indoles was prepared in moderate to very good yields and tested for their inhibitory activity and selectivity. Four compounds with good inhibitory activity were found. The most active derivative, **4b**, with an IC_{50} of 0.11 µM showed selectivity towards the human CK2, as judged by low inhibition of 22 different human kinases. Competitive inhibition of compound **4b** was demonstrated. Inhibition constants were determined by the standard radiometric activity assay and a novel capillary electrophoresis based method, giving comparable K_i of 0.06 and 0.15 µM, respectively. Membrane permeability of the strongest inhibitor **4b** could be demonstrated by theoretical predictions

Table 2
Inhibition[a] of 22 different protein kinases by indeno[1,2-*b*]indole **4b** and the known inhibitor ellagic acid (**EA**)

Kinase	AKT1	ARK5	Aurora-A	Aurora-B	B-RAF	CDK2/CycA	CDK4/CycD1	EGF-R	EPHB4	ERBB2	FAK
4b	>35	>35	>35	>35	>35	>35	>35	>35	>35	>35	>35
EA	3.34	0.51	2.48	2.50	1.76	3.39	1.09	0.69	1.83	3.54	3.11
Kinase	IGF1R	SRC	VEGF-R2	VEGF-R3	INS-R	MET	PDGFR -β	PLK1	SAK	TIE2	COT
4b	>35	>35	>35	>35	>35	>35	>35	>35	>35	>35	>35
EA	0.25	0.80	0.79	1.54	0.34	0.58	1.30	3.34	2.97	0.26	2.37

[a] Given are IC_{50} in μM.

and the cytotoxic potential of this compound towards an esophageal cancer cell line. Taken together, the results show that indeno [1,2-*b*]indoles, such as **4b**, are a promising starting point for the further development of potent and selective CK2 inhibitors, leading to potential drugs for the treatment of malignancies and non-cancer related diseases. Synthesis and evaluation of further derivatives, in particular *N*-isopropyl substituted derivatives, is in progress.

4. Experimental section

4.1. General

Melting points: Büchi melting point apparatus by Dr. Tottoli; not corrected. IR spectra: Perkin Elmer FT-IR 1600, using KBr discs. NMR spectra: Bruker AC 200F (^{1}H: 200 MHz/^{13}C: 50 MHz) with TMS as the internal standard and Varian AS 400 Mercuryplus (^{13}C: 100 MHz) with the solvent peak as the internal standard in the designated solvents, using δ (ppm) scale; signals, labeled by $*$ exchanged by addition of D_2O. ^{13}C NMR spectra of **5j** could not be obtained due to insufficient solubility in all standard deuterated solvents. EI mass-spectra: Finnigan 4200 quadrupole mass-spectrometer, equipped with a MASPEC data system; 70 eV ionizing potential. Microanalyses: Perkin Elmer Elemental Analyzer 2400/II. MPLC or flash chromatography was performed on 230–400 mesh silica (Merck). Solvents were purified by standard methods and dried over molecular sieves or sodium. Yields refer to the amount of the products after one recrystallization and are not optimized.

4.2. Synthesis of 7,8-dihydroindeno[1,2-*b*]indole-9,10(5*H*,6*H*)-diones (4)[23,24,40]

The synthesis and characterization of compounds **4** of Table 1 has been previously described by Hemmerling and Reiss.[23] We describe here again the methods to obtain compounds **4** that were shown to be pharmacologically most active (**4b**, **4d**). We also describe the compounds that served as reactants (**4c**, **4j**, **4n**) in the reactions to prepare compounds **5**.

General Procedure A: Compound **3** (5.0 mmol) was dissolved in 15 mL DMF and 3 mL acetic acid was added. After addition of TMTA (20 mmol) the solution was stirred for the time indicated. After this time a precipitate has deposited, which was separated by suction. The filtrate was evaporated in vacuo, the oily residue diluted with H_2O, and the solution was basified with $NaHCO_3$. Extraction with $CHCl_3$ gave a second crop, which was purified by MPLC on SiO_2/EtOAc-hexane or $CHCl_3$-EtOAc.

General Procedure B: Compound 3 (5.0 mmol) was dissolved in 15 mL DMF and 3 mL acetic acid was added. After addition of TMTA (20 mmol) the solution was stirred for the time indicated. After this time, the solution was poured into 500 mL H_2O. The suspension was stirred for 3 h and the solids separated by filtration. The solid was air dried and crystallized. The filtrate was evaporated to a small volume and worked up as described under General Procedure A.

4.2.1. 5-Isopropyl-7,8-dihydroindeno[1,2-*b*]indole-9,10(5*H*,6*H*)-dione (4b)

General Procedure B. Reaction time: 3 h. Yield: 89%. Mp: 201 °C (EtOAc). IR (KBr): ν (cm^{-1}) = 2944, 1707, 1665, 1609, 1520, 1485, 1426, 1319, 1297. ^{1}H NMR (200 MHz, TMS, $CDCl_3$): δ (ppm) = 1.65 (d, J = 7 Hz, 6H, $2CH_3$), 2.1–2.2 (m, 2H, H-7), 2.4–2.5 (m(t), 2H, CH_2), 2.8–2.9 (m(t), 2H, CH_2), 4.5– 4.7 (m, 1H, CH), 7.0–7.5 (m, 4H, Ar-H). EI-MS (70 eV): m/z (%) = 279 (49) [M^+], 278 (24), 264 (10), 237 (11), 223 (10), 209 (25), 208 (22), 181 (43), 180 (18), 153 (22), 152 (19), 130 (15), 126 (13), 125 (10), 102 (11), 78 (14), 77 (16), 75 (13), 69 (11), 51 (13), 43 (98), 42 (16), 41 (100). Anal. Calcd for $C_{18}H_{17}NO_2$ (279.34): C, 77.40; H, 6.13; N, 5.02. Found: C, 76.93; H, 6.00; N, 4.90.

4.2.2. 5-Benzyl-7,8-dihydroindeno[1,2-*b*]indole-9,10(5*H*,6*H*)-dione (4c)

General Procedure B. Reaction time: 3 h. Yield: 95%. Mp: 185 °C (EtOAc). IR (KBr): ν (cm^{-1}) = 1703, 1666, 1607, 1526, 1499, 1453, 1443, 1431, 1293. ^{1}H NMR (200 MHz, TMS, $CDCl_3$): δ (ppm) = 2.0–2.2 (m, 2H, H-7), 2.41 (m(t), 2H, CH_2) 2.62 (m(t), 2H, CH_2), 5.17 (s, 2H, NCH_2), 6.68–6.72 (m, 1H), 7.00–7.2 (m, 4H), 7.29–7.41(m, 4H). ^{13}C NMR (50 MHz, TMS, $CDCl_3$): δ (ppm) = 21.70, 22.85, 37.71, 49.14, 117.24, 117.88, 119.81, 123.43, 125.91, 125.98, 128.21, 128.25, 129.28, 132.44, 134.71, 134.89, 138.66, 150.66, 153.01, 184.18, 192.25. EI-MS (70 eV): m/z (%) = 327 (38) [M^+], 326 (12), 299 (4), 298 (3), 272 (3), 271 (5), 270 (6), 250 (5), 208 (5), 180 (4), 152 (4), 92 (8), 91 (100). Anal. Calcd for $C_{22}H_{17}NO_2$ (327.38): C, 80.71; H, 5.23; N, 4.28. Found: C, 80.81; H, 5.20; N, 4.24.

4.2.3. 5-Phenethyl-7,8-dihydroindeno[1,2-*b*]indole-9,10(5*H*,6*H*)-dione (4d)

General Procedure A. Reaction time: 5 h. Yield: 62%. Mp: 189–190 °C (EtOAc). IR (KBr): ν (cm^{-1}) = 1697, 1659, 1606, 1524, 1499, 1476, 1296. ^{1}H NMR (200 MHz, TMS, DMSO-d_6): δ (ppm) = 1.7–1.9 (m, 2H, CH_2), 2.2–2.4 (m, 4H, $2CH_2$), 2.9–3.1 (m, 2H, CH_2), 4.3–4.4 (m, 2H, NCH_2), 7.0–7.4 (m, 9H, Ar-H). EI-MS (70 eV): m/z (%) = 341 (100) [M^+], 285 (12), 250 (8), 249 (10), 237 (38), 209 (37), 194 (19), 121 (25), 105 (19), 104 (15), 91 (11). Anal. Calcd for $C_{23}H_{19}NO_2$ (341.40): C, 80.92; H, 5.61, N, 4.10. Found: C, 80.92; H, 5.39; N, 4.04.

4.2.4. 5-(3,4-Dimethoxyphenethyl)-7,8-dihydroindeno[1,2-*b*]indole-9,10(5*H*,6*H*)-dione (4j)

General Procedure B. Reaction time: 8 h. Yield: 90%. Mp: 189–190 °C (DMF/H_2O). IR (KBr): ν (cm^{-1}) = 1699, 1663, 1605, 1516, 1502, 1450, 1430, 1292, 1264, 1245. ^{1}H NMR (200 MHz, TMS, $CDCl_3$): δ (ppm) = 1.8–1.95 (m, 2H, CH_2), 2.07 (t, J = 6.0 Hz, 2H, CH_2), 2.35 (t, J = 6.0 Hz, 2H, CH_2), 3.02 (t, J = 6.0, 2H, CH_2), 3.74, 3.82 (2 s, 6H, $2OCH_3$), 4.16 (t, J = 6.0, 2H, NCH_2), 6.42 (d, J = 1.7,

1H, H2′), 6.53 (dd, J_1 = 1.7 Hz, J_2 = 8.0 Hz, 1 H, H6'), 6.75 (d, J = 8.0 Hz, 1H, H5), 6.85–7.5 (m, 4H, Ar-H). EI-MS (70 eV): m/z (%) = 401 (22) [M+], 400 (7), 399 (10), 165 (25), 164 (29), 163 (5), 151 (100), 150 (12), 149 (17), 107 (14), 105 (11), 102 (11). Anal. Calcd for $C_{25}H_{23}NO_4$ (401.45): C, 74.79; H, 5.77; N, 3.49. Found: C, 74.63; H, 5.84; N, 3.37.

4.2.5. 5-Benzyl-7-phenyl-7,8-dihydroindeno[1,2-*b*]indole-9,10(5*H*,6*H*)-dione (4n)

General Procedure A. Reaction time: 12 h. Yield: 80%. Mp: 252 °C (Toluene). IR (KBr): ν (cm^{-1}) = 1705, 1669, 1605, 1499. ^{1}H NMR (200 MHz, TMS, $CDCl_3$): δ (ppm) = 2.64–3.01 (m, 4H, H-6, H-8), 3.42–3.57 (m, 1H, H-7), 5.22 (s, 2H, NCH_2), 6.73–6.82 (m, 1H, H-4), 7.06–7.52 (m, 13H, Ar-H). ^{13}C NMR (50 MHz, TMS, $CDCl_3$): δ (ppm) = 29.8, 41.4, 44.7, 49.2, 117.2, 117.8, 120.0, 123.9, 125.3, 125.9, 126.8, 127.2, 128.2, 128.4, 128.5, 128.8, 129.0, 129.4, 132.4, 134.6, 134.8, 138.7, 142.5, 149.2, 153.4, 184.1, 191.0. EI-MS (70 eV): m/z (%) = 403 (4) [M^+], 312 (3), 299(12), 271 (13), 208 (13), 180 (4), 152 (5), 104 (4), 91 (100). Anal. Calcd for $C_{28}H_{21}NO_2$ (403.49): C, 83.35; H, 5.25; N, 3.47. Found: C, 83.54; H, 5.27; N, 3.33.

4.3. Synthesis of 9-hydroxyindeno[1,2-*b*]indole-10(5*H*)-ones (5)

4.3.1. 9-Hydroxy-5-isopropylindeno[1,2-*b*]indole-10(5*H*)-one (5b)

Compound **4b** (1.0 g, 3.6 mmol) was dissolved in 1,4-dioxane (150 mL) and a solution of DDQ (0.91 g, 4.0 mmol) was added. The mixture was heated to 70 °C and stirred for 24 h with TLC monitoring. Half of the solvent was then evaporated in vacuo and after cooling to rt the solid that separated was removed by suction. The filtrate was evaporated to dryness and the solid residue subjected to MPLC at silica (eluent: EtOAc) gave 0.8 g of **5b**. Yield: 65%. Mp: 182 °C (MeOH). IR (KBr): ν (cm^{-1}) = 2438, 1662, 1604, 1492, 1449, 1429, 1420, 1230. ^{1}H NMR (200 MHz, TMS, $CDCl_3$): δ (ppm) = 1.72 (d, J = 7 Hz, 6H, 2 CH_3), 4.79–4.93 (m, 1H, CH), 6.66–6.70 (m, 1H, Ar-H), 6.75* (s, 1H, OH), 6.9–7.5 (m, 6H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.80, 156.12, 151.22, 142.70, 140.28, 134.51, 132.47, 129.38, 124.26, 122.43, 120.06, 113.21, 109.33, 107.76, 104.73, 49.21, 21.22. EI-MS (70 eV): m/z (%) = 277 (99) [M^+], 262 (11), 235 (100), 220 (12), 207 (12), 206 (22), 179 (30), 178 (16), 177 (11), 151 (16), 150 (10), 76 (22). Anal. Calcd for $C_{18}H_{15}NO_2$ (277.32): C, 77.96; H, 5.45; N, 5.05. Found: C, 78.18; H, 5.66; N, 5.00.

4.3.2. 5-Benzyl-9-hydroxyindeno[1,2-*b*]indole-10(5*H*)-one (5c)

Preparation according to **5b** with **4c**. Reaction temperature: 65 °C. Reaction time: 72 h. Yield: 54%. Mp: 198–199 °C (EtOH). IR (KBr): ν (cm^{-1}) = 3426, 1666, 1604, 1523, 1504, 1488, 1448, 1440, 1430, 1422, 1238. ^{1}H-NMR (200 MHz, TMS, $CDCl_3$): δ (ppm) = 5.49 (s, 2 H, NCH_2), 6.54* (s, 1H, OH), 6.7–7.6 (m, 12H, Ar-H). ^{13}C NMR (50 MHz, TMS, $CDCl_3$): δ (ppm) = 49.23, 103.43, 108.16, 113.12, 115.38, 118.83, 123.54, 125.66, 126.34, 128.27, 129.20, 129.56, 132.44, 135.33, 135.69, 140.35, 144.02, 149.93, 156.80, 185.97. EI-MS (70 eV): m/z (%) = 325 (20) [M^+], 248 (7), 234 (5), 206 (4), 177 (3), 151 (5), 92 (8), 91 (100). Anal. Calcd for $C_{22}H_{15}NO_2$ (325.37): C, 81.21; H, 4.65; N, 4.30. Found: C, 80.98; H, 4.91; N, 4.37.

4.3.3. [2-(3,4-Dimethoxyphenyl)-ethyl]-9-hydroxyindeno[1,2-*b*]indole-10(5*H*)-one (5j)

Preparation according to **5b** with **4j**. Reaction temperature: 65 °C. Reaction time: 48 h. Yield: 58%. Mp: 137–138 °C (Toluene/Cyclohexane). IR (KBr): ν (cm^{-1}) = 3422, 1669, 1604, 1516, 1448, 1430, 1262, 1237, 1158, 1028. ^{1}H NMR (200 MHz, TMS, CD_2Cl_2): δ(ppm) = 3.09 (t, J = 6.6 Hz, 2H, CH_2), 3.62, 3.69 (2s, 6H, 2OCH_3), 4.40 (t, J = 6.7 Hz, 2H, NCH_2), 6.4 (m, 10H, Ar-H). EI-MS (70 eV): m/z (%) = 399 (62) [M^+], 248 (26), 165 (18), 151 (100). Anal. Calcd for $C_{25}H_{21}NO_4$ (399.44): C, 75.17; H, 5.30; N, 3.51. Found: C, 75.44; H, 5.24; N, 3.53.

4.3.4. 5-Benzyl-9-hydroxy-7-phenylindeno[1,2-*b*]indole-10(5*H*)-one (5n)

Preparation according to **5b** with **4n**. Reaction temperature: 60 °C. Reaction time: 48 h. Purification by MPLC on silica: (Eluent: EtOAc/hexane = 40:60 (v/v)). Yield: 74%. Mp: 254–255 °C (CH_3CN) IR (KBr): ν (cm^{-1}) = 3426, 1667, 1636, 1601, 1576, 1519, 1505, 1496, 1482, 1444, 1428, 1421, 1352, 1273. ^{1}H NMR (200 MHz, TMS, $CDCl_3$): δ (ppm) = 5.47 (s, 2H, NCH_2), 6.47* (s, 1 H, OH), 6.94–7.55 (m, 16H, Ar-H). ^{13}C NMR (50 MHz, TMS, DMSO-d_6): δ (ppm) = 47.8, 101.4, 107.2, 112.1, 113.8, 119.0, 122.4, 126.4, 126.5, 127.1, 127.5, 128.8, 128.8, 129.5, 132.4, 134.1, 136.8, 137.0, 140.1, 140.5, 144.9, 151.3, 157.7, 182.8. EI-MS (70 eV): m/z (%) = 401 (10) [M^+], 310 (19), 282 (10), 253 (5), 226 (4), 151 (2), 102 (5), 92 (7), 91 (100), 77 (7), 65 (20). Anal. Calcd, for $C_{28}H_{19}NO_2$ (401.46): C, 83.77; H, 4.77; N, 3.49. Found: C, 83.87; H, 4.85; N, 3.49.

4.4. Preparation of recombinant human CK2 enzyme and testing of inhibitors

As described earlier,[41] α-subunit (CSNK2A1) and β-subunit (CSNK2B) of protein kinase CK2 were expressed separately in *E. coli* (BL21(DE3) pT7-7). The starter cultures were grown overnight at 37 °C until the stationary phase was reached. With the separate starter cultures for each subunit, fresh medium was inoculated and cultivated until an OD_{500} of 0.6. For induction of protein expression IPTG was added 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 (6000×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 for both subunits were combined and subjected to a three-column purification. Activity of the fractions was determined by measurements of the conversion of a peptide substrate (RRRDDDSDDD). 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. For testing of inhibitors, 0.5 μL (0.25 U/mL) of CK2 holoenzyme was preincubated at room temperature for 10 min. The inhibitors (in DMSO) were then added to a final concentration of 10 μM in 20 μL of kinase buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT). DMSO alone was used as negative control. The reaction was started by the addition of 30 μL assay buffer (25 mM Tris/HCl, pH 8.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 100 μM ATP, 0.19 mM peptide substrate, 0.6 μCi [γ-^{32}P]ATP). Final concentrations in the reaction mixture were 60 μM for the cosubstrate ATP and 114 μM for the substrate peptide. The reaction was carried out at 37 °C for 15 min then the mixture was spotted onto a P81 ion exchange paper (Whatman). After washing thrice with excess phosphate (85 mM H_3PO_4) and subsequently with ethanol, the filter was dried and the adhering radioactivity measured by a scintillation counter (Packard). Initial inhibition at a fixed concentration of 10 μM was determined relative to the controls. Pure solvent without inhibitor was used as negative control (0 % inhibition), assays without CK2 were used as positive control (100 % inhibition). For IC_{50} determination inhibition was measured at final concentrations ranging from 0.01 to 30 μM in appropriate

intervals. IC_{50} were calculated from the resulting dose-response curves at least in triplicate in independent experiments.

4.5. Mode of inhibition

For the determination of the mode of inhibition, the recently established capillary electrophoretic CK2 activity assay was used.[31] Enzymatic reactions were performed as described in Section 4.2.2, but without the use of the [γ-^{32}P] labeled ATP in the assay buffer. Total reaction volume was scaled up to 200 μL, composed of 80 μL CK2-supplemented kinase buffer and 120 μL assay buffer. Reactions were stopped by the addition of 4 μL EDTA (0.5 M) and the reaction mixture was analyzed by a PA800 capillary electrophoresis from Beckman Coulter (Krefeld, Germany). For electrophoretic separation, 2 M acetic acid was used as the electrolyte. The separated substrate and product peptide were detected and quantified using a DAD-detector at 214 nm. Measurements were performed using six ATP concentrations ranging from approximately a tenth to ten times the K_m (75 μM) and for each ATP concentration IC_{50} were determined using nine inhibitor concentrations ranging from 0.001 μM to 100 μM. For the analysis of inhibition modality IC_{50} were plotted versus the ATP concentration.[32]

4.6. Determination of K_i

The K_i of **4b** for the radiometric measurements was calculated from IC_{50} with the Cheng–Prusoff equation for competitive inhibition ($IC_{50} = K_i(1+([ATP]/K_m))$.[33]

For determination of K_i the method described by Copeland was used.[34] Reaction velocities were determined at different inhibitor and cosubstrate concentrations with the CE-Method described in Section 4.2.3. Reaction velocities were plotted in a Lineweaver–Burk plot against the varied ATP concentrations where $-1/K_m^{app}$ are given by the intersections with the abscissa. K_m^{app} were then plotted against the corresponding inhibitor concentrations and $-K_i$ is given by the intersection of the linear regression with the abscissa.

4.7. Cell permeability

Fragment-based partition coefficients (*C*log*P*) and topological polar surface areas (TPSA) were calculated by the molinspiration web services.[35]

The ability of the compounds to permeate cells was evaluated indirectly with the Crystal Violet staining method as previously described.[42,43] With this method, growth inhibition of adherent cancer cell lines is determined in 96-well microtiter plates. The human esophageal cancer cell line used (KYSE-70) was obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures) and was grown in streptomycin and penicillin G supplemented RPMI 1640 medium containing 10% FCS (Sigma). Cells were incubated for 96 h with the compounds dissolved in DMF. Final concentration of the solvent was 0.1%. After incubation, cells were fixed 20 min with 1% glutaraldehyde and then stained for half an hour with 0.02% Crystal Violet in water. Following washing for half an hour in water, the residual cell-bound dye was dissolved with ethanol/water (7:3 (v/v)) and the absorption (570 nm) was measured with an Anthos 2010 plate reader (Anthos, Salzburg, Austria).

4.8. Kinase selectivity

The selectivity of **4b** towards CK2 was investigated using a commercial radiometric protein kinase assay, which tested this inhibitor against a panel of 22 purified recombinant human kinases (^{33}PanQinase® Activity Assay, ProQinase, Freiburg, Germany). The assay was conducted as previously described.[44] Briefly, inhibition was measured in a 50 μL reaction volume in 96-well FlashPlates from Perkin–Elmer/NEN (Boston, MA). The reaction contained 20 μL assay buffer, 5 μL aqueous ATP solution, 5 μL inhibitor (in 10% DMSO), 10 μL of the substrate, and 10 μL purified recombinant human protein kinase. The assay for all enzymes contained 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 μM sodium orthovanadate, 1.2 mM dithiothreitol, 50 μg/mL PEG_{20000}, and 1 μM [γ-^{33}P]ATP (approx. 5×10^5 cpm/well). Used substrates: GSK3(14-27) for AKT1; tetra(LRRWSLG) for Aurora A and B; MEK1 KM for B-RAF; Histone H1 for CDK2/CycA; RB-CTF for CDK4/CycD1; $Poly(E,Y)_{4:1}$ for EGF-R, EPHB4, ERBB2, FAK, IGF1-R, SRC, VEGF-R2, VEGF-R3 and TIE2; $Poly(A,E,K,Y)_{6:2:5:1}$ for INS-R, MET and PDGF-Rβ; Casein for PLK1; p38-alpha-KRKR for SAK; Autophosphorylation was measured for ARK5 and COT. All protein kinases were expressed in Sf9 insect cells as His-tagged or GST-fusion proteins and purified by affinity chromatography. Reactions were incubated at 30 °C for 80 minutes. The reaction was quenched with 50 μL of 2% (v/v) H_3PO_4, plates were aspirated and washed twice with 200 μL of 0.9% (w/v) NaCl or 200 μL H_2O. Incorporation of $^{33}P_i$ was measured with a microplate scintillation counter (Microbeta Trilux, Wallac). Assays were performed with a BeckmanCoulter/Sagian robotic system.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.02.017. These data include MOL files and InChiKeys of the most important compounds described in this article.

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