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Novel indeno[1,2-*b*]indoloquinones as inhibitors of the human protein kinase CK2 with antiproliferative activity towards a broad panel of cancer cell lines

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ABSTRACT

We previously reported indeno[1,2-*b*]indoles as a novel class of potent inhibitors of the human protein kinase CK2. In the present study we prepared two novel quinoid derivatives, the indeno[1,2-*b*]indoloquinones **6b** and **6c**, and demonstrated inhibition of the human CK2 by the compounds. Furthermore, we showed substantial antiproliferative activity of both compounds towards a broad panel of human cancer cell lines in the low micromolar range. Whereas the earlier indeno[1,2-*b*]indoles have been shown to be selective for CK2, the indeno[1,2-*b*]indoloquinones **6b** and **6c** also inhibited the AMPK activated protein kinase ARK5, potentially contributing to the anti-cancer effects of the compounds. In addition, with compound **6b** we found a very potent inhibitor of the leukemia-associated receptor tyrosine kinase FLT3, with an IC_{50} of 0.18 μM.

1. Introduction

Protein kinases act as key regulators of numerous cellular processes such as transcription, metabolism, differentiation, cell–cell communication, migration, cell cycle control and apoptosis. They mediate most of the signal transduction in eukaryotes by phosphorylation of client proteins at their serine, threonine or tyrosine residues [1]. Except for a small number of dual specificity kinases that are capable to phosphorylate all three residues, protein kinases are predominantly specific either for serine and threonine or for tyrosine residues only [2]. The human protein kinase CK2, earlier reported to be solely a Ser/Thr kinase, is one of these dual specificity kinases that is also able to phosphorylate tyrosine residues [3] with an enormous list of over 400 potential substrates [4]. Moreover, it also shows dual cosubstrate specificity, as it is able to use ATP or GTP as the phosphate group donor [5,6]. CK2 has been reported to play a part in various diseases as viral infections, parasitosis, inflammatory processes, angiogenesis related diseases, neurodegenerative diseases and in particular in cancer [7,8]. Its implication in cancer has been underlined by a number of facts and in a variety of cancers an elevated activity of CK2 has been reported [9,10]. Furthermore, an induction of apoptosis in cancer cells by the downregulation of CK2 activity (e.g. by CK2 inhibitors, CK2α siRNA) has been shown [11], emphasizing the importance of the development of CK2 inhibitors for the treatment of cancers.

We recently found that indeno[1,2-*b*]indoles (Fig. 1A) are potent inhibitors of the human protein kinase CK2, with an IC_{50} of 0.11 μM for the strongest inhibitor [12]. The earlier prepared indeno[1,2-*b*]indoles have also shown to be moderate inhibitors of the growth of esophageal cancer cells [12]. Additionally, as recently reported by Kashyap et al., structurally closely related indenoindolones (Fig. 1B) show higher anti-cancer activity than the chemotherapeutic drugs etoposide and 5-fluorouracil (5-FU) in kidney cancer cells [13].

To expand the structural diversity of our earlier reported indeno[1,2-*b*]indoles as inhibitors of the human CK2, we prepared two novel derivatives of the compounds, the quinoid indeno[1,2-*b*]indoloquinones (Fig. 2), by a new and convenient synthesis protocol.

We evaluated their potential as inhibitors of the human CK2 and their ability to inhibit the growth of different tumor cell lines. Furthermore, we investigated their selectivity towards a panel of 23 other kinases.

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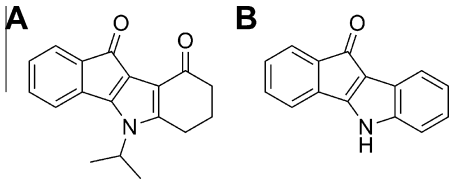

Fig. 1. (A) Compound **4b** as the most potent CK2 inhibitor of the indeno[1,2-*b*]indole type. (B) A structurally closely related indenoindolone with higher anticancer potency than 5-FU and etoposide.

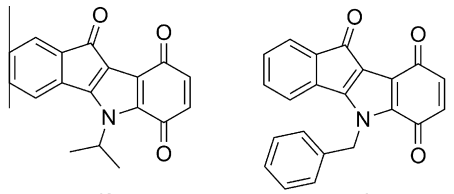

Fig. 2. The two novel indeno[1,2-*b*]indoloquinones **6b** and **6c**.

2. Materials and methods

2.1. Synthesis

2.1.1. General

Melting points: Büchi melting point apparatus, uncorrected. NMR spectra: Bruker AC 200F (^{1}H: 200 MHz/13C: 50 MHz) with TMS as the internal standard using δ (ppm) scale. IR spectra: Perkin Elmer FT-IR 1600. Mass-spectra: Finnigan 4200 quadrupole mass-spectrometer, equipped with a MASPEC data system. HRMS: Bruker micrOTOF-Q II (APCI) and Finnigan MAT 8200 (EI). MPLC was performed on 230–400 mesh silica (Merck) with EtOAc as the eluent. Solvents were purified by standard methods and dried over molecular sieves. Yields refer to the amount of the products after one recrystallization and are not optimized. Synthesis and characterization of the precursors **4** and **5** has been published earlier [12,14].

2.1.2. 5-Isopropyl-5H-indeno[1,2-b]indole-6,9,10-trione ***6b***

Compound **5b** (1.5 g, 5.4 mmol) was dissolved in dry DMF (100 ml) and Salcomine (80 mg, Merck) was added. The mixture was treated with dry O_2 at 70 °C. After 8 h the mixture was filtered and the filtrate evaporated to dryness. The residue was dissolved in $CHCl_3$ and purified by MPLC to give **6** as a red powder (2.4 mmol, 45%). Mp: 254 °C (CH_3CN); ^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.70 (d, J = 7 Hz, 6H, $2CH_3$), 5.6–6.1 (broad, 1 H, CH), 6.62, 6.63 (AB, 2H, H-7, H-8), 7.25–7.66 ppm (m, 4H, Ar-H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 21.8, 51.1, 122.4, 125.5, 130.8, 134.1, 134.5, 135.0, 136.4, 138.7, 140.9, 155.6, 178.8, 182.5, 184.2 ppm; IR (KBr): ν = 1715, 1662, 1649, 1599, 1518, 1441, 1289 cm^{-1}; EI-MS (EI, 70 eV): *m/z* (%) = 291 (57)[M$^+$], 276 (10), 249 (100), 221 (21), 195 (10); HRMS (APCI): m/z [M + 1]$^+$ calcd: 292.0968, found: 292.0970.

2.1.3. 5-Benzyl-5H-indeno[1,2-b]indole-6,9,10-trione ***6c***

Preparation according to **6b** with **5c**. Yield: 62%; mp: 224–226 °C (EtOH); ^{1}H NMR (200 MHz, CD_2Cl_2): δ = 5.84 (s, 2H, CH_2), 6.54, 6.59, 6.60, 6.65 (AB, 2H, H-7, H-8), 7.1–7.6 ppm (m, 9H, Ar-H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 50.7, 119.6, 120.8, 122.7, 124.7, 126.6, 128.4, 129.2, 130.3, 133.4, 133.5, 133.7, 134.8, 136.3, 137.2, 139.8, 155.8, 178.1, 181.6, 183.4 ppm; IR (KBr): ν = 1719, 1665, 1646, 1526, 1461, 1386, 1252 cm^{-1}; EI-MS (70 eV): *m/z* (%) = 339 (10) [M$^+$], 151 (3), 127 (4), 114 (2), 91 (100), 65 (23); HRMS (EI): m/z [M]$^+$ calcd.: 339.0895, found: 339.0896.

2.2. Testing of inhibitors of the human CK2 [12]

The preparation of the human recombinant CK2 holoenzyme was performed according to a protocol described earlier [15]. For testing, 0.5 µl (0.25 U/ml) of CK2 holoenzyme was incubated at room temperature for 10 min. Stock solutions of the Inhibitors (in DMSO) were diluted 1:1000 in 20 µl of kinase buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) to the final concentrations. The reaction was started by the addition of 30 µl assay buffer (25 mM Tris/HCl, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 100 µM ATP, 0.19 mM peptide substrate (RRRDDDSDDD), 0.6 µCi [γ-^{32}P]ATP). The reaction was performed at 37 °C for 15 min and subsequently spotted onto a P81 ion exchange paper (Whatman). After washing with excess phosphate (three times with 85 mM H_3PO_4) and subsequently with ethanol, the filter was dried and radioactivity measured by a scintillation counter (Packard). For IC_{50} determination, inhibition was measured at ten final concentrations from 0.01 to 30 µM in triplicate in independent experiments. IC_{50} were calculated from the resulting dose-response curves.

2.3. Cell culture

As previously described [16,17], measurement of growth inhibition of adherent cell lines was performed by the use of Crystal Violet staining in 96-well microtiter plates. Eight human cancer cell lines (5637, SISO, KYSE-70 used in primary testing, additionally MCF-7, DAN-G, LCLC, A427, RT-4 used in secondary testing) were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures). These were grown in RPMI 1640 medium containing 10% FCS (Sigma), supplemented with penicillin G and streptomycin. Cells were incubated for 96 h with the compounds in DMF with a final solvent concentration of 0.1% in each well. Preincubated cells were fixed (20 min, 1% glutaraldehyde) and subsequently stained for 30 min with 0.02% Crystal Violet in water. After washing for 30 min in water, the remaining cell-bound dye was redissolved with ethanol/water (7:3) and the absorption at 570 nm was measured with an Anthos 2010 plate reader (Anthos, Salzburg, Austria).

2.4. Selectivity studies

A radiometric protein kinase assay (^{33}PanQinase® Activity Assay, ProQinase, Freiburg, Germany) was used for measuring the kinase activity as described by Aly et al. [18]. Therefore, inhibition was measured in a total reaction volume of 50 µl in 96-well FlashPlates. The reaction was composed of 20 µl assay buffer, 5 µl aqueous ATP solution, 5 µl inhibitor (dissolved in 10% DMSO), 10 µl purified recombinant human protein kinase and 10 µl of the substrate. The reaction for all enzymes contained 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, 50 µg/ml PEG_{20000}, and 1 µM [γ-^{33}P]ATP (approx. 5×10^5 cpm/well). Substrates used for the enzymatic reactions were as follows: 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 FLT3, INS-R, MET and PDGFR-β; Casein for PLK1; p38-alpha-KRKR for SAK; Autophosphorylation was used for ARK5 and COT. Protein kinases were expressed as His-tagged or GST-tagged fusion proteins in Sf9 insect cells and purified by affinity chromatography. Reactions were incubated for 80 min at 30 °C. The reactions were quenched with 50 µl of 2% (v/v) phosphoric acid, plates were aspirated and washed two times 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).

3. Results and discussion

As described earlier, we prepared the precursors **4** and **5** by a convenient straightforward synthesis protocol [12,14]. Condensation of indanetrione hydrate **1** with the 3-(isopropylamino)cyclohex-2-enones **2** gave the *vic*-dihydroxyindeno[1,2-*b*]indoles **3**, which were then converted to the 5-isopropy-5,6,7,8-tetrahydro-indeno[1,2-*b*]indole-9,10-diones **4** by the means of tetramethyl-thionylamide (TMTA). The 9-Hydroxy-5*H*-indeno[1,2-*b*]indole-10-ones **5** were then prepared by the oxidation of **4** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in 1,4-dioxane [12], with subsequent oxidation with molecular oxygen in the presence of Salcomine [19] to give the 5*H*-indeno[1,2-*b*]indole-6,9,10-triones **6** (Fig. 3).

Two novel indeno[1,2-*b*]indoloquinones **6** were synthesized and tested for their inhibition of the human CK2 (Table 1). For IC_{50} determination, inhibition was measured at final concentrations ranging from 0.01 to 30 μM in appropriate intervals and IC_{50} were calculated from the resulting dose-response curves. Pure DMSO was used as negative control (0% inhibition); reactions devoid of CK2 were used as positive control (100% inhibition). For the known inhibitor Emodin, with a reported IC_{50} of 0.89 μM [20], we determined an IC_{50} of 0.46 μM. Both new compounds showed inhibitory activity towards the recombinant CK2 holoenzyme. The *N*-isopropyl substituted indeno[1,2-*b*]indoloquinone **6b** revealed an IC_{50} of 5.05 μM and the *N*-benzyl derivative **6c** was three times more potent with an IC_{50} of 1.49 μM.

For further evaluation, synthesized compounds were investigated in cell proliferation experiments. For primary testing, relative growth inhibition of three adherent tumor cell lines (5637, SISO and KYSE-70) at a concentration of 20 μM was evaluated. We previously reported that the indeno[1,2-*b*]indole **4b** is a strong CK2 inhibitor but only a moderate inhibitor of the growth of the esophageal KYSE-70 line (25% inhibition at 20 μM) [12]. In contrast, we found in this study that the indeno[1,2-*b*]indoloquinones showed nearly complete inhibition of growth (88–100%) of the three tumor lines at a concentration of 20 μM.

We then determined the GI_{50} (concentration required for 50% inhibition of growth) of these compounds towards eight adherent tumor cell lines. This included two bladder cancer cell lines (5637 and RT-4), a cervical cancer (SISO), an esophagus cancer cell line (KYSE-70), a breast cancer cell line (MCF-7), a pancreas line (DAN-G) and two lung cancer lines (LCLC and A427). Measurements were performed at five different inhibitor concentrations. GI_{50} were obtained by a linear least-squares regression of the growth values vs. the logarithm of the inhibitor concentration (Table 2).

Table 1
Inhibition of the human CK2 by indeno[1,2-*b*]indoloquinones.

Compound	R[a]	IC_{50} (μM)[b]
6b	$CH(CH_3)_2$	5.05
6c	$CH_2C_6H_5$	1.49

[a] According to Fig. 3.
[b] Determinations were performed in triplicate in independent experiments.

6b showed very good activity towards six out of eight cell lines with GI_{50} of 1.3 to 1.7 μM (5637, SISO, KYSE-70, MCF-7, LCLC, A427), whereas DAN-G and RT-4 were unaffected by this compound (GI_{50} > 10 μM). The same growth inhibition pattern was obtained with compound **6c**, but with somewhat higher GI_{50} ranging from 2.8–4.1 μM with tumor cell lines 5637, SISO, KYSE-70, MCF-7, LCLC and A427, respectively. No activity was seen towards tumor cell lines DAN-G and RT-4 (GI_{50} > 20 μM).

Due to the unaffected growth of some cell lines (GI_{50} > 10 μM) a general cytotoxic effect of the compounds (e.g. by ROS formation or Michael type reactions due to the quinoid substructure [21]) appears to be unlikely and mechanisms specific to tumor growth like kinase inhibition seem to account for the observed inhibitory activity. However, we did not see a clear correlation between the degree of inhibition of the CK2 by the compounds and the antiproliferative effects. The weaker inhibitor of CK2 (**6b**, with an IC_{50} of 5.05 μM) was the more potent inhibitor of growth in the cancer cell lines (GI_{50} of 1.3–1.7 μM). In contrast, the stronger inhibitor of CK2 (**6c**, with an IC_{50} of 1.49 μM) was a weaker inhibitor of cell growth in the cancer lines tested (GI_{50} of 2.8–4.1 μM).

To exclude a difference in the cell permeability of the compounds as the cause for the lower antiproliferative effects of **6c**, we predicted permeability properties by three different approaches. Fragment-based partition coefficients (*C*logP) and topo-

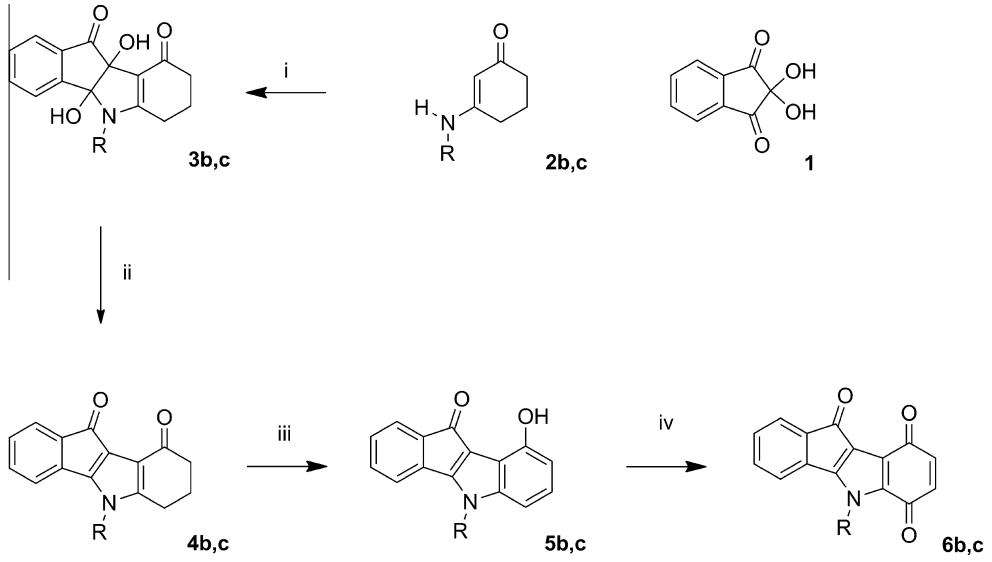

Fig. 3. Preparation of the indeno[1,2-b]indoloquinones **6b** and **6c**. For R refer to Table 1. *Reagents and conditions*: (i) MeOH, r.t., 3–8 h; (ii) DMF, acetic acid, TMTA, r.t., 3 h; (iii) DDQ, 1,4-dioxane, 65–70 °C, 24–72 h; (iv) O_2, Salcomine, DMF, 70 °C, 8 h.

Table 2
Determination of GI_{50} in cell culture.

	6b	6c
Cell line	GI_{50} in μM[a]	
5637	1.7 ± 0.4	2.8 ± 0.6
SISO	1.5 ± 0.2	2.8 ± 0.6
KYSE-70	1.5 ± 0.4	2.8 ± 0.3
MCF-7	1.3 ± 0.1	3.3 ± 0.4
DAN-G	>10	>20
LCLC	1.5 ± 0.2	4.1 ± 1.0
A427	1.4 ± 0.2	3.7 ± 0.7
RT-4	>10	>20

[a] Given is the concentration for growth inhibition of 50% of the corresponding cell line ± SD. GI_{50} are the average of three to seven independent experiments.

Table 3
*C*logP, TPSA and Caco-2 cell permeability coefficients of the tested compounds.

	*C*logP[a]	TPSA[a] in Å^2	$P_{Caco\text{-}2}$[b] in nm/s
6b	2.9	56.1	13
6c	3.7	56.1	22

[a] Calculated by the molinspiration web services [22].
[b] Calculated by the PreADMET webserver [23].

logical polar surface areas (TPSA) were calculated using the Molinspiration web services [22] and permeability coefficients towards Caco-2 cells ($P_{Caco\text{-}2}$) were determined with the PreADMET webserver (Table 3) [23].

The data suggest that there are only minor differences in the membrane permeability of the compounds. Low membrane permeability is predicted for any compounds with *C*logP ⩾ 5 [24], TPSA ⩾ 140 Å^2 [25,26] and $P_{Caco\text{-}2}$ < 4 nm/s [27], which means that both derivatives should be able to permeate cell membranes. Compared to **6b** with a $P_{Caco\text{-}2}$ of 13 nm/s, an even higher $P_{Caco\text{-}2}$ of 22 nm/s is predicted for the less active derivative **6c**, making it unlikely that membrane permeability properties are the cause for the differing effects of the two compounds in cell culture.

To evaluate potential inhibition of other kinases beyond CK2, we tested the compounds in a selectivity study towards a panel of 23 different kinases (Table 4).

Seven kinases (ARK5, FLT3, SRC, VEGF-R2, VEGFR-3, INS-R and SAK) were inhibited by the indeno[1,2-*b*]indoloquinone **6b** with IC_{50} below 10 μM. Thereof, ARK5 was inhibited with a considerable IC_{50} of 0.57 μM and for FLT3 an even three times lower IC_{50} of 0.18 μM was found. Compound **6c** inhibited 5 kinases (ARK5, EGF-R, IGF1-R, SRC and VEGF-R2) with IC_{50} below 10 μM. The strongest inhibition was observed with ARK5 and SRC with IC_{50} of 3.7 and 2.4 μM, respectively.

Besides inhibition of CK2, inhibition of ARK5 by the indeno[1,2-*b*]indoloquinones could contribute to the antiproliferative effects of the compounds in the cell culture experiments. The stronger inhibitor of ARK5 (**6b**, with an IC_{50} of 0.57 μM) was the more potent inhibitor of growth in the cancer cell lines (GI_{50} of 1.3–1.7 μM). Correspondingly, the weaker inhibitor of ARK5 (**6c**, with an IC_{50} of 3.7 μM) inhibited the growth of the cancer lines to the same extent (GI_{50} of 2.8–4.1 μM). Thus, ARK5 seems to be a potential candidate contributing to the effects observed in the tumor lines. ARK5 is a family member of the AMP activated Ser/Thr protein kinases (AMPKs) and is activated by the oncogene Akt [28]. Little is reported about the protein kinase ARK5, but it has been shown by Suzuki and coworkers to be involved in tumor malignancy through the repression of caspase 8 activity, resulting in the prevention of cell death [29]. Compared to the other tested bladder cell line 5637, decreased expression of apoptosis-related genes in RT-4 has been shown [30]. This would explain the insensitivity of the bladder cancer cell line RT-4 towards the inhibitors. Besides its role in the prevention of cell death, it was suggested that ARK5 plays a role in metastasis [31], and ARK5 mediated invasion of cancers has been shown [32], making this kinase an attractive target for the development of novel therapeutics for the treatment of cancers.

The insensitivity of the pancreatic adenocarcinoma line DAN-G towards the compounds could be due to a high expression of the multidrug resistance associated protein 5 (MRP5). A significant correlation between the expression of MRP5 in eight pancreatic adenocarcinoma cell lines (Colo-357, T3M4, Aspc-1, DAN-G, Panc-1, Patu-89O2, Patu-8988T and Patu-8988S) and the sensitivity of these cell lines towards the chemotherapeutic agent 5-fluorouracil (5-FU) has been reported. DAN-G showed the lowest sensitivity towards 5-FU while expressing high levels of MDR5 [33]. This would explain the insensitivity of DAN-G cells towards the inhibitors.

With an IC_{50} of 0.18 μM, **6b** is also a potent inhibitor of the human receptor tyrosine kinase FLT3. The inhibition of FLT3 does not explain the antiproliferative effects of the compounds due to two reasons: firstly, derivative **6c** strongly inhibited the growth of the cancer cell lines, but did not inhibit the kinase FLT3 (IC_{50} > 20 μM) and secondly, expression of FLT3 in human cancers has been shown to be restricted to cancer cells of the hematopoietic system [34]. The human FLT3 (FMS-like tyrosine kinase 3) belongs to the family of subclass III receptor tyrosine kinases, along with the platelet-derived growth factor receptor (PDGFR), the steel factor receptor (cKIT) and the macrophage colony-stimulating factor receptor (M-CSFR also named FMS) [35]. The receptor tyrosine kinase FLT3 plays a crucial role in hematopoietic malignancies. Elevated levels of FLT3 have been found in 100% of patients with B-lineage acute lymphoid leukemia (B-lineage ALL), in 88% with acute myeloid leukemia (AML) and in 25% with T-cell acute lymphoid leukemia (T-ALL) [36]. Gain of function mutations of FLT3 are most frequently in AML patients [37], which are found in 25% [35] to 35% [38] of all cases. Although FLT3 was recognized as a potential target for the treatment of AML a decade ago [39], few potent and selective inhibitors are available, with exceptions being MLN-518 and AC220 [40]. Thus, compound **6b** seems to be an interesting starting point for the development of further FLT3 inhibitors for the treatment of acute leukemias.

Table 4
Inhibition of 23 different protein kinases by indeno[1,2-*b*]indoloquinones **6b** and **6c.**

Kinase		AKT1	ARK5	Aurora-A	Aurora-B	B-RAF	CDK2/CycA	CDK4/CycD1	EGF-R	EPHB4	ERBB2	FAK	FLT3
IC_{50} (μM)	**6b**	n.i.[a]	0.57	20	19	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	0.18
	6c	n.i.	3.7	n.i.	n.i.	n.i.	n.i.	n.i.	7.4	n.i.	n.i.	n.i.	n.a.
Kinase		IGF1-R	SRC	VEGF-R2	VEGF-R3	INS-R	MET	PDGFR-β	PLK1	SAK	TIE2	COT	
IC_{50} (μM)	**6b**	10	5.6	6.4	3.3	5.5	n.i.	n.i.	n.i.	5.9	11	n.i.	
	6c	7.2	2.4	9.6	n.i.	19	n.i.	n.i.	n.i.	12	14	13	

[a] n.i.: no inhibition, IC_{50} > 20 μM.

In conclusion, we prepared two novel indeno[1,2-*b*]indoloquinones with considerable inhibitory activity towards the human protein kinase CK2. The compounds showed significant antiproliferative activity towards a broad panel of human tumor cell lines. Besides the inhibition of CK2, both compounds inhibited the human protein kinase ARK5, which may be an additional cause for the antiproliferative effects of the compounds. In addition, compound **6b** turned out to be a strong inhibitor of the human receptor tyrosine kinase FLT3. The results indicate that indeno[1,2-*b*]indoloquinones are a valuable starting point for the development of CK2-inhibitors with antiproliferative activity towards a variety of cancer cells. Additionally, the indeno[1,2-*b*]indoloquinone **6b**, could provide a new lead structure for future FLT3 inhibitors for the treatment of acute leukemias. Further derivatives of the indeno[1,2-*b*]indoloquinones will be synthesized and tested for inhibition of the human protein kinase CK2 and their antiproliferative effects in human cancer cells.

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