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7,7′-Diazaindirubin—A small molecule inhibitor of casein kinase 2 in vitro and in cells

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ABSTRACT

Aza- and diaza-bisindoles were synthesized by coupling of 7-azaisatin, 7-azaoxindol, 7-azaindoxyl acetate, and their non-aza counterparts, respectively. Whereas 7,7′-diazaindigo (**10**) and 7,7′-diazaisoindigo (**11**) did not show antiproliferative activity in several human tumor cell lines up to 100 μM, 7-azaindirubin (**12**) and 7′-azaindirubin (**13**) were more active than the parent molecule, indirubin, in LXFL529L cells (human large cell lung tumor xenograft), and 7,7′-diazaindirubin (**14**) was exhibiting substantially enhanced growth inhibitory activity in these cells. In the NCI 60 cell line panel, **14** displayed antiproliferative activity preferentially in certain melanoma and non-small cell lung cancer cells. In contrast to the potent serine/threonine/tyrosine kinase inhibition observed for indirubins, kinase inhibition profiling of **14** in 220 kinases revealed largely a loss of kinase inhibitory activity towards most kinases, with retained inhibitory activity for just a few kinases. At 1 μM concentration, especially casein kinases CK1γ3, CK2α, CK2α2, and SIK were inhibited by more than 50%. In cell-based assays, **14** markedly affected CK2-mediated signaling in various human tumor cells. In MCF7 cells, **14** induced cell cycle arrest at G1 and G2/M and apoptosis, whereas CK2-deficient MCF7 cells were resistant. These findings reveal a novel key mechanism of action for **14**, suggesting primarily CK2 inhibition to be causally related to growth inhibition of human tumor cells.

1. Introduction

Indigo, 2-(1,3-dihydro-3-oxo-2*H*-indol-2-ylidene)-1,2-dihydro-3*H*-indol-3-one (Fig. 1), is one of the oldest and most famous dyes of the world.[1] It has been produced for milleniums by artisan processes from several plants, such as *Indigofera tinctoria*, *Polygonum tinctorium*, and *Isatis tinctoria*, containing indigo precursors. The chemical synthesis of indigo has emerged in the last decades of the 19th century and is based on the oxidation of indoxyl formed during the process.[2]

Isoindigo, so-called indigo brown, is a 3,3′-linked indigoid bisindole (Fig. 1), that has typically been synthesized by acid condensation of oxindole and isatin.[3] 1-Methylisoindigo has been described as being active in the treatment of chronic myeloic leukemia (CML) in China.[4] It is currently under investigation concerning its mode of action.[5]

Indirubin, the 2′,3-isomer (Fig. 1), has been used to treat various chronic diseases, including CML in China. It represents a lead structure for antitumor agents featuring potent inhibitory activity towards a series of serine/threonine kinases[6,7] and tyrosine kinases[8,9] or activating the Ah receptor.[10] Recent results suggest involvement of further mechanisms in the regulation of cell fate induced by indirubins.[11] Crystal structures of specific indirubins bound in the ATP-binding pocket revealed the lipophilic interactions and hydrogen bonds formed between 1-NH, 2-C=O, and 1′-NH and the protein backbone to be of major relevance for binding.[6,12] To increase solubility and bioavailability, hydrophilic substituents were introduced into positions 5 and 3′ of the indirubin core.[13,14] Whereas substitutions at these positions did not

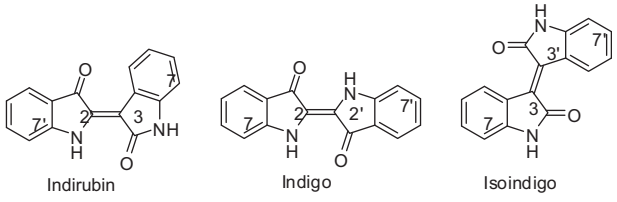

Figure 1. Chemical structures of indigo, indirubin and isoindigo.

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compromise kinase inhibitory activity, modifications at 1-NH and 1′-NH position, relevant for the formation of hydrogen bonds, are considered to counteract binding affinity.[12] The synthesis of those alkylated 7- and 7′-azaindirubins was reported previously.[15–17]

Indirubins have been shown to be prone to CYP450 mediated metabolic transformations, forming hydroxylated indirubins with decreased activity.[18] Insertion of N-atoms into the indirubin core was envisaged to increase metabolic stability by impeding ring hydroxylation and reducing the π-electron density. We therefore embarked on the synthesis of azaindirubins. As expected, when the 7-aza or 7′-aza analogs were tested for microsomal stability, the aza-insertion was found to provide protection against CYP-mediated ring hydroxylation, leaving the nonaza part or a side chain still susceptible to oxidative biotransformation.[19]

2. Results

2.1. Chemistry

The synthesis of indirubins is based on basic or acid condensation of indoxyl and isatin derivatives, depending on the chemical nature of the substituents.[20] The basic condensation was first reported by Baeyer in 1881[21] and was considerably improved by Russell and Kaupp in 1969 using the more stable 3-indoxyl acetate as coupling module.[22] The condensation in acetic acid was reported as early as 1921 by Martinet and Dornier for the preparation of indirubin-5-sulfonate.[23]

Previous syntheses of 5′- and 7′-substituted indirubins achieved by our group prompted us to explore 1-, 3-, or 1,3-diacetylindoxyl compounds as coupling modules.[24] Thus, for the synthesis of 7′-azaindirubin we first followed a synthetic approach to 1-acetyl-7-aza-3-indolinone, according to Desarbre and Merour (see Scheme 1).[25] Formylation and subsequent acetylation of 7-azaindole **1** produced 1-acetyl-3-formyl-7-azaindole **2** which on oxidation with *m*-CPBA in a Dakin reaction provided 1-acetyl-7-aza-3-indoxyl **3**. However, the poor overall yield of about 8% in 3 steps led us to explore another synthetic approach.

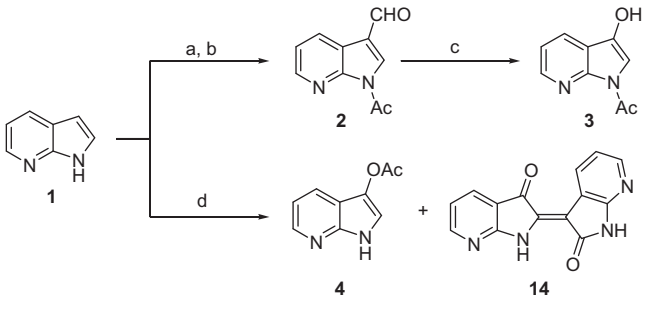
Thallium(III) acetate has been reported to act as a multifaceted oxidant of promise for oxidation of heterocycles.[26] For instance, the sole product isolated from the oxidation of indole with thallium(III) acetate in acetic acid containing a small amount of water was 3-indoxyl acetate in 43% yield.[27] We adopted this method for the oxidation of 7-azaindole **1** (Scheme 1). Due to the sluggish reactivity because of the electron deficient aromatic system, reaction temperature and time had to be raised to yield 32% of 7-aza-3-indoxyl acetate **4**.

Under such conditions the formation of a minor byproduct, representing about 10% of the raw product, was observed. This compound was identified by NMR and proven by synthesis to be 7,7′-diazaindirubin **14**. When **1** (1 equiv) was treated with thallium(III)-acetate (0.55 equiv) in glacial acetic acid at 90 °C in absence of water for 12 h, **14** was obtained as the main reaction product, albeit at a low yield (11%). Under these conditions **4** was produced as minor byproduct. Separation of **14** from **4** was achieved by recrystallization from ethyl acetate. As reported in the literature, another access to **4** was provided by reaction of **1** and [bis(acetoxy)iodo]-benzene. The yield of this small scale synthesis was given to be 43%.[28]

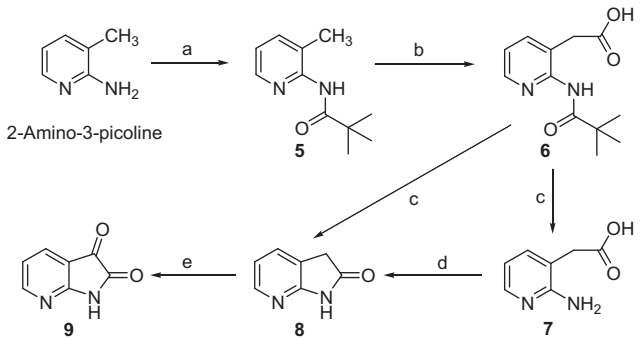
Previously reported syntheses of 7-azaisatins, e.g. by oxidation of **1** using indium-(III)-chloride and 2-iodoxybenzoic acid[29] or using *N*-bromosuccinimide and DMSO, described the way to 1-substituted 7-azaisatins.[16,30,31] The latter method had also been applied for the preparation of 1-unsubstituted 7-azaisatin **9**, reporting 10% of raw yield.[16] However, in our hands this method at best yielded traces of **9** and attempts to isolate **9** from the mixture were unsuccessful. Recently, synthesis of **9** was reported by oxidation of **1** using pyridinium chlorochromate and a polyaniline salt catalyst.[32] Compelling spectroscopic evidence in support of the identity of **9** has not been reported, though. Furthermore, a lengthy and cumbersome preparation procedure for **9** (at low yield) was reported already in 1941.[33] We set out to prepare **9** by oxidation of 7-aza-2-indolinone **8**,[34–37] inspired by a report on the oxidation of α-methylene ketones with NBS/DMSO.[38]

The synthesis of **8** is based on a method reported by Ting et al.[34] (Scheme 2). Pivaloyl protected 2-amino-3-picoline **5** was lithiated and converted with carbon dioxide into the 3-carboxymethylpyridine derivative **6**. Hydrolysis with 6 N HCl afforded a mixture of 2-amino-3-carboxy-methylpyridine **7** and the cyclized product **8**. Isolated **7** was cyclized in amyl alcohol using 4-toluene sulfonic acid (PTSA) to produce **8** in a moderate (40% overall) yield.[39] For the synthesis of **9**, we modified the NBS/DMSO-oxidation method reported for the oxidation of 1-indanone.[40] NBS and **8** were dissolved in DMSO and this solution was added dropwise to hot DMSO providing **9** after workup in 65% yield.

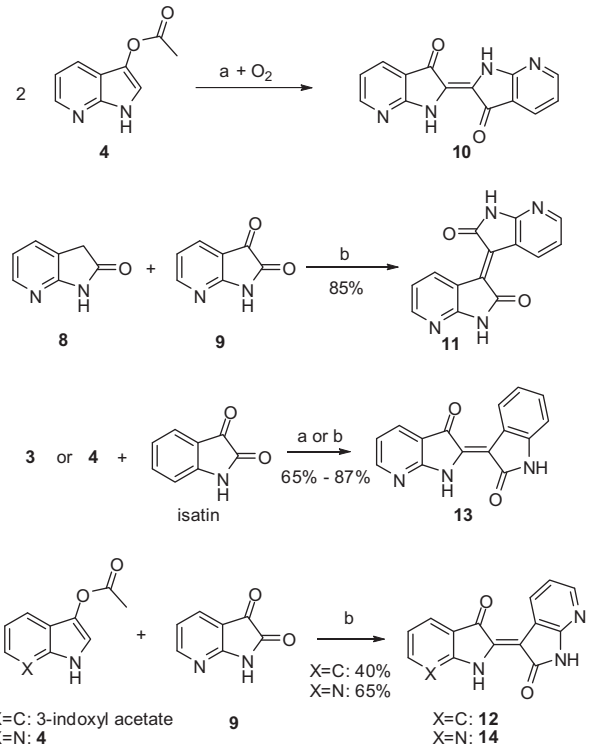
Syntheses of 7,7′-diazaindigo **10**, 7,7′-diazaisoindigo **11**, 7-azaindirubin **12**, 7′-azaindirubin **13**, and 7,7′-diazaindirubin **14** are shown in Scheme 3. We generated **10** by oxidation of **4** with air in methanolic alkaline solution. Acid condensation of **8** and **9** afforded **11** in high yield. Condensation of **3** or **4** with isatin, was achieved in both, acid and basic conditions to obtain **13**. Exclusively acid conditions were used for the condensation of **9** with indoxyl acetate to arrive at **12** thus avoiding the risk of basic lactam ring opening. Compound **14** was formed by oxidation of **1** with thallium(III) acetate and could be isolated by repeated crystallization at a low yield (11%). However, a substantially improved yield was achieved, by acid condensation of **4** and **9**, providing 62% of **14**.



Scheme 1. Oxidation of 7-azaindole **1** to synthesize 1-acetyl-7-aza-3-indolinone **3**, and synthesize 7-aza-3-indoxyl acetate **4**, concomitant formation of 7,7′-diazaindirubin **14**. Reagents and conditions: (a) hexamethylenetetramine, 33% AcOH; (b) Ac_2O, NEt_3, DMAP; (c) *m*CPBA, CH_2Cl_2; (d) $Tl(OAc)_3$, AcOH, H_2O.



Scheme 2. Synthesis of 7-azaisatin (**9**). Reagents and conditions: (a) PvCl, NEt_3, CH_2Cl_2; (b) *n*BuLi, CO_2, H^+; (c) 6 N HCl, reflux; (d) PTSA, $C_5H_{11}OH$, reflux; (e) NBS, DMSO, red. pressure.



Scheme 3. Synthesis of 7,7′-diazaindigo **10**, 7,7′-diazaisoindigo **11**, 7-azaindirubin **12**, 7′-azaindirubin **13**, and 7,7′-diazaindirubin **14**. Reagents and conditions: (a) basic conditions: Na_2CO_3/MeOH or (b) acid conditions: HCl concd/AcOH.

2.2. Biological activity

2.2.1. Antiproliferative activity in human tumor cell lines

Newly synthesized aza-bisindoles were tested in human cancer cell lines, including LXFL529L (large cell lung tumor xenograft cells), MCF7 (breast adenocarcinoma cells) and HT29 (colon adenocarcinoma cells) using the SRB assay as described in the Section 4. IC_{50} values are summarized in Table 1.

The compounds were tested at various concentrations for their anti-proliferative effects in LXFL529L, MCF7 and HT29 cells using the SRB assay as described in the Experimental section. The IC_{50} (μM) values were calculated from dose–response curves of at least three independent experiments.

Neither 7,7′-diazaindigo **10** nor 7,7′-diazaisoindigo **11** were found to inhibit tumor cell growth. Introduction of N-atoms either in position 7 **12** or 7′ **13** resulted in distinctly enhanced activity in LXFL592L cells as compared to indirubin (IC_{50} 9.9 μM)[20], whereas antiproliferative activity in MCF7-cells (IC_{50} 4.0 μM)[20] remained similar. However, **14** with N-atoms in 7- and 7′-position was more active by two orders of magnitude in LXFL592L cells (IC_{50} 0.06 μM). This increase was not seen in MCF7 cells (IC_{50} 0.5 μM), suggesting cell line specific targets for **14**. In the NCI 60 cell line panel **14** showed high activity in melanoma and non-small cell lung cancer cells, and in some colon and CNS cancer cells, with GI_{50} values (50% inhibition of cell growth) of 0.5 μM and below. In contrast, leukemia, ovarian, renal, prostate and breast cancer cell lines were less sensitive (Fig. 2 and SI Table 1).

Table 1
Tumor cell growth inhibition of aza-bisindoles (IC_{50} (μM))

	LXFL529L	MCF7	HT29
10	>100	>100	>100
11	>100	>100	>100
12	2.9 ± 0.06	3.0 ± 0.21	>10
13	4.4 ± 0.38	5.0 ± 0.22	>10
14	0.06 ± 0.01	0.5 ± 0.13	2.0 ± 0.05

Indirubin: IC_{50} (LXFL529L) 9.9 μM; IC_{50} (MCF7) 4.0 μM [20].

2.2.2. 7,7′-Diazaindirubin inhibits casein kinase 2

To gain first insight into potential mechanisms of action, inhibitory activity of **14** was tested in a limited spectrum of kinases (30 protein kinases) at 1 and 10 μM, respectively, shown before to be affected by substituted indirubins.[13] These kinases were not found to be targets for **14** additionally profiled (SI Table 2). Extended testing in a panel of 220 protein kinases (1 μM) revealed most kinases largely unaffected, except for casein kinases CK1γ3, CK2α, CK2α2 and Salt-inducible kinase, abbreviated as SIK (Fig. 3 and SI Table 3).

SIK is a serine/threonine kinase and belongs to AMP-activated protein kinase (AMPK) family.[41] Recent evidences showed SIK is involved in cardiogenesis,[41] adrenocorticotropin-induced steroidogenesis[42] and transforming growth factor-beta (TGFβ)-mediated fibrosis.[43] More recent reports also identified SIK as a class II histone deacetylase kinase contributing to skeletal myocytes survival,[44] which might be regulated by LKB1 (liver kinase B).[45] Since casein kinase 2 has been reported to play an important role in tissue development and tumorgenesis,[46] we focused on CK2 in our further experiments. The effect of **14** on SIK will be reported in a separated paper. The casein kinase family including CK1 and CK2 are highly conserved serine/threonine protein kinases. CK1 has six isoforms, α, γ1, γ2, γ3, δ and ε,[47] while tetrameric CK2 is composed of two catalytic (α and α2) and two regulatory (ß) subunits.[48] Both of them regulate diverse cellular processes such as DNA damage, DNA replication and signal transduction.[49,50] In Wnt signaling, activated CK2 was reported to stabilize β-catenin and enhance signaling output.[51] MCF7 cells were treated with **14** (1 μM), cells harvested at indicated time points and subjected to Western blot (Fig. 4A). A marked reduction in the level of β-catenin was detected as early as 15 min after beginning of incubation. CK2 can facilitate the phosphorylation of Akt at S473 in response to proliferation and active Akt participates in phosphorylation of GSK3β at serine 9.[52] In good agreement, we observed the reduction of phosphorylated Akt (S473) 30 min after treatment with **14**, and a 30 min-delayed decrease of GSK3ß (serine 9). To examine whether CK2-mediated signaling is affected by **14**, MCF7, Panc1 (human pancreatic cancer) and HEK293 (human embryo kidney) cells were incubated with **14**. Phosphorylation and activation of Akt was suppressed (Fig. 4B), apparently to a greater extent in Panc1 and HEK293 cells than in MCF7 cells. Taken together, these results demonstrate the potent inhibitory effects of **14** on CK2-dependent cellular signaling.

2.2.3. 14 induced cell cycle arrest at G1 and G2/M phase and apoptosis

The impact of **14** on cell cycle progression was studied using FACS (fluorescence-activated cell sorting). Cellular DNA was stained with propidium iodide (PI) 24 h after incubation. Treatment with **14** induced accumulation of cells at G1 phase and a concomitant decrease at S-phase (Fig. 5) suggesting blockage of cellular entry into S-phase. A slight increase of cell population at G2/M phase was observed, too.

Induction of programmed cell death was studied by propidium iodide (PI) uptake and annexin V staining in MCF7 cells incubated with **14** for 24 h. As depicted in Figure 6 (upper), cells showed a higher level of annexin V and PI double positive in comparison to the DMSO treatment, suggesting **14** to induce late-stage apoptosis, in good agreement to the result that **14** induced PARP cleavage, a hallmark of cell apoptosis detected by Western blot (Fig. 4A). This induction of apoptosis was not seen in CK2-deficient MCF7 cells (Fig. 6 lower), generated by transfection with CK2 siRNA as previously reported.[46] These results suggest a key role for CK2 inhibition in this context.

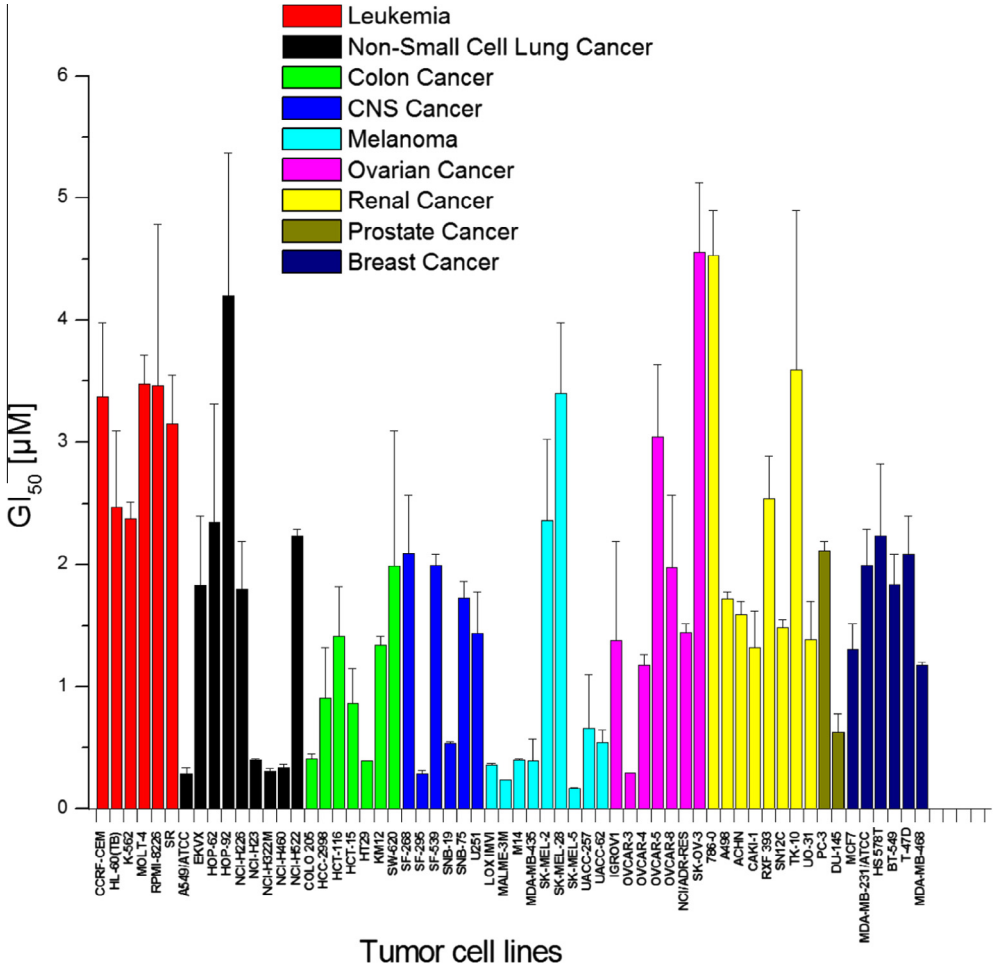


Figure 2. Tumor cell growth inhibition of **14** tested in the NCI 60 cell line panel. GI_{50} values are mean of two series of tests. The NCI decision for further investigation of compound **14** on hollow fiber assay and xenograft study is pending.

3. Conclusion

Aza-bisindoles were synthesized and their anti-proliferative activity was determined in human tumor cells. The aza-isosteric (7, 7′ and 7,7′) analogs of indirubin, initially designed to impede CYP450 mediated metabolic hydroxylations at the indirubin core, had marked antiproliferative effects on human tumour cells. Growth inhibitory activity of 7,7′-diazaindirubin was found to be two orders of magnitude higher in LXFL529L cells, as compared to indirubin. Whereas most indirubins had been found to exhibit pan kinase inhibition, **14** was found rather selectively to inhibit casein kinases, including CK1γ3, CK2a, CK2α2. Treatment with **14** led to inactivation of Akt in three human tumor cell lines, implicating **14** to potently inhibit CK2 under cellular conditions. In MCF7 cells, **14** induced cell cycle arrest at G1 and G2/M phases and cell apoptosis, concomitant with PARP cleavage, while CK2-depleted MCF7 cells were resistant.

4. Experimental section

4.1. Materials and methods

4.1.1. Cell culture

LXFL529L cells were grown at 37 °C in RPMI 1640 (Invitrogen, Karlsruhe, Germany), Panc1, HEK293, MCF7 and HT29 cells were grown at 37 °C in DMEM (Invitrogen, Karlsruhe, Germany), each supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL), streptomycin (100 mg/mL), in a humidified atmosphere of 5% CO_2. The cells were tested routinely for absence of mycoplasm contamination.

4.1.2. Transfection

The sequence of CK2 was previously reported (sense: TCAAGATGACTACCAGCTGTT)[41] and synthesized by riboxx. Riboxx®FECT reagent was used to get maximal transfectional efficiency according to the manufacturer's instructions. Nontargeting siRNA was used as control. After 48 h transfection in 10% FCS, cells were treated with compounds as indicated in context.

4.1.3. Sulforhodamine B assay (SRB assay)

Effects on cell growth were determined according to the method of Skehan et al.[53] with slight modifications. Briefly, cells were seeded into 24-well plates and allowed to grow for 24 h before treatment. Thereafter, cells were incubated with the respective drug for 3 days in serum containing medium. Incubation was stopped by addition of trichloroacetic acid (50% solution). After 1 h at 4 °C, plates were washed four times with water. The dried plates were stained with a 0.4% solution of sulforhodamine B. The dye was eluted with Tris-buffer (10 mM, pH 10.5) and quantified photometrically at 570 nm. Cytotoxicity was determined as percent survival, determined by the number of treated over control cells × 100 (% T/C).

4.1.4. Western blot

Cell extracts were homogenized in urea-lysis buffer (1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea, 1 mM Na_3VO_4,

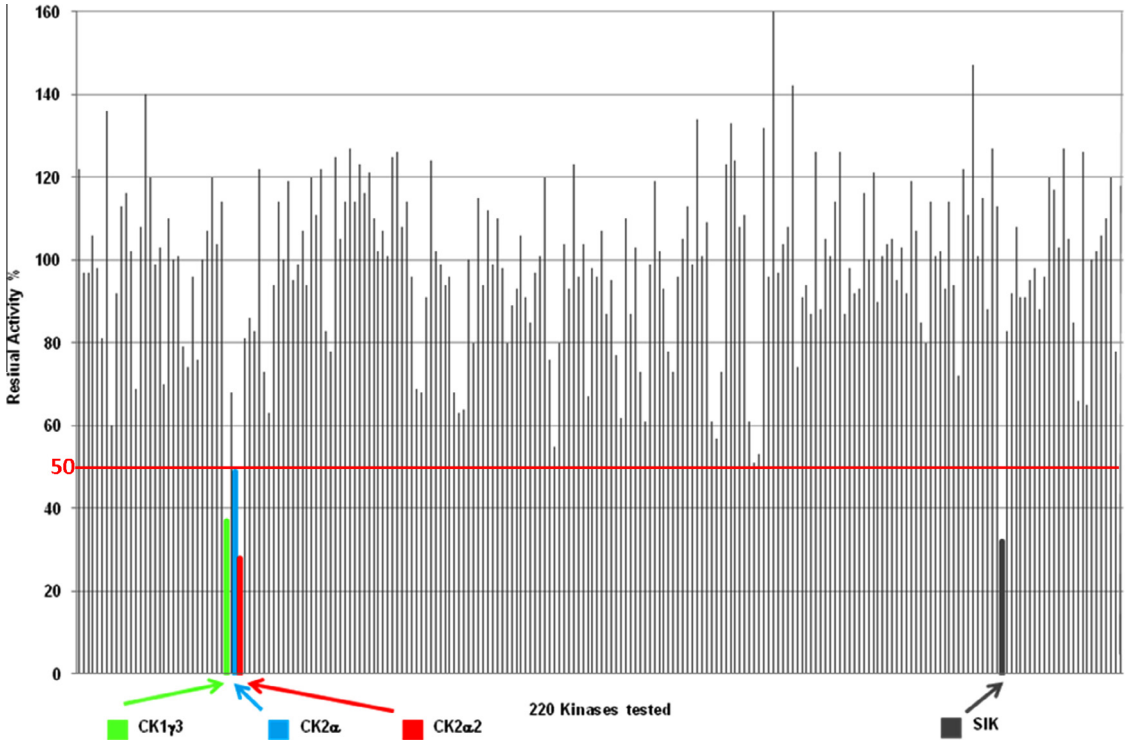


Figure 3. Kinase profiling assay with 220 kinases at 1 μM of **14**. Kinases with a residual activity <50% are shown prominently.

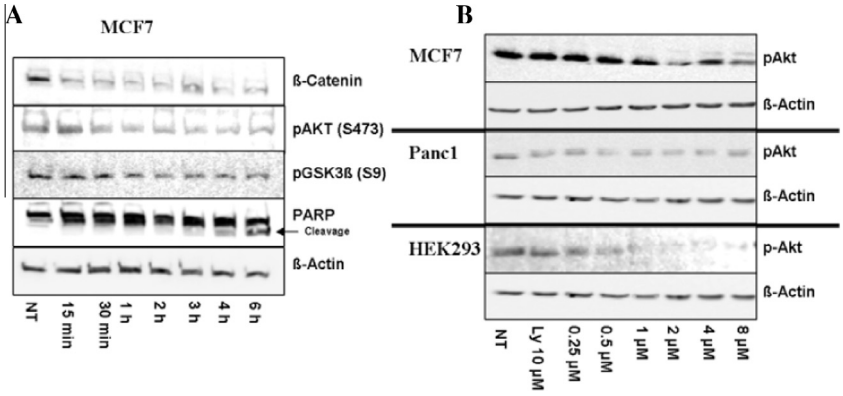


Figure 4. Compound **14** interferes with CK2-mediated signaling and induces PARP cleavage. (A) MCF7 cells were treated with **14** (1 μM) for indicated times and cell lysates were subjected to Western blot. DMSO was used as control (nontreatment, NT). β-Actin was used as loading control. (B) Compound **14** inhibits activation of Akt in MCF7, Panc1 and HEK293 cells in a concentration-dependent manner. The cells were treated with **14** for 2 h. Casein kinase inhibitor Ly294002 (Ly) was y used as a positive control.

10 μg/mL pepstatin, 100 μM PMSF and 3 μg/mL aprotinin in PBS). Enhanced chemiluminescence (ECL) Western blot analysis was performed. 20–40 μg of total protein was resolved on 10% SDS–PAGE gels and immunoblotted with specific antibodies. Primary antibodies pAkt (S473), β-catenin, pGSK3β (S9), PARP and β-actin were obtained from cell signaling (NEB, Germany) and incubated at a 1:1000 dilution in TBS (pH 7.5) with 0.1% Tween-20 and 5% BSA/nonfat milk with gentle agitation overnight at 4 °C. The proper secondary antibodies were incubated in TBS (pH 7.5) with 5% BSA/nonfat milk and 0.1% Tween-20 at a 1:5000 dilution for 1 h at room temperature.

4.1.5. Apoptosis assay and cell cycle analysis

MCF7 cells were cultured in DMEM containing 10% FBS and 1% PS for 24 h and then incubated with indicated concentration of **14** for 24 h. The cells were spun down after harvest, the supernatant was discarded. For apoptosis assay, cells were resuspended in 50 μL of annexin V binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$), incubated with 5 μL of FITC-conjugated annexin V (BD Bioscience, Germany) for 15 min in the dark at room temperature. Afterwards, 450 μL of annexin V binding buffer and 1.25 μL of propidium iodide (PI, 1 mg/mL) were added, incubated for 10 min in the dark at room temperature and analyzed on FACS (fluorescence-activated cell sorting). For cell cycle analysis, the pellets were fixed in 70% Ethanol for at least 24 h, washed two times with ice-cold PBS and resuspended in 500 μL of PBS. The suspension was incubated with RNase A (50 μg/mL) for 1 h at 37 °C and sequentially stained with PI (50 μg/mL) for 5 min and analyzed on FACS.

4.1.6. Reagents

Solvents and reagents obtained from commercial suppliers were at least of reagent grade and were distilled or dried according to prevailing methods prior to use, if necessary. The syntheses

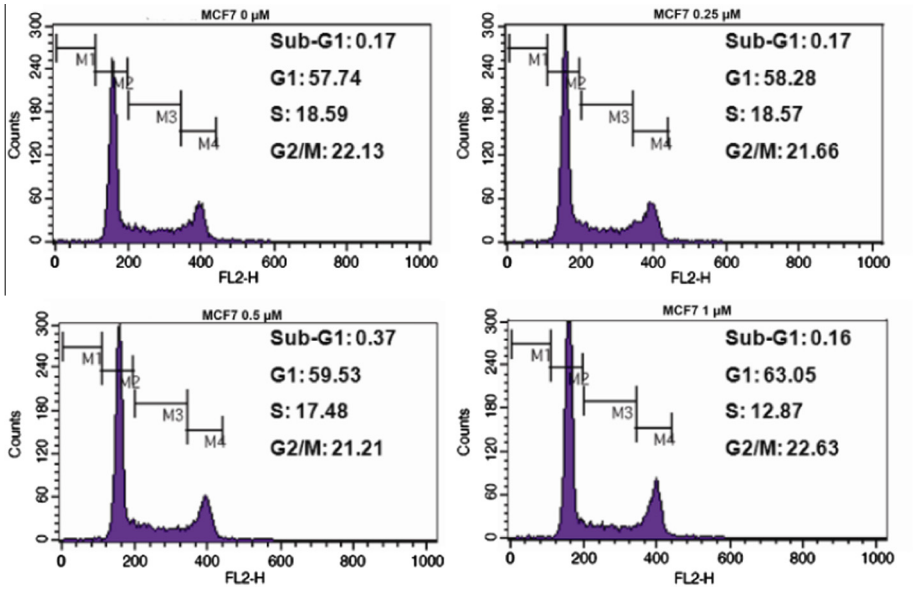


Figure 5. Compound **14** induces cell cycle arrest at G1 and G2/M phases. MCF7 cells were incubated with various concentrations of **14** for 24 h, fixed with 70% ethanol, stained with PI and analyzed by FACS.

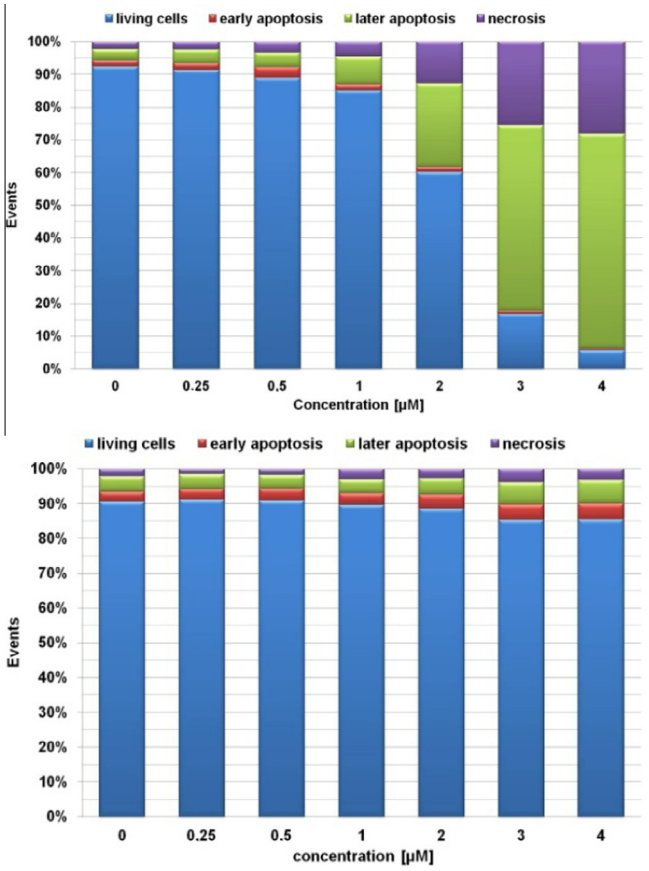


Figure 6. Annexin V/PI assay of MCF7 cells and CK2-depleted MCF7 cells. Cells were incubated with various concentrations (μM) of **14** for 24 h, labelled with FITC-conjugated Annexin V and PI, and analyzed by FACS. Upper: **14** induced apoptosis in MCF7 cells. Lower: CK2-depleted MCF7 cells were resistant to **14**.

were done under argon atmosphere, when required. Argon 4.8 was purchased from Air Liquide and was dried over phosphorus pentoxide. For monitoring the reactions, Alugram SIL G/UV_{254} sheets for TLC (Macherey &Nagel) were used. Column chromatography was accomplished using silica gel 60 (Macherey & Nagel, 0.063–0.200 mm), for flash chromatography silica gel 60 (Macherey & Nagel, 0.040–0.063 mm) was used.

4.1.7. Analytical methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-400 (^{1}H NMR: 400 MHz, ^{13}C NMR: 100 MHz) or on a Bruker AMX-600 (^{1}H NMR: 600 MHz, ^{13}C NMR: 150 MHz) at 298 K. Chemical shifts are reported in ppm from tetramethylsilane with solvent as the internal standard (^{1}H $CDCl_3$: δ 7.26; ^{13}C $CDCl_3$: δ 77.0; ^{1}H DMSO-d_6: δ 2.49; ^{13}C DMSO-d_6: δ 39.5).

Elemental analyses were performed on an Element Analyzer Perkin Elmer EA 240 or 2400 CHN in the University of Kaiserslautern, Dept. of Chemistry.

4.1.8. 3-Formyl-7-azaindole

Hexamethylenetetramine (1.79 g, 12.8 mmol) was added at once to 7-azaindole **1** (1.0 g, 8.5 mmol) in acetic acid (33%, 15 mL). The mixture was refluxed for 4 h, poured into water (25 mL) and cooled to 4 °C. The fine crystalline precipitate was collected, washed with water and dried in vacuo. Yield of 3-formyl-7-aza-indole: 674.3 mg (4.6 mmol, 54%). ^{1}H NMR (400.13 MHz): 12.68 (s, 1H), 9.91 (s, 1H), 8.46 (s, 1H), 8.39 (dd, 1H, $^{3}J_{H,H}$ = 7.8 Hz, $^{4}J_{H,H}$ = 1.6 Hz), 8.36 (dd, 1H, $^{3}J_{H,H}$ = 4.7 Hz, $^{4}J_{H,H}$ = 1.6 Hz), 7.27 (dd, 1H, $^{3}J_{H5,H4}$ = 7.8 Hz, $^{3}J_{H5,H6}$ = 4.7 Hz).

4.1.9. 1-Acetyl-3-formyl-7-azaindole (2)

Triethylamine (350 μL) and DMAP (25 mg) were added to a suspension of 3-formyl-7-azaindole (310 mg, 2.1 mmol) in acetic anhydride (10 mL) and stirred for 3 h at room temperature. The white precipitate was collected, washed with water till neutral, and dried in a desiccator over KOH. Additional product was isolated by keeping the mother liquor at 4 °C. Yield: 300.0 mg (1.6 mmol, 76%). ^{1}H NMR (400.13 MHz, DMSO-d_6, δ = 2.49 ppm): 10.08 (s, 1H), 8.95 (s, 1H, H2), 8.52 (dd, 1H, $^{3}J_{H,H}$ = 4.8 Hz, $^{4}J_{H,H}$ = 1.7 Hz), 8.48 (dd, 1H, $^{3}J_{H,H}$ = 7.8 Hz, $^{4}J_{H,H}$ = 1.7 Hz), 7.46 (dt, $^{3}J_{H5,H4}$ = 7,8 Hz, $^{3}J_{H5,H6}$ = 4.8 Hz), 3.03 (s, 3H).

4.1.10. 1-Acetyl-7-aza-3-indoxyl (3)

865 mg (7.6 mmol) of solid 3-chloroperoxybenzoic acid (70%) were added at 0 °C to **2** (500 mg, 2.7 mmol) in dichloromethane

(21 mL) and stirred for 2 h at 0 °C and for 24 h at room temperature. The mixture was poured into 35 mL of aqueous sodium sulfite (10%). The layers were separated and the aqueous layer was extracted two times with 30 mL of dichloromethane each. The combined organic layers were dried over magnesium sulfate, concentrated and stored at −20 °C. The colourless crystalline product was collected, washed with cold ethyl acetate and dried in vacuo. Yield: 82.7 mg (0.5 mmol, 17%). ^{1}H NMR (400.13 MHz, DMSO-d_6, δ = 2.49 ppm): 8.66 (s, 1H), 8.49 (dd, 1H, $^3J_{H,H}$ = 4.8 Hz, $^4J_{H,H}$ = 1.5 Hz,), 8.08 (s, 1H), 8.05 (dd, 1H, $^3J_{H,H}$ = 7.9 Hz, $^4J_{H,H}$ = 1.7 - Hz), 7.39 (dd, 1H, $^3J_{H5,H4}$ = 7.7 Hz, $^3J_{H5,H6}$ = 4.8 Hz), 2.98 (s, 3H).

4.1.11. 7-Aza-3-indoxyl acetate (4)

Under argon atmosphere at room temperature, a solution of thallium(III) acetate (3.82 g, 10.0 mmol) in acetic acid (51 mL) and water (2.5 mL) was added dropwise to a solution of **1** (1.075 g, 9.1 mmol) in glacial acetic acid (7.5 mL). The mixture was refluxed for 1 h, poured onto crushed ice (200 g) and neutralized with sodium carbonate at 0 °C. The precipitate was collected, dissolved in hot ethyl acetate and cooled to 0 °C. The residue was discarded and the solvent was removed in vacuo to yield 7-azaindoxyl-3-acetate (507 mg, 2.9 mmol, 32%) as a reddish solid. ^{1}H NMR (400 MHz, DMSO-d_6, δ = 2.49 ppm): 11.58 (s, 1H), 8.24 (dd, 1H, $^3J_{H,H}$ = 4.4 Hz, $^4J_{H,H}$ = 1.3 Hz), 7.83 (dd, 1H, $^3J_{H,H}$ = 8.3 Hz, $^4J_{H,H}$ = 1.8 Hz), 7.42 (s, 1H), 7.07 (dd, 1H, $^3J_{H,H}$ = 4.4 Hz, $^3J_{H,H}$ = 7.9 - Hz), 2.31 (s, 3H). ^{13}C–{^{1}H} NMR (150 MHz, DMSO-d_6, δ = 39.5 ppm): 168.7, 144.9, 143.3, 127.2, 125.7, 115.4, 115.0, 112.3, 20.4. $C_9H_8N_2O_2$. Found: C 60.90, H 4.29, N 16.42; requires: C 61.36, H 4.58, N 15.90.

4.1.12. 2-(Trimethylacetylamino)-3-methylpyridine (5)

Trimethylacetyl chloride (13.26 g, 0.11 mol) in methylene chloride (20 mL) was slowly added to a solution of 2-amino-3-methylpyridine (10.81 g, 0.1 mol) and triethylamine (12.63 g, 0.12 mol) in 150 ml of methylene chloride at 0 °C. The mixture was stirred in an ice bath for 1 h and further at room temperature for 2 h. Subsequently, the mixture was washed three times with an equal volume of aqueous sodium hydrogen carbonate (5%). The organic layer was dried with magnesium sulfate and concentrated to yield a colourless solid (14.52 g, 0.076 mol, 76%).

^{1}H NMR (400 MHz, DMSO-d_6, δ = 2.49 ppm): 9.55 (s, 1H), 8.25 (d, 1H, $^3J_{H,H}$ = 4.7 Hz), 7.64 (d, 1H, $^3J_{H,H}$ = 7.4 Hz), 7.19 (dd, 1H, $^3J_{H,H}$ = 7.4 Hz, $^4J_{H,H}$ = 4.7 Hz), 2.10 (s, 3H),1.22 (s, 9H). ^{13}C–{^{1}H} NMR (150 MHz, DMSO-d_6, δ = 39.5 ppm): 176.5, 150.6, 145.7, 139.0, 129.9, 121.9, 38.7, 27.3, 17.4.

4.1.13. 3-(2-Trimetylacetylamino)pyridyl acetic acid (6)

50 mL of *n*-butyllithium in hexane (1.6 M, 80 mmol) were slowly added within 1 h to a solution of **5** (6.15 g, 32 mmol) in dry THF (90 mL) at −78 °C in a period of 1 h. The mixture was stirred for 1 h at −78 °C and for 3 h at −20 °C. Powdered dry ice was added to this suspension at −78 °C. The mixture was allowed to warm up to room temperature, diluted with water and adjusted to pH 5 with 4 N HCl. It was extracted three times with an equal volume of ethyl acetate. The combined organic layers were dried over magnesium sulfate and the solvent was removed to yield **6** as a whitish solid.

^{1}H NMR (400 MHz, DMSO-d_6, δ = 2.49 ppm): 12.31 (s, 1H, COOH), 9.61 (s, 1H), 8.32 (dd, 1H, $^3J_{H,H}$ = 4.8 Hz, $^4J_{H,H}$ = 1.7 Hz), 7.71 (dd, 1H, $^3J_{H,H}$ = 7.4 Hz, $^4J_{H,H}$ = 1.7 Hz), 7.25 (dd, 1H, $^3J_{H,H}$ = 7.5 Hz, $^4J_{H,H}$ = 4.8 Hz), 3.50 (s, 2H), 1.2 (s, 9H). ^{13}C–{^{1}H} NMR (150 MHz, DMSO-d_6, δ = 39.5 ppm): 176.8, 172.0, 150.6, 146.7, 139.9, 127.7, 121.8, 38.7, 36.8, 27.1.

4.1.14. 2-Amino-3-pyridylacetic acid (7)

The residue **6** was dissolved in 6 N HCl and refluxed overnight. Adjustment with 6 N NaOH to pH 5 produced a precipitate, that was collected by filtration to afford **7** as a white solid (2 g, 12.0 mmol, 38%). ^{1}H NMR (400 MHz, DMSO-d_6, δ = 2.49 ppm): 7.83 (dd, 1H, $^3J_{H,H}$ = 4.8 Hz, $^4J_{H,H}$ = 1.4 Hz), 7.26 (dd, 1H, $^3J_{H,H}$ = 7.2 Hz, $^4J_{H,H}$ = 1.4 Hz,), 6.50 (dd, 1H, $^3J_{H,H}$ = 7.2 Hz, $^4J_{H,H}$ = 5.1 Hz), 5.70, (s, 3H) 3.42 (s, 2H).

4.1.15. 7-Azaoxindole (8)

The mother liquor of the synthesis of **7** was extracted three times with an equal volume of ethyl acetate and the combined organic phases were dried over sodium sulfate. Removal of the solvent afforded the product **8** as a white solid. A suspension of **7** (2.0 g, 12.0 mmol) in amyl alcohol was refluxed with a catalytic amount of *p*-toluenesulfonic acid for 48 h. After removal of the solvent from the clear solution, the residue was purified by column chromatography on silica gel using ethyl acetate/hexane (3:1) as eluent to provide **8** as a white solid (0.5 g, 3.7 mmol, 31%). The total yield was 40%. ^{1}H NMR (400 MHz, DMSO-d_6, δ = 2.49 ppm): 10.96 (s, 1H), 8.02 (d, 1H, $^3J_{H,H}$ = 5.3 Hz), 7.52 (d, 1H, $^3J_{H,H}$ = 7.0 Hz), 6.91 (dd, 1H, $^3J_{H,H}$ = 7.0 Hz, $^4J_{H,H}$ = 5.3 Hz), 3.53 (s, 2H). ^{13}C–{^{1}H} NMR (150 MHz, DMSO-d_6, δ = 39.5 ppm): 176.6, 150.8, 146.1, 131.8, 120.4, 117.3, 35.2. $C_7H_6N_2O$. Found: C 62.75, H 4.65, N 20.97; requires: C 62.68, H 4.51, N 20.88.

4.1.16. 7-Azaisatin (9)

Dried DMSO (6 mL) was heated to 120 °C and a solution of **8** (500 mg, 3.7 mmol) and *N*-bromosuccinimide (690.6 mg, 3.9 mmol) in dry DMSO (2 mL) was added dropwise. The mixture was stirred at 120 °C for 30 min, adjusted to pH 5–6 with aqueous $NaHCO_3$ (5%) and extracted with ethyl acetate. The solvent was removed and the residue was purified by column chromatography using ethyl acetate: hexane (3:1) as eluent to afford **9** as a yellow solid (259.2 mg, 1.76 mmol, 65%). ^{1}H NMR (400 MHz, DMSO-d_6, δ = 2.49 ppm): 11.6 (s, 1H), 8.38 (dd, 1H, $^3J_{H,H}$ = 3.3 Hz, $^4J_{H,H}$ = 0.9 Hz), 7.87 (dd, 1H, $^3J_{H,H}$ = 5.0 Hz, $^4J_{H,H}$ = 1.1 Hz), 7.01 (dd, 1H, $^3J_{H,H}$ = 4.8 - Hz, $^4J_{H,H}$ = 3.5 Hz). ^{13}C–{^{1}H} NMR (150 MHz, DMSO-d_6, δ = 39.5 ppm): 183.0, 164.0, 160.0, 155.2, 132.6, 119.0, 112.9. $C_7H_6N_2O_2$. Found: C 56.66, H 2.83, N 18.72; requires: C 56.76, H 2.72, N 18.91.

4.1.17. 7,7′-Diazaindigo (10)

Under normal atmosphere condition, a suspension of 7-aza-3-indoxyl acetate (200 mg, 1.1 mmol) was stirred in 25% aq ammonium hydroxide (10 mL). After 24 h water (50 mL) was added. The precipitate was filtered and dried to give **10** as dark blue solid (68 mg, 23.4%).

^{1}H NMR (600 MHz, 2 N DCl: DMSO-d_6 (1:3), δ = 2.49 ppm): 8.63 (d, 1H, $^3J_{H,H}$ = 5.0 Hz), 8.22 (d, 1H, $^3J_{H,H}$ = 3.8 Hz), 7.38 (t, 1H, $^3J_{H,H}$ = 4.4 Hz). $C_{14}H_8N_4O_2{\cdot}0.2\ H_2O$. Found: C 63.04, H 2.90, N 20.74; requires: C 62.78, H 3.16, N 20.92.

4.1.18. 7,7′-Diazaisoindigo (11)

Under argon atmosphere, 7-azaisatin (67.1 mg, 0.45 mmol) and 7-azaindole (97.3 mg, 0.45 mmol) were suspended in acetic acid (4 mL) and conc. HCl (340 μL). The mixture was stirred at room temperature for 12 h and diluted with water (30 mL). The precipitate was filtered and dried to yield a red solid (113.8 mg, 0.43 mmol, 95.8%).

^{1}H NMR (400 MHz, DMSO-d_6, δ = 2.49 ppm): 11.6 (s, 1H), 9.30 (d, 1H, $^3J_{H,H}$ = 5.4 Hz), 8.20 (dd, 1H, $^3J_{H,H}$ = 3.4 Hz, $^4J_{H,H}$ = 1 Hz), 7.07 (dd, 1H, $^3J_{H,H}$ = 5.1 Hz, $^3J_{H,H}$ = 3.2 Hz). $C_{14}H_8N_4O_2{\cdot}0.25\ H_2O$. Found: C 62.67, H 2.97, N 20.95; requires: C 62.57, H 3.19, N 20.85.

4.1.19. 7-Azaindirubin (12)

Under argon atmosphere, 7-azaisatin (67.1 mg, 0.45 mmol) and 3-indoxyl acetate (70 mg, 0.4 mmol) were suspended in a mixture of acetic acid (4 mL) and conc. HCl (340 μL). The mixture was refluxed for 3 h and diluted with water (30 mL). The precipitate was filtered and purified by column chromatography on silica gel using ethyl acetate: hexane (3:1) as eluent to yield a reddish solid (40 mg, 0.15 mmol, 33.8%).

^{1}H NMR (600 MHz, DMSO-d_6, δ = 2.49 ppm): 11.42 (s, 1H), 11.07 (s, 1H), 8.90 (d, 1H, $^3J_{H,H}$ = 4.4 Hz), 8,10 (d, 1H, $^3J_{H,H}$ = 2.4 Hz), 7.66 (d, 1H, $^3J_{H,H}$ = 5.0), 7,59 (t, 1H, $^3J_{H,H}$ = 5.5 Hz), 7,40 (d, 1H, $^3J_{H,H}$ = 5,3 Hz), 7,03–7.06 (m, 2H, $^3J_{H,H}$ = 7,6 Hz). ^{13}C–{^{1}H} NMR (150 MHz, DMSO-d_6, δ = 39.5 ppm): 188.8, 170.4, 154.7, 152.7, 146.5, 139.3, 137.4, 131.2, 124.5, 121.7, 118.9, 117.5, 116.1, 113.7, 103.8. $C_{15}H_9N_3O_2$. Found: C 68.41, H 3.48, N 15.94; requires: C 68.44, H 3.48, N 15.96.

4.1.20. 7′-Azaindirubin (13)

Under argon atmosphere at room temperature, a suspension of 7-aza-3-indoxyl acetate (200 mg, 1.1 mmol), isatin (183 mg, 1.2 mmol) and sodium carbonate (264 mg, 2.5 mmol) in degassed methanol (25 mL) was stirred for 5 h. The reddish precipitate was filtered off, washed with methanol and water and dried to afford 7′-azaindirubin (251 mg, 1 mmol, 87%).

^{1}H NMR (600 MHz, DMSO-d_6, δ = 2.49 ppm): 11.02 (s, 1H), 10.78 (s, 1H), 8.68 (d, 1H, $^3J_{H,H}$ = 7.7 Hz), 8.48 (d, 1H, $^3J_{H,H}$ = 3.5 Hz), 8.10 (d, 1H, $^3J_{H,H}$ = 6.2 Hz), 7.29 (t, 1H, $^3J_{H,H}$ = 7.6 Hz), 7.11 (dd, 1H, $^3J_{H,H}$ = 5.0 Hz, $^3J_{H,H}$ = 7.3 Hz), 7.04 (t, 1H, $^3J_{H,H}$ = 7.6 Hz), 6.92 (d, 1H, $^3J_{H,H}$ = 7.6 Hz). ^{13}C–{^{1}H} NMR (150 MHz, DMSO-d_6, δ = 39.5 ppm): 186.1, 171.1, 162.9, 155.7, 141.4, 137.6, 133.6, 130.2, 124.9, 121.6, 119.2, 117.8, 112.9, 110.0, 108.2. $C_{15}H_8N_3O_2$. Found: C 68.16, H 3.57, N 16.03; requires: C 68.44, H 3.45, N 15.96.

4.1.21. 7,7′-Diazaindirubin (14)

Method 1: Under argon atmosphere at 90 °C, a solution of thallium(III) acetate (1.91 g, 5 mmol) in glacial acetic acid (25 mL) was added dropwise within 2 h to a solution of **1** (1.075 g, 9.1 mmol) in glacial acetic acid (7.5 mL). The mixture was stirred overnight at 90 °C, cooled with ice and the solvent was removed by evaporation. The residue was extracted with ethyl acetate, filtered and the product was purified by repeated crystallization from ethyl acetate (red violet crystals, yield: 130 mg, 10.9%).

Method 2: Acid condensation. 7-Azaisatin (67.1 mg, 0.45 mmol) and 7-azaindoxyl-3-acetate (70 mg, 0.4 mmol) were suspended under argon atmosphere in acetic acid (4 mL) and conc. HCl (340 μL). The mixture was stirred at room temperature for overnight and diluted with water (30 ml). The precipitate was filtrated and purified by recrystallization from 1 N HCl/EtOH (75 mg, 0.28 mmol, 62.2%).

^{1}H NMR (400 MHz, DMSO-d_6, δ = 2.49 ppm): 11.58 (s, 1H), 10,74 (s, 1H), 8.84 (dd, 1H, $^3J_{H,H}$ = 7.9 Hz, $^4J_{H,H}$ = 1.7 Hz), 8.50 (dd, 1H, $^3J_{H,H}$ = 4.8 Hz, $^4J_{H,H}$ = 1.7 Hz), 8.15–8.11 (m, 2H), 7.15–7.07 (m, 2H). ^{13}C–{^{1}H} NMR (150 MHz, DMSO-d_6, δ = 39.5 ppm): 186.3, 170.5, 163.4, 155.8, 155.4, 147.8, 138.7, 133.8, 131.5, 118.2, 117.9, 115.5, 112.9, 105.7. $C_{14}H_8N_4O_2 \cdot 0.67\ H_2O$. Found: C 60.89, H 3.35, N 19.85; requires: C 60.87, H 3.41, N 20.28.

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Supplementary data

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