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Identification of a water-soluble indirubin derivative as potent inhibitor of insulin-like growth factor 1 receptor through structural modification of the parent natural molecule

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ABSTRACT. Indirubins have been identified as potent ATP-competitive protein kinase inhibitors. Structural modifications in the 5- and 3'-position have been extensively investigated, but the impact of substituents in 5'-position is not equally well-studied. Here, we report the synthesis of new indirubin-3'- and 5'-derivatives in the search of water-soluble indirubins by

introducing basic centres. Anti-proliferative activity of all compounds in tumor cells was evaluated along with kinase inhibition of selected compounds. The results show the 3'-position to tolerate large substituents without compromising activity, whereas bulk and rigid substituents in 5'-position appear unfavorable. Screening molecular targets of water soluble 3'-oxime ethers revealed **6ha** as preferential inhibitor of insulin-like growth factor 1 receptor (IGF-1R) in a panel of 22 protein kinases and in cells. Consistently, **6ha** inhibited tumor cell growth in the NCI 60 cell line panel and induced apoptosis. The results indicate that the 5'-position provides limited space for chemical modifications and identify **6ha** as a potent water-soluble indirubin-based IGF-1R inhibitor.

Introduction

The indigoid bisindoles (Figure 1) indirubin, indigo and isoindigo originate from two indole moieties connected in 3,2'-, 2,2'-, or 3,3'-position.[1] The dark-red indirubin is a minor constituent of the blue natural dye indigo and has been recognized as the main active agent of the traditional formulation *Dangui Luhui Wan* in Traditional Chinese Medicine (TCM).[1] Indirubin has been used in China for treatment of a series of diseases including Chronic Myelogenous Leukemia (CML).[1] Indirubin and its derivatives raised sustained scientific interest due to the discovery of their high affinity binding to the ATP-binding site of protein kinases involved in tumorigenesis, e.g. CDKs[2] and GSK3ß.[3] VEGFR-2,[4] c-Src,[5-7] and CK2.[8] Recently, our group revealed that indirubins are able to inhibit TGFß/BMP signaling by chemically knocking down Smad protein expression.[9] Moreover, *N*-methylisoindigo has been found to reduce the population of CD133 positive cancer stem cells in primary pancreatic cancer cells through interference with cellular metabolic signaling pathways.[10] These new developments provide promising perspectives for clinical application of indigoid bisindoles.

The main obstacle for clinical application of indirubin is very poor solubility in most solvents, probably caused by strong binding forces in the crystal lattice.[11] Studies on crystal structures of indirubins co-crystallized with CDK2,[12] CDK2/CyclinA,[12] CDK5/p25[3] and GSK3ß,[3] revealed positions 5 and 3' to best tolerate structural modifications to increase solubility and cytotoxicity.[13] Recently, we reported the synthesis of indirubin-5-carboxamides with basic centers in the amide substituent leading to enhanced anti-proliferative effects and target specificity against GSK3ß activity.[14] Their corresponding quaternary ammonium and hydrochloride salts provide compounds with significantly improved water-solubility, while maintaining cytotoxicity in cancer cells.[14] The solubility of 6-bromo substituted indirubins

designed as GSK3ß inhibitors was improved by chemical modifications in 3'-position.[15] The 5'-position opening out into the solvent canal should provide further options to increase solubility and cytotoxicity.[12] Molecular modeling binding studies of 5-nitro-5'-hydroxyindirubin-3'-oxime with CDK2 revealed an additional H-bond between the hydroxy group and Asp86 of the enzyme affording improved CDK2 inhibitory activity,[16] inhibition of tumor cell growth and induction of apoptosis.[17] 5'-Methoxyindirubin was shown to induce apoptotic cell death in human neuroblastoma cells.[18] Synthesis and biological activity of 5'-carboxamido substituted indirubins with increased water solubility supposedly acting as structural analogues of sunitinib have been reported by Wang et al.[19] Indirubins with 7-bromo or 7-trifluoromethyl substituent and polar acidic substituents in 5'- or 6'-position were shown to bind in an inverse manner to the binding site of DYRK kinases.[20] Additionally, self-emulsifying drug formulations have been developed, which significantly increased the bioavailability of indirubin derivatives *in vitro* and *in vivo*.[21-23]

Here we concentrated on chemical modifications of substituents in 3'- and 5'-position of the indirubin core. With one exception in the literature,[19] merely indirubins with small-sized 5'-substituents have been synthesized and investigated. We modified 5'-carboxy substituted indirubins in a manner previously described for the 5-position.[14] For the 3'-position which is directed towards the ribose/triphosphate canal, hence providing copious space for structural modification, an extended range of substituents, varying length and functionality, was synthesized. All compounds were tested for anti-proliferative activity in cancer cells. Promising compounds were selected for evaluating *in vitro* the inhibitory effect on diverse kinases. Solubility data were collated and structure activity relationship was discussed.

Results and Discussion

Chemistry

Synthesis of indirubin-3'-derivatives

For the synthesis of the indirubin-3'-oxime derivatives (Scheme 1) we first followed the process described by Baeyer,[24] modified by Russell and Kaupp,[25] consisting of basic condensation of isatins (**2a-2d**) with 3-acetylindoxyl (**3**) to obtain indirubins (**4a-4d;** Scheme 1, reaction a). In a recent paper, Saito *et al*. reported a series of methoxyindirubins and their anti-proliferative effects in human neuroblastoma cells,[18] in which however the synthetic method was not completely described. We prepared 5-methoxyisatin (**2b**) as well as 5-hydroxyisatin (**2d**) by direct ortho metalation of Boc-protected *p*-aniline (**8b, 8c**) using *t*-BuLi, subsequent reaction with diethyl oxalate, deprotection and cyclization via adding HCl (Scheme 2). In the case of **2d**, OH had to be initially protected with a triisopropylsilyl group to avoid side reactions (Scheme 2, reaction a). Heating with hydroxylamine hydrochloride in pyridine converted the indirubins (**4a-4d**) into their corresponding oximes (**5a-5d**; Scheme 1, reaction b), which in turn were substituted with a set of halogenides using 1,1,3,3-tetramethyl-guanidine (TMG) as base to get the products in high yields (**6a-6q**, Scheme 1, reaction c).[14] For the compounds **6d** and **6e,** butane-triol (**9**) and pentane-triol (**12**) were first protected with acetone (Scheme 3, reaction a). Compound **12** is commercially unavailable and was prepared in a cyclization of L-glutamic acid (**10**) with sodium nitrite, followed by reduction of 5-oxotetrahydrofuran-2-carboxylic acid (**11)** using $LiAlH_4$ (Scheme 3, reaction c and d).[26] The resulting dioxolanes (**13a**, **13b**) were halogenated by perbromomethane and triphenylphosphine in bromoform (**14a, 14b**; Scheme 3, reaction b).[27] Afterwards halogenides (**14a**, **14b**) were introduced into the indoxyl moiety and the protecting group was hydrolyzed in 1 N HCl (Scheme 1, reaction c).

To achieve enhanced water solubility, a polar substituent, *N*-methylpiperazine was connected to indirubin-3'-(2-bromoethyl)-oximether (**6o**) or 5-methoxy-indirubin-3'-(2-bromoethyl)-

oximether (**6r**; Scheme 1, reaction d). Treatment with THF containing saturated HCl converted **6h** and **6k** to the corresponding hydrochloride (**6ha** and **6ka**; Scheme 1, reaction e).

Synthesis of indirubin-5'-derivatives

Our approach to the synthesis of indirubin-5'-derivatives (**23**) is outlined in Scheme 4. These indirubins (**23**) were prepared by the acidic condensation of various 1-acetyl-3-indoxyl acetates (**3, 22**) with isatins (**2**) in moderate to good yields (50 - 90%; Scheme 4, reaction j).[8, 13] Substituted 3-indoxyl acetates (**22**) were synthesized from *N*-phenyl-glycine-*o*-carboxylic acids (**21**) by heating with sodium acetate in acetic anhydride (Scheme 4, reaction i).[28] The obtained 5'-indirubins (**23**) could be readily converted into 5'-substituted indirubin-3'-oximes (**24**) using hydroxyl-amine hydrochloride in pyridine as mentioned above (Scheme 4, reaction k).

The synthetic route to **21** (Scheme 4) depended on the substituent R_1. 4-Aminoisophthalic acid (**16**) was prepared via oxidation of 2,4-dimethyl-1-nitrobenzene in an aqueous solution of $KMnO_4$ at 85°C followed by catalytic hydrogenation with palladium on carbon (Pd/C; Scheme 4 I, reaction a and b).[29] Hydrogenation of 5-methoxy-2-nitrobenzoic acid afforded 2-amino-5-methoxybenzoic acid (**17**; Scheme 4 II, reaction b). The reaction of **16** or **17** with chloroacetic acid and sodium carbonate at 100°C provided the required **21a** (Scheme 4 I and II, reaction c).[28] Acetyl-protected *N*-phenylglycine-*o,p*-dicarboxylic acid **21b** was synthesized by reaction of 2,4-dimethylaniline (**18**) with chloroacetic acid,[14] acetylation and subsequent oxidation with permanganate (Scheme 4 III, reaction d). This compound is applicable for the cyclization, too.

Alternatively, 2-chlorobenzoic acids (**19, 20**) were transformed into **21a** via copper catalyzed nucleophilic aromatic displacement (Scheme 4 IV and V, reaction f).[30] For the synthesis of 5'-hydroxyindirubin, 3-amino-5-chloro-benzoic acid was diazotized to produce 5-chloro-3-

hydroxybenzoic acid[31] that was subsequently protected by a benzyl group to yield **20** (Scheme 4 V, reaction g and h).

To improve solubility and bioactivity, we placed a basic center in the amide group of indirubin-5'-carboxamides (**23e-g**; Scheme 5). Activation of 5'-carboxyindirubin (**23d**) with pentafluorophenyl trifluoroacetate and subsequent amidation were performed analogously to the method reported for the synthesis of indirubin-5-carboxamides.[14] 5'-Aminoindirubin (**23b**) was synthesized by Pd/C hydrogenation of **23a** (Scheme 6).

Biological activity

Antiproliferative activity in human tumor cell lines

Recently, several compounds with distinct chemical backbones have been identified as pan-assay interference compounds (PAINS), which often give false positive signals in various biological assays in high-throughput screening.[32-34] We recruited a recently developed software, called cAPP, and performed in silico screening of our indirubin derivatives using available PAINS filters.[35] The results confirmed that the basic structure of 3,2'-bisindole is not related to any reported PAINS. The result was listed in supporting information (SI. Table 1).

As next, we determined the anti-proliferative effects of compounds in LXFL529L (large cell lung carcinoma cell line) and/or MCF7 (human breast adenocarcinoma cells) in the sulforhodamine B (SRB) assay. The IC_{50}-value, structure and solubility were summarized in Table 1. Indirubin-3'-ethyloxime ether (**6a**) showed an IC_{50} value of 7.6 μM. Replacing the ethyl group with propane-1,2-diol resulted in 10-fold higher cytotoxicity (**6c**, also known as E804,[21, 23] 0.9 μM). Interestingly, a positive relationship between toxicity and length of alkyl chain was

observed for compounds carrying an alkane diol-group in 3'-position (**6d**, 0.65 μM and **6e**, 0.56 μM), while removal of one hydroxyl group from **6d** reduced toxicity (**6b**, 2.6 μM). Replacing diol of **6c** with *β*-D-glucopyranose gave the compound **6f** with a comparable activity (1.1 μM), but 15-fold higher solubility. Introducing basic substituents into 3'-position led to indirubin-3'-{2-[*p*-(*N,N*-dimethyl)aminoanilino]-ethyl}-oxime ether (**6g**, 5.1 μM) which showed 6-fold less inhibition, as compared to **6c** while **6h** (0.9 μM) with a methylpiperazine substituent showed a similar IC_{50} value.

In view of the fact that 5- and 3'- position share the ribose/triphosphate canal, using two large substituents at both positions could significantly reduce bioactivity.[14] We therefore incorporated small groups including methyl-, hydroxyl- and methoxy-group into the 5-position. In comparison to **6f** lacking a substituent in 5-position, additional methyl (**6m**, 2.0 μM) or hydroxyl (**6n**, 1.2 μM) substituents attenuated solubility, whereas a methoxy group (**6j**) slightly increased the solubility (1.5-fold). Remarkably, the compounds **6l** (also known as E738)[7, 9] and **6k** carrying additional basic substituent in 3'-position exhibited similar cytotoxicity as their respective counterparts **6c** and **6h**, but showed 25- and 200-fold higher aqueous solubility, respectively. Transformation **6h** and **6k** into hydrochloride salts achieved two well water soluble indirubins **6ha** and **6ka** (solubility >25 g/L) without loss of cytotoxicity.

In comparison to 3'- and 5-indirubins, modifications in 5'-position did not notably improve cytotoxicity or solubility (Table 2). The solubility of 5'-nitroindirubin **(23a)** was too low (<< 0.1 mg/L) to determine an IC_{50} value using the SRB-assay, although the anti-cancer activity of its 5-analogue (5-nitroindirubin) was reported in cells and animal model.[36] Appreciable cytotoxicity was achieved by hydrogenation of **23a.** The compound **23b** exhibited an IC_{50} of 7.6 μM. Our result showed that introduction of basic centers in 3'-position (compounds **6g**, **6h** and **6k**)

remarkably improved cytotoxicity and/or water solubility. Moreover, we previously reported that indirubin-5-caboxamides with similar basic centers showed enhanced solubility and anti-tumor activity.[14] Nevertheless, similar modifications in 5'-position failed to increase solubility and activity (**23e-g**). Additionally, 5'-carboxyindirubin **(23d)** was inactive regardless of further adding a carboxyl group in 5-position **(23h)** or conversion into oxime **(24d).** Derivatives carrying small substituents, like amino, hydroxy and methoxy in 5'-position (**23b**, **23c**, **23i**), illustrated good anti-proliferative effect, while converting **23c** into its 3'-oxime **24c** further increased cytoxicity by about 3 times.

Inhibition of protein kinases *in vitro*

On the basis of chemical structure and cytotoxicity, several compounds were selected for evaluating inhibitory effects on recombinant kinases including CDK1/CyclinB, CDK2/CyclinA, CDK2/CyclinE, CDK6/CyclinD, and GSK3ß. The IC_{50} values were listed in Table 3. Focusing on CDK2/CycE, **6c** and **6d** showed similar effects (IC_{50}: 0.21 μM and 0.23 μM). A methoxy group in 5-position significantly increased activity (**5b**: 0.054 μM; **6j**: 0.09 μM and **6l**: 0.043 μM), while dramatic ablation of activity was observed by replacement or withdrawal of the methoxy group (**6f** and **6m**). Analysis from molecule modeling suggested that the hydrochloride product of **6h** (**6ha)** should not be expected to effectively inhibit CDKs and GSK3ß (SI. Figure 1). We profiled **6ha** and **6l** against a panel of 22 protein kinases at 1 μM as previously described (Figure 2A).[14] As expected (Figure 2B), **6l** was the potent multi-kinase inhibitor and inhibited 12 out of 22 protein kinases (residual activity < 50%). The result also confirmed that CDKs and GSK3ß was not targeted by **6ha**, although its 6-bromo-substitueted counterpart had shown GSK3ß, CDK1 and CDK5 inhibition with IC50 values of 0.005 μM, 0.3 μM and 0.5 μM respectively.[15] Intriguingly, we observed potent an inhibitory effect (~30% residual activity) on

insulin-like growth factor receptor1 (IGF-1R). This was confirmed by using the ADP Glo® assay (Promega, Germany), showing an IC_{50} value of 169 nM (Figure 2C).

In comparison to indirubin, which was previously reported to inhibit activity of various CDK/Cyclin complexes at around 10 μM ($IC_{50\text{-}CDK1/CycB1}$: 10 μM; $IC_{50\text{-}CDK2/CycA}$: 7.5 μM; $IC_{50\text{-}CDK2/CycE}$: 2.2 μM; $IC_{50\text{-}CDK4/CycD1}$: 12 μM and IC $IC_{50\text{-}CDK5/p35NCK}$: 5.5 μM), but not IGF-1R ($IC_{50\text{-}IGF\text{-}1R}$: >1000 μM),[2] **6ha** showed highly selective inhibitory effect on IGF-1R. Recently, Anastassiadis et al. systematically analyzed the activity of 178 commercially available protein kinase inhibitors on a panel of 300 protein kinases.[37] To get more insight into the selectivity of our compounds, we generated the heat map (Figure 2B) to compare inhibitory effects of **6ha** and **6l** with relevant compounds provided in this study, which included three indirubin derivatives (**25**, **6c** and **26**), previously characterized IGF-1R-related inhibitors (**27-31**), and compounds inhibiting 50% of IGF-1R activity (**32-37**) in that study,[37] whose structures were listed in figure 2D. We found that indirubin derivatives generally targeted Auroras, CDKs, CHK2, FGF-R1 and GSK3ß with exception of **6ha** (Figure 2B). Of note, the reported IGF-1R or Akt inhibitors could not block the activity of IGF-1R in their study. Indeed, potent antagonists of IGF-1R were CDK inhibitors (Figure 2B). Taken together, **6ha** possessed a distinct pattern of protein kinase profiling as compared to other compounds in our analysis and showed the unique inhibitory activity.

6ha inhibited IGF-1R mediated signaling in various cell lines

IGF is a growth factor whose effects are transmitted by its receptor IGF-1R. Ligand binding activates the phosphatidylinositol 3-kinase (PI3K)—protein kinase B (PKB/Akt) signaling pathway.[38] In tumor cells, this signal cascade is normally hyperactivated to meet the special

requirement of tumor cells in metabolism, proliferation, survival, and motility.[38] The cells originated from IGF-1R knock-out embryos do not undergo transformation to malignancy implicating inhibition of activated IGF-1R as a prerequisite in tumorigenesis.[39] Therefore, the development of IGF-1R inhibitors may offer novel perspectives for basic cancer research and clinical therapy.[40] To evaluate the effect of **6ha** in cells, we performed immunocytochemistry in MCF7 cells (breast cancer cells) treated with **6ha** for 1 h. A specific antibody against phosphorylated IGF-1R at Tyr 1161 (p-IGF-1R) was used, whose activity is required for fully active IGF-1R and related downstream signaling pathway. As showed in figure 3A, the majority of IGF-1R detected by the antibody was localized on the membrane (mock treatment). Upon treatment the signal was completely dismissed. Ly294002 (**38**, Figure 2D), a PI3K inhibitor,[41] was used as positive control (10 μM) and showed mild inhibitory effect. As next, we performed immunoblot and further confirmed that phosphorylation of IGF-1R was sufficiently blocked by **6ha** even at a concentration of 0.5 μM for 1 h treatment in MCF7 cells (Figure 3B).

Moreover, we evaluated effects of **6ha** on the downstream pathway in various cancer cells treated for 1 h by detecting the level of phosphorylated Akt (S473), a well-known target molecule of the IGF-PI3K-related signaling pathway.[38] Dose-dependent inhibition was observed in all four cell lines tested, including MCF7, HeLa (cervical cancer cells), MDA MB231 (breast cancer cells) and Panc1 (pancreatic cancer cells). In comparison to the positive control, **38** at a concentration of 10 μM, the effect of **6ha** was comparable to better in certain cells (Figure 4A). In time-dependent study the activity of Akt was shut down by **6ha** as early as 15 min after incubation of MCF7 cells (Figure 4B).

6ha induced PARP cleavage, cell apoptosis and cell arrest

We found cleaved PARP in MCF7 treated with **6ha** for 6 h, implicating the induction of cell apoptosis (Figure 4C).[10] Here, a commercial Src inhibitor (Srci, 4-(4'-phenoxyanilino)-6,7-dimethoxyquinazoline, 10 μM) was used as the positive control. In good agreement, apoptotic cells were visualized by FITC conjugated annexin v and propidium iodide (PI), and quantified by fluorescence activated cell sorting (FACS) as previously described.[10] Bortezomib (Bz, [(1R)-3-methyl-1-({(2S)-3-phenyl-2-[(pyrazin-2-ylcarbonyl)amino]propanoyl}amino)butyl]boronic acid), a proteasome inhibitor,[42] was used as positive control. The result confirmed that **6ha** induced cellular apoptosis from a concentration of 0.5 μM (Figure 5). Analysis of DNA content revealed gradually elevated cell cycle arrest majorly at G2/M phase in MCF7 cells treated with **6ha** for 24 h and 48 h (Figure 6), consistent with a previous report that inhibition of IGF-1R caused cell apoptosis and cell arrest in G2/M.[43]

Activity of **6ha** was further evaluated by measuring the anti-proliferative effect on the NCI 60 panel of cell lines. A high activity of **6ha** in all NCI 60 cell lines (Figure 7 and supplementary information) with GI_{50} value (50% growth inhibition) between 3.19 μM (BT-549) to 0.08 μM (KM12) was observed. Of note, GI_{50} values in MCF7 and MDA MB231 are 0.59 μM and 2.14 μM respectively, in consistent with the premise of **6ha** blocking Akt phosphorylation similarly in these two cell lines (Figure 4A).

Conclusion

Taken together, the results show that bulky and rigid substituents are not suitable for the 5'-position, bringing about reduced toxicity and water solubility. The conversion of indirubin-3'-derivatives to stable and water-soluble hydrochloride salts was achieved. Compound **6ha** surprisingly effected potent inhibition of IGF-1R without notably inhibiting CDKs and GSK3ß in

a panel of 22 protein kinases. Further analyses identified that **6ha** inactivated IGF und related signalling in various tumor cells, and induced cell apoptosis and cell arrest at G2/M phases in MCF7 cells. In the NCI60 cell line panel, the compound generally inhibited the growth of malignant tumor cells at low micromolar concentrations.

Experimental Section

Cell culture

LXFL529L cells were grown at 37°C in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS). MCF7, Panc1, HeLa and MDA MB231 cells were cultivated in DMEM supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 (cell culture media and supplements were obtained from Invitrogen, Karlsruhe, Germany). Cells were tested routinely for absence of mycoplasm contamination. **38** was purchased from Calbiochem (Germany) and Srci from Merck (Germany). Compounds were dissolved in DMSO to obtain 10 mM stock solution and further dilutions were prepared in respective medium.

Sulforhodamine B assay

Effects on cell growth were determined according to the method of Skehan et al.[44] with slight modifications. Briefly, cells were seeded into 24-well plates with a density of 10,000 cells/well 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

calculated as percent survival, determined by the number of treated over control cells. GI_{50} of NCI60 was performed by the US National Cancer Institute.

Solubility in water

Standard solutions of the indirubin compounds were prepared in ethanol and maxima of absorbance were defined. Solubility was determined spectrophotometrically at maximum of absorbance by calibration method.[14]

Immunoblot

MCF7 cells were cultured in DMEM with 10% FBS for 24 h and then incubated with compounds as indicated in the text. Cells were lysed and proteins were analyzed by western blot with ECL detection as described before.[33] For each sample 40 µg of total protein were resolved with 10% SDS-PAGE and immunoblotted with antibodies specific for p-IGF-1R (Y1161), p-Akt (S473), Akt and PARP (Cell Signaling Technologies, Germany) and ß-Actin antibody for control (Sigma Aldrich, Germany). Primary antibodies were incubated at a 1:1000 dilution in TBS (pH 7.5) with 0.1% Tween-20 and 5% BSA/milk with gentle agitation overnight at 4°C. Secondary antibodies (Sigma Aldrich) were incubated in TBS (pH 7.5) with 5% BSA/Milk and 0.1% Tween-20 at a 1:10000 dilution for 1 h at room temperature.

Immunocytochemistry

Immunocytochemistry was performed as previously described.[45-46] Briefly, MCF7 cells were incubated with 6ha (1 µM) for 1 h. 0.1% DMSO was used as mock and **38** (10 µM) as positive control. Cells were fixed with 4% PFA at RT for 15 min, permeabilized and blocked with blocking buffer (5% goat serum and 0.3% Triton X-100 in PBS) for 1 hr. Cells were incubated

with p-IGF-1R antibody (1:200) in the antibody dilution buffer (1% BSA and 0.2% Triton X-100 in PBS) at 4°C overnight. The secondary antibody (Goat anti-rabbit Alexa Flour® 594) Dianova, Germany) was added and incubated for 1 hr. Hoechst 33258 (1 µg/mL in PBS) was used to visualize nuclei.

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 concentrations of **6ha** for 24h and harvested. Cells were collected by centrifugation and supernatant was discarded. For apoptosis assay, cells were resuspended in a solution of 5 µL FITC-conjugated annexin v (BD Bioscience, Germany) in 50 µL annexin v binding buffer (10 mM HEPES pH7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) and incubated for 15 min in the dark at room temperature. Afterwards, a solution of 1.25 µL propidium iodide (PI, 1 mg/mL) in 450 µL annexin v binding buffer were added, incubated for 10 min in the dark at room temperature. Then cells were immediately analyzed by flow cytometry (FACS, Fluorescence-activated cell sorting) using FACSCalibur (Becton Dickinson) and CellQuest Pro (BD) analysis software. For cell cycle analysis, the cell pellets were fixed in 70% Ethanol for at least 24 h, washed twice with ice-cold PBS and resuspended in 500 µL PBS. The suspension was incubated with RNase A (50 µg/mL) for 1 h at 37 °C and subsequently stained with PI (50 µg/mL) for 5 min and analyzed by FACS.

***In Vitro* Kinase assay**

In vitro inhibition of isolated or recombinant kinases, CDK1/CyclinB, CDK2/CyclinA, CDK2/CyclinE, CDK6/CyclinD, and GSK-3ß (Upstate, Lake Placid, USA , # 14-475), was analyzed following the manufacturer's protocol with 0.4 µCi [g-32P] ATP per sample vial.

Briefly, kinase assay mixtures were incubated for 10 min at 30 °C. Thereafter, reaction solutions (20 µL) were spotted on P81 phosphocellulose squares. After washing (3x, 5min with 0.75% phosphoric acid) phosphocellulose sheets were rinsed with acetone and dried. The squares were then transferred into scintillation vials and substrate phosphorylation was measured by β-counting. The IC_{50} values were determined by linear regression of the data points comparing drug-treated samples with solvent control. Each measurement was done in duplicate in at least three independent experiments. Kinase profiling assay for **6ha** at 1 µM was performed by ProQinase (Germany) as previously reported.[14] IGF-1R activity was determined in ADP Glo assay (Promega, Germany) according to the manufacturers protocol.

Synthesis

The syntheses of intermediates were described in supporting information

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 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/UV254 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.

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). Chemical shifts are reported in ppm from tetramethylsilane with solvent as the internal standard (^{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.

HPLC was employed to confirm the purity of compounds >96%.

General procedure for the synthesis of indirubin derivatives (4)

Under argon atmosphere at room temperature, a suspension of 3-indoxyl acetate (1.1 mmol), isatin (1.2 mmol) and sodium carbonate (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 the indirubin.

5-Methoxyindirubin (4b) (92.4%)

^{1}H-NMR (400 MHz; DMSO-d_6) 3.77 (s, 3H), 6.79 (d, 1H, ^{3}J = 8.4 Hz), 6.85 (dd, 1H, ^{3}J = 8.4 Hz, ^{4}J = 2.4 Hz), 7.01 (t, 1H, ^{3}J = 7.7 Hz), 7.41 (d, 1H, ^{3}J = 8.1 Hz), 7.56 (t, 1H, ^{3}J = 8.4 Hz), 7.65 (d, 1H, ^{3}J = 7.7 Hz), 8.48 (d, 1H, ^{4}J = 2.6 Hz), 10.69 (s, 1H), 11.01 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 55.4, 107.2, 109.8, 110.5, 113.4, 115.1, 119.0, 121.3, 122.2, 124.4, 134.8, 137.1, 138.4, 152.5, 154.3, 171.0, 181.8. Anal. Calcd for $C_{17}H_{12}N_2O_3$: C 69.9, H 4.1, N 9.6; Found: C 69.7, H 3.7, N 10.0.

5-Methylindirubin (4c) (92.0%)

^{1}H-NMR (400 MHz; DMSO-d_6) 2.33 (s, 3H), 6.79 (d, 1H, ^{3}J = 7.9 Hz), 7.00-7.08 (m, 2H), 7.42 (d, 1H, ^{3}J = 7.9 Hz), 7.58 (m, 1H), 7.64 (d, 1H, ^{3}J = 7.6 Hz), 8.63 (s, 1H), 10.79 (s, 1H), 11.00 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 21.0, 106.8, 109.2, 113.3, 119.9, 121.1, 121.5, 124.2, 125.1, 129.7, 129.7, 137.0, 138.1, 138.7, 152.4, 171.9, 188.5. Anal. Calcd for $C_{17}H_{12}N_2O_2$: C 73.9, H 4.4, N 10.1; Found: C 73.8, H 4.3, N 10.2.

5-Hydroxyindirubin (4d) (94.3%)

^{1}H-NMR (400 MHz; DMSO-d_6) 6.67 (m, 2H), 7.00 (t, 1H, ^{3}J = 7.3 Hz), 7.40 (d, 1H, ^{3}J = 8.1 Hz), 7.56 (t, 1H, ^{3}J = 7.3 Hz), 7.64 (d, 1H, 3J = 7.3 Hz), 8.31 (s, 1H), 9.02 (s, 1H), 10.57 (s, 1H), 10.98 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 107.5, 109.8, 112.2, 113.4, 116.2, 119.0, 121.1, 122.1, 124.3, 133.6, 137.1, 138.2, 152.1, 152.5, 170.2, 188.6. Anal. Calcd for $C_{16}H_{10}N_2O_3$: C 69.1, H 3.6, N 10.1; Found: C 69.0, H 3.8, N 9.9.

General procedure for the synthesis of indirubin-3'-oxime (5) (yield) A mixture of indirubin(**1**, **4b-d**) (1.1 mmol) and hydroxylamine hydrochloride (24.4 mmol) in pyridine (7 mL) was refluxed for 4 h. The mixture was diluted with 2 N HCl (50 mL) to yield the corresponding **5** as a reddish solid.

Indirubin-3'-oxime (5a) (78.9%)

^{1}H-NMR (400 MHz; DMSO-d_6) 6.90 (d, 1H, ^{3}J = 7.6 Hz), 6.95 (m, 1H), 7.01-7.05 (m, 1H), 7.13 (m, 1H), 7.40-7.41 (m, 2H), 8.24 (d, 1H, ^{3}J = 7.6 Hz), 8.65 (d, 1H, ^{3}J = 7.2 Hz), 10.72 (s, 1H), 11.73 (s, 1H), 13.48 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 98.9, 108.9, 111.5, 116.5, 120.4, 121.5, 122.7, 123.1, 125.9, 128.0, 132.0, 138.3, 144.8, 145.3, 151.2, 171.0. Anal. Calcd for $C_{16}H_{11}N_3O_2 \cdot 0.25\ H_2O$: C 68.2, H 4.1, N 14.9; Found: C 68.4, H 4.1, N 14.8.

5-Methoxy-indirubin-3'-oxime (5b) (95.0%)

1H-NMR (400 MHz; DMSO-d6) 3.76 (s, 3H), 6.72 (dd, 1H, 3J = 7.6 Hz, 4J = 2.5 Hz), 6.73 (d, 1H, 3J = 8.3 Hz), 7.00-7.04 (m, 1H), 7.38-.739 (m, 2H), 8.24 (d, 1H, 3J = 7.4 Hz), 8.33 (d, 1H, 4J = 2.1 Hz), 10.52 (s, 1H), 11.76 (s, 1H), 13.49 (s, 1H). 13C-{1H}-NMR (150 MHz; DMSO-d6) 55.5, 99.5, 108.8, 109.6, 111.2, 111.5, 116.5, 121.4, 123.5, 128.0, 132.0, 132.3, 144.8, 145.3, 151.4, 154.2, 171.1. Anal. Calcd for $C_{17}H_{13}N_3O_3 \cdot H_2O$: C 62.8, H 4.7, N 12.9; Found: C 63.0, H 4.4, N 13.2.

5-Methyl-indirubin-3'-oxime (5c) (56.0%)

^{1}H-NMR (400 MHz; DMSO-d_6) 2.33 (s, 3H), 6.76 (d, 1H, ^{3}J = 7.6 Hz), 6.93 (d, 1H, ^{3}J = 7.6 Hz), 7.01 (m, 1H), 7.38 (m, 2H), 8.23 (d, 1H, ^{3}J = 7.4 Hz), 8.48 (s, 1H), 10.59 (s, 1H), 11.71 (s, 1H), 13.40 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 21.0, 99.4, 108.7, 111.7 116.8, 121.6, 122.9, 124.0, 126.6, 128.2, 129.2, 132.2, 136.4, 145.1, 145.3, 151.5, 171.3. Anal. Calcd for $C_{17}H_{13}N_3O_2 \cdot 0.25\ H_2O$: C 69.0, H 4.6, N 14.2; Found: C 69.3, H 4.5, N 14.2.

5-Hydroxy-indirubin-3'-oxime (5d) (88.2%)

^{1}H-NMR (400 MHz; DMSO-d_6) 6.59 (dd, ^{1}H, 3J = 7.6 Hz, ^{4}J = 2.5 Hz), 6.65 (d, 1H, ^{3}J = 8.3 Hz), 7.00 (m, 1H), 7.40-7.35 (m, 2H), 8.12 (d, 1H, ^{4}J = 2.1 Hz), 8.22 (d, 1H, ^{3}J = 7.5 Hz), 8.71 (s, 1H), 10.41 (m, 2H), 13.45 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 99.8, 108.6, 111.1, 111.3, 112.6, 116.6, 121.3, 123.6, 128.1, 131.2, 132.0, 144.8, 145.0, 151.4, 151.6, 171.1. Anal. Calcd for $C_{16}H_{11}N_3O_3 \cdot H_2O$: C 61.7, H 4.2, N 13.5; Found: C 61.7, H 4.1, N 13.9.

General procedure for the synthesis of indirubin-3'-oxime ethers (6, except 6d, 6e)

1,1,3,3-Tetramethylguanidine (582 µL) and alkyl halogenide (5.4 mmol) were added to indirubin-3'-oxime (0.5 mmol) in ethanol (7 mL) with stirring. The mixture was refluxed for 2 h, cooled to 0°C and diluted with water (20 mL) and 1N HCl (20 mL). The precipitate was filtered, washed with water and dried to afford **6** except for 6d and 6e.

Indirubin-3'-ethyl-oxime ether (6a) (86.0%)

^{1}H-NMR (400 MHz; DMSO-d_6) 1.50 (t, 3H, ^{3}J = 7.1 Hz), 4.63 (m, 2H), 6.89 (d, 1H, ^{3}J = 7.6 Hz), 7.00 (m, 2H), 7.14 (dt, 1H, ^{3}J = 7.6 Hz, ^{4}J = 1.0 Hz), 7.40 (m, 2H), 8.13 (d, 1H, ^{3}J = 7.6 Hz), 8.61 (d, 1H, ^{3}J = 7.7 Hz), 10.74 (s, 1H), 11.97 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 14.4, 72.1, 100.0, 108.9, 111.7, 116.1, 120.6, 121.4, 122.3, 123.1, 126.4, 128.2, 132.7, 138.6, 144.2, 145.4, 151.0, 170.8. Anal. Calcd for $C_{18}H_{15}N_3O_2 \cdot H_2O$: C 66.9, H 5.3, N 13.0;. Found: C 67.3, H 4.9, N 13.0.

Indirubin-3'-(4-hydroxybutyl)-oxime ether (6b) (68.0%)

^{1}H-NMR (400 MHz; DMSO-d_6) 1.62 (m, 2H), 1.93 (m, 2H), 3.48 (m, 2H), 4.50 (m, 1H), 4.61 (m, 2H), 6.90 (d, 1H, ^{3}J = 7.6 Hz), 6.96-7.05 (m, 2H), 7.12-7.16 (m, 1H). 7.38-7.44 (m, 2H), 8.11 (d, 1H, ^{3}J = 7.6 Hz), 8.61 (d, 1H, ^{3}J = 7.8 Hz), 10.74 (s, 1H), 11.68 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 25.7, 29.2, 60.7, 76.7, 100.2, 109.2, 112.0, 116.4, 120.9, 121.7, 122.6, 123.5, 126.6, 128.3, 133.0, 138.9, 144.2, 145.6, 151.3, 171.1. Anal. Calcd for $C_{20}H_{19}N_3O_3$: C 68.8, H 5.5, N 12.0;. Found: C 68.5, H 5.4, N 11.8.

Indirubin-3'-(2,3-dihydroxypropyl)-oxime ether (E804, 6c) (44.0%)

^{1}H-NMR (400 MHz; DMSO-d_6) 3.52 (m, 2H), 3.97-4.04 (m, 1H), 4.49-4.53 (m, 1H), 4.63-4.68 (m, 1H), 4.80 (m, 1H), 5.11 (d, 1H, ^{3}J = 5.1 Hz), 6.90 (d, 1H, ^{3}J = 7.8 Hz), 6.96-7.05 (m,

2H), 7.12-7.16 (m, 1H), 7.39-7.45 (m, 2H), 8.18 (d, 1H, ^{3}J = 7.6 Hz), 8.62 (d, 1H, ^{3}J = 7.8 Hz), 10.75 (s, 1H), 11.68 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 63.0, 70.2, 78.9, 100.3, 109.1, 111.9, 116.5, 120.9, 121.6, 122.5, 123.6, 126.5, 128.6, 133.0, 138.8, 144.2, 145.6, 151.4, 171.1. Anal. Calcd for $C_{19}H_{17}N_3O_4$: C 65.0, H 4.9, N 12.0;. Found: C 64.8, H 4.8, N 12.4.

General procedure for the synthesis of (6d, 6e)

A suspension of **6p** oder **6q** (2.0 mmol) in 1N HCl (100 mL) was stirred at room temperature over night. The mixture was filtered and dried to afford **6d** or **6e**.

Indirubin-3'-(3,4-dihydroxybutyl)-oxime ether (6d) (62.6% two steps)

^{1}H-NMR (400 MHz; DMSO-d_6): 1.81-2.15 (m, 2H), 3.32 (dd, 1H, ^{2}J = 10.7 Hz, ^{3}J = 5.8 Hz), 2.48-2.50 (m, 2H), 3.40 (dd, 1H, ^{2}J = 10.7 Hz, ^{3}J = 5.8 Hz), 3.69-4.7 (m, 3H), 6.89 (d, 1H, 3J = 7.4 Hz), 6.97-7.04 (m, 2H), 7.14 (dt, 1H, ^{3}J = 7.8 Hz, 4J = 1,2 Hz), 7.39-7.42 (m, 2H), 8.13 (d, 1H, ^{3}J = 7.4 Hz), 8.63 (d, 1H, ^{3}J = 7.9 Hz), 10.58 (s, 1H), 11.76 (s, 1H). ^{13}C{^{1}H}-NMR (600 MHz; DMSO-d_6): 33.0, 65.9, 68.0, 73.4, 99.9, 108.9, 111.7, 116.1, 120.6, 121.4, 122.3, 123.2, 126.3, 128.1, 132.7, 138.6, 143.9, 146.4, 151.0, 170.8. Anal. Calcd for $C_{20}H_{19}N_3O_4 \cdot H_2O$: C 62.7, H 5.5, N 11.0; Found: C 63.0, H 5.1, N 10.8.

Indirubin-3'-(4,5-dihydroxypentyl)-oxime ether (6e) (13.7%, two steps)

^{1}H-NMR (400 MHz; DMSO-d_6): 1.24-5.05 (m, 11H), 6.89 (d, 1H, ^{3}J = 7.7 Hz), 6.97-7.03 (m, 2H), 7.14 (t, 1H, ^{3}J = 7.6 Hz), 7.39-7.43 (m, 2H), 8.11-8.18 (m, 1H), 8.59-8.62 (m, 1H), 10.75 (s, 1H), 11.69 (s, 1H). ^{13}C{^{1}H}-NMR (600 MHz; DMSO-d_6): 25.1, 33.0, 65.9, 68.0, 73.4, 100.0, 109.0, 111.8, 116.3, 120.7, 121.5, 122.4, 123.3, 126.4, 128.5, 132.8, 138.6, 144.4, 145.4, 151.1,

170.9. Anal. Calcd for $C_{21}H_{21}N_3O_4 \cdot 0.33\ H_2O$: C 65.4, H 5.7, N 10.9; Found: C 65.0, H 5.3, N 10.9.

Indirubin-3'-(2-ß-D-glucopyranosylethyl)-oxime ether (6f) (54.0%)

^{1}H-NMR (400 MHz; DMSO-d_6) 2.96-3.18 (m, 4H), 3.43-3.45 (m, 1H), 3.66-3.68 (m, 1H), 4.02-4.03 (m, 1H), 4.22-4.24 (m, 1H), 4.30 (d, 1H, ^{3}J = 7.7 Hz), 4.49 (t, 1H, ^{3}J = 5.8 Hz), 4.75 (t, 2H, ^{3}J = 4.9 Hz), 4.90 (d, 1H, ^{3}J = 5.0 Hz), 4.93 (1H, ^{3}J = 4.9 Hz), 5.04 (d, 1H, ^{3}J = 4.9 Hz), 6.89 (d, 1H, ^{3}J = 7.4 Hz), 6.97-7.03 (m, 2H), 7.14 (t, 1H, ^{3}J = 7.4 Hz), 7.37-7.45 (m, 2H), 8.21 (d, 1H, ^{3}J = 7.7 Hz), 8.59 (d, 1H, ^{3}J = 7.7 Hz), 10.75 (s, 1H); 11.68 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 61.1, 61.3, 67.1, 70.0, 72.4, 73.6, 76.2, 77.2, 100.4, 103.4, 109.1, 111.9, 116.4, 120.9, 122.6, 123.5, 126.5, 126.6, 132.8, 138.9, 144.1, 145.7, 151.7, 171.1. Anal. Calcd for $C_{24}H_{25}N_3O_8 \cdot H_2O$: C 57.5, H 5.4, N 8.4;. Found: C 57.7, H 5.6, N 8.5.

Indirubin-3'-{2-[p-(N,N-dimethyl)-amino-anilino]-ethyl}-oxime ether (6g) (84.6%)

^{1}H-NMR (400 MHz; DMSO-d_6): 2.67 (s, 6H), 3.53 (t, 2H, ^{3}J = 5.8 Hz), 4.69 (t, 2H, ^{3}J = 5.8 Hz), 6.57-6.63 (m, 4H), 6.70-6.89 (m, 4H), 7.14 (t, 1H, ^{3}J = 7.4 Hz), 7.42-7.34 (m, 2H), 8.13 (d, 1H ^{3}J = 7.4 Hz), 8.61 (d, 1H, ^{3}J = 7.9 Hz), 10.78 (s, 1H), 11.69 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz, DMSO-d_6): 39.4-39.9, 100.2, 109.0, 111.7, 113.5, 115.4, 116.3, 120.1, 121.5, 122.4, 123.3, 126.4, 128.7, 132.8, 138.7, 140.1, 144.0, 145.5, 151.5, 170.9. Anal. Calcd for $C_{26}H_{25}N_5O_2 \cdot 0.5H_2O$: C 69.6, H 5.8, N 15.6; Found: C 69.9, H 6.1, N 15.4.

Indirubin-3'-[2-(4-methylpiperazino)-ethyl]-oxime ether (6h) (94.2%)

^{1}H-NMR (400 MHz; DMSO-d_6): 2.12 (s, 3H), 2.48-2.50 (m, 8H), 2.87 (t, 2H, ^{3}J = 3.9 Hz), 4.67 (t, 2H, ^{3}J = 3.9 Hz), 6.89 (d, 1H, 3J = 4.8 Hz), 6.96-7.02 (m, 2H), 7.14 (dd, 1H, ^{3}J = 5.0 Hz,

^{4}J = 0.6 Hz), 7.40-7.42 (m, 2H), 8.16 (d, 1H, ^{3}J = 5.0 Hz), 8.60 (d, 1H, ^{3}J = 5.2 Hz), 10.76 (s, 1H), 11.68 (s, 1H). ^{13}C{^{1}H}-NMR (150 MHz; DMSO-d_6): 45.3, 53.7, 54.5, 56.1, 73.9, 100.1, 108.7, 111.2, 116.1, 120.2, 121.0, 122.2, 122.9, 126.0, 128.0, 132.4, 138.5, 143.8, 145.3, 151.1, 170.7. Anal. Calcd for $C_{23}H_{25}N_5O_2$: C 68.5, H 6.3, N 17.4; Found: C 68.2, H 6.2, N 17.2.

Indirubin-3'-[2-(4-methylpiperazino)-ethyl]-oxime ether hydrochlorid (6ha) (82.2%)

^{1}H-NMR (400 MHz; D_2O/DMSO-d_6): 2.81 (s, 3H), 3.44-3.64 (m, 10H), 4.88 (s, 2H), 6.93 (d, 1H, ^{3}J = 4.8 Hz), 6.94-7.04 (m, 2H), 7.15 (t, 1H, ^{3}J = 7.4 Hz), 7.22 (d, 1H, ^{3}J = 7.8 Hz), 7.41 (t, 1H, ^{3}J = 7.4 Hz), 8.15 (d, 1H, ^{3}J = 7.4 Hz), 8.47 (d, 1H, ^{3}J = 7.8 Hz). ^{13}C{^{1}H}-NMR (150 MHz; DMSO-d_6): 44.4, 51.6, 50.8, 56.8, 71.2, 100.4, 110.9, 112.2, 117.1, 122.7, 123.4, 123.4, 124.8, 127.8, 129.9, 134.5, 138.7, 145.9, 146.0, 153.7, 172.7. $C_{23}H_{25}N_5O_2 \cdot 2HCl$: C 58.0, H 5.7, N 14.7; Found: C 57.7, H 5.8, N 14.3.

5-Methoxy-indirubin-3'-(2-ß-D-glucopyranosylethyl)-oxime ether (6j)

^{1}H-NMR (400 MHz; DMSO-d_6) 2.99-3.13 (m, 4H), 3.42-3.45 (m, 1H), 3.65-3.69 (m, 1H), 3.78 (s, 3H), 4.01-4.05 (m, 1H), 4.21-4.24 (m, 1H), 4.29 (d, 1H, ^{3}J = 7.7 Hz), 4.48 (t, 1H, ^{3}J = 5.5 Hz), 4.74 (t, 2H, ^{3}J = 4.8 Hz), 4.91 (d, 1H, ^{3}J = 4.8 Hz), 4.94 (d, 1H, ^{3}J = 4.0 Hz), 5.03 (d, 1H, ^{3}J = 4.4 Hz), 6.74 (dd, 1H, ^{3}J = 7.4 Hz, ^{4}J = 2.2 Hz), 6.79 (d, 1H, ^{3}J = 8.1 Hz), 7.01 (t, 1H, ^{3}J = 7.3 Hz), 7.45-7.38 (m, 2H), 8.23 (d, 1H, ^{3}J = 7.7 Hz), 8.27 (d, 1H, ^{4}J = 2.2 Hz), 10.59 (s, 1H), 11.75 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 55.4, 61.1, 61.2, 66.6, 70.0, 73.4, 76.0, 76.7, 77.0, 100.9, 103.1, 108.7, 109.3, 111.2, 111.6, 121.5, 123.0, 128.9, 132.5, 132.9, 143.8, 145.4, 151.8, 154.1, 171.0. Anal. Calcd for $C_{25}H_{27}N_3O_9 \cdot 0.5H_2O$: C 57.5, H 5.4, N 8.0;. Found: C 57.3, H 5.2, N 8.0.

5-Methoxy-indirubin-3'-[2-(4-methylpiperazino)-ethyl]-oxime ether (6k) (76.1%)

^{1}H-NMR (400 MHz; DMSO-d_6): 2.12 (s, 3H), 2.48-2.50 (m, 8H), 2.87 (t, 2H, ^{3}J = 3.9 Hz), 3.77 (s, 3H), 4.68 (t, 2H, ^{3}J = 3.9 Hz), 6.74 (dd, 1H, ^{3}J = 5.5 Hz, ^{4}J = 1.8 Hz), 6.79 (d, 1H, ^{3}J = 5.5 Hz), 7.02 (m, 1H), 7.39-7.43 (m, 2H), 8.18 (d, 1H, ^{3}J = 5.0 Hz), 8.29 (d, 1H, ^{4}J = 1.8 Hz), 10.59 (s, 1H), 11.74 (s, 1H). Anal. Calcd for $C_{24}H_{27}N_5O_3$: C 66.5, H 6.3, N 16.2; Found: C 66.4, H 6.2, N 16.0.

5-Methoxy-indirubin-3'-[2-(4-methyl-piperazino)-ethyl]-oxime ether hydrochloride (6ka) (88.6%)

1H-NMR (400 MHz; D2O/DMSO-d6): 2.78 (s, 3H), 3.31-3.71 (m, 10H), 3.79 (s, 3H), 4.93 (s, 2H), 6.74-6.82 (m, 2H), 7.02 (m, 1H), 7.40-7.45 (m, 2H), 8.23-8.26 (m, 2H), 10.64 (s, 1H), 11.76 (s, 1H). Anal. Calcd for $C_{24}H_{27}N_5O_3 \cdot 2HCl \cdot H2O$: C 55.0, H 6.0, N 13.4; Found: C 54.6, H 5.9, N 13.0.

5-Methoxy-indirubin-3'-(2,3-dihydroxypropyl)-oxime ether (6l) (81.3%, E738)

^{1}H-NMR (400 MHz; DMSO-d_6) 3.51 (t, 2H, ^{3}J = 5.5 Hz), 3,79 (s, 3H), 3.97 (m, 1H), 4.53 (dd, 1H, ^{2}J = 10.6 Hz, ^{3}J = 4.3 Hz), 4.64 (dd, 1H, ^{2}J = 10.6 Hz, ^{3}J = 4.3 Hz), 4.77 (t, 1H, ^{3}J = 5.9 Hz), 5.10 (d, 1H, ^{3}J = 5.5 Hz), 6.73 (dd, 1H, ^{3}J = 8.2 Hz, ^{4}J = 2.4 Hz), 6.79 (d, 1H, ^{3}J = 8.6 Hz), 7.03 (m, 1H), 7.42 (m, 2H), 8.19 (d, 1H, ^{3}J = 7.4 Hz), 8.30 (d, 1H, ^{4}J = 2.7 Hz), 10.58 (s, 1H), 11.76 (s, 1H). ^{13}C{^{1}H}-NMR (600 MHz; DMSO-d_6): 55.5, 62.8, 69.8, 78.5, 100.8, 108.6, 109.3, 111.7, 112.9, 116.3, 121.5, 123.0, 128.6, 132.5, 132.9, 144.0, 145.4, 151.5, 154.2, 171.0. Anal. Calcd for $C_{20}H_{19}N_3O_5$: C 63.0, H 5.0, N 11.0; Found: C 62.8, H 5.0, N 10.9.

5-Methyl-indirubin-3'-(2-ß-D-glucopyranosylethyl)-oxime ether (6m)

^{1}H-NMR (400 MHz; DMSO-d_6) 2.33 (s, 3H), 2.99-3.14 (m, 1H), 3.41-3.46 (m, 1H), 3.65-3.67 (m, 1H), 3.78 (s, 3H), 4.01-4.05 (m, 1H), 4.21-4.24 (m, 1H), 4.29 (d, 1H, ^{3}J = 7.7 Hz), 4.48 (t, 1H, ^{3}J = 5.5 Hz), 4.74 (t, 2H, ^{3}J = 4.8 Hz), 4.91 (d, 1H, ^{3}J = 4.8 Hz), 4.94 (d, 1H, ^{3}J = 4.0 Hz), 5.03 (d, 1H, ^{3}J = 4.4 Hz), 6.79 (d, 1H, ^{3}J = 8.0 Hz), 7.00-7.08 (m, 2H), 7.42 (d, 1H, ^{3}J = 7.9 Hz), 7.58 (m, 1H), 7.64 (d, 1H, ^{3}J = 7.7 Hz), 8.63 (s, 1H), 10.78 (s, 1H), 11.00 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 21.1, 61.1, 61.2, 66.6, 70.0, 73.4, 76.0, 76.7, 77.0, 99.5, 109.0, 116.9, 121.8, 129.7, 124.2, 126.5, 128.2, 126.9, 129.5, 132.5, 136.7, 145.1. 145.2, 151.4, 171.2. Anal. Calcd for $C_{25}H_{27}N_3O_8$: C 60.4, H 5.5, N 8.5;. Found: C 60.0, H 5.8, N 8.6.

5-Hydroxy-indirubin-3'-(2-ß-D-glucopyranosylethyl)-oxime ether (6n)

^{1}H-NMR (400 MHz; DMSO-d_6) 3.00-3.14 (m, 1H), 3.40-3.46 (m, 1H), 3.65-3.70 (m, 1H), 3.79 (s, 3H), 4.00-4.05 (m, 1H), 4.21-4.25 (m, 1H), 4.30 (d, 1H, ^{3}J = 7.7 Hz), 4.47 (t, 1H, ^{3}J = 5.5 Hz), 4.75 (t, 2H, ^{3}J = 4.8 Hz), 4.90 (d, 1H, ^{3}J = 4.8 Hz), 4.94 (d, 1H, ^{3}J = 4.0 Hz), 5.00 (d, 1H, ^{3}J = 4.4 Hz), 6.60 (d, 1H, 3J = 7.6 Hz), 6.65 (d, 1H, ^{3}J = 8.3 Hz), 6.92-7.02 (m, 1H), 7.40-7.35 (m, 2H), 8.13 (d, 1H, ^{4}J = 2.1 Hz), 8.22 (d, 1H, ^{3}J = 7.5 Hz), 8.71 (s, 1H), 10.38 (s, 1H), 10.41 (s, 1H). ^{13}C-{^{1}H}-NMR (150 MHz; DMSO-d_6) 61.1, 61.2, 66.6, 70.0, 73.2, 76.1, 76.9, 77.2, 100.8, 109.6, 110.1, 111.3, 112.5, 118.6, 121.5, 122.6, 129.1, 131.5, 132.3, 145.0, 145.2, 152.4, 153.6, 171.3. Anal. Calcd for $C_{24}H_{25}N_3O_9$: C 57.7, H 5.1, N 8.4; Found: C 57.4, H 5.0, N 8.8.**General procedure for the synthesis of indirubin-5'-derivatives (23) (yield)**

Under argon atmosphere at room temperature, a suspension of 3-indoxyl acetate (1.1 mmol), isatin (1.2 mmol) and sodium carbonate (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 indirubine.

5'-Nitroindirubin (23a) (87.5 %):

^{1}H-NMR (400 MHz, DMSO-d_6): 6.89 (d, 1H, ^{3}J = 7.9 Hz), 7.03 (t, 1H, ^{3}J = 7.5 Hz), 7.29 (t, 1H, ^{3}J = 7.3 Hz), 7.58 (d, 1H, ^{3}J = 9.0 Hz), 8.36 (d, 1H, ^{4}J = 2.2 Hz), 8.41 (dd, 1H, ^{3}J = 8.9 Hz, ^{4}J = 2.2 Hz), 8.75 (d, 1H, ^{3}J = 7.7 Hz), 10.98 (s, 1H, NH), 11.57 (s, 1H, NH). ^{13}C-{^{1}H}-NMR (600 MHz, DMSO-d_6): 110.1. 110.2, 114.0, 119.1, 120.4, 121.2, 121.7, 125.6, 130.8, 132.0, 137.9, 141.4, 142.0, 156.0, 170.4, 187.0. Anal. Calcd for $C_{16}H_9N_3O_4$: C, 62.6; H, 3.0; N, 13.7. Found: C, 62.2; H, 2.9; N, 13.5.

5'-Aminoindirubin (23b) (50.0 %):

^{1}H-NMR (400 MHz, DMSO-d_6): 6.90 (d, 1H, ^{3}J = 7.7 Hz), 7.01 (dt, 1H, ^{3}J = 7.5 Hz, ^{4}J = 0.7 Hz), 7.26 (dt, 1H, ^{3}J = 7.7 Hz, ^{4}J = 1.1 Hz), 7.48 (d, 1H, ^{3}J = 8.8 Hz), 7.51 (dd, 1H, ^{3}J = 8.6 Hz, ^{3}J = 1.8 Hz), 7.59 (d, 1H, ^{4}J = 1.8 Hz), 8.74 (d, 1H, ^{3}J = 7.7 Hz), 10.02 (s, 2H), 10.94 (s, 1H), 11.10 (s, 1H). ^{13}C-{^{1}H}-NMR (600 MHz, DMSO-d_6): 107.8, 109.9, 114.7, 118.6, 119.5, 121.5, 125.0, 126.0, 129.9, 131.6, 138.4, 141.4, 151.4, 151.6, 170.9, 188.0. Anal. Calcd for $C_{16}H_{11}N_3O_2$: C, 69.3; H, 4.0; N, 15.2. Found: C, 69.2; H, 3.9; N, 15.3.

5'-Hydroxyindirubin (23c) (58.0 %):

^{1}H-NMR (400 MHz; DMSO-d_6): 6.88 (d, 1H, ^{3}J = 7.8 Hz), 6.97 (d, 1H, ^{4}J = 2.7 Hz), 6.99 (t, 1H, ^{3}J = 7.8 Hz), 7.03 (dd, 1H, ^{3}J = 8.5 Hz, ^{4}J = 2.4 Hz), 7.22 (m, 1H), 7.23 (d, 1H, ^{3}J = 8.5 Hz), 8.71 (d, 1H, ^{3}J = 7.5 Hz), 9.6, (s, 1H), 10.77 (s, 1H), 10.84 (s, 1H). ^{13}C-{^{1}H}-NMR (600 MHz, DMSO-d_6): 105.7, 109.2, 109.7, 114.5, 119.7, 121.4, 121.8, 124.6, 125.3, 129.0, 139.4, 140.8, 146.1, 152.6, 171.2, 189.2. Anal. Calcd for $C_{16}H_{10}N_2O_3$: C, 69.1; H, 3.6; N, 10.1. Found: C, 69.0; H, 3.7; N, 10.1.

5'-Carboxyindirubin (23d) (70.9 %):

^{1}H-NMR (400 MHz; DMSO-d_6): 6.88 (d, 1H, ^3J = 7.9 Hz), 7.02 (d, 1H, ^3J = 7.8 Hz), 7.26 (d, 1H, ^3J = 7.8 Hz), 7.47 (d, 1H, ^3J = 9.1 Hz), 8.09 (m, 2H), 8.76 (d, 1H, ^3J = 7.8 Hz), 10.93 (s, 1H), 11.23 (s, 1H), 12.87 (s, 1H). ^{13}C-{^{1}H}-NMR (600 MHz, DMSO-d_6): 108.2, 109.6, 113.2, 118.9, 121.2, 121.3, 123.8, 125.0, 125.5, 129.8, 137.7, 138.1, 141.3, 154.9, 166.4, 170.6, 187.7. Anal. Calcd for $C_{17}H_{10}N_2O_4 \cdot 0.5\ H_2O$: C, 64.8; H, 3.5; N, 8.9. Found: C, 64.9; H, 3.3; N, 8.8.

General procedure for the synthesis of indirubin-5'-carboxamides (23e, 23f and 23g) (yield)

Under argon a solution of **23k** (0.43 mmol), amino compound (0.6 mmol) and DMAP (0.49 mmol) in dried dioxane (12 mL) was refluxed for several hours till no starting material was detected by TLC. After cooling the mixture was diluted with 0.1 N HCl (100 mL). The precipitate was filtered and dried in vacuo to afford indirubin-5'-carboxamides

5'-(4-Methylpiperazinocarbonyl)-indirubin (23e) (72.5 %):

^{1}H-NMR (400 MHz; DMSO-d_6): 2.19 (s, 3H), 2.31 (s, 4H), 3.48 (m, 4H), 6.90 (d, 1H, ^3J = 7.4 Hz), 7.02 (t, 1H, ^3J = 7.4 Hz), 7.25 (d, 1H, ^3J = 7.8 Hz), 7.46 (d, 1H, ^3J = 8.2 Hz), 7.60-7.62 (m, 2H), 8.76 (d, 1H, ^3J = 7.8 Hz), 10.92 (s, 1H), 11.12 (s, 1H). Anal. Calcd for $C_{22}H_{20}N_4O_3 \cdot HCl$: C, 62.12; H, 5.0; N, 13.2. Found: C, 62.3; H, 5.0; N, 13.2.

5'-{*N*-[2-(Dimethylamino)-ethyl]-aminocarbonyl}-indirubin (23f) (37.2 %):

^{1}H-NMR (400 MHz; DMSO-d_6): 2.16-2-40 (m, 10H), 6.90 (d, 1H, ^3J = 7.4 Hz), 7.02 (t, 1H, ^3J = 7.4 Hz), 7.26 (t, 1H, ^3J = 7.4 Hz), 7.45 (d, 1H, ^3J = 8.7 Hz), 8.06 (d, 1H, ^3J = 8.2 Hz), 8.17 (s,

1H), 8.46 (s, 1H), 8.77 (d, 1H, ^3J = 7.8 Hz), 11.15 (s, 2H). ^{13}C-{^{1}H}-NMR (600 MHz, DMSO-d_6): 37.4, 45.3, 58.3, 108.2, 109.6, 113.2, 118.9, 121.2, 121.3, 123.8, 125.0, 125.5, 129.8, 137.7, 138.1, 141.3, 154.9, 166.4, 170.6, 187.7. Anal. Calcd for $C_{21}H_{20}N_4O_3 \cdot HCl$: C, 61.1; H, 5.1; N, 13.6. Found: C, 61.3; H, 5.3; N, 13.5.

5'-{*N*-[4-(4-Methylpiperazino)-phenyl]-aminocarbonyl}-indirubin (23g) (57.9 %):

^{1}H-NMR (400 MHz; DMSO-d_6): 2.23 (s, 3H), 2.54 (m, 4H), 3.30 (m, 4H), 6.97 (m, 3H), 7.09 (t, 1H, ^3J = 7.8 Hz), 7.33 (g, 1H, ^3J = 8.2 Hz), 7.55 (d, 1H, ^3J = 8.2 Hz), 7.66 (d, 2H, ^3J = 9.0 Hz), 8.21 (d, 1H, ^3J = 7.8 Hz), 8.38 (s, 1H), 8.84 (d, 1H, ^3J = 7.8 Hz), 10.13 (s, 1H), 11.01 (s, 1H), 11.31 (s, 1H). ^{13}C-{^{1}H}-NMR (600 MHz, DMSO-d_6): 45.8, 48.8, 54,6, 107.9, 109.7, 113.2, 115.4, 118.7, 121.3, 121.4, 121.5, 123.7, 125.0 , 127.7, 129.8, 131.0, 136.7, 138.4, 141.3, 147.5, 154.2, 163.7, 170.8, 188.2. Anal. Calcd for $C_{28}H_{25}N_5O_3 \cdot 3HCl$: C, 57.1; H, 4.8; N, 11.9. Found: C, 57.3; H, 4.6; N, 11.8.

5,5'-Dicarboxyindirubin (23h) (77.6 %):

^{1}H-NMR (400 MHz; DMSO-d_6): 6.96 (d, 1H, ^3J = 7.9 Hz), 7.48 (d 1H, ^3J = 8.8 Hz), 7.88 (dd, 1H, ^3J = 7.9 Hz, ^4J = 1.3 Hz), 8.10-8.12 (m, 2H), 9.42 (d, 1H, ^3J = 0.9 Hz), 11.27 (s, 1H), 11.36 (s, 1H), 12.56 (s, 2H). ^{13}C-{^{1}H}-NMR (600 MHz, DMSO-d_6): 107.0, 109.4, 113.5, 119.1, 121.2, 123.8, 123.9, 125.7, 126.6, 131.5, 138.0, 138.9, 144.7, 155.2, 166.5, 167.5, 172.1, 187.9. Anal. Calcd for $C_{18}H_{10}N_2O_6 \cdot H_2O$: C, 58.7; H, 3.3; N, 7.6. Found: C, 58.4; H, 3.5; N, 7.8.

5,5'-Dimethoxyindirubin (23i) (88.1 %)

^{1}H-NMR (400 MHz; DMSO-d_6): 3.76-3.77 (m, 6H), 6.78 (d, 1H, ^3J = 8.6 Hz), 6.83 (d, 1H, ^3J = 2.7 Hz), 7.18-7.21 (m, 2H), 7.34 (d, 1H, ^3J = 8.6 Hz), 8.47 (d, 1H, ^3J = 2.4 Hz), 10.66 (s, 1H),

10.84 (s, 1H). Anal. Calcd for $C_{18}H_{14}N_2O_4 \cdot 0.33H_2O$: C, 65.9; H, 4.5; N, 8.5. Found: C, 65.9; H, 4.7; N, 8.3.

Pentafluorophenyl-indirubin-5'-carboxylate (23k) :

Under argon atmosphere a solution of indirubin-5'-carboxylic acid (450 mg, 0.96 mmol), pentafluorophenyl trifluoroacetate (800 mg, 2.86 mmol), pyridine (0.25 mL) and catalytic amount of dimethylaminopyridine (DMAP) in dried DMF (15 mL) was stirred at room temperature for 4 h, diluted with ethyl acetate and washed with 0.1 N HCl (100 mL). After removal of solvent, the residue was dried in vacuo to yield a reddish solid **23k** (439.8 mg, 0.93 mmol, 97.0 %). ^{1}H-NMR (400 MHz; DMSO-d_6): 6.89 (d, 1H, ^3J = 7.8 Hz), 7.02 (t, 1H, ^3J = 7.4 Hz), 7.28 (t, 1H, ^3J = 7.0 Hz), 7.62 (d, 1H, ^3J = 8.7 Hz), 8.27 (dd, 1H, ^3J = 8.7 Hz, ^4J = 1.6 Hz), 8.34 (d, 1H, ^4J = 1.6 Hz), 8.75 (d, 1H, ^3J = 7.8 Hz), 11.06 (s, 1H), 11.52 (s, 1H).

General procedure for the synthesis of 5'-indirubin-3'-oxime (24)

A mixture of indirubin (1.1 mmol) and hydroxylamine hydrochloride (24.4 mmol) in pyridine (7 mL) was refluxed for 4 h. The mixture was diluted with 0.1N HCl (50 mL) to yield a reddish solid **24**

5'-Hydroxyindirubin-3'-oxime (24c) (93.0 %):

^{1}H-NMR (400 MHz; DMSO-d_6): 6.81 (d, 1H, ^4J = 1.7 Hz), 6.93 (d, 1H, ^4J = 1.7 Hz), 6.92 (dd, 1H, ^3J = 5.6 Hz, ^4J = 0.8 Hz), 7.08 (m, 1H), 7.25 (d, 1H, ^3J = 5.6 Hz), 7.72 (d, 1H, ^3J = 1.5 Hz), 8.58 (d, 1H, ^3J = 5.1 Hz), 9.20 (s, 1H), 10.64 (s, 1H,), 11.58 (s, 1H), 13.44 (s, 1H). Anal. Calcd for $C_{16}H_{11}N_3O_3 \cdot H_2O$: C, 61.7; H, 4.2; N, 13.5. Found: C, 61.8; H, 4.5; N, 13.1.

5'-Carboxylindirubin-3'-oxime (24d) (87.0 %):

^{1}H-NMR (400 MHz; DMSO-d_6): 6.89-6.97 (m, 2H), 7.15 (m, 1H), 7.47 (d, 1H, ^{3}J = 8.3 Hz), 8.00 (dd, 1H, ^{3}J = 8.3 Hz, ^{4}J = 1.5 Hz), 8.66 (d, 1H, ^{3}J = 7.8 Hz), 8.79 (d, 1H, ^{4}J = 1.5 Hz), 10.79 (s, 1H,), 11.89 (s, 1H), 12,87 (s, 1H), 13.71 (s, 1H). ^{13}C-{^{1}H}NMR (600 MHz, DMSO-d_6): 100.6, 109.1, 111.3, 116.5, 120.6, 122.4, 123.5, 123.5, 126.7, 129.4, 133.7, 138.9, 144.7, 148.2, 150.5, 167.0, 170.8. Anal. Calcd for $C_{17}H_{11}N_3O_4 \cdot 2HCl$: C, 51.8; H, 3.3; N, 10.7. Found: C, 51.7; H, 3.6; N, 10.5.

ASSOCIATED CONTENT

Supporting Information. The supporting information is available free of charge, including in silica screening of PAINS using cAPP, Molecular Modeling, raw data of inhibitory effect of compounds in kinase profiling, raw data of FACS analyses, GI50 of 6ha in the NCI60 cell line panel, and molecular formula strings, as well as Synthesis of intermediates and references of supporting information.

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Author Contributions

X.C designed experiment, synthesized compounds, performed biological assessments and wrote the manuscript. K-H.M designed experiment, synthesized compounds and wrote the manuscript.

S.V performed biological assessments. J.Z, S.M, A.T and J.C synthesized compounds. S.W helped to design biological assessment. G.E designed experiment and wrote the manuscript. All authors read the manuscript.

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ABBREVIATIONS

CD133, prominin-1; CDK, cyclin-dependent kinases; CK2, casein kinase 2; CML, chronic myelogenous leukemia; c-Src, proto-oncogene tyrosine-protein kinase Src; DYRK, Dual specificity tyrosine-phosphorylation-regulated kinase 1A; FACS, fluorescence activated cell sorting; GSK3, glycogen synthase kinase 3; IGF-1R: insulin growth factor 1 receptor; LXFL529L: large cell lung carcinoma cell line; SRB, sulforhodam B; TCM, traditional Chinese medicine; TLC, thin layer chromatography; TMG, 1,1,3,3-tetramethyl-guanidine; VEGFR, vascular endothelial growth factor.

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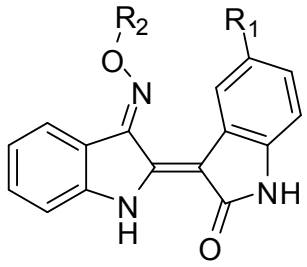
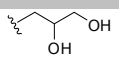
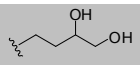
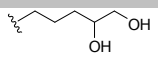
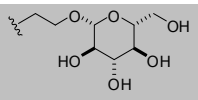
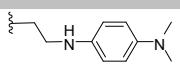
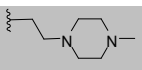
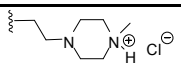
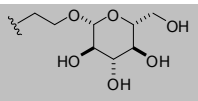
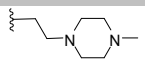
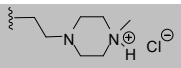
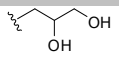
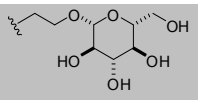
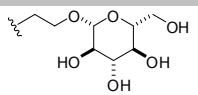
Tabel 1: Structure, cytotoxicity and solubility of novel indirubin-3'-derivatives

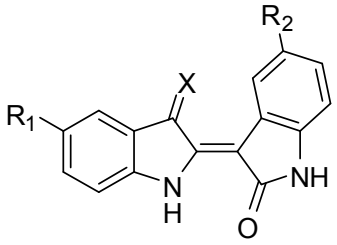


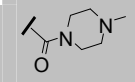
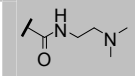
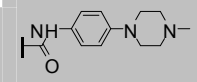
Compds	R1	R2	Solubility mg/L	Cytotoxicity IC50, [μM]	
				LXFL	MCF7
6a	H		n.d.	7.6±0.06	n.d
6b	H		0.1±0.01	2.6±0.03	n.d
6c	H		2±0.2	0.9±0.1	0.1±0.042
6d	H		3±0.2	0.65±0.21	n.d
6e	H		2±0.1	0.56±0.12	n.d
6f	H		30±1.7	1.1±0.17	1.2±0.085
6g	H		1.4±0.2	5.1±0.43	n.d
6h	H		2±0.3	0.86±0.06	0.8±0.042

6ha	H		>25 000	1.1±0.11	0.85±0.06
6j	OMe		50±3.4	1.1±0.09	0.7±0.048
6k	OMe		400±26.9	1.2±0.065	n.d
6ka	OMe		>25 000	1.2±0.05	0.89±0.12
6l	OMe		50±4.2	0.53±0.18	0.3±0.022
6m	Me		2.0±0.1	2.0±0.17	1.5±0.021
6n	OH		5±0.6	1.2±0.11	n.d
Indirubin[4)]					9,91±1,19
Bz[4)]					0.025±0.006

(1) A series of 3'-indirubins were tested at various concentrations for their effects on LXFL529L cells (human large cell lung cancer cells) and/or MCF7 cells (human breast adenocarcinoma cells) using the SRB assay (see Experimental Section). The IC_{50} [μM] values were calculated from dose-response curves. Solubility [mg/L] was spectrophotometrically determined as described previously.[14] (2) MCF7; (3) n.d.: not determined. (4) Indirubin and bortezomib (Bz, [(1R)-3-methyl-1-({(2S)-3-phenyl-2-[(pyrazin-2-ylcarbonyl)amino]propanoyl}-amino)butyl]boronic acid**)** was used as positive control.[42]

Table 2: Structure, cytotoxicity and solubility of novel indirubin-5'-derivatives



Compds	R1	R2	X	Solubility mg/L	Cytotoxicity IC50, [μM]
23a	NO_2	H	O	<0.1	n.d.
23b	NH_2	H	O	n.d.	7.6±0.058
23c	OH	H	O	7.3±0.7	10.7±0.093
23d	COOH	H	O	278±20.9	>50
23e		H	O	23.9±1.9	17.9±0.54
23f		H	O	16.1±1.3	5.43±1.69
23g		H	O	4.2±0.5	10±1,02
23h	COOH	COOH	O	0.6±0.1	>100
23i	OCH3	OCH3	O	n.d.	6.0±0,31
24c	OH	H	NOH	2.7±0.3	3.6±0.74

24d	COOH	H	NOH	<1	>10

n.d.: not determined.

Table 3. Inhibitory effects of indirubin-3'- derivatives (**6**) on protein kinases[1)]

	CDK1/ CycB	**CDK2/ CycA**	**CDK2/ CycE**	**CDK6/ CycD**	**GSK3ß**
5b	0.91±0.12	0.21±0.12	0.054±0.0015	0.16±0.01	0.09±0.01
6c	1.65±0.36	0.54±0.13	0.21±0.04	0.075±0.01	0.11±0.02
6d	n.d.[3)]	n.d.	0.23±0.02	n.d.	n.d.
6f	>10	≥10	1.70±0.421	1.35±0.3	0.81±0.02
6j	5.58±1.29	0.84±0.14	0.09±0.01	0.41±0.1	0.1±0.01
6l	n.d.	n.d.	0.043±0.005	n.d.	n.d.
6m	>10	9.30±1.52	0.92±0.1	1.65±0.03	0.29±0.03
1[4,6]	20±1.9	2.2±0.3	7.5±0.59	n.d.	0.6±0.04
PC[2)]	7.89±0.37	3.49±1.28	0.62±0.17	>10	n.d.

(1) A series of indirubin derivatives was tested at various concentrations in five kinase assays, described in the experimental section. EC_{50} values [μM] were calculated from dose-response curves. 2) PC = Positive control roscovitin.[37] (3) n.d.: not determined.

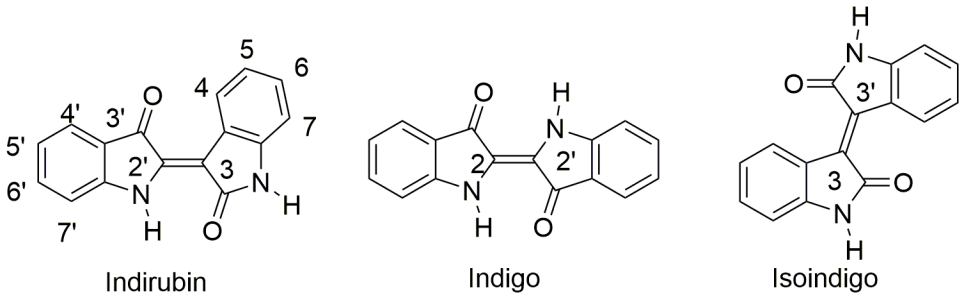


Figure 1: Structures of indirubin, indigo and isoindigo

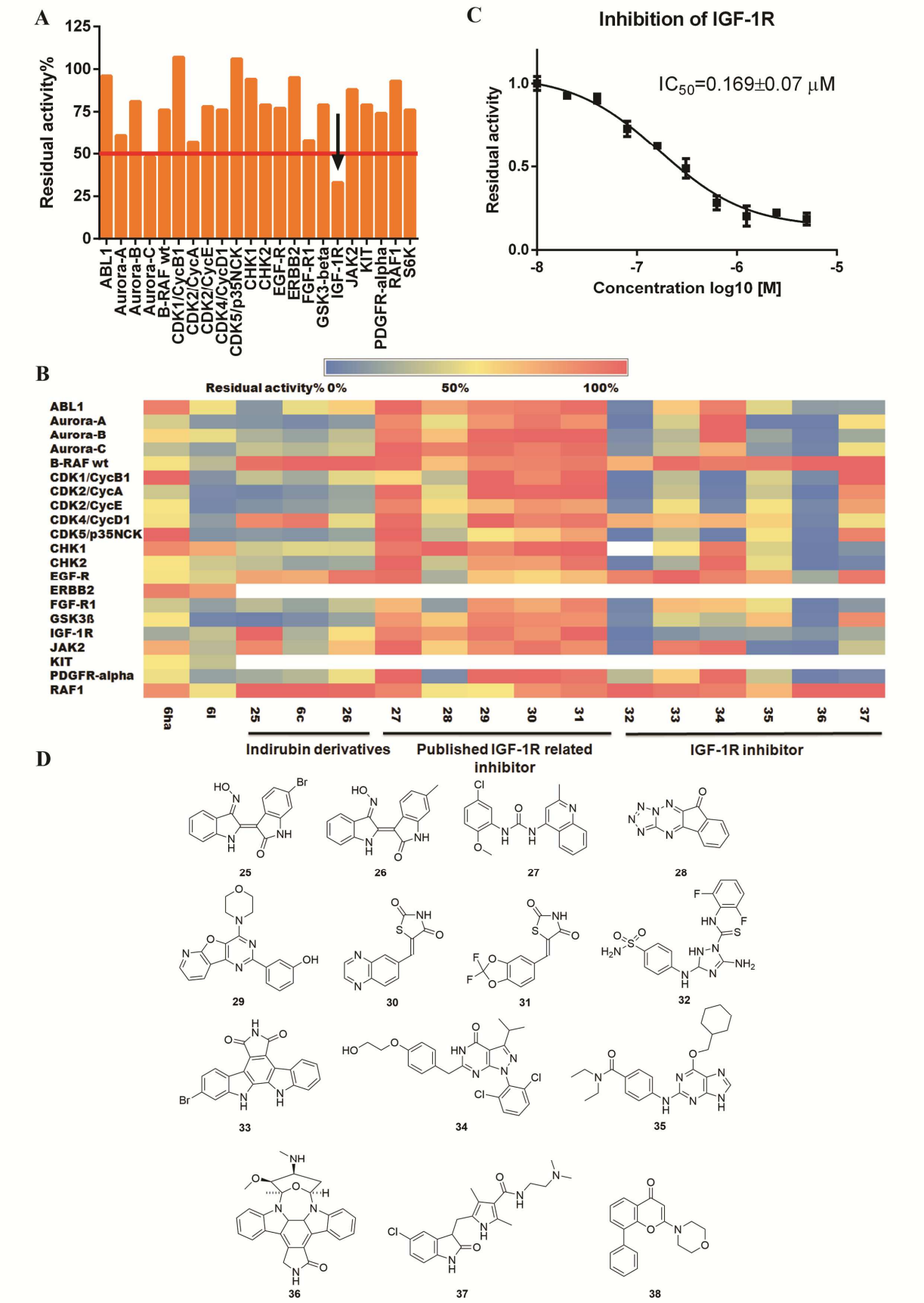

A
Residual activity%
125
100
75
50
25
0
ABL1
Aurora-A
Aurora-B
Aurora-C
B-RAF wt
CDK1/CycB1
CDK2/CycA
CDK2/CycE
CDK4/CycD1
CDK5/p35NCK
CHK1
CHK2
EGF-R
ERBB2
FGF-R1
GSK3-beta
IGF-1R
JAK2
KIT
PDGFR-alpha
RAF1
S6K
C
Inhibition of IGF-1R
IC50=0.169±0.07 μM
Residual activity
1.0
0.5
0.0
-8
-7
-6
-5
Concentration log10 [M]
B
Residual activity% 0%
50%
100%
ABL1
Aurora-A
Aurora-B
Aurora-C
B-RAF wt
CDK1/CycB1
CDK2/CycA
CDK2/CycE
CDK4/CycD1
CDK5/p35NCK
CHK1
CHK2
EGF-R
ERBB2
FGF-R1
GSK3ß
IGF-1R
JAK2
KIT
PDGFR-alpha
RAF1
6ha
6i
25
6c
26
27
28
29
30
31
32
33
34
35
36
37
Indirubin derivatives
Published IGF-1R related inhibitor
IGF-1R inhibitor
D
25
26
27
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Figure 2: **6ha** inhibited IGF-1R *in vitro*. A) Kinase profiling assay was performed by ProQinase. The residual activities of 22 kinases were measured. The residual activity of of IGF-1R was 33% in comparison to DMSO as control und was indicated with black arrowhead. B) Heat map analysis of protein kinase profiling of **6ha** and **6l** with compounds selected from published work by Anastassiadis et al,[37] including 3 indirubin derivatives (**25**, **6c** and **26**), previously reporter inhibitors targeting IGF-1R related protein kinases (**27-31**), and inhibitors showing inhibitory effect on IGF-1R. C) IC_{50} value of inhibiting IGF-1R *in vitro* was determined as 169±70 nM using ADP Glo assay. D) Structures of protein kinase inhibitors used as references.

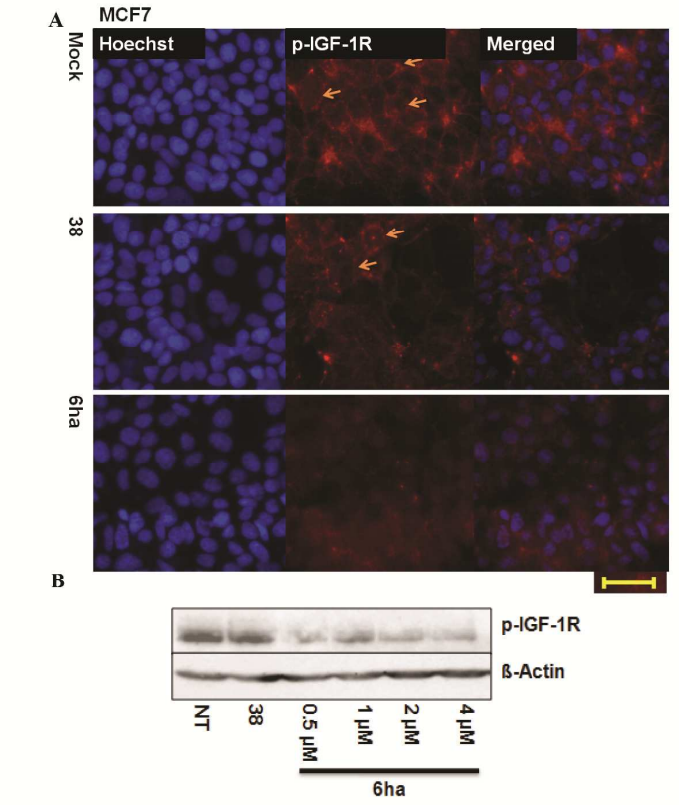


Figure 3: **6ha** inhibited activity of IGF-1R in cells. A) Inhibitory effect of **6ha** on phospho-IGF-1R in MCF7. Cells were incubated with **6ha** (1 µM) for 1 h. Immunocytochemistry was carried out to examine the activity of IGF-1R by using specific antibody against phosphorlyed IGF-1R

(Y1161). **38** was used as positive control. Nucleus was visualized by Hoechst dye. Scale bar. 40 µm. B) **6ha** inhibited the phosphorylation of IGF-1R in MCF7 cells. Cells were incubated with increasing concentration of **6ha** for 1 h. The phosphorylation of IGF-1R was examined by immunoblot. **38** was used as positive control and ß-Actin as loading control.

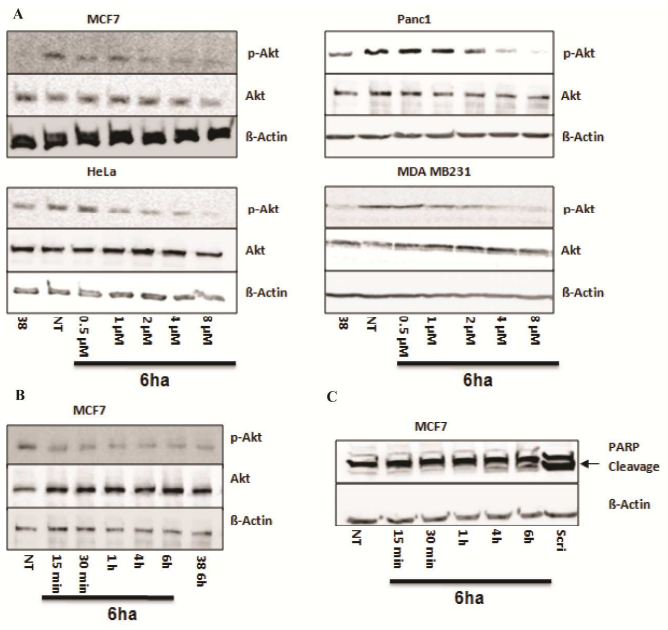


Figure 4: **6ha** inhibited IGF-1R associated signalling in various cancer cells. A) **6ha** inhibited activity of Akt in a concentration dependent fashion in MCF7, Panc1, HeLa and MDA MB231 cells. Cells were incubated with increasing concentrations of **6ha** in corresponding medium containing 10% FBS for 24 h. The whole cell lysates were subjected to immunoblot and detected with p-Akt (S 473) and total Akt antibodies. B) MCF cells were incubated with **6ha** (1 µM) and harvested at 15 min, 30 min, 1 h, 4 h, 6 h. The inhibitory effect was measured by immunoblot using p-Akt (S 473) antibody. C) PARP cleavage occurred in **6ha**-treated MCF7 cells. DMSO was used as non-treatment (NT). **38** or Srci was used as positive controls, as well as ß-Actin as loading control.

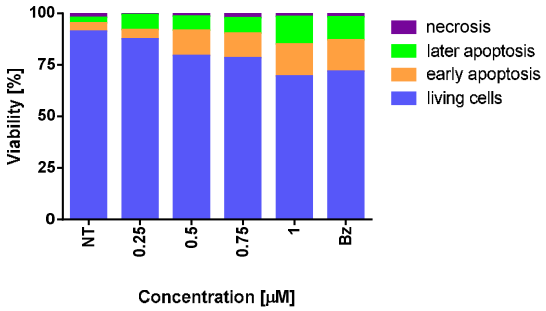


Figure 5: **6ha** induced cell apoptosis in annexin v/PI assay quantify by FACS. MCF7 cells were incubated with various concentrations of **6ha** for 24 h, labeled with FITC-conjugated annexin v and PI and analyzed by FACS. Bortezomib was used as positive control.

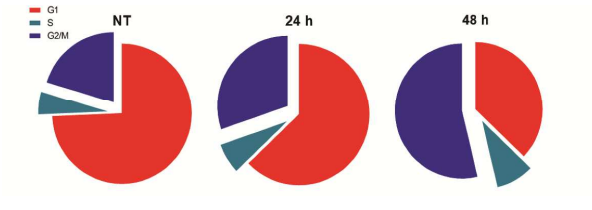


Figure 6: **6ha** induced cell arrest majorly at G2/M phase. MCF7 cells were incubated with 1 µM of **6ha** for 24h and 48 h, fixed with 70% Ethanol, stained with PI and analyzed by FACS.

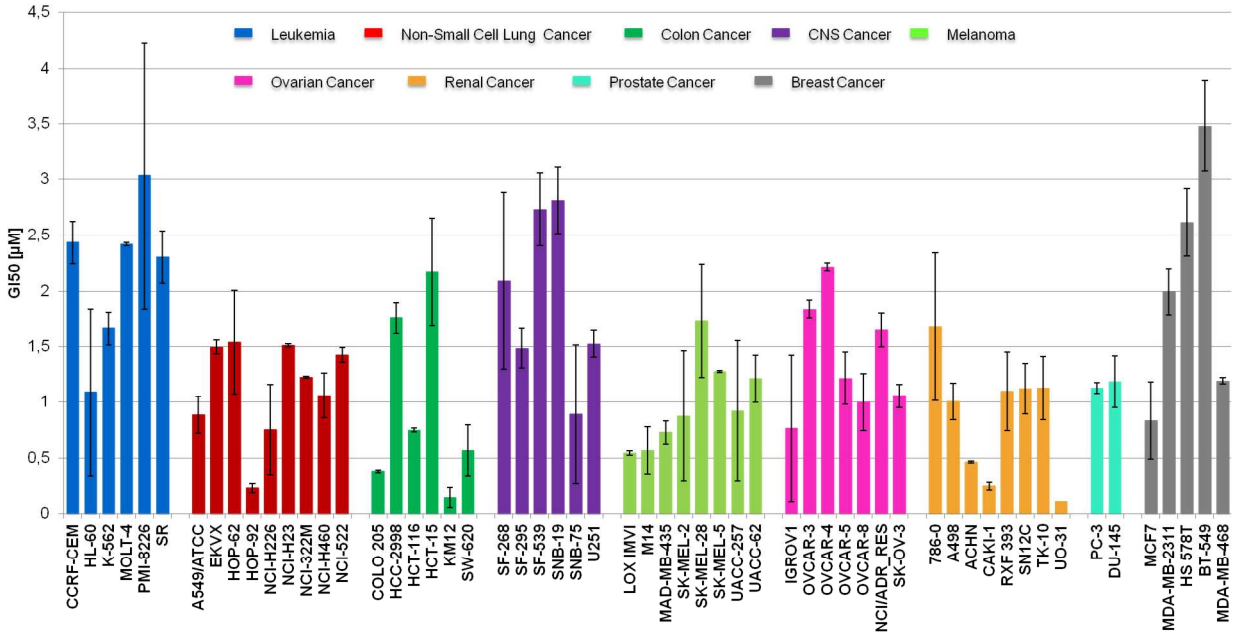
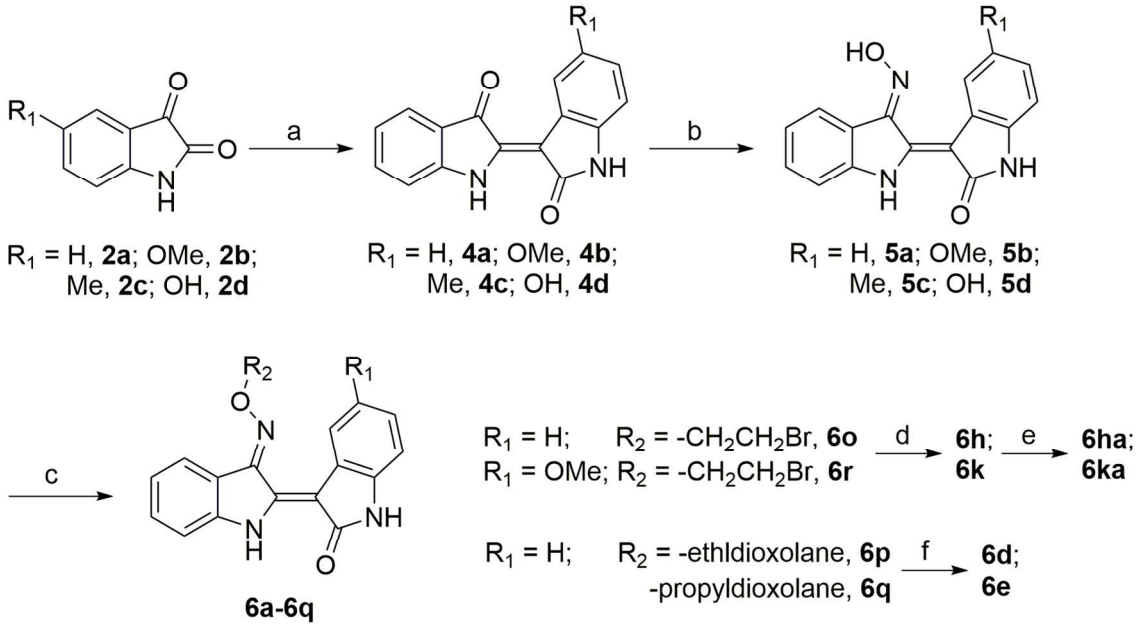
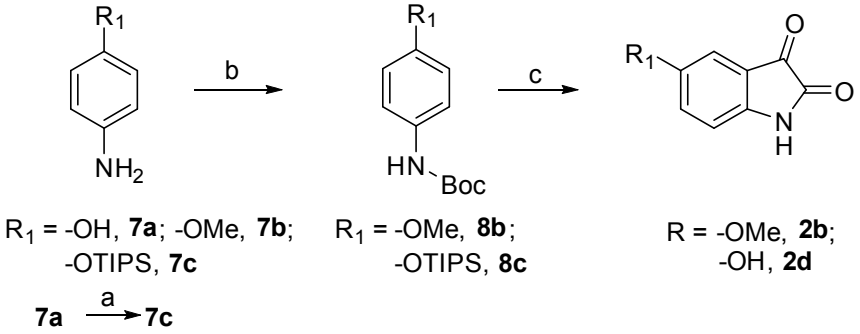


Figure 7: Tumor cell growth inhibiton of **6ha** tested in the NCI 60 cell line panel. GI_{50} values are mean of two series of tests.

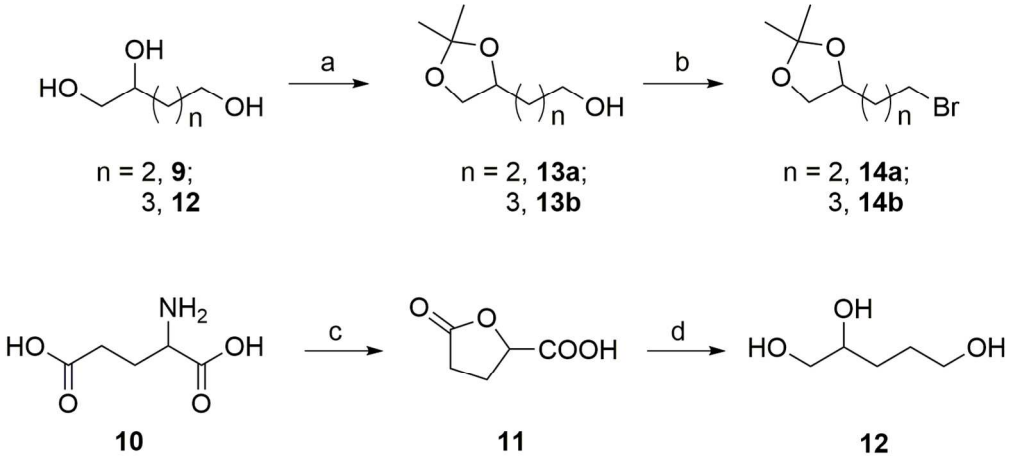


Scheme 1. Syntheses of indirubins (**4**), indirubin-3'-oximes (**5**) and indirubin-3'-oximethers (**6**). Reactants and conditions: a) indoxyl-3-acetate (**3**), MeOH, Na_2CO_3, RT; b) $NH_2OH{\cdot}HCl$,

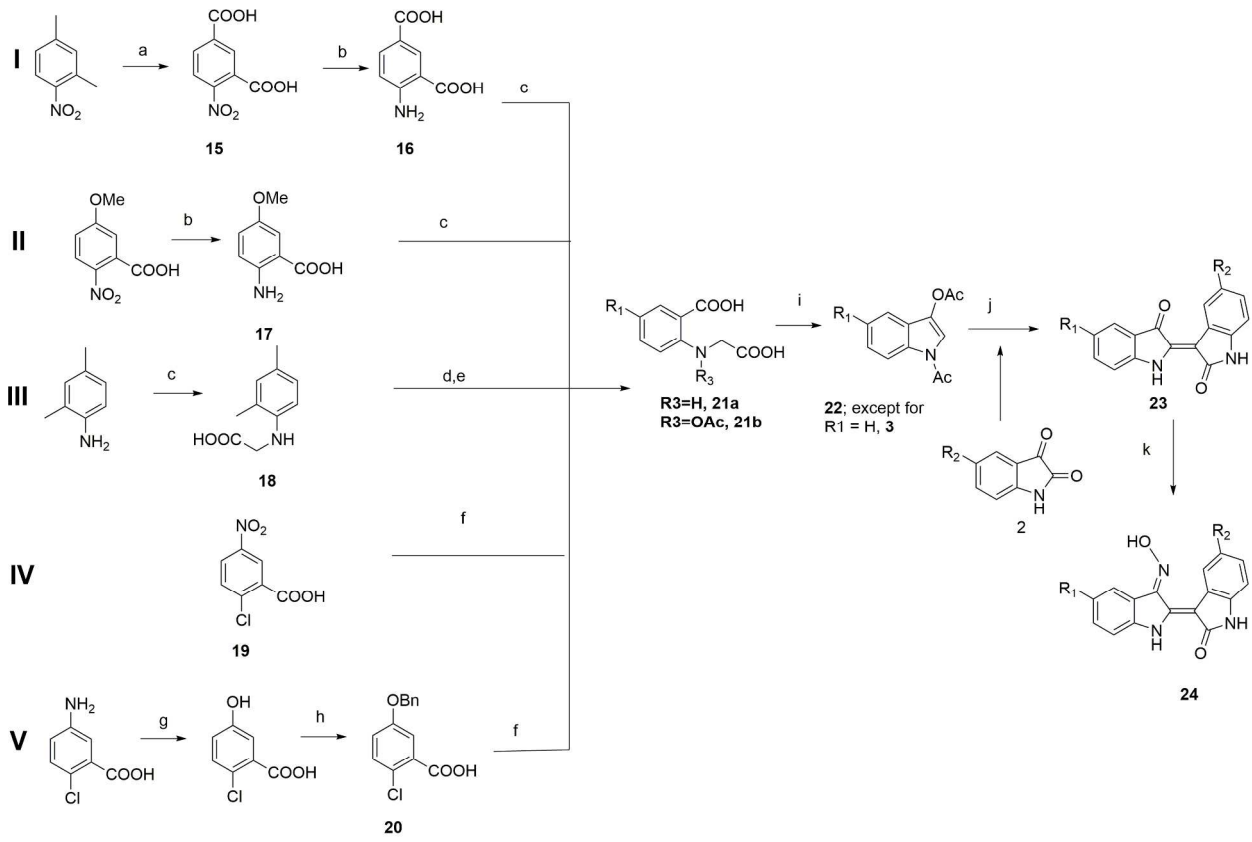
pyridine, ΔT; c) halogenide, 1,1,3,3-tetramethylguanidine (TMG), EtOH, ΔT; d) methylpiperazine, DMF; e) THF saturated HCl; f) 1N HCl.



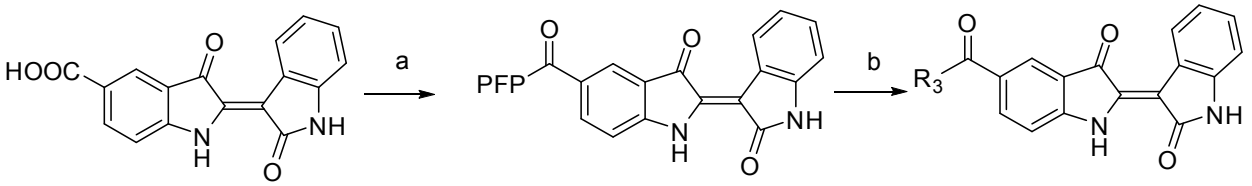
Scheme 2. Syntheses of isatins (**2b** and **2d**). Reactants and conditions: a) triisopropylsily chloride (TIPS-Cl), imidazole, CH_2Cl_2, RT; b) Boc_2O, THF, RT; c) THF, *t*-BuLi; $(COOEt)_2$, -78 °C – 0 °C; HCl.



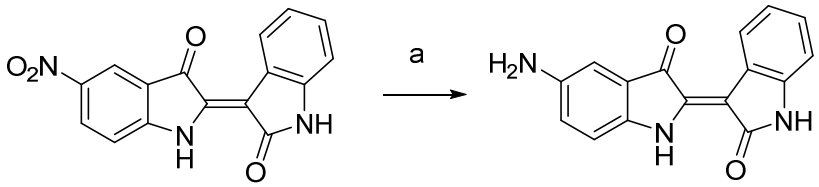
Scheme 3. Synthesis of bromo-alkyl-dioxolane **(14)**. Reactants and conditions: a) acetone, *p*-TsOH; b) $CHBr_3$, PPh_3, $CHCl_3$; c) $NaNO_2$, conc. HCl, H_2O, 0°C 4h, RT 24h; d) $LiAlH_4$; dried THF, RT 24h.



Scheme 4: Synthesis of phenylglycin-*o*-carbocxylic acid- (**21a; 21b**) (a: R_3=H; b: R_3=Ac), indoxyl- (**22**), isatin- (**2**) and indirubin-derivatives **(23)** and **(24)**. Reactants and conditions: a) $KMnO_4$, H_2O, 85°C; b) H_2, Pd/C, EtOH, conc. HCl; c) $ClCH_2COOH$, Na_2CO_3, H_2O, ΔT; d) $KMNO_4$, Na_2CO_3, H_2O, 45°C-55°C, 70°C-80°C; e) acetic anhydrided, RT, NaOH, conc. HCl; f) H_2NCH_2COOH, Na_2CO_3, Cu, ΔT, conc. HCl; g) H_2SO_4, $NaNO_2$, H_2O; h) NaOH, Bn-Cl, ΔT; i) NaOAc, acetic anhydride, ΔT; j) acetic acid, conc. HCl; k) $H_2NOH{\cdot}HCl$, pyridine.

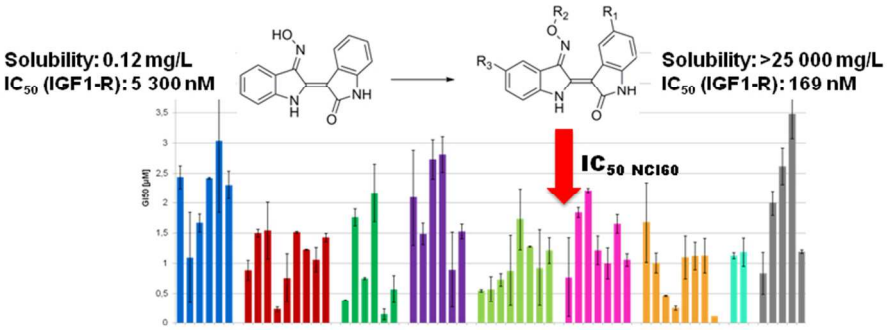


Scheme 5: Synthesis of indirubin-5'-carboxamide **(23e, 23f, 23g)**. Reactants and conditions: a) Pentafluorophenyl trifluoroacetate (PFP-trifluoroacetate), DMAP, pyridine, DMF; b) DMAP, DMF.



23a **23b**

Scheme 6: Synthesis of 5'-aminoindirubin (**23b**), Reactants and conditions: a) H_2, Pd/C, MeOH, H_2O, 1 N HCl.



Indirubins are potent ATP-competitive protein kinase inhibitors. In comparison to well-studied chemical modifications in the 5- and 3'-position, 5'-indirubin is rarely published. Here, we report the synthesis of new indirubin-3'- and 5'-derivatives. Structure-activity relationship demonstrated that bulky and rigid substituents in 5'-position appear unfavorable. Screening molecular targets of water soluble 3'-oxime ethers showed 6ha as preferential inhibitor of IGF-R1. Consistently, 6ha inhibited tumor cell growth in the NCI 60 cell line panel and induced apoptosis.

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