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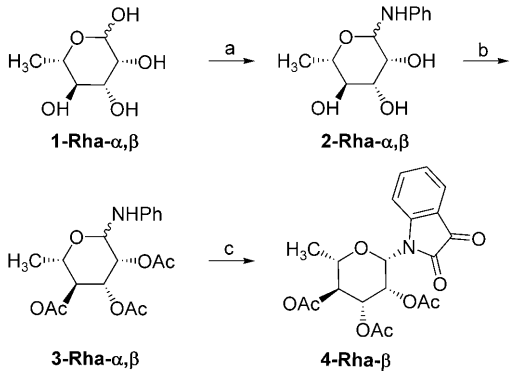
Synthesis of Thia-Analogous Indirubin *N*-Glycosides and their Influence on Melanoma Cell Growth and Apoptosis

Manfred Kunz,[a] Katrin M. Driller,[b] Martin Hein,[b] Stephanie Libnow,[b] Ina Hohensee,[a] Robert Ramer,[c] Burkhard Hinz,[c] Anja Berger,[d] Jürgen Eberle,[d] and Peter Langer*[b, e]

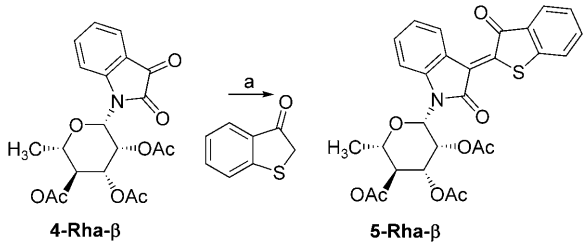
Glycosylated indoles possess remarkable pharmacological activity against different malignant tumor cells. Prominent derivatives with antitumor activity include the natural products staurosporine, K-252d, rebeccamycin and tjipanazoles.[1] Indigo, indirubin, and isoindigo contain a bis-indole framework and are found in a number of natural products. We previously reported the synthesis of indigo-*N*-glycosides (blue sugars).[2] This type of core structure is present in the akashines A–C, which were isolated by Laatsch et al. from *Streptomyces sp. GW48/1497*.[3] In contrast to the inactive parent indigo, the akashines show a remarkable antitumor activity against various human cancer cell lines. Indirubin, the red isomer of indigo, is the active ingredient in the traditional Chinese medicinal recipe Danggui Longhui Wan, which has been used for the treatment of myelocytic leukemia.[4] This substance and its substituted derivatives are potent inhibitors of several kinases involved in intracellular signaling pathways, such as GSK-3β and cyclin dependent kinases (CDKs).[5,6] Recently, we reported the synthesis of indirubin-*N*-glycosides (red sugars),[7] which showed considerable antiproliferative activity against various human cancer cell lines. Sassatelli et al. described the preparation of isoindigo-*N*-glycosides, which also possess considerable antiproliferative activity and kinase inhibitory potency.[8] Notably, both the deprotected and protected isoindigo-*N*-glycosides are of pharmacological relevance. For example, the biological activity of 'Natura', acetyl-protected β-D-xylopyranosyl-*N*-isoindigo, was reported to be higher than the activity of its deprotected analogue.[9] Herein, we report the first synthesis of thia-analogues of indirubin-*N*-glycosides and their influence on melanoma cell growth, apoptosis and intracellular signaling.

Compounds with a reactive methylene group like indoxyl, 3-coumaranone or thiaindane-3-one are known to give condensation products with isatin, where only the reactive β-carbonyl group of the isatin is involved in the reaction. *N*-Glycosylated isatins are also suitable for such condensation reactions.[7,8] As starting materials in the synthesis of thia-analogous indirubin-*N*-glycosides, we used isatin-*N*-glycosides with different carbohydrate moieties and thiaindane-3-one. In the case of the sugar L-rhamnose, acetyl-protected *N*-(β-L-rhamnopyranosyl)-isatin (**4-Rha-β**) was prepared from the acetyl-protected aniline-*N*-rhamnoside (**3-Rha-α,β**), which was obtained by reaction of L-rhamnose with aniline and subsequent acetylation (Scheme 1).[7,10] Furthermore, the corresponding isatin-β-*N*-glycosides of D-mannose, D-glucose and D-galactose were prepared under similar conditions.[7] Thiaindane-3-one was prepared from thiosalicylic acid and ethyl acetoacetate in the presence of sulfuric acid according to a procedure published by Friedländer.[11]

Reaction of the acetyl-protected isatin-*N*-glycosides (**4-β**) with thiaindane-3-one under mildly acidic conditions resulted in the formation of thia-analogous indirubin-*N*-glycosides in good to very good yields (see example given in Scheme 2).[12]



Scheme 1. Synthesis of the acetyl-protected *N*-β-L-rhamnopyranosylisatin (**4-Rha-β**). *Reagents and conditions*: a) $PhNH_2$, EtOH, 20 °C, 12 h, 96%; b) Ac_2O, pyridine, 0→4 °C, 8–12 h, 85%; c) oxalyl chloride, $AlCl_3$, 55 °C, 1.5 h, 63%.



Scheme 2. Synthesis of 3-[thiaindan-3′-on-2′-(*Z*)-ylidene]-1-(2″,3″,4″-tri-*O*-acetyl-β-L-rhamnopyranosyl)oxindol (**5-Rha-β**). *Reagents and conditions*: a) Ac_2O, AcOH, NaOAc, 80 °C, 1 h.

[a] *Prof. Dr. M. Kunz, I. Hohensee*
Comprehensive Center for Inflammation Medicine
University of Schleswig-Holstein, Campus Lübeck, 23562 Lübeck (Germany)

[b] *K. M. Driller, Dr. M. Hein, Dr. S. Libnow, Prof. Dr. P. Langer*
Institute of Organic Chemistry, University of Rostock
Albert-Einstein-Str. 3a, 18059 Rostock (Germany)
Fax: (+49) 381-4986-412
E-mail: peter.langer@uni-rostock.de

[c] *Dr. R. Ramer, Prof. Dr. B. Hinz*
Institute of Toxicology and Pharmacology, University of Rostock
18055 Rostock (Germany)

[d] *A. Berger, Dr. J. Eberle*
Department of Dermatology and Allergy, Skin Cancer Center Charité
Charité-Universitätsmedizin Berlin, 10117 Berlin (Germany)

[e] *Prof. Dr. P. Langer*
Leibniz Institute of Catalysis e.V., 18059 Rostock (Germany)

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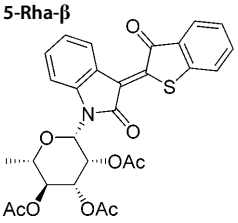
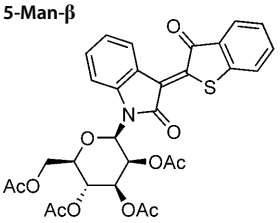
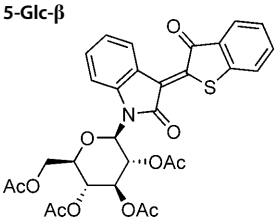
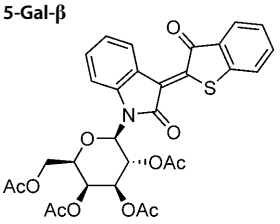
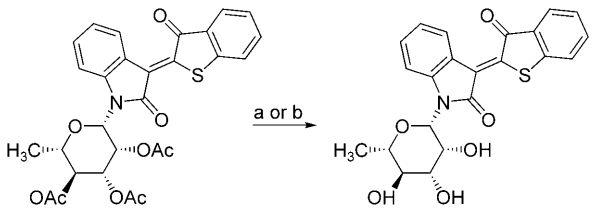
Interestingly, only one π-diastereomer could be isolated (*Z*-isomer). The condensation reaction was carried out with the isatin-*N*-glycosides of L-rhamnose (**4-Rha-β**), D-Mannose (**4-Man-β**), D-glucose (**4-Glc-β**) and D-galactose (**4-Gal-β**). The yields and reaction times are given in Table 1.

Table 1. Results of the synthesis of the thia-analogous indirubin-*N*-glycosides of L-rhamnose, D-mannose, D-glucose und D-galactose.

Glycoside	Product (*Z*-Isomer)	Yield [%]	T_{rxn} [h]
β-L-rhamnose	**5-Rha-β** 	90	1
β-D-mannose	**5-Man-β** 	95	1
β-D-glucose	**5-Glc-β** 	72	1
β-D-galactose	**5-Gal-β** 	93	1

In the case of the sulfur-analogous indirubin derivatives, deprotection of the acetyl groups could be carried out under base catalysis (Zemplén conditions),[13,8c] and resulted in the formation of the desired products **6-β** in 56–84% yield (see example given in Scheme 3).

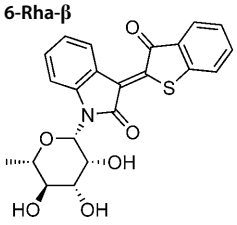
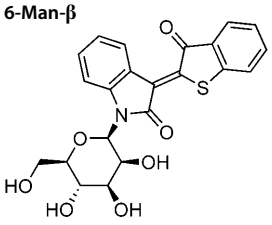
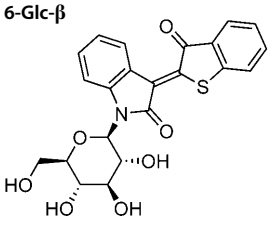
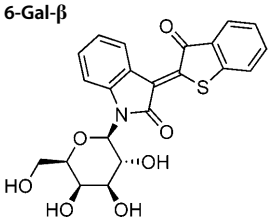
Deacetylation is quite fast under the chosen basic conditions and no side reactions (ring opening reactions) at the thiaindan-3-one carbonyl group (C-3) were observed. An alternative method for the deprotection using acidic conditions (1% methanolic HCl)[14] was also tested. Cleavage of the acetyl groups was possible but longer reaction times (3–5 days) were required and the deprotection product was isolated in lower yields (65% in the case of the deprotection of **5-Rha-β**). Table 2 shows the results of the deprotection of the acetylated compounds **5-β** under Zemplén conditions.



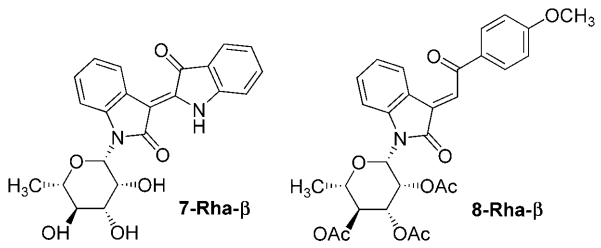
5-Rha-β **6-Rha-β**

Scheme 3. Deacetylation of thia-analogous indirubin-*N*-glycosides (synthesis of the deprotected 3-[thiaindan-3′-one-2′-(*Z*)-ylidene]-1-(β-L-rhamnopyranosyl)oxindol, **6-Rha-β**). *Reagents and conditions*: a) NaOMe, anhyd MeOH, RT; b) 1% HCl in anhyd MeOH, RT.

Table 2. Results of the deacetylation of the thia-analogous indirubin-*N*-glycosides.

Glycoside	Product (*Z*-Isomer)	Yield [%]	T_{rxn} [h]
β-L-rhamnose	**6-Rha-β** 	78	3
β-D-mannose	**6-Man-β** 	84	12
β-D-glucose	**6-Glc-β** 	75	12
β-D-galactose	**6-Gal-β** 	56	12

Two additional compounds were synthesized and used as reference compounds in the cell-based assays. Compound **7-Rha-β** is a glycosylated indirubin[7] and compound **8-Rha-β** is a related derivative, which could be synthesized by aldol addition of 4-methoxyacetophenone to **4-Rha-β** and subsequent dehydration.[15]



As mentioned above, indirubin glycosides are strong inhibitors of intracellular signaling kinases and may affect the cell proliferation of a variety of different tumor cells.[16,17] Here, the antiproliferative activities of thia-analogous indirubin-*N*-glycosides **6-β** were tested in four different metastatic melanoma lines (SK-Mel-19, SK-Mel-29, SK-Mel-103, SK-Mel-147). For this purpose, IC_{50} values of each of the tested thia-analogous indirubin-*N*-glycosides were determined using a colorimetric assay with WST-1 as a substrate (Table 3). The IC_{50} values showed slight variations between the different cell lines; **6-Gal-β** and **6-Glc-β** were less active in SK-Mel-19 cells compared with the other cell lines tested. **7-Rha-β** and **8-Rha-β** were used as control substances. The IC_{50} values of **7-Rha-β**, which had no effect on melanoma cell proliferation in preliminary experiments (data not shown), were far greater than those of other substances and can be considered as inactive. **8-Rha-β** was less active in the SK-Mel-103 and SK-Mel-147 cell lines, but had comparable activity to compounds **6-β** in SK-Mel-19 and SK-Mel29 cells. Altogether, compounds **6-β** appear to be active in melanoma cells.

Table 3. Inhibition of metastatic melanoma cell lines by different glycosylated indirubins and analogous compounds.

Compd	IC_{50} values [μM]			
	SK-Mel-19	SK-Mel-29	SK-Mel-103	SK-Mel-147
6-Rha-β	12.08 ± 1.21	6.29 ± 1.29	10.38 ± 1.16	7.52 ± 1.14
6-Man-β	5.81 ± 1.17	3.96 ± 1.25	6.69 ± 1.17	4.28 ± 1.13
6-Gal-β	24.36 ± 1.52	5.57 ± 1.33	6.94 ± 1.39	6.27 ± 1.24
6-Glc-β	17.10 ± 1.35	8.39 ± 1.26	6.06 ± 1.19	4.98 ± 1.17
7-Rha-β	65.39 ± 1.17	86.60 ± 1.53	383.30 ± 2.29	n.d.
8-Rha-β	13.93 ± 1.10	11.92 ± 1.14	46.41 ± 1.20	27.17 ± 1.23

Similar results were recently obtained for related glycosylated indirubin derivatives tested in other human cancer cell lines, such as 5637 (bladder), A-427 (small-cell lung), Kyse-70 (esophageal) and MCF-7 (breast), with IC_{50} values ranging from 0.67 μM to 15.28 μM.[16] Interestingly, the IC_{50} values of these compounds were in the range of well-known cytostatic agents, such cisplatin and chlorambucil.[16] These findings suggest indirubin-*N*-glycosides as active agents against malignant melanoma and possibly also other malignant tumor types.

Impaired apoptosis regulation is a critical feature of metastasizing tumors such as malignant melanoma. In order to test the hypothesis that thia-analogous indirubin-*N*-glycosides induce apoptosis, the four melanoma cell lines were treated with **6-Rha-β** and **8-Rha-β** (10 μM) and assayed for sub-G1 cell populations after PI staining, indicating DNA fragmentation due to apoptosis. Both glycosylated indirubin analogues significantly increased the numbers of sub-G1 cells in the four melanoma cell lines after 48 h and 72 h. The percentage of apoptotic cells after 72 h reached more than 20% and was five- to tenfold that of the untreated control (Figure 1 a). At the same time, the number of cells in G1 was significantly reduced (Figure 1 b). Apoptosis was also investigated by a second assay monitoring DNA fragmentation via an ELISA. In line with the subG1 populations, DNA fragmentation revealed largely parallel results, namely significantly increased apoptosis after 48 h and 72 h of treatment with the glycosylated indirubin analogues (Figure 1 c).

The effects of glycosylated indirubin analogues on melanoma cell proliferation were monitored by real-time cell analysis (RTCA). Whereas the cell density of untreated SK-Mel-103 and SK-Mel-147 in culture continuously increased during the observation period of 70 h after seeding, **6-Rha-β** and **8-Rha-β**-treated cells showed dramatically decreased attached cell numbers and cell density after only 10 h (Figure 2).

The cell lines SK-Mel-19 and SK-Mel-29 responded similarly, however, the effect of **8-Rha-β** was somewhat less pronounced (data not shown). Thus, the data show that thia-analogous indirubin-*N*-glycosides not only inhibit cell growth but also induce early apoptotic events in melanoma cells. In support of these findings, induction of apoptosis in tumor cells by indirubin derivatives was shown in a series of earlier reports. Indirubin-3-monooxime induced apoptosis in Hep-2 laryngeal carcinoma cells.[18] Moreover, indirubin-3′-oximes potently blocked constitutive Stat3 signaling in human breast and prostate cancer cells. Consecutive down-regulation of the Stat3 target genes encoding antiapoptotic proteins Mcl-1 and survivin was accompanied by induction of apoptosis in these cells.[19]

Melanoma cell growth and survival involves different intracellular signaling pathways, such as Ras/Raf/MEK/ERK and PI(3)K pathways.[20] It is well accepted that the transcription factor cJun is a downstream target of Ras/Raf/MEK/ERK and JNK/SAPK pathways.[21] Moreover, cJun plays an important role in melanoma biology.[22,23] To address whether thia-analogous indirubin-*N*-glycosides may influence cJun and JNK phosphorylation/activation as a possible explanation for their growth inhibitory effects, melanoma cell lines were treated with 10 μM **6-Rha-β**, and phosphorylation of dually phosphorylated cJun on Ser 63 and Ser 73, and dually phosphorylated JNK1/2/3 on Thr 183 and Tyr 185, was analyzed by immunoblotting (Figure 3).

Indeed, melanoma cell lines SK-Mel-19 and SK-Mel-29 revealed dramatic reductions in JNK and cJun phosphorylation after 48 h and 72 h, respectively. In contrast, the negative control indirubin **7-Rha-β** only effected JNK phosphorylation, and had no effect on cJun phosphorylation. **8-Rha-β** had a less pronounced effect on JNK phosphorylation compared with **6-Rha-β**, but again significantly reduced cJun phosphorylation. These findings support the role of cJun, but not of JNK, in melanoma cell biology. Obviously, cJun and JNK phosphorylation/activation at the tested amino acid sites occur independently. The

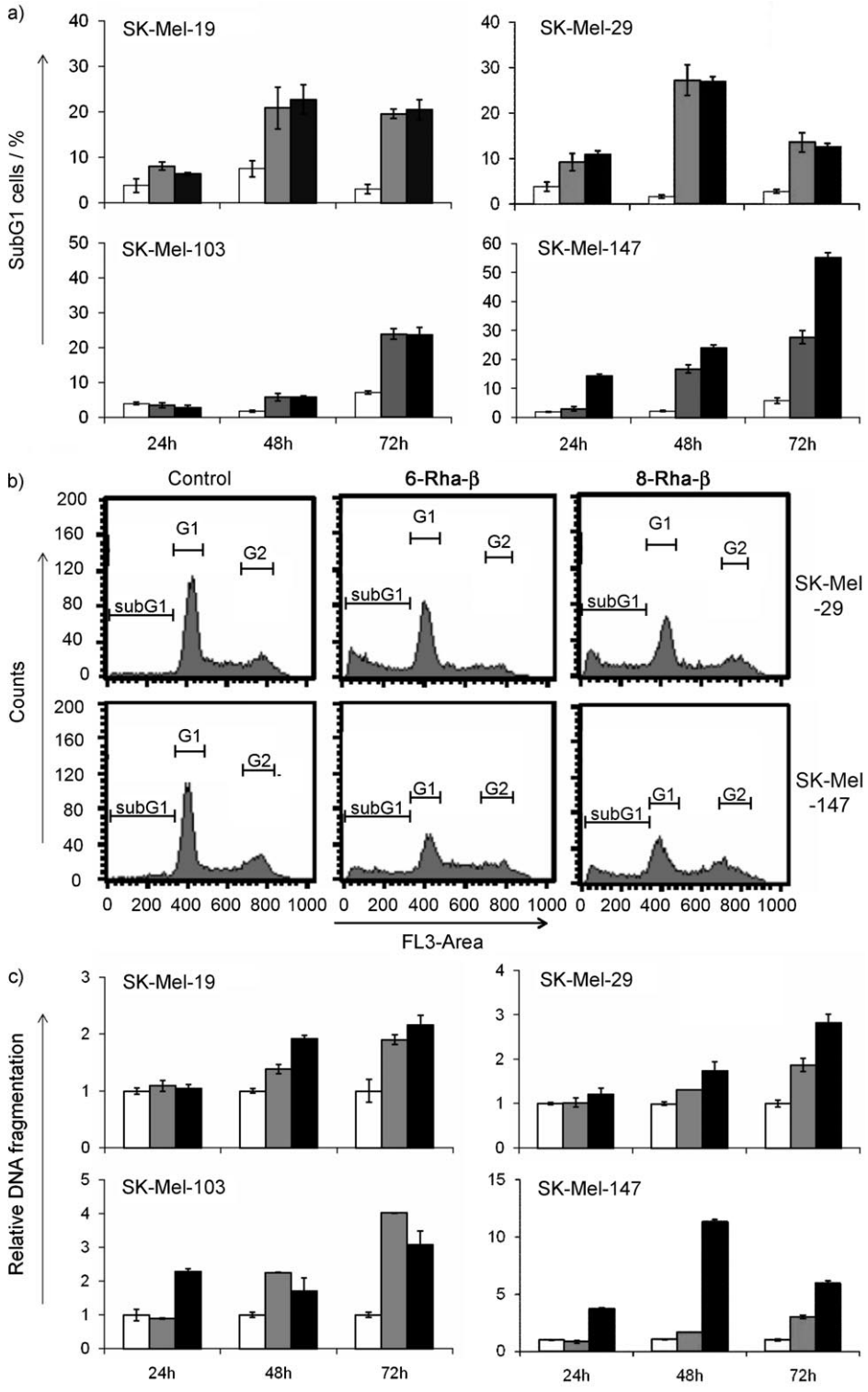


Figure 1. Induction of apoptosis in melanoma cells by glycosylated indirubin analogues. a) Four melanoma cell lines (SK-Mel-19, SK-Mel-29, SK-Mel-103 and SK-Mel-147) were treated with 10 μM of **6-Rha-β** (■) or **8-Rha-β** (■), or vehicle control (□). After incubation for 24, 48 and 72 h, respectively, cell cycle analyses were performed using PI staining and flow cytometry. The sub-G1 cell populations indicating apoptotic cells (in percent) are shown. Data shown reflect the means ±SD of one representative experiment with triple values. The whole experiment has been performed three times showing highly comparable results. b) Representative cell cycle analyses are shown for the cell lines SK-Mel-29 and SK-Mel-147 after 48 h (treated versus untreated). c) The amount of DNA fragmentation reflecting apoptosis was also determined by an alternative assay (cell death detection ELISA, as described in the Supporting Information). As before, cells were treated with 10 μM of **6-Rha-β** (■) or **8-Rha-β** (■), or vehicle control (□). Relative values are given as compared with untreated controls. Data shown reflect the means ±SD of one representative experiment with triple values. The whole experiment has been repeated once showing highly comparable results.

particular role of the cJun phosphorylation at Ser63 and Ser73 for cJun activity, has been described.[24]

Taken together, the presented data support the important role for cJun phosphorylation in cell proliferation. However, additional mechanisms involved in the antiproliferative activity of indirubin-*N*-glycosides and thia-analogous compounds cannot be ruled out.

Based on these findings, it might be reasonable to combine indirubin derivatives with other chemical agents, for example, those targeting the mammalian target of rapamycin (mTOR), a downstream component of PI(3)K signaling. When used alone, rapamycin had little effect on malignant melanoma.[25] More recent developments, such as the small-molecule MEK inhibitor AZD6244, are currently being evaluated in clinical trials in patients with advanced melanoma.[26] However, data from these trials have not been published so far. These compounds might also be good candidates for combination therapy with indirubins since they may act synergistically by addressing different signaling pathways.

In conclusion, the first synthesis of thia-analogues of indirubin-*N*-glycosides has been reported. Our current studies suggest that the strategy outlined herein is quite general and can be successfully applied to the synthesis of a variety of derivatives containing different carbohydrate moieties. Notably, it is also possible to prepare oxa-analogous indirubin-*N*-glycosides by condensation of *N*-glycosylated isatines with 3-coumaranone, but the deprotection of the acetyl groups seems to be more difficult due to side reactions.

These derivatives are active against malignant melanoma cells in vitro and are consequently good candidates for preclinical

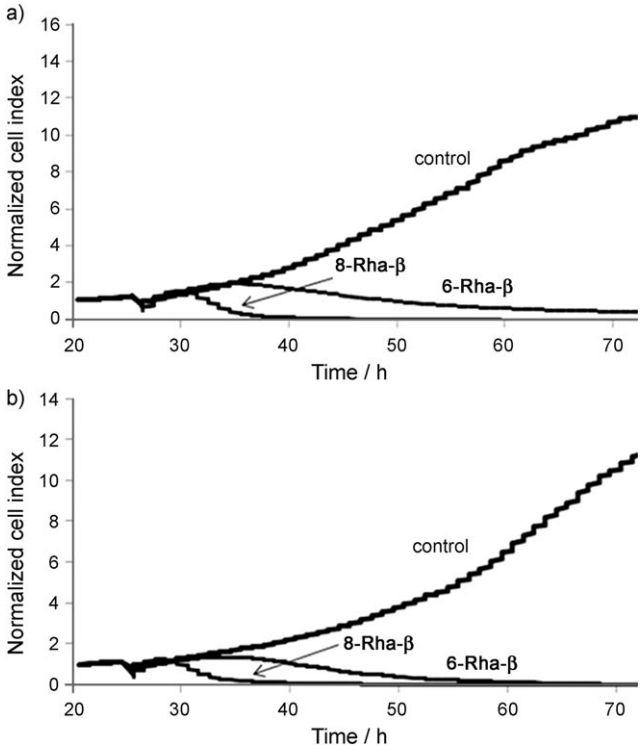


Figure 2. Glycosylated indirubin analogues interfere with cell confluence of melanoma cells. Melanoma cell lines a) SK-Mel-103 and b) SK-Mel-147 were seeded at a density of 5000 cells per microtiter well. Cells were treated with indirubin analogues **6-Rha-β** and **8-Rha-β** (μM) 26 h after seeding and monitored for 70 h. The normalized cell index gives a relative measurement of cell numbers (set to 1 at 26 h). The experiment was performed twice, each in triplicate. Data correspond to the means of one representative experiment.

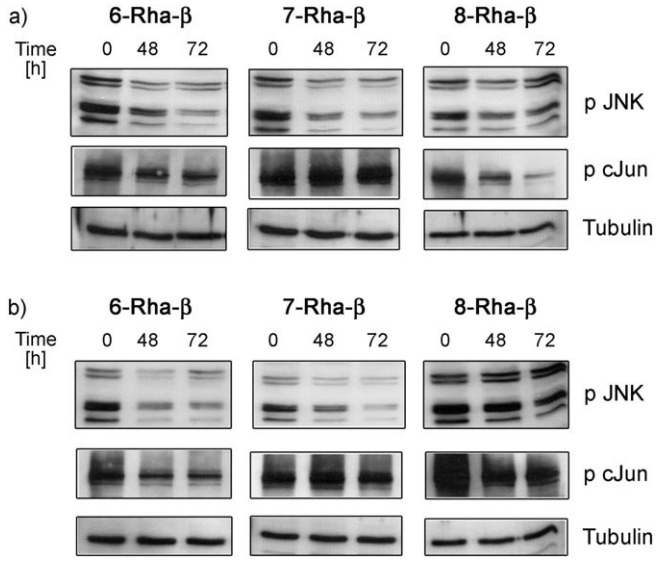


Figure 3. Glycosylated indirubin analogues interfere with phosphorylation of intracellular signaling kinase JNK and transcription factor cJun. a) SK-Mel-19 and b) SK-Mel-29 cells were treated with 10 μM **6-Rha-β**, **7-Rha-β** and **8-Rha-β**. Expression of dually phosphorylated JNK1/2/3 and cJun was analyzed by immunoblotting using appropriate antibodies. Staining of β-tubulin was used as loading control.

and perhaps subsequent clinical studies. They not only stop melanoma cell proliferation at concentrations similar to currently used chemotherapeutic agents, but also induce a significant rate of apoptosis. Finally, the thia-analogues of indirubin-*N*-glycosides interfere with a well-defined intracellular signaling pathway active in melanoma cells.

Experimental Section

The Supporting Information contains:

1) Detailed experimental procedures for the synthesis of compounds **5-β** and **6-β**.
2) Analytical data (^{1}H NMR, ^{13}C NMR, MS, HRMS and melting points, where possible) of compounds **5-β** and **6-β**.
3) Growth conditions of melanoma cell lines.
4) Determination of IC_{50} values of indirubins for different melanoma cell lines, immunoblotting procedures and apoptosis assays.

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Keywords: antitumor agents · apoptosis · carbohydrates · indirubin analogues · kinases

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