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Synthesis and biological evaluation of new derivatives of emodin

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Dedicated to Professor Reinhard Troschütz, Universität Erlangen, on the occasion of his 60th birthday

Abstract—Drugs containing an anthraquinone moiety such as daunorubicin (Daunoblastin®) and mitoxantrone (Onkotrone®) constitute some of the most powerful cytostatics. They suppress tumor growth mainly by intercalation into DNA and inhibition of topoisomerase II, and are suspected to generate free radicals leading to DNA strand scission. We established a novel strategy for obtaining new highly functionalized derivatives of emodin (1,3,8-trihydroxy-6-methyl-anthraquinone). Using emodin, DIB, and an appropriate amine as starting materials, we obtained a wide range of emodin-related structures by one-pot synthesis. Several of these derivatives showed stronger cytotoxic and cytostatic activity than emodin. In particular, compound **6** was highly effective on the HepG2 tumor cell line, but did not show any cytotoxicity on normal hepatocytes. In addition to this favorable feature, compound **6** revealed interesting binding properties to a recombinant fragment of the multi-drug-resistance transporter, pgp, and reversed the multi-drug-resistance phenotype of H4-II-E cells, thus making this compound a promising potential anti-tumor drug.

1. Introduction

Emodin (**I**) occurs in different plants (rhubarb, aloe) and is a widespread dye in fungi and lichens. It belongs to the anthraquinone group along with daunorubicin (**II**) and mitoxantrone (**III**), which constitute some of the most powerful cytostatics (Fig. 1).

Emodin itself is a tumor cell-growth inhibitor.[1] Emodin possesses diuretic[2] and vasodilatatoric[3] effects as well as antibiotic,[4,5] anti-viral,[6,7] and anti-neoplastic activity.[8,9] It is also supposed to sensitize cancer cells.[10] Thus, emodin sensitizes HER/neu-overexpressing cancer cells to chemotherapeutic agents such as cisplatin, doxorubicin, etoposide, and paclitaxel.[11] Furthermore, emodin induces apoptosis in CH27 and H460 lung carcinoma cell lines due to an increase in cytochrome *c* of the cytosolic fraction and activation of caspase-3, caspase-9, and caspase-8. PKC isoenzyme expression is involved in emodin-induced apoptosis of CH27 and H460 lung carcinoma cells, and appears to be associated with the increased expression of cellular Bak and Bax proteins, whereas no modulation of endogenous Bcl-XL protein expression has been observed.[12,13] We established a novel strategy to obtain new derivatives of emodin (1,3,8-trihydroxy-6-methyl-anthraquinone). These derivatives

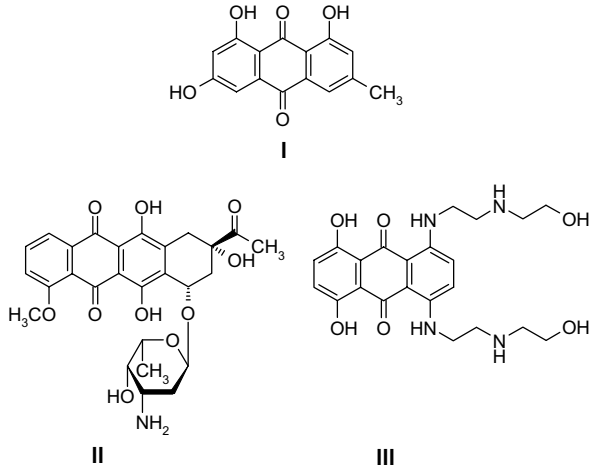

Figure 1. Emodin (**I**), daunorubicin (**II**), and mitoxantrone (**III**).

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were screened against the HepG2 hepatoblastoma cell line and normal hepatocytes for their cytotoxic and cytostatic properties and for their binding capacity to a recombinant fragment of the multi-drug-resistance transporter, pgp.

2. Chemistry

Compounds **1**–**13** were synthesized using a novel synthetic strategy (Figs. 2–4). Emodin (**I**) was reacted with DIB in the presence of an appropriate amine. Under this conditions emodin underwent an oxidizing amination by substitution of the hydrogen in position 4 by an amine-N, yielding 4-amino-emodin derivatives in one step. Depending on the structure of the used amine further reactions occurred, broadening the range of structures.

The poor solubility of emodin and DIB in common solvents was circumvented by using the amine itself as the solvent. Depending on the amine, the reaction times ranged from a matter of hours to days at room temperature. Reactions could be easily followed by TLC, because of the big differences of color and luminescence properties of the reaction products (Table 1). Temperature increase did not shorten reaction times, but did lead to lower yields in most cases. The structures thus obtained can be divided in three classes: amines (Fig. 2), annelated dihydrooxazole derivatives (Fig. 3) and annelated tetrahydropyrazine derivatives (Fig. 4). 4-Aminoemodines **3**–**8** were formed by converting emodin (**I**) with DIB in the presence of the appropriate amine, similar to reactions with acetophenone derivatives described by Wells et al.[14] We assume that in our case the primary step is the addition of DIB at OH-3 followed by amine attachment with dearomatization. After splitting of iodobenzene and two molecules of acetic acid rearomatization occurs (Scheme 1).

This is strengthened by the observation that similar compounds lacking the OH-3 group (chrysophanol, chrysazine) or displaying a methoxy group instead of the OH-3 group (physcione) do not react.[15] Some amines underwent further reactions: Ring-like amines as piperidine not only gave rise to the primary formed 4-(*N*-piperidinyl) derivative **7**, but also to the dihydrooxazole derivative **10** at prolonged reaction times. The smaller analogue pyrrolidine only resulted in the dihydrooxazole derivative **9**. We assume that in this cases a second DIB molecule is used for the oxidation of the amine moiety. When 1,2-diamines were used, annelated tetrahydropyrazine derivatives were formed. So ethylenediamine gave 4-[*N*-(2-aminoethyl)-amino] derivative **3**, which was converted to tetrahydropyrazine **11** at

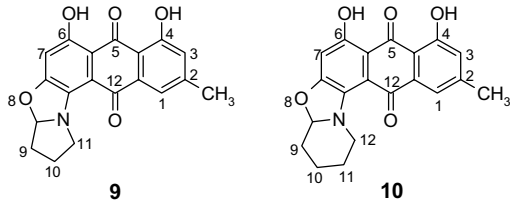


Figure 3. Synthesized 4-amino-emodin derivatives (annelated dihydrooxazole derivatives).

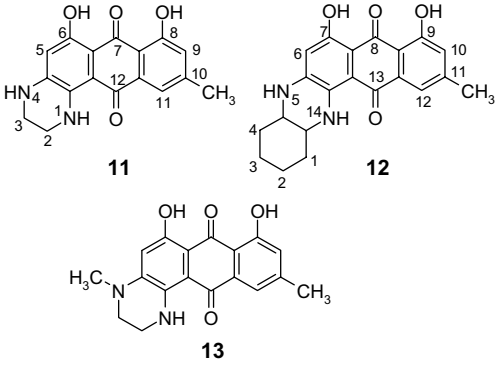


Figure 4. Synthesized 4-amino-emodin derivatives (annelated tetrahydropyrazine derivatives).

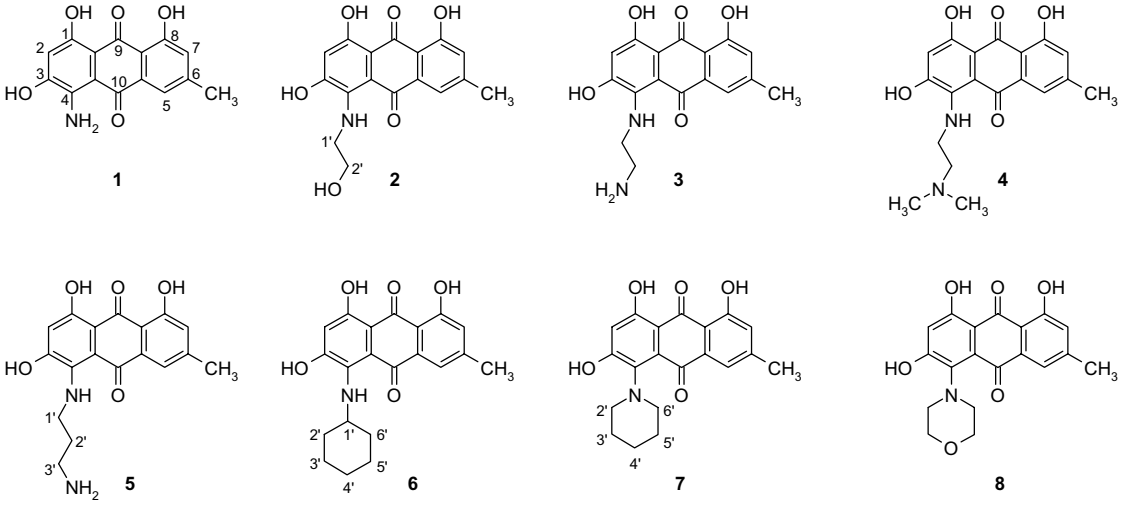
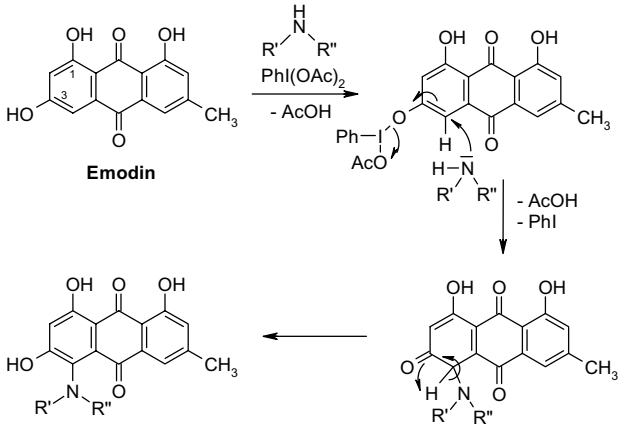


Figure 2. Synthesized 4-amino-emodin derivatives (amines).

Table 1. UV–vis data of synthesized emodin derivatives (in methanol)

Compound	λ_{max} in nm (ε)
4	220 ($20 * 10^3$), 274 ($17 * 10^3$), 546 ($12 * 10^3$), 587 ($14 * 10^3$)
6	225 ($16 * 10^3$), 261 ($18 * 10^3$), 554 ($14 * 10^3$), 595 ($16 * 10^3$)
9	223 ($20 * 10^3$), 258 ($23 * 10^3$), 520 ($9 * 10^3$)
11	226 ($18 * 10^3$), 277 ($17 * 10^3$), 514 ($7 * 10^3$), 550 ($16 * 10^3$), 592 ($18 * 10^3$)



Scheme 1. Possible mechanistic formation pathway of 4-amino-emodin derivatives.

prolonged reaction times. The space-demanding *trans*-1,2-diaminocyclohexane gave rise to **12**. While due to sterical reasons 1,3-diamines as **5** did not cyclize. An unexpected reaction was the cleavage of C–O– and C–NH– bonds by DIB: Similar to piperidine morpholine yield the 4-(*N*-morpholinyl) derivative **8**, but prolonged reaction times led to 4-[*N*-(2-hydroxyethyl)-amino]-emodin **2** by C–O– bond cleavage. When piperazine was used, the C–NH– bond was cleaved leading to the primary amine **3**, which was converted to tetrahydropyrazine derivative **11** as described above for the reaction of ethylenediamine. The same reaction occurred when *N*-methyl-piperazine was used and the *N*-methyl-tetrahydropyrazine derivative **13** was obtained, no primary product could be isolated in considerable amounts. Extensive structure investigations using NMR-coupling techniques were performed to confirm this product. Due to the lack of hydrogens and the many quaternary carbon atoms we still had doubts concerning the substitution pattern. Finally, X-ray diffraction analysis gave definitive confirmation of the substructure (Fig. 5).[16]

In all cases long reaction times or elevated temperatures led to degradation and formation of 4-amino-emodine **1** in traces. Emodin and an excess of DIB in boiling piperidine for 10 h is a possible preparation of **1**. Due to the incomplete consumption of emodin and the formation of highly oxidized degradation products middle and low yields[17] were observed (13–76%). Therefore, remaining emodin was recovered by chromatographic workup. The degradation products were removed by extractive workup. Analysis of the washing waters showed com-

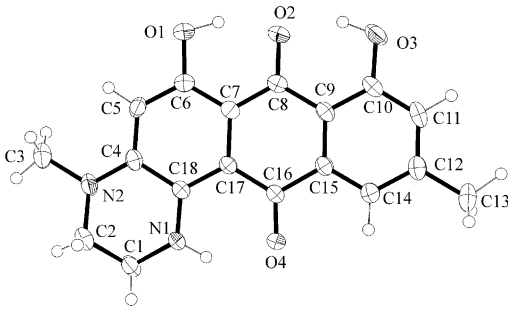


Figure 5. X-ray crystallographic structure of **13**.

plex mixtures of very hydrophilic compounds, no main components could be observed. Due to the 1,4- and 1,2-hydro-quinone substructure all compounds are quite susceptible to degradation and should be handled under mild conditions such as low temperatures, fast isolating and cleaning procedures, and short storage times for solutions prepared. However, using this synthetic strategy, it is possible now to obtain a wide range of highly substituted emodin derivatives in only one step!

To alter the solubility and the pharmacokinetic properties amides **14**–**16** were synthesized by reaction of compound **5** with the appropriate carbonic acid anhydride in dry methanol, from which the crude product can be isolated by filtration (Fig. 6).

All 4-amino-emodin derivatives **1**–**16** show strong color intensity (see Table 1 for example). What has to be considered using fluorescence-based biological assays.

Synthesis of tetranitro-emodin derivatives **17** and **18** were carried out with good yields using standard procedures (H_2SO_4/HNO_3). Acetylation of the steric hindered

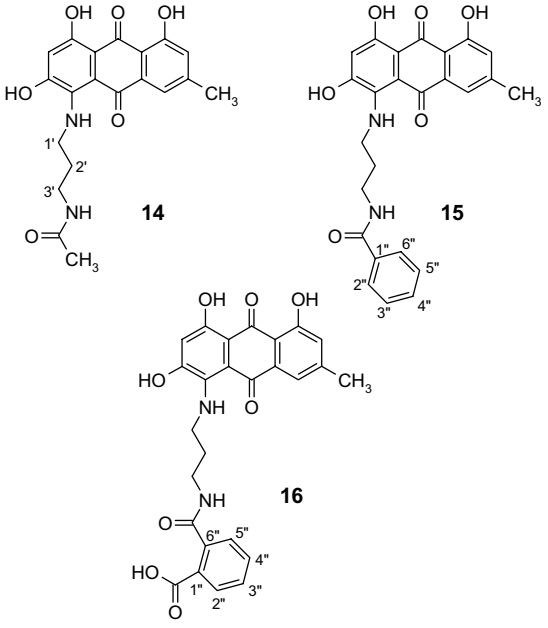


Figure 6. Synthesized amides of compound **5**.

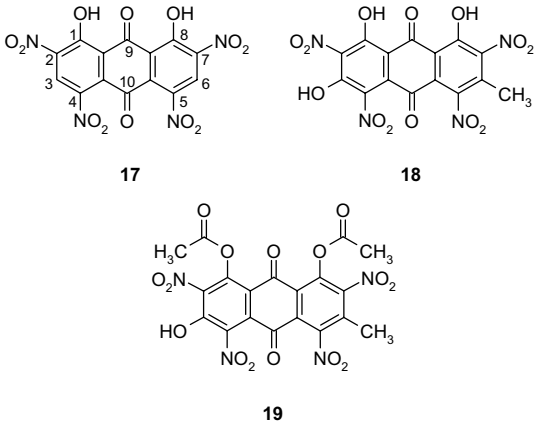


Figure 7. Synthesized tetranitro-emodin derivatives.

18 in acetic anhydride produced diacetylated compound **19** (Fig. 7).

3. Biological results

Cytostatic potential of the 4-amino-emodin and tetranitro-emodin derivatives was determined by measuring their cytotoxicity against normal and transformed hepatocytes and their inhibition of thymidine incorporation in growing cells. Cytotoxicity was determined using the MTT assay. Since some of the compounds showed considerable color and/or fluorescence, interference with this assay was unavoidable; however, appropriate measures were taken to obtain correct results. Proper determination of EC_{50} values was only impossible under conditions of limited solubility in culture medium (Table 2).

Table 2. Cytotoxicity of synthesized emodin derivatives on primary rat hepatocytes and HepG2 cells

Compound	Limit of solubility (μM)	Cytotoxicity EC_{50} values (μM)	
		Hepatocytes	HepG2 cells
Emodin	>400	38.2 ± 3.7	42.9 ± 1.1
1	>400	11.7 ± 0.4	17.3 ± 0.9
2	~400	32.4 ± 1.1	~70
3	200–400	>400	>400
4	~500	140 ± 1.5	>200
5	200–400	>400	~300
6	~200	>200	8.4 ± 0.5
7	>800	74.7 ± 1.6	88.6 ± 1.7
8	>400	13.8 ± 1.3	34.1 2.0
9	>400	360 ± 20	>200
10	>800	35–40	17.9 ± 0.7
11	>400	>400	>400
12	~400	>400	>400
13	~400	>400	>400
14	>400	>400	>400
15	>400	>400	>400
16	~300	12.7 ± 0.3	~200
17	200–400	>200	>200
18	~200	75 ± 8	~150
19	>200	53 ± 8	~150

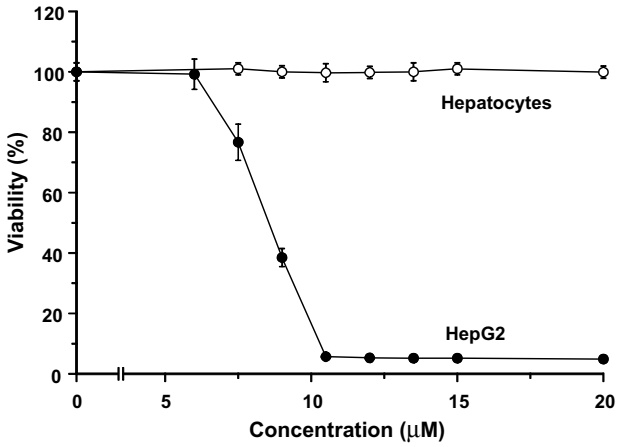


Figure 8. Cytotoxicity of **6** compared for HepG2 cells (closed circles) and rat hepatocytes (open circles) in the relevant concentration range. Cytotoxicity was determined by MTT assay. Values are presented as the mean ± SD of three independent determinations.

Cytotoxic influence of these compounds on primary rat hepatocytes and HepG2 cells was considerably diverse (Table 2). Three compounds, **1**, **6**, and **10**, were respectively 2.5, 5.1, and 2.4 times as cytotoxic to HepG2 hepatoblastoma cells as emodin based on EC_{50} values. Most remarkably, normal primary hepatocytes were almost unaffected (by a factor of at least 0.04-fold) by compound **6** (Fig. 8), whereas the other two also acted somewhat more vigorously on these cells than did emodin.

Several compounds, **2**, **7**, **8**, **16**, **18**, and **19**, showed the reverse characteristic and were slightly more cytotoxic on the normal than on the transformed hepatocellular phenotype (Table 2). In these cases, however, only compound **8** was generally more cytotoxic than emodin. Modifications in the emodin structure leading to compounds **3**, **4**, **5**, **9**, **11**, **12**, and **13** completely destroyed the cytotoxic potential of emodin. There is no apparent simple structure–function relationship. In particular, no clear distinction could be made between 4-amino-emodin and tetranitro-emodin derivatives. Among 4-amino-emodin derivatives **1**–**16**, voluminous or possibly charged structural additions to the 4-amino group seemed unfavorable. Whether the 4-amino group used either as a primary or secondary amine might retain the possibility of forming a hydrogen bond in order to enhance efficacy remains to be determined. A similar ranking as for the cytotoxic effect was apparent with respect to the inhibition of [^{3}H]-thymidine incorporation into HepG2 cell DNA (Table 3).

Of course, inhibition was generally observed at lower concentrations than the cytotoxic effect. Some compounds, namely **1**, **2**, **6**, and **10**, exerted a stronger inhibition than emodin **I**. Again, compound **6** appeared to be the most effective growth inhibitor at approximately five times more effective. When compared to mitoxanthrone and losoxanthrone, the cytotoxic and cytostatic effects of compound **6** were stronger, too.[18,19] Since this compound does not affect the normal hepatocellular phenotype, a feature that is often overlooked in searches

Table 3. Effects of synthesized emodin derivatives on [^{3}H]-thymidine incorporation into HepG2 cell DNA

Compound	^{3}H-Thymidine incorporation into DNA (% control)	
	10 μM	40 μM
Emodin	86.9 ± 3.6	22.5 ± 3.0
1	15.6 ± 1.2	10.2 ± 0.9
2	81.5 ± 0.1	11.3 ± 0.1
3	91.5 ± 2.0	77.3 ± 1.8
4	90.8 ± 3.1	28.2 ± 1.2
5	86.3 ± 1.7	42.8 ± 1.8
6	35.5 ± 3.4	4.9 ± 0.1
7	65.6 ± 0.7	22.7 ± 0.2
8	56.9 ± 0.7	21.2 ± 0.1
9	91.4 ± 0.9	30.4 ± 0.8
10	77.2 ± 1.1	10.9 ± 0.3
11	83.4 ± 0.9	84.5 ± 1.9
12	99.4 ± 1.2	98.9 ± 0.1
13	103 ± 4	101 ± 1
14	86.5 ± 3.7	72.8 ± 2.7
15	62.9 ± 2.0	20.1 ± 1.0
16	95.1 ± 1.6	65.1 ± 0.7
17	101 ± 2	73.8 ± 3.3
18	79.2 ± 0.8	35.5 ± 0.2
19	47.3 ± 0.7	19.9 ± 0.2

for new cytostatic drugs (!), this compound is an interesting candidate for such a drug, and worth for a further characterization through in vitro and in vivo studies. Further evaluation of these emodin derivatives was made with respect to possible binding and inhibition of multi-drug-resistance transporters. In order to facilitate these studies, interaction between these compounds and a recombinant cytosolic C-terminal fragment of pgp representing the second ATP-binding loop was measured by quenching the intrinsic fluorescence (Table 4).

Table 4. Interaction of synthesized emodin derivatives with recombinant pgp

Compound	Fluorescence quenching of recombinant pgp	
	EC_{50} (μM)	Max. quenching (%)
Emodin	18.9 ± 5.2	79.0
1	19.1 ± 2.4	100.0
2	27.5 ± 4.7	92.0
3	33.8 ± 17.9	100.0
4	24.8 ± 5.4	77.3
5	24.8 ± 4.5	100.0
6	14.0 ± 3.0	77.9
7	30.8 ± 9.1	96.0
8	9.1 ± 2.7	56.0
9	41.3 ± 13.4	100.0
10	26.5 ± 5.4	75.0
11	37.2 ± 14.2	100.0
12	18.7 ± 6.8	47.0
13	26.8 ± 5.2	90.2
14	17.7 ± 1.3	100.0
15	19.1 ± 3.0	100.0
16	17.1 ± 2.5	100.0
17	16.0 ± 1.0	100.0
18	12.0 ± 0.7	100.0
19	9.5 ± 0.4	98.8

Emodin binding to the recombinant cytosolic fragment of pgp revealed an EC_{50} value of 18.9 and a maximal quenching of 79%. Most derivatives were less effective than emodin in terms of EC_{50} values, although some exerted a higher maximal quenching of 100%. Apparently, tetranitro derivatives **17**, **18**, and **19** were more effective than 4-amino derivatives **1**–**16**. In particular, derivative **19** was almost twice as strong as emodin, and additionally led to 100% quenching. Therefore, this compound may represent a potentially interesting pgp inhibitor, but this would have to be substantiated in further experiments. Interestingly, derivative **6** also turned out to bind slightly better than emodin. Thus, in addition to its favorable cytostatic features, this derivative also seems to retain the potential to inhibit pgp transporters. This was demonstrated using H4-II-E cells, which show a multi-drug-resistance phenotype preventing cellular accumulation of doxorubicin. In the presence of 40 μM compound **6** or emodin, this phenotype was reversed and accumulation of doxorubicin amounted to 31.5 ± 1.7% and 64.0 ± 3.9% ($N = 3$), respectively. Cyclosporine A (1 μM) served as control and its effect was set 100%. The combination of these properties is unusual and renders this compound a very promising potential anti-tumor drug.

4. Conclusion

A novel synthetic approach to highly functionalized derivatives of emodin facilitates preparation of highly functionalized emodin derivatives in only one step. Using emodin, DIB, and an appropriate amine as starting materials, we were able to produce a wide range of emodin-related structures in a high regioselective manner by one-pot synthesis. Some of these emodin derivatives showed stronger cytotoxic and cytostatic activity than emodin and/or bound more effectively to the second ATP-binding loop of pgp. Most interestingly, compound **6** discriminated well between hepatoma cells and primary hepatocytes and retained the capacity to reverse the multi-drug-resistance phenotype. This compound, therefore, features unmatched properties of a potential anti-tumor drug.

5. Experimental

5.1. Chemistry

Melting points were measured in open capillaries in an oil bath and are uncorrected. ^{1}H and ^{13}C nuclear magnetic resonance spectra were obtained with a Varian Gemini-300 spectrometer. The chemical shifts were recorded in parts per million (ppm) with the chemical shifts of the remaining protons of the deuterated solvents serving as internal standards. H, H-COSY, HMBC, HMQC, and NOE experiments were acquired using a Bruker DRX instrument (600 MHz). High-resolution mass spectra were measured on a 7T APEX II FT-ICR-ESI mass spectrometer (Bruker Daltonics). Colorimetric measurements were carried out with a Shimadzu-UV-Vis spectrometer. IR spectra were

obtained with a Perkin–Elmer FT-IR PC16 spectrometer. Thin-layer chromatography was performed on Silica Gel 60 F_{254} plates (Merck) and visualized by color and under UV light (254 and 366 nm). Merck Silica Gel 60 (0.040–0.063 mm) was used for column chromatography.

5.2. Synthesis of 4-amino-emodin derivatives

5.2.1. 4-Amino-emodin (1). (Diacetoxyiodo)benzene (370 mg, 1.15 mmol) was added to a solution of emodin (270 mg, 1.0 mmol) in piperidine (30 mL) and refluxed for 10 h. Piperidine was removed in vacuo. The residue was extracted with ethyl acetate (3×100 mL). The organic layer was washed in 0.05 M HCl (150 mL) and water (3×100 mL) and evaporated. The crude product was purified by column chromatography in two steps first on silica gel (petroleum ether/acetone/ethyl acetate 1:1:1), then on Sephadex LH-20 with methanol to give compound **1**. Violet powder (57 mg, 20%), mp > 350 °C; $R_f = 0.6$ (ethyl acetate/ethanol/water 20:2:1) pink, orange fluorescence; IR (KBr) cm^{-1}: 3422, 2993, 1718, 1685, 1588, 1508, 1387, 1295, 1264, 1206; ^1H NMR (methanol-d_4) δ (ppm) 2.47 (s, 3H, CH_3), 6.50 (s, 1H, H-2), 7.01 (s, 1H, H-7), 7.64 (s, 1H, H-5); ^1H NMR (DMSO-d_6) 2.41 (s, 3H, CH_3), 6.25 (s, 1H, H-2), 6.96 (s, 1H, H-7), 7.52 (s, 1H, H-5), 12.40 (OH), 13.48 (OH); ^{13}C NMR (acetone-d_6) δ 21.5 (CH_3-6), 101.0 (C-9a), 106.2 (C-4a), 106.8 (CH-2), 114.6 (C-8a), 119.3 (CH-5), 121.8 (CH-7), 135.5 (C-10a), 140.8 (CNH_2-4), 147.5 (CCH_3-6), 157.0 (CH-3), 161.2 (COH-1), 162.3 (COH-8), 187.7 (CO-9), 182.0 (CO-10); HRMS calcd for $C_{15}H_{12}NO_5^+$: 286.07100, found 286.07109.

5.2.2. 4-[*N*-(2-Hydroxyethyl)-amino]-emodin (2). Second isolated product of the reaction mixture of **8**. Violet crystals (19 mg, 12%), mp 225–228 °C; $R_f = 0.6$ (ethyl acetate/ethanol/water 20:2:1) violet, red fluorescence; IR (KBr) cm^{-1}: 3422, 2932, 1585, 1457, 1420, 1383, 1306, 1265, 1206, 1028, 779, 767; ^1H NMR (DMSO-d_6) δ 2.42 (s, 1H, CH_3), 3.56–3.60 (t, 2H, $^3J = 5.1$ Hz, H-2′), 3.76–3.80 (t, 2H, $^3J = 5.1$, H-1′), 6.45 (s, 1H, H-2), 7.01 (s, 1H, H-7), 7.55 (s, 1H, H-5), 11.31 (br s, 1H, OH), 12.39 (s, 1H, OH-8), 13.73 (s, 1H, OH-1); ^{13}C NMR (DMSO-d_6) δ 21.8 (CH_3-6), 48.4 (CH_2-2′), 61.0 (CH_2-1′), 105.5 (C-4a), 108.0 (CH-2), 108.5 (C-9a), 113.8 (C-8a), 118.9 (CH-7), 120.9 (CH-5), 135.1 (C-10a), 142.9 (CNH-4), 146.8 (CCH_3-6), 160.0 (COH-3), 161.0 (COH-1), 161.3 (COH-8), 184.9 (CO-9), 179.6 (CO-10); HRMS calcd for $C_{17}H_{16}NO_6^+$: 330.09722, found 330.09727.

5.2.3. 4-[*N*-(2-Aminoethyl)-amino]-emodin (3). (Diacetoxyiodo)benzene (354 mg, 1.1 mmol) was added to a solution of emodin (270 mg, 1.0 mmol) in ethylenediamine (40 mL) and stirred for 5 h at room temperature. The reaction was monitored by TLC. The solution was poured into a mixture of ice (200 g) and 10 M HCl (60 mL), neutralized with saturated $NaHCO_3$ solution (200 mL) and extracted with ethyl acetate (3×100 mL). The organic layer was washed in water (3×100 mL), dried over Na_2SO_4, and evaporated at 40 °C. The crude product was purified by adsorptive filtration on silica gel (short column), the first eluate (ethyl acetate) contained traces of compound **11**, the second eluate (methanol) compound **3**. Violet crystals (80 mg, 24%), mp 178–185 °C; $R_f = 0.2$ (ethyl acetate/ethanol/water 20:2:1) violet, red fluorescence; IR (KBr) cm^{-1}: 3421, 1600, 1544, 1499, 1404, 1358, 1319, 1283, 1209, 753; ^1H NMR (methanol-d_4) δ 2.42 (s, 3H, CH_3-6), 3.19 (t, 2H, $^3J = 5.7$ Hz, CH_2-2′), 4.14 (t, 2H, $^3J = 5.7$ Hz, CH_2-1′), 5.96 (s, 1H, H-2), 6.86 (s, 1H, H-7), 7.56 (s, 1H, H-5); ^1H NMR (DMF-d_7) δ 2.39 (s, 3H, CH_3-6), 2.58 (m, 2H, CH_2-2′), 4.26 (m, 2H, CH_2-1′), 5.69 (s, 1H, H-2), 6.85 (s, 1H, H-7), 7.60 (s, 1H, H-5), 12.21 (br s, 1H, NH-1), 13.62 (br s, 1H, OH), 14.88 (br s, 1H, OH); ^{13}C NMR (DMF-d_7) δ 21.8 (CH_3-6), 42.5 (CH_2-2′), 44.6 (CH_2-1′), 101.3 (C-9a), 106.1 (C-2), 116.5 (C-8a), 117.9 (C-5), 119.1 (C-4a), 119.9 (C-7), 121.7 (CNH-4), 135.9 (C-10a), 143.4 (CCH_3-6), 149.8 (C-3), 161.7 (COH-8), 169.0 (COH-1), 176.9 (CO-9), 180.6 (CO-10); HRMS calcd for $C_{17}H_{17}N_2O_5^+$: 329.11320, found 329.11335.

5.2.4. 4-[*N*-(2-Dimethylaminoethyl)-amino]-emodin (4). (Diacetoxyiodo)benzene (354 mg, 1.1 mmol) was added to a solution of emodin (270 mg, 1.0 mmol) in *N*,*N*-dimethylethylenediamine (40 mL) and stirred for 4 days at room temperature. The reaction was monitored by TLC. The solution was poured into a mixture of ice (200 g), 10 M HCl (60 mL), neutralized with saturated $NaHCO_3$ solution (200 mL), and extracted with ethyl acetate (3×100 mL). The organic layer was washed with water (3×100 mL), dried over Na_2SO_4, and evaporated at 40 °C. The crude product was purified by adsorptive filtration on silica gel (short column); the first eluate (ethyl acetate) contained traces of emodin and compound **1**, the second eluate (methanol) compound **4** which was lyophilized to remove solvent residues. Violet crystals (58 mg, 15%); $R_f = 0.3$ (methanol) violet, red fluorescence; IR (KBr) cm^{-1}: 3432, 1577, 1534, 1458, 1394, 1353, 1279; ^1H NMR (methanol-d_4) δ 2.38 (s, 6H, $N(CH_3)_2$), 2.44 (s, 3H, CH_3-6), 2.73 (t, 2H, $^3J = 6.6$ Hz, CH_2-2′), 4.31 (t, 2H, $^3J = 6.6$ Hz, CH_2-1′), 5.89 (s, 1H, H-2), 6.86 (s, 1H, H-7), 7.63 (s, 1H, H-5); ^{13}C NMR (methanol-d_4) δ 22.0 (CH_3-6), 44.4 (CH_2-2′), 45.7 ($N(CH_3)_2$), 61.4 (CH_2-1′), 102.6 (C-9a), 107.9 (CH-2), 109.1 (8a), 117.3 (C-4a), 118.7 (CH-5), 120.4 (C-7), 121.3 (CNH-4), 136.9 (C-10a), 144.6 (CCH_3-6), 150.8 (COH-3), 162.2 (COH-8), 169.5 (COH-1), 177.1 (CO-9), 180.9 (CO-10); HRMS calcd for $C_{19}H_{21}N_2O_5^+$: 357.14450, found 357.14523.

5.2.5. 4-[*N*-(3-Aminopropyl)-amino]-emodin (5). (Diacetoxyiodo)benzene (354 mg, 1.1 mmol) was added to a solution of emodin (270 mg, 1.0 mmol) in propylenediamine (40 mL) and stirred for 16 h at room temperature. The reaction was monitored by TLC. The solution was poured into a mixture of ice (200 g), 10 M HCl (60 mL), neutralized with saturated $NaHCO_3$ solution (200 mL), and extracted with ethyl acetate (3×100 mL). The organic layer was washed with water (3×100 mL), dried over Na_2SO_4, and evaporated at 40 °C. The crude product was purified by adsorptive filtration on silica gel (short column), the first eluate (ethyl

acetate) contained traces of emodin and compound **1**, the second eluate (methanol) **5**. Violet crystals (260 mg, 76%), mp 180 °C (decomposition); $R_f = 0.2$ (ethyl acetate/ethanol/water 20:2:1) violet, red fluorescence; IR (KBr) cm^{-1}: 3440, 2924, 1589, 1526, 1420, 1383, 1276, 1208, 422; ^1H NMR (methanol-d_4) δ 2.04 (t, 2H, $^3J = 3.0$ Hz, CH_2-2′), 2.39 (s, 3H, CH_3-6), 3.16 (t, 2H, $^3J = 3.0$ Hz, CH_2-3′), 4.12 (br s, 2H, CH_2-1′), 5.96 (s, 1H, H-2), 6.80 (s, 1H, H-7), 7.46 (s, 1H, H-5). ^{13}C NMR (methanol-d_4) δ 20.9 (CH_3-6), 28.8 (CH_2-2′), 36.9 (CH_2-3′), 40.7 (CH_2-1′), 102.5 (C-9a), 107.9 (CH-2), 110.1 (C-8a), 115.5 (C-4a), 118.2 (CH-5), 120.3 (CH-7), 120.3 (CNH-4), 135.5 (C-10a), 144.6 (*C*CH_3-6), 148.2 (COH-3), 161.3 (COH-8), (COH-1), 172.4 (CO-9), 181.6 (CO-10). HRMS calcd for $C_{18}H_{19}N_2O_5^+$: 343.12885, found 343.12879.

5.2.6. 4-(*N*-Cyclohexyl-amino)-emodin (6). (Diacetoxyiodo)benzene (354 mg, 1.1 mmol) was added to a solution of emodin (270 mg, 1.0 mmol) in cyclohexylamine (40 mL) and stirred for 8 days at room temperature. The reaction was monitored by TLC (ethyl acetate/ethanol/water 20:2:1). When the consumption of emodin was complete, the solution was poured into a mixture of ice (400 g), 10 M HCl (40 mL), neutralized with saturated $NaHCO_3$ solution (200 mL), and extracted with ethyl acetate (3 × 100 mL). The organic layer was washed with water (3 × 100 mL), dried over Na_2SO_4 and evaporated at 40 °C. The crude product was purified by recrystallization from ethanol by adding water dropwise. Red needles (56 mg, 15%), mp 213–228 °C; $R_f = 0.7$ (chloroform/methanol 14:1) violet, red fluorescence; IR (KBr) cm^{-1}: 3440, 2926, 1630, 1586, 1522, 1457, 1362, 1302, 1263, 1241, 1207; ^1H NMR (acetone-d_6) δ 1.35 (m, 1H, H-4′a), 1.39 (br s, 2H, H-5′b, 3′b), 1.42 (br s, 2H, H-2′b, 6′b), 1.60 (br s, 1H, H-4′b), 1.80 (br s, 2H, H-5′a, 3′a), 2.10 (br s, 2H, H-2′a, 6′a), 2.47 (s, 3H, CH_3-6), 4.47 (br s, 1H, CH-1′), 6.62 (s, 1H, H-2), 7.07 (s, 1H, H-7), 7.71 (s, 1H, H-5); ^{13}C NMR (DMSO-d_6) δ 21.7 (CH_3-6), 23.8 (CH_2-3′, 5′), 25.2 (CH_2-4′), 34.4 (CH_2-2′, 6′), 52.9 (CH-1′), 105.4 (C-9a), 108.2 (CH-2), 108.8 (C-4a), 114.0 (C-8a), 119.0 (CH-5), 121.0 (CH-7), 135.1 (C-10a), 142.1 (CNH-4), 146.9 (*C*CH_3-6), 161.0 (COH), 161.0 (COH), 161.4 (COH), 179.9 (CO-10), 184.9 (CO-9); HRMS calcd for $C_{21}H_{18}NO_5^+$: 368.14925, found 368.14908.

5.2.7. 4-(*N*-Piperidinyl)-emodin (7). (Diacetoxyiodo)benzene (190 mg, 0.6 mmol) was added to a solution of emodin (135 mg, 0.5 mmol) in piperidine (20 mL) and stirred for about 8 h at room temperature. The solution was poured into a mixture of ice (200 g), 10 M HCl (30 mL), and extracted with ethyl acetate (3 × 100 mL). The organic layer was washed with water (3 × 100 mL), dried over Na_2SO_4, and evaporated at 20 °C. The crude product was purified by column chromatography in two steps first on silica gel (petroleum ether/acetone/ethyl acetate 1:1:1), then on Sephadex LH-20 (methanol). Orange-brown crystals (27 mg, 15%), mp 140 °C (decomposition); $R_f = 0.8$ (ethyl acetate/ethanol/water 20:2:1) yellow, no fluorescence; IR (KBr) cm^{-1}: 3441, 2934, 1560, 1498, 1412, 1363, 1283, 1260; ^1H NMR ($CDCl_3$) δ 1.60–1.84 (m, 6H, H-3′, H-4′, H-5′), 2.48 (s, 3H, CH_3-6), 2.63–2.66 (s, 2H, H-2′, H-6′), 6.82 (s, 1H, H-2), 7.08 (s, 1H, H-7), 7.58 (s, 1H, H-5), 12.10 (s, 1H, OH-8), 13.13 (s, 1H, OH-1); ^{13}C NMR ($CDCl_3$) δ 22.5 (CH_3-6), 23.9 (CH_2-4′), 27.7 (CH_2-3′, CH_2-5′), 49.1 (CH_2-2′, CH_2-6′), 98.6 (C-4a), 106.9 (CH-2), 109.2 (C-9a), 113.4 (C-8a), 120.8 (CH-5), 124.0 (CH-7), 130.3 (C-10a), 134.6 (CNH-4), 148.2 (*C*CH_3-6), 162.0 (COH-8), 163.4 (COH-1), 164.6 (COH-3), 183.5 (CO-10), 190.7 (CO-9); HRMS calcd for $C_{20}H_{20}NO_5^+$: 354.13360, found 354.13360.

5.2.8. 4-(*N*-Morpholinyl)-emodin (8). (Diacetoxyiodo)benzene (190 mg, 0.6 mmol) was added to a solution of emodin (135 mg, 0.5 mmol) in morpholine (25 mL) and stirred for 48 h at room temperature. Morpholine was removed in vacuo, and the residue was extracted with ethyl acetate (3 × 100 mL). The organic layer was washed with 0.05 M HCl (150 mL) and water (3 × 100 mL). The solvent was evaporated and the crude product purified by column chromatography in two steps first on silica gel (petroleum ether/acetone/ethyl acetate 1:1:1), then on Sephadex LH-20 (methanol). Violet crystals (35 mg, 20%), mp 205 °C; $R_f = 0.9$ (ethyl acetate/ethanol/water 20:2:1) yellow-orange, no fluorescence; IR (KBr) cm^{-1}: 3423, 3924, 2863, 1676, 1628, 1490, 1449, 1327, 1228, 1108; ^1H NMR ($CDCl_3$) δ 2.48 (s, 3H, CH_3), 3.80–4.04 (m, 8H, H-2′, H-3′, H-5′, H-6′), 6.85 (s, 1H, H-2), 7.10 (s, 1H, H-7), 7.60 (s, 1H, H-5), 9.58 (br s, 1H, OH-3), 12.02 (s, 1H, OH-8), 13.10 (s, 1H, OH-1); ^{13}C NMR ($CDCl_3$) δ 22.4 (CH_3), 47.8 (CH_2-3′, CH_2-5′), 68.3 (CH_2-2′, CH_2-6′), 107.4 (CH-2), 109.8 (C-9a), 113.2 (C-8a), 121.1 (CH-5), 124.2 (CH-7), 131.1 (COH-4), 132.4, 134.2 (C-4a, C-10a), 148.6 (*C*CH_3-6), 162.0 (COH-3), 162.8 (COH-1), 164.3 (COH-8), 183.5 (CO-10), 191.0 (CO-9); HRMS calcd for $C_{19}H_{18}NO_6^+$: 356.11268, found 356.11235.

5.3. Synthesis of annelated dihydrooxazole derivatives

5.3.1. 4,6-Dihydroxy-2-methyl-8a,9,10,11-tetrahydro-8-oxa-11a-aza-pentaleno[1,2-*a*]anthracen-5,12-dion (9). (Diacetoxyiodo)benzene (190 mg, 0.6 mmol) was added to a solution of emodin (135 mg, 0.5 mmol) in pyrrolidine (20 mL) and stirred for 2 days at room temperature. The reaction was monitored by TLC (ethyl acetate/ethanol/water 20:2:1). When the consumption of emodin was complete, the solution was poured into a mixture of ice (200 g) and 10 M HCl (40 mL) and extracted with ethyl acetate (3 × 100 mL). The organic layer was washed with water (3 × 100 mL), dried over Na_2SO_4 and evaporated at 40 °C. The crude product was purified by column chromatography on silica gel (ethyl acetate). Violet crystals (30 mg, 18%), mp 148–158 °C; $R_f = 0.9$ (ethyl acetate/ethanol/water 20:2:1) pink, orange fluorescence; IR (KBr) cm^{-1}: 2920, 1619, 1851, 1619, 1586, 1481, 1429, 1358, 1284, 1248, 1215, 1176, 1057, 777; ^1H NMR ($CDCl_3$) 1.89 (m, 1H, H-10a), 1.96 (m, 1H, H-10b), 1.98 (m, 1H, H-9a), 2.49 (s, 3H, CH_3), 2.49 (br s, 1H, H-9b), 3.01 (m, 1H, H-11a), 6.06 (br s, 1H, H-8a), 6.46 (s, 1H, H-7), 7.08 (s, 1H, H-3), 7.65 (s, 1H, H-1), 12.39 (s, 1H, OH-4), 13.52 (s, 1H, OH-6); ^{13}C NMR ($CDCl_3$) δ 22.3 (CH_3-2), 23.8 (CH_2-10), 32.6 (CH_2-9), 56.4 (CH_2-11), 102.1 (CH-7), 105.4

(CH-8a), 107.8 (C-5a), 108.5 (CO-7a), 114.4 (C-4a), 120.4 (CH-1), 123.6 (CH-3), 130.4 (C-11c), 134.2 (C-12a), 137.6 (C-11b), 147.9 (CH-2), 162.3 (COH-4), 164.8 (COH-6), 180.8 (CO-12), 189.2 (CO-5); HRMS calcd for $C_{19}H_{17}NO_5^+$: 338.10230, found 338.10239.

5.3.2. 4,6-Dihydroxy-2-methyl-9,10,11,12-tetrahydro-8a*H*-8-oxa-12a-aza-indeno[1,2-*a*]-anthracen-5,13-dione (10). Second isolated product of the reaction mixture leading to 4-(*N*-piperidinyl)-emodin **7**. Violet crystals (28 mg, 16%), mp 118–120 °C; $R_f = 0.9$ (ethyl acetate/ethanol/water 20:2:1) violet, red fluorescence; IR (KBr) cm^{-1}: 3441, 2937, 2857, 1618, 1492, 1445, 1364, 1293, 1255, 1214, 833, 770; ^1H NMR ($CDCl_3$) δ 1.65–1.84 (m, 4H, H-10, H-11), 2.34 (m, 2H, H-9), 2.48 (s, 3H, CH_3), 3.27–3.34 (t, 1H, H-12, $^3J = 12$ Hz), 5.03–5.07 (d, 1H, H-12, $^3J = 10.2$ Hz), 5.66 (d, 1H, H-8a, $^3J = 9.3$ Hz), 6.44 (s, 1H, H-7), 7.03 (s, 1H, H-3), 7.57 (s, 1H, H-1), 12.40 (s, 1H, OH-4), 14.16 (s, 1H, OH-6); ^{13}C NMR ($CDCl_3$) δ 22.4 (CH_3), 25.3 (CH_2-10), 30.0 (CH_2-11), 32.6 (CH_2-9), 48.4 (CH_2-12), 98.5 (CH-8a), 100.3 (C-12c), 102.0 (CH-7), 107.4 (C-5a), 114.4 (C-4a), 119.9 (CH-1), 122.5 (CH-3), 135.4 (C-13a), 137.3 (C-12b), 147.4 (*C*CH_3-2), 162.0 (COH-4), 162.0 (C-7a), 164.2 (COH-6), 181.1 (CO-13), 187.8 (CO-5); HRMS calcd for $C_{20}H_{18}NO_5^+$: 352.1185, found 352.1182.

5.4. Synthesis of annelated tetrahydropyrazine derivatives

5.4.1. 6,8-Dihydroxy-10-methyl-1,2,3,4-tetrahydro-1,4-diaza-benzo[*a*]anthracen-7,12-dione (11). (Diacetoxyiodo)benzene (180 mg, 0.56 mmol) was added to a solution of emodin (135 mg, 0.5 mmol) in ethylenediamine (20 mL) and stirred for 4 days at room temperature. The reaction was monitored by TLC (ethyl acetate/ethanol/water 20:2:1). Once the consumption of emodin was complete, the solution was poured into a mixture of ice (200 g) and 10 M HCl (40 mL), neutralized with saturated $NaHCO_3$ solution (200 mL), and extracted with ethyl acetate (3×100 mL). The organic layer was washed with water (3×100 mL), dried over Na_2SO_4, and evaporated at 40 °C. The crude product was purified by column chromatography on silica gel (ethyl acetate). To remove solvent residues, the product was dissolved in methanol (30 mL), and water (50 mL) was added followed by evaporation at 40 °C and lyophilization. Black powder (160 mg, 45%), mp 271–276 °C; $R_f = 0.8$ (ethyl acetate/ethanol/water 20:2:1) violet, red fluorescence; IR (KBr) cm^{-1}: 3372, 1586, 1526, 1475, 1390, 1347, 1327, 1274, 1199, 1175; ^1H NMR ($CDCl_3$) δ 2.37 (s, 3H, CH_3-10), 3.62 (br s, 2H, CH_2-3), 3.70 (br s, 2H, CH_2-2), 4.97 (br s, 1H, NH-4), 6.18 (s, 1H, H-5), 7.02 (s, 1H, H-9), 7.67 (s, 1H, H-11), 10.55 (br s, 1H, NH-1), 12.74 (s, 1H, OH-9), 13.81 (s, 1H, OH-6); ^1H NMR (DMF-d_7) δ 2.44 (s, 3H, CH_3-10), 3.60 (s, 2H, CH_2-3), 3.70 (s, 2H, CH_2-2), 6.23 (s, 1H, H-5), 7.00 (s, 1H, H-9), 7.59 (s, 1H, H-11), 8.18 (s, 1H, NH-4), 10.73 (s, 1H, NH-1), 12.84 (s, 1H, OH-8), 13.96 (s, 1H, OH-6); ^{13}C NMR (DMF-d_7) 22.9 (CH_3-10), 39.2, 39.4 (CH_2-3, CH_2-4), 101.3 (CH-5), 103.8 (C-6a), 107.4 (C-12a), 115.5 (C-7a), 119.05 (CH-11), 121.6 (CH-9), 135.7 (C-11a), 138.7 (C-4a), 146.5 (*C*CH_3-10), 148.3 (C-12b), 162.1 (COH-8), 163.4 (COH-6), 181.6 (CO-12), 183.4 (CO-7). HRMS calcd for $C_{17}H_{15}N_2O_4^+$: 311.10263, found 311.10298.

5.4.2. 7,9-Dihydroxy-11-methyl-1,2,3,4,4a,5,14,14a-octahydro-naphto[2,3-*a*]phenazine-8,13-dione (12). (Diacetoxyiodo)benzene (354 mg, 1.1 mmol) was added to a solution of emodin (270 mg, 1.0 mmol) in (+/−)*trans*-1,2-diaminocyclohexane (40 mL) and stirred for 7 days at room temperature. The reaction was monitored by TLC. The solution was poured into a mixture of ice (200 g), 10 M HCl (60 mL), neutralized with saturated $NaHCO_3$ solution (200 mL), and extracted with ethyl acetate (3×100 mL). The organic layer was washed with water (3×100 mL), dried over Na_2SO_4, and evaporated at 40 °C. The crude product was purified by column chromatography on silica gel (ethyl acetate). Violet crystals (218 mg, 60%), mp 145–155 °C; $R_f = 0.8$ (ethyl acetate/ethanol/water 20:2:1) violet, pink fluorescence; IR (KBr) cm^{-1}: 3422, 2929, 2858, 1586, 1513, 1355, 1311, 1270, 1225, 1196, 1178, 636; ^1H NMR (DMSO-d_6) δ 1.23–1.40 (m, 2H, H-1′, 4′), 1.39 (br s, 2H, H-2b, 3b), 1.78 (br s, 2H, H-2a, 3a), 2.02 (br s, 2H, H-1″, 4″), 2.40 (s, 3H, CH_3-11), 3.08 (br s, 2H, H-14a, 4a), 6.10 (s, 1H, H-6), 6.97 (s, 1H, H-10), 7.45 (s, 1H, H-12), 8.14 (s, 1H, NH-5), 10.41 (s, 1H, NH-14), 12.72 (s, 1H, OH-9), 13.80 (s, 1H, OH-7); ^{13}C NMR (DMSO-d_6) δ 21.7 (CH_3-11), 23.5, 23.6 (CH_2-2, 3), 29.5, 29.6 (CH_2-1, 4), 53.6, 53.8 (CH-14a, 4a), 100.6 (CH-6), 102.8 (C-7a), 106.4 (C-13a), 114.5 (C-8a), 118.5 (CH-12), 121.2 (CH-10), 134.5 (C-12a), 137.7 (C-13b), 145.8 (*C*CH_3-11), 147.5 (C-5a), 160.9, 162.2 (COH-7, 9), 180.8 (CO-13), 182.3 (CO-8); HRMS calcd for $C_{21}H_{21}N_2O_4^+$: 365.14958, found 365.14978.

5.4.3. 6,8-Dihydroxy-4,10-dimethyl-1,2,3,4-tetrahydro-1,4-diaza-benzo[*a*]anthracen-7,12-dione (13). (Diacetoxyiodo)benzene (180 mg, 0.56 mmol) was added to a solution of emodin (135 mg, 0.5 mmol) in *N*-methylpiperazine (20 mL) and stirred for 9 days at room temperature. The reaction was monitored by TLC (ethyl acetate/ethanol/water 20:2:1). Once the consumption of emodin was complete, the solution was poured into a mixture of ice (200 g) and 10 M HCl (40 mL), neutralized with saturated $NaHCO_3$ solution (200 mL), and extracted with ethyl acetate (3×100 mL). The organic layer was washed with water (3×100 mL), dried over Na_2SO_4, and evaporated at 40 °C. The crude product was purified by column chromatography on silica gel (ethyl acetate). Dark violet powder (160 mg, 60%), mp 200 °C (decomposition); $R_f = 0.6$ (ethyl acetate/ethanol/water 20:2:1) violet, red fluorescence; IR (KBr) cm^{-1}: 3440, 2924, 1586, 1529, 1474, 1414, 1383, 1346, 1330, 1314, 1284, 1260, 1182; ^1H NMR ($CDCl_3$) δ 2.46 (s, 3H, CH_3-10), 3.04 (s, 3H, CH_3-4), 3.51 (s, 2H, CH_2-3), 3.68 (s, 2H, CH_2-2), 6.00 (s, 1H, H-5), 6.95 (s, 1H, H-9), 7.79 (s, 1H, H-11), 10.94 (br s, 1H, NH-1), 12.76 (s, 1H, OH-8), 13.88 (s, 1H, OH-6); ^{13}C NMR ($CDCl_3$) δ 22.5 (CH_3-10), 39.2 (CH_3-4), 40.1 (CH_2-2), 48.2 (CH_2-3), 101.4 (CH-5), 104.3 (C-6a), 107.1 (C-12a), 115.2 (C-7a), 119.2 (CH-11), 121.7 (CH-9), 135.4 (C-11a), 138.8 (C-12b), 146.0 (*C*CH_3-10), 146.2 (CN-4a), 161.9 (COH-6), 162.1 (COH-8), 182.2 (CO-12), 184.9

(CO-7); HRMS calcd for $C_{18}H_{17}N_2O_4^+$: 325.11828, found 325.11928.

5.5. Synthesis of amides of compound 5

5.5.1. Amide (14). Acetic anhydride (46 μL, 0.5 mmol) was added to a solution of **5** (170 mg, 0.5 mmol) in dry methanol (15 mL) and stirred at room temperature for 2 h. Water (20 mL) was added and methanol was evaporated in vacuo. The violet precipitate was filtered off and dried in vacuo. The crude product was purified by column chromatography on silica gel (ethyl acetate). Dark violet powder (86 mg, 45%), mp 183–194 °C; $R_f = 0.5$ (ethyl acetate/ethanol/water 20:2:1) violet, red fluorescence; IR (KBr) cm^{-1}: 3384, 3110, 2955, 1636, 1584, 1527, 1458, 1340, 1508, 1261, 1206; ^1H NMR (pyridine-d_5) δ 2.05 (s, 3H, Ac), 2.10 (m, 2H, $^3J = 7$ Hz, CH_2-2′), 2.27 (s, 3H, CH_3-6), 3.65 (dt, 2H, $^3J = 6$ Hz, $^3J = 7$ Hz, CH_2-3′), 4.21 (t, 2H, $^3J = 7$ Hz, CH_2-1′), 6.69 (s, 1H, H-2), 7.08 (s, 1H, H-7), 7.99 (s, 1H, H-5), 8.72 (br s, 1H, N*H*Ac); ^{13}C NMR: due to the very low solubility in suitable solvents, the need for long recording times, and the instability of compound **14**, no satisfactory ^{13}C NMR could be obtained; HRMS calcd for $C_{20}H_{21}N_2O_6^+$: 385.13996, found 385.13963.

5.5.2. Amide (15). Benzoic acid anhydride (113 mg, 0.5 mmol) was added to a solution of **5** (170 mg, 0.5 mmol) in dry methanol (15 mL) and stirred at room temperature for 2 h. Water (20 mL) was added and methanol was evaporated in vacuo. The violet precipitate was filtered off and dried in vacuo. The crude product was purified by column chromatography on silica gel (ethyl acetate). Dark violet powder (100 mg, 45%), mp 191–200 °C; $R_f = 0.6$ (ethyl acetate/ethanol/water 20:2:1) violet, red fluorescence; IR (KBr) cm^{-1}: 3424, 2932, 1636, 1586, 1526, 1490, 1458, 1420, 1358, 1259, 1203; ^1H NMR (DMSO-d_6) δ 1.91 (m, 2H, CH_2-2′), 2.44 (s, 3H, CH_3-6), 3.34 (dt, 2H, $^3J = 6.0$ Hz, $^3J = 6.0$ Hz, CH_2-3′), 3.92 (br s, 2H, CH_2-1′), 6.45 (s, 1H, H-2), 7.04 (s, 1H, H-7), 7.34–7.53 (m, 3H, H-3′, 4′, 5′), 7.58 (s, 1H, H-5), 7.86 (d, 2H, $^3J = 7.0$ Hz, H-2′, 6′), 8.56 (t, 1H, $^3J = 6.0$ Hz, CONH), 11.34 (br s, 1H, NH-1′), 12.45 (s, 1H, OH-8), 13.83 (s, 1H, OH-1); ^{13}C NMR (DMSO-d_6) δ 21.7 (CH_3-6), 31.1 (CH_2-2′), 37.0 (CH_2-3′), 43.7 (CH_2-1′), 105.6 (C-9a), 108.2 (CH-2), 108.5 (C-8a), 113.9 (C-4a), 118.9 (CH-5), 121.0 (CH-7), 127.1 (CH-2″, 6″), 128.2 (CH-3″, 5″), 131.0 (CH-4″), 134.5 (C-1″), 135.1 (C-10a), 143.1 (C-4), 146.7 (*C*CH_3-6), 161.0 (COH-3), 161.5 (COH-1), 161.5 (COH-8), 166.2 (CONH), 179.8 (CO-10), 184.7 (CO-9); HRMS calcd for $C_{25}H_{23}N_2O_6^+$: 447.15506, found 447.15497.

5.5.3. Amide (16). Phthalic acid anhydride (74 mg, 0.5 mmol) was added to a solution of compound **5** (170 mg, 0.5 mmol) in dry methanol (15 mL) and stirred at room temperature for 2 h. Water (20 mL) was added and methanol was evaporated in vacuo. The violet precipitate was filtered off and dried in vacuo. The crude product was purified by column chromatography on silica gel (ethyl acetate). Dark violet powder (196 mg, 80%), mp 155–165 °C; $R_f = 0.3$ (ethyl acetate/ethanol/water 20:2:1) violet, red fluorescence; IR (KBr) cm^{-1}: 3408, 3110, 2950, 1700, 1583, 1527, 1457, 1420, 1380, 1261, 1205, 779; ^1H NMR (DMSO-d_6) δ 1.88 (m, 2H, CH_2-2′), 2.43 (s, 3H, CH_3-6), 3.34 (dt, 2H, $^3J = 6.0$ Hz, $^3J = 6.0$ Hz, CH_2-3′), 3.92 (br s, 2H, CH_2-1′), 6.42 (s, 1H, H-2), 7.00 (s, 1H, H-7), 7.55 (s, 1H, H-5), 7.43–7.80 (m, 4H, H-3′, 4′, 5′, 6′), 8.41 (t, 1H, $^3J = 6.0$ Hz, CONH), 11.34 (br s, 1H, NH-1′), 12.44 (s, 1H, OH-8), 13.84 (s, 1H, OH-1); ^{13}C NMR (DMSO-d_6) 21.8 (CH_3-6), 30.9 (CH_2-2′), 36.7 (CH_2-3′), 43.5 (CH_2-1′), 105.2 (C-9a), 107.9 (C-4a), 108.4 (CH-2), 113.9 (C-8a), 118.6 (CH-5), 120.8 (CH-7), 127.6 (CH-3″), 129.0 (CH-5″), 129.2 (CH-6″), 130.5 (C-1″), 131.2 (CH-4″), 135.0 (C-10a), 138.0 (C-2″), 143.1 (C-4), 146.6 (*C*CH_3-6), 160.6 (COH-3), 161.0 (COH-1), 161.6 (COH-8), 167.9 (COOH), 168.6 (CONH), 179.6 (CO-10), 184.3 (CO-9); HRMS calcd for $C_{26}H_{23}N_2O_8^+$: 491.14489, found 491.14502.

5.6. Synthesis of tetranitro-emodin derivatives

5.6.1. 1,8-Dihydroxy-2,4,5,7-tetranitro-anthraquinone (17). Concentrated HNO_3 (4 mL) was added to a solution of 1,8-dihydroxy-anthraquinone (240 mg, 1.0 mmol) in concentrated H_2SO_4 (5 mL) (CAUTION, ice bath). After 2 h stirring at room temperature the mixture was poured into ice (100 g). The yellow precipitate was filtered off, washed with water (20 mL), and dried in vacuo. The crude product was purified by adsorptive filtration on silica gel (short column, methanol). Yellow powder (310 mg, 74%), mp > 200 °C (decomposition); $R_f = 0.2$ (ethyl acetate/ethanol/water 20:2:1) red, no fluorescence; IR (KBr) cm^{-1}: 3075, 1639, 1587, 1418, 1370, 1252, 1106, 716; ^1H NMR (DMSO-d_6) 8.66 (s, 2H, H-3 and H-6); ^{13}C NMR (DMSO-d_6) δ 121.2 (C-8a, C-9a), 127.0 (C-3, C-6), 133.4 (C-4, C-5), 134.8 (C-4a, C-10a), 143.1 (C-2, C-7), 160.1 (C-1, C-8), 181.4 (C-9), 184.9 (C-10); HRMS calcd for $C_{14}H_3N_4O_{12}^-$: 418.97530, found 418.97481.

5.6.2. 1,3,8-Trihydroxy-6-methyl-2,4,5,7-tetranitro-anthraquinone (18). Concentrated HNO_3 (4 mL) was added dropwise to a solution of emodin (270 mg, 1.0 mmol) in concentrated H_2SO_4 (5 mL) (CAUTION, ice bath). After 2 h of stirring at room temperature, the mixture was poured into ice (100 g) and extracted with ethyl acetate (3 × 100 mL). The organic layer was washed with water (3 × 100 mL), dried over Na_2SO_4, and evaporated. The crude product was purified by adsorptive filtration on silica gel (short column, ethyl acetate) and lyophilized. Red powder (300 mg, 67%), mp > 200 °C decomposition; $R_f = 0.4$ (ethyl acetate/ethanol/water 20:2:1) red, no fluorescence; IR (KBr) cm^{-1}: 3432, 1618, 1534, 1420, 1376, 1248, 1186; ^1H NMR (methanol-d_4) δ 2.08 (s, 3H, 6-CH_3), ^{13}C NMR (methanol-d_4). Due to the lack of protons, even prolonged recording times show only the methyl carbon: δ 11.4 (6-CH_3); HRMS calcd for $C_{15}H_5N_4O_{13}^-$: 448.98532, found 448.98341.

5.6.3. 1,8-Diacetyl-3-hydroxy-6-methyl-2,4,5,7-tetranitro-anthraquinone (19). Some drops of concentrated H_2SO_4 were added to a solution of **18** (113 mg,

0.25 mmol) in acetic anhydride (15 mL). The solution was stirred at room temperature for two weeks, poured into ice (100 g), and extracted with ethyl acetate (3×100 mL). The organic layer was washed with water (3×100 mL), dried over Na_2SO_4, and evaporated. The crude product was purified by adsorptive filtration on silica gel (short column, ethyl acetate). Orange powder (34 mg, 25%), mp > 200 °C decomposition; $R_f = 0.4$ (ethyl acetate/ethanol/water 20:2:1) orange-yellow, no fluorescence; IR (KBr) cm^{-1}: 3440, 1794, 1696, 1636, 1598, 1548, 1367, 1274, 1245, 1175; ^1H NMR (DMF-d_7) δ 2.36 (s, 3H, CH_3CO), 2.38 (s, 3H, CH_3CO), 2.47 (s, 3H, CH_3-6); ^{13}C NMR (DMF-d_7) δ 12.8 (CH_3-6), 20.7, 20.9 (CH_3CO), 168.0, 168.2 (CH_3CO); HRMS calcd for $C_{19}H_9N_4O_{15}^-$: 533.00699, found 533.00640.

6. Biological assays

Primary rat hepatocytes and HepG2 hepatoblastoma cells were used for studying cytotoxicity and inhibition of proliferation. Primary hepatocytes were isolated and cultured in serum-free modified Williams medium E as described previously.[20] Test compounds were added after 2 h of cultivation and the cytotoxic response was determined after 24 h using the MTT assay.[21] HepG2 cells were maintained as described[18,22] and incubated with the test compounds similarly as primary cells for measuring cytotoxicity. EC_{50} values were determined by curve fitting of concentration versus viability plots (cf. Fig. 8), and are given as means ± SD. Where solubility of the respective compound was limited, EC_{50} values particularly in the upper range could not be determined exactly and are given as rough estimates. Anti-proliferative effect was determined by measuring the inhibition of [^{3}H]-thymidine incorporation into HepG2 cell DNA as described by Gebhardt.[23] The recombinant cytosolic C-terminal fragment of *p*-glycoprotein (pgp) representing the second ATP-binding loop was expressed as a His-tag fusion protein similarly as described by Di Pietro.[19,24] After purification, aggregated forms of the His-tagged fragment were separated by size-exclusion chromatography. Measurements of fluorescence quenching of the single tryptophane residue present were performed only with the strictly monomeric form of the pgp fragment. Fluorescence measurements were performed at 25 °C. Tryptophane-specific intrinsic fluorescence of a 1.2 μM recombinant fragment in 0.5 mL of 50 mM sodium phosphate (pH 6.8) containing 300 mM NaCl and no additional detergents was scanned between 300 and 400 nm at 295 nm excitation. Values at 340 nm were corrected for contributions of buffer, test compounds, and their solvent DMSO, which never exceeded 2%. Concentration of test compounds ranged from 5 to 50 μM. EC_{50} values and maximal quenching related to fluorescence decrease were determined by curve fitting (Hill equation) on a personal computer. The potency of compounds to reverse the multi-drug-resistance phenotype was determined by measuring cellular accumulation of doxorubicin in hepatoma cells H4-II-E similarly as described.[25] Briefly, 0.6 million cells were cultured in DME medium supplemented with 10% FCS for 24 h and the coincubated with 40 μM test compound and 10 μM doxorubicin in serum-free DME medium for 24 h. After two washing steps in ice cold phosphate buffered saline cells were lysed in 5 mM Tris–HCl by sonification for 5 s. Fluorescence of doxorubicin was measured at 590 nm upon excitation at 485 nm with Mithras LB 940 multi-label reader (Berthold Technologies, Bad Wildbach, Germany). Values were corrected for the intrinsic fluorescence of the derivatives determined by incubating cells under same conditions but without doxorubicin and expressed as percentage of the enhancement of doxorubicin uptake produced by 2 μM cyclosporine A.

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