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ORIGINAL ARTICLE

New ferrocene modified lawsone Mannich bases with anti-proliferative activity against tumor cells

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Abstract Lawsone (**1a**) is a known naphthoquinone dye from the Henna plant *Lawsonia inermis*. Out of a series of four new ferrocene modified Mannich bases of **1a**, the 2-pyridyl derivative **2a** was distinctly more active than its analogs **2b–d** in breast, prostate and pancreatic cancer cells. **2a** also exhibited greater antiproliferative effects when compared with the known anticancer active Mannich bases **1b** and **1c** in the androgen-receptor negative PC-3 prostate and Pgp-expressing KB-V1/Vbl cervix carcinoma cell lines. Compound **2a** reached sub-micromolar activities in these aggressive cancer cells and, thus, features a promising drug candidate for the efficient treatment of hormone- or multidrug-resistant cancer types.

1. Introduction

Naphthoquinones feature a large group of plant secondary metabolites with a broad range of properties including antioxidant, anti-inflammatory, anticancer, and antibacterial activities [1–4]. Lawsone, i.e., 2-hydroxy-1,4-naphthoquinone, (**1a**, Fig. 1) is a constituent of the Henna plant (*Lawsonia inermis*) which has been used in Ayurveda and Unani medicine for centuries mainly for the treatment of skin diseases [5,6]. A natural lawsone derivative (arabinosyl ester) was recently isolated from *Amomum subulatum* fruits growing in Sikkim, India [7]. Lawsone is a potentially useful starting material for the preparation of other *p*-quinones with proven or conceivable bioactivity such as atovaquone or lapachol [6]. The readiness with which lawsone derivatives undergo redox reactions and chelation of metal ions is likely responsible for at least a few of their biological activities [8–10]. Mannich bases have raised interest in the field of drug design and Mannich bases from lawsone have earlier been investigated as potential antimalarial agents [11,12]. A series of anticancer active 3-aminomethylnaphthoquinones (Mannich bases **1b** and **1c**) derived from lawsone **1a** together with their *N*,*N*-chelate platinum complexes was disclosed (Fig. 1) [13–15].

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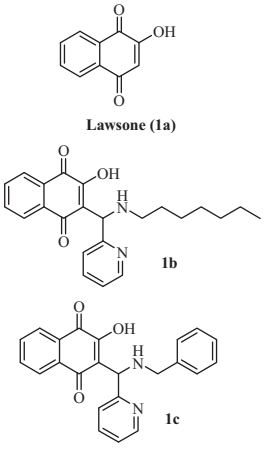


Figure 1 Chemical structures of lawsone (**1a**) and of two published anticancer active Mannich bases **1b** and **1c**.

The anticancer potential of ferrocenes as redox-sensitive phenyl surrogates and isosters has been reviewed thoroughly [16,17]. In addition, enhanced tumor selectivity of the non-discriminating fungal cytotoxin illudin M by esterification with ferrocene-1,1′-dicarboxylate was observed [18–20]. Concerning pathological bugs and parasites, ferrocene-modified aminohydroxynaphthoquinones derived from lawsone exhibited parasite growth inhibitory activity against *Toxoplasma gondii* [21].

For the current study, we further developed the anticancer active lawsone Mannich base motifs of **1b** and **1c** by attachment of ferrocene scaffolds. First, we modified the *N*-alkyl side chain by a ferrocenylmethyl moiety. Second, we replaced the 2-pyridyl group by other aryl moieties like 4-pyridyl, 3,4-difluorophenyl or ferrocenyl groups. The growth inhibitory activity of the new ferrocene-lawsone conjugates was determined in various cancer cell types that are difficult to tackle by approved anticancer drugs.

2. Experimental

2.1. General

Melting points were recorded using a Gallenkamp apparatus and are uncorrected. IR: Perkin-Elmer Spectrum One FT-IR spectrophotometer equipped with an ATR sampling unit. NMR: Bruker Avance 300 spectrometer; chemical shifts are given in parts per million (δ) downfield from Me_4Si as internal standard; coupling constants (J) are given in Hz. MS: Varian MAT 311A (EI). Microanalyses indicated by the symbols of the elements were within $\pm 0.2\%$ of the theoretical values for all new compounds. The starting compounds and pure solvents were purchased from the usual sources and were used without further purification. (Ferrocene-1-yl)-methylamine was prepared according to a literature procedure starting from commercially available ferrocene-1-yl carboxaldehyde [22].

2.2. Chemistry

*2.2.1. 3-[(Ferrocen-1-ylmethylamino)(2-pyridyl)methyl]-2-hydroxy-1,4-naphthoquinone (**2a**)*

2-Hydroxy-1,4-naphthoquinone (101 mg, 0.58 mmol) was suspended in EtOH (15 mL), ferrocene-1-ylmethyl amine (150 mg, 0.7 mmol) was added and the resulting solution was stirred at room temperature for 5 min. Pyridine-2-carboxaldehyde (73 μL, 0.76 mmol) was added and the reaction mixture was stirred at room temperature for 5 h. The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 157 mg (0.33 mmol, 57%); amber solid of mp 175–177 °C; ν_{max} (ATR)/cm^{-1} 3083, 3009, 2948, 2634, 1674, 1609, 1587, 1555, 1517, 1472, 1434, 1397, 1377, 1358, 1334, 1274, 1257, 1224, 1209, 1172, 1150, 1107, 1078, 1042, 1026, 994, 966, 937, 914, 901, 874, 832, 819, 797, 774, 742, 723, 696, 665; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.92 (1 H, d, J = 13.1 Hz), 4.1–4.2 (3 H, m), 4.2–4.3 (5 H, m), 4.3–4.4 (1 H, m), 4.4–4.5 (1 H, m), 5.83 (1 H, s), 7.1–7.2 (1 H, m), 7.3–7.4 (2 H, m), 7.5–7.6 (2 H, m), 7.84 (1 H, dd, J = 7.7 Hz, 1.0 Hz), 7.93 (1 H, dd, J = 7.7 Hz, 1.0 Hz), 8.4–8.5 (1 H, m), 9.7–9.9 (1 H, br s); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 46.0, 58.5, 68.9, 69.1, 69.2, 69.4, 69.6, 110.5, 122.2, 123.0, 125.6, 126.3, 131.1, 131.6, 133.6, 134.3, 137.7, 147.5, 155.5, 171.3, 181.4, 184.5; m/z (%) 478 (3) [M$^+$], 460 (9), 411 (26), 304 (100), 265 (26), 239 (63), 215 (72), 199 (82), 174 (45), 121 (44), 105 (30), 56 (22). Anal $C_{27}H_{22}FeN_2O_3$ calcd. C, 67.8, H, 4.64, N, 5.86. Found C, 67.5, H, 4.56, N, 5.77.

*2.2.2. 3-[(Ferrocen-1-ylmethylamino)(4-pyridyl)methyl]-2-hydroxy-1,4-naphthoquinone (**2b**)*

2-Hydroxy-1,4-naphthoquinone (61 mg, 0.35 mmol) was suspended in EtOH (15 mL), ferrocene-1-ylmethyl amine (90 mg, 0.42 mmol) was added and the resulting solution was stirred at room temperature for 5 min. Pyridine-4-carboxaldehyde (43 μL, 0.43 mmol) was added and the reaction mixture was stirred at room temperature for 5 h. The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 106 mg (0.22 mmol, 63%); red-brown solid of mp 175–177 °C; ν_{max} (ATR)/cm^{-1} 3071, 2952, 2606, 1673, 1589, 1522, 1474, 1419, 1374, 1335, 1270, 1222, 1242, 1159, 1106, 1068, 1024, 993, 969, 937, 872, 818, 810, 731, 694, 663; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.9–4.0 (2 H, m), 4.1–4.2 (5 H, m), 4.2–4.4 (4 H, m), 5.47 (1 H, s), 7.4–7.5 (2 H, m), 7.59 (1 H, dd, J = 7.3 Hz), 7.71 (1 H, dd, J = 7.4 Hz), 7.83 (1 H, d, J = 7.3 Hz), 7.92 (1 H, d, J = 7.4 Hz), 8.5–8.6 (1 H, m), 9.6–9.8 (1 H, br s); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 44.8, 55.9, 68.7, 70.2, 70.6, 76.7, 97.1, 109.7, 122.1, 125.1, 125.5, 131.0, 131.6, 133.8, 134.6, 146.7, 149.6, 170.7, 178.3, 184.0; m/z (%) 411 (36), 304 (100), 265 (43), 215 (59), 199 (96), 121 (70), 56 (24). Anal $C_{27}H_{22}FeN_2O_3$ calcd. C, 67.8, H, 4.64, N, 5.86. Found C, 67.7, H, 4.53, N, 5.75.

*2.2.3. 3-[(Ferrocen-1-ylmethylamino)(3,4-difluorophenyl)methyl]-2-hydroxy-1,4-naphthoquinone (**2c**)*

2-Hydroxy-1,4-naphthoquinone (101 mg, 0.58 mmol) was suspended in EtOH (15 mL), ferrocene-1-ylmethyl amine (150 mg, 0.7 mmol) was added and the resulting solution was stirred at room temperature for 5 min. 3,4-Difluorobenzaldehyde (84 μL, 0.76 mmol) was added and the reaction mixture was stirred at room temperature for 5 h. The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 290 mg (0.57 mmol, 98%); red-brown solid of mp 197–198 °C; ν_{max} (ATR)/cm^{-1} 3134, 3089, 2968, 2563, 1682, 1611, 1591, 1579, 1509, 1474, 1429, 1381, 1353, 1347, 1320, 1276, 1227, 1242, 1193, 1153, 1117, 1103, 1041, 1030, 999, 951, 931, 922, 885, 852, 819, 800,

786, 767, 737, 702, 693, 653; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.8–3.9 (2 H, m), 4.1–4.2 (5 H, m), 4.2–4.3 (4 H, m), 5.43 (1 H, s), 7.3–7.4 (2 H, m), 7.5–7.6 (2 H, m), 7.71 (1 H, ddd, J = 7.6 Hz, 1.4 Hz), 7.83 (1 H, dd, J = 7.6 Hz, 1.0 Hz), 7.91 (1 H, dd, J = 7.6 Hz, 1.0 Hz); m/z (%) 411 (2), 339 (8), 270 (100), 242 (19), 215 (92), 137 (37), 121 (24), 104 (28), 76 (26). Anal $C_{28}H_{21}F_2FeNO_3$ calcd. C, 65.52, H, 4.12, N, 2.73. Found C, 65.30, H, 4.02, N, 2.66.

2.2.4. 3-[(Heptylamino)(1-ferrocenyl)]-2-hydroxy-1,4-naphthoquinone (**2d**)

2-Hydroxy-1,4-naphthoquinone (217 mg, 1.25 mmol) was suspended in EtOH (15 mL), heptylamine (204 µL, 1.37 mmol) was added and the resulting solution was stirred at room temperature for 5 min. Ferrocene-1-yl carboxaldehyde (321 mg, 1.5 mmol) was added and the reaction mixture was stirred at room temperature for 5 h. The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 150 mg (0.31 mmol, 25%); brown solid of mp > 130 °C (dec.); ν_{max} (ATR)/cm^{-1} 3087, 2927, 2861, 1674, 1594, 1565, 1530, 1468, 1365, 1337, 1277, 1220, 1157, 1105, 1054, 1022, 1000, 963, 915, 895, 822, 759, 738, 664; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.7–0.8 (3 H, m), 1.0–1.6 (10 H, m), 2.9–3.0 (2 H, m), 3.8–3.9 (2 H, m), 4.0–4.1 (5 H, m), 4.1–4.2 (2 H, m), 6.82 (1 H, s), 7.4–7.5 (1 H, m), 7.5–7.7 (1 H, m), 7.7–7.8 (1 H, m), 8.1–8.2 (1 H, m); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.9, 22.4, 28.7, 31.5, 66.2, 68.6, 69.1, 123.9, 125.4, 126.9, 130.8, 131.9, 133.3, 134.1, 174.1, 183.6, 185.8; Anal $C_{28}H_{31}FeNO_3$ calcd. C, 69.28, H, 6.44, N, 2.89. Found C, 69.03, H, 6.19, N, 2.72.

2.3. Biological studies

2.3.1. Cell lines and culture conditions

BxPC-3 pancreas cancer cells (gemcitabine-sensitive) and MDA-MB-231 breast cancer cells (triple-negative) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 µg/mL of streptomycin. The prostate cancer cell line PC-3 (androgen receptor/AR-negative, ATCC, Manassas, VA, USA) was maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Prostate epithelial cells RWPE-1 (ATCC, Manassas, VA, USA) were cultured in keratinocyte serum free medium (Life Technologies, Carlsbad, CA, USA) with 0.05 mg/mL bovine pituitary extract and 5 ng/mL human recombinant epidermal growth factor. The human melanoma cell line 518A2 (Department of Radiotherapy and Radiobiology, University Hospital Vienna), the human colon adenocarcinoma cell line HT-29 (University Hospital Erlangen, Germany), and the KB-V1/Vbl cervix cancer cell line (Institute of Pharmacy, University of Regensburg, Germany) were grown in DMEM or RPMI (HT-29) medium, supplemented with 10% FBS, 1% Antibiotic–Antimycotic solution (both from Gibco, Darmstadt, Germany) and 250 µg/mL gentamycin (SERVA, Heidelberg, Germany). All cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C.

2.3.2. MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] (ABCR) was used to identify viable cells which reduce it to a violet formazan [23]. Cells (3×10^3/well) were seeded and cultured for 24 h on 96-well microplates. Incubation (5% CO_2, 95% humidity, 37 °C) of cells following treatment with the test compounds (dilution series from 5–40 µM in DMSO) was continued for 72 h. 25 µL of an MTT stock solution, containing 5 mg/mL in phosphate-buffered saline (PBS), was added to a final concentration of 0.05% and incubated for a further 2 h at 37 °C. The supernatant was withdrawn and the formazan was dissolved in DMSO (100 µL). The absorbance at 595 nm was measured on an Ultra Multifunctional Microplate Reader (Tecan, Durham, NC, USA).

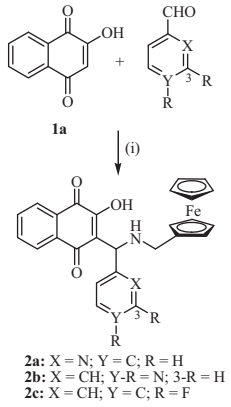
3. Results and discussion

3.1. Chemistry

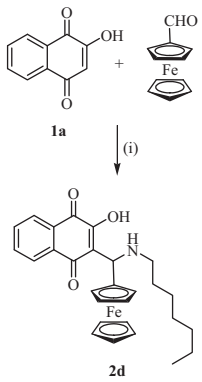
The new Mannich bases **2a**–**d** were prepared via Mannich reaction of lawsone (**1a**), aryl carboxaldehyde (2-pyridylcarboxaldehyde for **2a**, 4-pyridylcarboxaldehyde for **2b**, and 3,4-difluorobenzaldehyde for **2c**) and ferrocene-1-yl methylamine (Scheme 1). Analogously, compound **2d** was obtained from the reaction of **1a** with ferrocene-1-yl carboxaldehyde and heptylamine (Scheme 2). The racemic products precipitated from the reaction mixture as amber or brown solids after a few hours. ^{1}H NMR spectra revealed the particularly significant C*H*NHR signal (5.43–5.83 ppm for **2a**–**c**, 6.82 ppm for **2d**) aside the characteristic ferrocene protons (4.0–4.5 ppm). The ^{13}C NMR spectra of **2a** and **2b** exhibited ferrocene signals (68–77 ppm) aside CH_2 (44–46 ppm) and CH signals (55.9–58.5 ppm). In addition, the ^{13}C NMR spectrum of **2d** showed characteristic carbon signals of the heptylamine residue (13.9–28.7 ppm).

3.2. Biological evaluation

The antiproliferative activity of **1b**, **1c**, and **2a**–**d** was initially tested against the triple-negative MDA-MB-231 breast cancer, the BxPC-3 pancreas cancer, and the PC-3 (AR-) prostate cancer cells (Fig. 2). Compound **2a** was the most active ferrocene



Scheme 1 Reagents and conditions: (i) (Ferrocene-1-yl)-methylamine, EtOH, r.t., 5 h, 57–98%.



Scheme 2 Reagents and conditions: (i) Heptylamine, EtOH, r.t., 5 h, 25%.

derivative of this series **2a**–**d** in the three cancer cell lines (**2a** > **2b** > **2d** > **2c**). Compound **2a** also exceeded the activity of the known lawsone Mannich bases and close analogs **1b** and **1c** in the PC-3 cells. Prostate cancer cells generally exhibit high ROS levels leading to aggressive cancer phenotypes [24,25]. The enhanced activity by compound **2a** in the PC-3 prostate cancer cell line may be at least in parts due to activation of the ferrocene fragment by reactive oxygen species (ROS). The formation and stabilization of eventually emerging molecular radicals by the different scaffolds used in this study can influence the anticancer activity of the respective ferrocene derivatives **2** [26,27]. To confirm the cancer cell-specific activity of the most active compound **2a**, we tested it against the control prostate epithelial cell line, RWPE-1. We observed that this compound did not significantly inhibit the proliferation of these non-malignant cells (Fig. 3).

Further to this, the most active ferrocene derivative **2a** was tested in four aggressive, mutant and/or drug-resistant human cancer cell lines (518A2 melanoma, vinblastine-resistant KB-V1/Vbl cervix carcinoma, HCT-116 colon carcinoma, and HT-29 colon carcinoma) and compared with the activity of the lawsone Mannich base positive controls **1b** and **1c** (Table 1). Indeed, derivative **2a** revealed distinct activity in these cancer cell lines as well. **2a** exceeded the activity of **1b** and **1c** in KRAS-mutant HCT-116 colon carcinoma cells and multidrug-resistant KB-V1/Vbl cervix carcinoma cells. The activity of **2a** is particularly high (IC_{50} = 0.19 μM) in the Pgp-transporter (P-glycoprotein) overexpressing KB-V1/Vbl cervix cancer cells. The high efficacy of **2a** against these

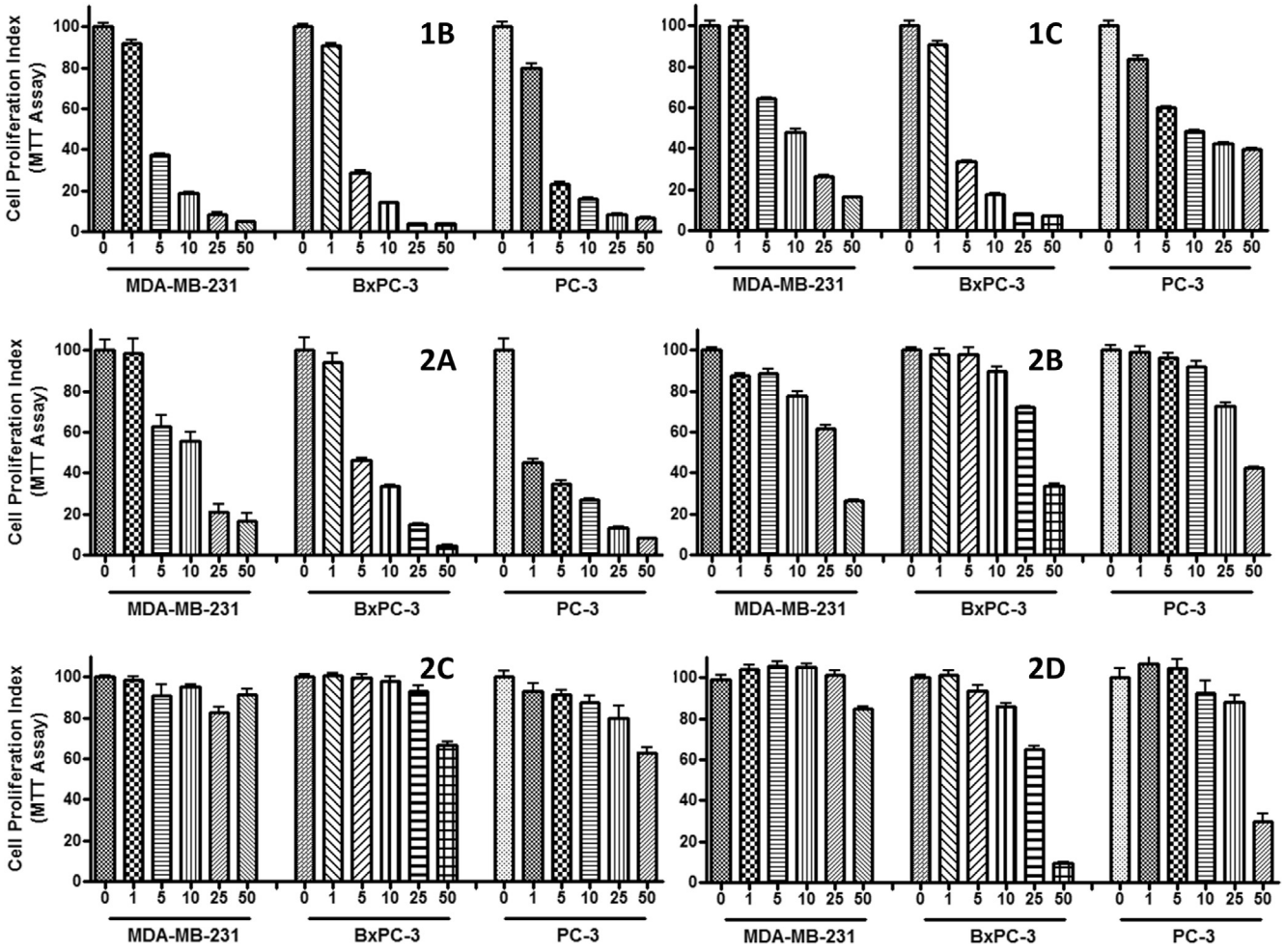


Figure 2 Growth inhibitory activity (MTT assay) of compounds **1b**, **1c**, and **2a**–**d** in triple-negative MDA-MB-231 breast carcinoma, BxPC-3 pancreas carcinoma, and androgen receptor-negative PC-3 prostate carcinoma cells. *X*-axis: concentrations in μM.

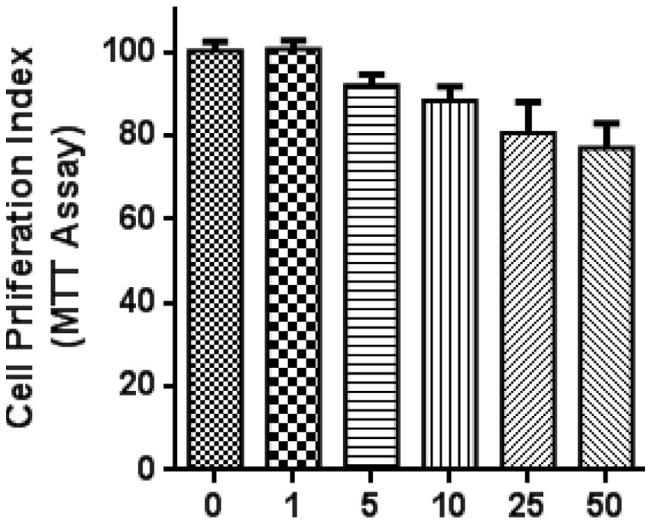


Figure 3 Growth inhibitory activity (MTT assay) of compound **2a** against prostate epithelial RWPE-1 cells. *X*-axis: concentrations in μM.

Table 1 Inhibitory concentrations IC_{50} (72 h) [μM] of compounds **1b**, **1c**, and **2a** from MTT tests against cells of 518A2 melanoma, vinblastine-resistant KB-V1/Vbl cervix carcinoma, HCT-116 colon carcinoma, and HT-29 colon carcinoma. Mean of three values, standard deviation < ±15%.

Compd./cell line	**1b**	**1c**	**2a**
518A2	2.58	2.33	2.60
KB-V1/Vbl	0.41	0.93	0.19
HCT-116	6.39	5.02	4.24
HT-29	5.12	2.39	3.58

Pgp-positive tumor cells is in line with a recent report of the natural naphthoquinone and Pgp-substrate plumbagin (isolated from *Plumbago* species) that was turned into a very active compound against Pgp-expressing cells after conjugation with a ferrocene scaffold [28].

4. Conclusions

A series of four new ferrocene-modified lawsone Mannich bases **2a**–**d** was prepared by a simple one-step three-component reaction protocol from lawsone **1a** and appropriately substituted aryl aldehydes and alkyl amines. Ferrocene **2a** was the most active Mannich base of this series. The combination of lawsone with a 2-pyridyl moiety and a ferrocene-1-yl methylamine scaffold as in **2a** seems to be optimal concerning anticancer activity. In addition, compound **2a** was more active than known anticancer active lawsone Mannich bases **1b** and **1c** against prostate cancer PC-3 (AR-negative) and KB-V1/Vbl (Pgp-positive) cervix carcinoma cells. In these hormone- and multidrug-resistant cancer cell lines **2a** exhibited excellent sub-micromolar activity and the effect of the ferrocene fragment became particularly visible. The possibilities for further structural fine-tuning render **2a** a promising lead compound for the development of new drugs for the treatment of hormone-refractory prostate carcinomas that don't respond to hormone therapy anymore, as well as for the treatment of multidrug-resistant tumors with elevated Pgp-levels.

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