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Cancer Selective Metallocenedicarboxylates of the Fungal Cytotoxin Illudin M

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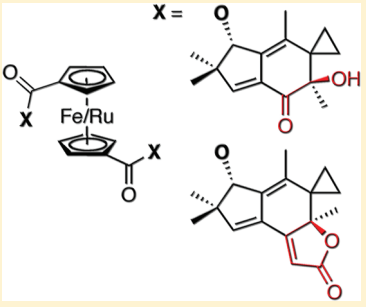
Supporting Information

ABSTRACT: The diester **2a** obtained from 1,1′-ferrocenedicarboxylic acid and the highly and indiscriminately cytotoxic fungal metabolite illudin M (**1**) displayed antiproliferative activity at submicromolar IC_{50} (72 h) values against a panel of eight cancer cell lines. Compound **2a** was about 40 times less toxic than **1** to nonmalignant human foreskin fibroblasts (HF). The analogous bis(illudinyl M) 1,1′-ruthenocenedicarboxylate (**2b**) exhibited submicromolar IC_{50} (72 h) values only against MDA-MB-231 and MCF-7/Topo breast carcinoma and HL-60 leukemia cells. Cytotoxicity studies in the presence of inhibitors of c-Jun N-terminal kinase (JNK) or extracellular signal-regulated kinase (ERK) revealed that the high efficacy of **2a**, but not that of **2b**, against HCT-116 colon cancer cells depends on active JNK/ERK signaling. A new illudin M lactone **5** was of low anticancer activity, but its ruthenocene diester **6b** also reached single-digit micromolar IC_{50} (72 h) values in HCT-116, MCF-7, and HL-60 leukemia cells while not affecting HF. Compounds **2a** and **6b** were tolerated by mice symptom-free at single doses as high as 25 mg/kg body weight, which is evidence for them being chemically stable under physiological conditions. Compound **2a** displayed a manageable in vivo toxicity profile when given repeatedly in high doses.



INTRODUCTION

The sesquiterpene illudin M (**1**) was first isolated from culture broths of the bioluminescent Jack o' Lantern mushroom (*Omphalotus olearius*, formerly *Clitocybe illudens*) growing in the east of North America.[1] After activation of its enone by NADPH-dependent oxido-reductases or glutathione (Nu^1), **1** can react with bionucleophiles (Nu^2) such as DNA, RNA, and proteins by opening the spirocyclopropane and thus induce apoptotic cell death (Scheme 1).[2] Compound **1**, like its congener illudin S, and the related semisynthetic irofulven[3] are known to inhibit the synthesis of DNA by blocking the cell cycle at the G_1-S phase interface. This effect presumably involves DNA damage[4] and is associated with a loss of RNA polymerase II activity.[5] It can be removed only via the transcription-coupled nucleotide excision repair.[6] Although the illudins are highly efficacious against cancer cells, their indiscriminate toxicity has prevented clinical applications.[7] Occasionally, illudin derivatives with reduced toxicity and improved therapeutic indices were reported.[8] Irofulven even underwent several phase II clinical trials but proved largely ineffective, except for some prostate and pancreatic cancers.[9]

The selectivity of **1** for cancer over normal cells might be increased by raising the threshold for the initial Michael addition of the mentioned bionucleophiles glutathione and oxido-reductases, which are abundant only in cancer cells. This can be achieved by shielding the enone of **1** sterically or electronically, for instance by attaching a late transition metallocene. Synergisms are to be expected since ferrocene derivatives have been frequently reported to show anticancer activity of their own,[10,11] which is thought to originate from redox-sensitive signaling.[12] Jaouen's tamoxifen analogue ferrocifen, which selectively targets breast cancers, is a prominent example.[13] We recently reported on a ferrocene diester (**2a**) of **1** that was efficacious against 518A2 melanoma cells without affecting normal human foreskin fibroblast (HF).[14] It also turned out to be stable for days in the presence of cell-free cell extracts and thus can be considered a genuinely new drug rather than a prodrug of **1**. Further to this, we now investigated the impact of **2a** and of an analogous ruthenocene diester **2b** on tumor cells of the colon, pancreas, breast, cervix, and leukemia entities. We also include the syntheses and activity studies of a new illudin M lactone and its diesters with ferrocene- and ruthenocene-dicarboxylic acids as well as toxicity studies in mice.

RESULTS AND DISCUSSION

Chemistry. The metallocene diesters **2a**[14] and **2b** were synthesized by Yamaguchi esterification of **1** with commercially available 1,1′-ferrocenedicarboxylic acid or with 1,1′-ruthenocenedicarbocyclic

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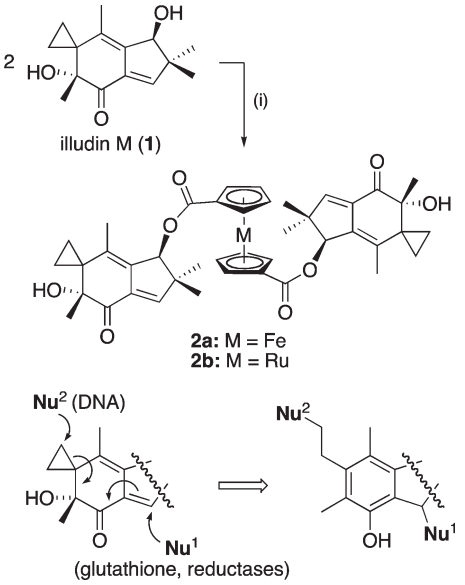
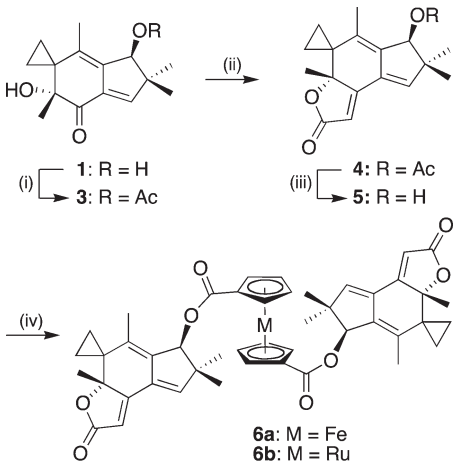
Scheme 1. Synthesis of Bis(illudinyl M) Metallocenedicarboxylates 2 and Mechanism of Illudin Action[a]



[a] Reagents and conditions: (i) 1,1′-Metallocenedicarboxylic acid, Et_3N, $C_6H_2Cl_3COCl$, DMAP, DMF/toluene, room temperature, 16 h, 50%.

Scheme 2. Synthesis of Illudin M Lactone 5 and Its Metallocenedicarboxylates 6[a]



[a] Reagents and conditions: (i) Ac_2O, pyridine, room temperature, 16 h, 86%. (ii) Ph_3PCCO, toluene, μw, 120 °C, 3 h, 45%. (iii) K_2CO_3, MeOH, room temperature, 1 h, 90%. (iv) 1,1′-Metallocenedicarboxylic acid, Et_3N, $C_6H_2Cl_3COCl$, DMAP, DMF/toluene, room temperature, 16 h, 60% (**6a**), 50% (**6b**).

acid, obtained by lithiation of ruthenocene with *n*-BuLi and subsequent carboxylation with dry ice (Scheme 1).[15]

A new lactone **5** of illudin M was prepared by a microwave-assisted domino addition−Wittig olefination of the α-hydroxy ketone fragment of illudin M acetate **3** with the cumulated phosphorus ylide Ph_3PCCO,[16,17] followed by hydrolytic deprotection of the secondary alcohol of **4** (Scheme 2). Analogously to the synthesis of **2**, the 1,1′-metallocenedicarboxylates **6** were obtained by Yamaguchi esterification of lactone **5**.

Biological Evaluation. The antiproliferative activities of compounds **2**, **5**, and **6** were evaluated in cells of HCT-116 and HT-29 colon carcinomas, multidrug resistant MCF-7/Topo breast and KB-V1/Vbl cervix carcinomas, 518A2 melanoma, HL-60 leukemia, and in nonmalignant HF cells, and they were compared with the activity of **1**. The compounds **2** were also tested on gemcitabine sensitive BxPC-3 pancreatic cancer cells and MDA-MB-231 breast carcinoma cells. The ferrocene conjugate **2a** was efficacious against all of these cancer cell lines at submicromolar IC_{50} (72 h) values, while its ruthenocene analogue **2b** was distinctly less active on average, yet reached submicromolar IC_{50} (72 h) values against the two breast carcinoma and the leukemia cells (Table 1). Cell cycle analyses revealed that **2a** and **2b** initiated identical G_1−S phase interface blockades in HCT-116 cells which differed not much from that caused by **1** (cf. Supporting Information). More important with respect to potential clinical applicability is the vastly improved therapeutic index of **2a** when compared with that of **1**. This index is a figure representing the ratio of IC_{50} (72 h) values against HF and the individual cancer cell lines. The greater this figure, the more selective for cancer vs normal cells the respective compounds are. A 20-fold increase in the therapeutic indices of **2a** over that of **1** was observed for cells of carcinomas HCT-116, MDA-MB-231, and BxPC-3 while merely minute index improvements were found for cells of HL-60 leukemia and the carcinomas HT-29 and KB-V1/Vbl (Table 2). The ruthenocenedicarboxylate **2b** displayed not only greater IC_{50} (72 h) values than **2a** but also lower therapeutic indices, which were generally not much different from those of **1**. The lactone **5** was much less active than **1** and its esters **2**, presumably due to an overly high threshold for the preactivating attack of a nucleophile or reductase on the now extended Michael system of the 2,4-dienoate moiety of **5**. ^{1}H NMR studies of **5** in the presence of an excess of the S-nucleophile *N*-acetylcysteine (NAC) revealed that after 72 h only 20% of the lactone had undergone such a Michael addition (cf. Supporting Information). A similar reaction between **1** and NAC was finished after 30 h including the cyclopropane ring-opening to give the aromatic end product as exemplified in Scheme 1. Interestingly, the antiproliferative activity of lactone **5** was significantly enhanced upon its esterification with the 1,1′-metallocenedicarboxylic acids. In contrast to the order of activities of the illudin M esters **2**, the ruthenocene diester **6b** was distinctly more active than the ferrocene diester **6a** in most tested cancer cells. The ruthenocene derivatives **2b** and **6b** were also conspicuously efficacious against HL-60 leukemia cells with **6b** featuring a huge therapeutic index >40 in this cell line.

We also tested the activities of the new illudin M derivatives against HCT-116 ($p53^{-/-}$) cells and found them differing not much from those against the HCT-116 (wt) cells. This means that the growth inhibition of HCT-116 colon cancer cells by the new metallocene illudin M conjugates operates by a p53-independent mechanism, which is typical of the effect of previously studied illudin derivatives in various cancer cell lines.[18] However, irofulven-initiated cell death of certain cancer cells had been found to be mediated by extracellular signal-regulated kinase (ERK) and c-Jun *N*-terminal kinase (JNK) kinases,[19] and so, we checked the influence of selective inhibitors of ERK and JNK on the activity of the compounds **2** and **6** against HCT-116 cells. The efficacy of ferrocene derivative **2a** was most active in the HCT-116 (wt) cells dropped by a factor of 3 upon addition of the JNK inhibitor SP600125 and by a factor of 2 on addition of the ERK inhibitor PD98059 (Tables 1 and 3). This pattern was

Table 1. Inhibitory Concentrations[a] IC_{50} [μM] of Compounds 1, 2, 5, and 6 When Applied to HF and Various Cancer Cells

	compounds					
cell lines	1	2a	2b	5	6a	6b
HF	0.13 ± 0.02[b]	5.3 ± 0.2	5.4 ± 0.1	>100	>100	>100
HCT-116 (wt)	0.01 ± 0.00	0.21 ± 0.03	1.1 ± 0.1	20 ± 1	11 ± 3	6.4 ± 0.4
HCT-116 ($p53^{-/-}$)	0.02 ± 0.01	0.34 ± 0.02	1.2 ± 0.2	27 ± 1	21 ± 1	5.5 ± 0.4
HCT-116 (JNK^-) [d]	0.01 ± 0.00	0.63 ± 0.12	1.4 ± 0.1	39 ± 4	32 ± 2	13 ± 1
HCT-116 (ERK^-)[d]	0.04 ± 0.00	0.42 ± 0.01	0.93 ± 0.03	24 ± 2	28 ± 2	6.7 ± 0.4
HT-29	0.07 ± 0.01	0.78 ± 0.13	2.8 ± 0.1	>50	>50	20 ± 1
MCF-7/Topo	0.01 ± 0.00	0.10 ± 0.02	0.57 ± 0.05	>50	11 ± 1	6.8 ± 2.8
MDA-MB-231	0.06 ± 0.01	0.10 ± 0.02	0.80 ± 0.15	c	c	c
KB-V1/Vbl	0.02 ± 0.00	0.59 ± 0.04	3.2 ± 0.1	>50	>50	>50
518A2	0.03 ± 0.02[b]	0.19 ± 0.03	2.4 ± 0.2	33 ± 2	40 ± 6	24 ± 1
HL-60	0.02 ± 0.01[b]	0.79 ± 0.20	0.87 ± 0.17	32 ± 4	34 ± 1	2.4 ± 0.3
BxPC-3	0.05 ± 0.01	0.13 ± 0.02	2.5 ± 0.2	c	c	c

[a] Values are derived from dose−response curves obtained by measuring the percentage of viable cells relative to untreated controls after 72 h of exposure of test compounds using an MTT assay; human cancer cell lines: HCT-116 colon (wt or $p53^{-/-}$), HT-29 colon, MCF-7/Topo breast, KB-V1/Vbl cervix, 518A2 melanoma, HL-60 leukemia, BxPC-3 pancreas, and MDA-MB-231 breast cancer. Values represent means of four independent experiments. [b] Values taken from ref 19. [c] Not measured. [d] JNK^-: + SP600125; ERK^-: + PD98059.

Table 2. Therapeutic Index IC_{50} (HF)/IC_{50} (Cancer Cells) of Compounds 1, 2, 5, and 6

	compounds					
cell lines	1	2a	2b	5	6a	6b
HCT-116 (wt)	1.3	25.2	4.9	>5	>9	>15
HT-29	1.9	6.8	1.9	a	a	>5
MCF-7/Topo	13	53	9.5	a	>9	>15
MDA-MB-231	2.2	53	6.8	a	a	a
KB-V1/Vbl	6.5	9.0	1.7	a	a	a
518A2	4.3	28	2.3	>3	>2.5	>4.2
HL-60	6.5	6.7	6.2	>3.1	>2.9	>42
BxPC-3	2.6	41	2.2	a	a	a

[a] Nondeterminable.

Table 3. Sensitivity Factors [a] of Compounds 1, 2, 5, and 6 from MTT Assays of Table *1*

cell line/compds	1	2a	2b	5	6a	6b
$p53^{-/-}$	1.5	1.6	1.1	1.3	1.9	0.9
JNK^-	1.1	3.2	1.4	2.0	2.9	2.0
ERK^-	3.5	2.0	0.8	1.2	2.5	1.1

[a] Defined as the ratio of IC_{50} (72 h) values for inhibitor-treated or $p53^{-/-}$ HCT-116 cells to untreated HCT-116(wt) cells.

mirrored by the results for ferrocene derivative **6a** albeit on a lower activity level. The activity of parent compound **1** against HCT-116 cells was diminished by a factor of 4 upon addition of the ERK inhibitor but remained unaltered in the presence of the JNK inhibitor. Possibly, the redox-active ferrocene hub of **2a** and **6a** contributes to the redox-sensitive JNK/ERK-signaling pathway of apoptosis induction. In contrast, complex **2b** did not lose considerably in activity against HCT-116 cells upon JNK/ERK inhibition. The induction of apoptosis in HCT-116 cells was ascertained by mitochondrial membrane potential assays using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzamidazolocarbocyanin iodide (JC-1) (cf. Supporting Information) and confirmed the results obtained from the cytotoxicity assays. The compounds most active in the latter also led to most intense apoptosis and JNK inhibition diminished strongly the apoptosis rate induced by complex **2a**.

Finally, the induction of apoptosis in BxPC-3 and MDA-MB-231 carcinoma cells by the diesters **2** was evaluated in more detail by Histone/DNA ELISA assays. The apoptosis rates in MDA-MB-231 cells thus obtained for **1**, **2a**, and **2b** were distinctly higher than the respective rates in BxPC-3 cells (Figure 1), which again correlates fairly with the respective IC_{50} values of the cytotoxicity assays. For instance, the apoptosis-inducing activity of **2a** in MDA-MB-231 cells was ∼70% greater than that in BxPC-3 cells at doses close to the IC_{50} values. Because sustained JNK/ERK signaling is crucial for apoptosis in MDA-MB-231 breast cancer cells,[20] the results that we obtained from the inhibition of these pathways in HCT-116 colon cancer cells might also go a long way toward explaining the strong impact of **2a** on the MDA-MB-231 cells.

Compounds **2a** and **6b** were also tested in terms of in vivo applicability. Two mice received a single dose of 10 mg/kg body weight of **2a** or **6b**, respectively, and showed no signs of toxicity over the following observation period of 10 days, while we observed that a comparable dose of **1** resulted in intolerable toxicity. Then, the same mice were treated with a single dose of 25 mg/kg body weight of **2a** or **6b**, and again, no signs of toxicity occurred. This is strong evidence for the long-term chemical stability of these esters under physiological conditions. Next, we tested the feasibility of repeated applications to establish a toxicity profile. A third mouse received 4 × 25 mg/kg body weight of compound **2a** weekly for two consecutive weeks. As shown in Figure 2, this schedule was well tolerated and induced only a transient marginal loss of body weight of maximal 8%. No cumulative toxicity became visible, and the mouse quickly regained its original body weight once the treatment was ceased. This profile of a toxicity, which is manageable even for high and repeated doses, advocates further in vivo tests of **2a** with different cancer models.

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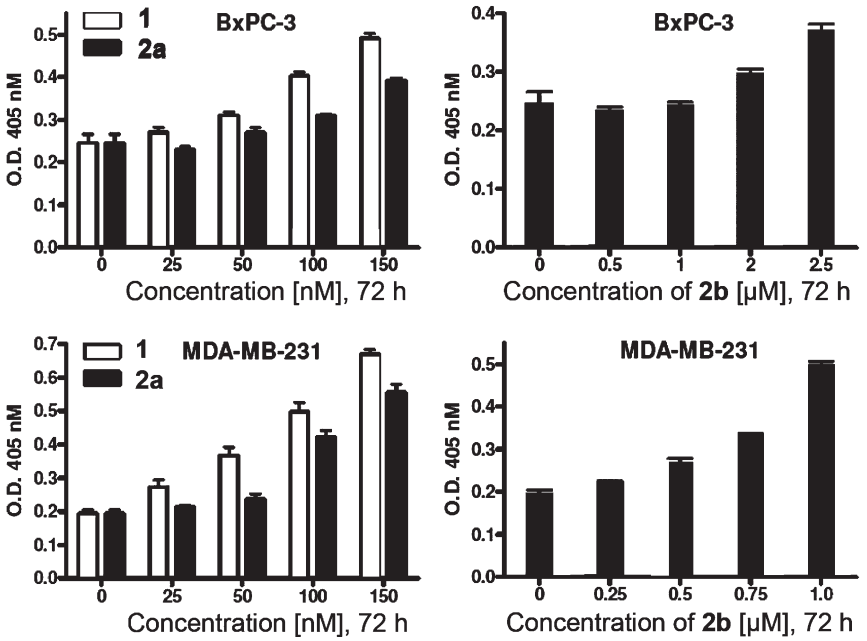

Figure 1. Apoptosis induction by compounds **1**, **2a**, and **2b** in BxPC-3 pancreas and MDA-MB-231 breast carcinoma cells as detected by the histone/DNA ELISA assay.

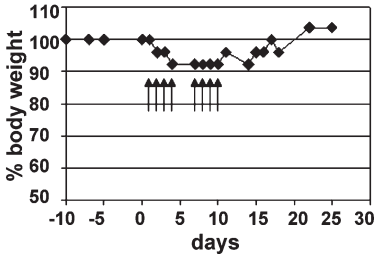

Figure 2. Toxicity profile of **2a** in mouse upon administration of 4 × 25 mg/kg body weight weekly for two consecutive weeks as indicated by arrows.

CONCLUSIONS

The central metal of the new diesters of metallocenedicarboxylic acids with illudin M or its lactone plays a pivotal role regarding the magnitude, selectivity, and mechanism of their anticancer effects. The illudin M ferrocenedicarboxylate **2a** retained submicromolar IC_{50} (72 h) values against all of the eight tested cancer cell lines while being 40 times less toxic than **1** to normal fibroblasts. Averaged over all cancer cell lines the therapeutic index of **2a** is more than five times as great as that of **1** itself. The cytotoxic effect of **2a** on HCT-116 colon cancer cells was shown to be dependent on JNK/ERK signaling. The illudin M ruthenocenedicarboxylate **2b** was less active than the iron anlogue **2a** but still reached submicromolar IC_{50} (72 h) values against the breast carcinoma cell lines MDA-MB-234 and MCF-7/Topo. Its efficacy against HCT-116 cells was not attenuated upon JNK/ERK inhibition.

While esterification of **1** with the metallocenedicarboxylic acids generally lowered the anticancer potential, esterification of these diacids with the largely ineffective illudin M lactone **5** afforded compounds **6** of enhanced antiproliferative efficacy. The ruthenocenedicarboxylate **6b** was generally more active than the iron analogue **6a** and reached single-digit micromolar IC_{50} (72 h) values against HCT-116 (wt), MCF-7/Topo, and HL-60 cells.

Both complexes **2a** and **6b** were tolerated by mice symptom-free at single doses as high as 25 mg/kg body weight, which is evidence for them being chemically stable under physiological conditions. In addition, compound **2a** that had shown a superior in vitro profile also displayed a manageable in vivo toxicity profile when given repeatedly in high doses. This bodes well for further in vivo screenings of its antitumoral activity in various cancer models. It will also be interesting to see whether the ruthenocene derivatives **2b** and **6b** exhibit any antimetastatic effects as is known of other ruthenium complexes.[21−23] First, in vitro "blot rolling" and "wound healing" assays are already underway.

EXPERIMENTAL SECTION

Chemistry. 1,1′-Ruthenocenedicarboxylic acid,[15] Ph_3PCCO,[16] and ferrocene diester **2a**[14] were prepared according to literature. Illudin M (**1**) was isolated from the culture medium of *O. olearius*, obtained from the German Collection of Biological Material (Braunschweig, Germany) as previously described.[24] All tested compounds were >95% pure by elemental analysis.

Bis(illudinyl M) 1,1′-Ruthenocenedicarboxylate (2b). A solution of 1,1′-ruthenocenedioic acid (120 mg, 0.37 mmol) in dry *N*,*N*-dimethylformamide (DMF) (2 mL) was treated with triethylamine (115 μL, 0.80 mmol) and 2,4,6-trichlorobenzoyl chloride (125 μL, 0.80 mmol), and the resulting mixture was stirred at room temperature for 20 min. A solution of **1** (200 mg, 0.80 mmol) and *N*,*N*-dimethylaminopyridine (DMAP) (195 mg, 1.60 mmol) in dry toluene (2 mL) was added, and the mixture was stirred at room temperature for 16 h. Ethyl acetate was added, and the resulting organic mixture was washed with water and dried over Na_2SO_4. The volatiles were removed on a rotary evaporator, and the residue thus obtained was purified by column chromatography on silica gel 60. Yield, 142 mg (0.18 mmol, 50%). R_f 0.47 (ethyl acetate/hexane 1:2). Yellow solid of mp 85 °C.

3-*O*-Acetyl illudin M (3). A mixture of **1** (150 mg, 0.60 mmol), pyridine (5 mL), and acetic anhydride (2.5 mL) was gently stirred for

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16 h. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel 60 to give **3** as a pale yellow oil. Yield, 160 mg (86%); R_f 0.55 (ethyl acetate/hexane 1:2).

***O*-Acetyl Illudin M Lactone (4).** A mixture of **3** (100 mg, 0.35 mmol), dry toluene (2 mL), benzoic acid (5 mg, 0.04 mmol), and Ph_3PCCO (320 mg, 1.05 mmol) was filled in a vial which was sealed, placed in a 300 W CEM Discover microwave oven, and irradiated for 3 h so as to maintain a temperature of 120 °C. The resulting brown residue was cooled to room temperature, concentrated, and purified by column chromatography on silica gel 60 to leave **4** as a yellow oil. Yield, 50 mg (45%); R_f 0.33 (ethyl acetate/hexane 1:4).

Illudin M Lactone (5). A mixture of acetate **4** (100 mg, 0.32 mmol), methanol (2.5 mL), and K_2CO_3 (50 mg, 0.36 mmol) was stirred at room temperature for 1 h and concentrated, and the residue was purified by column chromatography on silica gel 60. Yield, 78 mg (90%); R_f 0.25 (ethyl acetate/hexane 1:2); off-white solid of mp 92 °C.

Bis(illudin M Lactone) 1,1′-Ferrocenedicarboxylate (6a). Analogously to **2b**, compound **6a** (50 mg, 60%) was obtained from 1,1′-ferrocenedicarboxylic acid (28 mg, 0.10 mmol), triethylamine (35 μL, 0.25 mmol), 2,4,6-trichlorobenzoyl chloride (38 μL, 0.25 mmol), lactone **5** (50 mg, 0.20 mmol), and DMAP (50 mg, 0.40 mmol) in dry DMF/toluene (4 mL, 1:1) as an orange-brown solid of mp 88 °C; R_f 0.40 (ethyl acetate/hexane 1:1).

Bis(illudin M Lactone) 1,1′-Ruthenocenedicarboxylate (6b). Analogously to **2b**, compound **6b** (40 mg, 50%) was obtained from 1,1′-ruthenocenedicarboxylic acid (35 mg, 0.10 mmol), triethylamine (35 μL, 0.25 mmol), 2,4,6-trichlorobenzoyl chloride (38 μL, 0.25 mmol), lactone **5** (50 mg, 0.20 mmol), and DMAP (50 mg, 0.40 mmol) in dry DMF/toluene (4 mL, 1:1) as a yellow solid of mp 84 °C; R_f 0.38 (ethyl acetate/hexane 1:1).

Biological Studies. *Cell Lines and Culture Conditions.* BxPC-3 cells and MDA-MB-231 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 100 units/mL of penicillin, and 100 μg/mL of streptomycin in a 5% CO_2 atmosphere at 37 °C. The HL-60 cells were obtained from the German Collection of Biological Material. The human melanoma cell line 518A2 was obtained from the Department of Radiotherapy, Medical University of Vienna, Austria. The KB-V1/Vbl and the MCF-7/Topo cells were obtained from the Institute of Pharmacy of the University Regensburg, Germany, and the colon HT-29 cells, HCT-116 cells and HCT-116 ($p53^{-/-}$) cells from the University Hospital Erlangen, Germany. The HL-60 and the colon cancer cells HT-29 and HCT-116 were grown in Roswell Park Memorial Institute medium 1640 (RPMI-1640) supplemented with 10% FCS, 100 IU/mL penicillin G, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, and 250 μg/mL gentamycine (all from Gibco, Egenstein, Germany). The 518A2 and the KB-V1/Vbl cells were cultured in DMEM containing 10% FCS, 100 IU/mL penicillin G, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, and 250 μg/mL gentamycine. The MCF-7/Topo cells were grown in Eagle's minimal essential medium (EMEM; Sigma) supplemented with 2.2 g/L $NaHCO_3$, 110 mg/L sodium pyruvate, and 5% FCS. The cells were maintained in a moisture saturated atmosphere (5% CO_2) at 37 °C. The HF cells were cultured in DMEM with 10% FCS, 10^7 U/L penicillin, and 10 mg/L streptomycin. They were serially passaged following trypsinization with 0.05% trypsin/0.02% EDTA (PAA Laboratories, Cölbe, Germany). Mycoplasma contamination was routinely monitored, and only mycoplasma-free cultures were used.

Determination of Tumor Cell Growth. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; ABCR) was used to identify viable cells that reduce it to a violet formazan.[25] HL-60 leukemia cells (5×10^5 cells/mL), the adherent 518A2 melanoma, HT-29, HCT-116, KB-V1/Vbl, MCF-7/Topo, and HF cells (5×10^4 cells/mL), and BxPC-3 cells (3×10^3 cells/well) and MDA-MB-231 cells (3×10^3 cells/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 ranging from 0.0001 to 100 μM in dimethylsulfoxide) was continued for 24, 48, or 72 h. Solvent controls were incubated under identical conditions. In the case of BxPC-3 and MDA-MB-231 cells, 25 μL of MTT stock solution, containing 5 mg/mL in phosphate-buffered saline (PBS), was added to a final concentration of 0.05% and incubated for further 2 h at 37 °C. The supernatant was aspirated, and the formazan was dissolved in isopropanol or dimethylsulfoxide (100 μL). The absorbance at 595 nm was measured on an Ultra Multifunctional Microplate Reader (TECAN, Durham, NC). In the case of the other cells, a 5 mg/mL stock solution of MTT in PBS was added to a final MTT concentration of 0.05% (HL-60, 518A2) or 0.1% (HT-29, KB-V1/Vbl, MCF-7/Topo). After 2 h, the precipitate of formazan crystals was redissolved in a 10% solution of sodium dodecylsulfate in dimethylsulfoxide containing 0.6% acetic acid in the case of the HL-60 cells. For the adherent cells, the microplates were swiftly turned, flicked, and blotted to discard the medium prior to adding the solvent mixture. The microplates were gently shaken in the dark for 30 min and left in the incubator overnight to ensure a complete dissolution of the formazan. The absorbance at 570 and 630 nm was measured using an automatic ELISA microplate reader (MWG-BIOTECH). All experiments were carried out at least in triplicate, and the percentage of viable cells quoted was calculated as the mean ± SD with respect to the controls set to 100%. For the inhibition of JNK and ERK HCT-116 cells were incubated for 24 h, and 50 μL of the medium was removed from each well. Then, SP600125 or PD98059 (LC Laboratories) in DMEM was added to reach a final concentration of 20 or 50 μM, respectively, and the cells were incubated for 2 h to enable inhibitor uptake, before the compounds were added, and the procedure was continued as described above.

Histone/DNA ELISA for the Detection of Apoptosis. The Cell Death Detection Kit (Roche, Palo Alto, CA) was used to detect apoptosis in BxPC-3 and MDA-MB-231 cells treated with test compounds as described previously.[26] Briefly, cells were treated with test compounds for 72 h. The cytoplasmic histone/DNA fragments from these cells were extracted and incubated in microtiter plate modules coated with antihistone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments followed by color development with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate for peroxidase. The spectrophotometric absorbance of the samples was determined by using an Ultra Multifunctional Microplate Reader (TECAN) at 405 nm.

Animal Studies. In vivo toxicity and tolerability of compounds were studied in nude mice (Harlan and Winkelmann, Borchen, Germany). All experiments were performed according to institutional guidelines. Two mice were administered a single dose of 10 mg/kg body weight of **2a** or **6b**, respectively, and then observed for 10 days. Then, these mice were treated with a single dose of 25 mg/kg body weight of **2a** or **6b** and were observed. A third mouse received 4 × 25 mg/kg body weight of compound **2a** weekly for two consecutive weeks. The body weight of the mice was assessed 2−3 times weekly and daily while under therapy. For injections, the compounds were formulated in 10% Tween80/10% ethanol/80% normal saline and were administered intraperitoneally.

■ ASSOCIATED CONTENT

Supporting Information. Instruments used, microanalytical and spectroscopic data of all new compounds, ^{1}H NMR monitoring of the reaction of **1** and **5** with NAC, JC-1 assays, and cell cycle analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); DMAP, *N*,*N*-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; EMEM, Eagle's minimal essential medium; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; HF, human foreskin fibroblasts; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide; JNK, c-Jun *N*-terminal kinase; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; PBS, phosphate-buffered saline; RPMI-1640, Roswell Park Memorial Institute medium 1640

REFERENCES

(1) Anchel, M.; Hervey, A.; Robbins, W. J. Antibiotic substances from *Basidiomycetes*. VII. *Clitocybe illudens*. *Proc. Natl. Acad. Sci. U.S.A.* **1950**, *36*, 300–305.

(2) McMorris, T. C.; Anchel, M. The structures of the basidiomycete metabolites illudin S and illudin M. *J. Am. Chem. Soc.* **1963**, *85*, 831–832.

(3) McMorris, T. C.; Yu, J.; Lira, R.; Dawe, R.; MacDonald, J. R.; Waters, S. J.; Estes, L. A.; Kelner, M. J. Structure-activity studies of antitumor agent irofulven (hydroxymethylacylfulvene) and analogues. *J. Org. Chem.* **2001**, *66*, 6158–6163.

(4) Kelner, M. J.; McMorris, T. C.; Beck, W. T.; Zamora, J. M.; Taetle, R. Preclinical evaluation of illudins as anticancer agents. *Cancer Res.* **1987**, *47*, 3186–3189.

(5) Escargueil, A. E.; Poindessous, V.; Soares, D. G.; Sarasin, A.; Cook, P. R.; Larsen, A. K. Influence of irofulven, a transcription-coupled repair-specific antitumor agent, on RNA polymerase activity, stability and dynamics in living mammalian cell. *J. Cell Sci.* **2008**, *8*, 1275–1283.

(6) Jaspers, N. G. J.; Raams, A.; Kelner, M. J.; Ng, J. M. Y.; Yamashita, Y. M.; Takeda, S.; McMorris, T. C.; Hoeijmakers, J. H. J. Antitumor compounds illudin S and irofulven induce DNA lesions ignored by global repair and exclusively processed by transcription- and replication-coupled repair pathways. *DNA Repair* **2002**, 1027–1038.

(7) Kelner, M. J.; McMorris, T. C.; Taetle, R. Preclinical evaluation of illudins as anticancer agents: Basis for selective cytotoxicity. *J. Natl. Cancer Inst.* **1990**, *82*, 1562–1656.

(8) Schobert, R.; Knauer, S.; Seibt, S.; Biersack, B. Anticancer active illudins: Recent developments of a potent alkylating compound class. *Curr. Med. Chem.* **2011**, *18*, 790–807.

(9) Senzer, N.; Arsenau, J.; Richards, D.; Berman, B.; MacDonald, J. R.; Smith, S. Irofulven demonstrates clinical activity against metastatic hormone-refractory prostate cancer in a phase 2 single-agent trial. *Am. J. Clin. Oncol.* **2005**, *28*, 36–42.

(10) Fouda, M. F. R.; Abd-Elzaher, M. M.; Abdelsamaia, R. A.; Labib, A. A. On the medicinal chemistry of ferrocene. *Appl. Organomet. Chem.* **2007**, *21*, 613–625.

(11) Gasser, G.; Ott, I.; Metzler-Nolte, N. Organometallic anticancer compounds. *J. Med. Chem.* **2011**, *54*, 3–25.

(12) Kovjazin, R.; Eldar, T.; Patya, M.; Vanichkin, A.; Lander, H. M.; Novogrodsky, A. Ferrocene-induced lymphocyte activation and antitumor activity is mediated by redox-sensitive signaling. *FASEB J.* **2003**, *17*, 467–469.

(13) Top, S.; Vessières, A.; Leclercq, G.; Quivy, J.; Tang, J.; Vaissermann, J.; Huché, M.; Jaouen, G. Synthesis, biochemical properties and molecular modelling studies of organometallic specific estrogen receptor modulators (SERMs), the ferrocifens and hydroxyferrocifens: evidence for an antiproliferative effect of hydroxyferrocifens on both hormone-dependent and hormone-independent breast cancer cell lines. *Chem.—Eur. J.* **2003**, *9*, 5223–5236.

(14) Knauer, S.; Biersack, B.; Zoldakova, M.; Effenberger, K.; Milius, W.; Schobert, R. Melanoma-specific ferrocene esters of the fungal cytotoxin illudin M. *Anti-Cancer Drugs* **2009**, *20*, 676–681.

(15) Rausch, M. D.; Fischer, E. O.; Grubert, H. The aromatic reactivity of ferrocene, ruthenocene and osmocene. *J. Am. Chem. Soc.* **1960**, *82*, 76–82.

(16) Bestmann, H.-J.; Sandmeier, D. Eine neue Synthese des (Triphenylphosphoranyliden)ketens, seines Thioanalogen und stabiler Propadienylidentriphenylphosphorane. *Chem. Ber.* **1980**, *113*, 274–277.

(17) Schobert, R.; Gordon, G.; Mullen, G.; Stehle, R. Microwave-accelerated Claisen rearrangements of allyl tetronates and tetramates. *Tetrahedron Lett.* **2004**, *45*, 1121–1124.

(18) Poindessous, V.; Koeppel, F.; Raymond, E.; Comisso, M.; Waters, S. J.; Larsen, A. K. Marked activity of irofulven toward human carcinoma cells: Comparison with cisplatin and ecteinascidin. *Clin. Cancer Res.* **2003**, *9*, 2817–2825.

(19) Wang, W.; Waters, S. J.; MacDonald, J. R.; Roth, C.; Shentu, S.; Freeman, J.; Von Hoff, D. D.; Miller, A. D. Irofulven (6-hydroxymethylacylfulvene, MGI-114)-induced apoptosis in human pancreatic cancer cells is mediated by ERK and JNK kinases. *Anticancer Res.* **2002**, *22*, 559–564.

(20) Petterson, F.; Couture, M.-C.; Hanna, N.; Milller, W. H., Jr. Enhanced retinoid-induced apoptosis of MDA-MB-231 breast cancer cells by PKC inhibitors involves activation of ERK. *Oncogene* **2004**, *23*, 7053–7066.

(21) Levina, A.; Mitra, A.; Lay, P. A. Recent developments in ruthenium anticancer drugs. *Metallomics* **2009**, *1*, 458–470.

(22) Lipponer, K. G.; Vogel, E.; Keppler, B. K. Synthesis, characterization and solution chemistry of indazolium *trans*-tetrachlorobis(indazole)-ruthenate(III), a new anticancer ruthenium complex. IR, UV, NMR, HPLC investigations and antitumor activity. Crystal structures of 1-methylindazolium *trans*-tetrachlorobis-(1-methyl-indazole)ruthenate(III) and its hydrolysis product *trans*-monoaqua-trichlorobis(1-methylindazole)-ruthenate(III). *Metal-Based Drugs* **1996**, *3*, 243–260.

(23) Scolaro, C.; Bergamo, A.; Brescacin, L.; Delfino, R.; Cocchietto, M.; Laurenczy, G.; Geldbach, T. J.; Sava, G.; Dyson, P. J. In vitro and in vivo evaluation of ruthenium(II)-arene PTA complexes. *J. Med. Chem.* **2005**, *48*, 4161–4171.

(24) Schobert, R.; Biersack, B.; Knauer, S.; Ocker, M. Conjugates of the fungal cytotoxin illudin M with improved tumour specificity. *Bioorg. Med. Chem.* **2008**, *16*, 8592–8597.

(25) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63.

(26) Ahmad, A.; Banerjee, S.; Wang, Z.; Kong, D.; Sarkar, F. H. Plumbagin-induced apoptosis of human breast cancer cells is mediated by inactivation of NF-kappaB and Bcl-2. *J. Cell. Biochem.* **2008**, *105*, 1461–1471.