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ELECTRONIC SUPPLEMENTARY INFORMATION

Dual Anticancer Activity in a Single Compound: Visible-Light-Induced Apoptosis by an Antiangiogenic Iridium Complex

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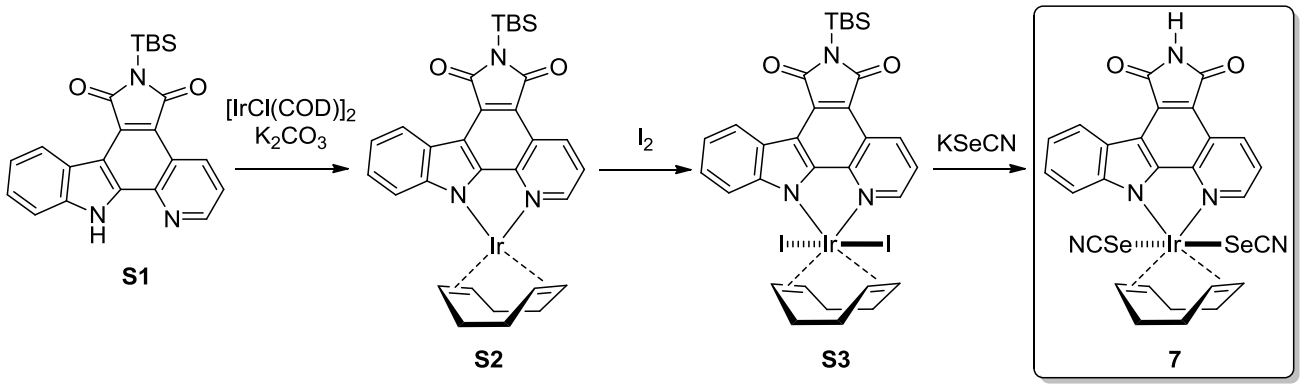
Content:

1. Materials and Methods

All reactions were carried out under a nitrogen atmosphere. Solvents were distilled under nitrogen from calcium hydride (CH_3CN, CH_2Cl_2), magnesium chips (MeOH), or sodium/benzophenone (THF). DMF was used as HPLC grade without further drying. Iodomethane was washed with saturated aqueous solutions of $Na_2S_2O_3$ and $NaHCO_3$ and purified by distillation. All other reagents were purchased from Aldrich or ABCR and used without further purification. Column chromatography was performed with silica gel (230-400 mesh). ^{1}H, ^{13}C, and ^{77}Se NMR spectra were recorded on a Bruker DRX (400 MHz), a Bruker DRX (500 MHz), and a Bruker Avance (500 MHz) spectrometer at ambient temperature. NMR standards used are as follows: (^{1}H NMR) DMSO-d_6 = 2.50 ppm, $CDCl_3$ = 7.26 ppm, CD_2Cl_2 = 5.32 ppm; (^{13}C NMR) DMSO-d_6 = 39.52 ppm, $CDCl_3$ = 77.16 ppm, CD_2Cl_2 = 53.84 ppm; (^{77}Se NMR) external standard was $SeMe_2$ = 0.0 ppm. ^{1}H NMR data are reported as follows: chemical shift in ppm downfield from tetramethylsilane (δ scale), multiplicity (s =singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (Hz), and integration. ^{13}C NMR chemical shifts are reported in ppm downfield from tetramethylsilane (δ scale). ^{77}Se NMR chemical shifts are reported in ppm downfield from $SeMe_2$ (δ scale). IR spectra were obtained on a Bruker Alpha-P series FT-IR spectrometer. High-resolution mass spectra were recorded on a Finnigan LTQ-FT instrument using APCI technique.

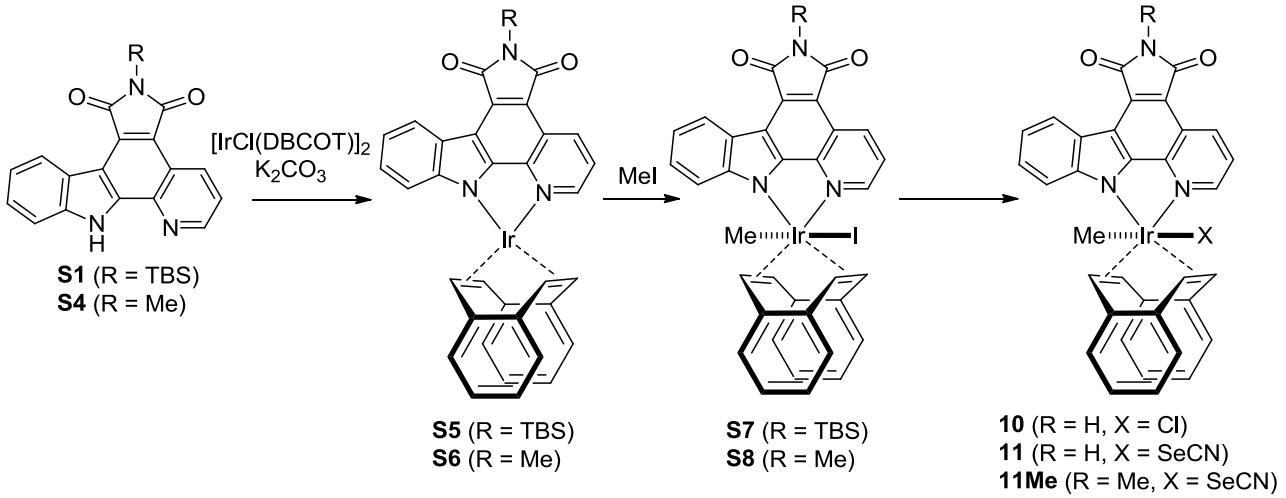
2. Synthesis of Iridium Complexes



Scheme S1. Overview of the synthesis of the iridium(III) complex **7**.

Iridium(III)-complex 7. Diiodo complex **S3** (synthesized according to Scheme S1)[1] (30.0 mg, 31.4 µmol) was dissolved in DMF (4 mL), KSeCN (45.3 mg, 314 µmol) was added and the reaction mixture was stirred at room temperature for 12 h before the solvent was removed under reduced pressure. The resulting red solid was dissolved in acetone, adsorbed onto silica gel and subjected twice to silica gel chromatography (first column: toluene-acetone 4:1, second column: chloroform) to afford complex **7** (14.1 mg, 56%) as a bright orange solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.39 (s, 1H), 9.29 (d, *J* = 8.2 Hz, 1H), 8.77 (d, *J* = 7.8 Hz, 1H), 8.56 (d, *J* = 5.3 Hz, 1H), 8.04 (dd, *J* = 8.4 Hz, *J* = 5.4 Hz, 1H), 7.51 (t, *J* = 7.2 Hz, 1H), 7.38 (t, *J* = 7.5 Hz, 1H), 7.09 (d, *J* = 8.4 Hz, 1H), 6.20-6.18 (m, 2H), 5.42-5.41 (m, 2H), 3.10-3.02 (m, 2H), 2.94-2.87 (m, 2H), 2.73-2.60 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.3, 169.8, 153.5, 148.9, 146.0, 142.8, 136.6, 130.9, 127.4, 125.3, 124.8, 124.0, 121.5, 120.5, 116.8, 116.5, 113.5, 101.9, 90.2, 87.3, 31.6, 29.5. ^{77}Se NMR (76 MHz, $CDCl_3$): δ 233.89. IR (film) ν (cm^{-1}) 3398, 3208, 3042, 2967, 2115, 1755, 1690, 1628, 1587, 1526, 1495, 1422, 1406, 1343, 1227, 1050, 1025, 1006, 821, 743, 709, 641, 499. HRMS (APCI) calcd. for $C_{26}H_{20}IrN_4O_2Se^+$ $(M\text{-}SeCN)^+$ 693.0276, found 693.0375.

[1] A. Wilbuer, D. H. Vlecken, D. J. Schmitz, K. Kräling, K. Harms, C. P. Bagowski, E. Meggers, *Angew. Chem. Int. Ed.* **2010**, *122*, 3928.



Scheme S2. Overview of the synthesis of the iridium(III) complexes **10**, **11**, and **11Me**. Complex **11** has been reported.[2]

Dichloro complex 10. Iodomethane complex **S7**[2] (16.9 mg, 19.9 µmol) was dissolved in THF (4 mL), TBACl (55.4 mg, 199 µmol) was added, and the reaction mixture was stirred at room temperature for 10 min before the solvent was removed under reduced pressure. The resulting red solid was kept at room temperature overnight and afterwards dissolved in CH_2Cl_2, adsorbed onto silica gel and subjected to silica gel chromatography (hexane-ethyl acetate 1:1) to afford complex **10** (11.1 mg, 76%) as a dark red solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.30 (s, 1H), 9.34 (d, *J* = 8.0 Hz, 1H), 8.84 (d, *J* = 5.0 Hz, 1H), 8.79 (d, *J* = 7.9 Hz, 1H), 8.02 (dd, *J* = 8.4 Hz, *J* = 5.3 Hz, 1H), 7.73 (d, *J* = 8.4 Hz, 1H), 7.64 (t, *J* = 7.2 Hz, 1H), 7.44 (t, *J* = 7.5 Hz, 1H), 7.37-7.35 (m, 1H), 7.30-7.28 (m, 1H), 7.22-7.20 (m, 2H), 7.17-7.15 (m, 1H), 7.12-7.10 (m, 2H), 7.06-7.03 (m, 2H), 6.76 (d, *J* = 8.7 Hz, 1H), 6.65 (d, *J* = 8.9 Hz, 1H), 6.27 (d, *J* = 8.9 Hz, 1H), 1.89 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.5, 170.0, 153.3, 146.4, 145.8, 143.3, 142.4, 141.8, 141.7, 141.3, 136.3, 131.3, 128.7, 128.2, 127.5, 127.4, 127.3, 126.7, 126.7, 126.5, 124.6, 123.7, 122.7, 121.6, 120.0, 115.4, 114.8, 113.5, 87.0, 86.4, 84.4, 84.0, 7.3. IR (film) ν (cm^{-1}) 3171, 3021, 2919, 2725, 2257,

[2] L. Feng, Y. Geisselbrecht, S. Blanck, A. Wilbuer, G. E. Atilla-Gokcumen, P. Filippakopoulos, K. Kräling, M. A. Celik, K. Harms, J. Maksimoska, R. Marmorstein, G. Frenking, S. Knapp, L.-O. Essen, E. Meggers, *J. Am. Chem. Soc.* **2011**, *133*, 5976.

1752, 1704, 1523, 1494, 1416, 1341, 1294, 1228, 1148, 1017, 816, 750, 638, 489. HRMS (APCI) calcd. for $C_{34}H_{24}IrClN_3O_2^+$ $(M+H)^+$ 734.1174, found 734.1185.

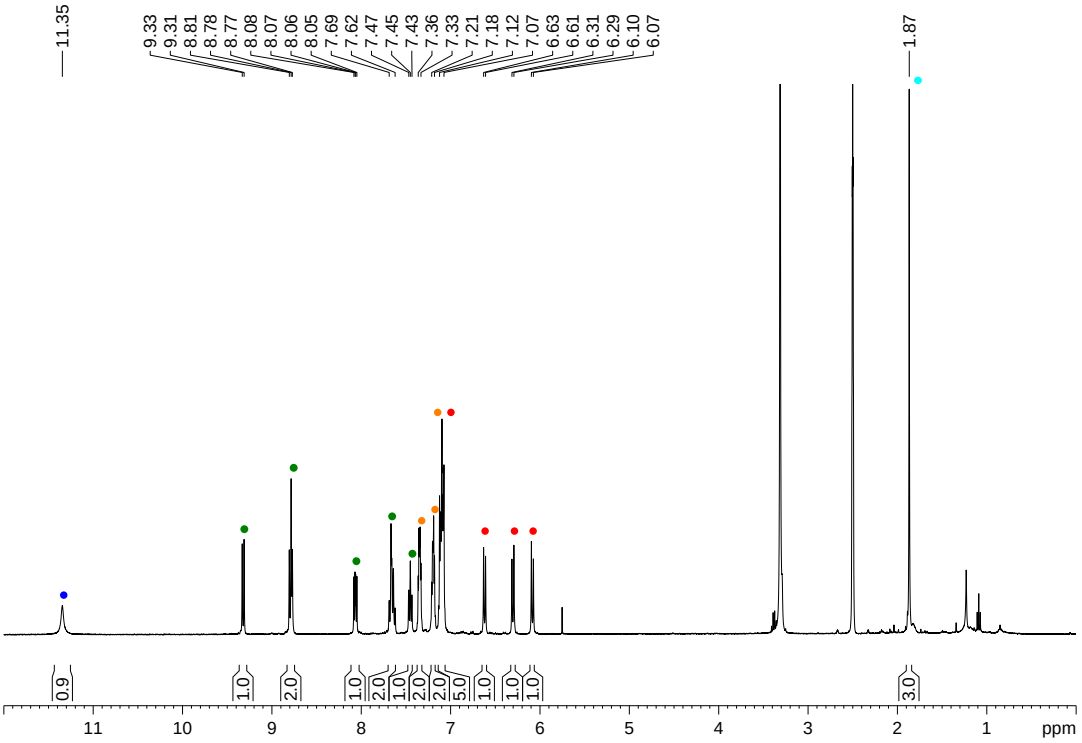
Iridium(III)-complex S8. Pyridocarbazole **S4**[1] (30.0 mg, 99.6 µmol), $[IrCl(DBCOT)]_2$ (47.3 mg, 54.8 µmol) and K_2CO_3 (9.6 mg, 69.7 µmol) were dried *in vacuo* for 30 min and then suspended in MeCN-MeOH (2:1, 5 mL). After 14 h stirring at room temperature the purple suspension was centrifuged (4000 rpm, 3 min, 4 °C) and washed with MeCN-MeOH (2:1, 2 mL) and Et_2O (2 mL) to obtain complex **S6** (63.2 mg, 91%) as a purple solid. Because of solubility issues the crude material was carried on to the next step without further purification. Iridium(I)-complex **S6** (63.0 mg, 90.4 µmol) was suspended in CH_2Cl_2 (18 mL) and iodomethane (200 µL, 3.21 mmol) was added in the dark at room temperature. The reaction mixture was stirred in the dark for 120 h. The solvent was removed under reduced pressure and the crude product was adsorbed onto silica gel and subjected to silica gel chromatography using ethyl acetate-hexane 2:1 as eluent to afford comlex **S8** (46.5 mg, 61%) as an intensive red solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.43 (dd, *J* = 8.3 Hz, *J* = 0.8 Hz, 1H), 9.00 (d, *J* = 7.6 Hz, 1H), 8.30 (d, *J* = 4.5 Hz, 1H), 7.70 (dd, *J* = 8.5 Hz, *J* = 5.3 Hz, 1H), 7.69-7.64 (m, 1H), 7.62 (d, *J* = 8.6 Hz, 1H), 7.54 (d, *J* = 8.3 Hz, 1H), 7.48 (t, *J* = 7.3 Hz, 1H), 7.21-7.18 (m, 2H), 7.13-7.01 (m, 6H), 6.69 (d, *J* = 8.8 Hz, 1H), 6.44 (d, *J* = 8.6 Hz, 1H), 5.55 (d, *J* = 8.8 Hz, 1H), 3.30 (s, 3H), 2.17 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.9, 169.2, 154.1, 147.3, 145.9, 144.1, 143.3, 142.3, 141.5, 139.9, 137.1, 131.7, 128.8, 128.5, 127.9, 127.8, 127.8, 127.1, 126.2, 126.0, 125.9, 124.1, 122.8, 122.6, 121.1, 117.2, 114.9, 112.8, 81.7, 79.8, 79.4, 78.2, 24.1, 18.9. IR (film) ν (cm^{-1}) 3017, 2921, 1755, 1700, 1585, 1496, 1440, 1412, 1378, 1334, 1249, 1230, 987, 744, 672, 611. HRMS (APCI) calcd. for $C_{35}H_{25}IrN_3O_2^+$ $(M-I)^+$ 712.1572, found 712.1569.

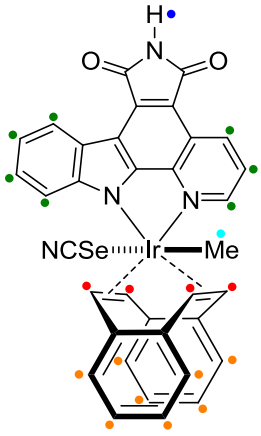
Iridium(III)-complex 11Me. Iridium(III)-complex **S8** (26.0 mg, 31.0 µmol) was dissolved in THF (10 mL), cooled to 0 °C, and $AgBF_4$ (9.1 mg, 46.5 µmol) was added. The reaction mixture was stirred for 5 min at 0 °C before KSeCN (8.0 mg, 62.0 µmol) was added and the suspension was

stirred for 1 h slowly warming up to room temperature. The solvent was removed under reduced pressure and the crude product was adsorbed onto silica gel and subjected to silica gel chromatography using ethyl acetate-hexane 2:1 as eluent to afford complex **11Me** (20.4 mg, 81%) as an intensive red solid. ^{1}H NMR (400 MHz, CD_2Cl_2): δ 9.47 (dd, *J* = 8.4 Hz, *J* = 0.9 Hz, 1H), 8.98 (d, *J* = 8.0 Hz, 1H), 8.18 (dd, *J* = 5.3 Hz, *J* = 0.8 Hz, 1H), 7.90 (dd, *J* = 8.4 Hz, *J* = 5.3 Hz, 1H), 7.68-7.63 (m, 1H), 7.52-7.46 (m, 2H), 7.28-7.19 (m, 4H), 7.14-7.10 (m, 4H), 6.94 (d, *J* = 8.6 Hz, 1H), 6.50 (d, *J* = 8.7 Hz, 1H), 5.99 (d, *J* = 8.8 Hz, 1H), 5.60 (d, *J* = 8.9 Hz, 1H), 3.28 (s, 3H), 1.99 (s, 3H). ^{13}C NMR (100 MHz, CD_2Cl_2) δ 169.9, 169.4, 153.7, 147.3, 143.5, 143.1, 142.7, 142.4, 142.1, 141.1, 137.5, 131.7, 129.1, 128.9, 128.3, 128.3, 127.9, 127.8, 127.7, 127.5, 127.2, 126.3, 124.5, 123.6, 123.2, 121.4, 117.5, 116.1, 112.8, 105.2, 84.6, 84.3, 82.4, 79.7, 24.2, 15.6. ^{77}Se NMR (76 MHz, $CDCl_3$): δ 59.43. IR (film) ν (cm^{-1}) 3061, 3016, 2922, 2111, 1756, 1700, 1586, 1474, 1412, 1379, 1335, 1230, 988, 904, 743, 672. HRMS (APCI) calcd. for $C_{35}H_{25}IrN_3O_2^+$ $(M\text{-}SeCN)^+$ 712.1572, found 712.1575.

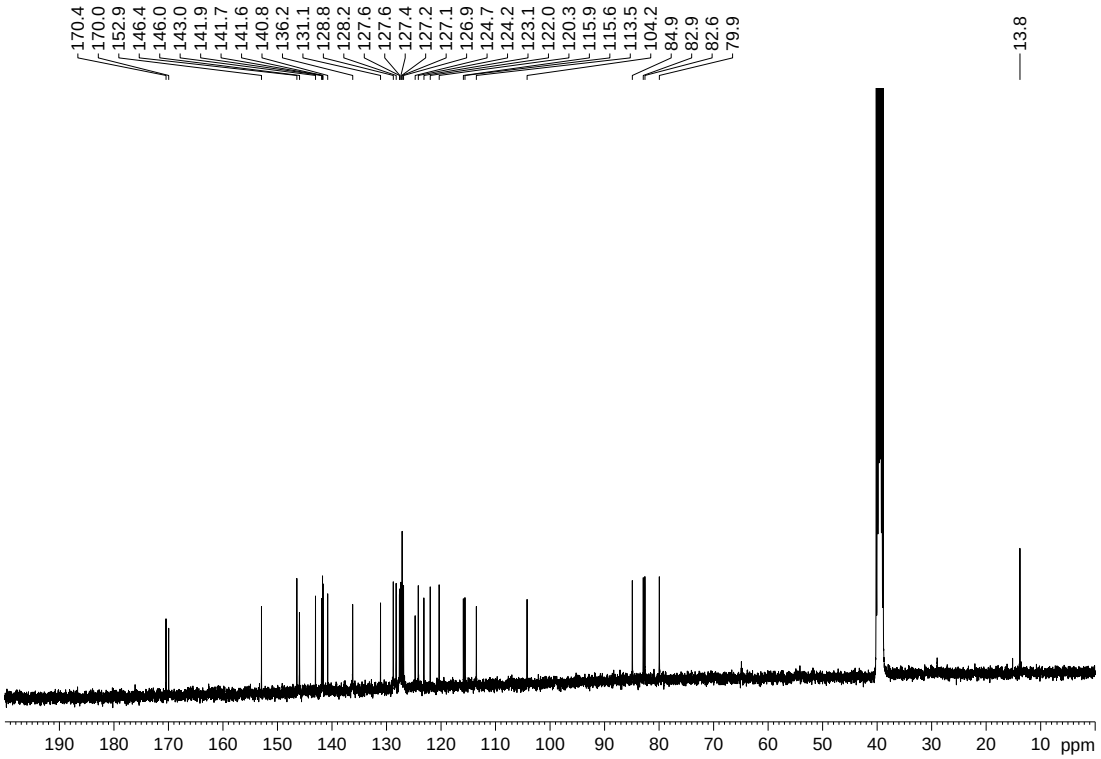
3. Proof of Purity of Iridium Complex 11 and Proton Assignment

^{1}H-NMR of complex **11**:

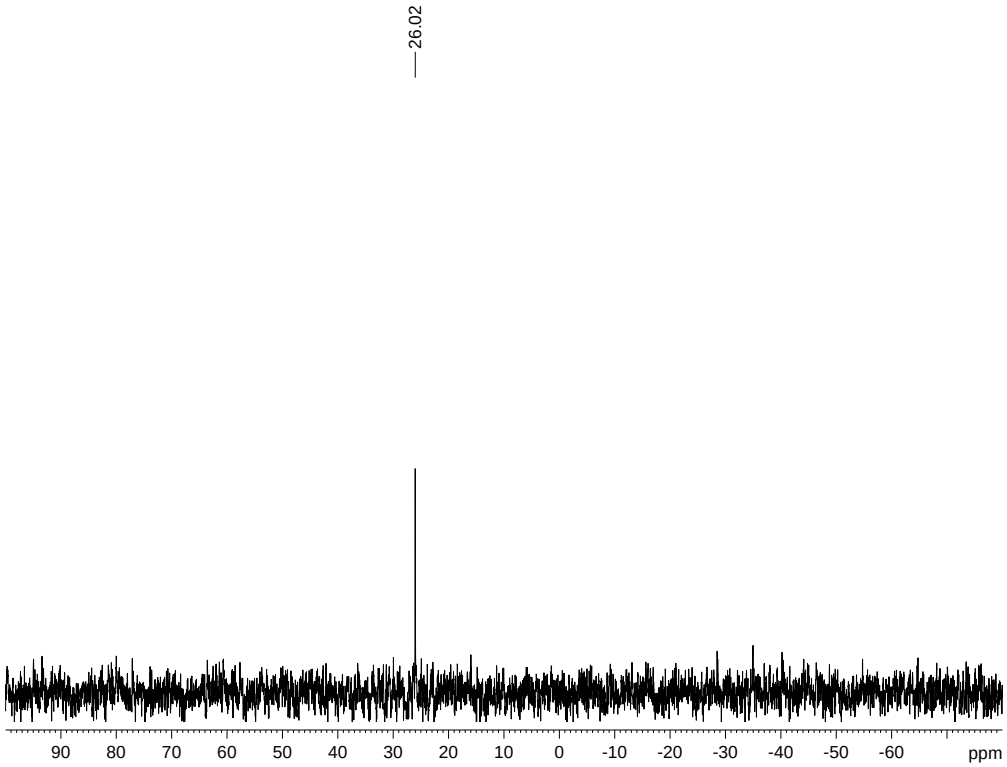




^{13}C-NMR of complex **11**:



^{77}Se-NMR of complex **11**:



HPLC chromatograph of complex **11**:

Complex **11** was eluted with a normal phase silica gel column (Merck Purospher STAR, LiChroCART 250 x 4.6 mm, Si, 5 µm) on an Agilent 1200 Series HPLC system with quaternary pump. Flow rate: 0.75 mL/min; with UV-detection: 520 nm; gradient: 10 min hexanes/acetone 70:30, then over 10 min gradient to hexanes/acetone 50:50.

Result: Purity of complex **11** > 98%.

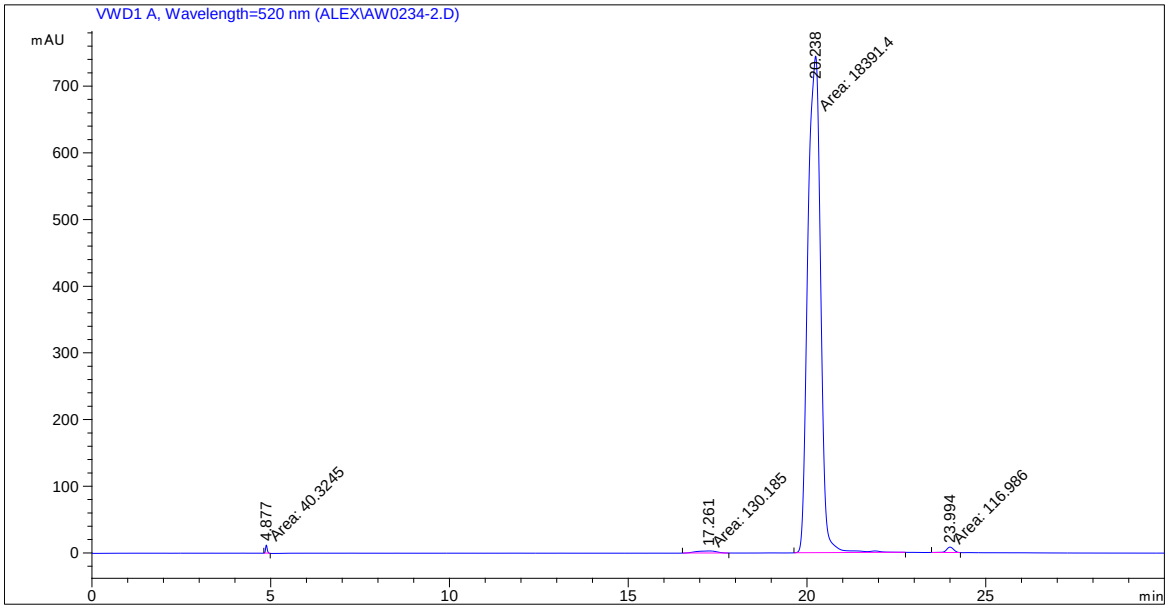


Figure S1. HPLC trace of complex **11**.

4. Biological Experiments

4.1. Protein Kinase Inhibition Properties of Complexes 11 and 11Me

Kinases (VEGFR3, Pim1) and substrates (Jak3tide, S6) were purchased from Millipore, or MoBiTec. Assays for VEGFR3 (human) and Pim1 (human): Various concentrations of inhibitor were incubated at room temperature in 20 mM MOPS/NaOH, 10 mM $Mg(OAc)_2$, 10% DMSO (resulting from the inhibitor stock solution), pH 7.0, in presence of substrate (JAK3tide for VEGFR3: 100 µM, S6 for Pim1: 50 µM) and kinase (VEGFR3: 8.8 nM, Pim1: 1.6 nM) that was diluted with 20 mM MOPS/NaOH, 1mM EDTA, 0.01% Brij-35, 5% glycerol, 0.1% 2-mercaptoethanol and 1mg/mL BSA. After 45 min, the reaction was initiated by adding ATP to a final concentration of 100 µM, including approximately 0.1 µCi/µL [γ-^{33}P]ATP. Reactions were performed in a total volume of 25 µL. After 180 min for VEGFR3 and 45 min for Pim1, the reaction was terminated by spotting 17.5 µL on a circular P81 phosphocellulose paper (diameter 2.1 cm, Whatman) followed by washing four times (five minutes each wash) with 0.75% phosphoric acid and twice with acetone. The dried P81 papers were transferred to a scintillation vial, 5 mL of scintillation cocktail were added and the counts per minute (CPM) determined with a Beckmann Coulter™ LS6500 Multi-Purpose Scintillation Counter. IC_{50} values were defined to be the concentration of inhibitor at which the CPM was 50% of the control sample, corrected by the background. All measurements were performed two times in duplicate.

Table S1. IC_{50} values of **11** and **11Me** against VEGFR3 and Pim1.[a]

Complex	VEGFR3	Pim1
11Me	4.6 µM	>30 µM
11	42 nM[2]	333 nM[2]

[a]Measured at 100 µM ATP.

4.2. Cell Culture Experiments

Human HeLa cells were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 1% of a mixture of penicillin and streptomycin (10000 Units penicillin and 10 mg/mL streptomycin, Sigma-Aldrich). Cells were maintained in 25 cm^2 flasks in a 5% CO_2-humidified atmosphere at 37°C. Passage takes place every 2–3 days.

4.3. Cell Viability Assays

The toxicity of the photoactivated compounds was determined using the MTT cell viability assay. For this purpose the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen) was dissolved in phosphate buffered saline (5 mg/ml) and further diluted in culture medium (1:11). 24 h after the addition of the metal complexes the culture medium with the dissolved inhibitors was removed from the cells and replaced by 200 µL diluted MTT reagent. After 3 h of incubation at 37 °C and 5% CO_2, 155 µL of the dilution were rejected and 90 µL of DMSO were added. To completely dissolve the Formazan salts plates were incubated for 10 min on a shaker and afterwards quantified by measuring the absorbance at 535 nm with a Spectramax M5 microplate reader (Molecular Devices). The cell viability was calculated as percentage of untreated control cells. All indicated values concerning cell viability are average values of 18 individual wells prepared in two independent experiments.

4.4. Determination of Photoinduced Cytotoxicities

For the investigation of photoinduced cytotoxicity of the metal complexes, cells were cultured in 96 well plates in a number of 9000 cells per well. After an initial incubation period of 24 h at 37 °C and 5% CO_2, the designated metal compound was added. For this purpose the inhibitor dissolved in DMSO (Sigma-Aldrich) was first diluted in culture medium to the desired concentration and then transferred into the culture plate. The amount of DMSO was kept constant at 1% for all tests.

Before irradiation the inhibitor treated cells were incubated in the dark for 1 h. Irradiation takes place by a Hg/Xe arc lamp from Newport. To better understand the photoactivity of our compounds we used different light transmission ranges with lower wavelength borders from 330-505 nm. This transmission ranges were determined with sorting filters form Newport. After the irradiation, cells were put back into the incubator until the next day. 24 h after the addition of the complexes, the photoinduced cytotoxicity of the compounds was determined using the MTT cell viability assay.

4.5. Photoinduced Cytotoxicity of Complex 11 after Medium Exchange

In order to investigate the contribution of intracellular compound **11** on the photoactivated cytotoxic effect, the culture medium with the dissolved complex **11** (1 μM) was removed after 1 h of incubation and replaced by fresh medium. Subsequently, the cells were irradiated with light ($\lambda \geq$ 450 nm) for 1 h. Cytotoxicity was determined 24 h after compound administration by the MTT assay which revealed a significant cytotoxicity of complex **11** in the experiment with medium exchange.

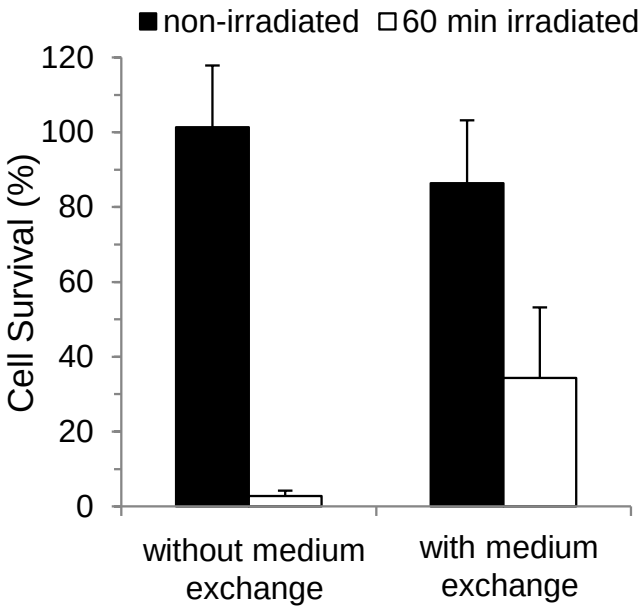


Figure S2. Comparison of photoinduced cytotoxicity of complex **11** with and without medium exchange before irradiation.

4.6. Caspase 3/7 Assays

Measuring the activity of caspases is a current approach to detect apoptosis in cell culture. In this study the activity of the caspases 3 and 7 was determined using the Apo-ONE® Homogeneous Caspase-3/7 Assay from Promega. 18,000 cells/well were cultured on opaque 96 well plates and incubated for 24 h at 37 °C and 5% CO_2. Compound **11** was diluted in culture medium to a concentration of 1 µM with a final DMSO concentration of 1%. At the end of the incubation period the existing culture medium was removed and 100 µl of the diluted complex **11** were added. After 1 h of incubation in the dark samples were irradiated for 45 min ($\lambda \geq 450$ nm). 5 h after the irradiation lysis buffer and the precursor of the fluorescent dye (Z-DEVD-R110) from Promega were added according to the supplier's instruction in a 100:1 mixture. After an incubation of 1 h on an orbital shaker and an additional incubation for 8 h in the dark, fluorescence was detected at the microplate reader (Spectramax M5, Molecular Devices) (Ex 490 nm / Em 525 nm). In the presence of active caspases 3 and 7 the protecting peptides (DEVD) of the non-fluorescent precursor are cleaved releasing the fluorescent leaving group rhodamine 110. The results indicate a nearly 3-fold increase in the fluorescence for cells treated with complex **11** and light compared to the irradiated control without **11**. Because the fluorescence signal is proportional to the caspase activity, these results suggest a significant increase in the activity of these enzymes after addition and photoactivation of **11**. Based on this fact we conclude that the photoinduced cytotoxicity we see for compound **11** is the result of an apoptotic cell response.

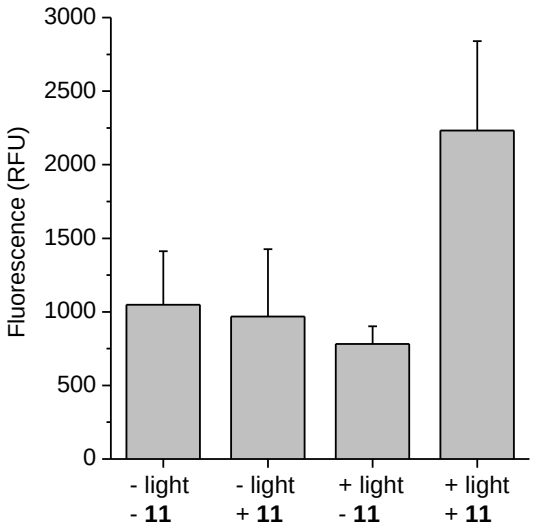


Figure S3. Photoinduced apoptosis with iridium complex **11**. Determination of the activity of the caspases 3 and 7 in HeLa cells 5 h after the addition of 1 µM of inhibitor **11** and the following period of 45 min irradiation at a lower transmission range of 450 nm. Caspase activity was measured by the Apo-ONE® Homogeneous Caspase-3/7 Assay from Promega according to the supplier's instruction. The determined fluorescent signal is proportional to the caspase activity. The illustrated results represent average values of three independent measurements. Error bars indicate standard deviations.

4.7. Propidium Iodide Staining and Flow Cytometry

Apoptosis and cell cycle progression was observed through propidium iodide (PI)-staining of the cells and following flow cytometry. HUVEC cells were plated in twelve-well plates (8 x 10^4 cells per well) with EBM-2 medium (Lonza, Walkersville, USA) and incubated for 24 hours. After treating the cells with 5 µM of **11** or **11Me** for 24 hours, respectively, floating and attached cells were harvested, centrifugated, and resuspended in a hypotonic solution containing 0.1 % sodium citrate, 0.1 % Triton X-100, and 50 µg/mL propidium iodide. Cells were incubated with PI solution for at least 1 hour on ice before analyzing them with Attune acoustic focusing cytometer (Applied Biosystems, Carlsbad, USA).

4.8. 3D-Angiogenesis Assays

Microvascular endothelial cells are known to build 3-dimensional capillary-like structures in a gel-matrix based *in vitro* assay. We took advantage of this ability to demonstrate the anti-angiogenic properties of compound **11**. Human Umbilical Vein Endothelial Cells **(**HUVEC) embedded in a collagen matrix were purchased from PromoCell in a ready-to-use assay kit. These cells that exist as small aggregates of 400-500 cells called spheroids were treated with compound **11** and **11Me** (3 µM or 5 µM) diluted in culture medium and stimulated by the administration of 25 ng/mL VEGF. Afterwards the assay plate was placed in a humidified incubator at 37 °C and 5% CO_2 for 48 h. The concentration of DMSO was 1% for all tests. After 48 h the cumulative sprout length of randomly selected spheroids was determined by light microscopy (Axiovert 200M, Zeiss). The results of the positive and negative control are each based on 10 individual spheroids while the average cumulative sprout length of the samples treated with **11** and **11Me** are based on 24 and 23 evaluated spheroids.

4.9. ROS Experiments

The presence of reactive oxygen species (ROS) after photosensitization was tested by the fluorescent dye carboxy-H_2DCFDA {5-(and-6)-carboxy-2´,7´-dichlorodihydrofluorescein diacetate}. HeLa cells were cultured in a black 96 well plate with optical bottom in a number of 18.000 cells per well. After an initial incubation time of 24 h, the culture medium was replaced by 100 µl phosphate buffered saline (PBS + Ca^{2+}/Mg^{2+}) (Invitrogen) supplemented with 10 µM carboxy-H_2DCFDA (Invitrogen), 1 µM of compound **11** and 1% DMSO. After 30 min, cells were washed twice with PBS and maintained in medium for the following 45 min irradiation at a lower wavelength border of 450 nm. Immediately after irradiation, fluorescence readings were performed with a Spectramax M5 microplate reader (Molecular Devices) at an excitation wavelength of 495 nm and emission wavelength of 529 nm. The non-fluorescent carboxy-H2DCFDA permeates live cells and is deacetylated by nonspecific intracellular esterases. In the presence of nonspecific ROS

the reduced fluorescein compound is oxidized and emits bright green fluorescence. Our measurements exhibited a low fluorescent signal in cells treated with **11** which significantly increased after irradiation. However, the irradiation of cells without **11** showed nearly the same increase which leads to the conclusion that most of the generated ROS is due to the irradiation with light and is not induced by the photoactivation of **11**. Because we know that the irradiation with visible light of $\lambda \geq 450$ nm does not induce cell death, we further conclude that the photoinduced cytotoxicity we detected for **11** has nothing to do with an increased intracellular ROS level.

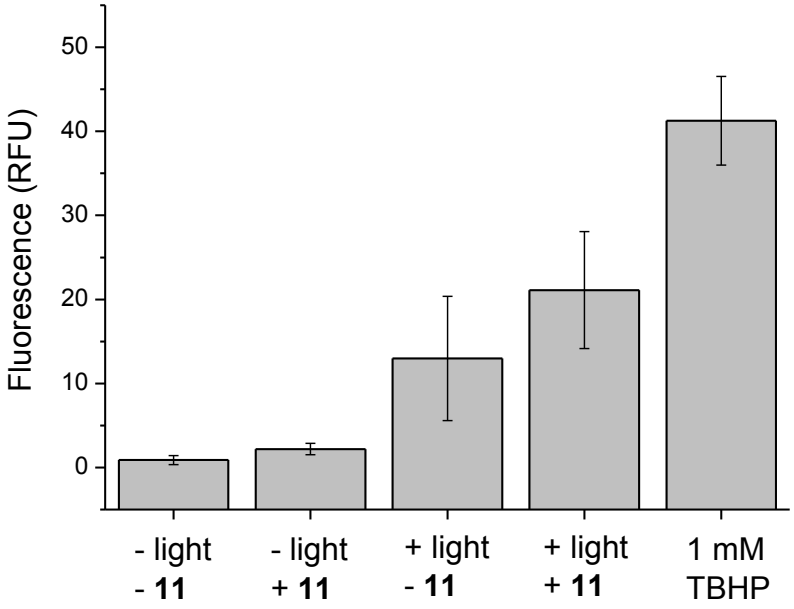


Figure S4. Detection of reactive oxygen species (ROS) in inhibitor treated, light treated, and inhibitor plus light treated HeLa cells. Cells were preincubated with 10 µM carboxy-H_2DCFDA and 1 µM of compound **11** followed by 45 min irradiation ($\lambda \geq 450$ nm). The cells of the negative control were treated with an adequate amount of medium plus 1% DMSO. As a positive control cells were incubated 45 min with 1 mM *tert*-butylhydroperoxide (TBHP). The fluorescent signal of the oxidized reagent carboxy-DCF is proportional to the amount of ROS in the sample. All test conditions were measured six times in quadruplicate. Error bars indicate standard deviations.