

Half-Sandwich Ir(III) and Os(II) Complexes of Pyridyl-Mesoionic Carbenes as Potential Anticancer Agents

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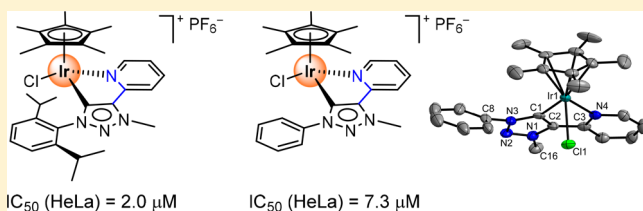
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Supporting Information

ABSTRACT: A series of cationic chlorido arene-iridium(III) and arene-osmium(II) complexes with bidentate pyridyl functionalized mesoionic carbenes (MIC) of the 1,2,3-triazol-5-ylidene type have been prepared. The variations in the ligand structures include the position of the pyridyl substituent relative to the triazolylidene ring (N-wingtip vs C-wingtip), phenyl versus ethyl substituents, and incorporation of several functional groups at the phenyl substituents. Five complexes have been characterized by X-ray structural analysis. All complexes, including osmium(II) and ruthenium(II) analogues having a pyrimidyl in place of the pyridyl group, have been studied for their cytotoxic activity on a human cervical carcinoma HeLa cell line. Two of the compounds, Ir³ and Ir⁹, were the most cytotoxic with IC₅₀ values of 7.33 μM and 2.01 μM, respectively. Examination of their cytotoxic effect on different cell lines revealed that they preferentially kill cancer over normal cells. The Ir⁵ and Ir⁹ compounds arrested cells in G2 and induced a dose-dependent increase in SubG0/G1 cell population. Apoptosis, as the primary mode of cell death, was confirmed by Annexin V/PI staining, detection of cleaved PARP, and caspases 3 and 7 activity upon treatment of HeLa cells with both compounds. The higher toxicity of Ir⁹ is probably due to its increased accumulation in the cells compared to Ir⁵. The role of glutathione (GSH) in the protection of cells against Ir⁵ and Ir⁹ cytotoxicity was confirmed by pretreatment of cells either with buthionine sulfoximine (inhibitor of GSH synthesis) or N-acetyl-cysteine (precursor in GSH synthesis).



INTRODUCTION

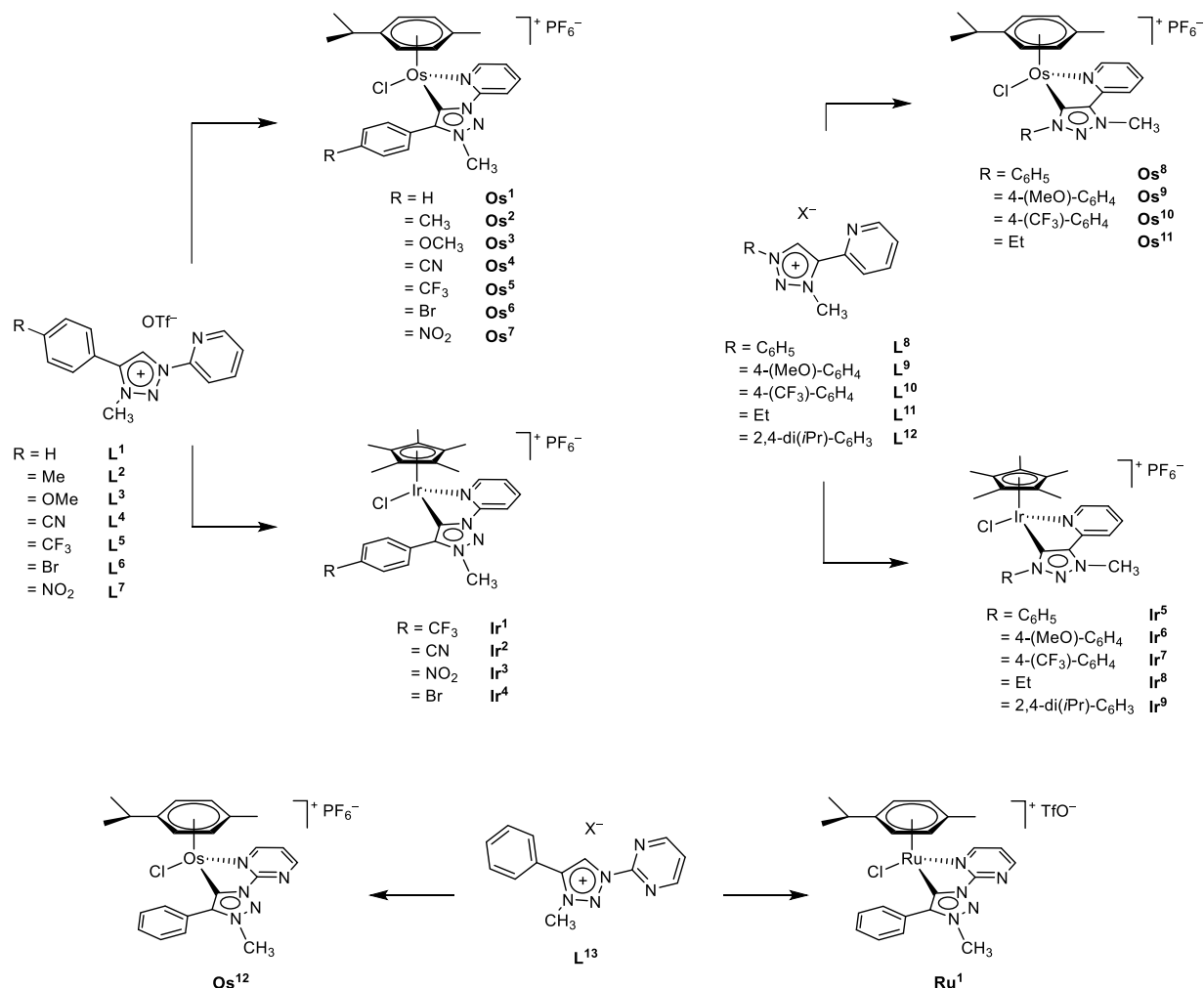
Cancer is one of the leading causes of death worldwide, treated mostly by surgery, radiotherapy, and chemotherapy.¹ Despite a broad use of well-known metal-based anticancer drugs, such as cisplatin and its derivatives, two main disadvantages of chemotherapy remain a problem in successful treatment of a variety of tumor types. Chemotherapeutics are largely inefficient against drug-resistant tumors and are followed by severe side effects including nephrotoxicity, hepatotoxicity, ototoxicity, and cardiotoxicity.² This urges a need for investigation of new compounds that will be highly toxic against tumor cells and nontoxic for the healthy cell population.

Recently, organometallic compounds have gained interest as anticancer agent candidates, owing to their increased structural variety, diverse stereochemistry, and modular way of ligand selection compared to organic molecules. Despite possessing a carbon–metal bond, these compounds are often kinetically stable, uncharged, and relatively lipophilic. Their metal atoms can be in a low oxidation state, making them suitable for application in medicinal chemistry.³

Mesoionic carbenes (MIC) of the 1,2,3-triazol-5-ylidene type are currently highly popular ligands in organometallic chemistry.^{4,5} Apart from the fact that their use as a ligand class is barely 10 years old, an important reason for their popularity is their modular synthesis by Click-reaction. Thus, introducing additional heteroatom donors on such ligands and tuning of the steric and electronic properties is often easier compared to the most other classes of N-heterocyclic carbenes (NHC). Hence, metal complexes of such ligands were successfully used in a variety of homogeneous catalytic processes. Additionally, metal complexes of these ligands have been successfully investigated for their intriguing redox properties, such as electrocatalysts, and for their photochemical properties. One field of research, where metal complexes of MICs are slowly but surely displaying promise, is their potential use in anticancer research.^{5,6} This is perhaps not that surprising considering the potential that metal complexes of various other NHC ligands have shown in medicinal chemistry. Recently, we

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Scheme 1. Synthesis of Os(II), Ir(III), and Ru(II) Complexes



have shown that 1,2,3-triazolium salts, precursor for the 1,2,3-triazol-5-ylidene MIC ligands, also possess anticancer activity.⁷

In the past years, we have been involved in the synthesis of pyridyl-substituted MIC ligands and their metal complexes, which are potent catalysts for a variety of homogeneous catalytic processes.⁸ An important discovery in terms of ligand synthesis was the development of methodologies for selective alkylation of pyridyl-triazoles.⁹ Herein, we report on the synthesis of a series of Os(II) and Ir(III) complexes, as well as one Ru(II) analogue with pyridyl-MICs. We present a comprehensive study involving 12 Os(II), 8 Ir(III) complexes, and 1 Ru(II) complex, along with their cytotoxic properties. The effects of introduced ligands and metal centers on cytotoxic properties as well as their mechanisms of action in cells are discussed.

RESULTS AND DISCUSSION

Synthesis and Characterization. The ligand precursors, triazolium salts, were synthesized following procedures that were recently reported by us.^{9,10} The variations in ligand precursor structures of L¹–L¹² include the position of the pyridyl substituent (N vs C-wingtip of the triazolylidene ring), phenyl versus ethyl substituents, and incorporation of several functional groups on the phenyl substituents (Scheme 1). Triazole rings in L¹–L⁷ are characterized by the N1-pyridyl substituent (N-wingtip), whereas those in L⁸–L¹² have the

chelating pyridine at C5 (C-wingtip). Ligand precursors L¹–L¹² were used for the synthesis of osmium(II) (Os¹–Os¹²) and iridium(III) half-sandwich complexes (Ir¹–Ir⁹). Transmetalation protocol⁸ with Ag₂O turned out to be the route of choice affording the products from moderate 50% yields to higher. Additionally, in the particular case of ligand precursor L¹³, pyrimidyl was used as a heterocycle (N-wingtip) instead of pyridyl to construct complexes Os¹² and Ru¹.

The complexes were characterized via ¹H and ¹³C NMR spectroscopy and mass spectrometry. The disappearance of the signal corresponding to the triazolium C–H proton in the ¹H NMR spectrum of the complexes was already a first indication of the formation of the complexes. In the ¹³C NMR spectra, the peak corresponding to the metal-bound MIC-C was observed between 159.6 and 160.9 ppm for the Os(II) complexes and between 156.3 and 158.4 ppm for the Ir(III) complexes. Molecular peaks corresponding to the cations were observed for all the complexes in their HRESI mass spectra.

Crystal Structures. We were successful in obtaining single crystals of compounds Os⁸, Os¹¹, Ir⁵, Ir⁶, and Ir⁷ that were suitable for X-ray diffraction studies (Figure 1). The two osmium complexes and Ir⁶ crystallized as dichloromethane solvates. All the metal centers were coordinated in a piano-stool type of coordination. The Cp* ligands were bound in an η⁵ way to the Ir(III) centers, and the cymene ligands were bound in an η⁶ fashion to the Os(II) centers. The three “legs”

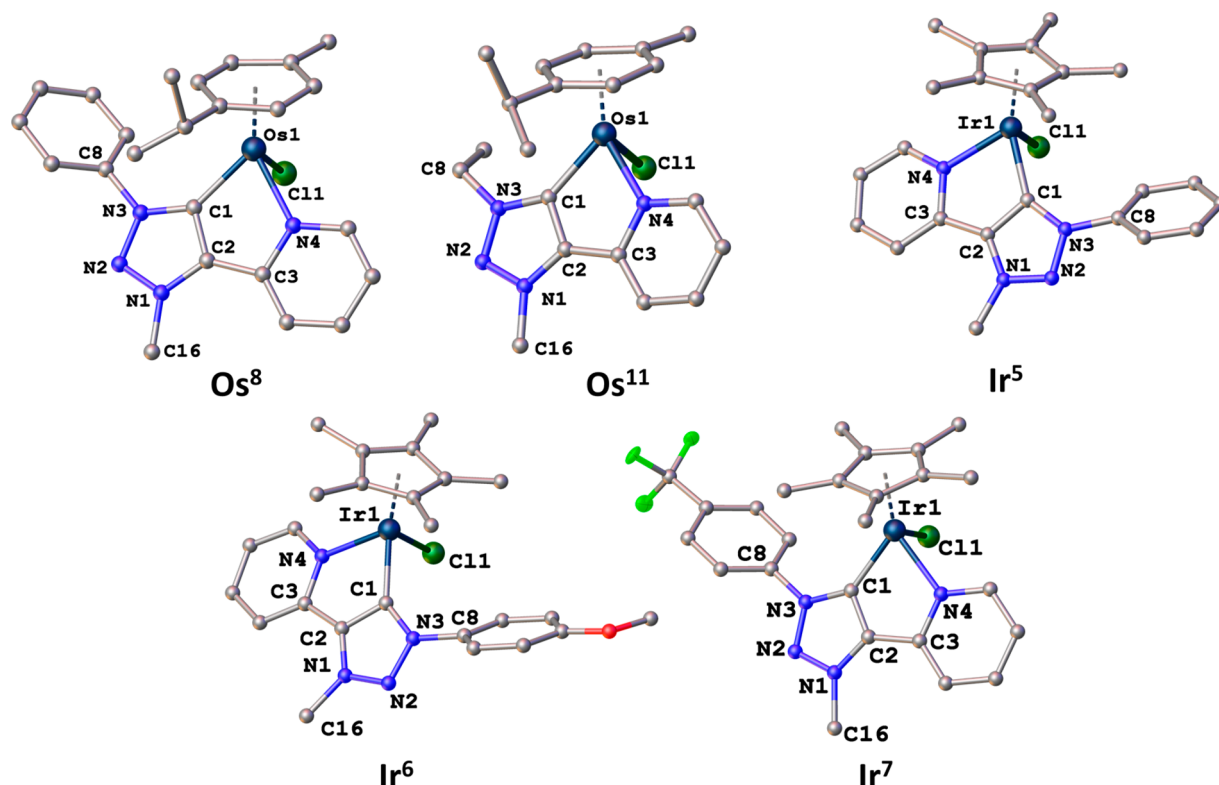


Figure 1. Ball and stick models of Os^8 , Os^{11} , Ir^5 , Ir^6 , and Ir^7 . Hydrogen atoms, counterions, and solvent molecules are omitted for clarity. Selected bond lengths and angles are reported in Table 1.

of the piano-stool are then made up of the pyridyl-N and MIC-C of the chelating ligands and an additional chloride ligand. As it has been observed earlier, the bond lengths inside the triazolylidene rings point to a delocalized situation (Table 1). The metal-C(MIC) and the metal-N(pyridine) distances are all in the expected range.^{4c,e,f} The aryl substituents on the triazolylidene rings are twisted out of plane as seen from angles close to 50° between the two rings (Table 1).

Effect of the Complexes on Cell Viability. This work was stimulated by recent reports on cytotoxic properties of the ligand precursors, 1,2,3-triazolium salts,⁷ as well as organometallic compounds of triazolylidene structure.^{5,6} Some triazolium salts exhibited high cell-type dependent cytotoxicity against different tumor cells. Those did not bind double stranded DNA, but induced formation of reactive oxygen species, which further triggered cell death.⁷ An excellent activity profile against a range of cell lines has been shown for half-sandwich Ru(II) and Os(II) organometallics having triazolylidene as a monodentate MIC ligand.^{6a} The structures of the half-sandwich $\text{Os}(\text{II})$, $\text{Ir}(\text{III})$, and $\text{Ru}(\text{II})$ compounds Os^1 – Os^{12} , Ir^1 – Ir^9 , and Ru^1 are distinctly different from the compounds mentioned above, possessing bidentate pyridyl-triazolylidene ligand that chelates the metal in $N(\text{pyridine})^{\wedge}\text{C}(\text{MIC})$ mode. In Os^{12} and Ru^1 the pyridyl dent is replaced by pyrimidyl.

First, all the complexes were evaluated for their cytotoxicity potential on human cervical carcinoma (HeLa) cells, the cell model system that was previously identified as suitable for the screening of new compounds.^{11,12} The results of the screening are collected in Table 2.

Cytotoxicity of complexes Os^1 , Os^3 – Os^5 , Os^7 , Os^8 , Os^{11} , Ir^1 – Ir^3 , Ir^8 , and Ru^1 could not be determined due to their poor solubility. Compound Ir^9 with IC_{50} of $2.01 \mu\text{M}$ was the

Table 1. Bond Lengths (\AA), Angles, and Dihedral Angles (deg)

atoms and planes	Os^8	Os^{11}	Ir^5	Ir^6	Ir^7
<i>Bond lengths</i>					
M1–Cl1	2.40(1)	2.401(2)	2.40(1)	2.40(1)	2.39(1)
M1–C1	2.02(1)	2.040(6)	2.03(1)	2.01(1)	2.01(1)
M1–N4	2.15(1)	2.124(4)	2.13(1)	2.12(1)	2.14(1)
C1–C2	1.38(2)	1.398(7)	1.39(1)	1.38(1)	1.34(1)
C2–C3	1.45(2)	1.430(8)	1.44(1)	1.44(1)	1.47(1)
C2–N1	1.36(2)	1.358(7)	1.35(1)	1.35(1)	1.36(1)
N1–N2	1.32(2)	1.323(6)	1.32(1)	1.31(1)	1.30(1)
N2–N3	1.33(1)	1.343(6)	1.36(1)	1.35(1)	1.34(1)
N3–C1	1.35(1)	1.353(7)	1.36(1)	1.36(1)	1.38(1)
N1–C16	1.45(2)	1.462(7)	1.47(1)	1.47(1)	1.47(1)
N3–C8	1.46(1)	1.465(7)	1.44(1)	1.44(1)	1.44(1)
C3–N4	1.38(2)	1.385(7)	1.38(1)	1.37(1)	1.36(1)
M1–center	1.70(1)	1.699(1)	1.82(1)	1.82(1)	1.83(1)
<i>Bond angles</i>					
C1–M1–N4	75.9(4)	76.4(2)	76.7(3)	76.3(2)	75.9(4)
C1–M1–Cl1	85.9(3)	85.4(2)	88.2(2)	89.7(2)	87.9(3)
N4–M1–Cl1	84.8(3)	84.6(1)	85.6(2)	87.4(2)	86.8(2)
N3–C1–C2	102.9(1)	105.5(5)	102.3(6)	102.9(5)	102.0(8)
<i>Dihedral angles</i>					
Tz–Py	5.9(4)	2.0(1)	7.4(2)	8.5(3)	10.2(3)
Tz–Ar	48.1(5)	–	44.1(2)	53.0(3)	56.4(3)

most toxic followed by Ir^5 (IC_{50} of $7.33 \mu\text{M}$). Both compounds are Cp^* –iridium complexes of very similar structure having

Table 2. Effect of the Compounds on Viability of HeLa Cells^a

compound	IC ₅₀ (μM)
Os ¹	nd
Os ²	33.01 ± 6.34
Os ³	nd
Os ⁴	nd
Os ⁵	nd
Os ⁶	24.42 ± 5.56
Os ⁷	nd
Os ⁸	nd
Os ⁹	89.41 ± 13.80
Os ¹⁰	100.88 ± 11.61
Os ¹¹	nd
Os ¹²	52.81 ± 5.97
Ir ¹	nd
Ir ²	nd
Ir ³	nd
Ir ⁴	28.52 ± 8.56
Ir ⁵	7.33 ± 0.28
Ir ⁶	17.52 ± 0.64
Ir ⁷	45.02 ± 3.07
Ir ⁸	nd
Ir ⁹	2.01 ± 0.28
Ru ¹	nd

^and = not determined due to insufficient solubility.

the N-wingtip ligand series, while only differing in the phenyl moiety being unsubstituted in Ir⁵ and 2,6-diisopropyl substituted in Ir⁹.

On the basis of the results from Table 2, compounds Ir⁵ and Ir⁹ were selected for screening on tumor cells of different origins and one normal cell line. These included HeLa cells, laryngeal carcinoma (HEp2) cells, large cell lung carcinoma (H460) cells, colorectal carcinoma (HCT-116) cells, ovarian cancer (MES-OV) cells, and normal primary keratinocytes. The results collected in Table 3 demonstrate similar toxicity of

Table 3. Effect of Ir⁵ and Ir⁹ on Viability of Different Cell Lines

cell line ^a	Ir ⁵ (IC ₅₀ (μM)) ^b	Ir ⁹ (IC ₅₀ (μM)) ^b
HeLa	7.33 ± 0.28	2.01 ± 0.28
HEp2	13.30 ± 1.20	4.66 ± 0.59
H460	6.00 ± 0.90	3.2 ± 0.89
HCT-116	6.50 ± 0.81	3.02 ± 0.17
MES-OV	7.6 ± 0.86	4.08 ± 0.5
keratinocytes	<100	<100

^aHeLa = cervical carcinoma cells; HEp2 = laryngeal carcinoma cells; H460 = large cell lung carcinoma cells; HCT-116 = colorectal carcinoma cells; MES-OV = ovarian cancer cells; Keratinocytes = normal primary keratinocytes. ^bThe data are shown as mean values of three experiments (±SD).

both compounds on all tested tumor cell lines independent from their origin, with Ir⁹ being consistently more cytotoxic than Ir⁵. One of the most important features of a compound to be considered as potential anticancer agent is selectivity, i.e., as high as possible cytotoxicity against tumor as compared to the nontumor cells. It is noteworthy that all examined tumor cell lines proved to be considerably more sensitive to both compounds as compared to the normal cell line, normal

primary keratinocytes (Table 3). The therapeutic index was not calculated since the IC₅₀ doses of both compounds in keratinocytes could not be determined.

Cell Cycle and Cell Death Analysis. To shed light into the mode of action that underlies the antiproliferative or/and toxic activity of Ir⁵ and Ir⁹, we first investigated their effect on the cell cycle. The flow cytometric analysis upon propidium iodide staining of cells treated with different concentrations of Ir⁵ and Ir⁹ during 72 h (Figure 2A) or with 7 μM Ir⁵ and 2 μM Ir⁹ during 24–72 h (Figure 2B) was performed. It seems that both compounds tend to arrest cells in the G2 phase of the cell cycle, and push the cells further into cell death. But, it is interesting to notice that IC₅₀ dose of Ir⁵ induces accumulation of cells in the S phase of cell cycle, which is not a case for IC₅₀ of Ir⁹. The S phase arrest implies problems with DNA replication that triggers a “checkpoint”—a cascade of signaling events that puts the phase on hold until the problem is resolved. It seems that these cells are unable to progress through S phase when the checkpoint is inhibited and often enter directly into premature mitosis.¹³ Further investigation is needed to understand why this effect is seen only upon Ir⁵ treatment and not after the treatment with Ir⁹. The SubG0/G1 cell population, which is measured in cell cycles analysis as well, is characterized with a lower amount of DNA. The cells that entered apoptosis from the G2/M phase of the cell cycle or have lost DNA for any other reason, e.g., death by some other form of oncosis, will appear in the SubG0/G1 region. The Ir⁵ and Ir⁹ induce dose- and time-dependent increase of SubG0/G1 cell population (Figure 2A and 2B).

To shed more light on the type of cell death, HeLa cells were treated with Ir⁵ (7 μM) or Ir⁹ (2 μM), following the Annexin V-FITC and PI staining 24–72 h after. The analyzed data shown in Figure 3A indicated that the majority of cells die by apoptosis, which we further confirmed by detection of cleaved PARP, a well-accepted apoptotic marker,¹⁴ in the treated cells. As shown in Figure 3B, the treatment of HeLa cells with 7 μM of Ir⁹ induced cleavage of PARP already 24 h after the treatment. Due to the lower toxicity, the cleaved PARP was not detected upon cell treatment with the same concentration of Ir⁵ (7 μM). However, when the equitoxic doses of Ir⁵ (33 μM) and Ir⁹ (10 μM) were used the cleavage of PARP was detected for both compounds after 72 h treatment (Figure 3C). In addition, 72 h upon cell treatment with 7 μM and 33 μM of Ir⁵, and 2 μM and 10 μM of Ir⁹, the activity of executor caspases 3 and 7 was measured. The results showed that both compounds activated caspases 3/7, although the activation was more visible upon Ir⁵ treatment (Figure 3D). It is likely that Ir⁹ induces the activity of other caspases such as caspase 1.¹⁵ The possible involvement of autophagy and necroptosis as cell death models was checked by pretreatment of cells with bafilomycin A (BAF A), a known inhibitor of the late phase of autophagy,¹⁶ and with the inhibitor of necroptosis, necrostatin-1 (Nec-1).¹⁷ Afterward, the cells were treated with different concentrations of either Ir⁵ or Ir⁹. Both inhibitors failed in protection of the cells from cell death, indicating that neither autophagy nor necroptosis are involved in cell death induced by Ir⁵ or Ir⁹ (Figure 3E).

One of the possibilities for higher toxicity of Ir⁹ compared to Ir⁵ could be increased accumulation of Ir⁹ in the cells due to the small structural difference. In order to test this hypothesis, we treated HeLa cells with different concentrations of both compounds during 3 h and measured the accumulation of iridium by high resolution inductively coupled plasma mass

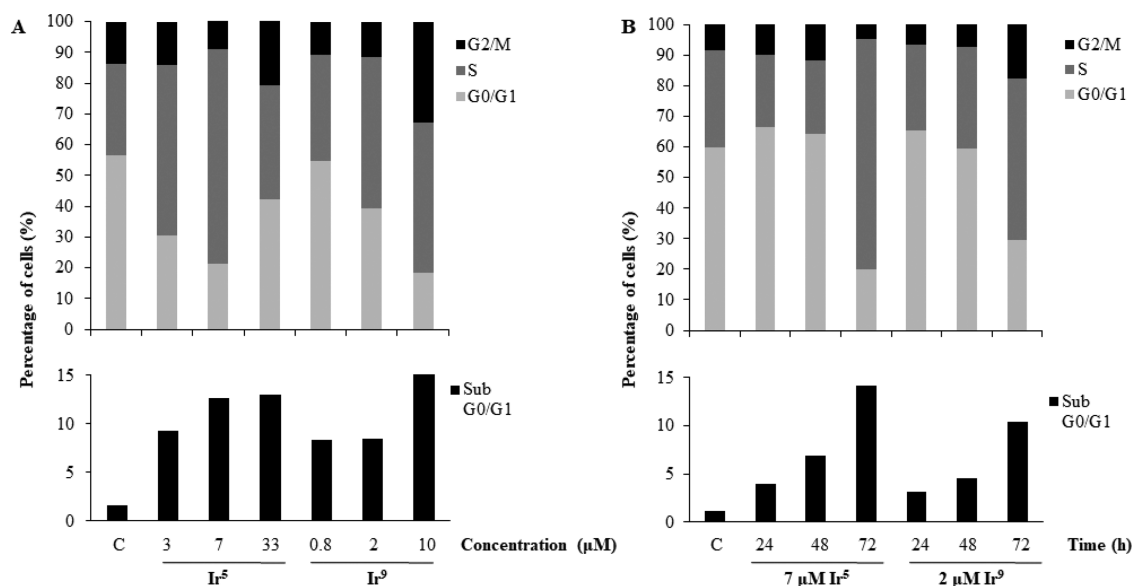


Figure 2. Effects of Ir⁵ and Ir⁹ on cell cycle. HeLa cells were treated either with different concentrations of Ir⁵ and Ir⁹ during 72 h (A) or with equitoxic doses of Ir⁵ (7 μM) and Ir⁹ (2 μM) during 24–72 h (B). A cell cycle distribution was assessed as described in the Experimental Section. Data of one from three independent experiments are presented.

spectrometry (HR ICPMS). The accumulation of both compounds in the cells increased with the time, but the content of iridium upon Ir⁹ was higher than the one measured upon Ir⁵ treatment (Figure 4). The higher level of Ir⁹ in the cell, compared to the level of Ir⁵, could be one of the reasons for its greater toxicity.

The Role of Glutathione (GSH) in Cell Response to Ir⁵ and Ir⁹. The role of GSH as a cell defense mechanism is well described in the literature.^{18,19} Previously, we described GSH as an important factor in the protection of cell upon induction of reactive oxygen species (ROS),^{20,21} and upon treatment with newly synthesized¹² or newly isolated compounds.²² Recently, we identified a triazolium salt of 4-(4-methoxyphenyl)-3-methyl-1-(2-picolyl)-1*H*-1,2,3-triazolium hexafluorophosphate(V) structure, which induced ROS and showed that GSH is involved in reduction of ROS-induced toxicity.⁷ Here we show that pretreatment of HeLa cells with buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis, decreased cell survival upon treatment with Ir⁵ and Ir⁹ (Figure 5A). The data imply a role of GSH in cell defense against damages induced by both investigated compounds. The important role of GSH in cell-stress protection was further confirmed by pretreatment of cells with *N*-acetyl-cysteine (NAC), a precursor of GSH synthesis. Increased GSH amount in the cell protects it against either Ir⁵ or Ir⁹ toxicity (Figure 5B). The protection role of GSH is more expressed in HeLa cells upon Ir⁹ treatment as compared to Ir⁵. In order to investigate the possibility of ROS-induced toxicity by Ir⁵ and Ir⁹, we stained cells upon treatment with fluorescent dye 5-(and-6)-chloromethyl-29,79-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA). The data obtained indicate that neither Ir⁵ nor Ir⁹ induce ROS (Figure 5C). This was further confirmed by pretreatment of cells with a well-accepted ROS scavenger tempol, which did not change survival rate of cells compared to the one treated only with compounds Ir⁵ and Ir⁹ (Figure 5D). It is known that GSH has a role in other physiological processes such as nutrient metabolism, defense by detoxification, and regulation of cellular metabolic functions ranging from gene expression, DNA, and protein synthesis to

signal transduction, cell proliferation, and apoptosis.²³ Previously, we showed importance of GSH detoxification role in cell defense against toxicity of several compounds.²⁴ Elucidating the mechanisms through which GSH is involved in defense against chlorido arene-iridium(III) complexes with bidentate pyridyl functionalized mesoionic carbenes (MIC) of the 1,2,3-triazol-5-ylidene type will be interesting for future investigation.

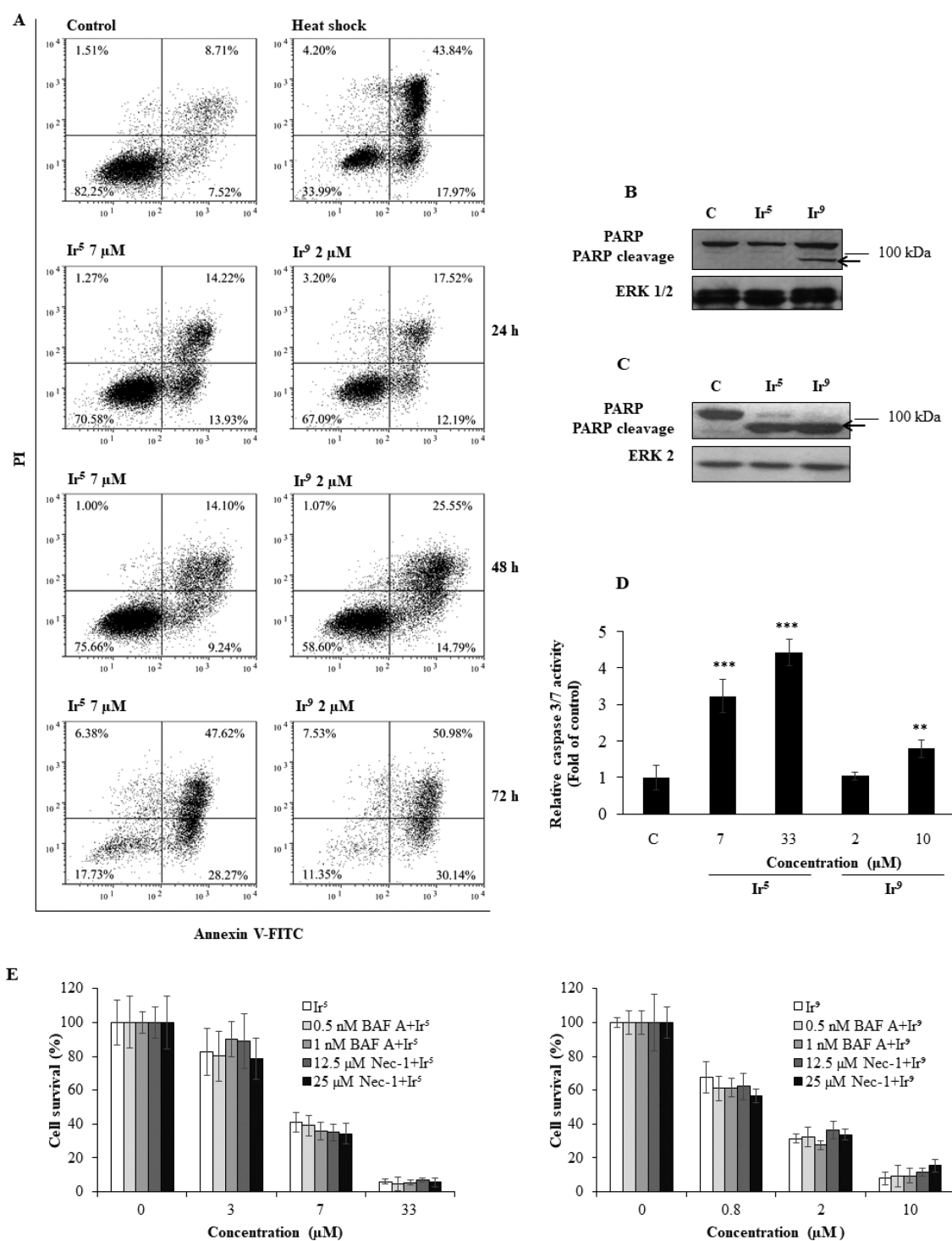
CONCLUSION

We have synthesized 12 osmium(II), 9 iridium(III), and 1 ruthenium(II) chloride arene organometallic compounds that are additionally coordinated with bidentate pyridyl and pyrimidyl functionalized mesoionic carbenes (MIC) of the 1,2,3-triazol-5-ylidene type. All new compounds have been fully characterized by means of standard analytical and spectroscopic techniques. For five of them we were able to provide X-ray structural analysis. The complexes have been tested for their anticancer activity, and it is noteworthy that this is the first report on biological activity of this type of organometallic compounds. Ir⁵ and Ir⁹ displayed the highest effect on cell viability in HeLa cells. They had a similar effect on different tumor cell lines, but they were more cytotoxic against tumor cells than normal cells. Moreover, both complexes induced programmed cell death. GSH plays an important role as a protection mechanism in Ir⁵- and Ir⁹-induced cell death. Organoiridium complexes seem very interesting for further investigation as potential anticancer agents due to their selective highly toxic effect on different tumor cell types.

EXPERIMENTAL SECTION

Chemistry. The reagents and solvents were used as obtained from the commercial sources (Sigma-Aldrich, Fluka, Alfa Aesar). Ag₂O²⁵ triazolium salts L¹–L¹³,¹⁰ complexes Os¹–Os⁷,^{8b} Os¹²,^{8d} Ir¹–Ir⁴,^{8b} Ir⁸,^{8c} and Ru^{18d} were prepared as described previously.

NMR spectra were measured with a Jeol ECS 400 spectrometer at 25 °C. Proton and carbon spectra were referenced to Si(CH₃)₄ as the internal standard. Chemical shifts are given on the δ scale (ppm).



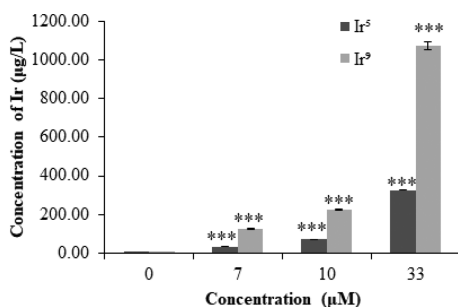


Figure 4. The accumulation of Ir⁵ and Ir⁹ in cells. HeLa cells were treated with different concentrations of Ir⁵ or Ir⁹ for 3 h. The cells were collected, and the amount of iridium was measured by HR ICPMS. The data of three independent experiments are presented. Significance was determined between control (nontreated) and Ir⁵, respectively, Ir⁹ treated cells (***) $P < 0.001$.

Coupling constants (J) are given in Hertz. The multiplicities are indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet), sept (septet), and m (multiplet). An Agilent 6210 ESI-TOF spectrometer and Agilent 6224 time-of-flight (TOF) mass spectrometer equipped with a double orthogonal electrospray source at atmospheric pressure ionization (ESI) coupled to an Agilent 1260 HPLC were used for recording HRMS spectra.

X-ray data were collected with a Bruker Smart AXS. Data were collected at 140(2) K using graphite-monochromated Mo $K\alpha$ radiation ($K\alpha = 0.71073$ Å). The strategy for the data collection was evaluated using the Smart software. The data were collected by the standard “omega scan techniques” and were scaled and reduced using SAINT+ software. The structures were solved by direct methods using SHELXS-97 and refined by full matrix least-squares with SHELXL-97, refining on F^2 .²⁶

General Procedure for the Preparation of Complexes Os⁸–Os¹¹ and Ir⁵–Ir⁷, Ir⁹. The corresponding ligand precursor (2 equiv, 0.2 mmol) was mixed with basic silver(I) oxide (7 equiv, 0.7 mmol, 163 mg) and potassium chloride (20 equiv, 2 mmol, 155 mg) and was dissolved under nitrogen in dry acetonitrile (10 mL). The reaction mixture was stirred under the exclusion of light for 2 days. Afterward, the corresponding chloro-bridged metal dimer precursor (1 equiv, 0.1 mmol) was added and stirring was continued for additional 2–3 days. The remaining silver(I) oxide and silver(I) chloride that formed were filtered off through a pad of Celite, and all volatiles were removed in vacuo. The crude product was then dissolved in methanol (5 mL). KPF₆ (8 equiv, 0.8 mmol, 147 mg) was added, and the solution was stirred for 20 min. Slow addition of water (80 mL) resulted in precipitation of the desired complexes, which were collected by filtration and air-dried. If the precipitation from water was not successful, the product was extracted with dichloromethane (3 × 20 mL). Combined organic layers were dried over Na₂SO₄ and concentrated to ca. 5 mL volume. The product was precipitated by the addition of hexane (50 mL) and collected by filtration. The complexes were obtained in moderate yields of 53% and higher.

Osmium Complexes. Os⁸. From L⁸ (65 mg) and [Os(*p*-Cym)Cl₂]₂ (79 mg). Green solid. Yield: 65% (0.13 mmol, 96.4 mg). ¹H NMR (400 MHz, CD₂Cl₂) $\delta = 9.18$ (td, $J = 5.7, 0.7$ Hz, 1H, Py-*H*), 8.01 (td, $J = 8.1, 0.7$ Hz, 1H, Py-*H*), 7.96 (ddd, $J = 8.1, 1.4, 0.6$ Hz, 1H, Py-*H*), 7.86 (ddd, $J = 5.5, 2.4, 1.1$ Hz, 2H, Ar-*H*), 7.67–7.61 (m, 3H, Ar-*H*), 7.37 (ddd, 1H, Py-*H*), 5.40 (dd, $J = 13.7, 5.8$ Hz, 2H, Cym-*CH*), 5.23 (d, $J = 5.6$ Hz, 1H, Cym-*CH*), 4.84 (d, $J = 5.6$ Hz, 1H, Cym-*CH*), 4.53 (s, 3H, N-CH₃), 2.24 (sept, $J = 6.9$ Hz, 1H, CH(CH₃)₂), 2.08 (s, 3H, Cym-CH₃), 0.86 (d, $J = 6.9$ Hz, 3H, CH(CH₃)₂), 0.84 (d, $J = 6.9$ Hz, 3H, CH(CH₃)₂). ¹³C NMR (100 MHz, CD₂Cl₂) $\delta = 160.9$ (Tz-*C*), 157.8; 148.6; 147.5, 139.7, 137.8, 131.4, 130.0, 126.3, 125.4, 121.2 (all Ar/Py-*C*), 99.1, 98.4, 81.9, 77.7, 77.6, 72.4 (all Cym-*C*), 39.2 (N-CH₃), 31.4 (CH(CH₃)₂), 22.6 (CH(CH₃)₂), 21.7 (CH(CH₃)₂), 18.6 (Cym-CH₃). HRMS (ESI+) m/z calcd. for C₂₄H₂₆ClN₄O₈⁺ [M]⁺ = 597.1461, found 597.1464.

Os⁹. From L⁹ (71 mg) and [Os(*p*-Cym)Cl₂]₂ (79 mg). Gray-green solid. Yield: 53% (0.106 mmol, 82 mg). ¹H NMR (400 MHz, CD₂Cl₂) $\delta = 9.17$ (dt, $J = 5.7, 1.1$ Hz, 1H, Py-*H*), 8.03–7.98 (m, 1H, Py-*H*), 7.94 (dt, $J = 8.1, 1.1$ Hz, 1H, Py-*H*), 7.77 (d, $J = 9.0$ Hz, 2H, Ar-*H*), 7.36 (ddd, $J = 7.4, 5.7, 1.5$ Hz, 1H, Py-*H*), 7.09 (d, $J = 9.0$ Hz, 2H, Ar-*H*), 5.44 (dd, $J = 5.8, 1.1$ Hz, 1H, Cym-*CH*), 5.38 (d, $J = 5.9$ Hz, 1H, Cym-*CH*), 5.28 (d, $J = 5.9$ Hz, 1H, Cym-*CH*), 4.86 (dd, $J = 5.5, 1.1$ Hz, 1H, Cym-*CH*), 4.51 (s, 3H, N-CH₃), 3.87 (s, 3H, O-CH₃), 2.24 (sept, $J = 7.0$ Hz, 1H, CH(CH₃)₂), 2.09 (s, 3H, Cym-CH₃), 0.86 (d, $J = 6.9$ Hz, 3H, CH(CH₃)₂), 0.85 (d, $J = 6.9$ Hz, 3H, 21.7 (CH(CH₃)₂). ¹³C NMR (100 MHz, CD₂Cl₂) $\delta = 160.9$ (Ar-*C*), 160.8 (Tz-*C*), 160.0, 157.0, 147.9, 147.5, 138.9, 129.7, 125.9, 125.4, 120.4, 114.9 (all Ar/Py-*C*), 98.2, 97.9, 81.0, 76.9, 76.8, 71.2 (all Cym-*C*), 55.1 (O-CH₃), 38.3 (N-CH₃), 30.6 (CH(CH₃)₂), 21.8 (CH(CH₃)₂), 21.0 (CH(CH₃)₂), 17.8, 13.06 (Cym-CH₃). HRMS (ESI+) m/z calcd. for C₂₅H₂₈ClN₄O₈⁺ [M]⁺ = 627.1566, found 627.1576.

Os¹⁰. From L¹⁰ (78 mg) and [Os(*p*-Cym)Cl₂]₂ (79 mg). Yellow solid. Yield: 61% (0.122 mmol, 98 mg). ¹H NMR (400 MHz, CD₂Cl₂) $\delta = 9.17$ (d, $J = 5.8, 1.0$ Hz, 1H, Py-*H*), 8.11 (d, $J = 8.8$ Hz, 2H, Ar-*H*), 8.03 (dd, $J = 7.9, 1.3$ Hz, 1H, Py-*H*), 7.96 (d, $J = 8.1$ Hz, 1H, Py-*H*), 7.91 (d, $J = 8.9$ Hz, 2H, Ar-*H*), 7.38 (ddd, $J = 7.3, 5.9, 1.4$ Hz, 1H, Py-*H*), 5.43 (d, $J = 5.9$ Hz, 1H, Cym-*CH*), 5.39 (d, $J = 5.8$ Hz, 1H, Cym-*CH*), 5.32 (d, $J = 5.9$ Hz, 1H, Cym-*CH*), 4.89 (d, $J = 5.6$ Hz, 1H, Cym-*CH*), 4.55 (s, 3H, N-CH₃), 2.27 (sept, $J = 6.9$ Hz, 1H, CH(CH₃)₂), 2.09 (s, 3H, Cym-CH₃), 0.88 (d, $J = 6.9$ Hz, 3H, CH(CH₃)₂), 0.87 (d, $J = 6.9$ Hz, 3H, CH(CH₃)₂). ¹³C NMR (100 MHz, CD₂Cl₂) $\delta = 160.2$ (Tz-*C*), 156.9, 147.7, 147.0, 140.0, 128.1, 126.5 (q, $J = 4$ Hz, Ar-*C*), 125.6, 125.4, 125.3, 120.6, (all Ar/Py-*C*), 99.0, 97.5, 80.8, 77.1, 77.0, 72.0 (all Cym-*C*), 38.6 (N-CH₃), 30.6 (CH(CH₃)₂), 21.9 (CH(CH₃)₂), 20.9 (CH(CH₃)₂), 17.8 (Cym-CH₃). HRMS (ESI+) m/z calcd. for C₂₅H₂₅ClF₃N₄O₈⁺ [M]⁺ = 665.1335, found 665.1340.

Os¹¹. From L¹¹ (55 mg) and [Os(Cym)Cl₂]₂ (79 mg). Yellow solid. Yield: 69% (0.137 mmol, 95 mg). ¹H NMR (400 MHz, CD₂Cl₂) $\delta = 9.23$ –9.17 (m, 1H, Py-*H*), 8.05–7.99 (m, 1H, Py-*H*), 7.94–7.88 (m, 1H, Py-*H*), 7.41–7.34 (m, 1H, Py-*H*), 5.95 (d, $J = 5.6$ Hz, 1H, Cym-*CH*), 5.85 (d, $J = 6.1$, 1H, Cym-*CH*), 5.78 (d, $J = 5.8$, 1H, Cym-*CH*), 5.49 (d, $J = 5.8$, 1H, Cym-*CH*), 4.78–4.57 (m, 2H, N-CH₂CH₃), 4.45 (s, 3H, N-CH₃), 2.36 (sept, $J = 6.9$ Hz, 1H, CH(CH₃)₂), 2.21 (s, 3H, Cym-CH₃), 1.66 (t, $J = 7.4$ Hz, 3H, N-CH₂CH₃), 0.98–0.90 (m, 6H, CH(CH₃)₂). ¹³C NMR (100 MHz, CD₂Cl₂) $\delta = 159.6$ (Tz-*C*), 157.7, 151.8, 148.7, 147.2, 139.6, 126.0, 120.8 (all Ar/Py-*C*), 97.7, 96.9, 81.1, 79.3, 77.1, 72.0 (all Cym-*C*), 49.4 (N-CH₂CH₃), 38.9 (N-CH₃), 31.5 (CH(CH₃)₂), 22.3 (CH(CH₃)₂), 18.6 (CH(CH₃)₂), 15.3 (N-CH₂CH₃). HRMS (ESI+) m/z calcd. for C₂₀H₂₆ClN₄O₈⁺ [M]⁺ = 549.1455, found 549.1453.

Iridium Complexes. Ir⁵. From L⁸ (65 mg) and [IrCp*Cl₂]₂ (80 mg). Bright-yellow solid. Yield: 63% (0.126 mmol, 93 mg). ¹H NMR (400 MHz, CD₂Cl₂) $\delta = 8.76$ (dt, $J = 5.7, 1.1$ Hz, 1H, Py-*H*), 8.13–8.06 (m, 2H, Py-*H*), 8.00–7.96 (m, 2H, Ar-*H*), 7.67–7.61 (m, 3H, Ar-*H*), 7.50 (ddd, $J = 6.8, 5.7, 2.2$ Hz, 1H, Py-*H*), 4.61 (s, 3H, N-CH₃), 1.42 (s, 15H, Cp*-CH₃). ¹³C NMR (100 MHz, CD₂Cl₂) $\delta = 156.5$ (Tz-*C*), 153.7, 148.7, 148.5, 140.2, 138.0, 131.1, 130.0, 126.3, 125.3, 121.3 (all Ar/Py-*C*), 91.2 (Cp*-*C*), 39.3 (N-CH₃), 8.7 (Cp*-CH₃). HRMS (ESI+) m/z calcd. for C₂₄H₂₇ClIrN₄⁺ [M]⁺ = 599.1553, found 599.1508.

Ir⁶. From L⁹ (71 mg) and [IrCp*Cl₂]₂ (80 mg). Bright-yellow solid. Yield: 73% (0.143 mmol, 112 mg). ¹H NMR (400 MHz, CD₂Cl₂) $\delta = 8.76$ (dd, $J = 5.8, 0.5$ Hz, 1H, Py-*H*), 8.08 (ddd, $J = 7.9, 1.6, 0.3$ Hz, 1H, Py-*H*), 8.01 (d, $J = 8.1$ Hz, 1H, Py-*H*), 7.88 (d, $J = 8.9$ Hz, 2H, Ar-*H*), 7.49 (ddd, $J = 7.4, 5.9, 1.4$ Hz, 1H, Py-*H*), 7.09 (d, $J = 7.0$ Hz, 2H, Ar-*H*), 4.57 (s, 3H, N-CH₃), 3.88 (s, 3H, O-CH₃), 1.44 (s, 15H, Cp*-CH₃). ¹³C NMR (100 MHz, CD₂Cl₂) $\delta = 161.6$ (Ar-*C*), 156.3 (Tz-*C*), 153.8, 148.8, 148.2, 140.1, 131.0, 126.7, 126.3, 122.6, 121.0, 114.9 (all Ar/Py-*C*), 91.2 (Cp*-CH₃), 55.9 (O-CH₃), 39.1 (N-CH₃), 8.8 (Cp*-CH₃). HRMS (ESI+) m/z calcd. for C₂₅H₂₉ClIrN₄O⁺ [M]⁺ = 629.1659, found 629.1616.

Ir⁷. From L¹⁰ (78 mg) and [IrCp*Cl₂]₂ (80 mg). Bright-yellow solid. Yield: 75% (0.15 mmol, 122 mg). ¹H NMR (400 MHz,

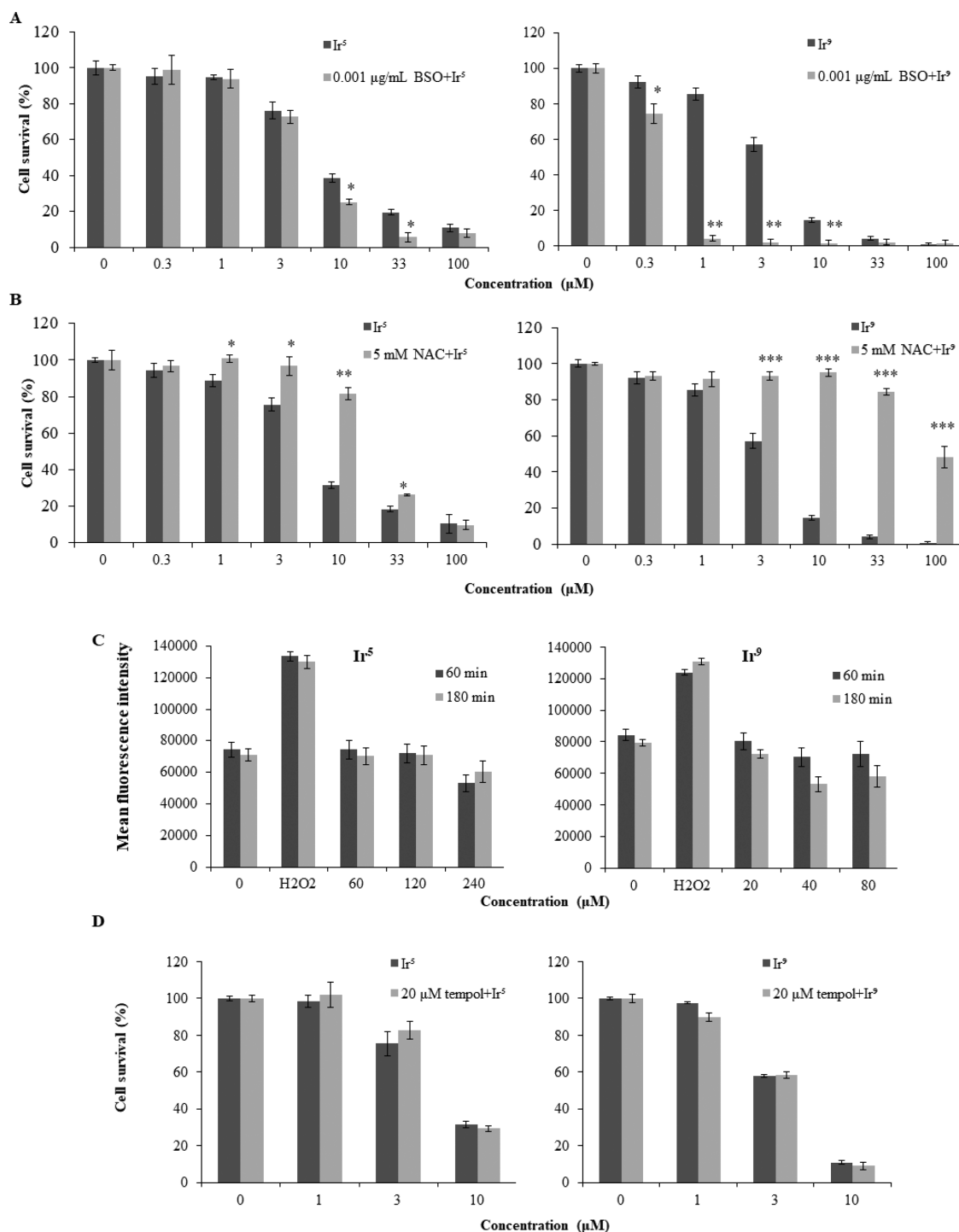


Figure 5. The role of GSH level on cell survival upon treatment with Ir⁵ and Ir⁹. Eight hours after seeding cells were pretreated for 16 hours with 0.001 μg/mL BSO (A) or 24 h after the seeding with 5 mM of NAC for 2 h (B). Afterward different concentrations of Ir⁵ or Ir⁹ were added. The cell survival was determined 72 h later by MTT assay. Each point represents the mean ± SD of at least three independent experiments. All data are expressed as the average percentage of survival values relative to an untreated control ± SD or samples treated with either BSO or NAC alone. The significance in differences is indicated (**P* < 0.05; ***P* < 0.01; ****P* < 0.001). Twenty-four hours after seeding the cells were treated with different concentrations of Ir⁵ or Ir⁹ during either 60 or 180 min. H₂O₂ treatment (0.01%, 30 min) was used as a positive control for formation of ROS (C). Each point represents the mean ± SD of two independent measurements performed in quadruplicates. Twenty-four hours after seeding cells were pretreated for 2 h with 20 μM tempol. Afterward different concentrations of Ir⁵ or Ir⁹ were added. The cell survival was determined 72 h later by MTT assay. (D) Each point represents the mean ± SD of at least three independent experiments. All data are expressed as the average percentage of survival values relative to an untreated control ± SD or samples treated with tempol alone.

CD₂Cl₂) δ = 8.77 (ddd, *J* = 5.7, 1.5, 0.7 Hz, 1H, Py-*H*), 8.21 (d, *J* = 8.3 Hz, 2H, Ar-*H*), 8.10 (ddd, *J* = 8.0, 1.4, 0.3 Hz, 1H, Py-*H*), 8.04 (td, *J* = 8.1, 1.4 Hz, 1H, Py-*H*), 7.91 (d, *J* = 8.8 Hz, 2H, Ar-*H*), 7.51

(ddd, *J* = 6.0, 1.4, 0.3 Hz, 1H, Py-*H*), 4.61 (s, 3H, N-CH₃), 1.43 (s, 15H, Cp*-CH₃). ¹³C NMR (100 MHz, CD₂Cl₂) δ = 157.2 (Tz-*C*), 153.8, 148.8, 148.6, 140.7, 140.2 (all Ar/Py-*C*), 132.9 (q, *J* = 33 Hz,

Ar-C), 127.2 (q, $J = 4$ Hz, Ar-C), 126.4, 126.1 (all Ar/Py-C), 123.5 (q, $J = 273$ Hz, CF₃), 121.2 (Ar/Py-C), 91.4 (Cp*-C), 39.4 (N-CH₃), 8.7 (Cp*-CH₃). HRMS (ESI+) m/z calcd. for C₂₅H₂₆ClF₃IrN₄⁺ [M]⁺ = 667.1427, found 667.1391.

Ir⁹. From L¹² (82 mg) and [Ir(Cp*)Cl₂]₂ (80 mg). Yellow solid. Yield: 75% (0.15 mmol, 124 mg). ¹H NMR (400 MHz, CD₂Cl₂) δ = 8.79–8.71 (m, 1H, Py-H), 8.14–8.08 (m, 1H, Py-H), 8.08–8.04 (m, 1H, Py-H), 7.60 (t, $J = 8.0$ Hz, 1H, Ar-H), 7.55–7.52 (m, 1H, Py-H), 7.44–7.39 (m, 2H, Ar-H), 4.61 (s, 3H, N-CH₃), 3.17 (sept, $J = 6.8$ Hz, 1H, CH(CH₃)₂), 2.35 (sept, $J = 6.8$ Hz, 1H, CH(CH₃)₂), 1.41 (s, 15H, Cp*-CH₃), 1.34 (d, $J = 6.8$ Hz, 3H, CH(CH₃)₂), 1.27 (d, $J = 6.8$ Hz, 3H, CH(CH₃)₂), 1.19 (d, $J = 6.8$ Hz, 3H, CH(CH₃)₂), 0.78 (d, $J = 6.8$ Hz, 3H, CH(CH₃)₂). ¹³C NMR (100 MHz, CD₂Cl₂) δ = 158.4 (Tz-C), 153.3, 149.6, 148.7, 146.8, 146.4, 139.9, 134.4, 132.1, 126.5, 124.6, 124.4, 121.2 (all Ar-C), 91.1 (Cp*-C), 39.4 (N-CH₃), 29.0 (CH(CH₃)₂), 27.9 (CH(CH₃)₂), 27.0 (CH(CH₃)₂), 25.3 (CH(CH₃)₂), 21.7 (CH(CH₃)₂), 21.6 (CH(CH₃)₂), 8.8 (Cp*-CH₃). HRMS (ESI+) m/z calcd. for C₃₀H₃₉ClIrN₄⁺ [M]⁺ = 683.2492, found 683.2464.

Biology. Cell Culture. Human cervical carcinoma HeLa and laryngeal carcinoma HEP2 cells were obtained from cell culture bank (GIBCO BRL-Invitrogen, USA). Large cell lung carcinoma H460 and colorectal carcinoma HCT-116 cell lines were obtained from American Type Culture Collection (ATCC, USA). Human ovarian cancer cell line MES-OV was obtained from Prof. Sikic group (Stanford University, USA). Normal human skin keratinocyte line was obtained from the foreskin of healthy boys, aged 3–8 years. Foreskin samples were noninflamed and the children were free of any therapy at least 1 month before the surgery.²⁷ The cells were obtained at the Neurochemical Laboratory, Department of Chemistry and Biochemistry, School of Medicine, University of Zagreb. All cell lines were grown as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) in a humidified atmosphere of 5% CO₂ at 37 °C and were subcultured every 3–4 days.

Cytotoxicity Assay. Cytotoxic activity of organometallic complexes was determined by MTT assay²⁸ adjusted accordingly. In short, the cells were seeded into 96-well tissue culture plates. The next day different concentrations of compounds were added to each well in quadruplicate. Upon 72 h incubation at 37 °C, the medium was aspirated and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye (Sigma-Aldrich) was added. Three hours later, formazan crystals were dissolved in DMSO, the plates were mechanically agitated for 5 min, and the optical density at 545 nm was determined on a microtiter plate reader (Awareness Technology Inc., USA). The cytotoxicity was expressed either as IC₂₀, IC₅₀, or IC₈₀ (survival rate 80, 50, and 20% compared to nontreated, control cells, which survival was set at 100%).

Cell Cycle and Cell Death Analysis. HeLa cells were seeded into tissue culture plates and treated with different concentrations of compounds for the indicated time. Thereafter, both adherent and floating cells were collected, washed with PBS and fixed overnight in 96% ethanol at –20 °C. Fixed cells were treated with RNase A (0.1 mg/mL, Sigma-Aldrich) for 30 min in water bath (37 °C) and afterward stained with propidium iodide (50 μ g/mL, Sigma-Aldrich) for 30 min in the dark. The DNA content was analyzed on flow cytometer (FACS Calibur, Becton Dickinson, USA). Data were analyzed for cell cycle distribution in FlowLogic software (Inivai Technologies, Australia) using cell cycle specific gating protocol and Watson pragmatic convolution.

In order to determine the type of cell death, HeLa cells were treated with different concentrations of investigated compounds. After specific time, both adherent and floating cells were collected, washed two times with PBS and stained with Annexin V and/or PI (propidium iodide) according to the producer's protocol (Thermo-Fisher Scientific, USA). The DNA content and Annexin V fluorescence were analyzed by flow cytometry (FACS Calibur, Becton Dickinson). Data were analyzed with FlowLogic software (Inivai Technologies, Australia) using specific gating protocol. After determination of protein concentration by Bradford analysis,²⁹ the

30 μ g of whole cell proteins were loaded on SDS polyacrylamide gel. Well accepted marker of apoptosis, cleaved PARP,¹⁴ was determined by using specific anti-PARP antibody (Cell Signaling Technology, USA). As internal loading controls ERK1/2 or ERK2 were used (Santa Cruz Technology, USA). In addition, the activities of caspases 3 and 7 were determined. Cells were seeded into tissue culture plates and after 1 day treated with different concentrations of investigated compounds. Upon 72 h incubation Caspase Glo 3/7 Mix (Promega, USA) was added, and the producer's protocol was followed. Luminometric signal was read on Fluoroskan Ascent FL (Thermo Fisher Scientific, USA) spectrometer. The possible role of autophagy and necroptosis as cell death models was checked as previously described.²⁴

Accumulation of Iridium in the Cells. The total accumulation of iridium was measured as described previously for measurement of total cell platination²¹ with modifications. In short, upon treatment of cells with different concentrations of Ir⁵ or Ir⁹ during 3 h, the cells were rinsed with ice-cold PBS and harvested into 10 mL of ice-cold PBS using a rubber policeman. After centrifugation, the cells were resuspended in PBS, an aliquot was used for determination of cell number, and the rest was digested in 70% nitric acid. Cell lysates were heated for 2 h at 75 °C, diluted in 5% nitric acid and assayed for iridium content. The amount of iridium was measured by a validated high-resolution inductively coupled plasma mass spectrometry (HR ICPMS) using the Element 2 (Thermo Finnigan, Germany). Calibration standards were prepared from single-element standard diluted in hydrochloric acid and water (1000 μ g/mL; Agilent, USA).

The Role of Glutathione in Cell Response to Ir⁵ and Ir⁹. The role of glutathione (GSH) in cell response to Ir⁵ and Ir⁹ was investigated by MTT assay by pretreatment of cells with specific inhibitor of GSH synthesis, buthionine sulfoximine (BSO) or precursor in GSH synthesis *N*-acetyl-cysteine (NAC). To examine the influence of GSH depletion on response of HeLa cells to selected compounds, 8 h after the seeding the cells were pretreated with 0.001 μ g/mL of BSO. Sixteen hours later the cells were treated with different concentrations of either Ir⁵ or Ir⁹, and the cytotoxicity effect was determined 72 h later. To determine the influence of increase of GSH synthesis on cells response to selected compounds, 5 mM NAC was added 2 h before treatment of cells with different concentrations of either Ir⁵ or Ir⁹. Generation of ROS was determined using the fluorescent dye 5-(and-6)-chloromethyl-29,79-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) (Life Technologies, USA) according to the previously described protocol.¹⁹ Possible role of ROS in induction of cells death was additionally checked by 2 h pretreatment with 20 μ M tempol, well accepted antioxidant³⁰ and then treated with different concentrations of either Ir⁵ or Ir⁹. The cytotoxicity effect was determined 72 h later by MTT assay.

Statistical Analysis. Data were analyzed by unpaired Student's *t* test and expressed as a mean \pm standard error of the mean. Data were considered significant when *P* values were lower than 0.05, and in the figures, these are designated as **P* < 0.05, ***P* < 0.01, or ****P* < 0.001. Experiments were performed in triplicate and repeated at least twice.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.organomet.9b00327](https://doi.org/10.1021/acs.organomet.9b00327).

Crystallographic details, ¹H and ¹³C NMR spectra for all new compounds (PDF)

Accession Codes

CCDC 1044233–1044236 and 973835 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by

contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

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