Ru(III) Complexes with Lonidamine-Modified Ligands

Ilya A. Shutkov 1, Yulia N. Okulova 1, Vladimir Yu. Tyurin 1, Elena V. Sokolova 2, Denis A. Babkov 2, Alexander A. Spasov 2, Yulia A. Gracheva 1, Claudia Schmidt 3, Kirill I. Kirsanov 4, Alexander A. Shil 4, Olga M. Redkozubova 6, Elena F. Shevtsova 7, Elena R. Milaeva 1, Ingo Ott 3 and Alexey A. Nazarov 1,*

1 Department of Medicinal Chemistry & Fine Organic Synthesis, Lomonosov Moscow State University, 1/3 Leninskiy Gory, 119991 Moscow, Russia; ilya-shutkov@med.chem.msu.ru (I.A.S.); nosovayulia@yahoo.com (Y.N.O.); olivecc@mail.ru (V.Y.T.); jullina74@mail.ru (Y.A.G.); milaeva@med.chem.msu.ru (E.R.M.)
2 Scientific Center for Innovative Drugs, Volgograd State Medical University, 39 Novorossiyskaya Street, 400087 Volgograd, Russia; sokolova210795@gmail.com (E.V.S.); denis.a.babkov@gmail.com (D.A.B.); aspasov@mail.ru (A.A.S.)
3 Institute of Medicinal and Pharmaceutical Chemistry, Technische Universität Braunschweig, 55 Beethovenstrasse, 38106 Braunschweig, Germany; cla.schmidt@tum.de (C.S.); ingo.ott@tu-braunschweig.de (I.O.)
4 Blokhin Cancer Research Center, 24 Kashirskoye Shosse, 115478 Moscow, Russia; kkirsanov85@yandex.ru (K.I.K.); shtilaa@yahoo.com (A.A.S.)
5 Institute of Medicine, RUDN University, 6 Miklukho-Maklaya St., 117198 Moscow, Russia
6 Neurobotics, Zelenograd pas. 4922, 4-2-477, 124498 Moscow, Russia; redkozubova@gmail.com
7 Institute of Physiologically Active Compounds, Russian Academy of Sciences, 1 Severnaya Proezd, 142432 Chernogolovka, Russia; e.f.shevtsova@gmail.com
* Correspondence: nazarov@med.chem.msu.ru

Abstract: A series of bifunctional Ru(III) complexes with lonidamine-modified ligands (lonidamine is a selective inhibitor of aerobic glycolysis in cancer cells) was described. Redox properties of Ru(III) complexes were characterized by cyclic voltammetry. An easy reduction suggested a perspective for these agents as their whole mechanism of action seems to be based on activation by metal atom reduction. New compounds demonstrated a more pronounced antiproliferative potency than the parental drug; individual new agents were more cytotoxic than cisplatin. Stability studies showed an increase in the stability of complexes along with the linker length. A similar trend was noted for antiproliferative activity, cellular uptake, apoptosis induction, and thioredoxin reductase inhibition. Finally, at concentrations that did not alter water solubility, the selected new complex evoked no acute toxicity in Balb/c mice.

Keywords: lonidamine; redox balance; cell death; antiproliferative activity; thioredoxin reductase

1. Introduction

Since the discovery of the antitumour activity of cisplatin, organic complexes with other metals such as ruthenium, gold, osmium, gallium, rhodium, titanium, etc., have been investigated. Ruthenium compounds became the most promising because of their different mode of action and relatively low general toxicity. The Ru(III) compounds KP1019 (eventually changed to the sodium salt known as KP1339; BOLD 100) and NAMI-A (Figure 1) became the first metal-based non-platinum drugs that entered clinical trials [1–7].

Compound NAMI-A exhibits an antimitastatic activity but is less active toward primary tumours [8], which has been attributed to a specific mode of action [9–11]. KP1339 was found to be active against drug-resistant cell lines [12–15].

One significant metabolic characteristic of malignant cells is their activated glycolysis [16,17]. Hexokinase II (HKII) catalyses the first stage of aerobic glycolysis, thereby inducing glycolysis and limiting its rate [18,19]. Lonidamine (Figure 2) is an inhibitor of mitochondrial hexokinase. This agent stimulates lactate production in normal cells and...
inhibits glycolysis in malignant counterparts [20,21]. Lonidamine is widely investigated in clinical trials for the treatment of different types of cancer [22–24] and has recently been recognised as a drug candidate for COVID-19 patients [25], alongside some metal-based compounds [26].

Figure 1. The structures of Ru(III) complexes in clinical trials.

Figure 2. Structure of 1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxylic acid (lonidamine).

Modifying the known metal-based drugs by introducing a biologically active molecule is a promising approach in medicinal chemistry to improve cytotoxicity, selectivity, and the twin-drug effect [27–30]. Recently, we introduced lonidamine in Pt(IV) or Ru(II) moieties and obtained compounds with increased activity and selectivity [31–34]. Pt(IV) compounds showed a significant increase of antiproliferative activity superior to cisplatin and lonidamine [32,35], and Ru(II) complexes were specifically active against glioblastoma cell lines [31]. Thioredoxin reductases (TrxR) belong to the thioredoxin system along with NADPH and thioredoxin (Trx). TrxR enzymes are overexpressed in cancer cells, ensuring the resistance of their phenotype to high ROS levels [36,37]. Thus, TrxR is a target for developing new metal-based anticancer agents, including ruthenium and gold complexes [37–40].

In this study, we describe the synthesis of Ru(III) complexes with lonidamine-modified imidazole ligands and report their cytotoxicity, electrochemical behaviour, stability, lipophilicity, intracellular accumulation, as well as mechanisms of cell death and in vivo tolerance.

2. Results and Discussion

2.1. Synthesis

Ligands (9–14) were obtained by the reaction of 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbonyl chloride (2) with corresponding imidazolamines (3–8) in CH$_2$Cl$_2$ using an excess of amine or triethylamine as an HCl acceptor (Scheme 1). Products were isolated by column chromatography on silica gel and characterised by both NMR spectroscopy ($^1$H and $^{13}$C[$^1$H]) and elemental analyses.
Ru(III) complexes (15–20), the analogues of NAMI complexes, were prepared as described [41] by substituting DMSO in Na[Ru(DMSO)$_2$] with the imidazole moiety of new ligands (Scheme 2).
The stability of Ru(III) complexes (15–20) was investigated in a solution resembling physiological conditions (20 mM of phosphate buffer, pH 7.4, and 100 mM of NaCl at 37 °C) by UV-vis spectrophotometry (Table 1). The half-transformation time \( t_{1/2} \) was calculated based on changes in the electronic absorption spectrum (Figure S1) and defined as the value of \( t \) at the \( A_{\text{lin}}/2 \) point. \( \Delta A(t) \) was plotted against \( \lambda_{\text{max}} \); the initial section was approximated as a linear function. We found that the stability of complexes increased along with the length of the alkyl linker. Complex 20 was the most stable \( (t_{1/2} \sim 35 \text{ min}) \), whereas 15–17 were unstable \( (t_{1/2} \sim 5 \text{ min}) \).

| Compound | 15 | 16 | 17 | 18 | 19 | 20 |
|----------|----|----|----|----|----|----|
| Linker, \( n \) | 2  | 3  | 4  | 6  | 8  | 12 |
| \( t_{1/2} \), sec | 360 ± 20 | 330 ± 20 | 360 ± 20 | 650 ± 30 | 1390 ± 70 | 2150 ± 110 |
| Log P * | * | * | * | 0.6 ± 0.1 | ±0.08 | 1.3 ± 0.2 |

* complexes are unstable in aqueous solutions. Results are expressed as mean ± SD from three independent experiments.

The lipophilicity of complexes 15–20 was studied using a standard shake-flask method in a mixture of water/n-octanol. For the quantification of compounds in the water phase, UV-vis spectrophotometry was used. Fast hydrolysis of 15–17 complicated the measurements, therefore we failed to obtain the results. Among other complexes, as expected, 20 was the most lipophilic (Table 1).

2.2. Electrochemical Studies of Ru(III) Complexes

The Ru(III) complexes are kinetically inert compared to the Ru(II) counterparts. It is assumed that the mechanism of action of Ru(III) complexes includes a reduction to Ru(II) compounds, which are more labile in substitution reactions and react with specific regions of proteins [12,42]. Reduction of complexes under physiological conditions occurs in the presence of glutathione, ascorbic acid, and cysteine. To be activated by reduction, the Ru(III) complexes should have biologically attainable reduction potentials (approximately \(-0.4–0.9 \text{ V with respect to the Ag/AgCl reference electrode}\)).

The redox behaviour of ligand 10 and complexes 15–20 was studied on Pt and glassy carbon (GC) electrodes in \( \text{CH}_3\text{CN} \) and \( \text{CH}_2\text{Cl}_2 \). In the case of the ligand, redox transitions were not observed at any applied potentials, up to the discharge of the \( \text{n-Bu}_4\text{NBF}_4 \) background electrolyte. At the same time, complexes 15–20 were electroactive and exhibited two redox processes with respect to the metal centres in the range of +2 V to −2 V.

Potentials of redox transitions are summarised in Tables 2 and 3; representative voltammograms are shown in Figures 4 and 5. In the anodic region, voltammograms of complexes demonstrated two oxidative responses. In both \( \text{CH}_3\text{CN} \) and \( \text{CH}_2\text{Cl}_2 \) using as a solvent, a one-electron reversible peak was recorded in the 1.19–1.3 V range corresponding to the oxidation of Ru (III) to Ru (IV; Figures 4 and 5). The two-electron quasi-reversible peak appeared at more positive potentials, namely 1.60–1.97 V, in the case of \( \text{CH}_3\text{CN} \) (Table 2), which has not been described for Ru complexes. Explanation of the nature of the second peak requires more investigation because such peaks were not observed on voltammograms of ligands.
Table 2. Redox potentials of complexes determined with a Pt and the glassy carbon (GC) working electrodes in CH$_3$CN (C = 10$^{-3}$ M, 0.5 M n-TBABF$_4$, scan rate 100 mV/s, and Ag/AgCl (sat.)).

| Complex | $E_{\text{ox}}$, V | $E_{\text{red}}$, V | $E_{\text{ox}}$, V | $E_{\text{red}}$, V |
|---------|------------------|-----------------|------------------|-----------------|
|         | $E_{\text{1ox}}$ | $E_{\text{2ox}}$ | $E_{\text{1ox}}$ | $E_{\text{2ox}}$ |
| 15      | 1.290/1.185      | 1.850/1.630     | 1.270/1.17       | 1.890/1.650     |
| 16      | 1.300/1.190      | 1.830/1.630     | 1.280/1.195      | -0.360/-0.190   |
| 17      | 1.290/1.190      | 1.820/1.610     | 1.300/1.196      | 1.870/1.640     |
| 18      | 1.280/1.170      | 1.820/1.620     | 1.310/1.192      | 1.860/1.600     |
| 19      | 1.320/1.190      | 1.840/1.620     | 1.308/1.200      | 1.950/1.620     |
| 20      | -                | -               | 1.490/1.137      | -0.250/-0.190   |

Table 3. Redox potentials of complexes determined with a Pt and the glassy carbon (GC) working electrodes in CH$_2$Cl$_2$ (C = 10$^{-3}$ M, 0.5 M n-TBABF$_4$, scan rate 200 mV/s, and Ag/AgCl (sat.)).

| Complex | $E_{\text{ox}}$, V | $E_{\text{red}}$, V | $E_{\text{ox}}$, V | $E_{\text{red}}$, V |
|---------|------------------|-----------------|------------------|-----------------|
|         | $E_{\text{1ox}}$ | $E_{\text{2ox}}$ | $E_{\text{1ox}}$ | $E_{\text{2ox}}$ |
| 15      | 0.410/0.350      | 1.290/1.170     | -                | 1.157/1.080     |
| 16      | 0.470/0.340      | 1.280/1.190     | -0.540/-0.410    | -0.490/-0.380   |
| 17      | 0.580/0.370      | -0.650/-0.430   | 1.150/1.430      | -0.690/-0.290   |
| 18      | 0.570/0.340      | 1.430/1.270     | -0.420/-0.280    | 1.150/1.350     |
| 19      | 0.250/0.150      | 1.390/1.160     | -0.550/-0.420    | 1.190/1.410     |
| 20      | -                | 1.410/1.290     | -                | 1.470/1.111     |

Figure 4. The cyclic voltammogram of complex 16 in the anodic range (sweep rate 100 mV/s, C = 1 × 10$^{-3}$ M, n-Bu$_4$NBF$_4$, and rel. Ag|AgCl|KCl(sat.)).

Figure 5. Cyclic voltammogram of complex 16 on the GC electrode (sweep rate 100 mV/s, C = 1 × 10$^{-3}$ M, n-Bu$_4$NBF$_4$, and rel. Ag|AgCl|KCl(sat.)).
Moreover, in the anode region of CVA in CH\textsubscript{2}Cl\textsubscript{2}, compounds 15–19 showed quasi-reversible peaks of low intensity in the 0.15–0.58 V region (Table 3). According to the literature [43], this peak can be due to the redox transition of Ru(II) into Ru(III) with a changed ligand environment.

In the negative potential range of complexes 15–20, a one-electron peak was observed at values from −80 to −310 mV on the cathodic scan when measured on the Pt electrode and at −128 to −410 mV on the GC electrode in CH\textsubscript{3}CN. These values correspond to the process of the reduction of Ru (III) in Ru (II) [41,42,44,45]. The peak of Ru(II)→Ru(III) oxidation appeared during the reverse scan of the potential (Figure 5).

The quasi-reversible nature of the peaks (the difference between cathodic and anodic potentials is ΔE = 150–260 mV on direct and reverse scans) points to the changed geometry of the complexes. Based on CVs, one may conclude that the length of the hydrocarbon linker in the ligand insignificantly affects the values of redox potentials. The redox behaviour of complexes also weakly depended on the nature of the working electrode and the solvent, although in the case of CH\textsubscript{2}Cl\textsubscript{2}, the Ru(III)/Ru (II) reduction was observed at bigger negative potentials (Table 3). An easy reduction of complexes 15–20 indicates the promise of their use as antitumour compounds whose efficacy is based on metal atom reduction.

2.3. Cellular Ruthenium Accumulation

The cellular accumulation of ruthenium complexes was studied by atomic absorption spectrometry. The MCF-7 breast carcinoma cells were incubated with 16 and 20 for 1–24 h in DMEM with or without fetal bovine serum to reduce the binding of complexes with proteins. The accumulation was dependent on the linker’s length. Complex 20 (n = 12) accumulated more readily than 16 (n = 3). The ruthenium complex entered the cells relatively fast; by 4 h, no notable further increase of the Ru content was observed (Figure 6). Notably, the cellular Ru accumulation was affected by the presence of serum in the cell culture media. In particular with complex 16, only very low uptake was observed when serum was present in the culture medium. For both complexes 16 and 20, significantly higher cellular ruthenium concentrations were determined when the experiments were done with serum-free cell culture medium. The negative effect of serum on the cellular Ru accumulation has been reported for other ruthenium species; however, significant effects were detectable only after longer exposure (24 h) [46].

![Figure 6. Ruthenium content in MCF7 cells.](image)

2.4. Inhibition of TrxR

Complexes 16 and 20 inhibited the activity of isolated rat TrxR1 at micromolar concentrations: IC\textsubscript{50} (16) = 20.4 ± 0.1 μM and IC\textsubscript{50} (20) = 8.5 ± 0.3 μM. We demonstrated the efficacy of 16 and 20 as modulators of intracellular TrxR1 using rat liver extracts and varying concentrations of each complex (Figure 7 and Table S1; see Experimental for details) [47].
Both compounds were micromolar TrxR1 inhibitors, with compound 20 being ca. two-folds more potent compared to 16. The efficacy of each compound was independent of the DTNB concentration (p = 0.75 for 16; p = 0.99 for 20), which indicates a non-competitive mechanism of inhibition. This is in line with previously reported metal-based non-competitive TrxR1 inhibitors (gold [48] and gadolinium [49] compounds or lanthanum chloride [50]). Inhibition constants were estimated according to the Cheng–Prusoff equation as $K_i = 30.7 \mu M$ for 16 and $K_i = 15.3 \mu M$ for 20.

Figure 7. TrxR1 inhibition in rat liver extracts.

2.5. Cell Death Studies

The antiproliferative activity of new ligands and complexes was determined against human cell lines, including colon adenocarcinoma SW480, breast adenocarcinoma MCF-7, lung adenocarcinoma A549, neuroblastoma SHSY5Y, and non-tumourigenic HaCaT (Table 4). In general, complexes were more cytotoxic compared to ligands, lonidamine, and, in some cases, cisplatin. Complex 20 was the most potent. We chose this compound and the synthetically accessible complex 16 for mechanistic studies.

Table 4. Antiproliferative activity against human tumour cells.

| Compound | Linker, n | IC50, µM | A549 | MCF-7 | SH-SY5Y | SW480 | HaCaT |
|----------|-----------|----------|------|-------|---------|-------|-------|
| cisplatin N/A | 23 ± 6.5 | >30 | 9.5 ± 0.1 | 21.7 ± 0.5 | 10 ± 5 |
| lonidamine N/A | >90 | 30 ± 10 | >30 | >90 | 3 ± 1 |
| 9 | 2 | 50 ± 16 | 35 ± 6 | ND | 48 ± 10 | 23 ± 2 |
| 10 | 3 | >90 | >90 | ND | >90 | 39 ± 4 |
| 11 | 4 | 25 ± 7 | 22 ± 6 | ND | 28 ± 8 | 45 ± 3 |
| 12 | 6 | 24 ± 10 | 17 ± 2.5 | ND | 16 ± 6 | 40 ± 4 |
| 13 | 8 | 15 ± 6 | 10 ± 2 | ND | >90 | 45 ± 2 |
| 14 | 12 | 15.0 ± 2.2 | 20.8 ± 4.7 | ND | 8.4 ± 1.6 | ND |
| 15 | 2 | >30 | 20.4 ± 0.1 | 25.8 ± 4.5 | 21.4 ± 1.8 | ND |
| 16 | 3 | >30 | 23.0 ± 0.5 | 26.1 ± 5.5 | 25 ± 7.1 | ND |
| 17 | 4 | >30 | >30 | 27.7 ± 3.3 | 21 ± 1.8 | ND |
| 18 | 6 | 12.9 ± 1.0 | 22.0 ± 0.7 | 5.1 ± 2.5 | 19.6 ± 3.9 | ND |
| 19 | 8 | 5.9 ± 2.6 | 17.06 ± 0.3 | 2.64 ± 1.5 | 9.3 ± 0.1 | ND |
| 20 | 12 | 8.1 ± 1.1 | 9.2 ± 0.1 | 6.1 ± 0.3 | 9.6 ± 1.3 | ND |

Results of MTT assays after a 72 h cell exposure. Shown are mean ± SD from three independent experiments. ND, not determined.

Mechanisms of cell death, that is, Annexin V/7-AAD reactivity and caspase 3/7 activation [51], were determined for 16 and 20 in HCT116 cells using the Muse® Annexin V & Dead Cell Kit (Luminex corp., Austin, TX, USA) and flow cytometry. Cells were incubated with 16, 20, or cisplatin (reference drug) for 24 h at concentrations corresponding to 2xIC50 values (obtained in MTT assays; Table 4). The percentages of Annexin V-positive cells in response to 16 and 20 were higher than cisplatin (Figure 8). Complex 20 with the C12 linker induced Annexin V positivity slightly more efficiently than 16 with the C3 linker (27.4 ± 2.1% vs. 19.7 ± 0.6%).
Caspase activation was studied using the Muse® Caspase-3/7 Kit (Luminex corp., Austin, TX, USA). Similarly to Annexin V reactivity (Figure 9), complexes activated caspases 3/7 processing more potently than cisplatin. Complex 20 with the longer linker was the most active.

To visualise the activity of complex 20 in caspase activation, the fluorescent kit CellEvent™ Caspase-3/7 Green ReadyProbes™ Reagent was applied. After incubation of cells with 20, the kit reagent was added and the formation of bright green fluorescent cells with activated caspases was observed (Figure S2).

2.6. Tolerance of 16 In Vivo

Finally, we tested the acute toxicity of complex 16 in Balb/c mice after a single bolus i.p. injection of the compound dissolved in saline. The range of doses was 70–110 mg/kg; higher doses were not achievable due to limited water solubility. Animals were monitored for 21 days after injection. As shown in Table 5, no deaths were detected after injections of 70 mg/kg or 80 mg/kg. Mice in these cohorts had normal hair cover; no changes of nutritional behaviour were registered over the entire period of observation. In contrast, doses >80 mg/kg were lethal for individual animals. Tremor and dyspnœa were registered within the initial 1–2 h after injection of 110 mg/kg. These manifestations gradually
subsided; however, mice became less active. Deaths were registered over the next 1–2 days (Table 5). In each group, the survived animals remained without visible changes for up to 21 days after the injection of 16.

Table 5. Drug-induced lethality in Balb/c mice injected with complex 16.

| Single Dose, mg/kg i.p. | 70 | 80 | 90 | 100 | 110 |
|------------------------|----|----|----|-----|-----|
|                       | 0/6* | 0/6 | 2/6 | 2/6 | 4/6 |

*died/survived (n = 6).

3. Materials and Methods

Reagents were purchased from Aldrich unless specified otherwise. All solvents were purified and degassed prior to use. NMR spectra were recorded on a Bruker FT-NMR Avance III 500 MHz instrument at 500.32 (1H), 125.81 (13C) MHz. Two-dimensional NMR measurements were carried out using standard pulse programs. Chemical shifts were referenced relative to the solvent signal for 1H and 13C spectra. ESI mass spectra were recorded on a LC/MSn ion trap mass spectrometer amaZon SL (Bruker, Bremen, Germany) with MeOH as a solvent. Elemental analysis was performed at Moscow State University with the MicroCube Elementar analyser. Melting points were determined with a Stuart Scientific SMP3 apparatus and uncorrected. UV-vis spectra were recorded on Thermo Scientific Evolution 300. Source of cell lines: initially, all cell lines were purchased from ATCC (Manassas, VA, USA) and routinely propagated by the authors according to the manufacturer’s protocols.

3.1. N-(2-(1H-Imidazol-1-yl)ethyl)-1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamide (9)

Oxalyl chloride (4.5 mL, 0.0525 mol) and a catalytic amount of DMF were added to the solution of lonidamine (893 mg, 2.78 mmol) in DCM (50.0 mL). The reaction mixture was refluxed for 1 h. The solvent and oxalyl chloride were removed in vacuum. The obtained chloroanhydride without purification was dissolved in DCM (50.0 mL) and triethylamine (774 µL, 5.56 mmol), and then 3 (300 mg, 2.70 mmol) was added with stirring. The reaction mixture was stirred for 8 h, then washed by NaHCO3 solution (2 × 100 mL) and NaCl solution (2 × 100 mL), and dried on Na2SO4. The solvent was removed and the product was purified by column chromatography on silica gel (eluent: CH2Cl2:EtOH 12:1, Rf = 0.4). Yield: 387 mg (34.6%), m.p. 120–121 °C, elem. anal. calc. (%) for C20H17Cl2N5O: C 57.98, H 4.14, and N 16.90. Found: C 58.19, H 4.14, and N 16.58.

1H NMR (400.13 MHz, CDCl3) δ: 8.39 (d, 1H, J = 8.2 Hz, H4), 7.53 (s, 1H, H20), 7.48–7.30 (m, 4H, H5, H6, H7, H12), 7.19 (t, 1H, J = 5.7 Hz, N H), 7.13 (d, 1H, J = 8.4 Hz, H14), 7.10 (s, 1H, H19), 6.99 (s, 1H, H18), 6.69 (s, 1H, J = 8.3 Hz, H15), 5.65 (s, 2H, H9), 4.26 (t, 2H, J = 5.8 Hz, H17), and 3.82 (q, 2H, J = 5.9 Hz, H16).

13C{1H}NMR (100.61 MHz, CDCl3) δ: 162.8(C1), 141.2(C2), 137.7(C8), 137.4(C20), 134.6(C10), 133.3(C11/C13), 132.1(C15), 129.9(C19), 129.5(C12), 127.6(C14), 127.5(C6), 123.3(C4), 122.9(C3), 122.8(C5), 119.0(C18), 109.4(C7), 50.1(C9), 46.5(C17), and 40.3(C16).

3.2. N-(3-(1H-Imidazol-1-yl)propyl)-1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamide (10)

Oxalyl chloride (1.6 mL, 0.0187 mol) and a catalytic amount of DMF were added to the solution of lonidamine (300 mg, 0.94 mmol) in DCM (16.0 mL). The reaction mixture was refluxed for 1 h. The solvent and oxalyl chloride were removed in vacuum. The
obtained chloroanhydride without purification was dissolved in DCM (16.0 mL) and triethylamine (261 µL, 1.88 mmol), and then 4 (114 mg, 0.91 mmol) was added with stirring. The reaction mixture was stirred for 8 h, then washed by NaHCO₃ solution (2 × 30 mL) and NaCl solution (2 × 30 mL), and dried on Na₂SO₄. The solvent was removed and the product was purified by column chromatography on silica gel (eluent: CH₂Cl₂:EtOH 12:1, Rf = 0.4). Yield: 270 mg (69.2%), m.p. 121–122 °C, elem. anal. calc. (%) for C₂₁H₁₉Cl₂N₅O: C 58.89, H 4.47, and N 16.35. Found: C 58.55, H 4.57, and N 16.06.

1H NMR (400.13 MHz, CDCl₃) δ: 8.38 (d, 1H, J = 7.9 Hz, H₄), 7.51 (s, 1H, H₂₀), 7.40–7.27 (m, 4H, H₅, H₆, H₇, H₁₂), 7.17–7.05 (m, 2H, H₁₄, N H), 7.03 (s, 1H, H₁₉), 6.95 (s, 1H, H₁₈), 6.62 (d, 1H, J = 8.5 Hz, H₁₅), 5.61 (s, 2H, H₉), 4.04 (t, 2H, J = 7.0 Hz, H₁₇), 3.50–3.45 (m, 2H, H₁₆), and 2.15–2.08 (m, 2H, H₂₁).

13C{¹H} NMR (100.61 MHz, CDCl₃) δ: 162.8(C₁), 141.2(C₂), 138.1(C₈), 137.1(C₂₀), 134.5(C₁₀), 133.2(C₁₁, C₁₃), 132.3(C₁₅), 129.6(C₁₉), 129.5(C₁₂), 129.3(C₁₄), 127.6(C₄/C₆), 127.5(C₄/C₆), 127.3(C₃/C₅), 122.9(C₃/C₅), 118.9(C₁₈), 109.3(C₇), 50.0(C₉), 44.6(C₁₇), 36.2(C₁₆), and 31.5(C₂₁).

3.3. N-(4-(1H-Imidazol-1-yl)butyl)-1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamide (11)

Oxalyl chloride (4.5 mL, 0.0525 mol) and a catalytic amount of DMF were added to the solution of lonidamine (829 mg, 2.58 mmol) in DCM (40.0 mL). The reaction mixture was refluxed for 1 h. The solvent and oxalyl chloride were removed in vacuum. The obtained chloroanhydride without purification was dissolved in DCM (40.0 mL) and triethylamine (718 µL, 5.16 mmol), and then 5 (348 mg, 2.50 mmol) was added with stirring. The reaction mixture was stirred for 8 h, then washed by NaHCO₃ solution (2 × 80 mL) and NaCl solution (2 × 80 mL), and dried on Na₂SO₄. The solvent was removed and the product was purified by column chromatography on silica gel (eluent: CH₂Cl₂:EtOH 12:1, Rf = 0.4). Yield: 841 mg (76.1%), m.p. 98–100 °C, elem. anal. calc. (%) for C₂₂H₂₁Cl₂N₅O: C 59.74, H 4.79, and N 15.83. Found: C 60.13, H 4.91, and N 15.66.

1H NMR (400.13 MHz, CDCl₃) δ: 8.36 (d, 1H, J = 8.0 Hz, H₄), 7.61 (s, 1H, H₂₀), 7.43–7.22 (m, 4H, H₅, H₆, H₇, H₁₂), 7.14–6.99 (m, 3H, H₁₄, H₁₉, NH), 6.90 (s, 1H, H₁₈), 6.59 (d, 1H, J = 8.3 Hz, H₁₅), 5.60 (s, 2H, H₉), 3.99 (t, 2H, J = 6.9 Hz, H₁₇), 3.47 (q, 2H, J = 6.3 Hz, H₁₆), 1.93–1.79 (m, 2H, H₂₁/H₂₂), and 1.66–1.54 (m, 2H, H₂₁/H₂₂).

13C{¹H} NMR (100.61 MHz, CDCl₃) δ: 162.5(C₁), 141.1(C₂), 138.2(C₈), 136.9(C₂₀), 134.4(C₁₀), 133.1(C₁₁, C₁₃), 132.3(C₁₅), 129.4(C₁₉), 129.3(C₁₂/C₁₉), 128.7(C₁₄), 127.6(C₆), 127.4(C₄), 123.0(C₃/C₅), 122.9(C₃/C₅), 118.9(C₁₈), 109.2(C₇), 50.0(C₉), 46.7(C₁₇), 38.0(C₁₆), 28.3(C₂₁/C₂₂), and 26.9(C₂₁/C₂₂).

3.4. N-(6-(1H-Imidazol-1-yl)hexyl)-1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamide (12)

Oxalyl chloride (4.0 mL, 0.0466 mol) and a catalytic amount of DMF were added to the solution of lonidamine (700 mg, 2.18 mmol) in DCM (40.0 mL). The reaction mixture was refluxed for 1 h. The solvent and oxalyl chloride were removed in vacuum. The obtained chloroanhydride without purification was dissolved in DCM (40.0 mL) and triethylamine (607 µL, 4.36 mmol), and then 6 (355 mg, 2.12 mmol) was added with stirring. The reaction mixture was stirred for 8 h, then washed by NaHCO₃ solution (2 × 80 mL) and NaCl solution (2 × 80 mL), and dried on Na₂SO₄. The solvent was removed and the
product was purified by column chromatography on silica gel (eluent: CH₂Cl₂:EtOH 12:1, Rf = 0.4). Yield: 247 mg (24.8%), elem. anal. calc. (%) for C₂₄H₂₅Cl₂N₂O*0.1CH₂Cl₂: C 60.44, H 5.30, and N 14.62. Found: C 60.48, H 5.09, and N 14.32.

**1H NMR** (400.13 MHz, CDCl₃) δ: 8.43 (d, 1H, J = 8.2 Hz, H₄), 7.50–7.27 (m, 5H, H₅, H₆, H₇, H₁₂, H₂₀), 7.14–7.01 (m, 3H, H₁₄, H₁₉, NH), 6.90 (s, 1H, H₁₈), 6.62 (d, 1H, J = 8.4 Hz, H₁₅), 5.66 (s, 2H, H₉), 3.93 (t, 2H, J = 7.1 Hz, H₁₇), 3.49 (q, 2H, J = 6.9 Hz, H₁₆), 1.87–1.74 (m, 2H, H₂₁/H₂₄), 1.72–1.59 (m, 2H, H₂₁/H₂₄), and 1.52–1.30 (m, 4H, H₂₂, H₂₃).

**13C{¹H} NMR** (100.61 MHz, CDCl₃) δ: 162.4 (C₁), 141.2 (C₂), 138.5 (C₈), 137.0 (C₂₀), 134.5 (C₁₀), 133.2 (C₁₁, C₁₃), 132.4 (C₁₅), 129.5 (C₁₉/C₁₂), 129.4 (C₁₉/C₁₂), 129.3 (C₁₄), 127.6 (C₆), 127.4 (C₄), 123.1 (C₃/C₅), 118.7 (C₁₈), 109.2 (C₇), 50.0 (C₉), 46.9 (C₁₇), 38.8 (C₁₆), 31.0 (C₂₁–C₂₄), 26.4 (C₂₁–C₂₄), and 26.3 (C₂₁–C₂₄).

### 3.5. N-(8-(1H-Imidazol-1-yl)octyl)-1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamide (13)

Oxalyl chloride (3.5 mL, 0.0408 mol) and a catalytic amount of DMF were added to the solution of lonidamine (678 mg, 2.11 mmol) in DCM (35.0 mL). The reaction mixture was refluxed for 1 h. The solvent and oxalyl chloride were removed in vacuum. The obtained chloroanhydride without purification was dissolved in DCM (35.0 mL) and triethylamine (587 µL, 4.22 mmol), and then 7 (400 mg, 2.05 mmol) was added with stirring. The reaction mixture was stirred for 8 h, then washed by NaHCO₃ solution (2 × 70 mL) and NaCl solution (2 × 70 mL), and dried on Na₂SO₄. The solvent was removed and the product was purified by column chromatography on silica gel (eluent: CH₂Cl₂:EtOH 12:1, Rf = 0.4). Yield: 419 mg (41.0%), elem. anal. calc. (%) for C₂₆H₂₉Cl₂N₂O*0.2CH₂Cl₂: C 61.05, H 5.75, and N 13.59. Found: C 61.22, H 5.49, and N 13.23.

**1H NMR** (400.13 MHz, CDCl₃) δ: 8.44 (d, 1H, J = 8.1 Hz, H₄), 7.49–7.27 (m, 5H, H₅, H₆, H₇, H₁₂, H₂₀), 7.14–7.00 (m, 3H, H₁₄, H₁₉, NH), 6.90 (s, 1H, H₁₈), 6.61 (d, 1H, J = 8.4 Hz, H₁₅), 5.66 (s, 2H, H₉), 3.91 (t, 2H, J = 7.1 Hz, H₁₇), 3.48 (q, 2H, J=6.8 Hz, H₁₆), 1.82–1.71 (m, 2H, H₂₁/H₂₆), 1.70–1.59 (m, 2H, H₂₁/H₂₆), and 1.45–1.25 (m, 8H, H₂₂–H₂₅).

**13C{¹H} NMR** (100.61 MHz, CDCl₃) δ: 162.3 (C₁), 141.2 (C₂), 138.6 (C₈), 137.0 (C₂₀), 134.5 (C₁₀), 133.2 (C₁₁, C₁₃), 132.4 (C₁₅), 129.4 (C₁₂/C₁₄/C₁₈/C₁₉), 129.3 (C₁₂/C₁₄/C₁₉), 127.6 (C₆), 127.4 (C₄), 123.2 (C₃/C₅), 122.9 (C₃/C₅), 118.7 (C₁₈), 109.2 (C₇), 50.0 (C₉), 47.0 (C₁₇), 39.0 (C₁₆), 31.0 (C₂₁–C₂₆), 29.8 (C₂₁–C₂₆), 29.0 (C₂₁–C₂₆), 28.9 (C₂₁–C₂₆), 26.8 (C₂₁–C₂₆), and 26.5 (C₂₁–C₂₆).

### 3.6. N-(12-(1H-Imidazol-1-yl)dodecyl)-1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamide (14)

Oxalyl chloride (1.8 mL, 0.0210 mol) and a catalytic amount of DMF were added to the solution of lonidamine (396 mg, 1.24 mmol) in DCM (20.0 mL). The reaction mixture was refluxed for 1 h. The solvent and oxalyl chloride were removed in vacuum. The obtained chloroanhydride without purification was dissolved in DCM (20.0 mL) and triethylamine (345 µL, 2.48 mmol), and then 8 (302 mg, 1.20 mmol) was added with stirring. The reaction mixture was stirred for 8 h, then washed by NaHCO₃ solution (2 × 40 mL) and NaCl solution (2 × 40 mL), and dried on Na₂SO₄. The solvent was removed and the product was purified by column chromatography on silica gel (eluent: acetone, Rf = 0.4). Yield: 665 mg
(47.9%), m.p. 86–88 °C, elem. anal. calc. (%) for C₃₀H₃₇Cl₂N₅O: C 64.97, H 6.73, and N 12.63. Found: C 65.02, H 6.72, and N 12.85. ¹H NMR (400.13 MHz, CDCl₃) δ: 8.45 (d, 1H, J = 8.2 Hz, H₄), 7.50–7.30 (m, 5H, H₅, H₆, H₇, H₁₂, H₂₀), 7.12 (dd, 1H, J = 8.3, 2.1 Hz, H₁₄), 7.07 (s, 1H, H₁₉), 7.03 (t, 1H, J = 5.6 Hz, NH), 6.92 (s, 1H, H₁₈), 6.62 (d, 1H, J = 8.3 Hz, H₁₅), 5.69 (s, 2H, H₉), 3.94 (t, 2H, J = 7.1 Hz, H₁₇), 3.50 (q, 2H, J = 6.9 Hz, H₁₆), 1.82–1.73 (m, 2H, H₂₁/H₃₀), 1.71–1.62 (m, 2H, H₂₁/H₃₀), and 1.46–1.23 (m, 16H, H₂₂–H₂₉).

13C{¹H} NMR (100.61 MHz, CDCl₃) δ: 162.3(C₁), 141.2(C₂), 138.6(C₈), 137.1(C₂₀), 134.4(C₁₀), 133.1(C₁₁, C₁₃), 132.5(C₁₅), 129.5(C₁₂/C₁₄/C₁₉), 129.2(C₁₂/C₁₄/C₁₈/C₁₉), 127.6(C₆), 127.4(C₄), 123.2(C₃/C₅), 123.0(C₃/C₅), 118.8(C₁₈), 109.2(C₇), 50.0(C₉), 47.0(C₁₇), 39.1(C₁₆), 31.1(C₂₁–C₃₀), 29.8(C₂₁–C₃₀), 29.5(C₂₁–C₃₀), 29.4(C₂₁–C₃₀), 29.3(C₂₁–C₃₀), 29.1(C₂₁–C₃₀), 27.0(C₂₁–C₃₀), and 26.5(C₂₁–C₃₀).

3.7. Na[trans-Ru(DMSO)(C₂₀H₁₇Cl₂N₅O)Cl₄] (15)

Compound 9 (100 mg, 0.24 mmol) in ~3 mL of acetone was added to the solution of [Ru(DMSO)₂Cl₄]⁻Na⁺ (102 mg, 0.24 mmol) in acetone (40.0 mL). The reaction mixture was stirred for 10 h. The solution was filtered and evaporated up to a minimum volume. The product was purified by column chromatography on silica gel (elucent: acetone, Rf = 0.5). Yield: 107 mg (58.5%), m.p. 181–183 °C (decomp.), elem. anal. calc. (%) for C₂₂H₂₃Cl₆N₅NaO₂RuS: C 34.85, H 3.06, N 9.24, and S 4.23. Found: C 35.07, H 3.38, N 8.99, and S 4.36, ESI-MS: m/z: 737 [M–Na⁺]⁻.

3.8. Na[trans-Ru(DMSO)(C₂₁H₁₉Cl₂N₅O)Cl₄] (16)

Compound 10 (100 mg, 0.23 mmol) in ~3 mL of acetone was added to the solution of [Ru(DMSO)₂Cl₄]⁻Na⁺ (98 mg, 0.23 mmol) in acetone (40.0 mL). The reaction mixture was stirred for 10 h. After 5 h, a precipitate was formed. The solution was filtered and the product was washed by acetone and dried. Yield: 125 mg (69.4%), m.p. 203–206 °C (decomp.), elem. anal. calc. (%) for C₂₃H₂₅Cl₆N₅NaO₂RuS: C 35.77, H 3.26, N 9.07, and S 4.15. Found: C 35.60, H 3.65, N 8.97, and S 3.90, ESI-MS: m/z: 749 [M–Na⁺]⁻.

3.9. Na[trans-Ru(DMSO)(C₂₂H₂₁Cl₂N₅O)Cl₄] (17)

Compound 11 (100 mg, 0.23 mmol) in ~3 mL of acetone was added to the solution of [Ru(DMSO)₂Cl₄]⁻Na⁺ (95 mg, 0.23 mmol) in acetone (40.0 mL). The reaction mixture was stirred for 10 h. The solution was filtered and evaporated up to a minimum volume. The product was purified by column chromatography on silica gel (elucent: acetone, Rf = 0.5). Yield: 74 mg, (41.6%), m.p. 157–159 °C (decomp.), elem. anal. calc. (%) for C₂₄H₂₇Cl₆N₅NaO₂RuS: C 36.66, H 3.46, N 8.91, and S 4.08. Found: C 36.60, H 3.65, N 8.97, and S 3.84, ESI-MS: m/z: 765 [M–Na⁺]⁻.

3.10. Na[trans-Ru(DMSO)(C₂₄H₂₅Cl₂N₅O)Cl₄] (18)

Compound 12 (100 mg, 0.21 mmol) in ~3 mL of acetone was added to the solution of [Ru(DMSO)₂Cl₄]⁻Na⁺ (90 mg, 0.21 mmol) in acetone (40.0 mL). The reaction mixture was stirred for 10 h. The solution was filtered and evaporated up to a minimum volume. The product was purified by column chromatography on silica gel (elucent: acetone, Rf = 0.5). Yield: 113 mg (65.3%), m.p. 90–92 °C (decomp.), elem. anal. calc. (%) for C₂₆H₂₇Cl₆N₅NaO₂RuS*: C 39.92, H 4.27, N 8.03, and S 3.68. Found: C 40.07, H 4.36, N 7.76, and S 3.29, ESI-MS: m/z: 791 [M–Na⁺]⁻.
3.11. Na[trans-Ru(DMSO)(C$_{26}$H$_{29}$Cl$_{2}$N$_{2}$O)Cl$_{4}$] (19)  
Compound 13 (100 mg, 0.20 mmol) in ~3 mL of acetone was added to the solution of [Ru(DMSO)$_{2}$Cl$_{4}$]$^{-}$ $\cdot$ Na$^{+}$ (85 mg, 0.20 mmol) in acetone (40.0 mL). The reaction mixture was stirred for 10 h. The solution was filtered and evaporated up to a minimum volume. The product was purified by column chromatography on silica gel (eluent: acetone, R$_{f}$ = 0.5). Yield: 121 mg (71.6%), m.p. 78–80 $^\circ$C (decomp.), elem. anal. calc. (%) for C$_{28}$H$_{35}$Cl$_{6}$N$_{5}$NaO$_{2}$RuS*0.5C$_{3}$H$_{6}$O: C 40.65, H 4.39, N 8.04, and S 3.68. Found: C 40.62, H 4.54, N 7.67, and S 3.43, ESI-MS: $m/z$: 819 [M–Na$^{+}$]–.

3.12. Na[trans-Ru(DMSO)(C$_{30}$H$_{37}$Cl$_{2}$N$_{5}$O)Cl$_{4}$] (20)  
Compound 14 (70 mg, 0.13 mmol) in ~3 mL of acetone was added to the solution of [Ru(DMSO)$_{2}$Cl$_{4}$]$^{-}$ $\cdot$ Na$^{+}$ (53 mg, 0.13 mmol) in acetone (30.0 mL). The reaction mixture was stirred for 10 h. The solution was filtered and evaporated to a minimum volume. The product was purified by column chromatography on silica gel (eluent: acetone, R$_{f}$ = 0.5). Yield: 70 mg (59.8%), m.p. 120–122 $^\circ$C (decomp.), elem. anal. calc. (%) for C$_{32}$H$_{43}$Cl$_{6}$N$_{5}$NaO$_{2}$RuS: C 42.77, H 4.82, N 7.79, and S 3.57. Found: C 43.04, H 5.23, N 8.01, and S 3.34, ESI-MS: $m/z$: 875 [M–Na$^{+}$]–.

3.13. Electrochemical Activity  
All electrochemical measurements were carried out under argon at room temperature. Cyclic voltammetry (CV) experiments were performed in KO$_{2}$64 PAR three-electrode microcells in CH$_{3}$CN solution with 0.05 M of Bu$_{4}$NBF$_{4}$ as a supporting electrolyte using an IPC-Win potentiostat. The number of transferred electrons was determined by comparing to the height of the Fc$^{2+}$/Fc$^{3+}$ wave for the same concentration. A glassy carbon (GC) working electrode (diameter 2 mm), a platinum wire auxiliary electrode, and an aqueous Ag/AgCl/KCl (sat.) reference electrode was used. Solvents were routinely distilled and dried prior to use.

3.14. Stability  
The stability of Ru(III) complexes was studied by electron absorption spectroscopy in 20 mM of phosphate buffer, pH 7.4, and 100 mM of NaCl. The working solution (2 mL, 200 $\mu$M complex) was prepared by diluting 10 $\mu$L of the original 40 mM solution in DMSO and 1.99 mL of phosphate buffer. UV-vis spectra were recorded every 60 s in the range of 280–600 nm at 37 $^\circ$C. The half-transformation time was t$_{1/2}$. For $\lambda_{max}$, $\Delta A(t)$ was plotted, where $\Delta A = A_{0}–A_{i}$, an initial section, was approximated as a linear function. At $\Delta A_{line}$, point calculated as t = t$_{1/2}$.

3.15. Lipophilicity  
$N$-octanol was saturated with water and with water saturated with n-octanol; mixture400 mL of n-octanol (water) and 100 mL of water (n-octanol) was stirred for 24 h; and then fractions were separated. The sample of the complex was dissolved in n-octanol and a series of solutions in octanol (300, 250, 3 $\times$ 200, 150, 100, and 50 $\mu$M) was prepared. Absorption spectra were recorded and the calibration curve for maximum absorption was plotted. From 200 $\mu$M solutions, the mixtures with water were prepared (1:1, 1:2, 2:1 v/v) and shaken for 15 min. The organic phase was separated by centrifugation. The concentration of the complex in $n$-octanol was determined from the calibration curve and lipophilicity (logP) was calculated as logP = log [(C$_{0}$–C$_{aq}$)/C$_{aq}$].

3.16. Cellular Accumulation of Ruthenium Determined by Atomic Absorption Spectrometry  
The intracellular accumulation of metal-containing compounds was determined as described [46,52,53]. The MCF-7 breast carcinoma cell line (CLS, Eppelheim, Germany) was propagated in Dulbecco’s modified Eagle medium supplemented with 10% foetal bovine serum (Biochrom GmbH, Germany) and 50 mg/mL gentamicin at 37 $^\circ$C, as well as 5% CO$_{2}$ in a humidified atmosphere. Cells were grown until 80% confluence in 75 cm$^{2}$
flasks. Stock solutions of 16 and 20 (10 mM in DMSO) were diluted with the full medium or serum-free medium immediately prior to cell exposure. Cells were treated with 16 or 20 (20 µM each) at 37 °C, 5% CO₂, for up to 24 h, washed twice with PBS, and isolated by scraping off and centrifuged at 1000 × g for 5 min.

For metal and protein quantification, pellets were resuspended in 250 µL of deionised water and lysed for 30 min with sonication. The protein content in lysates was determined by the Bradford method. For ruthenium measurements, a contrAA 700 high resolution continuum-source atomic absorption spectrometer (Analytik Jena AG, Jena, Germany) was used. Samples of the respective complex were used as standards. Calibration was done in a matrix-matched manner, that is, all samples and standards were adjusted to the same protein concentration of 1 mg/mL by dilution with water. Triton-X 100 (1%, 10 µL) and nitric acid (13%, 10 µL) were added to each standard sample (100 µL). Samples were injected (25 µL) into coated graphite tubes (Analytik Jena AG, Jena, Germany) and thermally processed as described with minor modifications [53]. Drying steps were adjusted, the atomisation temperature was set to 2400 °C, and the reading time was increased to 7 s. Ruthenium was quantified at 349.8945 nm. The mean integrated absorbance of triple injections was used throughout the measurements. Results (average of the two experiments) were expressed as nmol metal/mg protein.

3.17. Inhibition of TrxR
3.17.1. Inhibition of Purified Protein

The activity of TrxR [40, 54] was determined in a microplate format. Commercially available rat liver TrxR (Sigma Aldrich, St. Louis, MO, USA) was diluted with distilled water to 3.5 Unit/mL. In total, 25 µL aliquots of this solution were mixed with 25 µL of a potassium phosphate buffer, pH 7.0, with or without tested compounds. Fifty µL of 0.5% v/v dimethyl formamide in buffer was served as a blank. An additional control experiment revealed that the test compounds did not exhibit any absorption at the respective wavelength or reduce the DTNB in the absence of the enzyme. For this purpose, 25 µL of the highest test concentration of each compound and 25 µL of phosphate buffer (no enzyme) were mixed. Samples were incubated with moderate shaking for 75 min at 37 °C in a 96-well plate. To each well, 225 µL of the reaction mixture (500 µL of potassium phosphate buffer, pH 7.0, 80 µL of 100 mM ethylenediaminetetraacetic acid (EDTA), 20 µL of 0.2% bovine serum albumin, 100 µL of 20 mM NADPH (nicotinamide adenine dinucleotide phosphate), and 300 µL of water) was added; the reaction was initiated by the addition of 25 µL of 20 mM DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) solution in ethanol. The formation of 5-TNB was monitored at 405 nm 10 times at 35 s intervals by a VICTOR X4 Plate Reader (Perkin Elmer). The increase of the 5-TNB concentration over time showed a linear trend (r² ≥ 0.99); enzymatic activities were calculated as slopes (an increase of absorbance per second). IC₅₀ values were calculated as the concentration of the compound that decreased the enzymatic activity of the control (no compound) by 50%. Values are mean ± SD of the three independent measurements.

3.17.2. Inhibition of Intracellular TrxR1

The TrxR1 enzyme was measured in the extracts from the liver of male white outbred rats homogenised in 50 mM of PBS containing 1 mM of EDTA and centrifuged for 15 min at 10,000 rpm at 4 °C. The supernatant was adjusted to 7.5 µg/mL of protein as determined with pyrogallol red. Then, 40 µL of the enzyme solution was mixed with 10 µL of the test compounds in a 96-well clear flat bottom plate. After 10 min at 25 °C, 30 µL of PBS containing 0.7 mg/mL of BSA and 0.8 mM of NADPH was added. After 15 min at 25 °C, 20 µL of DTNB solution was added to the desired final concentrations. Kinetic studies were carried out by assaying TrxR1 at various concentrations of DTNB. The optical density of the samples was measured with an Infinite M200 Pro reader (Tecan, Grödig, Austria) at 412 nm every 30 s for 20 min. The activity of TrxR1 was calculated as a reaction slope relative to the control wells using Prism 8.0 (GraphPad, Inc., San Diego, CA, USA).
3.18. Cell Death Studies

The antiproliferative activity was studied by MTT assays as published previously [35]. For flow cytometry studies, cells were plated into 6-well plates (Eppendorf, Germany; $4 \times 10^5$ cells in 2 mL of DMEM) and incubated for 24 h. Solutions of complexes in DMSO were prepared immediately prior to the day of the experiments. Cells were treated with either 20 $\mu$M of cisplatin, 50 $\mu$M of 16, or 20 $\mu$M of 20. Concentrations corresponded to twofold IC$_{50}$ values based on MTT assays. Cells were incubated for 24 h, pooled, washed with cold PBS, and resuspended in DMEM. Aliquots of cells were processed as recommended in the Muse Annexin V&Dead Cell Kit or Muse Caspase-3/7 Kit (Luminex). Measurements were carried out on a Muse Cell Analyser, Luminex corp., TX, USA.

3.19. In Vivo Acute Toxicity

The Balb/c female mice (8–10 weeks old, weight 20–22 g) were bred and hosted at the animal facility of the Blokhin Cancer Center [55]. Mice were kept at $21 \pm 1$ °C, 50–60% humidity; food and water were added ad libitum. All manipulations were performed in accordance to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123). Compound 16 was injected i.p.; 70–110 mg/kg single bolus administration in 200 $\mu$L of saline. Each cohort contained six mice. Animals were monitored for 21 days after injection. General behavioural activity, nutritional habits, and the integrity of hair cover were the criteria of acute toxicity.

4. Conclusions

Complexes in which the ruthenium fragment and lonidamine were connected by an imidazole linker were obtained and described. These complexes showed micromolar cytotoxicity and lipophilicity between 0.5 and 1.5. Complexes were more active than the corresponding ligands, the parent drug, and, in certain cases, cisplatin. The cytotoxicity increased along with the linker’s length. The most stable and biologically active complex was 20 and its half-transformation time was ~35 min. The intracellular accumulation of ruthenium complexes was fast and dependent on the length of the linker. The caspase 3/7 mediated apoptosis is a major mode of cell death induced by lonidamine-Ru complexes. Together with the tolerance of 16 in Balb/c mice, our data suggest a perspective of the new chemotype in search of antitumour drug candidates.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms222413468/s1.

Author Contributions: Conceptualisation, A.A.N.; funding acquisition, E.R.M. and A.A.N.; investigation, I.A.S., Y.N.O., V.Y.T., E.V.S., D.A.B., Y.A.G., C.S., K.I.K., A.A.S. (Alexander A. Shtil), O.M.R., and E.F.S.; supervision, A.A.S. (Alexander A. Spasov), E.R.M., I.O., and A.A.N.; writing—original draft preparation, D.A.B., K.I.K., A.A.S. (Alexander A. Shtil), E.F.S., E.R.M., I.O., and A.A.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research study was funded by the Russian Science Foundation (grant number 19-13-00084, synthesis), Russian Foundation for Basic Research (grant number 19-53-12042, uptake and thioredoxin reductase experiments), and No. 19-03-00394 (cell studies). Financial support by Deutsche Forschungsgemeinschaft (DFG, project number 409903581) is gratefully acknowledged.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of National Medical Research Center of Oncolog of the Ministrv of Health of the Russian Federation (protocol № 2021-17 February 15 2021).

Acknowledgments: We acknowledge the unique scientific facility of Transgenebank for using its equipment and and the “Centre for Collective Use of IPAC RAS” for use of equipment.

Conflicts of Interest: The authors declare no conflict of interest.
47. Bakulina, O.; Bannykh, A.; Jovanović, M.; Domračeva, I.; Podolski-Renić, A.; Žalubovskis, R.; Pešić, M.; Dar’in, D.; Krasavin, M. Design, synthesis, and biological evaluation of novel derivatives of dithiodiglycolic acid prepared via oxidative coupling of thiols. J. Enzym. Inhib. Med. Chem. 2019, 34, 665–671. [CrossRef]

48. Omata, Y.; Folan, M.; Shaw, M.; Messer, R.L.; Lockwood, P.E.; Hobbs, D.; Bouillaguet, S.; Sano, H.; Lewis, J.B.; Wataha, J.C. Sublethal concentrations of diverse gold compounds inhibit mammalian cytosolic thioredoxin reductase (TrxR1). Toxicol. In Vitro 2006, 20, 882–890. [CrossRef]

49. Hashemy, S.I.; Ungerstedt, J.S.; Avval, F.Z.; Holmgren, A. Motexafin Gadolinium, a Tumor-selective Drug Targeting Thioredoxin Reductase and Ribonucleotide Reductase. J. Biol. Chem. 2006, 281, 10691–10697. [CrossRef]

50. Citta, A.; Folda, A.; Scutari, G.; Cesaro, L.; Bindoli, A.; Rigobello, M.P. Inhibition of thioredoxin reductase by lanthanum chloride. J. Inorg. Biochem. 2012, 117, 18–24. [CrossRef]

51. Vermes, I.; Haanen, C.; Steffens-Nakken, H.; Reutellingsperger, C. A novel assay for apoptosis Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J. Immunol. Methods 1995, 184, 39–51. [CrossRef]

52. Schatzschneider, U.; Niesel, J.; Ott, I.; Gust, R.; Alborzinia, H.; Wölfl, S. Cellular Uptake, Cytotoxicity, and Metabolic Profiling of Human Cancer Cells Treated with Ruthenium(II) Polypyridyl Complexes [Ru(bpy)2(N N)]Cl2 with NN = bpy, phen, dpq, dppz, and dppn. ChemMedChem 2008, 3, 1104–1109. [CrossRef]

53. Appold, M.; Mari, C.; Lederle, C.; Elbert, J.; Schmidt, C.; Ott, I.; Stühn, B.; Gasser, G.; Gallei, M. Multi-stimuli responsive block copolymers as a smart release platform for a polypyridyl ruthenium complex. Polym. Chem. 2017, 8, 890–900. [CrossRef]

54. Schmidt, C.; Karge, B.; Misgeld, R.; Prokop, A.; Franke, R.; Brönstrup, M.; Ott, I. Gold(I) NHC Complexes: Antiproliferative Activity, Cellular Uptake, Inhibition of Mammalian and Bacterial Thioredoxin Reductases, and Gram-Positive Directed Antibacterial Effects. Chem. Eur. J. 2017, 23, 1869–1880. [CrossRef]

55. Grin, A.M.; Tikhonov, I.S.; Petrova, S.A.; Pogortilyy, A.V.; Noev, N.A.; Tatarskiy, V.V.; Shpakovsky, B.D.; Milaeva, R.E.; Kalinina, V.E.; Chernov, N.N.; et al. New Derivatives of Bacteriopurpurin with Thiolated Au (I) Complexes: Dual Darkand Light Activated Antitumor Potency. Anti-Cancer Agents Med. Chem. 2020, 20, 49–58. [CrossRef]