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New Ruthenium(II)−Letrozole Complexes as Anticancer Therapeutics

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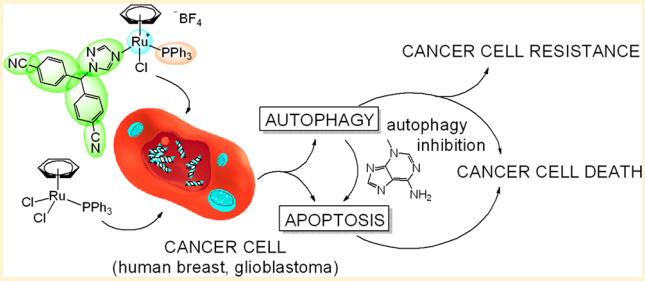
Supporting Information

ABSTRACT: Novel ruthenium−letrozole complexes have been prepared, and cell viability of two human cancer cell types (breast and glioblastoma) was determined. Some ruthenium compounds are known for their cytotoxicity to cancer cells, whereas letrozole is an aromatase inhibitor administered after surgery to post-menopausal women with hormonally responsive breast cancer. A significant *in vitro* activity was established for complex **5**·Let against breast cancer MCF-7 cells and significantly lower activity against glioblastoma U251N cells. The activity of **5**·Let was even higher than that of **4**, a compound analogous to the well-known drug RAPTA-C. Results from the combination of **5**·Let (or **4**) with 3-methyladenine (3-MA) or with curcumin, respectively, revealed that the resultant cancer cell death likely involves **5**·Let-induced autophagy.



INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among women, accounting for 23% of total cancer cases in women, and 14% of cancer deaths.[1] The development of cancer therapies over the past few decades has focused principally on chemotherapy combined with other treatment approaches such as surgery, radiotherapy, and targeted therapy. A major drawback of chemotherapy is that the drugs used are also toxic to normal cells, leading to numerous undesirable side effects (bone and back pain, blood clots, weakness, fatigue, stroke, hair loss, etc.). Moreover, the use of a single agent often fails to achieve complete cancer remission,[2] largely due to the development of cancer cell resistance. An appealing strategy to increase the survival chances of breast cancer victims is to create multitasking drugs, able to promote cell death by simultaneously targeting selected signal transduction molecules. In addition to possible synergistic effects resulting from the combined use of two drugs (which can lead to shorter-term treatments), another great advantage of this approach is the potential to gain some control of the hydrophilicity and the size of the entire delivery system, increasing drug efficiency and reducing side effects. We have undertaken the preparation of ruthenium−letrozole multitasking drugs; the selection of the two components is justified below.

Cell Killing Agent: Ruthenium. A well-known example of a cell killing drug is cisplatin, a transition metal-based complex that has been administered to cancer patients for decades and is known to interact with DNA.[3] During the last 20 years, ruthenium complexes of various kinds have emerged in the literature, mainly designed to replace cisplatin; it was believed that the interaction of ruthenium complexes with DNA would differ from that of cisplatin (or carboplatin and oxaliplatin), due to the different geometries that they adopt.[4] Ruthenium-based drugs are very appealing to study, not only to better understand their modes of action but also because they were found to have certain advantages over platinum-based drugs: (i) they are active against cisplatin resistant cell lines, notably the breast T47D cells and the ovarian A2780cisR cells, and (ii) they have a low occurrence of side effects due to their higher selectivity for cancer cells compared with normal cells.[4] Like cisplatin, ruthenium complexes interact with DNA but, as anticipated, they interact differently. Interestingly, some ruthenium compounds bind more strongly, leading to an adduct that is more resistant to cell repair mechanisms.[5] Ruthenium drugs are also believed to induce cancer cell death by other mechanisms; for instance, they are believed to enter cancer cells by mimicking iron, a ruthenium congener of the periodic table, (i) by binding to human serum proteins found at the cells surface[6a] and (ii) by interacting with mitochondria.[6b] Another mode of action for cancer therapeutics is the so-called "activation by reduction" mechanism, which has been under debate for decades but which only applies to Ru(III) drugs.[7] It is noteworthy that two Ru(III) complexes have entered phase II clinical trials: KP1019 and NAMI-A (Figure 1a). KP1019 was developed for solid tumors, whereas NAMI-A has been prepared as an antimetastatic drug.[4] Also, several Ru(II) complexes bearing an arene ligand are under preclinical evaluation, such as RAPTA-C and Ru-arene-en (Figure 1a).

Cell Growth Inhibiting Agent: Letrozole. Estrogen receptor positive (ER+) is a very common type of breast cancer and is diagnosed when estrogen receptors α (ERα) are found in excess in the tumor cells of the victim. After surgical

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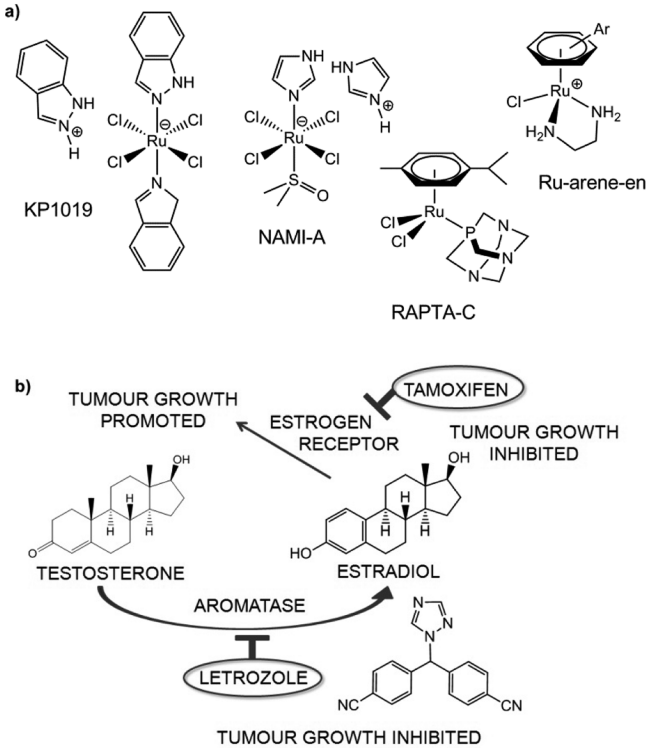

Figure 1. (a) Ruthenium compounds for cancer therapy and (b) inhibition of aromatase by letrozole.

removal of breast tumor(s), it is usual to administer a cell growth inhibiting drug in order to avoid ER+ cancer recidivism. A well-known example of this type of drug is tamoxifen (prodrug), which acts as a selective estrogen receptor down-regulator since its mode of action is to bind to the estrogen receptors and consequently to prevent tumor growth (Figure 1b). Another way of achieving the same goal is simply by blocking the action of an enzyme called aromatase, a member of the cytochrome P450 family, consisting of an iron center surrounded by a porphyrin and a cysteine unit.[8] This enzyme catalytically converts androgens to estrogens, notably testosterone into estradiol, an estrogen responsible for the growth of breast cancer tumors. In post-menopausal women, the production of estrogens is no longer governed by the ovaries and mainly relies on the catalytic action of aromatase. Letrozole (Femara) (Figure 1b) is an example of drugs that can coordinate to the aromatase iron center and deactivate it by preventing androgens from approaching its catalytic site. Letrozole was approved by the FDA a decade ago as a first-line treatment due to its proven superiority to the widely used prodrug tamoxifen[9] and is commonly administered to post-menopausal women suffering from an ER+ breast cancer.

Herein, we report the preparation of the first ruthenium–letrozole complexes; the special feature is their potential to simultaneously kill cancer cells (ruthenium) and inhibit the growth of the surviving ones (letrozole). We also report our results on their toxicity against human breast MCF-7 cancer cells and compare the results with the ones obtained for human glioblastoma brain U251N cancer cells, one of the deadliest forms of cancer because of its highly invasive nature.[10] We have also investigated the combination of our most potent ruthenium drugs with a well-known autophagy inhibitor, 3-methyladenine (3-MA), and a drug that is known to mainly induce cell death via autophagy, curcumin.

RESULTS AND DISCUSSION

Complex Synthesis and Characterization. Cationic ruthenium–letrozole complex **2a**, in which two letrozole ligands are coordinated to the metal via only one nitrogen atom, was obtained with 44% yield when dichloro(benzene)-ruthenium(II) dimer **1** was heated with 4 equiv of letrozole (Figure 2). Interestingly, when only 2 equiv of the aromatase inhibitor was used instead, compound **2a** was again obtained as the major species. To our knowledge, this is the first example of the coordination of letrozole to a group 8 metal, which is of great importance to further understand how this drug might also coordinate to iron, a congener of ruthenium in the periodic table, which is the metal responsible for the catalytic activity of the aromatase enzyme. Only two reports of letrozole–metal

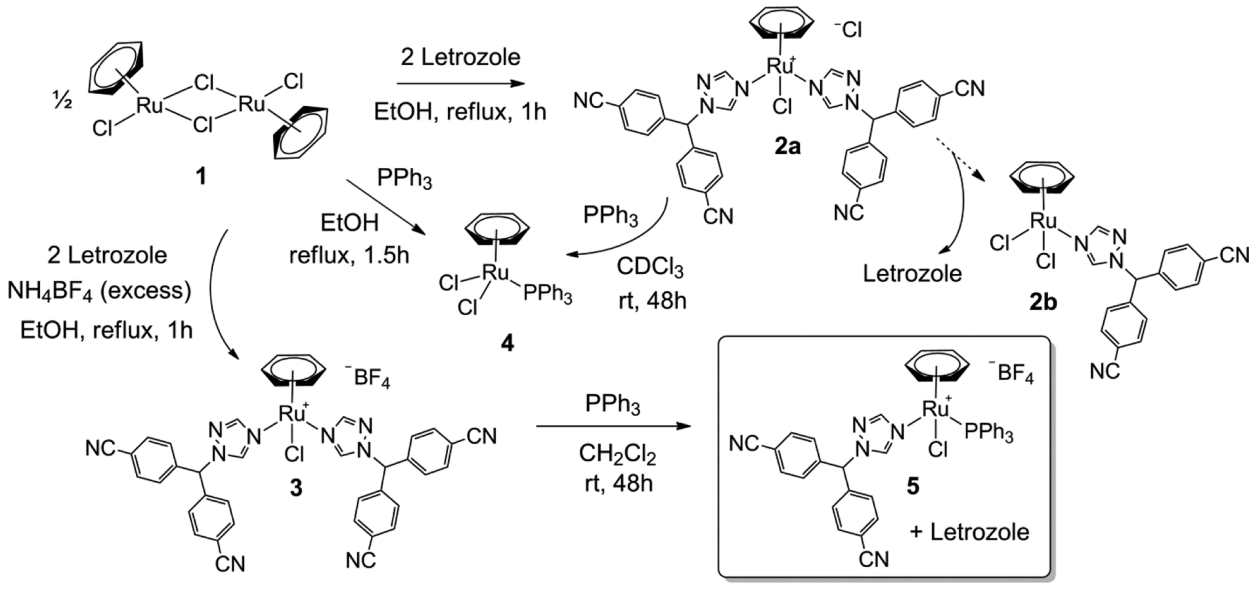

Figure 2. Synthesis of letrozole–ruthenium complexes.

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interactions were found in the literature; they showed that the enzyme inhibitor can coordinate to Cu(I) in a tetradentate fashion to afford a polymer, $\{[Cu(LTZ)](BF_4)\cdot CHCl_3\}_n$,[11] or can coordinate to Cu(II), Ni(II), or Co(II) by only one nitrogen of the triazole ring,[12] as observed in the present study. The nature of **2a** was unambiguously confirmed by high-resolution (and high-accuracy) mass spectrometry, as well as by elemental analysis. Characterization of the complex by NMR spectroscopy was less straightforward since the letrozole ligands in **2a** were found to be relatively labile in solution. For instance, when a ^{1}H NMR spectrum of **2a** was recorded in CD_2Cl_2, 50% of the molecules were found to undergo replacement of one letrozole ligand, most likely by the chloride counterion. As a consequence an equimolar mixture of cationic complex **2a**, neutral complex **2b** (the new species obtained from the displacement of one letrozole unit), and letrozole was produced. Markedly, when spectra of **2a** were recorded in coordinating solvents such as DMSO-d_6 or methanol-d_4, one letrozole from all the molecules was found to readily leave the coordination sphere of the ruthenium, leading to the exclusive observation, by ^{1}H NMR, of signals similar to the ones previously attributed to **2b**. It is noteworthy that in those coordinating solvents, the complete displacement of both letrozole ligands was also observed over time. The lability of the aromatase inhibitor(s) in **2a** was also observed in the solid state by X-ray crystallography studies. When single crystals were grown over 12 h by the diffusion of pentane into a freshly prepared and concentrated solution of **2a** in $CDCl_3$, two types of crystals were found to coexist, corresponding to **2a** and **2b** (Figure 2). The ORTEP (Oak Ridge thermal-ellipsoid plot) diagram for the solid-state structure analysis of complex **2b** is presented in Figure 3.[13] Crystals of **2a** were less easy to handle than those of **2b**, losing their crystalinity within a few minutes when removed from the $CDCl_3$/pentane solvent mixture at room temperature. This observation is consistent with the high content of residual electronic density found in the structure of **2a**, which might be due to the presence of solvent molecules within the crystal structure. For comparison purposes, the synthesis of a cymene analogue was attempted using the same procedure as the one used to prepare **2a**, starting with dichloro(*p*-cymene)ruthenium(II) dimer ($[RuCl_2(cymene)]_2$). Unfortunately, the solid obtained was only soluble in DMSO-d_6 and ^{1}H NMR spectroscopy revealed the presence of two free letrozole ligands per cymene unit, which might indicate that the steric hindrance of the arene ring is a factor enhancing letrozole ligand lability in solution.

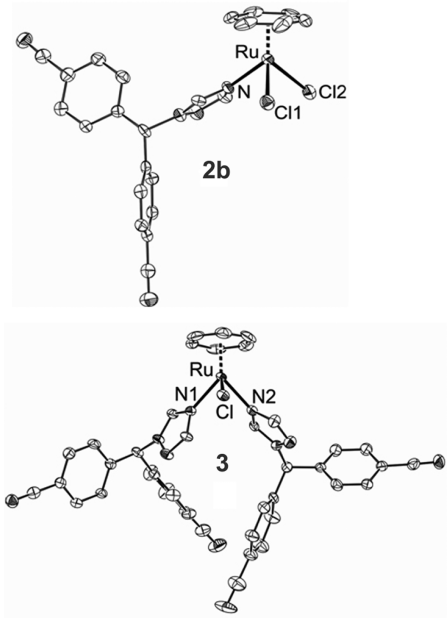

Figure 3. ORTEP diagrams for complexes **2b** and **3**. Thermal ellipsoids are shown at the 50% probability level.

Coordination of phosphorus ligands to transition metals favors hydrophilicity and promotes interactions with DNA,[14] leading to enhancement of the complex cytotoxicity. For example, the introduction of 1,3,5-triaza-7-phosphatricyclo[3.3.1.1]decane (PTA) into organometallic Ru(II)−arene systems resulted not only in formation of water-soluble complexes but also in selective antimetastatic activity. Also, it has been shown that when modified with a triphenylphoshine ligand, the RAPTA structure forms a complex with enhanced cytotoxicity and cellular uptake, and with higher affinity toward DNA.[15] We reasoned that it would be of interest to study the effect of the substitution of one of the letrozole ligands in **2a** by a triphenylphosphine. When the reaction of complex **2a** with 1 equiv of triphenylphosphine was followed by $^{31}P\{^1H\}$ NMR spectroscopy, it was noted that the singlet resonance corresponding to the free triphenylphosphine disappeared and that a new singlet resonance at 23 ppm emerged, which was found to correspond to the neutral **4** complex (Figure 2). The expected monosubstituted cationic complex might have formed but probably underwent a substitution reaction of the remaining letrozole ligand by the chloride counterion. The identity of the complex was confirmed by comparing its NMR spectra with the ones displayed by the product obtained from the well-known reaction of **1** with 2 equiv of triphenylphosphine. Since the presence of the chloride counterion in **2a** was most likely the main factor preventing us from preparing a complex bearing both a letrozole and a triphenylphosphine, we attempted the preparation of a new cationic letrozole−ruthenium complex, taking advantage of the less coordinating nature of the tetraphenylborate or tetrafluoroborate counterions. Complex **2a** reacted sluggishly with sodium tetraphenylborate or sodium tetrafluoroborate under the conditions used but the desired analogue of **2a**, complex **3**, was successfully prepared with 73% yield when **1** was heated with 4 equiv of letrozole and an excess of ammonium tetrafluoroborate for 1 h (Figure 2). As expected, the letrozole ligands in that complex were less labile in solution than the ones in **2a**. Fortunately, it was possible to achieve the synthesis of our target 18-electron compound **5** (Figure 2), by substituting only one letrozole ligand from **3**, by a triphenylphosphine. Complexes **3** and **5** were characterized by NMR spectroscopy, high-resolution (and high-accuracy) mass spectrometry, and by elemental analysis.[16] Their nature was unambiguously confirmed by X-ray crystallography, and the ORTEP diagram for the state structure analysis of complex **3** is presented in Figure 3.[13]

Cytotoxicity Studies. The *in vitro* toxicity of letrozole complexes **2a**, **3**, and **5**·Let and the letrozole free complex **4** (25 μM) was assessed against the human breast cancer MCF-7 cells (after 24 h), by the MTT assay and cell counting (Figure 4a, Figure S1, Supporting Information). The two analogues **2a** and **3**, each bearing two letrozole ligands, did not significantly reduce mitochondrial metabolic activity (**2a**, 82% ± 3%; **3**, 79% ± 3%; $p < 0.001$), compared with the effect of equimolar or

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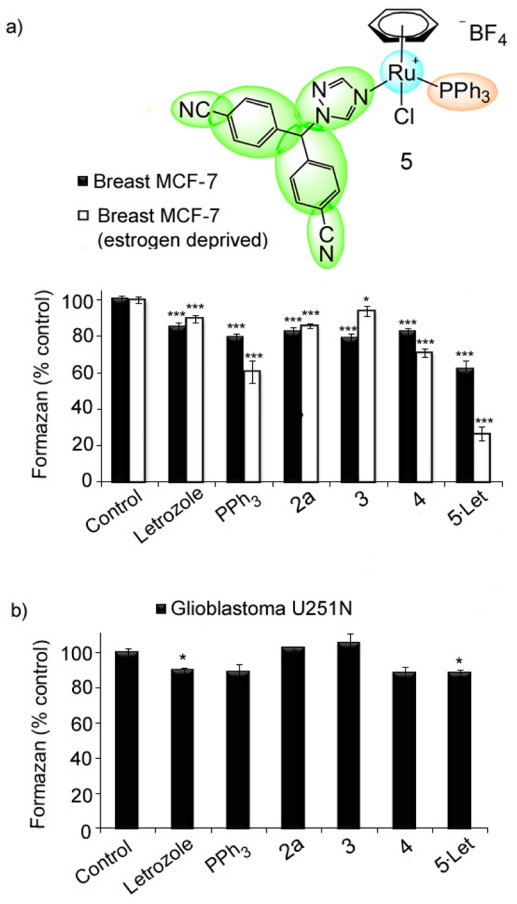

Figure 4. Mitochondrial metabolic activity determined by MTT assay in human breast cancer cells (MCF-7) (a) and glioblastoma cancer cells (U251N) (b), treated with **2a** (25 μM), **3** (25 μM), **4** (25 μM), **5**·Let (25 μM), triphenylphosphine (25 μM), and letrozole (50 μM). Results from estrogen-deprived MCF-7 cells are depicted with open bars, and those from MCF-7 cells grown in the presence of serum are depicted as filled bars. All values are expressed as means (from at least three independent experiments) ± SEM relative to untreated controls (100%). Significant differences: *$p < 0.05$; ***$p < 0.001$.

even double (50 μM) letrozole concentrations. Similar results were obtained for **4** and PPh_3. In contrast, **5**·Let significantly reduced mitochondrial metabolic activity (62% ± 5%, $p < 0.001$), suggesting an additive toxic effect between triphenylphosphine and two letrozole ligands, under comparable testing conditions. Interestingly, when estrogen-deprived conditions were used, the cytotoxicity of complexes **2a** and **3** (as well as the one corresponding to twice the concentration of letrozole tested alone) was found to be approximately equal or even slightly lower compared with the previous conditions used (Figure 4a). A plausible explanation for this behavior might be the low hydrophilicity of these compounds under the conditions used or simply that the cells developed a letrozole resistance. It should be stressed that the mitochondrial metabolic activity decreased significantly for the other compounds tested under estrogen-deprived conditions. This observation highlights the role played by estrogens in the growth of MCF-7 hormonally responsive cancer cells but also the role played by the triphenylphosphine moiety, which is present in the other compounds tested. This ligand is known to enhance the anticancer properties of metallic complexes but also proved to be surprisingly cytotoxic when tested alone (Figure 4a, 61% ± 6%, $p < 0.001$).This cytotoxicity is even higher than that observed for **4**, a compound analogous to RAPTA-C, which is a well-known antimetastatic ruthenium drug under preclinical evaluation (Figure 4a, 71% ± 2%, $p < 0.001$). Among all the drugs tested under estrogen-deprived conditions, **5**·Let remained the most potent drug for which the mitochondrial metabolic activity decreased dramatically (Figure 4a, 27% ± 4%, $p < 0.001$). This result might indicate a killing effect from the ruthenium moiety, the compound being 2 times more cytotoxic than one would have expected from the additive effect of the ligands tested alone, under those conditions. Moreover, this result demonstrates the importance of fine-tuning the drug solubility and size to favor the most appropriate interactions of the metal with biological molecules for optimizing a killing effect. The IC_{50} value for **5**·Let in MCF-7 cells is 36 ± 6 μM (for 24 h), which is in the range of the IC_{50} values reported for other Ru(II) complexes against human cancer cells. For instance, an IC_{50} value of 19.58 ± 2.37 μM (72 h) was reported for complex [Ru(η^6-*p*-cymene)(curcuminato)-Cl],[17a] an IC_{50} value of 11.3 ± 1.4 μM (72 h) was reported for complex [RuCl(Tp)(PTA)(PPh_3)] (Tp = trispyrazolylborate),[17b] and IC_{50} values in the range of 1.8 ± 0.5 to 115.0 ± 0.6 μM (96 h) were reported for complexes [Ru(η^6-*p*-cymene)-Cl_2{$Ph_2P(CH_2)_nS(O)x$Ph-κ-P}] ($x = 0-2$; $n = 1-3$),[17c] against MCF-7 breast cancer cells.

Compounds tested in MCF-7 cells were also tested in glioblastoma brain U251N cells (after 24 h exposure, Figure 4b). None of the compounds were cytotoxic, except letrozole (50 μM) and **5**·Let (25 μM), which displayed a small decrease in mitochondrial metabolic activity. These *in vitro* results, however, cannot be directly translated to the effectiveness in animals with glioblastoma xenographs or ectopic tumors. For example, ruthenium complex NAMI-A (Figure 1a) was poorly effective *in vitro*, but turned out to be remarkably effective in mice, *in vivo*.[18]

Combination Studies. Cell death occurs as a consequence of three different processes: apoptosis, autophagy, and necrosis.[19] It is important to learn more about the mechanism(s) by which individual anticancer drugs operate to create powerful anticancer treatments, especially ones using specific drug combinations. Numerous anticancer drugs are known to induce cell death by apoptosis, while some others are known to induce cell death via an autophagy pathway,[20] which is a self-cannibalization process that also can contribute to tumor resiliency.[21] Since inhibition of autophagy might prevent the growth of established tumors, the study of autophagy inhibitors used in combination with drugs that trigger "cytoprotective" autophagy holds promise for improved treatment of cancer. We have investigated whether an autophagy process might be triggered by our two most potent ruthenium drug candidates, **4** and **5**·Let. To do so, we assessed their *in vitro* toxicity in combination with a well-known autophagy inhibitor, 3-methyladenine (3-MA), against the previously investigated cells, breast MCF-7 (Figure 5a) and glioblastoma brain U251N (Figure 5b,c), using the MTT assay. After 24 h, the combination of complex **5**·Let (0–100 μM) with 3-MA (1 mM) did not lead to any enhancement of cytotoxicity against breast MCF-7 cells (Figure 5a). This implies that complex **5**·Let does not induce a "cytoprotective" autophagy process, as was reported for other Ru(II) drugs, which used in combination with autophagy inhibitors enhanced their cytotoxicity effect.[22] The fact that no additive effect is noted indicates that an autophagy killing process might have

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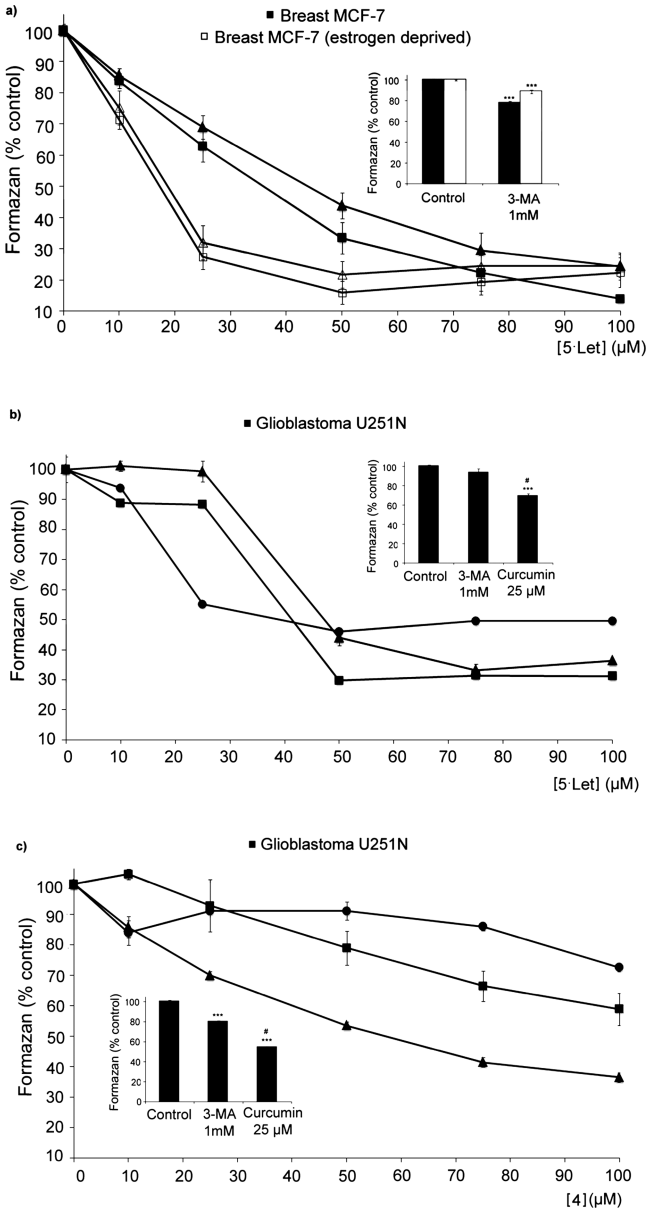

Figure 5. Mitochondrial metabolic activity determined by MTT assay, in human breast cancer cells (MCF-7) (a) and glioblastoma cancer cells (U251N) (b, c), treated with complex **4** or **5**·Let cotreated with 3-methyladenine (1 mM) and curcumin (25 μM), respectively. The insets display the results obtained upon cell exposure to 3-methyladenine (3-MA) and curcumin without any Ru complex under comparable conditions. Ru complex (■), Ru complex + 3-methyladenine (1 mM) (▲), Ru complex + curcumin (25 μM) (●). Results for estrogen-deprived MCF-7 cells are plotted with open symbols, and those for MCF-7 cells grown in the presence of serum are plotted with filled symbols. All values are expressed as means (from representative experiments) ± SEM relative to control (100%). Significant differences in the insets: $^{***}p < 0.001$ (compared with untreated control) and $^{\#}p < 0.001$ (compared with 3-MA treatment). For complete statistical details, see Supporting Information (statistical analysis section).

been triggered by **5**·Let. The same result was also noted for the combination of complex **4** with 3-MA, against the same cell line (results not shown). In the case of glioblastoma U251N cells, the combination of complex **5**·Let (0−100 μM) and 3-MA (1 mM) did not display any additive effect (Figure 5b), while complex **4** showed a considerable additive effect when combined with the same autophagy inhibitor, over the same range of concentrations (Figure 5c). This result suggests that **4** itself most likely does not induce autophagy, while **5**·Let might act by inducing cell killing autophagy (Figure 6). To clarify these findings, we investigated the effect of the combination of the same two ruthenium complexes with curcumin in glioblastoma cells (U251N), a compound which is known to promote cell death through induction of autophagy in glioma.[23] The combination of complex **5**·Let with curcumin (25 μM) showed a large synergistic effect at 25 μM (Figure 5b) whereas the combination of **4** (25 μM) with curcumin (Figure 5c) did not lead to a comparable effect. These findings suggest different modes of cell death induced by the two ruthenium complexes, and further studies should elucidate signal transduction pathways involved in these modes of cell death.

EXPERIMENTAL SECTION

Complex Synthesis and Characterization. All reagents were used as received from commercial vendors. Letrozole was purchased from AK Scientific, while $RuCl_3 \cdot xH_2O$, 1,4-cyclohexadiene, ammonium tetrafluoroborate, and triphenylphosphine were acquired from Sigma Aldrich. Experiments were performed under an argon atmosphere, and solvents were dried on activated molecular sieves columns using a solvent purification system. All NMR spectra were recorded at room temperature on a 200−500 MHz Varian spectrometer. ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra were referenced to solvent resonances, whereas $^{31}P\{^{1}H\}$ NMR spectra were referenced to an external 85% H_3PO_4 sample (0 ppm). All chemical shifts and coupling constants are expressed in ppm and Hz, respectively. The elemental analyses were performed by the Laboratoire d'Analyse Élémentaire (Département de Chimie, Université de Montréal). The high-resolution and high-accuracy mass spectra (ESI-MS) were obtained using an Exactive Orbitrap spectrometer from ThermoFisher Scientific (Department of Chemistry, McGill University). High-performance liquid chromatography (HPLC, Waters Instrument, reversed phase Symmetry C18 column 5 μm, 4.6 mm × 150 mm, detection 254 nm, mobile phase ethanol/methanol/2-propanol, flow rate 1.0 mL/min, retention time 9−11 min) was used to confirm the purity (≥95%) of the ruthenium complexes.

*[Ru(C$_6$H$_6$)(η^1-Let)$_2$Cl]Cl, **2**.* Letrozole (0.228 g, 0.80 mmol) was added to a degassed suspension of **1** (0.100 g, 0.20 mmol) in ethanol (35 mL), and the mixture was heated under reflux. After 1 h, the mixture was allowed to cool to room temperature and was filtered on a glass frit. The yellow-greenish precipitate that was collected was dissolved in a minimum amount of methanol, and the solution was filtered on a filter paper. When hexanes was added to the concentrated filtrate, the desired product precipitated. Compound **2** was obtained as a dark yellow powder (0.144 g, 44%) after it was collected on a filter paper and washed with hexanes.[24] ^{1}H NMR (CD_2Cl_2, 500 MHz) δ 5.70 (s, C_6H_6, 6H, **2b**), 5.88 (s, C_6H_6, 6H, **2a**), 7.03 (s, C*H*, 1H, **2b**), 7.30−7.42 (m, Ar*H*, 8H, **1a** or **1b**), 7.37 (s, C*H*, 2H, **2a**), 7.48−7.58 (m, Ar*H*, 8H, **2a** or **2b**), 7.64−7.73 (m, Ar*H*, 8H, **2a** or **2b**), 8.35 (s, $H_{triazole}$, 2H, **2a**), 8.40 (s, $H_{triazole}$, 1H, **2b**), 8.88 (s, $H_{triazole}$, 1H, **2b**), 10.47 (s, $H_{triazole}$, 2H, **2a**). $^{13}C\{^{1}H\}$ NMR (CD_2Cl_2, 125 MHz, **2a** and **2b**): δ 66.5−66.6 (CH), 83.3−85.8 (C_6H_6), 113.1−113.3 (C_{Ar}CN), 118.2−118.3 (CN), 129.1−129.8 (C_{Ar}), 132.7−133.1 (C_{Ar}), 141.0−144.2 (CHC_{Ar}), 151.9−154.0 ($C_{triazole}$). Found (%): C, 53.09; H, 3.44; N, 14.83. $C_{40}H_{28}Cl_2N_{10}Ru_1 \cdot 5H_2O$ requires C, 52.70; H, 4.17; N, 15.37. ESI-MS *m*/*z* (+): 785.1 M^+ (or $[Ru(C_6H_6)(\eta^1\text{-Let})_2Cl]^+$), 1607.2 $[M^+ + M^+ + Cl^-]^+$.

*[Ru(C$_6$H$_6$)(η^1-Let)$_2$Cl]BF$_4$, **3**.* Letrozole (0.114 g, 0.40 mmol) was added to a degassed suspension of **1** (0.050 g, 0.10 mmol) and NH_4BF_4 (0.052 g, 0.50 mmol) in ethanol (10 mL), and the mixture was refluxed for 1 h. The mixture was allowed to cool to room temperature and was then filtered. The precipitate was washed with methanol, followed by hexanes. A minimum of dichloromethane was added on the filter paper to dissolve the precipitate, and the filtrate was

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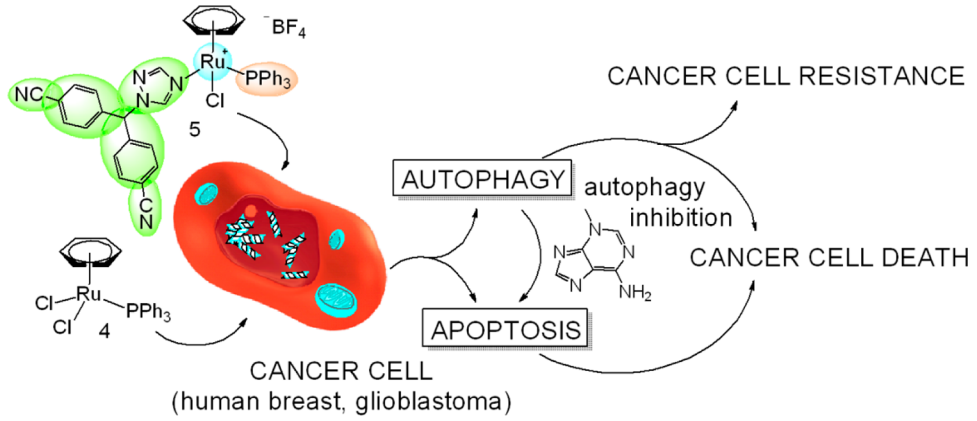

Figure 6. Different pathways by which complexes **4** and **5**·Let might induce cancer cell death.

collected in a separate flask. A dark yellow powder was obtained after the product was precipitated with hexanes and collected on a filter paper. Compound **3** was obtained as pale yellow crystals (0.130 g, 73%) from the diffusion of pentane into a concentrated dichloromethane solution of the powder. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.91 (s, C_6H_6, 6H), 7.03 (s, C*H*, 2H), 7.23 (d, J = 8, Ar*H*, 4H), 7.43 (d, J = 8, Ar*H*, 4H), 7.52 (d, J = 8, Ar*H*, 4H), 7.67 (d, J = 8, Ar*H*, 4H), 8.22 (s, $H_{triazole}$, 2H), 9.37 (s, $H_{triazole}$, 2H). ^{13}C{^{1}H} NMR ($CDCl_3$, 125 MHz): δ 66.9 (*C*H, 2C), 85.6 (C_6H_6, 6C), 113.5 (C_{Ar}CN, 2C), 113.6 (C_{Ar}CN, 2C), 117.8 (*C*N, 2C), 118.0 (*C*N, 2C), 129.1 (C_{Ar}, 4C), 129.2 (C_{Ar}, 4C), 132.9 (C_{Ar}, 4C), 133.1 (C_{Ar}, 4C), 140.8 (CHC_{Ar}, 4C), 147.6 ($C_{triazole}$, 2C), 152.0 ($C_{triazole}$, 2C). Found (%): C, 53.79; H, 2.98; N, 14.98. $C_{40}H_{28}B_1F_4Cl_1N_{10}Ru_1\cdot H_2O$ requires C, 53.92; H, 3.37; N, 15.72. ESI-MS m/z (+): 785.1 M^+ (or $[Ru(C_6H_6)(\eta^1\text{-Let})_2Cl]^+$), 1657.2 $[M^+ + M^+ + BF_4^-]^+$.

[Ru(C$_6$H$_6$)(η^1-Let)$_2$(PPh$_3$)](BF$_4$)(Cl), ***5***. Triphenylphosphine (0.040 g, 0.15 mmol) was added to a solution of **3** (0.100 g, 0.11 mmol) in dichloromethane (6 mL), and the mixture was stirred at ambient temperature for 48 h. The solvent was then concentrated, and the product was purified by column chromatography (silica gel) using dichloromethane, followed by acetone. Compound **5** (along with one molecule of letrozole)[16] was obtained as a light yellow powder (0.098 g, 75%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.79 (s, C_6H_6, 6H), 6.84 (s, C*H*, 1H), 7.16 (s, C*H*, 1H), 7.22−7.38 (m, Ar*H*, 21H), 7.57−7.73 (m, Ar*H*, 10H), 8.07 (br s, $H_{triazole}$, 1H), 8.18 (br s, $H_{triazole}$, 1H), 8.38 (s, $H_{triazole}$, 1H), 9.30 (s, $H_{triazole}$, 1H). ^{13}C{^{1}H} NMR ($CDCl_3$, 125 MHz): δ 65.7 (s, *C*H, 1C), 66.6 (s, *C*H, 1C), 90.7 (d, J = 3, C_6H_6, 6C), 112.8 (s, C_{Ar}CN, 1C), 113.1 (s, C_{Ar}CN, 1C), 113.4 (s, C_{Ar}CN, 2C), 118.0 (s, *C*N, 2C), 118.2 (s, *C*N, 1C), 118.3 (s, *C*N, 1C), 129.0 (d, J = 10, C_{PPh3}, 6C), 129.1 (s, C_{Ar}, 4C), 129.1 (s, $C_{Ar,}$ 8C), 129.9 (s, C_{Ar}, 4C), 131.6 (d, J = 2, C_{PPh3}, 3C), 132.7 (d, J = 14, C_{PPh3}, 3C), 133.1 (s, C_{Ar}, 8C), 133.8 (d, J = 10, C_{PPh3}, 6C), 141.3 (s, CHC_{Ar}, 1C), 141.7 (s, CHC_{Ar}, 1C), 141.9 (s, CHC_{Ar}, 2C), 149.3 (s, $C_{triazole}$, 2C), 152.1 (s, $C_{triazole}$, 2C); ^{31}P{^{1}H} NMR ($CDCl_3$, 200 MHz): δ 35.0; Found (%): C, 58.77; H, 3.54; N, 11.95. $C_{58}H_{43}B_1F_4Cl_1P_1N_{10}Ru_1\cdot 3H_2O$ requires C, 58.56; H, 4.12; N, 11.78. ESI-MS m/z (+): 762.1 M^+ (or $[Ru(C_6H_6)(\eta^1\text{-Let})(PPh_3)Cl]^+$), 1611.2 $[M^+ + M^+ + BF_4^-]^+$.

X-ray Diffraction Studies. Suitable crystals for X-ray diffraction data were obtained by slow diffusion of pentane into concentrate dichloromethane/chloroform-*d* solutions of **2a**, **3**, and **5**·Let, respectively. Data were collected at 150 K using a Bruker SMART APEX II CCD X-ray diffractometer. Structure resolution and refinement were performed with SHELXTL. H atoms were calculated and constrained as riding on their bound atoms. CCDC 890790 and 890791 (**2b** and **3**) 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.

Materials and Methods for Biology Experiments. 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), lipopolysaccharides from *Escherichia coli* O111:B4 (LPS), curcumin, antimycin A, and 3-methyladenine were purchased from Sigma-Aldrich. Estrogen receptor positive MCF-7 cell line was donated by Dr. Ursula Stochaj (Department of Physiology, McGill University, Montreal, Canada), and U251N glioblastoma cell line was donated by Dr. Josephine Nalbantoglu (Departments of Neurology & Neurosurgery and Medicine, McGill University, Montreal, Canada).

Cell Culture and Media. U251N glioblastoma and MCF-7 breast adenocarcinoma were routinely cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco), supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco). In addition, MCF-7 were supplemented with 1% sodium pyruvate (Gibco). MCF-7 cells that were treated under estrogen-deprived conditions were washed three times with 1× phosphate buffered saline (PBS) and prestarved for 3 h in serum-free, phenol-red-free medium. Their treatment was performed using the same medium. For all experiments, cells were seeded in 24-well plate (Corning) or 96-well plate (Sarstedt) at a density of 3.5×10^4 cells/well (U251N) or 5.0×10^4 cells/well (MCF-7) and maintained at 37 °C, 5% CO_2 in a humidified atmosphere and were grown in serum containing media for 24 h before cell treatments to attain confluency.

Ruthenium Drug Solutions. Ruthenium drug **2a**, **3**, **4** and **5**·Let stock solutions (1 mM) were prepared using dimethylsulfoxide (DMSO). Each stock solution was diluted in culture medium to obtain working concentrations. The final (DMSO) concentration never exceeded 0.5%, which was not toxic to the cells under the conditions used.

Determination of Mitochondrial Metabolic Activity (MTT Assay). Mitochondrial metabolic activity of cells was measured using MTT assay. Cells were preliminarily treated with ruthenium complexes **2a**, **3**, **4**, and **5**·Let solutions (in culture medium) at concentrations between 0.01 and 100 μM. Based on preliminary results, further experiments were performed at 10, 25, 50, 75, and 100 μM. Experiments were performed 24, 48, or 72 h after each treatment. After the treatments were performed, medium was removed and replaced with medium containing 500 μg/mL MTT. The cells were then incubated for 30−90 min at 37 °C in order for the formazan salts to form. Medium was removed, and cells were lysed using 500 μL of DMSO and mixed gently for 5 min. The dissolved formazan crystals were added in triplicate in a clear bottom 96-well plate (Sarstedt) and quantified by measuring the absorbance of the solution at 595 nm using Benchmark microplate reader (Bio-Rad). The extent of formazan conversion is expressed in percentage relative to the untreated control. Results are expressed as mean ± SEM obtained from at least three independent experiments performed in triplicate.

Determination of Cell Death by Counting. Cell viability was confirmed by cell counting using Hoechst 33258 (Sigma) Nuclear Staining. Following treatments in 24-well plates, cells were fixed and labeled with the fluorescent dye. Five images were taken per well using Leica Application Suite with Leica DFC350FX monochrome digital camera on Leica DM 4000B inverted fluorescent microscope with

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DAPI-1160A filter and 20× objective. The cells were subsequently counted using ImageJ with Cell Counter plugin.

Statistical Analysis. All data are presented as the means ± standard errors of the mean (SEM). A Student unpaired *t* test or ANOVA coupled to Bonferroni correction were used for analyzing the significance of the difference between the means. A *p*-value <0.05 (*) was considered statistically significant. All experiments were performed at least two or three times.

CONCLUSIONS

This study provides novel letrozole−ruthenium complexes as chemotherapeutic agents. Letrozole coordinates to ruthenium(II) in a monodentate fashion via one nitrogen atom of the triazole ring, similar to the coordination mode observed for copper(II), nickel(II), or cobalt(II). However, this coordination mode is different than the one noted for copper(I), where letrozole binds to the metal by its four nitrogen atoms. Letrozole ligand lability was found to be enhanced by the coordinative nature of the complex counterion and by the presence of alkyl substituents on the arene ring. Biological experiments show that **5**·Let is an effective cytotoxic agent against the human breast cancer cells (MCF-7) and also, although to a much smaller extent, against human glioblastoma cells (U251N). The activity of **5**·Let was significantly higher than that of **4**, a compound analogous to RAPTA-C, a ruthenium drug presently in preclinical studies. Results from the combined exposure of cancer cells to **5**·Let (or **4**) and 3-methyladenine (3-MA) or curcumin point toward the critical role of autophagy contributing to the **5**·Let-induced cell death. Taken together, these studies show the effectiveness of novel Ru−letrozole complexes in significantly reducing viability of different human cancer cell types.

ASSOCIATED CONTENT

Supporting Information

Supplementary experimental details, Figure S1 showing results of MTT assay for MCF-7 cells, and statistical details for Figure 5. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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