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A fluorine containing bipyridine cisplatin analog is more effective than cisplatin at inducing apoptosis in cancer cell lines

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Abstract—Novel cisplatin analogs dichloro[4,4′-bis(4,4,4-trifluorobutyl)-2,2′-bipyridine]platinum (**1**) and *fac*-tricarbonylchloro[4,4′-bis(4,4,4-trifluorobutyl)-2,2′-bipyridine]rhenium (**3**) were synthesized and evaluated for their cytotoxicity. While **3** was not cytotoxic, **1** was 14 to 125 times more lethal than cisplatin in breast, prostate, and lung cancer cell lines. Compound **1** was able to induce apoptosis and the presence of the platinum atom was essential to its function as a cytotoxin.

1. Introduction

cis-Diaminedichloroplatinum (II) (cisplatin) was approved by the U.S. Food and Drug administration in 1979 for the treatment of cancer.[1] With annual sales of about $500 million, cisplatin is still one of the most effective drugs to treat testicular, ovarian, bladder, and neck cancers.[2] Cisplatin treatment has dose-limiting side-effects including nausea, emetogenesis, hair loss, neuropathies, ototoxicity, renal toxicity, and platelet reduction.[3] Another problem with cisplatin is that cancer cells can develop a resistance to it, which renders it useless as a chemotherapeutic agent.[4] To address the side effects as well as the development of resistance, new platinum containing compounds are continually being developed. About 35 platinum complexes have entered clinical trials in order to circumvent the side-effects and the problem of tumor resistance to cisplatin.[5] In addition, an effort was made to utilize $PtCl_2$ complexes of 2,2′-bipyridine featuring extended fluorinated alkyl groups at the 4,4′ positions.[6] The studies focused on the function of the complexes in a liposomal delivery scheme and were found to be not more cytotoxic than cisplatin.[7]

Although numerous studies have been reported on the molecular mechanisms of cisplatin, their mechanisms are not yet completely understood. Once in solution, the chloride ligands of cisplatin are replaced by water to give $Pt(NH_3)_2(OH_2)_2$. The water ligands are labile and can be easily displaced. This allows cisplatin to form adducts with DNA or proteins. Only about 1% of the cisplatin added to a cell will end up in the nucleus where it predominately makes 1,2 (GpG) intrastrand crosslinks.[8] These DNA adducts are thought to be the primary cause of cisplatin cytotoxicity. Solution structure of cisplatin bound DNA dodecamer has shown that cisplatin induces a 60–80° bend in DNA. The bend is in the direction of the major groove and causes a widening of the minor groove.[9] The platinum center of cisplatin can also bind intracellular proteins, which may lead to another mechanism of cell death. Cisplatin adducts have been found to prevent chromatin remodeling necessary for DNA transcription, by creating cisplatin DNA–protein crosslinks with histones.[10]

Clearly the interaction of cisplatin with various targets is stressful to cells and one way they respond is by the synthesis of the heat shock proteins.[11] Heat shock proteins are expressed by all known cells (bacterial, plant, animal, and human) and generally function as molecular chaperons.[12] Several heat shock proteins including hsp70 and hsp27 are capable of inhibiting apoptosis and thus provide protection against a wide range of chemotherapeutic agents.[13]

In this study we explore the cytotoxicity of cisplatin analogs, four 4,4′-dialkyl-2,2′-bipyridine complexes of

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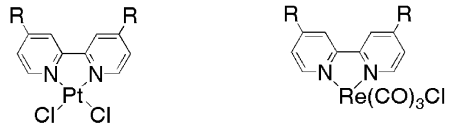


1: R = -$(CH_2)_3CF_3$
2: R = -CH_3

3: R = -$(CH_2)_3CF_3$
4: R = -CH_3

Scheme 1.

Pt(II) (**1** and **2**), and of Re(I) (**3** and **4**) (Scheme 1). As shown in Scheme 1, a comparatively short partially fluorinated ligand is contrasted to a non-fluorinated one. The corresponding rhenium complexes are known to be somewhat substitutionally inert at ambient conditions. Thus they do not have the ability to bind to DNA as observed for the platinum complexes. This comparison of platinum analogs and rhenium analogs will allow us to investigate the role of the metals in the cytotoxic cellular response. The selected cancer cell lines are well established and varied to allow the examination of the role of hsp27 protection against these agents in several human breast cancer cell lines.

2. Results

The ability of **1**, **2**, and cisplatin to induce cell death in the MDA-MB-231 cell line is shown in Figure 1A. Both **1** and **2** are more than 100 times more lethal than cisplatin, the EC_{50} values are listed in Table 1. The cell lines DC4 and DB46 were engineered from the MDA-MB-231 cell line by inserting a control plasmid in the DC4 cell line and the same plasmid plus the coding sequences for the human heat shock protein 27 (hsp27) in the DB46 cell line.[14] This results in the DB46 having a constitutive expression of hsp27 that is equal to the level seen in the DC4 cell line following a heat shock.[15] Therefore, these two cell lines can be used to determine the effect of hsp27 expression. As can be seen in Figure 1B and C, Hsp27 expression did not result in any protection against **1** or **2**. It is interesting to note that these two cell lines were more sensitive to cisplatin than the parental cell line. To determine the role of the 2,2′-bipyridine ring system, Re complexes **3** and **4** were prepared. The Re complexes failed to induce any significant cell death, Figure 1B and C. Compounds **1** and **2** were more lethal than cisplatin in MDA-MB-435 cells, while **1** was more lethal than cisplatin in DU-145 and A549 cell lines Figure 2A–C, respectively. The EC_{50} values for these graphs listed in Table 1 indicate that **1** was 60 times more lethal than cisplatin while **2** was 29 times lethal than cisplatin in the MDA-MB-435 cells. In the DU-145 cell line **1** was 50 times more lethal than cisplatin while in the A549 cell line it was 14 times more lethal.

The ability of cisplatin, **1**, and **2** to affect the cell cycle was investigated using propidium iodide staining and analysis via flow cytometry. Cells were treated with either 1 μM (low dose) or 100 μM (high dose) of the agent for 24 h and then cell cycle distribution was determined. Figure 3A indicates that in the MDA-MB-231 cell line, cisplatin had very little effect while 100 μM of

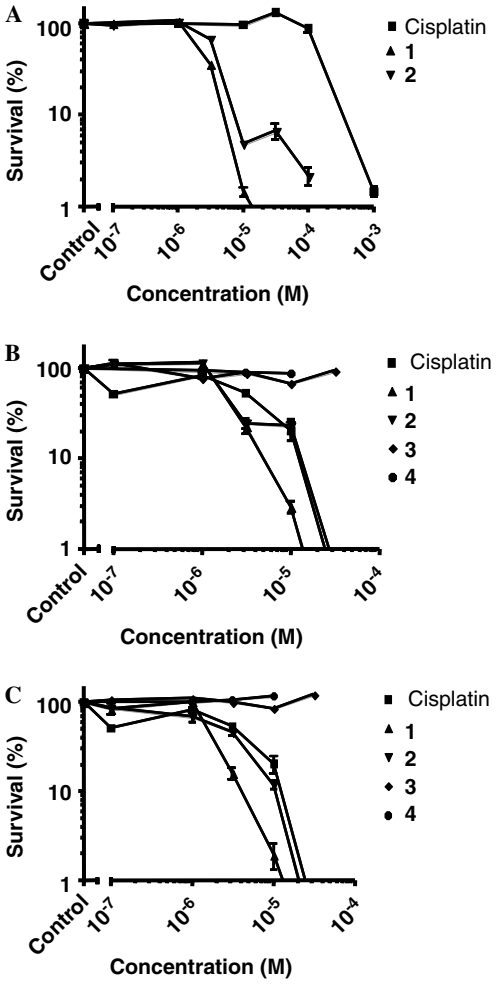


Figure 1. Cell survival curves from breast cancer cell lines. Cells were treated with the compounds indicated for 1 h at 37 °C, harvested, counted, and plated into 60 mm dishes with 5.0 ml of fresh media. The resultant colonies were stained 11–14 days following drug exposure. (A) MDA-MB-231. (B) DC4. (C) DB46. A typical result is shown. The error bars (sometimes smaller than the symbols) represent the standard deviation from the triplicate data set.

Table 1. EC_{50} values for cisplatin, **1** and **2**

EC_{50} values (μM)	Cisplatin	**1**	**2**
DC4	5.0	1.6	3.1
DB46	4.3	3.9	2.3
MDA-MB-231	237.1	1.9	5.1
MDA-MB-435	132.0	2.2	4.5
DU-145	180.0	3.4	N.D.
A549	100.8	7.0	N.D.

N.D., not determined.

1 was able to increase apoptosis to 8.2% compared to a control value of 1.6%. Compound **1** was also able to decrease G2 from 16.3% to 10.9% compared to control values. Apoptosis increased in the presence of 100 μM of **2** from 1.6% to 14.7% compared to control values but had a smaller effect on G2, 16.3–13.6%. When the same agents were added to the MDA-MB-435 cell line, panel B, 100 μM of **1** increased apoptosis from 2.7% to 10.3% over control values while **2** increased the

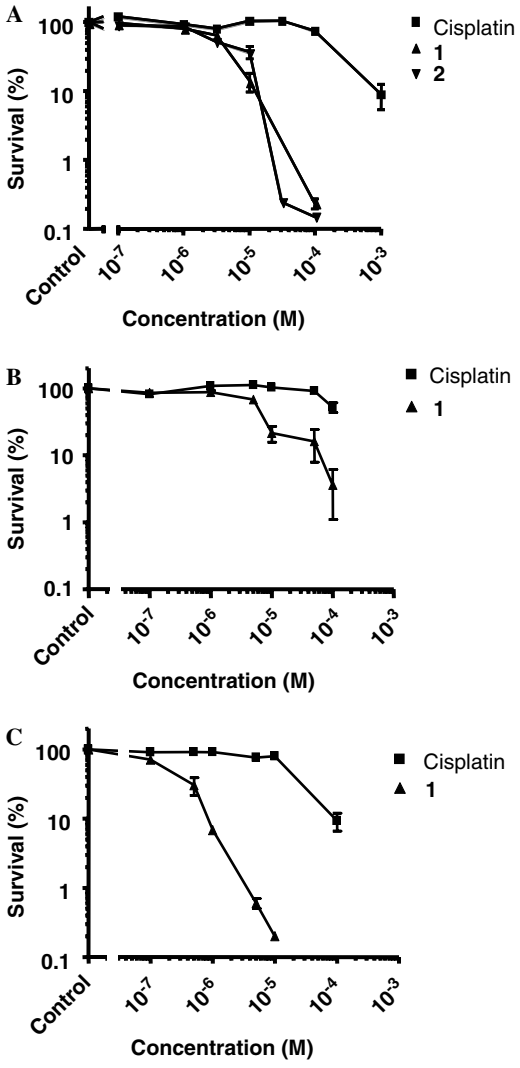


Figure 2. Cell survival curves from cancer cell lines. Cells were treated with the compounds indicated for 1 h at 37 °C, harvested, counted, and plated into 60 mm dishes with 5.0 ml of fresh media. The resultant colonies were stained 11–14 days following drug exposure. (A) MDA-MB-435. (B) DU-145. (C) A549. A typical result is shown. The error bars (sometimes smaller than the symbols) represent the standard deviation from the triplicate data set.

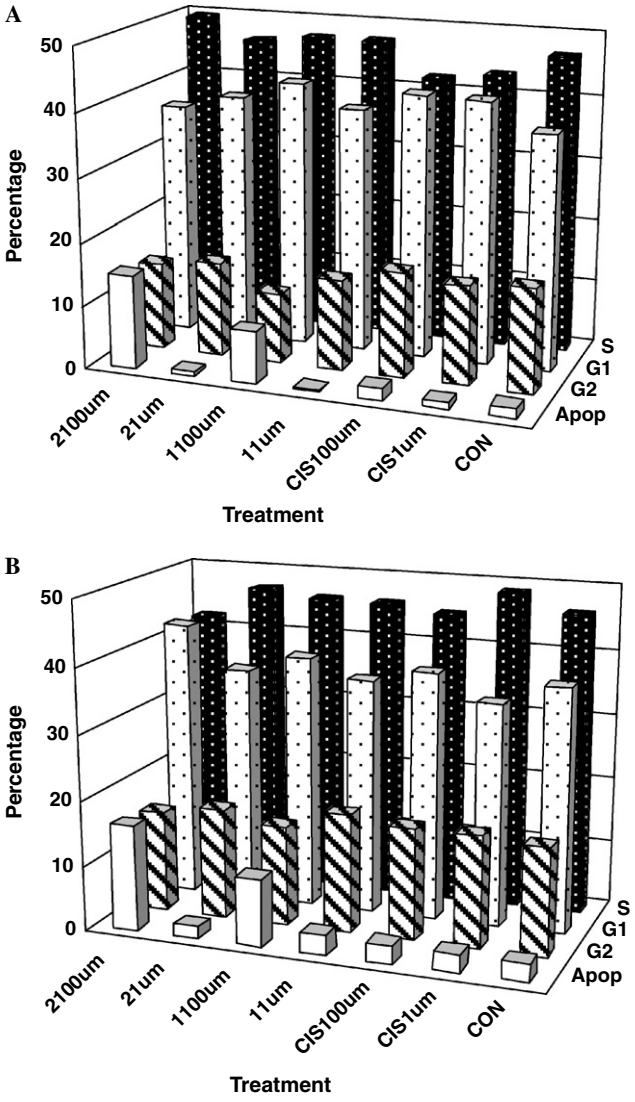


Figure 3. Cell cycle distribution. Cells were treated with the compounds indicated for 1 h at 37 °C, then the compounds were removed, the cells washed once with 5.0 ml PBS and then 5.0 ml of fresh media was added. The samples were fixed with 70% ethanol 24 h after drug exposure. (A) MDA-MB-231. (B) MDA-MB-435. Values from a typical experiment are shown.

percent apoptosis to 16.2%. No other changes were seen with **1** while 100 μM of **2** increased G1 from 37.4% to 42.5%.

Annexin-V-FITC staining was also used to evaluate the effects of cisplatin, **1**, and **2** on cell lines. Figure 4A indicates in DC4 cells treated with the EC_{50} values of cisplatin, **1**, and **2** for 1 h; very little increase in apoptosis over control values was observed, except for the **1** treated cells. Virtually no necrosis at 24 or 48 h after treatment was observed. Similar results are seen in the DB46 cell line, Figure 4B.

To further characterize cellular responses to cisplatin, **1**, and **2**, DC4 and DB46 cell lines were exposed to the EC_{50} values of these agents for 1 h, incubated for 24, 48 or 72 h, and stained with Hoechst and PI. The DC4 cell line showed little apoptosis induction (Fig. 5A) following exposure to cisplatin but significant apoptosis 24 h after exposure to **1** (74%) or **2** (54%). Longer incubation times after exposure to these agents resulted in a lower percentage of cells expressing apoptosis. Similar results were seen in the DB46 cell line however, apoptosis induction was less than that observed in the DC4 cell line, **1** (44%) and **2** (19%) (Fig. 5B).

To better understand how cisplatin, **1**, and **2** interact with cells, aphidicolin was used to synchronize the cell cycle prior to exposing cells to these agents. Aphidicolin inhibits DNA polymerase delta and epsilon, which lead to a block in G1/S phase, so following its removal cells would typically cycle into the G2 phase. Figure 6 indicates that in MDA-MB-231 (panel A) and MDA-MB-435 (panel B) most of the cells (>60%) are in the G2 phase of the cell cycle following release from aphidicho-

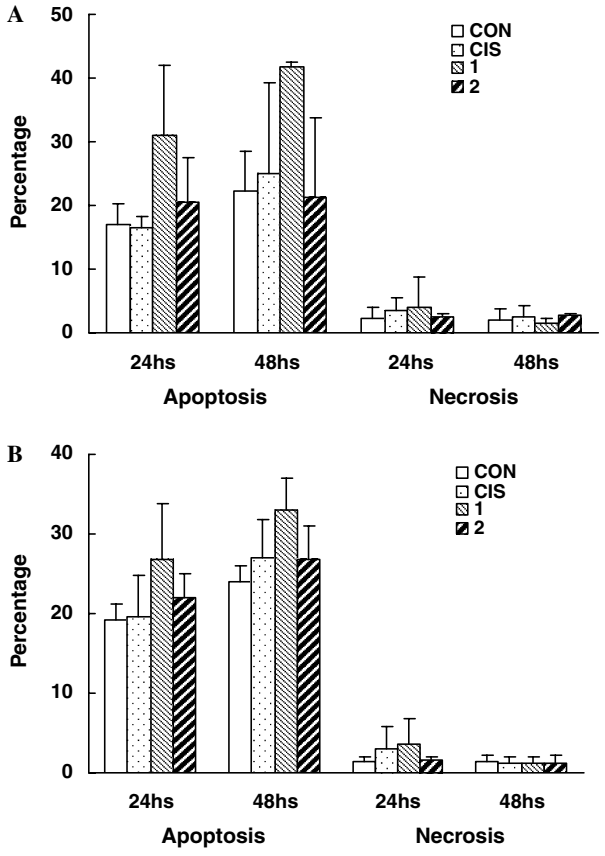


Figure 4. Annexin-V staining. Cells were treated with the compounds indicated for 1 h at 37 °C, washed once with 5 ml PBS and then 5.0 ml of fresh media was added. Then 24 or 48 h following drug treatment cells were harvested and stained with Annexin-V. (A) DC4. (B) DB46. Average values from three experiments are shown. The error bars represent the standard deviation.

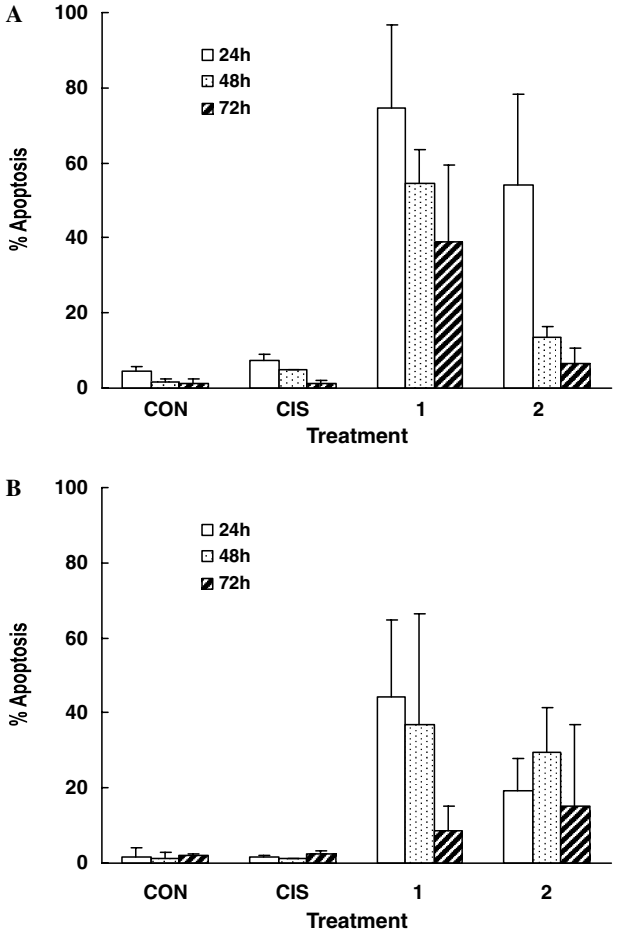


Figure 5. Fluorescent microscopy. Cells were treated with the compounds indicated for 1 h at 37 °C, washed once with 5 ml PBS and then 5.0 ml of fresh media was added. Then 24, 48 or 72 h following drug treatment cells were harvested and stained with PI and Hoechst. (A) DC4. (B) DB46. Average values from three experiments are shown. The error bars represent the standard deviation.

lin (con). Cisplatin had very little effect on the synchronized MDA-MB-231 cells, Figure 6A. The fluorine substituted Pt complex **1** blocked the MDA-MB-231 cells in both the G1 (38.9–10.6%) and S (38.8–24.6%) phases leading to a decrease in the G2 phase of 22.3% compared to 64.8% in the control treated cells. The methyl substituted Pt complex **2** also blocked both the G1 (45.4–10.6%) and S (32.8–24.6%) phases leading to a decrease in the G2 phase (21.9% from 64.8%) in this cell line. In the MDA-MB-435 cells, cisplatin treatment resulted in an increase in cells in the S phase from 19.7% to 27.0%. Treatment with **1** blocked, or held, cells in G1 48.3% compared to 13.9% in control treated MDA-MB-435 cells, Figure 6B. This blockage thus led to a decrease in cells in the S (19.7–11.4%) and G2 (66.5–40.3%) phases. Compound **2** blocked MDA-MB-435 cells in the S phase 70.0% compared to 19.7% in the control treated cells.

3. Discussion

The novel platinum complex **1** and **2** were more efficient than cisplatin (anywhere from 14 to 125 times depending on the cell line) in inducing cell death in breast, lung, and prostate cancer cell lines. This increased efficiency in cell killing was not observed in two genetically engineered breast cancer cell lines grown in the presence of G418 sulfate. Both **1** and **2** are capable of inducing apoptosis but not necrosis. Apoptosis induction was confirmed by DNA analysis via flow cytometry, Annexin-V staining, and fluorescent microscopy. There are several lines of experimental evidence to indicate that **1** and **2** are having slightly different effects on breast cancer cell lines. These differences include (a) Annexin-V staining in DC4 cells induces more apoptosis at 48 h in cells treated with **1** compared to cells treated with **2**, (b) fluorescent microscopy demonstrated more apoptosis when cells are treated with **1** than with **2** at 24 h (DB46 cell line), 48 or 72 h (DC4 cell line), and (c) cell cycle distribution following an aphidicholin block indicates differences between **1** and **2** treated cell lines. For these reasons, it appears that while both **1** and **2** can induce cell death, they have different mechanisms whereby they achieve apoptosis.

Hsp27 has been reported to provide protection against chemotherapeutic agents that induce apoptosis. Human

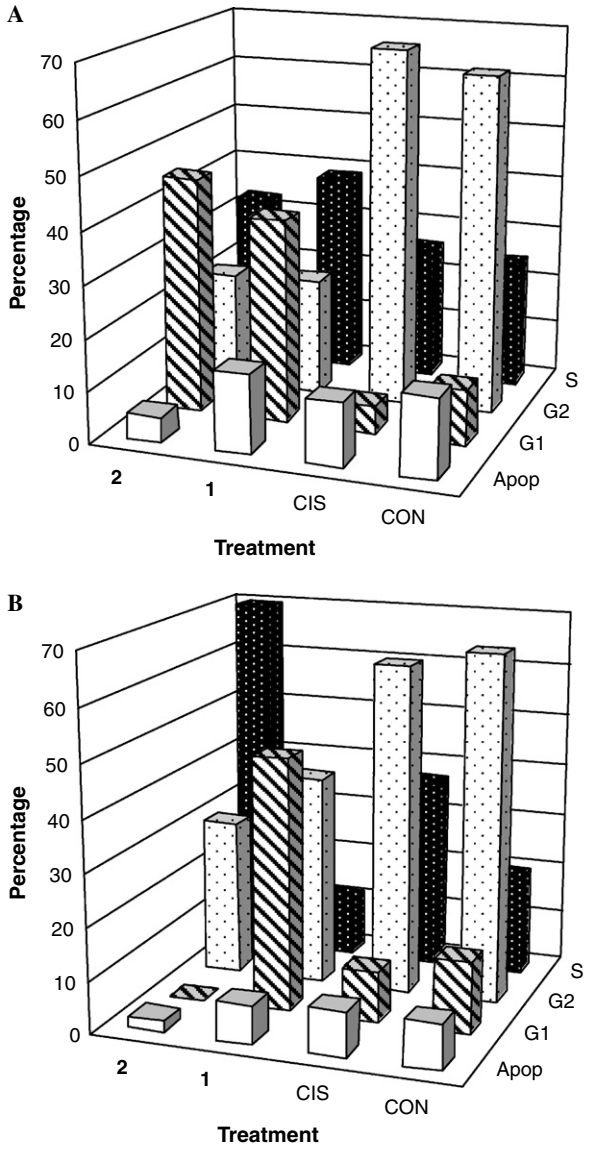


Figure 6. Cell cycle distribution following an aphidicholin block. Cells were treated with aphidicholin for 12 h, followed by exposure to the indicated compounds at their EC_{50} values for 1 h, then the samples were washed three times with 5.0 ml PBS and resuspended in 5.0 ml of fresh media. Twelve hours after removal of aphidicholin the samples were fixed in 70% ethanol and analyzed for DNA distribution. (A) MDA-MB-231. (B) MDA-MB-435. A typical result from a single experiment is shown.

testis tumor cells over-expressing hsp27 were more resistant to cisplatin as were human colorectal cells that over-expressed hsp27.[16,17] The expression of hsp27 was upregulated in a cisplatin resistant ovarian tumor cell line.[18] It is clear that most cell lines that over-express hsp27 are also resistant to cisplatin cytotoxicity. The finding that the DB46 cell line (which expresses high constitutive levels of hsp27) had a similar sensitivity to cisplatin, as did the DC4 cell line (which expresses a normal low level of hsp27), was surprising. In these engineered human breast cancer cell line DB46 is more resistant to doxorubicin, staurosporine, and sodium butyrate, agents that induce apoptosis.[15,19,20] Perhaps culturing the engineered cells in G418 sulfate (to maintain plasmid integrity) renders them more sensitive to cisplatin or cisplatin may be reacting with the G418 sulfate to increase its cytotoxicity.

Cisplatin makes intrastrand 1,2 (GpG) and 1,3 (GpXpG) adducts and interstrand G-G crosslinks.[21] The 1,2 adducts account for 90% of lesions while the 1,3 adducts account for an additional 5% of lesions.[22] These adducts distort the DNA structure and if not repaired lead to cell death via an apoptotic pathway.[23,24] The novel cisplatin analog investigated in this study (**1**) was also able to induce apoptosis. The cisplatin-DNA intrastrand lesions are typically repaired by the nucleotide excision repair (NER) pathway.[25,26] The NER enzymes recognize bulky distortions in the shape of the DNA double helix, which leads to the removal of a short single-stranded DNA segment that includes the lesion. This creates a single-strand gap in the DNA, which is subsequently filled in by DNA polymerase, using the undamaged strand as a template.[27] It is interesting to speculate that since **1** and **2** contain a 2,2′-bipyridine ligand with substituents they might inhibit the binding of the NER proteins thus preventing or inhibiting the repair of the platinum adducts leading to more cell death than that observed with cisplatin.

Cells have the ability to discriminate between adducts from various cisplatin analogs.[28] Oxaliplatin is a second-generation platinum complex in which the two amine groups are replaced by 1,2-diaminocyclohexane. The proteins involved in NER do not discriminate between the adducts formed by cisplatin or oxaliplatin, but the proteins involved in mismatch repair (MMR) can.[29–31] The MMR system can recognize and repair erroneous insertion, deletion or mis-incorporation of bases that occur during DNA replication and recombination as well as repairing some forms of DNA damage. There are two types of MMR, long patch (which can excise tracts up to a few kilobases in length) and short tract (which typically removes about 10 nucleotides). The binding of the MMR complex to Pt–DNA adducts increases the cytotoxicity of the adducts by activating downstream signaling pathways leading to apoptosis.[32–34] In eukaryotes, the initial step in MMR is when MSH2 forms a heterodimer with MSH6 or MSH3 to bind to distinct but overlapping spectra of mismatches.[35] MSH2 binds with greater affinity to cisplatin adducts than to oxaliplatin adducts, causing the cisplatin adducts to be repaired at a greater rate than the oxaliplatin adducts.[36] Since both **1** and **2** contain a 2,2′-bipyridine ligand with substituents which is larger than the diaminocyclohexane ring of oxaliplatin, it is reasonable to assume that MSH2 heterodimer would also not bind very tightly to the adducts formed by **1** and **2**.

The mechanism whereby the novel compounds **1** and **2** kill cancer cells may be by the formation of intrastrand adducts in a manner analogous to cisplatin. To investigate the role of the metal atom, synthesis of compounds with identical bipyridine ligands that contained rhenium instead of platinum was pursued. The platinum atom acts as an alkylating agent, while the rhenium atom does not.[37] Rhenium atoms complexed to tamoxifen do

demonstrate limited cytotoxicity, but cytotoxicity was not observed for compounds **3** and **4** in this study.[38] These results indicate that the platinum atom plays a major functional role in the toxicity of these compounds.

Cisplatin has a broad spectrum of activity and is used not only as first-line chemotherapy against epithelial malignancies of lung, ovarian, bladder, testicular, head and neck, esophageal, gastric, colon, and pancreatic but also as second- and third-line treatment against a number of metastatic malignancies including cancers of the breast, melanoma, prostate, mesothelioma, malignant gliomas, and others.[39] The novel platinum compounds **1** and **2** also have a broad spectrum of activity and are capable of killing established cancer cell lines derived from lung, breast, and prostate. Like other powerful anticancer agents, cisplatin has many serious side effects. Its main dose-limiting side effect is nephrotoxicity, which evolves slowly after initial and repeated exposure. The kidney accumulates cisplatin to a higher degree than other organs perhaps via mediated transport.[40] The side effects caused by **1** and **2** will need to be determined in future studies to evaluate the potential clinical feasibility of these compounds. Since these compounds have shown much lower LD_{50} values than cisplatin, lower doses should be possible to produce the same amount of tumor cytotoxicity while avoiding the side effects that limit the usefulness of cisplatin.

4. Conclusion

In summary, the novel fluoroalkyl substituted 2,2′-bipyridine platinum complex **1** is anywhere from 14 to 125 times more lethal than cisplatin. Compound **1** was capable of killing breast, prostate, and lung cancer cell lines via apoptosis. The presence of the Pt atom is essential for cytotoxicity since replacing it with Re atom resulted in complexes that were not cytotoxic. Further studies will need to be conducted to determine if NER or MMR is inhibited by **1**. We are also conducting a study on chemical structure and activity relationship to determine the role of the 4,4′-substitutions on the 2,2′-bipyridine ligand.

5. Experimental

5.1. Reagents

Minimum essential medium (MEM), fetal bovine serum, phosphate-buffered saline (PBS), trypsin–EDTA, and G418 sulfate were purchased from Invitrogen, Inc. (Carlsbad, CA). RPMI 1640 media were purchased from ATCC (Manassas, VA). Cisplatin and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Aphicholin was purchased from Alexis Biochemicals (San Diego, CA). All reagents and enzymes used for flow cytometry were of analytical grade from Sigma Inc. unless otherwise noted. The 4,4′-dimethyl-2,2′-bipyridine and $Re(CO)_5Cl$ were purchased from Alfa Aesar (Ward Hill, MA). Diethyl ether, methanol, Na_2SO_4, *n*-propanol, $SnCl_2$, 1,5-cyclooctadiene, 4,4′-dimethyl 2,2′-bipyridine, ethanol, benzene, toluene, acetonitrile, and hydrochloric acid were purchased from commercial suppliers and used as received.

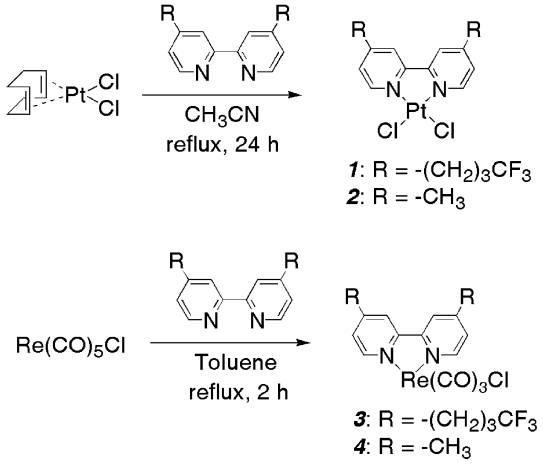
Dichloro(1,5-cyclooctadiene)platinum, (CAS [12080-32-9]),[41] dichloro[4,4′-dimethyl-2,2′-bipyridine]platinum (CAS [54822-56-9]),[42] and 4,4′-bis(4,4,4-trifluorobutyl)-2,2′-bipyridine[43] were prepared by literature procedures. Tetrahydrofuran (THF) was dried over sodium and distilled under nitrogen just prior to use.

NMR spectra were obtained using a Bruker AMX 400 spectrometer at 298 or 320 K: ^{1}H NMR, 400 MHz; $^{13}C\{^{1}H\}$ NMR, 100.65 MHz; ^{19}F NMR, 376.477 MHz. ^{1}H and ^{13}C NMR chemical shifts are reported in parts per million downfield from tetramethylsilane. ^{19}F NMR chemical shifts are referenced to an external sample of $CFCl_3$.

Scheme 2 contains the synthetic scheme for **1**, **2**, **3**, and **4**.

5.2. Synthesis of 2,2′-bipyridine cisplatin analogs

5.2.1. Synthesis of dichloro[4,4′-bis(4,4,4-trifluorobutyl)-2,2′-bipyridine]platinum (1). 38.0 mg (0.094 mmol) of dichloro(1,5-cyclooctadiene)platinum, 38.9 mg (0.104 mmol) of 4,4′-bis(4,4,4-trifluorobutyl)-2,2′-bipyridine, and 15 mL of acetonitrile were added to a 100 mL round-bottomed flask. The mixture was refluxed for 24 h. Upon cooling the solvent was evaporated and remaining volatiles were removed via vacuum. The residue was then dissolved in acetonitrile, and product was precipitated with diethyl ether. 37 mg of yellow solid was recovered (0.0467 mmol, 61% yield). mp/decomp. 260–261 °C. Anal. Calcd for $C_{18}H_{18}Cl_2F_6N_2Pt$: C, 33.66; H, 2.82; N, 4.36. Found: C, 33.81; H, 2.80; N, 4.25. ^{1}H NMR (400 MHz, 295 K, acetone-d_6): δ 9.51 (d, J_{HH} = 6.0 Hz, 2H, –C*H* 6/6′), 8.47 (s, 2H, –C*H* 3/3′), 7.71 (d, J_{HH} = 6.0 Hz, 2H, –C*H* 5/5′), 3.00 (t, J_{HH} = 8.8 Hz, 4H, $-CH_2CH_2CH_2CF_3$), 2.38 (m, 4H, $-CH_2CH_2CH_2CF_3$), 2.10 (m, 4H, $-CH_2CH_2CH_2CF_3$). $^{13}C\{^{1}H\}$ NMR (100.62 MHz, 298 K, acetone-d_6): δ



Scheme 2.

157.9 (*C*C, 2/2′,2C), 155.9 (*C*CH_2-, 4/4′, 2C), 149.0 (*C*H, 6/6′, 2C), 128.0 (*C*H, 3/3′, 2C), 125.0 (5/5′,2C), 128.4 (q, J_{FC} = 275.5 Hz,, 2C of *C*F_3), 35.0 (–*C*$H_2CH_2CH_2CF_3$, 2C), 33.5 (q, J_{FC} = 28.5 Hz, –CH_2CH_2*C*H_2CF_3, 2C), 22.7 (q, J_{FC} = 3.1 Hz, –CH_2*C*$H_2CH_2CF_3$, 2C). ^{19}F NMR: (376.47 MHz, 298 K, acetone-d_6): δ −66.5 (t, J_{HF} = 11.2 Hz, 6F of C*F*$_3$).

5.2.2. Dichloro(4,4′-dimethyl-2,2′-bipyridine)platinum (2). Compound was synthesized by the method of Bielli.[42] 15 mL of a 1 M HCl solution was added to 60.0 mg (0.32 mmol) of 4,4′-dimethyl-2,2′-bipyridine and the mixture was stirred under N_2 atmosphere until dissolved. 132 mg (0.32 mmol) of K_2PtCl_4 was dissolved in 5 mL of H_2O and added slowly to the 4,4′-dimethyl-2,2′-bipyridine solution via a syringe. The reaction mixture was allowed to stir for 24 h, filtered, and rinsed with H_2O, affording a yellow solid. ^{1}H NMR (400 MHz, 320 K, DMSO-d_6): δ 9.28 (d, J_{HH} = 5.8 Hz, 2H, C-*H* 6/6′), 8.39 (s, 2H, C-*H* 3/3′), 7.65 (d, J_{HH} = 5.3 Hz, 2H, C-*H* 5/5′), 2.50 (s, 6H, C*H*$_3$-Ar). ^{13}C{^{1}H} NMR (100.65 MHz, 320 K, DMSO-d_6): δ 156.0 (2C, 2/2′) 152.3 (2C, 4/4′), 147.3 (2C, 6/6′), 127.7 (2C, 3/3′), 124.4 (2C, 5/5′), 20.8 (2C, *C*H$_3$-Ar).

5.2.3. Synthesis of *fac*-tricarbonylchloro[4,4′-bis(4,4,4-trifluorobutyl)-2,2′-bipyridine]rhenium (3). 49.0 mg (0.266 mmol) of 4,4′-bis(4,4,4-trifluorobutyl)-2,2′-bipyridine was added to a 50 mL round-bottomed flask containing 98 mg (0.271 mmol) of $Re(CO)_5Cl$. 20 mL of toluene was added and the solution was refluxed for 2 h and then allowed to cool to ambient temperature. The precipitate was filtered and collected, affording 95 mg of product (0.139 mmol, 52.4% yield). Anal. Calcd for $C_{21}H_{18}ClF_6N_2O_3Re$: C, 36.98; H, 2.66; N, 4.11. Found: C, 37.16; H, 2.68; N, 4.06. mp 238–240 °C. ^{1}H NMR (400 MHz, 298 K, $CDCl_3$): δ 8.97 (d, J_{HH} = 5 Hz, 2 H, C-*H* 6/6′), 7.52 (s, 2H, C-*H* 3/3′), 7.38 (d, J_{HH} = 5 Hz, 2H, C-*H* 5/5′), 2.92 (t, J_{HH} = 8.0 Hz, 4H, $CF_3CH_2CH_2C$*H*$_2$-Ar), 2.24 (m, 4H, CF_3C*H*$_2CH_2CH_2$-Ar), 2.04 (m, 4H, CF_3CH_2C*H*$_2CH_2$-Ar). ^{13}C{^{1}H} NMR (100.62 MHz, 298 K, $CDCl_3$): δ 197.1 (2C, *C*O-Re), 189.5 (1C, *C*O-Re), 155.7 (2C, 2/2′), 153.7 (2C, 4/4′), 153.1 (2C, 6/6′), 128.0 (q, J_{FC} = 276 Hz, 2C, *C*$F_3CH_2CH_2CH_2$-Ar), 127.0 (2C, 3/3′), 122.9 (2C, 5/5′), 34.2 (2C, $CF_3CH_2CH_2$*C*H_2-Ar), 33.2 (q, J_{FC} = 28.9 Hz, 2C, CF_3*C*$H_2CH_2CH_2$-Ar), 22.4 (2C, CF_3CH_2*C*H_2CH_2-Ar). ^{19}F NMR (376.47 MHz, 298 K, $CDCl_3$): δ −71.2 (t, 6F, J_{HF} = 10 Hz, 6F of *C*F_3).

5.2.4. *fac*-Tricarbonylchloro(4,4′-dimethyl-2,2′-bipyridine)rhenium (4) (CAS [103667-38-5]). Compound was synthesized by the method of Van Wallendael.[44] 0.2 g (1.085 mmol) of 4,4′-dimethyl-2,2′-bipyridine and 0.672 g (1.86 mmol) of $Re(CO)_5Cl$ were combined in a 250 ml flask. Then, 50 mL of benzene was added, and the solution was refluxed for 4 h. After cooling the mixture was filtered, affording 461 mg of a yellow solid (0.9408 mmol, 86.7% yield). ^{1}H NMR (400 MHz, 320 K, $CDCl_3$): δ 8.88 (d, J_{HH} = 5.0 Hz, 2 H, C-*H* 6/6′), 7.94 (s, 2H, C-*H* 3/3′), 7.31 (d, J_{HH} = 4.4 Hz, 2H, C-*H* 5/5′), 2.55 (s, 6H, C*H*$_3$-Ar). ^{13}C{^{1}H} NMR (100.65 MHz, 320 K, $CDCl_3$): δ 200.6 (2C, *C*O-Re), 197.6 (1C, *C*O-Re), 156.0 (2C, 2/2′), 153.0 (2C, 4/4′), 151.4 (2C, 6/6′), 128.3 (2C, 3/3′), 124.1 (2C, 5/5′), 22.0 (2C, *C*H$_3$-Ar).

5.3. Cell culture

MDA-MB-231 and MDA-MB-435 and A549 cells were grown in MEM supplemented with 10% fetal bovine serum, 50 mM HEPES buffer (pH 7.4), penicillin (100 U/ml), streptomycin (100 mg/ml). DC4 and DB46 cells were grown in the same media to which G418 sulfate (600 μg/ml) was added. The DU-145 cell line was grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 50 mM HEPES buffer (pH 7.4), penicillin (100 U/ml), streptomycin (100 mg/ml). All cell lines were maintained at 37 °C, 100% humidity and CO_2/air (5%/95%) atmosphere.

5.4. Clonogenic survival

Drugs were dissolved in DMSO. Following one hour of drug exposure at 37 °C, cells were washed with PBS, harvested by trypsinization, resuspended in supplemented media, counted with a Coulter counter, and plated into 60 mm dishes. Cells were allowed to grow for 11–14 days and colonies containing more than 50 cells were counted by hand after staining with 0.5% crystal violet in 95% ethanol. The number of colonies that formed in the experimental plates was normalized to the control group. All experiments were preformed in triplicate and results shown are typical.

5.5. Flow cytometry

All acquisitions were performed on a BD FACS Calibur instrument.

5.5.1. Annexin-V. After drug incubation, cells were washed with PBS and harvested using trypsin. Cells were centrifuged at 1000*g* and resuspended with MEM supplemented with serum. The cells were washed a second time and then centrifuged. Pellets were then washed in 2.0 mL × 1 Annexin-V binding buffer (BD Bioscience, San Jose, CA) and centrifuged at 500*g* for 5 min. The pellets were treated with Annexin-V-FITC conjugate (BD Bioscience, San Jose, CA) and incubated in the dark for 15 min. Just before acquisition, the volume of cells-conjugate mixture was adjusted by addition of 1× Annexin-V binding buffer. Acquisition to discriminate between apoptotic and necrotic or dead cells was done by staining the cells-conjugate mixture with 10 μL PI (50 μg/mL) solution (BD Bioscience, San Jose, CA). Acquisitions were done on FACS Calibur Cytometer on the FL1 (Annexin) and FL3 (PI) channels with threshold and Duplet Discriminating Module (DDM) set at FL1. The level of shift in events distribution in the Annexin-V only and Annexin-V-PI populations in comparison to control is indicative of degree of effectiveness of the treatment agents. A quantitative measure of these event shifts was accomplished by gating.

5.5.2. DNA. MDA-MB-231 and MDA-MB-435 cells that were to be analyzed with propidium iodide for

cell cycle distribution were washed with PBS after being centrifuged. They were then resuspended in 1 mL of 70% EtOH at −20 °C and stored at 4 °C until they were analyzed. Ethanol fixated cells were cleared of excess fixative by a single wash with 3–5 ml of cold PBS and centrifuged at 500*g* for 5 min. The pellet was resuspended with 1.0% Triton X-100 in PBS. RNAse solution (1.0 mg/mL) was added and the mixture incubated at room temperature for 10–15 min before staining with 100 μg/mL PI solution. Final PI concentration in the test mixture was 50 μg/mL. Cytometry acquisition was done on BD FACS Calibur with argon laser set at 488 nm on the linear Flow Channel 2 (FL-2) with Doublet Discriminatory Module and Threshold set on FL-2. Modfit 3.0 was used to assign distribution curves to data analyzed with propidium iodide and flow cytometry.

5.5.3. Aphidicholin. Cells were seeded into T-25 flasks containing supplemented media and after 24 h were washed once with 5 ml of serum-free media and resuspended in 5 ml of serum-free media containing 2 μg/ml of aphidicholin dissolved in DMSO. After 24 h, the aphidicholin containing media were removed, the cells were washed three times each with 5 ml of supplemented media and resuspended in 5 ml of supplemented media containing the desired drug. After 12 h of incubation in the drug containing media the cells were fixed in 70% ethanol and analyzed for DNA content by flow cytometry.

5.6. Microscopy

MDA-MB-231 and MDA-MB-435 cells were grown on four chambered glass microscope slides for two days prior to treatment. Cells were treated in the same manner as described for clonogenic survival. After incubation cells were stained with Hoechst 33342 and propidium iodide for 15 min. In three separate fields, pictures were taken of the bright field, and then the same image was photographed with a red filter and then a blue filter. The total number of cells was counted in the bright field and this was compared to the number of cells staining positive for each dye. Cells staining positive for both Hoechst and propidium iodide were included only in the count with propidium iodide.

5.7. Statistics

All statistics were preformed on a PC using Graphpad software.

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