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Novel 4,4′-diether-2,2′-bipyridine cisplatin analogues are more effective than cisplatin at inducing apoptosis in cancer cell lines

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ABSTRACT

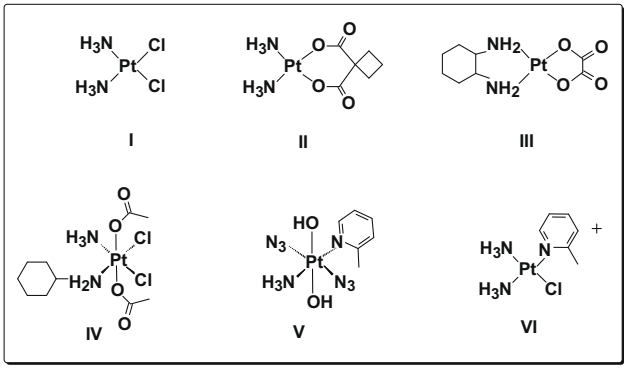
The synthesis and characterization of dichloro(4,4′-bis[methoxy]-2,2′-bipyridine)platinum (**1**) and dichloro(4,4′-bis[3-methoxy-*n*-propyl]-2,2′-bipyridine)platinum (**2**) are described. As analogues to CDDP, these 4,4′-disubstituted 2,2′-bipyridine complexes exhibit decreased EC_{50} values of 10–100 times in cancer cell lines of the lung, prostate, and melanoma with several combinations of complex and cell line less than 10 μM. Flow cytometry data indicate 'blocks' of MDA-MD-435 cycle by **1** (G2/M) and **2** (S). Observed cell survival trends in the presence of **1**, **2** under ionizing radiation mimic those of CDDP. Preliminary structure activity relationships are discussed for the 4,4′-substitutions made on the bipyridine ring.

1. Introduction

The successful application of Cisplatin (CDDP) as an effective cure for testicular cancer has driven the development of structurally similar complexes for treatment of other cancerous tissues.[1] Knowledge of activity modes of CDDP and cellular response to nuclear adducts has been extended significantly recently.[2–4] The side effects and reduced utility of CDDP in the many other cell lines have fueled research both on the activity of CDDP and the development of novel Pt complexes for treatment of solid tumors.[5]

The chronological order of the three FDA approved drugs CDDP **I** (1978), Carboplatin **II** (1989) and Oxaliplatin **III** (2002) also serves as a clear progression in the implementation and development of organic architecture which blossoms today (Scheme 1).[5] From completely inorganic **I** substitution of chlorides for the 1,1-cyclobutane-dicarboxylate afforded **II**, and oxalate in combination with an 1,2-diaminocyclohexane (DACH) replacing the ammonia ligands yielded **III**. Examples of octahedral Pt(IV) complexes in trials include Satraplatin **IV** (phase III—prostate)[6] and FM165 **V** (utility: sub dermal activation with light).[7] Substitution of the amine nitrogen atoms for *imine* nitrogens serves as a reasonable extension and generation of complexes with potential intercalative function as is the case for Picoplatin **VI** (ZD0473) which has reached Phase III trials.[5] The structure of Picoplatin differs from CDDP through replacement of one NH_3 group with 2-picoline (2-methylpyridine). The replacement affords a complex that (1) bears a predisposition to functioning as an intercalator, and (2) features reduced rates of hydrolysis and essentially modulation of alkylation activity (due to the steric hinderance of the methyl group).[8] Picoplatin's observed activity supports further design of platinum complexes with imine ligands and by logical extension simple 2,2′-bipyridine ligands (diimines).

In this study we explore the cytotoxicity of two 4,4′-substituted-2,2′-bipyridine complexes of Pt(II) as CDDP analogues where the substituents terminate with the $-OCH_3$ moiety a methyl ether functional group. Our report details the synthesis of these CDDP



Scheme 1. Selected platinum complexes: cisplatin **I**, carboplatin **II**, oxaliplatin **III**, satraplatin **IV**, FMV 165 **V**, and picoplatin **VI**.

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analogues and their effect upon the vitality of established cancer cells lines (lung [A549], prostate [DU-145] and melanoma [MDA-MB-435] as observed through clonogenic assay and flow cytometric methods. The extent to which the complexes sensitize cancer cells to the effects of ionizing radiation is also probed. Discussion of the ether terminated derivatives' activity leads to a conclusion featuring a preliminary structure–activity relationship.

2. Experimental section

The following chemicals were purchased from Alfa Aesar: 2,2′-bipyridine, 4,4′-dimethyl-2,2′-bipyridine, 2.5 and 2.9 M *n*-BuLi (in hexanes), diisopropyl amine, 2-bromoethyl methyl ether, and acetone-d_6 (1% TMS). 4,4′-dimethoxy-2,2′-bipyridine, 1,5-cyclooctadiene, $SnCl_2$ and DMSO-d_6 (0.1% TMS) were purchased from Aldrich. K_2PtCl_4 and $CDCl_3$ (0.03% TMS) were purchased from Acros. All materials purchased were above 95% purity and were used without further purification unless noted in the procedure. Procedures were carried out under argon in oven dried glassware utilizing anhydrous THF (distilled from Na). Minimum Essential Medium (MEM), Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), trypsin-EDTA, Penicillin–Streptomycin and G418 sulfate were purchased from Invitrogen, Inc. (Carlsbad, CA). RPMI 1640 medium was purchased from ATCC (Manassas, VA). HEPES, crystal violet, CDDP and Dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). All reagents and enzymes used for flow cytometry were of analytical grade from Sigma, Inc. unless otherwise noted.

2.1. Chemical characterization instrumentation

NMR spectra were obtained utilizing either a Varian 400 MHz VnmrJ 2.1A (Palo Alto, CA.) or a JEOL ECX 300 MHz system with Delta software version 4.3.6 (Peabody, MA). Either residual solvent or TMS were used as the internal chemical shift reference. GC/MS data were obtained utilizing Varian 4000 GC/MS/MS (Palo Alto, CA.) Elemental Analysis was determined by Desert Analytics, Tucson, AZ, USA and within the UNLV Chemistry department utilizing an Exeter CE 440 (UK).

2.2. Preparation of 4,4′-bis[3-methoxy-*n*-propyl]-2,2′-bipyridine

A three-neck 250 ml RBF was charged with a stir bar, 1.54 mL [11 mmol] of diisopropyl amine and 20 mL of THF under an argon atmosphere at 0 °C in a brine bath. *n*-BuLi (2.5 M) 4.4 mL [11 mmol] was added, via syringe, dropwise. After approximately 20 min 0.920 g [5.0 mmol] of 4,4′-dimethyl-2,2′-bipyridine dissolved in 30 mL of anhydrous THF was added dropwise via addition funnel affording a dark red colored solution. 10 min. after completing the previous addition 1.902 mL [20 mmol] 2-bromo-ethyl methyl ether was added. After 24 h, the product mixture had a yellow oily appearance. To this mixture 15 mL of distilled water was added and the product was extracted with diethyl ether. The ether fractions were combined and dried with Na_2SO_4. After evaporation of all volatiles a white crystalline solid was obtained: 0.5229 g [1.743 mmol] (35% yield) mp 38–40 °C.

Anal. Calcd for: $C_{18}H_{24}N_2O_2$: C, 71.97; H, 8.05; N, 9.33. Found: C, 71.82; H, 7.75; N, 9.41. NMR: ^{1}H (400 MHz, 298.1 K, acetone-d_6) δ 8.52, (dd, $^3J_{HH}$ = 4.80 Hz, $^4J_{HH}$ = 0.80 Hz, 2H, Ar-H 6,6′), 8.33 (s, 2H, Ar-H 3,3′), 7.21 (dd, $^3J_{HH}$ = 4.80 Hz, $^4J_{HH}$ = 2.00 Hz, 2H, Ar-H 5,5′), 3.35, (t, J = 6.40 Hz, 4H, Ar-$CH_2CH_2CH_2OCH_3$), 3.27 (s, 6H, Ar-$CH_2CH_2CH_2OCH_3$), 2.73 (t J = 8.00 Hz, 4H, ArCH_2), 1.86 (m, 4H, Ar-$CH_2CH_2CH_2OCH_3$); ^{13}C{^{1}H} NMR: (100 MHz, 298.1 K, acetone-d_6) δ 156.3 (2C, 2/2′) 152.1 (2C, 4/4′), 149.3 (2C, 6/6′), 124.2 (2C, 3/3′), 120.9 (2C, 5/5′), 71.4 (2C, Ar-$CH_2CH_2CH_2OCH_3$) 57.9 (2C, Ar-$CH_2CH_2CH_2OCH_3$) 31.8 (2C, Ar-$CH_2CH_2CH_2OCH_3$) 30.5 (2C, Ar-$CH_2CH_2CH_2OCH_3$); MS (EI: 70 eV, *m/z*) 301 (M+1, 34%), 255 (M+–CH_2OCH_3, 64%), 242 (M+–$CH_2CH_2OCH_3$, 100%), 184 (M+–CH_2-CH_2OCH_3, 32.5%).

2.3. Preparation of dichloro(4,4′-bis[methoxy]-2,2′-bipyridine)-platinum (1)

A 100 mL RBF was charged with 0.216 g [1.0 mmol] of 4,4′-dimethoxy-2,2′-bipyridine, 0.374 g [1.0 mmol] of (COD)$PtCl_2$ and 20 mL of acetonitrile then heated at reflux for 24 h. After cooling the mixture to ambient temperature all volatiles were removed and acetonitrile was added to the crude solid product. The solid component of the resulting heterogeneous mixture was isolated by filtration (fine porosity glass frit), rinsed with copious amounts of water and allowed to air dry. The resulting bright yellow powder weighed 0.3075 g [0.6377 mmol] (64% yield) mp 318–320 °C. Anal. Calcd for $C_{12}H_{12}Cl_2N_2O_2Pt$: C, 29.89; H, 2.51; N, 5.81. Found: C, 29.68; H, 2.51; N, 5.66. NMR: ^{1}H (400 MHz, 298.1 K, DMSO-d_6) δ 9.068, (m, $^3J_{PtH}$ = 38 Hz $^3J_{HH}$ = 6.80 Hz, 2H, Ar-H 6,6′), 8.129, (d, $^4J_{HH}$ = 2.80 2H, Ar-H 3,3′), 7.360, (dd, $^3J_{HH}$ = 6.80 Hz, $^4J_{HH}$ = 2.80 Hz, 2H, Ar-H 5,5′), 4.021, (s, 6H, Ar-OCH_3); NMR: ^{13}C {^{1}H} (100 MHz, 298.1 K, DMSO-d_6) δ 168.443 (2C, 2,2′) 158.584 (2C, 4/4′) 149.692 (2C, 6/6′) 113.468 (2C, 3/3′) 111.340 (2C, 5/5′) 57.875 (2C, Ar-OCH_3): NMR: ^{195}Pt {^{1}H} (64.6 MHz, 295 K, DMSO-d_6) δ −2311.

2.4. Preparation of dichloro(4,4′-bis[3-methoxy-*n*-propyl]-2,2′-bipyridine)platinum (2)

Complex **2** was prepared in the same manner as complex **1** utilizing 0.1059 g [0.353 mmol] of 4,4′-bis(*n*-propyl-4-methoxy)-2,2′-bipyridine and 0.1321 g [0.353 mmol] of (COD)$PtCl_2$. 0.1738 g [0.307 mmol] of a bright yellow powder was obtained (87% yield) mp 177–180 °C. Anal. Calcd for $C_{18}H_{24}Cl_2N_2O_2$ Pt: C, 38.17; H, 4.27; N, 4.95. Found: C, 38.24; H, 4.05; N, 5.12. NMR: ^{1}H (400 MHz, 298.1 K, DMSO-d_6) δ 9.307, (d $^3J_{HH}$ = 6.40 Hz, 2H, Ar-H 6,6′), 8.523, (d, $^4J_{HH}$ = 1.60 Hz, 2H, Ar-H 3,3′), 7.703, (dd, $^3J_{HH}$ = 6.00 Hz, $^4J_{HH}$ = 1.60 Hz, 2H, Ar-H 5,5′), 3.400 (t, J = 6.40 Hz, 4H, Ar-$CH_2CH_2CH_2OCH_3$), 3.37 (s, 6H, Ar-$CH_2CH_2CH_2OCH_3$), 2.828 (t J = 7.60 Hz, 4H, Ar-CH_2), 1.976 (m, 4H, Ar-$CH_2CH_2CH_2OCH_3$). NMR: ^{13}C{^{1}H} (100 MHz, 298.1 K, DMSO-d_6) δ 157.378 (2C, 2/2′) 157.073.1 (2C, 4/4′) 148.308 (2C, 6/6′) 127.904 (2C, 3/3′) 124.704 (2C, 5/5′) 71.4 (2C, Ar-$CH_2CH_2CH_2OCH_3$) 57.9 (2C, Ar-$CH_2CH_2CH_2OCH_3$) 31.8 (2C, Ar-$CH_2CH_2CH_2OCH_3$) 30.5 (2C, Ar-$CH_2CH_2CH_2OCH_3$) NMR: ^{195}Pt {^{1}H} (64.6 MHz, 295 K, DMSO-d_6) δ −2320.

2.5. Cell culture

Human cancer cell lines A549 (lung carcinoma), DU-145 (prostate carcinoma) and MDA-MB-435 (melanoma) were obtained from American Culture Type Collection (ATCC). MDA-MB-435 and A549 cells were grown in MEM supplemented with 10% FBS, 25 mM HEPES buffer (pH 7.4), Penicillin (100 μ/ml) and Streptomycin (100 mg/ml). The DU-145 cell line was grown in RPMI 1640 supplemented with 10% FBS, 25 mM HEPES buffer (pH 7.4), Penicillin (100 U/ml) and Streptomycin (100 μg/ml). All cell lines were maintained at 37 °C in humidified, 5% CO_2 atmosphere.

2.6. Drug preparation

Cis-diamminedichloroplatinum (II) (CDDP) was dissolved in DMSO to obtain a stock solution of 50 mM. Compounds **1** and **2** were dissolved in DMSO to obtain stock solutions of 5 mM. All solutions were sterilized by filtration.

2.7. Clonogenic survival assay

Drugs were dissolved in DMSO. Following 1 h of drug exposure at 37 °C, cells were washed with PBS, harvested by trypsinization, resuspended in supplemented medium, counted with a Coulter Z1 particle counter, and plated into 60 mm dishes. Cells were allowed to grow for 11–14 days, stained with 0.5% crystal violet in 95% ethanol and colonies containing 50 or more cells were manually counted. The average count of the control was set as 100% survival and the average count of the treated groups were calculated as a percent of the control. All experiments were performed in triplicate and results shown are typical. Ionizing radiation was generated using a Faxitron Model 650 with a Cobalt-60 radiation source operating at a 24 inches SID Shelf Position 2, a beam diameter of 17.4 inches and 60 kVp which gives a dose rate of 1 Gy/min. For the combination treatment of the drugs with IR, cells were first treated for 1 h with the drug immediately followed by irradiation.

Combination index (CI) values were calculated using the formula CI = (D1/D×1) + (D2/D×2), where D×1 is the concentrations of the drug and D×2 is the IR doses which are required to produce an ×% survival. D1 and D2 are concentrations of drug and IR doses in combination which are required to produce the same ×% survival as the agents alone. CI values <1 indicates treatments are synergistic, CI = 1 indicates treatments are additive and CI >1 indicates treatments are antagonistic.[9]

2.8. Flow cytometry

For propidium iodide (PI) staining, 40–80% confluent cells were incubated at 37 °C with 5% CO_2 for 1 h with EC_{50} concentration or 1×10^{-4} M of CDDP analogues. After 1 h, cells were washed with 5 mL PBS and fresh medium added. Cells were incubated at 37 °C with 5% CO_2 for 24 h and 48 h, at which point cells were harvested, counted and centrifuged at 500*g* for 5 min. Cells were then washed two times with 5 mL PBS. After washing, cells were fixed by resuspending in 0.1 mL PBS and 1 mL of cold 95% ethanol was slowly added drop wise with gentle vortexing. Fixed cells were stored at 4 °C until analysis. For analysis, fixed cells were washed once with 1–2 mL PBS and centrifuged at 500*g* for 5 min. Cells were resuspended in 100 μL of 1.0% Triton X-100 buffer solution. Then 100 μL of a 1.0 mg/mL RNAse solution was added and allowed to stand at room temperature for 10–15 min. While in the dark, 200 μL of a 100 μg/mL PI stain was added to make a final concentration of 50 μg/mL and gently vortexed. Cell mixture was incubated at room temperature for 30 min. Cytometry acquisition was done on Becton Dickinson FACS Calibur with the argon laser set at 488 nm on the linear Flow Channel 2 (FL-2) with Doublet Discriminatory Module (DDM) and Threshold set on FL-2.

For Annexin V-FITC/PI staining, the same procedure was used as for PI staining up to the washing point before fixation. After harvesting and counting, cells were centrifuged at 500*g* for 5 min. and washed once with 5 mL Ca^{2+} and Mg^{2+} free PBS. Pellets were then washed in 2.0 mL 1X 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 1X Annexin-V binding buffer. Acquisition to discriminate between apoptotic and necrotic 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.

2.9. Statistical analysis

GraphPad Prism 4 was used to graph survival curves. ModFit version 3.0 was used for flow cytometry analysis. Microsoft Office Excel was used to run Student's *t*-Test; values with $p < 0.05$ were considered significant. For Table 1 the Student's *T*-tests were used to verify significant differences among the EC_{50} values within the cell lines.

3. Results

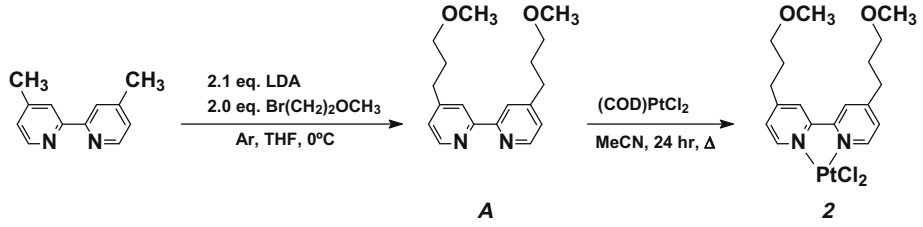
The CDDP analogues **1** and **2** were generated by reaction of their corresponding bipyridine ligand with (1,5-cyclooctadiene)platinum dichloride in acetonitrile at reflux (last step (Scheme 2)).[10] Thus complex **1** was prepared from 4,4′-dimethoxy-2,2′-bipyridine. Complex **2** was prepared from 4,4′-bis[3-methoxy-*n*-propyl]-2,2′-bipyridine, a molecule which was unknown prior to this report (Scheme 3).[10] Synthesis of bipyridine ligand **A** is accomplished through what is a typical S_N2 substitution reaction. Deprotonation of 4,4′-dimethyl-2,2′-bipyridine with lithium diisopropylamide (LDA) and quenching with 2-bromo-ethyl methyl ether yields the bipyridine in modest yield. The fluorinated ligand corresponding to platinum complex **3** (vide infra) was generated by a similar route (Scheme 2).[11]

Complexes **1** and **2** are isolated as light yellow solids having modest solubility in polar organic solvents facilitating characterization by NMR. ^{1}H NMR spectroscopy of each complex features a downfield coordination chemical shift of more than 0.5 ppm for the 6,6′-hydrogen resonances and indicative of complex formation.

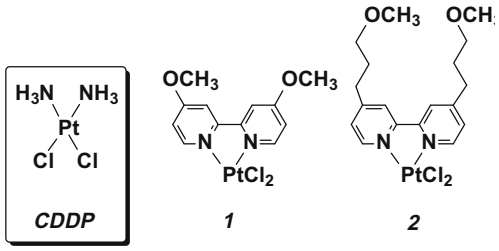
Table 1
EC_{50} values for CDDP analogues [substituents given in brackets]

Agent (μM)	A549	DU-145	MDA-MB-435
CDDP	900 ± 100	500 ± 200	310 ± 60
1 [–OCH_3]	>100	26 ± 3*	6 ± 3*
2 [–$(CH_2)_3OCH_3$]	25 ± 5*	22 ± 2*	3 ± 1*
3 [–$(CH_2)_3CF_3$]	7.0	3.4	2.16 ± 1.63*
4 [–H]	>100	50 ± 20*	20 ± 1*

* Statistically significant compared to CDDP within cell line with $p < 0.05$.



Scheme 2. Synthesis of **2**.



Scheme 3. The structures of CDDP and analogues **1** and **2**.

Coupling of the 6/6′ ^{1}H to ^{195}Pt was observed; $^{3}J_{PtH}$ = 38 Hz. The ^{195}Pt {^{1}H} spectra exhibit a single resonance at −2311 ppm for (**1**) and −2320 for (**2**). These data are similar to those of the parent complex (2,2′-bipyridine)$PtCl_2$ (**4**) (Scheme 4), δ −2315, and in positions relative to **4** that are intuitive based on the expected electronic impact of the substituents.[12] Finally, these chemical shifts confirm the oxidation levels of **1** and **2** as Pt(II). For comparison the resonance for the Pt(IV) complex (2,2′-bipyridine)$PtCl_4$, is observed at −311 ppm (noteworthy is the authors' mention of this Pt(IV) species being unstable in DMSO).[12]

The cytotoxicity of the CDDP analogues were evaluated in a series of established cancer cell lines including A549, DU-145, and MDA-MB-435. The EC_{50} values for all cell line/complex combinations are contained in Table 1. For all cells, CDDP was used in the final concentrations of 1×10^{-7} M, 1×10^{-5} M, 1×10^{-4} M and 1×10^{-3} M. Complexes **1** and **2** were used in the final concentrations of 1×10^{-7} M, 1×10^{-6} M, $1 \times 10^{-5.5}$ M, 1×10^{-5} M, $1 \times 10^{-4.5}$ M and 1×10^{-4} M, higher concentrations were not used due to limited availability of the complexes.

A549 cell survival was unaffected by CDDP over the concentration range 1×10^{-7}–1×10^{-4} M CDDP and **1** had little to no effect on cell survival in the same range (Fig. 1, panel A). However, at 1×10^{-3} M CDDP, cell survival was reduced to 53.3 ± 7.2%. Complex **2** was the most lethal complex tested with an EC_{50} of 24.6 ± 5.2 μM. Since CDDP has been reported to sensitize cells to ionizing radiation, the ability of **2** to sensitize cells to ionizing radiation was also explored. Fig. 1, panel B shows the effects of CDDP and **2** on the radio sensitivity of A549 cells. Complex **2** was able to sensitize the cells. None of the treatments resulted in synergy between the complexes and ionizing radiation as determined by inspection of the calculated combination index (CI).

DU-145 cell survival was unaffected by CDDP over the concentration range of 1×10^{-7}–1×10^{-4} M (Fig. 2, panel A). Upon treatment with 1×10^{-3} M CDDP, cell survival was reduced to 19.4 ± 4.1%. Complexes **1** and **2** both had EC_{50} values around 22 μM. Fig. 2, panel B shows the ability of **2** to act as a radiosensitizer in DU-145 cells. At the EC_{50} concentration **2** was more effective than CDDP as a radiosensitizer. The calculated CI value of 0.64 is evidence that **2** was acting synergistically with the ionizing radiation.

MDA-MB-435 cells displayed very similar survival responses for compounds **1** and **2** and their EC_{50} values ranged from 6.3 μM to 3.3 μM, respectively (Fig. 3, panel A). Both compounds were more effective than CDDP. CDDP was the least lethal compound tested and displayed its characteristic survival curve with an EC_{50} value of 312.0 ± 57.3 μM. CDDP and compound **2** used at their EC_{50} were both radiosensitizers in the MDA-MB-435 cells (Fig. 3, panel B). No evidence for synergy with ionizing radiation was observed with either compound.

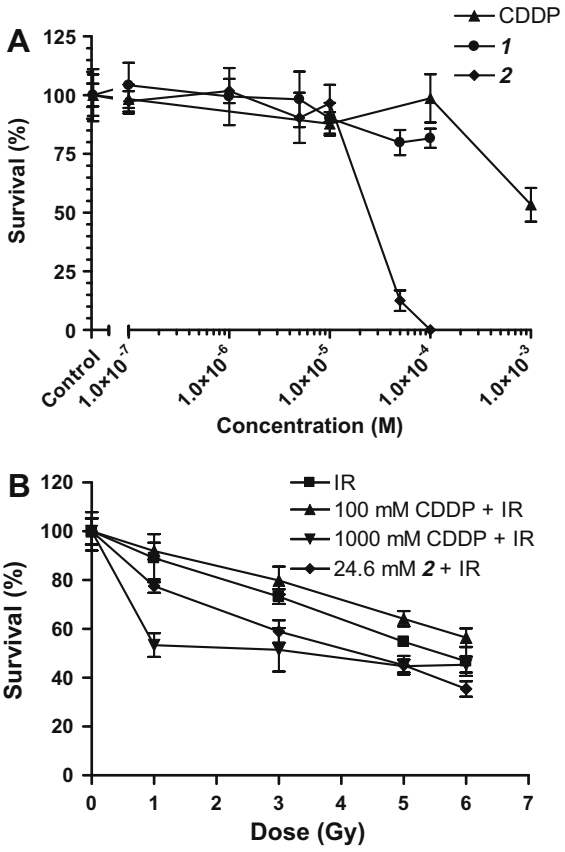
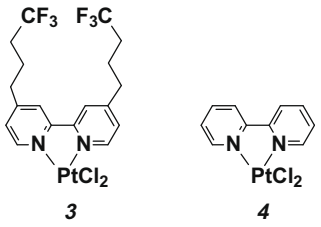


Figure 1. A-549 cell survival (A) survival with drug alone and (B) survival in combination with IR.

A comparison of the EC_{50} values for both analogues and cell lines is found in Table 1. Both analogues were more lethal than CDDP in all cell lines examined (lung, prostate and melanoma). The A549 cell line was the most resistant cell line among those evaluated with the analogues while the MDA-MB-435 cells line was the most sensitive.

The ability of compounds **1** and **2** to inhibit the cell cycle was investigated. The cells were treated with the EC_{50} concentrations of the CDDP or the analogues for 1 h and then stained with PI to assess the cell cycle distribution 24 h and 48 h after treatment. In cases in which the EC_{50} value was not obtained, 1×10^{-4} M was used. In A549 cells, at both 24 h (Fig. 4, panel A) and 48 h (Fig. 4, panel B) after treatment with compounds **1** or **2** the distribution of DNA was not statistically different than what was observed in control cells except for a decrease in the G2/M peak with compound **2** at 24 h after exposure. In the DU-145 cell line, 24 h following treatment there was no statistically significant difference between the control and treatment groups (Fig. 5, panel A). However, there was a small difference in the amount of apoptosis with the control displaying 1.43% while compound **1** had 4.18% 24 h after treatment (Fig. 5, panel B). Compound **2** caused a significant decrease in the amount of DNA in the S phase in DU-145 cells 48 h after treatment, 28.39% versus 36.79% in the control group. In MDA-MB-435 cells, the only significant cell cycle block 24 h



Scheme 4. Structure of related analogue **3** and parent bipyridine complex **4**.

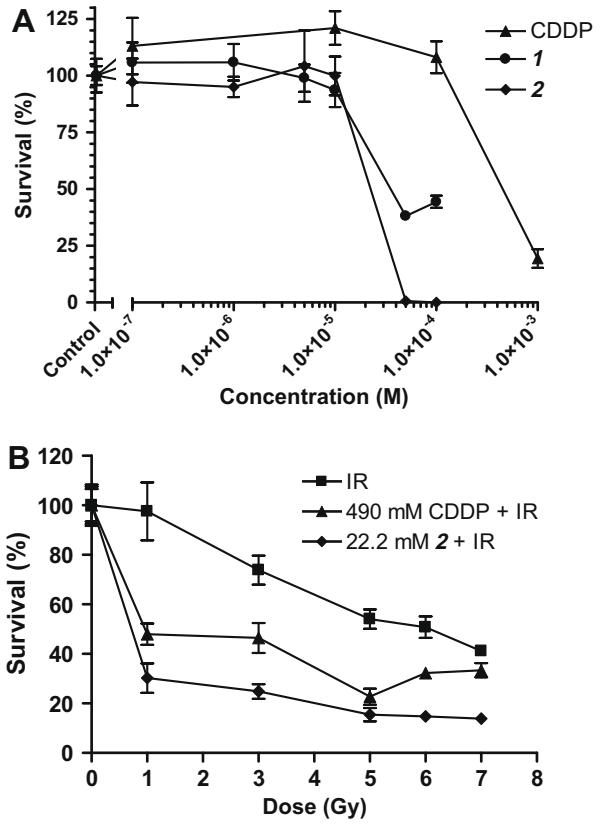


Figure 2. DU-145 cell survival (A) survival with drug alone and (B) survival in combination with IR.

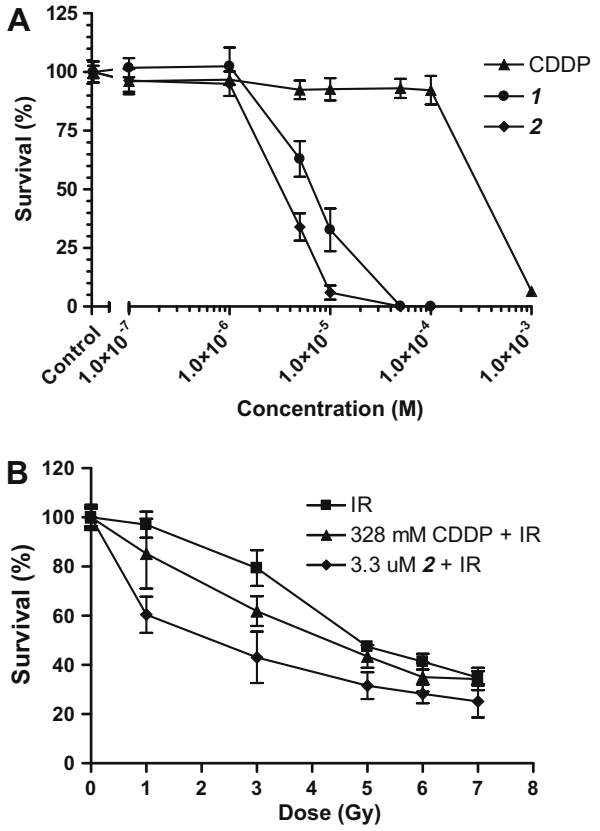


Figure 3. MDA-MB-435 cell survival (A) survival with drug alone and (B) survival in combination with IR.

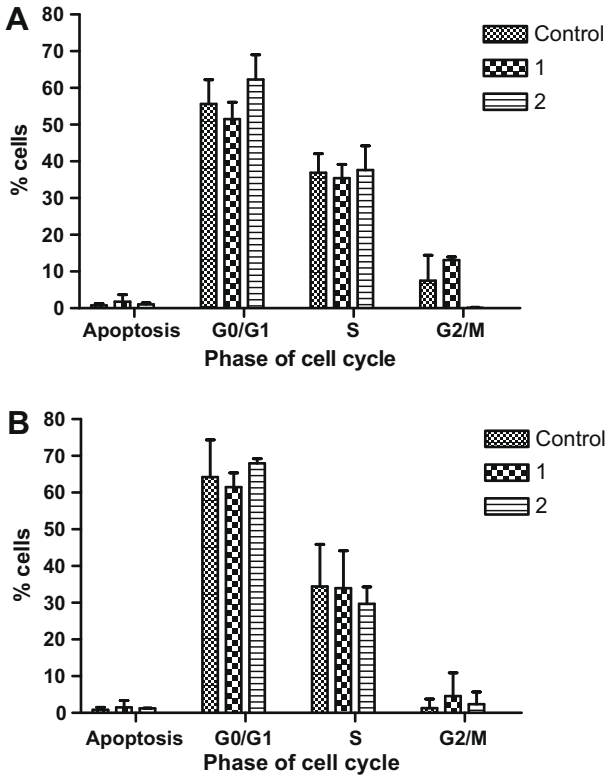


Figure 4. Flow cytometry: A549 (A) 24 h and (B) 48 h.

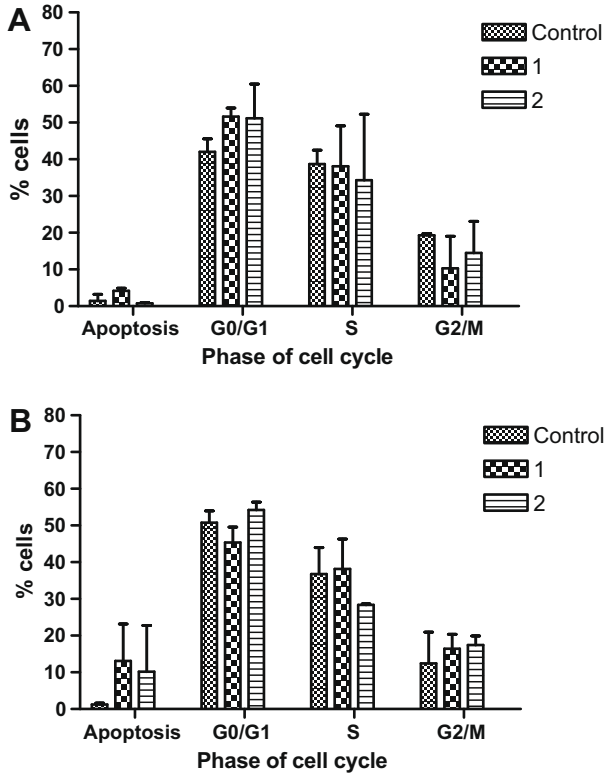


Figure 5. Flow cytometry: DU-145 (A) 24 h and (B) 48 h.

after treatment was caused by compound **1**, 50.61% versus only 25.05% in the control group at G2/M phase (Fig. 6, panel A). At 48 h following treatment compound **2** caused a block in the S

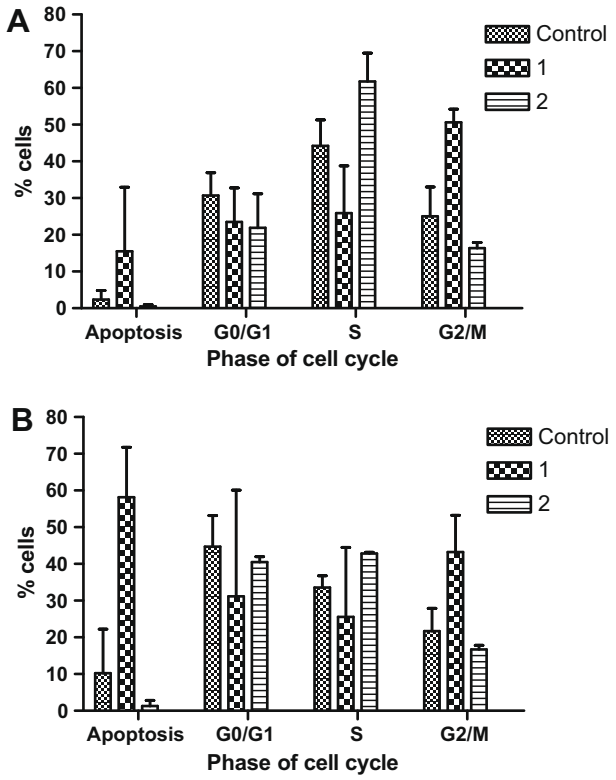


Figure 6. Flow cytometry: MDA-MB-435 (A) 24 h and (B) 48 h.

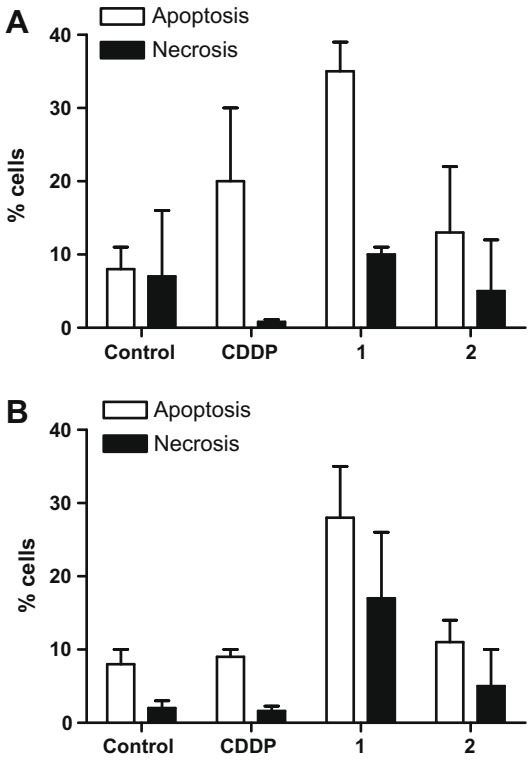


Figure 7. MDA-MB-435 flow cytometry with Annexin V (A) 24 h and (B) 48 h.

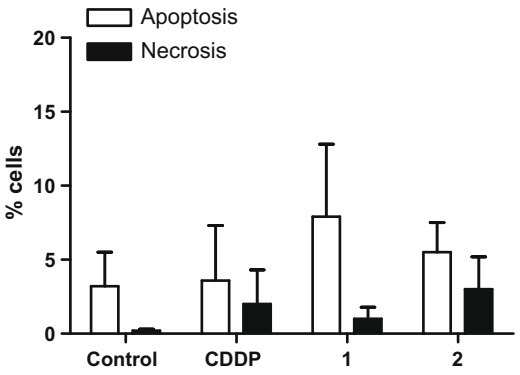


Figure 8. MDA-MB-435 cell death determined by fluorescent microscopy. Cells were treated with EC_{50} concentration of CDDP, **1**, or **2**, for 1 h and images were taken at 48 h.

phase of the cell cycle, 42.82% versus 33.60% in the control group (Fig. 6, panel B).

To determine the mode of cell death, Annexin V-FITC staining in MDA-MB-435 cells was utilized. The cells were treated with the EC_{50} concentration of CDDP or the novel analogues for 1 h and analyzed by flow cytometry 24 h or 48 h following treatments. Figure 7 shows that Annexin V staining detects low levels of both necrosis and apoptosis in the control cells. Treatment with CDDP or compound **2** did not result in a significant change in apoptosis or necrosis either 24 h (panel A) or 48 h (panel B) following treatments. Compound **1** produced a statistically significant increase in apoptosis at both 24 h (35.2 ± 3.8% vs 8.5 ± 2.5% in control cells) and 48 h (27.9 ± 7.1% vs 8.2 ± 2.2% in control cells). Data from fluorescent microscopy confirmed the results obtained with flow cytometry (Figs. 7 and 8).

4. Discussion

The activity of CDDP is well studied and a concise review is available.[2,3] CDDP makes intrastrand 1,2 (GpG) and 1,3 (GpXpG) adducts and interstrand G-G crosslinks.[13] These adducts distort the DNA structure and if not repaired lead to cell death via an apoptotic pathway.[14] The Pt-DNA intrastrand lesions are typically repaired by the nucleotide excision repair pathway (NER).[15] 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.[16] It is interesting to speculate that the introduction of the 2,2′-bipyridine ligand in complexes **1, 2** and those in our preliminary reports dichloro(4,4′-bis-(4,4,4-trifluorobutyl)-2,2′-bipyridine)platinum **3** and dichloro(2,2′-bipyridine)platinum **4** (Scheme 4) 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 CDDP.[17,18]

From the survival assay data in Table 1 it is apparent that the bipyridine complexes as a class possess greater activity as cytotoxins than CDDP. Secondly substitution at the 4,4′-position of the bipyridine ligand with ether, alkyl, or fluoroalkyl groups results in enhanced cytotoxicity as observed for analogues **1**–**3** when compared to unsubstituted complex **4**. Comparison of the complexes based on the substituent differences leads to a preliminary structure–activity relationship.

The steric character of the substituent on the aromatic structure of the bipyridine ligand does appear to correspond to differences in activity. Complexes **3** and **4** feature ligands with identical electronic character (calculated vertical ionization potentials and observed electrochemical reduction potentials) yet **3** has EC_{50}s far smaller than **4** in all lines and a qualitatively much larger steric size.[11]

The comparison of the two ether substituted complexes **1** and **2** provide a preliminary evaluation of the impact of hydrocarbon content on activity as both are methyl ethers. The structure of the ether complexes **1** and **2** differ by 6 methylene (–CH_2–) units in total. Additionally this difference in structure represents a larger steric profile for **2** if conformational freedom is considered for rotations around each C–C and C–O bond. With respect to activity, the EC_{50} values for **2** are lower than those of **1** across all cell lines. More hydrocarbon content (methylene groups, CH_2) appears to correspond to an enhancement in cytotoxic character for the complex. This relationship appears to hold for a comparison of **1** and **3**.

Subtle differences in functional group can lead to significant change in activity. Both complexes **2** and **3** feature an *n*-propyl group with different termini, methoxy and trifluoromethyl, respectively. The observed enhanced activity of **3** when compared to **2** supports a long standing theme in biological chemistry surrounding the special character of fluorine.[19]

Finally, **1–3** all have similar activity in MDA-MB-435 (most sensitive line to platinum) and enhanced levels of activity relative to both the parent unsubstituted bipyridine complex **4** and CDDP.

Substitution is important to generating potent cytotoxins within this class of platinum complex. Larger substituents appear to be a common characteristic of most potent cytotoxins. In fact, the substituent size in **2** and **3** appears to be a key to activity as these are the complexes with significant potency in the most insensitive line A549.

The CDDP analogues **1** and **2** produced several results that were similar to CDDP. The lack of any consistent effect on the cell cycle distribution is not surprising since CDDP fails to yield any reproducible cell cycle effects.[20] CDDP is known to act synergistically with ionizing radiation and complex **2** also displayed slightly synergistic interactions with ionizing radiation.[21–25]

DNA damage by cisplatin has been linked to the generation of many cellular responses involving interactions of numerous signaling proteins, including pathways such as p53-dependent apoptosis, Fas/Fas ligand-dependent apoptosis, and the mitogen-activated protein kinase (MAPK) signaling pathways.[26,4] Data from the literature indicate cisplatin-induced apoptotic signaling varies depending on the cell line.[26] The cell lines used in our work have differences in p53 expression with MDA-MB-435 and DU145 having mutant p53 while A549 having wild-type p53. Our data indicates that MDA-MB-435 and DU145 are more sensitive to treatment with cisplatin and the novel analogues than A549; this might suggest differences in mechanism of cell death. Flow cytometry data demonstrate a higher level of apoptosis in MDA-MB-435 and DU145 than in A549 after drug treatment. 'Given the differences in drug sensitivity with and without wild-type p53, our data suggest that p53 plays a protective role in defending the cells against the action of the drugs or that the p53 pathway antagonizes the mechanism of action of the drug.'

5. Conclusion

This study characterized the ability of two CDDP analogues to kill lung, prostate and melanoma cancer cells. Both of the compounds showed some ability to kill cancer cells, although their cytotoxicity varied from cell line to cell line. Both of the CDDP analogues appeared to kill the cancer cells predominately by apoptosis although some necrosis was observed. Complex **2** was the most lethal of the two ether complexes and was anywhere from 22 to 93 times more lethal than CDDP in the three cancer cell types investigated. The complexes **1** and **2** only caused a block in the cell cycle for MDA-MB-435. Complex **2** had a slightly synergistic interaction with ionizing radiation to kill prostate and functioned as a sensitizer in breast and lung cancer cell lines.

The structural variation represented in the CDDP analogues **1–4** utilized in this study allows for generation of preliminary structure–activity relationships for the molecular motif. (I) The 2,2′-bipyridine ligand structure appears to have a general impact on activity of the Pt center. The replacement of the ammonia ligands on CDDP with the 2,2′-bipyridine ring structure (complex **4**) resulted in a 10-fold increase in lethality in prostate and melanoma cancer cell lines. Further the addition of a pair of substituents at the 4,4′ positions on the bipyridine ring system could result in up to another 2–3-fold increase in lethality. (II) The functional group class and length of substituent lead to line specific responses. The position of a methoxy functional group (**1** and **2**) was not important in melanoma (MDA-MB-435) or prostate (DU-145) cancer cell lines, but did play a role in lung (A549) cancer cells such that the longer chain length (**2**) was more lethal. Interestingly, **3** has a greater potency in the same line and possesses an aliphatic chain like **2**.

The results presented here detail the activity of two ether substituted 2,2′-bipyridine complexes of Pt(II) **1** and **2**. With respect to CDDP, both **1** and **2** function as more potent cytotoxins for multiple cancer cell lines. Upon comparison to earlier work with the bipyridine structural motif an immerging structure–activity relationship is observed. Further studies are underway with this class of complex to determine if they can provide effective tumor killing in patients while being used at lower doses than CDDP to avoid nephrotoxicity.

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This manuscript is dedicated in memory of Dr. Stephen W. Carper. He was a man passionate about his research and had a great impact on the lives of his students. He is dearly missed by those who knew him.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.12.047.

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