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Anti-HIV and Cytotoxic Activities of Ru(II)/Ru(III) Polypyridyl Complexes Containing 2,6-(2′-Benzimidazolyl)-pyridine/chalcone as Co-Ligand

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Abstract—Ru(II)/Ru(III) polypyridyl complexes containing 2,6-(2′-benzimidazolyl)-pyridine or chalcone as co-ligands were synthesized and characterized previously (Mishra, L.; Sinha, R. *Indian J. Chem., Sec. A* **2001**, in press. Mishra, L.; Sinha, R. *Indian J. Chem., Sec. A*, 39A, **2000**, 1131). Their interaction with aqueous buffered calf thymus DNA was measured. (Novakova, O.; Kasparkova, J.; Vrana, O.; van Vliet, P. M., Reedijk, J.; Brabec, V., *Biochem. 34*, **1995,** 12369 and these results prompted additional screening for anti-HIV (human immunodeficiency virus) activity against DNA replication in H9 lymphocytes and cytotoxic activity against eight tumor cell lines. The most active compounds were **17** in the former assay (EC_{50} <0.1 μg/mL and TI >23.1) and **3**, **8**, **10**, and **14** in the latter assay, especially selectively against the 1A9 ovarian cancer cell line ($IC_{50}=4.1$, 3.8, 3.6, and 2.5 μg/mL, respectively).

Introduction

Rosenberg's discovery[1] of the important Pt-containing antitumor drug cisplatin led to the search for other metal ions that could complex with biomolecules and exhibit carcinostatic properties. Accordingly, because ruthenium complexes can interact with nucleic acids, especially DNA,[2] novel ruthenium complexes are being investigated by many research groups.[3] Recently, Ru(II) complexes have been shown to be potent antitumor drugs[4] and powerful immunosuppressants.[5] Ru(II) complexes have also been reported as cytotoxic and anti-HIV agents.[6]

Several DNA binding agents were also found to inhibit HIV-1 integrase, probably due to a nonspecific interaction with the DNA binding domain of the enzyme,[7] and many other biologically active ligands have been reported to behave as HIV-inhibitors[8] (i.e., inhibiting various steps in the viral life cycle).

Chalcones have been recognized for their antiviral,[9] antifungal,[10] antibacterial,[11] and antitumor[12] activities. Similarly, benzimidazole is an important bioactive moiety that complexes with transition metal ions in metalloproteins.[13] Thus, benzimidazole may impart bioactivity whenever attached as a ligand or co-ligand.[14] Bis-benzimidazoles have been reported to possess potential cytotoxic effects, especially against ovarian carcinoma cell lines.[15] The head-to-tail bis-benzimidazole compound Hoechst-33248 and several of its derivatives[16] belong to a super-family of anticancer drugs. It is known that each benzimidazole subunit of Hoechst-33258 interacts with two A-T base pairs by means of a pair of cross-strand H-bonds.[17,18]

Based on the above literature precedents and the importance of ligand-ruthenium systems, we chose to investigate two categories of target ligands, 2,6-bis-(benzimidazolyl)-pyridine and chalcone[19,20] and prepared their RuII/III polypyridyl complexes. In this report, target compounds were evaluated as cytotoxic agents and against replication of HIV.

Synthesis

The ligands L^1H, L^2, L^3H_2, and L^4 were prepared according to reported procedures.[21] $Ru(bpy)_2Cl_2$ and

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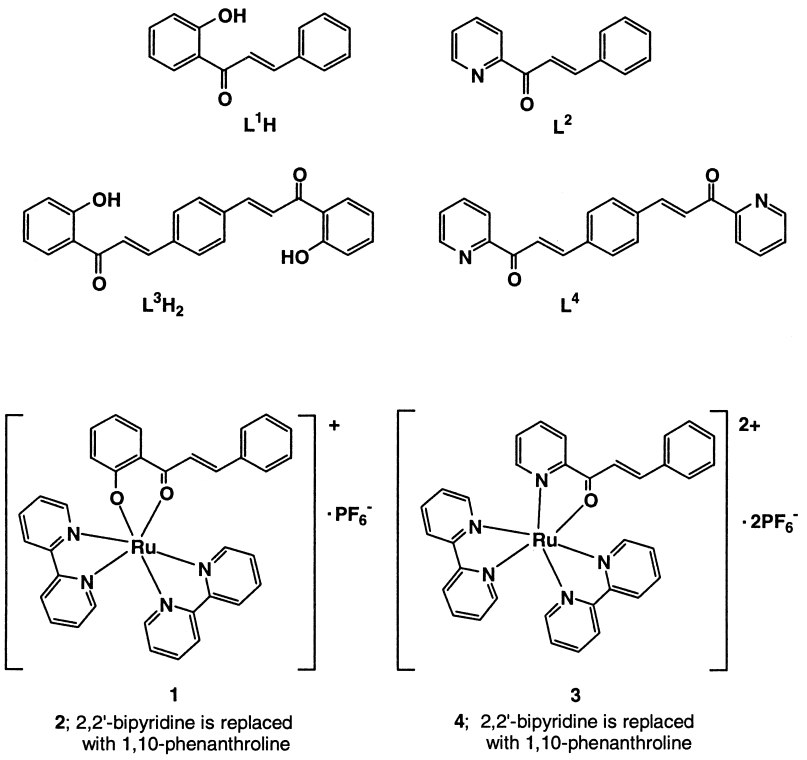

Figure 1. Structures of free ligands L^1H, L^2, L^3H_2 and L^4 and complexes **1**–**4**..

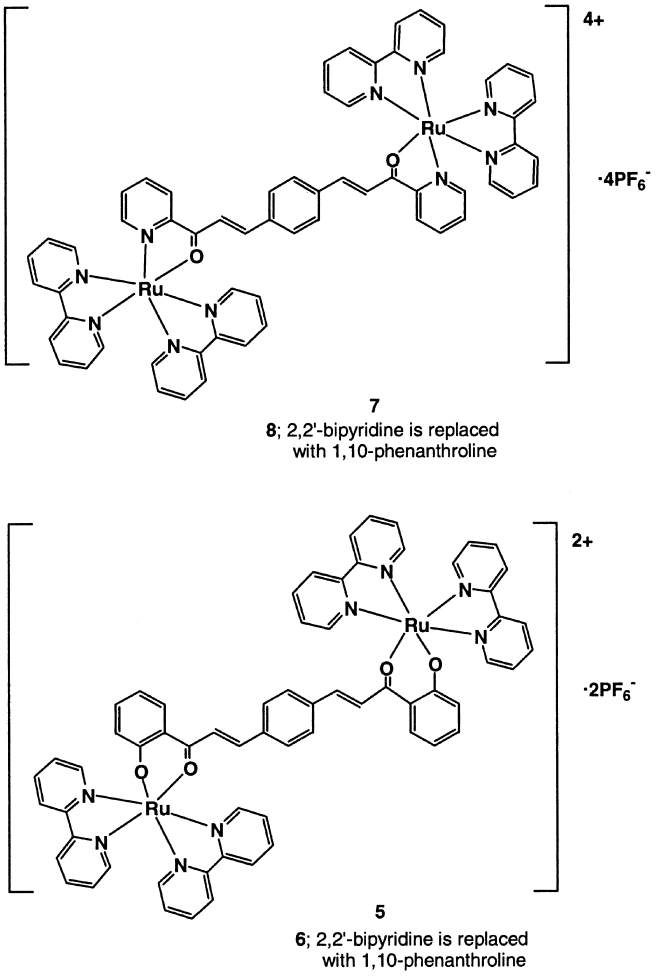

Figure 2. Structures of complexes **5**–**8**.

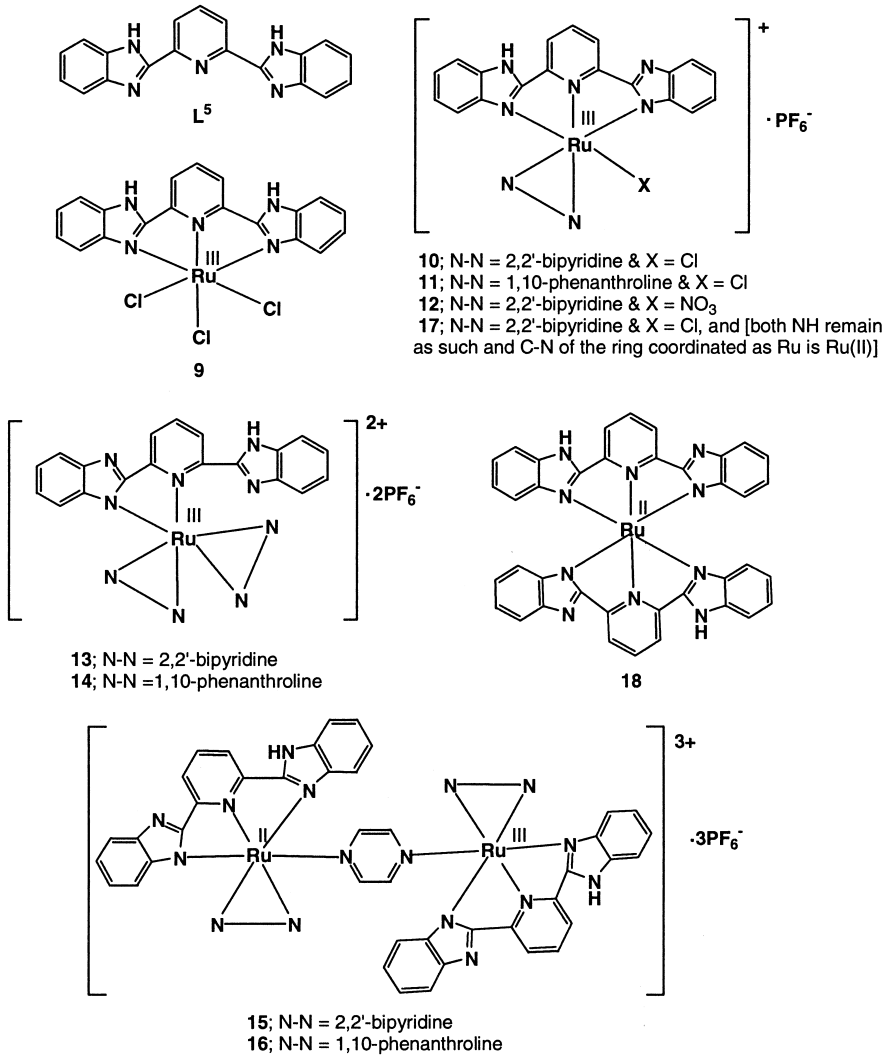

Figure 3. Structures of free ligand L^5 and complexes **10**–**18**.

$[Ru(phen)_2Cl_2]$[22] were added to a solution of the ligands under N_2 atmosphere, and the resulting mixture was stirred for 48 h. Excess of a methanol solution of NH_4PF_6 was added to precipitate the product. Purification by standard methods[19,20] gave the pure complexes **1**–**8**. The ligand L^5 was synthesized by a reported method[23] and refluxed with $RuCl_3 3H_2O$ to yield **9**, which was used to synthesize complexes **10**–**18**.[20]

Assay of Cytotoxic Activity

Compounds were assayed for cytotoxic activity at the School of Pharmacy, University of North Carolina, using a reported procedure.[24] All stock cultures were grown in T-25 flasks containing 4 mL of RPMI-1640 medium supplemented with 25 mM HEPES, 0.25% (w/v) sodium bicarbonate, 10% (v/v) fetal bovine serum, and 100 μg/mL kanamycin. Freshly trypsinized cell suspensions were seeded in 96-well microtitre plates at densitites of 2500–10,000 cells per well with compounds from DMSO-diluted stock. Upon dilution into culture medium, the final DMSO concentration was $\leq$2% DMSO (v/v), a concentration without effect on

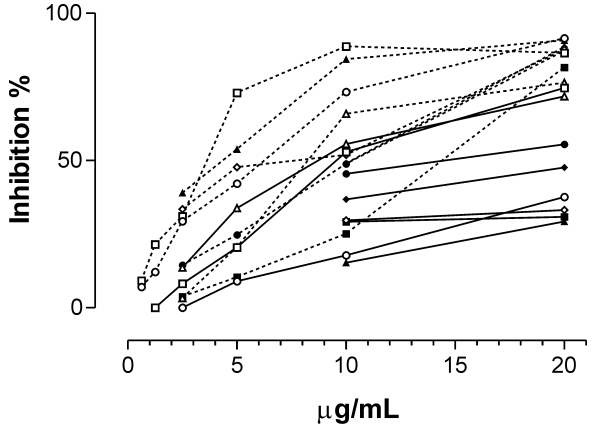

Figure 4. Dose-dependent action of L^5 and compound **10** as inhibitors of human tumor cell line replication. Cells were treated with compounds as described under Experimental. Values are the mean of replicate-treatments that differed no more than 5%. The IC_{50} values interpolated from the data are given in Table 1. The L^5 treatments are denoted by the solid line. Dotted lines represent dose responses for compound **10**. Cell lines are: KB (open circles); 1A9 (open squares); KB-VIN (open triangles); MCF-7 (open diamonds); A549 (closed circles); U87-MG (closed squares); HCT-8 (closed triangles) and HOS (closed diamonds). In general, cell lines were significantly more susceptible to the complex (**10**) than the free ligand (L^5).

Table 1. Cytotoxicity (IC_{50} μg/mL) results against different tumor cells[a,b]

Compound	Cell line							
	HCT-8	1A9	HOS	KB	KB-VIN	MCF-7	A549	U87-MG
L[2]	0.8	4.9	>20 (45)	18.0	18.0	>20 (42)	17.5	>20 (40)
1	>20 (7)	>20 (42)	NA	>20 (6)	NA	>20 (8)	>20 (39)	NA
2	NA	>20 (27)	NA	>20 (12)	>20 (8)	>20 (10)	>20 (7)	>20 (14)
3	1.0	4.1	15.0	9.5	9.0	18.0	8.0	>20 (48)
4	17.0	14.0	>20 (36)	>20 (30)	>20 (37)	>20 (25)	>20 (33)	>20 (18)
5	NA	>20 (16)	NA	>20 (10)	>20 (10)	NA	>20 (11)	NA
6	NA	14.2	NA	>20 (25)	>20 (9)	>20 (14)	>20 (20)	>20 (11)
7	14.8	5.0	12.0	16.2	15.5	>20 (47)	17.2	16.3
8	11.5	3.8	15.0	14.8	13.0	>20 (25)	15.2	13.2
L[5]	>20 (29)	9.5	>20 (48)	>20 (38)	8.5	>20 (33)	14.0	>20 (31)
9	>20 (16)	11.0	>20 (37)	>20 (38)	10.5	>20 (33)	17.7	>20 (30)
10	4.4	3.6	10.2	6.4	8.2	8.0	11.0	14.5
11	NA	18.0	>20 (16)	>20 (19)	>20 (36)	>20 (20)	>20 (26)	>20 (25)
12	>20 (13)	9.0	NA	>20 (46)	>20 (8)	18.0	>20 (44)	>20 (31)
13	>20 (6)	<10 (63)	NA	>20 (46)	>20 (8)	>20 (35)	>20 (30)	>20 (27)
14	>20 (20)	2.5	>20 (18)	>20 (15)	19.5	>20 (36)	>20 (43)	>20 (29)
15	>20 (44)	8.7	16.5	15.5	7.8	>20 (43)	10.8	>20 (45)
16	NA	>20 (25)	NA	>20 (15)	NA	>20 (15)	>20 (15)	>20 (12)
17	15.0	5.2	<10(52)	12.6	4.8	13.0	7.5	12.5
18	>20 (38)	5.5	15.0	15.4	4.9	19.0	8.6	14.8

[a]Cytotoxicity as IC_{50} for each cell line is the concentration that causes a 50% reduction in absorbance at 562 nm relative to untreated cells using SRB assay.
[b]NA, inactive, < or = 5% inhibition; HCT-8, ileothecal carcinoma (ATTC# CCL224); 1A9, ovarian carcinoma (a generous gift of Dr. P. Ginnakakal, NIH, MD), HOS; chemically transformed osteosarcoma; KB, nasopharyngeal carcinoma; KB-VIN, MDR-KB sub-line; MCF-7, breast carcinoma (ATCC# HTB22); A549, lung adenocarcinoma (ATCC# CCL185); U87-MG, glioblastoma (ATCC# HTB14).

Table 2. Anti-HIV activity of free ligands and Ru polypyridyl complexes[a]

Compound	IC_{50} (μg/mL)	EC_{50} (μg/mL)	Therapeutic index (TI)
L[2]	0.21	No suppression	No suppression
1	21.40	No suppression	No suppression
2	22.70	No suppression	No suppression
3	0.212	<0.1	>2.12
4	16.40	No suppression	No suppression
5	>100	No suppression	No suppression
6	21.20	No suppression	No suppression
7	2.30	No suppression	No suppression
8	2.28	No suppression	No suppression
L[5]	21.00	No suppression	No suppression
9	24.70	No suppression	No suppression
10	2.40	No suppression	No suppression
11	25.40	No suppression	No suppression
12	20.60	No suppression	No suppression
13	20.40	No suppression	No suppression
14	0.222	No suppression	No suppression
15	2.26	No suppression	No suppression
16	23.40	No suppression	No suppression
17	2.31	<0.1	>23.1
18	2.27	No suppression	No suppression
AZT	500	<0.001	>500,000

[a]AZT, azido-thymidine; IC, inhibitory concentration; EC, effective concentration.

cell replication. After 3 days in culture, cells attached to the plastic substractum were fixed with cold 50% trichloroacetic acid and then stained with 0.4% (w/v) sulforhodamine B (SRB) (Sigma Chemical Co., St. Louis, MO, USA). The absorbancy at 562 nm was measured using a microplate reader (Molecular Devices, Menlo Park, CA, USA) after solubilizing the bound dye. The IC_{50} is the concentration of agent that reduced cell growth by 50% over a 3 day assay period.

Assay of Anti-HIV Activity

Anti-HIV activity was evaluated at Panacos Pharmaceuticals. The biological evaluation of HIV-1 inhibition was carried out according to established protocols.[26] The T cell line, H9, was maintained in continuous culture with complete medium (RPMI 1640 with 10% fetal calf serum supplemented with L-glutamine at 5% CO_2 and 37 °C). Aliquots of this cell line were only used in experiments when in log-phase growth. Test samples were first dissolved in DMSO. The following drug concentrations (100, 20, 4, and 0.8) were routinely used for screening, and for active compounds, addition dilutions were prepared for subsequent testing so that an accurate EC_{50} value (defined below) could be achieved. As the test samples were being prepared, an aliquot of the H9 cell line was infected with HIV-1 (IIIB isolate) while another aliquot was mock-infected with complete medium. The stock virus used for these studies typically had a $TCID_{50}$ value of 10^4 Infectious Units/mL. The appropriate amount of virus for a multiplicity of infection (moi) between 0.1 and 0.01 Infectious Units/cell was added to the first aliquot of H9 cells. The other aliquot received only culture medium, and these mock-infected cells were used for toxicity determinations (IC_{50}, defined below). After a 4 h incubation at 37 °C and 5% CO_2, both cell populations were washed three times with fresh medium and then added to the appropriate wells of a 24-well plate containing the various concentrations of the test drug or culture medium (positive infected control/negative drug control). In

addition, AZT was also assayed during each experiment as a positive drug control. The plates were incubated at 37 °C and 5% CO_2 for 4 days. Cell-free supernatants were collected on day 4 for use in the in-house p24 antigen ELISA assay. P24 antigen is a core protein of HIV and therefore is an indirect measure of virus present in the supernatants. Toxicity was determined by performing cell counts with a Coulter Counter on the mock-infected H9 cells that had either received culture medium (no toxicity), test sample, or AZT. If a test sample had suppressive capability and was not toxic, its effects were reported in the following terms: IC_{50}, the concentration of test sample which was toxic to 50% of the mock-infected H9 cells; EC_{50}, the concentration of the test sample which was able to suppress HIV replication by 50%, and therapeutic index (TI), the ratio of IC_{50} to EC_{50}.

Results and Discussion

The synthesis and characterization of the ligands and complexes were reported previously.[19,20] Based on the spectroscopic and analytical data, the proposed structures for the complexes are depicted in Figures 1–3.

The cytotoxicity data (IC_{50}) of the free ligands (L^2 and L^5) and complexes (**1**–**18**) against different tumor cell lines are shown in Table 1. In general, neither the ligands or their complexes were potent cytotoxic agents (potent agents are defined as having IC_{50} values less than 4.0 μg/mL); however, some interesting tumor cell selectivity trends were noted. Several complexes of both ligand L^2 and L^5 (**3** and **8** of the former, **10** and **14** of the latter) show a selective cytotoxicity profile against 1A9 (ovarian cancer) cell growth. One compound (**3**) (IC_{50} 1.0 μg/mL) in this group and the corresponding free ligand (L^2) (IC_{50} 0.8 μg/mL) also demonstrated significant activity against HCT-8 (ileothecal carcinoma) cell growth and compound **10** (IC_{50} 4.4 μg/mL) had borderline activity in this cell line. Comparison of free ligands and complexes led to these observations. Among the more active compounds, complex **3** was equipotent with its ligand L^2 in the 1A9 and HCT-8 cell lines and more cytotoxic in A549 and both KB cell lines. In addition, complex **10** was more cytotoxic than its corresponding ligand L^5 in several cell lines (Fig. 4) and **14** was more cytotoxic in only the 1A9 cell line. In summary, from these results, we feel that certain compounds may possess tissue-selective cytotoxic activity and may warrant further investigation as anticancer drug leads.

The anti-HIV data of the ligands (L^2 and L^5) and complexes (**1**–**18**) are given in Table 2. Only compound **17** showed potent anti-HIV activity with an EC_{50} of < 0.1 μg/mL and a TI of > 23.

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