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SYNTHESIS AND IMMUNOSUPPRESSIVE ACTIVITY OF RUTHENIUM COMPLEXES

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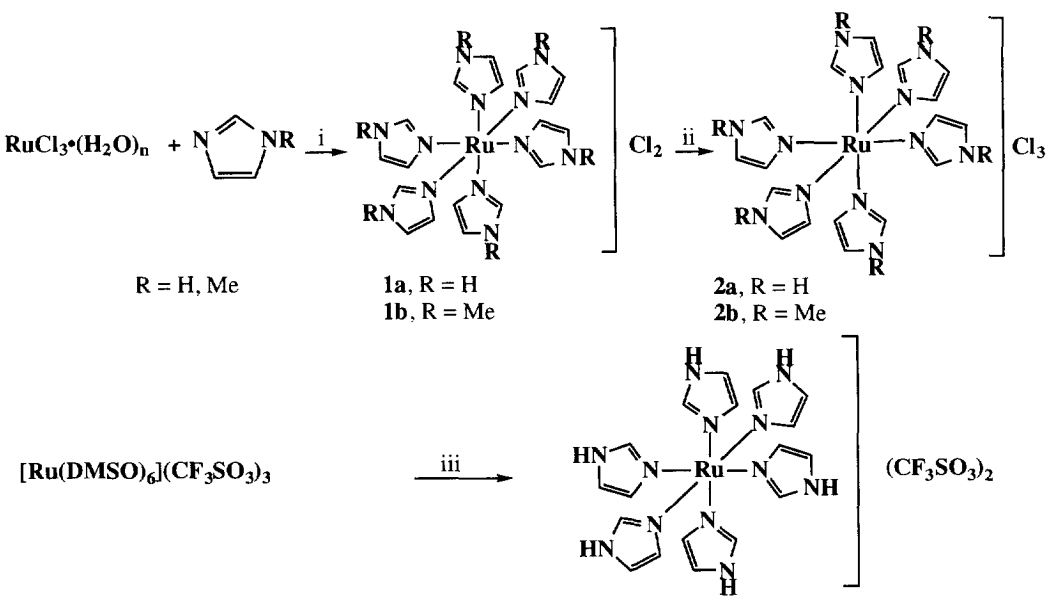
Abstract: The syntheses and immunosuppressive activity of ruthenium complexes are described. One of the complexes (**1a**) was shown to be a potent inhibitor of human T-lymphocyte proliferation with an IC_{50} of 5 nM. The activity of these complexes compares favorably to the well known immunosuppressants Cyclosporin A and Rapamycin.

The investigation of new drugs in the immunosuppressive field represents a very active area of research since current therapy has not sufficiently addressed the important issues of toxicity and efficacy.[1] We have sought to discover new classes of compounds that target the T-cell in order to provide therapeutic alternatives to current approaches. Recently, utilizing a T-cell screening program, we have discovered a new class of compounds that specifically inhibits the proliferation of human T-lymphocytes in vitro in the low nanomolar range. Here, we report the synthesis and preliminary in vitro data of ruthenium complexes that may provide a new therapeutic class for the treatment of autoimmune diseases and for the prevention of graft rejection in transplantation.

Complexes **1a–c** and **2a–b** were prepared according to Scheme 1. Reaction of $RuCl_3 \cdot xH_2O$ in boiling Im or 1-MeIm afforded $[Ru(Im)_6]Cl_2$ (**1a**)[2] and $[Ru(1\text{-}MeIm)_6]Cl_2$ (**1b**),[3] respectively, as the main products.[4] Oxidation of complexes **1a–b** with hydrogen peroxide in the presence of chloride afforded $[Ru(Im)_6]Cl_3$ (**2a**)



Scheme 1: (i) neat, reflux; (ii) H_2O_2, HCl (0.25 M); (iii) imidazole, MeOH, reflux.

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and $[Ru(1\text{-}MeIm)_6]Cl_3$ (**2b**). The triflate salt of complex **1a**, $[Ru(Im)_6](CF_3SO_3)_2$ (**1c**), was prepared by reaction of $[Ru(DMF)_6](CF_3SO_3)_3$[5] with excess imidazole in boiling methanol.

Immunosuppressant drugs such as CsA, FK506, and Rapa are known to inhibit the proliferation of human and animal T-lymphocytes in response to a variety of stimuli. The immunosuppressive activity of new compounds is typically determined by measuring the inhibition of proliferation of T-cells that have been exposed to antigens (e.g., tetanus toxoid).[6] Complexes **1a–c** and **2a–b** exhibit a typical dose–response curve for the inhibition of antigen-dependent lymphocyte proliferation[7] in response to TT as shown in Figure 1. Complexes **1a–c** and **2a–b** are potent inhibitors of human T-cell proliferation with IC_{50}'s in the low nanomolar range (**1a**, 5 ± 3; **1b**, 60 ± 30; **1c**, 4 ± 6; **2a**, 5 ± 3; **2b**, 80 ± 70 nM) and are comparable to other known potent immunosuppressants such as CsA (60 ± 40 nM) and Rapa (0.5 ± 0.7 nM).[8]

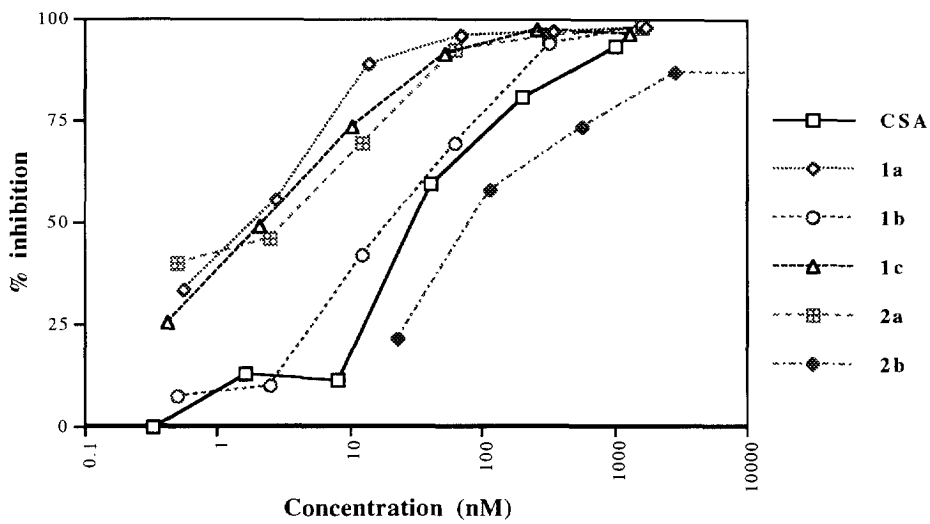

Figure 1. Inhibition curve of human T-lymphocyte proliferation after exposure to tetanus toxoid.

The data suggest that the activity of these complexes may be independent of the oxidation state of the metal center and the nature of the counterion. For example, the inhibitory activities of the Ru(II) and Ru(III) hexakis-imidazole complexes **1a** and **2a** (5 and 5 nM, respectively) and **2a** and **2b** (60 and 80 nM, respectively) are the same within experimental error. The solubility[9] of the complexes could be modulated by changing the counterion without deleterious effects on the activity. For example, the chloride salt **1a** and the triflate salt **1c** demonstrated nearly identical activity (5 and 4 nM, respectively) within experimental error.

In summary, we have identified a novel series of ruthenium complexes that are potent inhibitors of human T-cell proliferation. They are synthesized in a straightforward manner in good yield and are easily prepared in multigram quantities. Preliminary biological data suggest that these complexes are specific inhibitors of T-cells, though the mechanism of action is currently unknown.[10] Lack of drug sensitivity in several cell lines suggests that the compounds are not generally cytotoxic.[11] We are continuing our study of the synthesis and the activity of other homoleptic ruthenium complexes, as well as their mechanism of action. These complexes possess the potential to

serve as a new class of drugs for the treatment of autoimmune diseases and for prevention of allograft rejection in organ transplantation.

Experimental Section:

$[Ru(Im)_6]Cl_2$ (1a): A mixture of $RuCl_3 \cdot xH_2O$ (15.93 g, 76.9 mmol) and Im (89.29 g, 1.31 mol) was refluxed for 1 h. The mixture was cooled to room temperature, dissolved in warm water (55 °C, 1.5 L) and filtered over celite. The solvent was removed to dryness, the solid was redissolved in water (500 mL) and acetone (400 mL) added. The solution was filtered, the solid washed with acetone (2 x 50 mL) and then dried under vacuum. The solid was redissolved in a warm aqueous solution (55 °C) of imidazole (10 g in 850 mL H_2O), and then cooled slowly to 4 °C. The solid was filtered, washed with cold water (3 x 20 mL) and acetone (3 x 25 mL), and then dried under vacuum. The product was obtained as a greenish gray solid (13.952 g, 31% yield) after triple crystallization from a warm aqueous (55 °C) solution of imidazole. Anal. Calcd. (found) for $RuC_{18}H_{24}N_{12}Cl_2$: C, 37.25 (37.35); H, 4.17 (4.11); N, 28.96 (29.07); Cl, 12.22 (12.26); Ru, 17.41 (17.73). MS (FAB^+) *m/e* calcd. for $RuC_{18}H_{24}N_{12}$ (M^+) 508, found 508.

$[Ru(1\text{-}MeIm)_6]Cl_2 \cdot 3H_2O$ (1b): A mixture of $RuCl_3 \cdot xH_2O$ (10.043 g, 48 mmol) and 1-MeIm (70 mL, 0.88 mol) was refluxed for 1 h. The mixture was cooled to room temperature, and acetone (1 L) was added to the mixture. The mixture was filtered and the solid was dried under vacuum. The product was redissolved in MeOH (80 mL) and filtered over celite to remove a black impurity. The product was obtained as a light-yellow solid (19.58 g, 56.8% yield) after multiple crystallizations from MeOH/Ether. Anal. Calcd. (found) for $RuC_{24}H_{36}N_{12}Cl_2 \cdot 3H_2O$: C, 41.11 (41.31); H, 5.89 (5.71); N, 23.39 (23.91); Cl, 9.87 (10.04), Ru 14.06 (13.85), % H_2O 7.52 (7.82). MS (FAB^+) *m/e* calcd. for $RuC_{24}H_{36}N_{12} \cdot 2H_2O$ (M^+) 630, found 630.

$[Ru(Im)_6](CF_3SO_3)_2$ (1c): A mixture of $[Ru(DMF)_6](CF_3SO_3)_3$ (0.1880 g, 0.191 mmol) and Im (0.2763 g, 4.06 mmol) was dissolved in anhydrous methanol (10 mL). The mixture was degassed and then refluxed overnight. The mixture was cooled to room temperature, and the solvent removed to dryness. The product was obtained as a crystalline solid (0.121 g, 76% yield) after crystallization from MeOH/ether. Anal. calcd. (found) for $RuC_{20}H_{24}N_{12}F_6S_2O_6 \cdot 1/2MeOH$: C, 29.89 (30.22); H, 3.18 (2.97); N, 20.41 (20.42).

$[Ru(Im)_6]Cl_3 \cdot H_2O$ (2a): H_2O_2 (0.5 mL, 30%) was added to a solution of **1a** (0.206 g, 0.355 mmol) and Im (0.557g, 8.19 mmol) in HCl (0.25 M, 30 mL). The mixture was stirred at room temperature overnight, filtered and acetone (1 L) was added. The solution was centrifuged and the solid was dried under vacuum. The product was obtained as a crystalline solid (0.144 g, 64% yield) after crystallization from MeOH/ether. Anal. Calcd. (found) for $RuC_{18}H_{24}N_{12}Cl_3 \cdot H_2O$: C, 34.11 (34.08); H, 4.13 (4.26); N, 26.51 (26.41); Cl, 16.78 (16.81).

$[Ru(1\text{-}MeIm)_6]Cl_3 \cdot 2H_2O$ (2b) was prepared in a similar manner as **2a**. It was obtained as a crystalline solid (62% yield) after crystallization from H_2O/acetone. Anal. Calcd. (found) for $RuC_{24}H_{36}N_{12}Cl_3 \cdot 2H_2O$: C, 39.18 (38.90); H, 5.48 (5.57); N, 22.83 (22.71); Cl, 14.45 (14.51).

References

1. For example, see: (a) Kahan, B. D.; Chang, J. Y.; Sehgal, S. N. *Transplantation* **1991**, *52*, 185. (b) Suthanthiran, M.; Strom, T. B. *J. Clin. Immunol.* **1995**, *15*, 161.

2. Anderson, C.; Beauchamp, A. L. *Inorg. Chem.* **1995**, *34*, 6065. The X-ray structure of $[Ru(Im)_6]CO_3 \cdot 5H_2O$ was reported. It was obtained as an insoluble solid from the decomposition of $(ImH)_2[RuCl_5(Im)]$ in the presence of imidazole. No analytical data were reported.
3. Clarke, M. J.; Bailey, V. M.; Doan, P. E.; Hiller, C. D.; LaChance-Galang, K. J.; Daghlian, H.; Mandal, S.; Bastos, C. M.; Lang, D. *Inorg. Chem.* **1996**, *35*, 4896. The X-ray structure of complex **1b** was reported. No synthetic details or analytical data were reported.
4. Abbreviations used: Im, imidazole; 1-MeIm, 1-methylimidazole; DMF, dimethylformamide; py, pyridine; MeOH, methanol; CsA, cyclosporin A; Rapa, rapamycin; TT, tetanus toxoid, MLR, mixed lymphocyte reaction.
5. Judd, R. J.; Cao, R.; Biner, M.; Armbruster, T.; Burgi, H.; Merbach, A. E.; Ludi, A. *Inorg. Chem.* **1995**, *34*, 5080.
6. For example, see: (a) Chang, J. Y.; Sehgal, S. N.; Bansbach, C. C. *TIPS* **1991**, 218. (b) Mattila, P. S. *Biochem. Soc. Trans.* **1996**, *24*, 45. (c) Schreiber, S. L.; Albers, M. W.; Brown, E. J. *Acc. Chem. Res.* **1993**, *26*, 412. (d) Kay, J. E.; Benzie, C. R.; Goodier, M. R.; Wick, C. J.; Doe, S. E. A. *Immunology* **1989**, *67*, 473. (e) Crawford, D. J. K.; Maddocks, J. L.; Jones, D. N.; Szawlowski, P. *J. Med. Chem.* **1996**, *39*, 2690. (f) Gordaliza, M.; Faircloth, G. T.; Castro, M. A.; Miguel del Corral, J. M.; Lopez-Vazquez, M. L.; San Feliciano, A. *J. Med. Chem.* **1996**, *39*, 2865. (g) Batt, D. G.; Copeland, R. A.; Dowling, R. L.; Gardner, T. L.; Jones, E. A.; Orwat, M. J.; Pinto, D. J.; Pitts, W. J.; Magolda, R. L.; Jaffee, B. D. *Bioorg. Med. Chem. Lett.* **1995**, *14*, 1549. (h) Sehgal, S. N.; Molnar-Kimber, K.; Ocain, T. D.; Weichman, B. M. *Med. Res. Rev.* **1994**, 14, 1.
7. The experimental methods used for the determination of the inhibition of proliferation of T-lymphocytes driven by tetanus toxoid and the MLR assays were modified from the following: Coligan, J. E.; Kruisbeek, A. M.; Margulies, D. H.; Shevach, E. M.; Strober, W. *Current Protocols in Immunology*, Vol. 1, CURRENT PROTOCOLS: USA, 1994. **PBL Antigen Specific Proliferation Assay:** lymphocytes were prepared by first separating them from the blood samples of several donors by Ficoll gradient separation. The isolated lymphocytes were then grown in RPMI 1640 medium containing 5% human AB serum, glutamine (2 mM), penicillin/streptomycin, 100 U/mL/100 μg/mL sodium pyruvate (1 mM) and HEPES buffer (10 M). For assay purposes, PBL's were incubated at a sensity of 10^5 per 200 μL of medium per well of a 96-well plate. Tetanus toxoid (TT; Connaught Labs, Willow Dale, ON) was used as a stimulating antigen at a concentration of 5 LF/mL. The test wells containing PBL's, were exposed to tetanus toxoid antigen, along with various dilutions of the ruthenium complexes solutions. Subsequently, TT antigen/ruthenium complexes exposed PBL's were pulsed with 1 μCi/well of ^{3}H-thymidine on day 5 using a standard procedure known in the art. The cells were then harvested 16 h later onto a glass fiber filter using a TOMTEC cell harvester. Thymidine incorporation was measured by liquid scintillation counting using a Beta plate counter.
8. The IC_{50} is an average from the following number of assays (samples were run in triplicate each time): **1a**, 41; **1b**, 24; **1c**, 3; **2a**, 8; **2b**, 7; **4**, 2; CsA, 10; Rapa, 6. The immunosuppressive activity was also determined by using the MLR assay. Complexes **1a** and **1b** exhibited IC_{50}'s values in the low nanomolar range [8 ± 8 (15) and 80 ± 40 (10) nM, respectively] comparable to CsA [50 ± 30 (7) nM], where (n) is the number of experimental determinations (samples were run in triplicate each time). **MIXED LYMPOHCYTE REACTION:** blood samples were drawn from two donors and the lymphocytes were separated out by Ficoll gradient. The isolated lymphocytes from donor 1 were left untreated and the cells from donor 2 were suspended in Hanks Balanced Salt Solution at 10^7/mL and treated with 50 μg/mL mitomycin C for 30 min at 37 °C. The PBLs were cultured in RPMI 1640 medium containing 5% human AB serum, glutamine (2 mM), penicillin/streptomycin (50 μ/mL/50 μg/mL, sodium pyruvate (1 mM) and HEPES buffer (10 mM). For assay purposes, 10^5 PBLs from each donor were added to each well of a 96-well plate in a total volume of 200 μL along with various dilutions of test compound. The cultures were pulsed with 1 μCi ^{3}H-thymidine on day 5 and harvested 16 h later onto a glass fiber filter. Thymidine incorporation was measured by liquid scintillation counting using a Beta plate counter.
9. Complex **1a** is more soluble in water (1 mg/mL) than in ethanol (0.6 mg/mL), while complex **1c** is more soluble in ethanol (4 mg/mL) than in water (0.5 mg/mL).
10. A short communication describing preliminary biological activity for complex **1a** was recently published. See: Ocain, T. D.; Bastos, C. M.; Gordon, K. A.; Granstein, R. D.; Jenson, J. C.; McAuliffe, D. J.; Newcomb, J. R. *Transplantation Proceedings*, **1996**, *28*, 3032.
11. For example, the IC_{50} on the human cervical carcinoma cell line, HeLa, was > 1μM (data not shown).