Division of Chemistry[1], National Institute of Education, Nanyang Technological University, Singapore and Division of Medicinal Chemistry[2], School of Pharmacy, University of North Carolina, Chapel Hill, NC, USA

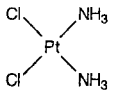
Cytotoxicity of rhenium(I) alkoxo and hydroxo carbonyl complexes in murine and human tumor cells

Y.-K. YAN[1], S. E. CHO[2], K. A. SHAFFER[2], J. E. ROWELL[2], B. J. BARNES[2] and I. H. HALL[2]

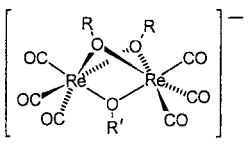
The rhenium(I) alkoxo/hydroxo carbonyl complexes were shown to be very potent in suspended tumor cell lines in suppressing growth but were more selective in inhibiting the growth of cultures from solid tumors. Their mode of action in L1210 lymphoid leukemia cells indicated that they were not alkylating agents but interfered with nucleic acid metabolism at multiple enzyme sites, e.g. dihydrofolate reductase, PRPP-amido transferase, thymidine kinase, with DNA strand scission after 60 min incubation. These compounds did not function mechanistically exclusively as cisplatin derivatives causing intrastrand linkages of DNA but rather they mimicked the metal complexes of aminecarboxyboranes, furan oximes, N-substituted thiosemicarbazones, trifluoromethyl borons and ferratricarbadecarbanyl complexes acting as antimetabolites.

1. Introduction

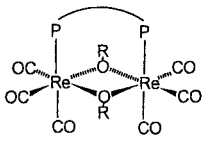
The success of the anti-tumor agent cisplatin [1] has stimulated the search for new inorganic and organometallic complexes having cytotoxic properties [2–4]. Cisplatin and its related cytotoxic platinum complexes are characterized by the presence of two *cis*-anionic ligands on the metal atom, and the lability of these *cis* ligands appears to be correlated with the cytotoxicity of the complexes [5]. The generally accepted mechanism of action of cisplatin involves the preferential binding of the platinum atom, after elimination of the chloride ligands, to the N(7) position of guanine residues in DNA, thereby forming inter- and intrastrand cross-links [5]. Many other anti-tumor agents based on the *cis*-dihalometal motif have been discovered, such as $[R_2SnX_2L_2]$, [R = alkyl, phenyl; X = halide; L = unidentate ligand (e.g. pyridine); L_2 = bidentate ligand (e.g. 2,2′-bipyridyl)] and $[Cp_2MCl_2]$, (Cp = η^5-cyclopentadienyl; M = Ti, V, Nb, Mo) [2]. In this paper, we report anti-tumor activities for a range of anionic and neutral rhenium(I) alkoxo/hydroxo carbonyl complexes $[Re_2(\mu\text{-}OR)_3/CO)_6]^-$ (R = H, **1**; R = Me, **2**; R = Et, **3**), $[Re_2(\mu\text{-}OH)(\mu\text{-}OPh)_2(CO)_6]^-$ **4**, $[Re_2(\mu\text{-}OPh)_3(CO)_6]^-$ **5**, $[Re_2(\mu\text{-}OR)_2(\mu\text{-dppf})(CO)_6]$ [dppf = 1,1′-bis(diphenylphosphino)ferrocene; R = H, **6**, R = Me, **7**; R = Ph, **8**] and *fac*-$[Re(OPh)(\eta^2\text{-dppf})(CO)_3]$ **9**. Complexes **1**–**8** contain hydroxo and alkoxo ligands in *cis*-positions [6–10]. The alkoxo/hydroxo ligands in **1** and **2** have been shown by electrospray mass spectrometry (ESMS) studies to be easily cleaved in the presence of protic compounds [11]. There is also ESMS evidence that complex **7** is hydrolyzed in wet acetonitrile to give $[Re_2(\mu\text{-}OH)_3(CO)_6]^-$ (**1**) as one of the major products [12]. Facile alkoxide-exchange reactions of the complexes *fac*-$[Re(OR)(L)_2(CO)_3]$ (L = phosphines or arsines, R = Me, Et, Pr^i), which are analogous to **9**, with alcohols and phenols have also been reported [13]. The present study involves the evaluation of these compounds to determine if they interact with DNA in a similar fashion as cisplatin (after displacement of the OH/OR ligands) or if they are similar to other metal complexes and interfere with nucleic acid metabolism functioning as antimetabolites [4].



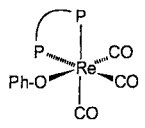
cisplatin



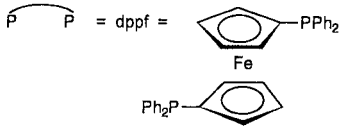
R = R′ = H (**1**), Me (**2**), Et (**3**), Ph (**5**); or R = Ph, R′ = H (**4**)



R = H (**6**), Me (**7**), Ph (**8**)



9



2. Investigations and results

2.1. Cytotoxicity

The rhenium alkoxo complexes demonstrated potent cytotoxicity in murine and human cultured cancer cells (Table 1). Compounds **2**–**8** were significantly active against the growth of murine L1210 lymphoid leukemia growth, i.e. $ED_{50} \leq 4$ mg/ml. All of the compounds were active against murine P388 lymphocytic leukemia growth. In human $Tmolt_3$ T cell leukemia compounds **1**–**5** and **9** were active and in human $Tmolt_4$ all of the compounds were active in suppressing growth. In human Hl-60 leukemia compounds **1**, **2**, **4**, and **6**–**9** inhibited cell growth. In the HuT-8 lymphoma screen all of the compounds demonstrated significant ED_{50} values. THP-1 acute monocytic leukemia growth was reduced by compounds **1**, **2**, and **5** to **9**. The suspended HeLa-S^3 uterine carcinoma growth was inhibited by all of the compounds with ED_{50} values of less than 2 μg/ml. The inhibition of the growth of cell cultures derived from solid human tumors was more selective and somewhat disappointing. Melanoma Sk-2 growth was reduced effectively by compounds **2**, **5**, **7** and **9**. MCF-7 breast effusion tumor growth was reduced signifi-

Table 1: Cytotoxicity of rhenium alkoxo complexes in murine and human cultured tumors (ED_{50} µg/ml, n = 4)

	IL1210 Leukemia	P388 Leukemia	$Tmolt_3$ Leukemia	$Tmolt_4$ Leukemia	HI-60 Leukemia	HuT-78 Lymphoma	THP-1 Acute monocytic	HeLa-S^3 uterine	KB Nasopharynx	Colon SW480	Lung A549	Lung-MB-9812	HeLa solid uterine
Compd.													
1	2.39	2.41	2.56	3.51	3.86	3.99	3.72	1.51	7.16	12.04	9.80	14.61	8.12
2	2.38	3.72	2.16	3.42	3.01	2.99	3.27	1.21	8.03	10.80	8.91	13.39	7.61
3	2.87	3.86	3.85	2.74	4.48	3.34	4.67	1.59	6.98	11.86	9.21	17.88	7.75
4	2.85	3.12	3.56	2.78	3.32	3.34	4.34	1.74	12.69	10.98	9.22	15.82	7.08
5	3.04	3.08	3.00	3.22	5.99	3.29	3.32	1.12	8.82	11.13	6.08	17.81	7.43
6	2.27	3.33	8.71	2.38	3.40	3.87	3.86	1.59	10.04	9.22	5.04	12.36	6.92
7	2.12	2.80	4.85	2.83	3.09	3.11	3.53	1.82	6.12	8.39	7.85	14.81	6.33
8	2.91	2.62	8.00	2.59	3.25	2.76	3.19	1.82	4.39	9.72	8.74	13.65	8.00
9	4.58	3.12	3.44	2.14	3.17	3.35	2.64	1.97	9.99	9.82	7.98	14.65	7.21
std													
6-MP	2.43	2.04	0.43	2.67	6.36	1.63	3.34	2.12	5.74	3.61	4.71	4.29	5.61
Ara-C	2.07	0.79	1.29	2.36	3.90	2.50	2.54	2.13	2.84	3.42	5.62	6.16	4.74
HU	2.67	1.30	4.47	6.68	5.22	3.87		1.96	5.27	7.33	8.89	7.18	8.12

Saso-2 bone	Melanoma	HCT-8 Ileum	MCF-7 Breast	Caki Kidney	A431 skin	Glioma U-87-MG
12.50	4.57	10.41	13.35	7.32	9.64	10.41
9.41	3.59	7.81	7.38	6.13	11.50	7.81
6.34	4.12	7.78	4.02	6.75	10.72	7.78
7.04	5.08	14.31	3.20	7.48	10.59	14.31
6.00	3.91	7.45	4.97	6.68	10.13	7.45
15.15	6.81	8.14	11.41	8.59	10.17	8.04
11.05	3.85	8.50	16.6	6.09	12.09	8.50
9.31	4.02	8.57	15.5	5.72	11.52	8.57
9.40	3.58	7.56	13.20	5.66	11.94	7.56
7.16	6.86	1.15	8.83	7.01	9.13	4.46
8.90	10.53	2.54	12.45	1.38	0.86	1.88
		1.77			2.87	2.27

$ED_{50} < 4$ µg/ml required for significant activity

cantly by compound **4**. The compounds demonstrated no activity against the growth of human KB nasopharynx, adenocarcinoma colon SW480 or ileum HCT-8, lung A-549, lung MB-9812, HeLa solid uterine carcinoma, Saos-2 osteosarcoma, clear cell renal Caki, A431 epidermoid skin, and glioma U-87-MG cells. Since these compounds possessed high molecular weights compared to the clinical drug standards conversion of the ED_{50} values to µM showed that they may be more promising as effective therapeutic agents.

2.2. Mode of action study

Compounds **1**, **4**, **7** and **8** were selected as been representative of this chemical class with good cytotoxic activity. The mode of action study in L1210 lymphoid leukemia

Table 2: Effects of compound 1 in L1210 leukemia cell metabolism over 60 min (n = 6)

Assay	Percent of control			
	Control	25 µM	50 µM	100 µM
DNA synthesis	100 ± 5^a	$23 + 3^*$	$21 + 2^*$	$18 + 3^*$
RNA synthesis	100 ± 6^b	$109 + 5$	$105 + 4$	$103 + 5$
Protein synthesis	100 ± 5^c	$44 + 4^*$	$39 + 4^*$	$31 + 3^*$
DNA polymerase α	100 ± 6^d	$203 + 8^*$	$99 + 5$	$91 + 6$
mRNA polymerase	100 ± 7^e	$85 + 6$	$77 + 5^*$	$66 + 5^*$
rRNA polymerase	100 ± 4^f	$122 + 6$	$97 + 5$	$95 + 6$
tRNA polymerase	100 ± 7^g	$121 + 5$	$118 + 4$	$109 + 5$
Ribonucleoside reductase	100 ± 5^h	$107 + 6$	$74 + 4^*$	$68 + 4^*$
Dihydrofolate reductase	100 ± 5^i	$115 + 6$	$83 + 5$	$57 + 3^*$
Purine synthesis	100 ± 5^j	$120 + 6$	$106 + 5$	$85 + 4$
PRPP amido transferase	100 ± 6^k	$122 + 5$	$110 + 4$	$99 + 5$
IMP dehydrogenase	100 ± 5^l	$117 + 6$	$110 + 5$	$97 + 6$
Pyrimidine synthesis	100 ± 6^m	$159 + 8^*$	$105 + 6$	$96 + 6$
Carbamyl phosphate synthetase	100 ± 7^n	$75 + 5^*$	$58 + 4^*$	$54 + 5^*$
Aspartate transcarbamylase	100 ± 6^o	$103 + 6$	$103 + 4$	$88 + 4$
Thymidylate synthetase	100 ± 5^p	$125 + 4$	$83 + 5$	$77 + 4^*$
Thymidine kinase	100 ± 6^q	$145 + 6^*$	$140 + 5^*$	$41 + 3^*$
Thymidine monophosphate kinase	100 ± 7^r	$120 + 5$	$96 + 6$	$50 + 4^*$
Thymidine diphosphate kinase	100 ± 6^s	$85 + 6$	$72 + 4^*$	$62 + 4^*$
d[ATP]	100 ± 5^t			$90 + 6$
d[GTP]	100 ± 6^u			$85 + 4$
d[CTP]	100 ± 5^v			$85 + 5$
d[TTP]	100 ± 4^w			$63 + 4$

* $P \leq 0.001$; control values based on 10^6 L1210 cells

[a] 26152 dpm
[b] 4851 dpm
[c] 7461 dpm
[d] 47804 dpm
[e] 4239 dpm
[f] 1502 dpm
[g] 6400 dpm
[h] 2744 dpm
[i] 0.868 OD units
[j] 92551 dpm
[k] 0.121 OD units
[l] 76058 dpm
[m] 19758 dpm
[n] 0.392 mol citrulline
[o] 1.064 mol N-carbamyl asparate
[p] 18463 dpm
[q] 1317 dpm
[r] 1179 dpm
[s] 1891 dpm
[t] 6.17 pmol
[u] 5.27 pmol
[v] 6.87 pmol
[w] 6.94 pmol

Table 3: Effects of compound 4 on L1210 leukemia cell metabolism over 60 min (n = 6)

Assay	Percent of control			
	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5^{a}	$87 + 5$	$67 + 4^{*}$	$57 + 4^{*}$
RNA synthesis	100 ± 6^{b}	$117 + 6$	$87 + 6$	$85 + 7$
Protein synthesis	100 ± 5^{c}	$77 + 5^{*}$	$57 + 4^{*}$	$44 + 3^{*}$
DNA polymerase α	100 ± 6^{d}	$119 + 7$	$88 + 5$	$47 + 4^{*}$
mRNA polymerase	100 ± 7^{e}	$74 + 5^{*}$	$56 + 4^{*}$	$56 + 5^{*}$
rRNA polymerase	100 ± 4^{f}	$97 + 5$	$90 + 5$	$88 + 4$
tRNA polymerase	100 ± 7^{g}	$91 + 6$	$80 + 4^{*}$	$77 + 4^{*}$
Ribonucleoside reductase	100 ± 5^{h}	$108 + 6$	$100 + 6^{*}$	$81 + 5^{*}$
Dihydrofolate reductase	100 ± 5^{i}	$114 + 5$	$74 + 4^{*}$	$61 + 4^{*}$
Purine synthesis	100 ± 5^{j}	$112 + 4$	$89 + 4$	$59 + 3^{*}$
PRPP amido transferase	100 ± 6^{k}	$116 + 6$	$109 + 5$	$57 + 4^{*}$
IMP dehydrogenase	100 ± 5^{l}	$90 + 6$	$71 + 4$	$60 + 4^{*}$
Pyrimidine synthesis	100 ± 6^{m}	$70 + 5^{*}$	$55 + 4^{*}$	$47 + 4^{*}$
Carbamyl phosphate synthetase	100 ± 7^{n}	$100 + 4$	$95 + 5$	$87 + 5$
Aspartate transcarbamylase	100 ± 6^{o}	$70 + 4$	$60 + 4^{*}$	$52 + 4^{*}$
Thymidylate synthetase	100 ± 5^{p}	$132 + 6^{*}$	$129 + 5^{*}$	$81 + 4^{*}$
Thymidine kinase	100 ± 6^{q}	$11 + 3^{*}$	$9 + 2^{*}$	$6 + 2^{*}$
Thymidine monophosphate kinase	100 ± 7^{r}	$51 + 3^{*}$	$29 + 3^{*}$	$23 + 2^{*}$
Thymidine diphosphate kinase	100 ± 6^{s}	$69 + 4^{*}$	$41 + 3^{*}$	$27 + 3^{*}$
d[ATP]	100 ± 5^{t}			$88 + 4$
d[GTP]	100 ± 6^{u}			$71 + 3^{*}$
d[CTP]	100 ± 5^{v}			$73 + 4^{*}$
d[TTP]	100 ± 4^{w}			$96 + 5$

cells (Tables 2–5) showed that compounds **1**, **4**, **7** and **8** preferentially inhibited DNA synthesis after 60 min in a concentration dependent manner. RNA synthesis was only reduced significantly with compound **7** by 22% at 100 μM. Protein synthesis was inhibited 56% to 75% by the four compounds after 60 min. Since the synthesis of DNA was inhibited by these compounds, a number of enzymes involved in nucleic acid metabolism were examined. DNA polymerase α activity was reduced 53% by compound **4** and 66% by compound **7**. m-RNA polymerase activity was inhibited 34% to 44% by the four compounds whereas r-RNA polymerase activity was significantly suppressed 19% to 21% by compounds **7** and **8** and t-RNA polymerase activity was reduced 23% by compound **4** and 19% by compound **7**. Ribonucleotide reductase activity was suppressed 19%, 20%, 32% and 82% by compounds **4**, **7**, **1** and **8**, respectively. Dihydrofolate reductase activity was suppressed 39%, 43%, 44% to 70% by compounds **4**, **1**, **7** and **8**, respectively, after 60 min at 100 μM. Purine de novo synthesis was reduced 41% by compounds **4** and **8**, and 39% by compound **7**. These same compounds reduced the activity of the rate limiting enzyme of the pathway PRPP-amido transferase by 32% to 43%. IMP dehydrogenase activity was inhibited 40% by compounds **4** and 25% by compound **7**. Pyrimidine de novo synthesis was blocked marginally by compounds **4**,

Table 4: Effects of compound 7 on L1210 leukemia cell metabolism over 60 min (n = 6)

Assay	Percent of control			
	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5^{a}	$33 + 4^{*}$	$32 + 3^{*}$	$32 + 3^{*}$
RNA synthesis	100 ± 6^{b}	$126 + 5$	$106 + 4$	$78 + 4^{*}$
Protein synthesis	100 ± 5^{c}	$42 + 3^{*}$	$27 + 3^{*}$	$25 + 4^{*}$
DNA polymerase α	100 ± 6^{d}	$80 + 4^{*}$	$45 + 4^{*}$	$34 + 4^{*}$
mRNA polymerase	100 ± 7^{e}	$84 + 5$	$72 + 5^{*}$	$63 + 4^{*}$
rRNA polymerase	100 ± 4^{f}	$105 + 5$	$95 + 5$	$79 + 4^{*}$
tRNA polymerase	100 ± 7^{g}	$98 + 6$	$95 + 5$	$81 + 5^{*}$
Ribonucleoside reductase	100 ± 5^{h}	$97 + 5$	$85 + 4$	$80 + 5^{*}$
Dihydrofolate reductase	100 ± 5^{i}	$80 + 4^{*}$	$64 + 4^{*}$	$56 + 3^{*}$
Purine synthesis	100 ± 5^{j}	$126 + 6^{*}$	$108 + 5$	$69 + 5$
PRPP amido transferase	100 ± 6^{k}	$104 + 5$	$84 + 5$	$68 + 4^{*}$
IMP dehydrogenase	100 ± 5^{l}	$86 + 5$	$80 + 4^{*}$	$75 + 4^{*}$
Pyrimidine synthesis	100 ± 6^{m}	$85 + 5$	$74 + 5^{*}$	$71 + 4^{*}$
Carbamyl phosphate synthetase	100 ± 7^{n}	$77 + 5^{*}$	$68 + 5^{*}$	$56 + 4^{*}$
Aspartate transcarbamylase	100 ± 6^{o}	$88 + 6$	$77 + 5^{*}$	$65 + 4^{*}$
Thymidylate synthetase	100 ± 5^{p}	$91 + 6$	$67 + 5^{*}$	$51 + 4^{*}$
Thymidine kinase	100 ± 6^{q}	$86 + 5$	$18 + 3^{*}$	$13 + 2^{*}$
Thymidine monophosphate kinase	100 ± 7^{r}	$77 + 5^{*}$	$58 + 5^{*}$	$58 + 4^{*}$
Thymidine diphosphate kinase	100 ± 6^{s}	$98 + 5$	$76 + 5^{*}$	$74 + 48$
d[ATP]	100 ± 5^{t}			$84 + 4$
d[GTP]	100 ± 6^{u}			$73 + 4^{*}$
d[CTP]	100 ± 5^{v}			$71 + 3^{*}$
d[TTP]	100 ± 4^{w}			$85 + 4$

Table 5: Effects of compound 8 on L1210 leukemia cell metabolism over 60 min

Assay	Percent of control			
	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5^a	$99 + 5$	$74 + 4^*$	$52 + 4^*$
RNA synthesis	100 ± 6^b	$343 + 9^*$	$284 + 6^*$	$201 + 5^*$
Protein synthesis	100 ± 5^c	$70 + 4^*$	$42 + 3^*$	$40 + 3^*$
DNA polymerase α	100 ± 6^d	$141 + 6^*$	$124 + 4$	$124 + 5$
mRNA polymerase	100 ± 7^e	$61 + 5^*$	$59 + 5^*$	$58 + 4^*$
rRNA polymerase	100 ± 4^f	$106 + 5$	$82 + 4$	$81 + 4^*$
tRNA polymerase	100 ± 7^g	$99 + 6$	$98 + 5$	$93 + 5$
Ribonucleoside reductase	100 ± 5^h	$115 + 6$	$57 + 4^*$	$18 + 3^*$
Dihydrofolate reductase	100 ± 5^i	$83 + 4$	$63 + 4^*$	$30 + 4^*$
Purine synthesis	100 ± 5^j	$96 + 6$	$73 + 5^*$	$59 + 4^*$
PRPP amido transferase	100 ± 6^k	$132 + 7^*$	$71 + 5^*$	$68 + 5$
IMP dehydrogenase	100 ± 5^l	$116 + 6$	$102 + 5$	$98 + 5$
Pyrimidine synthesis	100 ± 6^m	$120 + 5$	$80 + 5^*$	$79 + 5^*$
Carbamyl phosphate synthetase	100 ± 7^n	$80 + 6$	$70 + 5^*$	$66 + 5$
Aspartate transcarbamylase	100 ± 6^o	$101 + 5$	$94 + 6$	$68 + 5^*$
Thymidylate synthetase	100 ± 5^p	$88 + 6$	$77 + 5^*$	$61 + 4^*$
Thymidine kinase	100 ± 6^q	$156 + 6^*$	$85 + 5$	$84 + 4$
Thymidine monophosphate kinase	100 ± 7^r	$133 + 5^*$	$73 + 4^*$	$64 + 4^*$
Thymidine diphosphate kinase	100 ± 6^s	$79 + 5^*$	$68 + 4^*$	$62 + 4^*$
d[ATP]	100 ± 5^t			$95 + 4$
d[GTP]	100 ± 6^u			$92 + 5$
d[CTP]	100 ± 5^v			$70 + 4^*$
d[TTP]	100 ± 4^w			$76 + 4^*$

7 and **8** by 21%, 19% and 53% after 60 min. Carbamyl phosphate synthetase activity was inhibited 34% by compound **8**, 35% by compound **7** and 46% by compound **1**. Aspartate transcarbamylase activity was suppressed 32% by compound **8**, 35% by compound **7** and 48% by compound **4**. Thymidylate synthetase activity was blocked 23% by compound **1**, 29% by compound **4**, 39% by compound **8**, and 49% by compound **7**. Thymidine kinase activity was reduced 59% by compound **1**, 87% by compound **7** and 94% by compound **4**. TMP kinase activity was reduced 36% b compound **8**, 42% by compound **7**, 50% by compound **1**, and 77% by compound **4**. TDP kinase activity was inhibited 26% by compound **7**, 38% by compounds **1** and **8**, and 73% by compound **4**. d[GTP] pools were reduced 27% and 29% by compounds **7** and **4**. d[CTP] Pools were reduced 27% to 30% by compounds **4**, **7** and **8**. d[TTP] Pools were reduced 37% by compound **1** and 24% by compound **8** after 24 h incubation at 100 μM.

L1210 DNA strand scission was observed after 24 h incubation in the presence of the compounds at 100 μM (Fig.). ct-DNA viscosity studies showed that the control required 416.5 s to move through the reservoirs, compound **1** 398.6 s, compound **4** 391 s, compound **7** 393.7 s, and compound **8** 389.6 s. UV absorption from 220 to 340 nm of ct-DNA incubated for 24 h showed that the agents caused a slight hyperchromic shift which probably was not significant with regards to their mode of action. Tm values for ct-DNA denaturation were as follows: Tm for the control was 94.5 °C, for compound **1** 83 °C, for compound **8** 68 °C, for compound **4** 63 °C and for compound **3** 58 °C.

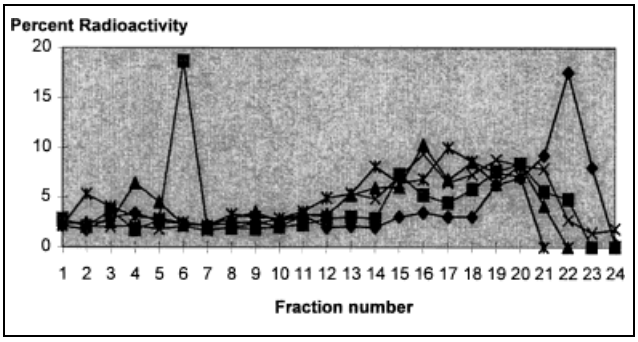


Fig.: L1210 DNA strand scission after 24 h at 100 μM
—◆— Control, —■— Drug 1, —▲— Drug 8, —×— Drug 7, —✳— Drug 4

3. Discussion

3.1. Cytotoxicity

The rhenium(I) alkoxo/hydroxo carbonyl complexes were very effective in reducing the growth of the suspended murine and human leukemias and lymphomas as well as the HeLa suspended uterine carcinoma cells. Their ED_{50} values for the inhibition of growth of these tumors were in the same range as those afforded by the standard clinical drugs. The ability of the agents to inhibit the growth of tumor derived from human solid cancers was not as impressive. Compounds **5**, **7** and **9** were effective in inhibiting SK-2 melanoma growth with improved ED_{50} values compared to the standard drugs' ED_{50} values. Only compound **4** was effective against the growth of MCK-7 breast effusion growth; this is important because the standard drugs are not effective against the tumor growth and new drugs are being sought to treat breast cancer in the clinic.

3.2. Mode of action

Whilst it would be premature to draw any definite conclusions on the molecular mechanism responsible for the activity of compounds **1**–**9**, some general observations can be made. The mode of action study in L1210 lymphoid leukemia cells indicates that the rhenium(I) alkoxo/hydroxo carbonyl complexes preferentially inhibit DNA synthesis and were not alkylating agents causing SN1 or SN2 attack of the nucleotide bases or acting like the cisplatin derivatives causing intrastrand linking between guanosine

bases. This does not rule out some other types of interaction with DNA bases since the Tm values were lower than the control values, DNA viscosity was reduced and DNA fragmentation was evident after incubation with the compounds. In accordance with our initial hypothesis that the compounds might attack DNA like cisplatin, the complexes **1**–**9** may bind to the nitrogenous bases of DNA after displacement of the alkoxide or hydroxide ligands. In this regard, it is significant that the hydroxide ligands of complex **1** are readily exchangeable with alcohols, phenol, thiols, primary amides, and in particular, adenine and thymine, as shown by ESMS studies [11, 15]. There is also ESMS evidence that complex **7** undergoes hydrolysis and phosphine elimination in wet acetonitrile to give $[Re_2(\mu\text{-}OH)_3(CO)_6]^-$ as one of the major products [12]. It is thus probable that complexes **6**–**8** are converted into $[Re_2(\mu\text{-}OH)_3(CO)_6]^-$ in the cells, prior to further transformation *via* ligand exchange. The eliminated dppf from complexes **6**–**8** probably also plays a part in the cytotoxic activity of the complexes, since dppf itself exhibits some anti-tumor activity [16]. It is noteworthy that complexes **6**–**8** do, on the whole, appear to be significantly more cytotoxic than **1**–**5**, on a molar basis, against most of the cell lines tested (the exceptions are $Tmolt_3$ leukemia, HeLa-S^3 uterine, Saos-2 bone and MCF breast). The $[Re^I(CO)_3]$ moiety most probably remains intact throughout; its high stability can be inferred from its widespread occurrence amongst Re(I) carbonyl complexes [17]. The resistance of the $[Re^I(CO)_3]$ moiety towards release of CO molecules is also established by ESMS studies [11].

It is also interesting to note that although complexes **1**–**5** are anionic and are thus expected to be repelled by the negatively-charged DNA, these complexes show strong cytotoxicity. Moreover, complexes **1** and **4** have been shown to disrupt DNA metabolism. This could mean that the complexes are converted into neutral or cationic species *via* ligand exchange reactions *in situ*, or that they act indirectly on DNA by inhibiting the enzymes involved in DNA synthesis.

3.3. Pharmacology

Examination of the effects of the rhenium(I) alkoxo/hydroxo carbonyl complexes on enzyme activities involved in DNA synthesis showed that the agents caused multiple effects and one single enzyme was not the target of the agents. Furthermore, depending on the functional moieties of the agent some enzyme activities were inhibited and others elevated. This type of phenomena has been observed with a number of metal complexes of iron, cobalt and copper [18–24]. It should also be pointed out that cisplatin derivatives inhibit the activities of multiple enzymes involved in nucleic acid synthesis in P388 leukemic cells, e.g. DNA polymerase α, thymidine kinase, ribonucleotide reductase, etc. [25, 26]. Dihydrofolate reductase activity was reduced significantly by all four compounds. Suppression of this enzyme would interfere with one carbon transfer significantly for purine, and moderately for pyrimidine, syntheses, Indeed purine and pyrimidine de novo syntheses were reduced within 60 min incubation of the drugs in this manner. In addition if the agent also inhibited the activity regulatory enzymes of the individual pathways then the affected pathway would be suppressed to a greater magnitude as observed with compounds **4**, **7**, and **8**. These derivatives reduced the activity of PRPP-amido transferase and/or IMP dehydrogenase activities, regulatory enzymes for the purine pathway and inhibited carbamyl phosphate synthetase and/or aspartate transcarbamylase activities, the regulatory enzymes for the pyrimidine pathway. Inhibition of these two de novo pathways would theoretically reduce nucleotides for both RNA and DNA syntheses. It should be noted that the ribonucleotide pools in mammalian tumors are 9 : 1 compared to the deoxyribonucleotide pools; therefore, the effects of an agent should appear first in DNA synthesis as opposed to RNA synthesis as observed after 60 min incubation of the rhenium(I) alkoxo/hydroxo carbonyl complexes. Interestingly, the d[NTP] pool levels were moderately reduced, also reflective of the suppression of these two de novo pathways by the agents.

On the other hand, compounds **4** and **7** also inhibited the activity of DNA polymerase α activity, which would cause an elevation of d[NTP] pools since the deoxyribonucleotides would not be incorporated into a new stand of DNA and would accumulate over time. Thus, the effects of these agents on purine and pyrimidine pathways appears to be more important than the inhibition of DNA polymerase α activity although this latter effect of the compounds would be additive in the overall inhibition of DNA synthesis. The ability of the compounds to inhibit nucleoside kineases over 60 min would also lead to reduced d[NTP] pool levels and reduced DNA synthesis, exclusively. The inhibition of thymidylate synthetase activity appears to be afforded only by the bis(diphenylphosphino)ferrocene derivatives, thus blocking the conversion of d[UMP] to d[TMP]. The derivative with a phenyl group in the R position, compound **8**, markedly inhibited ribonucleotide reductase activity which would lower the conversion of ribonucleotides to deoxyribonucleotides for incorporation into DNA. Consequently, individual derivatives are affording slightly different effects as well as slightly different magnitudes on the individual enzymes of nucleic acid metabolism; yet, the additive effects of the compounds would warrant the overall suppression of DNA synthesis after 60 min incubation.

In conclusion, this study demonstrates that rhenium(I) alkoxo/hydroxo carbonyl complexes display significant antitumor activity which appear to be as antimetabolites as opposed to being exclusively alkylating agents. We plan to broaden the range of this new class of anti-tumor organometallic compounds by suitable choice of other ligands analogous to alkoxides, such as thiolates and aminoalkoxides in order to improve the antitumor activity. In addition, the amino group of the latter can potentially be made quaternary to generate cationic complexes, which may interact more strongly with DNA molecules. We also plan to study the cytotoxicity of analogues of compounds **6**–**8** with different diphosphine ligands.

4. Experimental

4.1. Source of compounds and materials

The compounds $[[Et_4N][Re_2(m\text{-}OR)_3(CO)_6]$, (R = H, **1**; R = Me, **2**; R = Et, **3**); $[Et_4N][Re_2(m\text{-}OH)(m\text{-}OPh)_2(CO)_6$ **4**; $[Me_3NH][Re_2(m\text{-}OPh)_3(CO)_6]$ **5**; $[Re_2(m\text{-}OR)_2$ (m-dppf) $(CO)_6]$ (R = H, **6**, R = Me, **7**, R = Ph, **8**) and *fac*-$[Re(OPh)(\eta^2\text{-}dppf)(CO)_3]$ **9** were synthesized according to published methods [10, 14]. All radioisotopes were purchased from New England Nuclear (Boston, MA) unless otherwise indicated. Radioactivity was determined in Fisher Scintiverse scintillation fluid with correction for quenching. Substrates and cofactors were obtained from Signa Chemical Co. (St. Louis, MO).

4.2. Cytotoxicity

Compounds **1**–**9** (Table 1) were tested for cytotoxic activity by homogenizing the drugs as a 1 mg/ml solution in 0.05% Tween 80/H_2O. These solutions were sterilized by passing them through an acrodisc (0.45 μm).

The following cell lines were maintained by literature techniques [27]: murine L_{1210} lymphoid leukemia and P388 lymphocytic leukemia, human $Tmolt_3$ and $Tmolt_4$ T cell leukemia, Hl-60 leukemia, Hut-78 cutaneous lymphoma, THP-1 monocytic leukemia, SW480 colorectal adenocarcinoma, HCT-8 ileocecal adenocarcinoma, MB-9812 lung bronchogenic, A-549 lung carcinoma, Saos-2 osteosarcoma, KB epidermoid nasopharynx, HeLa-S^3 suspended and HeLa solid cervical carcinoma, Sk-MEL-2 malignant melanoma, skin epidermoid A431, clear cell renal Caki, breast Mck-7 effusion, and U-87-MG glioma. The NCI protocol was used to assess the cytotoxicity of the test compounds and standard drugs in each cell line [27]. The number of cells were determined by the trypan blue exclusion technique [27] and the percent inhibition of growth for each concentration of the compound was calculated and averaged (N = 4). The percent inhibition was plotted against the log of the concentration of compound and the ED_{50} value estimated. Solid tumor cytotoxicity was determined with crystal violet/MeOH and read at 580 nm (Molecular Devices) [28]. ED_{50} values for cytotoxicity were expressed as ED_{50} = μg/ml, i.e. the concentration of the compound inhibiting 50% of cell growth (Table 1). A value of less than 4 μg/ml was required for significant activity for inhibition of cell growth.

4.3. Incorporation studies

The effects of drug on the incorporation of radiolabeled precursors ^{3}H-thymine (72 Ci/mmol), ^{3}H-uracil (12 Ci/mmol) or ^{3}H-leucine (120 Ci/mmol) into DNA, RNA or protein, respectively, for 10^6 L1210 cell leukemia cells at 25, 50 and 100 μM was determined for 60 min [29]. The acid insoluble labeled DNA, RNA or protein was collected on discs [GF/A] which were counted in a Packard beta counter. The incorporation of ^{14}C-glycine (53.0 mCi/mmol) into purines was obtained by the method of Cadman et al. [30]. Incorporation of ^{14}C-formate (53.0 mCi/mmol) into pyrimidines was determined by the method of Christopherson et al. [31]. The final purines or pyrimidines were separated by TLC from starting components using the appropriate Rf for standard nucleoside bases and counted.

4.4. Enzyme assays

Since the compounds **1**, **4**, **7** and **8** effectively inhibited RNA and DNA syntheses in L1210 cells, their effects on a number of enzymes involved in nucleic acid metabolism were determined at 25, 50 and 100 μM after 60 min incubations. DNA polymerase α activity was determined in cytoplasmic extracts isolated by Eichler et al.'s method [32, 33]. The DNA polymerase α assay was described by Sawada et al. [34] with ^{3}H-2-deoxyribothymidine-5′-triphosphate [TTP] [53 Ci/mmol). Messenger-, ribosomal- and transfer-RNA polymerase enzymes were isolated from nuclei with different concentrations of ammonium sulfate; individual RNA polymerase activities were determined using ^{3}H-uridine-5′-triphosphate [UTP] (35 Ci/mmol) [35, 36]. The following enzyme activities were determined using L1210 homogenates. Ribonucleotide reductase activity was measured using ^{14}C-cytidine-5′-diphosphate [CDP] (19.4 Ci/mmol) with dithioerythritol [37]. ^{14}C-2′-Deoxyribocytidine-5′-diphosphate was separated from the ^{14}C-CDP by TLC on polyethyleneimine cellulose [PEI] plates. Thymidine, thymidine-5′-monophosphate [TMP] and thymidine-5′-diphosphate [TDP] kinase activities were determined using ^{3}H-thymidine (58.3 mCi/mmol) in the medium of Maley and Ochoa [38] and separated by TLC. Carbamyl phosphate synthetase activity was determined by the method of Kalman et al. [39] and the product citrulline was determined colorimetrically [40]. Aspartate transcarbamylase activity was measured using the incubation medium of Kalman et al. [39]; the product carbamyl aspartate was determined colorimetrically by the method of Koritz et al. [41]. Thymidylate synthetase activity was analyzed by Kampf et al.'s method [42]. The ^3H_2O separated by charcoal was proportional to the amount of TMP formed from ^{3}H-2′-deoxyribouridine-5′-monophosphate [UMP] (20 Ci/mmol).

Dihydrofolate reductase activity was determined by the NADH disappearance spectrophotometric method of Ho et al. [43] at 340 nm. Phosphoribosyl-pyrophosphate [PRPP]-amidotransferase activity was determined by Spassova et al.'s method as the generation of NADH [44] and inosine-5′-monophosphate [IMP] dehydrogenase activity was analyzed with 8-^{14}C-IMP (54 mCi/mmol) (Amersham, Arlingtom Heights, IL) after separating ^{14}C xanthosine-5′-monophosphate [XMP] on [PEI] plates (Fisher Scientific) by TLC [45] which was then counted. Protein content was determined for the enzymatic assays by the Lowry technique [46].

4.5. DNA studies

After deoxyribonucleoside triphosphates [d[NTP]] were extracted [47] from L1210 cells, d[NTP] levels were determined by the method of Hunting and Henderson [48] with calf thymus DNA, *E. coli* DNA polymerase I, non-limiting amounts of the three deoxyribonucleoside triphosphates not being assayed, and either 0.4 mCi of (^{3}H-methyl)-dTTP (53.1 Ci/mmol) or (5-^{3}H)-dATP (30 Ci/mmol). Thus, 2′-deoxyriboadenosine-5′-triphosphate [dATP], 2′-deoxyriboguanosine-5′-triphosphate [dGTP], 2′-deoxyribocytidine-5′-triphosphate [dCTP] and thymidine-5′-triphosphate [dTTP] levels were determined after incubation with the drugs for 60 min at 100 mM.

The effects of compounds **1**, **4**, **7** and **8** on DNA strand scission were determined by the methods of Suzuki et al. [49], Pera et al. [50] and Woynarowski et al. [51]. L1210 lymphoid leukemia cells were incubated with 10 μCi [methyl-^{3}H]-thymidine (84.0 Ci/mmol) for 24 h at 37 °C. $Tmolt_3$ cells (10^7) were harvested and then centrifuged at 600 × g for 10 min in phosphate buffered saline [PBS]. They were later washed and suspended in 1 ml of PBS. Lysis buffer (0.5 ml; 0.5 M NaOH, 0.02 M EDTA, 0.01% Triton X-100 and 2.5% sucrose) was layered onto a 5-20% alkaline-sucrose gradient (5 ml; 0.3 M NaOH, 0.7 KCl and 0.01 M EDTA); this was followed by 0.2 ml of the cell preparation. After the gradient was incubated for 2.5 h at room temperature, it was centrifuged at 12,000 × g for 17 h at 8 °C. Fractions (0.2 ml) were collected from the bottom of the gradient, neutralized with 0.2 ml of 0.3 N HCl, and measured for radioactivity.

Calf thymus DNA thermal denaturation studies were conducted from 37 to 100 °C in order to determine the Tm values for the control and drugs at 100 μM for 24 h. Changes in DNA UV absorption from 220–340 nm, and ct-DNA viscosity studies were conducted after incubation of compounds **1**, **4**, **7** and **9** at 100 μM at 37 °C for 24 h [52].

4.6. Statistics

The mean and standard deviation are designated by "X ± SD." The probable level of significance (p) between test and control samples was determined by the Student's "t" test using the raw data.

Acknowledgements: We thank the National Institute of Education, Singapore (RP 15/95 YYK) for financial support and Mr Chenghua Jiang for providing samples of compounds **1**–**9**.

References

1 Sherman, S. E.; Lippard, S. J.: Chem. Rev. **87**, 1153 (1987) and references therein
2 Köpf-Maier, P.; Köpf, H.: Chem. Rev. **87**, 1137 (1987)
3 Sadler, P. J.: Adv. Inorg. Chem. **36**, 1 (1991)
4 Wasczcak, M. D.; Hall, I. H.; Carroll, P. J.; Sneddon, L. G.: Angew. Chem. Int. Ed. Engl. **36**, 2228 (1997)
5 Pasini, A.; Zunino, F.: Angew. Chem. Int. Ed. Engl. **26**, 615 (1987)
6 Alberto, R.; Egli, A.; Abram, U.; Hegetschweiler, K.; Gramlich, V.; Schubiger, P. A.: J. Chem. Soc. Dalton Trans. 2815 (1994)
7 Herrmann, W. A.; Mihalios, D.; Öfele, K.; Kiprof, P.; Belmedjahed, F.: Chem. Ber. **125**, 1795 (1992)
8 Beringhelli, T.; Ciani, G.; D'Alfonso, G.; Sironi, A.; Freni, M.: J. Chem. Soc. Dalton Trans. 1507 (1985)
9 Yan, K.; Chan, H. S. O.; Hor, T. S. A.; Tan, L. A.; Liu, K. L.; Wen, Y.-S.: J. Chem. Soc. Dalton Trans. 423. (1992) and Jiang, C.; Wen, Y.-S.; Liu, L.-K.; Hor, T. S. A.; Yan, Y.-K.: Organometallics **17**, 173 (1998)
10 Jiang, C.; Henderson, W.; Hor, T. S. A.; McCaffrey, L. J.; Yan, Y.-K.: J. Chem. Soc., Chem. Commun 2029 (1998)
11 Jiang, C.; Henderson, W.; Hor, T. S. A.; McCaffrey, L. J.; Yan, Y.-K.; manuscript in preparation
12 Simpson, R. D.; Bergman, R. G.: Organometallics **12**, 781 (1993)
13 Jiang, C.; Wen, Y.-S.; Liu, L.-K.; Hor, T. S. A.; Yan, Y.-K.: J. Organomet. Chem. **543**, 179 (1997)
14 Yan, Y.-K.; Jiang, C.; Hor, T. S. A.; Henderson, W.: Proceedings of the First Singapore, 7–9, p. 66, (Dec. 1998)
15 Scarcia, V.; Furlani, A.; Longato, B.; Corain, B.; Pilloni, G.: Inorg. Chim. Acta **153**, 67 (1988)
16 Hill, D. T.; Girard, G. R.; McCabe, F. L.; Johnson, R. K.; Stupik, P. D.; Zhang, J. H.; Reiff, W. M.; Egleston, D. S.: Inorg. Chem. **28**, 3529 (1989)
17 Boag, N. M.; Kaesz, H. D.; in: Wilkinson, G.; Stone, F. G. A.; Abel, E. W. (eds.): Comprehensive Organometallic Chemistry, 1st ed., vol. 4, ch. 30, p. 161, Pergamon, Oxford 1982
18 Miller, III, M. C.; Sood, A.; Spielvogel, B. F.; Hall, I. H.: Res. Commun. Pharm. Toxicol. **1**, 245 (1996)
19 Miller, III, M. C.; Sood, A.; Spielvogel, B. F.; Hall, I. H.: Appl. Org. Met. Chem. **12**, 87 (1998)
20 Miller, III, M. C.; Bastow, K. F.; Stienman, C. N.; Vance, J. R.; Song, S. S.; West, D. X.; Hall, I. H.: Arch. Pharm. **331**, 121 (1998)
21 Hall, I. H.; Miller, III, M. C.; West, D. X.: Metal-Based Drugs **4**, 89 (1997)
22 Hall, I. H.; Lee, C. C.; Ibranhim, G.; Khan, M. A.; Bouet, G. M.; Appl. Organ Met. Chem. **11**, 565 (1997)
23 Hall, I. H.; Taylor, K.; Miller, III, M. C.; Dothan, X.; Khan, M. A.; Bouet, G. M.: Anticancer Res. **17**, 2411 (1997)
24 Hall, I. H.; Warren, A. E.; Lee, C. C.; Wasczxak, M. D.; Sneddon, L. G.: Anticancer Res. **18**, 951, (1998)
25 Oswald, C. B.; Chaney, S. G.; Hall, I. H.: J. Pharm. Sci. **79**, 875 (1990)
26 Hall, I. H.; Holshouser, M. E.; Loeffler, L. J.: J. Pharm. Sci. **69**, 1160 (1980)

27 Geran, R. J.; Greenburg, N. H.; MacDonald, M. M.; Schumacher, A. M.; Abbott, B. J.: Cancer Chemo. Rep. **3**, 9 (1972)
28 Leibovitz, A. L.; Stinson, J. C.; McComb, W. B. III; McCoy, C. E.; Mazur, K. C.; Mabry, N. D.: Cancer Res. **36**, 4562 (1976)
29 Liao, L. L.; Kupchan, S. M.; Horwitz, S. B.: Mol. Pharmacol. **12**, 167 (1976)
30 Cadman, E.; Heimer, R.; Benz, C.: J. Biol. Chem. **256**, 1695 (1981)
31 Christopherson, R. I.; Yu, M. L.; Jones, M. E.: Anal. Biochem. **11**, 240 (1981)
32 Eichler, D. C.; Fisher, P. A.; Korn, D.: J. Biol. Chem. **252**, 4011 (1977)
33 Mamaril, F. P.; Dobrjasky, A.; Green, S.: Cancer Res. **30**, 352 (1970)
34 Sawada, H.; Tatsumi, K.; Sadada, M.; Shirakawa, S.; Nakamura, T.; Wakisaka, G.: Cancer Res. **34**, 3341 (1974)
35 Anderson, K. M.; Mendelson, I. S.; Guzik, G.: Biochem. Biophys Acta **383**, 56 (1975)
36 Hall, I. H.; Carlson, G. L.; Abernathy, G. S.; Piantadosi, C.: J. Med. Chem. **17**, 1253 (1974)
37 Moore, E. C.; Hurlbert, R. B.: J. Biol. Chem. **241**, 4802 (1966)
38 Maley, F.; Ochoa, S.: J. Biol. Chem. **233**, 1538 (1958)
39 Kalman, S. M.; Duffield, P. H.; Brzozwki, T. J.: J. Biol. Chem. **241**, 1871 (1966)
40 Archibald, M.: J. Biol. Chem. **156**, 121, (1944)
41 Koritz, S. B.; Gohen, P. P.: J. Biol. Chem. **209**, 145 (1954)
42 Kampf, A.; Barfknecht, R. L.; Schaffer, P. J.; Osaki, S.; Mertes, M. P.; J. Med. Chem. **19**, 903 (1976)
43 Ho, Y. K.; Hakala, T.; Zakrzewski, S. F.: Cancer Res. **32**, 1023 (1971)
44 Spassova, M. K.; Russev, G. C.; Golovinsky, E. V.: Biochem. Pharmacol. **25**, 923 (1976)
45 Becker, J. H.; Lohr, G. W.: Klin. Wschr. **57**, 1109 (1979)
46 Lowry, O. H.; Rosebrough, J.; Farr, A. L.; Randall, R. J.: J. Biol. Chem. **193**, 265 (1951)
47 Bagnara, A. S.; Finch, L. R.: Anal. Biochem. **45**, 24 (1972)
48 Hunting, D.; Henderson, J. F.: Can. J. Biochem. **59**, 723 (1982)
49 Suzuki, H.; Nishimura, T.; Muto, S. K.; Tanaka, N.: J. Antibacteriol. **32**, 875 (1978)
50 Pera, J. F., Sr.; Rawlings, C. J.; Shackleton J.; Roberts, J.: Biochem. Biophys. Acta **655**, 152 (1981)
51 Woynarowski, J. W.; Beerman, T. A.; Konopa, J.: Biochem. Pharmacol. **30**, 3005 (1981)
52 Zhao, Y.; Hall, I. H.; Oswald, C. B.; Yokoi, T.; Lee, K. H.: Chem. Pharm. Bull. **35**, 2052 (1987)

Received July 7, 1999
Accepted August 16, 1999

Iris H. Hall Ph.D
Professor
Medicinal Chemistry and Natural Products
School of Pharmacy
CB# 7360
University of North Carolina
Chapel Hill, N.C. 27599-7360
iris_hall@unc.edu