Novel *N*-Hydroxyguanidine Derivatives as Anticancer and Antiviral Agents[1]

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Hydroxyguanidine contains both features of guanidine and hydroxyurea and has antiviral and anticancer activities both in vitro and in vivo. In order to enhance the antiviral and anticancer activity of this compound, a new series of hydroxyguanidine derivatives with the following structures were synthesized: R=NNHC(=NH)NHOH, where R = aromatic or heterocyclic aldehyde. This series of compounds was prepared in order to alter the lipophilic/hydrophilic balance, as well as the electronic and steric properties of hydroxyguanidine. The anticancer activities of the compounds were tested against cultured L1210 cells. The ID_{50} values of the above compounds are in the range of 7.80–126 μM. They are about 10-fold more active than hydroxyurea and hydroxyguanidine. The antiviral activities were also tested by assaying the inhibition of transformation of chicken embryo fibroblasts infected with Rous sarcoma virus. The ID_{50} values of these new compounds are in the range of 2.76–195.2 μM. The most active ones are about 100-fold more active than hydroxyguanidine. At the ID_{50}, no apparent toxicity to the cells was noticed.

Hydroxyguanidine, a compound with the combined functional groups of the anticancer agent hydroxyurea and the antiviral agent guanidine, has been reported to have anticancer activity, especially against solid tumors like Walker 256 carcinoma in rats.[2]

In vitro, hydroxyguanidine has an ID_{50} of 2 μg/mL against Moloney sarcoma virus.[3] Young has reported that hydroxyguanidine is equal to hydroxyurea in its ability to inhibit DNA synthesis in HeLa cells.[4] The biochemical target is generally believed to be ribonucleotide reductase, an enzyme needed for the reduction of ribonucleotides required for de novo synthesis[4] of DNA.

Brockman et al. have reported that 2-formylpyridine thiosemicarbazone increased the life span of mice bearing L1210 leukemia.[5] Later, French and Blanz synthesized 1-formylisoquinoline thiosemicarbazone and other α-(N)-heterocyclic-substituted carboxaldehyde thiosemicarbazones.[6] These compounds have shown anticancer activity against a wide spectrum of transplanted rodent neoplasms, including sarcoma 180, Ehrlich carcinoma, leukemia L1210, Lewis lung carcinoma, hepatoma 129, hepatoma 134, adenocarcinoma 755, and B16 melanoma.[6,7] The α-(N)-heterocyclic-substituted carboxaldehyde thiosemicarbazones have demonstrated potential as both antineoplastic and antiviral agents. These agents have been shown to inhibit DNA synthesis as a consequence of inhibiting ribonucleoside diphosphate reductase.[8] However, low water solubilities and high toxicities have limited practical therapeutic applications of these compounds.

We report here 12 new hydroxyguanidines, which have been designed to combine structural features of hydroxyguanidine and carboxaldehyde thiosemicarbazones.[9] The hydroxy group was expected to enhance water solubility, and the ring substituent groups were chosen to provide a range of electronic and lipophilic/hydrophilic balance.

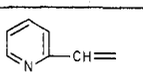
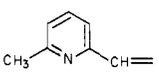
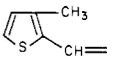
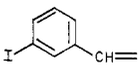
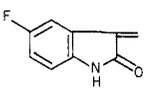
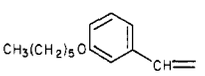
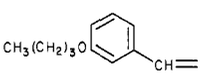
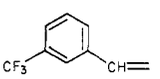
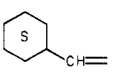
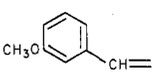
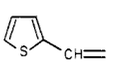
The in vitro activities of these compounds have been tested against both L1210 leukemic cultured cells and Rous sarcoma virus.

Results and Discussion

Table I summarizes the different effective doses of the substituted hydroxyguanidine derivatives in cultured L1210 leukemia cells after 48 h of incubation.

The ID_{50}s of the substituted 1-amino-3-hydroxyguanidine derivatives in the virus transformation and the L1210 leukemia studies were determined by using a computer plot employing Probit analysis.[10] Those outlier data points with deviations greater than two standard deviations (SD) in the preliminary calculations are not included in

Table I. Effective Doses of Substituted Hydroxyguanidine Derivatives in Cultured L1210 Leukemia Cells After 48 h of Incubation

	R=NNHC(=NH)NHOH[a]			
compd	R	ID_{16}, $\times 10^{-6}$ M	ID_{50}, $\times 10^{-6}$ M	ID_{84}, $\times 10^{-6}$ M
1		4.40	10.91	27.03
2		19.76	42.08	89.63
3		16.89	33.22	65.34
4		4.07	12.52	38.50
5		17.77	48.39	131.80
6	H_2	57.05	126.00	310.90
7		2.96	7.80	20.57
8		3.00	8.54	24.26
9		2.35	15.1	96.38
10		9.34	21.24	48.33
11		4.20	16.00	60.90
12		81.65	34.11	142.50
hydroxyguanidine sulfate		95.78	59.49	369.50
hydroxyurea		1.99	60.62	180.80

[a] The tosylates were used in the testing; see Table III for the details.

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Table II. Effective Doses of Substituted Hydroxyguanidine Derivatives on the Transformation of Chick Embryo Fibroblasts by RSV After 15 h of Incubation

compd	ID_{16}, $\times 10^{-6}$ M	ID_{50}, $\times 10^{-6}$ M	ID_{84}, $\times 10^{-6}$ M
1	1.01	2.76	7.55
2	36.01	114.10	361.30
3	5.89	18.46	57.86
4	0.90	3.45	13.33
5	5.36	18.27	62.25
6	15.63	195.20	2438.00
7	1.18	8.02	54.56
8	2.66	10.18	38.96
9	8.19	18.24	40.61
10	9.11	23.45	60.39
11	7.15	17.75	44.06
12	3.64	11.77	38.04
hydroxyguanidine sulfate	192.70	374.00	725.80

[a] ID_{16}, ID_{50}, and ID_{84}, which are determined by the same method described previously.[14] The ID_{50} values of these new compounds are in the range of 2.76 to 195.20 μM.

the final calculation. The correlation coefficients of all the data sets are equal to or greater than 0.85.

In L1210 leukemia cultured cell studies, the ID_{50} ranges from 7.80 to 126.0 μM (Table I). All the new derivatives, except compound **6**, are more active than the known compound hydroxyguanidine sulfate. The most active compound **7**, is about 10-fold more active than hydroxyurea and hydroxyguanidine sulfate against L1210 leukemia cultured cells. The most interesting characteristic of this series of compounds is that most of them show very steep dose–response curves. The ID_{50} of compound **7** is 7.80 μM, while the ID_{84} and ID_{16} are 20.57 and 2.96 μM, respectively. The ID_{50} of compound **1** is 10.91 μM, and the ID_{84} and ID_{16} are 27.03 and 4.40 μM, respectively.

Table II shows the effective doses of substituted hydroxyguanidine derivatives on the transformation of chicken embryo fibroblasts by RSV. There is no apparent cytotoxicity at the 50% inhibition level. The most active compounds are compounds **1** and **4**, while the least active compounds are **2**, **6** and **10**. It has been previously shown that in the thiosemicarbazone series, the substituents at the 6-position of the pyridine or the 3-position of the isoquinoline ring reduced or abolished the inhibitory activity for the enzyme RR.[9] This may also be true of hydroxyguanidine derivatives. Since viral transformation requires DNA synthesis, the viral RR production could be induced by RSV in vivo, which in turn could be inhibited by this new series of hydroxyguanidine derivatives. It is interesting that the effective dose range of most of these compounds studied are also very narrow. The ID_{50} of **1** is 2.76 μM, the ID_{84} is 7.55 μM, and the ID_{16} is 1.01 μM, with a range of less than 8 from ID_{16} to ID_{84}.

Among this series, compound **4**, with the highest lipophilicity and molecular weight, shows relatively high activity, which indicates that the bulk and the lipophilicity may play an important role in the absorption of the molecule across the membrane to reach the target site and also in contributing to the affinity for the hydrophobic region of the enzyme. On the other hand, compound **1** with a lower molecular weight and more hydrophilicity possesses the highest activities among the series, which indicates that other factors might be involved. In vitro, compound **1** has high activity as an anticancer and antiviral agent and it is relatively nontoxic in mice (LD_{50} = 315 mg/kg), while compound **4** has a LD_{50} of 190 mg/kg. Ribonucleotide reductase inhibition studies indicated that compounds **1** and **4** are 20–30 times more active than hydroxyguanidine.[11] Due to these properties, compound **1** may be a good chemotherapeutic agent; however, further studies are needed to confirm its antiviral and anticancer activities in vivo and to fully delineate the quantitative structure–activity relationships of these compounds.

Experimental Section

Synthesis and Physicochemical Properties of Substituted 1-Amino-3-hydroxyguanidine Derivatives. A total of 12 compounds were synthesized for the first time. It was carried out by preparing *S*-methylisothiosemicarbazide tosylate as an intermediate. Hydroxylamine was prepared by reacting hydroxylamine hydrochloride with potassium hydroxide in methanol. The desired 1-amino-3-hydroxyguanidine was formed by reaction of *S*-methylisothiosemicarbazide tosylate with hydroxylamine for 48 h at room temperature.

All these compounds were prepared with quite good yields (44–89%). The purity of these compounds were checked by TLC. The melting points of these compounds are within the 1–2 °C range. These values are shown in Table III. The percent of carbon, hydrogen, and nitrogen are all within 0.4% of theoretical values. Compounds **7–9** contain water of crystallization, resulting in lower melting points than the others.

Preparation of 1-Amino-3-hydroxyguanidine Tosylate as a Synthetic Intermediate. 1-Amino-3-hydroxyguanidine tosylate was prepared by a slight modification of the procedure of Houlihan et al.[12] A mixture of 0.5 mol of thiosemicarbazide and 0.5 mol of methyl *p*-toluenesulfonate in 500 mL of methanol was refluxed with stirring for 18 h. The reaction mixture was then evaporated to about 250 mL in vacuo, and 500 mL of ether was added. The remaining white precipitate was filtered and washed with ether. The resultant *S*-methylisothiosemicarbazide tosylate weighed 135.00 g with a melting point of 144–146 °C.

A cold solution of potassium hydroxide (0.5 mol) in 250 mL of methanol was added to a cold hydroxyamine hydrochloride solution (0.5 mol). The mixture was stirred for 1 h in a salt–ice bath. The potassium chloride precipitate was removed by filtration.

S-Methylisothiosemicarbazide tosylate (0.4 mol) was added to the filtrate. The reaction mixture was stirred at room temperature for 48 h, after which the mixture was evaporated to dryness in vacuo at 40 °C. The residue was dissolved in 800 mL of hot ethanol and cooled to room temperature. A small amount of insoluble material was filtered off by suction. The filtrate was concentrated in vacuo to about 200 mL. Ether (400 mL) was added and mixed thoroughly. The precipitate was filtered and washed with a mixture of ether and ethanol (2:1). The crystallized white 1-amino-3-hydroxyguanidine tosylate weighed 50 g. Its melting point was 136–137 °C, and it decomposed at 150–155 °C.

Preparation of Substituted 1-Amino-3-hydroxyguanidine Tosylate Derivatives. The final substituted 1-amino-3-hydroxyguanidine tosylate derivatives were prepared via Schiff's base formation by using 1-amino-3-hydroxyguanidine tosylate as the intermediate and reacting it with different aldehydes or ketones.[13]

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Table III. Summary of the Synthesis of Substituted 1-Amino-3-hydroxyguanidine Derivatives

no.	compound	recrystn solvent	mp, °C	yield, %
1	1-[(2-pyridylmethylene)amino]-3-hydroxyguanidine tosylate[a]	ethanol/ether (1:1)	167-169	46.11
2	1-[[(6-methyl-2-pyridyl)methylene]amino]-3-hydroxyguanidine tosylate[a]	ethanol/ether (1:1)	148-149	56.95
3	1-[[(3-methyl-2-thienyl)methylene]amino]-3-hydroxyguanidine tosylate[a]	ethanol/ether (1:1)	162-163	85.1
4	1-[(3-iodobenzylidene)amino]-3-hydroxyguanidine tosylate[a]	ethanol/ether/pet. ether (1:1:2)	193-194	88.96
5	1-[(5-fluoro-2-oxo-3-indolidene)amino]-3-hydroxyguanidine tosylate[b]	ethanol/ethyl acetate/pet. ether (1:1:2)	250-251	41.52
6	1-amino-3-hydroxyguanidine tosylate	ethanol/ether (2:1)	136-137	44.2
7	1-[[3-(hexyloxy)benzylidene]amino]-3-hydroxyguanidine tosylate	ethanol/water (1:1)	68-70	72.48
8	1-[(3-butoxybenzylidene)amino]-3-hydroxyguanidine tosylate hemihydrate[c]	ethanol/water (1:1)	78-80	83.75
9	1-(α',α',α'-trifluoro-3-toluidene)amino]-3-hydroxyguanidine tosylate hemihydrate[c]	ethanol/ether (1:1)	191-192	76.81
10	1-[(cyclohexylmethylene)amino]-3-hydroxyguanidine tosylate hemihydrate[b]	2-propanol/pet. ether (2:1)	112-114	69.3
11	1-[(3-methoxybenzylidene)amino]-3-hydroxyguanidine tosylate[b]	ethanol/water (1:2)	164-165	69.1
12	1-[(2-thienylmethylene)amino]-3-hydroxyguanidine tosylate[b,d]	ethanol/water (1:1)	153-155	56.11

[a] Analyzed at the California Institute of Technology, Pasadena, CA. [b] Analyzed at the Chun Shan Institute of Science & Technology, Taiwan, Republic of China. [c] Analyzed by the C. F. Geiger Co. [d] Anal. ($C_{13}H_{16}N_4O_4S_2$) C, H; N: calcd, 15.72; found, 15.17. [e] Analyses for all compounds were within ±0.4% of theoretical values, except for the N of compound 12.

L1210 Leukemia Cell Culture Growth Inhibition.[14] **Growth Medium.** Roswell Park Memorial Institute (RPMI) medium supplemented with necessary amino acids, vitamins, 10% fetal calf serum, and penicillin–streptomycin (1% each) was used.

Cell Culture Method. Cell cultures were seeded at 4.0–6.0 × 10^4 cells/mL in duplicates for each drug concentration in a plastic microtiter plate. The substituted derivatives were added to the cell cultures in 1:5 dilutions to get the desired drug concentration. Each compound was tested with at least six different concentrations. After 24–48 h of incubation in a humidified incubator supplied with 95% air and 5% carbon dioxide, the cells were harvested and counted with a Coulter counter. Each 0.1 mL of test culture fluid was suspended in a plastic vial containing 9.9 mL of isoton before the counting. An untreated set and Me_2SO-treated cultures were used as controls for each separate experiment.

Antiviral Testing via Focus Assay.[15] **Growth Medium.** Growth medium for cells was composed of 400 mL of 1X Hams F10 media, 40 mL of 10% Difco tryptose phosphate broth, 20 mL of bovine serum, 4 mL of heat-inactivated chicken serum, and 10 mL of 1% sodium bicarbonate.

Agar Medium. Agar medium for assays was composed of 100 mL of 2X Hams F10 media, 25 mL of Difco tryptose phosphate broth, 12.5 mL of bovine serum, 2.5 mL of heat-inactivated chicken serum, 5 mL of 1% sodium bicarbonate, and 100 mL of 1.8% Difco Bacto-Agar. The agar medium was prepared by bioling the agar until it melted (about 45 min) and then cooling it to 45 °C. The remaining media components were also warmed to 45 °C and mixed with the agar when it had cooled sufficiently.

Antiviral Assay. The antiviral assay was carried out by inhibition of viral focus formation. The Schmidt–Ruppin strain of Rous sarcoma virus (RSV) of subgroup D (SR-D) was used throughout the experiments. Virus stock solutions containing 1 × 10^6 virus particles per milliliter were stored at −80 °C. These assays were carried out in 60-mm plastic tissue culture petri dishes. A 1:20 dilution of the virus stocks was made prior to infection.

The test compounds were dissolved in dimethyl sulfoxide and diluted in growth medium to give less than 1% dimethyl sulfoxide. Each compound was prepared in 5 mL from 10^{-2} to 10^{-5} M concentrations by serial dilutions.

A monolayer of cells (5 × 10^5 cells/plate) was obtained by inoculating secondary chicken fibroblasts in 5.0 mL of growth medium per dish and incubating the culture at 37 °C. Medium on confluent cell sheets was taken off by suction, a series of cultures was exposed to 0.2-mL aliquots of each test compound, and 1.7 mL of medium was added to each culture dish. Each culture was then challenged with a 0.1-mL aliquot of virus (50 000 virus particles/mL), which contains approximately 150–200 focus-forming units of virus. Two dishes containing virus without exposure to drug were used as a control. A series of cultures that were exposed to 0.2-mL aliquots of each test compound and 1.8 mL of medium were used as a toxicity control.

The virus inoculum was evenly added to the cells by tilting and rotating the petri dish and then incubating it at 37 °C for 15 h. Medium was taken off again, and 5.0 mL of an agar overlay containing the same serial dilutions of each compound with 0.9% agar medium was added to each culture and allowed to solidify at room temperature.

The cultures were incubated for 3 days at 37 °C and then fed by adding an additional 5.0 mL of agar medium on top of the first agar layer. The assays were scored 7–10 days after infection.

In Vivo Toxicity Studies. C57 Bl/6J mice were used for this study. Six mice were used for each drug concentration. Compounds 1 and 4 were prepared in acacia suspension. Equal amounts of drug and acacia were weighed out, ground, mixed well, and then suspended in a volume of saline to give the desired concentration. The mouse was weighed, and the corresponding dose of drug was injected intraperitoneally. The toxic effects of these compounds and the lethality were then observed for 48 h.

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Registry No. 1, 85894-16-2; 2, 87861-77-6; 3, 85894-18-4; 4, 85894-20-8; 5, 87861-79-8; 6, 36826-58-1; 7, 87861-81-2; 8, 87861-83-4; 9, 87861-85-6; 10, 87861-87-8; 11, 87861-89-0; 12, 87861-91-4; thiosemicarbazide, 79-19-6; methyl tosylate, 80-48-8; *S*-methyl isothiosemicarbazide, 36778-68-4; hydroxylamine, 7803-49-8.

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