Design, Synthesis, Testing, and Quantitative Structure-Activity Relationship Analysis of Substituted Salicylaldehyde Schiff Bases of 1-Amino-3-hydroxyguanidine Tosylate as New Antiviral Agents against Coronavirus

Pou-Hsiung Wang,[†] James G. Keck,[‡] Eric J. Lien,*[,†] and Michael M. C. Lai[‡]

Section of Biomedicinal Chemistry, School of Pharmacy, and Department of Microbiology, School of Medicine, University of Southern California, Los Angeles, California 90033. *Received March 14, 1989*

Further modifications of the structural features of Schiff bases of hydroxyaminoguanidines (SB-HAG) led to nine substituted salicylaldehyde Schiff bases of HAG (SSB-HAG) derivatives and three other SB-HAG derivatives. These new compounds were tested for the first time against infection by a coronavirus, mouse hepatitis virus (MHV). The most active compound, **2** [1-[(3'-allyl-2'-hydroxybenzylidene)amino]-3-hydroxyguanidine], against the growth of MHV is about 376 times more active than hydroxyguanidine and about 564 times more active than HAG itself when the $TCID_{50}$ values are compared. Plaque assays of MHV released from cells treated with these compounds suggest that SSB-HAG tosylate may inhibit the transcription of viral RNAs in virus-infected cells. Quantitative structure-activity relationship (QSAR) analyses of two subsets show that the inhibitory activities correlate well with the electronic and the lipophilic parameters. The structural requirements for the antiviral activity of substituted SSB-HAG tosylate against coronaviral infection are stringent according to the inhibitory activities and QSAR analysis of these new compounds.

Hydroxyurea, hydroxyguanidine, and thiosemicarbazone derivatives have been reported to have antiviral as well as antitumor activities.[1-5] Several guanidine derivatives have been shown to be active against viral infections.[6,7] Substituted Schiff bases of hydroxyaminoguanidine (SB-HAG) contain the major functional group hydroxyguanidine; they also combine the structural features of hydroxyurea and thiosemicarbazone. A series of SB-HAG derivatives have previously been synthesized, tested, and shown to have antiviral and antitumor activities in our laboratories.[8,9]

Moore et al.[10,11] suggested that 1-formylisoquinoline thiosemicarbazone (IQ-1) and 5-hydroxypyridine thiosemicarbazone (5-HP) interact with ribonucleotide reductase (RR) by coordination of the iron of the non-heme iron subunit of RR. Cory's group reported that many SB-HAG derivatives are potent inhibitors of RR.[12,13] They have shown that the potency of hydroxyurea for inhibition of RR in the presence of the iron-chelating agents was synergistic.[14] They also reported that the terminal side of isoquinoline-SB-HAG may form a chelate with metal ions in the same way as hydroxyurea does.[15] Lien et al.[16] reported that the antitumor activity significantly correlated with the RR inhibition, but no correlation between RR inhibition and antiviral activity was found according to quantitative structure-activity relationship (QSAR) analysis of SB-HAG derivatives against the growth of L1210 cells and transformation of chicken fibroblasts by Rous Sarcoma virus (RSV) in vitro. Therefore, a different mechanism may be involved in antiviral action. It has been shown that the reverse transcriptase associated with RNA tumor viruses is required for the initiation of neoplastic transformation.[17] Zinc was found to be an intrinsic component of reverse transcriptase[18] and thus may be essential for the enzymatic activity. These SB-HAG compounds possess the same linkage [—CH=NNHC(=NH)NHOH] as guanoxabenz, which has been demonstrated to form a metal chelate complex with metal ions.[19] Both RR and reverse transcriptase require divalent metal ions for their enzyme activity. All of these data point to SB-HAG derivatives as potential compounds which may inhibit virus-induced or associated enzymes which require a metal ion as a cofactor for enzyme activities.

Coronaviruses are important pathogens in respiratory and gastrointestinal illnesses in humans and many other species of animals. Human coronaviruses are associated with common colds and lower respiratory tract and enteric diseases.[20-22] Coronavirus infection in avian and porcine

* To whom correspondence should be addressed.
[†] Section of Biomedicinal Chemistry.
[‡] Department of Microbiology.

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species can cause severe economic problems. The synthesis of viral RNAs in coronavirus-infected cells requires RNA-dependent RNA transcription, which involves a sequential process of the synthesis of (-)-RNA, the synthesis of subgenomic mRNAs, and the replication of genomic RNA.[23] The syntheses of viral RNAs in virus-infected cells requires virus-induced RNA-dependent RNA polymerases. Neither coronavirus virion particles nor unifected cells have detectable RNA-dependent RNA polymerases. Thus, this enzyme must be synthesized by the virus in infected cells. The enzyme requires an Mg^{2+} ion as cofactor for enzyme activity.[24,25] Currently, there are no effective drugs available for the treatment of coronavirus infection.

From the pharmacological point of view, a target compound should not only be active at the cell-free level but also should be able to reach the target site within the infected cells. Of the more than 20 SB-HAG derivatives previously studied in our laboratories,[8,9] 1-[(3',5'-dibromo-2'-hydroxybenzylidene)amino]-3-hydroxyguanidine is the only one that possesses an ortho OH group and has in vivo antitumor activity against P388, in vitro antitumor activity against L1210, and antiviral activity against RSV.[26] Therefore, a new series of substituted salicylaldehyde Schiff bases of HAG (SSB-HAG) tosylate were selected as new agents in the present study. In order to have a better understanding of the correlation between the biological activities and the physicochemical properties of these compounds, the molecular modifications cover a wide range of properties such as the lipophilicity, the size of the ring, the presence or absence of an ortho OH group, the presence of an electron-donating group or electron-withdrawing groups on the ring, the presence of a heterocyclic ring or aromatic ring, and the orientation of substituent on the ring. QSAR analysis based on the $TCID_{50}$ and selected physicochemical properties was performed by the method of least squares.

Results and Discussion

Chemistry. The substituted salicylaldehyde Schiff bases and other Schiff bases of 1-amino-3-hydroxyguanidine tosylate were prepared by reacting 1-amino-3-hydroxyguanidine tosylate with different aromatic or heterocyclic aldehydes to form the corresponding Schiff bases. A Dean–Stark distillation receiver was used to remove the water formed and to improve the yields of compounds with multiple hydroxy groups on the aromatic ring. The percent yields, molecular formulas, and melting points of SSB-HAG and SB-HAG derivatives are summarized in Table I.

Biological Study. The $TCID_{50}$s for the inhibition of virus production from mouse hepatitis virus (MHV) infected mouse astrocytoma (DBT) cells in tissue cultures were determined by plaque assay and are shown in Table II. Most compounds are more active than either hydroxyguanidine ($TCID_{50} = 1.97 \times 10^{-4}$ M) or HAG ($TCID_{50} = 2.57 \times 10^{-4}$ M). In this series, compounds **1**, **2**, **3**, **7**, and **13** are the most active, with $TCID_{50}$ values in the micromolar range. At higher concentrations compounds **1** (1.45×10^{-5} M), **2** (9.70×10^{-5} and 5.30×10^{-5} M), **7** (9.70×10^{-5} M), **10** (7.75×10^{-4} M), and **13** (9.70×10^{-5} M) reduced the number of virus particles produced by 1000-fold or 10000-fold at the respective concentrations indicated. At these concentrations, these compounds did not cause visible cytotoxicity. However, at even higher concentrations, for instance, 2.9×10^{-4} M or higher for compounds **2** and **7**, both infected cells and uninfected cells became visibly sick and were eventually killed. MHV infection causes a cytopathic effect (CPE), i.e., fusion of the infected cells which eventually resulted in cell death.[23] The MHV-induced cell fusion is caused by a virus-specific envelope protein, E2, which is found on the surface of the infected cells.[28] The fusion-inducing ability of the E2 is activated by cleavage of E2 by a cellular protease.[23,27] The data presented in this report showed that CPEs observed in the cultures are positively correlated with the number of virus particles produced from the infected cells, namely, the higher the percent of CPE, the more the release of mature virus particles. The compounds which more drastically reduced the number of virus particles also reduced virus-induced CPE. These results suggest that new substituted SSB-HAG tosylate may block the replication of virus at a step prior to the synthesis of viral proteins. Preliminary data showed that these compounds inhibited viral RNA synthesis.[29]

QSAR. The correlation between the inhibitory activities on MHV replication and the physicochemical parameters of 15 compounds was analyzed. The hydrophobic parameters examined were the following: Rm (a chromatographic parameter related to partition coefficient), substituent hydrophobic constant[30] π_R, and $\sum\pi_{3+5}$. The electronic parameters used were $\sum\sigma_m$ (the summation of Hammett substituent constants,[30] its sign is positive for electron-withdrawing group), μ_R (R group dipole moment[31]), and $\sum\vec{\mu}_{3+5}$ (the vector summation of group dipole moments at meta positions, by definition its sign is negative for electron-withdrawing group). The group dipole moments are measures of the separation of charge. The steric parameters used were log MR (molar refraction highly correlated with molar volume[30]), $\sum E_S^C$ (the summation of corrected Taft's constant[30]), $\sum Vef$ (the summation of the υ steric constant[33]), and Vw (the van der Waals volume[34]).

The physicochemical parameters and antiviral activities are summarized in Tables II and III. Stepwise F test was used to evaluate the statistical significance of each equation. The sequential F test was used to compare the statistical significance of adding a parameter to the subsequent equation. The group dipole moments (μ_R) of compounds **3** and **5** are not available for the literature, and therefore, are not included in the regression analysis for eq 2 and 3. The best equations correlating the biological activity with the physicochemical parameters are sum-

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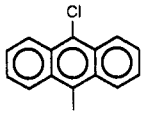
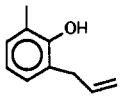
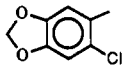
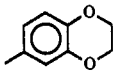
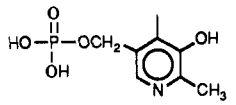
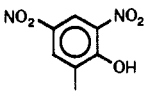
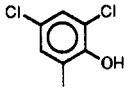
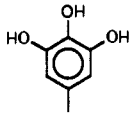
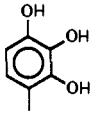
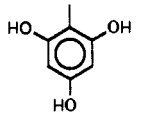
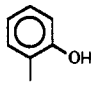
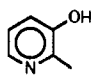
Table I. The Percent Yield and Melting Points of the Target Compounds Synthesized and Tested

$RCH{=}NNHC(=NH)NHOH{\cdot}CH_3C_6H_4SO_3H$

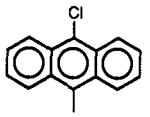
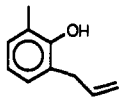
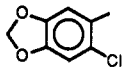
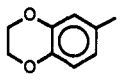
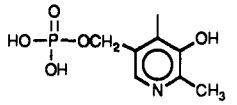
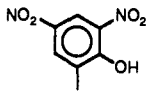
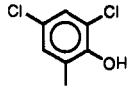
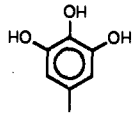
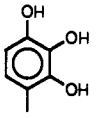
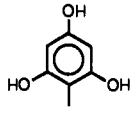
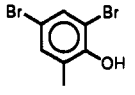
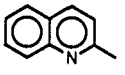
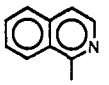
no.	R	recryst solvent	mp, °C	% yield	formula
1		EtOH–ether	183–185	52	$C_{23}H_{21}ClN_4O_4S$
2		EtOH–ether	162–164	70	$C_{18}H_{22}N_4O_5S$
3		EtOH–ether	134–136	68	$C_{16}H_{17}ClN_4O_6S$
4		EtOH–ether	194–196	55	$C_{17}H_{20}N_4O_6S$
5		EtOH–ether	209–211	69	$C_{16}H_{22}N_5O_9PS$
6		EtOH	224–226	65	$C_{15}H_{16}N_6O_9S$
7		EtOH–ether	208–210	75	$C_{15}H_{16}Cl_2N_4O_5S$
8		EtOH–ether	172–174 dec	4.5	$C_{15}H_{18}N_4O_7S{\cdot}1.5H_2O$
9		EtOH–ether	194–196	14.5	$C_{15}H_{18}N_4O_7S{\cdot}0.5H_2O$
10		EtOH–ether–H_2O	192–194	14.5	$C_{15}H_{18}N_4O_7S{\cdot}2H_2O$
11		EtOH–ether	176–178	57	$C_{15}H_{18}N_4O_5S$
12		EtOH	122–124	21	$C_{14}H_{17}N_5O_5S$

marized in Table IV. Equation 3 is better than eq 1 with higher r and lower s, and eq 3 is also better than eq 2 according to sequential F test at the 95 percentile level ($F_{1,10} = 5.11$; $F_{1,10,0.95} = 4.96$). Equation 3 reveals structural requirement on the inhibitory activities of SSB-HAG derivatives and SB-HAG derivatives against MHV replication. It can explain 66% of the variance in the data.

When the 15 compounds in Table I are divided into two subsets based on the presence or absence of ortho OH group for QSAR analyses, high correlations between the inhibitory activities and the physicochemical properties (lipophilic properties and electronic character) are revealed. Equation 4 can explain 76% of the variance in the data. Equations 5 and 6 have higher r and lower s; they are better than eq 4 based on sequential F tests at the 75 percentile level (i.e., $F_{1,6} = 2.86$ and 2.75, respectively, $F_{1,6,0.75} = 1.62$). Equations 5 and 6 can explain 83% of the variance in the data. Neither $\sum\bar{\mu}_{3+5}$ nor $\sum\sigma_m$ is highly correlated with $\sum\pi_{3+5}$ (see Table V), and thus, they can be used in the same equations. The signs and coefficients of the intercept and $\sum\pi_{3+5}$ in eq 4–6 are similar. Therefore, a change in $\sum\pi_{3+5}$ should have the same effect in these equations. Indeed, either eq 5 or eq 6 can be a better equation to present quantitative relationships between the activities and the physicochemical properties than eq 4. More data points are needed to establish the statistical significance at the 95 percentile level. Equations 5 and 6 show that the antiviral activities of SSB-HAG derivatives are negatively dependent on $\sum\sigma_m$, positively dependent on $\sum\pi_{3+5}$, and positively dependent on both $\sum\bar{\mu}_{3+5}$ and $\sum\pi_{3+5}$, respectively. These results indicate that the activity increases with increasing the summation of substituent hydrophobic constants. However, Figure 1 and eq 7 (shown in Figure 1) suggest that there may be a tendency toward a parabolic relationship between the biological activity and the substituent hydrophobic constant with an

Table II. Physicochemical Constants and Inhibitory Activity of MHV Growth by SSB-HAG and SB-HAG Derivatives

$RCH{=}NNHC(=NH)NHOH{\cdot}CH_3C_6H_4SO_3H$

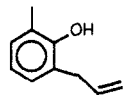
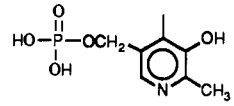
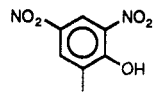
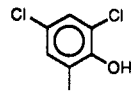
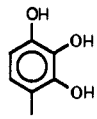
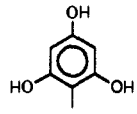
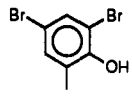
		inhibition of MHV growth				physicochemical constants of R			
			log (1/$TCID_{50}$)						
no.	R	$TCID_{50}$[b] ± D[c], μM	obsd	calcd[d]	Rm	π[e]	log MR	μ	ΔVw[f]
1		7.12 (±0.55)	5.17	5.14	−0.89	5.17	1.82	1.36	51.45
2		3.42 (±1.11)	5.47	4.84	−0.79	2.77	1.62	1.69	23.73
3		9.14 (±1.04)	5.04	4.71[g]	−0.66	2.79	1.57		13.69
4		46.10 (±14.30)	4.34	4.82	−0.66	2.01	1.57	1.32	13.61
5		40.80 (±6.40)	4.39	3.85[g]	0.10	−1.19	1.70		49.61
6		273.00 (±91.00)	3.56	3.89	−0.52	1.51	1.62	2.95	16.70
7		9.54 (±2.21)	5.02	4.88	−0.74	3.08	1.57	1.41	17.10
8		150.00 (±23.00)	3.82	4.14	−0.74	0.37	1.49	3.17	9.18
9		183.00 (±33.00)	3.74	3.91	−0.59	0.37	1.49	3.17	9.18
10		18.10 (±8.10)	4.74	4.28	−0.70	−0.13	1.49	2.70	9.18
11		76.00 (±22.80)	4.12	4.60	−0.59	1.46	1.43	1.53	0.00
12		89.90 (±26.70)	4.05	3.95	−0.30	0.48	1.40	2.00	−6.53
13[a]		9.50 (±2.58)	5.01	5.14	−0.89	3.48	1.66	1.35	22.30
14[a]		39.30 (±8.80)	4.41	4.40	−0.64	2.30	1.56	2.18	9.22
15[a]		47.70 (±6.60)	4.32	3.88	−0.42	2.08	1.56	2.60	9.22

[a] Compounds 13–15 were from Dr. A. T'ang et al.[9] [b] $TCID_{50}$ = the dose required to inhibit the growth of virus by 50% in tissue cultures. [c] D (standard deviation) = $(TCID_{84} - TCID_{16})/(\sqrt{2} \times 2n)$, where n = number of plates for each concentration of compound.[26] [d] Calculated from eq 3 in Table IV. [e] Calculated from log P_{corr} − π_H, for example (compound 11), log P_{corr} of phenol = 1.46, π_H = 0.00; compound 5 was calculated from π values. [f] ΔVw = Vw_R of compound − Vw_R of compound 11. [g] Calculated from eq 1 in Table IV.

ideal $(\sum\pi_{3+5})_0$ value of around 1.81. This result indicates that when the substituent hydrophobic constant has a value higher than 1.81, the biological activity may actually decrease due to excessive hydrophobic interactions with barriers like cell membranes or nonproductive interaction with nonspecific binding sites. Further study with more

Table III. Physicochemical Constants and Inhibitory Activity of MHV Growth by Substituted Salicylaldehyde Schiff bases of 1-Amino-3-hydroxyguanidine Tosylate

$$RCH{=}NNHC(=NH)NHOH{\cdot}CH_3C_6H_4SO_3H$$

no.	R	log (1/TCID$_{50}$) obsd	log (1/TCID$_{50}$) calcd[a]	physicochemical constants of subfunctional groups at 3 and 5 positions in R[b] $\sum E_s^C$	$\sum\sigma_m$	$\sum\pi$	$\sum$Vef	$\sum\mu^c$
2		5.47	5.07	-1.43	-0.11	1.10	0.69	0.76
5		4.39	4.66	0.32	-0.07	0.50	0.52	0.35
6		3.56	3.46	-4.40	1.42	-0.56		-5.06
7		5.02	4.99	-1.94	0.74	1.42	1.10	-1.94
9		3.74	3.75	0.55	0.12	-0.67	0.32	-1.60
10		4.74	4.33	0.00	0.00	0.00	0.00	0.04
11		4.12	4.33	0.00	0.00	0.00	0.00	0.04
12		4.05	4.33	0.00	0.00	0.00	0.00	0.04
13		5.01	5.18	-2.30	0.78	1.72	1.35	-1.92

[a] Calculated from eq 5 in Table IV. [b] Physicochemical constants were obtained or calculated from ref 29, 31, and 32. [c] $\sum\vec{\mu}_{3+5} = [(\vec{AO})^2 + (\vec{BO})^2 + 2(\vec{AO})(\vec{BO})\cos 120°]^{1/2}$ where AO and BO are the group dipole moments of subfunctional groups at positions 3 and 5, respectively.

Table IV. Best Equations for the Correlation of Biological Activity and Physicochemical Parameters

equation	n[a]	r[b]	s[c]	stepwise F test
All SSB-HAG and SB-HAG Derivatives				
(1) $\log(1/TCID_{50}) = 4.10 (\pm0.39)^d + 0.22\pi_R (\pm0.17)^d$	15	0.62	0.47	8.09
(2) $\log(1/TCID_{50}) = 5.63 (\pm0.89) - 0.56\mu_R (\pm0.40)$	13	0.69	0.44	10.25
(3) $\log(1/TCID_{50}) = 4.32 (\pm1.55) - 0.42\mu_R (\pm0.38) - 1.56Rm (\pm1.59)$	13	0.81	0.39	9.59
A Subset of SSB-HAG Derivatives				
(4) $\log(1/TCID_{50}) = 4.20 (\pm0.30) + 0.66\sum\pi_{3+5} (\pm0.34)$	9	0.87	0.39	21.14
(5) $\log(1/TCID_{50}) = 4.32 (\pm0.33) + 0.10\sum\vec{\mu}_{3+5} (\pm0.15) + 0.61\sum\pi_{3+5} (\pm0.32)$	9	0.91	0.31	14.80
(6) $\log(1/TCID_{50}) = 4.30 (\pm0.32) - 0.34\sum\sigma_m (\pm0.50) + 0.67\sum\pi_{3+5} (\pm0.31)$	9	0.91	0.31	14.60
A Subset of SB-HAG Derivatives without an Ortho OH group				
(8) $\log(1/TCID_{50}) = 3.82 (\pm0.52) + 0.29\pi_R (\pm0.19)$	6	0.91	0.24	18.28

[a] n = sample size. [b] r correlation coefficient. [c] s = standard error of estimate. [d] 95% confidence interval.

Table V. The Squared Correlation Matrix of Equations 5 and 6

	log (1/TCID$_{50}$)	$\sum\pi_{3+5}$	$\sum\sigma_m$	$\sum\tilde{\mu}_{3+5}$
log (1/TCID$_{50}$)	1	0.75	0.05	0.20
$\sum\pi_{3+5}$		1	0.004	0.04
$\sum\sigma_m$			1	0.91
$\sum\tilde{\mu}_{3+5}$				1

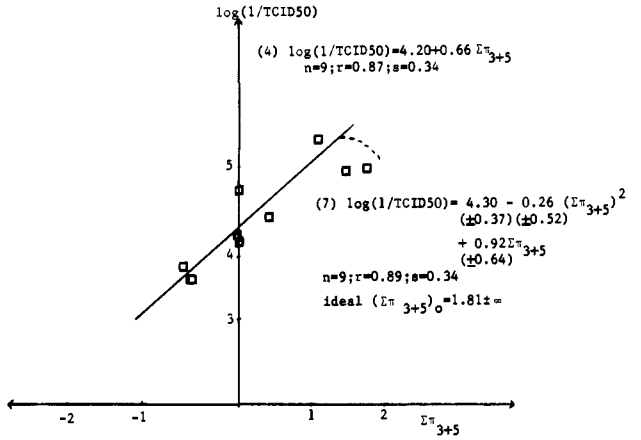


Figure 1. Dependence of the required dose (TCID$_{50}$) of the compound against MHV growth on relative hydrophobic constant of the functional group.

data points on the right hand side of the curve is needed to establish the possible parabolic relationship.

Both $\sum\tilde{\mu}_{3+5}$ and $\sum\sigma_m$ are highly correlated with each other according to the following equation:

$$\sum\tilde{\mu}_{3+5} = 0.01(\pm0.55) - 3.26(\pm0.92)\sum\sigma_m$$

$$n = 9,\ r = 0.95,\ s = 0.59,\ F_{1,7} = 69.76$$

Since both $\sum\tilde{\mu}_{3+5}$ and $\sum\sigma_m$ are electronic properties of the substituent, the high correlation indicates that eq 5 and 6 present basically the same information for small substituent groups. It is evident that the electronic properties and orientation of substituents are important for the antiviral activity. When larger groups are used, $\sum\sigma$ and $\sum\mu$ may give different results.[32]

QSAR analysis of a subset of SB-HAG derivatives without the ortho OH group indicates that eq 8 is the best equation with an r of 0.91 and s of 0.24 (in Table IV). This equation can explain 83% of the variance in the inhibitory activity of SB-HAG derivatives against coronaviral infection. The antiviral activity of this subset is positively dependent on the substituted hydrophobic constant (π_R) for the limited number of compounds studied.

In summary, the structural requirements for the antiviral activities of substituted salicylaldehyde Schiff bases of HAG tosylate are stringent according to the wide range of inhibitory activities between compounds **2** and **6** and QSAR analysis of a subset of SSB-HAG derivatives against copronavirus infection. It seems that the ortho OH group in SSB-HAG derivatives can form a fairly stable intramolecular hydrogen bond with the hydroxyaminoguanidine group in several possible ways. This intramolecular hydrogen bond may contribute to the greater stability of the Schiff base functional group and higher biological activity. In this case, different subsets (in Table IV) may require different equations to reflect the differences. The quantitative results of QSAR analyses support our hypothesis that the small size of the ring, the presence of an ortho OH group, the electronic character, the orientation of substituent on the ring, and the substituent hydrophobicity can all affect the antiviral activity. According to the QSAR analysis of substituted SSB-HAG tosylate, a compound having a substituent with increasing lipophilic properties and electron-donating properties at the 3- and 5-positions should have high antiviral activity. Parameter focusing[35] of the substituent hydrophobic constant and electron-donating constant (e.g. $\sum\sigma$) can be used to select effective substituents for further study.

Experimental Section

Chemistry. Corrected melting points of the compounds were determined in open capillary tubes with a Thomas-Hoover melting point apparatus. The identity and purity of the compounds are confirmed by IR and NMR as well as elemental analyses. Elemental analyses were performed by the Center of Instrumental Analysis, Dept. of Chemistry, National Taiwan University, Taipei, Taiwan, or by C. F. Geiger, Ontario, CA; results were within 0.4% of theoretical values. All compounds were dried in a drying apparatus with P_2O_5 before analysis.

IR spectra of the compounds were obtained with a Beckman IR 4210 spectrometer using KBr pellets. Proton NMR spectra were obtained with a GE QE-300MHz spectrometer by Pei Tsai, Dept. of Chemistry, University of Oregon, using DMSO-d_6 or MeOH-d_4 as the solvent.

Thin-layer chromatography was performed on the precoated silical gel F254 chromatographic plates (20 × 20 cm; 0.2 mm) (Aldrich) impregnated with silicone oil. The solvent system consisted of butanol–acetic acid–water (65:13:22).

Ultraviolet absorption spectra were obtained with a Varian/Cary Model 219 UV spectrophotometer.

Synthesis of SSB-HAG and SB-HAG Derivatives. Twelve new compounds were synthesized according to the procedure reported previously.[8,9] Thiosemicarbazide (0.5 mol) and methyl p-toluenesulfonate (0.5 mol) in methanol (500 mL) was refluxed for 18 h to form S-methylisothiosemicarbazide tosylate. S-Methylisosemicarbazide tosylate (0.4 mol) was reacted with hydroxylamine (0.5 mol) at room temperature for 48 h to give N-hydroxy-N-aminoguanidine tosylate, which was identified by measuring the melting point (136–137 °C).[8,9] The final substituted salicylaldehyde Schiff base and substituted Schiff base of HAG derivatives were prepared by reacting HAG tosylate with the appropriate aldehydes. A Dean–Stark distillation receiver was used to remove the water formed and to improve the yields of the compounds with more than one OH group on the benzene ring (compounds **8–10**) in the Schiff base formation. The aldehydes used to prepare the final SSB-HAG derivatives and SB-HAG derivatives were obtained commercially except for 3-hydroxy-2-pyridinecarbaldehyde, which was prepared in our laboratory. The percentage yields and melting points of the compounds synthesized are given in Table I.

3-Hydroxy-2-pyridinecarbaldehyde. Five grams of 3-hydroxy-2-(hydroxymethyl)pyridine hydrochloride dissolved in 30 mL of methanol was neutralized with 0.03 mol of potassium hydroxide in a salt–ice bath for 1 h and the precipitated KCl was removed by filtration. The methanol phase was evaporated to dryness in vacuo. The free 3-hydroxy-2-(hydroxymethyl)pyridine was suspended in 60 mL of chloroform and then was treated with 15 g of active MnO_2. The reaction mixture was allowed to stir under reflux for 3 h and then was filtered, and the MnO_2 cake was washed well with two 50-mL portions of chloroform. The chloroform extracts were concentrated to dryness in vacuo. The solid aldehyde was crystallized from petroleum ether (bp 60–110 °C). The yield for solid aldehyde was 1.6 g. The structure was confirmed by IR and NMR spectra.

Biological Procedures. Inhibition of MHV Replication in Tissue Culture. The test compounds were dissolved in 20% DMSO (analytic grade) in methanol (HPLC grade) to give concentrations between 3.2×10^{-2} and 3.2×10^{-3} M. DBT cells were grown as a monolayer on 60-mm plastic tissue-culture plates. Before virus infection, cell growth media (MEM) were gently removed by aspiration from the side of tissue culture plates. Virus inoculum (0.2 mL) was added to each cell monolayer at a multiplicity of infection of 1 to 5, and the samples were incubated at room temperature for 1 h. After virus adsorption, 3 mL of

(35) Magee, P. S. *Pestic. Chem.: Hum. Welfare Environ., Proc. 5th Int. Congr. Pestic. Chem.* **1982**, *1*, 251.

MEM supplemented with 5% FCS was added into each dish, and then 0.1 mL of test compound solution at given concentrations was added and incubated at 37 °C. The final concentration of DMSO in media was 0.15%, and those of test compounds ranged from 0.97 to 3030 μM. Each test was run in duplicate. The media were collected at 18 h postinfection and frozen for plaque assays of virus present in the media. The virion samples were thawed at room temperature and serially diluted into five different concentrations, with phosphate buffered saline (pH 7.0; PBS) solution. These serially diluted virus samples (0.2 mL) were then used to inoculate DBT cells separately. After virus absorption for 1 h at room temperature, 4 mL of 0.75% agarose in MEM solution was added to each culture and allowed to solidify at room temperature. The cultures were incubated for 48 h at 37 °C and 3 mL of neutral red was added to stain uninfected cells while the virus-infected cells appear as unstained plaques. Each plaque represents one virus particle. The number of virus particles was calculated by the following equation:

$$\text{no. of virus particles/mL} = \text{no. of virus particles in duplicated plates}/2 \times 5 \times \text{dilution factor}$$

The percent inhibition of virus growth was calculated as follows:

$$\%\ \text{inhibition} = (1 - T/C) \times 100\%$$

where T = number of virion particles/mL in treated samples and C = number of virion particles/mL in control group. The dose required for 50% inhibition of virus growth in the tissue culture ($TCID_{50}$) for each compound was calculated by using probit analysis.[36,37]

Acknowledgment. This paper was taken in part from the Ph.D. dissertation of P.H.W., University of Southern California, 1989.

Registry No. 1, 123541-05-9; 1 (free base), 123541-04-8; 2, 123541-07-1; 2 (free base), 123541-06-0; 3, 123541-09-3; 3 (free base), 123541-28-6; 4, 123541-11-7; 4 (free base), 123541-10-6; 5, 123541-13-9; 5 (free base), 123541-12-8; 6, 123541-15-1; 6 (free base), 123541-14-0; 7, 123541-17-3; 7 (free base), 123541-16-2; 8, 123541-19-5; 8 (free base), 123541-18-4; 9, 123541-21-9; 9 (free base), 123541-20-8; 10, 123541-23-1; 10 (free base), 123541-22-0; 11, 123541-25-3; 11 (free base), 123541-24-2; 12, 123541-27-5; 12 (free base), 123541-26-4; 13, 96826-63-0; 13 (free base), 96826-62-9; 14, 96826-58-3; 14 (free base), 96826-57-2; 15, 96826-69-6; 15 (free base), 96826-68-5; 10-chloro-9-anthracenecarboxaldehyde, 10527-16-9; 2-hydroxy-3-(2-propenyl)benzaldehyde, 24019-66-7; 6-chloro-1,3-benzdioxole-5-carboxaldehyde, 15952-61-1; 2,3-dihydro-1,4-benzodioxin-6-carboxaldehyde, 29668-44-8; 3-hydroxy-2-methyl-5-[(phosphonooxy)methyl]-4-pyridinecarboxaldehyde, 54-47-7; 2-hydroxy-3,5-dinitrobenzaldehyde, 2460-59-5; 3,5-dichloro-2-hydroxybenzaldehyde, 90-60-8; 3,4,5-trihydroxybenzaldehyde, 13677-79-7; 2,3,4-trihydroxybenzaldehyde, 2144-08-3; 2,4,6-trihydroxybenzaldehyde, 487-70-7; 2-hydroxybenzaldehyde, 90-02-8; 3-hydroxy-2-pyridinecarboxaldehyde, 1849-55-4; *n*-hydroxy-*n*-aminoguanidine tosylate, 36826-58-1; 3-hydroxy-2-(hydroxymethyl)pyridine, 14047-53-1.

(36) Finney, D. J. *Probit Analysis*; Cambridge University Press: Cambridge, UK, 1947; p 20.
(37) Miller, L. C.; Tainter, M. C. *Proc. Soc. Exp. Biol. Med.* **1944**, *57*, 261.

Potential Antisecretory Antidiarrheals. 2. α_2-Adrenergic 2-[(Aryloxy)alkyl]imidazolines†

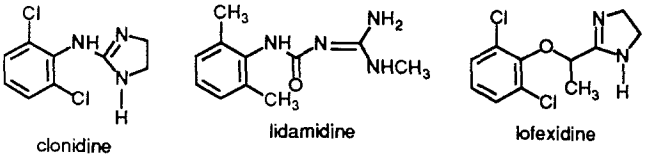
Alan E. Moormann,* Barnett S. Pitzele, P. H. Jones, Gary W. Gullikson, David Albin, Stella S. Yu, Robert G. Bianchi, Elizabeth L. Sanguinetti, Barbara Rubin, Maggy Grebner, Milagros Monroy, Peggy Kellar, and Jacquelyn Casler

Preclinical Research, G. D. Searle and Company, 4901 Searle Parkway, Skokie, Illinois 60077. *Received October 9, 1987*

Lofexidine, an α_2-agonist, has central hypotensive activity and peripheral intestinal antisecretory activity. Analogues were synthesized with increased polarity in an attempt to prevent penetration of the blood–brain barrier. The compounds were evaluated in the cholera toxin treated ligated jejunum of the rat and in the Ussing chamber with a rabbit ileum preparation. Active compounds were determined to be α_2-adrenergic agonists by yohimbine reversals of their Ussing chamber activities. The 2,6-dimethyl derivative of lofexidine, **4a**, was as active as lofexidine in vivo, but derivatives with 2,6-substituents larger than ethyl were inactive. (Aryloxy)alkyl derivatives which have an imidazoline and a methyl or larger group as part of the alkyl exhibited the best antisecretory activity. Compounds with substituents in the para position of the phenyl ring were generally inactive. 3-Amino-2,6-dimethyl derivative **21** was twice as active as **4a**. A 2-methyl substituent is required in the 3-amino series to retain good activity. 2-Methyl derivative **12a** had activity comparable to that of **4a**, while 6-methyl derivative **12f** was inactive. Substituents on the 3-amino group did not affect the activity, but substituting a hydroxyl for the amino group produced an inactive compound. Replacing the phenyl moiety with a 4-indole resulted in retention of activity, but other heterocycles were inactive. Compound **12a** was resolved and *d* isomer **32** was five times more potent than *l* isomer **33**. The more active compounds in the rat cholera toxin assay (RCTA), when evaluated in the dog, exhibited antisecretory activity but also exhibited central nervous system (CNS) effects, sedation and ataxia, at 10 mg/kg, and in spontaneously hypertensive rats at 50 mg/kg. A measure of polarity, log *P*, was calculated for the (aryloxy)alkyl groups. Regression analysis showed no correlation of antisecretory ED_{50} to the calculated log *P*. The active compounds did not show a separation of the central CNS effects from the peripheral antisecretory activity by increasing the polarity.

We have recently described a series of compounds which offer an alternate approach to antidiarrheal therapy.[1] Instead of focusing on the motility component of diarrhea, we investigated a new therapeutic strategy of using α_2-adrenergic agonists to block both secretion and motility.

Chart I



†This paper has been presented in part; see: Moormann, A. E.; Pitzele, B. S.; Jones, P. H.; Gullikson, G. W.; Bianchi, R. G.; Monroy, M.; Rubin, B.; Casler, J.; Grebner, M.; Yu, S. at the 190th National Meeting of the American Chemical Society, Chicago, IL, September 8–13, 1985; paper MEDI 72. Derivatives of Lofexidine as Intestinal α_2 Adrenergic Antisecretory Agents.

0022-2623/90/1833-0614$02.50/0