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Inhibition of murine coronavirus RNA synthesis by hydroxyguanidine derivatives

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Summary

A series of hydroxyguanidine derivatives, which are substituted salicylaldehyde Schiff-bases of 1-amino-3- hydroxyguanidine tosylate, were tested for the inhibition of RNA synthesis of mouse hepatitis virus (MHV). It was shown that these compounds could selectively inhibit virus-specific RNA synthesis. Every aspect of viral RNA synthesis, including synthesis of negative-stranded RNA, subgenomic mRNA transcription and genomic RNA replication, was inhibited to roughly the same extent. These compounds are the first known inhibitors of coronaviral RNA synthesis and should prove useful for understanding the mechanism of viral RNA synthesis.

Mouse hepatitis virus; Coronavirus

Coronaviruses are enveloped RNA viruses associated with respiratory and gastrointestinal illnesses in humans and many other species of animals (Wege et al., 1982). The prototype coronavirus, mouse hepatitis virus (MHV), causes hepatitis and neurological diseases in mice. These viruses employ a complex mechanism for the synthesis of viral RNA (Lai, 1987). Upon infection of a susceptible cell, the viral genomic RNA, which is a positive-sensed single-stranded RNA of more than 6×10^6 daltons (Lai and Stoleman, 1978), is released into the cytoplasm and used for the translation of a virus-specific RNA-dependent RNA polymerase (Brayton et al., 1982, 1984). This enzyme, in turn, transcribes the viral RNA into a negative-

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stranded RNA template (Lai et al., 1982). A modified or new RNA polymerase (Brayton et al., 1984) subsequently synthesizes virus-specific mRNAs via a unique mechanism of leader-primed transcription, i.e. a leader RNA is synthesized initially, dissociated from the template RNA and then rejoins the latter at the downstream transcriptional initiation sites to serve as the primer for mRNA synthesis (Baric et al., 1983; Makino et al., 1986b; Lai, 1987). Understanding of this complex transcription pathway mainly came from the sequence studies of MHV RNA genome (Shieh et al., 1987) and has been aided by genetic and biochemical studies of temperature-sensitive mutants (Baric et al., 1985; Leibowitz et al., 1982) which are defective in various aspects of MHV RNA synthesis. It has also been aided by studies of interaction between different MHV strains (Makino et al., 1986b). However, many aspects of this transcription pathway remain unclear.

Inhibitors of RNA synthesis have been useful for understanding mechanisms of RNA synthesis in several different virus systems. However, the only known inhibitor of RNA-dependent RNA synthesis carried out by RNA viruses is guanidine, which selectively inhibits poliovirus RNA synthesis (Caliguiri and Tamm, 1972). The sensitivity of poliovirus to this inhibitor appears to reside in the viral protein 2C, which may be directly or indirectly involved in viral RNA synthesis (Pincus et al., 1986). The mechanism of inhibition of RNA synthesis by guanidine is still unclear, but it appears that the initiation step of RNA synthesis is the major site of inhibition (Tershak, 1982). Similar inhibitors of RNA synthesis have not been available for any other RNA viruses. Our previous studies indicated that guanidine does not inhibit coronavirus RNA synthesis even at a concentration as high as 50 mM (S. Makino and M.M.C. Lai, unpublished observations). Recently, we have developed a series of hydroxyguanidine derivatives which are capable of inhibiting coronavirus replication in tissue culture (Wang et al., unpublished data). In this report, we show that these agents selectively inhibit RNA synthesis of MHV. Thus, they will be useful for studying the mechanism of coronavirus RNA synthesis.

The chemicals used in this study are substituted salicylaldehyde Shiff-bases of 1-amino-3-hydroxyguanidine (SSB-HAG) tosylate and other Schiff-bases of HAG (SB-HAG). The synthesis, chemical properties and inhibitory activities on MHV growth of these compounds have been described elsewhere (Wang et al., unpublished data). Some of these compounds have $TCID_{50}$ (the dose required for inhibition of the virus growth by 50% in tissue culture) in the range between 1 and 10 μM. At higher concentrations of these compounds, the virus yield was reduced 10000-fold relative to the control culture (Wang et al., unpublished data). No apparent cytotoxicity was observed at the concentrations studied. To examine the possible inhibitory activities of these compounds on MHV RNA synthesis, we selected compounds which have the highest activity in inhibiting virus growth (Wang et al., unpublished data) for further studies. The chemical structures of these compounds are summarized in Fig. 1.

The test compounds were dissolved in 5% DMSO (analytic grade) in methanol (HPLC grade) to give desired stock concentrations. DBT cells (Hirano et al., 1972) were grown in monolayers on 60 mm plastic tissue culture dishes. The JHM strain of MHV was added to culture at a multiplicity of infection (MOI) of 5 and allowed

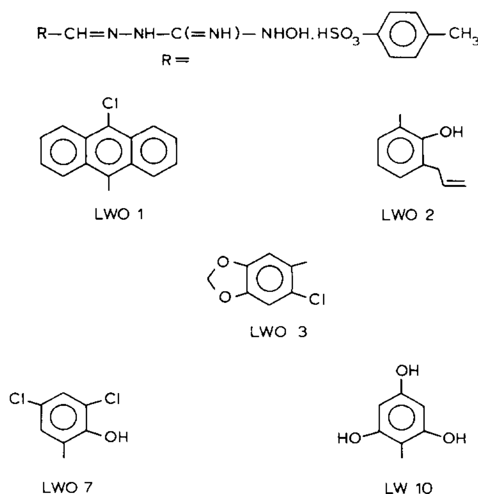

Fig. 1. The structure of test compounds used.

to adsorb at room temperature for 1 h. After virus adsorption, 3 ml of minimum essential media (MEM) supplemented with 5% fetal calf serum (FCS) was added in each dish, and then 0.1 ml of the test compound at given concentrations was added and incubated at 37 °C. The final concentration of DMSO in media was 0.15%, and those of the test compounds ranged from 1.45×10^{-5} M to 7.75×10^{-4}M. These concentrations had previously been determined to be the highest concentrations which could inhibit MHV growth without causing cytotoxicity (Wang et al., unpublished data). Each test was run in duplicate. A solvent-treated control set was included each time. Actinomycin D (2.5 μg/ml) was added at 4 h postinfection (p.i.) and [^{3}H]uridine (10 μCi/ml) was added at 5 h p.i. At 8 h p.i., the virus-induced cytopathic effect (CPE), as exhibited by cell fusion, of each plate was observed and recorded. Cells were lysed with 1% sodium dodecyl sulfate in phosphate-buffered saline (pH 7.0) and precipitated by the addition of trichloroacetic acid (TCA) to a final concentration of 5%. Table 1 shows that the virus-induced CPE of cells treated with the test compounds was significantly lower than that of infected cells treated with the solvent alone, indicating that these compounds inhibited the growth of virus. All of the compounds also significantly inhibited the DNA-dependent RNA synthesis of DBT cells. The only exception was compound LW03, which inhibited cellular RNA synthesis by less than 20%. However, the virus-specific RNA synthesis was invariably inhibited to a greater extent by all of the compounds tested. These results indicate that these compounds can inhibit viral RNA synthesis selectively.

To determine whether these compounds have differential inhibitory activities on the synthesis of different RNA species of MHV, we added the test compounds at different times p.i. As shown in Table 2, the compounds added at 0 h and at 2 h p.i. inhibited viral RNA synthesis to almost the same extent. However, all 4 compounds, when added at 4 h p.i., consistently inhibited viral RNA synthesis to a lesser extent. Since most of the virus-specific negative-stranded RNA synthesis takes place during

TABLE 1

Inhibition of cellular and virus-specific RNA synthesis by selected compounds

Compound	Final conc. (1×10^{-5} M)	CPE (% fused cells)	Inhibition (%) [a]	
			Cellular RNA synthesis	Viral RNA synthesis
solvent	–	75	0	0
LW01	1.45	25	63	90
LW02	5.30	0	53	91
LW03	1.45	5	18	86
LW07	9.70	1	68	95
LW10	77.50	0	73	94

The test compounds were added to the virus-infected cultures at 0 h p.i. and the extents of virus-induced CPE were observed at 8 h p.i. Actinomycin D was added at 2.5 μg/ml to inhibit cellular RNA synthesis at 4 h p.i. [^{3}H]uridine (10 μCi/ml) was added at 5 h p.i. and cells were harvested at 8 h p.i. [^{3}H]-uridine incorporation was determined by TCA precipitation and subsequent scintillation counting (Lai et al., 1981). A similar experiment was done on uninfected cells in the absence of actinomycin D to provide data points for the % inhibition of cellular RNA synthesis.

[a] Inhibition percentage $= \frac{\text{(cpm of solvent-treated cells – cpm of drug-treated cells)}}{\text{cpm of solvent-treated cells}} \times 100.$

the first 2–3 h p.i. (Brayton et al., 1982, 1984) and the positive stranded mRNAs are synthesized after 4 h p.i. (Lai et al., 1981), these results suggest that the negative-stranded RNA synthesis is more sensitive than the positive-stranded RNA synthesis to inhibition by these compounds. To determine whether these test compounds have differential effects on the synthesis of different viral positive-stranded RNA species, i.e., genomic vs subgenomic mRNA species, agarose gel electrophoresis of virus-specific RNAs in MHV-infected DBT cells treated with the test compounds was

TABLE 2

Kinetics of inhibition of MHV RNA synthesis by selected compounds

Compound	Final conc. (1×10^{-5} M)	Inhibition of RNA synthesis (%) [a]		
		T = 0 [b]	T = 2 [b]	T = 4 [b]
solvent	–	0	0	0
LW01	1.45	89	92	73
LW02	5.30	90	89	75
LW03	1.45	88	79	77
LW07	9.70	90	82	75
LW10	77.50	82	82	77

Actinomycin D was added 2.5 μg/ml at 5 h p.i. [^{3}H]uridine (10 μCi/ml) was added at 6 h p.i. and the cells were harvested at 8 h p.i. and processed for determination of TCA-precipitable counts as in Table 1.

[a] Inhibition percentage $= \frac{\text{(cpm of solvent-treated cells–cpm of drug-treated cells)}}{\text{cpm of solvent-treated cells}} \times 100.$

[b] T, hour p.i. when the compounds were added.

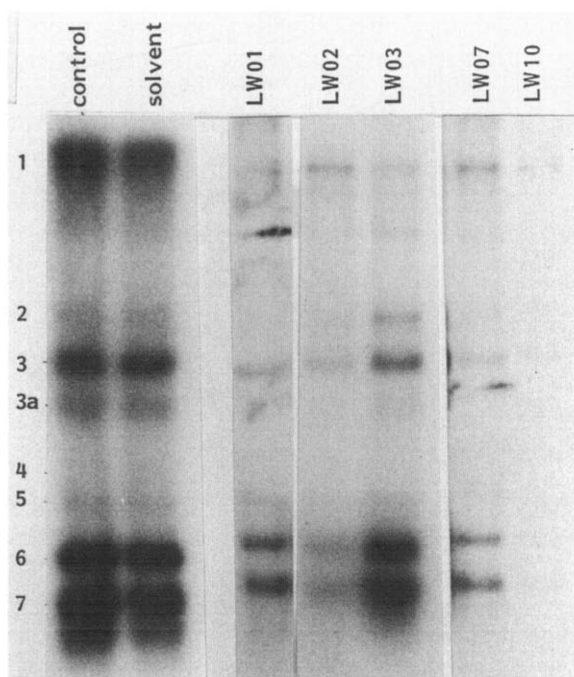

Fig. 2. Agarose gel electrophoresis of intracellular RNA from MHV-infected cells treated with test compounds. The test compounds at concentrations as described in Table 1 were added immediately after virus adsorption. Actinomycin D (2.5 μg/ml) was added at 4 h p.i. and [^{32}P]orthophosphate (200 μCi/ml) was added at 5 h p.i. RNA was extracted from cells at 8 h p.i. and analyzed by electrophoresis on a 1% agarose gel according to published procedures (Keck et al., 1988). The untreated and solvent-treated lanes were from a 2-h exposure at room temperature. The remaining lanes were from a 48-h exposure with an intensifying screen at $-70°$C. The viral RNA species are identified as described (Keck et al., 1988).

performed. The genomic-sized RNA synthesis represents viral RNA replication while subgenomic RNAs represent RNA transcription, which may be carried out by different RNA polymerases (Brayton et al., 1984). As shown in Fig. 2, all of the virus-specific mRNAs were detected in cells treated with different compounds although the amounts of these RNAs were significantly reduced as compared to the untreated virus-infected cells. (In Fig. 2, different lanes were exposed to X-ray films for different lengths of time in order to reveal individual RNA species.) The relative ratio of the genomic RNA to every subgenomic mRNA species remained the same for most of the compounds as in untreated cells. These results indicate that most of the test compounds inhibited both the replication of genomic RNA and the synthesis of subgenomic mRNAs to almost the same extent. However, in LW03-treated cells, the ratio of genomic RNA to subgenomic RNA species was slightly lower (Fig. 2), suggesting that LW03 might have a slightly differential effect on viral RNA replication and transcription.

These results indicate that these hydroxyguanidine derivatives can inhibit coronaviral RNA synthesis selectively, although most of them also have significant cytotoxicity toward the host cells. All of them inhibit every facet of viral RNA synthesis, including negative-stranded RNA synthesis, mRNA transcription and genome RNA replication. Since the synthesis of these different RNA species is probably carried out by different RNA polymerases or various modified forms of the same RNA polymerase (Brayton et al., 1982, 1984), these compounds are either not very selective or inhibit the core component of these RNA polymerases. The most likely targets for these compounds are virus-specific RNA-dependent RNA polymerases. Proof of this mechanism of inhibition requires the purified RNA polymerases, which is currently unavailable due to the extremely low quantity of these proteins in the virus-infected cells.

These compounds represent the first inhibitors of coronaviral RNA synthesis. They appear to be much more potent than guanidine used for the inhibition of poliovirus RNA synthesis. For instance, 2 mM of guanidine was required for the inhibition of poliovirus RNA synthesis (Pincus et al., 1986), while only 0.015 mM of LW01 was required to achieve 90% inhibition of coronavirus RNA synthesis (Table 1). As demonstrated for poliovirus RNA, the inhibitors of RNA synthesis are very useful for understanding the mechanism of RNA synthesis (Pincus et al., 1986; Tershak, 1982). The availability of these hydroxyguanidine derivatives should also prove useful for understanding the mechanism of coronaviral RNA synthesis. Approaches such as isolation of drug-resistant mutants, coupled with RNA recombination (Makino et al., 1986a) should provide a powerful tool for understanding the genetics and mechanism of RNA synthesis of MHV. It should be noted that the inhibition of cellular DNA-dependent RNA synthesis is a drawback of these compounds. However, the concentrations of the compounds used here represented at least 10-fold higher than the $TCID_{50}$ of these compounds. For instance, the $TCID_{50}$ of LW02 is 3.4 μM (Wang et al., unpublished data) while the concentration used here was 5.3×10^{-5} M, and the $TCID_{50}$ of LW07 is 9.5 μM (Wang et al. unpublished data) and the concentration used here was 9.7×10^{-5} M. Thus, it is not surprising that some cytotoxicity was noted. The reason for selecting such high concentrations in this study was to investigate the maximum possible activities of these compounds. This study indicates that it is possible to almost completely inhibit viral RNA synthesis despite some cytotoxicity. Thus, it is possible to isolate drug-resistant mutants with these compounds. Since some compounds (e.g. LW03) may have slightly differential inhibitory activities on different viral RNA species, the viral mutants resistant to different compounds may prove to be very useful for understanding the mechanism of RNA synthesis. Such an approach is currently in progress.

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References

Baric, R.S., Stohlman, S.A. and Lai, M.M.C. (1983) Characterization of replicative intermediate RNA of mouse hepatitis virus: presence of leader RNA sequences on nascent chains. J. Virol. 48, 633–640.

Baric, R.S., Stohlman, S.A., Razavi, M.K. and Lai, M.M.C. (1985) Characterization of leader-related small RNAs in coronavirus-infected cells: further evidence for leader-primed mechanism of transcription. Virus Res. 3, 19–33.

Brayton, P.R., Lai, M.M.C., Patton, C.D. and Stohlman, S.A. (1982) Characterization of two RNA polymerase activities induced by mouse hepatitis virus. J. Virol. 42, 847–853.

Brayton, P.R., Stohlman, S.A. and Lai, M.M.C. (1984) Further characterization of mouse hepatitis virus RNA-dependent RNA polymerases. Virology 133, 197–201.

Caliguiri, L.A. and Tamm, I. (1972) Guanidine. In: D.J. Bauer (Ed.), Chemotherapy of Virus Diseases, Vol. 1, pp. 181–230. Pergamon Press, Oxford, New York, Toronto.

Hirano, N., Fujiwara, K., Hino, S. and Matsumoto, M. (1974) Replication and plaque formation of mouse hepatitis virus (MHV-2) in mouse cell line DBT culture. Arch. Ges. Virusforsch. 44, 298–302.

Keck, J.G., Hogue, B.G., Brian, D.A. and Lai, M.M.C. (1988) Temporal regulation of bovine coronavirus RNA synthesis. Virus Res. 9, 343–356.

Lai, M.M.C. (1987) Coronavirus leader-RNA-primed transcription: an alternative mechanism to RNA splicing. Bioessays 5, 257–260.

Lai, M.M.C., Brayton, P.R., Armen, R.C., Patton, C.D., Pugh, C. and Stohlman, S.A. (1981) Mouse hepatitis virus A59: messenger RNA structure and genetic localization of the sequence divergence from the hepatotropic strain MHV3. J. Virol. 39, 823–834.

Lai, M.M.C., Patton, C.D. and Stohlman, S.A. (1982) Replication of mouse hepatitis virus: negative-stranded RNA and replicative form RNA are of genomic length. J. Virol. 44, 487–492.

Leibowitz, J.L., DeVries, F.R. and Haspel, M.V. (1982) Genetic analysis of murine hepatitis virus strain JHM. J. Virol. 42, 1080–1087.

Makino, S., Keck, J.G., Stohlman, S.A. and Lai, M.M.C. (1986a) High frequency RNA recombination of murine coronavirus. J. Virol. 56, 729–737.

Makino, S., Stohlman, S.A. and Lai, M.M.C. (1986b) Leader sequences of murine coronavirus mRNAs can be freely reassorted: evidence for the role of the free leader RNA in transcription. Proc. Natl. Acad. Sci., USA 83, 4204–4208.

Pincus, S.E., Diamond, D.C., Emini, E.A. and Wimmer, E. (1986) Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. J. Virol. 57, 638–646.

Shieh, C.-K., Soe, L.H., Makino, S., Chang, M.-F., Stohlman, S.A. and Lai, M.M.C. (1987) The 5′-end sequence of the murine coronavirus genome: implications for multiple fusion sites in leader-primed transcription. Virology 156, 321–330.

Sturman, L.S. and Holmes, K .V. (1983) The molecular biology of coronavirus. Adv. Virus Res. 28, 35–112.

Tershak, D.K. (1982) Inhibition of poliovirus polymerase by guanidine in vitro. J. Virol. 41, 313–318.

Wege, H., Siddell, S. and ter Meulen V. (1982) The biology and pathogenesis of coronaviruses. Curr. Top. Microbiol. Immunol. 99, 165–200.

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