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COMPARISON OF THE ANTIVIRAL EFFECTS OF SUBSTITUTED BENZIMIDAZOLES AND GUANIDINE IN VITRO AND IN VIVO

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6-[[(Hydroxyimino)phenyl]methyl]-1-[(1-methylethyl)sulfonyl]-1*H*-benzimidazole-2-amine (LY-122771-72) was substantially more active (and more toxic) than 2-(α-hydroxybenzyl)benzimidazole (HBB) and guanidine-HCl in a microtiter assay system employing the inhibition of the cytopathic effect produced by 13 enteroviruses. In cell cultures HBB and guanidine were mutually 'synergistic' for each other but neither had a similar effect on LY122771-72 or its close relative LY127123. A mixture of HBB and guanidine when injected into infant mice would save them from the lethal effects of infections with coxsackie A9 and echo 9 viruses. It was further found that the substituted benzimidazoles LY122771-72 and LY127123, when given daily for 9 days, could save significant numbers of mice from death caused by echo 9, coxsackie A9 and A16 viruses. HBB alone was significantly effective in the treatment of mice infected with echo 9 and coxsackie A9 but not coxsackie A16 virus. Guanidine alone was effective treatment for mice infected with either echo 9 or coxsackie A16 viruses with striking activity against this latter infection, requiring only a 4 day treatment starting 58 h after virus inoculation.

antipicornavirus enviroxime 2-(α-hydroxybenzyl)benzimidazole LY127123 LY122771-72

INTRODUCTION

There are few reports of compounds altering the course of disease in animals infected with human enteroviruses. Mice fed a diet containing 0.4% 2-(α-hydroxybenzyl)benzimidazole (HBB) were reported to be resistant to type 2 poliovirus-induced disease [11]. A propyl benzimidazole derivative, at the maximum tolerated dose, delayed death of mice infected with type A9 coxsackievirus but had little effect on the final death rate [19]. It was reported that guanidine hydrochloride might decrease the incidence of poliovirus type 1 disease in monkeys but this effect was complicated by the toxicity of the treatment [24]. Other studies found no effect in enterovirus-infected experimental animals [2, 4, 5, 16]. Derivatives of *N*-phenyl-*N'*-aryl- or alkylthioureas produced a protective effect in newborn mice infected with both coxsackieviruses types A and B [8] but in no case did many more than 50% of the mice survive. All mice treated with a mixture of

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guanide and HBB, however, were readily protected from lethal doses of coxsackievirus A9 or echovirus type 9 [5]. In this same study it was further shown, in brief experiments, that HBB alone protected mice from death induced by coxsackievirus A9 but had no effect on mice infected with echovirus 9.

The following study was undertaken to compare the effects of three substituted benzimidazoles and guanidine-HCl in enterovirus-infected cell cultures and infant mice.

MATERIALS AND METHODS

In vitro antiviral assays

Using procedures and media previously described [10], SW-13 cells [15] (American Type Culture Collection) and HeLa cells (used only for coxsackie A16 virus, V.V. Hamparian, Ohio State University) were planted in every other well of a 96-well microtiter plate (76-205-05, Linbro Scientific Co., New Haven, CT) in 0.1 ml of medium. The cultures were incubated at 35°C, after sealing with Mylar tape (76-402-05, Linbro), until the cell sheets were almost confluent at which time the medium was removed and replaced with 0.2 ml of maintenance medium.

Compounds were tested by the addition of 0.02 ml of each two-fold dilution, in serum-free cell culture medium, into four microcultures. The 48 cultures in each plate accommodated three dilutions each of three test compounds with enough cultures left for compound-free controls and a virus titration to extinction. The cultures were further incubated for 24 h and then all wells containing test compounds were infected with 0.02 ml containing approximately 100 $TCID_{50}$ (50% tissue culture infective doses) [23] of virus. The cell cultures were then incubated for 8–10 (HeLa) or 14 (SW-13) days, observed thrice weekly and virus-induced CPE (cytopathic effect) was recorded. The concentration of compound that completely inhibited virus-induced CPE for the entire incubation period, when converted to μg/ml, was considered the minimum inhibitory concentration. At the concentrations used these viruses produced complete destruction of the compound-free monolayers within 2–3 days.

Viruses

Coxsackieviruses types A9, B1, B2, B3, B4, echoviruses types 6, 7, 9 and polioviruses types 1 and 3 were all isolated from patient specimens using cultures of HeLa or rhesus monkey kidney cells in microcultures as previously described [10]. The Schmitt strain of coxsackievirus B6, the Griggs strain of coxsackievirus A9, the Lansing strain of poliovirus type 2, and the G-10 strain of coxsackievirus A16 were provided by the Research Resources Branch (RRB) of the National Institutes of Health (Bethesda, MD). The viruses were passaged in cell cultures 2–10 times prior to use in the antiviral tests. All viruses, whatever their source, were serotyped using specific antisera provided by the RRB.

Because of the extended studies using echo 9 and coxsackie A9 viruses from patient isolates five pools of each virus were further serotyped, using not only antiserum supplied by RRB but also that purchased from Microbiological Associates (Walkersville, MD). Since these viruses produced paralysis and death in infant mice after cell culture passage, it was not necessary to passage them further in infant mice as was the case with coxsackie A16 virus. This G-10 strain was of such low virulence that only undiluted cell culture virus pools produced any paralysis in infant mice, so it was necessary, using the sixth cell culture passage, to produce mouse muscle tissue pools. Virus was given by the subcutaneous (s.c.) route and infant mice sacrificed at the first sign of paralysis. A 20% homogenate was prepared using shoulder and limb muscle tissue and cell culture medium containing 10% fetal bovine serum. This pool, which produced 1 LD_{50} at a dilution of $10^{-4.5}$ when injected into infant mice was used to make a second mouse tissue pool with a titer of 1 LD_{50} at a dilution of $10^{-5.2}$ per 0.02 ml. Coxsackievirus A16 antisera (RRB) completely neutralized (at 50 units ml) 50 LD_{50} (50% lethal dose) [23] of the most recent tissue pool, while coxsackievirus A9 and echovirus type 9 antisera had no effect. Cell culture and mouse tissue virus pools were in all cases diluted in serum-free cell culture medium. All virus injections were subcutaneous (0.02 ml) in the midline over the shoulders of the mouse.

Mice

Non-inbred albino mice of the Ha/ICR strain (Sprague–Dawley, Madison, WI) were used as breeding stock. Pregnant mice were inspected at least twice daily and the mean age of the birth of a litter recorded as that time midway between inspections. Infant mice used in the following experiments were between 12 and 77 h of age and, as noted previously, quite sensitive to the lethal effect of the viruses used [12]. All litters used in a single experiment were distributed in such a manner that each experimental litter contained equal shares of mice born at the same time. The dams accepted all infant mice and appeared to cannibalize only those that were very ill or dead. Infected mice were observed once a day 7 days a week.

Nature of enteroviral disease

Echovirus type 9 and coxsackievirus type A9 produced a very similar disease involving spastic movements with a twitching tail on the 4th day after infection and frank paralysis from the 5th through the 8th day. After the 8th day no additional mice became paralyzed and by the 9th day surviving mice began to recover. It was not unusual for 20–30% of paralyzed control mice to recover and assume a normal weight by day 13. When the paralysis was severe the mice rapidly lost weight and died. The neck, shoulder and foreleg muscles consistently became paralyzed but hind limb paralysis often occurred. Death the first 4 days after infection was attributed to trauma. At the conclusion of the experiments on day 13 virtually all mice were either dead or healthy and the average weight

of the surviving mice was determined. All dead, missing and cannibalized mice from the 5th day onward were considered victims of the virus disease.

Mice infected with coxsackie A16 virus rarely survived the infection. Paralysis was detectable in the shoulder limb muscles and the mice rapidly died, frequently within 24 h of illness and even at times before illness was apparent. Ill or paralyzed mice were first detected on the 4th day after infection, but no deaths occurred at this time. Death occurred on the 5th day and continued into the 9th day. Any mice still healthy by the 9th day remained so until day 13 after virus infection when experiments were concluded.

Test compounds

The DL isomeric mixture of HBB was purchased from Aldrich Chemical Company (Milwaukee, WI, lot MC071857) as was guanidine-HCl (lot CD031497). Guanidine-HCl was completely soluble in distilled water at all concentrations used. HBB was solubilized for both in vivo and in vitro experiments in distilled water adjusted to pH 1 with 20% hydrochloric acid. The pH was then readjusted to 3.2 using a saturated sodium bicarbonate solution, a pH just short of producing any precipitate. For in vitro assays guanidine and HBB were further diluted in two-fold steps in serum-free cell culture medium.

LY122771-72, the *syn–anti* mixture of (6-[[(hydroxyimino)phenyl]methyl]-1-[(1-methylethyl)sulfonyl]-1*H*-benzimidazole-2-amine) and LY127123 (*trans* 1-[(methylethyl)sulphonyl]-6-(1-phenyl-1-propenyl)-1*H*-benzimidazole-2-amine) were provided by the medicinal chemists of the Lilly Research Laboratories both as the pure compounds and also adducted with PVP (polyvinylpyrrolidone) at the rate of 5 parts of PVP to every part of the test compound. For cell culture assays the pure compounds (PVP-free) were dissolved in dimethylsulfoxide (1 mg/ml, Aldrich Chemical Co.) and two-fold dilutions were made in cell culture medium free of serum. For animal experiments the PVP adducted test compounds were suspended in water. PVP is a well known, well tolerated blood expander that was shown to enhance the blood levels of LY122771-72 (J.F. Quay, J.F. Stucky II, C.J. Paget, K.S.E. Su and D.C. DeLong, Abstr. 18th I.C.A.A.C. 1978, abstr. 33) and also permits the preparation of a uniform suspension for injection. All batches of these compounds, adducted with PVP or not, were assayed in cell culture to be sure that they all produced the same inhibition of CPE at comparable concentrations. No activity was detected for PVP in vitro or in vivo. Because of limited quantities (*S,S*)-1,2-bis(5-methoxy-2-benzimidazolyl)-1,2-ethanediol dihydrochloride (A-37536, supplied by Abbott Laboratories, North Chicago, IL) was only tested in cell cultures but included for comparative purposes as still another established antipicornavirus compound [25]. The chemical structures other than A-37536 (which can be found in ref. 25) are illustrated in Fig. 1.

Treatment schedule

For all experiments except those summarized in Table 6, mice received 0.02 ml of

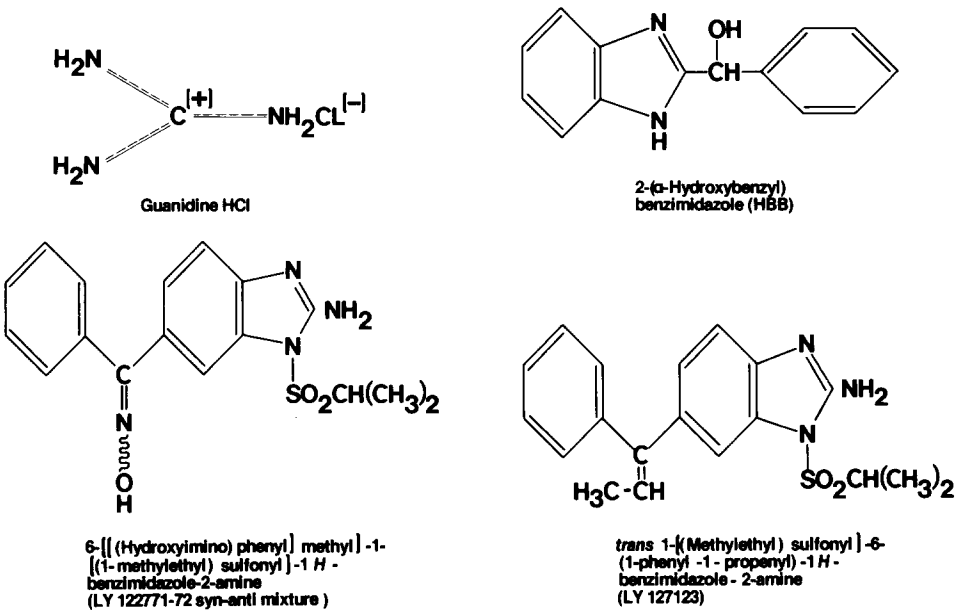

Fig. 1. Chemical structures of compounds with an effect on infections produced by coxsackie A9, A16 and echo 9 viruses in vitro and in vivo.

test compound 3 h before virus infection via the s.c. route, over the shoulders. Three hours after virus infection another 0.02 ml was injected with the next injection 7 h later. This latter injection was 0.03 ml per mouse as was the injection given 12 h later. From then on until the 8th day after virus infection, they received a treatment injection every 12 h, but it was 0.04 ml on days 2 and 3, then increased to 0.08 ml per mouse on days 4 through 8. All control mice were injected at the same times and with the same volumes using PVP, water at pH 3.2, or distilled water alone, depending on the experiment. Increasing the volumes of the injections was an arbitrary attempt to keep up with the rapid growth of the mouse for the first 4 days (about a 15–20% weight gain daily) after infection which has been shown to be the most rapid period for virus multiplication [5]. Daily weighings of 65 normal, infant mice showed that this strain averaged about 1.5 g when 24 h old, about 4 g on the 8th day and about 8 g on day 13, the day when the experiments were concluded. Handling infant mice was kept to a minimum; hence, they were not individually weighed twice a day as would be necessary to accurately determine the mg/kg/day of the compounds injected.

RESULTS

In vitro

Using 13 enterovirus serotypes it was found that guanidine was most active against

coxsackievirus A16, echovirus 6 and poliovirus type 2 (Table 1). HBB was somewhat more active against the enteroviruses than was guanidine, with the exception of coxsackievirus A16 and poliovirus type 1 where no HBB effect could be detected at concentrations tolerated by the cells. LY122771-72 was highly active against all the enteroviruses studied but least active against poliovirus type 2 and echovirus type 7. Similar activities were reflected by compounds LY127123 and A37536. All three of these complex benzimidazoles showed the least activity against poliovirus 2, while both guanidine and HBB produced their greatest effect on this virus. A37536 was very similar in its potency in cell cultures to LY122771-72, while LY127123 was somewhat less active. The inactivity in vitro of HBB against coxsackievirus A16 has been noted before and considered antiviral only to type 9 of the A coxsackieviruses [28].

No effect was detected with LY122771-72 at a maximum tolerated dose (4 μg/ml) on CPE produced by herpes simplex viruses, types 1 and 2, adenoviruses types 1, 2, 3 and 4, mumpsvirus, respiratory syncytial virus or parainfluenzavirus type 2. The antiviral activities of HBB and guanidine against many picornaviruses have been reported [6,9,26,28].

What Eggers and Tamm have called a mutual synergistic activity of HBB and guanidine [7] was confirmed (Table 2). As little as one-eighth the minimum active dose of guani-

TABLE 1

Comparative inhibition of enteroviral cytopathic effect by five compounds

Virus	Lowest concentration in μg/ml inhibiting CPE caused by 100 $TCID_{50}$				
	Guanidine-HCl	HBB	LY122771-72	LY127123	A37536
Coxsackie A9	167[a]	22	0.26	0.26	0.26
Coxsackie A16	42	>88	0.26	0.52	0.26
Coxsackie B1	167	44	0.26	0.26	0.13
Coxsackie B2	167	44	0.13	0.13	0.13
Coxsackie B3	167	44	0.26	0.26	0.26
Coxsackie B4	167	44	0.26	0.52	0.26
Coxsackie B6	83	44	0.13	0.13	0.13
Echo 6	42	22	0.26	0.26	0.26
Echo 7	167	44	0.52	1.04	0.52
Echo 9	83	22	0.26	0.52	0.26
Polio 1[b]	83	>88	0.26	0.26	0.26
Polio 2	42	11	0.52	1.04	1.04
Polio 3[b]	83	88	0.13	0.26	0.13
Tolerated level	250	88	4.5	2.0	9.0
Toxic level	334	176	9.0	4.5	>9.0

[a] From one to seven separate assays were performed for each determination with an overall average of 3. For coxsackie A9, A16 and echo 9 viruses the average was 4.

[b] These viruses were sensitive to 40°C.

TABLE 2

Enteroviral inhibitory concentrations of mixtures of HBB and guanidine and these compounds with LY122771-72 and LY127123

Compounds	Lowest concentration in μg/ml inhibiting CPE caused by	
	Echovirus 9	Coxsackievirus A9
HBB + 38.5 μg/ml guanidine	2.4[a,b]	4.8
HBB + 19.2 μg/ml guanidine	4.8	9.6
HBB + 9.6 μg/ml guanidine	2.4, 4.8	9.6
Guanidine + 4.8 μg/ml HBB	9.6	38.5
LY122771-72 + 38.5 μg/ml guanidine	0.48	0.24, 0.12
LY122771-72 + 19.2 μg/ml guanidine	0.48	0.48, 0.24
LY122771-72 + 4.8 μg/ml HBB	0.48	0.48, 0.24
LY127123 + 38.5 μg/ml guanidine	0.48	0.48, 0.24
LY127123 + 4.8 μg/ml HBB	0.48	0.48

[a] All concentrations were adjusted to reflect final culture volumes after addition of virus and two compounds (0.26 ml).

[b] From one to three assays were performed for each mixture with an overall average of 2.6 assays.

dine mixed with slightly less than one-fourth the minimum active dose of HBB resulted in complete inhibition of the CPE produced by 100 $TCID_{50}$ of echovirus 9. With coxsackievirus A9 'synergism' for HBB was seen best when one-fourth the minimum active dose of guanidine was used. When HBB was used at one-fourth the minimum inhibitory level, then from one-fourth to one-eighth the minimum inhibitory dose of guanidine was required to produce inhibition of the CPE caused by these viruses. Although the results can be somewhat variable, HBB and guanidine have no similar effect when mixed with either LY122771-72 or LY127123.

The highest tolerated doses of the various compounds were determined in cell culture by noting the absence of any change in the normal cell morphology (Table 1), a method well established by Sidwell and Huffman [27]. It was also determined that DMSO was well tolerated at a 4% level which is well above the concentrations used in tests with LY122771-72 or LY127123 (no more than 1.04%). These latter compounds were also tested as adducts with PVP and the cells tolerated the same concentrations.

Echovirus type 9 mouse infections

When HBB and guanidine were mixed together and infant mice treated using the previously described schedule, they were significantly protected from death caused by echovirus 9 (Table 3) as previously reported [5]. It has also been reported that neither HBB nor guanidine alone had an influence on mice infected with this virus [5], so it was a surprise when it was found that both were effective treatments where even a dose response could be established for HBB.

LY122771-72 and LY127123 were also active against echovirus type 9 infections in mice (Table 3). A low virus dose produced the superior result with LY122771-72. LY-127123 was somewhat more toxic than LY122771-72, but if either compound was used at levels higher than shown then frequently a few mice died early in the experiments.

TABLE 3

Effect of treatment with guanidine, HBB, LY122771-72, LY127123 and guanidine mixed with HBB on echovirus 9-induced deaths in infant mice

Compound	Mg/ml[a]	mM	No. dead/No. infected		*P* value[b]
			Treated	Control	
Guanidine	4.78	50	3/19[c]	15/20	<0.001
HBB	3.36	15	0/29[d]	25/31	<0.001
	1.68	7.5	3/23[d]	17/20	<0.001
	0.84	3.8	12/12[d]	9/10	NS[g]
HBB and guanidine	3.36 4.78	15 50	0/33[c]	20/45	<0.001
HBB and guanidine	0.84 2.39	3.8 25	0/10[e]	7/10	<0.001
LY122771-72	3.36	9.3	0/39[f]	24/45	<0.001
LY127123	2.50	7.3	3/17[d]	17/20	<0.001

[a] Each mouse received 1.06 ml s.c. in 9 days (see Methods for volumes used daily).
[b] Chi-square test, 95% confidence limit, one to three experiments for each determination.
[c] 2–4 LD_{50} s.c.
[d] 4 LD_{50} s.c.
[e] 2 LD_{50} s.c.
[f] 1 LD_{50} s.c.
[g] Not significant.

Coxsackievirus A9 mouse infections

As reported [5], HBB treatment alone did have activity on coxsackievirus A9 disease, while guanidine had no effect (Table 4). The Griggs strain of coxsackievirus A9 was introduced into these experiments but was used at times at unintentionally high levels yet HBB treatment still produced healthy mice. The mixture of guanidine and HBB was significantly active against either strain of this virus. Both LY122771-72 and LY127123 were effective in preventing some deaths caused by coxsackie A9 virus.

Coxsackie A16 mouse infections

Using an intensive treatment program (see Methods), guanidine was found to be quite active in preventing coxsackievirus A16-induced death (Table 5) and was more active than either LY122771-72 or LY127123. These latter compounds did, however, produce significant inhibition of death. HBB was inactive when used at a maximally tolerated dose.

Since it has been established that HBB enhanced the therapeutic activity of guanidine in mice infected with coxsackievirus A9 and echovirus 9, this combination was also tested in infections with coxsackievirus A16, even though HBB alone was inactive. Using 25 mM solutions of guanidine alone produced a significant activity which seemed improved when HBB was added ($P < 0.02$) (Table 5). Lowering the concentration of

TABLE 4

Effect of treatment with guanidine, HBB, LY122771-72, LY127123 and guanidine mixed with HBB on coxsackievirus A9-induced deaths in infant mice

Compound	Mg/ml[a]	mM	No. dead/No. infected[b]		*P* value[c]
			Treated	Control	
Guanidine	4.78	50	24/30	28/32	NS[d,e]
HBB	3.36	15	16/41	39/43	<0.005[f]
HBB and guanidine	3.36 4.78	15 50	4/30	36/43	<0.001[e]
LY122771-72	3.36	9.3	8/29	29/30	<0.001
LY127123	2.50	7.3	9/24	29/30	<0.001[e]

[a] Each mouse received 1.06 ml s.c. for 9 days (see Methods for volumes used daily).

[b] 10 LD_{50} s.c.

[c] Chi-square test, 95% confidence limit, three experiments for every determination.

[d] Not significant.

[e] One of these experiments was with the Griggs strain (50 LD_{50}).

[f] Two of these experiments were with the Griggs strain (50 LD_{50}).

guanidine to 12.5 mM produced no effect on the virus-induced death rate in the presence of HBB.

Comprehensive experiments were undertaken to find the minimal treatment schedule necessary to produce a statistically significant effect on the coxsackievirus A16 death rate, and it was found that treatment 3 h before virus infection could be eliminated (Table 6). It was then found, not surprisingly, that generally the earlier treatment was started, and the longer it was maintained, the greater the number of mice saved from death. When the first guanidine injection was delayed for 58 h after virus infection and then continued twice a day for 6 days, a highly significant therapeutic effect could be shown. Further, shortening the treatment time to only 5 days and even to 4 days still resulted in a significant effect against lethal doses of virus. Even a brief 3 day treatment affected the death rate, but the treatment had to be started the first day after virus infection.

Toxicity studies

Mice weighing about 1.4 g were injected a single time (s.c.) with two-fold concentrations of each compound to determine the 50% lethal dose (LD_{50}) [23]. The LD_{50} for

TABLE 5

Effect of treatment with guanidine, HBB, LY122771-72, LY127123 and guanidine mixed with HBB on coxsackievirus A16-induced deaths in infant mice

Compound	Mg/ml[a]	mM	No. dead/No. infected		*P* value[b]
			Treated	Control	
Guanidine	4.78	50	3/32[c]	29/30	<0.001
	2.39	25	23/30[c]	31/31	<0.005
HBB	3.36	15	18/19[d]	20/22	NS[g]
HBB and guanidine	3.36	15	14/31[e]	28/31	<0.001
	2.39	25			
LY122771-72	3.36	9.3	16/23[f]	22/22	<0.005
LY127123	2.50	7.3	15/23[f]	22/22	<0.005

[a] Each mouse received 1.06 ml s.c. for 9 days (see Methods for volumes used daily).
[b] Chi-square test, 95% confidence limit, two to three experiments for each determination.
[c] 11–33 LD_{50} s.c.
[d] 5–16 LD_{50} s.c.
[e] 5–33 LD_{50} s.c.
[f] 5–11 LD_{50} s.c.
[g] Not significant.

guanidine-HCl was 298 mg/kg, for HBB (acid-solubilized, pH 3.2) 129 mg/kg, for LY-122771-72 (adducted with 5 parts of PVP) 140 mg/kg and for LY127123 (5 parts of PVP) 131 mg/kg. These results for the various benzimidazoles were similar, but this did not determine what could be expected when multiple doses were given. Many experiments using twice daily injections indicated that concentrations of any of the benzimidazoles higher than shown in Tables 3–5 would kill at least a few mice and influence weight gain of surviving mice. It seems reasonable, therefore, to assume that these benzimidazoles were used at or near the maximum well-tolerated doses.

Prior experimentation defined those levels of guanidine that were well tolerated by the mice over a 9 day treatment period, but did not permit a judgment regarding the toxicity of this compound when given over a brief 4 day period, so experiments were performed to define, 58 h after virus, those tolerated levels. It was found that a 125 mM (12 mg/ml) solution injected twice with 0.04 ml s.c. on the 3rd day after infection and with 0.08 ml twice on the 4th day killed more than half the mice. Of the remaining mice, two retained a healthy appearance for the entire 13 days, 10 survived both the guanidine treatment and the coxsackievirus A16 infection.

When the mice were treated twice daily with a 100 mM solution for 4 days, then again more than half died prematurely but again the mice surviving this treatment also survived the virus infection. Experiments with mice injected with 75 mM solutions for the entire 4 day period produced highly significant antiviral activity and no premature deaths. These data suggest that successful guanidine treatment of coxsackievirus A16 disease even with 50 mM solutions of guanidine (Table 6) represents a rather narrow therapeutic index. It has been reported that 200 mM of guanidine mixed with 20 mM of HBB when given twice daily for 10 days had no effect on weight gain of infant mice [5].

DISCUSSION

The inhibition of rhinoviruses by LY122771-72 has been reported [3,29] and the inhibition of enteroviruses has been described (D.C. DeLong, J.D. Nelson, C.Y.E. Wu, B. Warren, J. Wikel, J. Chamberlin, D. Montgomery and C.J. Paget, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, S128, p. 234). There are no published reports regarding LY127123. Previous data show that dogs given i.v. doses of LY122771-72 had antiviral concentrations in the blood, but, when compared to brain, lung and liver, muscle tissue had the lowest concentration (J.F. Quay, J.F. Stucky II, C.J. Paget, K.S.E. Su, D.C. DeLong, and J.D. Nelson, Abstr. 18th I.C.A.A.C. 1978, Abstr. 33). As is well known, the viruses used in this study attack the mouse muscle tissue; so based on their limited in vivo activity it seems likely that LY122771-72 and LY127123 were not reaching the infected muscle in adequate concentrations. It was encouraging, however, that they showed as much activity as they did, considering the highly lethal nature of these infections.

There are a number of differences between the work reported here and that of Eggers [5]. The virus strains were different, the mice were likely a different strain and were

TABLE 6

Effect of varying guanidine treatment on death of infant mice infected with coxsackievirus A16

First injection after infection (h)	Total No. of successive days treated[a]	Total mg/mouse[b]	Virus LD_{50} used	No. dead/No. infected		P value[c,d]
				Treated	Control	
3	8.5	4.992	3 &10	0/21	20/22	<0.001
10	8	4.896	3	7/21	17/21	<0.005
58	6	4.224	3 & 5	5/20	15/20	<0.005
10	5	2.592	3 & 5	8/21	21/23	<0.001
34	5	3.072	3,5 &10	12/32	28/32	<0.001
58	5	3.456	3 & 5	6/24	15/24	<0.01
10	4	1.824	3,5 &10	6/32	24/27	<0.001
34	4	2.304	5	14/24	23/25	<0.01
58	4	2.688	5,10&10	19/29	28/30	<0.01
10	3	1.056	5	15/21	21/21	<0.01
34	3	1.536	5 &10	16/20	17/20	NS[e]

[a] Injections twice daily 12 h apart.

[b] Dosage in μg/mouse/injection: 96, day 0; 144, day 1; 192, days 2 and 3; 384, days 4–8.

[c] Chi-square analysis, 95% confidence limit.

[d] Two to three experiments for each determination.

[e] Not significant.

somewhat older herein, and the schedules and volumes of injections were different. Further, the HBB used by Eggers was the D isomer of the water-soluble hydrochloride. This D isomer was reported as more than two times more active than the L isomer [13]; however, the DL isomeric mixture used in these studies was used at a higher concentration (15 mM vs. 10 mM). Despite these and likely other differences between the two studies, a mixture of HBB and guanidine prevented death in mice infected with coxsackie A9 and echo 9 viruses.

When studying the effect of HBB or guanidine alone, however, some differences in results occur between this study and that of Eggers [5]. In this study, HBB was quite active against echovirus 9 infections in mice, contrary to the prior investigation [5] where HBB was shown to be active only against coxsackievirus A9 infections. For this reason a special effort was made in this study to confirm and reconfirm that the virus serotypes used were as stated.

Surprisingly, guanidine significantly increased the number of survivors in echovirus 9- and coxsackievirus A16-infected infant mice. Previously, no antiviral activity could be detected in laboratory animals [2,4,5,16] and it was presumed that failure to demonstrate an in vivo effect was due to the rapid development of resistance [16]. In the studies reported here increasing volumes of a 50 mM solution were injected for 9 days,

hence, mice received 20% more guanidine than previously reported (5.07 mg vs. 4.02 mg) [5].

Guanidine has been used in humans for certain nervous disorders [17,18,22], since it is a releaser of acetylcholine [1,20]. When guanidine was given orally to dogs or rats, blood levels were reached in 15–30 min and then sharply decreased during the next 4 h [4], suggesting that the compound might then be found in the tissues. It was also reported that rats excreted in the urine about one-half the guanidine in 2 h. This would seem to be incompatible with the results reported here.

In the coxsackie A16 virus studies effective guanidine treatment could be brief, only 4 days, and could be delayed until at least 58 h after virus infection, a time when pathology can already be detected in the mouse muscle tissue [14]. Effective dosages of guanidine were, however, close to the maximum tolerated levels and much above oral doses used in humans (up to 50 mg/kg) [22]. Based on its in vitro potency guanidine is not a highly active antiviral compound but yet has pharmacokinetic properties that permit it to produce its full potential in vivo.

LY122771-72 (enviroxime) is presently being studied in five locations for its potential use against rhinovirus (also picornaviruses) infections in humans. One report [21] indicates that enviroxime when given intranasally 'has potential for clinical use'. By the oral route it produced troublesome but not serious side-reactions. This illustrates that, although high doses of the compounds studied herein were required to influence lethal virus disease in mice, it may not require such concentrations against the much milder diseases produced by picornaviruses in humans.

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