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PREVENTION OF DEATH IN MICE INFECTED WITH COXSACKIEVIRUS A16 USING GUANIDINE HCl MIXED WITH SUBSTITUTED BENZIMIDAZOLES

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A significant reduction in the death rate of infant mice infected with ten 50% lethal doses (LD_{50}) of coxsackievirus A16 was observed when they were treated 58 h after infection with two injections of guanidine at 145 mg/kg per injection. Tremors occurred at this level but disappeared after treatment was discontinued. Tremors were apparent, but less severe at 97 mg/kg per injection and did not occur at 48 mg/kg per injection. No antiviral effect could be detected at either of these levels of guanidine. When an inactive level of guanidine (97 mg/kg per injection) was combined with 1.7 mg/kg per injection of LY122771-72, LY127123, or 2-(α-hydroxybenzyl)benzimidazole (HBB) and 17 mg/kg per injection of 2-guanidino-benzimidazole (GB), significant activity resulted with 2–8 treatments begun 58 h after infection. The same treatment schedule using 136 mg/kg per injection of LY122771-72, 90 mg/kg per injection of LY127123, 136 mg/kg per injection of HBB and 68 mg/kg per injection of GB produced no effect. Guanidine-associated tremors were also enhanced by the addition of the substituted benzimidazoles. When guanidine was reduced to 48 mg/kg per injection, 34 mg/kg per injection of LY122771-72 was required to produce a significant reduction in the death rate and no tremors were observed.

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

INTRODUCTION

Prevention of death of infant mice infected with enteroviruses has been rarely reported. It was most convincingly shown, however, that 2-(α-hydroxybenzyl)benzimidazole (HBB), combined with guanidine hydrochloride, was a very effective treatment for mice given lethal doses of echovirus 9 or coxsackievirus A9 [3]. In this same study the data suggest that even HBB alone was effective treatment for infant mice infected with coxsackievirus A9. Guanidine, as observed previously, was not found to be an effective treatment (see Ref. 7).

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The potent antiviral effect of combinations of HBB and guanidine both in cell cultures and in infant mice was confirmed [7]. It was further found, in this same study, contrary to all prior published data, that guanidine HCl alone was effective in treating infant mice lethally infected with echo 9 and coxsackie A16 viruses. Further, guanidine was very effective even when treatment was brief and delayed until 58 h after virus infection. In producing this striking antiviral effect, a dose of guanidine was used that did not affect the normal development and weight gain of the mice but which was, nonetheless, close to a lethal level.

The following study was undertaken in an effort to improve the ratio of the effective antiviral dose of guanidine and its toxic dose by combining it with each of four substituted benzimidazoles, two of which themselves had no demonstrable activity against coxsackievirus A16 infections in vitro or in vivo. As will be shown, all four enhanced the antiviral effect of guanidine.

MATERIALS AND METHODS

Mice

The source of the breeder mice, the production of litters and the care and handling of the mice have been previously described [6,7]. Mice were infected when 24–52 h of age. As described before, all litters were taken from the dams and then distributed in such a manner that each litter contained the same number of mice of the same ages. After infection the mice were inspected once each day for 13 days at which time the experiments were concluded.

Virus

The G-10 strain of coxsackievirus A16 was provided by the Research Resources Branch of the National Institutes of Health and grown in this laboratory in fetal fibroblasts. The virus was then passaged twice in infant mice by using muscle tissue harvests from paralyzed mice that had been infected by the subcutaneous (s.c.) route. A single pool of mouse muscle tissue (10%) in serum-free cell culture medium was used for all experiments reported herein. All mice received ten 50% lethal doses (10 LD_{50}) of this previously titrated pool, diluted in cell culture medium, by s.c. injection, over the shoulders (0.02 ml). The production of paralysis and death by coxsackievirus A16 has been described [6,7].

Test compounds

The source, preparation, full chemical names and structures of HBB, guanidine, LY122771-72 (6-[{(hydroxyimino)phenyl}methyl]-1, 1-{(methylethyl)sulfonyl}-1H-benzimidazole-2-amine, a 50:50 mixture of zinviroxime and enviroxime) and LY127123

(*trans*-1-{(methylethyl)sulphonyl}-6-(1-phenyl-1-propenyl)-1H-benzimidazole-2-amine) have been previously reported [6,7]. In addition, 2-guanidino-benzimidazole (GB) was, like HBB and guanidine, obtained from Aldrich Chemical Co. and used as a water solution. The use of polyvinylpyrrolidone (PVP) as a suspending menstrum for the insoluble benzimidazoles LY122771-72 and LY127123, its use as a control material as well as the method for solubilizing HBB have been described previously [6,7].

Treatment

All treatments were started 58 h after virus infection. Two to eight treatments were given, 12 h apart. Just before a treatment injection, the litter was weighed and the average mouse weight determined. Injections were made s.c., over the shoulders, at the rate of 0.04 ml per g body weight of the mouse. The volumes were changed with every injection to keep up with the rapid weight gain of the infant mice (15–20% per day). Control animals were injected with distilled water or PVP in the same manner. This method permitted treating the mice with approximately the same mg/kg of the test compounds and also indicated if the infant mice showed normal weight gain during the treatment period.

RESULTS

It has been previously reported that the 50% lethal dose for guanidine, when given by the s.c. route to infant mice in a single injection, was 298 mg/kg [7]. When 8 injections were given using 193 mg/kg per injection, premature deaths were, however, readily observed in virus infected mice (Table 1). With only 4 injections, this lethal effect was less obvious. Even at 145 mg/kg, given twice each day for 4 days, some premature deaths were detected. With only 4 treatments at this level, however, there was a striking decrease in the number of deaths due to coxsackievirus A16. Three treatments were equally effective and even with only 2 treatments an antiviral effect could be detected. What was observed, but not measurable, were tremors that occurred within 15 min after treatment and lasted until the next 12 h injection. Infant mice have some basic tremors but the tremors seen were clearly greater and could be expected since guanidine is a known releaser of acetylcholine [1]. When the dose was decreased to 97 mg/kg per injection, the antiviral effect could no longer be detected even with 8 treatments. The tremors still occurred, however, but in this case lasted only about 4 h. Despite the subjective nature of this observation it is clear that an undesirable, if perhaps harmless, side reaction occurs even at drug levels that are not therapeutic. At 48 mg/kg per injection tremors were not detected.

It was previously shown that LY122771-72 was very active in vitro against coxsackievirus A16 [6,7]. Activity could also be produced in mice infected with this same virus but the treatment had to be started very shortly after infection [7]. When a maximum tolerated dose of LY122771-72 (that is a dose that had no influence on weight gain) was

TABLE 1

Effect of guanidine treatment on coxsackievirus A16 induced deaths in infant mice

mg/kg per injection	No. of treatments	Dead/total infected		*P* value[a]
		Treated	Control	
193	8	17/18[b]	16/18	ns[d]
193	4	4/9[c]	9/9	<0.01
145	8	8/19[c]	16/18	<0.001
145	4	1/22	11/11	<0.001
145	3	0/10	10/10	<0.001
145	2	14/20	20/20	<0.01
97	3–8	53/55	51/52	ns
97 + PVP[e]	4–8	32/32	40/40	ns
48[f]	8	21/21	18/19	ns

[a] χ^2 test.
[b] Many premature (toxic) deaths.
[c] A few premature (toxic) deaths.
[d] Not significant.
[e] Polyvinylpyrrolidone.
[f] Only treatment not inducing tremors.

TABLE 2

Effect of guanidine mixed with LY122771-72 on coxsackievirus A16 induced deaths in infant mice

mg/kg per injection		No. of treatments	Dead/total infected		*P* value[a]
Guanidine	LY122771-72		Treated	Control	
0	136[b]	4 and 8	22/23	23/23	ns[c]
97	13.6	4	2/20	22/22	<0.001
97	13.6	3	0/10	10/10	<0.001
97	13.6	2	9/11	10/10	ns
97	6.8	4	0/10	10/10	<0.001
97	3.4	4	2/9	10/10	<0.001
97	1.7	4	13/23	21/21	<0.001
97	0.85	4	6/10	10/11	ns
73	27.2	4	3/13	12/12	<0.005
73	13.6	4	8/13	12/12	<0.05
73	6.8	4	8/10	10/11	ns
48[d]	34	8	11/28	28/28	<0.001
48	27.2	8	19/20	19/19	ns

[a] χ^2 test.
[b] Maximum tolerated dose.
[c] Not significant.
[d] Only concentration producing no obvious tremors.

used, it alone had no effect on infected mice when treatment was started 58 h after infection (Table 2). When LY122771-72 was combined with guanidine (at a level that does not produce an antiviral effect) then the combination was quite active, even with as little as 1.7 mg/kg per injection. If 13.6 mg/kg was used, then only three treatments were required to save all the animals but two treatments were ineffective.

Lowering guanidine levels to 73 mg/kg required a substantial increase in LY122771-72 and when guanidine was lowered even further, even more LY122771-72 was required. Again, no tremors were noted when guanidine was used at 48 mg/kg per injection. What was observed, again in a subjective manner, was that tremors caused by higher levels of guanidine were more severe and more prolonged when the benzimidazole was added.

In studying a closely related compound to LY122771-72, LY127123 (Table 3), it was again found that only 1.7 mg/kg per injection had to be combined with 97 mg/kg of guanidine to produce significant antiviral activity. In this case, however, lowering guanidine to 73 mg/kg with addition of 27.2 mg/kg of LY127123 produced no activity. This compound was somewhat more toxic than LY122771-72 so the maximum tolerated dose was lower but it, too, produced no activity despite the fact that it was quite active against coxsackievirus A16 in vitro and also active in vivo if treatments were begun soon after infection [6,7].

So far, HBB has not been demonstrated, using tolerated levels, to be active against coxsackievirus A16, either in vitro or in vivo [6,7] (Table 4). Yet here, too, when only 1.7 mg/kg was added to an inactive level (97 mg/kg) of guanidine, then significant activity could be detected using four treatments but not when guanidine was lowered to 73 mg/kg per injection. Again, in this case, as with LY127123, HBB enhanced the tremors. It

TABLE 3

Effect of treatment with guanidine mixed with LY127123 on coxsackievirus A16 induced deaths in infant mice

mg/kg per injection		No. of treatments	Dead/total infected		*P* value[a]
Guanidine	LY127123		Treated	Control	
0	90[b]	4	9/11	10/10	ns[c]
97	13.6	4	2/11	11/11	<0.001
97	13.6	3	4/11	11/11	<0.005
97	13.6	2	10/13	11/11	ns
97	6.8	4	4/11	10/10	<0.001
97	3.4	4	2/10	10/10	<0.001
97	1.7	4	7/21	21/21	<0.001
97	0.85	4	12/12	10/10	ns
73	27.2	4	8/11	10/10	ns

[a] χ^2 test.
[b] Maximum tolerated dose.
[c] Not significant.

TABLE 4

Effect of treatment with guanidine mixed with 2-(α-hydroxybenzyl)-benzimidazole (HBB) on coxsackievirus A16 induced deaths in infant mice

mg/kg per injection		Dead/total infected		*P* value[a]
Guanidine	HBB	Treated	Control	
0	136[b]	12/12	10/10	ns[c]
97	13.6	0/11	10/10	<0.001
97	13.6[d]	8/11	10/10	ns
97	6.8	14/21	21/21	<0.005
97	3.4	8/12	10/10	<0.05
97	1.7	4/10	10/10	<0.005
97	0.85	9/9	9/9	ns
73	27.2	10/10	9/9	ns

[a] χ^2 test.
[b] Maximum tolerated dose.
[c] Not significant.
[d] This experiment used 3 treatments. All the rest were 4 treatments.

should be noted that HBB-guanidine mixtures were not effective when only three treatments were given.

HBB is a widely accepted antiviral substance, active against many picornaviruses but only against coxsackievirus A9 of the coxsackieviruses [9]. Even though no activity was detected for HBB against coxsackievirus A16 in vivo or in vitro [6], the question has been raised whether HBB may still be antiviral against certain picornaviruses but at levels that are obscured by toxic effects on cells in culture [5]. Because of this possibility, still a fourth benzimidazole was studied. A literature search found no reference to 2-guanidinobenzimidazole having any activity against any virus nor was it found active in these studies either in vitro or in vivo against coxsackievirus A16 infections. Nonetheless, when combined with guanidine at inactive levels, in vivo activity could be detected (Table 5).

The 50% therapeutic level of each of the four benzimidazoles combined with 97 mg/kg of guanidine was calculated by the method of Reed and Muench [8]. LY122771-72 and LY127123 were similar and most effective with calculated 50% therapeutic levels of 2.0 and 1.8 mg/kg, respectively. The value for HBB was 6.2 while that for GB was 29.9. When guanidine was used at 73 mg/kg per injection, then LY122771-72 had a 50% therapeutic level of 15.8 mg/kg while the other three benzimidazoles were required in excess of 27.2 mg/kg per injection.

DISCUSSION

Of the four benzimidazoles tested, LY122771-72 seems to be the most active when combined with guanidine. Indeed, it seems that such a combination can aid in the

TABLE 5

Effect of treatment with guanidine mixed with 2-guanidino-benzimidazole (GB) on coxsackievirus A16 induced deaths in infant mice

mg/kg per injection		Dead/total infected		*P* value[a]
Guanidine	GB	Treated	Control	
0	68[b]	11/11	10/10	ns[c]
97	68	8/12[d]	12/12	<0.05
97	34	7/24	22/22	<0.001
97	17	6/11	10/10	<0.02
97	8.5	8/11	10/10	ns

[a] χ^2 test.
[b] This was a well tolerated dose started 10 h after infection then every 12 h for 15 treatments. All other results are for 4 treatments.
[c] Not significant.
[d] Some mice died prematurely (toxic).

elimination of an undesirable side effect of guanidine. The significant question now arises whether it is even necessary that the second agent be antiviral. The enhancement of and prolongation of guanidine-induced tremors, the low levels of the benzimidazoles required and the fact that at least two of the benzimidazoles have not been proven active against coxsackievirus A16 suggests at least a portion of the effect of the combined compounds involves an influence on the pharmacokinetics of guanidine. Although there is no evidence yet to support this concept, it still seems reasonable to assume that in some manner benzimidazoles are preventing the excretion of guanidine which normally is very rapid without any metabolic changes in the compound [2].

Realistically, guanidine is not a very potent antiviral substance because high, not far from lethal levels, are required in vitro and in vivo to produce antiviral effects. Guanidine seems, however, to exhibit superior pharmacokinetics in infant mice infected with coxsackie A16 virus, pharmacokinetics that may be enhanced by incorporation of benzimidazoles. Even with such advantages, it seems unlikely that guanidine has the inherent potency required of a useful antiviral drug. Nonetheless, some consideration should be given to the improvement of potential or actual antiviral drugs by the addition of a second well tolerated agent that can enhance the pharmacokinetics of an antiviral drug or even neutralize or block any undesirable side reactions.

Even though guanidine may not be useful in human enteroviral disease, the in vivo data resulting from treatment by this compound are encouraging. It is possible to wait for at least 58 h after virus infection, a time when virus has reached a level of 10^6 per g body weight of mouse [7], and still get an antiviral effect with as little as two treatments 12 h apart and all of this in highly lethal picornavirus infections, infections that are much more severe than observed in humans. Further, it is possible to enhance this effect by the addition of a second compound. When viewing such striking in vivo antiviral

effects, it is tempting to suggest that there seems no fundamental reason that enterovirus infections in humans cannot eventually be effectively treated with suitable drugs.

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