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Antibacterial Activity of 2',3'-Dideoxyadenosine In Vivo and In Vitro

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2',3'-Dideoxyadenosine (DDA) was shown not only to possess antibacterial activity in vitro against a variety of *Enterobacteriaceae*, but also to be effective in vivo. DDA was active in experimental mouse infections by the oral route against 5 *Salmonella* strains, 2 of 3 *Arizona* strains, 5 of 7 *Citrobacter* strains, 3 of 8 *Klebsiella* strains, 3 of 5 *Escherichia* strains, 1 of 3 *Shigella* strains, and 3 of 15 *Serratia* strains at concentrations generally well below the toxic level. Closely related compounds, with the exception of 2',3'-dideoxyinosine, were found to be inactive in vivo, indicating that a high degree of structural specificity was required for activity. The synthesis of deoxyribonucleic acid was inhibited by DDA in those strains susceptible in vitro to DDA, whereas ribonucleic acid and protein syntheses were not affected. The concentration of DDA which inhibited bacterial deoxyribonucleic acid synthesis by 50% was calculated based on the relative rates of deoxyribonucleic acid synthesis in the absence and in the presence of DDA. This value correlated well with the minimal inhibitory concentration determined by the in vitro broth dilution assay but not always with in vivo activity determined by the mouse protection test.

Adenine nucleoside and nucleotide analogs have been shown to be capable of interfering with deoxyribonucleic acid (DNA) synthesis in bacteria, bacteriophages, mammalian viruses, and cancerous and normal mammalian cells. One substance which has been well studied is 2',3'-dideoxyadenosine (DDA) (5, 8, 10, 12, 13, 17). Studies with DDA have been carried out primarily by using this substance as a tool to better understand the mechanism(s) by which DNA is synthesized in bacteria (8, 17), mammalian viruses (1), and bacteriophages (10, 12) and the effect of other adenosine nucleosides on DNA synthesis in mammalian cells (5, 13).

No information is available, however, concerning the potential use of DDA as an antibacterial agent. During the course of testing a series of purine nucleosides and nucleotides for antimicrobial activity, it was found that DDA not only possessed in vitro antibacterial activity but surprisingly was also effective in vivo in mouse protection studies against several *Salmonella* strains at doses well below the toxic level. Because of the in vivo activity, more extensive studies were carried out with DDA and related compounds to: (i) determine the structural specificity required for activity; (ii) investigate the spectrum of antibacterial activity of DDA; and (iii) determine whether a significant inhibition of DNA synthesis occurred only in bacterial strains which were susceptible in vitro or in vivo or both to DDA and, if so, was there a correlation between the inhibition of DNA synthesis and the observed in vitro or in vivo activity or both.

MATERIALS AND METHODS

Chemical agents and antibiotics. DDA, 2',3'-dideoxyinosine, 2',3'-dideoxyguanosine, *N,N*-dimethyl-2',3'-dideoxyadenosine, 2',3'-dideoxyadenosine-5'-monocarboxymethylphosphonic acid disodium salt (0.7 M hydrate), and 3'-deoxyadenosine (Cordycepin) were prepared in the Chemical Research Department of Hoffmann-La Roche Inc., Nutley, N.J. Adenosine-5'-phosphoric acid, adenosine, guanosine, and inosine were obtained from commercial suppliers. Commercial preparations of nalidixic acid (Mann Research Laboratories, New York, N.Y.), amoxicillin (Hoffmann-La Roche Inc.), mecillinam (Leo Pharmaceuticals, Copenhagen, Denmark), and gentamicin (Schering Corp., Bloomfield, N.J.) were used. All agents were dissolved or suspended in distilled water for in vitro testing and subcutaneous or intraperitoneal administration to mice and in 0.1% carboxymethyl cellulose for oral administration to mice. All agents were prepared on the day of testing and stored at 4°C until used. L-Leucine and uracil were obtained from Sigma Chemical Co., St. Louis, Mo., and Calbiochem, LaJolla, Calif., respectively. [^{14}C]leucine (354 mCi/mmol) and [^{14}C]uracil (56.5 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, Ill., and New England Nuclear Corp., Boston, Mass., respectively.

Bacteria. The following members of the *Enterobacteriaceae* were used: *Salmonella* (5 strains), *Shigella* (3 strains), *Citrobacter* (7 strains), *Arizona* (3 strains), *Escherichia* (5 strains), *Klebsiella* (8 strains), *Serratia* (15 strains), and *Proteus* (3 strains). In addition, one strain each of *Staphylococcus aureus*,

Streptococcus pyogenes, and *Streptococcus pneumoniae*, four strains of *Haemophilus influenzae*, and three strains of *Pseudomonas aeruginosa* were tested. The strains used were clinical isolates, except for the following: *Salmonella schottmuelleri*, *Salmonella typhi* P58A, *Salmonella typhimurium*, *Klebsiella pneumoniae* A, *Escherichia coli* 257, the three gram-positive strains, and one strain of *P. aeruginosa*.

In vitro tests. Serial twofold broth dilution tests were carried out in 1-ml volumes of the appropriate medium. All bacterial cultures were grown and tested in vitro in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.), with the following exceptions: the four *H. influenzae* strains were propagated and tested in brain heart infusion broth (BBL Microbiology Systems) containing 2.5% supplement C (Difco Laboratories, Detroit, Mich.), and for cultivation and testing of *S. pyogenes* and *S. pneumoniae*, TSB was supplemented with 10% goat serum. For members of the *Enterobacteriaceae*, *P. aeruginosa*, and *S. aureus*, the inoculum consisted of 0.05 ml of a 10^{-3} dilution of an overnight broth culture. For tests with *S. pyogenes*, *S. pneumoniae*, and *H. influenzae*, a 10^{-1} dilution was used. TSB, according to the manufacturer's specifications, is free of nucleosides but contains 1.3 g of L-leucine per liter. Additional studies were also carried out to determine the minimal inhibitory concentration (MIC) of selected strains in the chemically defined Davis A medium, which contains the following (in grams per liter): K_2HPO_4, 7; KH_2PO_4, 3; $(NH_4)_2SO_4$, 1; $MgSO_4$, 0.048; glucose, 10; and sodium citrate, 0.5.

After incubation overnight at 37°C, the tubes were examined for growth as indicated by turbidity, and the MIC value was determined. Tubes containing no visible growth were subcultured by streaking 0.05 ml of the broth onto the appropriate agar. The minimal bactericidal concentration (MBC) was the lowest concentration in a culture from which <10 colonies grew.

Effect of DDA on DNA, RNA, and protein syntheses. The procedure used for the determination of DNA, ribonucleic acid (RNA), and protein syntheses was that of Roodyn and Mandel (15).

Quantitation of inhibition of DNA synthesis. The degree of inhibition of DNA synthesis was quantitated by comparing the rate of DNA synthesis in the bacterial cultures exposed to DDA with that in the controls lacking DDA. The rates were calculated with data from samples taken during the first 20 or 30 min of incubation with the radioactive precursor, since the rate of incorporation was relatively constant up to these times. The inhibitory effect on DNA synthesis could be quantitated with an r value, which is the ratio of the rate of synthesis in the presence of DDA to that in its absence. The r values for all of the strains tested ranged from 0.09 to 0.83, corresponding to a 91 to 17% inhibition of DNA synthesis, respectively.

Correlation of DNA inhibition with DDA concentration. Cultures were grown in TSB or Davis A medium and exposed to different concentrations of DDA. DNA synthesis was monitored and compared with that of the control culture lacking DDA. The r values were calculated at each DDA concentration and plotted against the logarithm of the DDA concentration. The line fitted to each set of points by a least-squares analysis was then used for estimating the concentration of DDA in micrograms per milliliter which inhibited DNA synthesis by 50% (C_{50}).

In vivo tests. (i) Toxicity. The acute toxicity of DDA was determined in Swiss albino mice weighing 18 to 20 g by administering a single oral, intraperitoneal, or subcutaneous dose. The animals were observed for 72 h, and the 50% lethal dose was calculated by the method of Reed and Muench (14).

(ii) Systemic infections. All experiments were carried out in Swiss albino mice weighing 18 to 20 g. The animals were infected intraperitoneally with 0.5 ml of appropriate diluted suspensions of overnight cultures containing 100 to 1,000 minimal lethal doses. Infecting inocula for *S. pyogenes*, *S. pneumoniae*, and *K. pneumoniae* A were diluted in TSB. Infecting inocula for all other bacteria were prepared in 5% mucin.

The animals were treated orally or subcutaneously with graded doses of DDA or related substances in 1-ml amounts. Six animals were used for each dosage level, and each experiment in which activity was observed was repeated two or more times. The animals were treated at the following times relative to infection: immediately after infection and 5, 24, 29, 48, and 72 h postinfection.

The total number of mice surviving at each dose for 14 days was used to calculate the 50% protective dose (PD_{50}) by the method of Reed and Muench (14). The PD_{50} values, therefore, represent the average of at least three separate experiments.

RESULTS

Acute toxicity. The 50% lethal dose values in mice were 5,000, 1,320, and >500 mg/kg when DDA was administered by the oral, subcutaneous, and intraperitoneal routes, respectively. The acute toxicities of the other DDA-related compounds were not determined because of an insufficient amount of compound.

Cytoxicity. The results of cytoxicity studies (data not shown) with DDA on human amnion cells demonstrated that DDA was tolerated at ≥1,000 μg/ml.

Structural specificity for in vivo activity. The results of experiments on structural specificity for in vivo activity are shown in Table 1. DDA and 2',3'-dideoxyinosine were active orally. DDA showed the same effect subcutaneously against the two *Salmonella* species. 2',3'-Dideoxyguanosine, *N*,*N*-dimethyl-2',3'-dideoxyadenosine, 3'-deoxyadenosine (Cordycepin), and 2',3'- dideoxyadenosine-5'-monocarboxymethylphosphonic acid disodium salt were inactive at the highest doses tested by the oral route and also when tested by the subcutaneous route. In addition to these six substances, adenosine-5'-phosphoric acid, adenosine, guanosine, and inosine were evaluated in vivo for oral activity against the two *Salmonella* species. All substances were inactive at doses ranging from 250 to 1,000 mg/kg.

In vivo and in vitro activities. DDA was found to be orally active (PD_{50}, ≤250 mg/kg)

TABLE 1. *In vivo antibacterial activity of DDA and related substances*

Compound	PD_{50} (mg/kg)[a]			
	S. typhi		*S. schottmuelleri*	
	Oral	Subcutaneous	Oral	Subcutaneous
DDA	69	65	31	44
2',3'-Dideoxyinosine	68		108	
2',3'-Dideoxyguanosine	>250		>250	
N,N-Dimethyl-2',3'-dideoxyadenosine	>200	>200	>200	>200
3'-Deoxyadenosine (Cordycepin)	>250	>250	>250	>250
2',3'-Dideoxyadenosine-5'-monocarboxymethyl-phosphonic acid disodium salt	>250		>250	

[a] The treatment schedule was as described in the text. Three experiments were carried out with six animals per dose for the calculation of PD_{50} values.

against the 5 *Salmonella* strains, 2 of 3 *Arizona* strains, 5 of 7 *Citrobacter* strains, 3 of 8 *K. pneumoniae* strains, 3 of 5 *E. coli* strains, 1 of 3 *Shigella* strains, and 3 of 15 *Serratia marcescens* strains. No activity was seen against the three *Proteus*, four *Haemophilus*, and three *Pseudomonas* strains or the three gram-positive bacteria tested. In a limited number of tests in which DDA was administered by the subcutaneous route, the results were similar to those observed after oral treatment.

With the exception of 3 strains of *S. marcescens* and 1 strain of *E. coli*, the 18 strains which responded in vivo had MIC values of 62.5 to 250 μg/ml. The MBC values for the 18 strains were equivalent or at most fourfold higher than the MIC values. The one *E. coli* strain (503-455) had a PD_{50} value of 42 mg/kg and an MIC value of 500 μg/ml in TSB. The three *Serratia* strains (S966, S303, and S714) all had PD_{50} values of <150 mg/kg but MIC values of ≥1,000 μg/ml. Of the 37 strains that did not respond to DDA in vivo, the MIC values for 19 strains were 250 μg or less per ml, and the MBC values were equivalent or at most fourfold higher, whereas the MIC and MBC values for the remainder were >250 μg/ml.

Correlation of DNA inhibition with in vitro and in vivo activities. The effects of DDA on protein, RNA, and DNA syntheses were determined for 14 strains selected on the basis of their in vitro and in vivo responses to DDA (Table 2). In no case did DDA cause a significant inhibition of protein or RNA synthesis.

As shown in Table 2, the 14 strains selected could be divided into four general classes on the basis of their in vitro and in vivo susceptibilities to DDA. The organisms susceptible both in vitro and in vivo to DDA (strains A to F) had MIC values in both media ranging from 63 to 125 μg/ml, with the exception of *E. coli* 503-455, which in TSB had an MIC value of 500 μg/ml, and low r_{225} values (0.10 to 0.29) in both media and, hence, correspondingly low C_{50} values (19 to 60 μg/ml). The PD_{50} values were also relatively low (12 to 155 mg/kg). The second group of organisms (strains G to J) were classified as susceptible to DDA in vitro (MIC values, 16 to 125 μg/ml), with r_{225} values ranging from 0.09 to 0.33 and C_{50} values ranging from 17 to 78 μg/ml, but were resistant in vivo (PD_{50} values, >250 mg/kg). The third group of organisms (strains K and L) were quite interesting since the MIC values indicated resistance in vitro (≥1,000 μg/ml) and the r_{225} and C_{50} values were correspondingly high (0.43 to 0.56 and 144 to 337 μg/ml, respectively), but the two strains were susceptible in vivo (PD_{50} values, 73 and 104 mg/kg). The last group (strains M and N) could be classified as resistant to DDA in all tests. They had high PD_{50} values (>250 mg/kg), high MIC values (≥500 μg/ml), and high r_{225} values (0.46 to 0.83), with correspondingly high C_{50} values (181 to 1,750 μg/ml).

Although the addition of DDA had no apparent effect during the 1-h incubation period on the growth rate of the bacterial cultures as measured by optical density, it had a profound effect on their morphology, causing the formation of long filaments.

Synergy. When DDA was combined in a 1:1 ratio with nalidixic acid, amoxicillin, mecillinam, or gentamicin and tested in vivo against *Citrobacter freundii* 10 or *Shigella flexneri* LA, no synergy was observed with any of the combinations (data not shown).

DISCUSSION

In the testing of a series of adenosine nucleosides and nucleotides for antimicrobial activity, it was shown that DDA not only possessed activity in vitro against a variety of gram-negative bacteria but also was effective in vivo in mouse infections. The spectrum of in vivo activity was not sufficiently broad for DDA to compete with antimicrobial agents such as nalidixic acid, amoxicillin, and gentamicin. However, the doses at which protection was generally observed (i.e., from 12 to 219 mg/kg) were well below the acute

TABLE 2. *Susceptibility of bacterial strains to DDA in vitro and in vivo*

Susceptibility to DDA		Organism	MIC (μg/ml)		PD_{50} (mg/kg, orally)	Inhibition of DNA synthesis			
						r_{225}[a]		C_{50} (μg/ml)	
In vitro	In vivo		TSB	DAM[b]		TSB	DAM	TSB	DAM
S[c]	S	A. *Citrobacter diversus* CDC 1969-72	63	63	12	0.13	0.10	22	19
		B. *K. pneumoniae* 503-964	63	63	37	0.26	0.17	52	30
		C. *E. coli* 1	63	63	91	0.24	0.21	45	37
		D. *C. freundii* 10	125	125	69	0.15	0.20	25	35
		E. *E. coli* 9306	125	125	155	0.26	0.24	50	46
		F. *E. coli* 503-455	500	63	42	0.29	0.17	60	28
S	R	G. *Citrobacter* spp. TC-1	31	16	>1,000	0.26	0.16	50	27
		H. *C. freundii* CDC 6	63	16	>1,000	0.09	0.10	17	19
		I. *K. pneumoniae* 4964	125	125	>250	0.33	0.23	78	43
		J. *E. coli* 257	125	63	>500	0.25	0.16	48	26
R	S	K. *S. marcescens* S303	1,560	1,000	73	0.52	0.48	261	199
		L. *S. marcescens* S714	1,560	1,000	104	0.56	0.43	337	144
R[d]	R	M. *P. aeruginosa* 8710	1,560	500	>250	0.83	0.46	1,750	181
		N. *S. marcescens* S500	3,125	6,250	>250	0.64	0.68	552	678

[a] r_{225}, DNA synthesis in the presence of DDA at 225 μg/ml as a fraction of DNA synthesis in the absence of DDA.

[b] DAM, Davis A medium.

[c] Susceptible in vitro, ≤250 μg/ml; susceptible in vivo, ≤250 mg/kg.

[d] Resistant in vitro, >250 μg/ml; resistant in vivo, >250 mg/kg.

50% lethal dose (5,000 mg/kg) for the substance when administered orally. The relative nontoxic nature of the substance in mice and in human cell cultures and its intrinsic in vivo antibacterial activity suggested that, by combining DDA with another agent which selectively inhibited DNA synthesis (e.g., nalidixic acid) or protein synthesis (e.g., gentamicin) or with substances which would interfere with cell wall synthesis (e.g., amoxicillin and mecillinam) and so decrease penetration barriers, the activity of DDA in vivo might be broadened. Combinations of DDA with nalidixic acid, gentamicin, amoxicillin, or mecillinam at a ratio of 1:1 were not more effective than the single agents alone against a strain of bacteria susceptible (*C. freundii* 10) and resistant (*S. flexneri* LA) to DDA in vivo. Thus, the selective in vivo activity does not appear to be a phenomenon which can be enhanced by the alteration of bacterial cell permeability or metabolic activity.

Equally interesting were the highly selective structural requirements for in vivo activity. The natures of both the sugar moiety and the purine base were critical. The introduction of a 2'-hydroxyl group (i.e., 3'-deoxyadenosine) resulted in a loss of activity, as did the introduction of a 5'-phosphonic acid group (2',3'-dideoxyadenosine-5'-monocarboxymethylphosphonic acid disodium salt). The 6-amino group of the adenine moiety does not appear to be crucial for activity, since its replacement with a 6-oxo group (2',3'-dideoxyinosine) did not abolish activity. A possible alternative explanation is the metabolic interconversion of DDA and 2',3'-dideoxyinosine. Further modification of the purine portion (i.e., the addition of an amino group at the 2 position) resulted in an inactive compound (2',3'-dideoxyguanosine). Although the 6-amino group of DDA could be replaced with an oxo group, methylation of this group as in *N,N*-dimethyl-2',3'-dideoxyadenosine abolished the activity.

From the data presented, it is clear that the initial effect of DDA on bacterial macromolecular synthesis was the inhibition of DNA synthesis. It also appears that the inhibition of DNA synthesis is the most likely mechanism by which DDA inhibited growth in vitro, based on the following observations: (i) the inhibition of DNA synthesis by DDA could not be attributed to an inhibition of uptake of the radioactive precursor since the same precursor was used for monitoring RNA and DNA syntheses; (ii) agents which interfere with DNA synthesis characteristically produce the observed morphological effect of filamentation (7); and (iii) there was an excellent correlation between the inhibition of DNA synthesis and in vitro activity in terms of MIC values.

The relationship between DNA inhibition and

in vivo activity was much more variable. When in vitro activity as measured by MIC correlated with in vivo activity, there was an excellent agreement between DNA inhibition and in vivo activity. This suggests that, in those instances in which DDA was active, the effect seen in vivo may also be related to DNA inhibition. When DDA was active in vitro as measured by MIC but not in vivo, there was no agreement between DNA inhibition and in vivo activity. This would not be unexpected since many agents active in vitro are inactive or only partially active in vivo, where the dynamics of host defense, virulence of microorganisms, and pharmacological properties of the agent all play important roles. In the case of the two strains (*S. marcescens* S303 and S714) which responded in vivo but not at all in vitro, the mechanism by which DDA acts is unknown. One possible explanation is that a metabolite(s) of DDA produced in mice was active against these two strains.

Of the possible reasons why DDA is inhibitory to bacteria, but is, unlike most nucleoside analogs (4), not toxic in higher organisms (8, 13) except at high concentrations (6, 11), two seem to be most important: (i) DDA is rapidly phosphorylated to the active form, the dideoxynucleoside triphosphate, by kinases in bacteria (17), but only slowly, if at all, by those in animal tissues (3, 13; M. A. Waqar, R. G. Hughes, M. F. Manly, M. J. Evans, and J. A. Huberman, Abstr. Int. Congr. Biochem. 11th, Toronto, Ontario, abstr. no. 01-5-H18, p. 39, 1979), and (ii) there are differences in the intrinsic susceptibility of bacterial and mammalian DNA polymerases to dideoxynucleoside triphosphates. Bacterial polymerases are strongly inhibited by dideoxynucleoside triphosphates (2, 9, 16), whereas the mammalian polymerases involved in cellular DNA replication are relatively insusceptible (1, 16, 18, 19).

The results of this study show that DDA is a unique antibacterial agent among nucleoside inhibitors because of its (i) selective toxicity and (ii) limited but definite in vivo activity. It is also of interest that in vitro activity as measured by MIC determinations over an 18-h period correlated well with DNA inhibition measured within a 20- to 30-min time period.

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