Biochemical Pharmacology, 1959, Vol. 3, pp. 31–37. Pergamon Press Ltd., Printed in Great Britain.

CHEMICAL AND BIOLOGICAL STUDIES ON 1:2-DIHYDRO-*s*-TRIAZINES—XIV

INHIBITION OF *DIPLOCOCCUS PNEUMONIAE*, TYPE II, *IN VITRO* and *IN VIVO* SYNERGISM WITH SULPHADIAZINE*

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(*Received* 1 *May* 1959)

Abstract—A number of 1:2-dihydro-*s*-triazines effectively inhibit *Diplococcus pneumoniae*, type II, *in vitro*. Differences in biological activity could be correlated with certain variations in structure and substitution in the molecule. Maximum activity was observed with 3′:4′-dichlorophenyl derivatives which have 2:2-dimethyl, 2-(*n*-decyl) or 2-(*n*-tridecyl) substitution in the triazine ring.

The dihydrotriazines exhibited synergism with both sulphadiazine and aminopterin. Synergism between sulphadiazine and aminopterin also was observed. Inhibition analyses indicate that these three classes of inhibitor differ with respect to loci of activity. The pattern of reversal of inhibition is consistent with previous evidence that the 1:2-dihydro-*s*-triazines interfere with a diphosphopyridine nucleotide-mediated reduction concerned with the metabolism of pteroylglutamic acid or its derivatives.

Several of these compounds are more effective than sulphadiazine in the therapy of experimental *Diplococcus pneumoniae*, Type II, infections in mice, and exhibit a profound synergism with sulphadiazine *in vivo*; combinations of concentrations of both inhibitors which are ineffective alone protect mice. Similar synergism also was observed *in vivo* with combinations of sulphadiazine and aminopterin, as well as with combinations of 1:2-dihydro-*s*-triazine and aminopterin.

INTRODUCTION

INHIBITION of the response of *Lactobacillus arabinosus* (17-5) no. 8014† to *p*-aminobenzoic acid (PABA) by a series of 1:2-dihydro-*s*-triazines (D·HCl's), and the synergistic activity of these compounds with sulphadiazine in this system[1] and in murine toxoplasmosis[2], suggested that the D·HCl's might exhibit a similar activity against infection induced in experimental animals by appropriate bacterial agents; for example, those which respond to sulphonamide therapy. The present report is concerned with the inhibition of *Diplococcus pneumoniae in vitro* and *in vivo* by certain dihydrotriazines.

EXPERIMENTAL

A mouse-virulent, S-phase strain of *Diplococcus pneumoniae*, type II,‡ maintained

*This investigation was supported in part by research grants C-1921 (C-3) and CY3335 from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.

†American type culture collection number.

‡Obtained through the courtesty of V. J. Fields, Department of Bacteriology, Harvard Medical School.

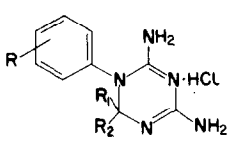
by mouse passage from 5% horse blood agar plates was used in these experiments. The methods of *in vitro* assay were those described for *Lactobacillus arabinosus*,[2] except that the medium was the tryptic digest broth described by Fields.[3] The basal medium contained, per millilitre, less than 0·001 μg of either pteroylglutamic acid (PGA) or citrovorum factor (CF), or both, as judged by microbiological assay with *Streptococcus faecalis*.

In the experiments, *in vivo*, mice of either sex, 20–25 g in weight, were infected intraperitoneally with 24 hr tryptic digest broth cultures of *D. pneumoniae* suitably diluted to contain in 0·1 ml the minimal dose which produced 100 per cent mortality in untreated mice within 72 hr. The D·HCl's, sodium sulphadiazine (sulpha) and aminopterin (4-aminopteroylglutamic acid) (4-APGA) were diluted appropriately with sterile glass-distilled water and the previously determined chronic maximum tolerated dose, and fractions thereof, were administered subcutaneously in approximately neutral solutions daily for 5 days, beginning on the day of infection. As recorded previously, the D·HCl's required careful administration to avoid untoward toxicity and cutaneous sloughing.[2] Each test group consisted of twenty or more treated and similar numbers of untreated mice. Mortality was observed daily for 10 days.

Structure–activity relationships

A comparison of the activity of representative dihydrotriazines is summarized in Table 1. Mono-halogen substitution in the phenyl ring had little effect, whereas

TABLE 1. INHIBITORY ACTIVITY OF 1:2-DIHYDRO-*s*-TRIAZINES *vs. D. pneumoniae*, TYPE II, *in vitro*



Compound no.	Structure			50% minimal inhibiting dose (μg/ml)
	R	R_1	R_2	
D-23·HCl	H	CH_3	CH_3	0·19
D-69·HCl	3′-Cl	CH_3	CH_3	0·175
D-20·HCl	4′-Cl	CH_3	CH_3	0·18
D-54·HCl	3′:4′-Cl_2	CH_3	CH_3	0·013
D-66·HCl	H	CH_3	C_2H_5	2·5
D-60·HCl	3′-Cl	H	C_2H_5	10+
D-43·HCl	4′-Cl	CH_3	C_2H_5	2·9
D-67·HCl	3′:4′-Cl_2	H	CH_3	1·25
D-75·HCl	3′:4′-Cl_2	CH_3	C_2H_5	0·31
D-68·HCl	2′-Cl	CH_3	CH_3	10+
D-98·HCl	2′-OCH_3	CH_3	CH_3	10+
D-40·HCl	3′-Cl	—$(CH_2)_5$—		10+
D-140·HCl	4′-SO_2NH_2	CH_3	CH_3	10+
Controls				
Na sulphadiazine (sulpha)				7·5
4-Aminopteroylglutamic acid (4-APGA)				3·0

di-halogen substitution resulted in a marked increase in activity. Those derivatives with asymmetrical substitution at the 2-position of the triazine ring were generally less active than the corresponding 2:2-dimethyl-substituted compounds. A compound incorporating the structural features of sulphanilamide into the D·HCl molecule (D-140·HCl)* was inactive, as it was in *Lactobacillus arabinosus*–PABA bioassay systems.[1]

Substitution at the *o*-position of the phenyl ring (D-68·HCl, D-98·HCl) or the presence of a large blocking group at the 2-position of the triazine ring (D-40·HCl) results in a marked decrease in activity (Table 1). Similarly, the anilinodihydrotriazines prepared by isomerization of the corresponding D·HCl derivatives[4, 5] also were inactive.

TABLE 2. EFFECT OF 2-(*n*-ALKYL) SUBSTITUTION AT 2-POSITION OF TRIAZINE RING ON ACTIVITY OF 1:2-DIHYDRO-*s*-TRIAZINES *vs. D. pneumoniae*, TYPE II, *in vitro*

Compound no.	Substitution* R_1	R_2	50% minimal inhibiting dose (μg/ml)
R=H			
D-133·HCl	H	C_2H_5	10·0
D-113·HCl	H	*n*-C_6H_{13}	2·2
D-121·HCl	H	*n*-$C_{13}H_{27}$	0·028
R=3′-Br			
D-130·HCl	H	C_2H_5	0·1
D-124·HCl	H	*n*-C_6H_{13}	0·28
D-122·HCl	H	*n*-$C_{11}H_{23}$	0·07
D-139·HCl	H	*n*-$C_{13}H_{27}$	0·028
R=3′-Cl			
D-114·HCl	H	C_2H_5	2·8
D-110·HCl	H	*n*-C_6H_{13}	0·28
D-109·HCl	H	*n*-$C_{11}H_{23}$	0·028
R=4′-Cl			
D-45·HCl	H	CH	3·1
D-84·HCl	H	*n*-C_6H_{13}	2·1
D-106·HCl	H	*n*-$C_{11}H_{23}$	0·028
R=3′:4′-Cl_2			
D-67·HCl	H	CH_3	1·25
D-95·HCl	H	*n*-C_6H_{13}	1·7
D-116·HCl	H	*n*-$C_{10}H_{21}$	0·35
D-108·HCl	H	*n*-$C_{13}H_{27}$	0·021

*See Table 1 for structure.

The activity of the various phenyl-substituted derivatives is increased markedly by the presence of an *n*-hexyl substituent at the 2-position of the triazine ring, while *n*-undecyl to *n*-tridecyl substitution results in a further increase in activity (Table 2). These changes in biological activity consequent on alterations in structure are in general similar to those observed in other microbiological systems.[6]

Reversal of inhibition

The pattern of the non-competitive reversal of the D·HCl inhibition of *D. pneumoniae*

*The synthesis of this and the other previously undescribed dihydrotriazines considered here will be reported elsewhere.

is identical with that observed in the *Lactobacillus arabinosus*–PABA bioassay system,[1] except that natural CF,* thymine and thymidine are relatively ineffective. Similarly, the pattern of reversal of sulpha inhibition resembles that described for *Lactobacillus arabinosus*,[1] except that in the case of *D. pneumoniae*, natural CF, thymine and thymidine are ineffective, while excess guanine or xanthine is highly effective.

The inhibition of *D. pneumoniae* by 4-APGA, however, is distinctly different from that observed in *Lactobacillus arabinosus* [1] and other microbiological systems,[7, 8] in that reversal is not effected by PABA, PGA and its derivatives, or by DPN and its precursors, or by biological reducing agents.[1] So far, reversal of 4-APGA inhibition of *D. pneumoniae* has been effected only by relatively high concentrations of pteroic acid.[1]*

Synergism

Graphic analyses[9, 10] of the effects of combinations of sulpha and D·HCl or 4-APGA, and D·HCl and 4-APGA on the growth of *D. pneumoniae in vitro* indicate synergistic activity (Fig. 1), as was observed in *Lactobacillus arabinosus*–PABA bioassay systems.[1] Thus, these inhibitors involve different loci of activity with *D. pneumoniae*, as has been observed in other microbiological systems.[1, 7, 8]

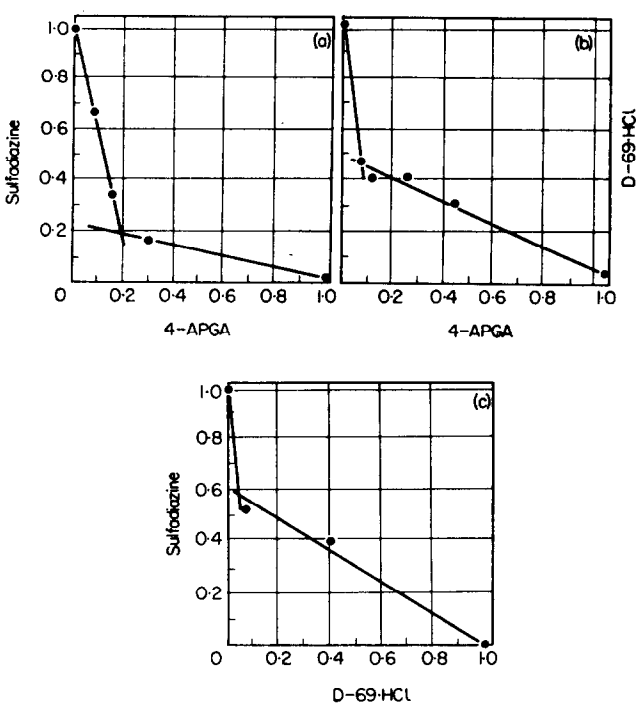


FIG. 1. Effect of combinations of inhibitors *vs. D. pneumoniae*, Type II, *in vitro*. (A) Sulphadiazine and 4-aminopteroylglutamic acid. (B) Sulphadiazine and 1:2-dihydro-*s*-triazine (D-69·HCl).* (C) 1:2-Dihydro-*s*-triazine (D-69·HCl)* and 4-aminopteroylglutamic acid.

Cf. Table 1 for structure.

*We wish to express our appreciation to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for generous samples of these compounds.

Activity in vivo

The activity exhibited by representative D·HCl's *vs. D. pneumoniae*, type II, infection in mice is summarized in Table 3. Both the 2:2-dimethyl derivatives, D-20·HCl

TABLE 3. ACTIVITY OF 1:2-DIHYDRO-*s*-TRIAZINES *vs.* EXPERIMENTAL *D. pneumoniae*, TYPE II, INFECTIONS IN MICE

Compound no.	Substitution*			Dose (mg/kg)†	% Mortality/days		
	R	R_1	R_2		2	4	10
D-23·HCl	H	CH_3	CH_3	65	100		
D-69·HCl	3′-Cl	CH_3	CH_3	180	50	100	
				90	100	100	
D-20·HCl	4′-Cl‡	CH_3	CH_3	75	50	100	
				50	60	60	60
D-54·HCl	3′:4′-Cl_2	CH_3	CH_3	120	30	100	
				100	55	55	55
				25	45	90	90
D-133·HCl	H	H	C_2H_5	40	80	100	
D-114·HCl	3′-Cl	H	C_2H_5	100	50	100	
D-60·HCl	4′-Cl	H	C_2H_5	25	80	100	
D-63·HCl	3′:4′-Cl_2	H	C_2H_5	100	90	90	100
D-95·HCl	3′:4′-Cl_2	H	*n*-hexyl	100	100		
				50	100		
D-116·HCl	3′:4′-Cl_2	H	*n*-decyl	50	50	100	
				25	35	75	75
				12·5	65	100	
D-108·HCl	3′:4′-Cl_2	H	*n*-tridecyl	20	85	100	
				10	70	85	85
				5	90	100	
D-68·HCl	2′-Cl	CH_3	CH_3	100	100		
D-40·HCl	3′-Cl	—$(CH_2)_5$—		100	100		
D-140·HCl	4′-SO_2NH_2	CH_3	CH_3	100	100		
Controls	Untreated			—	80	100	
	Sulphadiazine			250	0	100	

*Cf. Table 1 for structure.
†Experiments with higher doses complicated by toxicity of D·HCl; lower doses less effective.
‡Corresponding anilinodihydrotriazine isomer ineffective at 100 mg/kg.

(4′-chlorophenyl) and D-54·HCl (3′:4′-dichlorophenyl), were more active than the corresponding derivatives which bear asymmetrical 2-carbon substituents at the 2-position of the triazine ring. The 3′-chlorophenyl-2:2-dimethyl derivative (D-69·HCl) exhibited some protective activity for 48 hr, but thereafter was ineffective. D-140·HCl, which incorporates the structural features of sulphanilamide into the D·HCl molecule, was ineffective, as were those derivatives bearing *o*-phenyl substituents (D-68·HCl) or a large-blocking group at the 2-position of the triazine ring (D-40·HCl) (Table 3).

The increasing activity of the phenyl-substituted derivatives with increasing length of the *n*-alkyl substituent at the 2-position of the triazine ring observed *in vitro* (Table 2)[1, 6] was not apparent in these experiments with *D. pneumoniae in vivo*. Derivatives bearing *n*-alkyl substituents in general were less active than the corresponding 2:2-dimethyl compounds, with the exception of the *n*-decyl (D-116·HCl) and the *n*-tridecyl (D-108·HCl) derivatives (Table 3). The presence of *n*-decyl or *n*-tridecyl substituents at the 2-position of the triazine ring also resulted in maximum activity against *D. pneumoniae in vivo* (Table 2).

The various D·HCl derivatives also exhibit synergistic activity with sulpha or 4-APGA *in vivo*. As illustrated in Table 4, combination therapy with concentrations of both inhibitors, either of which is completely ineffective alone, will protect mice against experimental *D. pneumoniae*, type II, infection. Combinations of D·HCl and 4-APGA also exhibit a similar synergistic activity (Table 4).

TABLE 4. SYNERGISTIC ACTIVITY *vs.* EXPERIMENTAL *D. pneumoniae*, TYPE II, INFECTIONS IN MICE

Therapy	% Mortality, 10th day	
	Expected	Observed
Sulpha, 100 mg/kg	100	100
D-54·HCl, 3·125 mg/kg*	100	100
Sulpha, 100 mg/kg + D-54·HCl, 3·125 mg/kg	100	20
Sulpha, 50 mg/kg + D-54·HCl, 3·125 mg/kg	100	0
Sulpha, 25 mg/kg + D-54·HCl, 3·125 mg/kg	100	60
Sulpha, 6·25 mg/kg	100	100
4-APGA, 0.3 mg/kg	100	100
Sulpha, 6·25 mg/kg + 4-APGA, 0·1 mg/kg	100	0
D-54·HCl, 6·25 mg/kg	100	80
D-54·HCl, 6·25 mg/kg + 4-APGA, 0·2 mg/kg	100	10
D-54·HCl, 3·125 mg/kg + 4-APGA, 0·3 mg/kg	100	40
Untreated controls	100	100

*Cf. Table 1 for structure.

DISCUSSION

It is clear that the locus of D·HCl inhibition of *D. pneumoniae*, type II, both *in vitro* and *in vivo*, differs from that involved with inhibition by sulpha or 4-APGA. However, the precise mechanism of action of the D·HCl's is not immediately apparent. The pattern of reversal of inhibition *in vitro* is consistent with previous evidence [1,8] that the D·HCl's interfere with a DPN-mediated reduction concerned with the metabolism of PGA or its derivatives. Experiments indicate that this strain of *D. pneumoniae* converts PGA and pteroic acid to one or more substances showing citrovorum factor activity as assayed by the usual method of bioassay using *Pediococcus cerevisiae* (formerly termed *Leuconostoc citrovorum*). This conversion appears to be unaffected by sulpha, but is inhibited by the D·HCl's and 4-APGA. Thus, the failure of natural CF to reverse D·HCl (and also 4-APGA or sulpha) inhibition suggests that either PABA or its derivatives, or both, may be converted to an active structure which differs from that of natural CF, such as those tetrahydro-PGA derivatives described in other biological systems,[11–14] or that *D. pneumoniae* is impermeable to natural CF. CF has been reported to be similarly ineffective in reversing the sulpha inhibition of *E. coli*.[15, 16]

Pteroic acid reverses both sulpha and 4-APGA inhibition, but is no more effective than PGA in reversing D·HCl inhibition, as would be expected from the results obtained with pteroic acid in *Lactobacillus arabinosus* systems.[1] The relative ineffectiveness of thymine and thymidine in reversing inhibition induced by any of the three inhibitors considered here suggests that impaired synthesis of thymine-containing

compounds is not the growth-limiting factor under these experimental conditions. Further studies are now in progress.

Several of the D·HCl derivatives are more effective than sulpha in the therapy of *D. pneumoniae*, type II, infection in mice. Precise structure–activity studies *in vivo* are complicated by the inherent toxicity of many of the compounds in this series; however, it is apparent that the increasing activity *in vitro* consequent on increasing length of the *n*-alkyl substituent at the 2-position of the triazine ring (Table 2) does not obtain *in vivo* with the same strain of *D. pneumoniae*. Preliminary experiments indicate that this reduced activity *in vivo* may be due, at least in part, to the inactivating effect of serum on these *n*-alkyl derivatives.

The most interesting and probably the most significant aspect of these experiments *in vivo* is the profound synergism exhibited by combinations of D·HCl and sulpha. Synergistic activity with D·HCl and sulpha has been observed in experimental protozoan infections,[2,17] but such activity in experimental bacterial infections has not been reported previously in the literature. Biological activity resulting from combinations of small doses of inhibitors, any of which alone is devoid of activity even in much higher concentrations, as illustrated by the present studies, is unique. Studies on the usefulness of synergistic combinations of D·HCl and sulpha in other experimental systems are now in progress.

REFERENCES

1. G. E. Foley, E. J. Modest, J. R. Cataldo and H. D. Riley, *Biochem. Pharmacol.* **3**, 13 (1959).
2. W. D. Winter, Jr. and G. E. Foley, *Antibiot. & Chemother.* **6**, 444 (1956).
3. V. J. Fields, News Letter, *Soc. Amer. Bact.* **22**, 8 (1956).
4. E. J. Modest, *J. Amer. Chem. Soc.* **21**, 1 (1956).
5. E. J. Modest and P. Levine, *J. Amer. Chem. Soc.* **21**, 14 (1956).
6. E. J. Modest, G. E. Foley, W. D. Winter, Jr. and S. Farber, *Proc. Amer. Ass. Cancer Res.* **2**, 35 (1955).
7. G. E. Foley and E. C. Haley, *Antonie van Leeuwenhoek* **21**, 405 (1955).
8. G. E. Foley, E. J. Modest, S. Farber and E. C. Haley, *Antonie van Leeuwenhoek* **21**, 417 (1955).
9. J. H. Gaddum, *Pharmacology* (4th Ed.) p. 479. Oxford University Press (1953).
10. G. B. Elion, S. Singer and G. H. Hitchings, *J. Biol. Chem.* **208**, 477 (1954).
11. R. L. Kisliuk and W. Sakami, *J. Amer. Chem. Soc.* **76**, 1456 (1954).
12. G. R. Greenberg, *J. Amer. Chem. Soc.* **76**, 1458 (1954).
13. B. E. Wright, *Biochem. Biophys. Acta* **16**, 165 (1955).
14. J. C. Rabinowitz and W. E. Pricer, Jr., *J. Amer. Chem. Soc.* **78**, 5702 (1956).
15. J. Lascelles and D. D. Woods, *Brit. J. Exp. Path.* **33**, 288 (1952).
16. H. R. Alimchandani and A. Sreenivasan, *J. Bact.* **73**, 538 (1957).
17. R. E. Lux, *Antibiot. & Chemother.* **4**, 971 (1954).