

BULLETIN

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EFFECT OF METRONIDAZOLE ON VARIOUS STRAINS OF *E. HISTOLYTICA* IN VIVO

ANAGHA C. DESAI AND M. B. BHIDE

Department of Pharmacology, Haffkine Institute, Parel, Bombay 400 012

Recently few reports have been published on ineffectiveness of metronidazole in various clinical cases. One of the explanations offered is that the strains differ in their sensitivity to metronidazole. Five different strains of *E. histolytica* were isolated and the activity of metronidazole was assessed against all these strains in hepatic and caecal amoebiasis. One strain has shown partial resistance to metronidazole in caecal amoebiasis.

Introduction

Though metronidazole is found to be remarkably effective in the treatment of all forms of amoebiasis, the recent reports have pointed out few cases where the metronidazole is not effective even when given in higher doses (1, 2, 3). The object of this study was to determine the susceptibility of various strains of *E. histolytica* obtained from different sources.

Different strains of *E. histolytica* were isolated from stool samples. The activity of metronidazole was assessed against all these strains in hepatic and caecal amoebiasis in hamsters and rats respectively.

Material and Method

S₈₀, S₀ and S₅₉ were the three strains isolated from stool samples of patients admitted to J. J. and K. E. M. Hospitals. One strain was obtained from "Ciba-Geigy Research Centre" and one more was brought from C.D.R.I., Lucknow. All these strains were maintained in Dobell's diphasic medium (4) and L.M.S. medium (5).

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In vivo activity of metronidazole against caecal amoebiasis in albino rats was assessed by using Jones method (6) whereas its activity in hepatic amoebiasis in hamsters was determined by using Reinertson and Thompson method (7). The degree of infection was assessed in the individual animal by considering the size of abscess and the number of amoebae present in the smear.

Degree of infection	Macro and microscopical appearance of the liver
0	Liver appears normal, no amoebae were seen in the smear.
1	Liver appears normal, very few (1 to 2) amoebae were seen in the whole smear.
2	Liver showed dot like abscess with few amoebae in the smear.
3	Liver showed abscess of smaller magnitude and many amoebae were seen in the smear.
4	Liver showed abscess of greater magnitude and many amoebae in the smear.
5	The whole liver lobe or sometimes more than one lobe was covered with abscess and many amoebae were seen in the smear.

From the degree of infection (D.I.) average degree of infection was calculated (A.D.I.) and from this the percentage reduction in infection was calculated :

$$\% \text{Reduction} = \frac{\text{A.D.I. control} - \text{A.D.I. treated}}{\text{A.D.I. for control}} \times 100.$$

In the similar manner D.I. was assessed in rats considering the macro and microscopic appearance of caeca.

Results

The activity of metronidazole in five different strains against hepatic and caecal amoebiasis is given in Tables I and II respectively.

TABLE I
Effect of Metronidazole in Hepatic Amoebiasis

Strain No.	Source		*Dose mg/kg/day in two divided doses	%reduction in infection
S ₅₉	K. E. M. Hospital, Bombay	..	100 200	74 90
S ₈₀	K. E. M. Hospital, Bombay	..	100 200	84 96
S ₀	J. J. Hospital, Bombay	..	100 200	70 90
C	Ciba-Geigy Research Centre, Bombay	..	100 200	60 85
L	C. D. R. I., Lucknow	..	100 200	75 93

*Drug was administered in two divided doses. One dose was given 3 hours prior to the infection and the second dose was given immediately after the infection. The treatment was continued for 4 days.

TABLE II
Effect of Metronidazole in Caecal Amoebiasis

Strain No.	Source		Dose mg/kg/day single dose	%reduction in infection
S ₅₉	K. E. M. Hospital, Bombay	..	50 100 200	13 34.8 70
S ₈₀	K. E. M. Hospital, Bombay	..	50 100 200	40 83 100
S ₀	J. J. Hospital, Bombay	..	100 200	78 95
C	Ciba-Geigy Research Centre, Bombay	..	100 200	75 98
L	C. D. R. I., Lucknow	..	100 200	80 100

From the results obtained, it is clear that in hepatic amoebiasis, the activity of metronidazole varied from 60-84% at the dose of 100 mg/kg and from 85-96% at the dose of 200 mg/kg. The variation was more at 100 mg/kg dose.

In rats infected with different strains of *E. histolytica* (S_{80} , S_0 , C and L), metronidazole in doses of 100 mg/kg and 200 mg/kg for 6 days produced protection between 75-83% and 95-100% respectively. Metronidazole in the same dosages produced only 34.8 and 70% protection for strain S_{59} .

Discussion

Since the initial favourable reports of metronidazole in amoebiasis (8, 9, 10), several cases of failures with metronidazole have been reported in the literature (11-17) in spite of the use of standard doses of metronidazole (750 mg thrice a day for 10 days). One of the explanations offered for

this ineffectiveness is strain resistance, or variation.

Our present studies indicate that the explanation offered is possible and further work to confirm this is under progress.

Summary

Out of 5 strains studied, one strain showed partial resistance to metronidazole in caecal infection in the rat.

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*N*¹-*N*⁴-SUBSTITUTED PIPERAZINE DERIVATIVES AS ANTIAMOEBIIC AGENTS-II

ANAGHA C. DESAI AND M. B. BHIDE

Department of Pharmacology

AND

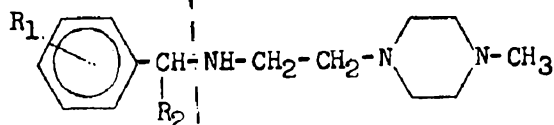
BHARATHI SHANKER

Department of Chemotherapy, Haffkine Institute, Parel, Bombay 400 012

*N*¹-Methyl-*N*⁴-[β (α-alkyl, *p*-chlorobenzylamino) ethyl]-piperazines are synthesised and tested against *E. histolytica* *in vitro* and *in vivo* against experimental amoebiasis. One of the compound (R-2768) of this series has shown effect comparable to metronidazole in hepatic infection of hamsters induced by different strains of *E. histolytica*. It potentiates the effect of metronidazole i.e. 10 % doses of two drugs combined produce effect equal to the effect produce by individual agent. However the therapeutic index of this combination showed no improvement over that of metronidazole.

Introduction

In an earlier paper (1) it was reported that *N*¹, *N*⁴-substituted piperazines of structure I possess significant *in vitro* antiamebic activity. The structure was further modified by increasing the carbon chain *R*₂ up to nine carbons and studied for *in vitro* activity. All these compounds were studied for their *in vivo* activity against experimental amoebiasis.



Out of all the compounds synthesised, compound R-2768 [*R*₁=4Cl and *R*₂=C₆H₁₃ (n)] has been found to be equally effective as compared with metronidazole at the same dose level (200 mg/kg) in hepatic infection in hamsters. To evaluate

it further, the present communication is intended to assess its effects on different strains of *E. histolytica*: its potentiating effect on metronidazole and its preliminary pharmacology and toxicology.

Material and Method

Synthesis of *N*¹-methyl-*N*⁴[β (*p*-chloro-α-heptyl benzylamino) ethyl]-piperazine-3-maleate:

β-*p*-chloro-α-heptyl benzylamino) ethyl chloride hydrochloride (0.05 moles), Et₃N (0.05 moles) and *N*-Methyl piperazine (0.1 mole) in EtOH (2.5 ml) was refluxed for 24 hours. The residue obtained after removal of solvent was basified with cold 5N NaOH solution and was extracted with CHCl₃. The combined CHCl₃ extracts

were dried (Na_2SO_4), concentrated and the residual viscous oil was subjected to fractional distillation *in vacuo*. The fraction distilling between 210-220°C at 3 to 5 mm was collected.

The maleate salt of the above base was prepared by adding the ethereal solution of the base to a solution of maleic acid in alcohol. The solid thus obtained was recrystallised from alcohol; m.p. 160-162°C; Yield 60% ($\text{C}_8\text{H}_8\text{N}_2$). Rest of the compounds were synthesised similarly.

'In vitro' Testing

The compounds were tested *in vitro* by using Woolfe's capillary method as described earlier (1).

'In vivo' Testing

All the compounds were studied *in vivo* at the dose of 200 mg/kg.

The activity of compounds against intestinal amoebiasis in albino rats was assessed by using Jones method (2). Weanling rats weighing between 18-20 gm were used for intracaecal infection. The volume of the inoculum injected varied from 0.25-0.5 ml depending upon the number of trophozoites present per ml of the inoculum and the virulence of the strain. Inoculum was adjusted to contain 12×10^4 cells/ml. Eight animals were used per group. Drug solutions were administered orally via a slightly bent metal tube affixed to a syringe. Five doses were given on five successive days. The first dose was given immediately after the infection. The animals were sacrificed at the end of the sixth day. Caecum was observed macro and microscopically. The degree was given as described by Jones. Compared with control, the percentage inhibition was calculated.

The activity of the compounds was determined against hepatic amoebiasis in hamsters by using Reinertson and Thompson

method (3). Hamsters of either sex weighing between 35-40 gm were used. Animals were anaesthetised with ether. Inoculum was adjusted to contain 12×10^4 cell/ml and 0.1 ml of this was injected intrahepatically.

The drug was given in two divided doses, the first dose was given 3 hours prior to the infection and the second immediately after the infection. Animals were sacrificed at the end of the 4 days and livers were observed macro and microscopically and the efficacy of the drug was determined (4). Percentage activity of the drug was calculated by comparing the average degree of infection of treated with control.

As the compound No. 7 (R-2768) has shown promising activity against hepatic amoebiasis, it was tested against the following strains *in vivo* and compared with metronidazole at different dose levels varied from 25 mg/kg to 400 mg/kg by using strain L:

L=Obtained from C.D.R. I, Lucknow.

C=Obtained from Ciba Research Centre, Bombay.

S_0 & S_{80} = Isolated from stool samples brought from J. J. Hospital, Bombay.

Toxicity Studies—Albino Swiss mice bred and reared at Haffkine Institute and weighing between 18-20 g were used. They were fasted overnight. The drugs suspended in one per cent gum acacia solution were administered orally. Following the administration of the compounds the animals were observed for the appearance of signs of toxicity for 2 hours, and thereafter at intervals up to 8 hours. The mortality was noted over a period of 7 days. LD_{50} was determined for R-2768 and metronidazole individually and in combination by using Litchfield and Wilcoxon method (5).

Effect on Cardiovascular and Respiratory System

Cats of either sex weighing between 2-3 kg were used. The cat was anaesthetised with pentobarbitone in 25% w/v urethane by intraperitoneal injection in a dose of 35 mg/kg. The trachea was cannulated to record respiration using a tambour. Blood pressure was recorded from carotid artery by using a mercury manometer. Drugs were injected through a cannula placed in the femoral vein. Drug was injected at different dose levels varying from 1 mg/kg-3 mg/kg body weight.

Effect on Smooth Muscle Preparations

(A) *Guinea-pig Ileum* (6)—A piece of guinea-pig ileum was suspended in an organ bath containing Tyrode's solution maintained at 37°C and bubbled with oxygen. Drugs were added into the bath

and responses recorded using a frontal writing lever.

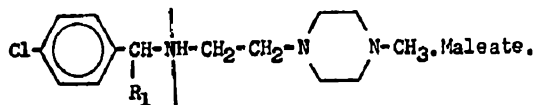
(B) *Guinea-pig Vas deferens* (7)—Guinea-pig vas deferens was mounted in an organ bath containing Tyrode's solution. Responses to adrenaline were recorded using frontal writing lever.

(C) *Guinea-pig Tracheal Chain Preparation* (8)—A tracheal chain preparation was maintained as described by Castillo and DeBeer. Responses were recorded using a frontal writing lever.

(D) *Rat Uterus* (9)—Rat uterus was mounted in an organ bath containing, DeJaln's solution. The responses to 5HT were recorded using frontal writing lever.

In all the experiments on isolated tissues the drugs under study were added 3 minutes, prior to the addition of the agonists.

TABLE I



No.	R ₁	Formula	M. P. °C*	%Yield†	Analysis
1	C ₇ H ₁₅ (n)	.. C ₃₈ H ₄₈ ClN ₃ O ₁₂	.. 160—62	60	C, H, N
2	C ₈ H ₁₇ (n)	.. C ₃₄ H ₅₀ ClN ₃ O ₁₂	.. 161—63	55	C, H, N
3	C ₉ H ₁₉ (n)	C ₃₅ H ₅₂ ClN ₃ O ₁₂	.. 160—62	60	C, H, N

* Melting points are uncorrected.

† Yields reported are of single experiment.

Results

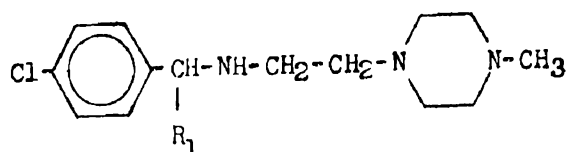
From Table II, out of ten compounds tested only one Compound No. 7 (R-2768 with 6 C atoms gave promising result. It was found to be active in hepatic (84%) and in caecal amoebiasis (78%).

Table III shows the activity of metronidazole and R-2768 with various strains

and comparison of these two at different doses. From the results it was observed that the activity varied with different strains. It was almost same in case of L S₀ and S₈₀ strains, whereas it was less in case of C strain. The same variation was also found with metronidazole at the same dose level (200 mg/kg).

TABLE II

In vivo and in vitro Activity (Dose = 200mg/kg/day)



No.	R ₁	In vitro M.I.C. μg/ml	Salt prepared	% Reduction in infection	
				Hepatic	Caecal
1	H	Inactive	3HCl	Nil	Nil
2	CH ₃	Inactive	3HCl	Nil	Nil
3	C ₂ H ₅	Inactive	3HCl	11.4	6.0
4	C ₃ H ₇ (n)	Inactive	3HCl	15.0	8.0
5	C ₄ H ₉ (n)	100	3HCl	32.0	25.0
6	C ₅ H ₁₁ (n)	50	Maleate	50.0	40.0
7	C ₆ H ₁₃ (n)	5	3-Maleate	84.0	78.7
8	C ₇ H ₁₅ (n)	5	3-Maleate	60.0	35.0
9	C ₈ H ₁₇ (n)	5	3-Maleate	44.0	30.0
10	C ₈ H ₁₈ (n)	5	3-Maleate	30.0	40.0
	Metronidazole	10		96.0	100

TABLE III

The Activity of Metronidazole and the Compound R-2768 against Hepatic Amoebiasis in Hamsters at Different Dose Levels and with Different Strains

Strain	Dose mg/kg	%Reduction in infection	
		Metronidazole	Compound R-2768
L	25	55.5	30.9
	50	61.0	33.3
	100	71.2	70.8
	200	93.2	84.8
	300	85.0	83.8
	400	68.5	62.0
C	200	85.0	63.0
S ₀	200	90	86
S ₈₀	200	96	84

TABLE IV

Compound	Dose mg/kg	%Reduction in infection
Metronidazole	100	66
	200	83
R-2768	100	60
	200	80
Metronidazole + R-2768	10 + 10	49
	20 + 20	74

When the above compound was tested in L strain and compared with metronidazole at various dose levels from 25-400 mg/kg it was found that the activity of the compound was 84.8 % and that of metronidazole 93.2 at the dose level of 200 mg/kg. In both cases the activity increased from 25 mg/kg onwards. This increase in activity was slow in case of metronidazole, whereas in case of this compound there was a sudden increase in response from 33 % at 50 mg/kg to 70 % at 100 mg/kg (Fig. 1). The activity was maximum at 200 mg/kg in both the cases and at higher doses again it started reducing. The reduction was same in both the cases. This may be explained as being due to the toxic effects of drugs. At higher doses animals died and this gave variable results.

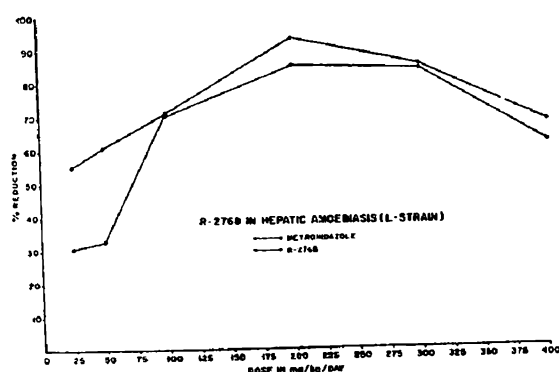


Fig. No. 1

The compound when tried in combination with metronidazole gave equal protection at lower doses compared to the protection given by metronidazole and the compound individually at higher doses (Table II).

Toxicity—The LD₅₀ of the compound R-2768 was 800 mg/kg and that for

metronidazole 4.5 gm/kg. The LD_{50} of the combination was 520 mg/kg.

Pharmacology—No behavioral changes were observed. The compound R-2768 causes an immediate and transient fall in B.P. roughly the fall due to 3 mg/kg of drug is equal to 1 μ g/kg of histamine. The compound had no effect on the response produced by either adrenaline, isoprenaline, acetylcholine or histamine. Neither propranolol nor mepyramine maleate nor atropine had any effect on the response of the drug.

It had no effect on respiration up to 3 mg/kg. It showed non-specific depressant action against histamine and acetylcholine on guinea-pig ileum at the dose level ranging from 0.1-1 μ g/ml.

It had no antagonistic effect at the dose of 0.5 μ g/ml on 5HT induced contractions on rat uterus.

It had no effect at the dose 10 μ g/ml on adrenaline induced contraction on vas deferens.

On tracheal chain the compound had potentiating action at the dose of 800 μ g/ml on isoprenaline induced relaxation of tracheal chain (maintained at high tone by Carbachol).

Discussion

Though number of drugs are available for the treatment of amoebiasis, each drug is plagued by individual limitations, some adverse actions, toxicity and other undesirable features. Today metronidazole and emetine are preferred but these two are also not ideal as they fail to cure number of cases (10-13). And also they exhibit some serious toxic effects like cardiotoxicity (14) or malignancy in experimental animal (15-18). It is also known that metronidazole though effective it has to be given in higher doses.

The compound R-2768 showed very good potentiating effect on metronidazole in hepatic amoebiasis. However the therapeutic index of the combination was not improved. So the combination would be of little use in therapeutics.

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ANTIFILARIAL ACTIVITY OF N¹, N⁴-DISUBSTITUTED PIPERAZINE DERIVATIVES

USHA A. SONURLIKAR, BHARATHI SHANKER AND M. B. BHIDE

Haffkine Institute, Parel, Bombay 400 012

Twenty-seven compounds of N¹, N⁴-disubstituted piperazine series were tested for their antimicrofilarial activity *in vitro* on microfilariae of *L. carinii* and *W. bancrofti*. Four compounds were effective at the concentration of 15.6 µg/ml and 31.2 µg/ml respectively. While these compounds were ineffective on microfilariae of *L. carinii* in cotton rats when fed orally with the dose of 60 mg/kg × 6. The higher dosages killed the animals.

Introduction

Diethylcarbamazine (DEC) was introduced as antifilarial agent (1). The need exists for a better drug because DEC has little or no effect on the adult worms (2, 3) and it also produces untoward reactions. Since then there is no notable introduction of a new drug against *W. bancrofti*. So new compounds of series N¹, N⁴-disubstituted piperazines were synthesised and screened against microfilariae of *L. carinii* and *W. bancrofti* *in vitro*.

Material and Method

Screening of Compounds against Microfilariae of 'L. carinii'—The compounds were screened against microfilariae of *L. carinii* by *in vitro* method (2) with the following modifications. Small test tubes were used in place of capillary tubes and to see the effect of the compounds the number of microfilariae were counted and the motility was noted every time. The effects were observed for 72 hours. Many of the compounds from the present series exhibited effect at very low concentration i.e. at 25 µg/ml within 5 minutes. So the method

was further modified as given below and followed routinely for all the compounds.

Accelerated Method—Microfilariae of *L. carinii* were suspended in the medium containing Tyrode with 20% horse serum. The final dilution of the compounds were also done in the same medium. 0.02 ml of the suspension of microfilariae was taken on the slide and 0.01 ml of the drug solution was added to it. They were mixed with glass rod and the microfilariae were observed under the microscope immediately. Ten microfilariae were observed for 5 minutes to see the effect of the compound. Number of motile microfilariae were noted at each minute. The concentration at which all the ten microfilariae became non-motile was considered as the effective concentration. The compounds were tested at the maximum concentration of 125 µg/ml and if found effective were tested at 62.5, 31.25, 15.6 and 7.8 µg/ml concentration to find the minimum effective concentration. Every time control was kept by adding 0.01 ml of medium in place of drug solution. Twenty-seven N¹, N⁴-disubstituted piperazine derivatives were tested by this method.

Screening of Compounds against Microfilariae of 'W. bancrofti'—Microfilariae of *W. bancrofti* were isolated and suspended in the MEM medium with 2% horse serum (4). For screening the compounds the procedure used for the microfilariae of *L. carinii* was followed. Compounds which were found active at the concentration of 15.6 µg/ml against microfilariae of *L. carinii* were used to test the activity against microfilariae of *W. bancrofti*.

Screening of Compounds against 'L. carinii' Infection in Cotton Rats—Cotton rats were infected with *L. carinii* (5) and those showing high microfilarial count i.e. more than 100 mf/10 mm³ were selected for study. Drugs which showed promising effects *in vitro* i.e. compound Nos. 9, 10 and 11 were chosen for *in vivo* studies. The compounds were fed orally to group of 6 cotton rats with the dose of 60 mg/kg/day for 6 days. The animals were bled through tail vein and the microfilariae were counted. If the drug is active microfilariae would disappear from the blood.

Results

Effect on Microfilariae of 'L. carinii'—Twenty-seven N¹, N⁴-disubstituted piperazines were tested by the accelerated method as described above. The structure of the compounds with the activity observed are listed in the Table.

Effect on the Microfilariae of 'W. bancrofti'—Four of the N¹, N⁴-disubstituted piperazines, compound Nos. 8, 9, 10, 11 which were found active against microfilariae of *L. carinii* at a concentration of 15.6 µg/ml were tested against microfilariae of *W. bancrofti*. All the four compounds were found effective at 31.2 µg/ml concentration against microfilariae of *W. bancrofti*.

Effect on 'L. carinii' Infection in Cotton Rats—None of the three compounds

showed any effect on microfilaria count upto the dose of 60 mg/kg/day for 6 days. Higher dosages killed the animals. However Hetrazan in the dose of 6 mg/kg/day × 6 causes disappearance of the microfilariae within three days.

Discussion

Many times correlation is not found between the *in vitro* and *in vivo* activity of the antifilarial compounds for example antifilarial drug DEC, which is reported as inactive *in vitro* and active *in vivo* (2). But it is not possible to test each and every compound for *in vivo* activity because of the cost of the animals and the amount of the compounds required. So the *in vitro* method is used for screening the compounds. The method was termed as the "Accelerated Method" because the time required to get the effect was reduced and amount of compound required was also very less. This method also gives the quantitative results as 10 microfilariae are counted and observed for the activity.

1. **Effect on Microfilariae of 'L. carinii'**—With X=NH and R₂=Phenyl group in the molecule, the position of the Cl atom on phenyl ring (R₁=Cl) at *ortho* or *para* position does not alter the activity but introduction of one more Cl atom (2, 4-dichloro, compound No. 5) improves the activity from 125 µg/ml to 62.5 µg/ml.

2. When R₂ was small alkyl group, like methyl or ethyl (compound Nos. 6 and 7), the compounds were found inactive.

3. When alkyl chain at R₂ was increased to five, six, eight and nine carbon atoms (compound Nos. 8 to 11), all these compounds showed activity at very low concentration viz. 15.6 µg/ml. They were also found effective against microfilariae of *W. bancrofti* at 31.2 µg/ml concentration.

4. When substitution on piperazine was varied from methyl to 2-hydroxyethyl

group (at R_2) and $n=3$, the compounds which exhibited activity at $15.6 \mu\text{g/ml}$ (compound Nos. 8 and 9, $n=2$) was reduced and found active at $62.5 \mu\text{g/ml}$ (compound Nos. 17 and 18).

5. When at R_2 *p*-(*t*-butyl) benzyl group was present (compound No. 19), the compound showed comparatively good activity at $62.5 \mu\text{g/ml}$ than with methyl (compound No. 1 active at $125 \mu\text{g/ml}$.)

6. When -X- is changed from -NH- to -O- or -S- no significant activity was observed but the compound No. 20, showed activity at $31.5 \mu\text{g/ml}$.

7. Compound Nos. 26 and 27 were found inactive.

Effect on 'L. carinii' Infection in Cotton Rats—Though the compounds were effective *in vitro*, they failed to produce any effect on microfilaria count in cotton rats.

Summary

Accelerated method was used for screening the compounds for antifilarial activity

in vitro. N^1 , N^4 -disubstituted piperazines were tested against microfilariae of *L. carinii* and *W. bancrofti*. Out of 27 compounds tested, 4 were found active at the concentration of $15.6 \mu\text{g/ml}$ against microfilariae of *L. carinii* and at the concentration of $31.2 \mu\text{g/ml}$ against microfilariae of *W. bancrofti*. The compounds were found active when the number of carbon atoms in the alkyl chain were increased. These compounds when fed orally did not exhibit any effect on microfilariae of *L. carinii* in cotton rats. All the compounds were found inactive when tested for antibacterial activity (6).

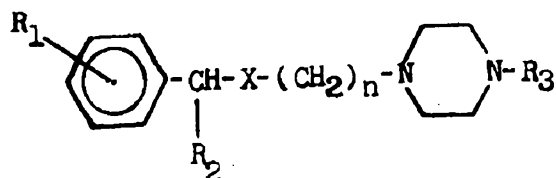
Acknowledgement

The authors thank Dr. W. N. Gatlewar, Officer-in-Charge, Department of Zoonosis for supplying microfilaria positive blood and infected rats, and Dr. B. B. Gaitonde, Director, Haffkine Institute, Bombay for providing the facilities to carry out to present work.

TABLE

In vitro Activity against Microfilariae of *L. carinii*

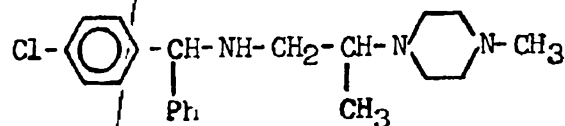
Structure I



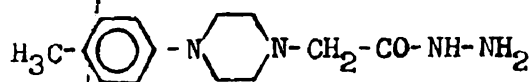
Serial No.	R_1	R_2	X	n	R_3	Salt	Concentration $\mu\text{g/ml}$
1	4-Cl	Ph	-NH-	2	CH_3	maleate	125
2	H	Ph	-NH-	2	CH_3	hydrochloride	125
3	2-Cl	Ph	-NH-	2	CH_3	hydrochloride	125
4	2-Cl	Ph	-NH-	2	CH_3	maleate	125
5	2, 4- Cl_2	Ph	-NH-	2	CH_3	maleate	62.5
6	H	CH_3	-NH-	2	CH_3	maleate	
7	4-Cl	C_2H_5	-NH-	2	CH_3	maleate	

Serial No.	R ₁	R ₂	X	n	R ₃	Salt	Concentration $\mu\text{g/ml}$
8	4-Cl	C ₆ H ₁₁ (n)	-NH-	2	CH ₃	maleate	15.6
9	4-Cl	C ₆ H ₁₃ (n)	-NH-	2	CH ₃	maleate	15.6
10	4-Cl	C ₈ H ₁₇	-NH-	2	CH ₃	maleate	15.6
11	4-Cl	C ₉ H ₁₉	-NH-	2	CH ₃	maleate	15.6
12	3-Cl	Ph	-NH-	3	CH ₂ CH ₂ OH	maleate	
13	4-Cl	H	-NH-	2	CH ₂ CH ₂ OH	maleate	
14	4-Cl	CH ₃	-NH-	2	CH ₂ CH ₂ OH	maleate	
15	4-Cl	C ₂ H ₅	-NH-	2	CH ₂ CH ₂ OH	hydrochloride	
16	4-Cl	C ₄ H ₉ (n)	-NH-	3	CH ₂ CH ₂ OH	maleate	
17	4-Cl	C ₅ H ₁₁ (n)	-NH-	3	CH ₂ CH ₂ OH	maleate	62.5
18	4-Cl	C ₆ H ₁₃ (n)	-NH-	3	CH ₂ CH ₂ OH	maleate	62.5
19	4-Cl	C ₆ H ₅	-NH-	2	<i>p</i> -(<i>t</i> -butyl) benzyl	maleate	62.5
20	4-Cl	C ₆ H ₅	-O-	2	C ₆ H ₅	maleate	31.5
21	4-Cl	C ₆ H ₅	-O-	2	2-Cl, C ₆ H ₅	Oxalate	
22	4-Cl	C ₆ H ₅	-O-	2	2-thiazolyl	maleate	125
23	4-Cl	C ₆ H ₅	-O-	4	2-pyridyl	maleate	125
24	4-Cl	C ₆ H ₅	-O-	4	2-pyrimidyl	oxalate	125
25	4-Cl	C ₆ H ₅	-S-	2	CH ₃ CHOH-CH ₂	maleate	125

26 Structure



27 Structure



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***N*¹, *N*⁴-DISUBSTITUTED PIPERAZINE DERIVATIVES AS ANTIFILARIAL ANTIAMOEBIC AND SPERMICIDAL AGENTS**

USHA A. SONURLIKAR, BHARATHI SHANKER, PUSHPA A. KIRKE
AND M. B. BHIDE

Haffkine Institute, Parel, Bombay 400 012

4-*N*¹, *N*⁴-disubstituted piperazines were studied for their activity against filariasis, amoebiasis, bacteria and sperms. It was shown that they possess broad spectrum activity in doses ranging from 5 to 50 mg/ml. All the compounds failed to exhibit any antibacterial activity upto 200 mg/ml. This is the first report to describe such a broad spectrum activity in any group of compounds.

Introduction

Diethylcarbamazine (DEC) is a piperazine derivative which is established as the drug of choice in filariasis due to *W. bancrofti*. In last decade many piperazine derivatives have been tried but with little success (1, 2). We are reporting here 4 compounds from *N*¹, *N*⁴-disubstituted piperazine series which show very interesting spectrum of activity (I) antifilarial, (II) antiamoebic and (III) spermicidal.

Material and Method

1. *Spermicidal Activity*—Compounds were diluted in Tyrode solution to give the required concentrations. Semen was obtained fresh from human volunteer and after diluting the same with Tyrode in 1 : 20, two drops were taken on a microscope slide and motility of the sperms was observed for 10 minutes. The microscope was kept in a temperature control unit, so that the temperature at the stage of the microscope was 37°C. On another slide semen was diluted with Tyrode solution containing drug and the motility of the sperms was observed for 10 minutes. The

minimum concentration which completely stopped the motility was described as the minimum inhibitory concentration.

2. *Antifilarial Activity*—Microfilariae of *W. bancrofti* and *L. carinii* were suspended in Tyrode solution and the effect of the compound i.e. stoppage of movements of microfilariae was observed for 5 minutes and minimum inhibitory concentration was found out (3).

3. *Antiamoebic Activity*—The minimum inhibitory concentration for *E. histolytica* was tested by Wolfe's method (4). The *in vitro* activity was observed at the end of 48 hours (5). Metronidazole and emetine were used as standards for comparison.

4. *Antibacterial Activity*—The antibacterial activity was tested against *Staphylococcus aureus*, *Salmonella typhi* and *Vibrio cholerae*. Nutrient broth was used as a culture medium for the above bacteria. The compounds were dissolved in distilled water and the sterilized solutions were added to the sterile medium in graded quantities to give final concentration (5) of 200, 100, 40 and 20 mg/ml. Control tubes without drug and with reference standard

were kept for comparison. The inoculum was added to the tubes and tubes were incubated for 48 hours at 37°C. The presence or absence of growth was observed visually at the end of incubation period. Penicillin and chloromycetin were used as the reference standards for *S. aureus* and *S. typhi*, *V. cholerae* respectively.

5. *Toxicity Studies*—Haffkine bred male albino mice weighing between 18 to 22 gm were used for the studies. Group of mice were fed or injected intraperitoneally (IP) with the various doses of drugs and the animal were observed upto 7 days for any mortality. The oral and IP LD₅₀ were 500 mg/kg and 250 mg/kg respectively.

Results

1. *Spermicidal Activity*—All the four compounds stopped the movements of the sperms within 10 minutes at the concentration of the 15.6 mg/ml. Compounds R-3504 and R-3536 showed activity within 2 minutes.

2. *Antifilarial Activity*—All the four compounds tested exhibited action within 5 minutes at 15.6 mg/ml and 31.2 mg/ml concentration against microfilariae of *L. carinii* and *W. bancrofti* *in vitro*.

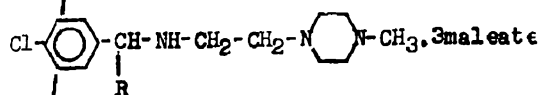
3. *Antiamoebic Activity*—Three of the compounds tested showed activity at 5 mg/ml concentration and compounds R-2916 at 50 mg/ml concentration against *E. histolytica* *in vitro*. Metronidazole and emetine were used as standards which were effective at 10 and 5 mg/ml concentration respectively.

4. *Antibacterial Activity*—The compounds exhibited no antibacterial activity upto 200 mg/ml concentration against *S. typhi*, *S. aureus* and *V. cholerae* when tested *in vitro*. Penicillin and chloromycetin were found active at 0.04 and 5 mg/ml respectively.

The structures of the compounds and the detailed results are given in the Table.

TABLE

Structure and Antifilarial, Antiamoebic and Spermicidal Activity of N¹, N⁴-disubstituted Piperazine Derivatives 'in vitro'



Serial No	Code No.	R	Minimum inhibitory concentration in mg/ml				
			Antifilarial		Antiamoebic	Antibacterial Inactive upto	Spermicidal
			<i>L. carinii</i>	<i>W. bancrofti</i>			
1	R-2916	C ₅ H ₁₁ (n)	15.6	31.2	50.0	200.0	15.6
2	R-2768	C ₆ H ₁₃ (n)	15.6	31.2	5.0	200.0	15.6
3	R-3504	C ₆ H ₁₇ (n)	15.6	31.2	5.0	200.0	15.6
4	R-3536	C ₉ H ₁₉ (n)	15.6	31.2	5.0	200.0	15.6

Discussion

From the results it can be seen that the compounds R-2768, R-3504 and R-3536 inhibited the motility of the microfilariae of *W. bancrofti* and *L. carinii*; *E. histolytica* and human sperms. This was not due to cytotoxic activity but was specific activity as the compounds did not exhibit any antibacterial activity. To our knowledge, this is the first series of the broad spectrum compounds having such a wide range of activity.

Summary

Interesting broad spectrum of activity i.e. antifilarial, antiamoebic and spermicidal was found in the four compounds of N¹, N⁴-disubstituted piperazines derivatives.

Acknowledgement

The authors are grateful to Dr. B. B. Gaitonde, Director, Haffkine Institute for providing excellent facilities.

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EFFECT OF HYPOVITAMINOSIS A AND D ON INFECTIVITY OF *N. DUBIUS* IN MICE

VIJAYALAKSHMI R. KAMATH,* D. M. RENAPURKAR AND M. B. BHIDE

Department of Zoonosis, Haffkine Institute, Parel, Bombay 400 012

Malnutrition is a condition prevalent throughout India and is one of the reasons for high prevalence of parasitic infections in many parts of the country. The purpose of the following study was to examine as to how artificially produced vitamin A and vitamin D deficiency affected the susceptibility of mouse to *N. dubius* strain. It was seen that vitamins A and D deficient diet-group of mice showed higher adult worm count in comparison to mice maintained on control diet.

Introduction

The rate of incidence and severity of helminthic infection in developing countries is more when compared to developed countries (1). It is an accepted fact that malnutrition predisposes a person to infection (2). Vitamin deficiency makes individuals susceptible to many human diseases. It was already known that inadequate supply of specific vitamins led to the establishment of many diseases and helminthic infestations are no exception.

Numerous attempts have been made to find out the particular dietary factors that affect the helminths. Vitamins A and D are responsible for keeping the epithelium healthy (3). So it was expected that their deficiency may lead to unhealthy epithelium and predispose the host to helminthic infestations. The following work was therefore carried out to see whether vitamins A and D play any role in the establishment of helminthic infestation in laboratory animals. The experiments were carried out by artificially producing vitamin A and vitamin D deficiency in the

mice and observing the influence on *N. dubius* infection.

Material and Method

The diet deficient in vitamins was prepared as follows :

1. Composition of basal diet (vitamin free):

Salt mixture	4%
Vitamin-free casein	18%
Fat	5%
Sugar	10%
Starch	63%

2. Composition of salt mixture :

Chemicals	Gm/1,000 gm
Sodium chloride ..	105.00
Potassium chloride ..	120.00
Potassium acid phosphate	310.00
Calcium phosphate ..	140.00
Magnesium sulphate anhydrous.	90.00
Calcium carbonate ..	210.00
Ferrous ammonium citrate.	14.00
Manganese sulphate anhydrous.	0.20
Alum potassium ..	0.09
Copper sulphate ..	0.39
Sodium fluoride ..	0.57
Potassium iodide ..	0.05

(Received for publication October 19, 1976)

*This paper forms a part of the work submitted for M.Sc. in Applied Biology.

3. Composition of vitamin B-complex Solution I (for One Litre):

Thiamin	..	53.33 mg.
Riboflavin	..	106.66 mg.
Niacin	..	666.66 mg.
Pyridoxin hydrochloride		53.33 mg.
Choline chloride	..	13.33 mg.

4. Composition of vitamin B-complex Solution II (for One Litre):

Para amino benzoic acid		3100.00 mg.
Biotin	..	8.00 mg.
Folic acid	..	80.00 mg.

5. Vitamin A, D and E Solutions :

The pure vitamins were dissolved in ground-nut oil to give:

Vitamin A	..	50.00 I.U./ml.
Vitamin D	..	50.00 I.U./ml.
Vitamin E	..	4.00 mg/ml.

Feeding Methods

The mice were kept in cages in groups of ten and were allowed to drink water *ad lib*. Each mouse received 7 gm of the basal diet containing 0.70 ml of B-complex Solution I, 0.25 ml of vitamin B-complex Solution II and 0.4 mg of vitamin E. The mice used for vitamin A deficiency studies were given vitamin D and the

mice for vitamin D deficiency were offered vitamin A. Control mice received the diet containing both vitamins A and D. The amount was 5.0 I.U. per mouse.

Infection and Egg Count

The mice were fed on the deficient diet for 15 days and then infected orally with 100 infective larvae of *N. dubius*. Once the infection was established, the egg count of each mouse was recorded at an interval of 5 days (4). The animals were sacrificed and adult worms counts taken on 10th day (5).

Results

During the period of experimentation the control animals were healthy while the vitamin deficient animals showed weakness, loss in weight and inaction. In addition to above observations animals with vitamin A deficient mice also showed progressive mortality (Table III).

From Table I, it can be seen that animals with vitamin deficiency showed higher egg count than the control mice. The values were statistically significant.

From the Tables II and III it can be seen that the worm load was significantly more in the vitamin deficient mice in comparison to the control mice.

TABLE I

Egg Count in Normal and Vitamin Deficient Mice (10 in each group)

Serial No.	Days after infection	Control mice	Vitamin A deficient mice	Vitamin D deficient mice	Significance between col. Nos. 3 and 4	Significance between col. Nos. 3 and 5
1	2	3	4	5	6	7
1	10	90.9 ± 8.0	127 ± 18.72	119.2 ± 11.22	Positive	Positive
2	15	88.6 ± 8.79	152 ± 13.27	130 ± 12.22	Positive	Positive
3	20	87.3 ± 10.98	192.2 ± 9.954	162 ± 15.87	Positive	Positive

TABLE II

Adult Worm Count of Control Mice and Vitamin A Deficient Mice

Serial No.	Days after infection	Control mice	Vitamin A deficient mice	Significance
1	2	3	4	5
1	10	52.6 (n=10)	66.00 $\pm$ 3.85 (n=10)	Positive
2	20	45.1 (n=10)	62.00 $\pm$ 5.56 (n=7)	Positive
3	30	45.4 (n=10)	59.2 $\pm$ 2.05 (n=4)	Positive
4	40	49.5 (n=10)	59.0 $\pm$ (n=1)	Positive

n=Number of mice.

TABLE III

Adult Worm Count of Control Mice and Vitamin D Deficient Mice

Serial No.	Days after infection	Control mice	Vitamin D deficient mice	Significance
1	2	3	4	5
1	10	54.20 (n=10)	68.3 $\pm$ 13.7 (n=10)	Positive
2	20	44.50 (n=10)	61.40 $\pm$ 4.92 (n=9)	Positive
3	30	45.40 (n=10)	66.2 $\pm$ 5.08 (n=10)	Positive
4	40	49.50 (n=7)	64.0 $\pm$ (n=1)	Positive

n=Number of mice.

Discussion

The egg count in both vitamins A and D deficient diet group was higher and it continued to increase as days advanced, while in the adequate diet group, the egg count was steady. However, the egg count in vitamin D deficient group was found to be lower than that in the vitamin A deficient diet group.

The number of adult worms present in the vitamins A and D deficient diet was more than that in the mice fed with standard diet. It appeared that the diet deficient in vitamins A and D diminished the resistance power of mice to *N. dubius* and resulted in higher worm load. Similar results were

described by others (6, 7) by using protein deficient diets.

This is the first report describing the effect of vitamin deficiency on *N. dubius* infestation.

Summary

The susceptibility to *N. dubius* infestation in mice increased when mice were fed with vitamin A and vitamin D deficient diet.

Acknowledgement

The authors sincerely thank Dr. B. B. Gaitonde, Director, Haffkine Institute, for providing facilities for carrying out this work.

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■ ■

RAT ILEAL LOOP MODEL FOR STUDY OF DRUG EFFECTS ON FLUID ACCUMULATION INDUCED BY VIBRIO CHOLERA AND CHOLERA TOXIN

B. B. GAITONDE AND S. S. JADHAV

Director's Research Cell, Haffkine Institute, Parel, Bombay 400 012

The rat ileal loop model has been studied. The choleraogenic toxin as well as the cholera organisms give a good dose response in this model. Prior vaccination with cholera vaccine suppresses fluid accumulation in the ileal loops. Berberine was found to be effective, while nicotinic acid did not show any potentiation of response when given with cholera toxin. Prostaglandin E_2 , although caused small fluid accumulation, did not potentiate the actions of cholera toxin. It is suggested that this model is reliable, cheap and reproducible. It can be used for study of mechanism of drug action. It is further postulated that change in PGE_2 and cAMP mechanism may not be responsible for fluid accumulation in rat ileal loop.

Introduction

After the discovery of vibrio cholera by Koch in 1883, many investigators attempted to develop animal models in wide variety of species. The rabbit ileal loop model was reported in 1953 which gave consistent results (1). Shortly thereafter, the suckling bunny model was reported in 1954 (2). An animal model using dogs with chronic Thiry-Vella loops of the jejunum or ileum was developed in 1968 (3). Rat was used as cholera model for the first time in 1968 (4). In 1969 the responses of the ligated ileal loops of various species of animals to vibrio and choleraogenic toxins was reported (5). In this report, we have investigated the rat ileal loop model for a study of drug effects and fluid accumulation induced by vibrio cholera or choleraogenic toxin.

Material and Method

Strain of pathogenic vibrio cholera, Inaba 569 B and crude toxin prepared from the

same, using Syncase medium, were used. The experimental inoculum was log phase vibrios grown in 2% peptone at 37°C for 4 hours. The crude choleraogenic toxin was prepared by growing Inaba 569 B in alkaline peptone water (6). The volume of injection in both the cases, i.e. vibrios and choleraogenic toxin did not exceed 0.2 ml for each dose. The doses used for injecting vibrios cholera Inaba 569 B were 1×10^2 , 1×10^4 , 1×10^6 , 1×10^8 organisms and doses for choleraogenic toxin were: 0.25 units, 0.5, 0.75 and 1.0 units (7).

Albino rats of either sex obtained from Haffkine Institute were used. They were deprived of food for 24 hours prior to inoculation, but had free access to water. Laparotomy was done under ether anaesthesia. Leaving 5 cm, from the ileo ceecal junction, 5 loops, each of 5 cm were prepared by ligation and washed with normal saline. The inoculation was done by method reported earlier (4).

To assess the influence of age and weight, if any, the animals were grouped according to their weights as follows:

- (1) 40-50 gm, (2) 80-100 gm, (3) 100-150 gm and (4) 200 gm and above.

The animals were sacrificed at various intervals, i.e. 2, 4, 8, 12, 24 hours after inoculation and the fluid was aspirated carefully by a tuberculin syringe from each loop and the fluid volume was recorded.

In each animal, one of the loops acted as control and received saline instead of cholera organisms or cholera toxin.

In another set of experiments, rats were immunised with cholera vaccine containing 12,000 million killed organisms per ml of all the cholera strains including El Tor biotype (Haffkine Institute) with a single injection of 0.5 ml or 0.75 ml. The loops were prepared 15 days later and fluid accumulation was studied after injection of organisms or cholera toxin as described above.

The effects of berberine sulphate, nicotinic acid, prostaglandin E_2 , imidazole and metronidazole on the fluid accumulation in the loops were evaluated. Berberine sulphate and nicotinic acid were dissolved in distilled water and were injected intraperitoneally half-an-hour prior to making loops in six animals. In second set of experiments, using 6 animals, all drugs including berberine and nicotinic acid were given in the intestinal loop after mixing with the toxin in a syringe. In the third set of experiments, the drugs were directly injected in the loops after one minute of the injection of the toxin. Metronidazole was given orally in a dose of 12.5 mg/kg half-an-hour before the preparation of loops.

Results

(I) *Response to Cholera Organisms*—The smallest dose of vibrios, i.e. 1×10^2 organisms induced fluid accumulation of $0.5 \text{ ml} \pm 0.016$, i.e. 10 times the amount recovered from control loops receiving saline injections. The maximum fluid accumulation was seen after an injection of 1×10^6 organisms (Table I). With the optimum dose of cholera vibrios, i.e. 1×10^6 , fluid started accumulating by 2nd hour and reached maximum between 12th hour and 14th hour.

TABLE I

Fluid Accumulation in Ileal Loop of Rats 14 hours after Injection of Various Doses of Vibrio Cholera

Serial No.	No. of animals	Dose/0.2 ml	Average fluid in ml/loop $\pm$ S. E.
1	5	1×10^2	0.5 ± 0.016
2	5	1×10^4	0.7 ± 0.031
3	5	1×10^6	1.1 ± 0.062
4	5	1×10^8	1.0 ± 0.062

(II) *Response to Cholera Toxin*—Dose response to cholera toxin is shown in Table II. The maximum fluid accumulation was seen after injecting 0.75 units of cholera toxin and this was comparable to that obtained after injecting 1×10^6 organisms.

TABLE II

Rat Ileal Loop Reaction to Cholera Toxin of 569B Inaba

Serial No.	No. of animals	Dose in units/0.2 ml	Average fluid in ml/loop $\pm$ S.E.
1	5	0.25	0.5 ± 0.031
2	5	0.5	0.9 ± 0.062
3	5	0.75	1.1 ± 0.062
4	5	1.0	1.1 ± 0.062

In order to assess whether fluid accumulation is influenced by age of the animal, cholera toxin was injected in 5 groups, each of 10 animals of different weight group (Table III). The loop which received injection of normal saline did not show any accumulation of fluid exceeding an average of 0.07 ml. Maximum fluid accumulation was seen in animals weighing between 100-150 gm weight.

TABLE III

Fluid Accumulation in Ileal Loop of Rats of Different Weights 14 hours after Injection of Various Doses of Vibrio Cholera

Serial No.	No. of animals	Average weight of rats in gm	Dose /0.2 ml	Average fluid in ml/loop $\pm$ S. E.
1	5	50	1×10^6	0.8 ± 0.031
2	5	75	1×10^6	0.9 ± 0.031
3	5	150	1×10^6	1.2 ± 0.062
4	5	200 & above	1×10^6	1.0 ± 0.062

(III) *Effect of Drugs on Fluid Accumulation*—Berberine was found to be effective in suppressing fluid accumulation in all the three sets of experiments—(1) when injected by intraperitoneal route, half-an-hour prior to making of loops (Table IV), (2) when mixed in the syringe with toxin (Table V) and (3) when toxin was administered one minute after berberine injection in the loops (Table VI).

Nicotinic acid in doses varying from 10-500 mg did not produce any fluid accumulation by itself. It did not influence the fluid accumulation caused by vibrio cholera or cholera toxin. Injection of Prostaglandin E_2 (100 μ g) does not result any substantial accumulation of fluid by itself. Further, the prostaglandin given along with toxin did not interfere with or potentiate the action of toxin. Imidazole and metronidazole have no influence on fluid accumulation in the ileal loops induced by vibrio cholera or cholera toxin.

TABLE IV

Fluid Accumulation in Ileal Loop of Rats 14 hours after Injection of Various Doses of Drugs and Vibrio Cholera administered half-an-hour before making the Loops

Serial No.	No. of animals	Drug and dose per loop	Dose/0.2 ml	Average fluid in ml/loop $\pm$ S.E.
1	5	Berberine 10 μ g alone without vibrio cholera	..	0.1 ± 0.031
2	5	Berberine 5 μ g	.. 1×10^6	0.5 ± 0.031
3	5	Berberine 10 μ g	.. 1×10^6	0.2 ± 0.031
4	5	Nicotinic acid 500 μ g alone without vibrio cholera		0.5 ± 0.031
5	5	Nicotinic acid 10 μ g	1×10^6	1.1 ± 0.062
6	5	Nicotinic acid 100 μ g	1×10^6	1.2 ± 0.062
7	5	Nicotinic acid 500 μ g ..	.. 1×10^6	1.2 ± 0.062
8	5	Prostaglandin E_2 100 μ g without vibrio cholera		0.2 ± 0.031
9	5	Prostaglandin E_2 10 μ g	1×10^6	1.1 ± 0.062
10	5	Prostaglandin E_2 100 μ g	.. 1×10^6	1.0 ± 0.031

TABLE V

Fluid Accumulation in Ileal Loop of Rats 14 hours after Injection of Various Doses of Drugs and Vibrio Cholera when administered simultaneously

Serial No.	No. of animals	Drug and dose per loop	V. Cholera Dose/0.2 ml	Average fluid in ml/loop $\pm$ S.E.
1	5	Berberine 10 μ g alone without vibrio cholera		0.1 $\pm$ 0.031
2	5	Berberine 5 μ g ..	.. 1 $\times$ 10 ⁶	0.4 $\pm$ 0.031
3	5	Berberine 10 μ g ..	.. 1 $\times$ 10 ⁶	0.1 $\pm$ 0.031
4	5	Nicotinic acid 500 μ g alone without vibrio cholera		0.8 $\pm$ 0.031
5	5	Nicotinic acid 10 μ g ..	.. 1 $\times$ 10 ⁶	1.1 $\pm$ 0.062
6	5	Nicotinic acid 100 μ g ..	.. 1 $\times$ 10 ⁶	1.2 $\pm$ 0.062
7	5	Nicotinic acid 500 μ g ..	.. 1 $\times$ 10 ⁶	1.2 $\pm$ 0.062
8	5	Prostaglandine E ₂ 100 μ g without vibrio cholera		0.15 $\pm$ 0.031
9	5	Prostaglandine E ₂ 10 μ g ..	.. 1 $\times$ 10 ⁶	1.0 $\pm$ 0.062
10	5	Prostaglandine E ₂ 100 μ g ..	.. 1 $\times$ 10 ⁶	1.0 $\pm$ 0.062

TABLE VI

Fluid Accumulation in Ileal Loop of Rats 14 hours after Injection of Various Doses of Drugs and Vibrio Cholera (Drugs were administered one minute after the Vibrio Cholera Injection).

Serial No.	No. of animals	Drug and dose per loop	Dose/0.2 ml	Average fluid in ml/loop $\pm$ S.E.
1	5	Berberine 10 μ g alone without vibrio cholera		0.1 $\pm$ 0.031
2	5	Berberine 5 μ g ..	.. 1 $\times$ 10 ⁶	0.4 $\pm$ 0.031
3	5	Berberine 10 μ g ..	.. 1 $\times$ 10 ⁶	0.1 $\pm$ 0.031
4	5	Nicotinic acid 500 μ g alone without vibrio cholera		0.5 $\pm$ 0.062
5	5	Nicotinic acid 10 μ g ..	.. 1 $\times$ 10 ⁶	1.1 $\pm$ 0.062
6	5	Nicotinic acid 100 μ g ..	.. 1 $\times$ 10 ⁶	1.2 $\pm$ 0.062
7	5	Nicotinic acid 500 μ g ..	.. 1 $\times$ 10 ⁶	1.2 $\pm$ 0.062
8	5	Prostaglandin E ₂ 100 μ g alone without vibrio cholera		0.1 $\pm$ 0.031
9	5	Prostaglandin E ₂ 10 μ g ..	.. 1 $\times$ 10 ⁶	1.0 $\pm$ 0.062
10	5	Prostaglandin E ₂ 100 μ g ..	.. 1 $\times$ 10 ⁶	1.1 $\pm$ 0.062

Discussion

Our results indicate that a dose of 1×10^8 organisms induces measurable fluid accumulation in the intestinal loops of rats. Further, Syncase toxin is as effective as the vibrio cholera injection. The effect of toxin 0.75 units is almost equivalent to that produced by vibrio cholera 10^6 organisms.

Vaccination with a standard cholera vaccine reduced accumulation of fluid. This is an evidence of a protective effect. In few animals which were vaccinated with 0.5 ml of vaccine prepared at Haffkine Institute, there was clear evidence of its protective effect on fluid accumulation in the loops.

One of the intriguing problems of the cholera toxin is its mode of activity. It has been postulated that cholera toxin probably acts at the level of intestinal mucosa and intestinal cells concerned in reabsorption or excretion. It was reported that berberine was effective in patients suffering from cholera (8). It was further postulated that berberine sulphate was active by virtue of its action directly on some mechanism concerned with fluid reabsorption (9). It did not cause a neutralization of toxin. In this report, we have confirmed that berberine has completely suppressed the activity of cholera toxin, even though the drug was given half-an-hour prior to the preparation of loops.

It is postulated that the exotoxin of vibrio cholera stimulates the adenylyl cyclase system of intestinal mucosal cells to increase their concentration of adenosine 3', 5' monophosphate (cAMP) (10, 11, 12). It is this increased intracellular cAMP which appears to be the basic biochemical change which leads to a net increase in water flux from the mucosal cell to the gut lumen (13, 14). Cyclic AMP concentration can be altered through (a) Adenylyl cyclase,

(b) directly through prostaglandin release, (c) Phosphodiesterase. Adenylyl cyclase is the molecular basis of B adrenergic receptors (15). As yet there is no evidence that B blockers can effectively suppress fluid accumulation. Similarly, nicotinic acid which is known to stimulate adenylyl cyclase also does not show any effect on fluid accumulation. Prostaglandins can stimulate cAMP formation and inhibition of prostaglandin synthesis by salicylates has already been suggested as a possible therapy (16). It has been further postulated that cholera toxin may be acting by releasing prostaglandins (17). Our data does not substantiate this hypothesis. Prostaglandin E_2 does not potentiate action of cholera toxin. Although it is true that small doses of prostaglandin E_2 by itself, caused fluid accumulation, this was not further increased by injection of cholera toxin. This evidence tends to support the views that indomethacin had no effect on toxin induced fluid accumulation or cAMP of mucosa (18).

In a recent report (19), it is suggested that imidazole a potent activator of phosphodiesterase may have beneficial effect in clinical cholera. In our experiments, imidazole and metronidazole do not show any effect on fluid accumulation in the ileal loops of rats. The elevation of phosphodiesterase levels causing activation by imidazole with a consequential probable reduction in cAMP does not inhibit action of choleratoxin and vibrio cholera.

Summary

The rat ileal loop model has been studied. The cholera toxin as well as the cholera organisms give a good dose response in this model. Prior vaccination with cholera vaccine suppresses fluid accumulation in the ileal loops. Berberine was found to be effective, while nicotinic acid did not show any potentiation of

response when given with cholera toxin. prostaglandin E_2 , although caused small fluid accumulation, did not potenti  te the actions of cholera toxin. It is suggested that this model is reliable, cheap and

reproducible. It can be used for study of mechanism of drug action. It is further postulated that change in PGE_2 and cAMP mechanism may not be responsible for fluid accumulation in rat ileal loop.

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SYNTHESIS AND STUDY OF 2-ARYL-4-[3'-(*p*-SUBSTITUTED BENZYLIDENEAMINO)-4'-METHOXY-PHENYL] THIAZOLES FOR IN VITRO ANTITUBERCULOSIS ACTIVITY-I

A. M. PATIL, D. T. CHAUDHARI, MADHUBEN R. PATEL AND S. S. SABNIS

Department of Chemotherapy, Haffkine Institute, Parel, Bombay 400 012 (India)

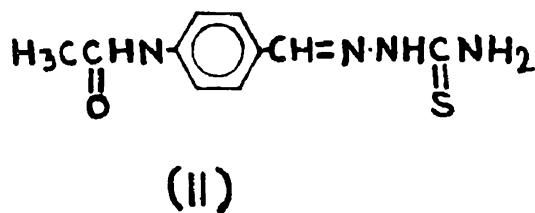
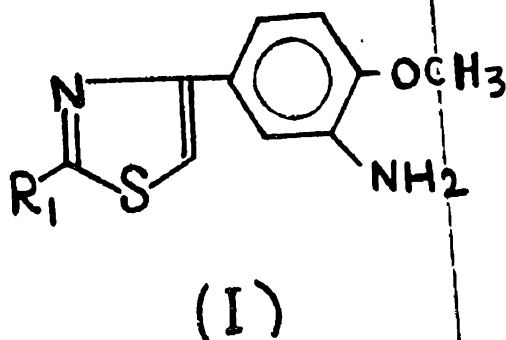
In view of antituberculosis activity displayed by various 4-(*p*-aminophenyl) thiazoles and 4-(3'-amino-4'-methoxyphenyl) thiazoles, various azomethine derivatives have been prepared in the present work from 4-(3'-amino-4'-methoxyphenyl) thiazoles and *p*-acetamidobenzaldehyde and *p*-N, N-dimethylamino-benzaldehyde. In all, nineteen derivatives have been synthesized and screened for *in vitro* antituberculosis activity. Nine compounds showed activity and inhibited the growth of H₃₇ strain of *M. tuberculosis* at concentration ranging from 5 to 2 µg/ml.

Introduction

Earlier, we reported the synthesis and antituberculosis activity of 4-(3'-amino-4'-methoxyphenyl)-thiazoles and 4-(*p*-aminophenyl) thiazoles (1, 2). Many of these thiazoles had minimum inhibitory concentration for *in vitro* antituberculosis activity as low as 0.2 µg/ml. In the present work, we have prepared azomethine derivatives from 4-(3'-amino-4'-methoxyphenyl) thiazoles (I) and some biologically important aldehydes like *p*-acetamidobenzaldehyde and *p*-N, N-dimethylaminobenzaldehyde.

The well-known antituberculous drug thiacetazone (II) has azomethine—CH=N—linkage formed by *p*-acetamidobenzaldehyde and thiosemicarbazide. Another drug used in the treatment of tuberculosis viz., rifampicin, contains an azomethine linkage formed by 3-formyl rifamycin and N-amino-N'-methylpiperazine. Rifampicin shows marked improvement in antituberculous activity over the parent molecules of rifamycins (3).

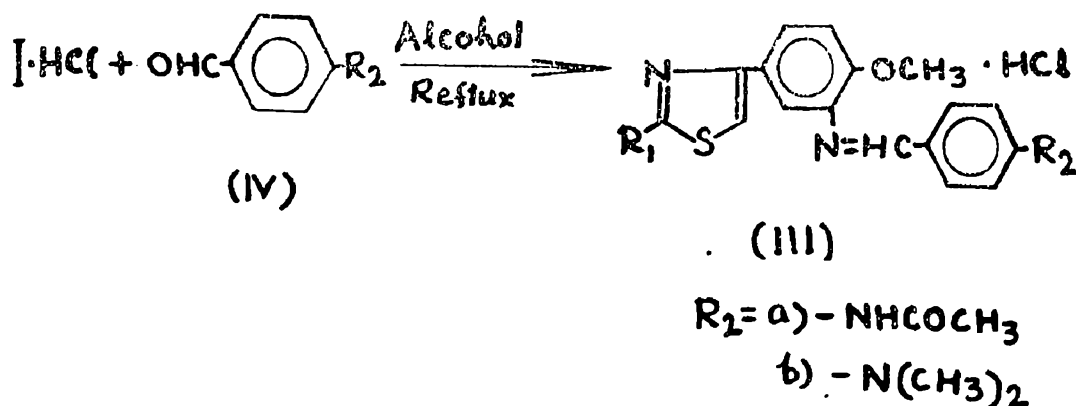
The so-called azomethines or Schiff bases might be inactive *per se* but may get



modified into active form by the process taking place in the host tissue under physiological conditions. With this approach, in 1953, a number of benzylideneamino compounds with azomethine linkage— $\text{CH}=\text{N}$ were studied and found active against tuberculosis in experimental test systems (4).

Chemistry

All the Schiff bases (III) were prepared by refluxing required thiazole hydrochloride (I) with *p*-acetamidobenzaldehyde (IVa) or *p*-N, N-dimethylaminobenzaldehyde (IVb) in alcohol and were isolated as hydrochloride salts (5).



4-(3'-amino-4'-methoxyphenyl) thiazoles having various substitutions at two positions of thiazole ring were prepared by previously reported methods (1).

Experimental

2-(3-methoxy-4-propoxyphenyl)4-[3'-(p-acetamidobenzylidene)-amino]-4-methoxyphenyl thiazole hydrochloride—To a solution of 2-(3-methoxy-4-propoxyphenyl)-4-(3'-amino-4'-methoxyphenyl) thiazole hydrochloride (0.81 g, 0.002 mole) in dry warm alcohol (10 ml) was added an alcoholic warm solution (8 ml) of *p*-acetamidobenzaldehyde (0.32 g, 0.002 mole). The mixture was refluxed for ten minutes. The resulting clear red coloured

solution on keeping at room temperature, deposited a crystalline solid. This was collected by filtration and washed with dry ethanol and ether to give analytically pure hydrochloride of Schiff base; yield : 0.88 g (80%); m.p. 230-232°.

All other Schiff bases reported in Table I were prepared in similar way.

Discussion

The *in vitro* tuberculostatic activity was determined on culture of *M. tuberculosis var hominis* (strain H₃₇ Rv) cultivated in Youman's medium containing 10% horse serum by a modified method of Doub and

Youman (6) and previously reported from this laboratory (7).

All the nineteen compounds (Table I) were tested *in vitro* test system and out of these, nine compounds showed activity and inhibited the growth of *M. tuberculosis* at a concentration ranging from 5 µg/ml to 2 µg/ml.

The compounds 1 and 2 showed the highest activity among these Schiff bases which were obtained by condensation of *p*-acetamidobenzaldehyde and thiazoles I, ($\text{R}_1 = \text{Ph}$; and PhCH_2 respectively). However, the replacement of *p*-acetamidobenzaldehyde by *p*-N, N-dimethylaminobenzaldehyde resulted in the compounds 12 and 13 which were devoid of activity.

In contrast to the above, Schiff bases obtained from some particular thiazoles and *p*-N, N-dimethylaminobenzaldehyde showed activity as in compounds 15 and 16; however, when same thiazoles were used for the formation of Schiff bases with *p*-acetamidobenzaldehyde, these did not show activity as in compounds 4 and 5, respectively.

Compounds 1, 2, 6, 8 and 10 obtained from *p*-acetamidobenzaldehyde were found to be active whereas corresponding compounds obtained from *p*-N, N-dimethylaminobenzaldehyde failed to show any activity except 7 and 8.

Definite correlation in structure activity relationship could not be drawn in these Schiff bases from two benzaldehydes IVa and IVb.

Summary

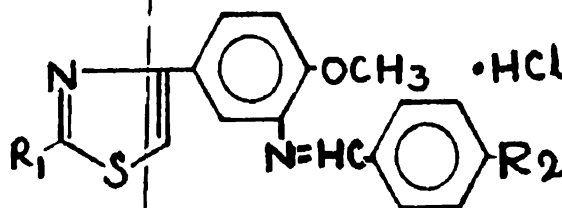
The azomethines prepared from *p*-acetamidobenzaldehyde, *p*-N, N-dimethylaminobenzaldehyde and 4-(3-amino-4-methoxyphenyl) thiazoles inhibited the growth of *M. tuberculosis* at the minimum concentration of 2 µg/ml *in vitro*. The compounds derived from *p*-acetamidobenzaldehyde were more active than corresponding compounds obtained from *p*-N, N-dimethylaminobenzaldehyde.

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TABLE I

2-Aryl-4- [3-(3-acetamidoand/or *p*-N, N-dimethylaminobenzylidene-amino)-4-methoxyphenyl] thiazoles Hydrochlorides



Serial No.	R ₁	°R ₂	M.p. °C*	Molecular formula†	Antituberculosis activity µg/ml‡
1	2	3	4	5	6
1	Phenyl	NHCOCH ₃	246-48	C ₂₅ H ₂₁ N ₃ O ₂ S. HCl	2
2	Benzyl	NHCOCH ₃	290 (§)	C ₂₈ H ₂₃ N ₃ O ₂ S. HCl	2
3	3, 4-Dimethoxyphenyl	NHCOCH ₃	246-48	C ₂₇ H ₂₅ N ₃ O ₄ S. HCl	..
4	3, 4, 5-Trimethoxyphenyl	NHCOCH ₃	218-20	C ₂₈ H ₂₇ N ₃ O ₅ S. HCl	..
5	3-Methoxy-4-Ethoxyphenyl	NHCOCH ₃	239-41	C ₂₈ H ₂₇ N ₃ O ₄ S. HCl	..
6	3-Methoxy-4-Propoxyphenyl	NHCOCH ₃	230-32	C ₂₉ H ₂₉ N ₃ O ₄ S. HCl	5
7	3, 4-Diethoxyphenyl	NHCOCH ₃	238-41	C ₂₉ H ₂₉ N ₃ O ₄ S. HCl	5
8	3-Ethoxy-4-Methoxyphenyl	NHCOCH ₃	218-16	C ₂₈ H ₂₇ N ₃ O ₄ S. HCl	5
9	3-Ethoxy-4-Propoxyphenyl	NHCOCH ₃	225-28	C ₃₀ H ₃₁ N ₃ O ₄ S. HCl	..

TABLE I—*contd.*

Serial No.	R ₁	°R ₂	Mp °C*	Molecular formula†	Antituberculosis activity µg/ml‡
1	2	3	4	5	6
10	3-Methoxy-4-Hydroxyphenyl	NHCOCH ₃	238-41	C ₂₀ H ₂₃ N ₃ O ₄ S. HCl	5
11	3-Ethoxy-4-Hydroxyphenyl	NHCOCH ₃	205-07	C ₂₇ H ₂₆ N ₃ O ₄ S. HCl	..
12	Phenyl	N(CH ₃) ₂	244-48	C ₂₅ H ₂₃ N ₃ OS. HCl	..
13	Benzyl	N(CH ₃) ₂	180-84	C ₂₆ H ₂₆ N ₃ OS. HCl	..
14	3, 4-Dimethoxyphenyl	N(CH ₃) ₂	233-36	C ₂₇ H ₂₇ N ₃ O ₂ S. HCl	5
15	3, 4, 5-Trimethoxyphenyl	N(CH ₃) ₂	229-31	C ₂₈ H ₂₉ N ₃ O ₄ S. HCl	5
16	3-Methoxy-4-Ethoxyphenyl	N(CH ₃) ₂	198-201	C ₂₈ H ₂₉ N ₃ O ₃ S. HCl	5
17	3-Methoxy-4-Propoxyphenyl	N(CH ₃) ₂	213-16	C ₂₉ H ₃₁ N ₃ O ₃ S. HCl	..
18	3, 4-Diethoxyphenyl	N(CH ₃) ₂	191-93	C ₂₉ H ₃₁ N ₃ O ₃ S. HCl	5
19	3-Ethoxy-4-Methoxyphenyl	N(CH ₃) ₂	205-07	C ₂₈ H ₂₉ N ₃ O ₃ S. HCl	..

* Melting points are capillary melting points and are uncorrected.

† Analysed correctly for C, H and N within $\pm 0.4\%$

‡ Minimum inhibitory concentration *in vitro* antituberculosis action for isoniazid=0.04 µg/ml and for *p*-aminosalicylic acid=0.4 µg/ml.

§ Decomposition.

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OBSERVATIONS ON PARTICLE SIZE AND ITS EFFECTS IN ACEDAPSONE INJECTIONS

M. H. SHAH, PUSHPA A. KIRKE AND HANSA SHAH

Department of Chemotherapy, Haffkine Institute, Parel, Bombay 400 012

In order to ensure precise dose from every ampoule of injection to achieve uniformity in blood levels of DDS, the uniform particle size of the DADDS (Acedapson) suspension injection became necessary. With about 90% of the particles between 1 to 6 μ size, the suspension was reasonably uniform. Such suspension gave blood levels of DDS significantly higher than the minimum inhibitory concentration for *M. leprae* even after 70 days.

Introduction

In case of an injectable suspension, absorption depends on many factors. The two most important ones are the nature of the substance and size of the particle. For example, Zinc Insulin lente, which is a suspension is absorbed within 24 hours while some other preparations require 3 to 8 days for complete absorption.

Because of the usually noticed drop-outs in the treatment of leprosy patients with Dapsone (DDS) a suspension formulation of 'Acedapson' was found useful (1). This could maintain the requisite blood levels of DDS for a long time. It has been reported (2) that the effective blood level is maintained for 77 days. Although by and large this is true, the particle size would determine the blood level. We should expect the duration to vary from individual to individual and also from sample to sample depending on the particle size. This aspect has not been reported with regard to acedapson injection so far.

Our initial preparation was formulated broadly on Shepard's formula (1, 2). We observed with our samples that the peak

level was reached on fourth day and then gradually fell. The fall was sharper after fourth week, but the level was still above MIC on 70th day (3). However, the blood levels showed fairly wide individual variations. This observation led us to examine the amount of the drug in each ampoule. Having observed significant variation in the amount of drug in each ampoule, it was thought necessary to control the particle size in such a manner that uniform amounts of the drug could be filled in each ampoule without difficulty and, once this is done, the only variable that remained was the individual variation of the rate of absorption.

With this objective in view, DADDS injections were prepared with the aid for micronization. The initial experience with this aid was not satisfactory. Besides, because of the possibility of contamination with fibres and dust particles it was thought worthwhile to prepare the powder with uniform small size crystals. For comparison, in two batches (Samples II and III) slightly bigger crystals were allowed to be formed, but the filling of uniform quantity in the ampoules was not easy in these cases. Hansolar^(R) is a vial of 3 adult

doses. In the three withdrawals from this vial using 20 gauge needle the amount of drug varied from -7% to $+18\%$ of the average.

Material and Method

DADDS is dissolved in dimethyl formamide. The solution is filtered after treating with charcoal (if found necessary) and the clear solution is poured in water with stirring. The stirring is then stopped and the precipitation is allowed to be complete (30 minutes) and then filtered. The material is sucked almost dry and then washed with distilled acetone and dried at $140-150^{\circ}\text{C}$. This powder is used for formulating the injection in the mixture of castor oil and benzyl benzoate essentially as used by Shepard (2) with additional 0.1% BHA. Such a formulation ensured reasonably uniform volume and drug content in each of the 2 ml ampoules.

For knowing the amount of the drug in each ampoule, the contents were shaken with 50 ml of ethyl ether and the solid was quantitatively filtered after 10 minutes using additional 10 ml of ethyl ether and weighed after drying (Table I). In this manner three ampoules, one from the first 25 ampoules, the second from the middle 25 and the third from the last 25, from a batch of about 450 ampoules were examined.

The drug which had dissolved in the vehicle was determined by addition of 25 ml of cold ethanol ($15-17^{\circ}\text{C}$) and filtered and again washed with 10 ml of the same cold ethanol (Table II). Two batches were examined. In the batch of 450 ampoules, two ampoules were selected for examination, one near 100th ampoule and second near 300th ampoule.

To examine the particle size, the contents of the ampoule were thoroughly shaken and using a capillary tube a sample

was taken on a slide. This slide was mounted on the microscope to measure the size of the particles. A total of 5 fields were examined. Practically all the particles in the field were measured and counted and then the average of five fields was taken (Table III). Five batches including Hansolar of Parke-Davis (V) were examined. Batches II and III were those where a little larger crystals were allowed to be formed.

Injections from one batch in which the drug content was reasonably uniform and the majority of the particles had uniform size, was utilized in four patients to study the DDS levels in their blood. Prior to administering the injection, initial zero DDS level was ensured. The DDS levels obtained between 59 and 71 days were estimated by the method of Ellard and Gammon (4). The results are shown in Table II.

Results and Discussion

Two batches were examined for the total drug content. The deviation from the average content was within $\pm 3.5\%$. This showed that a reasonably good uniformity was maintained in the amount of the drug in each ampoule.

For the amount of the drug in solution form in the ampoule, the results showed that the amount of drug that went into solution in the vehicle ranged from 16.7% to 19.5% i.e. over 80% the drug was in suspension form. The dissolved drug might be giving higher DDS-blood levels in the initial period.

When such injections were administered to eight patients (four patients received injections from batch No. 5 and four received from batch No. 6), one examined on 69th day showed abnormally high level and thus did not warrant consideration for knowing the blood levels of DDS. This

patient might have taken DDS tablet. Unfortunately, we could get blood sample from only one patient out of the four who received DADDS of batch No. 6. One patient refused to give blood sample and two did not report when called. The results on the four patients (three from batch No. 5 and one from batch No. 6) clearly showed that the DDS level in blood was not less than 18 ng/ml of serum of 63rd day. This level is much higher than the minimum inhibitory concentration which is less than 10 ng/ml of serum (5). This would broadly be true for majority of the

cases. A comparative study with Hansolar could not be done.

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TABLE I

Drug Contents in Acedapsone Suspension Ampoule

				Drug content	Deviation from average	% Deviation from average
<i>Batch A—</i>						
Ampoule I	..	..	..	200.5 mg	−2.3 mg	−1.15
Ampoule II	..	..	..	203.3 mg	+0.5 mg	+0.25
Ampoule III	..	..	..	204.5 mg	+1.7 mg	+0.85
Average	..	..	..	202.8 mg		
<i>Batch B—</i>						
Ampoule I	..	..	..	215.0 mg	+2.5 mg	−1.2
Ampoule II	..	..	..	217.0 mg	+4.5 mg	+2.1
Ampoule III	..	..	..	205.5 mg	−7.0 mg	−3.3
Average	..	..	..	212.5 mg		

TABLE II

DADDS Dissolved in Vehicle of Acedapsone Suspension Injection

				Undissolved drug	Average total content	Dissolved in vehicle	% in solution
<i>Batch A—</i>							
Ampoule I	..	..	..	166.1 mg			
Ampoule II	..	..	..	160.8 mg			
Average	..	..	..	163.5 mg	202.8 mg	39.3 mg	19.6
<i>Batch B—</i>							
Ampoule I	..	..	..	178.45 mg			
Ampoule II	..	..	..	176.00 mg			
Average	..	..	..	177.22 mg	212.5 mg	35.28 mg	16.9

TABLE III
Distribution and Particle Size of Acedapsone in Suspension Injections

Sample No.	Fields	1 to 3 μ	3 to 6 μ	7 to 12 μ	13 to 20 μ	More than 20 μ	Remarks
		No. (%)	No. (%)	No. (%)	No. (%)		
I	F ₁	300 (83.1)	36 (9.94)	26 (7.2)	None	None	
	F ₂	350 (81.5)	50 (11.6)	30 (7.0)	None	None	
	F ₃	330 (84.5)	37 (9.5)	24 (6.6)	None	None	
	F ₄	250 (85.9)	20 (6.9)	22 (7.2)	None	None	
	F ₅	200 (78.7)	32 (12.7)	22 (8.7)	None	None	
	Average (%)	286 (82.1)	37 (10.6)	25 (7.1)	None	None	
II	F ₁	11 (22)	18 (36)	10 (20)	10 (20)	1 (2)	
	F ₂	14 (26.9)	9 (17.3)	21 (40.4)	8 (15.4)	None	
	F ₃	17 (30.4)	13 (23.2)	20 (35.8)	6 (10.4)	None	
	F ₄	13 (22.2)	18 (30.5)	24 (40.7)	3 (5.1)	1 (1.75)	
	F ₅	17 (31.5)	20 (37.4)	11 (20.4)	5 (9.3)	1 (1.85)	
	Average (%)	14 (26.4)	15 (28.3)	17 (32)	6 (11.3)	0.6 (1.1)	
III	F ₁	15 (12.8)	20 (17.1)	60 (51.2)	16 (13.7)	6 (5.15)	
	F ₂	8 (10.8)	23 (30.1)	30 (40.5)	10 (13.5)	3 (4.5)	
	F ₃	11 (17.5)	8 (12.6)	35 (55.5)	6 (9.5)	3 (4.8)	
	F ₄	10 (17.3)	8 (13.8)	30 (51.7)	6 (10.2)	4 (7.0)	
	F ₅	6 (8.6)	24 (34.3)	28 (40)	8 (11.2)	4 (5.7)	
	Average (%)	10 (13.3)	15 (20)	37 (49.3)	9 (12)	4 (5.3)	
IV*	F ₁	80 (72.8)	24 (21.8)	6 (5.5)	None	None	
	F ₂	62 (67.3)	20 (21.7)	9 (9.8)	1 (1.8)	None	
	F ₃	55 (55.5)	39 (39.4)	3 (3.3)	2 (2.2)	None	
	F ₄	75 (61.4)	32 (26.27)	13 (10.6)	2 (1.6)	None	
	F ₅	66 (57.7)	32 (28.6)	16 (14.4)	None	None	
	Average (%)	68 (63.5)	29 (27.1)	9 (8.4)	1 (0.94)	0.2 (0.18)	
V (Hansolar Sample)	F ₁	16 (16)	15 (15)	37 (37)	24 (24)	8 (8)	
	F ₂	18 (20.7)	16 (18.4)	26 (29.9)	21 (24.1)	6 (6.9)	
	F ₃	23 (34.3)	12 (17.9)	12 (17.9)	14 (20.9)	6 (8.9)	
	F ₄	30 (34.9)	13 (15.1)	20 (23.2)	12 (13.9)	11 (12.8)	
	F ₅	15 (30.7)	5 (10.2)	16 (32.6)	7 (14.3)	6 (12.2)	
	Average (%)	20 (26.3)	12 (15.8)	22 (28.9)	15 (19.7)	7 (9.2)	

*Same as injection Batch No. 6 shown in Table IV.

TABLE IV

The DDS Levels in Patients given DADDs Injection

Serial No	Injection Batch No.	Age (Years)	Sex	Type of leprosy	Initial level	Concentration of DDS			
						ng/ml of serum after DADDs injection			
						59th day	62nd day	68th day	71st day
1	5	40	Male	TM ₂	Nil		23ng		25 ng
2	5	38	Male	B	Nil		24 ng		
3	5	30	Female	L ₂ P1	Nil	39 ng		25 ng	
4	5	17	Male	L1P1	Nil		*227ng		
5	6	26	Male	TM ₁	Nil		18		28

*This is abnormally high. He might have taken DDS tablets.

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ERYTHROCYTE ENZYMES AND GLUTATHIONE IN DECCANI SHEEP

ROSHAN S. PAVRI*, A. J. BAXI*, H. K. SONI† AND S. N. NAIK‡

*Blood Group Reference Centre (ICMR), Seth G. S. Medical College,
Haffkine Institute and Cancer Research Institute, Tata Memorial Centre,
Parel, Bombay 400 012*

Deccani breed sheep from Maharashtra were screened for potassium (K^+), glutathione (GSH), GSH stability and red cell enzymes, pyruvate kinase (PK), catalase and GSH-peroxidase. It was observed that GSH was highly stable in Deccani sheep. The changes in K^+ , GSH and red cell enzymes are discussed in light of our previous findings on G-6-PD deficiency in Indian sheep. It has been also shown that this breed consists of probably only low potassium (LK) group of animals.

Introduction

Domestic sheep is an interesting mammalian species in which a large number of polymorphic systems have been demonstrated (1, 2, 3, 4, 5, 6). Among the polymorphic systems, most studies have centred around low potassium (LK) and high potassium (HK), low glutathione (GSH^L) and high glutathione (GSH^H) genes in various sheep breeds. In India too, various sheep breeds studied so far have these inherited traits (6).

Deficiency of glucose-6-phosphate dehydrogenase (G-6-PD) in Indian sheep and other animal species has been demonstrated by us previously (7, 8). GSH has

been shown to be intimately connected with the maintenance of integrity of the red cell and becomes considerably unstable in G-6-PD deficient individuals (9, 10). In view of the above, it seemed desirable to study GSH levels and stability and other associated biochemical changes in Indian sheep. We present below the data on glutathione (GSH), pyruvate kinase (PK), catalase and GSH-peroxidase (GSH-Px) in relation to potassium type in Deccani breed from Maharashtra, Western India.

Material and Method

Thirty-four adult sheep, both males and females belonging to Deccani breed from a local farm were studied. Bloods were collected plain and in EDTA by jugular puncture. Investigations were carried out within 6 hours of collection.

Cations— K^+ and Na^+ were determined by flame photometry.

GSH and GSH Stability—GSH stability was adopted from Beutler (11) and GSH

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Present Addresses—

*Blood Group Reference Centre Unit at Haffkine Institute, Parel, Bombay 400 012 (India).

†Department of Pharmacology, Haffkine Institute, Parel, Bombay 400 012 (India).

‡Cancer Research Institute, Parel, Bombay 400 012 (India).

levels were determined by the DTNB method (12).

Red Cell Enzymes—PK was assayed according to the procedure of Swarup Mitra (13) as outlined by Bapat and Baxi (14). Catalase was determined by the method of Takahara (15) and GSH-Px analysed according to the modified method by Gross *et al.* (16) of the original method of Mills and Randall (17).

Hemoglobin and Hematocrit—These components were analysed by standard methods (18).

Result and Discussion

Cations—On the basis of data reported in Table I, it appears that all the animals studied belonged to the LK type. The mean value of Ke^+ , 0.113 ± 0.023 meq/gm. Hb was higher than that reported for several American breeds (19). This difference probably indicates the breed variation. It was difficult to ascertain whether these animals were heterozygotes or not in the absence of blood group M studies (20). The question as to whether the HK type exist among Deccani animals will need a study of a larger sample.

GSH and GSH Stability—GSH levels appeared to be widely varied in the LK group judging from our initial studies. We therefore divided the Ke^+ in two groups (within LK type) and analysed GSH levels accordingly. Mean GSH level in the 0 to 0.1 meq/gm. Hb group was lower than in the 0.1 to 0.24 meq/gm Hb group. This difference was significant ($p < 0.01$). However, pooled GSH mean indicates that this fits into GSH^h category. It is apparent that this breed is monomorphic with respect to GSH. These LK/ GSH^h results are in agreement with those reported by Kalla *et al.* (6) for some Rajasthani (Indian) breeds and Agar *et al.* (3) for Merino breeds. The correlation of LK with GSH^h (1, 3) is amply confirmed in the present series. Intra group differences of GSH levels and stability with respect to Ke^+ concentration are intriguing and difficult to explain at present (Table I). It may be that the GSH level may be greatly influenced by slightest differences in the Ke^+ concentration.

TABLE I

Data on GSH Levels and Stability in relation to Ke^+ Concentration in Deccani Sheep

Ke^+ (meq/gm Hb)	No. tested	GSH levels (mg%RBC)		GSH stability %	
		Range	Mean $\pm$ S.E.	Range	Mean $\pm$ S.E.
0 to 0.10	13	25.42 to 45.04	36.17* ± 1.78	33.34 to 82.57	63.69† ± 4.24
0.10 to 0.24	15	23.62 to 57.89	44.33 ± 2.04	57.17 to 92.42	72.85 2.71

* $p < 0.01$

† $p < 0.05$

Data on GSH stability in Indian sheep are probably reported for the first time. Results in Table I indicate that GSH stability is quite high in Deccani sheep. This observation is in sharp contrast to that reported in G-6-PD deficient man (9, 10, 14). Though the exact mechanism for the high GSH stability is unknown it seems

present study. Conceivably the higher catalase and GSH-Px levels (similar to humans) are probably concerned in removal of H_2O_2 more efficiently, thus affording a greater protection to the red cell. This postulate coupled with the stable GSH system seems to confer hemolysis resistant status to sheep in a general way.

TABLE II

Distribution of Erythrocyte Enzymes in Low Potassium (LK) Deccani Sheep

Erythrocyte enzyme	No. tested	Range	Mean $\pm$ S.E.
Pyruvate Kinase (Units/ml RBC/min)	34	25.00 to 45.00	35.32 $\pm$ 1.03
Catalase (K/gm Hb)	21	35.00 to 47.6	41.9 $\pm$ 0.78
GSH-Peroxidase (K/gm Hb)	21	46.00 to 71.00	57.76 $\pm$ 1.47

likely that the GSH system appears to compensate for low to zero G-6-PD activity in this species. This concept is further supported by the fact that hemolytic anemia could not be produced in sheep after primaquin treatment (21). Further studies to inquire into high GSH stability are clearly indicated.

Red Cell Enzymes—Levels of PK in Deccani sheep have been found to be similar to the humans. Our value is not comparable with that reported by Agar and Smith (22) since the units of activity are expressed differently. Again, this finding differs from that of G-6-PD deficient humans in whom there is a compensatory rise in PK (14). Findings on catalase activity in the present study are in agreement with those of Agar and Smith (22) for the LK type animals. It has not been possible to compare GSH-Px in HK-GSH^H types as these were not encountered in the

Higher mean hematocrit value (39.8, not reported in the Table) as compared to 31.8 reported by Agar *et al.* (4) suggest that geographic factors seem to play a role in maintenance of high PCV in our series as Deccani breed is predominantly highlandic. Further studies regarding hemocrit changes in the sheep breeds at different altitudes are suggested.

In conclusion, the Deccani breed has quite interesting and unique features outlined above. How far GSH stability influences associated biochemical topography remains to be determined.

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BOOK REVIEW

Book Review

Title : MOLECULAR AND CELLULAR MECHANISMS IN DISEASE

Editor : LANCKER, J. VAN, UNIVERSITY OF CALIFORNIA, LOS ANGELES,
CALIFORNIA, USA.

411 Figures, 68 Tables, XX, 1168 Pages, 1976

Cloth DM 362,—; US \$ 148.50

(in 2 parts, not available separately)

Berlin-Heidelberg-New York; Springer-Verlag ISBN 3-540-06932-1

Review : The modern pathologist has been consistently refusing to sever the umbilical cord which connects him to Virchow. It is naive to believe that every form of disease process has to have a corresponding morphological abnormality. Such alterations, when present do not necessarily explain, in every case, either in part, or *in toto*, the pathological mechanisms underlying a disease process. Perhaps gross and histological morbid anatomy has acquired such undue importance, because of the approach of the traditional books and teachers in pathology, who have consistently resisted change, for almost two decades now. Considering the tremendous advances made in molecular biology, it was refreshing to see a text written in a totally different vein—the author does not even attempt to provide the traditional fare. Instead he has made a commendable effort to explore, elaborate and present in a lucid manner molecular mechanisms underlying disease processes.

DR. R. S. KAMAT

Assistant Director, Clinical Pathology Department
Haffkine Institute, Bombay 400 012 (INDIA)

Book Review

Title : MOLECULAR BASIS OF MOTILITY

Editor : HEILMEYER, L.; RUEGG, J. C. AND WIELAND, T.

103 Figures, 22 Tables, IX, 222 Pages, 1976

Cloth DM 60,—; US \$ 24.60

Berlin-Heidelberg-New York; Springer-Verlag ISBN 3-540-07576-3

Review : So far, several studies including electron microscopy, X-ray analysis kinetics, protein chemistry and enzymology had been performed to investigate the structure and function of muscle and the molecular mechanism of movement.

For the first time have these studies been analysed together in order to present a comprehensive picture of the organization and function of the entire system of motility.

The colloquium has been divided under three headings, viz. mechanism of chemomechanical energy transformation, Ca^{++} action and the regulation of actomyosin ATPase and microtubules and microfilaments. All chapters present a vivid idea about the histochemical and biochemical mechanisms underlying muscle contraction. In the light of these results, a discussion on other contractile structures such as microtubules and microfilaments is also included.

The book will certainly stimulate the exchange of new concepts among specialists from different biomedical disciplines.

DR. N. G. CHANDERKAR

Officer-in-Charge, Radiation Biology Unit
Haffkine Institute, Bombay 400 012 (INDIA)

Book Review

Title : METABOLIC INTERCONVERSION OF ENZYMES 1975

Proceedings of Life Sciences

Fourth International Symposium held in Arad (Israel)

April 27—May 2 1975

Editor : SHALTIEL, S. AND REHOVOT, ISRAEL

ORGANIZED BY SHALTIEL, S.; GUTMAN, A.; LEVITZKI, A.; MOSES, S. W.; TIETZ DEVIR, A. AND GAIRON, S.

147 Figures, 66 Tables, XVII, 234 Pages. 1976

Cloth DM,—; US \$ 29·60

Berlin-Heidelberg-New York; Springer-Verlag ISBN 3-540-07888-6

Review : This volume contains some selected topics of current interest in the field, including glycogen metabolism and the mechanism of its regulation, involving multivalent phosphorylation at the molecular level; the role of cAMP dependent protein kinase, and hormones and phosphatases in a system of interconversion. Finding place also are the use of chemical and physicochemical probes in studying the architecture of interconvertible enzymes; the use of model systems for studying the structure and function of regulatory sites, and the assembly, function and regulation of the pyruvate dehydrogenase multienzyme system.

In the following sections, adenylation, ADP-ribosylation and their role in glutamine metabolism, in the functions of bacterial toxins, in mitochondria and during T-phage infections are discussed. Specific proteolysis is evaluated as a regulatory device and, also, the role of lysosomes in protein turnover. The comparison of *in situ* versus *in vivo* studies of enzymes is very informative. The final section deals with the unusual modulations of enzyme action and their possible function in ageing cells.

The subject matter in the book is well-treated with lucid presentation of figures and experimental data. The text is explicit, well-organised under sub-titles and altogether very readable. Finally the publishers deserve a word of praise for the excellence in presentation that would make this volume an useful, besides attractive, reference work for both students and specialists, alike, in the field.

DR. P. RAMKRISHNAN

Senior Scientific Officer CI-I, Department of Biochemistry
Haffkine Institute, Bombay 400 012 (INDIA)

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DIRECTIONS TO CONTRIBUTORS

Bulletin of Haffkine Institute is a triannual publication. It embodies review and research articles in biomedical sciences, mainly devoted to studies on Communicable Diseases.

Contributions should be precise and concise, highlighting original observations and inferences. The type-script (T.S.) should be sent in duplicate, typed in double space on only one side of the sheet with ample margins.

A brief summary not exceeding 200 words, should accompany the article.

The title should be informative and specific, followed by author's name and address.

A short title in not more than forty letters should also be suggested.

The text of the paper should be generally sub-divided into: Introduction, Materials and Methods, or Experimental, Results, Discussion, Acknowledgement and References.

'Short Abstract' of about 100 words suitable for inclusion in 'International Abstracts of Biological Sciences', etc. should be typed on a separate sheet, with author's name, address and title of the paper.

Results should be presented concisely in tables giving only important and relevant data and avoiding incorporation of elaborate observations.

Restraint should be exercised in submitting illustrative matter. Photographs should be well-defined and on glossy paper.

References in the text should be cited by numbers serially as they appear, and to be inserted in parenthesis, e.g. 'Plasmid-mediated resistances to these heavy metals have also been observed in enteric bacilli, especially in P factor carrying organisms (1), and have recently been studied by several workers (2, 3, 4). References at the end should be arranged numberwise serially as they figure in the text with author's name first and abbreviations according to Index Medicus. The following pattern may be followed:

- (i) Reuben, R.; Carey, D. E. and Meyers, R. M. (1968)—Japanese encephalitis studies in Vellore, S. India, *Indian J. Med. Res.*, **56**, 109-18.
- (ii) Cruickshank, R. (1968)—Medical Microbiology, 11th Ed. 180-4, E and S Livingstone Ltd., Edinburgh, p. 28.

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