ANTITRICHOMONAL AGENTS 5-NITROTHIAZOLES, 5-NITROPYRIDINES AND 5-NITROPYRIMIDINES

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Some 5-nitrothiazoles, 5-nitropyridines and 5-nitropyrimidines have been synthesised and evaluated against *Trichomonas vaginalis* and *Trichomonas foetus in vitro* and *in vivo* in the mouse, hamster and monkey. Although 2-amino-5-nitropyrimidine, 2-acetamido-5-nitropyrimidine and 2-trifluoroacetamido-5-nitropyrimidine were highly active in mice infected with *T. vaginalis* the activity was not superior to the standard 2-acetamido-5-nitrothiazole. The nitropyrimidines were highly species specific, being much more active *in vivo* against *T. vaginalis* than against *T. foetus*. One compound, 2-amino-5-nitropyrimidine was selected as the best of this series because of the high blood levels obtained after a single oral dose in a monkey, and its presence in a biologically active form.

Trichomonas vaginalis (Donné, 1836) and *Trichomonas foetus* are flagellate parasites of the genito-urinary tract of man and bovine, respectively. In the female, *Trichomonas vaginalis* causes an overt disease of the genito-urinary tract (Kean, 1955; Kean and Wolinska, 1956; Perl, Guttmacher and Raggazoni (1956), while the male serves primarily as an asymptomatic carrier (Perl and others, 1956). Evidence is accumulating that the parasite localises in Skene's glands, Bartholin's glands and the urinary tract (Kean, 1955; Kean and Wolinska, 1956; Perl and others, 1956), thus making it extremely difficult to eliminate the infection by local treatment.

2-Amino-5-nitrothiazole and a few of its acyl derivatives, in particular 2-acetamido-5-nitrothiazole (Cuckler, Kupferberg and Millman, 1955; Prince, 1960) are active in animals infected with *T. vaginalis* and *T. foetus*. The antibiotics Trichomycin and Fervenulin are also active (Hosaya, Soeda, Komatsu, Okada and Watanabe, 1953; Deboer, Dietz, Evans and Michaels, 1959–1960). Early clinical evaluation of these compounds was promising (Perl and others, 1956; Plentyl, Grey, Nelson and Da Lali, 1956; Magara, Nittono and Senda, 1955), but later they were shown to be without effect (Gardner and Dukes, 1956; Barnes, Bontwood, Haines, Lewington, Lister and Haram, 1957; Catterall and Nicol, 1957). Because of its toxicity, Fervenulin has not been tested in man. The only compound which has been found effective in laboratory animals (Cosar and Julou, 1959) and man is the recently discovered 1-(2'hydroxyethyl)-2-methyl-5-nitroimidazole. Cure rates above 85 per cent in females have been reported when the drug was used locally (Durel, Coutre, Collart and Girot, 1960) or systemically (Durel and others, 1960; Nicol, Barrow and Redmond, 1960; Rodin, King, Nicol and Barrow, 1960; Wilcox, 1960) and in males treated systemically (Nicol and

others, 1960; Rodin and others, 1960; Sylvestre, Gallai and Ethier, 1959; Sylvestre, Belanger and Gallai, 1960).

The need remains for other effective systemic treatments for this disease.

Chemical

The biological action of isosteric compounds often differs only in degree, therefore, we investigated isosteric compounds of 2-amino-5-nitrothiazole and 2-acetamido-5-nitrothiazole. The replacement of the sulphur atom of these thiazoles by the CH=CH and CH=N groups gave the isosteric compounds 2-amino-5-nitropyridine, 2-amino-5-nitropyrimidine and their corresponding acetyl derivatives. The *in vitro* antitrichomonal activities of 2-amino-5-nitrothiazole and its acetyl derivative are similar but in mice, the acetyl compound is much more active (Tables I and II). Whether this greater activity is due to more favourable distribution of the acetyl derivative in the animal body or whether the compound is actually more active in the body is not yet known. As a working hypothesis it was postulated that there might be a relation between the rate of deacylation and activity. For this reason the synthesis of the more rapidly hydrolysable trifluoroacetyl derivatives of 2-amino-5-nitrothiazole, 2-amino-5-nitropyridine and 2-amino-5-nitropyrimidine was undertaken.

In the evaluation of a series of 5-nitropyridine derivatives, 2-hydroxy-5-nitropyridine was found to be active in mice. This prompted the synthesis and evaluation of the isosteric compounds 2-hydroxy-5-nitrothiazole and 2-hydroxy-5-nitropyrimidine (Roblin, Winnech and English, 1942; Hale and Brill, 1912).

Experimental

2-*Trifluoroacetamido*-5-*nitrothiazole.* To a stirred suspension of 2-amino-5-nitrothiazole (14·5 g.; 0·10 mole) in 100 ml. of ether was added over 5 min. 50 g. (0·24 mole) of trifluoroacetic anhydride. After 15 min. the solution was treated with charcoal, filtered and distilled. The residual, slightly yellow product was washed with light petroleum and dried. Yield, 18·5 g.; m.p. 150–151°. Found: C, 25·21; H, 1·31; N, 17·40. $C_5H_2F_3N_3O_3S$ requires C, 24·98; H, 0·84; N, 17·43 per cent.

2-*Hydroxy*-5-*nitrothiazole.* This compound was synthesised as described by Babo and Prijs (1950), the product melted at 143–144° decomp. (Babo and Prijs gave m.p. 136° with decomp. at 142°). Found: C, 24·87; H, 1·77; N, 19·25; S, 21·57. $C_3H_2N_2O_3$ requires C, 24·66; H, 1·39; N, 19·17; S, 21·94 per cent.

2-*Acetamido*-5-*nitropyridine.* Acetylchloride (7·9 g.; 0·10 mole) was added dropwise over 30 min. to a cooled solution (10–15°) of 2-amino-5-nitropyridine (13·9 g.; 0·10 mole) in a mixture of dry acetone (100 ml.) and dry pyridine (10 ml.). Stirring was continued for another 30 min. at room temperature and for 15 min. at reflux temperature. The reaction mixture was poured into water (200 ml.) and the solid formed was removed by filtration. After recrystallisation from ethanol a slightly tan coloured

product was obtained melting at 198–199°. Tschitschibabin and Posdnjakow (1926) gave m.p. of 196° using acetic anhydride.

2-Trifluoroacetamido-5-nitropyrimidine. 2-Amino-5-nitropyrimidine (Roblin and others, 1942; Hale and Brill, 1912) (14·0 g.; 0·10 mole) was heated under reflux with trifluoroacetic anhydride (100 ml.) for 3 hr. with stirring. After cooling to room temperature the product was removed by filtration and recrystallised from toluene. The almost colourless product (12·9 g.) melted at 131–133·5°. Found: C, 30·45; H, 1·28; N, 23·50. $C_6H_3F_3N_4O_3$ requires C, 30·52; H, 1·28; N, 23·50 per cent.

Biological

The culture of *Trichomonas vaginalis* (Trussell and Plass, 1940) and *Trichomonas foetus* (Morgan and Campbell, 1946) made possible the *in vitro* testing of compounds for direct trichomonacidal properties. Effective contact agents have been found against *T. vaginalis* (Johnson and Trussell, 1943; Lynch, Holley and Salmirs, 1955; MacDonald and Tatum, 1948; Seneca and Ides, 1953; Trussell, 1947) and *T. foetus* (Morgan and Campbell, 1946; MacDonald and Tatum, 1948) *in vitro* and against *T. foetus in ovo* (Pierce and Morgan, 1950).

Further evaluation of compounds requires an *in vivo* system. The Rhesus monkey (*Macacus mulatta*) is the only species other than man which harbours *T. vaginalis* naturally and is susceptible to experimental vaginal infection. But few chemotherapeutic studies have been made with this animal.

Recently, Schnitzer, Kelly and Leiwant (1950) reported that white mice can be infected intraperitoneally, subcutaneously or intramuscularly with *T. vaginalis*, *T. foetus* and *T. gallinae*. Mice infected subcutaneously with either *T. foetus* or *T. vaginalis* have been used to study the effect of local treatment with either chemicals or antibiotics (Lynch and Stephens, 1955–56; Lynch, English, Morrison and Maven, 1954; Lynch, Holley and Margison, 1955). The systemic activity of compounds has been investigated in mice infected intraperitoneally with either *T. foetus* (Cuckler and others, 1955) or *T. vaginalis* (Bushby and Copp, 1955; Hosaya and others, 1953).

To ascertain whether drugs given systemically reach the vaginal secretions in an active form, a vaginal infection is required; this has been obtained by Kradolpher (1954) in golden hamsters with *T. foetus*.

Experimental

Materials

Cultures of *T. vaginalis** and *T. foetus* strain MSC† and strain L‡ were maintained at 37° in fluid thioglycollate medium§ and 5 per cent

* Obtained through the courtesy of Dr. A. C. Cuckler of the Merck Institute for Therapeutic Research, Rahway, New Jersey, U.S.A.

† Obtained through the courtesy of Dr. W. D. Lindquist of Michigan State University, East Lansing, Michigan, U.S.A.

‡ Obtained through the courtesy of Dr. D. T. Clark of Michigan State University, East Lansing, Michigan, U.S.A.

§ Fluid thioglycollate medium obtained from Baltimore Biological Laboratories Baltimore, Maryland, U.S.A. Medium 01–140.

horse-serum. For the cultivation of *T. vaginalis*, the pH was adjusted to 6·0. No adjustment in pH was required for the growth of *T. foetus*. Unless otherwise indicated, experiments with *T. foetus* were with the MSC strain.

Determination of in vitro *End Points*

Aliquots of logarithmically growing (24 hr.) flask cultures of either species were diluted with fluid thioglycollate medium to contain 110,000 cells per ml. checked by direct counts. The "medium-inoculum" was prepared by combining 10 volumes of diluted cells with 5 volumes of horse-serum and 85 volumes of fluid thioglycollate medium. Solutions or finely divided suspensions containing 2 mg. of drug per ml. were prepared. Serial two-fold dilutions of these were made in 0·85 per cent saline. 0·5 ml. of each drug dilution was added to an experimental tube. Control tubes received an equal volume of saline. The medium-inoculum was added to each tube in 4·5 ml. amounts using a Cornwall semi-automatic syringe. The cells were agitated during this process by a magnetic stirrer. The highest concentration of any chemical tested was 200 μg./ml. against an initial concentration of 10,000 cells/ml.

After 22 hr. incubation at 37°, while the cells were still growing logarithmically, determinations of cell populations were made by direct counts. The 90 per cent inhibitory level was calculated arithmetically using the expression per cent inhibition equals $(C - E)C \times 100$, where C = number of cells per ml. in a control tube and E = number of cells per ml. in an experimental tube. When the end point could not be calculated in this manner, the results were plotted on semi-logarithmic paper, and the end point read graphically.

Plasma Level Determinations

Plasma levels of 2-acetamido-5-nitropyrimidine were determined microbiologically by titration, in the way just described, against a standard. Controls included plasma obtained before the administration of drug, together with an estimate of the 90 per cent inhibitory level of this compound.

Evaluation of Compounds in Animals Infected by the Subcutaneous Route

Groups of 5 adult male albino mice (CF 1 strain) or female golden hamsters, were infected subcutaneously with 200,000, 24 hr. cells, contained in 0·2 ml. Compounds for treatment of these infections were homogenised in 50 per cent Plazmoid*. Beginning with the Tolerated Dose (in this study the T.D. is defined as the maximum amount of drug in mg./kg./day which can be administered for six consecutive days and still allow weight gains comparable to those of the control animals), drugs were given in serial two-fold dilutions either orally or intraperitoneally. The treatment was first given immediately after infection, and continued once daily for five days. Controls were dosed with 50 per cent Plazmoid. Since the pathogenicity of the cultures varied, an

* Sterile solution of 5 per cent gelatin.

estimate of the 50 per cent infective-dose (ID50), the number of organisms successfully infecting 50 per cent of the animals, was made for each individual experiment.

Animals were killed and examined seven days after infection. The presence of trichomonads in the lesions at the site of injection was verified by microscopic examination of an eosin stained wet smear.

The 50 per cent curative dose (CD50) and the ID50 were calculated according to the method of Reed and Muench (1938), while the ED50 was obtained graphically by the method of Litchfield and Wilcoxon (1949).

The relative activity of the compounds was obtained by comparing their Therapeutic Quotients. For our purpose we define the Therapeutic Quotient to be the Tolerated Dose of a compound divided by the CD50.

Evaluation of Compounds in Hamsters Infected Intravaginally

Four to six months before study, virgin female hamsters were infected intravaginally on three successive days with 100,000 to 200,000 (48 hr.) *T. foetus* cells contained in 0·05 ml. Vaginal smears for microscopic examination were made daily for 6 days before beginning treatment and only animals showing positive smears were used.

Groups of 5 animals were treated orally once daily for six consecutive days with suspensions of the drug in 50 per cent Plazmoid. The drug suspensions were so prepared that the animals in any one group received the same amount of a drug on a mg./kg. basis.

During the treatment and for two additional weeks, vaginal smears were examined daily. After a rest period of 2 weeks, this procedure was repeated for an additional week. Animals which became negative during the course of treatment and remained so during the entire follow-up period were considered to be cured.

Results and Discussion

A series of 2-substituted-5-nitropyridines, pyrimidines and thiazoles have been examined for their *in vitro* activity against *T. vaginalis* and *T. foetus*. *In vitro* end points are listed in Table I both in μg./ml. and in μMoles/l., since it is only on the latter basis that a direct comparison of activities can be made. With two exceptions (2-acetamido-5-nitropyridine and 2-hydroxy-5-nitropyrimidine), all of these compounds were more active *in vitro* against *T. vaginalis* than *T. foetus*. The 2-acetamido-5-nitropyridine was inactive, while in the pyrimidine and thiazole series the 2-hydroxy compounds were nearly so. Of the remaining compounds the activities are close together, so that there is only a ten-fold difference between the least and most active. Within the limitation of the few substituent groupings at C (2) studied no radical was found to enhance activity. Except in the pyridines, the difference in activity is not more than threefold. The activity of 2-amino-5-nitropyridine was 5 times as great as 2-hydroxy-5-nitropyridine and 6 times that of the trifluoroacetamido compound (Table I).

The most active compounds were 2-amino-5-nitropyridine, 2-amino-5-nitrothiazole and 2-acetamido-5-nitrothiazole.

There was almost a direct correlation of *in vitro* and *in vivo* results (Table II). All compounds inactive *in vitro* were inactive *in vivo*. In the pyridine and pyrimidine series activity followed the same pattern

TABLE I

THE EFFECT OF 5-NITROPYRIDINES, PYRIMIDINES AND THIAZOLES AGAINST *T. vaginalis* AND *T. foetus* IN VITRO

Compound	*T. vaginalis* End point		*T. foetus* End point	
	µg./ml.	µM/l.	µg./ml.	µM/l.
Pyridines				
2-Amino-5-nitropyridine	0·8	3·6	0·9	4·0
2-Acetamido-5-nitropyridine	+200	—	+200	—
2-Trifluoroacetamido-5-nitropyridine	5·0	21·2	6·2	26·4
2-Hydroxy-5-nitropyridine	2·5	17·8	3·1	22·0
Pyrimidines				
2-Amino-5-nitropyrimidine	2·1	15·0	2·7	19·2
2-Acetamido-5-nitropyrimidine	6·2	34·0	3·1	17·0
2-Trifluoroacetamido-5-nitropyrimidine	3·1	13·1	5·2	22·0
2-Hydroxy-5-nitropyrimidine	+200	—	150·0	1060·0
Thiazoles				
2-Amino-5-nitrothiazole	0·62	4·3	0·8	5·5
2-Acetamido-5-nitrothiazole	0·62	3·3	0·8	4·3
2-Hydroxy-5-nitrothiazole	62·0	424·0	140·0	960·0

TABLE II

THE ACTIVITY OF 5-NITROPYRIDINES, PYRIMIDINES AND THIAZOLES IN MICE INFECTED SUBCUTANEOUSLY WITH *T. vaginalis*

Compound	CD50*		TD†		TQ‡	
	Oral	I.P.	Oral	I.P.	Oral	I.P.
Pyridines						
2-Amino-5-nitropyridine	78·8§	77	200	250	2·5	3·2
2-Acetamido-5-nitropyridine	Inactive					
2-Trifluoroacetamido-5-nitropyridine	188§	194§	600	400	3·2	2·1
2-Hydroxy-5-nitropyridine	144§	134§	1000	400	6·9	3·0
Pyrimidines						
2-Amino-5-nitropyrimidine	35·3§	28·6§	250	200	7·1	7·0
2-Acetamido-5-nitropyrimidine	77·0	71·0	500	200	6·5	2·8
2-Trifluoroacetamido-5-nitropyrimidine	58·8§	50·0	500	200	8·5	4·0
2-Hydroxy-5-nitropyrimidine	Inactive					
Thiazoles						
2-Amino-5-nitrothiazole	—	70·6	—	100	—	1·4
2-Acetamido-5-nitrothiazole	11·6§	11·1§	100	100	8·6	9·0
2-Trifluoracetamido-5-nitrothiazole	Inactive					
2-Hydroxy-5-nitrothiazole	Inactive					

* CD50 Median curative dose mg./kg.
† TD = Tolerated dose.
‡ TQ = Therapeutic Quotient.
§ Geometric mean of more than one trial.

in vitro and *in vivo*. A comparison of the *in vivo* activity on a molar basis shows that the same number of moles of 2-amino-5-nitropyrimidine and 2-trifluoroacetamido-5-nitropyrimidine are required for cure. This was to be expected since experimentally it was found that the half-life of 2-trifluoro-acetamido-5-nitropyrimidine in solution is 4 min. Trifluoroacetic acid is not active systemically, nor does it influence the

CD50 of 2-amino-5-nitropyrimidine when the two are administered simultaneously at the same molar ratio as is found in 2-trifluoroacetamido-5-nitropyrimidine. While 2-amino and 2-acetamido-5-nitrothiazole were equally effective *in vitro*, the 2-acetamido compound was more than six times as active *in vivo* intraperitoneally.

However, *in vivo* chemotherapeutic efficacy is a resultant of the effect of the drug on the parasite and the host. There was a host variation in toxicity of compounds in the pyridine and pyrimidine series. When the Therapeutic Quotient of the compounds is calculated for the oral route, it is evident that 2-hydroxy-5-nitropyridine is twice as effective as other compounds in the pyridine series and that all the compounds in the pyrimidine series are of about the same efficacy. These, together with

TABLE III

THE EFFECT OF 5-NITROPYRIDINES, PYRIMIDINES AND THIAZOLES AGAINST *T. foetus* AND *T. vaginalis* IN LOCAL SUBCUTANEOUS LESIONS IN MICE

Compound	I.P.		Oral	
	T. vaginalis	*T. foetus*	*T. vaginalis*	*T. foetus*
Pyridines				
2-Amino-5-nitropyridine	77·0*	89·2	—	—
2-Acetamido-5-nitropyridine	Inactive	—	—	—
2-Trifluoroacetamido-5-nitropyridine	194	—	—	—
2-Hydroxy-5-nitropyridine	134	—	144·0	260·0
Pyrimidines				
2-Amino-5-nitropyridimidine	28·6	—	35·3	77·5
2-Acetamido-5-nitropyrimidine	71·0	—	71·0	177·0
2-Trifluoracetamido-5-nitropyrimidine	50·0	100·0	58·8	142·0
2-Hydroxy-5-nitropyrimidine	Inactive			
Thiazoles				
2-Amino-5-nitrothiazole	70·6	56·6	—	—
2-Acetamido-5-nitrothiazole	11·1	14·1	—	—
2-Trifluoroacetamido-5-nitrothiazole	Inactive	—	—	—
2-Hydroxy-5-nitrothiazole	Inactive	—	—	—

* CD 50 mg./kg.

2-acetamido-5-nitrothiazole, had Therapeutic Quotients of 6·5 or more against *T. vaginalis*. Compounds in the pyrimidine series exhibited species specificity, since they were almost three times as active against *T. vaginalis* as against *T. foetus*. (Table III.)

2-Hydroxy-5-nitropyridine and 2-acetamido-5-nitrothiazole were given by mouth to treat hamsters infected intravaginally with *T. foetus*. When hamsters were treated with 500 mg./kg. of the pyridine derivative it was consistently found that 100 per cent of the animals were cured, but none were cured by half this dose (Table IV). Both compounds were less active against the vaginal infection than against the local infection since it took almost 1·5 times as much of the pyridine derivative orally and almost four times as much of the thiazole compound given intraperitoneally to achieve the same level of cure (CD50) in the hamsters as in mice (Tables III and IV).

Even at an infecting dose of 1×10^6 organisms, hamsters were refractory to subcutaneous infection with the MSC strain of *T. foetus*. Three additional strains of recent isolation were obtained*. One of these,

* Through the courtesy of Dr. D. T. Clark of Michigan State University, East Lansing, Michigan, U.S.A.

strain L, infected hamsters when injected subcutaneously. When the infectivity of strain MSC and L was compared subcutaneously in mice it was found that the ID50 for the former was 66,600 and for strain L, 2,140.

In a single experiment mice and hamsters were infected subcutaneously with *T. foetus* (L) and treated orally with a 2-hydroxy-5-nitropyridine,

TABLE IV

A VAGINAL INFECTION IN HAMSTERS TREATED WITH 2-HYDROXY-5-NITROPYRIDINE AND 2-ACETAMIDO-5-NITROTHIAZOLE BY MOUTH

Compound	Dose mg./kg./day	No. cured / No. treated	Per cent cured	CD50
2-Hydroxy-5-nitropyridine ..	500	5/5	100	350
	250	0/5	0	—
2-Acetamido-5-nitrothiazole ..	100	5/5	100	50
	50	2/5	40	—
	25	1/4	25	—
Plazmoid	—	0/5	0	—

2-amino-5-nitropyrimidine or 2-acetamido-5-nitrothiazole. The pyridine and pyrimidine were found to be significantly less active in hamsters than in mice (Table V). The apparent differences in response to the subcutaneous and vaginal infections were entirely due to the host.

The specificity of 2-amino-5-nitropyrimidine against *T. vaginalis* and its stability in acid and basic solutions led us to study this compound in

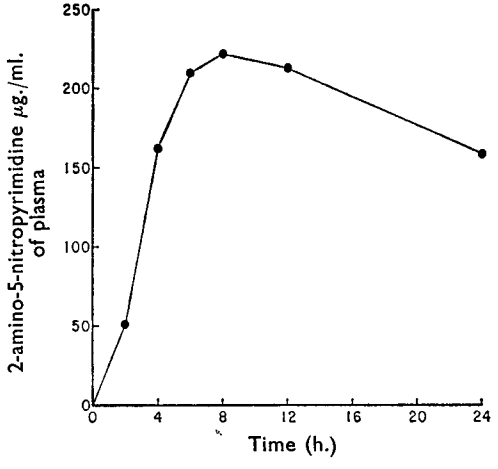

FIG. 1. The concentration of 2-amino-5-nitropyrimidine in μg./ml. of plasma after oral administration to a monkey.

more detail. Plasma concentrations were estimated microbiologically in one nonfasted monkey after oral administration of an aqueous suspension of the drug at 500 mg./kg. The data shown in Fig. 1 shows

TABLE V

THE EFFICACY OF THREE COMPOUNDS BY MOUTH IN MICE AND HAMSTERS INFECTED SUBCUTANEOUSLY WITH *T. foetus* (L)

Compound	ED50 and range mg./kg.		Significant (+) or not Significant (−)*
	Mice	Hamsters	
2-Amino-5-nitropyrimidine ..	108 (76–145)	196 (145–265)	+*
2-Hydroxy-5-nitropyridine ..	165 (126–214)	312 (268–350)	+
2-Amino-5-nitrothiazole	30 (20–45)	16 (7·6–33·5)	−

* Significance at the 5 per cent level of confidence.

that the drug, present in a microbiologically active form is slowly absorbed; probably from the intestine and is even more slowly excreted. The peak drug levels were obtained in 8 hr. The plasma level 24 hr. after drug administration is 50 times the amount of drug needed to kill the organism *in vitro*.

The stability of 2-amino-5-nitropyrimidine in acid, base and body fluids, its ability to produce good blood levels in monkeys, absorption in a biologically active form and ability to cure experimental infections in mice indicate that it should be an effective systemic treatment for the clinical disease evoked by *Trichomonas vaginalis*.

Qualitative differences in the carbohydrate metabolism of *T. vaginalis* and *T. foetus* exist but despite these, various investigators have used these species indiscriminately in the search for compounds which will inhibit *T. vaginalis*. Results from these experiments indicate that the two species probably utilise similar metabolic pathways *in vitro* but that *T. foetus* may change its metabolic pathway *in vivo*. If *T. foetus* only had been used to evaluate the compounds in the pyridine and pyrimidine series, their therapeutic potentialities would have been missed.

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