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THE MUTAGENIC ACTION OF NITROIMIDAZOLES
I. METRONIDAZOLE, NIMORAZOLE, DIMETRIDAZOLE AND RONIDAZOLE

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SUMMARY

In LURIA AND DELBRÜCK's fluctuation test, with streptomycin-resistance and streptomycin-dependence as markers, certain derivatives of 5-nitroimidazole having an antiprotozoal activity increased the mutation rate of *Klebsiella pneumoniae*, *Escherichia coli* K12 and *Citrobacter freundii* 425.

Among the substances tested, ronidazole showed the highest mutagenic activity, increasing the mutation rate of *Klebsiella pneumoniae* grown in 0.1 m*M* solutions by a factor of 26.5. The mutation frequency of bacteria grown in 0.1 m*M* solutions of metronidazole (Flagyl®), nimorazole (Naxogin®) and dimetridazole was increased between 3 and 7 times above the normal spontaneous mutation rate.

The possible implications of these findings in the application of the substances as human antitrichomonal drugs (metronidazole and nimorazole) or preparations to prevent infections by *Histomonas meleagridis* in poultry (dimetridazole and ronidazole) are discussed.

INTRODUCTION

The 5-nitroimidazoles are a group of substances some of which have applications for therapeutic and prophylactic or growth-promoting purposes.

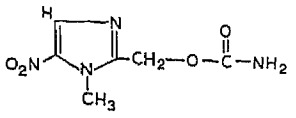
Metronidazole (since 1960) is the active principle of the well-known antitrichomonal drug Flagyl®. It is used to treat amoebic dysentery[3,7]. Metronidazole induces lung tumours in mice, when added to feeds at or above 0.06% (ref. 8). Its metabolism in mice is similar to that in humans[10].

Nimorazole is the active principle of Naxogin®, a recent drug for the treatment of *Trichomonas vaginalis* infections.

Dimetridazole is used as a feed additive at 0.05% to prevent infections by *Histomonas meleagridis* in poultry[6].

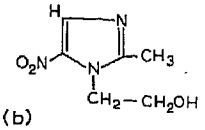
Ronidazole is an antibiotic used for poultry[9] and as a growth-promoting substance in pig breeding[2].

The chemical structures and molecular weights of these substances are given below.



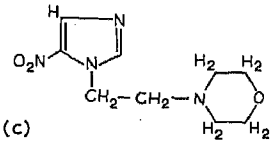
(a)

Pronidazole
(1-Methyl-5-nitroimidazole-2-yl)methylcarbamate. M.W., 200.



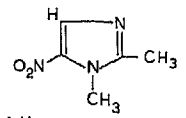
(b)

Metronidazole (Flagyl®, Clont®)
1-(2-Hydroxyethyl)-2-methyl-5-nitroimidazole. M.W., 171.



(c)

Nimorazole (Naxogin®)
1-(*N*-β-Ethylmorpholine)-5-nitroimidazole. M.W., 226.



(d)

Dimetridazole
1,2-Dimethyl-5-nitroimidazole. M.W., 141.

MATERIALS

Ronidazole (lot No. L-599278-00-F-49) was kindly supplied by Merck and Co. (Rahway, N. J.). The Badische Anilin and Soda Fabriken (Ludwigshafen, W. Germany) provided the dimetridazole. Metronidazole was a gift from Specia (Paris, France). Nimorazole and Naxogin® were received from Carlo Erba (Milan, Italy). Streptomycin sulphate was obtained from Mycofarm (Delft, The Netherlands). The Salmonella strains were kindly supplied by Prof. B. N. AMES[1].

METHODS

LURIA AND DELBRÜCK's fluctuation test[5] was applied in the following way. Nutrient broth containing the substance under test was seeded with 100 bacteria/ml and divided into 105 portions of equal volume in sterile culture tubes. After incubation of the mixture for 20 h at 37°, the total number of streptomycin-resistant and streptomycin-dependent bacteria was determined in 100 portions by the pour-plate technique using nutrient agar (pH 7.5) supplemented with streptomycin (100 μg/ml). The time of incubation was 3 days at 37°. One batch of agar was used for all experiments.

The numbers of bacteria present in the 5 remaining tubes were determined by tube dilution and plating with nutrient agar without streptomycin. From the number of portions without streptomycin-resistant or streptomycin-dependent mutants the mutation rate was calculated from the following formula:

$$M = \frac{^{10}\log A/B}{0.4343} \cdot \frac{^{e}\log 2}{N \times V}$$

in which M = mutation rate; A = number of portions without mutants; B = total number of portions poured with streptomycin agar; N = number of bacteria per ml; V = volume of a portion.

If mutants were present in more than 90 of the 100 portions, the mutation rate was calculated according to LEA AND COULSON[4] from the number of mutants present in the median portion. The following formula was used:

$$M = \frac{m}{N \times V}$$

in which M = mutation rate; m = number of mutations in the median portion; N = number of bacteria per ml; V = volume of a portion.

With the latter calculation, no correction for $^{e}\log 2$ had to be applied, because the results without applying this correction were similar to those obtained by applying the former formula.

As test organisms we used *Klebsiella pneumoniae*, requiring uracil and proline for growth, and also *Escherichia coli* K12 HfrH and *Citrobacter freundii* 425. As it is essential to the test to obtain a sufficient number of portions without mutants, the volumes per tube (portions) were 2.5, 1.5 and 0.5 ml respectively. *Salmonella typhimurium* strains his G46, TA 1530, TA 1531, TA 1532 and TA 1534 were also used to investigate the type of mutation induced by the substances tested. The mutation rates shown by these Salmonellae were usually too high for the application of standard fluctuation tests. Accordingly, tubes containing 10 ml broth and the substances under test were inoculated and incubated during 20 h at 37°. After the cultures had been washed once with 0.85% NaCl solution the number of mutants present was determined by dilution. The number of histidine revertants was determined by plating on a medium containing, per l, 15 g K_2HPO_4, 5 g KH_2PO_4, 2.5 g NH_4Cl, 0.5 g Na_2SO_4, 0.5 g NH_4NO_3, 5 g glucose, 15 g agar, 12.2 mg biotin, 50 mg $MgSO_4 \cdot 7H_2O$, 15 mg $MnCl_2 \cdot 4H_2O$, 5 mg $FeCl_3 \cdot 6H_2O$ and 2 mg $CaCl_2 \cdot 2H_2O$. The number of viable bacteria were counted with nutrient agar as described above.

RESULTS

To calculate the increases in mutation rates the average values for the spontaneous mutation rates obtained from the blanks of several experiments were used. These values are listed in Table I. The increase in mutation rate induced by the substances under test was calculated by dividing these mutation rates by the average levels of spontaneous mutation.

Ronidazole, even in low concentrations, exerted a pronounced mutagenic effect. With *Klebsiella pneumoniae*, the increase in mutation rate due to 0.01 mM solutions was 3.1 to 5.1 times the spontaneous mutation rate, and a solution of 0.1 mM caused an increase of even 26.5 times the normal level. With *Escherichia coli* and *Citrobacter freundii*, similar results were obtained. Some of the results are shown in Table II.

Dimetridazole, tested in the same way as ronidazole, was found to be less active (Table II).

TABLE I

AVERAGE OF SPONTANEOUS MUTATION RATES

Test organism	*Average mutation rates* ($\cdot 10^{-9}$)	*Standard deviation* ($\cdot 10^{-9}$)	*Number of experiments*
Klebsiella pneumoniae	0.1676	0.0304	26
Escherichia coli	0.3720	0.094	9
Citrobacter freundii	0.2616	0.076	10

With *Klebsiella pneumoniae*, solutions of 0.1 m*M* of dimetridazole increased the mutation rate by a factor of 3.4 to 4.1, whereas 1 m*M* solutions raised it to 32.2. Similar results were obtained with the two other test organisms *Escherichia coli* and *Citrobacter freundii*. Table II shows that, on the basis of molecular weight, dimetridazole had about one-third to one-fourth of the activity of ronidazole.

Dimetridazole is chemically closely related to the antitrichomonal drug metronidazole (Flagyl®). We therefore also tested the latter substance. The results in Table II show that the mutagenic activity of metronidazole is of about the same order as that of dimetridazole. For instance, concentrations of 0.1 m*M* induced mutation rates in *Klebsiella pneumoniae* between 5.2 and 9.7 higher than the spontaneous mutation rate. A concentration of 1 m*M* increased the mutation rate by a factor of 39.6.

Nimorazole, an antitrichomonal drug, also displayed a mutagenic effect. At the higher concentrations it was somewhat less active than dimetridazole and metronidazole. Thus, 2 m*M* of nimorazole increased the mutation rate of *Klebsiella pneumoniae* 21.8 times, whereas half this concentration (1 m*M*) induced increases between 5.7 and 9.4. However, at lower concentrations the mutagenic effect of nimorazole approached that of dimetridazole (Table II).

With the bacteria used in the fluctuation test probably only base-pair substitution mutations are detected, because in previous experiments with acridine orange (50 mg/ml) no increase in mutation rate was found. Therefore, a series of experiments was done with bacterial strains to find out whether these compounds induce base-pair substitution or frame-shift mutations. It is known that the former type of mutation may be found in *Salmonella typhimurium* strain TA 1530 and his G46, whereas the latter type may be detected in *Salmonella typhimurium* strains TA 1531, TA 1532 and TA 1534.

In these experiments only the number of mutants per million bacteria was determined. A mutation rate could not be calculated, because nothing is known about the differences in growth rate between mutated and non-mutated Salmonellae. Further, these differences may be increased by the growth inhibition caused by the substances in the concentrations used. So the only factor that could be determined with certainty was the frequency of mutants appearing in populations of the strains exposed to the substances. The results obtained with *Salmonella typhimurium* TA 1530 and his G46 are shown in Tables III and IV. With strains his G46 and TA 1530 a possible effect on the *uvr* repair system was detected. Tables III and IV show that TA 1530 is more sensitive than his G46 to nitroimidazoles. This agrees with the reported greater sensitivity of this strain to mutagenic agents[1] in comparison with his G46, so that it seems that the *uvr* system has not been influenced by these compounds.

Tables III and IV show that the nitroimidazoles also exerted a clear mutagenic

TABLE II

MUTAGENIC ACTION OF RONIDAZOLE, DIMETRIDAZOLE, METRONIDAZOLE AND NIMORAZOLE

Test organism	*Concentration (mM)*	*Mutation rate* ($\cdot 10^{-9}$)			
		Ronidazole	*Dimetridazole*	*Metronidazole*	*Nimorazole*
Klebsiella pneumoniae	2	–	–	–	3.828
	1	–	–	6.630	0.963
	0.5	–	3.380	1.351	0.594
	0.25	–	1.240	1.553	0.708
	0.1	4.446	0.688	1.031	0.577
	0.05	1.817	0.685	0.945	0.409
	0.025	1.205	0.325	0.331	0.379
	0.01	0.840	0.300	0.301	–
	Control	0.152	0.217	0.203	0.158
Escherichia coli	10	–	–	–	19.01
	5	–	–	–	4.16
	2.5	–	–	–	1.05
	2.0	–	–	6.49	–
	1	–	–	5.93	2.00
	0.5	–	6.54	5.18	0.84
	0.25	–	–	2.97	0.36
	0.1	8.59	2.60	1.62	0.22
	0.05	5.76	1.21	1.18	–
	0.025	3.39	–	1.23	–
	0.01	1.10	0.65	0.68	–
	Control	0.34	0.43	0.41	0.47
Citrobacter freundii	10	–	–	–	4.87
	2	–	–	3.81	–
	1	–	–	–	1.45
	0.5	–	2.22	–	1.13
	0.25	–	–	2.27	0.85
	0.1	4.05	0.76	1.68	–
	0.05	2.12	0.40	0.94	–
	0.025	1.52	–	0.72	–
	0.01	0.70	0.28	–	–
	0.005	0.32	–	–	–
	Control	0.20	0.13	0.23	0.35

TABLE III

MUTAGENIC ACTION OF NITROIMIDAZOLES ON *Salmonella typhimurium* TA 1530

Experiment	*Substance*	*Concentration (mM)*	*Number of histidine revertants per* 10^8 *bacteria*	*Increase*	*Number of streptomycin-resistant mutants per* 10^8 *bacteria*	*Increase*
I	Ronidazole	0.03	2.23	47×	6.93	8.8×
		control	0.047		0.82	
II	Dimetridazole	0.3	174	2300×	65	100×
		control	0.075		0.65	
III	Metronidazole	0.3	1.18	38×	28.3	3.1×
		control	0.031		9.2	
IV	Nimorazole	0.3	9.48	111×	4.16	4.4×
		control	0.085		0.95	
V	Nimorazole	0.1	0.521	9.1×	1.57	–
		control	0.057		1.30	

TABLE IV

MUTAGENIC ACTION OF NITROIMIDAZOLES ON *Salmonella typhimurium* LT2 his G46

Experiment	*Substance*	*Concentration (mM)*	*Number of histidine revertants per 10^6 bacteria*	*Increase*	*Number of streptomycin-resistant mutants per 10^6 bacteria*	*Increase*
I	Ronidazole	0.1	0.236	16.9×	2.85	3.1×
		control	0.014		0.92	
II	Dimetridazole	1	0.219	15.3×	3.20	5.7×
		control	0.014		0.56	
III	Metronidazole	1	0.29	24.3×	26.3	7.3×
		control	0.012		3.6	
IV	Nimorazole	3	0.909	55.8×	2.08	3.1×
		control	0.016		0.68	
V	Nimorazole	1	0.373	24.4×	–	–
		control	0.015		–	

TABLE V

MUTAGENIC ACTION OF DIMETRIDAZOLE ON *Salmonella typhimurium* TA 1531, TA 1532 AND TA 1534

Strain	*Concentration (mM)*	*Number of histidine revertants per 10^6 bacteria*	*Increase*	*Number of streptomycin-resistant mutants per 10^6 bacteria*	*Increase*
TA 1531	1	0.023	3.2×	1.60	–
	control	0.007		1.56	
TA 1532	0.1	0.434	4.4×	0.389	3.3×
	control	0.100		0.119	
TA 1534	0.3	30.9	320×	1.50	–
	control	0.097		1.60	

action upon these Salmonella strains. It may be concluded that the substances investigated induced base-pair substitution mutations in TA 1530 and his G46.

The possibility of frame-shift mutations was investigated with dimetridazole, with strains TA 1531, TA 1532 and TA 1534 of *S. typhimurium*. Table V shows that this substance also induced frame-shift mutations. However, more experiments are needed to confirm these findings.

The main results of all our studies are compiled in Table VI.

Comparison of the activities of the mutagenic agents shows that there is usually no linear relationship between the concentration and the increase in mutation rate. Deviations in linearity are especially obvious at lower concentrations.

The observation that in all the strains used the intrinsic mutagenic activity of nimorazole is lower at higher than at lower concentrations is not yet explainable. We are tempted to suggest that a charge transfer complex between the nimorazole molecules is involved.

Comparison of the mutagenic actions of 0.1 m*M* solutions of the nitromidazoles investigated shows that ronidazole is the most potent mutagenic substance in this series. However, the increases in mutation rates by 0.1 m*M* solutions of the other nitroimidazoles, namely metronidazole, dimetridazole and nimorazole, are of about the same order.

TABLE VI

COMPARISON OF THE MUTAGENIC ACTIVITY OF NITROIMIDAZOLES AT DIFFERENT CONCENTRATIONS

Substance and concentration (mM)	*Increase of mutation rate with Klebsiella pneumoniae*		*Increase of mutation rate after extrapolation to 1 mole/l*		
			Klebsiella pneumoniae	*Escherichia coli*	*Citrobacter freundii*
Ronidazole		*Average*			
0.1	26.5		265000	231000	155000
0.05	14.5; 10.8; 11.2; 10.6	11.9	238000	310000	162000
0.025	5.4; 7.2; 7.0	6.5	260000	364000	232000
0.01	3.1; 5.0; 5.1	4.4	440000	300000	270000
Metronidazole					
2			–	–	7300
1	39.6		40000	16000	–
0.5	8.1		16000	28000	–
0.25	9.3		37000	32000	35000
0.2	14.7		73000	–	–
0.1	6.2; 9.7; 5.6	7.2	72000	43000	64000
0.05	5.6; 4.9; 3.7	4.7	94000	64000	72000
Nimorazole					
10			–	5100	1900
5			–	2240	–
2	28.2; 17.4; 22.8	21.8	11000	–	–
1	9.4; 5.7	7.6	7600	5400	5500
0.5	2.7; 3.5	3.1	6200	4600	8600
0.25	3.7; 4.2	4.0	16000	–	13000
0.1	3.0; 3.4	3.2	32000	–	–
0.05	2.2; 2.4	2.3	46000	–	–
Dimetridazole					
1	27.8; 36.5	32.2	32000	–	–
0.5	20.2; 15.3	17.3	35000	34000	17000
0.25	7.4; 6.0	6.7	27000	–	–
0.2	4.9		25000	–	–
0.1	4.1; 3.4; 3.4	3.6	36000	70000	29000
0.05	4.1; 2.4	3.25	65000	64000	–

DISCUSSION

The nitroimidazoles investigated constitute an entirely new class of mutagenic agents.

The toxicology of this group of substances, which have wide application either in human therapy or as feed additives, has not been extensively studied up to now. One reason may be that serious (carcinogenic, teratogenic, *etc.*) side-effects due to the medical use of metronidazole and nimorazole have not yet been reported.

Regarding the results of our experiments and those of some others, however, the question may be raised whether the nitromidazoles are indeed so harmless as generally supposed. In the treatment of human trichomoniasis, giaridasis and amoebiasis in adult patients, daily doses of metronidazole ranging from 500 to 2000 mg for a period of 10 days are normal praxis. Ingestion of one tablet of metronidazole (Flagyl®) produces blood levels of approx. 5 μg/ml[11], a concentration which in our experiments increased the mutation rate by a factor of two. Daily quantities of 2000 mg, sometimes used in the treatment of certain forms of amoebiasis, may give rise to serum

levels as high as 30 to 45 μg/ml[3,7,12], corresponding to a potential risk of increase of the mutation rate by a factor of 5 to 10 (see Table VI). It is also noteworthy that metronidazole increases the incidence of lung tumours in mice[8], and that nothing is known about the potential mutagenic effect of the metabolites of metronidazole that appear in the urine of the patients.

It is concluded that metronidazole and related products should be used with caution, at least for the time necessary for a retrospective study of patients who have received these substances. Further, the application of nitroimidazoles for non-medical purposes should be reappraised in view of our findings.

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