Effect of the rat liver S9 fraction on the mutagenicity of azathioprine in the *Salmonella*/mammalian microsome assay[1]

I. Nepomnaschy, S.E. Sommer and R. Nagel[2]

Fundación de Genética Humana, Salta 661, 1074-Buenos Aires (Argentina), 7 December 1982

Summary: Azathioprine is a direct acting mutagen in *Salmonella typhimurium* TA100 and TA1535. Addition of rat liver S9 fraction with or without co-factors, or glutathione, causes a decrease in the mutagenicity of azathioprine in TA100 and an increase in TA1535, indicating the effect of SH groups.

In bacterial tests of mutagenicity addition of mammalian liver homogenate is the procedure routinely used to evaluate the effect of mammalian enzymes on the action of different drugs[3]. We now report that a chemical compound, glutathione, physiologically important for mammals and present in these organisms, can also exert an effect on the mutagenicity of the drug azathioprine, measured in the in vitro *Salmonella*-mammalian microsome assay.

The drug azathioprine, 6-[(1-methyl-4-nitroimidazole-5-yl) thio] purine, Imuran, is widely used as an immunodepressant in organ transplants and in the treatment of autoimmune diseases. There are reports on the carcinogenicity of azathioprine, mainly as an inducer of lymphomas[4]. It is mutagenic in *S. typhimurium* and *Klebsiella pneumoniae*[5-8], it induces sperm abnormalities, and its clastogenic effects can be measured by the micronuclei technique[9].

The results obtained with *S. typhimurium* vary according to the strain and the conditions employed[5,6]. We studied the modification of the mutagenicity of azathioprine by the rat liver S9 fraction with or without co-factors, or glutathione, in 2 *S. typhimurium* strains.

Materials and methods. S. typhimurium strains were provided by Dr B.N. Ames. Azathioprine was kindly provided by the Wellcome Foundation Ltd. All chemicals tested were dissolved in dimethyl sulfoxide. Mutagenicity assays were carried out according to the liquid incubation procedure of Ames et al.[10]. The S9 fraction was prepared from the livers of rats induced with phenobarbital; its protein content concentration, determined by the Lowry technique[11], was 30 mg/ml. The S9 mix, prepared according to Ames et al.[10], contained 200 µl of S9 fraction per ml. The concentrations of the mutagens are given as the concentration in the incubation mixture.

Results and discussion. Azathioprine showed a direct mutagenic effect in *S. typhimurium* strains TA100 and TA1535, carrying base-pair substitutions (table 1). Concentrations of azathioprine between 100 and 3000 nmoles/ml showed no mutagenicity in the frameshift-strains TA1537, TA1538 and TA98.

With strain TA100, addition of S9 mix resulted in a decrease in the number of *his*$^+$ revertants at the lower drug concentrations (table 1). This effect does not appear to be due to decreased viability in the presence of S9 mix, since the number of survivors of TA100 treated with azathioprine and S9 mix was similar to the number of survivors without S9 mix (table 1). On the other hand, strain TA1535 showed an increased mutagenic response in the presence of S9 mix (table 1). Higher concentrations of azathioprine, or lower concentrations of S9 fraction in the S9 mix, gave a lower deactivation with strain TA100. This suggests a saturation of the deactivation capacity of the liver fraction. Addition of S9 without the cofactors NADP and glucose-6-phosphate (S9) showed the same degree of deactivation as the S9 mix with strain TA100 and an intermediate effect with TA1535 (table 1). With this latter strain, the S9 mix is more efficient in activation than S9; this suggests that some enzymatic reaction might be involved in this effect, for example, the metabolic activation of 6-mercaptopurine (6MP) that could be generated by S9 mix (table 3).

Addition of glutathione at concentrations of 300–600 nmoles/ml to the mixture of bacteria and azathioprine (300–600 nmoles/ml) resulted in an effect similar to that of the addition of S9; glutathione when added alone at these concentrations had no effect on mutation rate (table 2). Therefore, we infer that glutathione and/or other nucleophilic agents present in the liver extract might be responsible, at least partially, for the effect observed with the addition of S9. In fact, the levels of

Table 1. Effect of S9 fraction with or without co-factors on *his*$^+$ reversion induced by azathioprine

Concentration of azathioprine	Strains TA100			TA1535		
nmoles/ml	− S9	+ S9m	+ S9	− S9	+ S9m	+ S9
0	105 ± 9	132 ± 11	130 ± 8	23 ± 3	15 ± 2	16 ± 2
75	165 ± 13	131 ± 13	128 ± 7	30 ± 7	35 ± 5	29 ± 6
150	677 ± 44[a]	148 ± 17[b]	140 ± 21	31 ± 6	36 ± 5	26 ± 5
300	848 ± 92[c]	244 ± 21[d]	253 ± 32	52 ± 9	116 ± 25	83 ± 11
750	1004 ± 96[e]	653 ± 71[f]	498 ± 57	81 ± 10	272 ± 41	132 ± 12
1500	1202 ± 115	1003 ± 98	1104 ± 88	353 ± 38	571 ± 60	494 ± 36
3000	ND	ND	ND	505 ± 63	782 ± 81	ND
2AA	55 ± 7	2304 ± 98	48 ± 8	ND	181 ± 26	ND

Mean number of *his*$^+$ revertants per plate; these values are the mean of triplicate plates from 3 experiments (± SE); ND, not determined; S9, S9 mix without co-factors; S9m, S9 mix; concentration of 2AA (2 amino-anthracene), 40 µg/ml; viable cells (×10^7/ml): [a] 4.2; [b] 3.9; [c] 3.9; [d] 4.0; [e] 2.8; [f] 4.2. These cells were scored after plating a suitable dilution of the incubation mixture in nutrient agar.

Table 2. Effect of glutathione on azathioprine mutagenicity

Strains	Azathioprine concentration (nmoles/ml)	Addition of glutathione (nmoles/ml) 0	150	300	600	1200
TA100	0	115 ± 10	ND	122 ± 8	110 ± 8	ND
	300	785 ± 52	560 ± 60	320 ± 40	297 ± 25	ND
TA1535	0	24 ± 3	ND	31 ± 3	29 ± 2	ND
	600	38 ± 12	48 ± 6	59 ± 7	68 ± 10	160 ± 35

Mean number of *his*$^+$ revertants per plate; these values are the mean of two experiments; ND, not determined.

glutathione present in our S9 preparations, calculated on the basis of reported values[12,13], should be about 265–388 nmoles/ml.

Glutathione splits azathioprine into 6MP and 1-methyl-4-nitroimidazole (MNI) and derivate molecules[14]. Hence, one possible explanation for the observed effects of S9 and glutathione, respectively, could be the partial or complete splitting of azathioprine to its component and derivative molecules. We therefore investigated the mutagenicity of 6MP and MNI under the same conditions as used with azathioprine. MNI was not mutagenic in strain TA1535 and its mutagenicity in strain TA100 was only manifested at concentrations 50 times higher that those used for azathioprine. 6MP was mutagenic in strains TA100 and TA1535 (table 3). At concentrations below 1500 nmoles/ml, strain TA100 was less sensitive to 6MP than to azathioprine, and TA1535 was more sensitive. Addition of S9 mix resulted in a slight increase in mutagenicity in both strains tested; addition of glutathione had no such effect.

These results show that azathioprine has mutagenic properties different from those of its component molecules. Also, though the effect of S9 mix on azathioprine mutagenicity probably result from other more complex reactions, these observations are consistent with the interpretation that the effect of S9 may be due to a partial or complete nucleophilic attack on the azathioprine molecule. This splitting of azathioprine could cause the deactivation observed with TA100 and the increase in mutagenicity with TA1535.

Future studies should elucidate whether glutathione or other nucleophilic agents present in mammalian cells could also modify the mutagenicity of azathioprine in animal models.

Table 3. Effect of 6MP in strains TA100 and TA1535

Concentration of 6MP nmoles/ml	Strains TA100		TA1535	
	− S9m	+ S9m	− S9m	+ S9m
0	123 ± 14	109 ± 12	21 ± 3	15 ± 3
75	ND	ND	25 ± 6	30 ± 5
150	256 ± 24	310 ± 27	66 ± 9	96 ± 12
300	304 ± 40	388 ± 39	96 ± 23	153 ± 38
600	332 ± 36	363 ± 38	187 ± 19	304 ± 31
1500	226 ± 29	305 ± 25	224 ± 20	341 ± 27

Mean number of *his*$^+$ revertants per plate ± SE; the values given are the mean of 3 experiments; ND, not determined; S9 m, S9 mix.

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2 To whom reprint requests should be addressed.

3 Ames, B.N., Durston, W.E., Yamasaki, E., and Lee, F.D., Proc. natl Acad. Sci. USA *70* (1973) 2281.
4 Casey, T.P., Blood *31* (1968) 369.
5 Herbold, B., and Buselmaier, W., Mutation Res. *40* (1976) 73.
6 Speck, W.T., and Rosenkranz, H.S., Cancer Res. *36* (1979) 108.
7 McMahon, R.E., Cline, J.E., and Thompson, C.Z., Cancer Res. *39* (1979) 682.
8 Voogd, C.E., Van der Stel, J.J., and Jacobs, J.J.J.A.A., Mutation Res. *66* (1979) 207.
9 Heddle, J.A., and Bruce, W.R., in: Cold Spring Harbor Conf. Cell Prolif., p.1549. Eds H.H. Hiatt, J.D. Watson and J.A. Winsten. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1977.
10 Ames, B.N., McCann, J., and Yamasaki, E., Mutation Res. *31* (1975) 347.
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
12 Meijer, J., De Pierre, J.W., and Rannug, U., Chem. biol. Interact. *31* (1980) 247.
13 Summer, K.H., Goggelmann, W., and Greim, H., Mutation Res. *70* (1980) 269.
14 Elion, G.B., and Hitchings, G.H., Handb. exp. Pharmak. *48* (1975) 404.

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Protection of rat gastric mucosa against ethanol injury by the new synthetic prostaglandin MDL 646[1]

G. Pallavicini, A. Bardoni, A. Profumo, A.A. Castellani, P. Schiatti and A. Glässer

Biochemistry Department, University of Pavia, 27100 Pavia (Italy) and Lepetit Research Labs., Pharmacology Dept., Via Durando, 38, Milan 20158 (Italy), 27 May 1983

Summary. Oral administration to fasted rats of absolute ethanol produces extensive necrotic lesions of gastric mucosa as well as a massive leakage of proteins and mucus glycoproteins into gastric lumen. When the new synthetic prostaglandin MDL 646, belonging to the PGE_1 series, is administered intragastrically (2 or 10 µg/kg) 30 min before ethanol administration, a significant protection of rat gastric mucosa against alcohol injury is observed.

Prostaglandins have been shown to protect both animal and human gastrointestinal mucosa against various noxious agents, such as ethanol, hydrochloric acid, boiling water and nonsteroidal antiinflammatory drugs[2-9]. This property of prostaglandins, which is independent of their acid-inhibiting activity, has been described as 'cytoprotection'[10,11]. Ethanol, because of its lipid solubility, diffuses particularly readily into the gastric mucosal cells and breaks the apical plasma membrane[12], thus giving rise to a massive leakage of proteins and mucus glycoproteins from the mucosa into the gastric lumen as well as extensive necrotic lesions of the gastric mucosa itself. In the present study we investigated the efficacy of a single intragastric administration of the new synthetic prostaglandin MDL 646 [11,15-dihydroxy-16-methyl-16-methoxy-9-oxoprost-13-en-1-oic acid, methyl ester (8*a*, 11*a*, 15R, 16R)], belonging to the PGE_1 series, in preventing EtOH-induced damage to the gastric mucosa of rat. Gastric lesions induced by ethanol were evaluated by direct examination of the mucosa under an illuminated magnifier, and leakage of mucosal proteins and mucus glycoproteins into the gastric lumen was assessed by assaying the non-dialyzable material from the gastric lumen for proteins and glucosamine, the latter as a marker for glycoproteins.

Materials and methods. Male Wistar rats (Charles River), 120–150 g, fasted for 24 h were used. Gastric lesions were induced by absolute ethanol, according to Robert's method[2]. A watery solution (2 ml/kg) of test compound MDL-646 (2 or 10 µg/kg) was given intragastrically 30 min before oral administration of absolute ethanol (1 ml/rat), whereas control animals received only the same volume of water. Animals were killed 1 or 6 h later by chloroform inhalation, their stomachs were removed after ligature of the esophagus and pylorus, rinsed externally with cold 0.9% NaCl and opened along the lesser curvature. Stomachs were then spread out and gastric lesions evaluated under an illuminated magnifier by the percentage of the mucosal area involved. The ratio, area in treated/area in control animals, was calculated.