Mutagenicity of Analgesics, Their Derivatives, and Anti-inflammatory Drugs with S-9 Mix of Several Animal Species

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Abstract

An investigation was undertaken to determine whether analgesics and their derivatives (13 compounds), and anti-inflammatory drugs (4 compounds) had mutagenicity. Rec-assay was used to clarify specific DNA-damaging properties, and the Ames test was used to find back-mutations, using S-9 fractions obtained from the liver of 4 animal species pretreated with polychlorobiphenyl.

In the Rec-assay, salicylic acid (2 mg), aspirin (5 mg), benzoic acid (4 mg), sulpyrine (0.4 mg), indomethacin (0.1 mg), oxyphenbutazone (0.1 mg) and diclofenac sodium (0.1 mg) showed a DNA-damaging tendency.

In the Ames test, mutagenicity of methyl salicylate was demonstrated using the *Salmonella typhimurium* TA98 strain upon addition of hamster S-9 mixture. Weak mutagenicity was also found using the TA100 strain with rat S-9 mixture for salicylic acid, sulpyrine, indomethacin and oxyphenbutazone, and with hamster S-9 mixture for methyl salicylate, acetaminophen and phenacetine.

Introduction

The mechanism of cancer induction has long been considered to be mutation caused by alteration of the primary structure of DNA^[1,2]. In recent years, an almost linear relationship has been obtained between mutagenic potency determined by the Ames test and carcinogenic potency in animals. It has also been demonstrated by Ames and his colleagues that 90% of known human carcinogens are mutagenic^[3,4].

Therefore, from the viewpoint of careful and safe drug use, it is important to detect the mutagenicity of drugs. We have demonstrated previously that sulpyrine possesses the strongest mutagenicity among analgesic drugs in widespread use^[5].

On the other hand, the metabolic pathways of compounds, as well as their mutagenic potency, vary in mammalian species^[6,7]. For example, it has been reported that mutagenicity of phenacetin, a component of many analgesic drugs, was detected in the TA100 system in the presence of liver S-9 from hamster, but not that from rat. Also, YAMAZOE et al.^[7] investigated the metabolic activation of

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Trp-P-2 in 5 experimental animal species in order to clarify whether N-hydroxylation was a common pathway for mutagenic activation, and to clarify the differences in activating enzymes among the species. In rats, the activity was low, and hepatic microsomes from hamster showed the highest activity, although there was no appreciable difference between the sexes. Therefore, S-9 mixes obtained from several species are an important factor for the efficient detection of mutagenicity^[8,9].

In this study, we tested various analgesics and their derivatives, and anti-inflammatory drugs for mutagenic activity by metabolic activation of the compounds using rat, mouse, guinea pig, and hamster liver microsomes in the Rec-assay (cold-incubation method with *Bacillus subtilis*) in order to clarify their DNA-damaging capacity and mutagenicity, and using the Ames test with a slight modification with *Salmonella typhimurium* strains TA98 and TA100.

Materials and Methods

1. Chemicals

The structures of the analgesics, anti-inflammatory drugs and their derivatives are listed in Fig. 1.

Salicylic acid, sodium salicylate, aspirin, methyl salicylate, salicylamide, benzoic acid, acetanilide, acetaminophen, phenacetin, antipyrine, aminopyrine, sulpyrine (SP), and indomethacin were purchased from Wako Pure Chemical Industries, isopropylantipyrine (IPA) was from Nihon Roche Co., oxyphenbutazone and diclofenac sodium were from Fujisawa Pharmaceutical Co., Ltd., and naproxen was from Tanabe Co. NADP and G-6-P were purchased from Sigma Chemical Co. Furyl furamide (AF-2) was kindly supplied by Dr. T. Kada, National Institute of Genetics, Mishima, Japan. All other chemicals, reagents and solvents were purchased from Wako Pure Chemical Industries.

Benzopyrene (BP), 4-nitroquinoline-1-oxide (4NQO), mitomycin C (MMC; Kyowa Hakko Co.), and AF-2 were used as positive controls.

2. Bacterial strains

Bacillus subtilis strains H17 (Rec⁺) and M45 (Rec⁻), used for the Rec-assay^[10], and *Salmonella typhimurium* strains TA98 and TA100, used for the mutation assay, Ames test^[11,12], were kindly provided by Dr. T. Kada of the National Institute of Genetics.

3. Rec-assay

Experimental procedures for the Rec-assay using the cold-incubation method were based mainly on those described already by KADA et al.^[10] Two strains of *B. subtilis* H17 and M45 were grown at 37°C in B-2 broth for 14 h. Each culture was streaked on the dry surface of a B-2 agar plate in a standard Petri dish as shown in Fig. 2, taking care not to let them touch one another. Test compounds, 0.05 ml (0.25~5 mg/disc), were dropped on a paper disc (diameter 8 mm), which had been placed at the starting point of the streaks. All the plates were kept at 4°C for 24 h, and then incubated at 37°C for 24 h. The length of the inhibition zone was then measured^[13].

When the inhibition zone of cellular growth was more than 2 mm of zone

difference between growth inhibition zones for Rec^+ and Rec^- strains, it was considered that the test compound had a damaging effect on cellular DNA (Fig.2).

Salicylate derivatives

Aniline derivatives

Pyrazolone derivatives

Anti-inflammatory drugs

Fig. 1 Chemical structures and names of analgesics, their derivatives, and anti-inflammatory drugs

Fig. 2 Procedure for the Rec-assay (cold-incubation method) using *Bacillus subtilis*

4. Preparation of S-9 mix

S-9 mix was prepared in the following manner^[11]: SD-strain male rats, 7 weeks old, weighing 180~200 g, ddy-strain male mice, 7 weeks old, weighing 18~25 g, Hartley-strain male guinea pigs, 5 weeks old, weighing 250~300 g, and male golden hamsters, 5 weeks old, weighing 80~100 g were obtained from Japan SLC Inc. Polychlorobiphenyl (PCB) was dissolved in corn oil and administered once to all the animals intraperitoneally at a dose of 500 mg/kg, 5 days before liver extraction.

The S-9 fraction was prepared aseptically by centrifugation of a liver homogenate (25% in 0.15 M KCl) at 4°C and 9,000 x g for 30 min, as described by AMES et al.^[11]

5. Ames test

The *Salmonella*/microsome mutagenicity test was carried out according to the procedure reported by AMES et al.^[11,12], with a slight modification. The test compounds, freshly dissolved in 0.1 ml (0.1~10 mg/plate) of DMSO, were preincubated at 37°C for 30 min in a shaking water bath with 0.5 ml of 1/15 M phosphate buffer (pH 7.4) or 0.5 ml S-9 mix and 0.1 ml of bacterial culture, as shown in Fig. 3. Then 3 ml of soft agar (45°C) was added by vortex action and the mixture was poured onto the surface of a minimal agar plate of Vogel-Bonner E medium (1.5% agar, 2% glucose). The plates were incubated at 37°C in the dark for 48 h, then His⁺ revertant colonies were counted. Test compounds inducing more than twice the number of His⁺ revertant colonies than that due to spontaneous mutation were considered to be mutagenic.

Fig. 3 procedure for the Ames test using *Salmonella typhimurium* TA98 and TA100

Results

1. Rec-assay

The results of the Rec-assay using the cold-incubation method for analgesics and their derivatives (13 samples), and anti-inflammatory drugs (4 samples) are shown in Table 1. The dose-response curves of salicylate derivatives (4 samples) are shown in Fig. 4. A trend of weak DNA damage was demonstrated by salicylic acid (more than 2 mg), aspirin (more than 5 mg) and benzoic acid (more than 4 mg), but methylsalicylate was negative. Salicylic acid was the most potent mutagenic compound among the salicylate derivatives.

Aniline derivatives such as acetanilide, acetaminophen and phenacetin had no DNA-damaging property at doses of 0.02~5 mg/disc. Among the pyrazolone derivatives, antipyrine, aminopyrine and IPA induced no DNA damage. SP was found to induce DNA damage at doses above 0.4 mg/plate, and the dose-response curve was nearly linear (Fig. 5).

The results for indomethacin, oxyphenbutazone and diclofenac sodium showed some representative differences in the inhibition zone at doses above 0.1 mg/disc in the Rec-assay. However, since both Rec⁺ and Rec⁻ strains showed growth inhibition, these compounds were thought to have possible cytotoxicity at concentrations higher than 1 mg/disc (Fig. 6).

Table 1 Rec-assay on analgesics, their derivatives, and anti-inflammatory drugs

Compound	Dose mg/disc	Length of inhibition zone, mm		Difference, mm (Rec ⁻ -Rec ⁺)
		H17 (Rec ⁺)	M45 (Rec ⁻)	
Control (DMSO)	0	0.0±0.0	0.0±0.0	0.0±0.0
Salicylate derivatives				
Salicylic acid	5	2.7±0.6	7.3±1.4	4.7±1.2*
Na salicylate	5	0.0±0.0	0.0±0.0	0.0±0.0
Aspirin	5	2.1±0.3	4.5±0.2	2.4±0.2*
Me. salicylate	5	0.0±0.0	1.2±0.3	1.2±0.3
Salicylamide	5	0.0±0.0	0.0±0.0	0.0±0.0
Benzoic acid	5	1.5±0.2	4.3±0.3	2.9±0.5*
Aniline derivatives				
Acetanilide	5	0.0±0.0	0.0±0.0	0.0±0.0
Acetaminophen	5	0.0±0.0	0.0±0.0	0.0±0.0
Phenacetin	5	0.0±0.0	0.0±0.0	0.0±0.0
Pyrazolone derivatives				
Antipyrine	5	0.0±0.0	0.0±0.0	0.0±0.0
Aminopyrine	5	0.0±0.0	0.0±0.0	0.0±0.0
IPA	5	0.0±0.0	0.0±0.0	0.0±0.0
Sulpyrine	5	0.0±0.0	21.5±0.7	21.5±0.7*
Anti-inflammatory drugs				
Indomethacin	5	8.3±0.3	12.8±0.8	4.5±0.6*
Oxyphenbutazone	5	12.5±0.5	15.7±0.1	3.2±0.3*
Diclofenac Na	5	4.7±1.3	12.7±1.6	8.0±1.7*
Naproxen	5	0.0±0.0	0.0±0.0	0.0±0.0
Positive control (μg)				
4NQO	0.2	0.0±0.0	25.1±3.1	25.1±3.1*
MMC	0.1	1.1±0.2	10.2±2.1	9.1±1.3*

* More than 2 mm zone difference
Mean ± SD for 5 plates, mm

Fig. 4 Rec-assay on salicylate derivatives

Fig. 5 Rec-assay on sulpyrine

Fig. 6 Rec-assay on anti-inflammatory drugs

2. Ames test

The results of the Ames test on His⁺ revertant colonies/plate using the TA98 system in the presence of S-9 mix obtained from the liver of rat, mouse, guinea pig or hamster are summarized in Table 2. Mutagenicity was demonstrated only for methyl salicylate using strain TA98 upon addition of S-9 mix from hamster.

Acetanilide, acetaminophen and phenacetin at doses of 0.1~10 mg/plate, did not induce His⁺ revertant colonies in the presence of S-9 mix obtained from the liver of all animal species.

All pyrazolone derivatives and anti-inflammatory drugs tested did not induce His⁺ revertant colonies in the presence or absence of S-9 mix obtained from the liver of all animal species.

4NQO, AF-2 and BP were used as the positive controls for the Ames test on His⁺ revertant colonies of TA98. For example, among 4 animal species, rat was most effective for increasing the number of His⁺ revertant colonies, at 234 ± 66 with 5 μg /plate BP, the order being rat > mouse > hamster > guinea pig.

Table 2 Ames test, reversion of *Salmonella typhimurium* TA98 induced by analgesics, their derivatives, and anti-inflammatory drugs

Compound	Dose mg/disc	-S-9	His ⁺ revertant colonies/plate			
			+S-9			
			Rat	Mouse	G. pig	Hamster
Control (DMSO)	0	24 ± 3	42 ± 6	28 ± 3	36 ± 5	38 ± 7
Salicylate derivatives						
Salicylic acid	0.1	0 ± 0	19 ± 3	21 ± 3	31 ± 2	42 ± 5
Na salicylate	0.1	13 ± 2	23 ± 2	29 ± 6	23 ± 1	39 ± 4
Aspirin	0.1	17 ± 3	31 ± 2	35 ± 3	27 ± 3	48 ± 5
Me. salicylate	0.1	0 ± 0	24 ± 3	36 ± 6	36 ± 5	$74 \pm 4^*$
Salicylamide	0.1	17 ± 4	31 ± 3	29 ± 2	26 ± 3	40 ± 6
Benzoic acid	0.1	0 ± 0	33 ± 4	26 ± 7	33 ± 4	28 ± 6
Aniline derivatives						
Acetanilide	0.1	18 ± 2	23 ± 2	36 ± 1	35 ± 3	45 ± 2
Acetaminophen	0.1	19 ± 4	29 ± 6	14 ± 1	23 ± 7	30 ± 2
Phenacetin	0.1	28 ± 4	21 ± 1	19 ± 0	39 ± 7	41 ± 1
Pyrazolone derivatives						
Antipyrine	1.0	18 ± 2	25 ± 5	38 ± 4	34 ± 3	45 ± 3
Aminopyrine	1.0	16 ± 2	30 ± 4	33 ± 2	29 ± 5	35 ± 5
IPA	1.0	11 ± 0	21 ± 5	22 ± 4	28 ± 3	39 ± 5
Sulpyrine	1.0	32 ± 8	49 ± 3	52 ± 2	54 ± 2	45 ± 6
Anti-inflammatory drugs						
Indomethacin	0.1	28 ± 3	38 ± 6	20 ± 2	37 ± 3	36 ± 3
Oxyphenbutazone	0.1	13 ± 1	31 ± 5	43 ± 5	41 ± 5	31 ± 3
Diclofenac Na	0.1	16 ± 2	33 ± 4	29 ± 8	33 ± 6	46 ± 5
Naproxen	0.1	21 ± 6	38 ± 6	37 ± 8	41 ± 2	39 ± 2
Positive control (μg)						
4NQO	0.5	$482 \pm 34^*$	21 ± 5	33 ± 3	26 ± 3	31 ± 3
AF-2	0.02	$240 \pm 31^*$	79 ± 11	$80 \pm 21^*$	$102 \pm 22^*$	67 ± 11
BP	5.0	31 ± 6	$234 \pm 66^*$	$81 \pm 39^*$	$123 \pm 36^*$	$165 \pm 45^*$

* More than twice the number of His⁺ revertants than that due to spontaneous mutation
Mean $\pm$ SD for 5 plates

The results of the Ames test in terms of the numbers of His⁺ revertant colonies/plate of TA100 in the presence of S-9 mix obtained from 4 animal species are summarized in Table 3.

Salicylic acid (0.1 mg) produced 446 ± 78 His⁺ revertant colonies upon addition of S-9 mix obtained from rat. The number of His⁺ revertant colonies observed in the presence of methyl salicylate (0.1 mg) was 400 ± 41 upon addition of S-9 mix obtained from hamster. Weak mutagenicity was demonstrated by acetaminophen in the presence of S-9 mix obtained from hamster. Phenacetin (0.1 mg) gave 400 ± 78 and 410 ± 33 His⁺ revertant colonies after addition of S-9 mix obtained from guinea pig and hamster, respectively (Fig. 7). Weak mutagenicity was demonstrated for sulpyrine (5 mg) with rat S-9 mix.

Faint mutagenicity was also found for indomethacin and oxyphenbutazone upon addition of S-9 mix obtained from rat. All other drugs were negative. 4NQO,

Table 3 Ames test, reversion of *Salmonella typhimurium* TA100 induced by analgesics, their derivatives, and anti-inflammatory drugs

Compound	Dose mg/disc	-S-9	His ⁺ revertant colonies/plate			
			Rat	Mouse	G. pig	Hamster
Control (DMSO)	0	141 ± 31	160 ± 33	158 ± 51	161 ± 13	182 ± 33
Salicylate derivatives						
Salicylic acid	0.1	198 ± 71	446 ± 78*	161 ± 45	170 ± 31	150 ± 11
Na salicylate	0.1	130 ± 30	110 ± 37	89 ± 11	180 ± 25	150 ± 38
Aspirin	0.1	120 ± 20	100 ± 30	150 ± 15	122 ± 30	113 ± 21
Me. salicylate	0.1	202 ± 61	200 ± 22	198 ± 27	150 ± 43	400 ± 41*
Salicylamide	0.1	150 ± 54	199 ± 71	200 ± 43	120 ± 50	207 ± 64
Benzoic acid	0.1	78 ± 31	199 ± 68	217 ± 48	130 ± 10	120 ± 31
Aniline derivatives						
Acetanilide	0.1	150 ± 34	167 ± 10	180 ± 20	177 ± 31	222 ± 43
Acetaminophen	0.1	190 ± 54	200 ± 11	190 ± 23	195 ± 19	380 ± 70*
Phenacetin	0.1	183 ± 21	240 ± 31	182 ± 33	400 ± 78*	410 ± 33*
Pyrazolone derivatives						
Antipyrine	1.0	170 ± 11	149 ± 18	150 ± 49	160 ± 32	200 ± 22
Aminopyrine	1.0	155 ± 22	167 ± 23	125 ± 29	133 ± 51	191 ± 51
IPA	1.0	155 ± 36	170 ± 66	166 ± 31	145 ± 40	172 ± 34
Sulpyrine	5.0	150 ± 28	366 ± 68*	168 ± 39	155 ± 33	200 ± 30
Anti-inflammatory drugs						
Indomethacin	0.1	250 ± 58	410 ± 85*	152 ± 32	200 ± 24	201 ± 25
Oxyphenbutazone	0.1	170 ± 66	392 ± 29*	182 ± 22	150 ± 19	168 ± 29
Diclofenac Na	0.1	16 ± 2	33 ± 4	29 ± 8	33 ± 6	46 ± 5
Naproxen	0.1	21 ± 6	38 ± 6	37 ± 8	41 ± 2	39 ± 2
Positive control (μg)						
4NQO	0.5	1233*	480*	517*	766*	500*
		± 216	± 78	± 116	± 163	± 138
AF-2	0.02	1700*	576*	539*	685*	780*
		± 263	± 103	± 113	± 186	± 261
BP	5.0	180	1350*	517*	1100*	850*
		± 36	± 186	± 134	± 267	± 161

* More than twice the number of His⁺ revertants than that due to spontaneous mutation
Mean ± SD for 5 plates

AF-2 and BP were used as the positive controls. For BP, among the 4 animal species, rat was most effective, increasing the number of His⁺ revertant colonies to 1350 ± 281 , and giving an order of rat > guinea pig > hamster > mouse.

Fig. 7 Effect on S-9 fractions obtained from PCB-treated guinea pig and hamster on mutagenicity of phenacetin in TA100

Discussion

We investigated whether widely used analgesics, their derivatives, and anti-inflammatory drugs possessed mutagenicity.

A trend of weak DNA damage was demonstrated for salicylic acid, which was the most potent DNA-damaging compound among the salicylate derivatives. Modification of the -COOH and -OH groups of salicylic acid decreased this DNA-damaging potency. Mutagenicity for salicylic acid was found by the Ames test using strain TA100 in the presence of S-9 mix obtained from rat, and for methyl salicylate using strains TA98 and TA100 in the presence of S-9 mix obtained from hamster. Several investigators^[14,15] have reported that all salicylate derivatives are teratogenic and show fetal toxicity in various animals. There is also evidence that aspirin causes congenital malformation in man^[16].

It may be concluded from the results of the present study and those by other investigators^[14-16] that salicylic acid, aspirin and methyl salicylate are potentially carcinogenic compounds.

Phenacetin and its metabolite acetaminophen cause serious side effects, such as renal papillary necrosis^[17-19], and phenacetin has been reported to produce tumors in the renal pelvis and urinary bladder of rats^[20]. In another case, ISAKA et al.^[21] described that the target of phenacetin-induced carcinogenesis was the nasal cavity and urinary tract. SUGIMURA et al.^[22], using strains TA98 and TA100 with

or without S-9 mix, reported that phenacetin itself had no mutagenicity. On the other hand, NOHMI et al.^[9] reported that phenacetin was clearly mutagenic in the TA100 system when S-9 mix from PCB-treated hamster instead of rat was used, and that phenacetin mutagenicity was due to the difference in deacetylation activity between rat and hamster liver microsomes.

In the present study, phenacetin and acetaminophen had no DNA-damaging capacity in the Rec-assay, but phenacetin showed mutagenicity to TA100 with S-9 mix obtained from guinea pig and hamster, and acetaminophen was mutagenic to TA100 in the presence of S-9 mix from hamster. Among the 4 animal species, hamster was the most effective in increasing the number of His⁺ revertant colonies (410 ± 33) for phenacetin at 0.1 mg/plate, giving the order: hamster > guinea pig > rat > mouse.

LIJINSKY et al.^[23] reported that several drugs containing secondary and tertiary amine groups reacted with sodium nitrite *in vitro* and *in vivo*, yielding N-nitroso compounds. For example, dimethylnitrosoamine (DMN) was readily formed from aminopyrine and nitrite in rat stomach, and caused hemorrhagic liver tumors in rats^[24]. On the other hand, ARISAWA et al.^[25] reported that at a concentration of 0.1 ~ 10 mg/ml and pH 1.5 ~ 4.5, antipyrine, aminopyrine and SP, but not IPA, were easily reactive with equal or 2-fold amounts of sodium nitrite to give various nitrosation products.

In this study, antipyrine, aminopyrine, IPA and SP were clearly demonstrated to have DNA-damaging properties at doses of more than 0.4 mg/disc by Rec-assay, with an almost linear dose-response curve. SP showed mutagenicity using the TA100 system with rat S-9 mix, but not with TA98. KUBOYAMA^[26] has already reported that SP and its metabolites have mutagenicity.

Conclusions

In order to study the mutagenicity of analgesics, their derivatives, and anti-inflammatory drugs, the Rec-assay (cold-incubation method) and Ames test were performed. In the Rec-assay, *Bacillus subtilis* H-17 (Rec⁺) and M-45 (Rec⁻) strains were used to assess the presence of DNA-damaging potential. In the Ames test, *Salmonella typhimurium* strains TA98 and TA100 were used to reveal back-mutation:

Salicylate derivatives, salicylic acid (2 mg), aspirin (5 mg), and benzoic acid (4 mg) showed specific DNA-damaging properties in the Rec-assay. Methyl salicylate reacted with strain TA98 in the presence of hamster S-9 mix, and salicylic acid and methyl salicylate were also demonstrated to be mutagenic using strain TA100 with S-9 mix from rat and hamster.

Weak mutagenicity was demonstrated by acetaminophen and phenacetine with hamster S-9 mix and by phenacetine with guinea pig S-9 mix in the Ames test.

All pyrazolone derivatives except SP produced no DNA damage or mutagenicity under the present experimental conditions. The dose-DNA damage curve for SP was nearly linear. Weak mutagenicity of SP was also found using strain TA100 with S-9 mix of rat.

When indomethacin, oxyphenbutazone and diclofenac sodium were tested,

not only the Rec⁻ strain but also Rec⁺ was affected at doses of more than 0.1 mg/disc, indicating that these drugs possessed strong cytotoxicity. In the Ames test, mutagenicity was demonstrated for indomethacin (0.1 mg) and oxyphenbutazone (0.1 mg) using strain TA100 with rat S-9 mix. Therefore, care should be taken with prolonged administration of salicylic acid, methyl salicylate, acetaminophen, phenacetin, SP, indomethacin, oxyphenbutazone and diclofenac sodium.

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