The Journal of Applied Pharmacology, 11, 190-195(2003)

Genotoxicity Study of AS6, a Triterpenoid Derivatives

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(Received April 16, 2003; Accepted July 21, 2003)

Abstract – To assess the genotoxicity of AS6, several classical toxicological tests were performed. In Ames test, AS6 did not show any transformation of revertant with or without S-9 metabolic activating system, indicating the lack of mutagenic effect of the compound. To assess clastogenic effect, *in vivo* micronucleus and *in vitro* chromosomal aberration assays were performed using male ICR mice and Chinese hamster lung (CHL) fibroblast cells, respectively. Chromosomal aberration was not induced regardless of the presence of S-9 metabolic activating system. In addition, AS6 did not cause any increase in the incidence of micronucleated polychromatic erythrocytes at any of the dose levels, suggesting little clastogenicity *in vitro* or *in vivo*. Taken together, these results demonstrate that AS-6 has no mutagenic effect in our test system.

Keywords □ AS6, Ames test, *In vivo* micronucleus assay, *In vitro* chromosomal aberration assay

INTRODUCTION

Asiaticoside (AS), a biologically active triterpenoid present in Centella asiatica (L.) Urban has been known to have a variety of biological effects such as anti-inflammatory, wound-healing, antiulcer, hepatoprotective, skin-tumor prevention, and immunomodulatory effects (Price et al., 1987; Nishino et al., 1988; Maquart et al., 1999; Tan et al., 1997). AS6 was synthesized by Dong Kook Pharmaceutical Co. and found to be effective in decreasing chemical-induced liver injury and also had the inhibitory effect in chronic liver cirrhosis. AS6 (mol. wt. 472.7 Da and melting point of 277-278°C) is white crystalline powder with the formula of [(3β, 4α)-3,23-dihydroxyurs-12-en-28-oic acid). In our previous 4 weeks repeated toxicity study in rats, AS6 has no effects on mortality, clinical signs, body weight, food and water consumption, ophthalmoscopy, urinalysis, hematology, serum biochemistry, necropsy findings, organ weights, and histopathology in any treatment group (Han *et al.*, 2003).

In this study we report the results of genotoxicity study performed as a part of the preclinical safety evaluation program for AS6. Genotoxicity tests can be defined as *in vitro* and *in vivo* designed to detect compounds, which include genetic damage directly or indirectly by various mechanisms. However, no single test is capable of detecting all relevant genotoxic agents. Therefore, the usual approach should be to carry out a battery of *in vitro* and *in vivo* tests for genotoxicity. Generally, the following standard test battery is recommended: 1) A test for gene mutation in bacteria; 2) An *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells or an *in vitro* mouse lymphoma tk assay; 3) An *in vivo* test for chromosomal damage using rodent hematopoietic cells. Therefore, to investigate the whole genotoxic profile of AS6, we carried out the Ames assay, in vitro chromosomal aberration assay using Chinese hamster lung (CHL) cells, and in vivo micronucleus assay using male ICR mice. This study was conducted according to the test guidelines from the KFDA and OECD guidelines for the testing of chemicals under modern Good Laboratory Practice Regulations.

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MATERIALS AND METHODS

Materials

AS6 (purity ≥ 99.3%) was supplied from Dong Kook Phar-

maceutical Co. (Jincheon, Korea). Most chemicals including positive controls such as 4-nitroquinoline-1-oxide (4NQO) and cyclophosphamide were obtained from Sigma (St. Louis, MO). MEM medium, fetal bovine serum, penicillin, and streptomycin were purchased from GIBCO BRL (Grand Island, NY). S9, which was prepared from male Sprague-Dawley rats induced with Aroclor 1254, was from Molecular Toxicology Inc. (Boone, NC) and cofactor for S9 mix was from Wako Pure Chem. Ind., Ltd. (Japan).

Animals

Approximately 5.5 weeks old specific pathogen free male ICR mice weighing 26 ± 2 g were obtained from Bio Genomics, Inc. (Seoul, Korea). The animals were housed in polycarbonate cages. An ambient temperature of 25 ± 2°C, relative humidity of 50 ± 2%, and photoperiod of 12 h was maintained throughout the study. Commercial pellet diet and water were provided *ad libitum*. Clinical signs of animals were checked and recorded once a day for 11 days quarantine and acclimatization period.

All animal experiments were conducted in facilities approved by the AAALAC International, and animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996).

Cell culture

In vitro chromosomal aberration test was performed using Chinese Hamster Lung cells (Koyama *et al.*, 1970), (CHL, modal chromosome number 25, population doubling time 12 to 15 hours), which were obtained from Division of Genetic Toxicology, KFDA. The cells were cultured in Minimum Essential Medium supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine and 10% fetal bovine serum. Subculturing was conducted every 2~3 days so as to prevent overgrowth.

Ames assay

Salmonella typhimurium strain TA98 and TA1537 (detect frameshift mutagens), and strain TA100, TA1535 and *Escherichia coli* WP2 uvrA (detect base-pair substitution mutagens) were used as test strains. All of the tester strains were purchased from Molecular Toxicology Inc. (Boone, NC). The mutation test was performed by the method of Maron and Ames (1983). A 0.1 ml aliquot of AS6 containing 0−5000 μg per plate, 0.5 ml of S-9 mix (or sodium-phosphate buffer, pH 7.4), and 0.1 ml inoculum of the test strain were added to each tube containing 2 ml of top agar. The contents of the test tubes were mixed well and the mixtures were poured onto the Vogel-Bonner minimal agar plates. Plates were incubated at 37°C for 48 h. The revertant colonies were counted using hand counter. Control plates were also set up with each assay. Triplicate plates were run for each assay and each experiment was repeated at least once. The formation of background lawn and other abnormalities were also checked.

***In vitro* chromosome aberration assay**

This test consisted of short-term and continuous (24 h) treatments. For short-term treatment, AS6 was treated for 6 h followed by a recovery period of 18 h, with and without rat liver S9. Two hours before the end of the cultivation, colchicine was added. The slides of CHL cells were prepared and stained according to the routine hypotonic-acetic acid-methanol-flame-drying-Giemsa schedule for metaphase plate analysis (Preston et al., 1987). At least 200 well-spread intact metaphases were scored per dose level under 1000 × magnification using a light microscope (Nikon Microphoto). Each type of aberration was recorded, and the number of aberrant metaphases (showing one or more aberrations, including/excluding gaps) and total aberrations (including/excluding gaps) were calculated. The results were expressed as number of findings per 200 metaphases. Regardless of the presence of aberration, an additional 100 metaphases were examined to determine the frequency of diploid (DP, 23-36 chromosomes), polyploid (PP, 37 ≤ chromosomes), and endoreduplication (ER). All aberrations were considered as equal regardless of the number of breaks involved, gaps were not included (Tice *et al.*, 1987). Blind scoring was done by a single observer.

***In vivo* micronucleus Assay**

A preliminary assay was done to select a maximum dose of AS6, which would be expected to produce lethality. AS6 was administered by gavage to groups of six mice at doses of 500, 1000 and 2000 mg/kg in 0.5% methylcellulose at a dose volume of 20 ml/kg. Groups of control mice received only the vehicle by gavage. Six mice were administered cyclophosphamide in normal saline (10 ml/kg) by ip injection at 70 mg/kg and served as positive controls. Mice were killed 24 h, which was selected as the time of peak response of micronuclei induction, after treatment with AS6. The mouse bone marrow micronucleus test was carried out according to the method of Schimd (1975). Bone marrow was prepared for evaluation with slight modifications of the method reported by Schmid. Following sacrifice of the animals, one femur was excised intact. The bone marrow

was expelled from the cavity by repeated gentle aspirations and flushings with fetal bovine serum. The cell suspension was centrifuged at approximately 1000 rpm for 5 min in a clinical centrifuge. The supernatant was decanted and the pellet resuspended in a small volume of serum. At least two slides of the cell suspension per animal were made. The air-dried slides were stained with May-Grunwald and Giemsa as described by Schimd. Smears were allowed to dry overnight before being coverslipped with mounting medium. Slides were then examined under 1000 X magnification. Small round or oval shaped bodies, size of which ranging about 1/5 to 1/20 of the diameter of polychromatic erythrocyte (PCE), were counted as micronuclei. A total of 2000 PCEs were scored per animal by the same observer for determining the frequencies of micronucleated polychromatic erythrocytes (MNPCEs). PCE/[PCE+NCE] ratio was calculated by counting 500 cells.

RESULTS

Ames test of AS6

The results of Ames test of AS6 are shown in Table I. There was no increase in the number of revertant colonies compared to its negative control at any dose in all of strains. In addition, the antibacterial effects such as decrease in the number of colonies were not observed in all of strains except TA1537 strain, in which AS6 caused 50% reduction in the number of colonies at 5000 mg/plate in the absence of S-9 mix.

In vitro chromosomal aberration test of AS6

The results of the *in vitro* chromosomal aberration assay of AS6 are shown in Table II. In the case of continuous treatment, total aberrations including gaps of AS6 was less than 2% in CHL cells up to 10 μg/ml. In the short-term treatment, total aberrations including gaps of AS6 was less than 3% without S-9 mix and less than 2% with S-9 mix, respectively. Since structural aberrations did not exceed 5% in either treatment, AS6 was concluded to be non-clastogenic in this assay.

In vivo micronucleus test of AS6

Table III shows the incidence of micronucleated polychromatic erythrocytes (MNPCEs) per 2000 polychromatic erythrocytes (PCEs). AS6 administration did not increase MNPCEs at any of the dose levels used when compared to vehicle controls. However, there was a statistically significant increase in number of MNPCEs in the positive control group (61.33, $P<0.01$). Vehicle control values on MNPCE in our study were within the range of historical data on micronucleus test in ICR mice. In addition, no remarkable effects of AS6 were seen on mean

Table I. Ames test for AS6

Tester Strain	Chemical Treated	Dose (μg/plate)	Colonies/plate(Mean) [Factor][a]			
			Without S-9 mix		With S-9 mix	
TA100	AS6	0	148 ± 8		150 ± 6	
		313	161 ± 7	[1.1]	171 ± 15	[1.1]
		625	168 ± 15	[1.1]	157 ± 8	[1.0]
		1250	157 ± 3	[1.1]	156 ± 5	[1.0]
		2500	157 ± 12	[1.1]	160 ± 4	[1.1]
		5000	144 ± 4	[1.0]	162 ± 18	[1.1]
TA1535	AS6	0	16 ± 5		15 ± 4	
		313	15 ± 4	[0.9]	11 ± 2	[0.7]
		625	16 ±2	[1.0]	11 ±3	[0.7]
		1250	14 ± 2	[0.9]	13 ± 3	[0.9]
		2500	11 ± 2	[0.7]	10 ± 2	[0.7]
		5000	10 ± 2	[0.6]	9 ± 1	[0.6]
TA98	AS6	0	20 ± 2		34 ± 1	
		313	19 ± 1	[1.0]	32 ± 3	[0.9]
		625	18 ± 4	[0.9]	32 ± 2	[0.9]
		1250	20 ± 4	[1.0]	30 ± 2	[0.9]
		2500	17 ± 1	[0.9]	28 ± 2	[0.8]
		5000	14 ± 2	[0.7]	21 ± 4	[0.6]

Table I. Continued

Tester Strain	Chemical Treated	Dose (μg/plate)	Colonies/plate(Mean)[Factor][a)] Without S-9 mix		With S-9 mix	
TA1537	AS6	0	10 ± 1		13 ± 1	
		313	10 ± 2	[1.0]	18 ± 2	[1.4]
		625	9 ± 3	[0.9]	18 ± 4	[1.4]
		1250	6 ± 1	[0.6]	13 ± 0	[1.0]
		2500	7 ± 2	[0.7]	10 ± 2	[0.8]
		5000	5 ± 2	[0.5]*	8 ± 1	[0.6]
E.coli	AS6	0	19 ± 5		25 ± 1	
WP2 uvrA		313	22 ± 3	[1.2]	25 ± 4	[1.0]
		625	22 ± 5	[1.2]	25 ± 0	[1.0]
		1250	22 ± 4	[1.2]	23 ± 2	[0.9]
		2500	20 ± 4	[1.1]	24 ± 4	[1.0]
		5000	21 ± 1	[1.1]	23 ± 3	[0.9]
Positive controls						
TA100	SA	0.5	673 ± 57	[4.5]		
TA1535	SA	0.5	411 ± 18	[25.7]		
TA98	4NQO	0.5	341 ± 20	[17.1]		
TA1537	9-AA	50	157 ± 21	[6.3]		
WP2 uvrA	4NQO	0.5	272 ± 11	[14.3]		
TA100	2-AA	0.4			707 ± 64	[4.7]
TA1535	2-AA	2			412 ± 40	[27.5]
TA98	2-AA	0.4	33 ± 3	[1.7]	389 ± 30	[11.4]
TA1537	2-AA	2			382 ± 7	[29.4]
WP2 uvrA	2-AA	4			230 ± 44	[9.2]

* Indicates significant difference at $p<0.05$ level when compared with the control group.
a) No. of colonies of treated plate/No. of colonies of negative control plate
SA, Sodium azide; 9-AA, 9-Aminoacridine; 2-AA, 2-Aminoanthracene; 4NQO, 4-Nitroquinoline-1-oxide

numbers of PCEs per 500 erythrocytes (PCE/[PCE+NCE]), an indicator of cytotoxicity. Although slight decreases were observed in the mean values for the PCE/[PCE+NCE] ratio in other groups treated with AS6, none of these changes was statistically significant.

DISCUSSION

Triterpenoids are an interesting group of compound that exists widely in plants. AS6, a triterpenoid compound with ursane structure, is a new hepatoprotective agent effective in decreasing chemical-induced liver injury and inhibiting chronic liver cirrhosis.

In the Ames test, AS6 does not have gene mutation activity. In addition, there was little clastogenicity seen *in vitro* chromosomal aberration assay. The analysis of standard genotoxicity test data that have been conducted for safety evaluation of chemical compounds usually reveals a fairly high percentage of positive results in the *in vitro* chromosomal aberration test (Muller and Sofuni, 2000). In particular, DNA double-strand breaks associated with cytotoxicity are produced by chemicals that are not rodent carcinogens or *Salmonella* mutagens, and not generally genotoxic in other systems (Galloway, 2000). Therefore, lowering the upper limits of test compound concentration irrespective of cytotoxicity may prove useful to ensure a sufficient reliability of genotoxicity testing with mammalian cells in vitro. Thus 50% cell growth inhibition concentration was used as a maximum concentration for *in vitro* chromosomal aberration test. From concentration-finding test, 50% cell growth inhibition concentrations were about 100 μg/ml with S-9

Table II. *In vitro* chromosomal aberration test for AS6

Conc. (μg/ml)	Relative Cell Counts (%)[a]	No. of Aberrant Metaphase	No. of Total Aberration	No. of findings/200 metaphases						
				Gap	Chromosome type		Chromatid type		Other	PP+ER
					DEL	EXC	DEL	EXC		
With S-9 mix (6 h)										
0	100	1/0[b]	1/0	1	0	0	0	0	0	0+0
25	116	1/0	1/0	1	0	0	0	0	0	1+0
50	113	2/1	2/1	1	0	0	0	0	0	0+0
100	55	1/0	1/0	1	0	0	0	0	0	0+0
CPA	105	33/29	39/36	3	0	0	1	35	0	0+0
Without S-9 mix (6 h)										
0	100	0/0	0/0	0	0	0	0	0	0	0+0
5.8	113	1/0	1/0	1	0	0	0	0	0	0+0
11.5	109	1/1	1/1	0	0	1	0	0	0	1+0
23	19	4/1	4/1	3	0	0	0	1	0	1+0
EMS	121	35/32	50/46	4	0	1	2	43	0	0+0
Without S-9 mix (24 h)										
0	100	0/0	0/0	0	0	0	0	0	0	0+0
2.5	110	0/0	0/0	0	0	0	0	0	0	0+0
5	116	0/0	0/0	0	0	0	0	0	0	0+0
10	24	2/1	2/1	1	0	0	0	1	0	0+0
EMS	115	52/44	60/47	13	0	0	3	44	0	0+0

[a] Relative Cell Counts = (Cell counts of treated flask/Cell counts of control flask)× 100
[b] Gaps included/excluded, 200 metaphases per culture.
DEL, Deletion; EXC, Exchange; PP, Polyploid; ER, Endoreduplication

Table III. *In vivo* micronucleus test for AS6

Dose (mg/kg)	No. of Animal	MNPCE/2000 PCEs (Mean±S.D.)	PCE/[PCE+NCE] (Mean±S.D.)
0	6	1.17 ± 0.75	0.51 ± 0.09
500	6	1.83 ± 0.41	0.43 ± 0.06
1000	6	1.17 ± 0.98	0.41 ± 0.03
2000	6	1.33 ± 1.21	0.48 ± 0.09
CPA	6	61.33 ± 13.66**[a]	0.35 ± 0.12**[b]

**indicates significant difference at $p<0.01$ level when compared with the control group.
[a]Mann-Whitney's U-test
[b]Student's t-test
Abbreviations
PCE: Polychromatic erythrocyte
NCE: Normochromatic erythrocyte
MNPCE: PCE with one or more micronuclei

mix and 23 μg/ml for short-term treatment, and 10 μg/ml for continuous treatment, respectively. Although, in the main study, it showed too potent cytotoxicity for 6 h treatment at 23 mg/ml and for 24 h treatment at 10 μg/ml in the absence of S-9 mix, AS6 was found as negative in the chromosome aberration assay in CHL cells up to maximum concentrations.

Our results clearly also demonstrate that AS6 does not induce micronuclei formation in bone marrow cells after single gavage dose to ICR mice. Although the current preferred method of staining for MN assay is acridine orange staining (Tinwell and Ashby, 1989), Giemsa was chosen to stain our slides because it is a permanent stain which does not fade even when exposed to strong light during the scoring period. Giemsa stain is acceptable by an international expert group that recently reviewed in vivo rodent erythrocyte micronucleus assay in the International Workshop on Genotoxicity Test Procedures (Hayashi *et al.*, 2000). On the other hand, the time of peak response of micronuclei induction should be selected as the sacrifice time of mutagens. According to the information of kinetics of erythropoiesis and the recommendations and observations made by several laboratories (Salamone and Heddle, 1983; Henderson *et al.*, 1984; Krishna, 2000), mice were killed 24 h, after treatment with AS6. In fact, in most cases approximately 24 h is more practical than 48 h.

From the above *in vitro* and *in vivo* results, AS6 is not considered to act as a genotoxic material.

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