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Genotoxicity on Structural Derivatives of Sophoricoside, a Component of *Sophora Japonica*, in Bacterial and Mammalian Cells

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Abstract

To develop the novel anti-allergic drug, many sophoricoside derivatives were synthesized. Among these derivatives, JSH-II-3, VI-3, VII-3, VIII-3, VII-20 and VII-20 (sodium salt) were selected and subjected to high throughput toxicity screening (HTTS) because they revealed strong IL-5 inhibitory activity and limitation of quantity. Single cell gel electrophoresis (Comet) assay, mouse lymphoma thymidine kinase ($tk^{+/-}$) gene assay (MOLY), chromosomal aberration assay in mammalian cells and Ames reverse mutation assay in bacterial system were used as simplified, inexpensive, short-term *in vitro* screening tests in our laboratory. Through the primary screening using the comet assay, we could choose the first candidates of sophoricoside derivatives with no genotoxic potentials as JSH-VI-3, VII-3, VII-20 and VII-20 (sodium salt). Also JSH- VII-3, VII-20 and VII-20 (sodium salt) are non-mutagenic in MOLY assay, while JSH-II-3 is mutagenic at high concentration with the presence of metabolic activation system in both comet assay and MOLY assay. The selected derivatives (JSH-VI-3, VII-3, VII-20 and VII-20 (sodium salt) are not mutagenic in *S. typhimurium* TA98 and TA100 strains both in the presence and absence of metabolic activation. From results of chromosomal aberration assay, 6 h treatment of JSH-VI-3, VII-3 and VII-20 (sodium salt) were not revealed clastogenicity both in the presence and absence of S-9 mixture. Therefore, we suggests that JSH-VI-3, VII-3, VII-20 and VII-20 (sodium salt), as the optimal candidates with both no genotoxic potential and IL-5 inhibitory effects must be chosen. To process the development into new anti-inflammatory drug of these derivatives, further investigation will need.

Keywords: sophoricoside derivatives, genotoxicity, comet assay, MOLY assay, chromosomal aberration assay, Ames reverse mutation assay, anti-inflammatory effect

Plant-based medicines have been used to treat diseases world-wide for thousands of years[1]. Even today, in most developing countries, herbs and other traditional systems of medicines are used by about 80% of the indigenous populations as their major source of primary healthcare[2]. However, most of the information about several medicinal herbs does not have any scientific data support, and some of them may have deleterious effects, for example, mutagenic, toxic and carcinogenic activities[3]. It has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that the majority of mutagens are carcinogens[4] and, for at least some compounds, mutagenic potency is closely correlated with carcinogenic potency[5]. It is, therefore, important to assess the potential mutagenic effect of natural products before they are considered acceptable for medical use[6]. So, we call attention to the control of such compounds since they may present genetic toxicity and moreover it is very important to check their cancer inducing potentiality by performing traditional and advanced genotoxicity battery including bacterial reversion, *in vitro* DNA break and forward mutation induction in mammalian cells, *in vitro* chromosomal aberration analysis, and *in vivo* micronucleus assay.

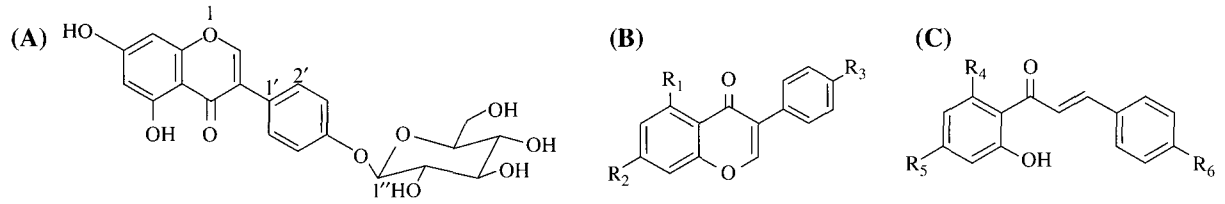
Eosinophilic inflammation is the main histological correlate of airway hyper-responsiveness and tissue injury in the pathogenesis of bronchial asthma[7,8]. Interleukin (IL)-5 appears to be one of the main proinflammatory mediators among a growing number of cytokines and chemokines that induce eosinophilic inflammation[9,10]. IL-5 is critically involved in eosinophilia-associated allergic inflammation. Interfering with the action of IL-5 represents one of the new immunomodulatory therapeutic strategies in the treat-

ment of allergic diseases including bronchial asthma. Compared to established immunosuppressive agents like corticosteroids, a major advantage of this strategy is the specificity of reducing eosinophilic inflammation, thus possibly acting nearly without side effects. However small organic compounds to inhibit IL-5 activity have been rarely found.

Sophoricoside (Fig. 1) was isolated from *Sophora japonica*, a plant of Leguminosae family, as inhibitors of IL-5 bioactivity[11], and showed differential inhibition on IL-3 and GM-CSF bioactivities[12]. Also, sophoricoside has been reported to have an anti-inflammatory effect on rat paw edema models and have an antioxidant effect evaluated by *in vitro* and *in situ* liver chemiluminescence[13,14]. This isoflavonoid is unrelated to the structural unit of IL-5 and is the first natural products to show the inhibitory activity against IL-5 bioactivity.

From structure activity relationship study of sophoricoside, we could design of potent derivatives (Fig. 1). The structural requirement of sophoricoside derivatives possessing the inhibitory activity against IL-5 was summarized as isoflavonone moiety is essential and glucosyl group is not essential, especially in the presence of cyclohexylmethoxy at position 5 as bulky hydrophobic group. Using this pharmacophore, novel isoflavones (JSH-II-3, VI-3, VII-3 and VIII-3) were designed, prepared and evaluated their inhibitory activity against IL-5. As another derivative, chalcone derivatives (JSH-VII-20 and its sodium salt form) were identified as novel skeleton for the inhibition of IL-5 activity.

However, it is very few reports on the toxicity, especially, genotoxicity of sophoricoside and its derivatives. Previous work by our group has shown that the parent compound, sophoricoside undergoes several genotoxic potentials leading to *thymidine kinase* gene mutation and micronucleus *in vivo*[15]. Our purpose was to demonstrate whether newly synthesized derivatives with anti-inflammatory effects have genotoxic properties. If anything among these derivatives has not genotoxicity, it should be considered as potentially ideal drug candidate through further investigations for their safety.



Sophoricoside

Compound		R						Inhibition
		R_1	R_2	R_3	R_4	R_5	R_6	IC_{50} (μM)
Isoflavone	JSH-II-3	benzyloxy	H	OH				15.3
	JSH-VI-3	H	benzyloxy	OH				36.3
	JSH-VII-3	$OCH_2C_6H_{11}$	H	OH				5.8
	JSH-VIII-3	H	$OCH_2C_6H_{11}$	OH				4.0
Chalcone	JSH-VII-20				$OCH_2C_6H_{11}$	H	COOH	9.4

Fig. 1. Structure of sophoricoside derivatives. (A) Sophoricoside, (B) isoflavone structures, (C) chalcone structures. IC_{50} is 50% inhibition concentration of IL-5 activity.

Table 1. Cytotoxicity of sophoricoside derivatives in L5178Y mouse lymphoma cell line and Chinese hamster lung (CHL) cell line in the presence and absence of S-9 metabolic activation system. IC_{20} (20% inhibition concentration of cell growth) and IC_{50} were calculated by the results of cell viability using trypan blue dye exclusion assay (unit = μg/mL)

	JSH-II-3		JSH-VI-3		JSH-VII-3		JSH-VIII-3		JSH-VII-20		JSH-VII-20 (sodium salt)	
	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9
IC_{20} (L5178Y)	32.8	79.0	29.8	14.5	33.0	42.0	13.9	18.0	31.8	19.5	90.9	127.3
IC_{50} (CHL)	–	–	115.0	61.9	12.0	32.7	–	–	–	–	55.7	64.2

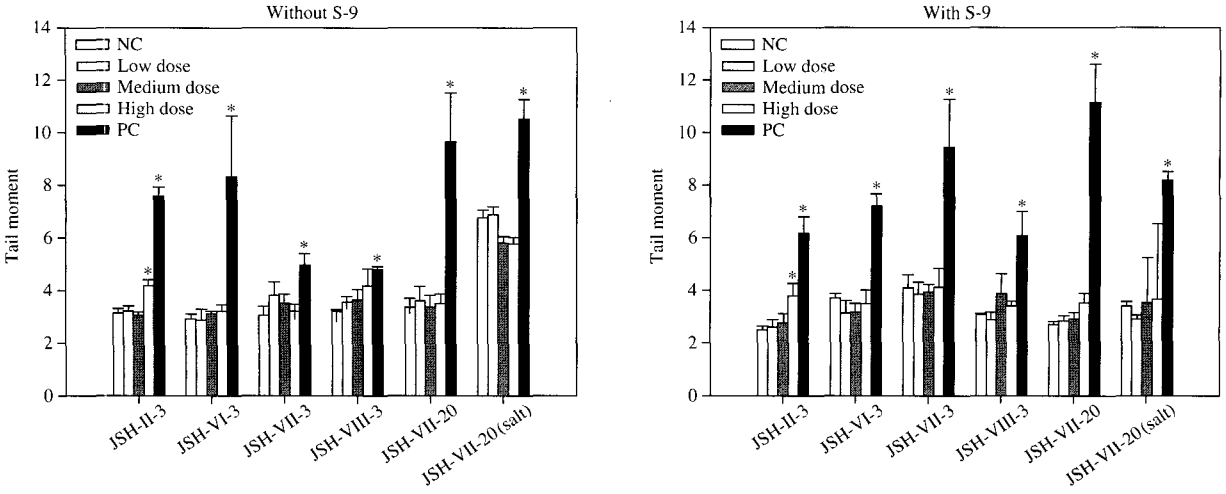


Fig. 2. Tail moment of JSH-II-3, VI-3, VII-3, VIII-3, VII-20 and VII-20 (Na-salt) at the absence and presence of S-9 metabolic activation system. Values are mean ± S.D. (n = 4). Positive controls were MMS (150 μM) in the absence and BaP (50 μM) in the presence of S-9 metabolic activation system, respectively. NC: DMSO, PC: +S9 (BaP-50), −S9 (MMS-150). * statistically significant ($P < 0.05$)

In this study, *in vitro* assay has been performed to determine these cytotoxic and genotoxic potentials. We adopted various methods to assess the genotoxicity of sophoricoside derivatives such as single cell gel electrophoresis (comet) assay[16-18], bacterial reverse mutation assay[19-21], chromosomal aberration assay with Chinese hamster lung cells[22] and thymidine kinase gene forward mutation assay with mouse lymphoma cells.

Cytotoxicity of Newly Synthesized Sophoricoside Derivatives

Cytotoxicity of L5178Y cells and CHL cells following exposure to a range of concentrations of sophoricoside derivatives was determined by trypan blue dye exclusion assay. The survival percentage relative to solvent control (DMSO) was determined as a percentage of the number of cells survived after treatment with or without metabolic activation system. All the derivatives studied decreased the viability of the cells studied proportionally to the concentration in both cell lines. And based on results of cytotoxicity assay, 20% inhibitory concentration (IC_{20}) on L5178Y cell growth of each compound was calculated (Table 1). These concentrations were considered to be in the acceptable range for conducting the Comet assay[23]. As shown in Table 1, exposure of 127.3-13.9 μg/mL derivatives for 2 h resulted in relative survival exceeded 80% compared to solvent control in mouse lymphoma L5178Y cells. Also, the 50% cell growth inhibition concentrations (IC_{50}) of derivatives were determined as 115-12 μg/mL in CHL fibroblast cells.

Screening of DNA Damage with Sophoricoside Derivatives Using the Single Cell Gel Electrophoresis (Comet) Assay

Firstly, we screened using the comet assay in L5178Y cells whether sophoricoside derivatives could induce subtle DNA damages, following guideline recommended by IWGTP. The comet assay, as a high throughput toxicity screening tool for detection of DNA damage in mammalian cells, is rapid, sensitive, visual and simple technique to quantify DNA strand breaks in individual cells. The intensity and the length of comet images were expressed in terms of the tail moment. Comet assay was carried out at IC_{20} values of each compound as maximum concentration. The results of the comet assay are shown in Fig. 2. The response of the positive control (150 μM MMS and 10 μM BaP) was significantly greater ($P < 0.001$) than solvent control in conditions without or with S-9 metabolic activation system (S-9), respectively. Derivatives like as JSH-VI-3, VII-3, VII-20 and VII-20 (sodium salt) did not induce a significant DNA damage at all the tested concentrations, while JSH-II-3 and VIII-3 produced a significant increase in tail moment values (Fig. 2). In detail, JSH-II-3 was induced DNA damage at 32.8 μg/mL and 79.0 μg/mL in the −S-9 and +S-9, respectively ($P < 0.05$) (Fig. 2). JSH-VIII-3 at 13.9 μg/mL in the −S-9 was induced a significant DNA strand breaks (Fig. 2). Through the primary screening using the comet assay, we could choose the first candidates of sophoricoside derivatives with no genotoxic potentials as JSH-VI-3, VII-3, VII-20 and VII-20 (sodium salt).

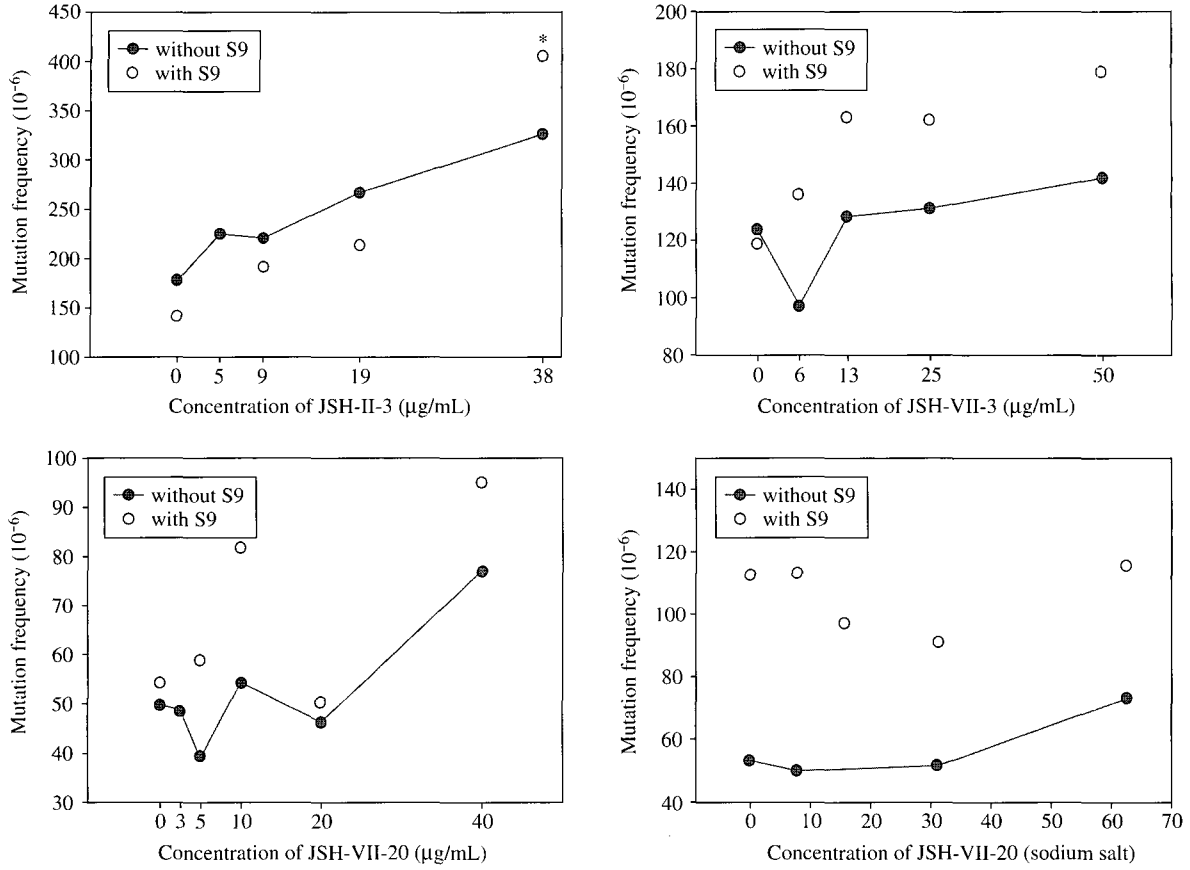


Fig. 3. Mutation frequencies by JSH-II-3, JSH-VII-3, JSH-VII-20 and JSH-VII-20 (sodium salt) in L5178Y cells in the absence and presence of metabolic activation system (3 h treatment). Total *tk* mutant frequency is displayed. Results are taken from one representative experiment. *statistically significant ($P<0.05$)

Detection of Gross Genetic Alteration on Sophoricoside Derivatives Using L5178Y *thymidine kinase (tk)*$^{+/-}$-3.7.2C mouse lymphoma Assay (MOLY)

Next, we investigated whether derivatives induce the base-pair as well as frameshift mutations or small deletions in L5178Y cells using MOLY assay. The MOLY assay detects a broader range of mutations in a more complex eukaryotic system for more sensitive detection of mutagens[24]. The *tk* mutant frequencies (including the small and large colony *tk* mutant frequencies) from one representative experiment with JSH-II-3, VII-3, VII-20 and VII-20 (sodium salt) are displayed in Fig. 3. Background mutant frequencies were within the historical control range, and positive controls gave large dose-dependent increases in mutant frequencies, meeting assay acceptance criteria. The mutant frequencies in the treated cultures were similar to the vehicle controls, and none of JSH-VII-3, VII-20 or VII-20 (sodium salt) with and without S-9 doses induced a mutant frequency over twice the background. However, clearly increased mutant frequency was found after treatment of JSH-II-3 at 38.0 μg/mL in the +S-9. Mutant frequencies were nearly three-fold increased compared to the untreated control, and cell survival was reduced to about 20%. Also, a dose response of mutagenic effect was observed at concentrations between 5 and 38 μg/mL of JSH-II-3. It is suggests that JSH-VII-3, VII-20 or VII-20 (sodium salt) are non-mutagenic in MOLY assay, while JSH-II-3 is mutagenic at high concentration with the presence of metabolic activation system in both comet assay and MOLY assay. Therefore, the safety of JSH-II-3 should be considered through further investigation and then it dropped out of candidate derivatives.

Assessment of Mutation Induction by Sophoricoside Derivatives in the Bacterial System Using Ames Reverse Mutation Assay

For the bacterial mutation screening, only two tester strains of *Salmonella typhimurium* (TA98 and TA100)

were used because these two strains detect the vast majority of bacterial mutagens in our experience. TA98 is sensitive to frameshift mutagens while TA100 is sensitive to base substitution mutagens[21]. Both tester strains contain the pKM101 plasmid, which makes them more sensitive than their counterparts TA1538 and TA1535[25]. Therefore, the use of only TA98 and TA100 for rapid screening tests is justified in terms of the lower usage of test compound and lower cost of testing. The mutagenic potential of JSH-VI-3, VII-3, VII-20 and VII-20 (sodium salt) was investigated with these two strains in the presence and in the absence of S-9 mixture. Positive controls specific to each of the two tester strains resulted in the expected increases in the number of histidine revertants. In observation of the background lawns of treated bacteria, JSH-VII-3 was cytotoxic at doses above 1,000 μg/plate in the absence and presence of S-9 mixture. JSH-VII-20 and JSH-VII-20 (sodium salt) at 5,000 μg/plate were a little cytotoxic. JSH-VI-3 was not observed cytotoxic effects in applied concentrations. So, we determined as optimal maximum concentrations of derivatives for this assay. As shown in Table 2, no significant increase of revertants in two strains at all concentrations of derivatives used. These results suggest that selected derivatives are not mutagenic in *S. typhimurium* TA98 and TA100 strains both in the presence and absence of metabolic activation.

Assessment of Clastogenicity of Sophoricoside Derivatives in the CHL Cells Using Chromosomal Aberration Assay

The types and frequencies of chromosomal aberrations seen in treated and control cultures for 6 h of JSH-VI-3, VII-3 and VII-20 (sodium salt) treatment are listed in Table 3. The solvent (DMSO)-treated control was revealed only 0-1% of spontaneous chromosomal aberrations in 200 metaphase cells. Cyclophosphamide (3 μg/mL) used as an indirect-acting mutagen that requires metabolic activation and mitomycin (0.3 μg/mL) as a direct-acting mutagen, induced remarkable chromosomal aberrations (about 20-40%) in CHL fibroblasts. Low frequencies of breaks and fragments were seen in derivatives-treated cultures and solvent controls, both with and without S-9 mixture. No statistically significant increases in the mean percentage of aberrant cells in the types of aberrations noted between derivatives-treated cultures and solvent-control were seen both with and without S-9 mixture (Table 3). In the JSH-VII-20 (sodium salt), cells with polyploidy types were observed. The

Table 2. Mutagenicity of sophoricoside derivatives in *Salmonella typhimurium* TA 98 and TA100 in the presence and absence of S-9 metabolic activation system

S-9 mix	His$^+$ revertants/plate (Mean±S.D.)										
	Compound dose (μg/plate)	JSH-VI-3		JSH-VII-3		Compound dose (μg/plate)	JSH-VII-20		Compound dose (μg/plate)	JSH-VII-20 (salt)	
		TA 98	TA 100	TA 98	TA 100		TA 98	TA 100		TA 98	TA 100
−	DMSO	23±2	211±15	23±4	216±13	DMSO	21±5	137±3	DMSO	43±7	194±19
−	16	26±3	209±10	23±5	210±3	10	22±4	−	40	38±5	209±9
−	31	27±3	211±6	25±5	212±17	40	32±3	−	79	45±8	195±3
−	63	29±2	215±13	27±4	215±41	157	24±4	−	157	39±10	212±14
−	125	32±8	225±5	25±4	201±29	313	−	147±18	313	33±3	171±31
−	250	40±2	203±11	22±7	177±24	625	18±4	146±9	625	28±7	193±6
−	500	41±8	213±23	21±5	189±27	1250	−	143±9	1250	21±2	174±28
−	1000	30±4	197±6	16±4	162±16	2500	16±3	144±6	2500	23±6	164±18
−						5000	−	119±12	5000	10±5	136±30
−	SA 1	−	1331±178	−	1176±110	SA 1		1259±91	SA 1		478±30
−	2-NF 0.2	329±19	−	349±45	−	2-NF 0.2	314±22	−	2-NF 0.2	414±52	−
+	DMSO	45±6	203±20	38±5	224±6	DMSO	45±3	181±18	DMSO	84±1	197±5
+	16	46±2	209±31	40±8	244±7	10	53±3	−	40	73±13	190±9
+	31	48±6	202±15	36±3	232±21	40	56±5	−	79	44±9	178±5
+	63	51±8	230±6	35±2	230±16	157	42±3	−	157	41±3	186±19
+	125	46±7	211±38	26±6	210±15	313	−	189±9	313	36±12	172±6
+	250	50±9	270±12	24±5	224±12	625	36±3	183±15	625	34±0	190±4
+	500	42±3	240±26	29±7	222±15	1250	−	177±3	1250	40±12	183±3
+	1000	24±3	215±20	22±3	162±13	2500	28±2	178±8	2500	32±3	210±5
+						5000	−	165±9	5000	16±2	205±1
+	2-AA 0.5	893±80	−	858±59	−	2-AA 0.5	1523±857	−	2-AA 0.5	1523±857	−
+	2-AA 1	−	1285±140	−	3341±274	2-AA 1	−	1844±318	2-AA 1	−	3348±177

DMSO: dimethyl sulfoxide, SA: Sodium Azide, 2-NF: 2-Nitrofluorene, 2-AA: 2-Aminoanthracene

Table 3. Chromosome aberrations induced by sophoricoside derivatives in CHL fibroblasts

Treatment			S-9 Mix	Chromosome aberrations/200 cells									
				Chromatid type		Chromosome type		Total aberration (%)	Extra aberration				nor
Compound	Conc. (μg/mL)	hr		Br	Ex	Br	Ex		ctg	csg	poly	endo	
DMSO	-	6	+	0	0	2	1	1.5	0	0	0	0	197
CP	5.0	6	+	18	34	2	2	28	3	0	0	0	145
JSH-VI-3	61.9	6	+	2	4	0	0	3	1	1	1	0	191
	31.0	6	+	3	0	0	0	1.5	1	0	0	0	196
	15.5	6	+	0	2	0	0	1	1	0	0	0	195
DMSO	-	6	-	1	0	0	0	0	1	0	0	0	199
MMC	0.1	6	-	11	42	0	0	26	6	0	0	0	148
JSH-VI-3	115	6	-	0	0	0	0	0	3	0	0	1	199
	57.5	6	-	1	0	0	0	0.5	1	0	0	0	198
	28.8	6	-	0	2	0	0	1	0	0	0	0	199
DMSO	-	6	+	2	1	0	0	1.5	1	1	1	0	194
CP	5.0	6	+	18	34	2	2	28	3	0	0	0	145
JSH-VII-3	32.7	6	+	0	1	0	0	0.5	2	0	0	0	197
	16.4	6	+	0	3	2	0	2.5	2	0	0	0	193
	8.2	6	+	1	0	0	0	0.5	3	0	0	0	196
DMSO	-	6	-	2	0	0	0	1.3	0	0	0	0	146
MMC	0.1	6	-	11	42	0	0	26	6	0	0	0	148
JSH-VII-3	12.0	6	-	1	2	0	1	2	0	1	0	0	196
	6.0	6	-	2	1	0	1	2	2	0	0	0	194
	3.0	6	-	1	0	1	0	1	0	0	0	0	197
DMSO	-	24	-	1	1	1	0	1.5	0	0	0	0	197
MMC	0.1	24	-	21	51	1	0	36.5	6	0	0	0	132
JSH-VII-3	12.0	24	-	0	0	1	0	0.5	1	0	0	0	198
	6.0	24	-	4	1	0	0	2.5	1	0	0	0	195
	3.0	24	-	1	1	0	0	1	0	0	0	0	198
D.W	-	6	+	1	0	0	0	0.5	3	1	1	0	194
CP	5.0	6	+	18	34	2	2	28	3	0	0	0	145
JSH-VII-20 (salt)	64.23	6	+	1	3	0	0	2	1	0	21	0	175
	32.12	6	+	3	1	0	0	3	1	0	26	0	169
	16.06	6	+	1	0	0	0	0.5	1	0	3	0	195
D.W	-	6	-	1	0	1	0	1	0	1	0	0	197
MMC	0.1	6	-	11	42	0	0	26	6	0	0	0	148
JSH-VII-20 (salt)	55.72	6	-	1	1	0	0	1	0	0	45	0	153
	27.86	6	-	0	0	0	0	0	0	0	3	0	197
	13.93	6	-	1	0	0	0	0.5	0	0	0	0	199

Conc.: concentration, Br: breakage, Ex: exchange, ctg: chromatid gap, csg: chromosome gap, poly: polyploid, endo: endoreduplicate, nor: normal, MMC: mitomycin C, CP: cyclophosphamide

mean percentage of aberrant cells without S-9 mixture ranged from 0.5 to 2%, compared with a mean solvent control value of 1%. For treatment with S-9 mixture, the mean aberrant cell percentage ranged from 0.5 to 2%, compared with a mean solvent control value of 0.5%. From these results, 6 h treatment of JSH-VI-3, VII-3 and VII-20 (sodium salt) were not revealed clastogenicity both in the presence and absence of S-9 mixture in this assay.

Conclusion

In the present study, a preliminary profile of the genotoxic potential of six sophoricoside derivatives was obtained using four *in vitro* screening tests: single cell gel electrophoresis (Comet) assay, a mammalian cell mutation screening test using L5178Y mouse lymphoma cells, a bacterial mutation screening test (screening Ames assay) and a chromosomal aberration screening test using CHL cells. The results of the present study show that the genotoxic activity of sophoricoside derivatives could have been easily detected if these simplified, inexpensive, short-term *in vitro* screening tests had been used during anti-inflammatory drug development, prior to distribution of it to general population. The genotoxic potential of two out of six derivatives of sophoricoside (JSH-II-3

and JSH-VIII-3) was detected with two of the four parameters measured, depending on the concentrations. In conclusion, through pharmacophore analysis of sophoricoside, a parent compound, novel isoflavones derivatives (JSH-II-3, VI-3, VII-3 and VIII-3) were designed, prepared and evaluated. As the results, these four compounds showed their inhibitory activity against IL-5 at uM level on cell based assay, while a compound JSH-VII-3 was investigated as possible preclinical candidates through toxicological data and physico-chemical properties, like solubility. And novel chalcones, which were identified as novel skeleton for the inhibition of IL-5 activity, were designed, prepared and evaluated. As the results, compound JSH-VII-20 and its sodium salt form showed their inhibitory activity against IL-5 at uM level on cell based assay and no genotoxic potential. Compound JSH VII-20 is considered as a possible preclinical candidate. Therefore, we suggests that JSH-VI-3, VII-3, VII-20 and VII-20 (sodium salt), as the optimal candidates with both no genotoxic potential and IL-5 inhibitory effects must be chosen.

Methods

Materials

Sophoricoside derivatives were synthesized and donated by Dr. Jung from Chungnam University. Stock solution of derivatives was prepared freshly in dimethylsulfoxide (DMSO) before use. Eagles minimum essential medium (EMEM), RPMI-1640, 0.25% trypsin-EDTA, trypan blue, colcemid, fetal bovine serum (FBS) and horse serum were the products of GIBCO® (California, USA). Low melting agarose was a product of Amresco (Solon, OH, USA). All other chemicals used were of analytical grade or the highest grade available. The preparation of rat liver S-9 fraction for metabolic activation system was previously reported[19,21]. The S-9 fraction prepared was stored immediately at −80°C before use.

Cell lines and Culture

A clonal sub-line of a Chinese hamster lung (CHL) fibroblast cells was obtained from the National Institute of Health Sciences, Tokyo, Japan. The karyotype of CHL cells consisted of 25 chromosomes. The cells had been maintained by 3-4 day passages and grown in a monolayer with EMEM supplemented with 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin. The mouse lymphoma L5178Y cell line ($tk^{+/-}$ 3.7.2c) was cultivated in 90% RPMI-1640 with 1 mM sodium pyruvate, 0.1% pluronic supplemented with 10% heat-inactivated horse serum and antibiotics. These cells were maintained at 37°C in humidified 5% CO_2 atmosphere.

Cytotoxicity (Cell Growth Inhibition)

Cytotoxicity of cells was checked by the trypan blue exclusion assay. For the determination of cell cytotoxicity, 1×10^5 CHL cells or 1×10^6 L5178Y cells were treated to various concentrations of sophoricoside derivatives in 12-well plate in the absence and presence S-9 metabolic activation system for 6 h or 2 h, respectively. After the staining of 0.4% trypan blue (Life Technologies, MD, U.S.A), the total number of cells and the number of unstained cells were counted in five of the major sections of a hemocytometer, and then average number of cells per section was calculated. Cell viability of treated chemical was related to controls that were treated with the solvent. All experiments were repeated twice in an independent experiment.

Single Cell Gel Electrophoresis (Comet) Assay

DNA damages were detected by the alkaline version of standard comet assay described by Singh *et al.*[16,17] with minor modifications[18,23]. For the comet assay, 8 $\times 10^5$ cells were seeded into 12 wells plate (Falcon 3043) and then treated with sophoricoside derivatives. At all doses of these derivatives used in the experiment, the cell viability exceeded 80%. In the experiments, parallel cultures were performed and benzo[a]pyrene (BaP) and methyl methanesulfonate (MMS) were used as a positive control in the presence or absence of S-9 mixture, respectively. After treatment with derivatives for 2 h, cells were centrifuged for 3 min at × 100 g (about 1,200 rpm), and gently resuspended with PBS and 100 μL of the cell suspension was immediately used for the test. Cells were mixed with 0.1 mL of 1% low melting point agarose (LMPA, Gibco BRL, Life Technologies, Inc., MD, USA) and added to fully frosted slide (Fisher Scientific, PA, USA), had been covered with a bottom layer of 100 μL of 1% normal melting agarose (Amresco, OH, USA). The cell suspension was immediately covered with cover glass and the slides were then kept at 4°C for 5 min to allow solidification. After removing the cover glass gently, the slides were covered with a third layer of 100 μL of 0.5% LMPA by using a cover glass and then the slide were kept again at 4°C for 5 min. The cells embedded in the agarose on slides were lysed for 1.5 h in reaction mixture of 2.5 M NaCl, 0.1 M Na_2-EDTA, 10 mM Tris-HCl (pH 10), 1% Triton X-100 at 4°C. Slides were then placed in 0.3 M NaOH containing 1 mM Na_2-EDTA (approximately pH 13) for 20 min to unwind

DNA before electrophoresis. Electrophoresis was conducted at 25 V (about 1 V/cm across the gels) and approximately 300 mA for 20 min at 4°C. All of the steps described above were conducted under yellow light or in the dark to prevent additional DNA damage. After the electrophoresis, the slides were washed gently to remove alkali and detergents that would interfere with ethidium bromide staining, by placing the slides vertically in glass jar containing 0.4 M Tris (pH 7.5) three times for 10 min. The slides were stained by 50 μL of ethidium bromide in distilled water solution on each slide, and then covering the slide with a cover glass. Image of 100 randomly selected cells (50 cells from each of two replicate slides) was analysed each sample. All experiments were repeated in an independent test. Measurement was made by image analysis with Komet 3.1 (Kinetic Imaging Limited, Liverpool, UK) system, determining the mean tail moment (percentage of DNA in the tail times tail length) of the 50 cells. Differences between the control and the other values were tested for significance using one way of analysis of variance (ANOVA).

L5178Y *Tk*$^{+/-}$-3.7.2C Mouse Lymphoma Assay (MOLY)

To prepare working stocks for gene mutation experiments, cultures were purged of $tk^{-/-}$ mutants by exposure for 1 day to THMG medium (culture medium containing 3 μg/mL thymidine, 5 μg/mL hypoxanthine, 0.1 μg/mL methotrexate and 7.5 μg/mL glycine) and then the cells were transferred to THG medium (THMG but without methotrexate) for 2 days. The purged cultures were checked for low background $tk^{-/-}$ mutants and stored in liquid nitrogen. Each experiment started with working stock. The cells were usually used on day 3 or 4 after thawing and during logarithmic growth. A single lot of post-mitochondrial supernatant fractions of rat liver homogenates (S-9) for exogenous metabolic activation had been made from the liver of phenobarbital- and 5,6-benzoflavone-pretreated Sprague Dawley rats. S-9 mixture was prepared just prior to use by combining 4 mL S-9 with 2 mL each 180 mg/mL glucose-6-phosphate, 25 mg/mL NADP and 150 mM KCl. The concentration of S-9 mixture was 5% during treatment and the final concentration of S-9 was 2%. For treatment, cells were centrifuged and suspended at a concentration of 0.5×10^6 cells in 10 mL of medium in 15 mL polystyrene tubes. All chemicals were tested with and without S-9 mixture. Sophoricoside derivatives at each concentration were added and these tubes were gassed with 5% CO_2 in air and sealed. The cell culture tubes were placed on a roller drum and incubated at 37°C for 3 h. At the end of the treatment period, the cell cultures were centrifuged and washed twice with fresh medium and resuspended in fresh medium. We conducted preliminary experiments to determine the solubility and cytotoxicity of the test chemicals. Cytotoxicity was determined by relative survival (RS) and relative total growth (RTG) following 3 h treatments at concentrations up to 5 mg/mL, usually regardless of solubility. The recommended highest concentration was one with a 10-20% RS and/or RTG. Mutant selection was performed using the modified microwell version of the assay as described by Clements *et al.*[26]. Simply, the treated cells in medium containing 3 μg TFT/mL for selection or without TFT for cloning efficiency were distributed at 200 μL/well into 96-well flat-bottom microtiter plates. For mutant selection, two plates were seeded with ~2000 cells/well. For cloning efficiency, two plates were seeded with ~1 cell/well. All plates were incubated in 5% CO_2 in air in a humidified incubator at 37°C. After 11-13 days incubation, clones were counted and the colony size distribution was determined. Mutant frequencies were calculated using a statistical package (MutantTM; UKEMS, York, UK) in accordance with the UKEMS guidelines[27].

Ames *Salmonella* Bacterial Mutagenicity Assay

This test performed essentially as described by Ames *et al.*[19,20]. Media and positive control chemicals were obtained from commercial sources and were the purest grades available. The dose range for test chemical was determined by performing a toxicity assay using strain *Salmonella typhimurium* TA 100 and half-log dose intervals of the test substance up to 5 mg/plate. Strain TA 100 was chosen as the representative tester strain because of its high spontaneous reversion rate. Spontaneous revertant numbers were counted and plotted against the dose of the test chemical to produce a survival curve for the his^+ genotype. The mutagenicity assay was performed by mixing one of the tester strains which was cultured overnight, with the test substance in the presence and in the absence of S-9 mixture condition, sodium phosphate buffer added instead of S-9 mixture both in negative and positive control in test tube. Then, incubating the mixture in water bath for 30 min at 37°C and after incubation, the mixture mixed with top agar containing a minimal amount of histidine and then poured onto the surface of a *r*-ray sterile Petri dish (Falcon, USA) containing 25 mL of solidified bottom agar. The finished plates were incubated for 48 h at 37°C, and revertant colonies were counted later. Negative control plates containing no added test

chemical but positive control plates containing appropriate amounts of chemicals known to be active were included with each tester strain (Table 1). All platings were done in triplicate, and the results were tabulated as the mean ± standard deviation for each condition. A response was considered to be positive in our criteria if there was a dose-dependent increase in revertants per plate resulting in at least a doubling of the background reversion rate for strains TA 98 or TA 100.

In Vitro Chromosomal Aberrations Assay in CHL Cells

The clastogenicity of sophoricoside derivatives were evaluated for their ability to induce chromosomal aberrations in CHL cells. The experiment was performed as described by OECD[28] and Ishidate and Odashima[22] with some minor modifications[29-35], which are briefly summarized as follows. Concentration selection for this assay was based on solubility (testing was performed up to precipitating concentrations, 5 mg/mL, whichever was lower), and determination of cytotoxicity. Three different doses, including the IC_{50} value as a maximum dose, were prepared and separately added to 3-day-old cultures (approximately 10^5 cells/60 mm dish). In the absence and in the presence of S-9 mixture, cultures were treated for 6 h with derivatives and then maintained for 18 h in the fresh medium to adjust a time equivalent to about 1.5 normal cell cycle lengths. Cyclophosphamide (CP) and mitomycin C (MMC) were used as a positive control in combination with or without S-9 mixture, respectively. After 22 h incubation, the treatment was followed by addition of medium containing colcemid at a concentration of 0.2 μg/mL. Then, 2 h further incubated in the presence of colcemid, metaphase cells were harvested by trypsinization and centrifugation. The cells were swollen by adding with hypotonic (0.075 M) KCl solution for 20 min at 37°C, and washed three times in ice-cold fixative (methanol : glacial acetic acid = 3 : 1). After centrifugation, the fixative was removed, and cell pellet suspensions were prepared by pipetting gently. A few drop of cell pellet suspension were dropped onto pre-cleaned glass microscope slides, and-dried in the air. Slides were stained with 5% Giemsa buffered solution at pH 6.8 for scoring of chromosome aberrations. The number of cells with chromosomal aberrations was recorded on 200 well-spread metaphase cells at the magnification of 1,000 with Axioscope microscope (Karl Zeiss, FRG). The classification of aberration types referred to JEMS-MMS[36]. Breaks less than the width of a chromatid were designated as gaps in our criteria, and it was not included as chromosomal aberration. The incidence of polyploid and endoreduplicated cells was also recorded when these events were observed. Solvent-treated cells served as controls in this experiment. CHL cells usually have less than 3.0% cells with spontaneous chromosome aberrations. Aberration frequencies, defined as aberrations observed divided by number of cells counted, were analyzed using Fishers exact test[37] with Dunnetts adjustment and compared with results from the solvent controls. Therefore, data from count up well-spread 200 metaphase cells were expressed as percentages, and then dose-dependent responses and the statistical significance in p-value will be considered as positive results.

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