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Effects of Nine Active Ingredients in Chinese Herbal Medicine Sho-saiko-to on 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide Mutagenicity

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The antimutagenic effects of nine active compounds in the Chinese herbal medicine "sho-saiko-to" on mutagenesis induced by a direct-acting mutagen, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) were investigated in *Salmonella typhimurium*, strain TA100. The active compounds examined were classified into two major groups, saponins and flavonoids, the former comprising glycyrrhizin, saikosaponins a, c and d, and ginsenosides Rb1 and Rg1, and the latter, baicalin, baicalein and wogonin. Saikosaponin a and ginsenoside Rb1 were found to reduce the mutagenicity of AF-2 significantly when applied post-AF-2-treatment in the *Salmonella* mutagenicity assay. Ginsenoside Rb1 also decreased the mutagenic activity of AF-2 in a simultaneous treatment protocol. The results indicate that saikosaponin a and ginsenoside Rb1 may enhance DNA repair, and ginsenoside Rb1 may also have the ability to inactivate the mutagenic activity of AF-2 directly. On the other hand, saikosaponin d and baicalin showed a slight enhancing effect. None of the compounds, except baicalein, showed any toxic effect on the test strain. These findings may be useful for the development of chemopreventive agents.

Key words: Medicinal herb — Active ingredient in sho-saiko-to — Antimutagenicity — Ames test

Sho-saiko-to, a traditional Chinese herbal medicine, is used to treat chronic hepatitis in Japan, because of its pharmacological activities, including antiallergy and antiinflammation. Chemopreventive effects of sho-saiko-to have been reported, e.g., curative effects on D-galactosamine-induced hepatic injury in rats,[1] inhibition of hepatocarcinogenesis induced by the chemical carcinogen N-nitrosomorpholine[2] or 2-acetylaminofluorene (AAF)[3] in rats, or suppression of tumor cell growth through activation of macrophages.[4,5] Yano *et al.* demonstrated the growth-inhibitory effects of sho-saiko-to in human hepatocellular carcinoma (HCC) cells by inducing apoptosis and arrest at the G_0/G_1 phase in *in vitro* studies.[6]

Sho-saiko-to is a mixture of crude extracts of several herbs, including *Bupleuri radix, Pinelliae tuber, Scutellariae radix, Zizyphi fructus, Ginseng radix, Glycyrrhizae radix,* and *Zingiberis rhizoma,* from which nine active compounds, saikosaponins a, c and d, ginsenosides Rb1 and Rg1, glycyrrhizin, baicalin, baicalein, and wogonin have been isolated and identified. Antitumor activities of these compounds have been reported. Glycyrrhizin has been shown to suppress tumor-promoting activity[7] or to inhibit proliferation of human hepatoma cells by interfering with the cell cycle at the G_0/G_1 phase.[8] Qain *et al.* revealed that baicalein inhibited DNA synthesis and thus suppressed proliferation of a human HCC cell line.[9] However, the antimutagenic activities of the nine active compounds of sho-saiko-to have not been reported. Therefore, in this work, we investigated whether these compounds have the potency to suppress mutagenicity induced by 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2), a potent *in vitro* mutagen and clastogen,[10,11] a weak *in vivo* clastogen[12] and a forestomach carcinogen,[13] in the *Salmonella* mutagenicity assay.[14,15]

MATERIALS AND METHODS

Chemicals Baicalin, baicalein, wogonin, glycyrrhizin, saikosaponins a, c and d, and ginsenosides Rb1 and Rg1 were purchased from Wako Pure Chemical Industries Ltd. (Osaka). Their structural formulas are shown in Fig. 1. AF-2 and 2-aminoanthracene (2AA) were obtained from Wako Pure Chemical Industries Ltd. Sho-saiko-to powder (TJ-9) was kindly provided by Tsumura & Co. (Tokyo).

Bacterial strains *Salmonella typhimurium* TA98 and TA100 were kindly provided by Prof. B. N. Ames (University of California, Berkeley, CA, USA).

Drug-metabolizing enzyme system S9 fraction was taken from the supernatant of liver homogenates of male Sprague-Dawley rats pretreated with phenobarbital sodium and 5,6-benzoflavone, as previously described.[15] The S9 mix was a mixture of 10% S9 and a solution of co-factors.[15]

Mutagenicity assay Sho-saiko-to mutagenicity was assessed in *S. typhimurium* TA98 and TA100 with and

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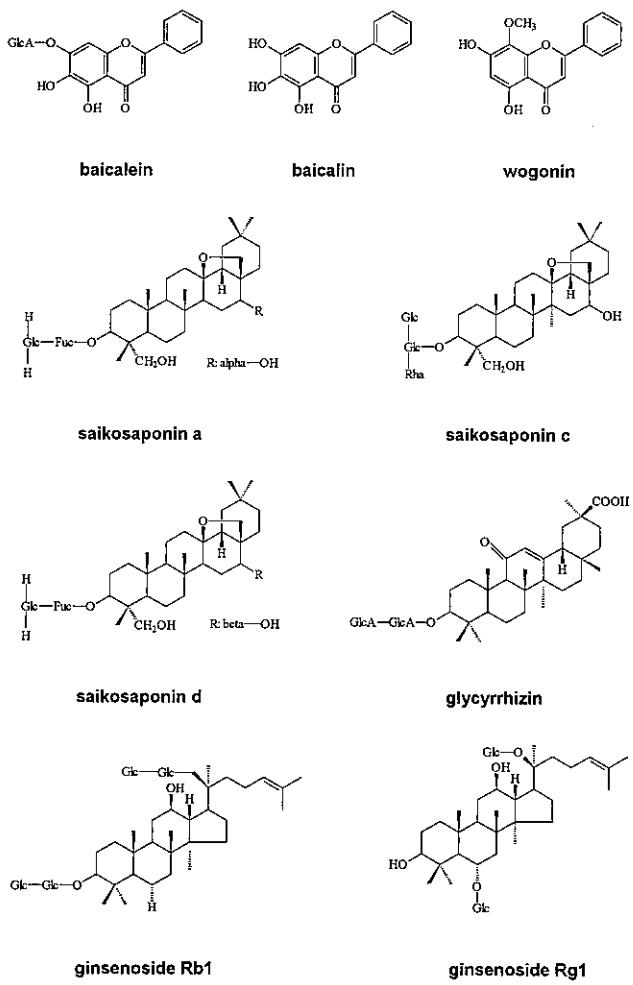

Fig. 1. Chemical structures of nine active ingredients in sho-saiko-to.

without S9 mix using the procedure of Maron and Ames[15] with the modification of preincubation.[16] S9 mix (or phosphate buffer, pH 7.4) (0.5 ml), 0.1 ml of bacterial culture and 0.1 ml of a sho-saiko-to suspension in dimethyl sulfoxide (DMSO) at graded dose levels were mixed and preincubated at 37°C for 20 min in a water bath. Molten soft agar, 2 ml, was added and the mixture was poured onto minimum glucose agar plates. The plates were incubated at 37°C for 48 h, and the number of His^+ revertant colonies was counted using a CA-9 colony analyzer (Toyo Sokki, Tokyo). The positive controls were AF-2 at concentrations of 0.1 and 0.01 μg/plate for TA98 and TA100, respectively, in the absence of S9 mix, and 2AA at concentrations of 0.5 and 1 μg/plate for TA98 and TA100, respectively, in the presence of S9 mix. DMSO was used as the solvent control. Triplicate plates were run and the mean value was calculated. If the number of revertant colonies was more than twice that of the solvent control and showed dose-dependency, the test results were judged positive.

Antimutagenic activity screening The antimutagenicity towards AF-2 was assessed in strain TA100. Each compound was applied at a concentration of 20 mg/ml, near the solubility limit in DMSO. Bacterial cells were subjected to two protocols. For simultaneous treatments with AF-2 and a test compound, a mixture containing 0.1 ml of AF-2 and 0.1 ml of the compound was first incubated at 37°C for 20 min. Then, 0.5 ml of phosphate buffer and 0.1 ml of bacterial culture were added and the mixture was incubated at 37°C for 20 min for the mutagenicity assay. For post-treatment assay, 0.5 ml of phosphate buffer, 0.1 ml of AF-2 and 0.1 ml of bacterial culture were mixed together and incubated at 37°C for 20 min. The mutagenized bacterial cells were washed once by centrifugation with phosphate buffer and incubated with 0.1 ml of a test compound solution at 37°C for 20 min. Each dose was tested in duplicate. Growth inhibition of the bacterial lawn was observed under a stereomicroscope, to assess toxicity. The mean number of spontaneous revertants was subtracted from the number of revertants induced by AF-2 in the presence, or in the absence of, test compounds. The mutagenicity inhibition rate (%) caused by a test compound was calculated according to the following formula: % inhibition = 100 − (number of induced revertants/plate of AF-2 + test compound)/(number of induced revertants/plate of AF-2 alone) × 100. The antimutagenic effect was qualitatively evaluated according to the following criteria proposed by Wall *et al.*[17]: inhibition values of 0–20% were classified as negative, 20–40% as weak, 40–60% as positive, 60–90% as strong, and >90% as toxicity suspected.

RESULTS

Mutagenicity of sho-saiko-to First, the mutagenicity of sho-saiko-to was examined (Table I). The number of revertants slightly increased as higher doses, such as 5 and 10 mg/plate, in a dose-dependent manner, but sho-saiko-to was judged to be non-mutagenic for both TA98 and TA100 with and without S9 mix, up to 10 mg/plate, because it did not induce dose-dependent increases in revertant colonies exceeding twice that of the solvent control.

Antimutagenic effect of nine active compounds against AF-2 mutagenesis The nine active compounds were screened in order to examine their effects on the mutagenicity of a direct-acting mutagen, AF-2, in a *S. typhimurium*, strain TA100. In the simultaneous treatment assay, as shown in Table II, ginsenoside Rb1 decreased

Table I. Mutagenicity of Sho-saiko-to

Dose (μg/plate)	TA100		TA98	
	−S9 mix	+S9 mix	−S9 mix	+S9 mix
0	111±9	114±9	32±16	43±11
313	114±19	132±16	28±3	34±4
625	114±15	153±7	24±8	35±8
1250	104±25	172±11	31±1	44±3
2500	108±16	157±9	36±7	45±8
5000	155±23	155±11	40±6	51±4
10000	213±26	188±17	51±8	56±7
AF-2	691±17	686±23	—	—
2AA	—	—	850±62	207±28

Results are the mean±SD of counts from triplicate plates. The zero dose indicates DMSO, used as a control. Positive controls consist of AF-2 at concentrations of 0.01 and 0.1 μg/plate for TA100 and TA98, respectively, in the absence of S9 mix and 2AA at concentrations of 1.0 and 0.5 μg/plate for TA100 and TA98, respectively, in the presence of S9 mix.

Table II. Inhibition of AF-2-induced Mutagenicity in *Salmonella typhimurium* TA100 by Simultaneous Treatment

Compound[a)]	Revertants/plate[b)]	% Inhibition[c)]
Baicalin	362, 414 (388)	−3.7
Baicalein	toxic	—
Wogonin	351, 406 (379)	−1.3
Glycyrrhizin	309, 371 (340)	0.19
Saikosaponin a	355, 393 (374)	0
Saikosaponin c	309, 355 (332)	11.2
Saikosaponin d	411, 493 (452)	−20.9
Ginsenoside Rb1	244, 293 (269)	28.1
Ginsenoside Rg1	307, 324 (316)	15.5

The number of spontaneous *his*$^+$ revertants of *S. typhimurium* TA100 has been subtracted.
a) Compounds were applied at a concentration of 2 mg/plate. None of the compounds, except baicalein, was toxic to the tester strain.
b) AF-2 was dissolved in DMSO at a concentration of 0.1 μg/ml. The number of *his*$^+$ revertants of TA100 induced by AF-2 was 374. Values in parenthesis are the mean of counts from duplicate plates.
c) % inhibition = 100 − (revertants/plate of AF-2 + test compound)/(revertants/plate of AF-2 alone) × 100.

AF-2 mutagenesis by approximately 30%, but this inhibitory effect was evaluated as weak according to the criteria proposed by Wall *et al.*[17] On the other hand, four compounds were antimutagenic in the post-treatment assay (Table III). Saikosaponin c and wogonin suppressed the mutagenicity of AF-2 by approximately 30% and were evaluated as weak inhibitors. Saikosaponin a and ginsenoside Rb1 both suppressed AF-2-induced mutagenesis by more than 50%. They were confirmed to

Table III. Inhibition of AF-2-induced Mutagenicity in *Salmonella typhimurium* TA100 by Post-treatment

Compound[a)]	Revertants/plate[b)]	% Inhibition[c)]
Baicalin	327, 399 (363)	−28.7
Baicalein	toxic	—
Wogonin	189, 213 (201)	28.7
Glycyrrhizin	234, 309 (272)	3.5
Saikosaponin a	122, 150 (136)	51.8
Saikosaponin c	188, 216 (202)	28.4
Saikosaponin d	214, 283 (249)	11.7
Ginsenoside Rb1	123, 152 (138)	51.1
Ginsenoside Rg1	247, 287 (267)	5.3

The number of spontaneous *his*$^+$ revertants of *S. typhimurium* TA100 has been subtracted.
a) Compounds were applied at a concentration of 2 mg/plate. None of the compounds, except baicalein, was toxic to the tester strain.
b) AF-2 was dissolved in DMSO at a concentration of 0.1 μg/ml. The number of *his*$^+$ revertants of TA100 induced by AF-2 was 282. Values in parenthesis are the mean of counts from duplicate plates.
c) % inhibition = 100 − (revertants/plate of AF-2 + test compound)/(revertants/plate of AF-2 alone) × 100.

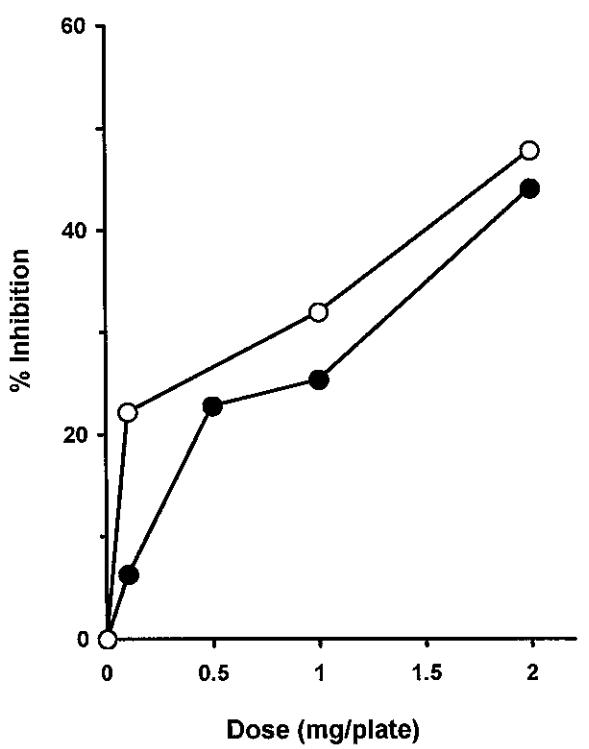

Fig. 2. Antimutagenicity of saikosaponin a (○) and ginsenoside Rb1 (●) against AF-2 (0.1 μg/ml)-induced mutation in *S. typhimurium* TA100. The % inhibition is calculated based on mutagenicity induced by AF-2 alone as 100%.

suppress AF-2-induced mutagenesis in a dose-dependent manner (Fig. 2). The inhibition % values at 2 mg/plate, however, were lower than those in Table III, probably because of the difference in the number of revertants in the vehicle control. No toxic effects on the bacterial tester strain were observed for any of the compounds, except baicalein, which showed strong cytotoxicity. Therefore, the observed decreases in the number of revertant colonies cannot be attributed to a decrease of cell viability.

DISCUSSION

In the present study, we examined the antimutagenic activities of nine active compounds in the traditional Chinese medicine sho-saiko-to against AF-2-induced mutagenesis in *S. typhimurium* TA100.

Kada *et al.*[18] classified antimutagens into 2 categories: desmutagens are defined as agents causing chemical or biochemical modifications before DNA damage, while bioantimutagens are agents interfering with cellular mutation fixation processes.

The data in Table II demonstrate that ginsenoside Rb1 reduced AF-2 mutagenesis in simultaneous treatment, suggesting that ginsenoside Rb1 probably has desmutagenic ability, inactivating AF-2 mutagenic activity by direct interaction. When incubated with AF-2-mutagenized bacterial cells (Table III), ginsenoside Rb1 and saikosaponin a were found to cause significant suppression of AF-2-induced mutation. This post-treatment procedure can identify antimutagenic activity that involves effects on the repair processes and replication of DNA in bacteria.[19, 20] Therefore, ginsenoside Rb1 and saikosaponin a could be bioantimutagens according to the definition of Kada *et al.*[18] Ohta *et al.*[21, 22] examined the antimutagenic mechanisms of vanillin and reported that vanillin inhibited mutagenesis induced by AF-2, 4-nitroquinoline 1-oxide or UV, the so-called SOS repair-dependent mutagenesis,[23] by enhancement of *recA*-dependent error-free recombination repair, but not by inhibition of error-prone SOS repair, using *Escherichia coli* mutant strains. The inhibitory profiles of ginsenoside Rb1 and saikosaponin a against AF-2 obtained in the present study were quite similar to that of vanillin, suggesting that ginsenoside Rb1 and saikosaponin a might also suppress AF-2-induced mutagenesis through promotion of post-replication repair.

Conversely, saikosaponin d and baicalin enhanced, rather than inhibited, AF-2-induced mutagenesis, producing a slight increase of revertant colonies in the sho-saiko-to mutagenicity test (Table I).

Okita *et al.* suggested that sho-saiko-to might contain chemopreventive agents, based on their finding that sho-saiko-to clearly inhibited hyperplastic nodule development induced by the mutagenic hepatocarcinogen AAF in rat liver.[3] The present results indicate possible protective actions of ginsenoside Rb1 and saikosaponin a against mutagenesis. It is generally accepted that multiple genetic alterations induced by mutations are important in carcinogenesis. Therefore, ginsenoside Rb1 and saikosaponin a in sho-saiko-to might participate in the chemopreventive effects of sho-saiko-to against carcinogenesis via prevention of mutation.

These findings may be useful for the development of new chemopreventive agents.

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