Tumor-promoting Activity of Staurosporine, a Protein Kinase Inhibitor on Mouse Skin

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Tumor-promoting Activity of Staurosporine, a Protein Kinase Inhibitor on Mouse Skin[1]

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ABSTRACT

Staurosporine, which is a potent inhibitor of protein kinases, such as protein kinase C, inhibited both inductions of adhesion of human promyelocytic leukemia cells (50% effective dose = 9.0 nM) and Epstein-Barr virus early antigen in Raji cells (50% effective dose = 3.4 nM) by teleocidin. However, staurosporine induced irritation on mouse ear and histidine decarboxylase activity in mouse skin. It did not induce ornithine decarboxylase activity in mouse epidermis. The two-stage carcinogenesis experiments of staurosporine were carried out at two different doses. Experiment 1 revealed that the group treatment with a single application of 100 μg of 7,12-dimethylbenz(*a*)anthracene, followed by repeated applications of 50 μg of staurosporine, resulted in 85.7% of tumor-bearing mice at Wk 30, whereas group treatment with staurosporine alone or 7,12-dimethylbenz(*a*)anthracene alone gave 6.7% and 0%, respectively. Experiment 2 showed that group treatment with 7,12-dimethylbenz(*a*)anthracene followed by applications of 10 μg of staurosporine resulted in 33% of tumor-bearing mice at Wk 30. In addition, staurosporine treatment reduced the percentages of tumor-bearing mice treated with teleocidin from 100% to 67% in Wk 15. These results demonstrated that staurosporine is a weak tumor promoter of mouse skin compared with teleocidin, but staurosporine has some potency to inhibit tumor promotion by teleocidin.

INTRODUCTION

TPA[3]-type tumor promoters, such as TPA, teleocidin, and aplysiatoxin, activate calcium-activated, phospholipid-dependent protein kinase (protein kinase C) and, thus, induce many biological activities and tumor promotion (1, 2). If inhibitors of protein kinase C block this phosphorylation, they should also inhibit the tumor promotion induced by TPA-type tumor promoters. In fact, palmitoylcarnithine, a protein kinase C inhibitor, has this effect (3, 4).

A potent protein kinase inhibitor, staurosporine (Fig. 1), was isolated from *Streptomyces* spp. by Tamaoki *et al.* (5). Staurosporine inhibited protein kinase C activity within a nM range of concentration, as well as the activity of cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase, and the $p60^{v\text{-}src}$ tyrosine kinase (5, 6). Although staurosporine did not selectively inhibit protein kinase C, the effects of staurosporine on various biological activities induced by phorbol esters had been reported. Staurosporine enhanced differentiation of HL-60 cells (7) and inhibited induction of EB virus EA in Raji cells (8) and NADPH-oxidase activation in human neutrophils (9). Two research groups independently found that staurosporine inhibited tumor promotion of TPA in two-stage carcinogenesis experiments on mouse skin (10, 11). Staurosporine induced morphological changes of primary mouse epidermal cells similar to phorbol 12,13-dibutyrate (9). Thus, the study with staurosporine suggested the presence of dual effects on tumor promotion.

Here, we report that staurosporine induced irritation on mouse skin and HDC activity in mouse skin and had a weak tumor-promoting activity on mouse skin initiated with DMBA, although staurosporine showed inhibition of biological activity of teleocidin in HL-60 cells and Raji cells. Staurosporine is a unique tumor promoter possessing dual effects: tumor-promoting activity and antipromoting activity on mouse skin.

MATERIALS AND METHODS

Materials

Staurosporine was kindly provided by Dr. H. Nakano, Tokyo Research Laboratories, Kyowa Hakko Kogyo, Tokyo, Japan. Teleocidin was isolated from *Streptomyces mediocidicus* (12). DMBA was purchased from Sigma Chemical Co., St. Louis, MO, and TPA was from LC Services Corporation, Woburn, MA. Sodium *n*-butyrate was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. [γ-^{32}P]ATP was from Amersham, United Kingdom, and [20-^{3}H]TPA, DL-[1-^{14}C]ornithine monohydrochloride, and L-[*carboxyl*-^{14}C]histidine were from New England Nuclear, Boston, MA. Antiserum against EB virus EA (1:160) was a generous gift from Dr. N. Yamamoto of Yamaguchi University, Japan. Anti-human IgG conjugated with fluorescein isothiocyanate was obtained from Dakopatts a/s, Glostrup, Denmark.

Animals

Female CD-1 mice were purchased from the Japanese Charles River Co., Ltd., Kanagawa, Japan, and kept as reported previously (13).

Assay of Inhibition of Activation of Protein Kinase C *in Vitro*

The effect of staurosporine on protein kinase C activated by 2.3 μM teleocidin was examined as described previously (14). Protein kinase C was purified from mouse brain by DEAE-cellulose column chromatography (15).

Assay of Inhibition of Induction of HL-60 Cell Adhesion

Adhesion of HL-60 cells cultured in RPMI-1640 medium with 10% fetal calf serum was induced by incubation with 4.5 nM teleocidin for 48 h (16). The inhibitory effect of various concentrations of staurosporine on cell adhesion was examined in the presence of teleocidin.

Assay of Inhibition of Induction of EB Virus EA

Raji cells (3×10^5 per ml) were incubated in 1 ml of RPMI-1640 medium containing 10% fetal calf serum with 2.3 nM teleocidin, 4 mM sodium *n*-butyrate, and 0.21 nM to 2.1 μM staurosporine. After 48-h incubation, EA-positive cells stained by the indirect immunofluorescence method were counted (17). Cell numbers were counted after staining with trypan blue.

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[3] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMBA, 7,12-dimethylbenz(*a*)anthracene; HL-60 cells, human promyelocytic leukemia cells; EB virus, Epstein-Barr virus; EA, early antigen; HDC, histidine decarboxylase; ODC, ornithine decarboxylase; ED_{50}, 50% effective dose.

Irritant Test on Mouse Ear

Various amounts of staurosporine in 10 μl of acetone were applied to the ears of 8-wk-old CD-1 mice. The extent of irritation was expressed as the minimum dose of the compound causing irritation, as described previously (18).

Induction of HDC in Mouse Skin

Staurosporine in 0.1 ml of acetone was applied to the skin of the backs of CD-1 mice, and a crude enzyme extract was obtained from the skin 18 h later as described previously (19). HDC activity was expressed as pmol of CO_2 released per mg of protein per 1 h of incubation.

Induction of ODC in Mouse Skin

Staurosporine in 0.2 ml of acetone was applied to the skin of the backs of CD-1 mice. After 4 h, a crude enzyme extract was prepared from the epidermis, and its ODC activity was measured as described previously (16). Enzyme activity was expressed as nmol of CO_2 per mg of protein per 30 min of incubation.

Two-Stage Carcinogenesis Experiments

Experiment 1. Initiation was achieved by a single application of 100 μg of DMBA dissolved in 100 μl of acetone to the skin of the backs of 8-wk-old female CD-1 mice (13). From 1 wk after initiation, 50 μg (107 nmol) of staurosporine dissolved in 100 μl of acetone were applied to the initiated skin parts of the mice, twice a wk, until Wk 30. Control groups were treated with DMBA alone or staurosporine alone. The percentages of tumor-bearing mice and the average numbers of tumors per mouse were determined weekly as described previously (20).

Experiment 2. The experiment was carried out by the same procedure. From 1 wk after initiation with 100 μg of DMBA, 10 μg (21 nmol) of staurosporine were applied, twice a wk. In addition, this experiment included a group treated with DMBA and staurosporine plus 2.5 μg (5.7 nmol) of teleocidin, which was applied 15 min after each application of staurosporine. Control groups treated with DMBA and teleocidin or DMBA alone were also observed. In both Experiments 1 and 2, each group consisted of 15 mice, because of the limited availability of staurosporine.

RESULTS

Inhibitory Effects of Staurosporine on Biological Activities Induced by Teleocidin. Based on our evidence that staurosporine inhibited the activation of protein kinase C by 2.3 μM teleocidin, and that the effective dose for 50% inhibition was 2.6 nM, comparable to the results of Tamaoki *et al.* (4), we then studied whether it inhibited the other biological activities induced by teleocidin. Treatment with 4.5 nM teleocidin for 48 h induced adhesion of HL-60 cells, and this induction was inhibited by staurosporine dose dependently at concentrations of 6 to 30 nM (Fig. 2). The ED_{50} value was 9.0 nM. In this experiment, the treatment with teleocidin and staurosporine did not show any toxicity to HL-60 cells at concentrations of staurosporine up to 30 nM, determined by trypan blue staining. Incubation of Raji cells with 2.3 nM teleocidin for 48 h induced expression of EB virus EA in 55% of the cells, whereas in the absence of teleocidin, less than 1% of the cells expressed EB virus EA. As Fig. 3 shows, incubation of the cells with staurosporine at concentrations of 0.21 nM to 2.1 μM together with 2.3 nM teleocidin and 4 mM sodium *n*-butyrate resulted in a dose-dependent decrease in the percentage of EA-positive cells, the ED_{50} value of staurosporine being 3.4 nM. The viability of Raji cells was more than 80% at concentrations of up to 1 μM staurosporine in the presence of 2.3 nM teleocidin and 4 mM sodium *n*-butyrate. These two experiments showed that staurosporine strongly inhibited two of the biological activities induced by teleocidin. However, a single application of 10 nmol of staurosporine clearly enhanced the effects of teleocidin on ODC activity (Table 1).

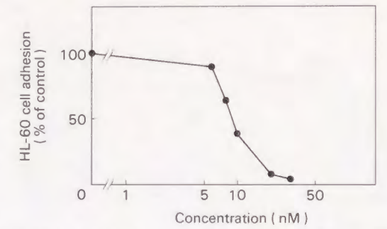

Fig. 2. Inhibitory effect of staurosporine on HL-60 cell adhesion of teleocidin. HL-60 cells were incubated with 4.5 nM teleocidin in the presence of various concentrations of staurosporine for 48 h. After incubation, adherent cells and unadherent cells were counted, and the percentage of adherent cells was calculated.

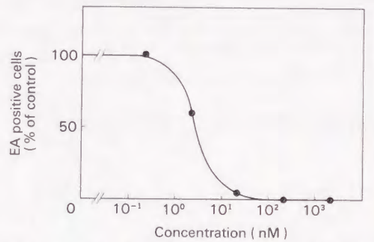

Fig. 3. Inhibitory effect of staurosporine on induction of EB virus EA by teleocidin. Raji cells were cultured with 2.3 nM teleocidin and 4 mM sodium *n*-butyrate in the presence of various concentrations of staurosporine. After 48-h incubation, cells were stained by an indirect immunofluorescence method, and the percentages of EA-positive cells were estimated after at least 500 cells were counted.

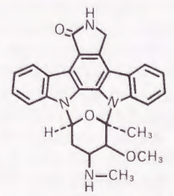

Fig. 1. Structure of staurosporine, isolated from *Streptomyces* spp. (4).

Biological Activities of Staurosporine. Staurosporine induced irritation of mouse ear, the dose causing redness of the ear 24 h after its application being 200 ng (0.43 nmol). Since that of teleocidin was 0.008 nmol (12), the potency of staurosporine for irritation on mouse ear was about 50 times weaker than that of teleocidin. In addition, applications of 170 nmol and 340 nmol of staurosporine induced HDC activity at levels of 196 and 173 pmol of CO_2/mg of protein per 60 min, respectively, which was about 10 times weaker than that of teleocidin (Table 2).

Staurosporine, at concentrations of up to 500 nmol, did not induce ODC activity in mouse epidermis 4 h after application, whereas teleocidin induced it as usual (Table 1). Furthermore, staurosporine did not induce HL-60 cell adhesion at a concentration of 50 nM. In Raji cells, staurosporine itself, at concentrations of up to 2.1 μM, did not induce EB virus EA in the presence of 4 mM sodium *n*-butyrate. Table 3 summarizes the effects of staurosporine.

Tumor-promoting Activity of Staurosporine. Since staurospor-

Table 1 *Effect of staurosporine on induction of ODC in mouse epidermis*

Compounds[a]	ODC activity[b] (nmol of CO_2/mg of protein/30 min)
Staurosporine	
1 nmol	0.12 ± 0.05[c]
10 nmol	0.18 ± 0.04
50 nmol	0.14 ± 0.05
100 nmol	0.18 ± 0.05
250 nmol	0.15 ± 0.02
500 nmol	0.12 ± 0.12
Teleocidin	
10 nmol	2.56 ± 0.50
10 nmol + staurosporine[d] 10 nmol	5.83 ± 1.16
10 nmol + staurosporine 100 nmol	3.74 ± 0.37
Acetone	0.19 ± 0.10

[a] Compounds were applied to the skins of backs of mice, and mouse epidermis was scraped 4 h after the application of compound.
[b] ODC activity was determined by the method described previously (16) and expressed as nmol of CO_2 per mg of protein per 30 min.
[c] Mean ± SD.
[d] Staurosporine was applied 15 min prior to teleocidin.

Table 2 *Induction of HDC activity in mouse skin*

Compounds[a]	HDC activity (pmol of CO_2/mg of protein/60 min)
Staurosporine	
1.7 nmol	0
17 nmol	0
170 nmol	196
340 nmol	173
Teleocidin	
17 nmol	211
Acetone (vehicle)	0

[a] Compounds dissolved in 0.1 ml of acetone were applied to the skins of backs of mice, and a crude enzyme fraction was obtained by the procedure described previously (19).

Table 3 *Biological activities of staurosporine*

Irritation[a]	HL-60 cell adhesion[b]	EB virus EA induction[c]	ODC induction[d]	HDC induction
+	−	−	−	+

[a] Staurosporine (0.43 nmol) induced irritation on mouse ear.
[b] Staurosporine at concentrations up to 50 nmol did not induce HL-60 cell adhesion.
[c] Staurosporine at concentrations up to 2.1 μmol in the presence of 4 mM sodium *n*-butyrate did not induce EB virus EA in Raji cells.
[d] ODC activity was not induced in mouse epidermis 4 h after application of compound.

ine showed positive responses in the irritation of mouse ear, induction of HDC on mouse skin, and stimulation of prostaglandin E_2 production in rat macrophages,[4,5] a tumor-promoting activity of staurosporine was examined in two-stage carcinogenesis experiments on mouse skin. In Experiment 1, the repeated applications of 50 μg of staurosporine to the skin of the backs of mice initiated with DMBA, twice a wk, produced the first tumor at Wk 11 and gradually increased the percentages of tumor-bearing mice (Fig. 4). At Wk 30, the percentages of tumor-bearing mice in the groups treated with DMBA and staurosporine, or with staurosporine alone, were 85.7% and 6.7%, respectively. The average numbers of tumors per mouse in these two groups were 1.9 and 0.1, respectively. The group treated with DMBA alone did not produce any tumors throughout this experiment. A weak tumor-promoting activity of staurosporine was statistically significant in both tumor-bearing mice (χ^2 test) and papillomas per mouse (F test).

Fig. 5 shows the results of Experiment 2. The repeated applications of 10 μg of staurosporine to the DMBA-initiated mouse skin induced tumors in 33% of the mice with an average of 0.5 papilloma per mouse in Wk 30. The amount of staurosporine (10 μg) was not sufficient to induce a significant tumor-promoting activity, but the results with 50 μg of staurosporine were statistically significant, as shown in Fig. 4.

Antipromoting Activity. Although staurosporine is a tumor promoter, it has inhibitory activities for activation of protein kinase C and induction of HL-60 cell adhesion and of EB virus EA. Therefore, we examined whether staurosporine inhibits the tumor promotion on mouse skin. As Fig. 5 shows, the group treated with DMBA and teleocidin plus staurosporine resulted in 67% tumor-bearing mice by Wk 15 and in 80% by Wk 30, whereas the group treated with DMBA and teleocidin induced tumors in 100% of the mice by Wk 15 and remained constant up to Wk 30. The staurosporine treatment reduced the average numbers of tumors per mouse from 6.1 to 3.9 in Wk 30. In the group treated with DMBA and teleocidin plus staurosporine, mice did not show any significant body weight loss throughout the experiment, and the skins of the backs of these mice were not irritated, compared with those in the group treated with DMBA and teleocidin. The inhibitory effect of staurosporine was statistically significant at around Wk 10 of tumor promotion. Although statistically significant inhibition was not obtained with 10 μg of staurosporine in later weeks of the experiment, a decreasing tendency in the percentages of tumor-bearing mice and in average numbers of tumors per mouse was apparent (Fig. 5). Thus, staurosporine slightly inhibited tumor promotion of teleocidin, even at the dose at which staurosporine itself induced tumors.

DISCUSSION

Staurosporine is a potent inhibitor of protein kinases, such as protein kinase C, cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase, and tyrosine kinase (6). Since staurosporine inhibited biological activities of teleocidin in cell lines such as HL-60 cells and Raji cells, it was expected to inhibit tumor promotion of the TPA-type tumor promoters. In fact, staurosporine slightly inhibited the tumor-promoting activity of teleocidin in a two-stage carcinogenesis experiment on mouse skin. During the preparation of this paper, two groups reported independently that staurosporine inhibited tumor promotion of TPA in two-stage carcinogenesis experiments on mouse skin. Although our results with staurosporine were not strongly inhibitory, due to limited amounts of the compound, our results well meet the results of these two reports (10, 11).

In addition to inhibition of tumor promotion, staurosporine itself had a tumor-promoting activity on mouse skin and was classified as an additional non-TPA-type tumor promoter, which does not bind to phorbol ester receptors (1, 2). Staurosporine induced irritation on mouse ear and HDC activity in mouse skin like the TPA-type tumor promoters. However, it did not significantly induce ODC activity in mouse skin. Recently, in collaboration with K. Ohuchi, we showed that staurosporine stimulated prostaglandin E_2 production in rat macrophages, and with L. Levine, we demonstrated the stimulation of 6-keto-prostaglandin $F_{1\alpha}$ production in rat liver cells. These results were well in agreement with our previous data that stimulation of arachidonic acid metabolism is the common

[4] K. Ohuchi *et al.*, manuscript in preparation.
[5] L. Levine *et al.*, manuscript in preparation.

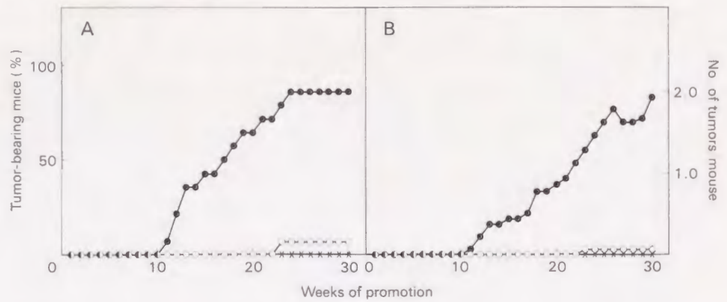

Fig. 4. Tumor-promoting activity of staurosporine in a two-stage carcinogenesis experiment. *A*, percentages of tumor-bearing mice; *B*, average numbers of tumors per mouse treated with DMBA and staurosporine (50 μg) (●), staurosporine alone (○), and DMBA alone (×).

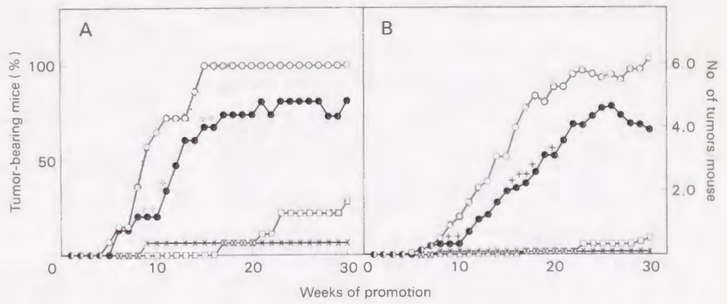

Fig. 5. Tumor-promoting activity and antipromoting activity of staurosporine. *A*, percentages of tumor-bearing mice; *B*, average numbers of tumors per mouse in the groups treated with DMBA and teleocidin (○), DMBA and teleocidin plus staurosporine (10 μg) (●), with DMBA and staurosporine (□), and DMBA alone (×). *, $P < 0.05$ by the χ^2 test; +, $P < 0.05$; ++, $P < 0.01$ by the F test.

effect induced by TPA-type and non-TPA-type tumor promoters (1, 2).

As to the ODC induction by staurosporine, it was reported that staurosporine did not induce ODC activity in primary mouse epidermal cells (9), and Verma *et al.* (21) also presented that staurosporine did not affect ODC activity in T24 cells. These results were in agreement with our data. In one exception, Yamamoto *et al.* (11) found that 100 nmol of staurosporine induced ODC activity in mouse skin 5 h after application.

Moreover, two research groups (10, 11) reported that staurosporine itself did not show any tumor-promoting activity in a two-stage carcinogenesis experiment. This discrepancy between our results might be simply due to the doses of DMBA and of staurosporine that were used. The results of our experiment indicated that the doses of staurosporine which other groups used were not sufficient to induce any tumor-promoting activity in two-stage carcinogenesis experiments.

Recently, evidence has been accumulating that inhibition of protein kinase C does not directly reflect inhibition of tumor promotion by the TPA-type tumor promoters. Sako *et al.* (9) reported that staurosporine did not effectively inhibit the effect of phorbol esters on epidermal growth factor binding or induction of ODC and of epidermal transglutaminase in mouse primary epidermal cells, and that staurosporine itself induced morphological changes in keratinocytes, resembling those induced by the phorbol esters.

Since staurosporine is a microbial alkaloid produced by *Streptomyces* spp. (5), staurosporine was, structurally, from a new family of tumor promoters than those already reported, such as phorbol esters, teleocidins, aplysiatoxins, palytoxin, thapsigargin, and okadaic acid. K-252a, which is a structurally related compound to staurosporine, is also an inhibitor of protein kinase C, cyclic AMP-dependent protein kinase, and cyclic GMP-dependent protein kinase (22). The irritant activity of K-252a on mouse ear was 40 times weaker than that of staurosporine; the dose causing redness of the ear was 8 μg (17 nmol). These results suggested that K-252a might be a weaker tumor promoter than staurosporine.

The antiinflammatory steroid, fluocinolone acetonide, is reported to be a very strong inhibitor of tumor promotion induced by TPA in mouse skin (23). However, Fukao *et al.* (24) recently reported that fluocinolone acetonide has a tumor-promoting activity in mouse skin initiated with 3-methylcholanthrene. Retinoic acid strongly inhibits skin tumor promotion by phorbol esters (25). However, retinoic acid itself induced papillomas in mouse skin initiated with DMBA (26). Further study revealed that retinoic acid has the ability to act as a first stage tumor promoter as well as a weak complete tumor promoter (27). Bryostatin 1 also shows both effects on tumor promotion in a multistage carcinogenesis experiment on mouse skin. Bryostatin 1 inhibits the tumor-promoting activity of TPA in SENCAR mice (28), and further study revealed that bryostatin 1 inhibits the first stage of tumor promotion (29, 30). On the other hand, bryostatin 1 itself is a second stage tumor promoter (30). Thus, like staurosporine, fluocinolone acetonide, retinoic acid, and bryostatin 1 have dual effects on tumor promotion and inhibition of tumor promotion. Staurosporine provides important new information for understanding the mechanisms of tumor promotion and inhibition of tumor promotion.

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