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Diarrhetic Shellfish Toxin, Dinophysistoxin-1, Is a Potent Tumor Promoter on Mouse Skin

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Dinophysistoxin-1, 35-methylokadaic acid, is a causative agent of diarrhetic shellfish poisoning. The biological activities and tumor-promoting activity of dinophysistoxin-1 were studied together with those of okadaic acid and 7-O-palmitoyl okadaic acid. Dinophysistoxin-1 is a skin irritant and induces ornithine decarboxylase in mouse skin with the same potency as okadaic acid. 7-O-Palmitoyl okadaic acid induced a lower activity than the other compounds. Dinophysistoxin-1 inhibited the specific [^{3}H]okadaic acid binding to a particulate fraction of mouse epidermis. The binding affinities of dinophysistoxin-1 and okadaic acid to a particulate fraction were almost the same. Dinophysistoxin-1 showed a tumor-promoting activity as strong as that of okadaic acid in a two-stage carcinogenesis experiment on mouse skin. The percentages of tumor-bearing mice in the groups treated with 100 μg of 7,12-dimethylbenz[*a*]anthracene (DMBA) followed by 5 μg of dinophysistoxin-1, twice a week, and with DMBA followed by 5 μg of okadaic acid twice a week were 86.7% and 80.0% in week 30, respectively. The average number of tumors per mouse was 4.6 in the former group and 3.9 in the latter. Dinophysistoxin-1 and okadaic acid act on cells through different pathways from the 12-O-tetradecanoylphorbol-13-acetate-type tumor promoters.

Key words: Dinophysistoxin-1 — Okadaic acid — Non-TPA type tumor promoter — Diarrhetic shellfish poisoning — Skin

Dinophysistoxin-1, which is 35-methylokadaic acid, was first isolated from the hepatopancreas of the mussel *Mytidus edulis,* as a causative agent of diarrhetic shellfish poisoning in Japan.[1] Like dinophysistoxin-1, okadaic acid was reported to be the toxic principle of a disease caused by ingesting European mussels.[2] Since dinophysistoxin-1 and okadaic acid are products of marine dinoflagellates, they accumulate in marine sponges and in mussels or scallops.[3] In addition to dinophysistoxin-1 and okadaic acid, acylated derivatives of okadaic acid have been associated with diarrhetic shellfish poisoning affecting people in several countries. Recently, we reported that okadaic acid induces ornithine decarboxylase (ODC) activity in mouse skin[4] and has a potent tumor-promoting activity in a two-stage carcinogenesis experiment on mouse skin, through different pathways from 12-O-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoters such as phorbol esters, teleocidin and aplysiatoxin.[5] Next, we thought it would be worthwhile to study the tumor-promoting activity of dinophysistoxin-1 on mouse skin. Dinophysistoxin-1 was isolated in large amounts from a black sponge, *Halichondria okadai,* to carry out a two-stage carcinogenesis experiment. This paper reports a potent tumor-promoting activity of dinophysistoxin-1 compared with that of okadaic acid. The relation between tumor-promoting activity and diarrhetic shellfish poisoning is discussed.

MATERIALS AND METHODS

Chemicals Dinophysistoxin-1 and okadaic acid (Fig. 1) were isolated from a black sponge, *Halichondria okadai,* collected off the coast of Mie Prefecture as reported previously.[4] 7-O-Palmitoyl okadaic acid (Fig. 1) is one of the acylated okadaic acids, which occur naturally in mussels and scallops. It was chemically synthesized from okadaic acid for the experiment. [27-^{3}H]Okadaic acid was synthesized as reported previously.[6] DMBA was

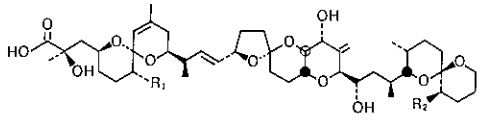
purchased from Sigma Chemical Co., St. Louis, Mo.

Irritant Test on Mouse Ear One μg of each of the test compounds in 10 μl of acetone was applied to the ears of 8-week-old female CD-1 mice, which were purchased from the Charles River Japan Inc., Kanagawa. The reddening of mouse ear was assessed 24 hr after the application as reported previously.[7]

Induction of ODC in Mouse Skin ODC activity was determined 4 hr after application of test compounds to the skin of the backs of 8-week-old female CD-1 mice as described previously.[8] Dinophysistoxin-1 was tested in the dose range of 0.1 nmol–36.7 nmol and okadaic acid between 0.1 nmol and 124.4 nmol. 7-O-Palmitoyl okadaic acid was only tested between 0.1 nmol and 19.2 nmol due to its limited availability.

Inhibition of Specific Binding of [^{3}H]Okadaic Acid [^{3}H]Okadaic acid binding was assayed by the filtration method, which will be reported in detail elsewhere (manuscript in preparation). Briefly, 150 μg of a particulate fraction of mouse epidermis was incubated at 37° for 20 min with 30n*M* [^{3}H]-okadaic acid and the test compound in 1 ml of 50 m*M* Tris-HCl buffer (pH 7.4), containing 2m*M* 2-mercaptoethanol. Specific binding of [^{3}H]okadaic acid was estimated as the difference between total binding and non-specific binding measured in the presence of a 500-fold excess of unlabeled okadaic acid.

Two-stage Carcinogenesis Experiment on Mouse Skin Carcinogenesis was initiated by a single application of 100 μg of DMBA dissolved in 0.1 ml of acetone to the skin of the backs of 8-week-old female CD-1 mice as reported previously.[9] From one week after initiation, either 5 μg of dinophysistoxin-1 or 5 μg of okadaic acid dissolved in 0.1 ml of acetone was applied to the same areas on the mice, twice a week. Control groups were treated with DMBA alone and dinophysistoxin-1 alone. Each group consisted of 15 mice. Tumor-promoting activity was evaluated macroscopically in terms of percentage of tumor-bearing mice and average number of tumors per mouse, since histological evaluation was not performed in this experiment. The percentages of tumor-bearing mice and average number of tumors per mouse were recorded at every week up to week 30 of tumor promotion. In this experiment, 5 μg each of dinophysistoxin-1 and okadaic acid were applied per application, because a potent tumor-promoting activity of okadaic acid had previously been shown with 10 μg per application.[4]



	R_1	R_2
Okadaic acid	OH	H
Dinophysistoxin-1	OH	CH_3
7-O-Palmitoyl okadaic acid	$O-\overset{\overset{O}{\|}}{C}(CH_2)_{14}CH_3$	H

Fig. 1. Structures of okadaic acid, dinophysistoxin-1 and 7-O-palmitoyl okadaic acid.

Results

Induction of Irritant Activity on Mouse Ear Like okadaic acid, dinophysistoxin-1 is a skin irritant on mouse ear. One μg of dinophysistoxin-1 gave as strong a positive response in an irritant test as okadaic acid, whose ID_{50}^{24} was 50 pmol per ear as reported previously.[4] However, 7-O-palmitoyl okadaic acid gave a weak response. Methylation at C-35 of okadaic acid did not cause any reduction in irritant activity, whereas acylation at C-7 of okadaic acid markedly reduced the potency of irritant activity.

Induction of ODC Activity in Mouse Skin Figure 2 shows the dose-response curves for ODC induction by dinophysistoxin-1 compared with those of okadaic acid and 7-O-palmitoyl okadaic acid. Dinophysistoxin-1, like okadaic acid, showed a bell-shaped dose-response curve with a maximum at about 10 μg (12.4 nmol) per application. The potency of ODC induction of dinophysistoxin-1 was almost as strong as that of okadaic acid. 7-O-Palmitoyl okadaic acid did not induce ODC activity at up to 20 μg (19.2 nmol) per application. Therefore, the results of ODC induction with dinophysistoxin-1 and okadaic acid correlated well with those of the irritant test on mouse ear.

Inhibition of Specific Binding of [^{3}H]Okadaic Acid to a Mouse Particulate Fraction Figure 3 shows that like unlabeled okadaic acid, dinophysistoxin-1 inhibited the specific binding of [^{3}H]okadaic acid to a particulate fraction of mouse epidermis. The activity of okadaic acid, expressed as the effective dose for 50% inhibition (ED_{50}), is 30n*M* and that of dinophysistoxin-1 is 21n*M*. Therefore, the binding affinities of okadaic acid and dinophysistoxin-1 to a particulate fraction were almost the same.

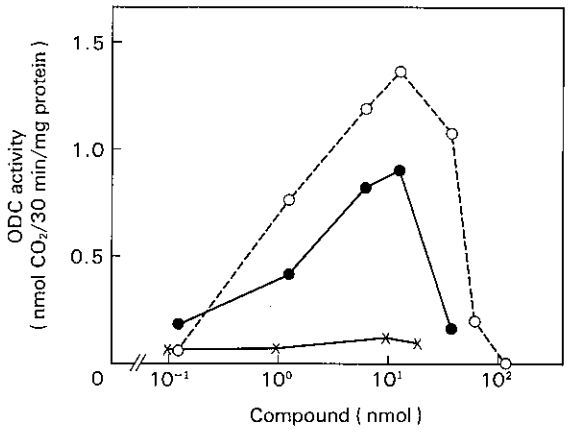


Fig. 2. Dose-response curve of ODC induction by dinophysistoxin-1 (●) compared with those of okadaic acid (○) and 7-O-palmitoyl okadaic acid (×).

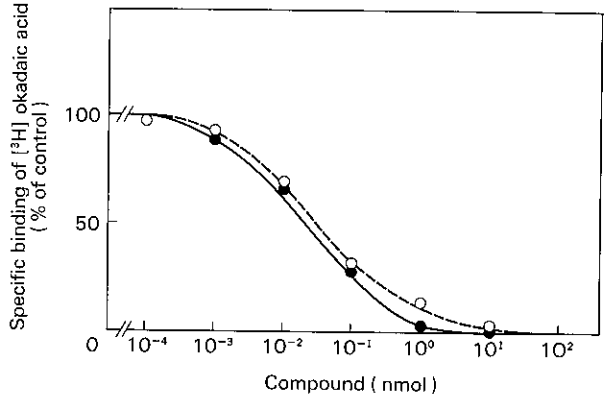


Fig. 3. Inhibition of specific [^{3}H]okadaic acid binding to a mouse skin particulate fraction by dinophysistoxin-1 (●) and okadaic acid (○).

Two-stage Carcinogenesis Experiment on Mouse Skin The tumor-promoting activity of dinophysistoxin-1 is illustrated in Fig. 4. The group treated with DMBA plus dinophysistoxin-1 showed the first tumors at week 6 of tumor promotion, as did the group treated with DMBA plus okadaic acid. The percentage of tumor-bearing mice in the group treated with DMBA plus dinophysistoxin-1 reached the maximum of 93.3% at week 11, and remained at 86.7% from week 12 to week 30 (Fig. 4A). The group treated with DMBA plus okadaic acid showed a similarly high percentage of tumor-bearing mice, in agreement with the previously reported results at 10 μg per application.[4] The groups treated with DMBA alone or dinophysistoxin-1 alone did not produce a significant number of tumors, like the group treated with okadaic acid alone, as reported previously.[4] Average number of tumors per mouse at week 30 was 4.6 for the group treated with DMBA plus dinophysistoxin-1 and 3.9 for the group treated with DMBA plus okadaic acid (Fig. 4 B). These results showed that dinophysistoxin-1 has a tumor-promoting activity as strong as that of okadaic acid, and is an additional tumor promoter of the okadaic acid class, because it also inhibits the specific binding of [^{3}H]okadaic acid to a particulate fraction of mouse epidermis.

Discussion

Dinophysistoxin-1, okadaic acid and acylated derivatives of okadaic acid have attracted our attention, because dinophysistoxin-1 and okadaic acid are causative agents of diarrhetic shellfish poisoning. Based on our

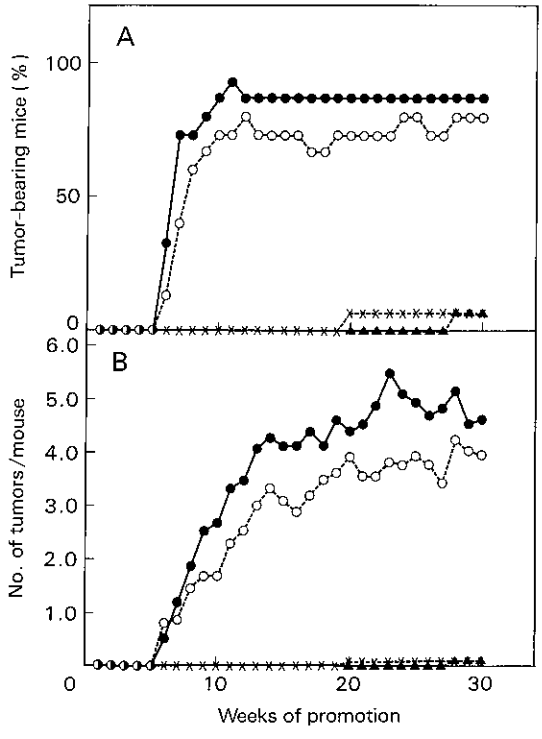


Fig. 4. Tumor-promoting activities of dinophysistoxin-1 and okadaic acid. A, percentage of tumor-bearing mice and B, average numbers of tumors per mouse. Groups treated with DMBA plus dinophysistoxin-1 (●), DMBA plus okadaic acid (○), DMBA alone (×) or dinophysistoxin-1 alone (▲).

recent findings that okadaic acid is a potent non-TPA type tumor promoter in a two-stage carcinogenesis experiment on mouse skin, we thought that the study of the other compounds of the okadaic acid class would be desirable. This paper shows that dinophysistoxin-1 has several biological activities and is a potent tumor promoter with the same specific activity as that of okadaic acid. Tumors of the groups treated with DMBA plus dinophysistoxin-1 or DMBA plus okadaic acid were not studied histologically. However, the macroscopic appearance of these tumors suggested the presence of papillomas in a high percentage, as previously reported.[4] Recently, we showed the specific binding of [^{3}H]okadaic acid to a particulate fraction of mouse skin (manuscript in preparation). The results of receptor binding studies with dinophysistoxin-1 indicated that it acts through the same pathway as okadaic acid. As described previously, okadaic acid does not bind to the phorbol ester receptors in cell membranes or activate protein kinase C *in vitro*. Dinophysistoxin-1 gave the same results. Therefore, dinophysistoxin-1 and okadaic acid might act similarly through a different pathway from TPA-type tumor promoters. Although we do not yet have direct evidence of whether or not compounds of the okadaic acid class are also tumor promoters of the digestive tract, the binding sites of okadaic acid are present in skin, the stomach, the small intestine and the colon, as well as other tissues (manuscript in preparation). Okadaic acid induced ODC in the glandular stomach of rats.[4] Terao *et al.* demonstrated that dinophysistoxin-1 produced severe injuries in the small intestine.[10] This recent evidence encourages the study of the tumor-promoting activity of okadaic acid and dinophysistoxin-1 in digestive organs. Although 7-O-palmitoyl okadaic acid was not examined in detail because of the limited amount available, 7-O-palmitoyl okadaic acid seems to be less active than dinophysistoxin-1 and okadaic acid. These results suggested that okadaic acid requires a free hydroxyl group on C-7 for potent activity. However, the relationship between the length of the acyl groups and the activity remains to be studied. Since tumor promoters of the okadaic acid class have a stronger tumor-promoting activity than other non-TPA type tumor promoters, such as palytoxin and thapsigargin, studies on the mechanism of action of the okadaic acid class will provide interesting information on tumor promotion.

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