J Cancer Res Clin Oncol (1994) 120:208–212

Journal of Cancer Research and Clinical Oncology

Increase of labeling indices in gastrointestinal mucosae of mice and rats by compounds of the okadaic acid type

H. Yuasa [1,3], K. Yoshida [1], H. Iwata [1], H. Nakanishi [1], M. Suganuma [2], M. Tatematsu [1]

[1] Laboratory of Pathology, Aichi Cancer Center Research Institute, Nagoya 464, Japan
[2] Cancer Prevention Division, National Cancer Center Research Institute, Tokyo 104, Japan
[3] Present address: Safety Research Laboratory, Tanabe Seiyaku Co., Ltd., Yodogawa-ku, Osaka, 530, Japan

Received: 17 May 1993/Accepted: 4 August 1993

Abstract. Effects of compounds of the okadaic acid type (okadaic acid, dinophysistoxin-1, calyculin A and tautomycin) on proliferation by digestive-tract epithelial cells were investigated in mice and rats. In mice, a single oral administration of these agents caused significant enhancement of BrdU labeling indices in a dose/response manner. Exceptions showing no response were limited to the pyloric mucosa for okadaic acid, the pyloric and fundic mucosa for calyculin A and the pyloric mucosa for tautomycin. Sequential analysis of labeling indices after a single oral administration of dinophysistoxin-1 revealed two peaks of cell proliferation at 18 h and 36 h in the esophagus, ileum and colon. The labeling indices of the forestomach, fundus, pylorus and jejunum, on the other hand, continuously increased from 6 h after the administration. Elevated proliferation was also observed in the skin after 30 h or after, but no effects on the liver or kidney were evident. A single oral administration of the okadaic acid type of compounds also dose-dependently enhanced cell proliferation of the rat digestive tract. These results strongly suggest that the okadaic acid class of compounds may exert promoting potential for the gastrointestinal mucosa when administered orally.

Key words: Okadaic aicd – Digestive tract – Gastrointestinal mucosae – Cell proliferation – Protein phosphatases 1, 2A

Introduction

Compounds of the okadaic acid type, which are all potent inhibitors of protein phosphatases 1 and 2A, are tumor promoters in various organs, such as mouse skin (Suganuma et al. 1988, 1990; Fujiki et al. 1988), rat glandular stomach (Suganuma et al. 1992 b) and rat liver (Nishiwaki-Matsushima et al. 1992). From these results, we hypothesized that the okadaic acid pathway, mediated through inhibition of protein phosphatases 1 and 2A, is a general mechanism of tumor promotion in human cancer development (Fujiki 1992). At present, four different structural forms of compounds of the okadaic acid type have been identified, as follows: okadaic acid, calyculins, microcystins and tautomycin (Fujiki and Suganuma 1993). Their potencies in terms of inhibition of protein phosphatases 1 and 2A are slightly varied, indicating differences in their chemical natures. For example, okadaic acid inhibited protein phosphatase 1 less strongly than protein phosphatase 2A, and the three other compounds inhibited the two protein phosphatases with this order of potency. Microcystin is the strongest inhibitor and tautomycin demonstrates the weakest inhibition (Suganuma et al. 1992 a). In particular, microsystin-LR has a specific organotrophy limited to the liver. Studies with these different types of compound should allow clarification of the significance of inhibition of protein phosphatases 1 and 2A in the cells and facilitate our understanding of the mechanisms of tumor promotion.

As a result of the finding that okadaic acid and dinophysistoxin-1, otherwise known as 35-methylokadaic acid, contained in the hepatopancreas of mussels and scallops, are causative agents of diarrhetic shellfish poisoning (Murata et al. 1982), the effects of okadaic acid on rat intestinal epithelium have been studied (Terao et al. 1986; Edebo et al. 1989). Recently we reported that okadaic acid administration in the drinking water significantly increases the development of neoplastic changes in the glandular stomach of rats initiated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Suganuma et al. 1992 b). As a preliminary to further two-stage carcinogenesis experiments, the present study of cell proliferation induction by various compounds of the okadaic acid class, given p.o., in the gastrointestinal tract, kidney, liver and skin was performed. This paper documents findings on the kinetics of BrdU labeling indices in various organs after a single oral administration of the agents to mice and rats, and on a unique biphasic response to dinophysistoxin-1.

Materials and methods

Chemicals

Okadaic acid, dinophysistoxin-1, calyculin A and tautomycin, more than 99.0% pure, were isolated from various sources, as reported previ-

Correspondence to: M. Tatematsu

ously (Fujiki and Suganuma 1993). Bromodeoxyuridine (BrdU) was obtained from Sigma Chemical Co., St. Louis, Mo., and monoclonal mouse antibody to BrdU was purchased from Dakopatts a/s, Denmark.

Animals

Eighty-eight male ICR mice, 5 weeks of age, and 40 male SD rats, 7 weeks of age, each specific-pathogen-free, were obtained from Charles River Japan Inc., Kanagawa, Japan. The animals were maintained on standard diets (Oriental MF for rats and Oriental NMF for mice) and tap water ad libitum, and housed in plastic cages in an air-conditioned room under a 12-h light/dark schedule. Before administration of okadaic-acid-type compounds, animals were fasted for 13–15 h.

Experiments with mice

Dose/response effects of okadaic acid, dinophysistoxin-1, calyculin A and tautomycin. Forty-six male ICR mice, 6 weeks of age, were divided into nine groups. Each group consisted of five or six mice. Okadaic acid, dinophysistoxin-1 and calyculin A were administered orally through a gastric tube at two different doses, 2 μg and 10 μg, and tautomycin at 50 μg and 300 μg. These amounts of the compounds were dissolved in a standard 0.2-ml volume of corn oil or sesame oil. Mice received BrdU at a dose of 75 mg/kg body weight intraperitoneally 17 h after the administration, and were sacrificed 1 h thereafter. The gastrointestinal tract, including the esophagus, forestomach, fundic and pyloric glandular stomach, jejunum, ileum, and proximal and distal colon, was removed, fixed in neutral buffered 10% formalin solution, and embedded in paraffin. Thin sections were stained with hematoxylin and eosin (H&E) and immunohistochemically with anti-BrdU monoclonal antibody. BrdU labeling indices were expressed as either the mean of positive nuclear counts per 20 pits in glandular tissues, or as numbers of positive cells per 1000 cells counted in other tissues.

Time-course of the effects of dinophysistoxin-1. Forty-two male ICR mice, 6 weeks of age, were used. Each group consisted of six mice. Dinophysistoxin-1, 10 μg/0.2 ml corn oil, was given orally to each mouse, and groups were sacrificed 6, 12, 18, 24, 30, and 36 h thereafter. The control group received vehicle alone. BrdU was administered (75 mg/kg b.w.) intraperitoneally to all animals 1 h before each sacrifice for labeling of S-phase cells. In addition to the organs mentioned in a dose/response study, skin samples, the kidneys and liver were subjected to histological examinations. Labeling indices were determined in the same manner as above.

Experiments with rats

Forty male SD rats, 8 weeks of age, were divided into nine groups consisting of four to six animals. Okadaic acid and calyculin A were administered at doses of 1, 10, and 50 μg, and tautomycin at 50 μg and 300 μg. Each compound dose was dissolved in 0.2 ml sesame oil. Animals received BrdU at a dose of 50 mg/kg body weight intraperitoneally 17 h after administration and were sacrificed 1 h thereafter. Control animals received the vehicle alone. Organs, including the forestomach, fundic and pyloric glandular stomach, jejunum, ileum, and proximal and distal colon, were processed for determination of labeling indices.

Statistical analysis

The mean values obtained were compared by Student's *t*-test. When variances between groups were different, the degree of freedom was corrected by the method of Cochran and Cox.

Results

Experiments with mice

Dose/response effects of okadaic acid, dinophysistoxin-1, calyculin A and tautomycin. Table 1 summarizes the average labeling indices of various tissues in the mouse gastrointestinal tract after administration of the higher doses of okadaic acid, dinophysistoxin-1, calyculin A and tautomycin. In general, these four compounds increased labeling indices in all parts but the pylorus. Although the effects of lower doses of the four compounds are not presented here, most of the tissues showed a dose dependence.

In H&E staining, edema or acute inflammation of the squamous mucosa of the esophagus and forestomach, and erosion of the fundic mucosa of mice treated with 10 μg okadaic acid animal or with the same dose of calyculin A were noted. No significant changes were evident in the other regions of the gastrointestinal tracts of mice treated with okadaic acid or calyculin A, or in any region of mice treated with dinophysistoxin-1 or tautomycin.

Table 1. Labeling indices of gastrointestinal tract epithelial cells in mice 18 h after oral administration of okadaic acid, dinophysistoxin-1, calyculin A and tautomycin

Cells	Control (corn oil) 0 μg (5)	Okadaic acid 10 μg (5)	Dinophysistoxin-1 10 μg (6)	Calyculin A 10 μg (5)	Tautomycin 60 μg (5)
Esophagus					
Proximal	4.0±1.7	9.2*±4.4	20.5**±4.4	10.0**±2.9	11.2***±0.4
Distal	2.7±1.4	8.2±5.8	22.3***±3.8	9.1*±4.7	12.3***±3.3
Forestomach	2.0±0.9	15.5±12.5	24.6*±14.0	23.2*±16.1	11.4***±0.4
Glandular stomach					
Fundus	3.0±1.1	7.0**±0.6	6.8**±1.5	3.9±0.7	5.6*±1.5
Pylorus	3.3±0.8	3.4±1.3	5.6*±2.1	3.0±0.7	3.8±1.2
Small intestine					
Jejunum	11.3±1.9	18.7***±1.5	21.4***±2.6	17.8***±1.3	20.0***±1.4
Ileum	10.9±1.8	14.5**±1.0	15.3**±2.9	14.2*±2.7	17.7**±1.1
Colon					
Proximal	1.2±0.8	6.6***±1.3	8.8**±3.7	6.2***±0.8	8.4**±2.8
Distal	1.5±0.7	13.4***±1.2	15.1***±2.6	11.2***±2.0	10.3**±3.4

Doses are in μg/mouse; numbers of mice are shown in parentheses
*,**,*** Significantly different from respective control value: * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Time-course of the effects of dinophysistoxin-1. Data for sequential changes in the labeling index of the mouse gastrointestinal tract, liver and kidney are summarized in Fig. 1. Within the 36-h observation period, the labeling indices of the esophagus, ileum and colon regions (see Fig. 1 A, C) demonstrated two peaks, the first appearing 18 h after the treatment, and the second at 36 h. In contrast, the labeling indices of the forestomach, fundus, pylorus and jejunum showed continuous significant plateau increases from 6 h to 18 h after the administration (see Fig. 1 A, B). In the skin, the labeling index gradually increased with time and with marked elevation after 30 h (see Fig. 1 D). Labeling indices of the liver and kidney did not significantly alter during the 36-h observation period.

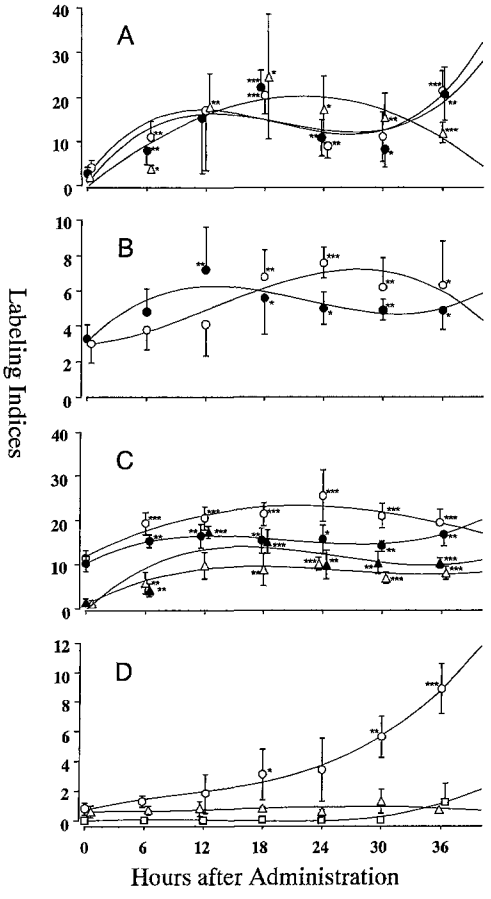

Fig. 1 A–D. Time course of the effects of dinophysistoxin-1. **A** The proximal esophagus (○), distal esophagus (●) and forestomach (△); cubic curves were fitted using the computer software "Cricket graph". **B** The fundic (○) and pyloric (●) areas of the stomach. **C** The jejunum (○), ileum (●), proximal colon (△) and distal colon (▲). **D** The skin (○), liver (□) and kidney (△). Significant differences from 0 h: *$P<0.05$; **$P<0.01$; ***$P<0.001$

Experiments with rats

The labeling indices of the various tissues in the rat gastrointestinal tracts after treatment with okadaic acid, calyculin A or tautomycin are tabulated in Table 2. These three compounds increased the labeling indices in almost all gastrointestinal mucosae in a dose-dependent manner. After treatment with tautomycin, cell proliferation in the colon was not altered, but a relatively strong influence was exerted on the stomach and small intestine.

No remarkable changes were recognized in any regions of the gastrointestinal tracts of rats treated with okadaic acid, calyculin A or tautomycin.

Discussion

The present study revealed clearly increased cell proliferation of various tissues of the alimentary canal of both mice and rats, 18 h after oral application of okadaic acid, dinophysistoxin-1, calyculin A or tautomycin. From 6 h after a single oral administration of dinophysistoxin-1, cell proliferation was in fact induced in the digestive tracts of the mice. Organ specificity was demonstrated by the contrasting lack of response in the liver and kidney while a proliferative reaction in the skin first became marked after 30 h.

In the esophagus, ileum and colon of the mouse, a first wave of cell division occurred after 18 h with a subsequent drop before rising again within 36 h. The first peak in labeling indices presumably relates to DNA synthesis synchro-

Table 2. Labeling indices of gastrointestinal tract epithelial cells in rats 18 h after oral administration of okadaic acid, calyculin A and tautomycin

Cells	Control (corn oil)	Okadaic acid			Calyculin A			Tautomycin	
	0 μg (3)	1 μg (4)	10 μg (5)	50 μg (5)	1 μg (4)	10 μg (4)	50 μg (3)	50 μg (4)	300 μg (5)
Forestomach	8.7±0.8	12.3*±2.4	17.7**±3.7	39.2**±11.3	10.7±2.6	6.7±3.1	26.5***±1.1	11.0±3.3	18.8*±5.4
Glandular stomach									
Fundus	2.5±0.8	3.9*±1.0	4.7**±0.8	6.5**±1.4	1.8±0.3	2.0±0.4	3.6±1.7	4.6*±0.7	8.4*±2.8
Pylorus	3.1±0.4	3.4±3.1	5.4**±0.9	8.5***±1.3	2.9±0.6	3.7±0.8	4.8**±0.1	3.9*±0.4	7.9***±0.4
Small intestine									
Jejunum	16.0±1.6	22.5**±2.4	23.7**±2.6	24.7***±1.9	17.7±3.3	20.9**±1.5	20.5*±3.2	21.5**±1.4	22.6***±1.0
Ileum	16.0±2.1	23.5**±3.1	25.5**±3.3	24.6***±1.2	18.4±2.0	20.8**±1.0	20.6*±1.6	21.6**±1.7	22.7***±1.0
Colon									
Proximal	8.1±1.5	15.3**±0.9	16.9**±1.7	17.5**±2.3	9.0±0.7	14.3**±1.4	17.0**±3.6	9.5±0.7	8.8±0.6
Distal	8.4±0.5	11.3±0.2	16.1**±0.7	17.8***±0.9	9.3±0.5	10.2±0.6	11.5*±0.5	8.4±0.2	8.7±0.2

Doses are in μg/mouse; numbers of mice are shown in parentheses
*,**,*** Significantly different from respective control value: * $P<0.05$; ** $P<0.01$; *** $P<0.001$

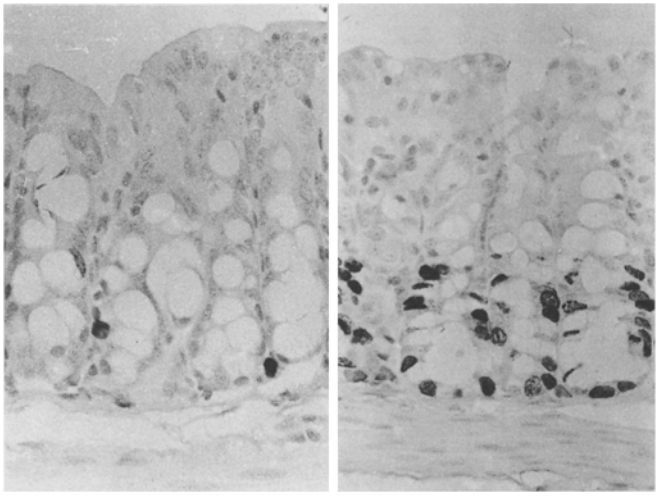

Fig. 2. Mouse proximal colon. Sporadic positive cells are present at the bottom of the colonic glands of a control animal (*left field*). A marked increase in the numbers of positive cells in the proliferative zone (*right field*) is apparent 18 h after treatment with 10 μg of dinophysistoxin-1. Anti-BrdU immunostaining. ×242

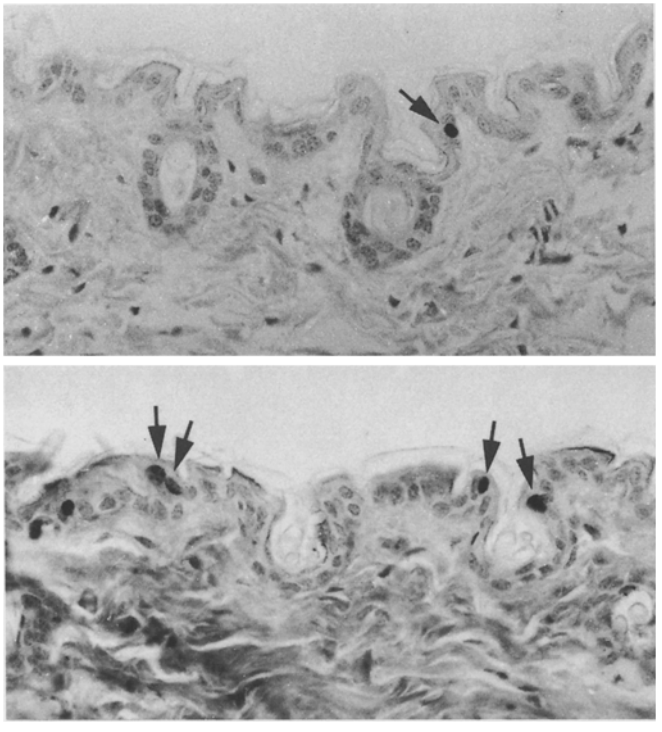

Fig. 3. Mouse skin. Very few positive cells (*arrow*) are noted in the squamous epithelium of the skin of a control animal (*upper field*). An increase in the number of positive cells (*arrows*) is apparent in the basal layer (*lower field*) 36 h after treatment with dinophysistoxin-1. Anti-BrdU immunostaining. ×300

nized by direct stimulation of the digestive cavities of the treated animals. The second peak could be associated with the cell proliferation cycle, which turns around within 16–24 h (Pardee et al. 1986). Persistence of the stimulation influence, inducing a second peak of labeling indices, has also been recognized in other systems, whereby okadaic acid caused sustained expression of c-*fos* and c-*jun* genes in cultured cells (Schonthal et al. 1991 a, b) and resulted in the expression of the c-*fos* gene with two peaks 6 h and 48 h after topical treatment on mouse skin (Holladay et al. 1992). In addition, persisting stimulation is also shown by partial hepatectomy, where a second peak of incorporation of tritiated thymidine is well known in the rat liver (Fabrikant 1967).

The fact that two peaks were not recognized in the stomach and jejunum suggests that continued exposure to the orally administered okadaic-acid-type compounds, partially remaining in the diet in the forestomach, resulted in a constant stimulation of these mucosae. A preliminary examination (unpublished data, H. Yuasa), after i.g. intubation of oil containing coloring matter (Oil red O) revealed material persisting in the forestomach and rectum after 3 h. This suggests that okadaic-acid-type compounds dissolved in oil had reached the colon within 3 h after dosing but also still remained in the forestomach.

With regard to the observed tissue specificity of dinophysistoxin-1, the earlier report that the steady-state level of c-*jun* gene expression in skin treated with okadaic acid was slightly increased from 12 h to 48 h after the treatment (Holladay et al. 1992) is of interest. This phenomenon might explain the delay in effective stimulation of cell proliferation by okadaic-acid-type compounds in this tissue. In addition, proliferating populations might be more sensitive to such compounds than normally non-proliferating cells like those in the liver and kidney. In cultured cells, such as C3H10T1/2 for example, non-dividing cells were found to be less vulnerable to okadaic acid toxicity than dividing cells (Herschman et al. 1989).

In the present study, the larger the doses of okadaic-acid-type compounds administered, the more enhanced was the cell proliferation. Tissue damage was detected in the esophagus of mice treated with okadaic acid, but an inverse relation to labeling indices was found for high and low doses. This could have reflected an inhibitory effect or direct toxicity to the mucosa. Large standard deviations for forestomach values of mice treated with okadaic acid, dinophysistoxin-1 and calyculin A might also be ascribed to variation in toxicity. It was earlier reported that intraperitoneal injection of dinophysistoxin-1 to suckling mice produces mucosal injuries in the small intestine (Terao et al. 1986) and okadaic acid was also described to be cytotoxic for C3H10T1/2 and 3T3 cells in vitro (Herschman et al. 1989). However, okadaic acid stimulated cell proliferation in the small and large intestine without any obvious degenerative changes in the present case. Furthermore the finding of strong effects of tautomycin as well as okadaic acid on the glandular stomach and small intestine of rats, but not on the large intestine, suggests a regional specificity in the cell-division-promoting and cytotoxic effects in the gastrointestinal tract.

In conclusion, the present finding of significant enhancement of cell proliferation in the various mucosae of the esophagus, stomach and intestine by compounds of the okadaic-acid-type suggests that they might have promoting potential for carcinogenesis involving those populations when administered orally. In addition, oral administration of okadaic-acid-type compounds might even exert a promoting influence on the skin.

Acknowledgements. This work was supported in part by a grant-in-aid for a comprehensive 10-year strategy for cancer control.

References

Edebo L, Lange S, Li XP, Allenmark S, Jennishe E (1989) Diarrhetic shellfish toxins induce rapid swelling of the intestinal epithelium and hypersecretion in the rat small intestine. In: Natori S, Hashimoto K, Ueno Y (eds) Mycotoxins and phycotoxins. Elsevier, Amsterdam, pp 437–444

Fabrikant JI (1967) The spatial distribution of parenchymal cell proliferation during regeneration of the liver. Johns Hopkins Med Bull 120:147–147

Fujiki H (1992) Is the inhibition of protein phosphatase 1 and 2A activities a general mechanism of tumor promotion in human cancer development? Mol Carcinog 5:91–94

Fujiki H, Suganuma M (1993) Tumor promotion by inhibitors of protein phosphatases 1 and 2A, the okadaic acid class compounds. Adv Cancer Res 61:143–194

Fujiki H, Suganuma M, Suguri H, Yoshizawa S, Takagi K, Uda N, Wakamatsu K, Yamada K, Murata M, Yasumoto T, Sugimura T (1988) Diarrhetic shellfish toxin, dinophysistoxin-1, is a potent tumor promoter on mouse skin. Jpn J Cancer Res (Gann) 79:1089–1093

Herschman HR, Lim RW, Branknow W, Fujiki H (1989) The tumor promoters 12-*O*-tetradecanoylphorbol-13-acetate and okadaic acid differ in toxicity, mitogenic activity and induction of gene expression. Carcinogenesis 10:1495–1498

Holladay K, Fujiki H, Bowden GT (1992) Okadaic acid induces the expression of both early and secondary response genes in mouse keratocytes. Mol Carcinog 5:16–24

Murata M, Shimatani M, Sugitani H, Oshima Y, Yasumoto T (1982) Isolation and structure elucidation of the causative toxin of the diarrhetic shellfish poisoning. Bull Jpn Soc Sci Fish 48:549–552

Nishiwaki-Matsushima R, Ohta T, Nishiwaki S, Suganuma M, Kohyama K, Ishikawa T, Carmichael WW, Fujiki H (1992) Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J Cancer Res Clin Oncol 118:420–424

Pardee AB, Coppock DL, Yang HC (1986) Regulation of cell proliferation at the onset of DNA synthesis. J Cell Sci Suppl 4:171–180

Schonthal A, Tsukitani Y, Feramisco J (1991 a) Transcriptional and posttranscriptional regulation of c-*fos* proto-oncogene expression by the tumor promoter okadaic acid. Oncogene 6:423–430

Schonthal A, Alberts AS, Frost JA, Feramisco JR (1991 b) Differential regulation of *jun* family gene expression by the tumor promoter okadaic acid. New Biol 3:977–986

Suganuma M, Fujiki H, Suguri H, Yoshizawa S, Hirota M, Nakayasu M, Ojika M, Wakamatsu K, Yamada K, Sugimura T (1988) Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. Proc Natl Acad Sci USA 85:1768–1771

Suganuma M, Fujiki H, Suguri HF, Yoshizawa S, Yasumoto S, Kato Y, Fusetani N, Sugimura T (1990) Calyculin A, an inhibitor of protein phosphatases, a potent tumor promoter on CD-1 mouse skin. Cancer Res 50:3521–3525

Suganuma M, Fujiki H, Okabe S, Nishiwaki S, Brautigan D, Ingebritsen SI, Rosner MR (1992 a) Structurally different members of the okadaic acid class selectively inhibit protein serine/threonine but not tyrosine phosphatase activity. Toxicon 30:873–878

Suganuma M, Tatematsu M, Yatsunami J, Yoshizawa S, Okabe S, Uemura D, Fujiki H (1992 b) An alternative theory of tissue specificity by tumor promotion of okadaic acid in glandular stomach of SD rats. Carcinogenesis 13:1841–1845

Terao K, Ito E, Yanagi T, Yasumoto T (1986) Histopathological studies on experimental marine toxin poisoning. I. Ultrastructural changes in the small intestine and liver of suckling mice induced by dinophysistoxin-1 and pectenotoxin-1. Toxicon 24:1141–1151