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A Serine Protease-inhibitory Benzamidine Derivative Inhibits the Growth of Human Colon Carcinoma Cells

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The inhibitory effect of a serine protease-inhibiting tetra-benzamidine derivative, TAPP-Br, on the cell growth of 8 human colon carcinoma cell lines was examined and the mechanism of the inhibition was analyzed. TAPP-Br inhibited the cell growth of all the colon carcinoma cell lines, and this effect was irreversible. The expression of mRNAs for nuclear oncogenes such as MYC, FOS and JUN was decreased by TAPP-Br after treatment for 3 h and the effect continued for 48 h. mRNA expression of epidermal growth factor receptor, transforming growth factor-β and type IV collagenase was suppressed at 48 h after the initiation of TAPP-Br treatment, suggesting an indirect action of TAPP-Br. TAPP-Br decreased protein kinase C activity in the particulate fraction, whereas it increased the enzyme activity in the soluble fraction. These findings overall suggest that the serine protease inhibitor, TAPP-Br, might inhibit the cell growth of colon carcinoma cell lines through suppressing the expression of genes whose promoter contains a 12-O-tetradecanoylphorbol-13-acetate-responsive element or serum-responsive element.

Key words: Protease inhibitor — Colon carcinoma — Cell growth inhibition

Recently, interest has been focused on the finding that tumor invasion and metastasis are associated with release of specific proteases such as type IV collagenase, stromelysin and urokinase-type plasminogen activator.[1-3] These proteases degrade the extracellular matrix to facilitate tumor cell invasion and proliferation, while some proteases stimulate tumor cell proliferation.[3,4] On the other hand, Ha-*ras* oncogene transformation of NIH3T3 cells is inhibited by such protease inhibitors as antipain, leupeptin and α_1-antitrypsin.[5] In addition, some protease inhibitors such as soybean-derived Bowman-Birk inhibitor and potato chymotrypsin inhibitor-1 suppress chemical- and radiation-induced carcinogenesis.[6-8]

A synthetic competitive inhibitor of serine protease, tetra-*p*-amidinophenoxypropane (TAPP),[4] was developed by Parsons.[9] TAPP and its halo derivatives inhibit the *in vitro* conversion of fibrinogen to fibrin and the hydrolysis of esters and anilides of amino acids catalyzed by various proteases such as trypsin, chymotrypsin, kallikrein and urokinase. This compound inhibits the growth of sarcoma 180 tumors implanted in hybrid mice and the production of "cancer coagulation factor" due to Walker carcinoma in Wistar rats.[9] The bromo derivative of TAPP, (TAPP-Br), has been shown to inhibit cell proliferation of B-lymphoid human tumor cell line, WI-L2, and to inhibit the expression of c-*myc* mRNA.[10]

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[4] The abbreviations used are: TAPP, tetra-*p*-amidinophenoxypropane; IP_3, inositol 1,4,5-triphosphate; TGF-α, transforming growth factor-α; TGF-β, transforming growth factor-β; EGF, epidermal growth factor; poly(A)$^+$, polyadenylated; cDNA, complementary DNA; kb, kilobase.

In this study, the inhibitory effect of TAPP-Br on cell proliferation of 8 human colon carcinoma cell lines was examined. Moreover, to elucidate the mechanisms of growth inhibition, we studied the effect of TAPP-Br on the expression of oncogenes, growth factors and their receptors.

MATERIALS AND METHODS

Synthesis of TAPP-Br TAPP-Br was kindly provided by Dr. R. Gambari (University of Ferrara, Italy). The structure of TAPP-Br is shown in Fig. 1. Synthesis, melting points, yields, crystallization solvents and analytical data have been reported elsewhere.[11,12] The method of preparing TAPP-Br has been described previously by Ferroni *et al.*[11] As a control, benzamidine (Sigma) was used.

Cell culture Eight cell lines derived from human colon carcinomas were used. Six of them (WiDr, LoVo, SW837, Colo201, Colo320DM and DLD-1) were provided by the Japanese Cancer Research Resources Bank. TCO cell line was established in our laboratory and HCT was obtained from the American Type Culture Collection (ATCC). These cell lines were routinely cultured in RPMI1640 (Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum (FBS, Whittaker M. A. Bioproducts Inc., Maryland), penicillin (0.1 mg/ml) and streptomycin (0.1 mg/ml) under a humidified 5% CO_2 atmosphere at 37°C.

Cell growth and DNA synthesis Cells were seeded on 22 mm dishes (5×10^4/dish) and cultured in the presence of various concentrations of TAPP-Br up to 10 μM. The

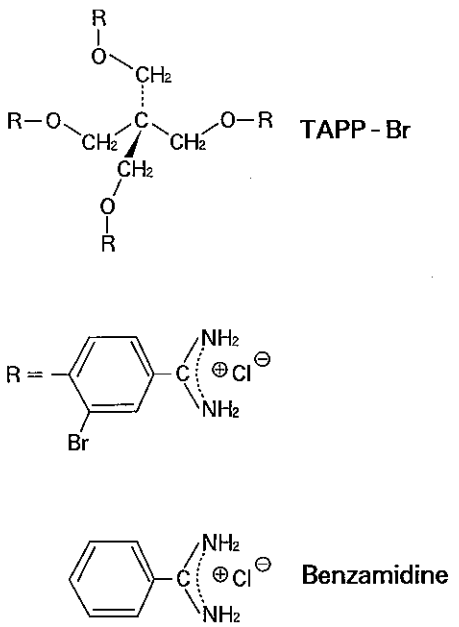

Fig. 1. Structures of TAPP-Br and benzamidine. TAPP-Br: 1,3-di-(*p*-amidinophenoxy)-2,2-bis(*p*-amidinophenoxymethyl)-bromine.

medium was changed every two days. Cell number was counted using a Neubauer-type counting plate. All the experiments were done in triplicate.

DNA synthesis was measured in terms of ^{3}H-thymidine incorporation. About 2×10^{4} cells were seeded on 12-well culture plates (Falcon, Becton Dickinson) and cultured in RPMI1640 medium with 10% FBS. After 24 h, the medium was exchanged with fresh medium supplemented with 0.5% FBS. After 24 h, the cells were cultured with 10 μM TAPP-Br or 10 μM benzamidine for 16 h. The cells were pulse-labeled with 0.2 μCi of ^{3}H-thymidine for 3 h. Radioactivity was determined by solubilizing cells with 1 N NaOH and 1 N NaCl, and counted in a liquid scintillator. These experiments were performed in triplicate.

Northern blot analysis RNA was extracted from cultured cells by the standard guanidine isothiocynate/cesium chloride method.[13] Ten μg of poly(A)$^{+}$-selected RNA was electrophoresed on 1.0% agarose/formaldehyde gel and blotted onto a zeta-probe nylon filter (BioRad, Richmond, CA). The filters were baked for 2 h at 80°C under vacuum. Hybridization using ^{32}P-labeled probe and washing were performed as described previously.[14] The filters were exposed to X-ray film.

The 1.9 kb human EGF cDNA insert from phEGF 15 was kindly provided by Dr. G. I. Bell,[15] 1.4 kb human transforming growth factor-α (TGF-α) cDNA[16] and 1.0 kb human TGF-β cDNA[17] by Dr. Ric Derynck, 2.4 kb human epidermal growth factor (EGF) receptor cDNA in pE7,[18] 0.4 kb human MYC cDNA, 0.95 kb human JUN cDNA, and 2.1 kb human FOS cDNA by the Japanese Cancer Research Resources Bank, human ERBB2 cDNA in pCER204[19] by Dr. T. Yamamoto, and human type IV collagenase cDNA by Dr. G. I. Goldberg.[20] β-Actin was purchased from Oncor Inc. (Gaithersburg, MD).

^{125}I-labeled anti-EGF antibody binding assay Membrane-bound EGF was assayed as described previously.[21] About 1×10^{4} cells were plated on a 6-well plate (Falcon, Becton Dickinson). The cells were then treated with 10 μM TAPP-Br or 10 μM benzamidine for 3 days. At 1 h before the assay, the medium was exchanged with RPMI-1640 containing 20 mM HEPES, pH 7. 4. The cells were incubated for 1 h at 37°C and then cooled on ice. The medium was exchanged with ice-cold binding buffer (RPMI1640 containing 0.5% BSA and 20 mM HEPES, pH 7. 4). For the binding assay, wells containing 0.1 μg of ^{125}I-labeled anti-EGF antibody (KEM-10) were set up in triplicate and incubated for 4 h at 4°C. The samples were washed three times with binding buffer, lysed with 1 N NaOH and then counted in a gamma counter. Non-specific binding was determined in the presence of 100 μg of unlabeled antibody.

Protein kinase C assay The cells were suspended in homogenized buffer containing 20 mM Tris HCl pH 7.5, 2 mM EDTA, 10 mM EGTA (glycoletherdiaminetetraacetic acid), 20 mM 2-mercaptoethanol, 0.25 M sucrose, 10 μM leupeptin, and 10 μM PMSF. They were sonicated and centrifuged at 100,000g for 1 h. The pellets were resuspended in homogenized buffer and stored at −80°C until use. Protein kinase C activity was measured by using a PKC enzyme assay system (Amersham, RPN 77). Radioactivities were counted in a gamma counter. These experiments were performed in duplicate. To examine whether TAPP-Br directly inhibits protein kinase C, the reaction mixture was incubated in the presence or absence of 1 μM TAPP-Br, 10 μM TAPP-Br or 10 μM benzamidine with non-treated particulate fraction.

Measurement of inositol 1,4,5-triphosphate (IP_3) The cells were washed with cold PBS, then 7.5% (w/v) ice-cold trichloroacetic acid was added. They were centrifuged at 2000g for 15 min at 4°C. The supernatants were extracted three times with water-saturated diethyl ether and neutralized with $NaHCO_3$. IP_3 was measured by a competitive binding assay using an IP_3 assay system (Amersham, TR 1000).

RESULTS

Inhibitory effect of TAPP-Br on cell growth The effect of TAPP-Br on the cell growth of a colon cancer cell line,

WiDr, is shown in Fig. 2. TAPP-Br inhibited the cell growth of WiDr cells in a dose-dependent manner up to 10 μM. Benzamidine did not show any effect on the cell growth at the same concentration. Therefore, using a concentration of 10 μM, the inhibitory effect of TAPP-Br was examined on 8 colon carcinoma cell lines. As shown in Table I, the growth of all 8 cell lines was significantly inhibited by treatment with TAPP-Br. Percentage growth inhibition on the 6th day was more than 50%. The effect of TAPP-Br on ^{3}H-thymidine incorporation was examined on WiDr cells. The radioactivity incorporated into the cells treated with TAPP-Br for 19 h was 265 ± 30 (cpm/10^5 cells; average $\pm$ SE of triplicate experiments), while that incorporated into the control cells (non-treated) was 1160 ± 23, the difference between the two being statistically significant ($P < 0.01$). Benzamidine treatment did not show any effect on the ^{3}H-thymidine incorporation (1140 ± 173). To ascertain whether the effect of TAPP-Br is reversible or not, WiDr cells were cultured in the presence of 10 μM TAPP-Br for 4 days and then cultured in the presence or absence of 10 μM TAPP-Br for an additional 6 days. When $66 \pm 1.9 \times 10^4$ (average $\pm$ SE of triplicate) cells were re-seeded at the 4th day, the numbers of cells after culture for a further 6 days in the presence and the absence of TAPP-Br were $147 \pm 9.9 \times 10^4$ and $153 \pm 2.8 \times 10^4$, respectively. Therefore, the inhibitory effect of TAPP-Br on cell growth was suggested to be irreversible.

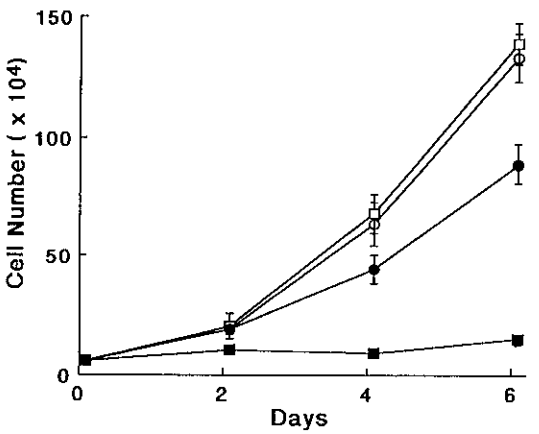

Fig. 2. Dose-dependent inhibition of cell growth of WiDr cells by TAPP-Br. Cells (5×10^4) were cultured in the absence (○) or presence of TAPP-Br at 2 μM (●) or 10 μM (■), or 10 μM benzamidine (□). The medium was changed every two days. Cell number was counted every two days. Values are the average $\pm$ SE of triplicate experiments.

Table I. Effect of TAPP-Br on Cell Growth of Colon Carcinoma Cell Lines

Cell lines	% Growth inhibition[a)]
WiDr	97.4%
Colo201	94.7%
Colo320	65.7%
SW837	54.9%
HCT	69.8%
LoVo	54.8%
DLD-1	66.1%
TCO	89.0%

a) The cells were cultured for 6 days in the presence or absence of 10 μM TAPP-Br in RPMI supplemented with 10%FCS. % Growth inhibition was calculated as follows:

$$\left(1 - \frac{\text{TAPP-Br-treated cell number} - \text{basal cell number}}{\text{control cell number} - \text{basal cell number}}\right) \times 100.$$

This experiment was performed in triplicate and the average is presented.

Effect of TAPP-Br on the expression of oncogenes, growth factors, and growth factor receptors We examined the effect of TAPP-Br on mRNA expression of oncogenes, growth factors and their receptors by WiDr cells. As shown in Fig. 3, the mRNA expressions of FOS, JUN, and MYC were decreased after treatment with TAPP-Br for 3 h, and the effect continued for 48 h. To get quantitative data, the autoradiographic intensities of the specific bands were measured by densitometric scanning and normalized with respect to the intensities of an

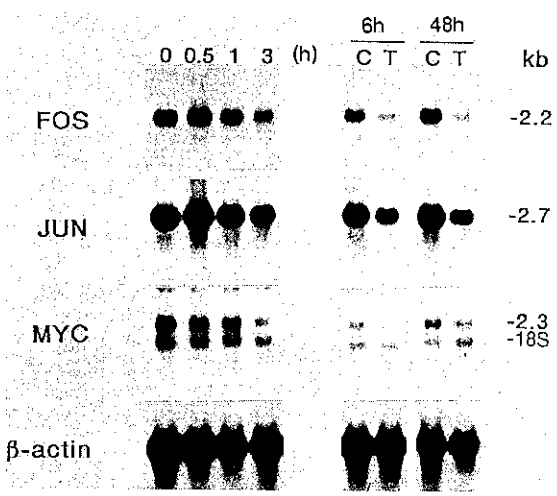

Fig. 3. Time course of effect of TAPP-Br on the expressions of FOS, JUN and MYC mRNA by WiDr cells. The cells were cultured with 10 μM TAPP-Br for 30 min, 1 h, 3 h, 6 h, 24 h, and 48 h. Poly(A)$^+$-selected RNA was extracted from the cells and subjected to Northern blot analysis as described in "Materials and Methods." Control represents the data obtained from cells cultured in 10% FCS without TAPP-Br (C, control; T, treated with 10 μM TAPP-Br).

internal control (β-actin). The ratio of T/C (TAPP-Br-treated cells/control cells) was then calculated. The values of the T/C ratio of FOS, JUN and MYC at 6 h were 0.23, 0.58 and 0.23, respectively. As shown in Fig. 4, on the other hand, the expression of mRNA for TGF-β, EGF receptor and type IV collagenase was not changed for the initial 6 h, the values of the T/C ratio being 0.92, 1.17 and 0.94, respectively. At 48 h after the initiation of TAPP-Br treatment, the expression of TGF-β, EGF receptor and type IV collagenase was decreased, the T/C ratios being 0.71, 0.56 and 0.56, respectively. The expression of mRNAs for EGF, TGF-α and ERBB2 was not changed at any time.

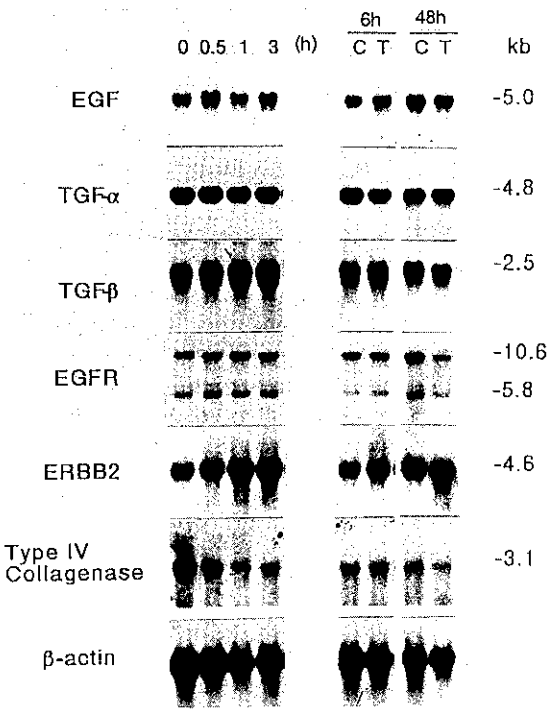

Fig. 4. Time course of effect of TAPP-Br on the expression of EGF, TGF-α, TGF-β, EGF receptor, ERBB2 and type IV collagenase mRNA by WiDr cells. Treatment with TAPP-Br and Northern blot analysis were performed as described in the legend to Fig. 3 (C, control; T, treated with 10 μM TAPP-Br).

The production of membrane-bound EGF precursor by WiDr was examined using ^{125}I-labeled anti-EGF antibody binding assay. The amounts of EGF precursor in the cells treated with TAPP-Br for 3 days and in non-treated cells were 130 ± 34 pg/10^6 cells and 42 ± 7.9 pg/10^6 cells, respectively, the difference being statistically significant ($P < 0.01$). The same amount of benzamidine did not cause any decrease in the level of EGF precursor.

Effect of TAPP-Br on protein kinase C activity and IP_3 production To examine the effect of TAPP-Br on protein kinase C signal transduction, the kinase activity was examined on WiDr cells after treatment with 10 μM TAPP-Br for 1 h or 3 days. Although total protein kinase C activity was not changed by TAPP-Br, the kinase activity of the particulate fraction was significantly decreased, whereas that of the soluble fraction was significantly increased (Table II; $P < 0.05$). A similar effect was noticed even on treatment for 1 h. Benzamidine treatment did not affect the total activity of protein kinase C or the localization of the kinase activity. Ten μM TAPP-Br did not show any inhibition of protein kinase C activity stimulated by TPA (data not shown).

The effect of TAPP-Br on IP_3 production by WiDr cells was examined. The levels of IP_3 in the cells treated with TAPP-Br for 3 days and in the non-treated cells were 0.92 ± 0.03 (average $\pm$ SE of duplicate) pmol/10^7 cells and 0.52 ± 0.03 pmol/10^7 cells, respectively, the IP_3 level in TAPP-Br-treated cells being significantly higher than that in non-treated cells ($P < 0.05$).

DISCUSSION

Inhibition of protease activity might be a possible strategy to reduce the metastatic potential of tumor cells,

Table II. Inhibitory Effect of TAPP-Br on Protein Kinase C Activity

	Protein kinase C activity[a]		
	Total activity	Soluble fraction	Particulate fraction
Control	6.98 ± 0.70[b]	3.34 ± 0.68	3.64 ± 0.76
Benzamidine	7.13 ± 0.65	3.69 ± 0.72 [c]	3.44 ± 0.77 [c]
TAPP-Br 1 h	6.39 ± 0.72	3.47 ± 0.67 [c]	2.91 ± 0.77 [c]
TAPP-Br 3 days	7.10 ± 1.10	4.99 ± 0.89	2.12 ± 0.98

a) Protein kinase C activity was measured in the particulate fraction and in soluble fraction obtained from WiDr cells after treatment with TAPP-Br (for 1 h or 3 days) or benzamidine for 3 days. Unit: (pmol/min/10^7 cells) $\times 10^{-3}$.
b) Values are the average $\pm$ SE of triplicate measurements.
c) Significantly different ($P < 0.05$).

since proteases play an important role in tumor invasion and metastasis by degrading the extracellular matrix.[3] As reported previously, production of type IV collagenase and stromelysin by gastric cancer cell lines is induced by EGF or TGF-α.[22] In this paper, we have shown the inhibitory effect of TAPP-Br on proliferation of all of the 8 colon cancer cell lines studied. TAPP-Br also inhibits the cell growth of leukemia, malignant melanoma, and malignant lymphoma,[10] and we recently found an inhibitory effect in esophageal and gastric cancer cell lines.

In the present study, mRNA expression of FOS, JUN, MYC, TGF-β, EGF receptor and type IV collagenase was found to be suppressed by TAPP-Br. These genes commonly have a TPA-responsive element (TRE) or serum-responsive element (SRE) in their promoter regions.[23-26] In the time course experiment, the expression of MYC, FOS and JUN was decreased at 3 h after the start of TAPP-Br treatment, while the expression of TGF-β, EGF receptor and type IV collagenase was decreased only after 48 h. It is well known that FOS and JUN form a heterodimeric protein complex that interacts with the AP-1 binding site and regulates the gene transcription.[27] On the other hand, decreased expression of TGF-β, EGF receptor and type IV collagenase was detected after 48 h, which is a sufficient period to allow synthesis of new protein. Therefore, the mechanism by which TAPP-Br decreases the expression of TGF-β, EGF receptor and type IV collagenase might be an indirect one through AP-1-dependent gene translation.

The expression of the genes containing SRE or TRE in their promoter is regulated by the signals from protein kinase C. Therefore, it is possible that TAPP-Br might suppress protein kinase C activity and decrease the gene expression. In the present study, we found that, although total protein kinase C activity was not changed by treatment with TAPP-Br for 3 days, the kinase activity in the particulate fraction was decreased, whereas that in the soluble franction was increased. The decreased activity in the particulate fraction was apparent at 1 h after the start of treatment. However we could not exclude the possibility that TAPP-Br may modulate the protein kinase C activity indirectly. We should examine the effect of TAPP-Br on the protein kinase C activity at early time points in more detail. Furthermore, to get more conclusive information, an experiment using cells whose protein kinase C is down-regulated should be done. As to IP_3 production, we found a decrease at 3 days after the initiation of the TAPP-Br treatment. IP_3 is known to increase transiently in response to some stimuli and then to decrease shortly afterwards. Therefore, we should examine in detail the effect of TAPP-Br on not only IP_3 production but also inositol phospholipid metabolism.

A relationship between oncogene expression and protease inhibition has been reported during chemical or radiation-induced carcinogenesis, which is suppressed by protease inhibitors such as antipain and leupeptin.[6, 8, 28] The expression of c-*fos* in BALB/c/3T3 is significantly decreased in the presence of antipain during carcinogenesis.[29] The c-*myc* RNA levels are reduced in proliferating normal mouse fibroblasts by antipain and leupeptin.[30] Although these studies did not deal with protein kinase C activity or other oncogenes and genes related to cell proliferation, overall they suggest that inhibition of cell proliferation and carcinogenesis by protease inhibitors might be commonly brought about by suppressing the expression of genes whose promoter contains SRE or TRE.

In the present study, TAPP-Br also inhibited the production of membrane-bound precursor of EGF, although the expression of EGF mRNA was not changed. Membrane-bound precursor of EGF is known to function in an autocrine or juxtacrine manner. EGF receptor kinase phosphorylates PLCγ upon EGF-binding and induces production of IP_3. Although it is not known at present how TAPP-Br suppresses the production of EGF precursor, a decrease in EGF precursor might be attributed to the reduction in protein kinase C activity.

In summary, it is suspected that the serine protease inhibitor, TAPP-Br, inhibits cell growth of all colon cancer cell lines through the suppression of TRE- or SRE-dependent gene expression. This compound is expected to be an effective tool for therapy of human colon cancer. Further study should be conducted to elucidate the precise point at which TAPP-Br affects phospholipid turnover.

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