Inhibition of Experimental Metastasis by an \( \alpha \)-Glucosidase Inhibitor, 1,6-Epi-cyclophellitol

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ABSTRACT

Isolated from a culture filtrate of *Phellinus* sp., cyclophellitol is a specific inhibitor of \( \beta \)-glucosidase, but unlike castanospermine, it does not inhibit experimental metastasis. However, its structural analogue, 1,6-epi-cyclophellitol, inhibited \( \alpha \)-glucosidase as well as \( \beta \)-glucosidase, and inhibited experimental metastasis. 1,6-Epi-cyclophellitol depressed \( \alpha \)-glucosidase activity in cultured B16/F10 cells after 48 h of incubation. Preincubation of B16/F10 cells for 48 h with 1,6-epi-cyclophellitol inhibited invasion of the cells in a Boyden chamber assay at the doses effective in inhibiting \( \alpha \)-glucosidase activity *in situ*. Pulmonary metastasis of B16/F10 cells in mice was inhibited by pretreatment of the cells with 1,6-epi-cyclophellitol in culture. The inhibitor reduced the collagen type I- and IV-mediated attachment of the cells, whereas it had no effect on laminin-mediated attachment. These results suggest that \( \alpha \)-glucosidase in tumor cells is essential for the metastatic process through the cellular interaction with collagen type I and IV.

INTRODUCTION

Carbohydrate residues on cell-surface glycoconjugates are suggested to modify malignant phenotypes (1). Glycosidase inhibitors were shown to perturb biosynthesis and oligosaccharide structure on the cell surface (2–6), changing the behavior of tumor cells. The v-sis-NRK cells lost serum and anchorage independence after treatment with castanospermine, an inhibitor of \( \alpha \)-glucosidases I and II and of \( \beta \)-glucosidase (7). Castanospermine also induced normal phenotypes in v-fms-transformed rat embryo cells (8).

Metastasis was also inhibited by disturbing the carbohydrate structure on the surface of neoplastic cells. For example, monoclonal antibodies against specific cell-surface carbohydrate structures were demonstrated to inhibit metastasis of highly metastatic variant of mouse melanoma cells (9). Liver metastasis of sarcoma L-1 tumor in mice was inhibited by blocking hepatocyte lectins with arabinogalactan infusions and \( \alpha \)-galactose (10). An inhibitor of glucosylceramide synthase was indicated to inhibit experimental metastasis of murine lung carcinoma cells (11).

Glycosidase inhibitors also suppress the metastatic potential of malignant cells by perturbing synthesis of the correct carbohydrate arrangement (12). Swainsonine (13) and mannostatin A (14, 15), which are \( \alpha \)-mannosidase II inhibitors, castanospermine (16), and NDZ2001 (17), a \( \beta \)-glucosidase inhibitor, have been reported to inhibit experimental metastasis.

Recently we isolated CPL (Fig. 1) from a culture filtrate of *Phellinus* sp. (18). CPL was demonstrated to be a specific and irreversible inhibitor of \( \beta \)-glucosidase, but it did not inhibit invasion of B16/F10 melanoma cells *in vitro* in our previous studies (19, 20).

In this paper we found that its synthetic epimer epi-CPL (Fig. 1) suppressed experimental metastasis, inhibiting both \( \alpha \)- and \( \beta \)-glucosidases.

MATERIALS AND METHODS

Epi-CPL was synthesized from cyclophellitol by Sharpless oxidation. CPL was prepared from the culture filtrate of *Phellinus* sp. as described previously (18). Nojirimycin was kindly supplied by Meiji Seika, Ltd. \( \alpha \)-Glucosidase, \( \alpha \)-fucosidase, \( \alpha \)-mannosidase, N-acetyl-\( \beta \)-D-glucosaminidase, and \( \beta \)-galactosidase were obtained from Boehringer Mannheim. Amyloglucosidase (exo-1,4- \( \alpha \)-o-glucosidase), \( \beta \)-glucosidase, \( \beta \)-xylosidase, p-nitrophenyl glycosides, 4-methylumbelliferyl glucosides, collagen type I and type IV, and laminin were purchased from Sigma. \( \beta \)-Glucuronidase was obtained from Funakoshi Pharmaceutical Co., Ltd. Polyvinylpyrrolidone-free polycarbonate filters (8 \( \mu \)m pore size) were obtained from Nucleopore. C57BL/6 mice were purchased from Charles River Japan.

Cell Conditions. B16/F10 murine melanoma cells (21) were supplied by Dr. S. Silagi through the Japanese Cancer Research Resources Bank. The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and kanamycin sulfate (0.2 mg/ml) at 37°C in a humidified atmosphere of 5% CO2/95% air.

Glycosidase Assay. Commercially available glucosidases in 25 mM sodium acetate buffer (pH 5.2; 20 \( \mu \)l) were incubated with epi-CPL or CPL for 10 min at 37°C. Each enzyme amount was set up to give an absorbance change of from 1.0 to 2.0 during the incubation period. p-Nitrophenyl glucosides (15 \( \mu \)mol) in 25 mM sodium acetate buffer (pH 5.2; 10 \( \mu \)l) were added to the enzyme/epi-CPL or CPL mixture and incubated for 10 min at 37°C. The enzyme reaction was stopped by addition of 150 \( \mu \)l of 0.4 mM glycine/NaOH buffer (pH 10.4), and liberated p-nitrophenol was detected at 410 nm following the method of Saul et al. (22).

Glucosidase Activity after Dialysis. Glucosidase activity after dialysis was detected as previously described (19). Epi-CPL was added to the \( \alpha \)-glucosidase solutions (500 \( \mu \)l) containing 30 \( \mu \)g/ml of yeast \( \alpha \)-glucosidase, 50 \( \mu \)g/ml bovine serum albumin, and 25 mM sodium acetate (pH 5.2). CPL or nojirimycin was added to the \( \beta \)-glucosidase solution (500 \( \mu \)l) containing 10 \( \mu \)g/ml almond \( \beta \)-glucosidase in 25 mM sodium acetate (pH 5.2). After incubation at room temperature for 60 min, 0.5 volume of each mixture was dialyzed against 2 liters of 25 mM acetate buffer for 12 h at 4°C. The rest stood for 12 h at 4°C without dialysis. p-Nitrophenyl glucopyranosides were added to the dialyzed and control samples and were tested for the enzyme activity as described above.

Glucosidase Activity in Melanoma Cells. Subconfluent cultures of B16/F10 cells were detached with 0.25% trypsin/0.02% EDTA, washed, and resuspended at \( 4 \times 10^6 \) cells/ml in Ca24, Mg2+ -free PBS. The cell suspension was stored at –80°C and homogenized by Ultraturrax immediately before use. The protein content of the cell lysate was measured by Bio-Rad protein assay kit. The glucosidase reaction was started by addition of Ultraturrax immediately before use. The protein content of the cell lysate was measured by Bio-Rad protein assay kit. The glucosidase reaction was started by addition of Ultraturrax immediately before use. The protein content of the cell lysate was measured by Bio-Rad protein assay kit. The glucosidase reaction was started by addition of Ultraturrax immediately before use. The protein content of the cell lysate was measured by Bio-Rad protein assay kit.

In Vitro Cell Invasion Assay. The assay of *in vitro* cell invasion was carried out essentially by the method described by Terranova et al. (24) and Albini et al. (25). Polycarbonate filters were coated with 15 \( \mu \)g collagen type I, 15 \( \mu \)g collagen type IV, and 15 \( \mu \)g laminin, in that order, and placed in a modified Boyden chamber. Conditioned medium (1.5 ml), obtained by incubation of NIH 3T3 cells in serum-free medium for 24 h, was placed in the lower compartment of the Boyden chamber. The B16/F10 cells (5 \( \times 10^5 \)) were removed from the bottom of flasks by incubation with 0.02% EDTA in Ca24, Mg2+-free PBS. The cell suspension was stored at –80°C and homogenized by Ultraturrax immediately before use. The protein content of the cell lysate was measured by Bio-Rad protein assay kit. The glucosidase reaction was started by addition of Ultraturrax immediately before use. The protein content of the cell lysate was measured by Bio-Rad protein assay kit.
Mg²⁺-free PBS for 20 min at 37°C and dispersed in 0.5 ml of Dulbecco's modified Eagle's medium containing 0.1% fetal bovine serum. The cell suspension was added to the upper chamber and incubated for 24 h at 37°C in a humid 5% CO₂ atmosphere. Then, the cells on the upper surface of the filter were completely removed by wiping, and the filters were fixed with methanol and stained with Harris' hematoxylin. The cells that had penetrated through the filter were counted in 10 fields under a microscope (×400). All the assays were done in triplicate.

Pulmonary Colonization Assay. Pulmonary colonization assay was carried out as described previously (13, 26). Seven mice (C57BL/6, 6 weeks old, female) group were inoculated with 1.25 × 10⁶ B16/F10 cells via a tail vein. Three weeks later the animals were sacrificed, and pulmonary metastasis was assayed by counting metastatic foci on the surface of the lungs.

Cell Attachment Assay. Cell attachment was assayed essentially as described by Inokuchi et al. (11). The cells were detached with EDTA and suspended in the culture medium, pelleted, and resuspended at 10⁵ cells/ml in the Dulbecco's modified Eagle's medium containing 0.1% fetal bovine serum. Fifty μl of the cell suspensions were placed in each well which was coated with collagen type I, type IV, or laminin, then the cells were incubated for 60 min for attachment. Then the medium was removed and the wells were washed with 50 μl PBS twice. Adhering cells were fixed with glutaraldehyde and stained with Giemsa. The fixed cells in at least five microscopic fields (×400 high power field) per well were counted. Each experiment was done in sextuple.

RESULTS

Inhibition of Glucosidases by Epi-CPL in Vitro. Epi-CPL inhibited both α- and β-glucosidases, but it showed no marked inhibitory activity against the other 6 enzymes tested (Table 1), whereas CPL inhibited only β-glucosidase (99%) and β-glucuronidase (66%). However, inhibition of β-glucosidase by epi-CPL (50% inhibitory concentration, 300 μM) was much weaker than that by CPL (50% inhibitory concentration, 1.4 μM) as seen in Fig. 2.

α-Glucosidase inhibitory activity of epi-CPL was increased by preincubation of the drug with the enzyme. Epi-CPL at 280 μM inhibited α-glucosidase only 20% without preincubation, but it inhibited the enzyme 81% with preincubation for 3 h at 8°C (Fig. 3). To investigate irreversibility of the inhibition we examined the influence of dialysis on α-glucosidase inhibition by epi-CPL. As shown in Table 2, inhibition of the enzyme by epi-CPL remained after dialysis. In contrast, inhibition of β-glucosidase by nojirimycin, which is a tightly bound inhibitor (27), was lost after dialysis.

Inhibition of B16/F10 Glucosidases in Cell Lysate by Epi-CPL. Epi-CPL effectively inhibited α-glucosidase activity in B16/F10 cell lysates (Fig. 4A). Epi-CPL treatment at 14 and 71 μM in culture medium for 48 h inhibited 50 and 90% of cellular α-glucosidase, respectively; it also inhibited β-glucosidase activity (Fig. 4B). On the other hand, CPL at 57 μM did not inhibit B16/F10 α-glucosidase at all (Fig. 4A), whereas it inhibited B16/F10 β-glucosidase completely (Fig. 4B).

Inhibition of Cell Invasion by Epi-CPL in Vitro. As shown in Fig. 5, Boyden chamber analysis showed that epi-CPL inhibited the invasiveness of B16/F10 cells at the same concentrations that inhibited cellular α-glucosidase activity (Fig. 4A). Greater than 80% inhibition of invasion was consistently observed with epi-CPL at 71 μM in multiple experiments, whereas CPL did not inhibit cell invasion at all, even at 570 μM. Neither epi-CPL nor CPL at 570 μM inhibited the growth of B16/F10 cells (data not shown).

Inhibition of Pulmonary Colonization by Epi-CPL. As shown in Fig. 6, when B16/F10 cells were treated with epi-CPL in cell culture, their metastatic activity was significantly decreased. The treated or untreated cells were injected into a lateral tail vein of C57BL/6 mice. Epi-CPL at 14 and 140 μM significantly inhibited the metastasis by about 50 and 80%, respectively.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>% of inhibition</th>
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</thead>
<tbody>
<tr>
<td>α-Glucosidase</td>
<td>Yeast</td>
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</tr>
<tr>
<td>β-Glucosidase</td>
<td>Almond</td>
<td>99%</td>
</tr>
<tr>
<td>Amyloglucosidase</td>
<td>Aspergillus niger</td>
<td>6%</td>
</tr>
<tr>
<td>α-Fucosidase</td>
<td>Beef kidney</td>
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</tr>
<tr>
<td>α-Mannosidase</td>
<td>Canavalia ensiformis</td>
<td>0%</td>
</tr>
<tr>
<td>N-Acetyl-β-D-galactosaminidase</td>
<td>Beef kidney</td>
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<td>β-Galactosidase</td>
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<tr>
<td>β-Glucuronidase</td>
<td>Escherichia coli</td>
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</tr>
<tr>
<td>β-Xylosidase</td>
<td>Aspergillus niger</td>
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Fig. 1. Structure of epi-CPL and CPL.

Fig. 2. Inhibition of glucosidases by epi-CPL and CPL. α-Glucosidase (yeast, 6.7 μg/ml) was preincubated with epi-CPL (●), and β-glucosidase (Escherichia coli, 6.7 μg/ml), with epi-CPL (●) or CPL (▲), for 10 min at 37°C, then enzyme activities were assayed.

Table 1 Effect of dialysis on glucosidase inhibition by epi-CPL

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>α-Glucosidase (%)</th>
<th>β-Glucosidase (%)</th>
<th>Nojirimycin (%)</th>
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<tr>
<td>Epi-CPL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialyzed</td>
<td>96.7</td>
<td>98.7</td>
<td>80.9</td>
</tr>
<tr>
<td>Control</td>
<td>86.9</td>
<td>96.7</td>
<td>57.1</td>
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</table>

Table 2 Effect of epi-CPL on sugar-hydrolyzing enzymes

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INHIBITION OF METASTASIS BY EPI-CYCLOPHELLITOL

Fig. 4. Effect of epi-CPL and CPL on α- and β-glucosidases in B16/F10 cells. The cells were cultured alone (○), or with 2.9 μM (●), 14 μM (▲), or 71 μM (△) epi-CPL or with 57 μM CPL (△) for 48 h, and collected. The activities of α-glucosidase (A) and β-glucosidase (B) in the cell lysate were assayed.

Fig. 5. Inhibition of B16/F10 cell invasion in vitro by preincubation with epi-CPL. The cells were cultured with epi-CPL or CPL for 48 h. and then used in an in vitro cell invasion assay. The results are expressed as the mean ± SD of 3 determinations. * and **, P < 0.05 and P < 0.01, respectively. The data are representative of 5 experiments.

Fig. 6. Inhibition of B16/F10 pulmonary invasion by preincubation of the cancer cells with epi-CPL. B16/F10 cells were cultured with epi-CPL for 48 h; then the cells were collected and injected into tail veins of mice. Results are expressed as the mean ± SD of 7 mice. * and **, P < 0.05 and P < 0.01, respectively. The data are representative of 2 experiments.

Effects of Epi-CPL on Adhesion of B16/F10 Cells. We used the mixture of collagen types I and IV, and laminin-coated membrane in a Boyden chamber assay. Therefore, we tested the effect of epi-CPL treatment on adhesion of the cells to collagen type I, type IV, and laminin. As shown in Fig. 7, adhesion of the cells to collagen type I and type IV was significantly reduced by epi-CPL but not by CPL. However, the adhesion to laminin was unaffected by epi-CPL.

DISCUSSION

We found that experimental metastasis of B16/F10 murine melanoma was suppressed by epi-CPL but not by CPL. Thus, the inhibition of metastasis by epi-CPL appears to be based upon its additional enzyme inhibitory activity, i.e., inhibition of α-glucosidase. We showed that α-glucosidase in B16/F10 cell lysate was inhibited by epi-CPL at the same concentrations that inhibited experimental metastasis of the same cells. Also, only epi-CPL but not CPL reduced the adhesion of B16/F10 cells to collagen types I and IV. These observations suggest that α-glucosidases in tumor cells are essential for metastasis, possibly modulating the cellular interaction with collagen type I and type IV.

It is possible that epi-CPL inhibits glucosidase I and/or II, which are N-linked oligosaccharide-processing enzymes to suppress metastasis like castanospermine (16). Castanospermine and deoxynojirimycin also inhibit α-glucosidase in human cell lysates, using 4-methylumbelliferyl-α-glucoside as substrate (19). Castanospermine-treated cells have been reported to attach laminin normally but it is not known whether castanospermine reduced cell attachment to substrate of collagen type I and type IV. A second possibility is that epi-CPL inhibits glycolipid hydrolysis. In fact, roles of the oligosaccharide moiety in metastasis were suggested by many observations (9, 10, 28–32), and inhibition of specific glycolipid hydrolysis appears to influence the metastatic potential by disturbing the balance of oligosaccharide organization. Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, an inhibitor of glucosylceramide synthase, inhibits experimental metastasis of murine lung carcinoma cells, and also reduces the cell attachment to laminin specifically (11). It is not known whether threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol reduced cell attachment to collagen type I and type IV.

Inhibitors of N-linked oligosaccharide processing have been reported to inhibit human melanoma cell invasion with a concomitant decrease in collagenase IV expression (33). It was also reported that inhibition of N-linked oligosaccharide processing in murine lymphoma and human melanoma was associated with enhanced tissue inhibitor of metalloproteinases gene expression (34). It is possible that epi-CPL also modify expression of collagenase or its inhibitor.

Epi-CPL is much less toxic than castanospermine. Epi-CPL at 570 μM did not inhibit the growth of B16/F10 cells in 4 days of culture. Although castanospermine has been shown to inhibit pulmonary colo-
nization of B16/F10 cells at several μM concentrations (16), we found that it inhibited the growth of the cell at 10 μM.

CPL is a potent and irreversible inhibitor of β-glucosidases in vivo (35). CPL was shown to be a time-dependent irreversible inactivator of both the Agrobacter sp. β-glucosidase and almond β-glucosidase by kinetic study (20). It is likely from its structure and from our dialysis experiment that epi-CPL is also an irreversible and long-lasting inhibitor and that it would be effective in vivo to suppress metastasis. Other derivatives of CPL may also contribute to the study of the involvement of carbohydrates in malignant cell movement and of the chemotherapy of cancer.

REFERENCES


24. Kojima, N., and Hakomori, S. Specific interaction between gangliotriaosylceramide (Gg3) and sialosylactosylceramide (Gm3) as a basis for specific cellular recognition between lymphoma and melanoma cells. J. Biochem. Chem., 264: 20159–20162, 1989.


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