Uptake and metabolism of BuCast: a glycoprotein processing inhibitor and a potential anti-HIV drug

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We have previously shown (Sunkara et al., 1989; Taylor et al., 1991) that 6-o-butanoyl castanospermine (BuCast) was a 30–50-fold better inhibitor of HIV syncytia formation than castanospermine (Cast). Radiolabeled Cast and BuCast were used to study the uptake and metabolism of these compounds in cultured cells and in mice. BuCast was preferentially taken up by cells compared to Cast. Approximately 30–50-fold higher radioactivity was found in cells treated with BuCast compared to cells treated with Cast during the initial 4–6 h of labeling. HPLC analysis showed that once BuCast was taken up by cells, it was rapidly converted to Cast. Mice given oral doses of BuCast had 5–10-fold higher levels of Cast in the plasma and tissues as compared to Cast treated mice. However, when the compounds were given i.v., the levels of plasma and tissue radioactivity obtained from Cast or BuCast were equivalent suggesting rapid conversion of BuCast to Cast in the blood. In mice orally treated with BuCast, HPLC analysis suggested that only Cast was found in the plasma and tissues. With multiple dosing of mice, additive results were obtained, suggesting that multiple doses may be used to obtain higher concentrations of the compound in the target cells. These data suggest that the lipophilic properties of butanoyl side chain on the Cast ring makes BuCast significantly better absorbed, and this may help to alleviate some of the gut toxicity associated with Cast treatment.

Key words: HIV/AIDS/glycoproteins/inhibitors

Introduction

During biosynthesis of N-linked glycoproteins, the oligosaccharide portion is assembled on the lipid carrier dolicholpyrophosphate. Lipid-linked oligosaccharide (Glc₃Man₉GlcNAc₂) is then transferred en bloc to the acceptor protein. The oligosaccharide portion then undergoes a series of processing reactions in which three terminal glucose and six mannoses are removed. For complex and hybrid glycans the core oligosaccharide structure (Man₃GlcNAc₂) is then processed further by addition of different sugars to give the final glycoprotein (For reviews, see Hubbard et al., 1985; Tyms et al., 1990; Elbein, 1991; Moremen et al., 1994). The first reaction in the processing pathway of Glc₃Man₉GlcNAc₂ is removal of the terminal α-1,2-linked glucose, which is catalyzed by α-glucosidase I. α-Glucosidase I has been the target for anti-HIV drug development. Inhibition of α-glucosidase I by alteration of gp120 of HIV has been shown to have anti-HIV potentials (Grunerts et al., 1987; Tyms et al., 1987; Walker et al., 1987). Recently, N-butyl-1-deoxynojirimycin (N-BuDNJ), an inhibitor of α-glucosidase I (IC₅₀ = 0.05 μg/ml, our unpublished data for isolated enzyme) was tested for safety and efficacy in AIDS patients. These studies showed that, although N-BuDNJ had gastrointestinal toxicity, possibly due to its potent inhibition of intestinal sucrase, based on suppression of HIV p24 antigen and increase in CD4 cell count, the study suggested that N-BuDNJ may possess anti-HIV activity (Fischel et al., 1994). These results suggest that other compounds of this class might be effective as anti-HIV agents. Cast is a potent inhibitor of isolated α-glucosidase I (IC₅₀ = 0.02 μg/ml; Kang et al., 1995). However, in cell culture studies, 500 times more Cast was required (IC₅₀ = 10 μg/ml) for inhibition of the enzyme in intact cells (Kang et al., 1995). Furthermore, the Cast concentration required for significant reduction in HIV infectivity was similar (IC₅₀ = 7 μg/ml; Sunkara et al., 1989; Taylor et al., 1991) to that required for inhibition of the intracellular enzyme. Cast also showed only moderate therapeutic activity (IC₅₀ = 1–2 μg/ml) against Moloney leukemia virus (MOLV) and Rauscher leukemia virus (RLV) infections (Ruprecht et al., 1989; Sunkara et al., 1989). Since lipophilicity might be an important attribute for increasing the efficacy of this type of inhibitor, particularly to improve cellular uptake, we synthesized a series of acyl derivatives of Cast in an attempt to improve their inhibitory activity against α-glucosidase I in cultured cells. Several of these compounds showed improved inhibition of α-glucosidase I in cultured cells (Kang et al., 1995) as well as improved inhibition of HIV infectivity in vitro (Sunkara et al., 1989; Tyms et al., 1990; Taylor et al., 1991). One analog, BuCast (MDL 28,574), had minimal inhibitory activity against sucrase (IC₅₀ = 12 μg/ml, B. L. Rhinehart, personal communication) in comparison to Cast (IC₅₀ = 0.004 μg/ml; Robinson et al., 1989). BuCast also proved to be a better anti-HIV agent in vitro and is currently in clinical trials as an anti-AIDS drug. This study was initiated to compare the uptake of Cast with BuCast by cultured cells and after oral dosing of mice.

Results

BuCast distribution—in vitro

Figure 1 demonstrates that in B₁₀F₁₀ and JM-1 cells incubated with radiolabeled compounds, the incorporation of ¹⁴C-labeled Cast into the cells was fairly linear with time up to 24 h. However, with BuCast, there was rapid uptake of radioactivity during the first 4–6 h of incubation. Cell associated radioactivity slowly decreased after 24 h. During the first 4–6 h, 30–50 times more radioactivity was incorporated in the cells incubated with BuCast than with Cast. The ratio of radioactivity incorporated from BuCast to...
radioactivity incorporated from Cast dropped to 7–10 times by 24–48 h in JM-1 cells (Figure 1B). In initial 4–6 h of incubation of 1 million JM-1 cells/ml with BuCast, 2 μmol of Cast per gram of cells accumulated in the cells compared to the accumulation of 0.075 μmol of Cast per gram of cells during incubation with Cast. The ratio of intracellular to extracellular (medium) radioactivity by 24–48 h of incubation with BuCast was 13–18 as compared to 2 with Cast. These results suggest preferential uptake of BuCast by the cells compared to Cast. Similar results were obtained when radiolabeled Cast or BuCast uptake was studied in H9 cells chronically infected with HIV (Taylor et al., 1994). When lysates from B6F10 cells incubated for 2 h with radioactive BuCast were analyzed by HPLC, the radioactivity was in the form of Cast rather than BuCast (Figure 2). Similar results were obtained when B6F10 cells incubated with BuCast for 30 min were analyzed. Small amount of radioactive background detected at fraction 10 in Figure 2 is probably due to BuCast contamination of HPLC column from the previous run since this was not routinely observed. In incubations of BuCast at 37°C with B6F10 Cell media, 30% of original BuCast was recovered at 2 h. These studies suggest that once BuCast is taken up by the cells it is rapidly converted into Cast.

**BuCast distribution—*in vivo***

Mice were given 84 μmol/kg as a single oral dose of 14C-labeled (5 μCi/mouse) Cast (16 mg/kg) or BuCast (25 mg/kg). Approximately 5 times more radioactivity was recovered in the plasma of BuCast treated mice than in the plasma of Cast treated mice at 2 and 5 min after the dose (Figure 3). At 5 min this represents 63 μM concentration of the compound in plasma of mice dosed with 25 mg/kg.
of BuCast. When plasma samples obtained 5 min after oral treatment of mice with BuCast were analyzed, only Cast was recovered. Similar results were obtained with rats (data not shown). These results suggest that BuCast may be preferentially absorbed from gastrointestinal tract compared to Cast and rapidly converted to Cast. It is not clear from these results whether conversion of BuCast occurs during absorption or immediately in the plasma. However, data from i.v. studies (see below) suggest that BuCast may be hydrolyzed immediately in the plasma. The rate of hydrolysis of BuCast to Cast in the fetal bovine serum varied with the pH, higher the pH, faster the hydrolysis. At neutral pH and 37°C in 10% fetal bovine serum, 60% of BuCast was converted to Cast in 30 min. Figure 4 shows incorporation of radioactivity into the tissues of orally dosed mice. Approximately 5–10 times more radioactivity was incorporated into thymus, spleen, liver, and brain tissues 5 min after dosing with BuCast than with Cast. However, by 2 h after dosing, the tissue levels of radioactivity obtained from Cast or BuCast had declined and were similar, suggesting rapid clearance of the drugs. Although considerably less radioactivity was recovered in the brain in comparison to other tissues, the data suggest that Cast crosses the blood brain barrier. When tissue samples of thymus and spleen from orally dosed mice with BuCast for 5 min were analyzed by HPLC no BuCast was present, only Cast was detected. Results similar to those with tissues described in Figure 4 were obtained when single cell suspensions made from tissues of orally dosed mice were analyzed for radioactivity, suggesting intracellular uptake of radioactivity after oral administration of Cast and BuCast (data not shown).

If BuCast is rapidly converted to Cast in the blood then equimolar amounts of Cast or BuCast given i.v. should result in similar tissue radioactivity. Figure 5 demonstrates that when equimolar doses of Cast or BuCast were given i.v. to mice, similar amounts of tissue and plasma radioactivity were recovered with either Cast or BuCast at five min post dose. With a 10-fold higher dose of Cast, approximately 10 times more radioactivity was recovered. Similar results were obtained when rats were given i.v. doses of Cast or BuCast (data not shown). Analysis of plasma of BuCast treated mice 5 min after i.v. dose suggested that that radioactivity was in the form of Cast. These data indicate that BuCast in the blood of mice is rapidly converted to Cast. These data support the hypothesis that the higher concentration of Cast in the plasma from mice orally dosed with BuCast was responsible for higher amounts of radioactivity in the tissues. Conversely, the data show that higher tissue radioactivity levels in BuCast dosed mice are not due to preferential tissue uptake of plasma BuCast.
Fig. 3. Increased (5-fold) plasma radioactivity following BuCast (vs Cast) after oral administration in mice. Mice were given a single dose of 5 μCi (84 μmol/kg) per 10 gm mouse of 14C labeled Cast (solid bars) and BuCast (open bars) by oral gavage, and blood was collected by puncturing the heart at the indicated time. Plasma was recovered by centrifugation, and an aliquot of plasma was counted to determine the radioactivity.

**Uptake into the target cells**

Figure 6 shows the uptake of radioactivity into lymphocytes of orally dosed mice, isolated by gradient centrifugation from peripheral blood (PBL), bone marrow, and thymus. Approximately a 5-fold greater level of radioactivity was observed following oral administration of BuCast compared to Cast at 5 min post dose. Similar results were obtained when lymphocytes were isolated at 2 and 15 min after oral administration of these compounds (data not shown). The ratio of BuCast to Cast radioactivity in the target cells (PBLs) was similar to that obtained in plasma and tissues (Figures 3 and 4). These data indicate that oral administration of BuCast will result in higher levels of Cast in the target tissues than can be obtained with equimolar oral doses of Cast.

**Effect of varying and multiple dosing**

Oral administration of 21, 42, and 84 μmol/Kg of BuCast to mice resulted in a dose dependent increase in radioactivity in the thymus, liver, brain, and spleen. In the thymus, 0.87, 1.75, and 2.33 pmol of compound per gram of tissue was recovered with the above doses. These results suggest that higher doses may be used to attain higher levels of compound in the tissues. In multiple dosing experiments 5 min after a single oral dose (84 μmol/kg, 5 μCi) of Cast or BuCast approximately 5-fold more radioactivity was incorporated into the thymus, spleen, liver, brain, and plasma with BuCast compared to Cast. A second equivalent dose of BuCast or Cast given 5 min after the initial dose, gave a 3–4-fold higher level of radioactivity than was obtained with a single dose (data not shown). These results suggest that repeated dosing with BuCast may be used to obtain higher tissue levels.

**Discussion**

The results presented above demonstrate that in mouse melanoma and human T cells, the uptake of Cast was linear with time. In incubations with BuCast, there was a rapid uptake of radioactivity into the cells in the initial stages of incubation resulting in a 30–50-fold higher amount of radioactivity with BuCast as compared to Cast. By 24–48 h, 7–10-fold higher radioactivity was observed in the cells labeled with BuCast as compared to Cast. A 30–50-fold higher intracellular drug concentration with BuCast as compared to Cast during the initial 4–6 h of incubation is consistent with the observed 30–50-fold higher activity of BuCast compared to Cast against HIV in cell culture assays (Sunkara et al., 1989; Tyms et al., 1990; Taylor et al., 1991). Preferential uptake of BuCast compared to Cast by cells in in vitro experiments was also demonstrated by higher uptake of BuCast by gastrointestinal absorption. Bioavailability studies indicate 5–10-fold higher levels of the active
metabolite Cast were achieved in the target lymphocytes, tissues, and plasma of mice after oral administration of BuCast compared to Cast, suggesting that BuCast was absorbed to a greater extent than Cast. This may be due to lipophilicity contributed by the side chain of BuCast. This study suggests that BuCast is rapidly converted to Cast in the plasma and increased amount of Cast in the blood of animals orally treated with BuCast may be responsible for higher levels of Cast obtained in the target cells. Although these results indicate a short half-life of Cast in the plasma of treated mice, clinical studies in progress with asymptomatic patients showed a more than 20 h half-life for Cast in the plasma of BuCast treated patients (data not shown). Superior oral bioavailability of BuCast compared to i.v. administration may be useful for long-term outpatient care. Repeated dosing with BuCast may be used
to achieve therapeutic levels of the compound. Results with varying doses suggest that absorption of these compounds may not be a saturable process. Therefore, higher doses may be used to obtain efficacious levels in the target tissues. Use of lower levels of BuCast than Cast to achieve equivalent plasma and tissue levels combined with relatively low inhibitory activity to BuCast against sucrase may significantly lower the potential gastrointestinal toxicity associated with glucosidase inhibitors. This should allow higher levels of the active inhibitor to be obtained with lower side effect liability.

**Materials and methods**

Cast was isolated from *Castanospermum australe* seeds at the Marion Merrell Dow Research Institute (Liu et al., 1986). BuCast was synthesized as described earlier (Liu et al., 1990). $^{14}$C-radiolabeled Cast and BuCast

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**Fig. 5.** Comparison of incorporation of radioactivity from $^{14}$C-labeled Cast or BuCast into mouse tissues and plasma. The animals were given a single dose of 1 μCi Cast or BuCast (8.4 μmol/kg) or 10 μCi Cast (84 μmol/kg) by i.v. Plasma and tissue samples were processed as in Figure 3 and 4, respectively, for radioactivity determination.
were prepared by a stepwise synthesis of Cast and BuCast with radiolabel at the C7 position of the Cast ring. For uptake studies in cells, mouse melanoma (B16F10) cells and human (JM-1) cells were used. For in vivo uptake studies, 4-5 week old C57 female mice weighing 10-12 g were obtained from Charles River Laboratories. Lympholyte-M was obtained from Cederlane Laboratories. Falcon Cellstrainers #2350 (Becton Dickinson Labware), IEC centra-8 centrifuge (International Equipment Company), Econolume scintillation fluid (ICN), cell culture media (GIBCO Laboratories), and a Tri-Carb 1900 CA scintillation counter (PACKARD) were used in these studies.

Cell culture studies

JM-1 cells were grown in suspension culture in RPMI-1640 media with HEPES buffer containing 12% heat-inactivated fetal calf serum, 20 mM L-glutamine and 1 x of penicillin/streptomycin antibiotic mixture. B16F10 cells were grown as monolayers in GIBCO's MEM-57 media containing 10% heat-inactivated fetal calf serum and penicillin/streptomycin antibiotic mixture. Cells were incubated with 1 ptCi/ml of 14C-labeled Cast or BuCast (specific activity 8-10 mCi/mmol). Following each incubation period the cells were washed six times with cold PBS to remove unincorporated radioactivity. The cells were lysed in 1% SDS for radioactivity determination or scraped with a rubber policeman for HPLC analysis. Samples were mixed with 20 ml of Econolume and counted for radioactivity determinations.

Animal studies

14C-labeled (5 μCi, Sp. Ac. 4 mCi/mmol) Cast or BuCast in equimolar doses of 84 μmol/kg (16 mg/kg Cast and 25 mg/kg BuCast) was administered orally to 4–5 week old mice for the indicated times. At the end of each experiment, tissue samples were removed, washed with cold phosphate-buffered saline (PBS) and processed to determine the radioactivity in the samples. For radioactivity determination in tissue samples, the tissues were homogenized in 1 ml of water and another 1 ml of 1% SDS was added to solubilize the cells. For radioactivity determination in the cells obtained from tissues, the tissues were homogenized in 4–5 ml of cold PBS, filtered through a nylon filter, centrifuged at 1000 r.p.m. in an IEC centrifuge for 5 min to recover the cell pellet, and then lysed in 1% SDS before counting. For HPLC analysis samples of plasma, tissues or cell pellets were extracted in 80% acetonitrile in water (see below).

For isolation of peripheral blood lymphocytes (PBLs), blood samples were diluted 2-3-fold in PBS at room temperature and the diluted blood was layered onto Lympholyte-M for gradient separation. For bone marrow (BM) PBL isolation, cells obtained from BM were filtered through a nylon filter as described above and separated on Lympholyte-M. For thymus PBLs, cells were recovered after homogenization and then separated on a Lympholyte-M gradient. For gradient separation of PBLs, the tubes were centrifuged at 1500 r.p.m. in an IEC centrifuge for 30 min at room temperature. PBLs at the interface were removed, washed with several volumes of cold PBS, pelleted by centrifugation, and lysed in 1% SDS for counting. For the repeated dosing, mice were orally dosed at 5 min intervals. At the end of the experiment (total 10 min for two doses) tissue and plasma samples were collected and analyzed as described above for the single dose experiments.

HPLC analysis

For HPLC analysis, cell pellets and tissue samples were extracted with 80% acetonitrile. Cell pellets were sonicated for 10 s in 1 ml of 80% acetonitrile. Whole tissues were homogenized in 1 ml (thymus and spleen), 2 ml (brain), or 3 ml (liver) 80% acetonitrile and allowed to sit at -20°C for 60 min. Samples were centrifuged at 1500 r.p.m. in an IEC centrifuge for 5 min and 80% of the supernatant was evaporated to dryness at 37°C under a stream of nitrogen. The samples were redissolved in 100 μl of mobile solvent (85:15:0.001 M ammonium acetate: acetonitrile, pH 5.0), filtered through 0.45 μM filters, and analyzed by HPLC. For calibration of HPLC columns, samples of 14C-radiolabeled Cast and BuCast were diluted to

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\text{Fig. 6. Incorporation of radioactivity from } ^{14}\text{C-labeled Cast (solid bars) or BuCast (open bars) into lymphocytes isolated from blood (PBL), bone marrow, or thymus. Mice were orally dosed as in Figure 3. Blood and tissues were removed 5 min after dosing and lymphocytes were isolated by gradient centrifugation and radioactivity was determined as described in Materials and methods.}
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800 cpm/ml. (1:250,000 dilution of ¹⁴C-labeled 10 mM samples) with mobile solvent. The samples were analyzed using a Zorbax Rx 4.6 x 150 mm column at a flow rate of 1 ml/min. The fractions were counted in 10 ml of counting fluid. Cast was eluted in fractions 3 and 4 and BuCast was eluted in fraction 10. The BuCast sample contained approximately 5–10% Cast as an impurity (data not shown).

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Abbreviations
Cast, castanospermine; BuCast, 6-o-butanoyl castanospermine; PBL, peripheral blood lymphocytes; BM, bone marrow; PBS, phosphate-buffered saline; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid; HPLC, high performance liquid chromatography.

References