Ceramide-β-d-Glucuronide: Synthesis, Digestion, and Suppression of Early Markers of Colon Carcinogenesis


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/22/5768

Cited Articles
This article cites by 28 articles, 9 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/22/5768.full.html#ref-list-1

Citing articles
This article has been cited by 15 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/59/22/5768.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.
Ceramide-β-D-Glucuronide: Synthesis, Digestion, and Suppression of Early Markers of Colon Carcinogenesis

Eva M. Schmelz, Anatolyi S. Bushnev, Dirck L. Dillehay, M. Cameron Sullards, Dennis C. Liotta, and Alfred H. Merrill, Jr.

Departments of Biochemistry [E. M. S., M. C. S., A. H. M.] and Chemistry [A. S. B., D. C. L.] and Department of Pathology and Division of Animal Resources [D. L. D.], Emory University, Atlanta, Georgia 30322-3050

ABSTRACT

Dietary sphingolipids inhibit chemically induced colon cancer in mice. The most likely mediators of this effect are the metabolites ceramide (Cer) and sphingosine, which induce growth arrest and apoptosis in transformed cells. Sphingolipids are digested in both the upper and the lower intestine; therefore, a more colon-specific method of delivery of sphingolipids might be useful. A Cer analogue with a β-D-glucuronic acid attached at the primary hydroxyl of N-palmitoyl-N-sphingosine (Cer-β-D-glucuronide) was synthesized and evaluated as a substrate for Escherichia coli β-glucuronidase and colonic digestion, as well as for suppression of early events in colon carcinogenesis in CF1 mice treated with 1,2-dimethylhydrazine. Purified β-glucuronidase (EC 3.2.1.31) and colonic segments (as a source of colonic enzymes and microflora) hydrolyzed Cer-β-glucuronide to release Cer, as analyzed by tandem mass spectrometry. More than 75% of the Cer-β-glucuronide was cleaved in an 8-h incubation with the colonic segments. When Cer-β-glucuronide was administered for 4 weeks as 0.025% and 0.1% of the diet (AIN 76A) to 1,2-dimethylhydrazine-treated mice, there were significant reductions in colonic cell proliferation, as determined by in vivo BrdUrd incorporation, and in the appearance of aberrant crypt foci. The effect of dietary Cer-β-glucuronide on aberrant crypt foci correlated significantly with the length of the colon, which suggests that Cer-β-glucuronide was most effective when there was a larger compartment for digestion. Thus, synthetic sphingolipids that target the colon for the release of the bioactive backbones offer a promising approach to colon cancer prevention.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer mortality in the United States (1), and considerable progress has been made in identifying genetic and environmental factors that influence colon cancer risk. Nonetheless, many of the effects of the diet on colon cancer remain poorly understood; hence, there have been few instances in which findings with these naturally occurring compounds have led to the development of more effective chemopreventive agents.

Recent studies have shown that dietary sphingolipids inhibit DMH-induced colon cancer in CF1 mice (2–4), presumably because they are digested to the lipid backbones Cer and sphingosine4 that inhibit cell growth and induce differentiation and apoptosis (5). However, substantial amounts of dietary sphingomyelin and other sphingolipids are digested and absorbed in the upper small intestine (6–8), which reduces the amount of bioactive molecules reaching the colon.

A strategy for colon-specific drug delivery that has been used with other compounds, such as dexamethasone, retinoic acid, and budesonide (9–11), is to prepare the conjugate with β-D-glucuronic acid. The resulting glycoside should be poorly digested in the upper intestine but sensitive to hydrolysis to the aglycon by bacterial β-glucuronidase in the colon.

In this study, we synthesized Cer-β-glucuronide (Fig. 1) and evaluated the ability of β-glucuronidase(s) from Escherichia coli or intestinal segments to release the head group. In addition, Cer-β-glucuronide was fed to DMH-treated mice to determine whether this sphingolipid analogue could suppress early markers of colonneoplasia (increased crypt cell proliferation and formation of ACF). Based on the findings of these studies, Cer-β-glucuronide is a promising candidate for colon-specific delivery of bioactive sphingolipids.

MATERIALS AND METHODS

Synthesis. Cer-β-glucuronide was synthesized as follows: O-(2,3,4-tri-O-pivaloyl-α-D-glucopyranosyl)methyl(trichloroacetimidate)-trichloroacetimidate was prepared from β-D-glucurono-6,3-lactone (Aldrich, Milwaukee, WI) by methylation of the lactone with sodium methoxide in methanol, followed by treatment of the formed methyl esters with pivaloyl chloride in pyridine (12). After selective hydrolysis of the glycosidic pivaloyl group of the protected β-glucuronic acid by treatment with hydrazine acetate (13) or saturated ammonia in methanol (14), potassium hydride was added to react the formed hemiacetal with deoxyuridine.

Ceramide-β-glucuronide (9–11), is to prepare the conjugate with β-D-glucuronic acid. The resulting glycoside should be poorly digested in the upper intestine but sensitive to hydrolysis to the aglycon by bacterial β-glucuronidase in the colon.

In this study, we synthesized Cer-β-glucuronide (Fig. 1) and evaluated the ability of β-glucuronidase(s) from Escherichia coli or intestinal segments to release the head group. In addition, Cer-β-glucuronide was fed to DMH-treated mice to determine whether this sphingolipid analogue could suppress early markers of colonneoplasia (increased crypt cell proliferation and formation of ACF). Based on the findings of these studies, Cer-β-glucuronide is a promising candidate for colon-specific delivery of bioactive sphingolipids.

MATERIALS AND METHODS

Synthesis. Cer-β-glucuronide was synthesized as follows: O-(2,3,4-tri-O-pivaloyl-α-D-glucopyranosyl)methyl(trichloroacetimidate)-trichloroacetimidate was prepared from β-D-glucurono-6,3-lactone (Aldrich, Milwaukee, WI) by methylation of the lactone with sodium methoxide in methanol, followed by treatment of the formed methyl esters with pivaloyl chloride in pyridine (12). After selective hydrolysis of the glycosidic pivaloyl group of the protected β-glucuronic acid by treatment with hydrazine acetate (13) or saturated ammonia in methanol (14), potassium hydride was added to react the formed hemiacetal with deoxyuridine.

Ceramide-β-glucuronide (9–11), is to prepare the conjugate with β-D-glucuronic acid. The resulting glycoside should be poorly digested in the upper intestine but sensitive to hydrolysis to the aglycon by bacterial β-glucuronidase in the colon.

In this study, we synthesized Cer-β-glucuronide (Fig. 1) and evaluated the ability of β-glucuronidase(s) from Escherichia coli or intestinal segments to release the head group. In addition, Cer-β-glucuronide was fed to DMH-treated mice to determine whether this sphingolipid analogue could suppress early markers of colonneoplasia (increased crypt cell proliferation and formation of ACF). Based on the findings of these studies, Cer-β-glucuronide is a promising candidate for colon-specific delivery of bioactive sphingolipids.
Louis, MO; 30 mg/kg body weight in 1 mL EDTA) once a week for 6 weeks while fed a semipurified AIN 76A diet (Dyets, Bethlehem, PA). This diet is essentially sphingolipid free (3). After a rest period of 1 week, the diets of the experimental groups were supplemented with 0.025% or 0.1% Cer-

Ronide (by weight), whereas the control group received the AIN 76A diet without supplementation. These amounts were chosen because sphingomyelin (2–4) and glycosphingolipids (25) at 0.025% and 0.1% of the diet significantly reduced ACF by 50–70%. One group of mice (untreated controls) was not injected with the carcinogen and was fed the unsupplemented control diet. After 4 weeks of consuming the different diets, the mice were killed by CO2 asphyxiation, and the colons were excised, opened longitudinally, rinsed, and fixed overnight in fresh 10% buffered formalin. The ACF were stained with 0.2% methylene blue for approximately 20 min and scored by light microscopy in a blinded manner.

**BrdUrd Staining of Proliferating Cells in Situ.** On the last day of the study, five mice/group (treated as described above) were injected i.p. with 1 mL of BrdUrd solution (In Situ Cell Proliferation Kit 1758756; Boehringer Mannheim, Indianapolis, IN) per 100 g of body weight to label proliferating cells during S phase. After 2 h, the mice were killed by CO2 asphyxiation, and the colons were removed, opened longitudinally, flushed with cold PBS, and fixed overnight in 10% neutral buffered formalin. After determination of the number and size of the ACF, the tissues were embedded in paraffin. Sections of 3–5 μm were deparaffinized with fresh xylene and rehydrated through graded alcohol (100%, 95%, 80%, 50%, and 30%). According to the manufacturer’s instructions, the colons were incubated with an alkaline phosphatase-conjugated anti-BrdUrd monoclonal antibody to detect incorporated BrdUrd. This antibody was visualized with Fast Red, which forms an insoluble colored compound precipitating at the site of the immunocomplex. The sections were covered with an aqueous mounting solution and examined by light microscopy at ×10 or ×40 in a blinded manner (50 fully visible crypts were scored per animal).

**Enzymatic Digestion of Cer-β-glucuronide by β-Glucuronidase.** Cer-β-glucuronide (15 μg in chloroform:methanol, 1:1, v/v), 15 μg of phosphatidylycerine (from egg yolk), and 30 μM sodium cholate (final concentration; both from Sigma) were placed in a glass test tube, the solvents were evaporated under nitrogen, and 100 μl of potassium phosphate buffer (0.02 M, pH 6.8) containing 0.001 M EDTA were added. After sonication for about 2 min (or until the solution was clear), β-glucuronidase from E. coli (Sigma) in phosphate buffer (1000 units in 100 μl) was added, and the mixture was incubated at 37°C for up to 8 h. At the indicated time points, the reaction was stopped with 1 mL of methanol. Intact Cer-β-glucuronide and the hydrolysis product Cer were analyzed by mass spectrometry using a Perkin-Elmer-Sciex API 3000 triple quadrupole mass spectrometer (as described below).

**Dietary Effects of Cer-β-glucuronide.** When Cer-β-glucuronide was solubilized with mixed micelles with phosphatidylycholine and chololate and incubated with β-glucuronidase from E. coli, cleavage of the head group was demonstrable but slow. After 8 h, approximately 20% of the substrate was lost, and the amount of Cer increased (Fig. 2). No change in Cer-β-glucuronide intensity was seen when it was incubated without enzyme for 24 h, suggesting that there is no nonenzymatic hydrolysis of Cer-β-glucuronide (data not shown). Although it is not possible to conclude that the appearance of Cer was stoichiometric with the disappearance of Cer-β-glucuronide (for the reasons noted above), the goal of this experiment was to determine whether the head group of Cer-β-
glucuronide could be hydrolyzed by purified *E. coli* β-glucuronidase, which was clearly the case. It might be possible to find more optimal conditions for the hydrolysis of Cer-β-glucuronide by this enzyme.

To evaluate the hydrolysis of Cer-β-glucuronide by the more complex components of the intestinal tract, the substrate was incubated with segments from mouse upper small intestine and the colon. With the segments of the small intestine, less than 10% of the Cer-β-glucuronide was hydrolyzed in the first 2 h of the incubation, and only 25% was cleaved after 8 h (Fig. 3A). With the colonic segments, approximately 45% of the Cer-β-glucuronide was cleaved after 2 h of incubation and 75% was cleaved after 8 h. There were concomitant increases in Cer (Fig. 3, A and B). It is noteworthy that the intensity of the Cer signal relative to the loss of Cer-β-glucuronide was lower for the colonic segments than for the purified enzyme or the upper small intestine (compare Fig. 2 and Fig. 3, A and B). This is presumably due to further hydrolysis of Cer to sphingosine, because we have shown previously that Cer is hydrolyzed in the intestinal tract (6).

**Biological Effects of Dietary Cer-β-glucuronide**

**Effect on Animal Weight Gain.** There was no difference in weight between the experimental groups at the end of the study (35.1 ± 1.87 and 35.2 ± 1.55 g for mice fed 0.025% and 0.1% Cer-β-glucuronide, respectively) or between the experimental groups and the control group (36.5 ± 1.46 g); there was also no difference in weight gain. Thus, the addition of Cer-β-glucuronide to the diet did not affect the growth of the animals.

**Suppression of ACF Formation.** No ACF were found in the colons of mice that were not injected with DMH (untreated control group). DMH induced 30.2 ± 4.2 ACF/animal in the control group, and Cer-β-glucuronide reduced the number of ACF by 30% and 37% (21.2 ± 2.9 and 19.3 ± 3.1 ACF/animal) at 0.025% and 0.1%, respectively. This reduction was statistically significant in the group fed 0.1% Cer-β-glucuronide (*P* = 0.049) and was marginally significant (*P* = 0.096) for the group fed 0.025% Cer-β-glucuronide (Fig. 4).

The crypt multiplicity of ACF, the number of aberrant crypts/focus (considered an indicator for the probability that adenomas and adenocarcinomas will develop from ACF), appeared to be lower for both groups fed Cer-β-glucuronide (1.8 ± 0.1, 1.6 ± 0.1, and 1.5 ± 0.4 aberrant crypts/focus for the control group and the groups fed 0.025% and 0.1% Cer-β-glucuronide, respectively), but this was not statistically significant for the 0.025% group (*P* = 0.337) and was only marginally significant for the 0.1% group (*P* = 0.080).

The number of ACF in the control group was positively correlated with body weight for the control group (*r* = 0.468; *P* = 0.043), but not for the groups fed 0.025% and 0.1% Cer-β-glucuronide (*r* = 0.196 and *P* = 0.588 and *r* = 0.152 and *P* = 0.246 for the 0.025% group and the 0.1% group, respectively). There was no correlation between the ACF size and mouse weight in any of the experimental groups.

There was a significant negative correlation between the number of ACF and the length of the colon in the group fed 0.1% Cer-β-glucuronide (*r* = −0.637; *P* = 0.047), with the mice with longer colons having fewer ACF (Fig. 5). No such correlation was seen for the control mice (*r* = 0.050; *P* = 0.890) or for the mice fed 0.025% Cer-β-glucuronide (*r* = 0.407; *P* = 0.277). The mean length of the colons did not differ between the Cer-β-glucuronide-fed mice and the controls (data not shown).
Inhibition of Proliferation. Cer-β-glucuronide reduced the number of BrdUrd-positive cells by 8% and 20% at 0.025% and 0.1%, respectively, \((P < 0.001)\) in the lower half of the colonic crypts (Fig. 6). Cer-β-glucuronide also suppressed proliferation in the upper half of the crypts by 50% and 70% \((P < 0.001)\). The number of ACF was not statistically related to the decrease of proliferation, possibly due to the small number of mice used for the determination. In the untreated controls, no proliferative cells were detected in the upper half of the colonic crypts.

DISCUSSION

Colonic bacterial enzymes are widely known to convert β-glucuronides to the aglycon upon reaching the colon. This can be used therapeutically by preparation of β-glucuronides as prodrugs that are not metabolized and taken up by the small intestine but are delivered to and activated in the colon, thereby avoiding loss of the compound (and possibly undesired effects in other parts of the intestine). This principle has been demonstrated with various hydrophobic agents, such as dexamethasone and retinoids (9–11).

This study was designed to evaluate Cer-β-glucuronide as a substrate for purified and colonic β-glucuronidase(s) and to determine the capacity of the released bioactive compounds to suppress the early stages of colon cancer. Cer-β-glucuronide was hydrolyzed by both purified \(E.\) coli β-glucuronidase and segments from the mouse intestine that should contain hydrolyses produced by the microflora. Involvement of animal hydrolase(s) is also possible, but hydrolases of microbial origin are most likely, because there was greater hydrolysis using segments from the colon than using those from the upper intestine. In the rodent, both regions contain some bacteria (9), which probably accounts for the slow cleavage of Cer-β-glucuronide in the segments of the upper small intestine.

Cer-β-glucuronide inhibited ACF formation by 30–37%, which is about half the suppression we have seen with other sphingolipids (2–4). The reason for a somewhat lesser effect is not clear but might reflect incomplete hydrolysis of Cer-β-glucuronide in the mouse colon due to the rapid gastrointestinal transit time for these animals (markers can be transported through more than 80% of the total length of the intestinal tract in 20–40 min; Refs. 26 and 27). This hypothesis is supported by the strong correlation between the length of the colon and the suppression of ACF. A longer colon may expose Cer-β-glucuronide to microbial enzymes for a longer period of time and may account for a stronger suppression of ACF. If so, a greater effect might be seen in species with a slower passage time through the gastrointestinal tract, such as humans, who sometimes retain 20% of meal components in the colon for 120 h (28).

The reduced proliferation in the colons of mice fed Cer-β-glucuronide provides an insight into the mechanism(s) whereby this compound reduces ACF formation. Sphingolipids function as lipid second messengers that regulate cell behaviors that are important in all stages of carcinogenesis: (a) growth inhibition, which they regulate by modulating the phosphorylation state of the Rb protein (29, 30), causing cell cycle arrest (31), as well as by down-regulation of c-myc (32–34) and inhibition of protein kinase C (35); (b) induction of differentiation (35); and (c) induction of apoptosis (32, 37, 38). It is noteworthy that Cer-β-glucuronide reduced proliferation most exten-
visively in the upper half of the colonic crypts, where proliferative cells are usually not encountered unless they have undergone DNA damage. This corresponds well with the our findings with milk sphingomyelin (2–4) and glycosphingolipids9 and further substantiates the ability of natural and synthetic sphingolipids to suppress colon carcinogenesis.

Based on these findings, Cer-β-glucuronide may be an effective chemopreventive (and possibly, chemotherapeutic) agent for human colon cancer.

REFERENCES


