Enigmol: A Novel Sphingolipid Analogue with Anticancer Activity against Cancer Cell Lines and In vivo Models for Intestinal and Prostate Cancer

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Abstract

Sphingoid bases are cytotoxic for many cancer cell lines and are thought to contribute to suppression of intestinal tumorigenesis in vivo by ingested sphingolipids. This study explored the behavior of a sphingoid base analogue, (2S,3S,5S)-2-amino-3,5-dihydroxyoctadecane (Enigmol), that cannot be phosphorylated by sphingosine kinases and is slowly N-acylated and therefore is more persistent than natural sphingoid bases. Enigmol had potential anticancer activity in a National Cancer Institute (NCI-60) cell line screen and was confirmed to be more cytotoxic and persistent than naturally occurring sphingoid bases using HT29 cells, a colon cancer cell line. Although the molecular targets of sphingoid bases are not well delineated, Enigmol shared one of the mechanisms that has been found for naturally occurring sphingoid bases: normalization of the aberrant accumulation of β-catenin in the nucleus and cytoplasm of colon cancer cells due to defect(s) in the adenomatous polyposis coli (APC)/β-catenin regulatory system. Enigmol also had antitumor efficacy when administered orally to Min mice, a mouse model with a truncated APC gene product (C57Bl/6 Min/+ mice), decreasing the number of intestinal tumors by half at 0.025% of the diet (w/w), with no evidence of host toxicity until higher dosages. Enigmol was also tested against the prostate cancer cell lines DU145 and PC-3 in nude mouse xenografts and suppressed tumor growth in both. Thus, Enigmol represents a novel category of sphingoid base analogue that is orally bioavailable and has the potential to be effective against multiple types of cancer. Mol Cancer Ther; 10(4); 648–57. ©2011 AACR.

Introduction

Sphingolipids are highly bioactive compounds that modulate many cell signaling pathways that are relevant to tumor biology and cancer control (1, 2). Much attention has been given to ceramide (Cer) and Cer analogues (3, 4) as inhibitors of cell growth and inducers of apoptosis (5), and to sphingosine 1-phosphate (S1P), which can inhibit apoptosis, induce cell migration and other pro-carcinogenic behaviors (1, 2). However, sphingosine, sphinganine, and other sphingoid bases (such as safinogol) also have the potential to be useful for cancer control, because they inhibit transformation of normal cells induced by irradiation (6) and chemical carcinogens (7), induce differentiation of transformed cells (8), and are growth inhibitory and cytotoxic for many cancer cell types (9–13). The mechanism(s) for these effects is unclear; however, sphingoid bases and analogues affect multiple signaling pathways (14) and important processes such as autophagy (15).

Because dietary sphingolipids are hydrolyzed to the sphingoid base backbones that are taken up by intestinal cells (16, 17), many of the studies of cancer suppression in vivo have focused on colon cancer. Orally administered sphingolipids decrease precancerous lesions and tumors in models of chemically induced and inherited colon cancer (18, 19), and sphingoid bases normalize one of the regulatory defects in colon cancer the accumulation of β-catenin in the nucleus and cytosol of intestinal cells (20, 21).

The effectiveness of sphingoid bases seems to be limited by their phosphorylation by sphingosine kinase—an enzyme that has been called an oncogene (22); indeed, studies with sphingosine kinase 1 knockout mice have found that this enzyme is required for
small intestinal tumor cell proliferation in multiple intestinal neoplasia mice (Min; ref. 23). On the basis of this rationale, one would predict that compounds that cannot be phosphorylated would be more effective in cancer suppression than the naturally occurring sphingoid base sphingosine.

This study describes the findings with a sphingoid base analogue, (25,35,55)-2-amino-3,5-dihydroxyoctadecane (named Enigmol), that cannot be phosphorylated by sphingosine kinase, and is also poorly N-acylated (24). Enigmol was compared with natural sphingoid bases for cytotoxicity for cancer cell lines, cellular uptake and metabolism, and reduction of nuclear β-catenin, which has previously been associated with suppression of colon cancer (20, 21), and then its efficacy was evaluated using mouse models for colon and prostate cancer. Enigmol displayed anticancer activity in all these systems and hence represents a novel category of sphingoid base analogue that is worthy of additional consideration in cancer control.

Materials and Methods

Additional information about the materials and methods has been provided in Supplementary files that are available online.

Sphingolipids and analogues

Enigmol [(25,35,55)-2-amino-3,5-dihydroxyoctadecane; Fig. 1B] was prepared using a highly diastereoselective chemical methodology developed in the Liotta laboratory (25) and references cited in Supplementary Materials and Methods), except for the studies using mouse xenografts with PC-3 cells, where it was obtained from the NCI under the RAND program. Other compounds and reagents were obtained commercially.

Cell culture and treatments

The cell lines referred to in this article and Supplementary Data were obtained from the American Type Culture Collection (ATCC) and grown in media standard for each cell type as described in the Supplementary files. The identity of the HT29, DU145, and PC-3 had been established by the ATCC by its criteria for authentication, which are described on their Web site (http://www.atcc.org/Science/CollectionsResearchandDevelopment/CellBiology/tabid/205/Default.aspx) and the lines had not been passaged in culture for greater than 6 months from their receipt or resuscitation.

For treatment of the cells in culture, the sphingoid bases were prepared as the 1:1 molar complex with fatty acid-free bovine serum albumin (BSA; Calbiochem; ref. 26). Unless otherwise noted, viability was measured using the WST-1 reagent (Roche). The NCI-60 Human Tumor Cell Line Screen was conducted by the NCI’s Developmental Therapeutics Program (NCI DTP; http://www.dtp.nci.nih.gov/branches/btb/ivclsp.html) using the BSA complex of Enigmol provided by our laboratory.

Analysis of sphingolipids by liquid chromatography, electrospray ionization tandem mass spectrometry

Lipid analysis was conducted by extracting the sphingolipids from the cells followed by liquid chromatography, electrospray ionization tandem mass spectrometry (LC ESI-MS/MS) in positive ion mode as described previously (27), with minor modifications described in Supplementary Materials and Methods.

Other assays

The localization of β-catenin in HT29 cells was determined using a primary anti–β-catenin antibody (Transduction Labs; 1:500 dilution) and Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) as the secondary antibody (20, 21).

Animals and treatments

Protocols involving animals were approved by the Institutional Animal Care and Use Committee and conducted according to National Research Council Guidelines. Male Min mice (C57Bl/6jMin/+ mice; 32 days of age; Jackson Laboratory) were fed a semipurified AIN 76A diet (Dyets) that is essentially sphingolipid free (28) supplemented with 0%, 0.025%, or 0.1% (w/w) Enigmol. At 39 days of age, the mice were changed to these diets for 45 days and then killed for analysis of the tissues.

In the first mouse xenograft study, athymic nu/nu Balb/c mice (Charles River Laboratories) of both sexes at 32 days of age were given subcutaneous injections of DU145 cells (5 × 10⁶/100 μL PBS) in the right and left flanks, then the groups received Enigmol i.p. (0 or 8 mg/kg mouse body weight in 200 μL olive oil) daily for 5 days. Tumors were measured with calipers (width and length) 2 times a week and the tumor volume was calculated as 0.5 × (length × width²). In the second, athymic (nu/nu) mice (from the NCI) received 5 × 10⁶ PC-3 cells, and when tumors became palpable, mice were assigned to groups (n = 15) that received 0 or 10 mg/kg Enigmol orally by daily gavage until termination of the experiment. For these studies, Enigmol was dissolved in a small volume of 100% ethanol and then diluted into olive oil; the daily dosage was delivered in 200 μL per mouse.

Results

The hypotheses of this study were that Enigmol will display potential anticancer activity with human cancer cell lines, have greater potency than sphingosine and sphinganine because it will undergo slower metabolism, modulate at least some of the regulatory processes that have been seen previously for sphingoid bases, and suppress tumorigenesis in vivo in animals models.

Results from an NCI-60 human tumor cell line screen

When Enigmol was tested by the NCI (http://dtp.nci.nih.gov/branches/btb/ivclsp.html; ref. 29), it displayed...
50% growth inhibition for all 57 cell lines at concentrations between 0.4 and 14 μmol/L and had LD<sub>50</sub> in the 5 to 10 μmol/L range for 5 of the cell lines, and possibly as many as 40 (the latter estimate includes cell lines that displayed ≥50% toxicity at 10 μmol/L but less toxicity at 100 μmol/L, which might reflect a solubility problem at such a high level, or interference from the equimolar fatty acid–free BSA that is also added to assist with solubilization). For more results from this screen, see the Supplementary Data available online.

Additional cellular studies using cancer cell lines

Starting with HT29 cells because sphingolipids are known to inhibit colon carcinogenesis (20, 21), the IC<sub>50</sub> for Enigmol was about 8 versus 25 μmol/L for sphingosine and sphinganine after 24 hours (Fig. 1C; it is worth mentioning that this IC<sub>50</sub> refers to this specific time after treatment, i.e., while ~8 μmol/L Enigmol caused a 50% reduction in 24 hours, there was almost complete elimination of viable cells between 48 and 72 hours). At the higher concentrations, cells that appeared to be dead had detached from the dishes and were seen floating in the medium; therefore, to confirm that these were not viable, floating cells from dishes treated with 20 μmol/L or greater Enigmol were pelleted and replated in new medium without Enigmol, and the dishes were examined for viable cells after 24 hours, but none were seen. Likewise, new medium was added to the original dishes to determine if the few cells that remained were viable, but no colonies of resistant clones or any evidence of viable cells were seen in the dishes after several days in culture.

As has been noted before (26), the presence of albumin and other serum proteins has a major impact on the IC<sub>50</sub> for sphingoid bases because they are bound by serum proteins. For example, in the case of Enigmol, when the ratio of Enigmol to BSA was increased from 1:1 to 2:1, the IC<sub>50</sub> decreased by about half; likewise, removal of FBS from the medium decreased the IC<sub>50</sub> by approximately 3-fold (data not shown). Therefore, all of the comparisons in these studies were made at the same Enigmol:BSA ratio (1:1) and with 10% FBS.

The mechanism(s) for cell death was not elucidated, but caspase activity was elevated by Enigmol (Supplementary Fig. S1) and Enigmol was also more potent than sphingosine in caspase activation.

Figure 1C also shows the greater toxicity of Enigmol versus sphinganine or sphingosine for the prostate cell line DU145. The effect of Enigmol on a number of other cell lines (PC3, LnCAP, HL60, and MCF-7 cells) was also

![Diagram of sphingolipid structures](image-url)
examined using the WST-1 assay (as in Fig. 1C) and all had IC₅₀ values in the range of 8 to 12 μmol/L (data not shown); therefore, this sphingoid base analogue affects a wide variety of cancer cell lines, as was indicated by the NCI-60 Human Tumor Cell Line Screen.

Enigmol uptake and metabolism

The greater toxicity of Enigmol is not due to increased uptake by cells (Fig. 2A); however, Enigmol persisted longer, as seen in the pulse-chase portion of the experiment (right graph of Fig. 2A)—that is, where the cells were treated with sphingosine or Enigmol for 1 hour and then the medium was removed and replaced with new medium minus these compounds and incubated for varying times and finally analyzed by LC ESI-MS/MS. While very little free sphingosine was found in the cells (only 10% at 3 hours and <2% at 6 hours), more than 50% of the Enigmol was present after 3 hours and about 25% after 12 hours. Under these conditions, Enigmol killed 98% ± 2% of the cells versus about 50% for sphingosine (Supplementary Fig. S2).

A substantial portion (~5 nmol/10⁶ cells) of the Enigmol that disappeared during the chase period (~6 nmol/10⁶ cells) could be accounted for as N-acyl-Enigmols (Fig. 2B), which is consistent with this compound being a modest substrate for Cer synthase(s) (ref. 24; the finding of multiple fatty acyl derivatives suggests that it is acylated by several Cer synthases, because each is relatively selective with respect to the fatty acyl coenzyme A that it uses; ref. 30). HT29 cells also produced small amounts (~0.1 ± 0.02 nmol/10⁶ cells at the 12-hour time point) of N,N,N-trimethyl-Enigmol, a type of sphingoid base metabolite that has recently been found with safingol (31).

As a weak Cer synthase inhibitor (12, 24, 32), Enigmol elevated sphinganine and sphinganine 1-phosphate (Sa1P) by several fold (Fig. 2C and D), but had little or no effect on endogenous cellular sphingosine (Fig. 2C). The latter is somewhat surprising because Enigmol inhibited sphingosine kinase in vitro (see Supplementary Fig. S3). Enigmol had little or no effect on the amounts of Cer, sphingomyelin, and mono-hexosylCer (Supplementary Figs. S4 and S5).

The sphingosine that was added to the cells resulted in extremely large increases in S1P (Fig. 2D), from 0.07 ± 0.01 to 7 ± 1 nmol S1P/10⁶ cells. There was also a large increase in Cer (from ~1 to 15 nmol/10⁶ cells at 12 hours; Supplementary Fig. S2) and, interestingly, the fatty acid
compositions of the Cer in sphingosine-treated cells differed considerably from the N-acyl-Enigmols in Enigmol-treated cells (Supplementary Fig. S6), therefore, it is possible that different Cer5 are involved. Sphingomyelins and monohexosylCers also increased when the cells were treated with sphingosine, but by much lower amounts (~1 nmol/10^6 cells each) than was seen with Cer (Supplementary Fig. S5). These results show that the addition of sphingosine alters many sphingolipid subspecies, with probably the most noteworthy being the 100-fold increase in S1P and 15-fold increase in Cer.

**Other effects of Enigmol on HT29 cells**

Because a large number of signaling pathways are affected by sphingoid bases, evaluation of all of them was beyond the scope of this study. We selected one in particular—that is, how Enigmol compares to sphingosine with respect to reduction of nuclear β-catenin—because this has been associated with colon cancer suppression by sphingoid bases (20). HT29 cells have large amounts of nuclear and cytosolic β-catenin (Fig. 3A) due to truncation of adenomatous polyposis coli (APC) protein (33), and at the concentration where sphingosine shows a noticeable effect on nuclear β-catenin (i.e., at ~30 μmol/L, middle panels of Fig. 3A), Enigmol eliminated most of the nuclear β-catenin. Close examination of the images also indicates that fluorescence at the cell–cell boundaries is enhanced by Enigmol and sphingosine (e.g., see arrows with @ symbol in panels d and g of Fig. 3A), which resembles the normal localization of β-catenin with E-cadherin in differentiated intestinal cells (20, 21).

The relative degree of nuclear staining for β-catenin is also depicted in Fig. 3B using 3 categories: positive, partially positive, or negative scored for 5 randomly selected fields of view with 10 to 25 cells per field of view. Essentially, all of the control cells were positive for nuclear β-catenin; Enigmol-treated cells displayed fewer nuclear β-catenin–positive cells at all time points, with only 2% positive and 3% partially positive at 12 hours. Sphingosine treatment also decreased the percentage that were nuclear β-catenin positive, but not as extensively as Enigmol. Note in particular that the loss of nuclear β-catenin is 10 times greater with Enigmol than sphingosine at 2.5 hours, which is a time point when HT29 cells contain essentially the same amounts of Enigmol and sphingosine (compare with Fig. 2A).

Regulation of β-catenin is a complex process, but a major factor in the aberrant accumulation of this protein in many colon cancer cells, including HT29 cells, is mutation of the APC gene, which participates in β-catenin turnover by facilitating its phosphorylation, ubiquitination, and proteasomal proteolysis (34). Western blot analysis (Fig. 4A, right) revealed that sphingosine reduces cytosolic β-catenin somewhat at 30 μmol/L whereas Enigmol (Fig. 4A, left) caused a substantial decrease in soluble (cytosolic) β-catenin within 2 hours, and its almost complete disappearance by 6 hours.

Some of the upstream regulators of β-catenin turnover are summarized in the scheme in Fig. 4—that is, β-catenin phosphorylation by casein kinase 1-α (CKIα) and glycogen synthase kinase 3-β (GSK-3β) as part of the destruction complex with APC and AXIN (34). To explore if these are involved, HT29 cells were treated with the inhibitor of proteasomal proteolysis MG-132 and the amounts of soluble β-catenin were compared by Western blot analysis. MG-132 completely blocked the disappearance of soluble β-catenin on Enigmol treatment (Fig. 4B), which suggests that Enigmol is acting by increasing proteasome-dependent turnover. Likewise, the phosphorylation state of β-catenin was affected by Enigmol as analyzed using phospho-specific antibodies for S33/S37/T41-β-catenin and T41/S45-β-catenin (Fig. 4C, right), which suggests that Enigmol
enhances the phosphorylation of β-catenin by CKI-α and/or GSK-3β, which occurs at these sites (34). GSK-3β is also phosphorylated on S9 by protein kinase C (35), which might also be affected because protein kinase C (PKC) is inhibited by sphingoid bases (36), and indeed, there was a decrease in phospho-S9-GSK-3β in cells treated with Enigmol (Fig. 4C, left).

These might not be the only mechanisms whereby Enigmol induces turnover of β-catenin in these cells because other proteases are also activated by sphingoid bases (as shown in Supplementary Fig. S1 for caspase activation by sphingosine and Enigmol). All in all, these results establish that Enigmol has effects similar to, but more potent than, sphingosine with respect to decreasing the amount of β-catenin in the nucleus and cytosol.

**Inhibition of adenoma formation in Min mice**

To determine if Enigmol has antitumor activity in vivo, Min mice (19, 20) were fed an essentially sphingolipid-free diet with no supplement (control; n = 9), 0.025% (w/w) Enigmol (n = 8), or 0.1% (w/w) Enigmol (n = 8). Mice fed the control diet had 75.0 ± 13.6 tumors per mouse, whereas those fed Enigmol had 52% and 37% fewer tumors per mouse for the 0.025% and 0.1% fed groups, respectively (P < 0.05 vs. control; Fig. 5A). Suppression by Enigmol was seen throughout the proximal, mid, and distal portions of the intestine (Fig. 5A). Because only a few tumors develop in the colon of Min mice, Enigmol did not have a statistically significant effect, although the averages seem lower (i.e., 0.63 ± 0.43 for 0.025% and 0.88 ± 0.44 for 0.1% vs. 1.0 ± 0.4 for the control, with P = 0.2 and 0.4, respectively).

Enigmol had no adverse effect on body weight (20.2 ± 0.9 g for the control mice and 20.2 ± 1.3 g) nor on any of the biomarkers for liver and kidney function (Fig. 5C) at 0.025% of the diet. However, 0.1% Enigmol reduced weight gain to 4.3 ± 0.7 g during the course of the 5.5-week study (vs. 6.0 ± 0.5 g for the control mice and 6.2 ± 0.4 g for the 0.025% Enigmol group, both of which were significantly different from the 0.1% group with P < 0.05) for a final weight of 18.0 ± 0.8 g (P < 0.05). We do not
know whether this is due to an effect of 0.1% Enigmol on food consumption, which was not recorded; however, we did not notice differences in the amounts of food left in the food containers. In addition, although the biomarkers for liver and kidney function were in the normal range, the average blood urea nitrogen level was significantly higher ($P < 0.05$) for mice given 0.1% Enigmol versus the control. Total serum protein and albumin levels were somewhat higher for the Enigmol-treated mice compared with the control, which is generally not thought to be an indication of toxicity, but may indicate dehydration (Fig. 5C, right panels). All together, these results establish that Enigmol can reduce intestinal tumorigenesis in this mouse model with no evidence of host toxicity at 0.025% of the diet, but the higher level of 0.1% may be more problematic.

**In vivo antitumor activity and toxicity of Enigmol in mouse xenografts for prostate cancer**

Although the focus of our studies has been on colon cancer due to the relatively well-established link for this cancer (18–21), the screening of Enigmol against other cancer cell lines indicates that it might have broader efficacy (Fig. 1C and Supplementary Data). To test this possibility, Enigmol was tested against the prostate cancer line DU145 in a mouse xenograft model ($nu/nu$ Balb/c mice), because it was toxic for these cells in culture (Fig. 1C). As shown in Fig. 6A, Enigmol significantly suppressed the growth (i.e., a $\geq$50% reduction in tumor size compared with the control) of tumors from these cells when injected i.p. at 8 mg/kg body weight for 5 days. At this dosage, there was no sign of host toxicity by histopathology or by clinical pathology for either study; the mean $\pm$ SD for 3 randomly selected male mice given Enigmol at this dosage were as follows: blood urea nitrogen (25 $\pm$ 2 mg/dL), creatinine (0.65 $\pm$ 0.49 mg/dL), ALT (alanine aminotransferase; 33 $\pm$ 9 U/L), albumin (2.6 $\pm$ 0.4 g/dL), and total serum protein (4.8 $\pm$ 0.3 g/dL), all within normal values.

In a second study (Fig. 6B), Balb/c $nu/nu$ mice implanted with PC-3 cells were assigned to 3 groups ($n = 15$) that received a daily oral gavage with vehicle alone (200 $\mu$L of olive oil) or 10 mg/kg Enigmol in 200 $\mu$L of olive oil until termination of the experiment. This also resulted in an approximately 50% reduction in tumor size for the Enigmol group versus the vehicle control. The animals in these groups displayed similar changes in weight during the treatment period; that is, the starting/ending weights were as follows: 22.8 $\pm$ 2.4 g/19.5 $\pm$ 2.4 g (loss of 3.3 g) for the controls versus 23.5 $\pm$ 3.5 g/20.8 $\pm$ 2.9 g (loss of 2.7 g) for the animals administered Enigmol at 10 mg/kg.

Therefore, these studies show that Enigmol has anticancer activity against 2 human prostate cancer cell lines in a mouse xenograft model and the latter study shows that an effect can be seen when Enigmol is administered orally.
Anticancer Activities of Enigmol

Blood and tissue levels of Enigmol

An analysis of the pharmacokinetics of Enigmol uptake and elimination will be published separately (37); however, it is worth mentioning here that the amounts of Enigmol in blood on the fourth day of administration of 4 mg Enigmol/kg i.p. was 0.41 nmol/L (n = 4), following the protocol used for Fig. 6A. In the study using PC-3 cells to prepare the xenografts (Fig. 6B), analysis of 3 of the tumors at the end of the study showed that they contained 0.18 ± 0.07 nmol of Enigmol/g of tissue. These results confirm that Enigmol appears in both blood and the tumors.

Discussion

These studies have established that Enigmol is toxic for numerous human cancer cell lines in the NCI-60 screen and suppressed tumor growth in mouse models for colon and prostate cancer. The toxicity for so many cell lines in the NCI-60 screen raised concern that Enigmol might be toxic for host and cancer cells; however, the in vivo studies suggested that tumors are most affected.

It is usually difficult to predict if compounds that display potential anticancer activity in studies of cells in culture will have efficacy in vivo, and this can be especially problematic when the agent is hydrophobic and its delivery to the target site might be limiting. Therefore, on a practical level, the most remarkable findings of these studies were that Enigmol suppresses tumor growth not only in a colon cancer model (Min mice), as has been seen before for dietary sphingolipids (20), but also in mouse xenografts with 2 prostate cancer cell lines, thereby extending the types of cancer that might be affected by this category of compounds, and that oral administration of Enigmol could affect the PC3 cell xenografts. These properties, plus the apparently low host toxicity of Enigmol when fed to animals at levels that decrease tumor growth, suggest that this compound has promise for cancer control.

The rationale behind the selection of this type of analogue was that sphingoid bases lacking the 1-hydroxy group cannot be metabolized by sphingosine kinase(s) and the 2,35 stereochemistry (compared to 2,3R for sphingosine) also makes Enigmol a poorer substrate for N-acylation (24, 38); therefore, Enigmol would be predicted to be more persistent as the free sphingoid base, which was borne out by the cell culture studies with HT29 cells and the appearance of free Enigmol in tumors in the mouse xenograft studies. A longer persistence of the free sphingoid base might also contribute to the cytotoxicity of the synthetic analogues safingol (39), N, N-dimethylsphingosine (40) and naturally occurring sphingoid base variants (41, 42), including one recently evaluated in a phase I trial (43).

The inability of Enigmol to undergo phosphorylation seems to be especially important because sphingosine kinase and S1P clearly play important roles in colon tumorigenesis (44, 45). Nonetheless, it does not seem that Enigmol acts via elimination of endogenous S1P (although it can inhibit sphingosine kinases in vitro), based on no measurable reduction in S1P in the cells (Fig. 2). This warrants further study, however, because the pertinent S1P might be localized in a specific sub-compartment, secreted from the cells, and/or involved in receptor cross-talk (46), that would not have been detected in our analysis. It was striking that the addition of sphingosine to HT29 cells elevated S1P levels by more than 100-fold, which might be a major factor in its lower toxicity, because S1P can sometimes protect cells against apoptosis. The addition of sphingosine also elevated Cer by more than 10-fold and sphingomyelin and glycosphingolipids by smaller but detectable amounts (Supplemental Figs. S2 and S3), which illustrates the complexity of studies of naturally occurring sphingolipids, because the added compound undergoes extensive metabolism to multiple bioactive species.

The mechanism(s) for the tumor suppression by Enigmol has been only partially defined by these experiments;
nonetheless, Enigmol can normalize one of the defects that is important in colon cancer—the aberrant appearance of β-catenin in the nucleus (20, 21) where it plays a major role in regulating proliferation via the T-cell factor/lymphoid enhancer transcription factor (47). Furthermore, these studies have established that Enigmol and, to a lesser extent sphingosine, increases proapoptotic turnover of β-catenin, apparently by increasing its phosphorylation by GSK-3β (and possibly CKI-α, although this was not tested), which precedes the polyubiquitination and degradation of β-catenin. The immediate target of Enigmol is not known but might include PKC, which is elevated in colon tumors (48) and is known to be inhibited by sphingoid bases (36); however, other protein kinases, such as PKB (protein kinase B)/Akt (49–51), might also play a role because they are also known to be affected by sphingoid bases. It is noteworthy that interference with these mitogenic signaling pathways might also provide a link between Enigmol and cell death, because Akt and other signaling pathways influence caspase activation and apoptotic cell death.

All in all, these studies suggest that Enigmol represents a novel category of sphingoid base analogue that is orally bioavailable and has the potential to be effective against multiple types of cancer.

Disclosure of Potential Conflicts of Interest

A.H. Merrill, Jr. has patents pertaining to sphingolipids, but none licensed to companies.

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