Structural and stereochemical studies of potent inhibitors of glucosylceramide synthase and tumor cell growth

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Abstract Analogs and homologs of PDMP were synthesized, based on its structure (D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol). This compound had previously been found to block the synthesis of GlcCer (glucosylceramide). Increasing the acyl chain length from 10 to 16 carbon atoms greatly enhanced the efficacy of the enzyme inhibitor, as did the use of a less polar cyclic amine, especially a pyrrolidine instead of a morpholine ring. Replacement of the phenyl ring by a chain corresponding to sphingosine also yielded a strongly inhibitory material. By using a chiral synthetic route, we showed that the isomers active against GlcCer synthase had the R,R-(D-*threo*)-configuration. However, strong inhibition of the growth of human cancer cells in plastico was produced by both the threo and erythro racemic compounds, showing involvement of an additional factor (beyond simple depletion of cell glycosphingolipids by blockage of GlcCer synthesis). The growth arresting effects could be correlated with increases in cellular ceramide and diglyceride levels. The aliphatic pyrrolidino compound was strongly inhibitory toward the glucosyltransferase and produced almost complete depletion of glycolipids, but did not inhibit growth or cause an accumulation of ceramide. Attempts were made to see whether the differences in growth effects could be attributed to the influence of the inhibitors on related enzymes (ceramide and sphingomyelin synthase and ceramidase and sphingomyelinase). While some stimulation of enzyme activity was noted, particularly at high inhibitor concentrations (50 μM), these findings did not explain the differing effects of the different inhibitors. The best inhibitors of GlcCer synthase compared favorably in efficacy with some cancer chemotherapeutic drugs in current use when tested with a battery of human cancer cells.—**Abe, A., N. S. Radin, J. A. Shayman, L. L. Wotring, R. E. Zipkin, R. Sivakumar, J. M. Ruggieri, K. G. Carson, and B. Ganem.** Structural and stereochemical studies of potent inhibitors of glucosylceramide synthase and tumor cell growth. *J. Lipid Res.* 1995. **36:** 611–621.

Supplementary key words PDMP • 1-phenyl-2-decanoylamino-3-morpholino-1-propanol • 1-pyrrolidino-1-deoxyceramide • glycolipid depletion • ceramide accumulation • diacylglycerol accumulation • cancer chemotherapy • sphingomyelin synthase • ceramide synthase • ceramide hydrolases • sphingomyelin hydrolases

The enzyme that makes glucosylceramide (GlcCer), ceramide:UDP-glc glucosyltransferase, plays a key role in the synthesis of hundreds of different glucosphingolipids (GSLs). The rate of reaction under physiological conditions may depend on the tissue level of UDP-glc, which in turn depends on the level of glucose in a particular tissue (1). In vitro assays based on endogenous ceramide yield lower synthetic rates than mixtures containing added ceramide, suggesting that the tissue level of ceramide is also normally rate-limiting (2).

Many researchers have found that the level of a particular GSL controls a variety of cell functions, such as growth, differentiation, adhesion between cells or between cells and matrix proteins, binding of microorganisms and viruses to cells, and metastasis of tumor cells. In addition, the GlcCer precursor, ceramide, may cause differentiation or marked inhibition of cell growth (3) and may be involved in the functioning of vitamin D_3, tumor necrosis factor-α, interleukins, and apoptosis (programmed cell death). The sphingols (sphingoid bases), precursors of ceramide and products of ceramide catabolism, have also been shown to influence many cell systems, possibly by inhibiting protein kinase C. It seems likely that all the GSLs undergo catabolic hydrolysis, so any blockage in the glucosyltransferase should ultimately lead to depletion of

Abbreviations: BSA, bovine serum albumin, fatty acid free; DAG, diacylglycerol or, more generally, diradylglycerol; GlcCer, glucosyl ceramide; GSL, glucosphingolipid, a metabolite of GlcCer; MDCK cells, Madin-Darby canine kidney cells; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PMMP is the myristoyl homolog of PDMP; SM, sphingomyelin; C, chloroform; M, methanol; W, water; TLC, thin-layer chromatography.

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the GSLs and profound changes in the functioning of a cell or organism.

An inhibitor of GlcCer synthase, PDMP (1R-phenyl-2R-decanoylamino-3-morpholino-1-propanol, previously identified as the D-*threo* isomer (4)), has been found to produce a variety of chemical and physiological changes in cells and animals (for reviews see refs. 5, 6). Particularly intriguing is the compound's ability to cure mice of cancer induced by Ehrlich ascites carcinoma cells (7), to produce accumulation of sphingosine and *N,N*-dimethylsphingosine (8), and to slow cell growth (9). Compounds with longer chain fatty acyl groups were found to be substantially more effective (10). The present paper describes the even stronger effects obtained with the pyrrolidine analogs of PDMP and glucosylceramide. In addition, we report a new, chiral synthesis and an unambiguous identification of the primary structure required for strong inhibition of GlcCer synthase.

MATERIALS AND METHODS

Materials

The aromatic inhibitors were synthesized by the Mannich reaction from 2-*N*-acylaminoacetophenone, paraformaldehyde, and a secondary amine (Table 1), as described before (4, 11). For those syntheses in which phenyl-substituted starting materials were used, the methyl group in the acetophenone structure was brominated and converted to the primary amine. The reaction produces a mixture of four isomers, due to the presence of two asymmetric centers. Bromination of *p*-methoxyacetophenone was performed in methanol. The acetophenones and amines were from Aldrich Chemical Co., St. Louis, MO. Miscellaneous reagents were from Sigma Chemical Co. and the sphingolipids used as substrates or standards were prepared here.

The aliphatic inhibitors were synthesized from the corresponding 3-*t*-butyldimethylsilyl-protected sphingols, prepared by enantioselective aldol condensation (12, 13) using a modification of the procedure of Nicolaou et al. (14). Each protected sphingol was first converted to the corresponding primary triflate ester, then reacted with morpholine or pyrrolidine. Subsequent *N*-acylation and desilylation led to the final products in good overall yield (15). The compounds can be called 1-morpholino-(or pyrrolidino)-1-deoxyceramides.

Labeled ceramide, decanoyl sphingosine, was prepared by reaction of the acid chloride and sphingosine (16) and NBD-SM (12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]-sphingosylphosphorylcholine) was from Molecular Probes, Inc., Eugene, OR. It should be noted that the latter substrate is probably heavily contaminated with the unnatural (*threo*) isomer, which is formed during the preparative acidic hydrolysis of SM (17). This presumption probably applies to all commercial preparations and publications using such materials.

Methods

TLC of the amines was carried out with HPTLC plates (E. Merck silica gel 60) and C–M–HOAc 90:10:10 (solvent A) or 85:15:10 (solvent B) or C–M–conc. ammonium hydroxide 30:10:1 (solvent C). The bands were stained with iodine or with Coomassie Brilliant Blue R-250 (18) and, in the latter case, quantified with a Bio-Rad Model 620 videodensitometer operated with reflected white light. The faster band of each PDMP analog, previously identified as the *erythro* form, corresponds to the 1S,2R and 1R,2S stereoisomers, and the slower band, previously identified as the *threo* form, corresponds to the 1R,2R and 1S,2S stereoisomers.

TLC of the cell lipids was run with C–M–W 24:7:1 (solvent D) or 60:35:8 (solvent E).

Growth of cell lines

Comparisons of different inhibitors with regard to suppression of human cancer cell growth were made by the University of Michigan Cancer Center in vitro Drug Evaluation Core Laboratory. MCF-7 breast carcinoma cells, HT-29 colon adenocarcinoma cells, H-460 lung large cell carcinoma cells, and 9L brain gliosarcoma cells were grown in RPMI 1640 medium with 5% fetal bovine serum, 2 mM glutamine, 50 units/ml of penicillin, 50 μg/ml of streptomycin, and 0.1 mg/ml of neomycin. UMSCC-10A head and neck squamous carcinoma cells were grown in minimal essential medium with Earle salts and the same supplements. Medium components were from Sigma Chemical Co. Cells were plated in 96-well microtiter plates (1000 cells/well for H-460 and 9L cells, and 2000 cells/well for the other lines), and the test compounds were added 1 day later. The stock inhibitor solutions, 2 mM in 2 mM BSA, were diluted with different amounts of additional 2 mM BSA, then each solution was diluted 500-fold with growth medium to obtain the final concentrations indicated in the figures and tables.

Five days after plating the H-460 and 9L cells, or 6 days for the other lines, cell growth was evaluated by staining the adhering cells with sulforhodamine B and measuring the absorbance at 520 nm (19). The absorbance of the treated cultures is reported as percent of that of control cultures, to provide an estimate of the fraction of the cells that survived, or of inhibition of growth rate.

For the experiments with labeled thymidine, each 8.5-cm dish contained 500,000 Madin-Darby canine kidney (MDCK) cells in 8 ml of Dulbecco modified essential supplemented medium. The cells were incubated at 37°C in 5% CO_2 for 24 h, then incubated another 24 h with medium containing the inhibitor–BSA complex. The control cells were also incubated in the presence of BSA. The cells were washed with phosphate/saline and trichloroa-

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cetic acid, then scraped off the dishes, dissolved in alkali, and analyzed for protein and DNA tritium. [Methyl-^{3}H]thymidine (10 μCi) was added 4 h prior to harvesting.

Assay of sphingolipid enzymes

The inhibitors were evaluated for their effectiveness against the GlcCer synthase of MDCK cell homogenates by incubation in a thermostatted ultrasonic bath (20) with octanoyl sphingosine and uridinediphospho[^{3}H]glucose (21). The lipoidal substrate (85 μg) was added in liposomes made from 0.57 mg dioleoylphosphatidylcholine and 0.1 mg of Na sulfatide. Confluent cells were washed, then homogenized with a micro-tip sonicator at 0°C for 3 × 30 sec; ~0.2 mg of protein was used in each assay tube. In the case of the aromatic inhibitors, the test compound was simply evaporated to dryness from solution in the incubation tube. This method of adding the inhibitor was found to give the same results as addition as a part of the substrate liposomes. The aliphatic inhibitors, which appeared to be less soluble in water, were added as part of the substrate liposomes.

Acid and neutral ceramidases were assayed under conditions like those above, but the medium contained 110 μM [1-^{14}C]decanoyl sphingosine (10^5 cpm) in 340 μM dioleoylphosphatidylcholine liposomes and 0.34 mg of MDCK cellular protein homogenate. The acid enzyme was incubated in 32.5 mM citrate-Na^+ (pH 4.5) and the neutral enzyme buffer was 40 mM Tris-Cl^- (pH 7.1 at 37°C). After 60 min in the ultrasonic bath, 3 ml of C-M 2:1, carrier decanoic acid, and 0.6 ml of 0.9% saline were added and the lipids in the lower layer were separated by TLC with C-HOAc 9:1. The liberated decanoic acid was scraped off the glass plate and counted.

Ceramide synthase was assayed with 1 μM [3-^{3}H]sphingosine (70,000 cpm, repurified by column chromatography), 0.2 mM stearoyl-CoA, 0.5 mM dithiothreitol, and ~300 μg of MDCK homogenate protein in 25 mM phosphate-K^+ buffer, pH 7.4, in a total volume of 0.2 ml. The incubation (for 30 min) and TLC were carried out as above and the ceramide band was counted.

Sphingomyelin synthase was evaluated with 44 μM [^{14}C]decanoyl sphingosine (10^5 cpm) dispersed with 136 μM dioleoyllecithin as in the ceramide synthase assay, and 5 mM EDTA and 50 mM HEPES-Na^+, pH 7.5, in a total volume of 0.5 ml. MDCK homogenate was centrifuged at 600 *g* briefly, then at 100,000 *g* for 1 h, and the pellet was suspended in water and sonicated with a dipping probe. A portion of this suspension containing 300 μg of protein was used. Incubation was at 37°C for 30 min, after which the lipids were treated as above, using C-M-W 60:35:8 for the isolation of the labeled decanoyl SM.

Acid and neutral SMase assays were based on the procedures of Gatt et al. (22), using liposomes containing NBD-SM dispersed like the labeled ceramide (10 μM substrate and 30 μM lecithin). The assay medium for the neutral enzyme also contained 50 mM Tris-Cl^- (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$ and 0.29 mg of MDCK cell protein in a total volume of 0.25 ml. Incubation was at 37°C for 30 min in the ultrasonic bath, then the fluorescent product, NBD-ceramide, was isolated by partitioning the assay mixture with 0.45 ml 2-propanol, 1.5 ml heptane, and 0.2 ml water. After centrifugation, a trace of contaminating NBD-SM was removed from 0.9 ml of the upper layer by washing with 0.35 ml water. The upper layer was analyzed with a fluorometer (460 nm excitation, 515 nm emission).

Acid SMase was assayed with the same liposomes in 0.2 ml of assay mixture containing 125 mM NaOAc (pH 5.0) and 61 μg of cell protein, with 60 min of incubation at 37°C. The resultant ceramide was determined as above.

RESULTS

Table 1 lists the aromatic compounds synthesized and their migration rates on silica gel TLC plates. Separation of the *threo*- and *erythro*-steroisomers by TLC was generally very good, except for BML-120, -121, and -122 in the acidic solvent. In the basic solvent BML-119 and BML-122 yielded poorly resolved double bands. BML-112 was unexpectedly fast-running, especially when compared with BML-120; both are presumably dihydrochlorides.

Table 2 describes four aliphatic inhibitors, which can be considered to be ceramide analogs in which the C-1 hydroxyl group is replaced by a cyclic amine. It should be noted that the carbon frameworks of compounds in Tables 1 and 2 are numbered differently, thus affecting comparisons of stereochemical configurations. The *threo*- and *erythro*-isomers separated very poorly on TLC plates. Like the aromatic inhibitors, however, the morpholine compounds ran faster than the pyrrolidine ones. The latter are presumably more strongly adsorbed by the silica gel because they are more basic.

Structure-activity correlations

The results of testing the compounds in an assay system for GlcCer synthase are listed in **Table 3.** Because of the preliminary, exploratory nature of this initial study, many of the inhibitors were tested as mixtures of DL-*erythro*- and DL-*threo*-isomers (column 4). Only the D-*threo* enantiomer in each mixture was predicted to be the actual enzyme inhibitor (4); the content of this isomer was calculated by measuring the proportions of the *threo*- and *erythro*-racemic mixtures by quantitative TLC. The DL-*threo* contents were found to range from 40 to 72%. Thus the comparisons, in the case of the mixtures, are only approximate; they were made in the course of the synthetic work primarily to direct further synthetic efforts. The separation of the *threo*- and *erythro*-forms is most conveniently ac-

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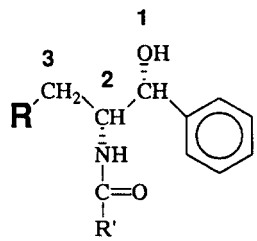
TABLE 1. Structures of the aromatic inhibitors

BML Number or Name	R Group	Phenyl Substituent	TLC hR_f Value[a]
PDMP[b]	morpholino		34(47)
PPMP	morpholino		(53)
112	N-phenylpiperazino		56
113	morpholino	*p*-fluoro	25
114	diethylamino		25
115	piperidino (pentamethyleneimino)		29
116	hexamethyleneimino		34
117[b]	morpholino	*p*-fluoro	41
118	piperidino	*p*-fluoro	26
119	pyrrolidino (tetramethyleneimino)		20–70(44)
120	1-methylpiperazino		7–62
121	3-dimethylaminopiperidino		1–30
122	N-methylethanolamino		6–71
123	azetidino (trimethyleneimino)		12
124	amino		15
125	morpholino	*p*-methoxy	37
126	pyrrolidino	*p*-methoxy	(50)

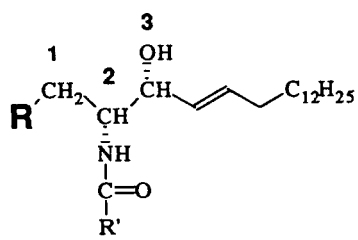


(1R,2R)-1-phenyl-2-acylamino-3-cyclic amino-1-propanol
R is the N-sustituted cyclic amine; R' is the alkyl residue of decanoic or palmitic acid.

[a]Only the relative R_f value of the faster-moving band is shown. The first value was obtained with solvent A, the second with solvent C, and the numbers in parentheses, with solvent B. In the case of BML-117, -125, and -126, a 20-cm high TLC plate was used to improve the separation.
[b]The fatty acid chain suggested by the R' group is decanoyl here, not palmitoyl.

TABLE 2. Characterization of the sphingosyl inhibitors

Number	R Group	Sphingol Structure	TLC hR_f Value[a]
IV-181A	morpholino	2R,3S	43
IV-206A	morpholino	2R,3R	40
IV-230A	pyrrolidino	2R,3S	31
IV-231B	pyrrolidino	2R,3R	31



(2R,3R)-2-palmitoyl-sphingosyl amine or
1-cyclic amino-2-hexadecanoylamino-3-hydroxy octadec-4,5-ene

Note that the backbone's numbering system is different in the aliphatic and aromatic series of inhibitors. Natural (D-*erythro*) sphingosine has the 2S,3R configuration.
[a]TLC solvent: C–M–HOAc 90:5:10. Similar but faster migrations were obtained with solvent A.

complished by crystallization, but the specific conditions needed vary critically for each substance; thus this problem was pursued only in the case of BML-119, a very strong inhibitor.

Comparison of PDMP (1R,2R-decanoate) and PPMP (1R,2R-palmitate), when evaluated at the same time in Expt. f, shows that an increase in the chain length of the N-acyl group from 10 to 16 carbon atoms distinctly improved the inhibitory activity against GlcCer synthase, as noted before (10). Accordingly, most of the other compounds were synthesized with the palmitoyl group for comparison with PPMP. The comparisons between the best inhibitors are clearer at the 5 μM level.

Replacing the oxygen in the morpholine ring of PPMP with a methylene group (BML-115) improved activity ~1.4-fold (calculated from the inhibitions at 5 μM in Expt. f and relative purities, and assuming that the percent inhibition is proportional to concentration in this region: 12.4/27 × 100/32 = 1.4). Previous comparison with mouse brain, human placenta, and human Gaucher spleen glucosyltransferase also showed that replacing the morpholino ring with the piperidino ring in a ketone ana-

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TABLE 3. Inhibition of ceramide glucosyltransferase of MDCK cell homogenates by different compounds

Inhibitor Number	% Inhibition at 80 μM	% Inhibition at 5 μM	% Active Isomer[h]
BML-113	60 ± 4.7[a]		29
BML-114	31 ± 2.9[a]		20
BML-115	84 ± 0.8[a]	12.4 ± 0.7[f]	27
	82 ± 0.3[b]		
BML-116	28 ± 3.2[a]		27
BML-117	35 ± 0.6[b]		36
BML-118	62 ± 0.4[b]	8.3 ± 1.4[f]	32
BML-119	94 ± 1.4[b]	51 ± 2.3[e]	29
	97 ± 0.1[c]	49 ± 0.8[f]	
	96 ± 0.1[d]		
BML-120	11 ± 3.0[c]		26
BML-121	11 ± 0.4[c]		28
BML-122	58 ± 1.6[d]		26
BML-123	86 ± 0.1[d]	15 ± 0.8[f]	33
BML-124	−2 ± 1.6[d]		15
BML-125		9 ± 3.0[e]	26
BML-126	60 ± 1.8[e]		34
		54 ± 0.3[f]	
PDMP	90 ± 0.8[a]	16 ± 1.8[f]	100
PPMP		32 ± 1.8[e]	100
		32 ± 0.7[f]	
IV-181A		12 ± 0.2[g]	100
IV-206A		73 ± 1.5[g]	100
IV-230A		19 ± 2.1[g]	100
IV-231B		87 ± 0.4[g]	100

Different samples were assayed as parts of different experiments, identified by superscripts *a–g*. Each inhibition determination (± SD) was carried out in triplicate. In comparing the activities, keep in mind the fact that most of the samples were not purified to remove the three less-active isomers and that the observed data were not corrected for the level of the primary enantiomer. BML-112 proved to have no inhibitory activity against GlcCer synthase of rabbit liver microsomes.

[h]Percent of the active D-stereoisomer in the synthesized sample, estimated by scanning the two stained bands, assuming the slower one was the (racemic) active form.

log of PDMP (1-phenyl-2-decanoylamino-3-piperidino-1-propanone) produced a much more active inhibitor (11).

Replacing the piperidine group with a 7-membered ring (BML-116) greatly decreased the activity, while use of a 5-membered ring (BML-119) quadrupled the effectiveness (50 vs. 12.4% inhibition). A 4-membered ring (BML-123) yielded a compound about as effective as the piperidino compound. The parent amine (BML-124), its *N,N*-diethyl analog (BML-114), and the sterically bulky *N*-phenylpiperazine analog (BML-112) displayed little or no activity.

Replacing a hydrogen atom with a fluorine atom in the *p*-position of the phenyl ring decreased the inhibitory power (BML-117 vs. PDMP and BML-118 vs. BML-115). Substitution of the *p*-position with an electron-donating moiety, the methoxy group, had a similar weakening effect in the case of the morpholino compound (BML-125 vs. PPMP). Comparison of the pyrrolidino compounds, which are more basic than the morpholino compounds, showed that the methoxy group enhanced the inhibitory power (BML-126 vs. BML-119). Attempts at preparing the *o*- and *m*-fluoro derivatives of BML-119 were unsuccessful.

Preparations of BML-119 were separated into *threo* and *erythro* racemic mixtures by HPLC on a Waters Microbondapak C_{18} column, using M–W–conc. NH_4OH 90:10:0.2 as the elution solvent. The material eluting earlier (but migrating more slowly on a TLC plate) was called BML-130; the later eluting material (faster by TLC) was called BML-129. Assay of GlcCer synthase with each preparation at 5 μM showed 15% inhibition by BML-129 and 79% inhibition by BML-130. TLC analysis of the two preparations revealed incomplete separation, which could explain the minor inhibition by BML-129. When the two stereoisomers were separated by preparative TLC, the difference in effectiveness was found to be somewhat higher, evidently due to the better separation by this method. Thus the slower-migrating stereoisomer accounted for all or nearly all of the inhibitory activity, as noted with PDMP (4).

Comparison of the two pairs of aliphatic inhibitors (bottom of Table 3) showed that the 2R,3R (D-*threo*) form is the primary inhibitor of glucosyltransferase. This finding is in agreement with our previous identification of the active PDMP isomer as being the D-*threo* enantiomer. However, unlike the aromatic analog, BML-129 (2R,3S/2S,3R), there was a relatively small but significant activity in the case of the (*erythro*) 2R,3S stereoisomer. The *erythro* form of PDMP was found to inhibit cell proliferation of rabbit skin fibroblasts almost as well as R,R/S,S-PDMP but it did not act on the GSLs (23). As noted with the aromatic analogs, the pyrrolidine ring was more effective than the morpholine ring (Table 3).

Comparison of the aliphatic and corresponding aromatic inhibitors can be made in the case of the optically active morpholine compounds PPMP and IV-206A, both of which have the R,R structure and the same fatty acid. Here it appears that the aliphatic compound is more effective (Table 3). However in a second comparison, at lower concentrations with the inhibitors incorporated into the substrate liposomes, the degree of inhibition was 77 ± 0.9% with 3 μM IV-231B and 89 ± 0.6% with 6 μM DL-*threo* BML-119.

Evaluations of cultured cell growth

Exposure of five different cancer cell lines to inhibitors at different concentrations for 4 or 5 days showed that the six BML compounds most active against GlcCer synthase were very effective growth inhibitors (**Table 4**). The IC_{50} values (rounded off to one digit in the table) ranged from 0.7 to 2.6 μM.

A set of typical activity/concentration curves for 9L gliosarcoma cells (**Fig. 1**) illustrates the relatively weak effectiveness of R,R-PPMP and even weaker effectiveness of R,R-PDMP. The three new compounds, however, are much better inhibitors of GlcCer synthase and growth.

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TABLE 4. Inhibition of tumor cell growth in vitro by various inhibitors

Cell Type	BML-115	BML-118	BML-119	BML-123	BML-126	BML-129	BML-130
MCF-7	2	2	2	2	1	3	2
H-460	2	2	1	1	1	2	3
HT-29	2		1	2	1	2	2
9L	2	2	1	2	2	2	2
UMSCC-10A	1		1	1	1	2	2

The values shown are IC_{50}s in micromolar concentrations, derived from 16 wells (one to three independent evaluations of 16 each).

These differences in growth inhibitory power correlate with their effectiveness in MDCK cell homogenates as GlcCer synthase inhibitors. Some differences can be expected due to differences in sensitivity of the synthase occurring in each cell type (the synthases were assayed only in MDCK cells). There may be differences in the rate of uptake of inhibitor by the intact cells.

A significant point is that growth inhibition by each of the most active BML compounds occurred in an unusually small range of concentrations (i.e., the slopes of the cytotoxic regions are unusually steep). Similar rapid drop-offs were seen in another series of tests with 9L cells, in which BML-119 yielded 71% of the control growth with 1 μM inhibitor, but only 3% of control growth with 3 μM. Growth was 93% of control growth with 2 μM BML-130 but only 5% of controls with 3 μM inhibitor. While some clinically useful drugs also show a narrow range of effective concentrations, this is a relatively uncommon relationship.

When the *erythro-* and *threo*-stereoisomeric forms of BML-119 (-129 and -130) were compared, they were found to have similar effects on tumor cell growth (Table 4). This observation is similar to the results with PDMP isomers in fibroblasts cited above (23). As enzymes are optically active and as stereoisomers and enantiomers of drugs can differ greatly in their effects on enzymes, it is likely that BML-129 and BML-130 work on different sites of closely related metabolic steps. Judging by several experiments in which BML-119 appeared to be more effective than either of the separated stereoisomers, it is possible that there is a synergistic action of using a mixture of the two isomers.

The two steroisomers were compared in greater detail with MDCK cells and found to inhibit growth and DNA synthesis with similar effectiveness (**Fig. 2** and **Fig. 3**). Thus the MDCK cells behaved like the human tumor cells with regard to IC_{50} and the narrow range of concentrations resulting in inhibition of protein and DNA synthesis.

Surprisingly, the aliphatic inhibitor IV-231B exerted no inhibitory effect on MDCK cell growth when incubated at 20 μM for 1 day or 1 μM for 3 days. Tests with a longer growth period, 5 days, in 5 μM inhibitor also showed no slowing of growth. The control cells, which contained BSA as the only additive to the medium, contained 3.31 $\pm$ 0.19 mg of protein, while the IV-231B/BSA-treated cells contained 3.30 $\pm$ 0.04 mg.

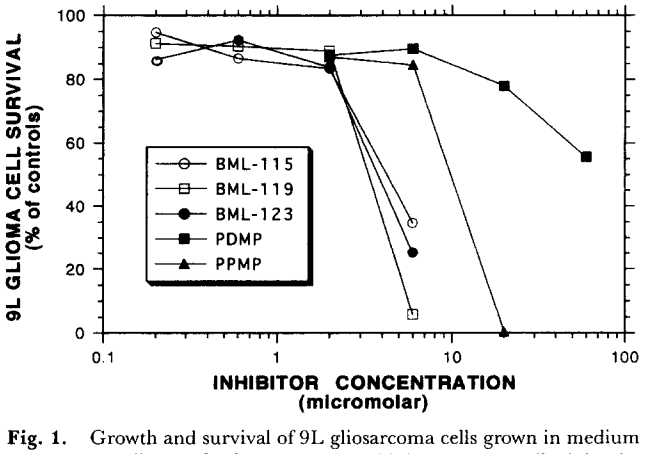

Fig. 1. Growth and survival of 9L gliosarcoma cells grown in medium containing different GlcCer synthase inhibitors, as described in the Methods section. The BML compounds were used as synthesized (mixtures of DL-*threo* and -*erythro* stereoisomers) while the PDMP and PPMP were optically resolved R,R-isomers. The concentrations shown are for the mixed racemic steroisomers, since later work (Table 4) showed that both forms were very similar in effectiveness.

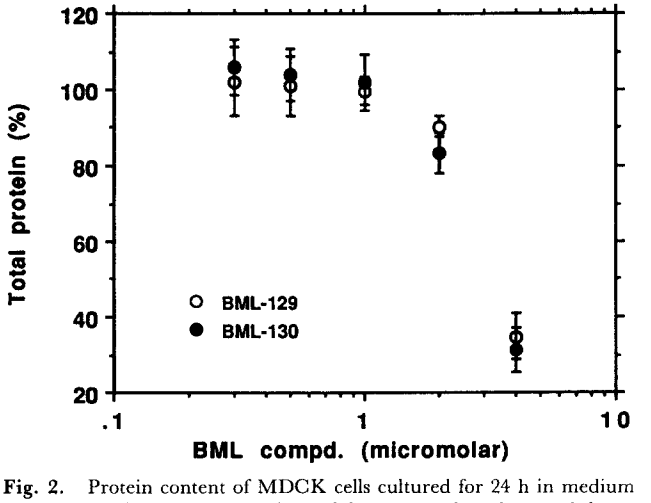

Fig. 2. Protein content of MDCK cells cultured for 24 h in medium containing different concentrations of the separated erythro- and threo-isomers of BML-119, as percent of the incorporation by cells in standard medium. Each point shown is the average of values from three plates, with error bars corresponding to one standard deviation.

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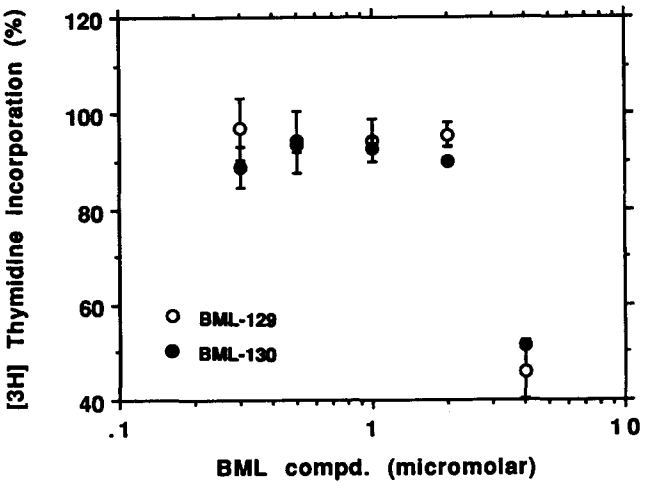

Fig. 3. [^{3}H]thymidine incorporation into DNA of MDCK cells incubated as in Fig. 2. The values are normalized on the basis of the protein content of the incubation dishes and compared to the incorporation by cells in standard medium.

Lipid changes induced in the cells

Examination by TLC of the alkali-stable MDCK lipids after a 24-h incubation disclosed that BML-130 was more effective than BML-129 in lowering GlcCer levels, as expected from its greater effectiveness in vitro as a glucosyltransferase inhibitor. The level of GlcCer, estimated visually, was greatly lowered by 0.3 μM BML-130 or 0.5 μM BML-129. The levels of the other lipids visible on the plate (mainly SM, cholesterol, and fatty acids) were changed little or not at all. BML-129 and the GlcCer synthase inhibitor, the slower-migrating BML-130, were readily detected by TLC at the various levels used, showing that they were taken up by the cells during the incubation period at dose-dependent rates. Lactosylceramide overlapped the inhibitor bands with solvent D but was well separated with solvent E, which brought the inhibitors well above lactosylceramide.

Ceramide accumulation was similar for both stereoisomers (photo not shown). An unexpected finding is that noticeable ceramide accumulation appeared only at inhibitor concentrations that were more than enough to bring GlcCer levels to a very low point (i.e., at 2 or 4 μM). The changes in ceramide concentration were quantitated in a separate experiment by the diglyceride kinase method, which allows one to also determine diacylglycerol (DAG) concentration (24). The results (**Table 5**) are similar to the visually estimated ones: at 0.4 μM BML-129 or -130 there was little effect on ceramide content but at 4 μM inhibitor, a substantial increase was observed. (While the duplicate protein contents per incubation dish were somewhat erratic in the high-dose dishes, in which growth was slow, the changes were nevertheless large and clear.) Accumulation of ceramide had previously been observed with PDMP, at a somewhat higher level of inhibitor in the medium (9). From the data for cellular protein per incubation dish, it can be seen that there was no growth inhibition at the 0.4 μM level with either compound but substantial inhibition at the 4 μM level, especially with the glucosyltransferase inhibitor, BML-130. This finding is similar to the ones made in longer incubations with human cancer cells.

In a separate study of ceramide levels in MDCK cells, BML-130 at various concentrations was incubated with the cells for 24 h. The ceramide concentration, measured by TLC densitometry, was 1.0 nmol/mg protein at 0.5 μM, 1.1 at 1 μM, 1.5 at 2 μM, and 3.3 at 4 μM. The results with BML-129 were virtually identical.

It is interesting that the accumulation of ceramide paralleled an accumulation of DAG, as observed before with PDMP (9). DAG is ordinarily considered to be an activator of protein kinase C and thus a growth stimulator, but the low level of GlcCer in the inhibited cells may counteract the stimulatory effect. Ceramide reacts with lecithin to form SM and DAG, so it is possible that the increased level of the latter reflects enhanced synthesis of the phosphosphingolipid rather than an elevated attack on lecithin by phospholipase D. Arabinofuranosylcytosine (ara-C), an antitumor agent, also produces an elevation in the DAG and ceramide of HL-60 cells (25).

It is also interesting that the addition of lecithin liposomes (500 μg of dioleoylphosphatidylcholine per 8 ml of medium) 1 h after adding the inhibitor/BSA dispersions, followed by 23 h more of incubation, largely prevented the inhibition of cell growth by both BML-129 and -130. The lecithin evidently was partially hydrolyzed to DAG, as shown in the control incubations (5.1 nmol DAG/mg protein without lecithin, 7.7 nmol/mg with lecithin). We had previously noted this effect with MDCK cells when lecithin liposomes, with or without octanoyl sphingosine or the octanoyl homolog of GlcCer, were in the medium (26).

TABLE 5. Effects of BML-129 and -130 on MDCK cell growth and the content of ceramide and diacylglycerol

Growth Medium	Protein	Ceramide	Diglyceride
	μg/dish	nmol/mg protein	
Controls	490	1.04	4.52
	560	0.96	5.61
0.4 μM BML-129	500	1.29	5.51
	538	0.99	5.13
0.4 μM BML-130	544	0.94	4.73
	538	0.87	5.65
4 μM BML-129	396	3.57	9.30
	311	3.78	9.68
4 μM BML-130	160	5.41	11.9
	268	3.34	8.71

Each value is the mean of two analyses of the cells from a single growth dish (the values from 4 μM inhibitors were based on the cells pooled from two dishes). The inhibitors were added to the medium as BSA complexes. The cells were grown in the test media for 24 h.

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TABLE 6. Effect of inhibitors on acid and neutral ceramidases and ceramide synthase of MDCK cells

Inhibitor Tested	Enzyme Activity (% of control)		
	Ceramidase pH 4.5	Ceramidase pH 7.1	Ceramide Synthase
D-*threo*-PDMP, 5 μM	97 ± 4	116 ± 19	99 ± 5
D-*threo*-PDMP, 50 μM	133 ± 13[a]	105 ± 11	66 ± 9[a]
BML-129, 5 μM	108 ± 8	100 ± 0	97 ± 0
BML-129, 50 μM	171 ± 26[a]	99 ± 2	102 ± 1
BML-130, 5 μM	107 ± 11	100 ± 15	108 ± 10
BML-130, 50 μM	160 ± 21[a]	100 ± 15	106 ± 29
IV-231B, 5 μM	106 ± 3	116 ± 20	90 ± 8
IV-231B, 50 μM	113 ± 8	112 ± 3	71 ± 18[a]

Each value is the average ± SD of the activities obtained with triplicate incubations, as a percent of the control (inhibitor-free) incubations. The specific activities of the control cells were 1.51 ± 0.06 nmol/h per mg protein for acid ceramidase, 1.88 ± 0.02 for neutral ceramidase, and 57 pmol/h per mg protein for ceramide synthase.

[a] Notable differences.

TLC of MDCK cells grown in the presence of 0.02–1 μM IV-231B for 3 days showed that the inhibitor indeed penetrated the cells and that there was a great depletion of GlcCer, but no ceramide accumulation. The depletion of GlcCer was evident even at the 0.1 μM level and virtually no GlcCer was visible at the 1 μM level; however, the more polar GSLs were not affected as strongly. After incubation for 5 days in 5 μM inhibitor, all the GSLs were virtually undetectable. The ceramide concentrations in the control and depleted cells were very similar: 13.5 ± 1.4 versus 13.9 ± 0.2 μg/mg protein.

An interesting observation is the striking increase in the ceramide content of both groups between 2 and 5 days of growth: almost 15-fold. This increase may be related to the fact that both groups of cells had approached confluency.

The lack of ceramide accumulation in cells exposed to the aliphatic inhibitor was examined further to see whether it might be due to differential actions of the different inhibitors on additional enzymes involving ceramide metabolism. For example, IV-231B might block ceramide synthase and thus prevent accumulation despite the inability of the cells to utilize ceramide for GlcCer synthesis. However, assay of ceramide synthase in cell membranes showed it was not significantly affected by 5 μM inhibitors (**Table 6**). There did appear to be moderate inhibition at the 50 μM level with PDMP and the aliphatic inhibitor.

Assay of the two kinds of ceramidase (Table 6) showed that there was no effect of either the aliphatic or aromatic inhibitors at the 5 μM level, at which point cell growth is completely stopped in the case of the pyrrolidino compounds. At the 50 μM level, however, the acid enzyme was stimulated markedly by the aromatic inhibitors, particularly the two steroisomeric forms of the pyrrolidino compound.

Sphingomyelin synthase was unaffected by PDMP or the aliphatic inhibitor but BML-129 and -130 produced appreciable inhibition at 50 μM (54% and 61%, respectively) (**Table 7**).

Neutral sphingomyelinase was distinctly stimulated by the aliphatic inhibitor, IV-231B, even at 5 μM (Table 7). From this one would expect that the inhibitor would produce accumulation of ceramide, yet it did not. The two pyrrolidino compounds produced appreciable stimulation at the 50 μM level. No significant effects were obtained with acid SMase.

An interesting kinetic feature of the SMase assays was that increasing the amount of membranous enzyme produced only a small increase in observed activity, under acidic conditions (31–305 μg of protein at pH 4.5). This was probably due to mixing of the exogenous, fluorescent

TABLE 7. Effect of inhibitors on acid and neutral sphingomyelinases and sphingomyelin synthase of MDCK cells

Inhibitor Tested	Enzyme Activity (% of control)		
	Sphingomyelinase pH 4.5	Sphingomyelinase pH 7.4	Sphingomyelin Synthase[a]
D-*threo*-PDMP, 5 μM	102 ± 3	121 ± 13	
D-*threo*-PDMP, 50 μM	100 ± 3	108 ± 8	
BML-129, 5 μM	108 ± 4	105 ± 11	84 ± 27
BML-129, 50 μM	97 ± 3	142 ± 11[b]	46 ± 11[b]
BML-130, 5 μM	109 ± 1	110 ± 7	87 ± 14
BML-130, 50 μM	114 ± 2	152 ± 14[b]	39 ± 18[b]
IV-231B, 5 μM	101 ± 7	131 ± 3[b]	
IV-231B, 50 μM	112 ± 11	120 ± 3[b]	

Each value is the average ± SD of the activities obtained with triplicate incubations, as a percent of the control (inhibitor-free) incubations. The specific activities of the control cells were 403 ± 23 pmol/h per mg protein for acid sphingomyelinase, 345 ± 28 for neutral sphingomyelinase, and 1.61 nmol/h per mg protein for sphingomyelin synthase.

[a] Data for PDMP and IV-231B are not shown here as they were tested in other experiments; no effect was seen.

[b] Notable differences.

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SM with the endogenous SM, so that the specific fluorescence of the substrate decreased as the amount of tissue was increased. In the case of neutral SMase assays, there was a somewhat closer proportionality between tissue weight and observed fluorescent ceramide formation. This suggests that much of the endogenous SM did not react with the neutral enzyme or compete with the NBD-SM. At the low pH, the membrane-bound SM may be liberated and capable of mixing with the fluorescent SM.

DISCUSSION

In previous searches for a potent inhibitor for GlcCer synthase (11), based on phenyl analogs of the ceramide structure, modifying the structure to include an amine group was found to enhance the compound's effectiveness. A similar observation with glycosidase inhibitors has been made in several laboratories (27–31), suggesting that the active sites of both glycosyltransferases and glycosidases have an important transition structure in common. The most intensively studied cyclic amine, morpholine, was positioned to interact with the glucose-binding site. A later study (10) showed the importance of using a longer chain fatty acyl moiety, one that more closely resembled the acyl groups commonly found in natural ceramide (C_{16}, C_{18}, C_{24}). The present study shows that the nature and size of the cyclic amine also exerts a strong influence, a 5-membered ring being most active. It also shows that the phenyl ring used in our earlier work to simulate the *trans*-alkenyl chain corresponding to that of sphingosine could, with benefit, be replaced with the natural alkenyl chain. Our success in substantially increasing the efficacy of our most-studied compound, 1R,2R-PDMP, emphasizes the likelihood that further advances can be made by synthesizing additional variants of the compounds described here.

Findings with the most active GlcCer synthase inhibitors in growth tests compare favorably with evaluations of some clinically useful chemotherapeutic agents on three of the tumor cell lines in the same Drug Evaluation Core Laboratory. The IC_{50} values were 0.2–6 μM for cisplatin, 0.02–44 μM for carboplatin, 0.03–0.2 μM for methotrexate, 0.07–0.2 μM for fluorouracil, and 0.1–1 μM for etoposide. Unlike these agents, our compounds yielded rather similar effects with all the cell types, including MDCK cells, and thus have wider potential chemotherapeutic usefulness. This uniformity of action is consistent with the idea that GSLs play a wide and consistent role in cell growth and differentiation.

An important observation from the MDCK cell study is that strong inhibition of cell growth and DNA synthesis occurred only at the same concentrations of aromatic inhibitor that produced marked ceramide accumulation. This observation supports the suggestion from Hannun's laboratory (3) that ceramide inhibits growth and enhances differentiation or cell death. It agrees with our own study with octanoyl sphingosine, a short chain ceramide that produced greatly elevated levels of natural ceramide and slowed growth (26). It is also in agreement with our finding (details not given here) that some synthetic, nonionic ceramide-like compounds did not inhibit GlcCer synthase even though they behave like ceramide in blocking growth (32). Compounds tested included 20 μM D-*erythro*-N-myristoyl-2-amino-1-phenyl-1-propanol, its L-enantiomer, the four stereoisomers of *N*-acetylsphinganine, and *N*-acetylsphingosine, kindly furnished by Dr. Y. A. Hannun. Furthermore, the lack of growth inhibition and ceramide accumulation in cells treated with the aliphatic inhibitor IV-231B is also consistent with the correlation between ceramide level and growth rate.

The accumulation of ceramide that occurred at higher levels of GlcCer synthase inhibitors could be attributed not only to blockage of ceramide utilization, but also to blockage of SM synthesis or ceramide hydrolase. This possibility is especially relevant to the R,S-, S,R-, and S,S-isomers, which seem to exert effects on sphingolipids without strongly inhibiting GlcCer synthesis. Our tests with both the DL-*erythro*-pyrrolidino inhibitor (BML-129) and the DL-*threo*-pyrrolidino inhibitor (BML-130), at a level producing strong growth inhibition, showed that neither material inhibited the enzymes tested (Tables 6 and 7) but they did cause growth inhibition as well as accumulation of ceramide. PDMP, at relatively high concentrations (50 μM), was found to inhibit SM synthase in growing CHO cells (33). In our test with MDCK homogenates, it did not inhibit this synthase, in agreement with our finding that labeled palmitate incorporation into SM was stimulated by PDMP (9).

It is curious that low concentrations of PDMP and related synthase inhibitors can produce considerable depletion of GSLs without producing strong inhibition of growth. A similar observation was made with fish embryos (34) and cultured Lewis lung carcinoma cells (35). In addition, mutant cell strains have been found that grow (slowly) without apparently making any sphingolipids at all (36, 37). This problem has been discussed (38). Perhaps most of the GSL molecules occur in aggregates, typically in plasma membranes or cytoskeletal filaments, and the rest of the molecules are diffusely distributed. The latter may carry out the GSL functions required for growth and development in a sheltered, culture-dish environment, while the aggregates may perform the GSL functions needed in a natural environment. In the case of cancer cells, which seem to evade the host's immunodefense system by secreting GSLs (39), even a modest blockage of GSL synthesis could be therapeutically useful.

A complicating observation that bears on the essentiality of GSLs is the discovery that the yeast mutant mentioned above (36) synthesizes novel inositol glycerophospholipids that presumably substitute for some sphin-

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golipid structures (40). Our lipid analyses, which involved alkaline methanolysis of ester-type lipids, could not detect such lipids, if indeed they were formed.

The failure of two strong inhibitors (BML-129 and -130) to appreciably affect growth and ceramide levels when added shortly before adding lecithin liposomes, is thought-provoking. Perhaps the liposomes withdrew the inhibitors from the cells and medium, but TLC showed disappearance of GlcCer and the presence of the amines in the cells at a level similar to that obtained without added lecithin. Another explanation is that the exogenous lecithin, acting as a substrate for SM synthase, stimulated conversion of accumulating ceramide to SM, thus minimizing the expected elevation in ceramide level. But a simultaneous, corresponding increase in DAG was not evident. Whatever the explanation, the data suggest that treatment of cancer patients with drugs like BML-130 ought to be accompanied by a treatment to lower body levels of lecithin and raise the levels of ceramide. Retinoic acid is a growth inhibitor of interest in cancer chemotherapy and a possible adjunct in the use of our inhibitors. It has been found to elevate ceramide and DAG levels (41) and possibly lower lecithin content (42).

D-*threo*-PDMP was found to be rather active in delaying tumor cell growth or in producing complete cures in mice (7) but high doses were needed. The new BML inhibitors are ≈30 times as active, judging by the data in Fig. 1, so the dosage levels should be more typical of clinically useful drugs. The need to use high doses with PDMP was due to rapid inactivation by cytochrome P450 (43) and it will be necessary to evaluate the resistance of the new inhibitors in this regard.

The mode of growth inhibition, GSL depletion, and ceramide accumulation by erythro isomers of the GlcCer synthase inhibitors remains a mystery. Possible sites of action include sphingosine metabolism, intracellular sphingolipid transport by GSL-binding proteins (44), the interactions between saposins and their respective sphingolipid hydrolases (45), the translocation of GlcCer from the cytoplasmic to the luminal side of Golgi membranes (46), and sphingolipid intramembrane distribution. Because of the structural similarities between the aromatic inhibitors and ceramide, one may postulate that they mimic ceramide's physiological effects, such as activating cellular kinases (47) or phosphatases (48).

We are indebted to Paul W. Chrenka, Jr. for determining the inhibition of cancer cells. This work was partially supported by Contract R43 CA 58159 from the National Cancer Institute, by the Glycolipid Research Fund, by the University of Michigan Renal Center Grant P50 DK39255 (to JAS); by Grant GM 35712 (to BG) from the National Institute of General Medical Sciences, and by the University of Michigan Comprehensive Cancer Center grant 2P30 CA 46592 (to LLW) from the National Cancer Institute, U. S. Public Health Service, DHHS.

Manuscript received 8 July 1994 and in revised form 12 September 1994.

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