α-Galactosylceramide and Novel Synthetic Glycolipids Directly Induce the Innate Host Defense Pathway and Have Direct Activity against Hepatitis B and C Viruses

Anand S. Mehta,1* Baohua Gu,1 Bertha Conyers,1 Serguey Ouzounov,1 Lijuan Wang,1 Robert M. Moriarty,2 Raymond A. Dwek,3 and Timothy M. Block1

Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, The Jefferson Center, Doylestown, Pennsylvania 18901; Department of Chemistry, University of Illinois at Chicago, Chicago, Illinois 60607; and Department of Biochemistry, The Glycobiology Institute, University of Oxford, Oxford, United Kingdom OX1 3QU

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α-Galactosylceramide is a glycolipid derived from marine sponges that is currently in human clinical trials as an anticancer agent. It has also been shown to be effective in reducing the amount of hepatitis B virus (HBV) DNA detected in mice that produce HBV constitutively from a transgene. It was assumed that all of the antiviral and antitumor activities associated with α-galactosylceramide were mediated through the activation of NK T cells. However, we report here an additional unpredicted activity of α-galactosylceramide as a direct antiviral agent and inducer of the innate host defense pathway. To exploit this activity, we have developed a new class of smaller, orally available glycolipids that also induce the innate host defense pathway and have direct activity against HBV and hepatitis C virus.

Hepatitis B virus (HBV) is the prototypic member of the Hepadnaviridae family of viruses that chronically infect >350 million people worldwide (1). The major complication is the development of primary hepatocellular carcinoma estimated to cause >500,000 deaths annually (1, 2). Although there is no cure for HBV infection, several therapeutic options now exist (9). However, the poor response rate and development of resistant mutants highlight the need for alternatives and complements to the conventional therapeutic regimens (13).

α-Galactosylceramide, shown in Fig. 1A, is a glycolipid derived from marine sponges that is currently in human clinical trials as an anticancer agent (4). It has also been shown to be effective in reducing the amount of HBV DNA detected in mice that produce HBV constitutively from a transgene (11). These long-chain alkylated sugars bind CD1 molecules on the plasma membranes of diverse cell types and are presented to subsets of CD4+ CD8− or CD4− CD8− T cells that express markers associated with NK cells and are referred to as NK T cells (18). NK T cells, when activated, secrete cytokines that have antiviral and antitumor properties and are thought to mediate important components of the non-major histocompatibility complex-dependent immune system.

Previous work showed that the synthetic glycolipid N-nonyl-deoxy-galactonojirimycin (NN-DGJ) could inhibit the production and secretion of HBV in vitro (16, 17). The exact molecular mechanism whereby NN-DGJ (and the chemical family it represents) induced its antiviral effect is not fully known but is analogous to the effect of inducers of the innate host defense pathway against HBV (8, 24). That is, it has been shown (similar to the effect of alpha interferon) (i) that although it is antiviral, NN-DGJ does not inhibit the viral polymerase, (ii) that NN-DGJ does not inhibit HBV surface protein (HBsAg) production or other viral gene product production, and (iii) that total viral RNA levels were not influenced by these compounds (17). Recent work has shown that the mechanism of action of these compounds is similar to that of alpha interferon (8) in that they either inhibit the formation of pregenomic RNA-containing capsids or accelerate their degradation (15). Further work characterizing the structural requirements for antiviral activity highlighted a need for both a sugar head group and an unbroken alkyl tail of at least eight carbons (16). Structure activity relationship analysis led to the development of an analogue of NN-DGJ, N-9-oxadecyl-6-methyl-DGJ, which was as potent and efficacious as the parent molecule but with a much improved toxicity profile. Similar to the structural requirements for α-galactosylceramide, structural analysis of the synthetic glycolipids also highlighted the importance of a galactose head group in the antiviral activity of these compounds (16). Synthetic glycolipids with alternative head groups, such as mannose or fucose, have reduced potency compared to those with the galactose head group (16).

In the case of α-galactosylceramide, it was assumed that all of the antiviral and antitumor activities were mediated through the activation of NK T cells (11, 12). However, we report here an additional unpredicted activity of α-galactosylceramide as a direct antiviral agent and inducer of the innate host defense pathway. To exploit this activity, we have developed a new class of smaller, orally available glycolipids (Fig. 1B and C) that also induce the innate host defense pathway and have direct activity against HBV and hepatitis C virus (HCV).

MATERIALS AND METHODS

Cells and compounds. HepG2 2.2.15 cells were kindly provided by George Acs (Mt. Sinai Medical College, New York, N.Y.) and maintained in the same way as...
HepG2 cells. The HCV subgenomic replicon cell line 9-13, a kind gift of R. Bartenschlager (14), was cultured in Dulbecco’s modified Eagle medium (In-vitrogen Corp., Carlsbad, Calif.) containing 10% fetal calf serum, 1% penicillin-streptomycin, 1% nonessential amino acids, and 0.5 mg of Geneticin/ml. The cells were maintained at subconfluence prior to splitting. α-Galactosylceramide was the kind gift of Kirin Brewery Co., Ltd. (Tokyo, Japan). All synthetic glycolipids presented in this study were provided by Synergy Pharmaceuticals (Edison, N.J.) and United Therapeutics (Silver Spring, Md.) according to the manufacturer’s directions. RNA samples were further purified using the Ambion (Austin, Tex.) DNA-free kit before reverse transcriptase (RT) PCR with PCR conditions and primers exactly as reported in the literature (10). PCR was performed in the absence of RT for 50 cycles to ensure no DNA contamination. Dilution experiments were used to ensure that the PCR was within the linear range of the assay. Southern blotting–RT-PCR of RNA from HepG2 cells treated with the appropriate concentration of N-9-oxadecyl-6-methyl-DGJ or with alpha interferon (2a/2b) for 16 h was also performed to allow the quantification of induction. Briefly, limited PCR was performed as described above for 5, 10, or 15 cycles, and the PCR products were transferred to nylon membranes. Hybridization was carried out using a 1,377-bp cDNA probe from nucleotides 1 to 1377 of the published OAS-40/46 gene (accession no. X02874). OAS-40/46-specific bands were identified and quantified by phosphorimager analysis (Bio-Rad). For analysis of interferon secretion, cells were treated as described above, and the amount of beta interferon secreted into the culture medium was determined using a commercially available human beta interferon enzyme-linked immunosorbent assay kit (PBL Biomedical Laboratories, Piscataway, N.J.) according to the manufacturer’s directions.

RESULTS

α-Galactosylceramide (Fig. 1) has been reported to have antiviral activity in a transgenic animal model of HBV (11). This antiviral activity is thought to involve the activation of intrahepatic NK T cells, which inhibit HBV through the secretion of gamma interferon. However, the direct activity of this compound against HBV was not tested and remained a possibility. Thus, the direct antiviral activities of α-galactosylceramide and our smaller orally available glycolipids were tested in tissue culture using the stable HBV-producing cell line HepG2 and our smaller orally available glycolipids were tested in tissue culture using the stable HBV-producing cell line HepG2. For the assays, the cells were treated in 24-well trays. Briefly, HepG2 cells were incubated with either α-galactosylceramide or the synthetic glycolipids shown in Fig. 1 for the desired length of time, and the total RNA was harvested using Tri-reagent (Gibco-BRL, Rockville, Md.) according to the manufacturer’s directions. RNA samples were further purified using the Ambion (Austin, Tex.) DNA-free kit before reverse transcriptase (RT) PCR with PCR conditions and primers exactly as reported in the literature (10). PCR was performed in the absence of RT for 50 cycles to ensure no DNA contamination. Dilution experiments were used to ensure that the PCR was within the linear range of the assay. Southern blotting–RT-PCR of RNA from HepG2 cells treated with the appropriate concentration of N-9-oxadecyl-6-methyl-DGJ or with alpha interferon (2a/2b) for 16 h was also performed to allow the quantification of induction. Briefly, limited PCR was performed as described above for 5, 10, or 15 cycles, and the PCR products were transferred to nylon membranes. Hybridization was carried out using a 1,377-bp cDNA probe from nucleotides 1 to 1377 of the published OAS-40/46 gene (accession no. X02874). OAS-40/46-specific bands were identified and quantified by phosphorimager analysis (Bio-Rad). For analysis of interferon secretion, cells were treated as described above, and the amount of beta interferon secreted into the culture medium was determined using a commercially available human beta interferon enzyme-linked immunosorbent assay kit (PBL Biomedical Laboratories, Piscataway, N.J.) according to the manufacturer’s directions.

Western blot analysis: Western blot analysis was done as described elsewhere (5). A monoclonal antibody to NS5A, a kind gift of C. Liu (University of Florida, Gainesville), was used to measure the viral-protein level.
nM to tissue culture cells inhibited the secretion of enveloped HBV with a 50% inhibitory concentration (IC₅₀) of 0.4 ± 1.1 nM. In this assay, alpha interferon is used as a control and also inhibits secretion effectively at a concentration of 10³ IU/ml, consistent with other reports (8). It was noted that the potency of α-galactosylceramide was dependent upon the formulation of its delivery; dissolution in the lipophilic solvent provided by the supplier (intended to promote intracellular delivery) actually reduced potency (data not shown), suggesting that a surface receptor is involved. Similar to α-galactosylceramide, and consistent with previous reports regarding this compound class (16, 17), the synthetic glycolipid N-9-oxadecyl-6-methyl-DGJ also exerted antiviral activity at various concentrations (Fig. 2B) with an IC₅₀ of 1 ± 3.6 μM.

The cytotoxicity profiles of α-galactosylceramide and the synthetic glycolipid N-9-oxadecyl-6-methyl-DGJ were examined in parallel (and under the same conditions), as were the antiviral profiles, and the results are shown in Fig. 2C and D. In these experiments, the cytotoxic concentration required to kill 50% of the cells with α-galactosylceramide is 8 μM, and that for N-9-oxadecyl-6-methyl-DGJ is >2,000 μM. Since the IC₅₀ for α-galactosylceramide and N-9-oxadecyl-6-methyl-DGJ were 0.4 ± 1.1 nM and 1 ± 3.6 μM, respectively, it is safe to say that the antiviral activities of these compounds occur at concentrations well below that at which toxicity was observed, thus demonstrating selectivity for viral functions.

As stated, neither α-galactosylceramide nor N-9-oxadecyl-6-methyl-DGJ had any detectable effect upon HBsAg production or secretion, core antigen production or secretion, or HBV polymerase activity at concentrations that were highly antiviral (11, 17). This indicates that the compounds were well tolerated at these concentrations and that the antiviral activity cannot be explained by a direct effect upon the synthesis of viral products.
but rather through the activation of a cellular defense mechanism.

Current experimental and therapeutic antivirals against HBV target either a specific viral protein, such as the viral polymerase, or activate components of the innate host defense pathway. As both α-galactosylerceramide and N-9-oxadecyl-6-methyl-DGJ do not have a detectable impact upon HBV-specific proteins, it appeared possible that they induced components of the innate host defense pathway. Thus, the abilities of α-galactosylerceramide and N-9-oxadecyl-6-methyl-DGJ to directly induce the innate host defense pathway in tissue culture were determined by analysis of the induction of the 2',5' OAS gene utilizing a southern blotting-based methodology (10, 20). As Fig. 3A shows, alpha interferon is a potent inducer of both the medium (p69) and small (p40) 2',5' OAS genes. In contrast, α-galactosylerceramide induced only the small 2',5' OAS gene expression over a wide dose range. Consistent with this result, the synthetic glycolipid N-9-oxadecyl-6-methyl-DGJ also induced only the small 2',5' OAS gene. Figure 3B shows the induction of the small 2',5' OAS gene utilizing a southern blotting–RT-PCR-based methodology. As the figure shows, N-9-oxadecyl-6-methyl-DGJ induced a 20-fold induction of the 2',5' OAS gene at 70 μM, with lower concentrations giving a dose-dependent 2- to 15-fold increase in 2',5' OAS gene expression.

The innate host defense pathway may also involve the induction and secretion of interferon (21). As shown in Fig. 4, beta interferon secretion was induced by both the exogenous addition of 10^7 U of alpha interferon (2a/2b)/ml or the addition of N-9-oxadecyl-6-methyl-DGJ. Again, the induction of components of the innate host defense pathway correlated with antiviral activity. N-7-oxanonyl-6-methyl-DGJ, which has limited antiviral activity at the doses used in this assay, does not induce any of the 2,5 OAS genes and was unable to induce the antiviral activity seen with this compound class correlates with concentrations that are antiviral induce the small 2',5' OAS gene (16), while compounds and doses with no antiviral activity do not (Fig. 2 and 3 and data not shown).

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production of beta interferon. Consistent with other reports (8), we have found that the level of beta interferon secreted after treatment with N-9-oxadecyl-6-methyl-DGJ (250 pg/ml or 10 to 20 U/ml), while consistent with the activation of the interferon pathway in these cell lines (3), is not sufficient to inhibit HBV directly (data not shown). However, this result provides further evidence that N-9-oxadecyl-6-methyl-DGJ induces the innate host defense pathway.

As N-9-oxadecyl-6-methyl-DGJ could induce the innate host defense pathway, it was of interest to determine its effect against other viruses, such as HCV. Clone 9-13 is an Huh7-derived cell line that constitutively expresses the bicistronic HCV subgenomic replicon and has been shown to be sensitive to alpha interferon (14). Therefore, as replication of HCV RNA in 9-13 cells is sensitive to alpha interferon and N-9-oxadecyl-6-methyl-DGJ induces an arm of the interferon pathway, it was hypothesized that N-9-oxadecyl-6-methyl-DGJ would have an antiviral effect upon HCV in this cell line. This possibility was tested by examining the amount of HCV RNA in 9-13 cells as a function of incubation in various concentrations of our lead compound, N-9-oxadecyl-6-methyl-DGJ. The results, shown in Fig. 5, demonstrate a clear, if subtle, dose-dependent reduction in the NS5A protein level as a function of drug treatment. A more dramatic reduction in the steady-state level of HCV RNA is observed after treatment with various concentrations of N-9-oxadecyl-6-methyl-DGJ, with an IC50 of 1.5 ± 4.0 μM. Beta-actin protein and RNA were used as controls in these experiments. Thus, as predicted, N-9-oxadecyl-6-methyl-DGJ is inhibitory for HCV.

DISCUSSION

In this report, we make several simple but surprising points regarding the abilities of certain glycolipid molecules, such as...
α-galactosylceramide and N-9-oxadecyl-6-methyl-DGJ, to directly (i) induce the small 2',5' OAS gene family, (ii) induce the secretion of beta interferon, and (iii) inhibit the production of HBV DNA and HCV RNA. Although induction of components of the innate host defense pathway is clearly observed with these compounds, the exact mechanism by which they inhibit HBV and HCV is still under investigation by our laboratory and many others. However, it does appear that it is not through the direct action of the 2',5' OAS gene family (7).

It was noted that N-7-oxanonyl-6-methyl-DGJ, which had very limited activity in the experiments described here (Fig. 2 and 3), was shown to antagonize an ion channel activity associated with the HCV p7 protein (19). Since p7 may be essential to the life cycle of HCV, one possibility is that the mechanism of antiviral action observed for the glycolipids studied here involved inhibiting p7 function. However, since N-9-oxadecyl-6-methyl-DGJ activated the small (p40) 2',5' OAS gene and inhibited HCV under conditions where p7 is not present, this cannot be the sole explanation for its antiviral action.

More generally, how the innate host defense pathway is activated is not fully known, but the rapid induction and high-dose desensitization seen with the glycolipids used here is consistent with a receptor-mediated mechanism (21). It was initially believed that the glycolipids might work through the Toll-like receptor (TLR) family. However, a key factor of TLR stimulation, NF-κB activation, was not detected with these compounds (data not shown). Thus, it is possible that the glycolipids activate host defense components through a TLR-distinct or NF-κB-independent mechanism. The analysis of a possible receptor is under way.

Activation of an innate host defense pathway, as shown here, is in some respects analogous to the phenomenon observed with double-stranded RNA (6, 24). In contrast to the situation with double-stranded RNA, however, activation with N-9-oxadecyl-6-methyl-DGJ and α-galactosylceramide appears to induce only a subset of interferon-specific transcripts and is associated with little or no toxicity (Fig. 2 and 3). In addition, these molecules are orally available and hence represent potentially orally available therapeutics. Indeed, one compound in this class is in phase 2 clinical trials for the treatment of chronic HCV infection.

In conclusion, we have shown that small, orally available glycolipid mimetics, such as N-methoxynonyl-6-methyl-DGJ, can directly activate cellular defense genes (such as the small 2',5' OAS gene) and reduce the amount of HBV and HCV replication without the recruitment of any cells other than those infected. Since the synthetic glycolipids that stimulate this response could be mimetic for pattern recognition molecules, we propose that hepatocytes have the ability themselves to autogenously recognize and react defensively to foreign pathogen molecules without assistance from any other immunological cells, and perhaps this represents a very primitive arm of the host defense system. Thus, these synthetic glycolipids represent a new class of orally available small molecules that may have therapeutic value in all cases where interferon induction is useful.

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REFERENCES


