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Use of a Prenylation Inhibitor as a Novel Antiviral Agent

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No specific therapy exists for hepatitis delta virus (HDV), which can cause severe liver disease. Molecular genetic studies have implicated the prenylation site of large delta antigen as a critical determinant of HDV particle assembly. We have established a cell culture model which produces HDV-like particles, and we show that delta antigen prenylation can be pharmacologically inhibited by the prenylation inhibitor BZA-5B. Furthermore, BZA-5B specifically abolishes particle production in a dose-dependent manner. These results demonstrate that the use of such a prenylation inhibitor-based antiviral therapy may be feasible and identify a novel class of potential antiviral agents.

Hepatitis delta virus (HDV) is a novel viral pathogen that is an important cause of acute and chronic liver disease in various parts of the world (4, 14, 16, 18, 24, 29). There is currently no effective treatment for HDV infections. Recent advances in our understanding of the viral life cycle have revealed new targets for antiviral therapy.

The HDV particle's core contains the 1.7-kb single-stranded circular genomic RNA (32) and the virally encoded small and large delta antigens. The particle core is encapsulated by a lipid envelope embedded with hepatitis B virus (HBV) surface antigen (HBsAg) proteins (2). HBsAg is provided by HBV, which accounts for the occurrence of HDV infections only in the presence of an HBV infection.

Infectious HDV particles can be produced in vitro by cells transfected with cloned DNAs containing portions of the HBV and HDV genomes (28, 33). Virus-like particles can also be produced in the absence of genome replication. Indeed, cotransfection into cultured cells of plasmids encoding only large delta antigen and HBsAg is sufficient for the production and release of particles (5, 31). Essential to large delta antigen's requisite role in HDV assembly are the last four amino acids at the carboxyl terminus (Cys-Arg-Pro-Gln-COOH), which together form a "CXXX box," a motif recognized by prenyltransferase enzymes as a substrate for covalent addition of a prenyl lipid to the CXXX box cysteine (13, 21, 26, 34). Prenyl lipid addition to delta antigen may help target the protein to cellular membranes containing HBsAg and may help trigger virus assembly (7, 10).

When the large delta antigen's CXXX box is destroyed by genetic mutation, prenylation is prevented and particle production is abolished (11, 19). While these studies demonstrated the critical role of prenylation in HDV virion morphogenesis, a strategy that uses mutagenesis to disrupt delta antigen prenylation in natural infections would be impractical. Therefore, we investigated whether virion assembly is similarly inhibited when delta antigen prenylation is prevented by pharmacologic means—by using a drug that specifically inhibits the enzyme responsible for the transfer of the prenyl lipid to delta antigen.

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Construction and characterization of a particle-producing cell line. We constructed a permanent cell line capable of continuously producing HDV-like particles. Briefly, a clone of NIH 3T3 cells stably transfected with pSVL-large, which expresses large delta antigen (11), and pHygro, which encodes hygromycin resistance, was further cotransfected with SV24H, which expresses HBsAg (3), and pRCCMV (Invitrogen), which encodes G418 resistance. Cells were transfected with Lipofectamine (Gibco BRL) according to the manufacturer's directions and selected with hygromycin B and G418. One of the resulting clones, termed LH, was selected for further characterization.

Confluent LH cells were washed twice with phosphate-buffered saline, harvested in cell lysis buffer (50 mM Tris [pH 8.8], 2% sodium dodecyl sulfate [SDS]), and analyzed for the presence of large delta antigen. Briefly, samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with 12% resolving gels, followed by transfer to Immobilon polyvinylidene difluoride membranes (Millipore), essentially as described previously (11). The blots were treated with a human antibody against delta antigen (11) and with alkaline phosphatase-conjugated rabbit antibody to human immunoglobulin G (Promega), followed by chemifluorescence development (with a kit from Amersham) and detection (STORM 840; Molecular Dynamics). The results (Fig. 1, lanes 2) confirm the presence of large delta antigen in LH cells. In addition, similar to other cell lines stably transfected with cDNA encoding HBsAg (17, 20, 27), LH cells abundantly express and constitutively secrete HBsAg into the media, as measured by a commercial assay (Auszyme; Abbott Laboratories). Finally, LH cells also produce and release HDV-like particles that contain both HBsAg and delta antigen. The particles can be isolated from clarified medium supernatants by either immunoprecipitation with a monoclonal antibody to HBsAg (Abbott Laboratories) (Fig. 1A, lane 1) or ultracentrifugation through a 20% sucrose cushion and collection of the pellet (25) (Fig. 1B, lane 1). The LH cell line is thus well suited to pharmacologic studies dependent on precise reproducibility and aimed at measuring the effect of various inhibitors on particle production.

Effect of BZA-5B on large delta antigen prenylation. We next wished to identify a compound capable of inhibiting large delta antigen prenylation. For this purpose, we chose to test BZA-5B, a drug originally synthesized as a specific prenyltransferase inhibitor and known to inhibit prenylation of the oncoprotein H-Ras^{V12} (22). BZA-5B can abrogate the prenylation-

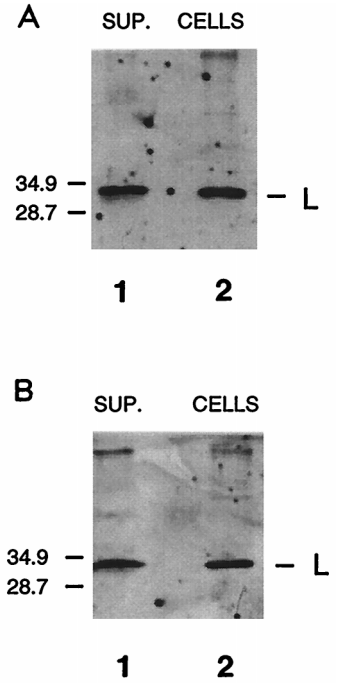

FIG. 1. LH cells produce HDV-like particles. (A) HDV-like particles (lane 1) were immunoprecipitated from clarified medium supernatants (Sup.) of LH cells with a monoclonal antibody to HBsAg and subjected to immunoblot analysis for the presence of large delta antigen. LH cells remaining on the dish were harvested in cell lysis buffer and an aliquot (lane 2) was included in the immunoblot analysis. (B) Particles (lane 1) were isolated by ultracentrifugation of clarified medium supernatants of LH cells and an aliquot was subjected to immunoblot analysis, along with a sample of LH cell lysate (lane 2). L, large delta antigen. The locations of prestained molecular mass markers are shown at the left (in kilodaltons).

dependent, H-Ras^{V12}-mediated transformation of Rat-1 cells without observed gross cellular toxicity (15).

To determine the effect of BZA-5B on large delta antigen prenylation, we performed in vitro translation-prenylation reactions, essentially as described previously (11), with the addition of BZA-5B. Briefly, combined in vitro transcription-translation reactions with rabbit reticulocyte lysates (Promega) were programmed with a plasmid encoding large delta antigen in the presence of [5-^{3}H]mevalonate (60 Ci/mmol [*R*, *S*]; American Radiolabeled Chemicals), a metabolic precursor of prenyl lipids. A carrier (final concentrations of 0.5 mM dithiothreitol [DTT] and 0.05% dimethyl sulfoxide [DMSO]) or various concentrations of BZA-5B dissolved in the carrier were included in the reactions. Aliquots of each reaction mixture were then subjected to SDS-PAGE and either fluorography (11) (Fig. 2A) or immunoblot analysis for delta antigen as described above (Fig. 2B).

In the absence of BZA-5B, [^{3}H]mevalonate was incorporated into large delta antigen (Fig. 2A, lane 3), indicating that it undergoes prenylation, as previously shown (11). BZA-5B had no apparent effect on the translation of large delta antigen (Fig. 2B), whereas profound inhibition of posttranslational prenyl lipid modification was observed at 5 μM BZA-5B. No prenylation was detectable at concentrations of 25 μM and above (Fig. 2A, lanes 4 to 7). Thus, BZA-5B appears to be a potent inhibitor of large delta antigen prenylation. These results are in good agreement with recent data showing that large delta antigen is prenylated by farnesyltransferase (23), the prenyltransferase most sensitive to BZA-5B (15). Our results also suggested that BZA-5B would be a good candidate for inhibiting HDV particle production.

Effect of BZA-5B on particle production. To test the hypothesis that an inhibitor of delta antigen prenylation can prevent virus-like-particle production, the effect of BZA-5B on LH-cell particle production was studied (Fig. 3). LH cells were seeded at low confluency in 100-mm-diameter dishes (15). Duplicate dishes were grown in media containing a carrier (0.5 mM DTT and 0.05% DMSO—to minimize oxidation and enhance cellular penetration of the compound) alone or a carrier plus various concentrations of BZA-5B. After four medium changes, made every other day, portions (2.5 ml) of the respective final clarified medium supernatants were quantitatively analyzed for the presence of HDV-like particles with the centrifugation-over-sucrose-cushion and immunoblot procedures described above (Fig. 3A). The large delta antigen bands were quantitated with the ImageQuant (Molecular Dynamics) software package. All quantitations were performed within the linear range of the chemifluorescence detection, as determined by serial dilutions of large delta antigen standards. As a control for nonspecific inhibition of protein synthesis and secretion, HBsAg was quantitated in duplicate aliquots (10 μl) of each medium supernatant sample with a commercial assay (Auszyme; Abbott Laboratories). After the medium supernatants were collected, the underlying LH cells were harvested and counted, and a fraction of them were subjected to immunoblot analysis (Fig. 3B). The percentage of control particles per cell and of HBsAg per cell was then calculated for each concentration of BZA-5B and plotted as a function of drug concentration (Fig. 3C).

As shown in Fig. 3A, a significant inhibition of particle production was observed with 10 μM BZA-5B compared to that in the control with the carrier alone (lane 2 versus lane 1). At 50 μM BZA-5B, particle production is reduced to below the level of detection (Fig. 3A, lane 4). The inhibition of particle formation was not due to a decrease in the cellular pool of delta antigen (Fig. 3B). To assess whether BZA-5B's inhibition of particle production was secondary to a more general effect on secretion or cell metabolism, rather than direct prenylation inhibition, we measured the HBsAg contained in the collected medium supernatants. HBsAg does not harbor a CXXX box and it is therefore not subject to prenylation, although the known constitutive secretion of HBsAg alone into the media could be

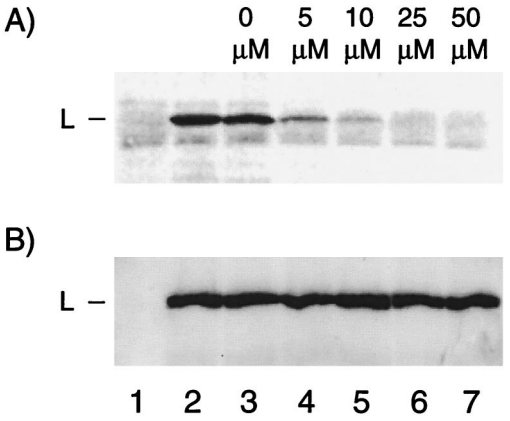

FIG. 2. BZA-5B inhibits prenylation of large delta antigen. Combined in vitro transcription-translation reactions were performed with rabbit reticulocyte lysates programmed with water (lanes 1) or a plasmid encoding large delta antigen (lanes 2 to 7) in the presence of [5-^{3}H]mevalonate and either water (lanes 2), a carrier (0.5 mM DTT and 0.05% DMSO) (lanes 3), or a carrier with 5, 10, 25, or 50 μM BZA-5B, as indicated. Aliquots (1 μl) were subjected to SDS-PAGE and either fluorography (A) or immunoblot analysis (B). L, large delta antigen.

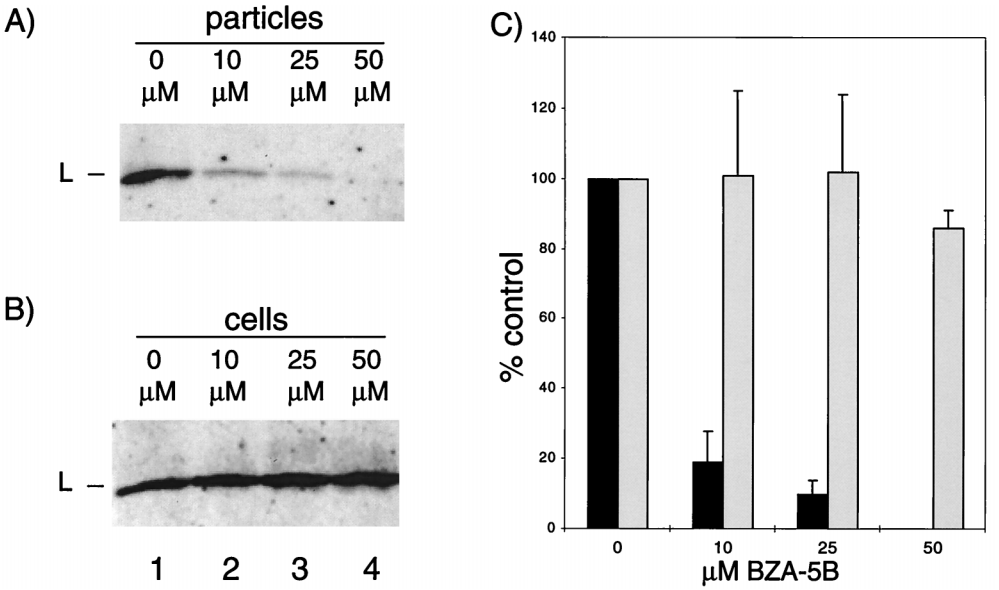

FIG. 3. BZA-5B inhibits HDV-like-particle production. Duplicate dishes of LH cells were grown in media containing a carrier (0.5 mM DTT and 0.05% DMSO) alone (lanes 1) or a carrier with either 10, 25, or 50 μM BZA-5B. Clarified medium supernatants were analyzed for the presence of HDV-like particles by quantitative immunoblot analysis (A). The underlying cells were harvested and counted, and the presence of large delta antigen was analyzed by immunoblotting (B). Only one of the duplicate set of blots is shown. (C) HBsAg was quantitated in duplicate aliquots of each medium supernatant sample, and the percentage of control HBsAg per cell (light bars) and of control particles per cell (dark bars), determined from the experiment whose results are shown in panel A, at each concentration of BZA-5B is plotted. Error bars represent the average deviations.

affected by nonspecific toxicity. As indicated in Fig. 3C, however, BZA-5B selectively abolished prenylation-dependent HDV-like particle release while exerting no such effect on the constitutive secretion of HBsAg.

As with most pharmacologic inhibitor experiments, it is possible that the inhibition of particle formation is unrelated to BZA-5B's effect on delta antigen prenylation. Since particle formation, however, can be similarly abolished when delta antigen prenylation is specifically prevented by a completely different and nonpharmacologic means—namely, genetic mutation of the prenylation site on delta antigen (11)—our data suggests that it is indeed the specific ability of BZA-5B to inhibit delta antigen prenylation (Fig. 2) that is responsible for preventing particle formation (Fig. 3).

These results have obvious implications for a new type of antiviral therapy based on prenylation inhibition. Such an antiviral strategy may be able to be applied to other viruses found to have similarly prenylated proteins. While delta antigen was the first viral protein shown to undergo prenylation, analysis of sequence data banks reveals the presence of CXXX box-containing proteins in numerous other viruses, including herpes simplex virus, cytomegalovirus, and hepatitis A virus (10).

As an initial viral target, HDV is particularly attractive because preventing prenylation of large delta antigen might have two antiviral consequences. First, as we show here, particle assembly could be blocked. Second, as a result of not being released in the form of viral particles, the large delta antigen concentration within infected cells may increase. Because large delta antigen is a potent *trans*-dominant inhibitor of HDV genome replication (6, 12), the antiviral effect of prenylation inhibition on assembly could thus be amplified by an additional suppression of viral genome replication.

Strategies designed to inhibit viral prenylation could affect host cell prenylation as well. Several factors, however, may help limit the potential for intolerable side effects. Normal cellular prenylation is accomplished by a family of prenyltransferases (34). Thus, selective inhibition of the prenyltransferase that modifies delta antigen may not affect host cell functions which depend on other prenyltransferases. Some substrates can be prenylated by more than one prenyltransferase (1, 30). Such potential cross-specificity may help mitigate unwanted prenylation inhibition of critical cellular proteins by BZA-5B. Indeed, BZA-5B is surprisingly well tolerated in a variety of experimental systems (9, 22); in our experiments, we observed no gross cellular toxicity and a mild (30 to 50%) inhibition of growth rate at the highest BZA-5B concentrations. In addition, viral assembly may be more sensitive than key host cell functions to the effects of prenylation inhibitors. It is possible that inhibiting the prenylation of only a fraction of the large delta antigen in a nascent virus particle may be sufficient for abrogating normal assembly of the entire particle. Ultimately, the true benefit-to-risk ratio will need to be determined for each clinical application. In the case of HDV, there are established animal models (8) which may now be suitable for further evaluating the proposed antiviral strategy.

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REFERENCES

1. **Armstrong, S. A., V. C. Hannah, J. L. Goldstein, and M. S. Brown.** 1995. CAAX geranylgeranyl transferase transfers farnesyl as efficiently as geranyl to Rho B. J. Biol. Chem. **270:**7864–7868.
2. **Bonino, F., B. Hoyer, J. W.-K. Shih, M. Rizzetto, R. H. Purcell, and J. L. Gerin.** 1984. Delta hepatitis agent: structural and antigenic properties of the delta-associated particle. Infect. Immun. **43:**1000–1005.
3. **Bruss, V., and D. Ganem.** 1991. Mutational analysis of hepatitis B surface antigen particle assembly and secretion. J. Virol. **65:**3813–3820.
4. **Casey, J. L.** 1996. Hepatitis delta virus. Genetics and pathogenesis. Clin. Lab. Med. **16:**451–464.
5. **Chang, F.-L., P.-J. Chen, S.-J. Tu, C.-J. Wang, and D.-S. Chen.** 1991. The large form of hepatitis δ antigen is crucial for assembly of hepatitis δ virus.

Proc. Natl. Acad. Sci. USA **88:**8490–8494.
6. **Chao, M., S.-Y. Hsieh, and J. Taylor.** 1990. Role of two forms of hepatitis delta virus antigen: evidence for a mechanism of self-limiting genome replication. J. Virol. **64:**5066–5069.
7. **Chow, M., C. J. Der, and J. E. Buss.** 1992. Structure and biological effects of lipid modifications on proteins. Curr. Opin. Cell Biol. **4:**629–636.
8. **Cova, L., I. Fourel, L. Vitvitski, V. Lambert, S. Chassot, et al.** 1993. Animal models for the understanding and control of HBV and HDV infections. J. Hepatol. **17**(Suppl. 3)**:**S143–S148.
9. **Dalton, M. B., K. S. Fantle, H. A. Bechtold, L. DeMaio, R. M. Evans, et al.** 1995. The farnesyl protein transferase inhibitor BZA-5B blocks farnesylation of nuclear lamins and p21ras but does not affect their function or localization. Cancer Res. **55:**3295–3304.
10. **Glenn, J. S.** 1995. Prenylation and virion morphogenesis, p. 83–93. *In* G. Dinter-Gottlieb (ed.), The unique hepatitis delta virus. R. G. Landes Company, Austin, Tex.
11. **Glenn, J. S., J. A. Watson, C. M. Havel, and J. M. White.** 1992. Identification of a prenylation site in delta virus large antigen. Science **256:**1331–1333.
12. **Glenn, J. S., and J. M. White.** 1991. *trans*-Dominant inhibition of human hepatitis delta virus genome replication. J. Virol. **65:**2357–2361.
13. **Glomset, J. A., M. H. Gelb, and C. C. Farnsworth.** 1990. Prenyl proteins in eukaryotic cells: a new type of membrane anchor. Trends Biochem. Sci. **15:** 139–142.
14. **Hoofnagle, J. H.** 1989. Type D (delta) hepatitis. JAMA **261:**1321–1325.
15. **James, G. L., J. L. Goldstein, M. S. Brown, T. E. Rawson, T. C. Somers, et al.** 1993. Benzodiazepine peptidomimetics: potent inhibitors of ras farnesylation in animal cells. Science **260:**1937–1942.
16. **Lai, M. M. C.** 1995. The molecular biology of hepatitis delta virus. Annu. Rev. Biochem. **64:**259–286.
17. **Laub, O., L. B. Rall, M. Truett, Y. Shaul, D. N. Standring, P. Valenzuela, and W. J. Rutter.** 1983. Synthesis of hepatitis B surface antigen in mammalian cells: expression of the entire gene and the coding region. J. Virol. **48:** 271–280.
18. **Lazinski, D. W., and J. M. Taylor.** 1994. Recent developments in hepatitis delta virus research. Adv. Virus Res. **43:**187–231.
19. **Lee, C.-Z., P.-J. Chen, M. M. C. Lai, and D.-S. Chen.** 1994. Isoprenylation of large hepatitis delta antigen is necessary but not sufficient for hepatitis delta virus assembly. Virology **199:**169–175.
20. **Liu, C. C., D. Yansura, and A. Levinson.** 1982. Direct expression of hepatitis B surface antigen in monkey cells from an SV40 vector. DNA **1:**213–221.
21. **Marshall, C. J.** 1993. Protein prenylation: a mediator of protein-protein interaction. Science **259:**1865–1866.
22. **Marsters, J. C., Jr., R. S. McDowell, M. E. Reynolds, D. A. Oare, T. C. Somers, et al.** 1994. Benzodiazepine peptidomimetic inhibitors of farnesyltransferase. Bioorg. Med. Chem. **2:**949–957.
23. **Otto, J. C., and P. J. Casey.** 1996. The hepatitis delta virus large antigen is farnesylated both in vitro and in animal cells. J. Biol. Chem. **271:**4569–4572.
24. **Rizzetto, M.** 1983. The delta agent. Hepatology **3:**729–737.
25. **Ryu, W.-S., M. Bayer, and J. Taylor.** 1992. Assembly of hepatitis delta virus particles. J. Virol. **66:**2310–2315.
26. **Schafer, W. R., and J. Rine.** 1992. Protein prenylation: genes, enzymes, targets, and functions. Annu. Rev. Genet. **30:**209–237.
27. **Simon, K., V. R. Lingappa, and D. Ganem.** 1988. Secreted hepatitis B surface antigen polypeptides are derived from a transmembrane precursor. J. Cell Biol. **107:**2163–2168.
28. **Sureau, C., and R. Lanford.** 1993. Analysis of hepatitis B virus envelope proteins in assembly and infectivity of human hepatitis delta virus, p. 45–51. *In* S. J. Hadziyannis, J. M. Taylor, and F. Bonino (ed.), Hepatitis delta virus: molecular biology, pathogenesis, and clinical aspects. Wiley-Liss, Inc., New York, N.Y.
29. **Taylor, J. M.** 1992. The structure and replication of hepatitis delta virus. Annu. Rev. Microbiol. **46:**253–276.
30. **Trueblood, C. E., Y. Ohya, and J. Rine.** 1993. Genetic evidence for in vivo cross-specificity of the CaaX-box protein prenyltransferases farnesyltransferase and geranylgeranyltransferase-I in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**4260–4275.
31. **Wang, C.-J., P.-J. Chen, J.-C. Wu, D. Patel, and D.-S. Chen.** 1991. Small-form hepatitis B surface antigen is sufficient to help in the assembly of hepatitis delta virus-like particles. J. Virol. **65:**6630–6636.
32. **Wang, K.-S., Q.-L. Choo, A. J. Weiner, J.-H. Ou, R. C. Najarian, et al.** 1986. Structure, sequence and expression of the hepatitis delta viral genome. Nature **323:**508–514.
33. **Wu, J.-C., P.-J. Chen, M. Y. P. Kuo, S.-D. Lee, D.-S. Chen, and L.-P. Ting.** 1991. Production of hepatitis delta virus and suppression of helper hepatitis B virus in a human hepatoma cell line. J. Virol. **65:**1099–1104.
34. **Zhang, F. L., and P. J. Casey.** 1996. Protein prenylation: molecular mechanisms and functional consequences. Annu. Rev. Biochem. **65:**241–269.