The Guanine Nucleoside Analog Penciclovir Is Active against Chronic Duck Hepatitis B Virus Infection In Vivo

ENJARN LIN, CAROLYN LUSCOMBE,† YAN YAN WANG, TIM SHAW, AND STEPHEN LOCARNINI*

Victorian Infectious Diseases Reference Laboratory, Fairfield Hospital, Fairfield, Victoria 3078, Australia

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Ducks congenitally infected with duck hepatitis B virus (HBV) were treated with the antiviral guanine nucleoside analog penciclovir for 4 weeks at a dose of 10 mg/kg of body weight per day. The effects of treatment on viremia and intrahepatic viral genome replication, transcription, and translation were examined. In seven of eight penciclovir-treated ducks, viremia was barely detectable after a week of treatment. After 4 weeks of treatment, molecular hybridization studies showed that intrahepatic viral DNA, RNA, and protein levels were significantly reduced compared with those in placebo-treated controls. Synthesis of all viral replicative intermediates, including the normally persistent viral supercoiled DNA species, was inhibited by penciclovir treatment. Examination of liver tissue sections after in situ DNA hybridization or immunohistochemical staining confirmed that viral DNA and protein synthesis had been profoundly inhibited in most hepatic parenchymal cells. However, small subpopulations of cells, in particular the small bile duct epithelial cells, remained strongly positive for duck HBV antigens and DNA despite treatment. There was no evidence of toxicity associated with penciclovir therapy. This study confirms the safety and potent antiepapnaviral activity of penciclovir in vivo but indicates that further improvements in antiviral therapy will be required to completely eliminate HBV infection.

Materials and Methods

Ducks and treatment protocol. One-day-old, female Pekin-Aylesbury crossbred ducklings congenitally infected with an Australian strain of DHBV were obtained commercially (1). Ducklings were bled at 1 week and again at 5 weeks of age. Sera were tested for DHBV DNA by dot blot hybridization (1), and 20 ducklings which had a stable intermediate virus titer were selected for the study. Treatment protocols were approved by the institutional Animal Ethics Committee. Treatments began when ducklings were 6 weeks old. Ducklings were randomly assigned to treatment groups as shown in Fig. 1. In addition, four ducks were sacrificed at the start of the study as pretreatment controls.

(i) Penciclovir-treated ducks. Eight ducks were treated for 4 weeks with penciclovir at the dosage of 10 mg/kg of body weight (1 g/kg·day)−1, given by intraperitoneal injection in two equal (morning and evening) doses dissolved in 2 ml of 1% (vol/vol) dimethylsulfoxide. Four penciclovir-treated ducks were sacrificed at the end of treatment, and the remaining four were sacrificed after 4 weeks of drug-free follow-up.

(ii) Placebo-treated ducks. Eight ducks were treated with isotonic saline containing 1% (vol/vol) dimethylsulfoxide instead of penciclovir. Four were sacrificed immediately after treatment (end of treatment controls), and the remaining four were kept without treatment for a further 4 weeks before sacrifice (follow-up controls). Placebo- and penciclovir-treated ducks and controls were housed separately. All ducks were weighed before treatment and weekly thereafter. Blood samples were taken from all ducks before treatment began and weekly thereafter for the duration of the study. Weekly serum or blood samples were tested for markers of liver, renal, and hematological function by standard clinical pathology procedures as described previously (16, 34). For autopsy, ducks were anesthetized with a lethal dose of sodium pentobarbital. Wedges of tissue were removed from each liver lobe and snap-frozen in liquid nitrogen or fixed in 3:1 (vol/vol) ethanol-acetic acid (16). Ducks were carefully inspected postmortem for pathological changes.

Preparation of probes. (i) Radiolabeled DHBV DNA probe. DHBV DNA was labeled with [α-32P]dCTP with a NEN Random Primer Plus extension kit (DuPont-NEN, Boston, Mass.) as described previously (22). (ii) DIG-labeled DHBV DNA probe. A digoxigenin (DIG)-labeled DHBV probe, used for in situ hybridization studies, was prepared with a random-primed DIG labeling kit (Boehringer-Mannheim, Germany) as described previously (8).
A cDNA derived from NS4 (a nonstructural region of hepatitis C virus) was similarly DIG labeled and used as a negative control.

Detection of markers of DHBV replication. (i) Viral DNA in serum. Viremia was monitored by dot blot hybridization after alkaline denaturation of sera as described previously (8, 34).

(ii) DHBV DNA in liver. Two different procedures were used to extract viral DNA from liver. For total DNA extraction, DNA was extracted from liver tissue and standardized amounts (1 or 10 µg) were analyzed by slot blot or Southern blot hybridization, respectively, as previously described (34). The number of viral genome equivalents per liver cell was estimated according to the method of Libbert et al. (7).

For SC viral DNA extraction, enrichment was achieved by extraction in the presence of 500 mM KCl as described elsewhere (27, 34). Cell numbers were standardized for each extraction by counting the number of ethidium bromide-stained nuclei in an aliquot of preextraction homogenate (16). From each extraction, DNA isolated from a total of 3 × 10⁶ nuclei was analyzed by Southern blot hybridization. Slot blot and Southern blot hybridizations and autoradiography were performed as previously described (27, 34). Autoradiographs were analyzed with the aid of an imaging densitometer (model GS670; Bio-Rad, Hercules, Calif.) and Molecular Analyst computer software.

(iii) DHBV RNA in liver. Total RNA was extracted from liver tissue with an RNA extraction kit (Pharmacia, Milwaukee, Wis.) according to the manufacturer's instructions. RNA samples were analyzed by slot blot hybridization as described previously (35).

Detection of DHBV-specific proteins by immunoblotting. Protein concentrations in sodium dodecyl sulfate-lysates of liver cells were estimated with a detergent-compatible assay (DC protein assay kit; Bio-Rad, Hercules, Calif.) and Molecular Analyst computer software.

Histological investigations. Liver tissue was processed for histology and histological detection techniques by standard procedures as described by Lascombe et al. (16).

In situ DNA hybridization. Paraﬁn-embedded tissue sections of approximately 3 µm in thickness were mounted on microscope slides, dewaxed, washed in ice-cold phosphate-buffered saline, and ﬁxed in 0.1% glutaraldehyde in preparation for prehybridization. The prehybridization solution (Dighyb) contained 1 mM Tris-HCl (pH 7.4), 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1 mM EDTA, 10% (vol/vol) dextran sulfate, and 50% (vol/vol) formaldehide. Sections were covered with prehybridization solution and incubated at 42°C. After 1 h, the prehybridization solution was removed and replaced by a hybridization solution consisting of 0.75 ng of DIG-labeled DNA probe ml⁻¹ in Dighyb. Coverslips were applied, and slides were heated to 95°C for 10 min to denature the probe. After overnight hybridization at 42°C, the coverslips were removed and then the sections were rinsed with 2× SSC before a 30-min wash in 0.1% SSC at 42°C. After blocking with aqueous 3% (vol/wt) egg albumin, bound DNA probe was reacted for 1 h at 37°C with an anti-DIG alkaline phosphatase conjugate diluted 1:600 in Tris-saline (0.1 M Tris-HCl [pH 7.6], 150 mM NaCl). Sections were then given two 10-min washes in TBS-T (Tris-buffered saline, 150 mM NaCl, 0.1 M Tris-HCl [pH 7.4], 0.3% [vol/vol] Tween 80) before application of color development solution from a DIG DNA detection kit (Boehringer Mannheim) essentially according to the manufacturer's instructions. Finally, sections were counterstained with nuclear fast red before dehybridization through graded ethanol to xylene. Coverslips were applied, and sections were examined by light microscopy under code.

Immunohistochemistry. DHBV-specific (envelope [PreS] and core) antigens in tissue sections were detected by immunoperoxidase staining as previously described (16).

Statistics. Data were analyzed with StatWorks, a computer software package from Cricket Software, Philadelphia, Pa. Unpaired t tests were used to determine significance. The test of significance was at the P < 0.05 level.

RESULTS

All ducks remained healthy throughout the study. Pathological changes were not seen in any of the ducks at autopsy, nor were abnormalities in laboratory markers of hematological, renal, or liver function observed (results not shown). Weight loss did not occur, and mean weights of both placebo- and penciclovir-treated groups increased comparably during the study (results not shown).

Markers of DHBV replication. (i) Serum DHBV DNA. Viremia was monitored by serum dot-blot hybridization (Fig. 2). A rapid and dramatic response to penciclovir occurred in all but one of the eight penciclovir-treated ducks, with serum DHBV DNA concentrations falling to undetectable levels in less than a week. During the follow-up period, serum DHBV DNA concentrations in penciclovir-treated ducks returned to near pretreatment levels. A single penciclovir-treated duck in the group which was not followed up remained strongly positive for serum DHBV DNA. Treatment failure in this duck was complete by all criteria (see Discussion), and data relating to this bird have been omitted from the calculations.

(ii) Liver DHBV DNA. The total viral DNA burden was quantitated by slot blot hybridization and densitometry. Results are summarized graphically in Fig. 3 (slot blots are not shown). In three of the four penciclovir-treated ducks which responded to treatment, viral DNA was reduced to around 10% of the age-matched control level. The numbers of viral genome equivalents were estimated to be 515 ± 30, 427 ± 63, and 544 ± 44 for pretreatment, end-of-treatment, and follow-up controls, respectively (Fig. 3). Southern analysis of total DNA (not shown) and SC DNA preparations (Fig. 4) showed that both relaxed circular and double-stranded linear forms of viral
DNA were reduced to barely detectable levels by 4 weeks of penciclovir therapy (Fig. 4, lanes 7 and 8). In three of the four end-of-treatment liver specimens, the level of SC DNA was reduced by an average of 53% (Fig. 3), a reduction which, although substantial, was not significant at \( P < 0.05 \), probably because of the small sample size. All intrahepatic viral DNA species were regenerated in the livers of penciclovir-treated ducks during the follow-up period, at the end of which the amount of SC DNA present was estimated by densitometry to average almost twice that detected in controls (Fig. 4, lanes 9 and 10, and Fig. 3).

(iii) Intrahepatic DHBV RNA. After 4 weeks of penciclovir therapy, a mean reduction in viral RNA of 60% compared with the level in matched placebo-treated controls was estimated from RNA blots; this decrease was almost significant (\( P = 0.07 \)). Intrahepatic viral RNA further decreased during the follow-up period, and the viral RNA load in penciclovir-treated ducks was significantly lower than that in controls after 4 weeks of follow-up (\( P < 0.05 \); blots not shown [results are summarized in Fig. 3]).

(iv) Immunobots. Immunoblot results are summarized in Fig. 3, and representative immunobots are shown in Fig. 5. After 4 weeks of therapy, reductions in viral envelope PreS and core protein were estimated by densitometry to be 86 and 85%, respectively (lanes 7 and 8 in Fig. 5a and b). Viral protein synthesis resumed during the follow-up period (lanes 9 and 10) but was still at a reduced level compared with that in controls (Fig. 3).

Histological investigations. (i) Histology. Differences in histological appearance between samples from penciclovir-treated ducks and those from control ducks were not seen at any stage of the study, but mild, variable steatosis was observed in some samples from both penciclovir- and placebo-treated ducks (results not shown).

(ii) In situ DNA hybridization. Representative results are shown in Fig. 6. Pretreatment pancreatic tissue, in which islets of Langerhans stain strongly positive for DHBV DNA (Fig. 6a), was used as an internal control (16). DHBV DNA was detectable in almost the entire hepatocyte population in all control ducks (Fig. 6b). Staining was mainly cytoplasmic, but both the cytoplasmic and nucleus were stained in isolated cells. In addition, many bile duct epithelial cells and small bile duct cells stained positive for DHBV DNA, suggesting that either active viral replication was occurring or had occurred in these cells. In comparison, there was evidence that profound inhibition of DHBV DNA replication had occurred after 4 weeks of penciclovir treatment, when viral DNA was barely detectable in most cells (Fig. 6c). By the end of the follow-up period, viral replication had resumed in many parenchymal cells, and the bile duct epithelial cells in particular were strongly stained (Fig. 6d).

(iii) Immunohistochemistry. When liver sections were stained for viral PreS1 and core antigen, the intensity of stain-
ing in controls was found to remain stable for the duration of the
study (results not shown). In contrast, sections from livers of
penciclovir-treated ducks showed reduced intensity of stain-
ing for both PreS and core antigen (results not shown), reflect-
ing what had been observed on immunoblots (Fig. 5). Rela-
tively more intense staining in sections of livers taken at the
end of the follow-up period confirmed that active viral protein
synthesis had resumed. Many bile duct epithelial cells as well as
isolated parenchymal cells in both placebo- and penciclovir-
treated ducks maintained strong positive staining for DHBV
antigens throughout the entire study period, confirming the
pattern seen after in situ DNA hybridization (16). The histo-
logical appearance of these cells makes it unlikely that they
were Kupffer cells which had taken up antigen or virus. Such
responses are almost identical to those observed during and
after ganciclovir treatment (16).

FIG. 5. Immunoblot analysis of liver cell lysates. Sample lanes are labeled as
described in the legend to Fig. 4. (a) Immunodetection with antiserum to DHBV
PreS antigen. (b) The same blot after being stripped and reprobed for DHBV
core antigen.

FIG. 6. Detection of DHBV nucleic acids in tissues of penciclovir- and placebo-treated ducks. (a) Pancreatic tissue from a pretreatment control used as a
hybridization control. Islets of Langerhans (arrow) are positive (darkerly stained), while most of the exocrine pancreas is negative. (b) Liver from a duck after 4 weeks
of placebo treatment. The parenchyma is positive for DHBV, and the bile duct epithelium (arrows) is strongly positive. (c) Liver from a penciclovir-treated duck at
the end of treatment. Most cells are negative, although the cytoplasm of a few cells is weakly positive. (d) Liver from a penciclovir-treated duck at the end of treatment
showing bile duct epithelial cells (arrow) still positive for DHBV.

DISCUSSION
The work described here confirms and extends the results of
earlier studies (25, 31) which identified penciclovir as a poten-
tial anti-hepadnaviral agent. Intraperitoneal injection of pen-
ciclovir into chronically DHBV-infected ducks rapidly and sig-
nificantly inhibited viral DNA replication, as indicated by the
reduction both of viremia and of markers of viral replication in
liver. The DHBV DNA remaining in the liver after 4 weeks of
therapy was mainly in the SC form, but it was reduced by more than half compared with that in the controls. When penciclovir treatment was stopped, rebound of viral replication, a common posttreatment finding (3, 16, 17, 34–36), was observed. This characteristic and to date unpreventable phenomenon has been attributed to the unusual resistance of the viral SC DNA species to conventional antiviral therapy (3, 17, 36). Besides isolated parenchymal foci and bile duct epithelial cells in the liver, extrahepatic foci in tissues including the pancreas and spleen are assumed to act as reservoirs of potentially infectious virus, even during otherwise effective chemotherapy (16, 17).

No evidence of penciclovir toxicity was found during this study. In in vitro experiments, penciclovir concentrations as high as 100 μg ml⁻¹ (395 μM) have been shown not to affect cellular DNA synthesis in uninfected cells (33). Furthermore, cumulative safety data from 13 separate clinical studies have shown that famciclovir, the well-absorbed oral form of penciclovir, has been well tolerated by more than 3,000 herpesvirus-infected individuals worldwide (24).

Penciclovir was originally developed as an anti-herpes virus agent for use against herpes simplex virus and varicella-zoster virus infections (33). In common with other nucleoside analogs, it is believed that phosphorylation to the triphosphate (PCV-TP) is mandatory for antiviral activity (26, 33). The initial phosphorylation (to the monophosphate) of nucleosides and their analogs is the most substrate-specific and critical step in the activation pathway (26), and penciclovir and other nucleoside analogs which are selective for herpesviruses are phosphorylated by a virally encoded deoxypyrimidine kinase (33). The corresponding di- and triphosphates may be produced by the action of cellular (deoxy)nucleotide kinases (33). In herpesvirus-infected cells, PCV-TP inhibits viral replication by causing premature chain termination after incorporation by the viral DNA polymerase into viral DNA (33). Analogous actions of PCV-TP against hepadnaviruses have generally been assumed, but with little substantive evidence to date.

There is no evidence that hepadnavirus genomes encode (deoxy)nucleoside kinases or that a cellular (deoxy)nucleoside kinase phosphorylates penciclovir (26). Nor is there any evidence that hepadnavirus genomes encode proteins analogous to the human cytomegalovirus UL-97 gene product, which can phosphorylate ganciclovir (14, 28). One activation mechanism may involve the ubiquitous cellular enzyme IMP-GMP 5’-nucleotidase, which is capable of phosphorylating several acyclic purine nucleoside analogs, including acyclovir and ganciclovir (9). Recent results (20) suggest that a cellular enzyme is probably responsible for penciclovir phosphorylation in hepatocytes, and the high level of activity of IMP-GMP 5’-nucleotidase in vertebrate livers tends to favor it as a candidate (26). Regardless of the phosphorylation mechanism, it seems reasonable to assume that PCV-TP is the active metabolite and that HBV polymerase is the major target. We have recently reported that PCV-TP inhibits HBV DNA polymerase in vitro (20) but found that the (R) enantiomer rather than the (S) enantiomer is the more potent inhibitor, whereas the opposite has been found for herpesviruses (33). We estimated a Ki of about 0.03 μM for (R)-PCV-TP as an inhibitor of dGTP incorporation by HBV DNA polymerase in vitro (20). This value is >6,000-fold lower than the Ki of 200 μM for PCV-TP as a competitive inhibitor of dGTP incorporation by primase-coupled human DNA polymerase α recently reported by Isley et al. (6), who also found that PCV-TP was a poor inhibitor of other human DNA polymerases (50% inhibitory concentrations were 450, 120, and 375 μM for polymerases α, δ, and ε, respectively). After incubation of HBV-transfected human hepatoma cells with [3H]penciclovir, we found intracellular concentrations of PCV-TP in the same range as the estimated Ki for HBV DNA polymerase (20), sufficient to account for selective inhibition of HBV DNA polymerase. Intracellular concentrations of PCV-TP were comparable in transfected and untransfected cells, suggesting that cellular enzymes catalyze penciclovir phosphorylation.

Addition of penciclovir to nascent HBV DNA chains would not necessarily cause immediate chain termination, since penciclovir possesses the equivalent of a 3’-hydroxyl group and HBV polymerases, unlike herpesvirus and cellular DNA polymerases, lack proofreading ability (5). By analogy with 2’-deoxyguanosine (23), incorporation of penciclovir into HBV DNA is likely to result in a destabilized or nonfunctional DNA product. The suppression of DHBV RNA synthesis seen during and after penciclovir treatment is consistent with this notion. Blockage of the priming of hepadnavirus reverse transcription (38) by penciclovir is an additional action mechanism which has only recently been reported (37). Other factors which probably contribute to the antihepadnaviral activity of penciclovir include the long intracellular half-life of PCV-TP (33) and the relative intracellular deficiency of guanine nucleotides (26).

The failure of penciclovir (this study) and the related analogs 2’-carboxyguanosine (17) and ganciclovir (16) to inhibit DHBV replication in bile duct epithelial cells and putative oval cells may be explained by the low level of cellular activity of the necessary phosphorylating enzyme(s), the identity of which remains unknown (see above). Similarly, the enzymatic machinery for drug uptake and activation may be inadequate or absent from cells at extrahepatic sites which remain infected despite therapy. It is more difficult to account for the total failure of penciclovir treatment to affect markers of DHBV replication in one of the eight treated ducks. High-performance liquid chromatography analysis of duck sera collected during the treatment period showed comparable penciclovir concentrations in all treated animals (data not shown). Further studies will be required to define the basis of this discrepancy.

The in vivo antiviral activity of penciclovir reported here is comparable to that of other guanine nucleoside analogs in similar trials against DHBV (4, 11, 16, 17, 34); however, penciclovir at nontoxic doses seems to be the best inhibitor of SC DNA generation identified to date. The half-life of the DHBV SC DNA in a primary hepatocyte culture has recently been estimated to be about 3 to 5 days (2). In the present trial, ducks were treated with penciclovir for 28 days, or 7 half-lives. Substantial reductions in intrahepatic DHBV SC DNA could be expected at the end of this period if the intracellular conversion pathway (32) was completely blocked by penciclovir. These results imply that complete elimination of hepadnavirus from immunotolerant hosts by treatment with antiviral nucleoside analogs alone is probably impossible, at least in the short term. Longer-term trials will be required to determine whether immunotolerance to HBV can be overcome, and in future, it is likely that penciclovir and other nucleoside analogs will be used in combination with other agents which stimulate host immune responses or inhibit viral replication by different mechanisms. For example, it would be interesting to investigate the activity of penciclovir in combination with interferon or interferon inducers (22), in combination with drugs targeted specifically at SC DNA (34), or in combination with both. Results from continuing studies of hepadnaviral SC DNA (21) should be a valuable aid in the rational development of antiviral strategies designed to eliminate it.
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