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In Vitro Antiviral Activity of Penciclovir, a Novel Purine Nucleoside, against Duck Hepatitis B Virus

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The in vitro antihepadnavirus activities of the purine nucleoside analogs ganciclovir {9-[2-hydroxy-1-(hydroxymethyl)ethoxymethyl]guanine} and penciclovir [9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine; BRL 39123] were compared in primary duck hepatocyte cultures congenitally infected with the duck hepatitis B virus (DHBV). Both compounds inhibited DHBV DNA replication to a comparable extent during continuous short-term treatment of the cultures. However penciclovir was more active both during longer-term continuous treatment (50% inhibitory concentrations: penciclovir, 0.7 ± 0.1 μM; ganciclovir, 4.0 ± 0.2 μM) and in washout experiments (50% inhibitory concentrations: penciclovir, 3.0 ± 0.4 μM; ganciclovir, 46 ± 1.5 μM) designed to compare the persistence of inhibitory activity after removal of the extracellular compound. The effects on viral protein synthesis were similar to the effects on viral DNA replication. These data suggest that penciclovir or its oral form, famciclovir, may have clinical utility in the treatment of chronic hepatitis B virus infection.

Attempts to develop therapy against hepatitis B virus (HBV) have been hampered by its extremely narrow host range (only humans and chimpanzees are susceptible to HBV infection) and by the inability of cell lines to support complete autonomous HBV replication (22). These problems have been overcome to some extent by the recent development of assay systems for identifying potential antihepadnavirus agents. Animal models of HBV infection, particularly those of the woodchuck and duck, have been used for in vivo testing and evaluation, while continuous HBV-transfected cell lines and primary hepatocytes have been used for in vitro screening. The utilities and validities of these systems seem to have been vindicated by findings that agents previously found to be active in clinical studies (2, 19, 20) are generally active in the test systems (8, 21, 23, 33, 34), supporting assumptions that drugs which show activity in the test systems may be clinically useful.

In the Infectious Diseases Reference Laboratory at Fairfield Hospital, we have used duck HBV (DHBV) as our HBV model and we routinely use both in vitro testing in primary duck hepatocytes (PDHs) to identify active compounds and in vivo testing in live ducks to develop effective therapeutic regimens. Following the observation that ganciclovir, an acyclic guanosine analog, significantly inhibits human HBV DNA replication in patients coinfected with the human immunodeficiency virus (20), we reported that it also inhibits DHBV replication in persistently infected PDHs both in cell culture (8) and in vivo (33, 34). Despite its efficacy, use of ganciclovir is associated with the necessity for parenteral administration, relapse of viral replication after drug withdrawal, and myelosuppression (2, 26, 34). The present study was undertaken, first, to test whether penciclovir has activity against DHBV in cultured PDHs and, second, to compare the relative efficacies of ganciclovir and penciclovir.

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MATERIALS AND METHODS

Animals. One-day-old Pekin-Aylesbury cross ducks congenitally infected with an Australian strain of DHBV were obtained from a commercial supplier (33). Viremia was monitored by dot blot hybridization of serum, and 7- to 14-day-old ducklings with stable viral titers of 5×10^8 to 10×10^8 viral genome equivalents per ml were selected for hepatocyte isolation.

Cell culture. Primary cultures of duckling hepatocytes were prepared as described previously (5, 30), except that feeder cell layers (a potential source of competing nucleosides) were not used. Six-well plastic culture plates (Greiner, Frickenhausen, Germany) were seeded with 2×10^6 to 2.5×10^6 hepatocytes per well, and cells were allowed to attach overnight before the first medium change (on day 1 postplating) and were maintained with medium changes every second day.

Antiviral drug treatment. For each experiment, triplicate sets of PDH monolayers were exposed to drug concentrations in the range 0 to 500 μM beginning on day 1 postplating. One set was harvested after 5 days of drug treatment. Treatment of the second set was stopped after 5 days, and the cells were incubated for an additional 5 days in drug-free medium. Treatment of the third set was continued until day 10 postplating, when both the second and third sets were harvested. Antiviral effects were assessed by monitoring viral DNA replication and viral protein synthesis.

Detection of DHBV DNA replication. Total cellular DNA was prepared as described by Tuttleman et al. (30). Briefly, cells were lysed in 100 mM Tris-HCl (pH 8.0) containing 0.5% sodium dodecyl sulfate (SDS), 10 mM EDTA, and 150 mM NaCl. Lysates were stored frozen at −70°C before processing for hybridization analysis essentially as described previously (5), except that a commercial slot blot apparatus was used to apply samples to the hybridization membranes and the DHBV probe was prepared in the Infectious Diseases Reference Laboratory at Fairfield Hospital as described below. A full-length clone of the Australian strain of DHBV (5a) ligated into plasmid pT3T7 (Pharmacia, Uppsala, Sweden) was propagated in *Escherichia coli*, and the plasmid was extracted by standard

techniques (24). The cloned DHBV DNA was removed from the plasmid by digestion with *Eco*RI (Amersham International, Amersham, England), separated by preparative gel electrophoresis, and purified by using the Prep-A-Gene DNA purification kit (Bio-Rad) according to the manufacturer's recommendations. DHBV DNA was labelled with [α-^{32}P] dCTP by using an NEN Random Primer Plus Extension Kit (NEN Research Products, DuPont, Wilmington, Del.) to a specific activity of 0.5×10^9 to 1×10^9 dpm/μg. Hybridization conditions were as described previously (23, 33), and radiolabelled DNA was detected with the aid of intensifying screens by autoradiography at -70°C on Kodak X-Omat film.

Detection of DHBV-specific protein synthesis. Procedures for antibody production have recently been described in detail elsewhere (21). Monoclonal antibodies to the pre-S1 protein and polyclonal rabbit antibodies to anti-DHBV antigen corresponding to the 13 carboxy-terminal amino acids of the DHBV core protein were used. Immunoblotting was performed as described previously (5), except that bound antibody was detected by enhanced chemiluminescence by using a kit purchased from Amersham International.

Assessment and analysis of antiviral effects. Autoradiographs and enhanced chemiluminescence-exposed films were analyzed by using an integrating densitometer (Cliniscan 2; Helena Laboratories, Beaumont, Tex.). Exposures and dilutions were chosen such that the resulting datum points lay within a range in which there was a linear relationship between the amount of bound probe and the resulting film density, i.e., the peak area calculated by the integrating densitometer. The amount of bound probe detected after drug treatment was expressed as a percentage of the amount detected in corresponding mock-treated controls. The amount of viral replication or viral protein synthesis (expressed as a percentage of that detected in appropriate controls; abcissa, linear scale) was plotted against the corresponding drug dose (ordinate, logarithmic scale). A computer graphics program (Cricket Graph version 1.3.2; Cricket Software, Malvern, Pa.) was used to analyze data and generated lines of best fit on the basis of least-squares regression analysis of data which lay in the linear dose-response range. The 50 and 90% inhibitory drug concentrations corresponding to specific endpoints (IC_{50}s and IC_{90}s, respectively) were calculated from the resulting computer-generated linear equations.

Cytotoxicity testing. On the day of harvest, one replicate from each set of PDH cultures was used to assess end-of-treatment cell survival. Cell viability was assessed by a neutral red uptake method essentially as described by Fautz et al. (11).

RESULTS

Cytotoxicity. Significant differences ($\geq$5%) in neutral red uptake between drug-treated and mock-treated cells were not found after any of the treatments with either ganciclovir or penciclovir (data not shown). However, this assay does not measure antiproliferative effects, since primary hepatocytes remain quiescent during culture under the conditions that we used.

Inhibition of DHBV DNA replication. In the short-term (5-day) dose-response test, in which penciclovir or ganciclovir was continuously present in the culture medium, both drugs had comparable activities, with IC_{50}s and IC_{90}s of 4 and 21 μM and 7 and 29 μM, respectively (Fig. 1, set 1). However, in a longer (10-day) conventional dose-response test, penciclovir was more active (IC_{50}, 0.7 $\pm$ 0.1 μM; IC_{90}, 4.0 μM compared with 4.0 $\pm$ 0.2 μM and 11.0 μM for ganciclovir [values are means $\pm$ standard deviations]) (Fig. 1, set 3). The difference in

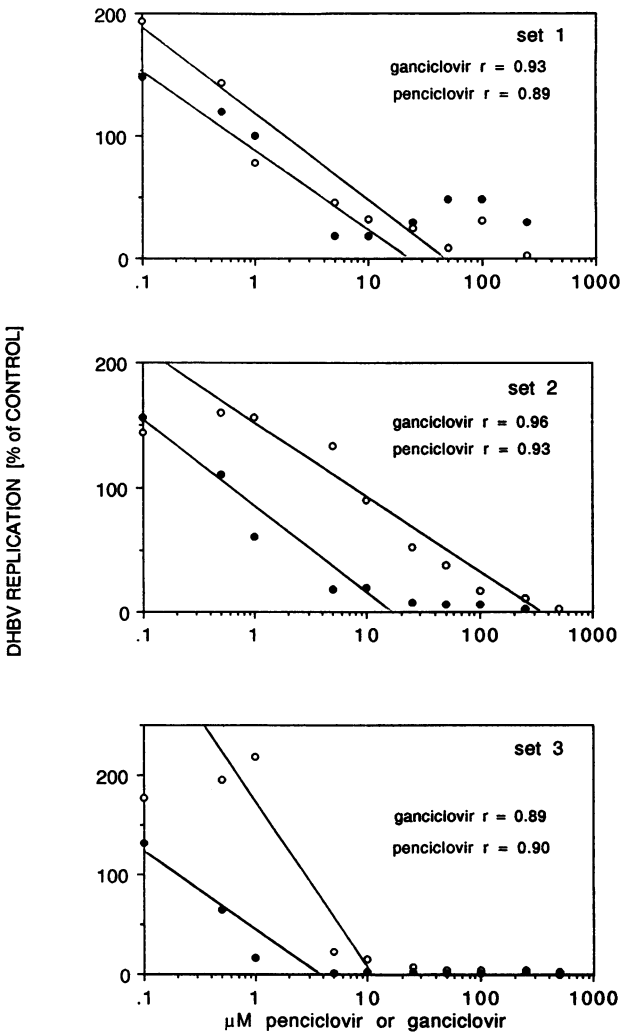

FIG. 1. Dose-response plots for inhibition of DHBV replication. After drug treatment, total cellular DNA was extracted and was used to prepare slot blots. Blots were probed for DHBV by the slot blot method described in the text, and the resulting autoradiographs were analyzed by using a scanning densitometer. Autoradiographic exposures and conditions were such that the relationship between the amount of probe bound and the resultant film density was linear. Film densities corresponding to drug-treated samples were expressed as percentages of the area under the corresponding control peaks. Least-squares analysis was used to fit regression lines to the linear part of each dose-response plot; IC_{50}s and IC_{90}s were calculated from the regression equations. Closed circles, penciclovir; open circles, ganciclovir.

antiviral activity was more dramatic in the washout experiments, in which drug treatment was stopped after 5 days but cell culture continued in drug-free medium for an additional 5 days; IC_{50}s and IC_{90}s were 3 $\pm$ 0.4 and 13 μM and 46 $\pm$ 1.5 and 214 μM for penciclovir and ganciclovir, respectively.

Inhibition of DHBV-specific protein synthesis. The effects on viral protein synthesis reflected inhibition of viral DNA replication, although the effects were slower to develop. In the short-term (5-day) tests, neither penciclovir nor ganciclovir caused significant inhibition of synthesis of either surface antigens (pre-S1 and pre-S2) or core antigen (data not shown). However, in the longer (10-day) dose-response tests (set 3),

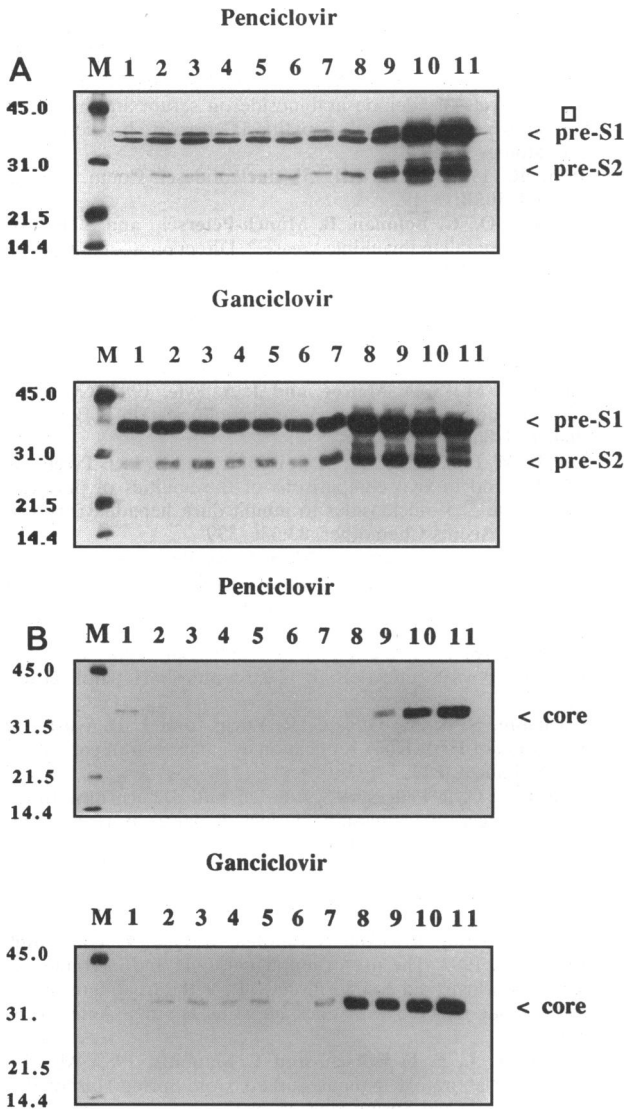

FIG. 2. Immunoblot analyses of inhibition of DHBV protein synthesis by penciclovir and ganciclovir in a washout experiment. Congentially infected PDHs were exposed to nucleoside analogs for 5 days and were then maintained for an additional 5 days in drug-free medium. After SDS-polyacrylamide gel electrophoresis, the proteins were transferred to nitrocellulose membranes and probed sequentially with a monoclonal antibody to pre-S1 protein (A) and polyclonal antibody to core protein (B). Bound antibodies were detected by enhanced chemiluminescence and were quantified by densitometry. The values for the inhibition of envelope protein synthesis are based on the averages of areas under the pre-S1 and the pre-S2 peaks. Drug concentrations corresponding to lanes 1 to 11 were 500, 250, 100, 50, 25, 10, 5, 1, 0.5, 0.1, and 0 μM, respectively. The molecular sizes of the markers (lane M) are in kilodaltons.

penciclovir was more active, showing an IC_{50} of 1.3 μM compared with an IC_{50} of 5.8 μM for ganciclovir for inhibition of envelope protein (pre-S1 plus pre-S2) synthesis and an IC_{50} of 0.2 μM for penciclovir compared with an IC_{50} of 1.4 μM for ganciclovir for inhibition of core antigen synthesis. In washout tests in which drug treatment was stopped after 5 days but cell culture was continued in drug-free medium for an additional 5 days (Fig. 2), penciclovir was again more active, having an IC_{50} of 2.9 μM compared with an IC_{50} for ganciclovir of 6.3 μM (pre-S1 plus pre-S2) and an IC_{50} of 0.4 μM for penciclovir compared with an IC_{50} of 3.8 μM for ganciclovir (core antigen).

DISCUSSION

The results presented here demonstrate that penciclovir and ganciclovir are potent inhibitors of DHBV DNA replication and viral protein synthesis. Several other purine nucleoside analogs have already been reported to inhibit hepadnavirus replication both in vitro and in vivo (12, 17, 28, 35). Ganciclovir, in particular, is known to inhibit replication of ground squirrel HBV (26), human HBV (20), and DHBV (23, 33, 34) in vivo and in vitro (8, 34). The in vitro antihepadnaviral activity of penciclovir, which was initially developed as an antiherpetic drug (6, 7, 31, 32), has been not been reported previously, although a recently published abstract reports that the drug has in vivo activity against acute DHBV infection (29). The reasons for the apparent stimulation of DHBV replication at the lowest drug concentrations (Fig. 1) are unknown, but this phenomenon has been common with all acyclic purine nucleoside analogs tested in the Infectious Diseases Reference Laboratory (25a).

Paradoxically, the selectivities of acyclic guanosine analogs as antiherpetic agents depend largely on phosphorylation by virus-encoded deoxypyrimidine (rather than purine) kinases. Until it was found that ganciclovir also had activity in deoxypyrimidine kinase-negative cells against herpesvirus species such as human cytomegalovirus and human herpesvirus type 6, which lack deoxypyrimidine kinases, it was believed that a viral or cellular deoxypyrimidine kinase activity was mandatory for antiviral activity (3). At least two deoxypyrimidine kinase-independent mechanisms of activation are now known, namely, phosphorylation by a virally encoded protein kinase, as has been demonstrated for ganciclovir and human cytomegalovirus (18, 27), and activation by a phosphotransferase activity associated with the ubiquitous enzyme IMP-GMP 5′-nucleotidase, as demonstrated by Keller et al. (16) for acyclovir, ganciclovir, and 9-β-D-arabinofuranosylguanine, as well as for purine dideoxynucleosides by Johnson and Fridland (15). Which mechanism(s) (if, indeed, either mechanism) activates ganciclovir in hepatocytes infected with hepadnaviruses remains unknown, although the high levels of IMP-GMP 5′-nucleotidase activity present in vertebrate livers, particularly in avian livers (13), would tend to favor the latter mechanism. If the 5′-nucleotidase is the activating enzyme, the antiviral activities of the purine nucleoside analogs may be increased by inhibitors of IMP dehydrogenase (1, 4), and experiments to test this prediction in the DHBV-PDH system are under way. Whatever the hepatic activation mechanism(s) for ganciclovir and penciclovir, the mechanism must be independent of deoxypyrimidine kinases, since such enzymes are neither encoded by hepadnaviruses (25) nor expressed in vertebrate hepatocyte cytoplasm, the only detectable deoxypyrimidine kinase being mitochondrial (10, 14).

Our data confirm the prediction that penciclovir, like ganciclovir, is activated in hepatocytes, but whether these drugs are activated by the same or a different mechanism(s) remains unknown. The greater antiviral potency of penciclovir relative to that of ganciclovir is probably at least partly due to the increased intracellular stability of its nucleotides (suggested by the results of the washout experiments); however, other factors such as phosphorylation efficiency or recognition by the viral polymerase may also be involved. In this context, it is of interest that the antiherpesvirus activity of penciclovir has been

found to be more persistent than that of acyclovir after drug withdrawal (7) and that this prolonged activity was attributed to the greater intracellular half-life of penciclovir nucleotides (9).

The data presented here indicate that, in cell culture studies, penciclovir is superior to ganciclovir as an anti-DHBV agent in terms of both its potency and its duration of action. Taken together with observations that (i) ganciclovir is active in vivo against hepadnaviruses in several species, including humans, and (ii) the antiviral concentrations of penciclovir can be generated from oral doses of famciclovir, our data strengthen the prediction that testing of penciclovir (via its orally active form, famciclovir) as an antiviral agent for the treatment of human HBV infections is warranted.

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