Interaction between ganciclovir and foscarnet as inhibitors of duck hepatitis B virus replication in vitro.

G Civitico, T Shaw and S Locarnini


Updated information and services can be found at:
http://aac.asm.org/content/40/5/1180

These include:
Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»
Interaction between Ganciclovir and Foscarnet as Inhibitors of Duck Hepatitis B Virus Replication In Vitro

GILDA CIVITICO,† TIM SHAW, AND STEPHEN LOCARNINI*

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

Received 12 October 1995/Returned for modification 27 December 1995/Accepted 5 March 1996

Safe and effective treatments for chronic hepatitis B virus (HBV) infection have yet to be developed. Both ganciclovir (9-[1,3-dihydroxy-2-propoxymethyl]guanine) and foscarnet (trisodium phosphonoformate hexahydrate) are potent inhibitors of hepadnavirus replication when used individually in vitro and in vivo. However, the clinical usefulness of each drug is reduced by dose-limiting toxicity, especially during long-term monotherapy. Here we demonstrate additive inhibition of duck HBV DNA replication in cultures of primary duck hepatocytes congenitally infected with duck HBV by combinations of ganciclovir and foscarnet at low, clinically achievable concentrations. These results suggest that the effects of ganciclovir and foscarnet against HBV may be additive in vivo.

Following the observation that ganciclovir, an acyclic guanosine analog, significantly inhibits human hepatitis B virus (HBV) DNA replication in patients coinfected with the human immunodeficiency virus (18), we reported that it also inhibits duck HBV (DHBV) replication in persistently infected primary duck hepatocytes (PDH) both in cell culture (5, 31) and in vivo (8, 20, 40, 41). Inhibition of DHBV replication in vitro by ganciclovir was independently confirmed by Yokata and colleagues (42). More recent work from our laboratory showed that ganciclovir also inhibits human HBV in vitro (24) and that it could successfully control severe recurrence of human HBV infection in liver transplant patients (1). Despite its efficacy as an ant hepadnaviral drug, ganciclovir use has some disadvantages. Ganciclovir is poorly absorbed orally, requiring parenteral administration (9); viral replication commonly recurs after drug withdrawal (8); and prolonged use frequently causes myelosuppression (9). Since ganciclovir is virustatic rather than virucidal, its use against chronic hepadnaviral infection must necessarily be long-term, exacerbating these problems.

Combination chemotherapy offers well-recognized advantages over monotherapy (see, e.g., references 3, 17, 26, 28, 34, and 36). In particular, toxicity may be reduced without sacrificing efficacy by choosing combinations of agents which act additively or synergistically but have different toxicity spectra. Other advantages include a reduced likelihood or rate of emergence of drug-resistant mutants and the potential for targeting different reservoirs of virus with individual drugs.

Foscarnet, like ganciclovir, has been shown to have activity against hepadnaviruses both in vitro (19, 22, 24) and in vivo (1, 22, 33). Like ganciclovir, it has the disadvantage of requiring parenteral administration, but its adverse side effects, which include anemia, hypercalcemia, and renal impairment, do not overlap with those of ganciclovir (9, 13, 21). Combinations of ganciclovir and foscarnet (13, 21, 34), as well as foscarnet and other acyclic purine nucleoside analogs (7), have been shown to act synergistically or additively against human herpesviruses in cell culture (13, 21, 34) and in experimental animals (13).

Here we report the results of studies designed to investigate the effects of combinations of ganciclovir and foscarnet on DHBV replication in congenitally infected PDH in vitro.

MATERIALS AND METHODS

Animals. One-day-old Pekin-Aylesbury crossbred ducklings congenitally infected with an Australian strain of DHBV were obtained from a commercial supplier (4). Viremia was monitored by dot blot hybridization, and 7- to 14-day-old ducklings having stable viral titers of at least 10⁹ viral genome equivalents per ml (15) were selected for hepatocyte isolation.

Cell culture. Primary cultures of duck hepatocytes were prepared essentially as described by Tuttlemann et al. (38), with minor modifications (4, 31). After isolation, hepatocytes were seeded into 6-well plastic multiplates (Greiner, Frickenhausen, Germany) at cell densities between 1.5 × 10⁴ and 2.5 × 10⁵ hepadocytes per well. 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. Ganciclovir was a gift from Syntex (Sydney, New South Wales, Australia); foscarnet was purchased from Sigma-Aldrich Pty Ltd. (Castle Hill, New South Wales, Australia). For each experiment, duplicate sets of PDH monolayers were exposed to ganciclovir concentrations in the range 0 to 50 μM or to foscarnet concentrations in the range 0 to 1.000 μM. Three separate experiments were performed with drug combinations. Concentrations of drugs used in combinations were 0, 0.5, 1, 2.5, 5, 6.25, 10, 12.5, and 25 μM for ganciclovir and 0, 0.1, 0.5, 2.5, 12.5, 50, 625, 1000, 250, and 500 μM for foscarnet, depending on the particular experiment. PDH monolayers were continuously exposed to drugs from day 1 postplating until day 10 postplating, when they were harvested. Antiviral effects were assessed by monitoring viral DNA replication and viral protein synthesis.

Detection of DHBV DNA replication and analysis of viral replicative species. Total cellular DNA was prepared as described by Tuttlemann et al. (38). Briefly, cells were lysed in 100 mM Tris-HCl (pH 8.0) containing 0.5% sodium dodecyl sulfate, 10 mM EDTA, and 150 mM NaCl. Lysates were stored frozen at −70°C before being processed for hybridization analysis as described previously (26, 31).

DHBV DNA replicative species were analyzed by Southern blot hybridization after electrophoresis through 1.0% agarose gels and vacuum transfer to positively charged nylon membranes (Boehringer, Mannheim, Germany), according to standard procedures (29). Probe preparation, hybridization conditions, and autoradiography were also as described previously (31).

Detection of DHBV-specific protein synthesis. A polyclonal rabbit antibody to DHBV core proteins (a gift from Alison Jilbert of the Institute for Medical and Veterinary Science, Adelaide, South Australia, Australia) was used to stain immunoblots, which were prepared as described previously (31). Bound antibody was detected by using an enhanced chemiluminescence kit (Amersham Australia, North Ryde, New South Wales, Australia) according to the manufacturer’s instructions.

Assessment and analysis of antiviral effects. Image densities resulting from autoradiographs (DNA) and enhanced chemiluminescence (protein) exposures were quantitated by using an imaging densitometer (model GS-67 with Molecular Analyst software; Bio-Rad Laboratories, Hercules, Calif.) (31). Inhibition of viral replication and protein synthesis was expressed as a percentage relative to values for drug-free controls. Data describing inhibition of DHBV DNA replication were plotted in three dimensions, and dose-response surfaces (28, 36)
were fitted and analyzed by using TableCurve3D, a graphics-statistics program from Jandel Scientific (San Rafael, Calif.). Drug combination effects were also analyzed by using the ComboStat program (3) (ComboStat Corp., Duluth, Ga.).

Cytotoxicity testing. On the day of harvesting, cell viability was assessed by a method based on neutral red uptake as described by Fautz and colleagues (10).

RESULTS

Cytotoxicity. There were no significant differences in neutral red uptake sites between drug-treated and untreated PDH after any of the treatments (data not shown). When examined by phase-contrast microscopy, the treated hepatocytes appeared healthy throughout the treatment period, and cell monolayers remained intact for the duration of the experiments. However, the neutral red uptake assay does not measure antiproliferative effects, as primary hepatocytes remain quiescent during culture under the conditions described (31).

Inhibition of DHBV replication by ganciclovir or foscarnet alone or in combination. Preliminary experiments using hepatocytes from three different ducks established reproducibility of inhibition of DHBV replication by ganciclovir and foscarnet. In these experiments, the 50% inhibitory concentrations (mean ± standard deviation [SD]) were 2.5 ± 0.3 and 70 ± 21 μM for ganciclovir and foscarnet, respectively. Three further experiments were performed with ganciclovir and foscarnet at different doses and dose combinations. Data from these experiments were plotted in three dimensions, and a dose-response surface was fitted to the data by using the TableCurve3D program (Fig. 1). The majority of individual experimental data points, as well as the fitted overall dose-response surface, lay within a 95% confidence interval of theoretically estimated additivity (Table 1). Analysis of subsets of data (each consisting of three or more data pairs in which ganciclovir and foscarnet were present at a fixed ratio) was done by using the ComboStat program. Combination indices produced by these analyses (3) were not significantly different from 1.0, also suggesting that the ganciclovir-foscarnet interaction was additive (data not shown).

Effects on DHBV DNA replicative species. DNA extracted from PDH after treatment with drugs at different ratios and concentrations was analyzed by Southern blot hybridization to investigate inhibition of DHBV DNA synthesis by ganciclovir and foscarnet in greater detail. In this study, only the relaxed circular, linear, and single-stranded replicative intermediates were detected (Fig. 2A). Both ganciclovir and foscarnet partially inhibited viral DNA synthesis when used alone, but combinations of 2.5 μM ganciclovir and 50 or 100 μM foscarnet were more effective than either drug alone at these concentrations, as confirmed by analysis of a lighter exposure of the autoradiograph shown in Fig. 2A. Results of densitometric analysis of the lighter exposure are shown in Fig. 2B. Almost complete suppression of DHBV DNA synthesis occurred when 2.5 μM ganciclovir was used in combination with 500 μM foscarnet, and although Fig. 2A suggests that at these concentrations, antiviral effects were not additive, the difference in...
image densities of the two right-hand bands is not significant. Neither drug showed any selectivity for a particular replicative species in this experiment.

Inhibition of DHBV-specific protein synthesis. Figure 3 shows the results of an immunoblot analysis of the effects of ganciclovir alone and in combination. A lighter exposure of the immunoblot shown in Fig. 3A was analyzed by densitometry, and data were plotted to produce Fig. 3B. Foscarnet alone caused a dose-dependent inhibition of DHBV core antigen synthesis. In the presence of 2.5 μM ganciclovir, the concentration of foscarnet required to inhibit viral core protein synthesis by 50% decreased from 60 to 10 μM, suggesting that inhibition of DHBV protein synthesis was additive; however, at the highest dose of foscarnet tested (500 μM), ganciclovir did not enhance the antiviral effect (see the two right-hand lanes and columns in Fig. 3A and B, respectively).

### DISCUSSION

Recent case reports have described the successful use of combinations of ganciclovir and foscarnet for treatment of aggressive cytomegalovirus infection in patients coinfected with human immunodeficiency virus (9) and for control of recurrent HBV infection after liver transplantation (1). Results presented here show that clinically achievable concentrations of ganciclovir and foscarnet (<12.5 and <250 μM, respectively) can act additively against DHBV in vitro, indicating that under some conditions, foscarnet and ganciclovir may act differently and independently to inhibit hepadnavirus replication.

Results presented here confirm previous observations from our laboratory and elsewhere that both ganciclovir and foscarnet are inhibitors of hepadnavirus replication and protein synthesis (1, 2, 8, 18, 19, 24, 31, 40, 42). On a molar basis, ganci-
clovir showed much greater activity against DHBV than did foscarnet, analogous to their effects on replication of herpesviruses (12, 21, 34). Ganciclovir triphosphate is known to compete with dGTP for incorporation into DNA, and although it does not necessarily cause immediate chain termination, incorporated ganciclovir inhibits further DNA synthesis (12, 14, 32). Foscarnet has been shown to inhibit both DHBV reverse transcriptase and DNA polymerase activity (11, 19, 23, 27). Our results also show that foscarnet and ganciclovir in combination can inhibit DHBV replication in PDH at lower drug concentrations than when either is used individually. At clinically achievable concentrations (<12.5 and <250 μM for ganciclovir and foscarnet, respectively), the two drugs in combination have effects that are approximately additive (Fig. 1 and Table 1).

A number of methods have been developed to analyze drug interactions, and different methods use different criteria to define theoretical additivity. Consequently, whether effects of drug combinations are defined as being synergistic, additive, or antagonistic may depend on the method used for analysis (see references 28 and 36 for critical reviews). Data reported here were derived from experiments designed on the checkerboard principle intended for three-dimensional analyses such as that described by Prichard and Shipman (28) (see legend to Fig. 1). The ComboStat program, which employs analyses based on the median-effect principle (3), became available after these experiments were completed. Although it was only possible to analyze subsets of data by using ComboStat, the results of the two types of analyses were essentially in agreement, with combination indices not significantly different from 1.0 (ComboStat results not shown). Effects of drug combinations on DHBV core antigen synthesis (Fig. 3) reflected effects on viral DNA replication, confirming lack of antagonism of low-dose ganciclovir-foscarnet combinations (1) and suggesting that foscarnet may be usefully used in combination with penciclovir, a newer orally available deoxyguanosine analog (16, 31), in future.

For hepadnaviruses, replication of which requires at least three functions (reverse transcription, RNase H, and DNA-dependent DNA polymerase activities), all of which are dependent on the virally encoded polymerase (32, 37), the mech-
anism(s) for apparent additivity or synergy between ganciclovir and foscarnet remains to be established. Although both foscar
net and ganciclovir have been reported to inhibit both DNA- and RNA-dependent hepapnarnavirus DNA polymerase activities in cell-free assays (11, 19, 27), these assays have invariably been performed with partially purified whole virus preparations, and to date it has been virtually impossible to study the effects of specific inhibitors on either of the viral polymerase-associated activities in complete isolation. Fur
thermore, potential inhibitory activities of ganciclovir which are independent of consequences of its incorporation into nas
cent viral DNA have not yet been thoroughly investigated. Such activities might include inhibition of priming of reverse tran
scription (35, 39) which is refractory to inhibition by pp
analog, as well as dideoxynucleotides, but susceptible to inhibi
tion by nucleotide analogs which, like ganciclovir triphos
phate, possess the equivalent of a 3′ hydroxyl group (35).
Elucidation of the mechanism(s) of action of any nucleoside anal
ogue which shows significant antihepadnaviral activity at nocytoxic concentrations is extremely important, since the lack of virus-specific enzymes for nucleoside salvage, together with characteristics of hepatic nucleoside metabolism, severely restricts the available drug choices (reviewed in references 22 and 32). Indeed, nucleoside analogs alone may never com
pletely suppress hepapnarnavirus DNA synthesis because of ca
tabolism, inefficient phosphorylation, and competition from endogenous nucleic acid precursors (32). In addition, the per
sistence of hepapnarnavirus supercoiled DNA in the nuclei of infected cells even during aggressive antiviral therapy (6, 8, 25, 41) is a problem which must be faced in future if eliminating the need for lifelong treatment is regarded as an eventual goal. Successful antiviral chemotherapy must be able to completely eliminate all viral subtypes from infected hepatocytes with
out unacceptable host toxicity. Such therapy will almost cer
tainly involve the use of different drug combinations (17, 26, 41). Experimental animal models will be needed for testing new therapies. Past experience supports the validity of the in vitro PDH-DHBV system for screening and assay of potential antihepadnaviral drugs. Data presented here indicate that the PDH-DHBV system can also be extended to study drug inter
actions. This system may be useful in future to predict inter
actions between drugs which have potential clinical application against severe HBV infection in vivo (1).

ACKNOWLEDGMENTS

We are grateful to Syntex Australia for the supply of ganciclovir used in this study. We also thank Patrick Edwards for preparation of radiolabelled probes and Su San Mok and Scott Bowden for helpful discussions and for critically reviewing the manuscript. This work was supported by grants from the Research and Educa
tion Foundation, Fairfield Hospital, and the National Health and Med
ical Research Council of Australia. G.C. was supported by a Research and Education Fellowship from the Senior Medical Staff, Fairfield Hospital.

REFERENCES