In Vitro Antihepadnaviral Activities of Combinations of Penciclovir, Lamivudine, and Adefovir

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Penciclovir \(\{9\text{-}[2\text{-hydroxy-1-(hydroxymethyl)-ethoxymethyl]guanine}\text{[PCV]}\}\), lamivudine \((\text{-1}\text{-}\beta\text{-L-2',3'-dideoxy-3'-thiacytidine}}\text{[3TC]})\), and adefovir \((9\text{-}[2\text{-phosphonylmethoxymethyl]adenine}\text{[PMEA]})\) are potent inhibitors of hepatitis B virus (HBV) replication. Lamivudine has recently received approval for clinical use against chronic human HBV infection, and both PCV and PMEA have undergone clinical trials against HBV in their respective prodrug forms \{famciclovir and adefovir dipivoxil \{bis\-(POM)-PMEA\}\}. Since multidrug combinations are likely to be used to control HBV infection, investigation of potential interactions between PCV, 3TC, and PMEA is important. Primary duck hepatocyte cultures which were either acutely or congenitally infected with the duck hepatitis B virus (DHBV) were used to investigate in vitro interactions between PCV, 3TC, and PMEA. Here we show that the anti-DHBV effects of all the combinations containing PCV, 3TC, and PMEA are greater than that of each of the individual components and that their combined activities are approximately additive or synergistic. These results may underestimate the potential in vivo usefulness of PMEA-containing combinations, since there is evidence that PMEA has immunomodulatory activity and, at least in the duck model of chronic HBV infection, is capable of inhibiting DHBV replication in cells other than hepatocytes, the latter being unaffected by treatment with either PCV or 3TC. Further investigation of the antiviral activities of these drug combinations is therefore required, particularly since each of the component drugs is already in clinical use.

It has been estimated that approximately 5% of the world’s human population is chronically infected with the hepatitis B virus (HBV) (23) and as a direct consequence are at dramatically increased risk of developing cirrhosis, hepatocellular carcinoma, or decompensated liver disease (23, 36). Antiviral chemotherapy remains the only option for controlling HBV infection in these individuals, for whom the HBV vaccines which are now available provide no benefit (23). Unfortunately, the development of effective chemotherapy for treatment of chronic HBV infection has proven difficult due to a variety of factors which are both virus and host dependent (32). At present, the only licensed treatment for chronic hepatitis B infection in most countries is alpha interferon, use of which is only partially effective and frequently limited by the occurrence of adverse side effects (23). Although the use of nucleoside and nucleoside analogs as antiviral agents has been similarly disappointing (9, 30), prospects for their future use have improved dramatically, as several of the more recently developed analogs have been found to be potent and selective inhibitors of HBV replication (10, 32). These analogs fall into two broad categories: (i) those which have modified cyclic or acyclic sugar configurations and (ii) those which have the “unnatural” L configuration. Of the recently developed analogs which have already been used clinically or are about to enter preliminary clinical trials against HBV, all representatives of the first category are purine derivatives, whereas all representatives of the second category are pyrimidine derivatives. Penciclovir \((9\text{-}[2\text{-hydroxy-1-(hydroxymethyl)-ethoxymethyl]guanine}\text{[PCV]})\), a deoxyguanosine analog, and lamivudine \((\text{-1}\text{-}\beta\text{-L-2',3'-dideoxy-3'-thiacytidine}}\text{[3TC]})\), a deoxycytidine analog, are representative of the first and second categories, respectively.

PCV was originally developed as an antiherpesvirus agent (33). Its antihepadnaviral activity was first demonstrated in the duck model of HBV infection (26, 31, 33), and it has already undergone clinical trials against chronic HBV infection in its orally available form, famciclovir (27). 3TC was originally developed as an inhibitor of reverse transcriptase and was later shown to possess potent antiviral activity (33); following successful clinical trials against chronic HBV infection (16, 24), it was approved for clinical use against chronic HBV in several countries, including the United States, Canada, and members of the European Union. Because PCV and 3TC have been in clinical use for some time as antiviral agents, respectively, there is already a considerable body of literature attesting to their safety and efficacy (33).

Treatment with either PCV or 3TC alone causes a rapid and substantial decrease in viremia in most chronically HBV-infected patients, and it has recently been shown that the decrease is sufficient to restore the antiviral T-cell response within 2 weeks of the initiation of treatment in a subset of 3TC-treated patients (6). We have previously shown that the in vitro anti-HBV (anti-DHBV) activities of PCV and 3TC are synergistic (11), and preliminary reports of the successful clinical use of this combination both simultaneously (16) and sequentially (37) have already appeared.

Adefovir \((9\text{-}[2\text{-phosphonylmethoxymethyl]adenine}\text{[PMEA]})\), an acyclic dAMP analog, is a third compound which, in its oral prodrug form, adefovir dipivoxil \{the \text{bis\-(pivaloyloxy-methyl)-}

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observed in human hepatocellular carcinoma cells stably transfected with HBV and in primary duck hepatocytes infected with DHBV (21, 22).

The encouraging results from preliminary clinical trials of PCV, 3TC, and PMEA are tempered by a large body of experience suggesting that long-term remissions of chronic HBV infection following chemotherapy are uncommon, with most patients experiencing a relapse or a rebound in viremia after drug administration is stopped (10, 23). This phenomenon is attributable to two important characteristics of chronic hepadnaviral infection: (i) the potential for reinitiation of infection by viral particles released from cell and tissue sites which may act as sanctuaries for potentially infectious virus, even during aggressive antiviral therapy (26, 29, 30), and (ii) the persistence of intranuclear viral supercoiled, covalently closed circular (CCC) DNA, the hepadnaviral genomic species which serves as the template for transcription of viral mRNA (mediated by host cell RNA polymerase II) but which is only indirectly involved in DNA replication and is minimally affected by deoxynucleoside triphosphate (dNTP) analogs (8). These characteristics ensure that chemotherapeutic control of chronic HBV infection is most likely to require long-term treatment. Such long-term treatment may be compromised by cumulative drug toxicity and also carries increased risks for development of viral resistance. Although at date no consistent dose-related toxic effects have been observed during clinical use of PCV, 3TC, or PMEA, strains of HBV resistant to PCV and 3TC have already been reported (1, 3, 40). In both cases resistant virus may become predominant after less than 12 months’ antiviral therapy in as many as 25% of treated patients, depending on the clinical setting (41). Consequently, it is becoming increasingly apparent that no single antiviral agent will be able to suppress chronic hepadnaviral infection in the long term (9–11). Preliminary indications of the efficacy of drug combinations can be obtained from in vitro experiments; accordingly, we have investigated the effects of combinations containing PCV, 3TC, and PMEA on DHBV replication in primary duck hepatocytes (PDH). Here we report that all possible combinations of these drugs behave additively or synergistically in this system. Although these observations are not necessarily predictive of in vivo efficacy, use of such combinations may also be beneficial in vivo, and their use may inhibit the rate of development of viral resistance. Indeed, since 3TC, PCV, and PMEA are already in clinical use, further investigation of possible interactions in terms of pharmacology, toxicology, and possible viral cross-resistance would be appropriate.

MATERIALS AND METHODS

Chemicals and drugs. All chemicals and reagents were of analytical grade or of the highest grade obtainable commercially. Lamivudine and penciclovir were provided by SmithKline Beecham Pharmaceuticals, King of Prussia, Pa., and PMEA was supplied by Gilead Sciences, Foster City, Calif. Stock solutions (50 mM) of 3TC and PCV were prepared in distilled water or dimethyl sulfoxide, respectively, and stored at room temperature in light-proof containers. Stock solutions of PMEA were prepared when required and were discarded after 1 day. Serial working dilutions of drugs and drug combinations at 100 times the final

FIG. 1. Drug combination assay protocol. (A) Diagram illustrating how combination assays were set up in 12-well cell culture plates. PDH were plated on day 1, and triplicate cultures exposed to either A, B, A plus B, or no drug (control), where A and B are the concentrations of drugs A and B, respectively. After 9 days' incubation, intracellular accumulation of DHBV RI was assayed by dot blot hybridization with a 32P-labeled full-length DHBV cDNA probe. (B) Autoradiograph showing an example of results obtained after processing cell lysates derived from six 12-well plates. In rows A to F, columns 1 through 5, 4 through 6, 7 through 9, and 10 through 12 correspond to triplicate cultures from a single plate. The total drug concentration (A + B) which is present in columns 7 through 9 is shown (as a micromolar concentration) on the right, at the end of each row. There were no samples in row G. Row H contained DHBV DNA standards. In this example, drug A was PMEA and drug B was PCV. They were present at equimolar concentrations. Note that dot blot data presented in tables and graphs were derived directly from scintillation counting, (which gives linear responses over a much wider range), not from densitometry of autoradiographs. (C) Intracellular DHBV levels during culture. PDH were plated on day 0 and incubated overnight in drug-free medium. They were harvested immediately after plating and at 2-day intervals thereafter until day 12, and lysates were assayed for DHBV DNA. Exponential curves were fitted to the data from days 4 onward and were used to estimate the doubling time (approximately 1.5 days) for intracellular DHBV in the absence of inhibitors of replication. In the presence of 1.0 μM penciclovir, a concentration which was not cytotoxic but was sufficient to completely block DHBV replication, intracellular DHBV DNA decreased with a half-life of 3.3 days, close to the estimated half-life of DHBV CCC DNA under similar conditions (see reference 8). (Data were pooled from three separate experiments; DHBV signals were standardized as fractions of the day 0 signal; error bars represent standard deviations.)
concentration were prepared in sterile isotonic saline and added to cell culture medium immediately before each medium change. The final concentration of dimethyl sulfoxide in cell culture medium never exceeded 0.2% (vol/vol). Drug concentrations, purity, and stability were checked by UV spectrophotometry and high-performance liquid chromatography (HPLC).

**PDH isolation and culture.** One-day-old Pekin-Aylesbury cross ducks congenitally infected with an Australian strain of DHBV were obtained from a commercial supplier (7, 31). DHBV-free ducklings were obtained from a different supplier, and DHBV-infected and uninfected ducklings were housed separately. Viremia was monitored by serum dot blot hybridization as previously described (7). Primary hepatocytes were obtained from livers of 7- to 14-day-old congenitally infected or virus-free ducklings and seeded into 12-well plastic culture plates (ICN Biomedicals, Aurora, Ohio) at a density of approximately 0.75 \( \times 10^6 \) to 1.0 \( \times 10^6 \) PDH per well (7, 29). PDH were allowed to attach overnight before the first medium change (on day 1 postplating) and were maintained at 37°C in a humidified incubator under 5% CO2 with medium changes every second day (7). Under these conditions, DHBV replicative intermediates (RI), including CCC DNA, accumulate intracellularly from day 3 or 4 onwards, paralleled by secretion of virions into the culture medium (7, 8, 11, 38, 39). The slight decrease in intracellular DHBV during the first 2 or 3 days in culture is due to loss of cells (see Fig. 1). Like primary hepatocytes of other species, PDH in culture show negligible mitotic activity. Intracellular CCC DNA, the least abundant intracellular DHBV RI, becomes easily detectable after about day 6 postplating (7, 8, 38, 39). Antiviral assays were performed on two separate occasions, using PDH from different ducklings for each assay. Antiviral effects were assessed by monitoring viral DNA replication and virus-specific protein synthesis at the end of treatment.

**Cytotoxic and cytostatic effects of treatment.** In experiments conducted in parallel with antiviral assays, PDH congenitally infected with DHBV were exposed continuously from day 1 postplating to PMEA, PCV, and 3TC alone or in combination at total concentrations of 0, 0.25, 0.5, 1.0, 2.0, or 4.0 mM for 9 days. On day 10, cell viability was assessed by microscopic examination immediately before each medium change and by neutral red uptake (31). Since PDH are quiescent, they cannot be used to assess potential cytostatic (antiproliferative) effects, for which a well-characterized human hepatocyte-derived cell line, Huh-7, was used. For these assays, Huh-7 cells were seeded into 96-well flat-bottom microtiter plates at a density of approximately 10^4 cells per well in Dulbecco's modified Eagle medium supplemented with 5% (vol/vol) fetal calf serum. They were allowed to attach for 4 h, after which the medium was replaced by fresh medium with or without PMEA, PCV, or 3TC alone or in combination at total concentrations of 0, 0.25, 0.5, 1.0, 2.0, or 4.0 mM (three sets of triplicates for each treatment). After 2, 4, or 6 days, cell viability was assessed by neutral red uptake. Huh-7 monolayers in drug-free control wells reached confluence on day 4. Neutral red uptake data were fitted to exponential decay equations of the form

\[ y = a \times 10^{-bx}, \]

where \( y \) is the percentage of dye uptake relative to the average value (defined as 100%) for untreated controls and \( x \) is the drug concentration.

The curve is described by the equation $y = k + a/(1 + c/x)$, where $x$ represents the dose (drug concentration) and $y$ represents the response (virus replication expressed as a percentage of that in untreated control cultures). $a$ and $b$ define the curves' maxima and slopes, respectively. $CC_{50}$ (the drug concentrations which reduced uptake to 50% of the control value) were estimated by solving each equation for the $a$ (drug concentration) value (corresponding to a y (percentage uptake) value of 0).

Antiviral activities of PMEA, PCV, and 3TC alone or in combination in congenitally infected PDH. Replicate sets of PDH monolayers congenitally infected with DHBV were continuously exposed to PCV, 3TC, and PMEA alone or in combination at various concentrations and concentration ratios for 9 days beginning on day 1 postplating. Total drug concentrations (in micromolar concentrations) were 10, 5, 0, and 10 halving dilutions from 2 to 0.0078125. Each concentration and concentration ratio was assayed in triplicate (Fig. 1). Cells were harvested on day 10 postplating, and antiviral effects were assessed by monitoring viral DNA replication and virus-specific protein synthesis. In experiments using PMEA in combination with PCV or 3TC, the drugs were present at fixed molar ratios (PMEA to PCV or PMEA to 3TC) of either 0:1, 1:9, 1:4, 1:1, 4:1, or 1:5. A wide range of concentrations and concentration ratios was used to increase the chances of detection of possible anomalies in combination behavior. In initial experiments in which all three drugs were present in combination, the concentration ratio was 1:1:1. The ratio was modified for subsequent experiments (see below).

Antiviral activities of PMEA, PCV, and 3TC alone or in combination in acutely infected PDH. One set of studies was carried out using acutely infected PDH, in which the viral load is lower and the effects of drugs on early replication events can be assessed more easily compared to those for chronically infected PDH. Uninfected PDH cultures were prepared as described above and allowed to attach overnight before infection with DHBV. Infection was achieved by removing the medium and then incubating the cell monolayers for 1 h with DHBV-positive serum diluted (antigen) that could be transformed into a detectable concentration of approximately 5 to 10 viral genome equivalents per cell (300 μg/ml). Pooled sera from 4- to 5-week-old ducklings with high DHBV titers were used as the inoculum. Mock infection of control PDH was performed using DHBV-free duck serum. After an hour, inocula were removed and replaced by fresh medium with or without test compounds at various concentrations as above. Cultures were maintained for as long as 9 days after infection.

Inhibition of DHBV-specific protein synthesis and CCC DNA production by PMEA, PCV, and 3TC alone or in combination. Two separate sets of samples were subjected to further analysis. They were derived from cells infected either acutely or chronically with DHBV. Drug concentrations were chosen so that each inhibitor was present at a concentration expected to cause 25 to 50% inhibition of viral DNA replication based on previous experience. Samples were prepared for assay of DHBV-specific protein synthesis and CCC DNA production by immunoblotting or Southern blotting, respectively. Further details are provided in the legend to Fig. 5.

Preparation of radioactive probe, detection of DHBV DNA replication, and analysis of viral replicative species. Southern hybridization was performed, and DNA dot blots were probed with a full-length DHBV DNA clone labeled with [α-32P]dCTP as described previously (7, 8, 31) by using the Random Primer Plus extension kit (Dupont-NEC, Boston, Mass.). Total cellular DNA was extracted from cell lysates and probed for DHBV DNA by dot blot hybridization as described previously (7, 31). Intracellular DHBV replicative species were analyzed by Southern blot hybridization after electrophoresis through 1.5% agarose gels and capillary transfer to positively charged nylon membranes (7, 31). Postcellular DHBV CCC DNA was extracted from lysates by a specific enrichment procedure (39) and analyzed by Southern blotting as previously described (7, 31). Hybridization conditions and autoradiographic procedures have been described in detail previously (7, 31).

Detection of DHBV-specific protein synthesis. Immunoblottig was performed as described elsewhere (31). Polyclonal rabbit antibodies to the carboxy-terminal part of the DHBV core protein or monoclonal antibodies to the DHBV pre-S antigen were used to stain immunoblots. Bound antibody was detected using an enhanced chemiluminescence (ECL) kit (Amersham Australia, North Ryde, New South Wales, Australia), according to the manufacturer's instructions. Detailed procedures have been reported previously (7, 31).

Quantitation of antiviral effects and data analysis. DNA dot blots were autoradiographed to visualize bound probe. The amount of bound probe on each blot was then quantitated directly by liquid scintillation counting using a Microplate Liquid Scintillation Counter (Top Count; Packard Instruments, Meriden, Conn.). Image densities in autoradiographs following Southern blot analysis or ECL (immunoblot) exposures following immunoblotting were quantitated by densitometry. Viral replication levels in drug-treated PDH were expressed as percentages relative to the mean values for drug-free controls. Two-dimensional dose-response plots for individual drugs and drug combinations were generated with the aid of TableCurve2D, a graphics-statistics software package from Jandel Scientific (San Rafael, Calif.).

Definitions and modelling of drug interactions. Various methods have been employed for the analysis of drug interactions, generating a large body of literature on the subject (for comprehensive reviews, see Berenbaum [5] and Greco et al. [19]), in which definition of the outcome expected from the use of drug combinations is crucial, because opposing phenomena are defined as significantly greater (anergy) or significantly less (antagonism) than the “expected” outcome. Two main alternative approaches, termed “Bliss independence” and “Lowe additivity,” have been generally accepted and widely used as the basis for pre-

FIG. 3. (A) Diagram illustrating parameters of the logistic dose-response curve. The curve is described by the equation $y = k + a/(1 + c/x)$, where $x$ represents the dose (drug concentration) and $y$ represents the response (virus replication expressed as a percentage of that in untreated control cultures). $k$ is a constant corresponding to the minimum value of $y$ (i.e., the lower plateau of the curve); $a$ defines the curves' amplitude, being the difference between the maximum and minimum values of $y$. The $b$ value defines the transition center, which is the $x$ value at half the amplitude. The value of $c$ controls the transition width (distances on the $x$ axis on either side of $b$ corresponding to the middle 2/3 of $a$). In this form of the equation, the slope of the curve becomes steeper as $c$ increases. Note that the $x$ axis is logarithmic and the $y$ axis is linear. In the assays described here, $k = 0$ and the amplitude $a$, representing the amount of viral replication in uninfected controls, is by definition 100%. $b$ therefore corresponds to the $EC_{50}$ (the drug concentration which reduces viral replication to 50% of the control value). (B) Application of dose-response curve analysis to a set of data. After 9 days' continuous exposure of chronically DHBV-infected PDH to either PMEA, 3TC, PCV, or equimolar mixtures, intracellular DNA was extracted and dot blots were prepared and probed as described in the text. The mean probe signal from drug-treated PDH was expressed as a percentage of the mean from untreated controls and plotted (on the $y$ axis) versus the total drug concentration (x axis). Each data point represents the mean of triplicates. This example shows data from experiment 2 (see Table 1), in which the total concentrations required to inhibit intracellular DHBV replication by 50% (EC_{50}) for PMEA, PCV, 3TC, PCV plus 3TC, and PMEA plus PCV plus 3TC were 0.17, 0.43, 0.64, 0.28, and 0.2 μM, respectively. The last two values correspond to 0.14 and 0.1 μM for each component.
dicting the effects of drug combinations. Although each has particular advantages and disadvantages, only the latter is free from implicit assumptions about mechanisms of action (5, 19).

In the Bliss independence model, the combined effect \( E_{xyz} \) of drugs in combination is the product of the individual effects \( E_x, E_y, \) and \( E_z \), respectively, and can be calculated from the equation \( E_{xyz} = E_x(E_yE_z) \), in which the effect \( E \) represents fractional viral replication rather than inhibition of replication.

An alternative model, known as Lowe additivity, is based on a definition of zero interaction which can be expressed as \( (d_xD_x + d_yD_y + d_zD_z) = 1 \), where \( D_x, D_y, \) and \( D_z \) are the doses of individual drugs required to produce the same effect as the effect produced by doses \( d_x, d_y, \) and \( d_z \) in combination. Antagonism \( (>1) \) or synergy \( (<1) \) is indicated if the sum of the expression (the "combination index") \( (d_xD_x + d_yD_y + d_zD_z) \) is significantly different from 1. Experimental data were analyzed using both Bliss independence and Lowe additivity models.

In addition, three-dimensional dose-response surfaces (19) which described the activity of each drug combination were generated by using the TableCurve3D program (Jandel Scientific), which fitted a surface to all data points for each set of experiments without reference to preconceptions of the nature of the drug interaction or shapes of individual dose-response curves. Coordinates of points on each dose-response surface were compared with predictions from the two main theoretical models. Further details are provided in the legends to Fig. 3 and 4 and in the footnote to Table 1.

RESULTS

Cytotoxic and cytostatic effects of drug treatment. PDH monolayers remained intact for the duration of all experiments, and on microscopic examination, there were no observ-

able differences between treated and untreated PDH in antiviral assays, nor were there measurable differences in neutral red uptake. Significant toxicity occurred only when PDH were exposed continuously to drug concentrations in the millimolar range. After 9 days’ continuous exposure, the concentrations required to cause a 50% reduction in cell viability as measured by neutral red uptake were estimated (by extrapolation) to be approximately 3, 5, and 7 mM for PMEA, PCV, and 3TC, respectively, and 3, 5, and 6 mM for PMEA, PCV, and 3TC, respectively, and

### Table 1. Anti-DHBV efficacies of PCV, 3TC, and PMEA alone and in combination in congenitally infected PDH

<table>
<thead>
<tr>
<th>Drug or combination and molar ratio</th>
<th>Equation parameter (mean ± SD)</th>
<th>Correlation coefficient ( r^2 )</th>
<th>C.I. (Lowe)</th>
<th>% Inhibition expected at EC(_{50}) (Bliss)</th>
<th>Interaction</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>( a )</td>
<td>( b )</td>
<td>( c )</td>
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<tr>
<td>Expt 1: PMEA in combination with PCV or 3TC</td>
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<tr>
<td>PMEA</td>
<td>100 ± 1.4</td>
<td>0.12 ± 0.01</td>
<td>1.5 ± 0.08</td>
<td>1.0</td>
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<tr>
<td>PCV</td>
<td>100 ± 5.1</td>
<td>0.13 ± 0.03</td>
<td>0.94 ± 0.15</td>
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<td></td>
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<tr>
<td>3TC</td>
<td>100 ± 4.5</td>
<td>0.20 ± 0.02</td>
<td>2.0 ± 0.38</td>
<td>0.97</td>
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<tr>
<td>PMEA-PCV (4:1)</td>
<td>100 ± 5.6</td>
<td>0.05 ± 0.01</td>
<td>0.87 ± 0.12</td>
<td>0.96</td>
<td>0.41 ± 0.13</td>
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<td>PMEA-PCV (1:1)</td>
<td>100 ± 5.0</td>
<td>0.04 ± 0.01</td>
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<td>0.97</td>
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<td>PMEA-PCV (1:4)</td>
<td>99.8 ± 5.5</td>
<td>0.05 ± 0.01</td>
<td>0.90 ± 0.12</td>
<td>0.93</td>
<td>0.39 ± 0.12</td>
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<td>PMEA-PCV (1:9)</td>
<td>100 ± 6.5</td>
<td>0.14 ± 0.04</td>
<td>0.87 ± 0.17</td>
<td>0.99</td>
<td>1.09 ± 0.33</td>
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<td>PMEA-3TC (4:1)</td>
<td>96.9 ± 4.8</td>
<td>0.06 ± 0.01</td>
<td>0.89 ± 0.12</td>
<td>0.97</td>
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<td>PMEA-3TC (1:1)</td>
<td>100 ± 5.0</td>
<td>0.13 ± 0.01</td>
<td>2.2 ± 0.40</td>
<td>0.98</td>
<td>0.87 ± 0.29</td>
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<tr>
<td>PMEA-3TC (1:4)</td>
<td>98.4 ± 3.6</td>
<td>0.12 ± 0.01</td>
<td>1.5 ± 0.16</td>
<td>0.99</td>
<td>0.68 ± 0.22</td>
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<tr>
<td>PMEA-3TC (1:9)</td>
<td>102 ± 4.5</td>
<td>0.13 ± 0.01</td>
<td>2.0 ± 0.28</td>
<td>0.98</td>
<td>0.69 ± 0.23</td>
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<td>Expt 2: PMEA in combination with both PCV and 3TC</td>
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<tr>
<td>PMEA</td>
<td>104 ± 4.2</td>
<td>0.17 ± 0.01</td>
<td>2.47 ± 0.30</td>
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<td>PCV</td>
<td>99.8 ± 4.7</td>
<td>0.43 ± 0.04</td>
<td>1.8 ± 0.3</td>
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<tr>
<td>3TC</td>
<td>102.7 ± 6.8</td>
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<td>1.7 ± 0.4</td>
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<tr>
<td>PCV + 3TC (1:1)</td>
<td>100 ± 2.31</td>
<td>0.28 ± 0.02</td>
<td>2.35 ± 0.23</td>
<td>0.99</td>
<td>0.54 ± 0.12</td>
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<tr>
<td>PMEA + PCV + 3TC (1:1:1)</td>
<td>99.9 ± 3.5</td>
<td>0.2 ± 0.03</td>
<td>0.7 ± 0.17</td>
<td>0.98</td>
<td>0.71 ± 0.29</td>
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*Congenitally DHBV-infected PDH were harvested after 10 days’ in vitro culture (i.e., after 9 days of continuous exposure to drugs. Calculations are based on relative signal intensities of probe signals from dot blots of total intracellular DNA probed with a full-length 32P-labeled DHBV cDNA probe. Parameters describing each dose-response curve were derived from analysis of 12 data points, each of which represented the mean of triplicate assays. Logistic dose-response curves of the form \( y = k + a/(1 + [x/b]^c) \) (see Fig. 3) were fitted to each data set with the aid of TableCurve2D, which also calculated the equation parameters and correlation coefficients. Listed \( b \) values correspond to EC\(_{50}\) in micromolar concentrations (see Fig. 3). For drug combinations, the \( b \) values listed correspond to the total drug concentration, not the concentration of any single component. EC\(_{50}\) for individual components of combinations can be calculated by dividing the \( b \) value by the fractional concentration of that component. Combination indices (C.I.) were estimated at the EC\(_{50}\) using the Lowe additivity formula (see the text). The percent inhibition expected at the observed EC\(_{50}\) for each drug combination was calculated using the Bliss independence formula (see the text). Errors in calculations were assumed to be additive, and interactions were defined as synergistic (SYN) if the C.I. was significantly less than 1 and/or the expected percent inhibition at the EC\(_{50}\) was significantly less than 50. The upper 95% confidence interval can be obtained by adding twice the standard deviation to each estimate. Synergy was significant at the \( P < 0.05 \) level for all but two combinations rated as synergistic. Synergy between PMEA and 3TC at ratios of 1:4 and 1:9 was significant at a \( P < 0.05 \) level based on the C.I. and at a \( P < 0.05 \) level based on the Bliss calculation. In the remaining three cases, estimates of C.I. and expected percent inhibition were not significantly different from 1 or 0, respectively, so the interaction was defined as additive (ADD). Most importantly, antagonism between drugs in combination was not observed (but see Fig. 3B).
CONCENTRATIONS which produced 50% inhibition of replication for 3TC alone and in all combinations caused dose-dependent combination in congenitally infected PDH.

Approximately additive cytostatic effects were also observed for two-drug combinations (data not shown). Bliss model. Approximately additive cytostatic effects according to either the Lowe or the Bliss independence model. By contrast, treatment with individual inhibitors had no significant effect on core antigen expression and barely significant effects (<20% reduction, slightly greater than 1 standard deviation) on pre-S antigen expression, although all combinations caused inhibition (approaching 50%) of both core and pre-S antigen synthesis (Fig. 5A).

**DISCUSSION**

Here we confirm and extend our earlier observations (11, 34; D. Colledge, S. Locarnini, and T. Shaw, Abstr. 38th Intersci. COLLEDGE ET AL. ANTIMICROB. AGENTS CHEMOTHER.

![Three-dimensional dose-response plot illustrating inhibition of DHBV DNA replication in chronically infected PDH by PMEA in combination with PCV (A) or 3TC (B) at molar ratios (PMEA to PCV or PMEA to 3TC) of 4:1, 1:1, 1:4, or 1:9. The amount of DHBV replication was expressed as a percentage relative to replication in untreated controls (set at 100%) and plotted on the y (vertical) axis against the concentration of each drug using the x axis for PMEA.](image)

**FIG. 4.** Three-dimensional dose-response plot illustrating inhibition of DHBV DNA replication in chronically infected PDH by PMEA in combination with PCV (A) or 3TC (B) at molar ratios (PMEA to PCV or PMEA to 3TC) of 4:1, 1:1, 1:4, or 1:9. The amount of DHBV replication was expressed as a percentage relative to replication in untreated controls (set at 100%) and plotted on the y (vertical) axis against the concentration of each drug using the x axis for PCV or 3TC and the y axis for PMEA. Although they do not show individual drug dose responses, logarithmic axes have been used because they show the response to drug combinations much more clearly than do linear axes. A best-fit dose-response surface (shown as a mesh) has been fitted to each set of points by unweighted nonlinear regression analysis with the TableCurve3D program, which was given the option of fitting dose-response surfaces “expected” on the basis of different drug interaction models. The best-fit surfaces in each case were found to be those predicted by the Bliss independence model of drug interaction. Each data point shown represents the average of three determinations. Points lying above or below the fitted surface suggest antagonism or synergy, respectively. Those which lie within 1 standard deviation of the fitted surface are indicated by open circles, and those which lie within 2 standard deviations of the fitted surface are indicated by filled circles. Most experimental data points lie within 1 standard deviation of the fitted dose-response surfaces, and no data points lie beyond 2 standard deviations.

Anti-DHBV activities of PMEA, PCV, and 3TC alone or in combination in acutely infected PDH. Acute infections were performed to determine the effects of drug treatments on early DHBV replication, namely, the events leading to the accumulation of DHBV CCC DNA by the direct conversion of DNA within infecting virus particles and the early intracellular recycling of immature progeny virions to the nucleus. Drug concentrations used in these experiments were identical to those used in corresponding experiments with chronically infected PDH. Because drugs are added at around the time of infection, the viral load is significantly lower in acutely infected cells than in chronically infected cells incubated ex vivo for equivalent times. As a consequence, drug responses are enhanced due to the absence of a pre-existing intracellular pool of viral DNA or protein (Fig. 5B).

**AntidiHBV activities of PMEA, PCV, and 3TC alone or in combination in acutely infected PDH.** Acute infections were performed to determine the effects of drug treatments on early DHBV replication, namely, the events leading to the accumulation of DHBV CCC DNA by the direct conversion of DNA within infecting virus particles and the early intracellular recycling of immature progeny virions to the nucleus. Drug concentrations used in these experiments were identical to those used in corresponding experiments with chronically infected PDH. Because drugs are added at around the time of infection, the viral load is significantly lower in acutely infected cells than in chronically infected cells incubated ex vivo for equivalent times. As a consequence, drug responses are enhanced due to the absence of a pre-existing intracellular pool of viral DNA or protein (Fig. 5B).

**DISCUSSION**

Here we confirm and extend our earlier observations (11, 34; D. Colledge, S. Locarnini, and T. Shaw, Abstr. 38th Intersci.
Conf. Antimicrob. Agents Chemother., abstr. 159, p. 360, 1998) by showing that at clinically achievable concentrations, the antiviral effects of all two-drug combinations containing PCV, 3TC, and PMEA are additive or synergistic. Furthermore, the anti-DHBV effect of combinations containing all three drugs is also approximately additive as measured by inhibition of intracellular DHBV replication, although this is not consistently reflected by comparable inhibition of virus-specific protein synthesis. Conclusions about the effects of drug combinations on viral DNA synthesis were similar regardless of the method used to analyze results, demonstrating that despite controversy over which drug interaction model(s) is appropriate for application in particular cases (5, 19), the different approaches lead to similar conclusions when applied to biological data, which is typically too noisy to provide the accuracy required to support subtle distinctions required by theoretical arguments.

Combination chemotherapy has a number of recognized advantages over monotherapy and in the future will probably become the most effective approach to control chronic human HBV infection (9–11). Rationally chosen drug combinations have the potential to minimize the risk of drug toxicity and to reduce the probability of development of viral resistance, both important considerations during long-term therapy (5, 9, 11, 19). Theoretically it should be possible to design therapeutic regimens using two or more drugs having complementary ac-

FIG. 5. Inhibition of viral CCC DNA (indicated by open circles) and protein synthesis (bar graphs) by PMEA, PCV, and 3TC alone and in combination. Concentrations of PMEA, PCV, and 3TC in these experiments were 0.1, 0.125, and 0.125 μM, respectively. Southern blots and immunoblots were prepared, stained, and analyzed as described previously (9, 26), and results are expressed as percentages of the average probe signal density from untreated controls. Values for each parameter are the means of duplicate or triplicate determinations, with standard deviations represented by error bars. Arrows above and below each bar for combinations indicate expected values for pre-S1 and core antigens, respectively, applying the Bliss independence formula to results for each drug alone. Results are from representative experiments using chronically (A) and acutely (B) infected PDH.
Hepadnaviruses depend completely on the host cell's enzymatic machinery for their supply of deoxynucleotides, since their genomes do not encode enzymes for deoxynucleoside salvage or dNTP synthesis. Pathways for deoxynucleoside salvage and de novo dNTP synthesis are relatively inactive in hepatocytes and presumably in other cells (such as pancreatic islet cells and bile duct epithelial cells) which are known to be susceptible to HBV infection, since these cell types turn over slowly and most are normally quiescent. Ideally, drugs used in combination should use independent processes for uptake and activation and have different mechanisms or sites of action. To minimize administration frequency and make patient compliance with dosage schedules easier, the active metabolites should have long, and preferably similar, intracellular half-lives, and it is also desirable that the agents be orally available and able to be administered simultaneously without adverse or unpredictable pharmacological and pharmacokinetic interactions.

PCV and 3TC appear to be transported into the cell and activated enzymatically by different sets of cellular enzymes. Both specifically inhibit the hepadnaviral DNA polymerase/reverse transcriptase by competing with the corresponding dNTPs for incorporation into nascent DNA and acting as chain terminators after incorporation. Specific interference with the unique dGTP-dependent step which primes hepadnaviral reverse transcription is an additional mechanism by which PCV exerts antihepadnaviral activity and which is not shared by 3TC. Both PCV (as famciclovir) and 3TC are orally available, and each has been used for HBV monotherapy with varying success. Regardless of more-consistent responses obtained with 3TC (23), emergence of resistance appears almost inevitable during long-term monotherapy (3, 33, 40), necessitating the development of new drugs and therapeutic regimes.

We have previously shown that the in vitro antihepadnaviral activities of PCV and 3TC are synergistic (11) and that each acted additively or synergistically in combination with PMEA (35; Colledge et al., 38th ICAAC) in the test system described here. PMEA has low oral bioavailability, a problem which has been overcome by the development of prodrugs including the bis(pivaloyloxymethyl) derivative (bis-[POM]-PMEA; adefovir dipivoxil), which has reached phase II and III trials against HBV (18, 20).
vivo evaluation of PMEA in the duck model of chronic HBV infection. Using immunohistochemical and in situ DNA hybridization techniques, it was found that treatment with PMEA reduced viral protein and DNA loads in bile duct epithelial cells (BDEC) as well as hepatocytes (30). This observation is significant since the reservoir of DHBV in BDEC is refractory to treatment with nucleoside analogs such as PCV and 3TC, presumably because BDEC lack the enzymatic machinery required for their uptake or phosphorylation (29, 30). Since it is a dAMP analog, PMEA is able to bypass the initial phosphorolysis site, which is critical and often limiting for the activity of most nucleoside analogs (32, 33) (Fig. 6). Cellular enzymes convert PMEA to its diphosphate, a dATP analog which acts as an obligatory chain terminator after incorporation into nascent DNA (2, 28). Its selectivity depends on relatively high affinity for HBV DNA polymerase/reverse transcriptase compared to host cell DNA polymerases (28).

Significantly, mutations in the HBV polymerase gene which confer resistance to 3TC do not confer cross-resistance to PMEA in vitro (40). In addition, PMEA has immunomodulatory activity and has been shown to stimulate interferon alpha production and natural killer cell activity (14, 28). Whether the results presented here are predictive of efficacy against human HBV infection remains to be established, since they may be influenced by species-dependent differences in the behavior of key cellular and/or viral enzymes (e.g., transmembrane transporters, cellular deoxynucleoside kinases, and viral DNA polymerase/reverse transcriptases).

Our results probably underestimate the potential efficacy of triple-drug combination for at least two main reasons. First, the immunostimulatory potential of PMEA cannot be reliably assessed in congenital DHBV infection because (i) immune responses are typically species-specific and (ii) ducks are, in any case, essentially immunotolerant of hepaviral infection. Secondly, the assay described here uses an isolated population of primary hepatocytes, relatively free of other liver cell types (7). Consequently, the putative advantages of PMEA—the ability to stimulate immunity and the ability to inhibit viral replication in hepatocytes as in other cells harboring virus—cannot be adequately assessed using this system.

Available evidence, although limited (2, 28, 32, 33), indicates that PCV, 3TC, and PMEA each use a different pathway for cellular uptake and activation (Fig. 6), and although the active anabolites of each must compete for recognition by the HBV DNA polymerase/reverse transcriptase, each pairs with a different template site, since each is an analog of a different dNTP. Neither PCV nor 3TC affects hepadnaviral replication in BDEC, a site where PMEA is active (30); nor does PCV or 3TC share the immune-stimulating capacity of PMEA (14). In addition, 3TC-resistant HBV mutants do not display significant cross-resistance to PMEA (40), and while 3TC resistance also confers resistance to PCV, the converse has not been observed (41). Together, these observations provide a sound rationale for the use of PCV, 3TC, and PMEA in combination, and further investigation of their combined anti-HBV activity is justified, particularly since each is already being used clinically. In particular, the issues of tissue-specific activity, possible cross-resistance, and potential for cumulative in vivo toxicity require further investigation.

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