Lobucavir is Phosphorylated in Human Cytomegalovirus-Infected and Uninfected Cells and Inhibits the Viral DNA Polymerase


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Lobucavir (LBV) is a deoxyguanine nucleoside analog with broad-spectrum antiviral activity. LBV was previously shown to inhibit herpes simplex virus (HSV) DNA polymerase after phosphorylation by the HSV thymidine kinase. Here we determined the mechanism of action of LBV against human cytomegalovirus (HCMV). LBV inhibited HCMV DNA synthesis to a degree comparable to that of ganciclovir (GCV), a drug known to target the viral DNA polymerase. The expression of late proteins and RNA, dependent on viral DNA synthesis, was also inhibited by LBV. Immediate-early and early HCMV gene expression was unaffected, suggesting that LBV acts temporally coincident with HCMV DNA synthesis and not through cytoxicity. In vitro, the triphosphate of LBV was a potent inhibitor of HCMV DNA polymerase with a Ki of 5 nM. LBV was phosphorylated to its triphosphate form intracellularly in both infected and uninfected cells, with phosphorylated metabolite levels two- to threefold higher in infected cells. GCV-resistant HCMV isolates, with deficient GCV phosphorylation due to mutations in the UL97 protein kinase, remained sensitive to LBV. Overall, these results suggest that LBV-triphosphate halts HCMV DNA replication by inhibiting the viral DNA polymerase and that LBV phosphorylation can occur in the absence of viral factors including the UL97 protein kinase. Furthermore, LBV may be effective in the treatment of GCV-resistant HCMV.

Lobucavir (LBV) \{\(1R-1\alpha, 2\beta, 3\alpha\)-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine; also known as cyclobut-G, BMS-180194, SQ-34,514, (R)-BHCG, and c-oxetanocin-G\} is a nucleoside analog of deoxyguanine which exhibits broad-spectrum antiviral activity against a wide variety of herpesviruses, hepatitis B virus, and human immunodeficiency virus (HIV) (4, 8, 13, 17, 19, 20, 32, 33, 37, 44, 56, 58). LBV is currently under investigation in clinical studies for the treatment of human cytomegalovirus (HCMV) retinitis in AIDS patients, recurrent herpes simplex virus (HSV) infections, and chronic hepatitis B virus (HVB) infections.

Previous reports have shown that LBV inhibits HSV type 1 (HSV-1) DNA replication and that the triphosphate of LBV (LBV-TP) is a chain termination inhibitor of HSV-1 and -2 DNA polymerases in vitro (13, 21, 53). Phosphorylation of LBV is initiated by herpesvirus thymidine kinase (TK) in vitro (13, 22) and intracellularly (24, 56). TK-deficient HSV-1 and -2 and varicella-zoster virus (VZV) have reduced sensitivities to LBV (13, 24, 36, 56). These results suggest that the mechanism of action of LBV against HSV-1 and -2 and VZV is inhibition of the viral DNA polymerase after phosphorylation by the virally encoded TK.

Although LBV has activity against HCMV and murine cytomegalovirus, neither of these viruses encodes the herpesvirus TK known to phosphorylate LBV (5, 40). Additionally, HV and HVB do not encode products that appear capable of mediating LBV phosphorylation. The cytomegaloviruses do, however, possess homologs of a herpesvirus-encoded protein kinase (6, 45) which mediates the phosphorylation of ganciclovir (GCV) (25, 48). Indeed, in the case of VZV, both the herpesvirus TK and protein kinase may independently enable the phosphorylation of LBV and GCV (24). This raises the hypothesis that the HCMV protein kinase, encoded by the UL97 gene, selectively phosphorylates LBV, thus enabling inhibition of the viral DNA polymerase.

In this study, we sought to determine the mechanism of LBV inhibition of HCMV replication and to test the above hypothesis. The events in the HCMV viral life cycle in LBV-treated cells were characterized in parallel with those in cells treated with the known HCMV DNA polymerase inhibitor, GCV (14, 28, 30). We also examined the intracellular levels of phosphorylated LBV and the activity of LBV-TP versus the HCMV DNA polymerase in vitro. Finally, we measured the activity of LBV against GCV-resistant HCMV isolates with mutations in the UL97 gene.

MATERIALS AND METHODS

Cells, viruses, infections. HCMV strain AD169 (kindly provided by Anamari Colberg-Poley, Children’s National Medical Center) was propagated in human foreskin fibroblast (HFF) cells (Viromed, Inc.), as described previously (42). Plaque reduction assays were performed in WI38 cells, as described previously (13). Clinical HCMV isolates (7, 12, 46) were kindly provided by Karen Biron (Glaxo-Wellcome). Experiments to examine virus macromolecular synthesis used HFF cells infected with HCMV (multiplicity of infection, 3 PFU per cell) and treated with LBV or GCV (synthesized as described in references 43 and 30, respectively) at 4 μg/ml, or 0.1% (vol/vol) of the dimethyl sulfoxide (DMSO) control. For DNA synthesis experiments, cells were treated immediately following a 1-h virus adsorption period. For protein and RNA expression studies, as described in Results, cells were treated approximately 24 h prior to, and immediately following, the virus adsorption period.

Viral DNA. Total infected cell DNA was prepared from cells 72 h postinfection (p.i.) by incubation of cells in 100 mM NaCl–10 mM Tris-HCl (pH 8)–25 mM EDTA–0.5% sodium dodecyl sulfate–100 μg of proteinase K per ml at 50°C for 2 h. The DNA was extracted with phenol, phenol-chloroform, and chloroform and was ethanol precipitated. DNA from 3 × 10⁶ cells was applied to GeneScreen Plus membranes (DuPont-NEN), as described by the manufacturer, with a slot blot vacuum apparatus. A DNA fragment from HCMV UL36 gene exon 1 (219-bp Apg-dSmal fragment from plasmid p279; 51) was labeled with [α-32P]dCTP by using a Bio-Rad Random Primer DNA labeling kit, as described by the manufacturer. Following hybridization of the blot with the labeled probe and autoradiography, as described previously (51), the autoradiograph was analyzed by scanning laser densitometry. The amounts of DNA in the drug-treated cul-
tures are presented as percentages of that synthesized in the DMSO-treated culture.

**Virally proteins.** Infected cell proteins were harvested and analyzed by Western immunoblotting, as described previously (42). Mouse monoclonal antibodies were from Advanced Biotechnologies, Inc. (anti-pp28 and anti-pp65) and The Goodwin Institute for Cancer Research, Inc. (anti-g). Polyclonal rabbit anti-peptide sera directed against the major capsid protein was generously provided by Wade Gibson (Johns Hopkins). Alkaline phosphatase-conjugated secondary antibodies, rabbit anti-mouse (Bio-Rad), anti-rabbit (Boehringer Mannheim) antibodies were detected with 5-bromo-4-chloro-3-indolylphosphate toluidinium-nitroblue tetrazolium (BCIP-NBT) (Kirkegaard and Perry).

**Virral RNA transcript analysis.** Total infected cell RNA was isolated as described previously (38). RNA (2.5 µg) was analyzed by Northern blot hybridization with 32P-labeled riboprobes by using probes to well-characterized transcripts from the UL36 to UL38 (51) and the major immediate-early (IE) regions (50), as described previously (51).

**Enzymes.** HCMV DNA polymerase was partially purified from HCMV-infected HFF cells by high-salt extraction and chromatography on Q-Sepharose and heparin-agarose. Fractions containing the HCMV DNA polymerase were identified by activity on an activated calf thymus DNA template in 100 mM (NH4)2SO4 as described previously (52).

2 ml of fresh media (containing 2% fetal bovine serum), then labeled with 50 µCi [3H]water for 24 h, two dishes each of mock- and HCMV-infected cells were refed with 50 µCi [3H]water and redissolved to 1 mM in sterile phosphate-buffered saline (PBS). In labeling experiments, 5 µCi [3H]LBV (2 mCi/ml, 11.9 Ci/mmol). After 2 days of labeling, cells were washed twice with PBS and extracted twice for 1 h with 1 ml of ice-cold 60% methanol, after which the extracts were taken to dryness under vacuum. Extracts were redissolved in 150 µl of distilled water, clarified by centrifugation, and analyzed by ion-pairing HPLC on a Vydac C18 reverse-phase column. Detection in series was through a Waters 441 UV detector (260 nm) and quantitated by UV spectroscopy (261 nm). Extracts were monitored from 24 to 72 h p.i.

**Synthesis of LBV-TP.** LBV triphosphate (LBV-TP) was synthesized with purified HSV-1 TK, as described previously (53). Briefly, 25 mM LBV was phosphorylated at 37°C in a cocktail consisting of 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 30 mM KCl, 2 mM diithiothreitol, 5 mM ATP, 30 mM creatine phosphate, 2 U of HSV-1 TK per ml, and 8 U of creatine kinase per ml. TK monophosphorylation of LBV was followed by ion-pair-high-pressure liquid chromatography (HPLC) analysis on an Applied Biosystems reverse-phase C18 column equilibrated with 10 mM KH2PO4 and 2 mM tetrabutylammonium phosphate and eluted with a 0 to 35% acetonitrile gradient. Within 24 h LBV monophosphate production was detected by spectral analysis with a Waters 991 photodiode array detector. Guanylate kinase (2 U/ml) and nucleoside-diphosphate kinase (1 U/ml; Sigma) were added successively for phosphorylation to the di- and triphosphates, respectively. Additional amounts of all three phosphorylating enzymes were added each day, and creatine kinase was added when ADP began to accumulate. Phosphorylation reactions were conducted for 24 h before LBV-TP was detected and were continued for 7 days. LBV-TP was collected by HPLC by repeated injection. Typical yields for LBV-TP were 1 to 2%. Pooled LBV-TP was concentrated by freeze-drying and collected as a single fraction by HPLC. High-concentration LBV-TP (quantitated by UV spectroscopy) was freeze-dried for 10 mM KH2PO4 and 2 mM tetrabutylammonium phosphate.

**In vitro phosphorylated LBV.** [3H]LBV (2 nCi/ml, 11.9 Ci/mmol; 97.4% radiochemically pure LBV, with 2.6% [3H]water as the only detectable radioactive impurity) was obtained from Amersham as a custom synthesis and chemical impurity) was obtained from Amersham as a custom synthesis and

**RESULTS**

**LBV prevents HCMV DNA synthesis.** LBV, a known inhibitor of the HSV-1 and HSV-2 DNA polymerases (13, 21, 53), was examined for its effect on HCMV DNA replication. HFF cells were infected with HCMV (AD169) and treated with 0.1% DMSO or 0.1% DMSO containing LBV or GCV (see Fig. 1 for structures) at 4 µg/ml, as this value represents the approximate 90% effective dose (ED90) in plaque-reduction assays (13) and is well below cytotoxic levels (56). At 72 h p.i. total DNA was isolated and analyzed by quantitative slot blot hybridization. The results of this experiment revealed that LBV and GCV reduced the amounts of viral DNA to 5.0 and 5.2% of the untreated control value, respectively. Therefore the reduction in viral DNA approximates the reduction of plaque formation by the drugs. We found that inhibition of viral DNA synthesis was increased if the drugs were added to cultures 24 h prior to infection (data not shown); therefore, subsequent inhibition experiments used this time for pretreatment. Although DNA synthesis was reduced, an examination of other markers of the viral replication cycle was needed to identify at which step the inhibition occurred.

**LBV prevents late HCMV protein expression.** To determine if inhibition was exclusive to viral DNA synthesis or included later events in the HCMV growth cycle, the expression of several distinct viral proteins was examined. Cell proteins were prepared at 8, 24, or 72 h p.i. and subjected to Western immunoblot assays for well-characterized viral proteins as described previously (42). The results (Fig. 2) show that pp65, glycoprotein B (gB), and the major capsid protein were detected at 8 h p.i. and may represent protein from input virions. The early-late pp65 and gB proteins are expressed at early times but accumulate to higher levels at late times after DNA replication (i.e., 72 h p.i.) (10, 23, 39). The results in Fig. 2 show that this increase was blocked by both LBV and GCV, indicating a lack of late gene expression. The true-late protein, pp28 and the major capsid protein, are strictly synthesized only after viral DNA replication (31, 41). The levels of these proteins were dramatically reduced in the presence of LBV or GCV relative to the control. These results suggest that LBV, like GCV, blocks HCMV replication prior to late gene expression.
LBV prevents expression of late but not early gene transcripts. A decrease in viral DNA synthesis or late protein expression could arise as a consequence of inhibition at earlier stages of infection or by cytotoxicity, as evidenced by an additional lack of IE and early viral gene expression. Therefore, viral gene expression that occurs prior to and after HCMV DNA replication was examined. Steady-state levels of representative viral RNAs for the IE (2.2-kb IE2 and 1.9-kb UL37x1), early (1.35-kb UL38), and true-late (1.5-kb IE2) transcripts were analyzed by Northern blot hybridization of total cell RNA. The temporal classes with which these transcripts are expressed have been well established (47, 51). The results (Fig. 3) show that the IE and early transcripts are expressed in LBV- or GCV-treated cells. However, expression of the true-late 1.5-kb IE2 transcript, whose expression is dependent upon viral DNA synthesis, is markedly reduced in both LBV- and GCV-treated cells. Therefore LBV and GCV halt virus replication between early and late gene expression, temporally coincident with DNA synthesis. Furthermore, expression of IE and early genes in LBV-treated cells argues against a cytotoxic mechanism of virus inhibition.

LBV is phosphorylated in mock- and HCMV-infected cells. The presence of intracellular phosphorylated LBV was examined to confirm that LBV-TP was indeed available to mediate the observed inhibition. Previously phosphorylated LBV was barely detectable, but not quantifiable, in infected cells but not in uninfected cells (56). Therefore, to determine the effect of HCMV infection on intracellular LBV phosphorylation, [3H]LBV with higher specific activity was used. Mock- and HCMV-infected HFF cells were labeled with [3H]LBV from 24 to 72 h.p.i., and the levels of phosphorylated LBV were determined. Phosphorylated LBV was found in both mock-infected and HCMV-infected cells (Fig. 4A and B, respectively). The elution of phosphorylated LBV anabolites was identical to that of phosphorylated LBV synthesized in vitro (data not shown). Nonphosphorylated [3H]LBV eluted from the HPLC column at 5.2 min, the diphosphate eluted at 17.9 min, and the triphosphate eluted at 21.9 min. The monophosphate was not present at detectable levels in mock-infected cells. In HCMV-infected cells (Fig. 4B), however, the presence of an additional very minor peak at 11.7 min indicated detectable levels of monophosphate. The total level of phosphorylated LBV was only modestly increased by HCMV infection (Fig. 4C). Nevertheless, an increased level of LBV-TP was always seen in infected cells (56). Viral factors, therefore, including the UL97 protein kinase, may be responsible for the small increase in LBV phosphorylation during infection.

LBV is a potent inhibitor of the HCMV DNA polymerase. LBV-TP was synthesized in vitro with HSV-1 TK, and HCMV DNA polymerase was partially purified from HCMV-infected cells, as described in Materials and Methods. The results of kinetic analyses presented in Fig. 5 show that LBV-TP proved to be a potent, competitive inhibitor of the HCMV DNA polymerase, with a \( K_i \) of approximately 5 nM. This potency is on the order of that of acyclovir triphosphate (29, 46, 54) and is similar to the potency of LBV-TP against HSV DNA polymerase (13, 53). Furthermore, LBV-TP is a more potent inhibitor of the HCMV DNA polymerase than of various cellular DNA polymerases: the \( K_i \) for HeLa polymerase alpha is reported to be 0.22 to 14 \( \mu \)M (13, 16, 21, 53); the \( K_i \) for polymerase gamma is 54 to 330 \( \mu \)M (16, 21); and polymerase beta is not inhibited at any concentration tested (16, 21). By using a mammalian cell volume of \( 10^{-9} \) ml (11), the concentration of LBV-TP in HCMV-infected cells was found to be 0.9 \( \mu \)M, far greater than the \( K_i \) value. Thus, LBV-TP, present in infected cells, is a potent inhibitor of the HCMV DNA polymerase.
cells, halts HCMV DNA synthesis by the viral DNA polymerase.

**LBV is active against GCV-resistant HCMV.** Prolonged treatment of HCMV disease in immunodeficient patients with GCV results in the development of drug-resistant virus (reviewed in references 1 and 9). The most common resistant isolates are deficient in GCV phosphorylation due to mutations in the UL97 kinase gene (reviewed in references 1 and 9). To determine if LBV may be effective in the treatment of HCMV disease caused by GCV-resistant virus, we measured the efficacy of LBV against GCV-resistant HCMV (12) deficient in GCV phosphorylation (46) due to mutations in the UL97 gene (7). The results in Table 1 show that these isolates are sensitive to LBV and are similar to previous findings with cyclobutyl-adenine (55). Therefore, changes in the UL97 kinase that result in GCV resistance do not alter sensitivity to LBV.

**DISCUSSION**

Through a series of experiments, we have demonstrated that inhibition of viral DNA synthesis is the mechanism by which LBV inhibits HCMV replication. LBV-TP is the likely direct mediator of this inhibition; it was a potent and competitive inhibitor of the viral DNA polymerase with respect to dGTP in vitro and was present intracellularly at concentrations far greater than its $K_i$. Inhibition of the HCMV DNA polymerase by LBV resulted in a lack of viral DNA synthesis and late gene expression, but not a lack of IE and early gene expression. Although the formal possibility exists that LBV inhibits other aspects of HCMV replication, the most likely mechanism of action is potent inhibition of the viral DNA polymerase.

The activity of LBV in cell culture seems to reflect the levels of intracellular triphosphate formation: The 50% effective concentration ($EC_{50}$) for HSV-1 is 50-fold greater than that for HCMV (0.06 versus 3 $\mu$M), and this is reflected in 100-fold-

**FIG. 3.** LBV inhibits the accumulation of late but not early viral transcripts. Total cellular RNA was isolated from treated, infected cells at various times (in hours) after infection or from mock-infected cells (M), as indicated above each lane. Northern blots were performed as described in Materials and Methods. The IE2 probe (50) (A) detects the 2.2-kb IE and the 1.5-kb true-late IE2 transcripts (47); the UL38 probe (B) detects the 1.9-kb IE UL37x1 transcript and the 1.35-kb early UL38 transcript (51).

**FIG. 4.** LBV is phosphorylated in mock- and HCMV-infected cells. HCMV-infected or mock-infected HFF cells were labeled with [3H]LBV from 24 to 72 h p.i. Extracts were prepared and analyzed by ion-pairing reverse-phase HPLC with a Vydac C18 column as described in Materials and Methods. LBV and phosphorylated LBV were detected by in-line scintillation counting, and the data was expressed as picomoles per 10^6 cells. Shown are the radioactivity levels of fractions from HPLC with mock-infected cells (A) and HCMV-infected cells (B). Tabulated data from panels A and B is presented in panel C. nd, not detected.

**FIG. 5.** LBV-TP competitively inhibits HCMV DNA polymerase activity with respect to dGTP. HCMV DNA polymerase activity was measured after a 30-min incubation in the presence of various concentrations of dGTP and LBV-TP, 5 $\mu$M dATP and dCTP, and 5.25 $\mu$M dTTP (specific activity of dTTP = 5 Ci/mmol). LBV-TP was used at 0 nM (squares), 6.25 nM (diamonds), 12.5 nM (circles), and 25 nM (triangles). The data was fit to the equation $y = V \cdot S/[K_m(1+I/K_i)+S]$. $V = \text{velocity in cpm} \times 10^{-14}/\text{min}$; $I = \text{nM LBV-TP}$; $S = \mu$M dGTP. The $K_m$ for dGTP was 0.21 ± 0.02 $\mu$M, and the $K_i$ for LBV-TP was 5.4 ± 0.6 nM.
greater levels of triphosphate (110 versus 0.9 pmol/10^6 cells) (reference 56 and this study). Interestingly, the potencies of LBV and GCV against HCMV in cell culture are similar (13, 56). A comparison of the LBV-TP K_i with that reported for GCV-TP reveals an approximately 2-log-unit more potent inhibition with LBV-TP (46). This apparent discrepancy is perhaps due to more efficient phosphorylation of GCV than LBV in HCMV-infected cells (56).

A significant finding of this report is that levels of intracellular phosphorylated LBV were changed very little by HCMV infection. While GCV phosphorylation increases 50- to 100-fold upon wild-type HCMV infection (2, 5, 56), the phosphorylation of LBV increases only modestly (less than 3-fold) (this study). While the phosphorylation of GCV in HCMV-infected cells is largely dependent upon the UL97 protein, our results suggest that LBV phosphorylation in HCMV-infected cells is relatively independent of viral factors, including the UL97 protein. Further evidence that LBV phosphorylation is not mediated by HCMV UL97 or other HCMV-specific factors comes from its broad spectrum of activity (4, 8, 13, 17, 19, 20, 32, 33, 36, 37, 44, 56, 58). The potency and levels of phosphorylation in HBV-infected 2.2.15 cells resemble those in HCMV-infected cells (EC_{50} = 2.5 μM [ref. 20]; LBV-TP level, approximately 0.25 pmol/10^6 cells [ref. 57]). Low levels of LBV-TP similar to those in uninfected or HCMV-infected HFF cells are also produced in HepG2 and CV-1 cells (57). Moreover, LBV activity against HBVs (8, 19, 20) and HIV (17), viruses devoid of known nucleoside kinases, suggests that a cellular kinase initiates the phosphorylation pathway to the triphosphate. The possibility exists, however, that the modest increase of phosphorylated LBV in HCMV-infected versus mock-infected cells results from an induction of a viral or cellular activity by virus infection.

The HCMV UL97 protein facilitates the intracellular phosphorylation of GCV in many cell types (18, 34). Cell-free phosphorylation of GCV, however, has only been reported from a bacterial extract containing a truncated, catalytic domain of the protein (25). One of many possible explanations discussed by Sullivan et al. (48) and others (18, 35) for the inability to demonstrate direct drug phosphorylation with purified enzyme, is the involvement of a cellular intermediary. Although both GCV and LBV serve as excellent substrates for the herpesvirus TK (13, 22, 24, 53, 56), the finding that HCMV infection and UL97 mutation have little effect on the efficacy and phosphorylation of LBV suggests that different cellular enzymes (intermediaries) could potentially convert the drugs to the monophosphate form. Although our results suggest that factors in uninfected cells phosphorylate LBV, the VZV UL97 homolog was able to render COS-1 cells somewhat more susceptible to LBV toxicity (24). Perhaps the VZV UL97 protein homolog phosphorylates LBV to a greater extent than do factors present in HCMV infection.

GCV-induced mutations in the UL97 gene most frequently do not result in cross-resistance to other antivirals with the exception of acyclovir (26, 46; reviewed in references 1 and 9). However, GCV-induced mutations within the viral DNA polymerase can result in cross-resistance to other nucleoside analogs (27, 49; reviewed in references 1 and 9). It will be of interest to determine the cross-resistance of such isolates to LBV. Interestingly, a study examining the cross-resistance of drug-resistant isolates of murine cytomegalovirus found that viruses resistant to cidofovir and foscarnet, as well as GCV, are not cross-resistant to LBV (44). The converse approach would be to determine the resistance profile of LBV-resistant HCMV. We have attempted to generate such virus by serial passage in increasing concentrations of LBV. After 19 passages to drug levels 7 to 15 times the EC_{90} for plaque reduction, plaque-purified viruses showed only a two- to fourfold increase in LBV EC_{90}. Phenotypic and genotypic analyses of these viruses will further our understanding of the potential utility of LBV in treating drug-resistant HCMV.

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