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Ribavirin and mycophenolic acid potentiate the activity of guanine- and diaminopurine-based nucleoside analogues against hepatitis B virus

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Abstract

Mycophenolic acid [the active metabolite of the immunosuppressive agent mycophenolate mofetil (MMF)] and ribavirin were found to potentiate the anti-HBV activity of the guanine-based nucleoside analogues penciclovir (PCV), lobucavir (LBV) and 3′-fluorodideoxyguanosine (FLG) and diaminopurine dioxolane (DAPD). Ribavirin and mycophenolic acid are both inhibitors of inosine 5′-monophosphate dehydrogenase and cause a depletion of intracellular dGTP levels. It may be assumed that the 5′-triphosphorylated derivatives of the guanine-based nucleoside analogues, in the presence of reduced levels of dGTP, inhibit more efficiently the priming reaction as well as the reverse transcription and DNA-dependent DNA polymerase activity of the HBV polymerase. This assumption is corroborated by the observation that exogenously added guanosine reversed the potentiating effect of ribavirin and mycophenolic acid on the anti-HBV activity of the guanosine analogues. Our observations may have implications for those (liver) transplant recipients that receive MMF as (part of their) immunosuppressive regimen and that, because of de novo or persistent infection with HBV, need specific anti-HBV therapy.

Keywords: HBV; Ribavirin; Mycophenolic acid; Transplantation

1. Introduction

We have previously reported that mycophenolic acid (MPA), the active metabolite of the immunosuppressive agent mycophenolate mofetil (MMF) and ribavirin markedly potentiate, both in cell culture and in animal models, the antiherpes virus activity of guanine-based nucleoside analogues [such as acyclovir (ACV), penciclovir (PCV) and ganciclovir (GCV)] (Neyts et al., 1998a,b; Neyts and De Clercq, 1998). MPA and ribavirin are potent inhibitors of inosine 5′-monophosphate dehydrogenase (IMP-DH), the enzyme responsible for the conversion of IMP through xanthine 5′-monophosphate (XMP) to guanosine 5′-monophosphate (GMP). The reason for the

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potentiating effect resides in a depletion of the intracellular dGTP pools by ribavirin and MPA and thus a decreased competition of the 5′-triphosphorylated metabolites of these drugs with dGTP in the DNA polymerization reaction. Organ transplant recipients that receive, as part of their immunosuppressive drug regimen, mycophenolate mofetil, may, because of the immunosuppression, develop opportunistic herpesvirus infections for which they need treatment with an antiherpes drug. MMF, administered concomitantly with ACV, PCV or GCV may thus result in enhanced antiviral activity against those viruses that fall within the activity spectrum of these compounds.

Guanine-based nucleoside analogues that have been, or are being, pursued for the treatment of HBV infections include famciclovir [the oral prodrug form of penciclovir or (9-4-hydroxy-3-hydroxymethylbut-1-yl)guanine], FLG [2′,3′-dideoxy-3′-fluoroguanosine], BMS200475 [1S-(1α,3α,4β)]-2-amino-1,9-dihydro-9-[4-hydroxy-3-(hydroxymethyl)-2-methylenecyclo-pentenyl]-6H-purin-6-one] (entecavir), and LBV [R-(1α,2β,3α)-9-[2,3-bis-(hydroxymethyl)cyclobutyl]guanine] (lobucavir) (Fig. 1). DAPD [(1)-β-D-2,6-diaminopurine dioxalane] is converted intracellularly by adenosine deaminase to the guanine dioxalane (Korba et al., 1996; Lofgren et al., 1996; Innaimo et al., 1997; Schroder et al., 1998; Chen et al.,

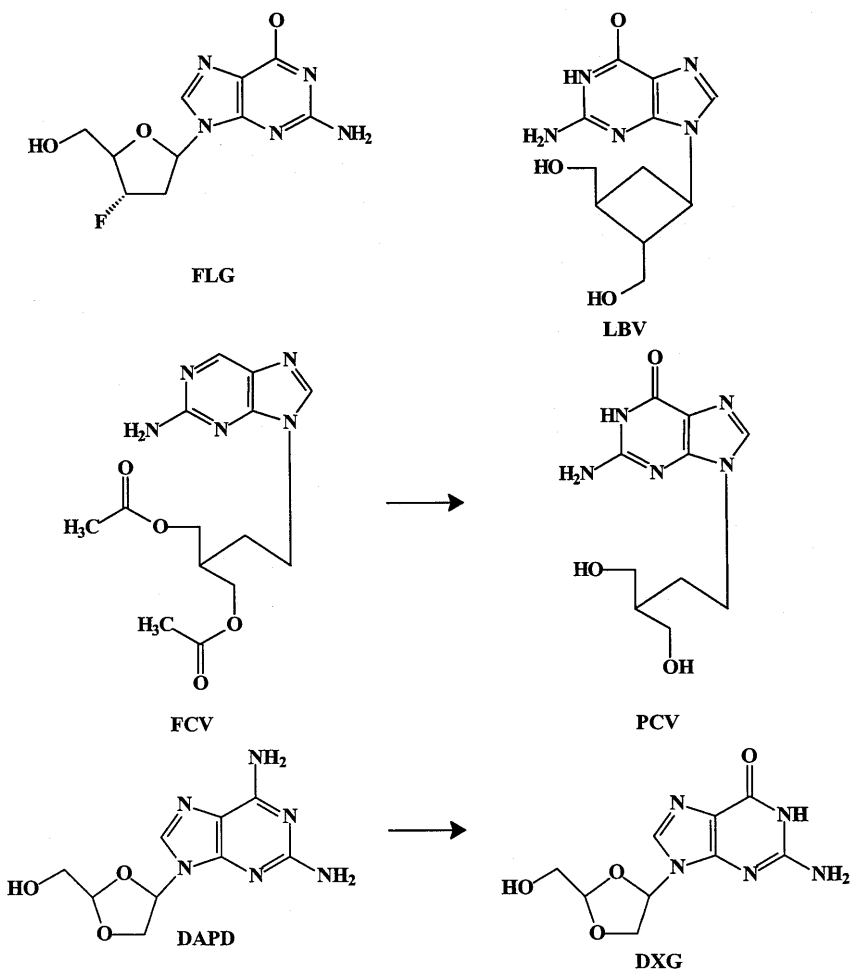

Fig. 1. Structural formulae of FLG, LBV, FCV, PCV, DAPD and DXG.

1999; Furman et al., 2000). After uptake into the cells, all these compounds are phosphorylated to their 5′-triphosphates, which selectively inhibit the viral DNA polymerase or reverse transcriptase (Dannaoui et al., 1997; Seifer et al., 1998; De Clercq, 1999; Yamanaka et al., 1999).

Famciclovir, either alone or in combination with other agents, results in a decrease of HBV DNA levels in patients with chronic HBV infection or in patients with HBV infection following liver transplantation (Rayes et al., 1999). Resistant virus may develop following long-term treatment with famciclovir (Xiong et al., 2000). DAPD, the prodrug of 1-β-D-dioxolane guanine (DXG), elicits anti-HBV activity in vitro (Ying et al., 2000), and in woodchucks experimentally infected with woodchuck hepatitis virus (WHV) (Schinazi et al., 1996). In this model the compound is as effective as lamivudine in reducing circulating viral DNA levels when administered for 12 weeks. FLG has been shown to be a potent inhibitor of HBV replication. In the duck hepatitis B virus (DHBV) model, it strongly suppressed serum DHBV DNA at doses as low as 1 mg/kg per day given twice daily for 7–10 days (Lofgren et al., 1996; Schroder et al., 1998). Lobucavir (LBV) entered phase II/III trials for the treatment of infections with CMV, but clinical studies with this compound were recently suspended (R.J. Colonno, personal communication). BMS-200475 (entecavir) is a carbocyclic 2′-deoxyguanosine analogue which differs from guanosine by replacement of the natural furanose oxygen on the sugar moiety with an exo-methenyl function. The compound has in vitro potency that is superior to that of lamivudine and adefovir against HBV (Yamanaka et al., 1999) and efficiently reduces levels of WHV DNA in chronically infected woodchucks (Genovesi et al., 1998). Entecavir is currently in phase II clinical study for the treatment of HBV infections.

In vitro studies have shown that penciclovir, lobucavir and BMS 200475 exert their anti-HBV activities via inhibition of three distinct phases of hepadnaviral replication: priming, reverse transcription and DNA-dependent DNA synthesis (Shaw et al., 1996; Dannaoui et al., 1997; Seifer et al., 1998; Zoulim, 1999). Unlike other reverse transcriptases, the hepadnavirus DNA polymerase uses a tyrosine residue near its amino terminus as a primer for reverse transcription. Viral DNA is initiated by the formation of a covalent bond between the polymerase and dGMP followed by the addition of 3 or 4 nucleotides (Zoulim and Seeger, 1994). Compounds such as PCV, LBV and BMS-200475 may in their 5′-triphosphate form compete with dGTP in the priming reaction. As a consequence, HBV DNA synthesis will not proceed or will do so inefficiently. The guanine-based nucleoside analogues may be expected to achieve a better anti-HBV efficacy when (i) levels of the competing substrate dGTP are reduced or; (ii) when the formation of 5′-phosphorylated derivative is enhanced. We have now investigated whether a reduction of the intracellular dGTP pools favors the anti-HBV activity of PCV, DAPD, FLG and LBV.

2. Materials and methods

2.1. Compounds

Penciclovir (PCV) was from Roche (Palo Alto, CA), lobucavir (LBV) from Bristol-Myers Squibb (Wallingford, CT), FLG from Medivir (Huddinge, Sweden), DAPD from Triangle Pharmaceuticals (Durham, NC), ribavirin from ICN (Costa Mesa, CA) and MPA was from Sigma (St. Louis, MO).

2.2. Cell and culture conditions

The antiviral activity was determined in the tetracycline-responsive HepAD38 and HepAD79 cells which are stably transfected with either a cDNA copy of the wild-type pregenomic RNA or with cDNA derived from a 3TC-resistant variant containing the M550V mutation in the DNA polymerase (Ladner et al., 1997, 1998). Cells were maintained in DMEM/F12 (50/50) medium supplemented with 10% FCS, 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml kanamycin (P/S/K), 400 μg/ml G418 and 0.3 μg/ml tetracycline. Cells were seeded in 12-well plates at a density of

0.1×10^6 cells/cm^2. After 3 days, when cells had reached confluency, cultures were washed five times with prewarmed PBS, whereupon they were further incubated with tetracycline-free DMEM/F12 medium supplemented with 10% FCS and serial dilutions of (combinations of) the different drugs. Three days after removal of the tetracycline-containing medium, the culture medium was removed and fresh medium, containing the appropiate drug combinations, were added. Total cellular DNA was extracted from the cultures 6 days after the start of the experiment by means of the Qiagen blood kit (Qiagen, Hilten, Germany).

2.3. Quantification of HBV DNA.

DNA was blotted onto a nylon membrane (Hybond-N, Amersham) and UV-cross-linked after which prehybridization was carried out for 1 h at 42°C followed by overnight hybridization at 42°C with 25 ng/ml of a digoxigenin-labelled HBV specific probe. The latter, spanning a 523 bp fragment in the core gene of the HBV genome, was generated in a PCR reaction using the primer pair: (1) 5′ CTG TGG AGT TAC TCT CGT TTT TGC 3′ and (2) 5′ CTA ACA TTG AGA TTC CCG AGA TTG 3′. The PCR reaction contained 5 μl 10 × PCR buffer (Gibco, Paisly, Scotland), 100 μM each of dATP, dGTP, dCTP, 67 μM of dTTP, 33 μM of dig-dUTP (Boehringer Mannheim, Germany), 1μM of each primer and 300 ng of template DNA, as well as 1 μl of Amplitaq DNA polymerase (5U/μl). The following PCR program was used: 1 cycle of 5 min at 94°C, 30 cycles of [30 s at 94°C, 60 s at 57°C and 30 s at 72°C] and 1 cycle of 7 min at 72°C. The fragment generated was gel-purified and stored at −20°C. Following hybridization the membranes were washed two times with 2 × SSC, 0.1% SDS for 10 min at room temperature followed by two washes of 15 min each in 0.1 × SSC, 0.1% SDS at 65°C. After incubation with 1% blocking buffer (Boehringer Mannheim, Germany) for 2 × 15 min, the membranes were incubated with an anti-digoxigenin antibody conjugated with alkaline phosphatase (anti-digoxigenin-AP, Fab fragments) (Boehringer Mannheim, Germany) for 1 h followed by detection of chemiluminescence by means of standard methods. The signal was quantified densitometrically, as described previously (Ying et al., 1999). The assay allows to detect immobilized HBV DNA in the range of 2.5–200 pg ($r = 0.95$) with a detection limit of 1 pg. The amount of DNA measured in the antiviral assays falls within this range (Ying et al., 1999).

3. Results

Ribavirin and mycophenolic acid, when tested at concentrations of respectively 50 and 5 μg/ml, had little, or no, effect on HBV replication in Hep AD38 cells. We choose 50 μg/ml ribavirin and 5 μg/ml of MPA as the highest concentration used, based on previous experiments with herpes simplex virus and human cytomegalovirus (Neyts et al., 1998a,b). Levels of HBV DNA in cells that had been treated with either ribavirin at 50 or 10 μg/ml were respectively 85 ± 12% and 95 ± 7% of the untreated controls. In cells that had been treated with MPA at either 5 or 1 μg/ml, levels of HBV DNA in Hep AD38 cells were respectively 80 ± 20% and 91 ± 8% of the untreated control.

Both ribavirin and MPA potentiated the anti-HBV activity of FLG and LBV (Table 1). For example at a concentration of 50 μg/ml, ribavirin resulted in a 7- and 20-fold increase in the antiviral potency of LBV (5 μg/ml) and FLG (5 μg/ml), respectively. Similarly, at 5 μg/ml, MPA afforded a 11- and 5-fold increase in the anti-HBV activity of LBV and FLG, respectively. Both ribavirin and MPA also potentiated the anti-HBV activity of the diaminopurine derivative DAPD, a compound which is converted intracellularly to dioxolane guanine. Although the enhancing effects of MPA and ribavirin on the anti-HBV activity of FLG, LBV and DAPD are quasi comparable, MPA tends to be a little stronger than ribavirin in its potentiating effect on the anti-HBV activity of PCV. We have as yet no explanation for this observation.

There have been conflicting reports on the anti-HBV activity of penciclovir. Some authors reported strong activity, whereas others observed only moderate to weak anti-HBV activity (De

Table 1
Effect of MPA or ribavirin on the anti-HBV activity of guanine-based nucleoside analogues in HepAD38 cells

Compound (μg/ml)		Percentage of untreated control[a,b]				
		As such	With ribavirin at 50 μg/ml	With ribavirin at 10 μg/ml	With MPA at 5 μg/ml	With MPA at 1 μg/ml
FLG	5	20 ± 5	1 ± 1**	6 ± 1*	4 ± 2**	6 ± 2*
	1	29 ± 9.2	7 ± 3***	29 ± 7^{NS}	16 ± 4*	20 ± 4^{NS}
	0.2	68 ± 11	15 ± 13*	38 ± 7*	58 ± 4^{NS}	40 ± 6^{NS}
LBV	5	23 ± 9	3 ± 2*	6 ± 4*	2 ± 1*	5 ± 4*
	1	40 ± 3	9 ± 1**	18 ± 3*	4 ± 2***	7 ± 3**
	0.2	62 ± 8	16 ± 3**	40 ± 5^{NS}	6 ± 3***	8 ± 5***
DAPD	25	44 ± 4	13 ± 3**	29 ± 5*	24 ± 5*	35 ± 3*
	5	74 ± 5	29 ± 5***	39 ± 7*	46 ± 3**	62 ± 5*
	1	100 ± 8	52 ± 6**	88 ± 9^{NS}	69 ± 4*	86 ± 7^{NS}
PCV	25	95 ± 6	49 ± 4***	59 ± 5**	29 ± 4***	33 ± 6**
	5	101 ± 4.6	60 ± 7*	73 ± 7*	44 ± 8*	62 ± 8*
	1	105 ± 6.2	82 ± 11^{NS}	90 ± 12^{NS}	64 ± 5**	81 ± 12^{NS}

[a] Levels of HBV DNA in Hep AD38 cells that had been treated with MPA alone at 5 μg/ml were 80 ± 20%; with MPA alone at 1 μg/ml, 91 ± 8%; with ribavirin alone at 50 μg/ml, 85 ± 12% and with ribavirin alone at 10 μg/ml, 95 ± 7%.
[b] Data are mean values ± SD for 3–4 independent experiments. Statistical significance was calculated by means of the two tailed Student's *t*-test. *$P<0.05$; **$P<0.01$; ***$P<0.005$; NS, not significant ($P>0.05$).

Clercq, 1999). In Hep AD38, we observed little, if any anti-HBV activity of PCV. However, when combined with either ribavirin or MPA, the anti-HBV activity of PCV became somewhat more prominent. For example, HBV DNA synthesis in HepAD38 cells was inhibited for 71% by the combination of 25 μg/ml PCV with 5 μg/ml MPA (Table 1).

We also verified the potentiating effect of ribavirin and mycophenolic acid with a lamivudine drug-resistant HBV variant. To this end, Hep AD79 cells were employed that produce the DNA polymerase M550V (notation M539V in the manuscript) variant of HBV (Ladner et al., 1998). In these experiments, lobucavir, which together with FLG proved to be the most sensitive to the potentiating effect of MPA and ribavirin, was used. As can be derived from Fig. 2, the anti-HBV activity of LBV in HepAD79 was markedly potentiated by both ribavirin and MPA, whereas both IMP-DH inhibitors as such had little or no inhibitory effect on viral DNA synthesis in this cell line.

To assess whether the depletion of intracellular dGTP pools was indeed responsible for the observed potentiating effect of ribavirin and MPA on the anti-HBV activity of the nucleoside analogues studied here, guanosine was added exogenously (at 100 μg/ml) to the cultures that had been treated with either the LBV + MPA combination or LBV + ribavirin combination. As can be inferred from Table 2, guanosine reversed the potentiating effect of ribavirin and MPA on the anti-HBV activity of lobucavir.

4. Discussion

Here we report that mycophenolic acid, the active metabolite of the immunosuppressive agent mycophenolate mofetil and ribavirin, at concentrations that have as such little or no effect on HBV replication, potentiate the in vitro anti-HBV (both wild-type virus and a lamivudine-resistant variant) activity of the guanine-based nucleoside analogues FLG, lobucavir and penciclovir and of DAPD, a diaminopurine derivative. PCV, LBV

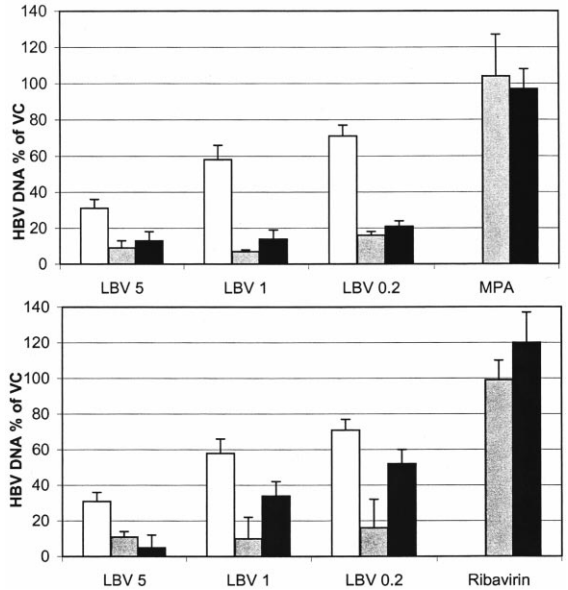

Fig. 2. Potentiating effect of MPA (upper panel) or ribavirin (lower panel) on the anti-HBV activity of LBV in HepAD79 cells which carry cDNA from a lamivudine-resistant HBV variant. LBV was used at 5, 1 and 0.2 μg/ml; MPA at 5 μg/ml (gray columns) or 1 μg/ml (black columns); and ribavirin at 50 μg/ml (grey columns) or 10 μg/ml (black columns). White columns: LBV without MPA or ribavirin.

and FLG are phoshorylated intracellularly to their respective 5′-triphosphate metabolites which then selectively inhibit the viral reverse transcriptase, including the short primer generation and the DNA polymerase function. The diaminopurine dioxolane DAPD is first converted to guanine dioxolane which is then further converted to its 5′-triphosphate derivative. A reduction in the levels of the competing natural substrate dGTP may be held responsible for the improved anti-HBV activity as noted here. This is corroborated by our observation that exogenously added guanosine reversed the potentiating effect of ribavirin and mycophenolic acid on the anti-HBV activity of these nucleoside analogues.

Table 2
Effect of exogenously added guanosine on the potentiating effect of MPA on the anti-HBV activity of LBV in HepAD38 cells

Condition	% HBV DNA[a]
LBV 5 μg/ml	21 ± 3
LBV 5 μg/ml + MPA 5 μg/ml	4.5 ± 3.5
LBV 5 μg/ml + MPA 5 μg/ml + guanosine 100 μg/ml	38 ± 20
LBV 5 μg/ml + ribavirin 50 μg/ml	4.7 ± 2.0
LBV 5 μg/ml + ribavirin 50 μg/ml + guanosine 100 μg/ml	12 ± 3
Guanosine 100 μg/ml	≥100

[a] Data are mean values ± standard deviation for two separate experiments.

We have previously reported that both ribavirin and mycophenolic acid markedly potentiate the anti-herpesvirus activity of acyclovir, ganciclovir, penciclovir, lobucavir and H2G [(R)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine] (Neyts et al., 1998a,b; Neyts and De Clercq, 1999). The effect observed in vitro also pertained to the in vivo situation in murine models for herpesvirus infections. Indeed, the combination of a subactive dose of acyclovir with MMF completely protected mice against virus-induced morbidity and mortality associated with intracutaneous HSV-1 infections, whereas neither compound used singly had a protective effect (Neyts et al., 1998a; Neyts and De Clercq, 1998).

Our present findings may have important clinical implications. Organ (i.e. liver) transplant recipients that do receive, as (part of) their immunosuppressive drug regimen, mycophenolate mofetil and that develop either de novo or persistent infection with HBV will need specific anti-HBV therapy. If these patients are being treated with guanine-based nucleoside analogues (such as penciclovir, entecavir, DAPD or FLG) as anti-HBV therapy, the concomitant administration of MMF may result in an increased antiviral efficacy. The concentrations required for mycophenolic acid to potentiate the antiviral activities of the drugs studied here is not more than 1 μg/ml, that is a concentration that can be easily attained in human plasma upon oral dosing of 1.5–3 g of MMF (Bullingham et al., 1996). One could also envisage the combined use of ribavirin with guanine-based anti-HBV nucleoside analogues. The use of ribavirin (in combination with interferon-α) has been well documented for the treatment of HCV infection (Poynard et al., 1998). Ribavirin

appears to have a beneficial effect on the liver function, and, when combined with interferon, results in improved antiviral efficacy. Because of (i) the potentiating effect of ribavirin on the anti-HBV activity of guanine-based nucleoside analogues as observed here; and (ii) the reported beneficial effect of ribavirin on the liver function observed in patients with chronic HCV hepatitis, combination therapy with ribavirin and guanine-based anti-HBV drugs may be an option to be further considered for the treatment of HBV infections.

In conclusion, we report that mycophenolic acid and ribavirin, both inhibitors of IMP-DH, potentiate the anti-HBV activity of guanine-based nucleoside analogues. Our findings may have implications for transplant recipients that would receive mycophenolate mofetil as (part of their) immunosuppressive therapy regimen together with guanine-based nucleoside analogues for the treatment of de novo or persistent HBV infections. Furthermore, the combined use of guanine-based nucleoside analogues and ribavirin could also be considered for the treatment of HBV infections at large.

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