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Ribavirin and mycophenolic acid markedly potentiate the anti-hepatitis B virus activity of entecavir

Chunxiao Ying [a], Richard Colonno [b], Erik De Clercq [a], Johan Neyts [a,*]

[a] *Rega Institute for Medical Research, Faculty of Medicine, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium*
[b] *Bristol-Myers Squibb Pharmaceutical Institute, Wallingford, CT, USA*

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Abstract

MPA [the active metabolite of the immuno-suppressive agent CellCept] and ribavirin markedly potentiate the anti-HBV activity of the guanine-based nucleoside analogue entecavir (ETV) against both wild-type HBV and a lamivudine-resistant variant. Ribavirin (in its 5′-monophosphate form) and MPA are inhibitors of IMP-dehydrogenase and cause depletion of intracellular dGTP pools. The active triphosphorylated form of ETV may inhibit more efficiently the priming reaction, reverse transcription and DNA-dependent DNA polymerase activity of the HBV polymerase in the presence of reduced levels of dGTP. The potential for enhanced ETV activity is supported by the observation that exogenously added deoxyguanosine reversed the potentiating effect of ribavirin and MPA. Our observations may have important implications for those (liver) transplant recipients that receive MMF as part of their immunosuppressive regimen and who, because of a *de novo* or a persistent infection with HBV need antiviral therapy such as ETV. Further studies will need to be conducted to determine if combining ribavirin (a compound used for the treatment of HCV infections) with ETV could have an advantage for the treatment of HBV infections, in particular in patients co-infected with HCV.

Keywords: HBV; Entecavir; CellCept; Ribavirin

1. Introduction

We reported previously that ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) and mycophenolic acid [MPA, 6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-methyl-4-hexenoic acid], the active metabolite of the immuno-suppressive agent mycophenolate mofetil (MMF) (Fig. 1) potentiate the anti-HBV activity of guanine- and diamino-purine-based nucleoside analogues, such as FLG (2′,3′-dideoxy-3′-fluoroganosine), lobucavir (LBV) [*R*-(1α,2β,3α)-9-[2,3-bis-(hydroxymethyl)cyclobutyl]guanine], penciclovir (PCV) [(9-4-hydroxy-3-hydroxymethylbut-1-yl)guanine] and DAPD [(1)-β-D-2,6-diaminopurine dioxalane] (Ying et al., 2000). MPA and ribavirin are potent inhibitors of inosine 5′-monophosphate dehydrogenase (IMP-DH), the enzyme responsible for the conversion of IMP through xanthosine 5′-monophosphate (XMP) to guanosine 5′-monophosphate (GMP). The reason for the 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.

Entecavir (ETV) [1*S*-(1α,3α,4β)]-2-amino-1,9-dihydro-9-[4-hydroxy-3-(hydroxymethyl)-2-methenylcyclo-pentyl]-6*H*-purin-6-one] is a carbocyclic 2′-deoxyguanosine analogue which differs from guanosine by replacement of the natural furanose oxygen on the cyclopentyl moiety with an exo methenyl function (Fig. 1). The compound has *in vitro* potency that is superior to that of lamivudine and adefovir against HBV (Yamanaka et al., 1999; Billich, 2001) and efficiently reduces levels of woodchuck hepatitis virus (WHV) DNA in chronically infected woodchucks (Colonno et al., 2001), infected ducklings (Marion et al., 2002; Foster et al., 2005) and transgenic mice (Julander et al., 2003). In addition, ETV is active against the YMDD lamivudine-resistant HBV variant (Levine et al., 2002). ETV has been approved in the US for the treatment of chronic HBV infections. The efficacy and safety of ETV for the treatment of chronic Hepatitis B has been established in three multinational phase III trials. In two trials in nucleoside-naïve HBeAg-positive and HBeAg-negative patients, ETV 0.5 mg demonstrated superiority to lamivudine

* Corresponding author. Tel.: +32 16 337341; fax: +32 16 337340.
E-mail address: Johan.Neyts@rega.kuleuven.be (J. Neyts).

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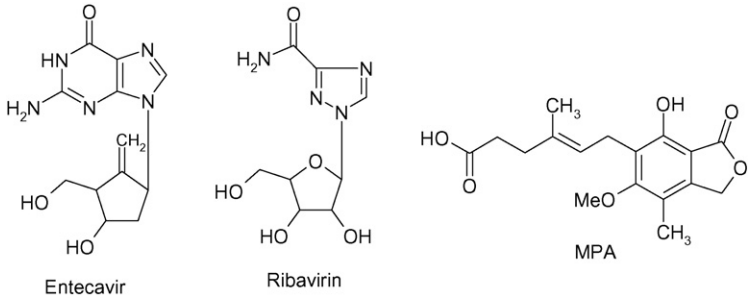

Fig. 1. Chemical structures of entecavir (ETV), ribavirin and mycophenolic acid (MPA).

for histological, virological, and biochemical endpoints over 48 weeks of treatment (Chang et al., 2004; Shouval et al., 2004). Entecavir treatment resulted in the reduction of HBV DNA levels to <300 copies/ml in 67% of HBeAg-positive patients and in 90% of HBeAg-negative patients. In lamivudine-refractory patients, 48 weeks of treatment with ETV 1.0 mg also resulted in superior histologic, virologic, and biochemical improvement compared to lamivudine: co-primary endpoints of histologic improvement and HBV DNA < 0.7 Mequiv./ml by bDNA assay with alanine aminotransferase <1.25 times the upper limit of normal were both achieved by 55% of patients on ETV (compared with 28% and 4%, respectively, of patients on continued lamivudine) (Sherman et al., 2004). In all three trials the safety profile of ETV was comparable to that of lamivudine. Resistance to ETV does not readily develop. At least three different concomitant mutations in the HBV-RT are required to achieve clinically relevant levels of ETV resistance (Tenney et al., 2004). Entecavir is considered as the most potent anti-HBV agent under development today (Shaw and Locarnini, 2004).

In vitro studies have shown that penciclovir (PCV), lobucavir (LBV) and ETV 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 synthesis is initiated by the formation of a covalent bond between the polymerase and dGTP followed by the addition of three or four nucleotides (Zoulim and Seeger, 1994). Compounds such as PCV, LBV and ETV may compete with dGTP in their fully phosphorylated form in the priming reaction, and 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 levels of the competing substrate dGTP are reduced. Here, we investigated whether MPA and ribavirin, two drugs that are being used in the clinical setting, may potentiate the anti-HBV activity of entecavir.

2. Materials and methods

2.1. Antiviral assay

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 rtM204 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, 400 μg/ml G418 (Invitrogen) and 0.3 μg/ml tetracycline (Sigma). Cells were seeded in 48-well plates at a density of 5×10^5 per well. After 2–3 days the cultures were induced for viral production by washing with prewarmed PBS and feeding with 200 μl of assay medium (medium without tetracycline and G418) with or without the antiviral compounds. Medium was replaced after 3 days.

2.2. Quantitative HBV PCR

The antiviral effect was quantified by measuring levels of viral DNA in the culture supernatant at day 6 post-induction, by a real time quantitative PCR (Q-PCR). The Q-PCR was performed with 3 μl of culture supernatant in a reaction volume of 25 μl using the TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ, USA) with forward primer 5′-CCG TCT GTG CCT TCT CAT CTG-3′ (final concentration 600 nM), reversed primer 5′-AGT CCA AGA GTY CTC TTA TRY AAG ACC TT-3′ (final concentration 600 nM), and Taqman probe 6-FAM-CCG TGT GCA CTT CGC TTC ACC TCT GC-TAMRA (final concentration 150 nM). The reaction was analyzed using a SDS 7000 (Applied Biosystems, Foster City, CA). A plasmid containing the full length insert of the HBV genome was used to prepare the standard curve. The amount of viral DNA produced in treated cultures was expressed as a percentage of the amount in mock-treated samples.

2.3. Cytotoxic and cytostatic assay

The effect of drug on exponentially growing HepG2 cells was evaluated. Briefly, cells were seeded at a density of 5000 cells/well (96-well plates). Six hours later serial dilutions of the (combinations of) compounds were added and cells were allowed to proliferate for 3 days in the absence or presence of compounds, after which time cell density was determined by means of the MTS method (Promega).

3. Results and discussion

The effect of combining ribavirin or MPA with ETV was evaluated over a range of exposures against both WT and lamivudine

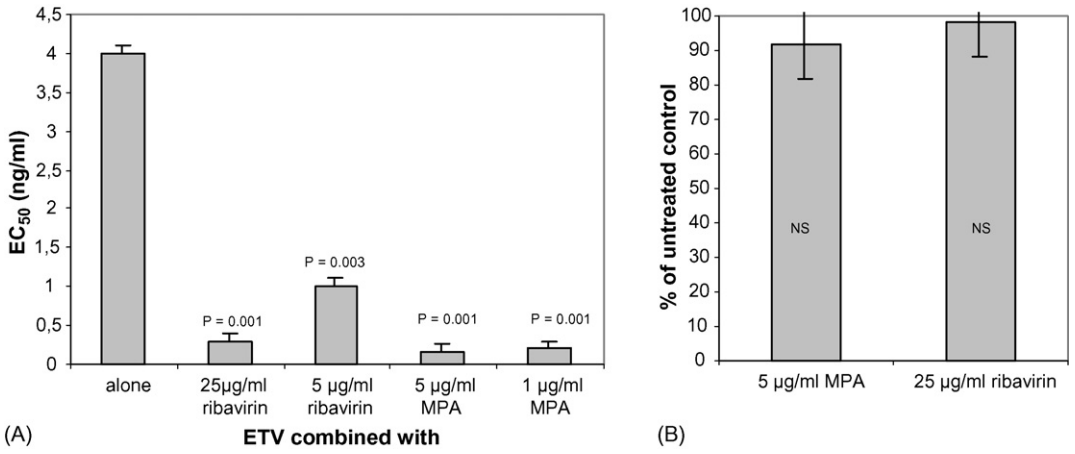

Fig. 2. (Panel A) Activity of ETV either in combination with MPA or ribavirin on the replication of a 3TC-resistant HBV variant (in HepAD79 cells). Data are mean ± S.D. for at least three independent experiments. (Panel B) MPA and ribavirin alone have no inhibitory effect on the replication of a 3TC-resistant HBV variant (in HepAD79 cells). Data are mean ± S.D. for at least three independent experiments.

Table 1
Activity of entecavir in combination with either ribavirin or MPA on HBV (wild-type) replication in HepAD38 cells

Conditions	EC_{50} (ng/ml)	Average fold increase
ETV alone	3 ± 1	
ETV + ribavirin 25 μg/ml	0.4 ± 0.1*	7.5
ETV + ribavirin 5 μg/ml	2 ± 1NS	1.5
ETV + MPA 5 μg/ml	0.1 ± 0.02*	30
ETV + MPA 1 μg/ml	0.2 ± 0.1*	15

At the concentrations used, ribavirin nor MPA alone caused any significant inhibitory effect on HBV replication. Data are mean values ± S.D. for at least three independent experiments. NS: not significant.
* $p < 0.05$.

resistant viruses. Both ribavirin (5 μg/ml) and MPA (1 μg/ml) markedly potentiated the anti-HBV activity of ETV against wild-type (WT) virus and against a lamivudine-resistant HBV variant (Table 1, Fig. 2). The 50% effective concentration (EC_{50}) of ETV against the WT virus was 3 ng/ml. When combined with ribavirin at 25 μg/ml, the anti-HBV potency of ETV increased 7-fold ($p < 0.001$). When ETV was combined with MPA at 5 μg/ml, a 30–40-fold increase in the antiviral activity of ETV was noted ($p < 0.001$). A marked potentiating effect of MPA and ribavirin (14–16-fold) on the anti-HBV activity of ETV was also observed against the lamivudine-resistant HBV variant (Fig. 2A). At the concentrations used, neither ribavirin nor MPA alone caused any significant inhibition of HBV replication (in either HepAD38 or HepAD79 cells) (Fig. 2B). Levels of HBV DNA in cultures treated with (i) 25 μg/μl ribavirin, (ii) 5 μg/ml ribavirin or (iii) MPA 5 μg/ml or (iv) MPA 1 μg/ml were 96%, 100%, 91% and 95%, respectively.

The effect of the combined treatment of ETV with either ribavirin or MPA on the proliferation of the parent cell line Hep G2 was subsequently studied (Table 2). At the highest concentration tested (0.2 μg/ml), ETV had no effect on cell proliferation. Apart from ribavirin concentrations at 25 μg/ml, other concentrations (i.e. concentrations that potentiate the antiviral activity of ETV) showed no obvious concentration-dependent cytotoxicity, whereas MPA at the highest tested concentration of 5 μg/ml resulted in a 31% decrease in total cell density. Neither the combination ribavirin + ETV nor the combination MPA + ETV resulted in a synergistic inhibitory effect on cell proliferation.

ETV is phosphorylated intracellularly to its 5′-triphosphate metabolite which then selectively inhibits the viral reverse transcriptase, including the short primer generation and the DNA polymerase function (Seifer et al., 1998). A reduction in the intracellular levels of the competing natural substrate dGTP may account for the improved anti-HBV activity noted here. This is supported by the observation that exogenously added deoxyguanosine [and to a lesser extent] also guanosine and gua-

Table 2
Effect of the combination of ETV with either MPA or ribavirin on uninfected HepG2 cells

ETV (μg/ml)	Alone	Cell density (% of untreated control)								
		+MPA				+Ribavirin				
		5 μg/ml	1 μg/ml	0.2 μg/ml	0.04 μg/ml	25 μg/ml	5 μg/ml	1 μg/ml	0.2 μg/ml	0.04 μg/ml
0.2	95 ± 7	68 ± 1	81 ± 4	111 ± 22	94 ± 6	98 ± 3	71 ± 2	79 ± 19	81 ± 10	84 ± 3
0.04	95 ± 5	71 ± 0	89 ± 2	84 ± 1	97 ± 1	102 ± 11	94 ± 5	86 ± 7	83 ± 8	90 ± 6
0.008	99 ± 9	71 ± 6	91 ± 9	101 ± 11	97 ± 8	106 ± 1	72 ± 2	85 ± 0	85 ± 12	94 ± 6
0.0016	95 ± 6	70 ± 4	88 ± 8	100 ± 1	85 ± 10	108 ± 1	73 ± 4	83 ± 1	91 ± 1	91 ± 1
0.00032	96 ± 5	69 ± 4	90 ± 11	97 ± 1	86 ± 17	107 ± 4	87 ± 1	78 ± 2	89 ± 1	98 ± 1
0	100	69 ± 3	87 ± 4	107 ± 11	102 ± 8	111 ± 2	88 ± 13	90 ± 4	95 ± 7	102 ± 4

Data are mean values ± S.D. for two independent experiments.

Table 3
Deoxyguanosine (dGuo) reverses the potentiating effect of MPA and ribavirine on the anti-HBV (wild-type) activity of ETV

Concentration ETV (μg/ml)	% viral DNA in cultures treated with ETV+				
		MPA 5	MPA 5 + dGuo100	Ribavirin 25	Ribavirin 25 + Guo100
1	3	0	4	0	2
0.2	10	0.1	16	0.1	4
0.04	16	–	–	3	8
0.008	27	4	25	8	20
0.0016	63	9	39	22	50
0.00032	91	21	56	55	65

dGuo when used at 100 μg/ml had no significant effect on HBV replication. "MPA 5" means MPA used at 5 μg/ml; "ribavirin 25" means ribavirin used at 25 μg/ml. "dGuo 100" means deoxyguanosine used at 100 μg/ml. At the concentration used, MPA nor ribavirin caused any significant inhibitory effect on HBV replication.

nine (data not shown), reverse the potentiating effect of ribavirin and MPA on the anti-HBV activity of the nucleoside analogue ETV (Table 3). Other nucleosides, i.e. adenosine (at a final concentration of 100 μg/ml) and thymidine (at a final concentration of 20 μg/ml) did not reverse the potentiating effect of MPA or of ribavirin on the anti-HBV activity of ETV (data not shown).

To further demonstrate that the observed potentiating effect is specific for guanine based nucleoside analogues, the anti-HBV activity of the combination of (i) adefovir with either ribavirin or MPA and (ii) lamivudine with either ribavirin or MPA was also investigated. Ribavirin (at 25 and 5 μg/ml) had no stimulatory effect (<2-fold) on the anti-HBV activity of adefovir or lamivudine (data not shown). Similarly, MPA at 5 or 1 μg/ml had minimal to no effect (<2-fold), on the antiviral activity of adefovir and lamivudine (data not shown).

Our findings may have potential clinical implications when ETV is used for the treatment of HBV infections in transplant recipients. Organ (i.e. liver) transplant patients that receive an immunosuppressive drug regimen which contains mycophenolate mofetil (MMF, CellCept®) and who develop either a *de novo* or a persistent infection with HBV, will need specific anti-HBV therapy. If these patients receive ETV as their anti-HBV therapy, the concomitant administration of MMF may result in enhanced antiviral efficacy. The threshold concentration required for MPA-directed potentiation of ETV is ≤1 μg/ml, which can be readily 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 ETV. Ribavirin is being extensively used in patients in combination with pegylated interferon-α for the treatment of chronic HCV infection (Poynard et al., 2003). Combination therapy involving ribavirin and ETV may therefore be an interesting option for the treatment of HBV infections, particularly in patients co-infected with HCV, although the adverse effects of ribavirin need to be considered. Median concentrations of ribavirin in plasma can be 40 μM (∼10 μg/ml) (Homma et al., 1999; Lindahl et al., 2004) which is in the same range as the concentration of ribavirin (25 and 5 μg/ml) that potentiate the anti-HBV activity of ETV. Animal studies suggest that even higher concentrations can be reached (in particular in the liver) with viramidine, a prodrug of ribavirin (Lin et al., 2004).

In conclusion, we report that MPA and ribavirin, two drugs that are being used in the clinical setting, potentiate the anti-HBV activity of ETV *in vitro*. These findings may have implications for transplant recipients who receive ETV for the treatment of *de novo* or persistent HBV infections and CellCept® (MMF) as, part of their, immunosuppressive therapy regimen. Furthermore, in patients co-infected with HBV and HCV, and that are being treated with the combination of (pegylated) interferon and Virazole® (ribavirin); the latter drug may enhance the efficacy of ETV against HBV.

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