In patients coinfected and treated for both HIV-1 and hepatitis C virus (HCV), administration of ribavirin (RBV) may result in altered intracellular drug levels of nucleoside reverse transcriptase inhibitors through inhibition of inosine 5′-monophosphate dehydrogenase. Drug interactions between tenofovir and RBV were studied in vitro in order to provide insights into the safety of co-administration of tenofovir disoproxil fumarate (DF) and RBV in HCV/HIV-1-coinfected patients. In accordance with previous in vitro studies, strongly increased anti-HIV activity was observed when RBV was combined with didanosine (ddI). In contrast, low-level anti-HIV antagonism was observed when RBV was combined with either tenofovir or abacavir. Significantly stronger anti-HIV antagonism was observed when RBV was combined with either zidovudine, stavudine, emtricitabine or lamivudine. Thus, although tenofovir and ddI are both adenosine analogues, their in vitro interactions with RBV are markedly different. These results suggest a low potential for increased toxicity upon co-administration of tenofovir DF with RBV in patients.

**Introduction**

Coinfection with hepatitis C virus (HCV) in HIV-1-infected patients is common. One study found that 16% of HIV-1-infected patients were coinfected with HCV, with as many as 72% of patients coinfected in cohorts of haemophiliacs or intravenous drug users [1]. The primary treatment against HCV is a combination of pegylated interferon-α with ribavirin (RBV) that HCV/HIV-1-coinfected patients receive in addition to their antiretroviral therapy for HIV-1. The exact mode of action of RBV is not fully understood but is thought to be due in part to inhibition of inosine 5′-monophosphate dehydrogenase (IMPDH), which is an enzyme involved in de novo guanine nucleotide synthesis [2]. The US Food and Drug Administration (FDA) issued a warning that increased didanosine (ddI) toxicity has been observed in HCV/HIV-1-coinfected patients receiving both ddI and RBV. Specifically, the US FDA found a significant increase in the risk of developing mitochondrial toxicity in HCV/HIV-1-coinfected patients receiving RBV and ddI concomitantly [3]. This finding was also documented by other groups [4–6], leading to the conclusion that co-administration of RBV and ddI should be avoided due to a high rate of clinically significant toxicity [4]. These findings are in agreement with an earlier study demonstrating increased activity of ddI in the presence of RBV [7] and with data showing that RBV increases the levels of the active metabolite of ddI, dideoxyadenosine triphosphate (ddATP), in vitro [8,9]. Alterations in levels of intracellular active metabolites of anti-HIV drugs can also be measured indirectly through altered anti-HIV activity in vitro. The purpose of this study is to investigate in vitro anti-HIV drug combinations of RBV with nucleotide or nucleoside reverse transcriptase inhibitors (NRTIs) in order to address the potential for intracellular drug interactions of RBV with these agents when co-administered in HCV/HIV-1-coinfected patients.

**Methods**

Drug combinations

Anti-HIV drug combination studies of RBV with either tenofovir (TFV), emtricitabine (FTC), ddI, zidovudine (ZDV), lamivudine (3TC), stavudine (d4T), abacavir (ABC) or nelfinavir (NFV) were performed. TFV and FTC were synthesized at Gilead Sciences (Foster City, CA, USA); RBV, ZDV and ddI were purchased from Sigma (St Louis, MI, USA); 3TC was obtained from Moravek Biochemicals (Brea, CA, USA); ABC was supplied by GlaxoSmithKline (Research Triangle Park, NC, USA); d4T was obtained from Bristol-Myers Squibb (New York, NY, USA); and NFV was obtained from Agouron Pharmaceuticals (San Diego, CA, USA). The cytotoxic effect of RBV in MT-2 cells was measured using an XTT assay at RBV concentrations ranging from 500 nM to 3.2 mM. The

© 2005 International Medical Press 1359-6535
five concentrations of RBV tested in the combinations ranged from 0.75–12 µM and were well below cytotoxic levels. For the anti-HIV compounds, six concentrations of drug were tested, starting at a maximum drug concentration of at least four times the expected effective concentration to inhibit 50% of viral replication (EC\textsubscript{50}) for each specific drug. In all cases, the concentrations tested were well below cytotoxic levels. For each drug combination, the compounds were prepared separately by twofold serial dilution and mixed in 96-well assay plates to create a two-dimensional matrix of diluted drugs. Each combination experiment was done in triplicate and a minimum of three independent experiments were conducted for each combination. A no-drug control was used for all the compounds tested.

Antiviral assay and statistical analyses
The antiviral effects of the drug combinations was determined using an XTT assay in MT-2 cells as described earlier \cite{10,11}. Briefly, 1.2 million MT-2 cells were infected with wild-type HIV-1 (HXB2D) and incubated in the assay plates for 5 days, starting at an initial concentration of approximately 17 000 cells/well. The antiviral effect of the combinations was measured by determining the HIV-1 cytopathic effect using the vital dye XTT. The data were analysed with the software MacSynergy II according to the method of Prichard \textit{et al.} \cite{12,13}. The software uses the independent effects definition of additive interactions. In this model, the inhibition observed in the drug combination is compared with the predicted theoretical inhibition obtained by simply adding the inhibitory effect of each drug alone. Any deviation from predicted is scored as synergy (positive deviation) or antagonism (negative deviation). The software calculates confidence intervals to assess the statistical significance of the deviations observed. In this study, data were analysed at the 95% confidence level. Synergy volumes were defined by the program as follows: values <25 µM\textsuperscript{2}\% reflect insignificant synergy (neither synergy nor antagonism); values ≥25 to <50 µM\textsuperscript{2}\% indicate minor synergy/antagonism; values ≥50 to <100 µM\textsuperscript{2}\% indicate moderate synergy/antagonism; and values ≥100 µM\textsuperscript{2}\% indicate strong synergy/antagonism. Three-dimensional mesh plots and EC\textsubscript{50} values were generated using SigmaPlot (SPSS, Inc, Chicago, IL, USA). Statistical significance of EC\textsubscript{50} fold-changes in the presence of RBV were calculated in Excel using two-tailed paired \textit{t}-tests.

Results
The cytotoxicity of RBV in MT-2 cells was analysed in four independent experiments. The concentration to inhibit 50% of cell growth (CC\textsubscript{50}) was measured at 70 µM (Figure 1) and was found to be approximately sixfold above the highest concentration of RBV, 12 µM, used in the combination experiments. The start of observed toxicity was found to be >12 µM in all of the CC\textsubscript{50} experiments.

The results for the combinations of RBV with the eight HIV-1 inhibitors are shown in Figure 2, showing a representative experiment for each of the drug combinations analysed. The values in Table 1 are the average synergy and antagonism values from three to five combination experiments and the standard deviations between experiments. RBV on its own did not exhibit any anti-HIV activity when tested at non-cytotoxic concentrations ranging from 0.75–12 µM in over 30 triplicate experiments. However, the presence of RBV had an effect on the anti-HIV activity of all the NRTIs analysed. The combination of RBV with ddI showed strong enhancement of the anti-HIV activity of ddI, while the combinations with either ZDV, d4T, FTC or 3TC showed strong antagonism of the anti-HIV activity of these NRTIs. RBV combined with either TFV or ABC resulted in moderate levels of anti-HIV antagonism. The anti-HIV activity of the protease inhibitor nelfinavir was not affected by RBV.

The effect of RBV on the anti-HIV EC\textsubscript{50} values for the NRTIs was also determined. The observed levels of RBV interaction in the drug combination studies correlated closely with the antiretroviral EC\textsubscript{50} observed for the NRTIs. Fold-changes in the anti-HIV EC\textsubscript{50} values for the antiretroviral drugs in the presence of RBV are shown in Figure 3. Combinations of RBV with ddI
Figure 2. Three-dimensional synergy plots

The level of synergy or antagonism found at the 95% confidence intervals for each of the combinations tested is shown. (A) ddI–RBV combination; (B) NFV–RBV combination; (C) TFV–RBV combination; (D) ABC–RBV combination; (E) 3TC–RBV combination; (F) FTC–RBV combination; (G) ZDV–RBV combination; (H) d4T–RBV combination. The colours indicate planes of 10% of volume of synergy or antagonism and change by increment of 10% with increasing synergy or antagonism.

ABC, abacavir; ddI, didanosine; FTC, emtricitabine; 3TC, lamivudine; NFV, nelfinavir; RBV, ribavirin; d4T, stavudine; TFV, tenofovir; ZDV, zidovudine.
resulted in an increase in ddI activity, while the combinations of RBV with either ZDV, d4T, 3TC, FTC, ABC or TFV resulted in a decrease in anti-HIV activity. At an RBV concentration of 3 µM, the anti-HIV activity of ddI was increased 2.5-fold, while the anti-HIV activity of ABC, TFV, 3TC, FTC, ZDV and d4T was decreased 1.4-, 1.6-, 3.2-, 3.2-, 3.5- and 3.4-fold, respectively, when compared with the no-RBV control. These changes in anti-HIV effect were further enhanced in the presence of 6 µM RBV and 12 µM RBV (Figure 3). For TFV, the EC50 values obtained without RBV or in the presence of either 3 µM, 6 µM or 12 µM RBV were 5.1, 8.0, 8.0 and 9.3 µM, respectively. These concentrations of RBV represent near-physiological plasma concentrations of RBV based on pharmacokinetic studies in patients where a Cmax of 3.9 µM has been reported [14]. The anti-HIV activity of NFV was not modified in the presence of RBV.

**Discussion**

Our findings with regards to cytotoxicity and lack of anti-HIV activity of RBV are in agreement with previous experiments in MT-4 cells [7,15]. In contrast to these findings, Klein et al. [16] found that RBV had some low-level anti-HIV activity described as >50 µM in an assay system using human cord blood mononuclear cells. Thus, the level of activity of RBV against HIV-1 may depend on the cell type used in the experiments and a clear delineation between antiviral effect and cytotoxicity must be made. In our experiments, cytotoxicity could result in the appearance of anti-HIV antagonism. However, the concentrations of RBV employed were below the levels of cytotoxicity.

In this study, the reported increases in ddI-associated toxicities with RBV co-administration were correlated with a strong enhancement of the anti-HIV activity of ddI when combined with RBV in vitro, in agreement with earlier studies [7,17]. In contrast, TFV anti-HIV activity was not enhanced with RBV, but low-level antagonism was observed. Although TFV and ddI are both adenosine analogues, their in vitro interactions with RBV are markedly different. It has been suggested that increased levels of the active metabolite of ddI, ddATP, in the presence of RBV is due to the increased level of inosine monophosphate (IMP) as a consequence of inhibition of IMP.

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>Synergy (µM±%)</th>
<th>Antagonism (µM±%)</th>
<th>Combined effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBV–ddI</td>
<td>359 ±111</td>
<td>4.8 ±7.1</td>
<td>Strong potentiation</td>
</tr>
<tr>
<td>RBV–NFV</td>
<td>6.9 ±2.9</td>
<td>9.0 ±8.2</td>
<td>No effect</td>
</tr>
<tr>
<td>RBV–ABC</td>
<td>3.6 ±5.3</td>
<td>64.1 ±23.0</td>
<td>Moderate antagonism</td>
</tr>
<tr>
<td>RBV–TFV</td>
<td>3.8 ±8.4</td>
<td>84.3 ±39.6</td>
<td>Moderate antagonism</td>
</tr>
<tr>
<td>RBV–3TC</td>
<td>0 ±0</td>
<td>185 ±88.7</td>
<td>Strong antagonism</td>
</tr>
<tr>
<td>RBV–FTC</td>
<td>2.0 ±3.5</td>
<td>246 ±184</td>
<td>Strong antagonism</td>
</tr>
<tr>
<td>RBV–ZDV</td>
<td>0 ±0</td>
<td>339 ±119</td>
<td>Strong antagonism</td>
</tr>
<tr>
<td>RBV–d4T</td>
<td>0.5 ±1.0</td>
<td>455 ±217</td>
<td>Strong antagonism</td>
</tr>
</tbody>
</table>

*Volume of synergy and antagonism were computed by the MacSynergy™ II program using a 95% confidence interval and are defined by the program as follows: values <25 µM% indicate insignificant synergy (additive); values ≥25 to <50 µM% indicate minor synergy/antagonism; values ≥50 to <100 µM% indicate moderate synergy/antagonism; and values ≥100 µM% indicate strong synergy/antagonism. ABC, abacavir; ddI, didanosine; FTC, emtricitabine; 3TC, lamivudine; NFV, nelfinavir; RBV, ribavirin; d4T, stavudine; TFV, tenofovir; ZDV, zidovudine.
dehydrogenase by RBV [8]. As IMP is the major phosphate donor for the initial phosphorylation step in the pathway from ddI to ddATP, this would result in increased ddATP levels that can be observed in vitro through increased anti-HIV activity and in vivo through increased mitochondrial toxicity. TFV, on the other hand, is a monophosphate molecule and therefore does not require initial phosphorylation in the manner of ddl. The results presented here are in support of the differential metabolic pathways for TFV and ddl based on their different initial phosphorylation requirements.

Thus, unlike the case of ddl, the results of this study suggest a very low potential for increased toxicity upon co-administration of TFV with RBV in patients. The result of low-level antagonism of TFV anti-HIV activity with RBV is of interest. Similar low levels of RBV-mediated antagonism were observed for ABC while much higher levels of antagonism were observed for ZDV, d4T, FTC and 3TC. The strong antagonistic effects seen for those four drugs in the presence of RBV correlates with previous reports that have shown that RBV could inhibit the phosphorylation of pyrimidine 2′,3′-dideoxynucleotides such as ZDV, d4T, FTC and 3TC in vitro [15,17–19]. Alternatively, the levels of antagonism observed for the various combinations could be due to differential toxicity between the drugs on their own and when they are in combination with RBV. This hypothesis appears unlikely based on the high level of antagonism observed for otherwise relatively non-toxic drugs such as 3TC and FTC. The mechanistic basis for the observation of low-level antagonism with tenofovir and abacavir is unknown, but may relate to a general perturbation of the intracellular phosphorylation pathways as a result of RBV activity.

To our knowledge, there has been no report of a negative clinical consequence of the antagonistic anti-HIV effect of RBV in combination with ZDV, d4T, FTC or 3TC. Two studies have shown that anti-HIV treatment that included the combination of d4T and 3TC or ZDV and 3TC was not adversely affected in patients receiving RBV as a part of their anti-HCV treatment [20,21]. The concomitant use of interferon with RBV to treat the HCV may also be playing a role in maintaining virological control of HIV. Overall, these clinical findings suggest that the in vitro antiretroviral drug antagonism observed in the combinations with RBV are not clinically significant for the combined treatment of HCV and HIV-1 in HCV/HIV-1 coinfected patients. Moreover, with the exception of ddl, none of the licensed NRTIs tested showed any synergistic activity with RBV that could be associated with increased toxicity in HCV/HIV-1 coinfected patients.

Acknowledgements

The authors wish to thank Adrian Ray for his editorial review and Margaret Benton and Susan Edl for technical assistance in the writing of this manuscript.

References


Received 20 October 2004, accepted 17 February 2005