Background: Today, treatment of chronic hepatitis C is based on a synergistic combination of pegylated interferon and ribavirin with antiprotease inhibitors. Haemolytic anaemia, which is the major side effect of ribavirin treatment, disrupts ribavirin treatment compliance and varies significantly from one patient to another. There is an individual susceptibility to ribavirin haemolysis. With a view to studying haemolysis, and thus optimizing the treatment response, we have developed a new in vitro tool for analysing the ribavirin-induced lysis of red blood cells.

Methods: Resuspended red blood cells were incubated with isotonic buffer and a range of concentrations of ribavirin. Haemolysis was quantified by spectrophotometric measurement of the supernatant at 540 nm. The assay was used to test the effects of various compounds and to investigate the susceptibility of patients to haemolytic anaemia.

Results: In our assay, the degree of haemolysis is dependent on the ribavirin concentration used and can be inhibited by the addition of dipyridamole (50% inhibitory concentration [IC₅₀] 30 μM), ATP or glutathione (IC₅₀ 1.63 mM and 767 μM, respectively). We observed a strong decrease in red blood cell haemolysis in the presence of the ribavirin prodrug viramidine (Taribavirin®). When testing the performance of this assay with blood from 24 patients before treatment, we observed a strong correlation between in vitro haemolysis before treatment and the decrease in haemoglobin levels seen in vivo during subsequent treatment (P<0.001).

Conclusions: With this new tool it is possible to better evaluate individual susceptibility to ribavirin-induced haemolysis before the start of treatment. In addition, this model will enable the mechanism of ribavirin-induced anaemia to be further explored and allow molecules that could reduce ribavirin haemolysis to be screened and tested in vitro. This approach could help optimize current and future therapeutic strategies involving ribavirin in the treatment of chronic hepatitis C.

Introduction

Approximately 180 million individuals worldwide are chronically infected with HCV and are at risk of morbidity and mortality from cirrhosis and hepatocellular carcinoma. Since the addition of ribavirin (RBV) to interferon therapy response rates have doubled, illustrating the importance of RBV in HCV treatment [1]. The bodyweight-adjusted dose of RBV appears to be crucial for optimizing virological response rates [2]. The early cessation or dose reduction of RBV can affect the treatment response [3]. Hence, RBV doses should be optimal throughout the course of treatment. It is not possible to increase the RBV dose indefinitely, however, as haemolytic anaemia (the major side effect of RBV) will compromise treatment compliance and outcomes. RBV-induced anaemia remains poorly described and limits the clinician’s ability to maximize the treatment response. RBV becomes concentrated in circulating red blood cells (RBCs) and induces an ATP deficiency [4]. This phenomenon leads to RBC toxicity through oxidative membrane damage and the inhibition of intracellular energy metabolism [5]. To maximize the patient benefit of RBV, a balance must be struck between the drug’s antiviral activity and its treatment-limiting side effect [6].

During combination therapy, the average drop in haemoglobin is approximately 2.5–3 g/dl. The drop is >4 g/dl in about 20% of patients and approximately 10% of patients present with severe anaemia (defined as an haemoglobin level <10 g/dl) [7]. RBV lacks a therapeutic window in which effective but
non-cytotoxic concentrations are reached and maintained. In most cases, toxicity follows on from efficacy. On an individual patient basis, however, RBV-plasma concentration is not always associated with a fall in haemoglobin levels. There is a strong individual susceptibility to haemolysis. Recently, two polymorphisms (rs1127354 and rs7270101) in the gene coding for inosine triphosphatase (ITPA) have been found to protect against RBV-induced anaemia [8]. However, only 30% of the population have ≥1 of these polymorphisms and a total lack of ITPA activity is found in just 2% of the population [9]. It is essential to develop strategies for maximizing treatment compliance and thus improve outcomes in current and future therapeutic strategies involving RBV. A better understanding of the mechanism of RBV-induced anaemia could help to counteract this side effect. In addition, a means of predicting haemolysis prior to treatment initiation would be of major therapeutic benefit. To this end, we have developed a new in vitro model of RBV-induced anaemia. With this model, it is now possible to estimate, in part, the risk of developing severe anaemia before starting treatment. Moreover, the model will be able to provide insights into the mechanism of RBV-induced haemolysis and should facilitate the discovery of new drugs to counteract this effect.

Methods

In vitro red blood cell haemolysis by ribavirin

To measure RBC haemolysis by RBV, blood was first collected in EDTA-coated tubes and samples centrifuged at 2,500 rpm for 10 min. RBCs were separated from plasma and the buffy coat and then washed twice with 4 volumes of phosphate-buffered saline (pH=7.4). Next, 300 μl of plasma and 300 μl of RBC suspension were placed in a polypropylene tube. To obtain a haematocrit of 15%, 1,400 μl of a mixture containing RBV and isotonic buffer (0.125 M sodium chloride, 0.005 M potassium chloride, 0.0012 M magnesium chloride, 0.0012 M potassium phosphate monobasic, 0.24 M sodium bicarbonate and 0.01 M glucose, as described by Grattagliano et al. [10]) were added. RBV was diluted in this buffer to concentrations ranging from 0 to 10 mM. The tubes were then closed and incubated horizontally with shaking (130 rpm) for various time intervals at 37°C.

The haemolysis assay

After incubation, tubes were centrifuged at 2,500 rpm for 10 min. Haemolysis was quantified by spectrophotometric measurement of cyanmethemoglobin in the supernatant at 540 nm. The percentage haemolysis in each tube was determined as follows: % haemolysis = \(\frac{A}{B}\) ×100, where A is the optical density at 540 nm (OD_{440}) in the supernatant for a tube at a given concentration of RBV and B is the OD_{440} for the supernatant from a positive control tube (containing water instead of isotonic buffer with the same haematocrit).

Reagents

Dipyridamole was purchased from Sigma–Aldrich (St Quentin Fallavier, France), RBV was provided by Carbosynth (Compton, UK). All compounds were dissolved in the isotonic buffer described above. Viramidine (Taribavirin®) was synthesized according to the method described in the literature [11].

Genotyping

Genomic DNA was extracted from whole blood samples using the QIAamp DNA blood mini kit (Qiagen, Courtabeuf, France), according to the manufacturer’s instructions. Each patient was genotyped (after having provided their written informed consent) for two ITPA polymorphisms [8] using an ABI TaqMan allelic discrimination kit and the ABI7900HT sequence detection system (both from Applied Biosystems, Courtabeuf, France). The ITPA activity was determined according to the method described by Maeda et al. [12].

Patients

Written informed consent was obtained from each study participant and ethical approval was obtained from our local investigational review board. A total of 24 adult patients infected with genotype 1 HCV were included in the study. Patients began combination therapy with pegylated interferon (PEG-IFN)-α2b and RBV for chronic hepatitis C infection between February 2010 and May 2011. Exclusion criteria included coinfection with HBV and/or HIV, abnormal RBCs (thalassaemia, sickle cell disease, pyruvate kinase deficiency or glucose-6-phosphate deficiency) and impaired kidney function. Patients were excluded if they had reduced the RBV dose during treatment or if erythropoietin had been administered. Patients received weekly injections of PEG-IFN-α2b and RBV was administered orally. The RBV dose was based on bodyweight and the mean value for the group of 24 patients was 15.04 ±1.51 mg/kg/day.

Four variables were recorded prior to treatment: age, creatinine clearance, complete blood count data and HCV RNA concentration. Haemoglobin levels were measured at four time points during the course of treatment (at weeks 4, 8, 12 and 24).

Statistical analysis

All experiments were performed in triplicate. R software was used to perform the statistical analysis. Student’s t-test and Wilcoxon’s test were used to compare mean values. The Pearson correlation coefficient was
determined in a linear regression analysis. The threshold for statistical significance was set to $P<0.05$.

**Results**

**Ribavirin-induced in vitro haemolysis of red blood cells**

The first objective of this study was to develop an *in vitro* assay of RBC haemolysis with increasing RBV concentrations. After various adjustments of the isotonic buffer composition and the haematocrit, the percentage haemolysis of RBCs was analysed at various incubation times. An RBV concentration range from 0 to 10 mM was chosen (Figure 1). The RBV concentration found in RBCs *in vivo* is approximately 1.5 mM [13]. Haemolysis was measured after 24, 48, 72 and 96 h incubation with RBV (Figure 1). We can observe that for each measurement point the variability is very low. In the absence of RBV, the percentage haemolysis after 24, 48, 72 and 96 h was 0.92% ±0.17%, 1.26% ±0.26%, 2.01% ±0.07% and 6.24% ±0.57%, respectively. The effect of RBV on RBCs can be detected from 48 h onwards. The repeatability of this model of haemolysis is very good with low intra- and interassay variability (approximately 5%). For RBV concentrations ranging from 0–5 mM, the linearity was good (Pearson coefficient correlation 0.952; five measurement points, data not shown). For the first time, these results demonstrated three key points: RBV causes lysis of RBCs *in vitro*, this phenomenon requires time to be triggered, as observed in patients and the haemolysis observed *in vitro* by RBV is linear and repeatable. For all further analyses, we chose to read haemolysis after 72 h incubation, which provides a good compromise between a detectable level of haemolysis and an incubation time that is not too long.

**Inhibition of haemolysis**

To cause haemolysis, RBV must accumulate inside RBCs. The human equilibrative nucleoside transporter is involved in the cellular import of natural and synthetic nucleotides and mediates RBV uptake by RBCs [14]. We found that the human equilibrative nucleoside transporter inhibitor dipyridamole significantly decreased RBV-induced haemolysis *in vitro* (Figure 2A). This effect was concentration dependent; there was a 50% inhibitory concentration (IC$_{50}$) of approximately 30 µM. These results confirm that haemolysis of RBCs *in vitro* is due to RBV and show that it is possible to inhibit haemolysis *in vitro* by disrupting RBV entry into RBCs.

The addition of ATP and glutathione at various concentrations was then tested in our experimental model in the presence of 2 mM RBV. As shown in Figure 2B, ATP and glutathione greatly reduce the haemolytic...
The effect of RBV (IC_{50} 1.63 mM and 767 μM, respectively). These results clearly demonstrate that the origin of RBV haemolysis is a decrease in the intracellular energy and antioxidant pool.

The effect of viramidine on red blood cell haemolysis \textit{in vitro}

Viramidine (Taribavirin®, a 3-carboxamidine prod-\textsuperscript{u}ug of RBV) is associated with a lower incidence of anaemia because of its liver-targeting properties \cite{15, 16}. We therefore synthesized viramidine \cite{11} and checked its activity against poliovirus. At a concentration of 1,000 μM, RBV and viramidine both decreased the poliovirus titre at 24 h by 2.83 log and 2.03 log, respectively (data not shown). When we used viramidine in the haemolysis assay, significantly lower haemolysis was observed (relative to RBV) at all concentrations tested (Figure 3). The mean reduction for viramidine concentrations between 0.5 and 10 mM was 62.4% ± 11.4%. These results suggest that it will be possible to use the haemolysis assay developed here both to better understand the mechanism of RBV-induced anaemia and to test molecules that could attenuate haemolysis.

Correlation between \textit{in vitro} haemolysis before treatment and \textit{in vivo} anaemia during treatment

We next investigated the putative link between \textit{in vitro} haemolysis and the decrease in haemoglobin levels observed during standard treatment with Peg-IFN-α2b and RBV. The \textit{in vitro} haemolysis assay was performed three times on samples from 24 patients before starting treatment. For each patient, the average maximum percentage haemolysis observed \textit{in vitro} at 72 h
was compared with the RBV dose-related decay in haemoglobin levels during treatment (Figure 4). The two parameters were well correlated, with a Pearson correlation coefficient of 0.727 (P=0.000188). Patients who subsequently showed a strong decrease in haemoglobin levels during treatment had the highest pretreatment levels of in vitro haemolysis in the presence of RBV (Figure 4). We conclude that the in vitro haemolysis assay performed prior to treatment can estimate individual susceptibility to RBV-induced anaemia.

ITPA polymorphism, in vitro haemolysis and anaemia during treatment

Finally, the 24 patients were screened for ITPA polymorphisms. A deficit in the activity of ITPA leads to reduced anaemia during RBV treatment. The probable ITPA activity was also calculated [12]. On this basis, patients were subvided into two groups (activity ≤30% or >30%) and compared in terms of their mean values. Hb, haemoglobin; RBV, ribavirin.

Table 1. Inosine triphosphatase polymorphisms, in vitro haemolysis and anaemia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ITPA activity ≤30% (n=6)</th>
<th>ITPA activity &gt;30% (n=18)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum haemolysis in vitro, %</td>
<td>29.62 ±7.69</td>
<td>35.17 ±7.93</td>
<td>0.1552</td>
</tr>
<tr>
<td>In vitro haemolysis without RBV, %</td>
<td>2.38 ±0.51</td>
<td>3.74 ±1.08</td>
<td>0.0006</td>
</tr>
<tr>
<td>Reduction in Hb at week 4, g/dl</td>
<td>0.83 ±0.46</td>
<td>2.23 ±1.22</td>
<td>0.0024</td>
</tr>
<tr>
<td>Reduction in Hb at week 4, %</td>
<td>5.46 ±3.01</td>
<td>13 ±8.76</td>
<td>0.0097</td>
</tr>
<tr>
<td>Maximum reduction in Hb during treatment, g/dl</td>
<td>2.77 ±0.89</td>
<td>3.38 ±1.27</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Data are mean ±sd unless otherwise indicated. Polymorphisms in the inosine triphosphatase (ITPA) gene were determined for each patient. The possible genotypes for each biallelic polymorphism are as follows: C/C, A/C and A/A (where A is the minor allele) for rs1127354 and A/A, A/C and C/C (where C is the minor allele) for rs7270101. ITPA activity was determined according to the method described by Maeda et al. [12]. On this basis, two groups of patients were derived (activity ≤30% or >30%) and compared in terms of their mean values. Hb, haemoglobin; RBV, ribavirin.

For each of the 24 patients, the haemolysis assay was performed three times on the same sample before the initiation of treatment. For a given patient, the average maximum percentage haemolysis observed in vitro at 72 h was compared with the reduction in haemoglobin levels during treatment (g/dl), relative to daily dose of ribavirin (mg/kg/day). Pearson’s coefficient and the P-value were calculated.
Discussion

To the best of our knowledge this is the first study to describe the \( \textit{in vitro} \) haemolysis of RBCs in the presence of RBV and isotonic buffer. The degree of haemolysis depends on the incubation time and concentration of RBV. Two previous studies induced haemolysis rapidly (after 4 h or 8 h) by using RBV in a hypotonic buffer [10,17]. This approach introduced variability that was not due to the RBV concentration and makes the technique difficult to use and reproduce. We also incubated RBCs in the presence of phosphate-buffered saline. We noted a strong haemolysis without RBV. With a new buffer, by testing longer incubation times under isotonic conditions we observed dose-dependent haemolysis from 48 h. De Franceschi et al. [5] have shown that the pool of ATP in RBCs decreases after 12 h incubation with RBV. This is probably why a delay in the onset of haemolysis was observed in our assay. After optimizing the incubation time, we adjusted the haematocrit of the assay to 15%. We obtained a plateau in haemolysis at 5 mM. This observation might be due to a saturation of RBV entry and phosphorylation in RBCs. A study of the metabolism of RBV in mice indicated that the plateau observed in the intra-erythrocyte concentration of the drug was due to ATP-dependent kinases [18]. Inside the RBC, RBV is converted to a triphosphate derivative by an ATP-dependent adenosine kinase [19].

To test the specificity of haemolysis, we supplemented the incubation buffer with the RBV uptake inhibitor dipyridamole [14]. Under these conditions, we observed strong inhibition of haemolysis (Figure 2A). Although dipyridamole is a licensed drug with antiplatelet and coronary dilatating properties, it cannot be used to reduce haemolysis \( \textit{in vivo} \) because it would also prevent RBV from entering hepatocytes. Nevertheless, our \( \textit{in vitro} \) observation proves that it is possible to pharmacologically counter the adverse effects of RBV on RBCs, as demonstrated with ATP and glutathione (Figure 2B). Our assay thus constitutes a new tool for the \( \textit{in vitro} \) screening of compounds that might counter the major adverse effect of RBV treatment.

We synthesized the nucleoside analogue viramidine from RBV, as described elsewhere [11]. Viramidine is an oral prodrug of RBV that is currently undergoing Phase III clinical trials. Viramidine might increase compliance with treatments of chronic HCV infection by reducing the need for anaemia-triggered dose reduction. Here, we demonstrated that haemolysis is much greater for RBV than for viramidine at a given drug concentration (Figure 3). These \( \textit{in vitro} \) results confirm for the first time that viramidine might have fewer adverse effects \( \textit{in vivo} \) in patients with chronic hepatitis C.

The pretreatment prediction of haemolysis is a major challenge in optimization of the treatment response. We observed a strong correlation between the peak \( \textit{in vitro} \) haemolysis in our assay (with samples collected before treatment) and the fall in haemoglobin seen during therapy (Figure 4). Hence, our assay could be used to screen for individuals at high risk of severe anaemia (typically older patients, patients with abnormal RBCs and those with renal and cardiovascular disorders). Patients with increased resistance to haemolysis \( \textit{in vitro} \) (in the presence of RBV) could be treated with higher initial doses of the drug, offering a greater likelihood of a sustained virological response. Indeed, a recent meta-analysis has concluded that the addition of ribavirin was a benefit in the treatment of hepatitis C patients with thalassaemia [20]. It would be interesting to use our assay in patients with haemoglobinopathies.

We observed a good correlation between \( \textit{in vitro} \) haemolysis and the decrease in haemoglobin during treatment. However, the correlation is not complete and could be explained by the individual effect of interferon on bone marrow. Another explanation is that, despite the fact that doses of RBV were weight-adjusted, plasma concentrations can fluctuate greatly. We did not need to measure RBV levels \( \textit{in vivo} \). Indeed, measuring RBV-induced anaemia is useful to detect low concentrations in non-responders [21,22], there is no single upper RBV concentration threshold for anaemic toxicity. Hence, anaemia prediction for a patient before treatment appears to be more relevant.

Lastly, we studied ITPA polymorphisms that were recently found to protect against anaemia in a genome-wide association study [8] of 1,602 European/American patients. However, the resulting difference in ITPA function explains only 30% of the variability in quantitative haemoglobin reduction; thus, ITPA activity is not sufficient for fully predicting susceptibility to anaemia for a given patient. The same researchers also showed that these ITPA polymorphisms were associated with a decrease in haemoglobin after 4 weeks of treatment, but not throughout [23]. This same observation was made in our study.

In conclusion, the assay presented here should be of use in establishing a patient’s susceptibility to anaemia and might facilitate screening for compounds that could counteract the effect of RBV on RBCs. Novel anti-HCV drugs (such as the protease inhibitors telaprevir and boceprevir) are likely to be used in combination with current treatments and are associated with increased rates of anaemia and frequent erythropoietin use [24]. Thus, RBV will remain an important drug for achieving a sustained virological response in HCV-infected individuals and the knowledge and prediction of anaemia will continue to be an important issue in therapy.
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Disclosure statement

The authors declare no competing interests.

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