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Macromolecular prodrugs of ribavirin combat side effects and toxicity with no loss of activity of the drug†‡

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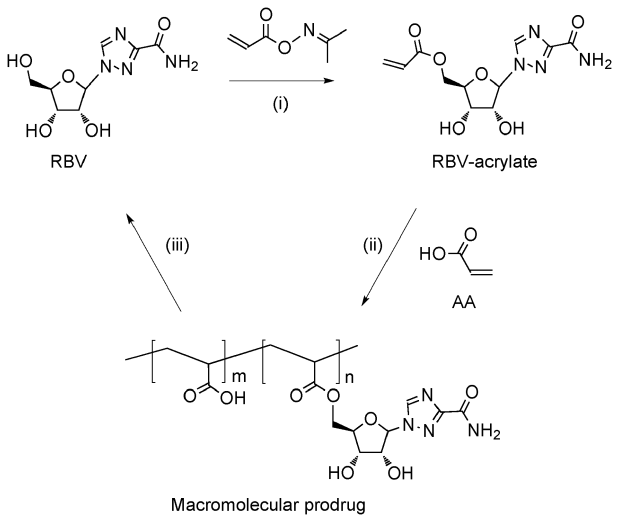
Chemi-enzymatic synthesis of ribavirin acrylate and subsequent RAFT co-polymerization with acrylic acid afforded a formulation of a broad spectrum antiviral drug which avoids accumulation in erythrocytes, the origin of the main side effect of ribavirin. In cultured macrophages the macromolecular prodrugs exhibited decreased toxicity while maintaining the anti-inflammatory action of ribavirin.

Macromolecular prodrugs (MP) are a powerful tool in biomedicine employed to optimize pharmacokinetics of therapeutically active compounds and increase the drug payload delivered to a desired site of action.[1] Typically, one or more drug molecules are conjugated to a polymer carrier, with the possibility to use the polymer molecular weight to control blood residence time and achieve accumulation of the MP at the site of action *via* active and passive targeting.[1] In turn, drug release kinetics is determined by the linkage between the drug and the polymer chain and a set of biodegradable linkages are described to achieve a release of the payload at the desired site in response to a particular stimulus.[2,3] While most MP are currently developed for anticancer treatment, similar design criteria are expected to deliver practical benefit in delivery of drugs to combat other diseases, specifically hepatitis C.[4] Ribavirin (RBV) is a broad spectrum antiviral drug prescribed against respiratory syncytial virus, influenza, herpes virus, HIV infections, Lassa fever, haemorhagic fever, and it remains the number one treatment against hepatitis C virus (HCV).[5,6] At prescribed doses, RBV has a modest efficiency, yet dose escalation is precluded by a dose-dependent toxicity of this drug. The main origin of toxicity is accumulation of RBV in the red blood cells (RBCs),[7] which results in a volume of distribution of 2000 L.[8] This implies that only a minor fraction of the drug reaches its site of action and strongly suggests that RBV would tremendously benefit from a targeted mode of delivery. Herein, we develop MP of RBV, reveal that these formulations overcome the main origin of hemolytic toxicity of RBV, and demonstrate that this is achieved without compromising efficacy of treatment in cell culture model tests. To the best of our knowledge, these results constitute the first example of multi-angle optimization of the pharmacokinetics of RBV using synthetic macromolecular prodrugs.



Scheme 1 Schematic illustration of the proposed synthesis of macromolecular prodrugs of ribavirin (RBV). Polymerizable acrylate ester of RBV was synthesized *via* a chemi-enzymatic approach using Nz435/CAL-B in dioxane (i) and used in RAFT polymerization with AA as comonomer to obtain macromolecular prodrug (ii). Synthesized polymers released pristine RBV upon hydrolysis (iii).

For the synthesis of MP, we developed an acrylate-based polymerizable form of ribavirin, (Scheme 1). Previous reports on RBV conjugation to drug carriers include the use of phosphoramidate linkages to hemoglobin[9,10] and synthesis of vinyl ester based

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RBV monomers.[11–13] Powerful in their own right, these schemes lack the versatility of acrylate monomers with regard to diversity in macromolecular design, in the latter case achieved through copolymerization with various monomers to a desired overall chain length using a desired content of the RBV monomer. RBV acrylate was synthesized *via* a chemi-enzymatic approach using acetone oxime acrylate, RBV, and novozyme 435 lipase. Optimized reaction and purification procedures afforded overall product recovery yields over 85%, which is important for the overall practical utility of the method.

As a partner to RBV acrylate in the synthesis of MP, we chose to use acrylic acid. Poly(acrylic acid) (PAA) has an extensive history of biomedical applications[14–19] yet surprisingly does not appear in the arsenal of tools of polymer therapeutics, *i.e.* polymer-conjugated drugs. A plausible reason to this is that well-defined samples of PAA are typically obtained *via* ester hydrolysis of the parent polymer, *e.g.* poly(*tert*-butyl acrylate),[20] a reaction which may not be compatible with hydrolytically unstable prodrugs and bioconjugates. Similarly, copolymerization with acrylic acid may also exert limitations on the range of monomers compatible with this strategy in the design of macromolecular prodrugs. In our hands, RBV acrylate underwent RAFT-controlled copolymerization with acrylic acid in DMF at 60 °C without signs of ester degradation. Resulting polymer contained RBV acrylate in a quantity matching well the feed of this drug containing monomer, revealed narrow polydispersity of the sample (1.16), and had molecular weight compatible with biomedical applications with regard to a possibility of renal elimination (27 kDa). To facilitate analysis of polymer association with mammalian cells, polymer samples were obtained using a monomer feed containing 1 mol% of fluorescein acrylate, resulting in fluorescently labeled samples of MP RBV.

To investigate the interaction of MP with mammalian cells, RBV MP was incubated with erythrocytes, hepatocytes and macrophages at polymer concentrations from 0.1 to 100 μg mL^{-1}. Following an incubation for 24 h, fluorescence of cells was quantified using flow cytometry, Fig. 1. In this assay, increase in fluorescence of individual cells is indicative of association and/or internalization of the polymer by the cells. For red blood cells, incubation with MP RBV resulted in negligible increase in the fluorescence of the cells and an overall minor fraction of cells testing positive for associated polymer. In contrast, cell lines with hepatic relevance exhibited a dose dependent interaction with macromolecular prodrugs. At 10 μg mL^{-1}, at least 50% of cultured hepatocytes and macrophages revealed fluorescence levels indicative of polymer binding and uptake, as well as an order of magnitude increase in the mean cell fluorescence (Fig. 1A). These data illustrate that MP RBV effectively eliminate the origin of the main side effect of RBV, *i.e.* uptake by the red blood cells, yet exhibit pronounced levels of interaction with hepatic cells.

To investigate intracellular activity of RBV upon delivery using acrylic acid based macromolecular prodrugs, we tested the hypothesis of the anti-inflammatory activity of RBV,[22] specifically *via* inhibition of production of nitric oxide in macrophages. This read-out system has relevance to viral hepatitis since liver fibrosis is hypothesized to be a body

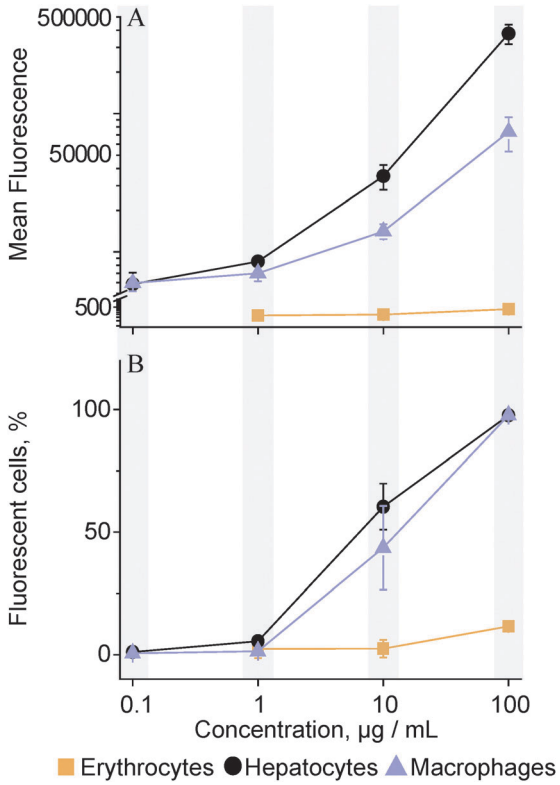

Fig. 1 (A) Mean fluorescence and (B) percent fluorescent cells for erythrocytes, hepatocytes and macrophages upon incubation with PAA–RBV for 24 h at polymer concentrations from 0.1 to 100 μg mL^{-1} quantified *via* flow cytometry. Results shown are the average of triplicate experiments, reported as mean ± SD (n = 3).

response to persistent liver inflammation, the latter resulting from viral infections. There is growing evidence that RBV does not elicit direct antiviral activity, as is done by its partner tandem drug, PEG interferon,[23] but contributes to anti-HCV treatment *via* immunomodulation and anti-inflammatory activity.[22,24] One of the plausible mechanisms of this action is *via* the inhibition of inosine-5′-monophosphate dehydrogenase and a resulting depletion of tetrahydrobiopterin.[25–27] The latter is a cofactor of inducible nitric oxide synthase and an overall effect of RBV should be a reduction in production of nitric oxide. Experimentally, this was verified in a single report and using endothelial cells,[26] yet remains unexplored in macrophages, mimics of liver resident Kuppfer cells.

A mammalian macrophage cell line was incubated with RBV and its macromolecular prodrugs for 24 h followed by stimulation with a potent pro-inflammatory endotoxin, bacterial lipopolysaccharide. Relative levels of nitric oxide were quantified *via* a Griess assay,[28] following further 24 h of cell culture. In agreement with the hypothesis put forward,[22] 10 μM RBV, thus being in the range of clinically prescribed concentration of RBV (9–18 μM (ref. 29)), afforded a 50% reduction in the synthesis of nitric oxide, Fig. 2. However, this treatment was also associated with a statistically significant reduction in cell viability by 20%. MP of RBV taken at 100 μg mL^{-1} concentration (corresponding to 67 μM of free RBV) exhibited a similar efficacy of treatment and afforded a 50% inhibition in the production of NO. In contrast to pristine drug, MP treatment was associated with no

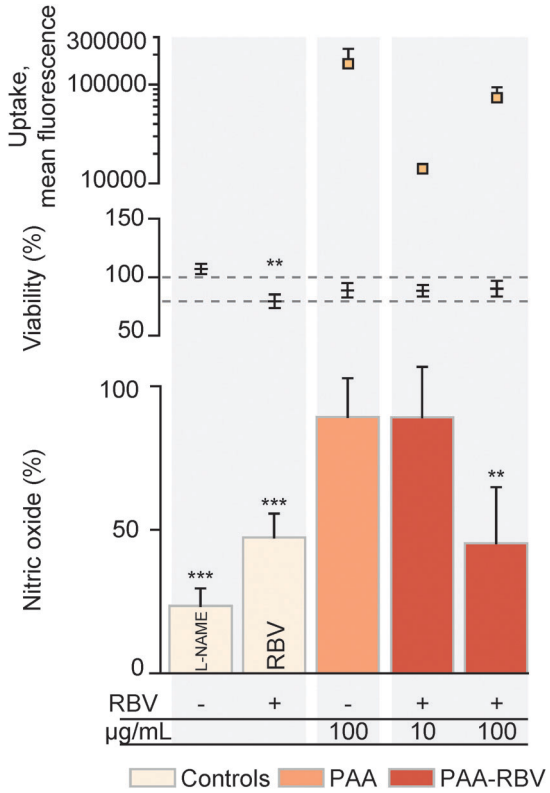

Fig. 2 Inhibition of NO production and associated cytotoxic effect quantified in stimulated RAW264.7 macrophages upon incubation with RBV (10 μM), L-NAME (1 mM, iNOS inhibitor[21]), and PAA–RBV at 10 and 100 μg mL^{-1}, as well as, mean cell fluorescence upon incubation with PAA and PAA–RBV for 24 h. Results shown are average of triplicate experiments, reported as mean ± SD (n = 3). Statistical significance is given in relation to the negative control. ** $P < 0.01$, *** $P < 0.001$.

statistically significant toxicity. Parent polymer, PAA, exhibited a similar level of interaction with macrophages as evidenced by comparable levels of attained mean cell fluorescence, but had a minor effect on the levels of nitric oxide produced by macrophages. We note that mechanism of action of RBV involves phosphorylation on the 5′ hydroxyl,[25] *i.e.* the same hydroxyl used for the synthesis of RBV monomer and the macromolecular prodrug. The data in Fig. 2 therefore strongly suggest that the drug was released from the polymer chain intracellularly and demonstrate that conjugation to a polymer carrier affords a significant therapeutic benefit *via* reduction of cytotoxicity of RBV without compromising efficacy of treatment.

Overall, results of this study demonstrate that conjugation to a polymer carrier prevents accumulation of RBV in erythrocytes and thus eliminates the origin of the main side effect of ribavirin. Further to this, in the form of PAA-based MP, delivery of RBV to cultured macrophages decreases cytotoxic effect without compromising efficacy of the treatment. In presenting this data, we reveal that polymer therapeutics have potential to significantly improve pharmacokinetics and pharmacodynamics of RBV-based therapies and present synthetic macromolecular prodrugs of ribavirin as a novel opportunity for treatment of liver inflammation, specifically associated with viral hepatitis.

Notes and references

1 R. Duncan, *Nat. Rev. Drug Discovery*, 2003, **2**, 347–360.
2 F. Kratz, I. A. Müller, C. Ryppa and A. Warnecke, *ChemMedChem*, 2008, **3**, 20–53.
3 C. A. Blencowe, A. T. Russell, F. Greco, W. Hayes and D. W. Thornthwaite, *Polym. Chem.*, 2011, **2**, 773–790.
4 J. Sanchis, F. Canal, R. Lucas and M. J. Vicent, *Nanomedicine*, 2010, **5**, 915–935.
5 N. J. C. Snell, *Expert Opin. Pharmacother.*, 2001, **2**, 1317–1324.
6 C. Caussin-Schwemling, C. Schmitt and F. Stoll-Keller, *J. Med. Virol.*, 2001, **65**, 14–22.
7 M. W. Fried, *Hepatology*, 2002, **36**, s237–s244.
8 P. Glue, *Semin. Liver Dis.*, 1999, **19**(Suppl 1), 17–24.
9 S. Brookes, P. Biessels, N. F. L. Ng, C. Woods, D. N. Bell and G. Adamson, *Bioconjugate Chem.*, 2006, **17**, 530–537.
10 G. A. Levy, G. Adamson, M. J. Phillips, L. A. Scrocchi, L. Fung, P. Biessels, N. F. Ng, A. Ghanekar, A. Rowe, M. X. Z. Ma, A. Levy, C. Koscik, W. He, R. Gorczynski, S. Brookes, C. Woods, I. D. McGilvray and D. Bell, *Hepatology*, 2006, **43**, 581–591.
11 B. K. Liu, N. Wang, Q. Wu, C. Y. Xie and X. F. Lin, *Biotechnol. Lett.*, 2005, **27**, 717–720.
12 X. Li, Q. Wu, Z. Chen, X. Gong and X. Lin, *Polymer*, 2008, **49**, 4769–4775.
13 X. Li, Q. Wu, M. Lu, F. Zhang and X. F. Lin, *J. Polym. Sci., Part A: Polym. Chem.*, 2008, **46**, 2734–2744.
14 A. Bernkop-Schnurch, *Adv. Drug Delivery Rev.*, 2005, **57**, 1569–1582.
15 M. Rutnakornpituk, N. Puangsin, P. Theamdee, B. Rutnakornpituk and U. Wichai, *Polymer*, 2011, **52**, 987–995.
16 V. Thilakarathne, V. A. Briand, Y. X. Zhou, R. M. Kasi and C. V. Kumar, *Langmuir*, 2011, **27**, 7663–7671.
17 J. M. Pelet and D. Putnam, *Bioconjugate Chem.*, 2011, **22**, 329–337.
18 V. A. Kabanov, *Pure Appl. Chem.*, 2004, **76**, 1659–1677.
19 M. A. C. Stuart, W. T. S. Huck, J. Genzer, M. Muller, C. Ober, M. Stamm, G. B. Sukhorukov, I. Szleifer, V. V. Tsukruk, M. Urban, F. Winnik, S. Zauscher, I. Luzinov and S. Minko, *Nat. Mater.*, 2010, **9**, 101–113.
20 W. Zhang, B. Fang, A. Walther and A. H. E. Müller, *Macromolecules*, 2009, **42**, 2563–2569.
21 S. Moncada, R. M. J. Palmer and E. A. Higgs, *Pharmacol. Rev.*, 1991, **43**, 109–142.
22 R. E. Kast, *Neoplasia*, 2003, **5**, 3–8.
23 E. De Clercq, *Nat. Rev. Drug Discovery*, 2007, **6**, 1001–1018.
24 R. G. Gish, *J. Antimicrob. Chemother.*, 2006, **58**, 488.
25 J. J. Feld and J. H. Hoofnagle, *Nature*, 2005, **436**, 967–972.
26 M. Michaelis, R. Michaelis, T. Suhan, H. Schmidt, A. Mohamed, H. W. Doerr and J. Cinatl Jr., *FASEB J.*, 2007, **21**, 81–87.
27 E. De Clercq, *Nat. Rev. Drug Discovery*, 2002, **1**, 13–25.
28 M. J. Moorcroft, J. Davis and R. G. Compton, *Talanta*, 2001, **54**, 785–803.
29 A. Tsubota, N. Akuta, F. Suzuki, Y. Suzuki, T. Someya, M. Kobayashi, Y. Arase, S. Saitoh, K. Ikeda and H. Kumada, *Intervirology*, 2002, **45**, 33–42.