Highly Active Macromolecular Prodrugs Inhibit Expression of the Hepatitis C Virus Genome in the Host Cells

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Ribavirin (RBV) is a nucleoside analogue with activity against various viruses (influenza, hepatitis C, Lassa fever virus, etc).[1,2] This drug has a good bioavailability; however, an intravenous administration has proven to be beneficial for an optimized treatment.[1] Unfortunately, this broad spectrum antiviral agent has a highly unfavorable pharmacokinetic (PK) profile, specifically accumulation in red blood cells (volume of distribution 2000 L) resulting in anemia.[3,4] A highly powerful tool to optimize PK of toxic drugs is that of macromolecular prodrugs (MP),[5–7] yet RBV presents scarce opportunities for conjugation. Indeed, the 5′-hydroxyl is not amenable for direct conjugation using linkages typically employed in the design of MP.[7] Of the latter, disulfide chemistry[8] is unique in that the prodrugs built around this linkage are stable in the blood stream yet degrade upon cell entry in response to the intracellular concentration of glutathione, GSH. To overcome the chemical incompatibility of RBV, a molecule without sulfhydryl functionality, and to introduce disulfide reshuffling as a mechanism for drug release, we employ the chemistry of self-immolative linkers (SIL),[9,10] **Figure 1**. We designed a methacrylate monomer to contain a disulfide linkage connected through an SIL to a carbonate with a functionality of RBV (for details on synthesis, see Supporting Information). The designed linker is poised to release the drug in response to a thiol trigger through the formation of a cyclic thiocarbonate, Figure 1B.[8,11]

The synthesized monomer was used to obtain a series of polymers through copolymerization with methacrylic acid via the reversible addition-fragmentation chain transfer mechanism, RAFT.[12,13] The labile disulfide monomer revealed no degradation during polymerization. Based on our prior report,[14] polymer average molar mass is a dominant factor defining the activity of the MP while drug loading plays a secondary role. Taking this into account, polymers were obtained using the same feed ratio of the RBV-containing monomer but with different ratio of the monomers to the RAFT agent. The resulting polymers had a similar drug loading ≈4 mol% and an average molar mass of 7, 14, and 23 kDa (labeled PMAA-2 through PMAA-4, respectively; for details of synthesis and characterization, see Supporting Information).

Characterization of drug release was performed in a variety of solution conditions using HPLC, **Figure 2** and Table S3 (Supporting Information). The synthesized polymers released the drug fast in the presence of dithiothreitol at concentrations equimolar to the content of RBV (Figure 2A). This illustrates the success of the proposed design, namely release of RBV triggered via thiol-disulfide exchange. RBV-containing polymers in which the drug is conjugated to the polymer directly through an ester linkage[14] (PMAA-5 and 6) released minute quantities of the drug under these conditions. SIL-containing polymers also exhibited a slow spontaneous drug release in phosphate buffer and in the presence of serum, yet this process was characterized by values of half-life well exceeding 48 h (panel B). These observations are in close agreement with the drug release kinetics reported previously for this type of the biodegradable linkage.[11] As a significantly more thorough test, RBV release was quantified using lysate of mammalian macrophage cells (panel B). Quantitative Ellman's test revealed that the content of GSH (more precisely, total thiol content) in the cell lysate was on a sub-millimolar level. Nevertheless, the lysate successfully triggered release of RBV from the disulfide-containing, SIL-equipped prodrugs. In contrast, ester-based MP revealed insignificant drug release in this experiment. Together, the HPLC characterization data illustrate that the proposed drug conjugation scheme makes up MP that are stable in serum and are highly responsive to the intracellular trigger, a key requirement for drug release upon cell entry.

The therapeutic effect associated with the designed MP was quantified in a hepatitis C virus subgenomic replicon system (Figure S3, Supporting Information) hosted within a hepatocyte cell lineage.[15,16] Previous reports on MP of RBV analyzed toxicity[17] or anti-inflammatory activity[14,18] of the released drug (in vitro) or quantified viral titer in blood (in vivo).[19,20] To the best of our knowledge, we present the first direct characterization of MP in their capacity to interfere with the proliferation of HCV as measured within the cells hosting infection.

The expression of the viral genome was conveniently quantified through monitoring co-expression of a protein, renilla luciferase, providing a read-out to screen the activity of antiviral (pro)drugs in a virus-free cell culture. Conventional treatment against HCV and viral hepatitis consists of two drugs, interferon-alpha (IFN-α) and RBV, the mechanism of action of RBV in this tandem is a subject of debate.[2] In the subgenomic

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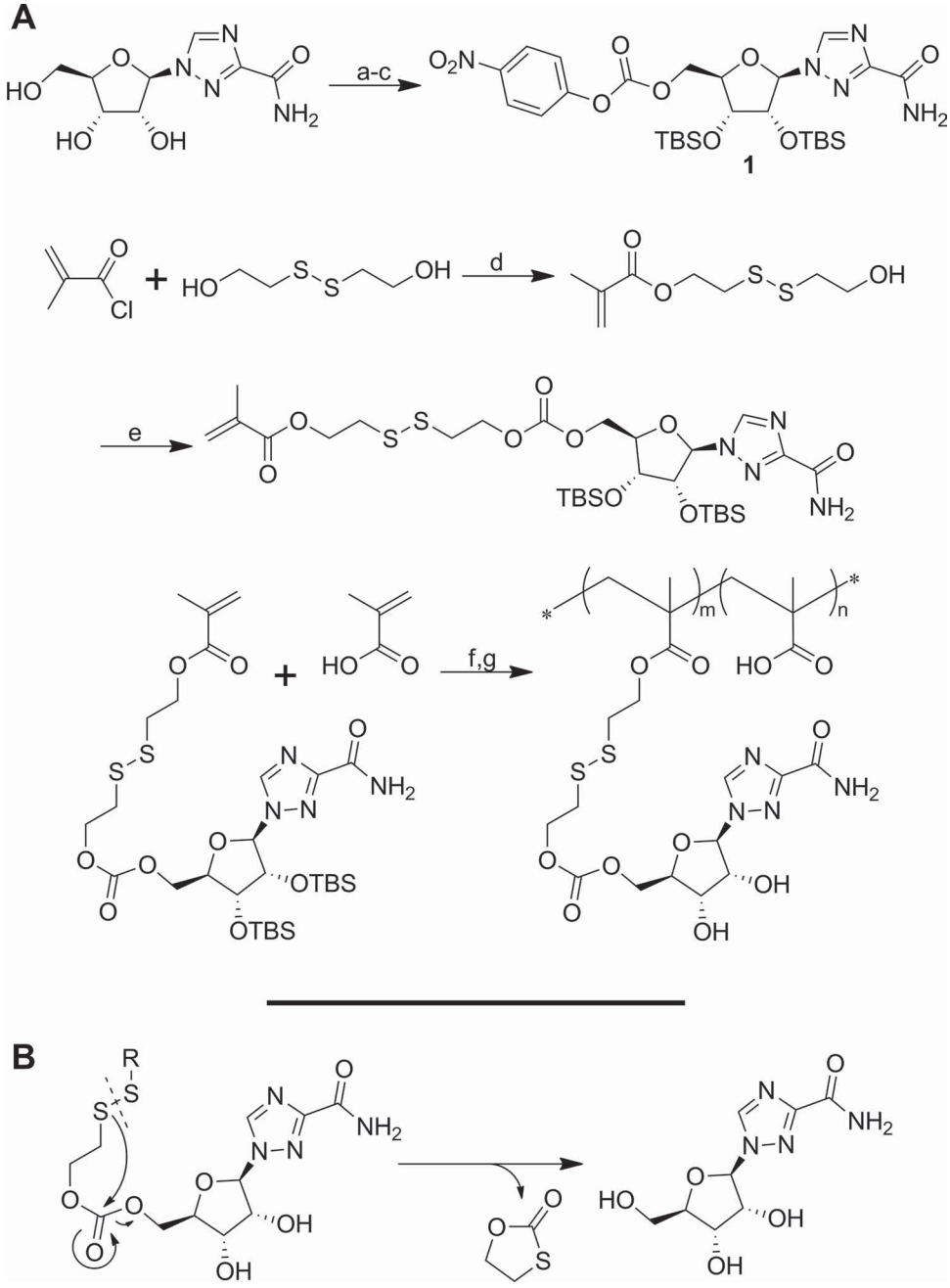

Figure 1. A) Synthesis of the disulfide SIL-RBV monomer and its copolymerization with methacrylic acid: a) TBSCl, imidazole, DMAP, DMF anh., 84%; b) HCl, MeOH, 73%; c) 4-Nitrophenyl chloroformate, TEA, THF, 85%; d) TEA, DCM anh., 76%; e) **1**, DMAP, DIEA, DCM, quant.; f) AIBN, 2-cyano-2-propyl-benzodithioate, DMF; g) TEA·3HF, DMF. B) Mechanism of RBV release through disulfide reshuffling under trigger of a thiol.

replicon system used herein, IFN-α proved to be highly effective and suppressed expression of the viral genome at concentrations as low as 1.4 U mL^{-1} (Figure S5, Supporting Information). In contrast and in accordance with the ineffectiveness of RBV mono-therapy in the clinic, RBV revealed an EC_{50} value of 80×10^{-6} M (see Figure S5, Supporting Information). This concentration is markedly higher than the toxicity associated IC_{50} value of RBV established in macrophages[18] (mimics to the liver resident Kupffer cells). Furthermore, this concentration is much higher than the therapeutically relevant content of the drug in patients' plasma ($6–20 \times 10^{-6}$ M).[1] Finally, toxicity-associated IC_{50} in the viral replicon system was only marginally higher (103×10^{-6} M) reflecting a very narrow therapeutic window of RBV, much similar to our previous report on the pharmacodynamics properties of RBV in macrophages.[18]

The synthesized MP with a sensitive trigger for an intracellular drug release was as efficacious as the pristine drug and at the same time less toxic, **Figure 3**A. Thus, MP with a molar mass 7 kDa (PMAA-2, marked "A" in Figure 3) was able to inhibit expression of the viral genome to the same extent as RBV

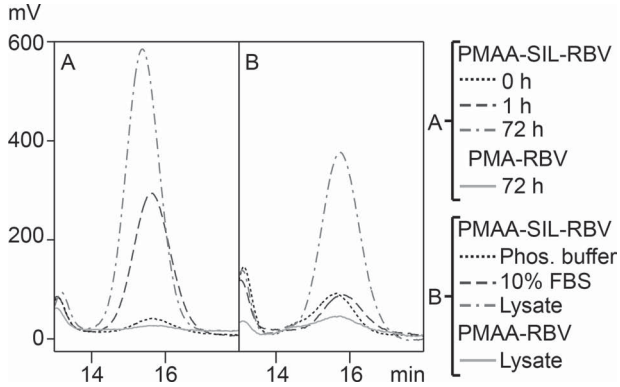

Figure 2. A) HPLC elution profiles illustrating release of RBV in phosphate buffer (200×10^{-3} M) in the presence of DTT (800×10^{-6} M) for MP based on thiol-sensitive, SIL-containing linkages (PMAA-SIL-RBV) and direct ester linkage between RBV and the polymer (PMA-RBV). B) HPLC elution profiles illustrating release of RBV from the SIL-containing MP in phosphate buffer (200×10^{-3} M), 10% fetum bovine serum (FBS), and in the milieu consisting of lysate of mammalian cells (Lysate) and release of RBV from the ester-based MP in the cell lysate; in all cases, the data are for 48 h incubation in the respective media at 37 °C. See Table S3 (Supporting Information) for numerical values.

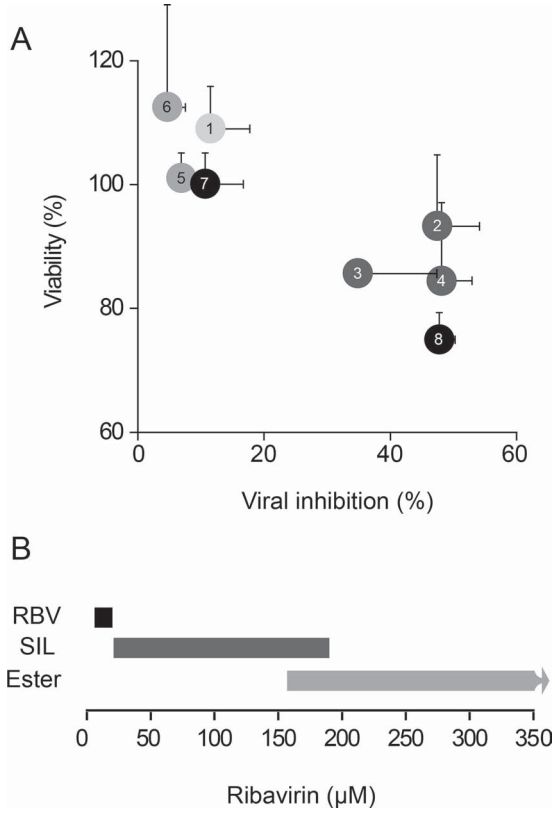

Figure 3. A) Inhibition of expression of the viral genome and cellular viability of the host cells upon a treatment with PMAA (1), MP based on the SIL linkage (4–5 mol% RBV, M_n 7 kDa (2), 14 kDa (3), and 23 kDa (4)), MP based on the ester linkage (7 mol% RBV, M_n 7 kDa (5), and 14 kDa (6)), or pristine RBV (10×10^{-6} M (7), 78×10^{-6} M (8)). See Table S2 (Supporting Information) for polymer characteristics. Results are the average ± SD of three independent experiments ($n = 3$). B) Schematic Illustration of the therapeutic windows corresponding to inhibition of the synthesis of nitric oxide (NO) in macrophages upon their stimulation toward an inflammatory response and treatment with RBV, a PMAA-SIL-RBV and a PMAA-RBV macromolecular prodrugs Full dose-response curves for all polymers in the replicon and NO assay are given in Figures S6 and S7 (Supporting Information).

at a concentration equal to its EC_{50} yet exhibited minor if any associated toxicity (statistical significance between the two treatments $p = 0.06$). This formulation was much more effective than 10×10^{-6} M RBV, the latter being the therapeutically relevant drug content ($p < 0.01$). Importantly, ester-based MPs exhibited only negligible activity in these experiments. The engineered drug release in response to the intracellular trigger was therefore highly important to achieve this level of therapeutic activity.

In separate experiments, we analyzed the newly synthesized MP in their capacity to deliver RBV to the macrophages. The read-out in this system relates to the anti-inflammatory activity of this drug, as was established in our previous publications.[14,18] HCV replication in the liver is associated with acute and chronic inflammation and these can lead to cirrhosis and liver cancer. The anti-inflammatory mode of action of RBV is hypothesized to be important in that it enables the host organism to fight the virus using tools of natural immunity.[21] Experimental dose-response curves (Figures S7, Supporting Information) were used to establish the EC_{50} and IC_{50} values related to this therapeutic effect and resulting therapeutic windows (range between EC_{50} to IC_{50} in equivalent concentrations of RBV, μM) are presented in Figure 3B.

The prodrugs with engineered intracellular drug release proved significantly more potent than their ester-based counterparts. For the SIL-containing MPs, EC_{50} values were only marginally higher than that for the pristine drug. With that, these MPs significantly decreased the toxicity of treatment and in doing so drastically expanded the therapeutic window. The ester-based MPs were also successful in achieving this, yet at an expense of ≈10-fold higher value of EC_{50}. These experiments further highlight the importance of the intracellular drug release engineered into MPs.

In this work, we engineered a highly sensitive response to intracellular thiols into the prodrugs of the thiol-free therapeutic RBV, a broad-spectrum antiviral drug. Using this tool, we designed MPs of RBV with potency near matching that of the pristine drug yet without associated toxicity. Together with our previous reports on hemocompatibility of MPs of RBV,[22,23] our efforts establish a novel platform for safe and efficient delivery of RBV that can also be adapted to other nucleoside analogue drugs for various therapies (antiviral, anticancer).

Supporting Information

Supporting Information (protocols for the syntheses, details of characterization (NMR, GPC), additional experimental results) is available from the Wiley Online Library or from the author.

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