Electronic Supplementary Information

Macromolecular Prodrugs of Ribavirin Combat Side Effects and Toxicity with No Loss of Activity of the Drug

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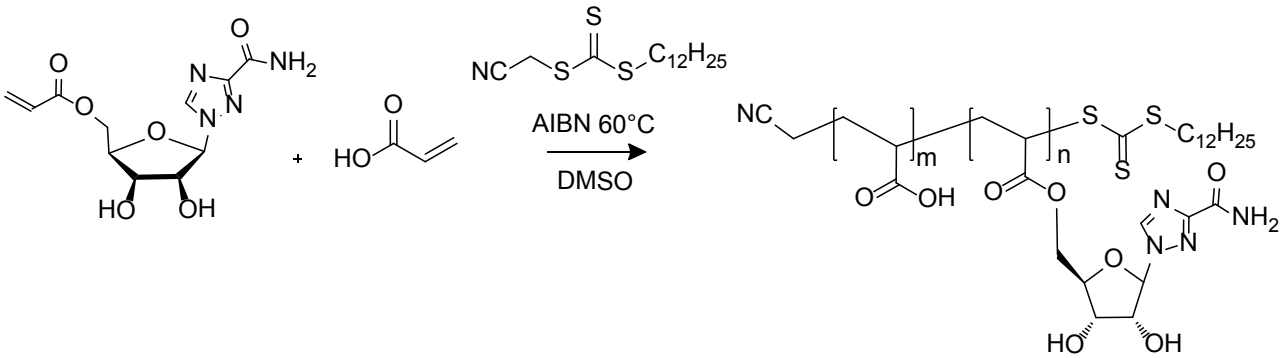
MATERIALS: All materials and chemicals were purchased from Sigma-Aldrich, unless stated otherwise, and used as received without purification. Trypsin-EDTA 0.05% was purchased from Invitrogen. High-purity water with a resistivity of 18.2 MΩ/cm was obtained from an in-line Thermo scientific, Barnstead Easypure II.

METHODS: Flow cytometry was performed using a BD Accuri® C6 flow cytometer using an excitation wavelength of 530 nm. In each case at least 1000 events were analyzed. Fluorescence measurements were conducted using an Enspire Perkin Elmer plate reader. Hep G2 (human liver carcinoma cell line) were cultured at 37°C with 5% CO_2, in full cell culture medium contain Minimum Essential Medium Eagle (MEME) supplemented with Fetal Bovine Serum (FBS, 10%), penicillin streptomycin (P/S, 1%), Non-Essential Amino Acids (NEA, 1%), L-Glutamine (2 mM). RAW264.7 (murine monocyte macrophage cell line) was cultured at 37°C with 5% CO_2, in full cell culture medium containing: Dulbecco's Modified Eagle's Medium (DMEM), FBS (10%) and P/S (1%). NMR spectra were obtained with a Varian Mercury 400 NMR spectrometer on samples dissolved in deuterated chloroform, unless stated otherwise. Chemical shifts are reported in ppm from external tetramethylsilane. MS-ESI: Mass spectra were obtained with a Bruker maXis Impact. Gel Permeation Chromatography (GPC) was performed on a system comprising a LC-20AD Shimadzu HPLC pump, a Shimadzu RID-10A refractive index detector and a DAWN HELEOS 8 static light scattering detector, equipped with a HEMA-Bio Linear column with 10 μm particles, a length of 300 mm and an internal diameter of 8 mm from MZ-Analysentechnik providing an effective molecular weight range of 1000−1.000.000. The eluent was 0.1 μm filtered MilliQ water at a flow rate of 1 mL/min with 300 ppm sodium azide.

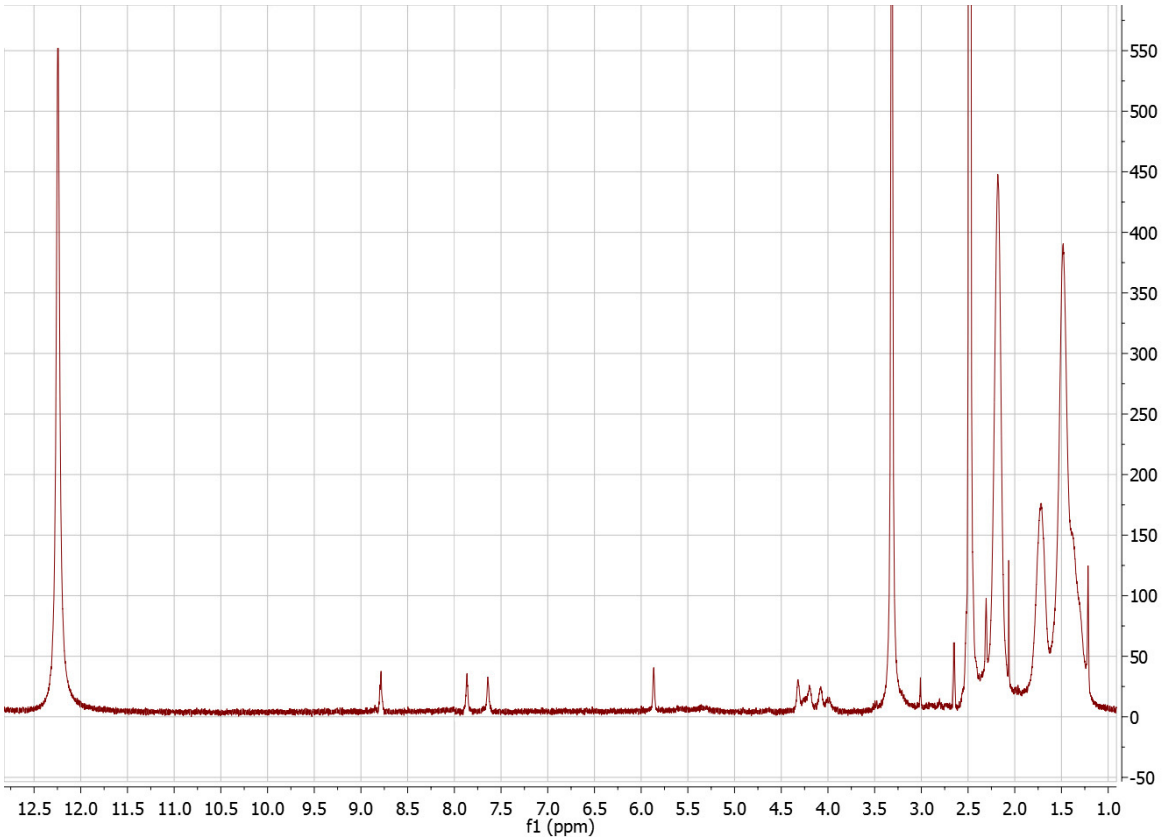
SYNTHESIS OF RBV ACRYLATE:

Via an adopted method[1] RBV acrylate was synthesized using a chemi-enzymatic approach with ribavirin (TCI, 3.0 g, 12 mmol), enzyme beads (Novozyme 435, 3.0 g) and a few crystals of di-tertbutyl methylphenol suspended in 150 mL dioxane. Acetoneoxime acrylate (6.75 g, 48 mmol) was added dropwise and the reaction was stirred at 50°C for 32 h. 500 mL MeOH was added and the mixture was filtered and reduced *in vacuo* followed by precipitation into diethyl ether. The precipitate is washed with 2x 50 mL pentane to yield RBV-5-O-acrylate as a white powder (1.2 g, 4.1 mmol, 34%).). ^{1}H-NMR (DMSO-d6), δ(ppm): 8.80 (s, 1 H, -N=CH-), 7.81 (s, 1 H, -NH2), 7.61 (s, 1 H, -NH2), 6.32 (dd, 1 H, J = 4 Hz, J = 16 Hz, CH2=), 6.13 (dd, 1 H, J = 12 Hz, J = 16 Hz, CH2=), 5.90 (dd, 1 H, J = 4 Hz, J = 12 Hz, J = 4 Hz, =CH-), 5.88 (s, 1 H, N-CH-O), 5.65 (s, 1 H, 3'-OH), 5.38 (s, 1 H, 2'-OH), 4.25 (m, 5 H, -CH2-, -CH-O, -CH-O). ^{13}C-NMR (DMSO-d6), δ(ppm): 165.71 (C=O), 160.75 (C3), 158.01 (C6), 145.90 (C5), 132.39 (CH2=), 128.40 (=CH-), 91.87 (1'C), 81.85 (4'C), 74.59 (2'C), 70.80 (3'C), 64.48 (5'C). MS-ESI: 298.0913.

SYNTHESIS OF PAA-RBV-FLUORESCEIN:



A schlenk tube was charged with Cyanomethyl dodecyl trithiocarbonate (12.5 mg, 0.039 mmol), AIBN (1.6 mg, 0.01 mmol), 1 eq. fluorescein acrylate (15.2 mg, 0.039 mmol), RBV-acrylate (206 mg, 0.703 mmol), acrylic acid (1.00 g, 13.8 mmol) and 3 mL DMF. The mixture was degassed by three freeze-pump-thaw cycles and polymerized for 7.5 h at 60°C. The conversion was determined by NMR to be 68%. The reaction mixture was precipitated into diethylether, redissolved in MeOH, and precipitated into diethylether again to yield 670 mg of the polymer. Mn from conversion was 17 kDa. Mn from GPC is 27 kDa with a PDI of 1.16 and a RBV-acrylate content of 4.5 mol%.



H^1 NMR ((CD3)2SO), δ (ppm):): 12.25 (bs, 1H, COOH), 8.80 (bs, 1 H, -N=CH-), 7.81 (bs, 1 H, -NH2,), 7.61 (bs, 1 H, -NH2) , 5.88 (bs, 1 H, N-CH-O), 4-4,4 (bm, (m, 5 H, -CH2-, -CH-O, -CH-O, -CH-)), 2.0-2.3 (bs, 1H, PAA polymer backbone), 1.25-1.75 (broad signal, 2H, PAA polymer backbone). RBV content is calculated from the ratio of the 8.80 ppm RBV signal to the polymer backbone.

SYNTHESIS OF PAA-FLUORESCEIN:

A schlenk tube was charged with Cyanomethyl dodecyl trithiocarbonate (13.2 mg, 0.042 mmol), AIBN (0.7 mg, 0.004 mmol), 1 eq. fluorescein acrylate (16.1 mg, 0.042 mmol), acrylic acid (1.00 g, 13.8 mmol) and 1 mL DMF. The mixture was degassed by three freeze-pump-thaw cycles and polymerized for 3 h at 65°C. The conversion was determined by NMR to be 63%. The reaction mixture was precipitated into diethylether, redissolved in MeOH, and precipitated into diethylether again to yield 506 mg of the polymer. Mn from conversion was 14 kDa. Mass from GPC-LS 19 kDa with a PDI of 1.09.

	RBV feed (%)	Conversion (% RBV)	Conversion (% AA)	RBV (mol%)	Mn (kDa)	PDI	Fluorescence (a.u.)
PAA	5	70	68	4.5	27	1.16	2.3
	0	---	63	---	19	1.09	2

CELL UPTAKE: Hep G2 cells (5E4 cells/ well) and RAW264.7 macrophages (2E4 cells/ well) were seeded in 96-well plates for pre-incubation in 24 h. Then, media was changed (90 µL) and substrates (10 µL) were added in different concentrations and incubated (24 h). Following incubation, the cells were trypsinized using Trypsin-EDTA 0.05% and analyzed by flow cytometry.

RBC ASSOCIATION: Human red blood cells obtained from Skejby Hospital blood bank were washed and mixed with cryopreservative (54.7 mM glycerol, 50 mM NaPO3, 0.37 mM NaCl)[2] and stored at -80°C. RBC were thawed in a 37°C water bath and subsequently washed with PBS (3x 10 mL, 5 min., 800 rpm). Then substrates (5 µL) were added to the RBC solution (45 µL) in eppendorf tubes and the samples were incubated overnight while shaken (37°C, 300 rpm). Blank: untreated cells. Negative control: cells with PBS. Positive control: 50% Milli-Q water. After incubation, the samples were washed gently with PBS (1x 1000 µL, 5 min., 800 rpm) and the supernatant was removed and the washed RBCs were diluted in PBS (1:2000 µL PBS) and analyzed by flow cytometry.

NITRIC OXIDE INHIBITION: For NO inhibition experiments RAW264.7 cells were seeded at a density of 2E4 cells/well in 100 µL media in 96-well plates. After 1 h respective concentrations of polymer and L-N^G-nitroarginine methyl ester (L-NAME) were added. Following incubation (24 h) media was renewed and cells were stimulated through the addition of 1 µg/mL lipopolysaccharide (LPS, E. coli 026:B6). After incubation (24 h) relative nitric oxide levels were determined by measuring nitrite levels through the Griess assay. In short, 50 µL media was transferred to a new 96-well plate and 50 µL sulfanilic acid (10 g/L, 5% phosphoric acid) were added. After incubation (5 min.) 50 µL N-1-napthylethylenediamine dihydrochloride (1 g/L) was added and absorbance was measured using EnSpire Multimode Plate Reader (548 nm). The nitrite levels were quantified against a freshly-prepared sodium nitrite standard curve and normalized within each experiment against the negative control consisting of LPS stimulated cells without the addition of any reagent/polymer.Viability of the cells was measured by quantifying metabolic activity through the PrestoBlue assay (Invitrogen) according to the manufacturer's instructions, and absorbance was determined by EnSpire Multimode Plate Reader (450 nm). Each experiment was normalized against a negative control where no reagent/polymer has been added to the cells. Positive control: 20% DMSO.

STATISTICAL ANALYSIS: Student's T-test was applied to determine statistical significance using Excel software. One-tailed unpaired t-test with 95% confidence interval was considered statistical significant if $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

1. F. Moris and V. Gotor, *The Journal of Organic Chemistry*, 1993, **58**, 653-660.
2. S. Henkelman, G. Rakhorst, J. Blanton and W. van Oeveren, *Materials Science & Engineering C-Biomimetic and Supramolecular Systems*, 2009, **29**, 1650-1654.