Water-soluble withaferin A polymer prodrugs via a drug-functionalized RAFT CTA approach

WATER-SOLUBLE WITHAFERIN A POLYMER PRODRUGS VIA A DRUG-FUNCTIONALIZED RAFT CTA APPROACH

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Abstract

To cope with the poor aqueous solubility of withaferin A, a broad-spectrum anti-cancer agent effective against therapy-resistant cancers, a polymer-drug conjugate was synthesized via a grafting-from-drug approach. Modification of withaferin A at the C27-OH with a chain transfer agent (CTA) for reversible addition-fragmentation chain transfer (RAFT) polymerization through a degradable ester bond was achieved with excellent control on the regioselectivity via a PFP-ester transesterification route. Subsequent RAFT polymerization with the hydrophilic N,N-dimethylacrylamide yielded a fully water-soluble conjugate. A decreased cytotoxicity *in vitro* indicated that withaferin A exists as a prodrug in the conjugate form and requires hydrolysis of the ester to regain its activity.

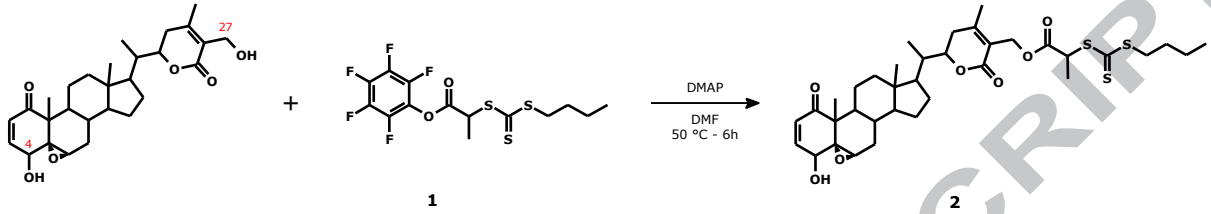
1 Introduction

Altering solubility and pharmacokinetics through chemical conjugation is a powerful method that is actively pursued for the formulation of both low and high molecular weight drugs.[1–4] Withaferin A is a natural steroidal lactone with anti-cancer properties derived from the medicinal plant Withania somnifera. A growing body of evidence has implicated the efficacy of WA to suppress the growth of various human cancer xenograft tumours.[5–7] Recently, withaferin A was shown to eradicate high-risk neuroblastoma by induction of ferroptosis through a double –edged mechanism.[8] Withaferin A is hydrophobic and notoriously poorly soluble in aqueous medium. Previously, we have succeeded in solubilizing withaferin A in the hydrophobic cavity of block copolymer micelles, albeit at a moderate concentration compared to other types of hydrophobic drugs (such as e.g. paclitaxel and amphotericin) reported by us and others.[9–11] Moreover, withaferin A loading in micelles required the use of relatively high volume fraction of organic solvents such as acetone and THF which pose translational challenges with regard to complete removal. Moreover, whereas physical encapsulation of hydrophobic molecules into self-assembled structures is a viable strategy to enhance drug dissolution, it is often incapable to alter the pharmacokinetic profile of the drug due to premature drug release upon dilution in a protein-rich environment such as the blood circulation.[3,12,13] Therefore, conjugation through a linker that can be cleaved under physiologically relevant conditions would be of interest. In this paper we report on such strategy by designing withaferin A-polymer prodrug conjugates by selectively addressing a hydroxyl group of withaferin A and conjugating this to a chain transfer agent (CTA) for reversible addition-fragmentation chain transfer (RAFT) polymerization. Such grafting-from-CTA approach allows for direct growth of a polymer chain from a drug molecule and has the major advantage of avoiding post-polymerization purification issue with regard to separating modified and un-modified drug.[11,14–16]

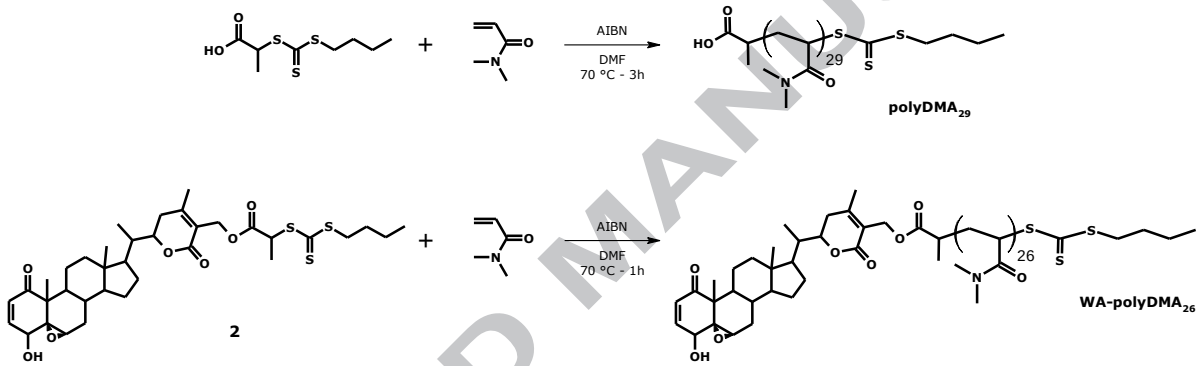
2 Results and discussion

The synthesis was started with the conjugation of withaferin A to the RAFT CTA, 2-(butylthiocarbonothioylthio)propanoic acid (PABTC), through a degradable ester bond. The choice for an ester bond was fostered by our previous work [14] on paclitaxel-polymer prodrug conjugates that showed highly efficient conversion of the polymer prodrug in inactive from into an active drug upon cellular uptake. As shown in **Scheme 1**, the ester linkage was obtained through a transesterification reaction with the pentafluorophenyl ester derivative of PABTC (**1**). This route was chosen since the common approach toward ester synthesis from carboxylic acids and hydroxyl groups using carbodiimide coupling reagents resulted in a substantial amount of regioisomers, conjugation at C27-OH or C4-OH, and bifunctional product (**Figure 1A**). Earlier reports mention that the biological activity of withaferin A was best preserved when modification was performed at the C27-OH position,[17] hence this was also our preferred route. Due to the lower reactivity of the pentafluorophenyl ester (PFP-ester) towards transesterification compared to carbodiimide routes, the reactivity of the hydroxyl group plays a key role in the conjugation reaction, resulting in a higher selectivity and preferential formation of the primary hydroxyl C27-OH adduct.[18] Via this route the C27-OH modified withaferin A CTA (WA-CTA, **2**) was obtained in excellent yield (83 %) as confirmed by NMR spectroscopy and mass spectrometry (**Figure 1B** and **Figure S4 and S5**). The regioselective modification at the C27-OH is evidenced by the proton shift at C27 from 4.36 ppm to 4.94 ppm upon esterification of the hydroxyl group whereas the C4-H proton (3.75 ppm) does not shift. Additional proof for the regioselectivity is given when compared to the spectrum for the C4-derivative (**Figure 1B**). When an ester is formed from the hydroxyl at the C4 position, a shift is observed for the C4-H proton from 3.75 ppm (d; J = 5.9 Hz, 1H) to 4.72 ppm (t, J = 6.1 Hz, 1H). In addition, the coupling of the C2 and C3 protons is altered from a doublet (J = 10 Hz) to a triplet (J = 9.5 Hz) for C2-H and from a dd (J = 10 Hz, 5.8 Hz) to a ddd (J = 9.7, 6.0, 3.3 Hz) for C3-H.

Scheme 1. Reaction scheme for the synthesis of withaferin A functionalised RAFT chain transfer agent (WA-CTA).



Scheme 2. Synthesis of DMA polymers by RAFT polymerisation.



The obtained C27-OH modified WA-CTA (**2**) was used for the RAFT polymerisation of N,N-dimethylacrylamide (DMA) to yield WA-polyDMA polymer-drug conjugate (**Scheme 2**). DMA was used as a hydrophilic monomer. Our choice for DMA is fostered by its relatively low molar mass compared to other hydrophilic (meth)acrylates and (meth)acrylamides, hence resulting in a relatively high drug load. Moreover, multiple studies have reported on the excellent biocompatibility and low immunogenicity of polyDMA.[19] A final chain length of 30 monomer units was targeted, based on prior research from our labs on polymeric prodrugs of the extremely hydrophobic anti-cancer drug paclitaxel, which pointed at 30 repeating units to be sufficient to endow the resulting structure with excellent water-solubility.[14,20] As a control polyDMA$_{29}$ was synthesized using non-functionalized PABTC as CTA. Polymer characterization is summarized in **Table 1**. An optimized protocol was used for the synthesis of WA-polyDMA as the method utilised for polyDMA synthesis, which is stopped at high conversion (>90%), resulted in a deviation towards high molar mass polymers in the molar mass distribution profile (**Figure S8**). During polymerization a side

reaction could occur due to the presence of an α,β-unsaturated carbonyl group in the C4 ring of withaferin A that is prone to nucleophilic attack[21] and shows resemblance to an acryloyl moiety. To avoid attack of active radical species on this position of withaferin A, the polymerisation was set with a monomer to CTA ratio of 44:1 and stopped at rather low monomer conversion (60 %) to ensure that polymerization of DMA would favour over the aforementioned side reaction (**Figure S8 and S9**). Of note, also the use of a low temperature azo-initiator could be considered to supress side reactions.

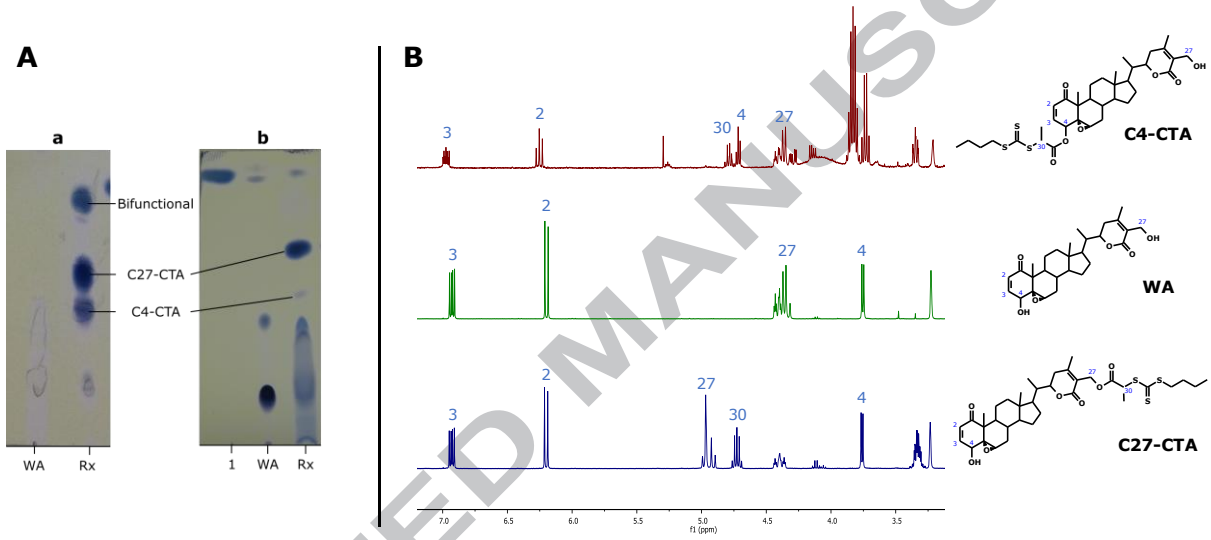


Figure 1. Regioselectivity in the synthesis of WA-CTA via carbodiimide (DIC) route or PFP-ester. **A**) TLC plates of reaction mixture via DIC coupling (a) or via PFP-ester CTA **1** (b), compounds are visualised with Hanessian's Stain. **B**) ^{1}H NMR spectra of the purified regioisomers. C4-CTA (red), WA (green) and C27-CTA (blue). A zoom region and annotation of the most relevant peaks for differentiation are given together with the corresponding molecular structures.

Table 1. Overview of polymer characterisation results from SEC and DLS analysis

Polymer	DP	M_n^{theor} (kDa)	M_n^{SEC} (kDa)	Đ	wt% WA	size (nm)	PdI
polyDMA$_{29}$	29	3.1	2.0	1.23	-	2.2 ± 0.8	0.24 ± 0.002
WA-polyDMA$_{26}$	26	3.3	3.4	1.13	14	4.2 ± 0.4	0.68 ± 0.203

M_N value is the number average molar mass calculated from either DP (M_N^{theor}) or by SEC analysis (M_N^{SEC}).

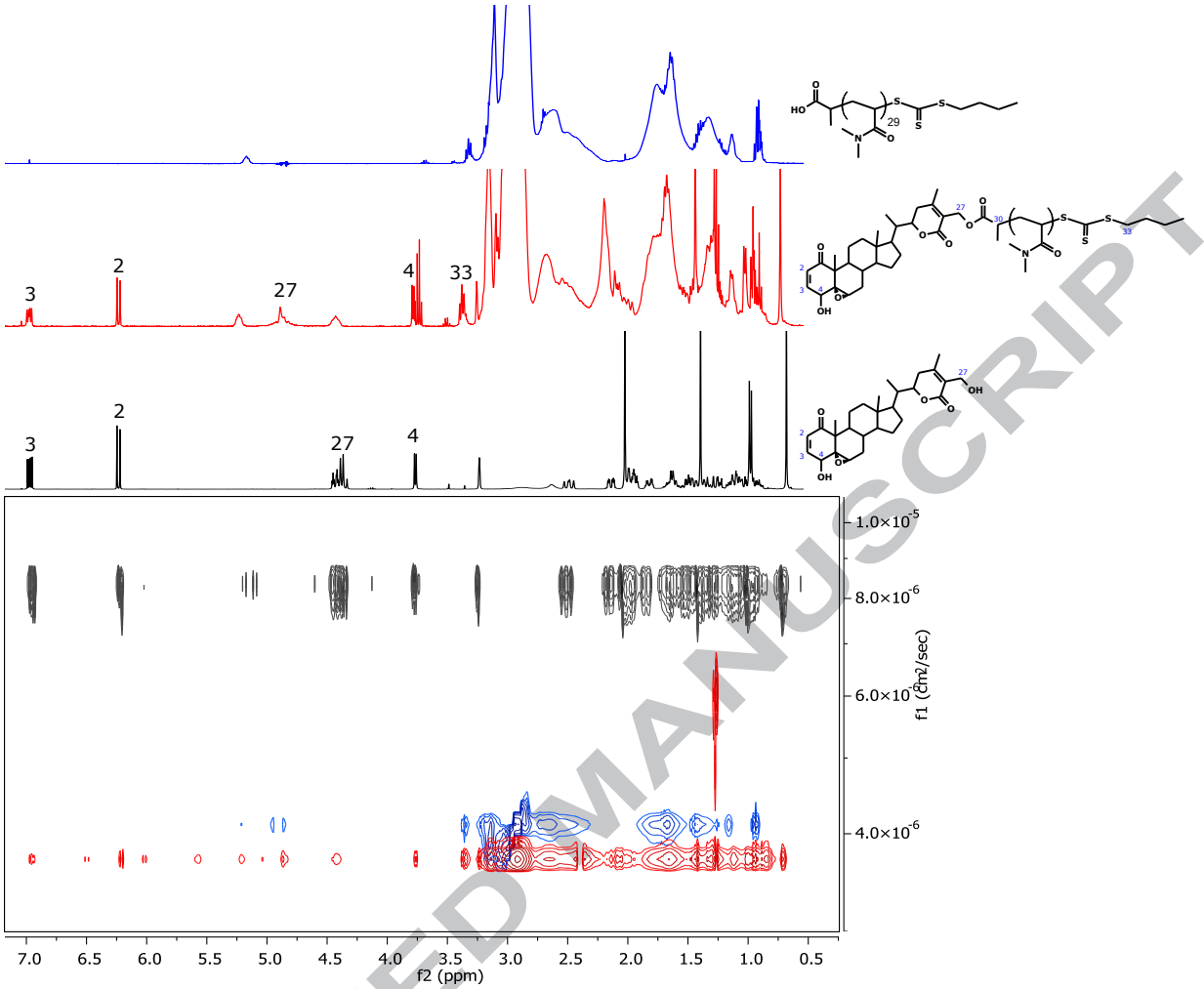


Figure 2. ^{1}H and DOSY NMR (400 MHz, $CDCl_3$) analysis of WA (black), WA-polyDMA$_{26}$ (red) and polyDMA$_{29}$ (blue).

Polymer analysis by size exclusion chromatography (SEC) indicated that narrowly dispersed polymers were obtained (**Figure 3A** and **Table 1**). A detailed NMR analysis via ^{1}H and DOSY NMR was performed to characterise the WA-polyDMA conjugate on end-group fidelity (**Figure 2**). Integration of WA peaks at the α-chain end (C2-H and C3-H, both 1H) and the CTA at the ω-chain end (C33-H, 2H) in the ^{1}H NMR spectrum (**Figure 2** and **Figure S6 and S9**) of WA-polyDMA$_{26}$ indicated that the withaferin A functionalisation is maintained during polymerisation. DOSY NMR analysis of withaferin A, polyDMA$_{29}$ and WA-polyDMA$_{26}$ showed no detectable free withaferin A in the WA-polyDMA polymer conjugate (**Figure 2**). Furthermore, as no resonance signals were observed in de WA-polyDMA spectra having a similar diffusion rate as polyDMA$_{29}$, we concluded that virtually all WA-polyDMA$_{26}$ polymers contained withaferin A.

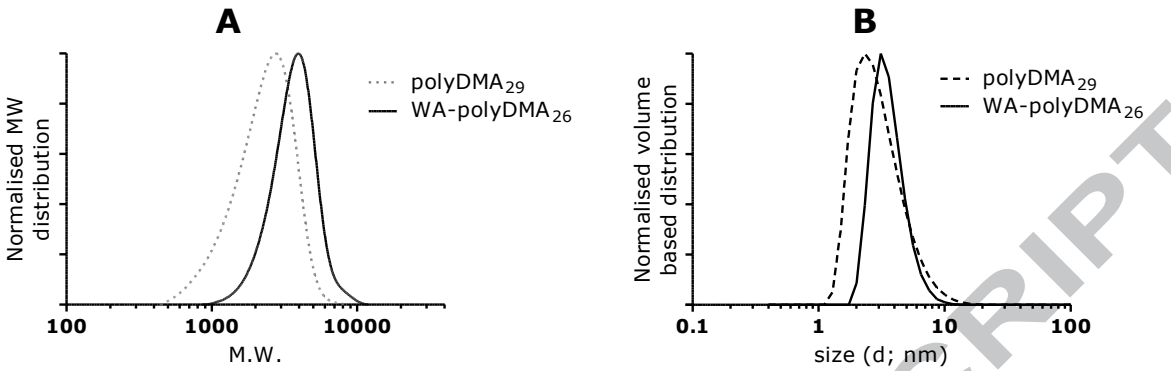


Figure 3. A) Molecular weight distribution profiles of both DMA polymers obtained by SEC analysis. **B)** Size distribution profiles of polyDMA$_{29}$ and WA-polyDMA$_{26}$ at 2 mg/mL in H_2O measured by DLS and presented as normalised values of volume based distribution.

Next, the behaviour of the polymer in aqueous media was assessed. Dynamic light scattering (DLS) analysis of the DMA polymers dissolved in water resulted in size distribution profiles and mean size values below 10 nm, this accompanied by very low scattering intensity values which are characteristic for soluble unimers (**Table 1** and **Figure 3B**). These observations allowed us to conclude that a water soluble withaferin A polymer conjugate was successfully obtained by polymerisation of DMA via a 'chain transfer from' WA approach.

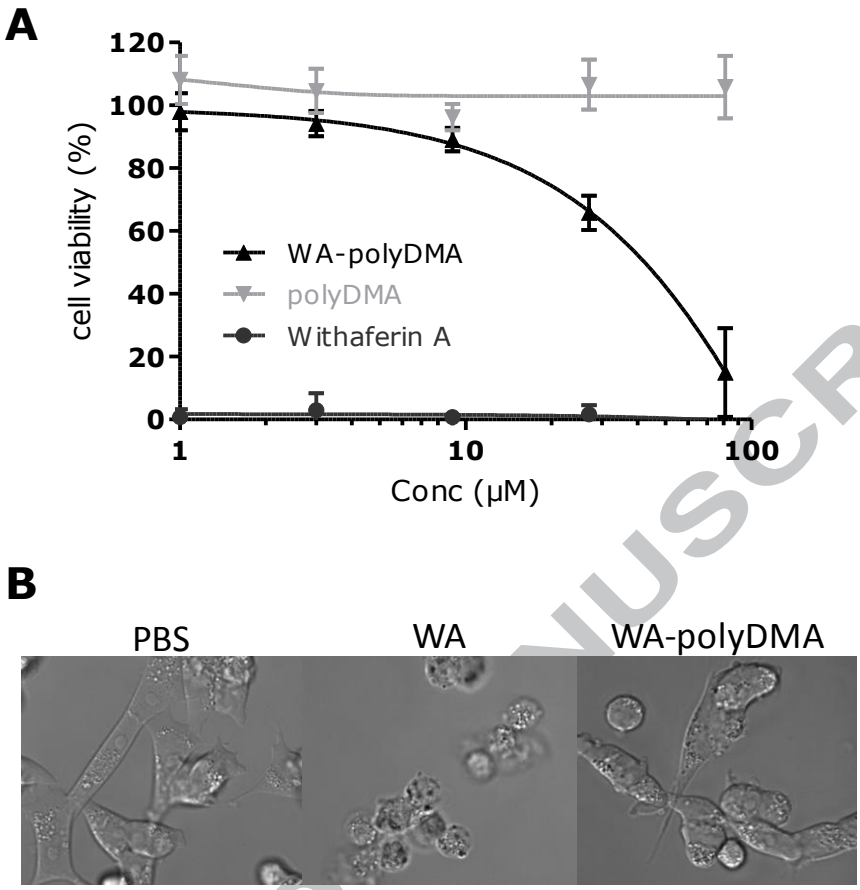


Figure 4. A) In vitro cytotoxicity of WA, WA-polyDMA and polyDMA in IMR-32 cells after 48 h incubation analysed by MTT assay. (n = 6). **B**) DIC images of IMR-32 cells after 30 h incubation with 50 μM of WA or WA-polyDMA or PBS as negative control, obtained by confocal microscopy.

Finally, we evaluated the *in vitro* cytotoxic activity of the WA polymer pro-drug against IMR-32 neuroblastoma cells by MTT assay.[8] In these experiments, we compared the performance of WA-polyDMA relative to withaferin A and non-functionalized polyDMA as a control. The results of the MTT assay after 48 h of drug-incubation are presented in **Figure 4A** and show that the WA-polyDMA construct was cytotoxic at higher concentrations. This property can be attributed to the biological effect of WA as the polyDMA control polymer did not give any toxicity within the tested experimental window. In comparison to unconjugated withaferin A, the potency of WA-polyDMA was significantly reduced. This can be due to either a lower extent of cellular uptake of WA-polyDMA, relative to WA in native form, or might indicate that covalent modification of withaferin A to a polymer yields an inactive prodrug, which becomes active after hydrolysis of the

ester linkage over time. The cytotoxic effect of withaferin A was also observed by the morphological changes of IMR-32 cells. Differential interference contrast (DIC) imaging was done after incubation with WA or WA-polyDMA (**Figure 4B**). In comparison to a PBS control, cells depict a clear difference in morphology when exposed to WA, evidenced by cell rounding and plasma membrane rupture. Although less pronounced, the start of this process could also be observed for cells treated with WA-polyDMA with the majority of the cells showing signs of plasma membrane rupture and some cell rounding. We attribute the lower biological activity of WA-polyDMA to the requirement for ester hydrolysis to liberate the WA in free form.

3 Conclusions

Summarizing, we have shown in this paper that site-selective modification of the hydrophobic anti-cancer drug withaferin A (WA) with a RAFT CTA can be achieved and used for the synthesis of a polymeric pro-drug. Using DMA as hydrophilic monomer, a dramatic increase in water-solubility relative to unformulated WA was achieved. *In vitro* cytotoxicity experiments showed a loss in activity of the polymer pro-drug, pointing at insufficient enzymatic or hydrolytic degradation of the ester bond between the WA and the polymer. Hence, this could open a window of opportunity to investigate other degradable spacers with higher susceptibility to intracellular degradation. These could include spacers that are susceptible to acid hydrolysis[20,22] or reduction[23], or ester spacers that yield better access to esterases. [24] Further improvements could include incorporation of a targeting moiety like anti-GD2 antibody[25] that specifically targets neuroblastoma.

4 Materials and methods

4.1 Materials

All chemicals and solvents were obtained from commercial sources and used as such unless otherwise noted. 2.2'-azobis(2-methylpropionitrile) (AIBN) as initiator was provided by WAKO Chemicals and purified by recrystallization from diethyl ether prior to use. *N,N*-dimethylacrylamide (Sigma Aldrich) was purified over inhibitor remover column prior to use. Withaferin A was provided to us by prof. dr. Wim Vanden Berghe. Dulbecco's phosphatebuffered saline (PBS), cell

culture media and supplements were obtained from ThermoFisher. IMR-32 cells were obtained from Jo Vandesompele, Ghent University Hospital, Medical Research Building, Ghent, Belgium.

4.2 Methods

Synthesis of Withaferin A RAFT CTA (WA-CTA, 2). To a stirred solution of PFP-CTA (**1**) (64.4 mg, 159 µmol) in 1 mL anhydrous DMF was added withaferin A (30 mg, 63.8 µmol) at room temperature followed by DMAP (1.56 mg, 12.7 µmol). The reaction was stirred at 50 °C for 6h protected from light, after which it was poured into EtOAc and washed multiple times with brine. The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude mixture was purified by silica column chromatography (Cylcohexane/EtOAc 1:1) to give 38 mg (86 %) of a yellow solid. ^{1}H NMR (400 MHz; $CDCl_3$, **Figure S4**) δ ppm: 6.93 (dd, *J* = 10.0, 5.8 Hz, 1H), 6.20 (d, *J* = 9.9 Hz, 1H), 5.03 – 4.85 (m, 2H), 4.73 (p, *J* = 7.3 Hz, 1H), 4.40 (ddt, *J* = 14.8, 13.1, 3.4 Hz, 1H), 3.76 (d, *J* = 5.8 Hz, 1H), 3.40 – 3.27 (m, 2H), 3.23 (t, *J* = 1.8 Hz, 1H), 2.56 – 2.46 (m, 1H), 2.15 (ddd, *J* = 14.8, 4.1, 2.5 Hz, 1H), 2.07 (d, *J* = 4.5 Hz, 3H), 2.04 – 2.01 (m, 1H), 2.01 – 1.91 (m, 2H), 1.83 (dd, *J* = 14.2, 3.8 Hz, 1H), 1.74 – 1.61 (m, 4H), 1.58 (d, *J* = 7.4 Hz, 3H), 1.52 (dd, *J* = 11.1, 4.0 Hz, 1H), 1.49 – 1.45 (m, 1H), 1.45 – 1.42 (m, 2H), 1.41 (s, 3H), 1.30 – 1.21 (m, 1H), 1.21 – 1.03 (m, 2H), 1.00 (d, *J* = 6.7 Hz, 3H), 0.93 (t, *J* = 7.3 Hz, 3H), 0.70 (d, *J* = 1.7 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$, **Figure S5**) δ ppm: 202.22, 170.97, 165.06, 157.54, 141.82, 132.21, 121.44, 78.24, 69.82, 63.80, 62.52, 59.21, 56.00, 51.93, 48.01, 47.61, 44.07, 42.53, 39.28, 38.71, 36.84, 31.10, 30.11, 29.84, 29.71, 26.84, 24.20, 21.99, 20.65, 17.37, 16.52, 13.52, 13.25, 11.55. m/z = 691.2835 $[M+H]^+$.

Synthesis of polyDMA$_{29}$. In a Schlenk tube N,N-dimethylacrylamide (115 mg, 1.16 mmol) and PABTC (9.22 mg, 38.7 µmol) were dissolved in 950 µL anhydrous DMF. AIBN (1.27 mg, 7.74 µmol) was added from a stock solution in DMF. The mixture was degassed by multiple freeze-vacuum-thaw cycles and immersed in a preheated oil-bath at 70 °C. After 3 h the reaction was quenched by cooling and exposure to air. A sample was taken before and after reaction and analysed by ^{1}H NMR to determine conversion. The polymer was purified by

repeated precipitation in cold diethylether to give 102 mg of a yellow solid. The purified polymer was analysed by SEC in DMAc.

Synthesis of WA-polyDMA$_{26}$. In a Schlenk tube N,N-dimethylacrylamide (115 mg, 1.16 mmol) and WA-CTA (**2**) (20 mg, 28.9 µmol) were dissolved in 950 µL anhydrous DMF. AIBN (0.95 mg, 5.8 µmol) was added from a stock solution in DMF. The mixture was degassed by multiple freeze-vacuum-thaw cycles and immersed in a preheated oil-bath at 70 °C. After 1 h the reaction was quenched by cooling and exposure to air. A sample was taken before and after reaction and analysed by ^{1}H NMR to determine conversion. The polymer was purified by repeated precipitation in a cold hexane/diethylether (1:1) mixture to give 79 mg of a yellow solid. The purified polymer was analysed by SEC in DMAc.

Dynamic light scattering. Polymer aggregation was analysed by dynamic light scattering (DLS). Polymers were dissolved in H_2O at 2 mg/mL aided by vortex. Measurements were done at 25 °C in triplicate.

In vitro cell culture. IMR-32 cells were cultured in RPMI-glutamax, supplemented with 10 % FBS, 1 % penicillin/streptomycin and 1 mM sodium pyruvate at 37 °C in a controlled, sterile environment of 95 % relative humidity and 5 % CO2.

MTT assay. *In vitro* cytotoxicity was assessed by MTT assay. Samples were tested for a withaferin A concentration ranging from 1 µM to 81 µM. Dilution series for polyDMA$_{29}$ and WA-polyDMA$_{26}$ were made from a stock solution in water, starting at 1.215 mM with 3-fold dilutions in sterile water. Due to poor water solubility of withaferin A, a dilution series was made in DMSO, starting at 12.15 mM with 3-fold dilutions in DMSO. IMR-32 cells were seeded in 96-well cell culture plates (20 000 cells per well in 140 µL culture medium) and incubated overnight. Next, cells were pulsed with 10 µL sample for polyDMA$_{29}$ and WA-polyDMA$_{26}$, with 1 µL sample + 9 µL H_2O for WA , 10 µL H_2O (negative control) or 100 µL DMSO (positive control) and incubated for 48 h at 37 °C. Cells were pulsed with 40 µL of 1 mg/mL MTT reagent dissolved in culture medium and incubated for 1 h at 37 °C. The formed formazan crystals were dissolved by addition of 100 µL 10% m/v SDS/0.01 M HCl solutions and incubated overnight. Quantification was done by measuring the absorbance at 590 nm using a microplate reader.

DIC imaging. IMR-32 cells were seeded on Willco-Dish glass bottom dishes (190 µL of 100 000 cells/mL) and incubated overnight. Next, cells were pulsed with WA-polyDMA$_{26}$ or WA reaching concentration of 50 µM WA or with sterile water as control and incubated at 37 °C. After 30 h microscopy images were collected.

APPENDIX A: SUPPLEMENTARY MATERIAL

Supplementary data associated with this article can be found in the online version.

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

Keywords

Withaferin A

Polymer conjugate

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SUPPORTING INFORMATION

5 Instrumentation

All ^{1}H-, ^{13}C-NMR spectra and 2D-NMR spectra were recorded on a Bruker 300 MHz, 400 MHz or 500 MHz FT NMR spectrometer. Chemical shifts (δ) are provided in ppm relative to TMS. Samples were prepared in given deuterated solvents and their signals referenced to residual non-deuterated signals of the solvent.

ESI-MS was performed on a Waters LCT Premier XETM Time of flight (TOF) mass spectrometer equipped with a standard electrospray ionization and modular

LockSpray TM interface. The purity of the products was assessed by HPLC and PDA detection (190-400 nm) using a reverse phase column (Phenomenex Luna 3 µm C18(2), 100 Å, 200 mm) with a linear gradient of 10-100 % B over 9 min, where A is 0.1 % formic acid in H_2O and B is 0.1 % formic acid in CH_3CN at a flow rate of 0.4 mL/min.

Molar mass distribution analysis were performed by size exclusion chromatography (SEC) measurements on a Shimadzu 20A system in N,N-dimethylacetamide (DMAc) as solvent containing 50 mM LiBr. The system was equipped with a 20A ISO-pump and a 20A refractive index detector (RID). Measurements were recorded at 50 °C with a flow rate of 0.700 mL/min. Calibration of the 2 PL 5 µm Mixed-D columns was done with poly(methyl methacrylate) (PMMA) standards obtained from PSS (Mainz, Germany). Samples were run with toluene as an internal standard.

Dynamic light scattering (DLS) was performed on a Zetasizer Nano S (Malvern Instruments Ltd., Malvern, U.K.) equipped with a HeNe laser (λ = 633 nm) and detection at scattering angle of 173°. Cumulants analysis of the data gave the z-average and polydispersity index and data fitting by CONTIN the particle size distribution.

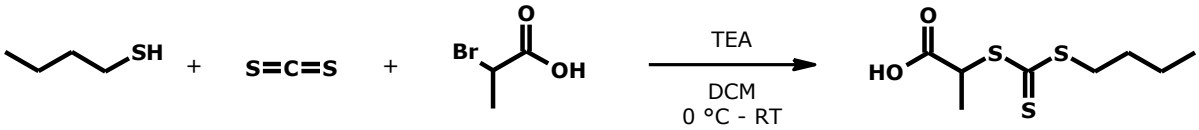
Cell culture plate readouts were collected on an Epoch 2 Microplate Spectrophotometer (Biotek, BioSPX, Drogenbos, Belgium).

Confocal microscopy images were taken by a Confocal Leica DMI6000 microscope coupled to an Andor DSD2 scanner and a Zyla5.5 CMOS camera. Two-dimensional cell cultures were imaged with a 1.40 B-NA, 63x oil immersion objective. Images were processed with ImageJ.

6 Synthesis methods

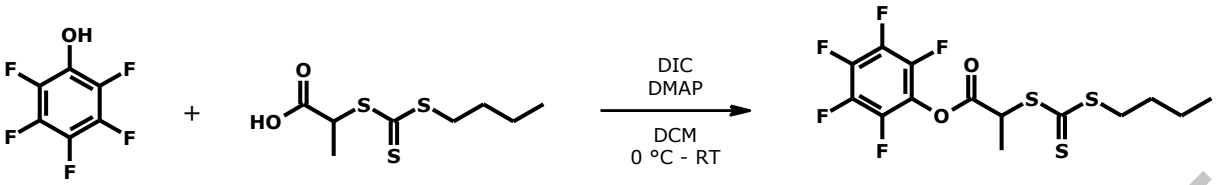
6.1 Synthesis RAFT CTA

Scheme S1. Synthesis of the RAFT CTA 2-(butylthiocarbonothioylthio)propanoic acid (PABTC).



Synthesis of 2-(butylthiocarbonothioylthio)propanoic acid (PABTC). The RAFT CTA 2-(butylthiocarbonothioylthio)propanoic acid (PABTC) was synthesized according to literature.[1] To 150 mL anhydrous DCM in a round bottom flask was sequentially added, 1-butanethiol (15 mL, 140 mmol) and trimethylamine (21.2 mL, 152 mmol) under inert atmosphere. The mixture was cooled on ice and carbon disulphide (9.15 mL, 152 mmol) dissolved in 150 mL anhydrous DCM was added dropwise under stirring and a distinct yellow color appeared. Once all reagent was added, the reaction mixture was allowed to warm to room temperature under continuous stirring. After 30 minutes, 2-bromopropanoic acid (13.7 mL, 152 mmol) dissolved in 75 mL DCM was added dropwise and the mixture was stirred for 2 h more. Next, the reaction mixture was concentrated, taken up in cyclohexane and extracted subsequently with 10 % HCl aqueous solution, deionized water and brine. All organic phases were collected and dried over Na_2SO_4 before being concentrated under vacuum. Recrystallization from hexane gave 21.7 g (65 %) of PABTC as yellow crystals. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 9.59 (br.s, 1H), 4.80 (q, *J* = 7.4 Hz, 1H), 3.31 (t, *J* = 7.5 Hz, 2H), 1.68 – 1.58 (m, 2H), 1.56 (d, *J* = 7.4 Hz, 3H), 1.44 – 1.29 (m, 2H), 0.87 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, CDCl3) δ ppm: 176.86, 47.55, 37.29, 30.02, 22.20, 16.73, 13.72. m/z = 239.061 $[M+H]^+$.

Scheme S2. Reaction scheme for the synthesis of pentafluorophenyl activated RAFT CTA (PFP-CTA, **1**).



1

Synthesis of pentafluorophenyl ester RAFT CTA (PFP-CTA, 1). In a round bottom flask PABTC (200 mg, 0.84 mmol), pentafluorophenol (169 mg, 0.92 mmol) and DMAP (10,2 mg, 83.7 µmol) were dissolved in 9 mL anhydrous DCM and cooled on ice. *N,N'*-diisopropylcarbodiimide (DIC) (116 mg, 0.92 mmol) was added and the reaction was stirred at room temperature for 3 h. Next, the reaction mixture was filtered, concentrated under reduced pressure and purified by column chromatography (DCM) to give 323 mg (95 %) of an orange oil. ^{1}H NMR (500 MHz; $CDCl_3$): δ ppm 5.11 (q, J = 7.48 Hz, 1H); 3.39 (t, J = 7.40 Hz, 2H); 1.77 (d, J = 7.48 Hz, 3H); 1.75 – 1.67 (m, 2H); 1.44 (dq, J = 14.9, 7.43 Hz, 2H); 0.95 (t, 7.3 Hz, 3H). ^{13}C NMR (400 MHz; $CDCl_3$): 167.92, 142.51, 142.51, 139.92, 139.92, 138.58, 136.81, 47.19, 37.39, 30.00, 22.20, 16.49, 13.71. ^{19}F NMR (470 MHz, $CDCl_3$): δ ppm -162, -157, -152. m/z = 422.127 $[M+H]^+$.

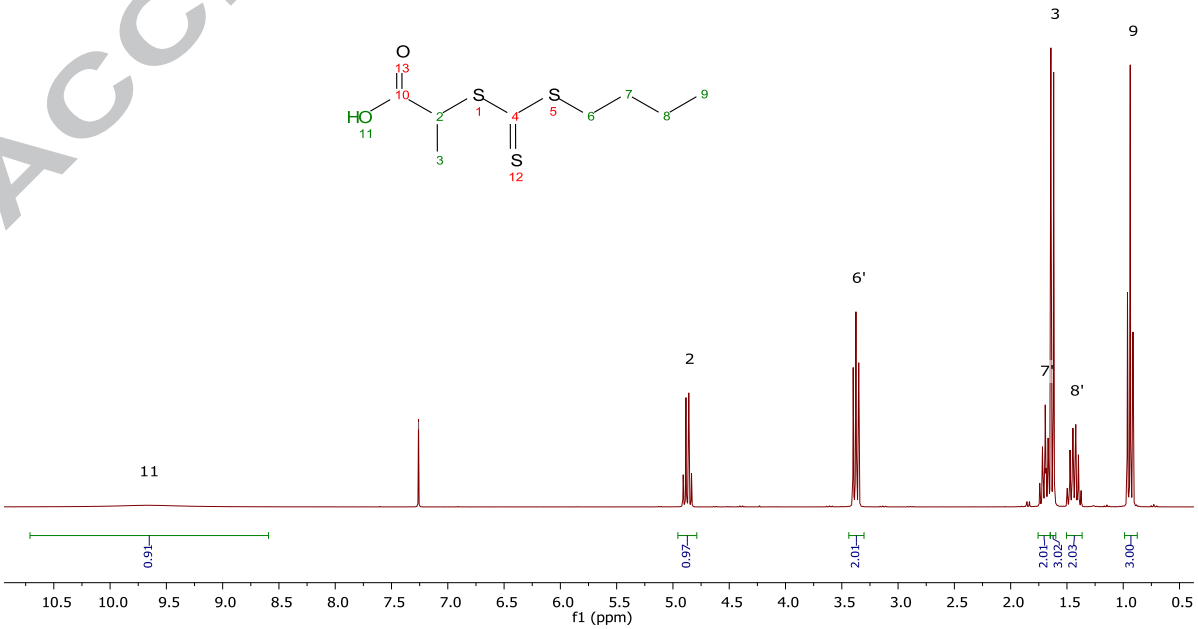


Figure S1. ^{1}H NMR (300 MHz, $CDCl_3$) of PABTC.

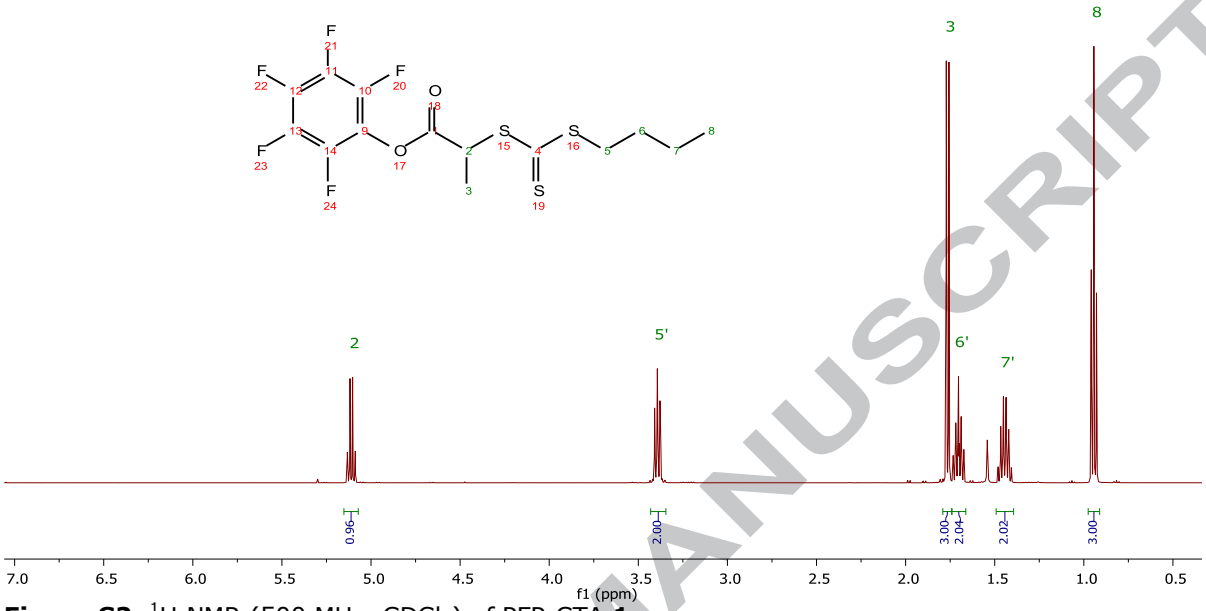


Figure S2. ^{1}H NMR (500 MHz, $CDCl_3$) of PFP-CTA **1**.

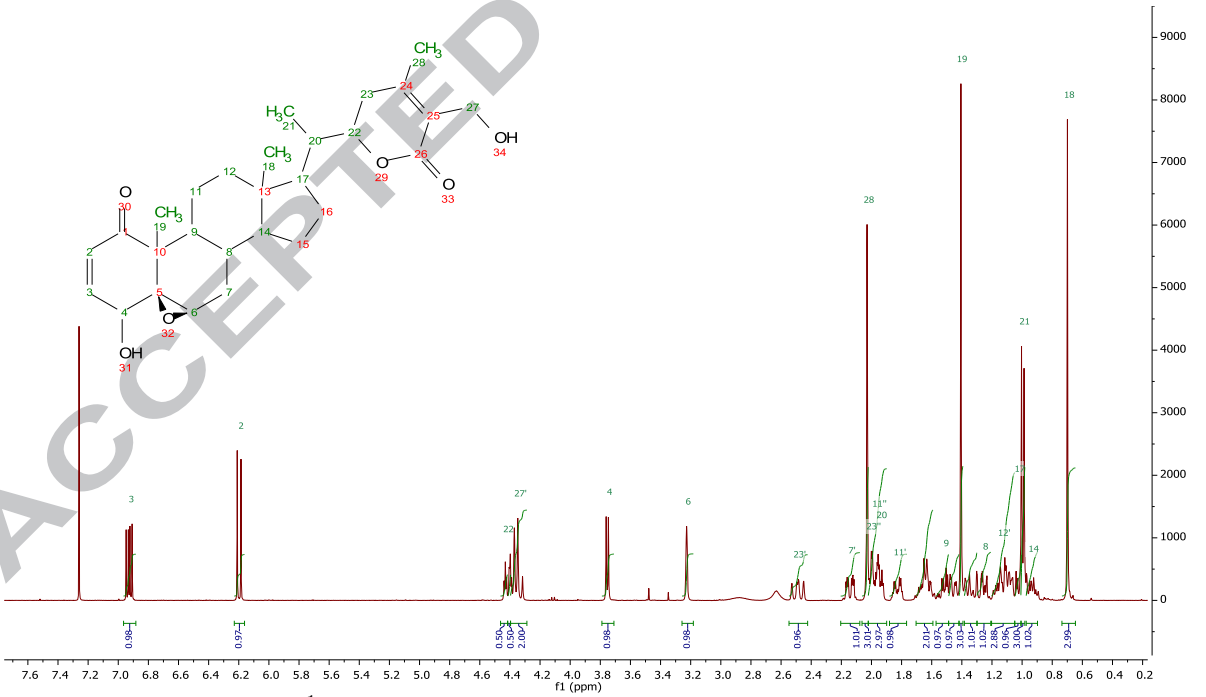


Figure S3. Withaferin A ^{1}H NMR (400 MHz; $CDCl_3$) δ ppm: 6.93 (dd, *J* = 10.0, 5.8 Hz, 1H, *3*), 6.20 (d, *J* = 10.0 Hz, 1H, *2*), 4.41 (dt, *J* = 13.2, 3.6 Hz, 1H, *22*), 4.36 (dd, *J* = 12.6, 9.2 Hz, 2H, *27*), 3.75 (d, *J* = 5.9 Hz, 1H, *4*), 3.23 (dd, *J* = 2.5, 1.5 Hz, 1H, *6*), 2.55 – 2.42 (m, 1H, *23'*), 2.14 (ddd, *J* = 14.8, 4.1, 2.5 Hz, 1H, *7'*), 2.03 (s, 3H, *28*), 2.02 – 1.99 (m, 1H, *23''*), 1.99 – 1.90 (m, 2H, *11'* + *20*), 1.83 (dq, *J* = 14.1, 3.7 Hz, 1H, *11''*), 1.70 – 1.59 (m, 2H), 1.51 (dd, *J* = 11.1, 4.0 Hz, 1H, *9*), 1.49 – 1.42 (m, 1H), 1.41 (s, 3H, *19*), 1.38 – 1.30 (m, 1H), 1.30 – 1.21 (m, 1H, *8*), 1.20 –

1.05 (m, 3H, *12*), 1.05 – 1.01 (m, 1H, *17*), 1.00 (d, *J* = 6.5 Hz, 3H, *21*), 0.97 – 0.90 (m, 1H, *14*), 0.70 (s, 3H, *18*). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm: 202.42, 167.14, 152.94, 142.04, 132.43, 125.83, 78.87, 70.03, 64.00, 62.68, 57.59, 56.20, 52.11, 47.82, 44.26, 42.72, 39.48, 38.92, 31.30, 29.96, 29.91, 27.41, 24.41, 22.28, 20.14, 17.55, 13.46, 11.75.

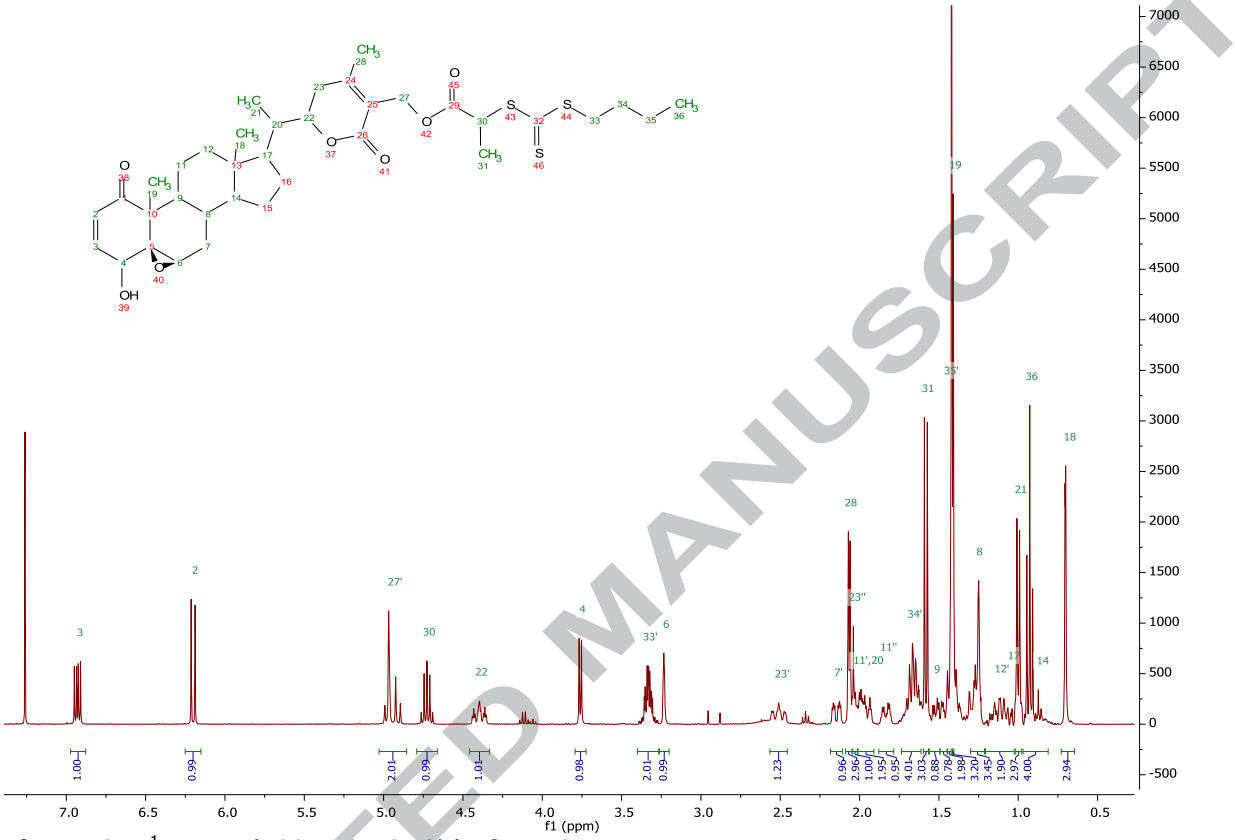


Figure S4. ^{1}H NMR (400 MHz; $CDCl_3$) of WA-CTA.

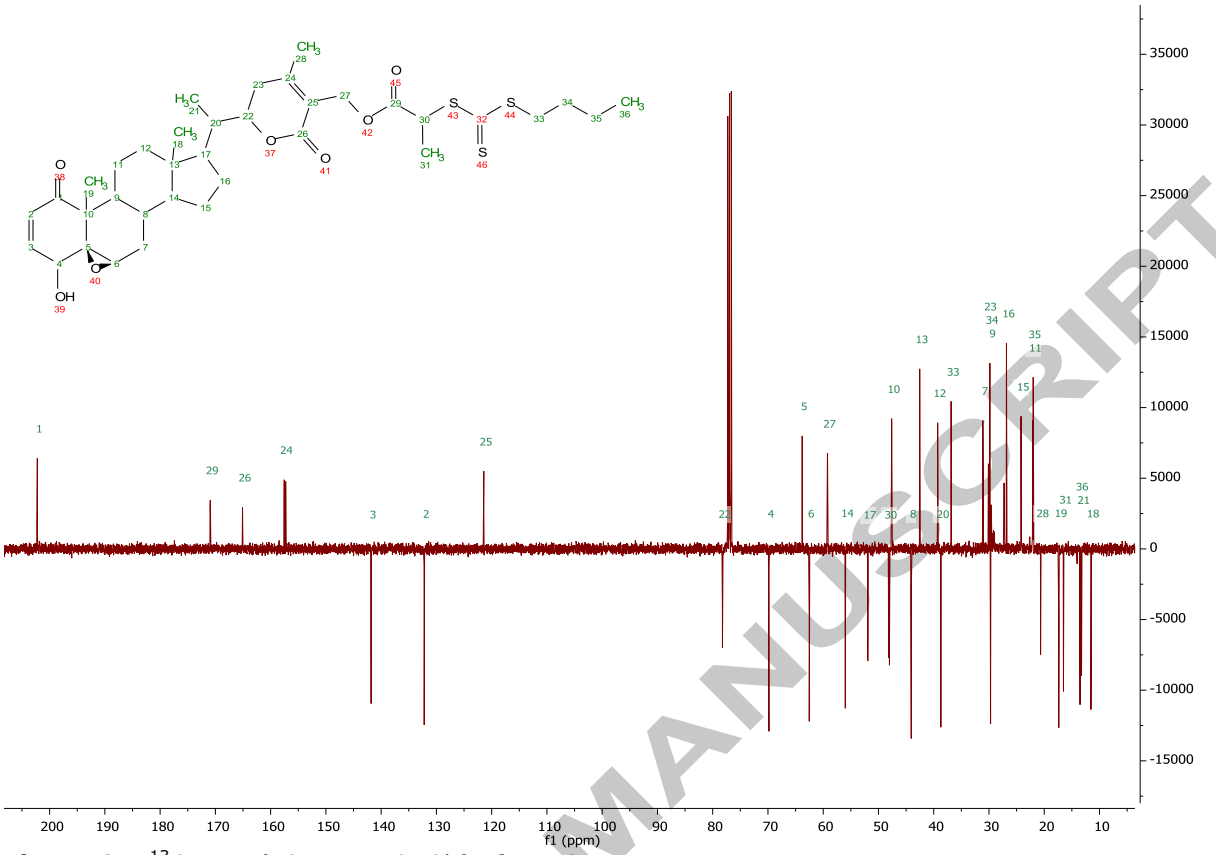


Figure S5. ^{13}C NMR (101 MHz, $CDCl_3$) of WA-CTA

6.2 RAFT polymers

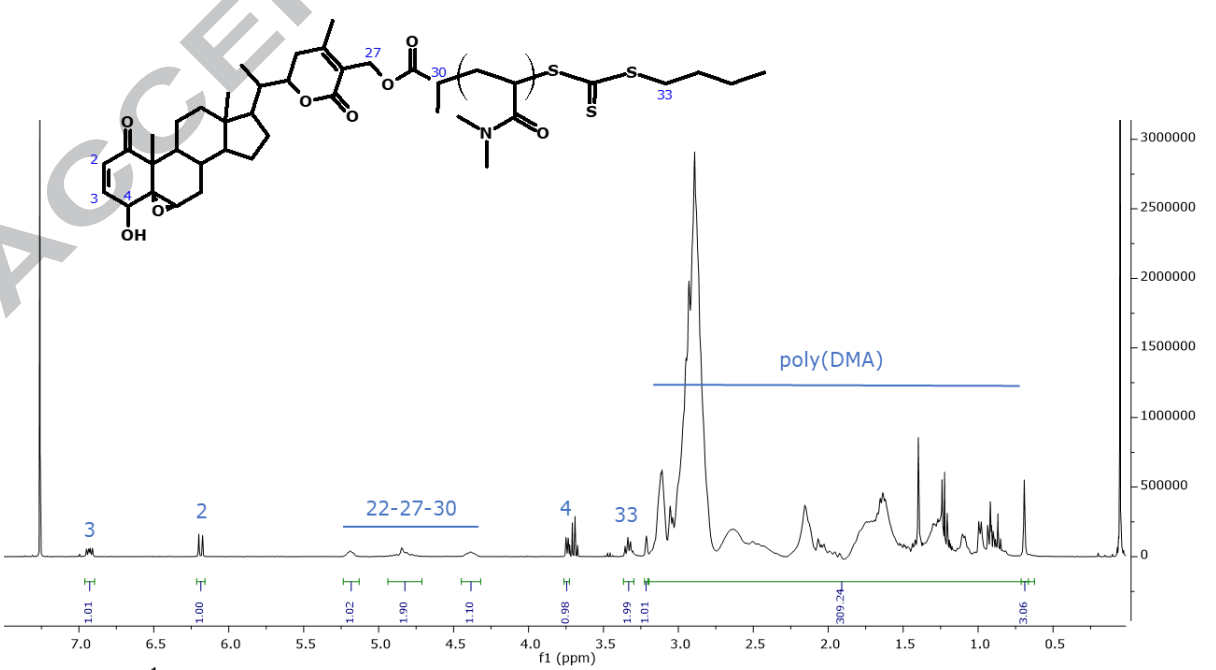


Figure S6. ^{1}H NMR (300 MHz; $CDCl_3$) of WA-polyDMA$_{26}$.

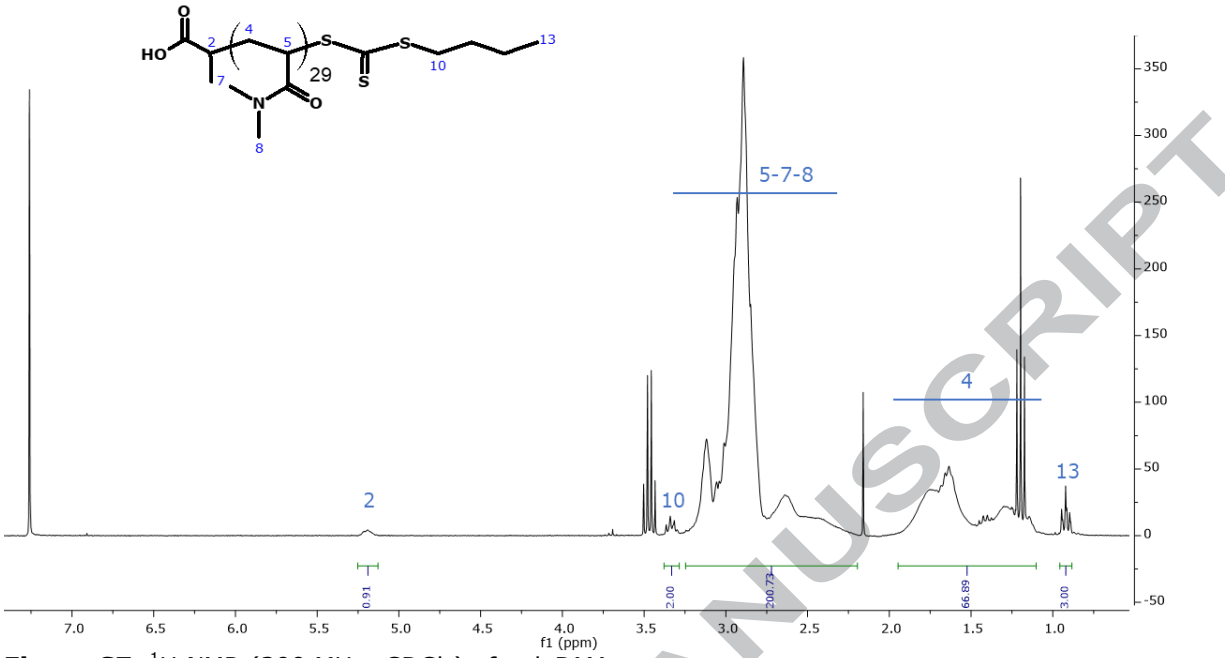


Figure S7. ^{1}H NMR (300 MHz; $CDCl_3$) of polyDMA$_{29}$.

6.3 Polymerisation conditions analysis

In the next part additional data is presented on the method development for RAFT polymerisation of WA-polyDMA. The results from SEC (**Figure S8**) and ^{1}H NMR (**Figure S9**) analysis are compared for a polymerisation stopped at high conversion (90 %, **P$_{90}$**) and the optimised conditions with lower conversion (60 %, **P$_{60}$**). The deviation at high molar mass in the distribution profile for **P$_{90}$** is an indication for poor control over the polymerisation. Since this was not observed for DMA polymerisation with PABTC (97 % conversion, **Figure 3A**) it could be attributed to the participation of WA in the polymerisation at high conversion, which was avoided by stopping polymerisation at lower conversion. An additional indication that WA did not fully survive polymerisation at high conversion was given by ^{1}H NMR analysis. When comparing the integration intensities for the α,β-unsaturated carbonyl group of the C4 ring for the **P$_{60}$** and **P$_{90}$** polymer, with intensities referenced to C18-H_3, they significantly differed from 1 in the **P$_{90}$** polymer, in comparison this was not the case for the **P$_{60}$** polymer.

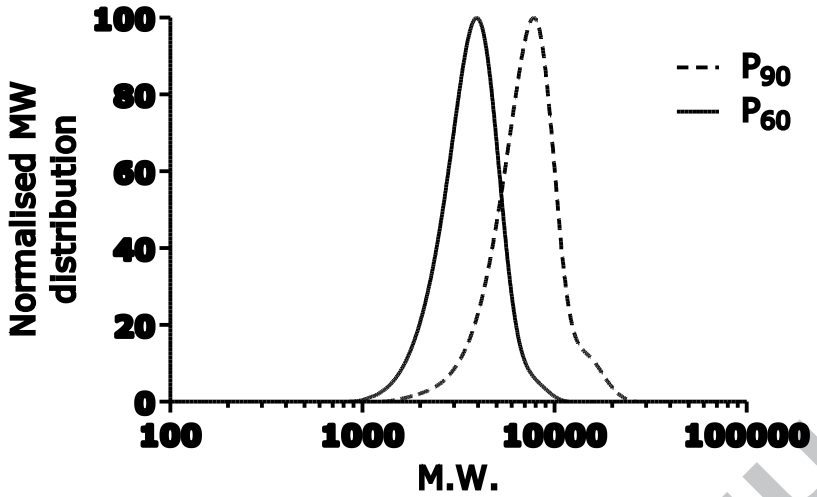


Figure S8. Molar mass distribution profiles of WA-polyDMA polymers stopped at 90% (P90) or 60% (P60) conversion.

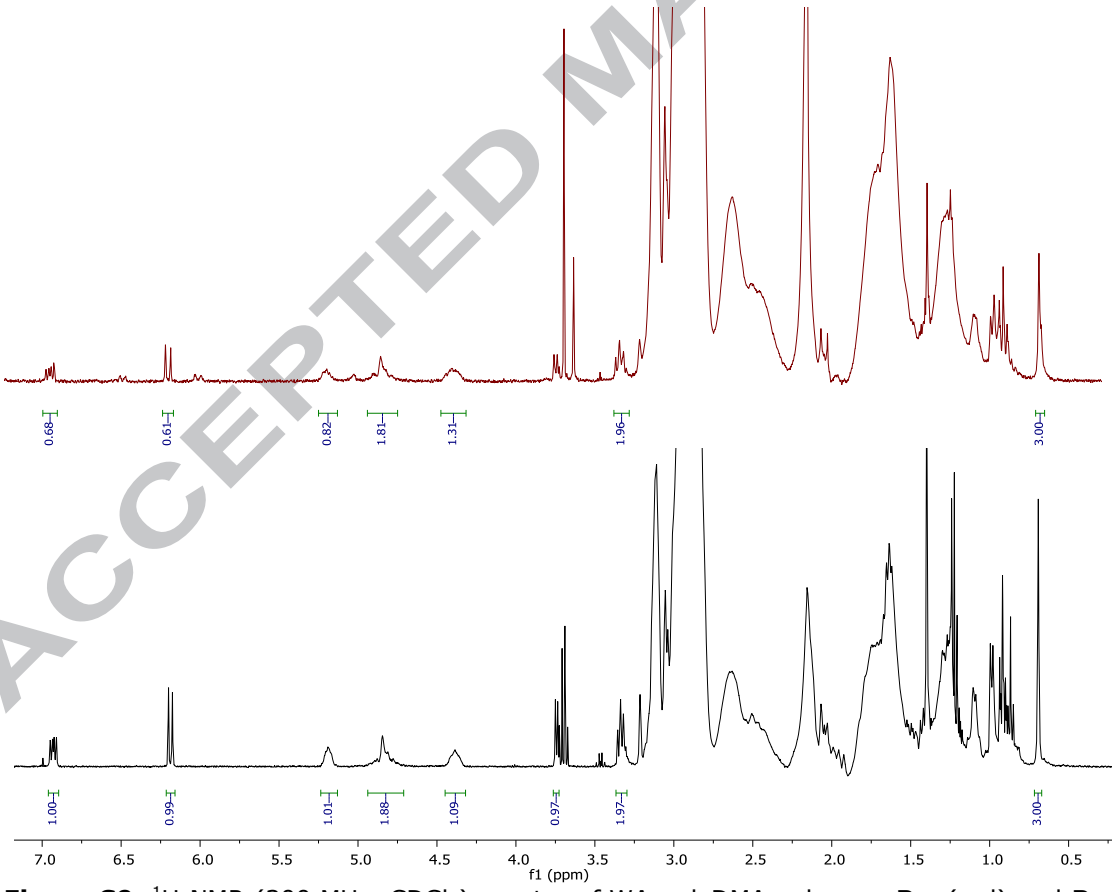


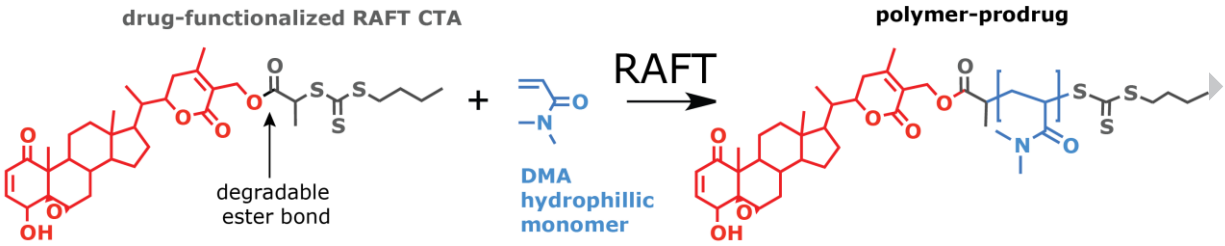
Figure S9. ^{1}H NMR (300 MHz, $CDCl_3$) spectra of WA-polyDMA polymers **P_{90}** (red) and **P_{60}** (black). Integration intensity is referenced to C18 (3H).

[1] D. Li, H. Li, L. Wu, Structurally dependent self-assembly and luminescence of polyoxometalate-cored supramolecular star polymers, Polym. Chem. 5 (2014) 1930–1937. doi:10.1039/C3PY01349A.

Highlights

- Solubility of the anti-cancer drug withaferin A is dramatically improved by polymer-conjugation
- Grafting-from chain transfer agent is a powerful route to well-defined polymer-drug conjugates
- Withaferin A-polymer conjugates are polymeric pro-drugs

Graphical abstract



withaferin A
hydrophobic anti-cancer drug