Supplementary Information

Polymeric prodrugs combination to exploit the therapeutic potential of antimicrobial peptides against cancer cells

Synthesis of the dual-release prodrug candidate

P18 was assembled, from a Rink Amide PEGA resin, with D-amino acids and extended with the GFLG motif, using L-phenylalanine and L-leucine, by automated synthesis as described for 1. Pegylation was performed manually on 0.1 mmol of resin-bound peptide, using (polydisperse) Fmoc-NH-PEG\(_{2000}\)-COOH (400 mg, 0.2 mmol) and HATU/DIEA coupling chemistry, with 1.9 equivalents of HATU (72 mg, 0.19 mmol) and 4 equivalents of DIEA (69.6 µl, 0.4 mmol) dissolved in DMF, and a reaction time of 3 hours. Fmoc-deprotection was carried out with a solution of 20% piperidine in DMF. The deprotection reaction was performed for 10 min and repeated twice for 5 min each. The resin was then washed 3 x 5 min with DMF and 1 x 5 min with DCM. Monitoring was performed by MALDI-TOF MS with analytical samples cleaved from the resin before and after Fmoc-deprotection (m/z: 5024.0430 and 4801.2998, M\(^+\), n=45, before and after Fmoc-deprotection, respectively). N-terminal elongation with a succinamide linker was then performed by reacting S-benzyl thiosuccinic acid (112.5 mg, 0.5 mmol), dissolved in DMF together with HOBT (74.9 mg, 0.49 mmol), HBTU (185.7 mg, 0.49 mmol) and DIEA (174 µl, 1 mmol), with the supported peptide for 3 hours. Monitoring was performed by MALDI-TOF MS with an analytical sample cleaved from the resin (m/z = 5052.4160). After a wash step, hydrazine hydrate (2 ml, 7.4 mmol) and 1,4-dioxane (2 ml) were added to the resin-tethered peptide and the reaction was performed for 2 hours. The resin was then washed with DCM (3 x 5 min each) and the reaction was repeated. Monitoring was performed by MALDI-TOF MS with an analytical sample cleaved from the resin (m/z = 4933.0936). The hydrazide-succinimide-PEG-peptide was then cleaved from the resin with a solution consisting of 5100 µl of TFA, 150 µl of EDT, 150 µl of thioanisole, 300 µl of water and 300 µl TIS. After 2.5 hours, the solution was filtered, the peptide was precipitated with diethyl ether, collected by centrifugation, washed twice with diethyl ether, dissolved in water and lyophilized overnight to yield a white powder. Analytical HPLC (SEC-Biosep S-2000) showed a peak (76.12% of all UV absorbing components) with retention time of 22.67 min for H\(_2\)N-NH-C(O)-(CH\(_2\))\(_2\)-C(O)-NH-PEG\(_{2000}\)-GFLG-P18-NH\(_2\). MALDI-TOF MS (m/z) (α-cyano-4-hydroxycinnamic acid): (n=54) 4716.9, (n=57) 4830.2, (n=60) 4933.0, (n=63) 5026.2. Conjugation of doxorubicin was carried out by reacting H\(_2\)N-NH-C(O)-(CH\(_2\))\(_2\)-C(O)-NH-PEG\(_{2000}\)-GFLG-P18-NH\(_2\) (10 mg, 2 µmol) with doxorubicin hydrochloride (11.5 mg, 2 µmol) in anhydrous methanol under argon and in the dark. The reaction was monitored by SEC-HPLC and performed for 24 hours. The methanol was then evaporated and the residue dried on a high vacuum line. The product was then purified by column chromatography (SEC) using Sephadex LH-20 and methanol as the mobile phase. The separation was monitored by SEC-HPLC with a flow rate of 0.5 mL/min and monitoring at wave-lengths of 510 nm and 214 nm from the PDA for doxorubicin and peptide amide bond absorptions, respectively. SEC-HPLC showed a peak (57.78 % of all UV absorbing components) with retention time of 25.72 min.
Fig. S1. Synthetic route to a dual-release anticancer prodrug candidate, based on a bi-functional PEG, containing an antimicrobial peptide (P18) and an anthracycline agent (doxorubicin). In blue is the P18 sequence, in turquoise the peptide termini, in orange the cathepsin B-sensitive linker, in green the PEG, in black the succinamidylhydrazone linker and in red doxorubicin (a). MALDI-TOF MS spectrum of the ultimate intermediate in the synthesis of the dual-release anticancer prodrug candidate (b). SEC-HPLC chromatogram (c). The signal at 510 nm indicated in the insert corresponds to the peak at a RT of 25.72 min and is in the region of maximum absorption of doxorubicin.
Fig. S2. MALDI-TOF MS spectrum of compound 1.

Fig. S3. Analytical RP-HPLC chromatogram of compound 1.
Fig. S4. MALDI-TOF MS spectrum of compound 2.

Fig. S5. RP-HPLC chromatogram of compound 2.
Fig. S6. MALDI-TOF MS spectrum of compound 3.

Fig. S7. RP-HPLC chromatogram of compound 3.
Fig. S8. SEC-HPLC chromatogram of compound 4. The signal at 520 nm indicated in the insert corresponds to the peak at a RT of 13.85 min and is in the region of maximum absorption of doxorubicin.

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Fig. S9. UV-VIS spectrum of compound 4 and free doxorubicin, showing the shift in the maximum absorption of doxorubicin as a hydrazone derivative.
Fig. S10. MALDI-TOF MS spectrum of compound 5.

Fig. S11. RP-HPLC chromatogram of compound 5.
Fig. S12. ESI-MS spectrum of compound 6, recorded on an Advion Expression Compact Mass Spectrometer.

Fig. S13. RP-HPLC chromatogram of compound 6.
Fig. S14. Cathepsin B proenzyme (37 kDa) expression in SK-OV-3 and A2780P cell lines (40 μg of cell lysate were separated by 12% SDS PAGE and analysed for cathepsin B expression by Western blotting (antibody used at 1:100 dilution)). Representative image of n=2.

Fig. S15. SEC-HPLC chromatogram of compound 2 before incubation with purified cathepsin B.
Fig. S16. SEC-HPLC chromatogram of compound 2 incubated with purified cathepsin B for 24 h. The peak at 14.5 min corresponds to intact 2 (see Fig. S14); peaks at higher RTs are attributed to pegylated and peptidic fragments of 2.

Fig. S17. ESI MS of compound 2 incubated with purified cathepsin B. EI-MS was recorded on a Waters Micro mass LCT at 80 eV. The signal at m/z = 589.52 is [M +4 H]⁴⁺ for G-(D)-P18-NH₂.
Fig. S18. Statistical analyses of the cell viability data, carried out using GraphPad Prism software and the two-tailed unpaired t test.