International Journal of
Molecular Sciences

MDPI

Article: Supplementary material

Targeting the Gastrin Releasing Peptide Receptor (GRP-R) in Cancer Therapy: Development of Bombesin Based Peptide-Drug Conjugates

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Table of Content

RP-HPLC chromatogram and ESI-HRMS spectrum of **G1** 2
RP-HPLC chromatogram and ESI-HRMS spectrum of **G2** 2
RP-HPLC chromatogram and ESI-HRMS spectrum of **G3** 2
RP-HPLC chromatogram and ESI-HRMS spectrum of **G4** 3
RP-HPLC chromatogram and ESI-HRMS spectrum of **G5** 3
RP-HPLC chromatogram and ESI-HRMS spectrum of **L1** 3
RP-HPLC chromatogram and ESI-HRMS spectrum of **L2** 4
RP-HPLC chromatogram and ESI-HRMS spectrum of **L3** 4
RP-HPLC chromatogram and ESI-HRMS spectrum of **L4** 4
RP-HPLC chromatogram and ESI-HRMS spectrum of **L5** 5
RP-HPLC chromatogram and ESI-HRMS spectrum of **L6** 5
RP-HPLC chromatogram and ESI-HRMS spectrum of **FP1** 5
RP-HPLC chromatogram and ESI-HRMS spectrum of **FP2** 6
Western Blot analysis of GRP-R protein level expression on MDA-MB-231, MDA-MB-453 and PC-3 human cancer cell lines 6
Dose-response curves of the PDCs and Dau on human fibroblasts MRC-5 7
Cellular uptake of the PDCs on MDA-MB-231, MDA-MB-453 and PC-3 human cancer cell lines (flow cytometry measurements) 7
Stability of the PDCs in cell culture medium 8
Stability of **L1**, **L5** and **L6** in mouse plasma (Extracted Ion Chromatograms) 8
Metabolites produced by **L1**, **L5** and **L6** in presence of lysosomal rat liver homogenate 9
Mice weight change during the tumour growth inhibition experiment 10

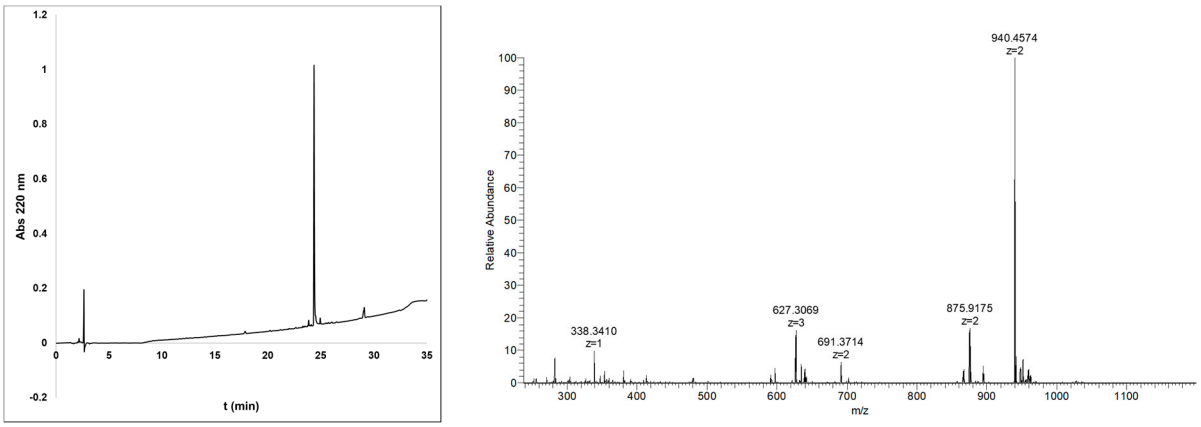


Figure S1. RP-HPLC chromatogram and ESI-HRMS spectrum of **G1**. *Rt* = 24.35 min. MW_{meas} / MW_{cal}: 1878.8988 / 1879.1050 g/mol. A signal diminished by about 129 g/mol from the expected MW is observed in all the MS spectra of the conjugates due to the cleavage of the Dau glycosidic bond during the MS measurement [1].

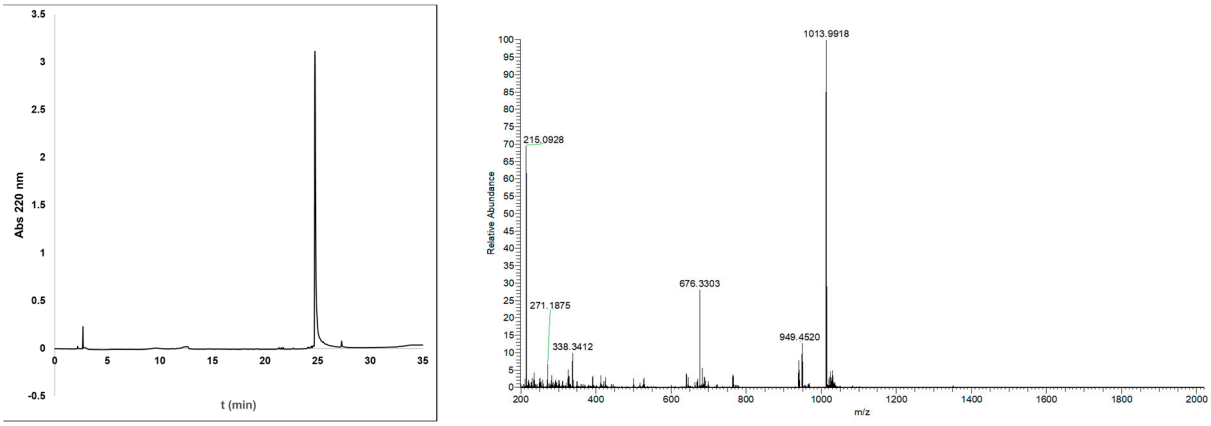


Figure S2. RP-HPLC chromatogram and ESI-HRMS spectrum of **G2**. *Rt* = 24.75 min. MW_{meas} / MW_{cal}: 2025.9836 / 2026.2820 g/mol.

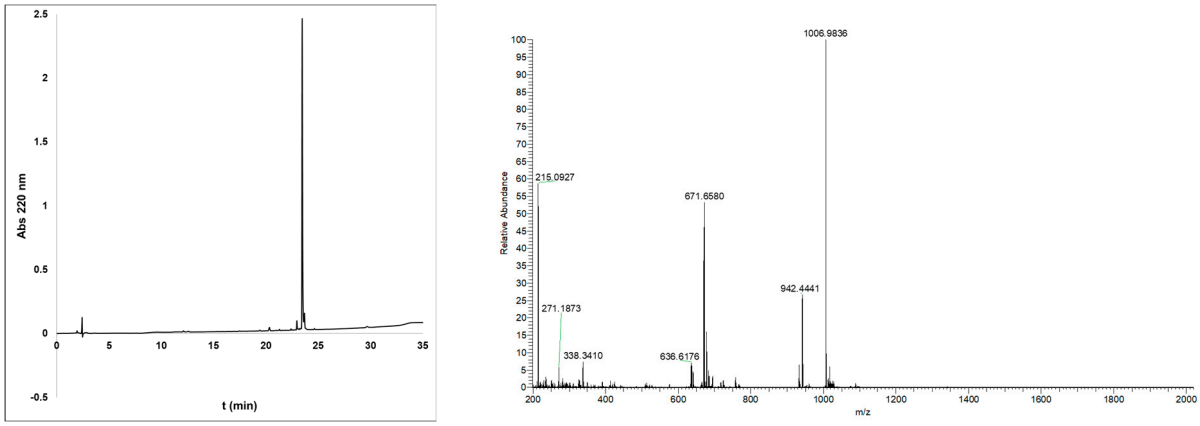


Figure S3. RP-HPLC chromatogram and ESI-HRMS spectrum of **G3**. *Rt* = 24.45 min. MW_{meas} / MW_{cal}: 2011.9672 / 2012.2550 g/mol.

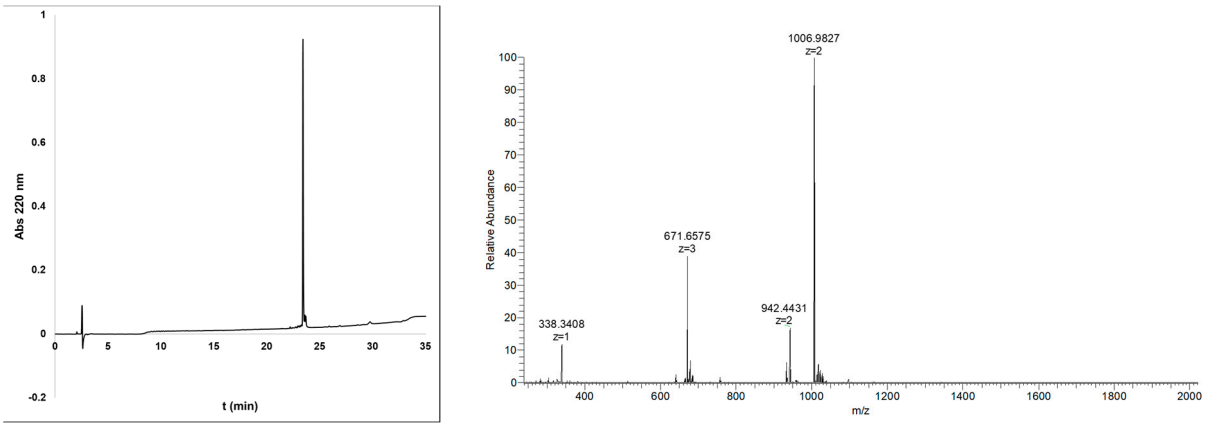


Figure S4. RP-HPLC chromatogram and ESI-HRMS spectrum of **G4**. *Rt* = 23.42 min. MW_{meas} / MW_{cal}: 2011.9494 / 2012.2550 g/mol.

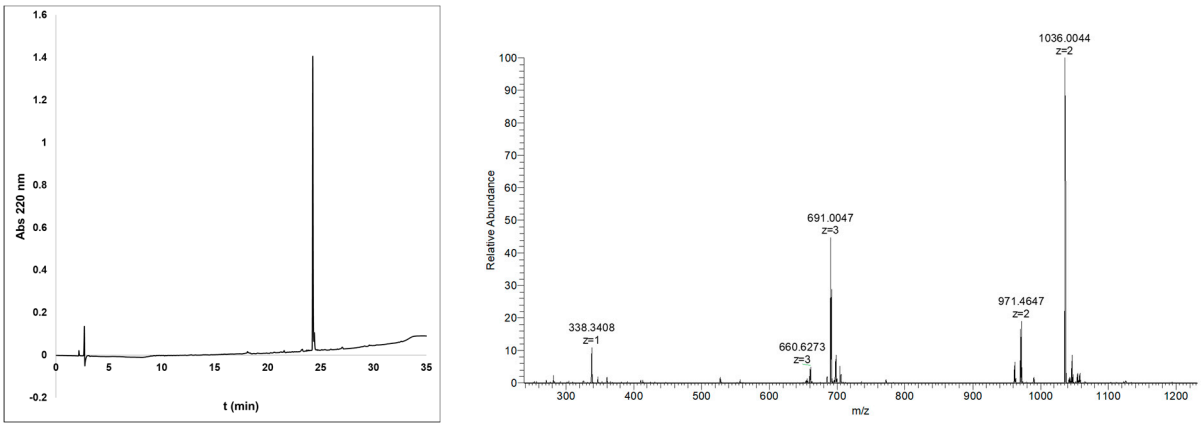


Figure S5. RP-HPLC chromatogram and ESI-HRMS spectrum of **G5**. *Rt* = 24.25 min. MW_{meas} / MW_{cal}: 2069.9928 / 2070.3350 g/mol.

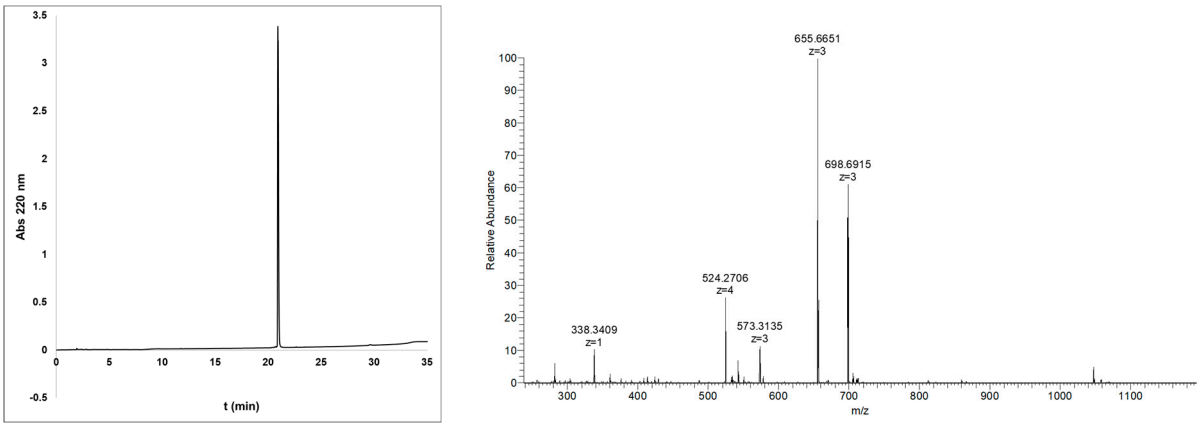


Figure S6. RP-HPLC chromatogram and ESI-HRMS spectrum of **L1**. *Rt* = 20.93 min. MW_{meas} / MW_{cal}: 2093.0528 / 2093.3780 g/mol.

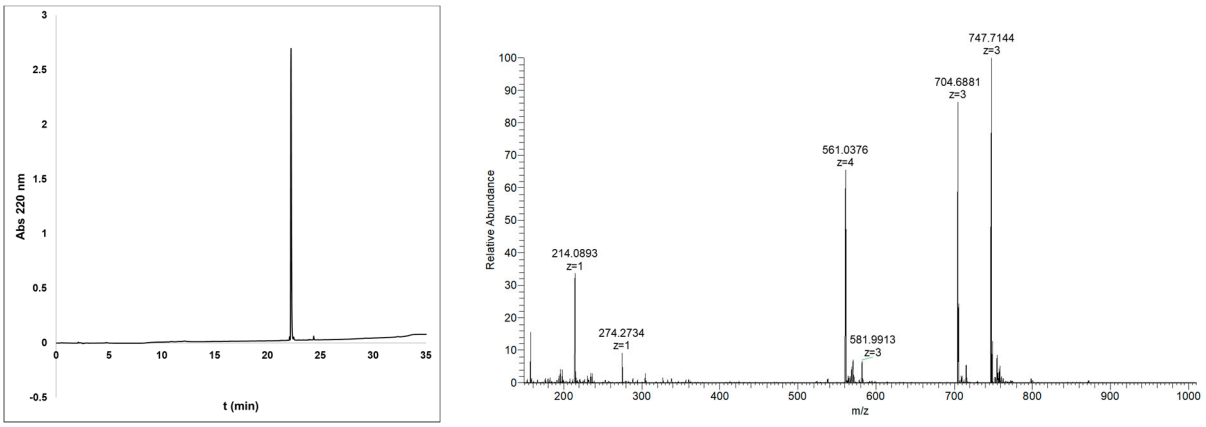


Figure S7. RP-HPLC chromatogram and ESI-HRMS spectrum of **L2**. *Rt* = 22.22 min. MW_{meas} / MW_{cal}: 2240.1192 / 2240.5550 g/mol.

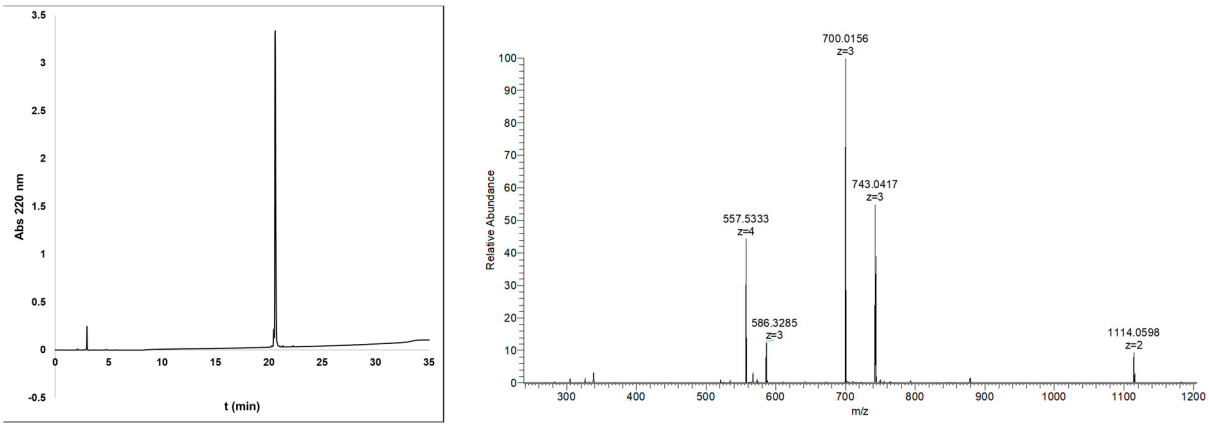


Figure S8. RP-HPLC chromatogram and ESI-HRMS spectrum of **L3**. *Rt* = 21.23 min. MW_{meas} / MW_{cal}: 2226.1036 / 2226.5280 g/mol.

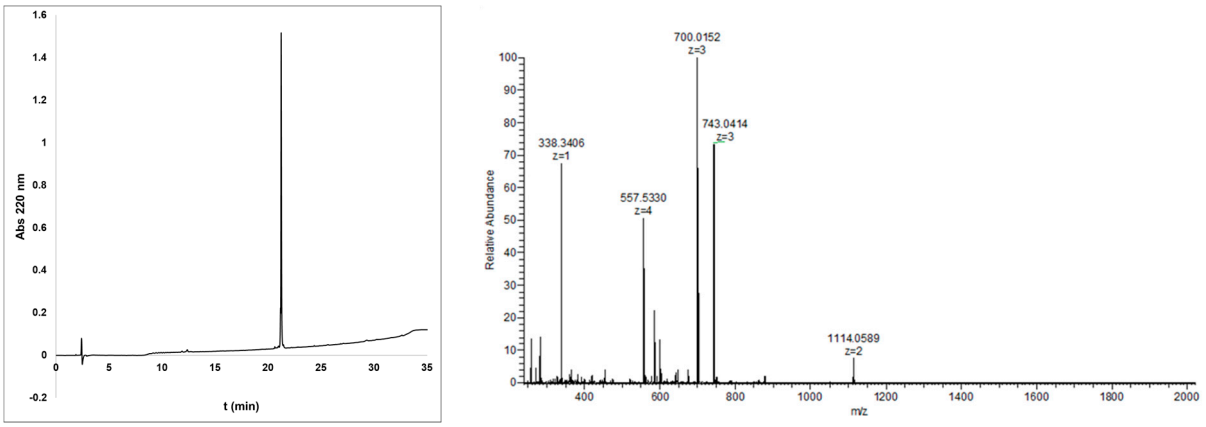


Figure S9. RP-HPLC chromatogram and ESI-HRMS spectrum of **L4**. *Rt* = 20.58 min. MW_{meas} / MW_{cal}: 2226.1018 / 2226.5280 g/mol.

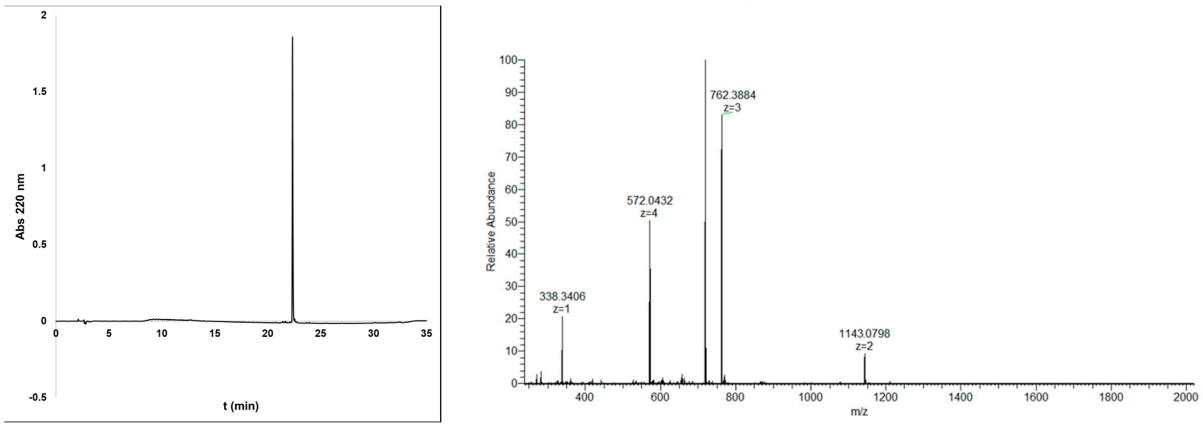


Figure S10. RP-HPLC chromatogram and ESI-HRMS spectrum of **L5**. *Rt* = 22.35 min. MW_{meas} / MW_{cal}: 2284.1462 / 2284.6080 g/mol.

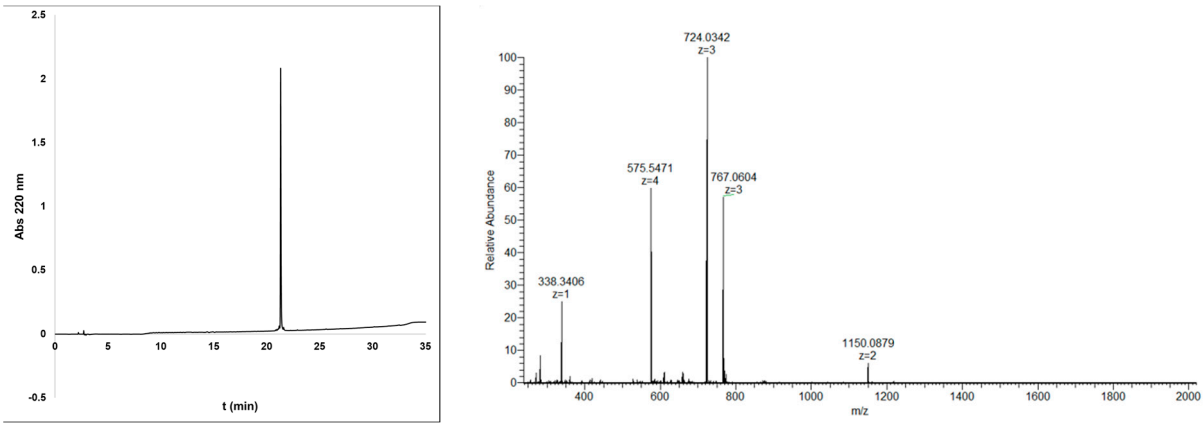


Figure S11. RP-HPLC chromatogram and ESI-HRMS spectrum of **L6**. *Rt* = 21.30 min. MW_{meas} / MW_{cal}: 2298.1598 / 2298.6350 g/mol.

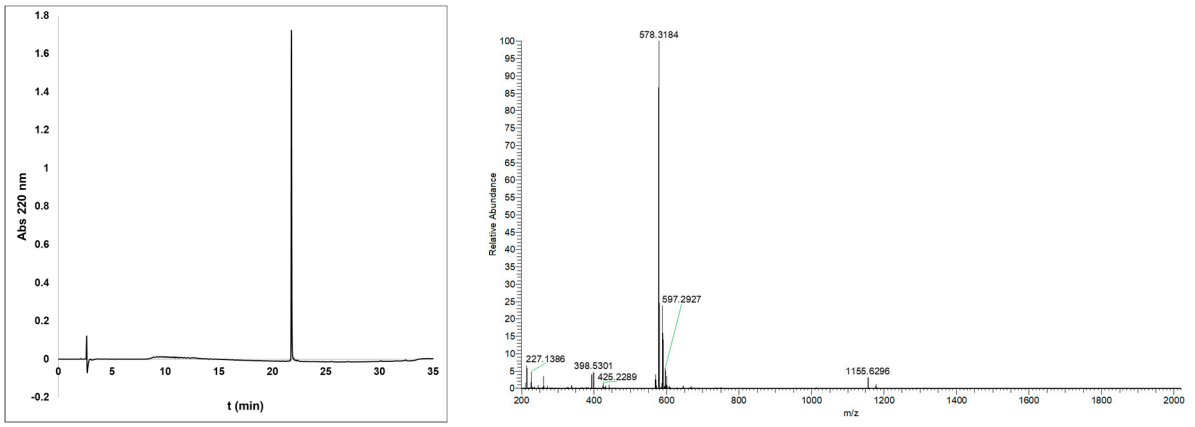


Figure S12. RP-HPLC chromatogram and ESI-HRMS spectrum of **FP1**. *Rt* = 21.76 min. MW_{meas} / MW_{cal}: 1155.6368 / 1155.3690 g/mol.

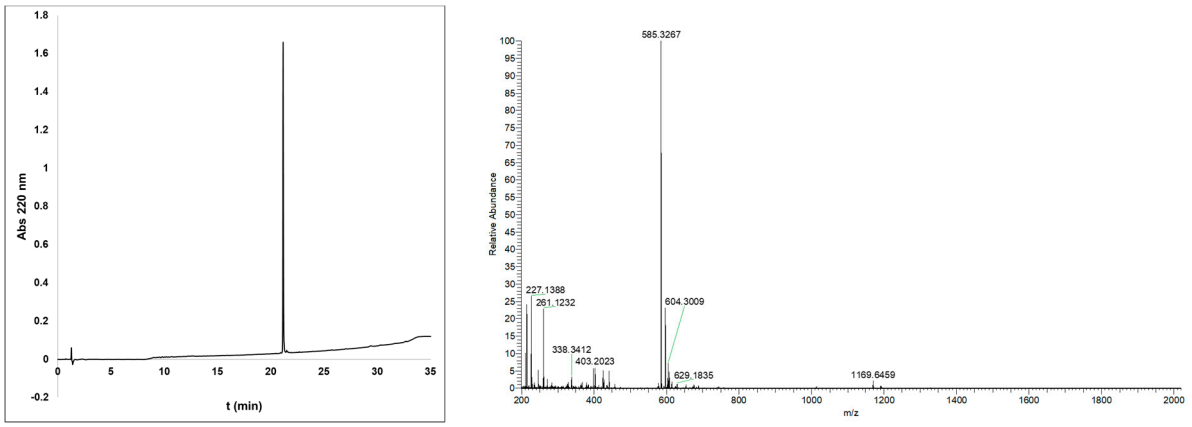


Figure S13. RP-HPLC chromatogram and ESI-HRMS spectrum of **FP2**. *Rt* = 21.17 min. MW_{meas} / MW_{cal}: 1168.6534 / 1169.3960 g/mol.

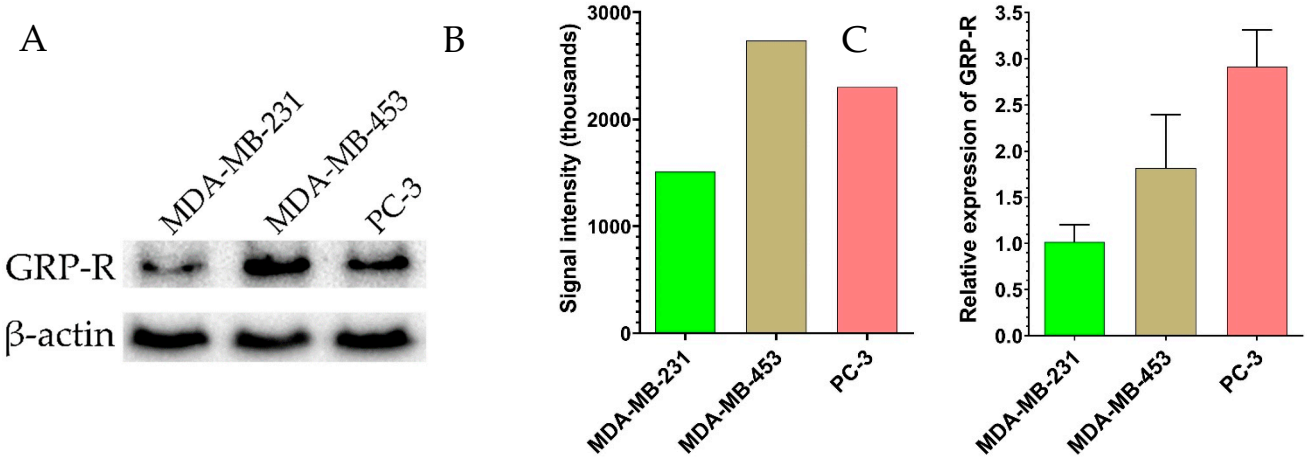


Figure S14. GRP-R expression levels in human cancer cell lines PC-3 (prostate), MDA-MB-453 and MDA-MB-231 (breast). (**A**) Western Blot and (**B**) quantification of the total GRP-R protein expression. The expression of β-actin was assessed as loading control and used for normalisation of the GRP-R content. (**C**) mRNA relative expression of GRP-R, normalised to the lowest expressing cell line, MDA-MB-231 (mean ± SD, n = 3).

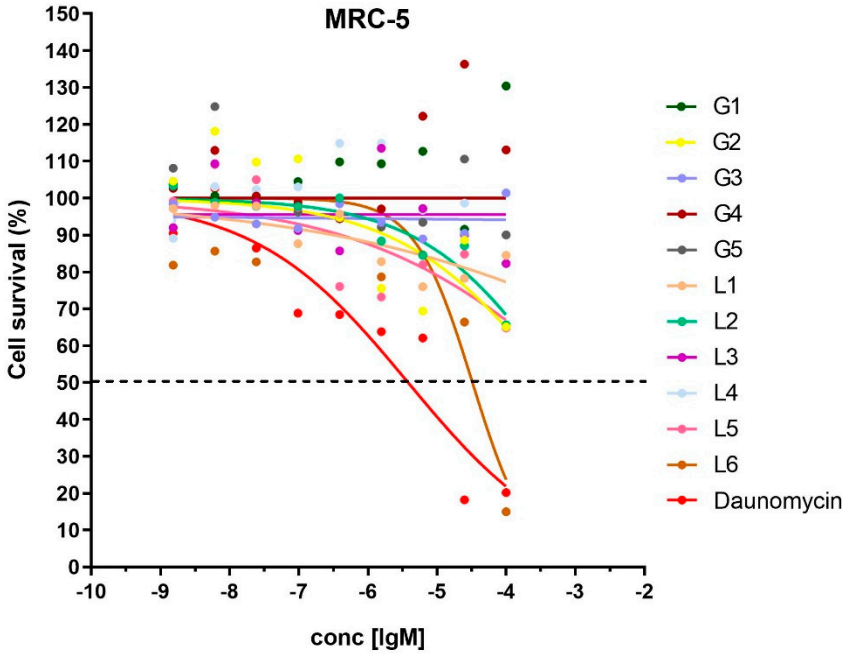


Figure S15. Dose-response curves of the PDCs and Dau on MRC-5 human fibroblast cell line. Only Dau reveals toxicity on the cells.

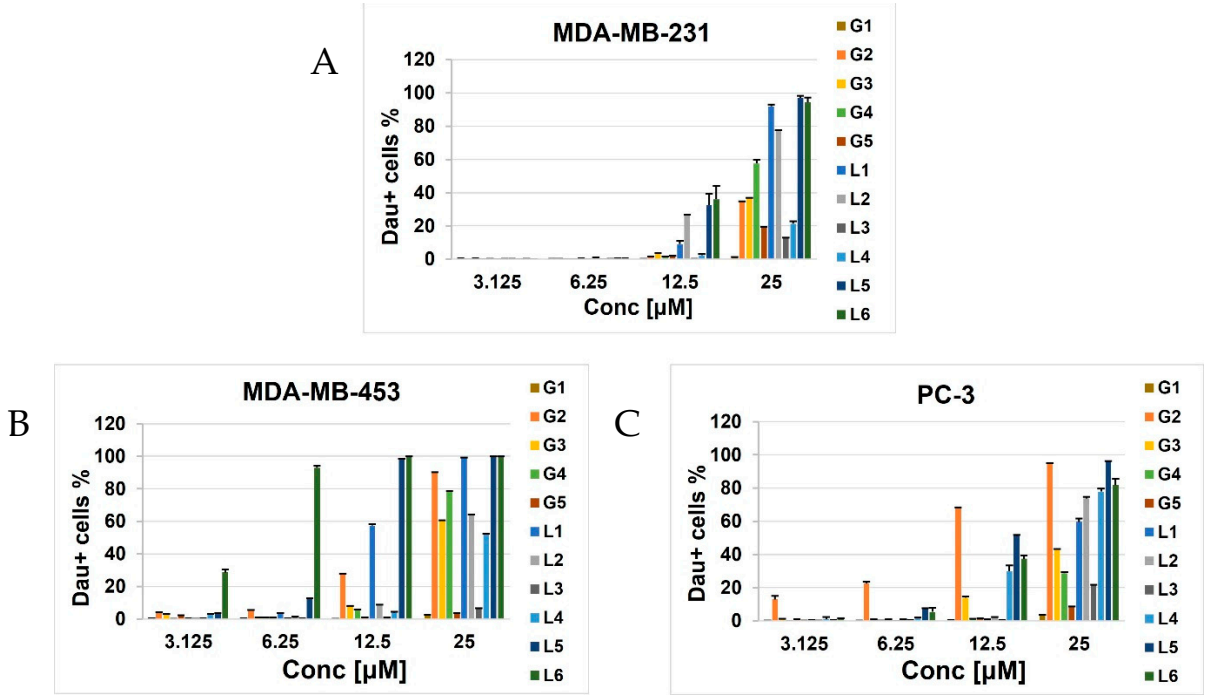


Figure S16. Flow cytometry measurements for the uptake of all the PDCs on MDA-MB-231 (**A**), MDA-MB-453 (**B**) and PC-3 (**C**) human cancer cell lines. The percentage of cells containing the Dau-conjugates was evaluated on four concentrations (%, mean ± SD, n= 2). Given that above 12.5 µM the internalisation may not only be receptor-mediated, this concentration was considered to compare the uptake of the bioconjugates.

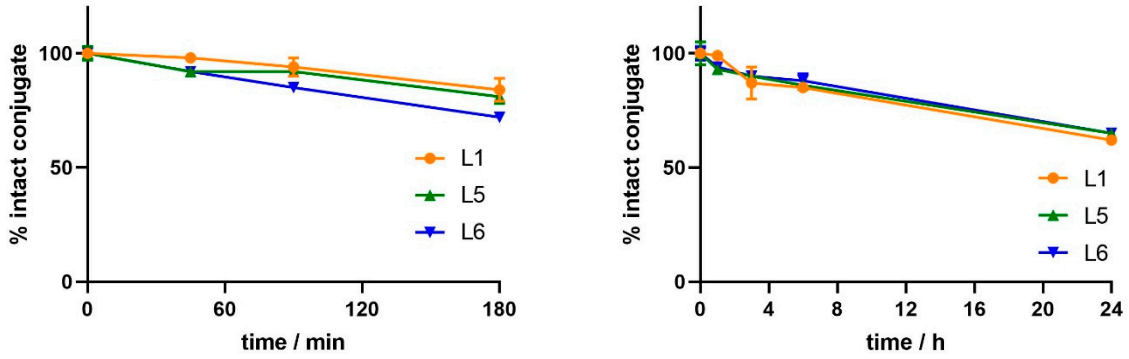


Figure S17. Stability of **L1**, **L5** and **L6** in serum free (**left**) and 2.5 % FBS containing (**right**) DMEM (%, mean ± SD, n = 2). The assays were performed over 3 h and 24 h, respectively, to monitor any possible loss of conjugate that could affect the cellular uptake and MTT assays, respectively.

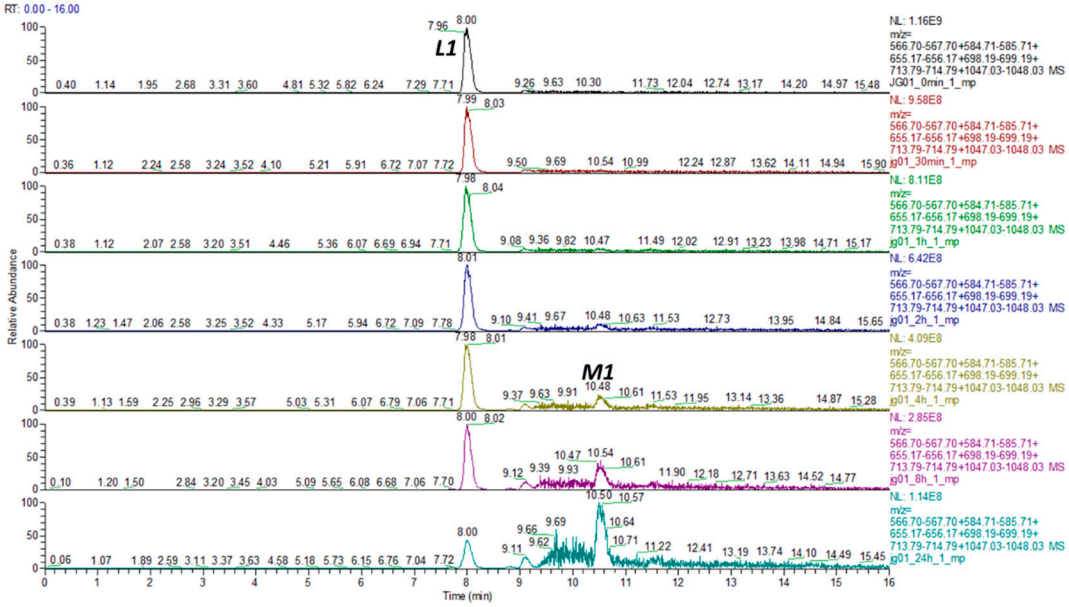


Figure S18. Plasma stability of **L1**. Mass Spectrometry Extracted Ion Chromatograms (EIC) at the collection time points (0 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h) for **L1** and the cytotoxic metabolite **Dau=Aoa-Leu-OH** (**M1**).

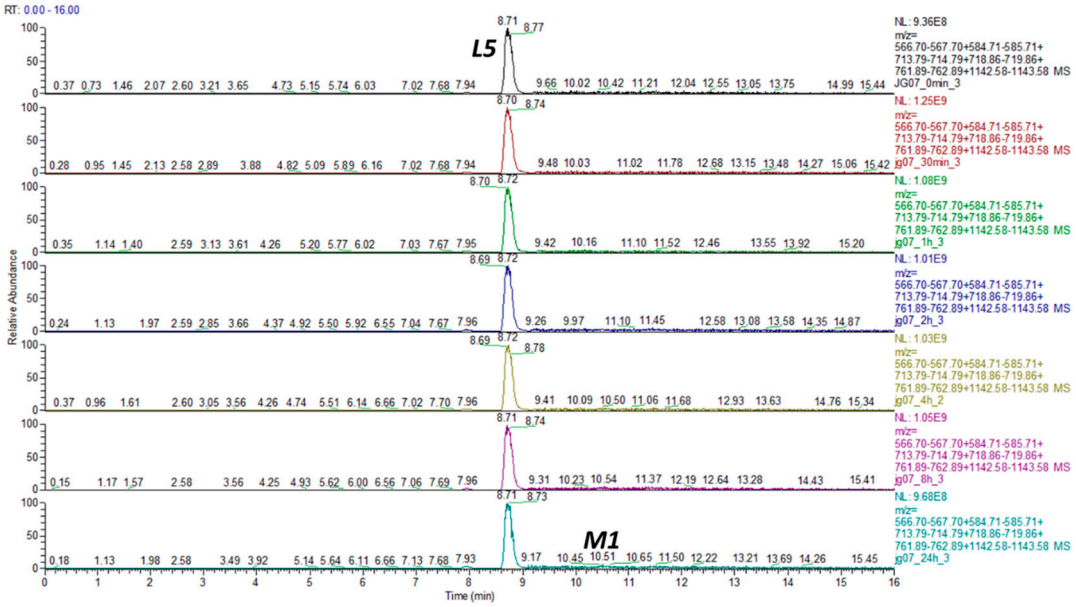


Figure S19. Plasma stability of **L5**. Mass Spectrometry Extracted Ion Chromatograms (EIC) at the collection time points (0 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h) for **L5** and the cytotoxic metabolite **Dau=Aoa-Leu-OH** (**M1**).

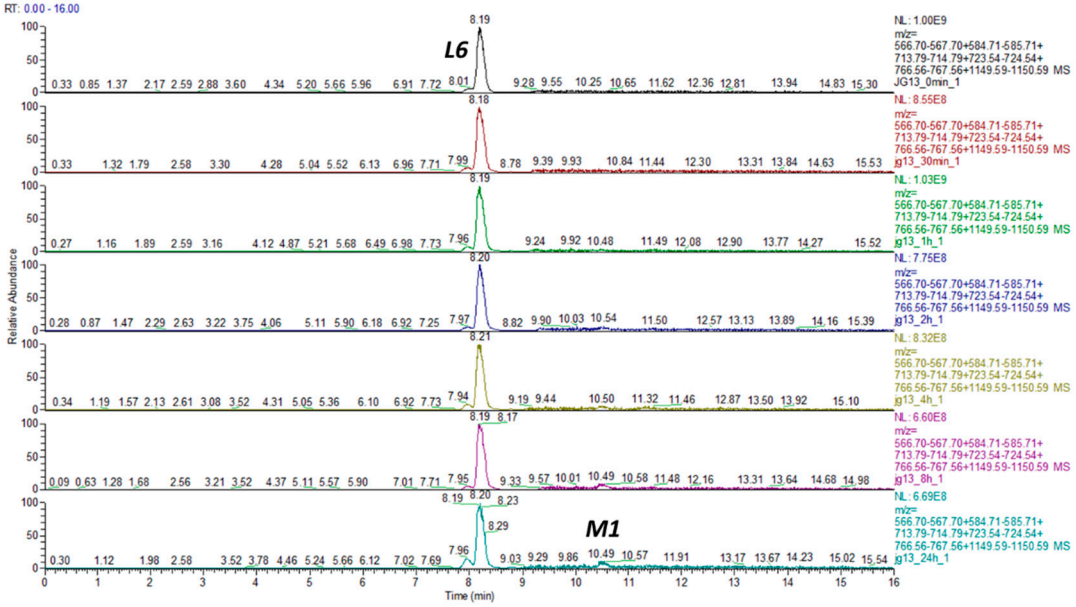


Figure S20. Plasma stability of **L6**. Mass Spectrometry Extracted Ion Chromatograms (EIC) at the collection time points (0 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h) for **L6** and the cytotoxic metabolite **Dau=Aoa-Leu-OH** (**M1**).

Table S1. Metabolites obtained by the degradation of **L1**, **L3**, **L5** and **L6** in rat liver lysosomal homogenate. Only the metabolites giving clearly visible peaks in the chromatograms are reported.

Code	Compound	Metabolite	MW_{meas} / MW_{cal} (g/mol)
L1	(Dau=Aoa)-LRRY-BBN(7-14)[Nle^{14}]	Dau=Aoa-LRR-OH	1025.49 / 1026.12
		Dau=Aoa-LR-OH	869.39 / 869.93
		Dau=Aoa-L-OH	713.29 / 713.74
L3	(Dau=Aoa)-LRRY-BBN[D-Phe^{6}, β-Ala^{11}, Aib^{13}, Nle^{14}]	H_2N-RY-fQWAV-β-Ala-H-Aib-Nle-NH_2	1374.74 / 1374.62
		H_2N-Y-fQWAV-β-Ala-H-Aib-Nle-NH_2	1218.64 / 1218.43
		H_2N-RY-fQW-OH	799.39 / 798.90
		Dau=Aoa-LR-OH	869.39 / 869.93
		Dau=Aoa-L-OH	713.29 / 713.74
L5	(Dau=Aoa)-LRRY-BBN[D-Phe^{6}, Sta^{13}, Leu^{14}]	H_2N-RY-fQWAVGH-Sta-L-NH_2	1431.80 / 1432.70
		H_2N-VGH-Sta-L-NH_2	580.38 / 580.73
		H_2N-GH-Sta-L-NH_2	481.31 / 481.60
		H_2N-RY-fQW-OH	798.39 / 798.90
		H_2N-RYfQ-OH	612.31 / 612.69
		Dau=Aoa-LR-OH	869.39 / 869.93
		Dau=Aoa-L-OH	713.29 / 713.74
L6	(Dau=Aoa)-LRRY-BBN[D-Phe^{6}, β-Ala^{11}, Sta^{13}, Nle^{14}]	H_2N-RY-fQWAV-βAla-H-Sta-Nle-NH_2	1445.80 / 1446.72
		H_2N-Y-fQWAV-βAla-H-Sta-Nle-NH_2	1290.68 / 1290.54
		H_2N-LRRY-fQWAV-βAla-H-NH_2	1446.78 / 1446.68
		H_2N-RY-fQW-OH	798.39 / 798.90
		Dau=Aoa-LR-OH	869.39 / 869.93
		Dau=Aoa-L-OH	713.29 / 713.74

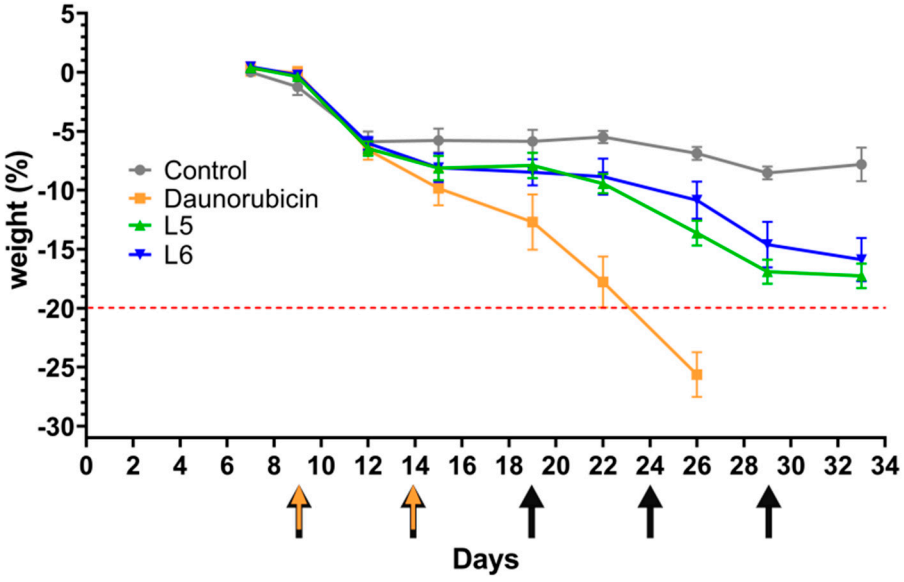


Figure S21. Mouse weight variation during the tumour growth inhibition experiment (%, mean ± SEM, n = 8). Administration regime: 0.9% saline (control group, black arrows), Dau (1 mg/kg, orange arrows) and PDCs (10 mg/kg calculated on Dau content, black arrows), every 5th day starting from Day 9.

References

1. Pethő, L.; Mező, G.; Schlosser, G. Overcharging Effect in Electrospray Ionization Mass Spectra of Daunomycin-Tuftsin Bioconjugates. *Molecules* **2019**, *24*, 2981. https://doi.org/10.3390/molecules24162981