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Supporting Information

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Lipid-Conjugation of Endogenous Neuropeptides: Improved
Biotherapy against Human Pancreatic Cancer

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Couvreur**

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Supporting Information

Lipid-Conjugation of Endogenous Neuropeptides: Improved Biotherapy Against Human Pancreatic Cancer

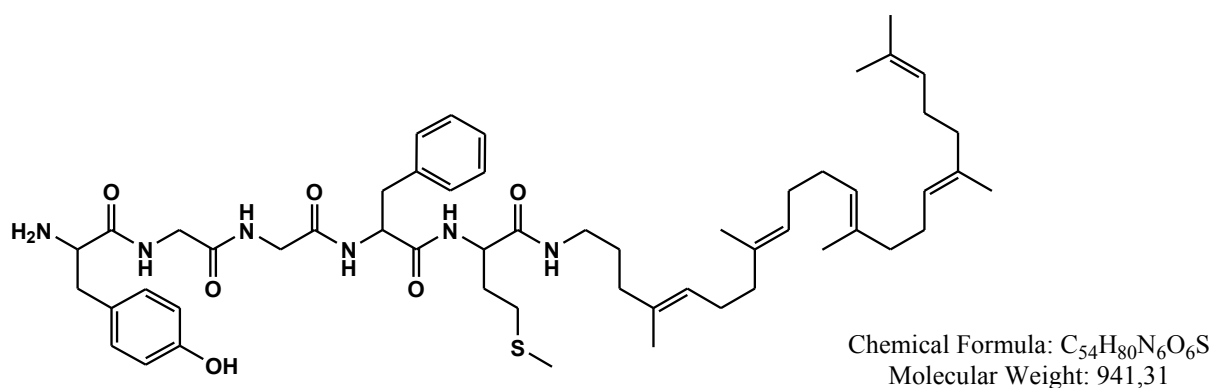
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Institut Galien Paris-Sud (UMR CNRS 8612)

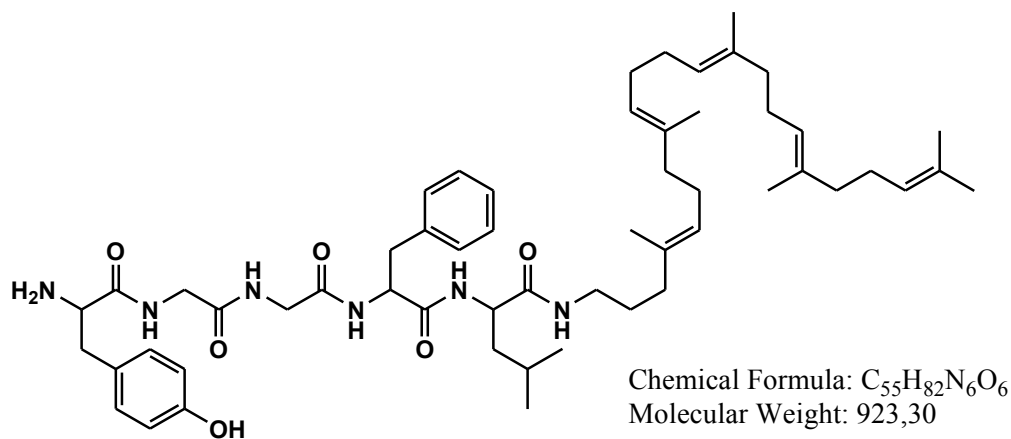
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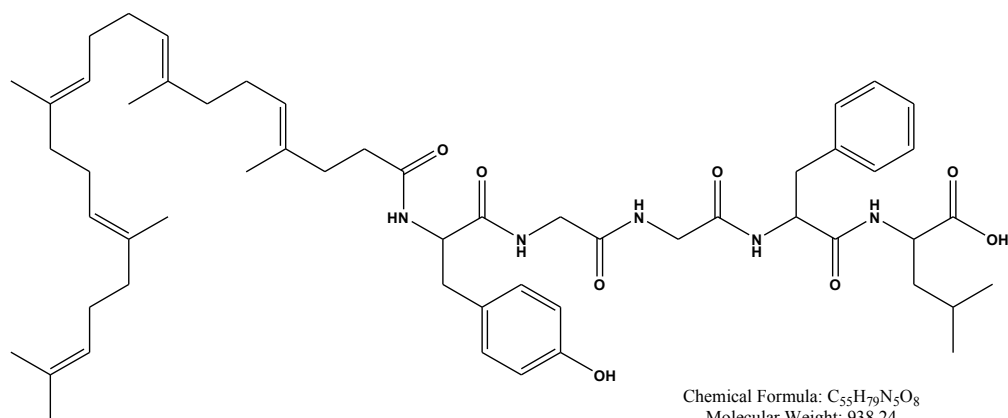
1. Detailed structures of the SQ-neuropeptide bioconjugates



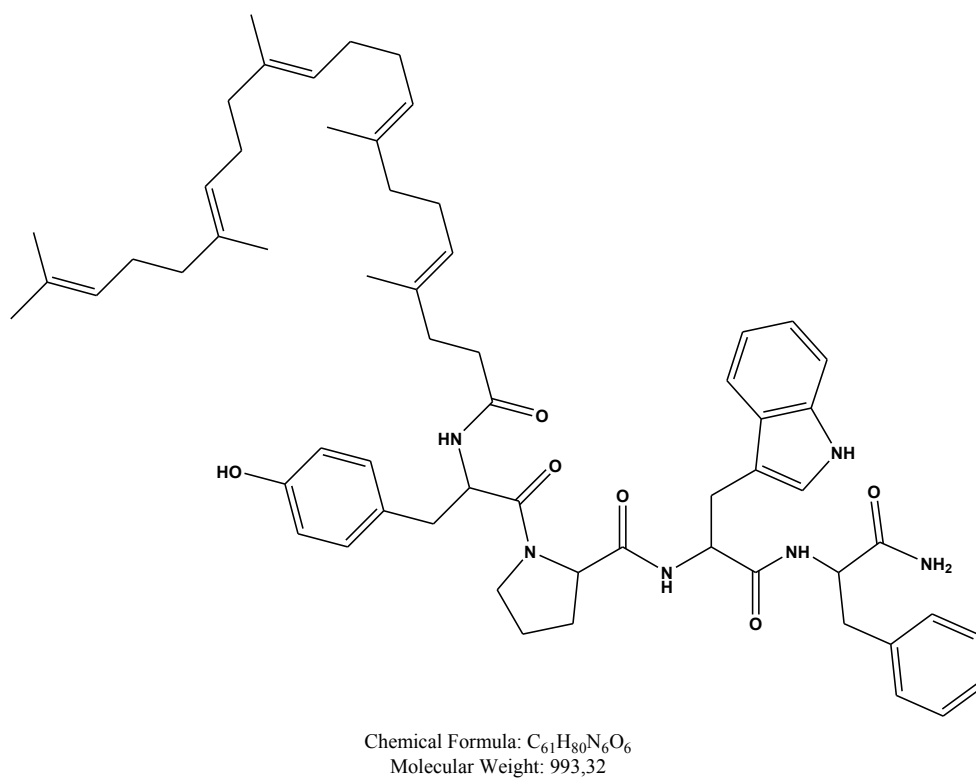
SQ-Met-Enk



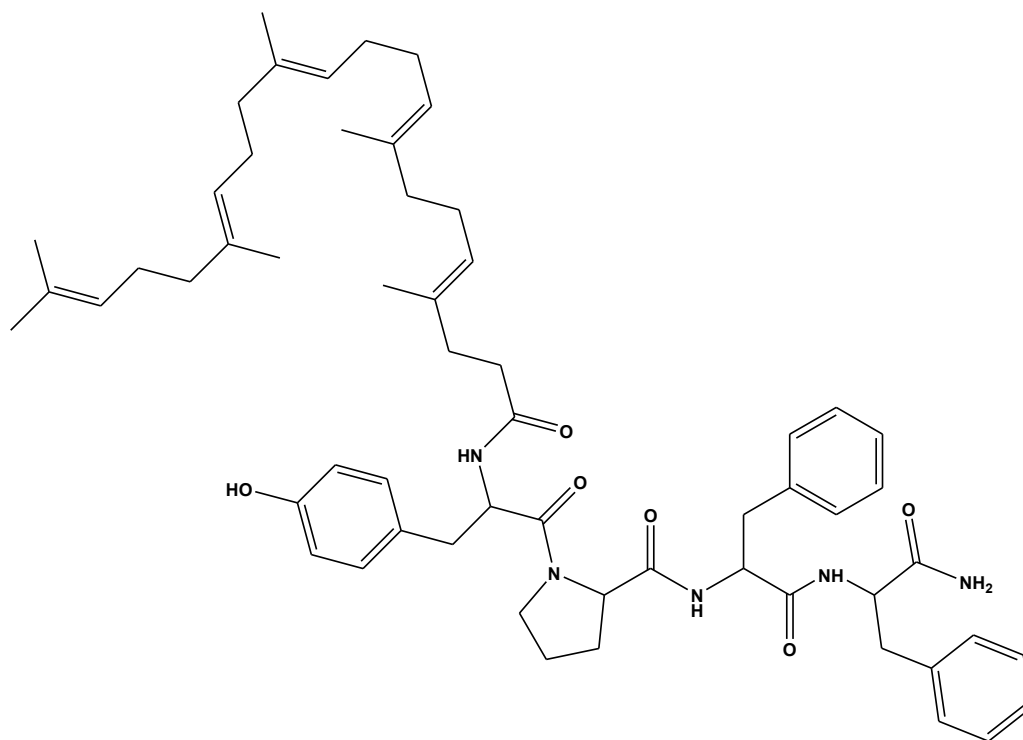
SQ-Leu-Enk



SQ-Leu-Enk (Alternate conjugate)



SQ-EM-1



Chemical Formula: C₅₉H₇₉N₅O₆
Molecular Weight: 954,29

SQ-EM-2

2. Experimental Section

Synthesis and characterization of SQ-NH₂: 1',2-trisnorsqualenamine (SQ-NH₂) was synthesized from 1,1',2-trisnorsqualene aldehyde (SQ-CHO)¹⁷ that was readily available in a few reaction steps starting from squalene (SQ) by oximation/reduction sequence.

1,1',2-trisnorsqualene oxime : SQCHO (4.75 g, 12.3 mmol) was first solubilized in a solvent mixture containing EtOH (27 mL) and H₂O (13 mL). To this, AcONa (1.51 g, 18.5 mmol) was added, followed by NH₂OH.HCl (1.28 g, 18.5 mmol). The reaction mixture was stirred overnight under nitrogen atmosphere at room temperature (RT). The reaction was concentrated under reduced pressure and the residue was transferred into water (200 mL). The mixture was extracted with ethyl acetate (3 x 100 mL). The extracts were combined and washed twice with water, dried on MgSO₄ and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel with an increasing ratio of ether (from 0.5% to 10%) in cyclohexane to obtain the pure SQ-oxime (SQCH=NOH) as a colorless oil (3.0 g, 61% yield). ¹H NMR (300 MHz, CDCl₃): δ = 7.40 (t, *J* = 5.8 Hz, 0.5 H,

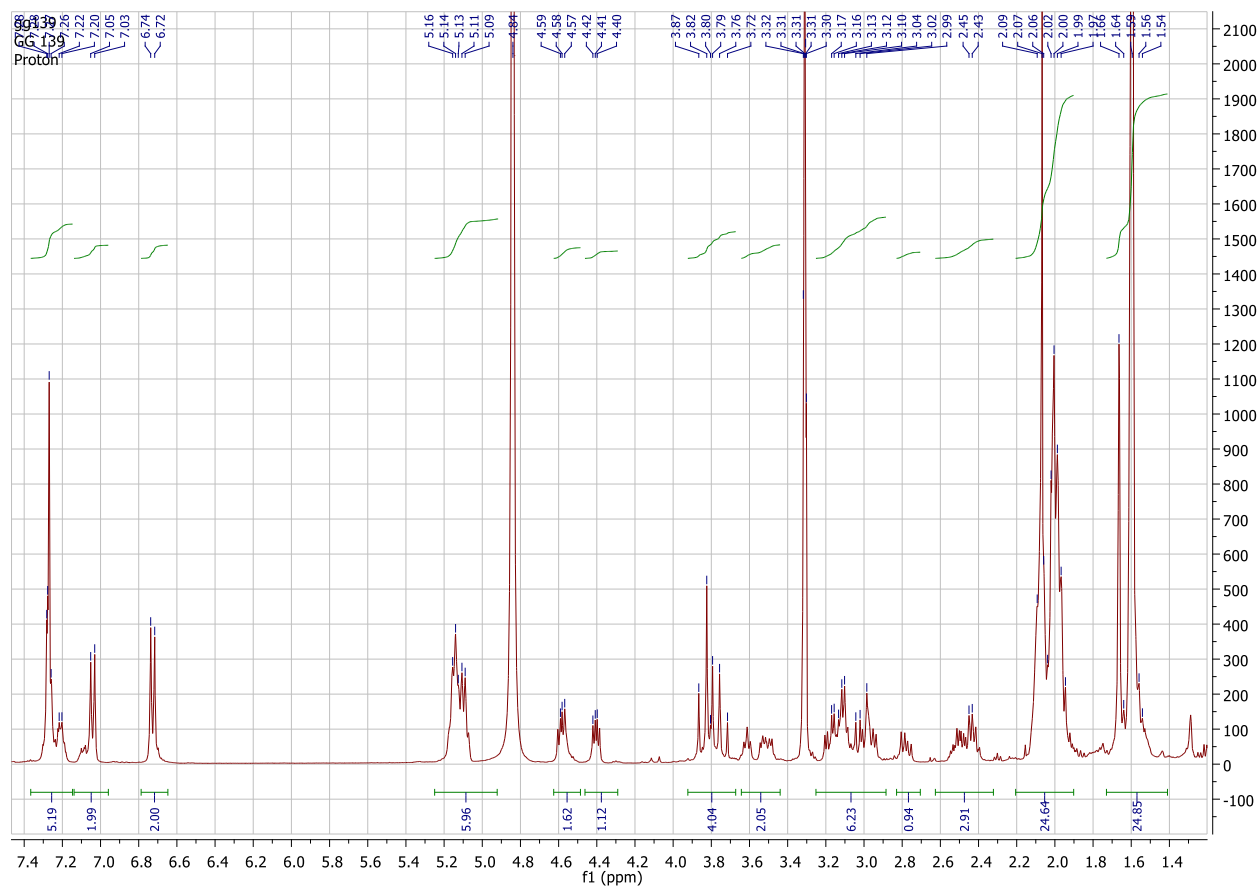
HO-N=CHCH₂, isomer *E* or *Z*), 6,69 (t, *J* = 5.3 Hz, 0.5 H, HO-N=CHCH₂, isomer *E* or *Z*), 5.21-5.06 (m, 5 H, CH₂C=CH(CH₃)), 2.52-2.45 (m, 1 H, HO-N=CHCH₂CH₂, isomer *E* or *Z*), 2.34-2.26 (m, 1 H, HO-N=CHCH₂CH₂, isomer *E* or *Z*), 2.18-1.98 (m, 18 H), 1.68 (s, 3 H, C(CH₃)₂=CH), 1.60 (s, 15 H, CH₂CH=C(CH₃)) ppm. ¹³C NMR (75 MHz, CDCl₃): δ = 151.6 (0.5 CH, HO-N=CHCH₂, isomer *E* or *Z*), 151.2 (0.5 CH, HO-N=CHCH₂, isomer *E* or *Z*), 134.7 (C, CH₂C(CH₃)=CH), 134.5 (2 C, CH₂C(CH₃)=CH), 133.1 (C, CH₂C(CH₃)=CH), 132.9 (C, CH₂C(CH₃)=CH), 130.7 (C, CH=C(CH₃)₂), 125.4 (CH, C(CH₃)=CHCH₂), 125.3 (CH, C(CH₃)=CH), 124.3 (2 CH, C(CH₃)=CH), 124.2 (CH, C(CH₃)=CH), 124.1 (CH, C(CH₃)=CH), 39.6 (2CH₂, =C(CH₃)CH₂CH₂), 39.5 (CH₂, =C(CH₃)CH₂CH₂), 36.4 (CH₂, =C(CH₃)CH₂CH₂), 35.5 (CH₂, =C(CH₃)CH₂CH₂), 28.1 (3CH₂, =C(CH₃)CH₂CH₂), 27.9 (CH₂, =C(CH₃)CH₂CH₂), 26.6 (2 CH₂, =C(CH₃)CH₂CH₂), 26.5 (CH₂, =C(CH₃)CH₂CH₂), 26.4 (CH₂, =C(CH₃)CH₂CH₂), 25.5 (CH₃, CH=C(CH₃)₂), 23.1 (CH₂), 17.4 (CH₃, CH=C(CH₃)CH₂), 15.8 (3 CH₃, CH=C(CH₃)CH₂), 15.6 (0.5 CH₃, CH=C(CH₃)), 15.6 (0.5 CH₃, CH=C(CH₃)) ppm. IR (neat): ν = 2916, 1450, 1382, 1083, 915, 737 cm⁻¹

1,1',2-trisnorsqualenamine : An ice-cooled slurry of LiAlH₄ (0.72 g, 18.9 mmol) in anhydrous THF (22 mL), was added dropwise to SQ-oxime (2.92 g, 7.3 mmol) that was pre-dissolved in anhydrous THF (20 mL). Once the addition was completed, the reaction mixture was brought to room temperature and stirred during 5 h under reflux under nitrogen atmosphere. Once the reaction was completed, THF (50 mL) was added followed by ethyl acetate (dropwise, 1 mL). The mixture was then cooled to 0 °C and 1N NaOH was carefully added until all the aluminate salts have precipitated. The mixture was stirred during 1 h and the precipitate was filtered out using Celite® and rinsed several times with THF. The filtrate was concentrated under vacuum, and the crude product was then purified using chromatography over silica gel, eluting with an increasing ratio of MeOH (from 0.5% to 10%) in DCM in the presence of 1% of triethylamine, to obtain the pure squalenamine (SQ-NH₂) as a colorless oil. ¹H NMR (300 MHz, CDCl₃): δ = 5.20-5.07 (m, 5 H, CH₂C=CH(CH₃)), 2.60 (t, *J* = 6.9 Hz, 2 H, CH₂CH₂NH₂), 2.14-1.85 (m, 20 H, CHCH₂CH₂C(CH₃)=CH), 1.68 (s, 3 H, (CH₃)₂C=CH), 1.60 (s, 15 H, CH₂CH=C(CH₃)) ppm. ¹³C NMR (75 MHz, CDCl₃): δ = 136.0 (2 C, CH₂C(CH₃)=CH), 135.8 (C, CH₂C(CH₃)=CH), 135.6 (C, CH₂C(CH₃)=CH), 132.0 (C, CH=C(CH₃)₂), 125.8 (CH, C(CH₃)=CHCH₂), 125.7 (2 CH, C(CH₃)=CHCH₂), 125.6 (CH, C(CH₃)=CHCH₂), 125.5 (CH, (CH₃)C=CHCH₂), 42.3 (CH₂, CH₂NH₂), 41.0 (3 CH₂, =C(CH₃)CH₂CH₂), 38.2 (CH₂, =C(CH₃)CH₂CH₂), 32.0 (CH₂, =C(CH₃)CH₂CH₂), 29.4 (2 CH₂, =C(CH₃)CH₂CH₂), 27.9 (CH₂, =C(CH₃)CH₂CH₂), 27.8 (CH₂, =C(CH₃)CH₂CH₂), 27.7 (CH₂, =C(CH₃)CH₂CH₂), 26.1 (CH₃, CH=C(CH₃)₂), 18.0 (CH₃, =C(CH₃)CH₂), 16.4 (3 CH₃,

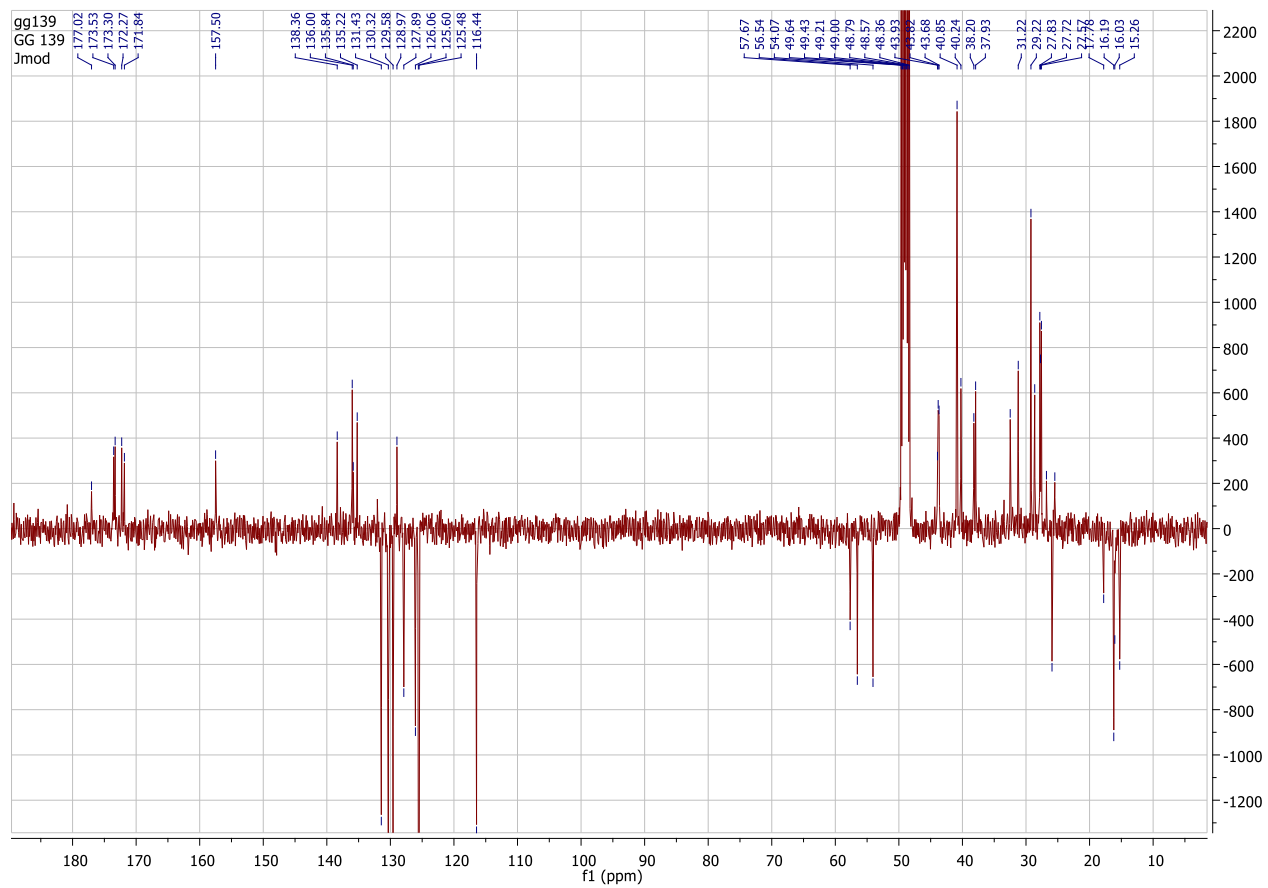
$\text{CH}=\text{C}(\text{CH}_3)\text{CH}_2$, 16.2 (CH_3 , $=\text{C}(\text{CH}_3)\text{CH}_2$) ppm. IR (film): $\nu = 2925, 2855, 1448, 1381, 1239, 1130, 1105, 833, 732 \text{ cm}^{-1}$.

3. NMR spectra of the bioconjugates:

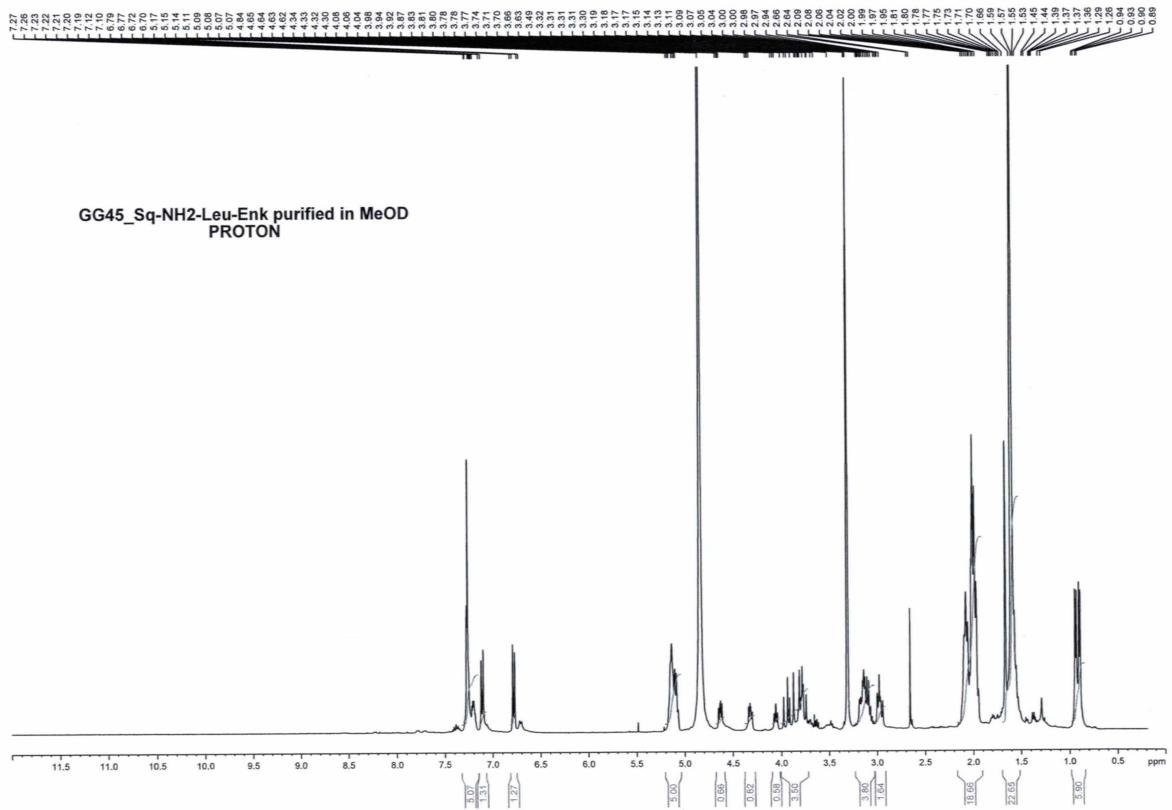
^1H spectrum of SQ-Met-Enk



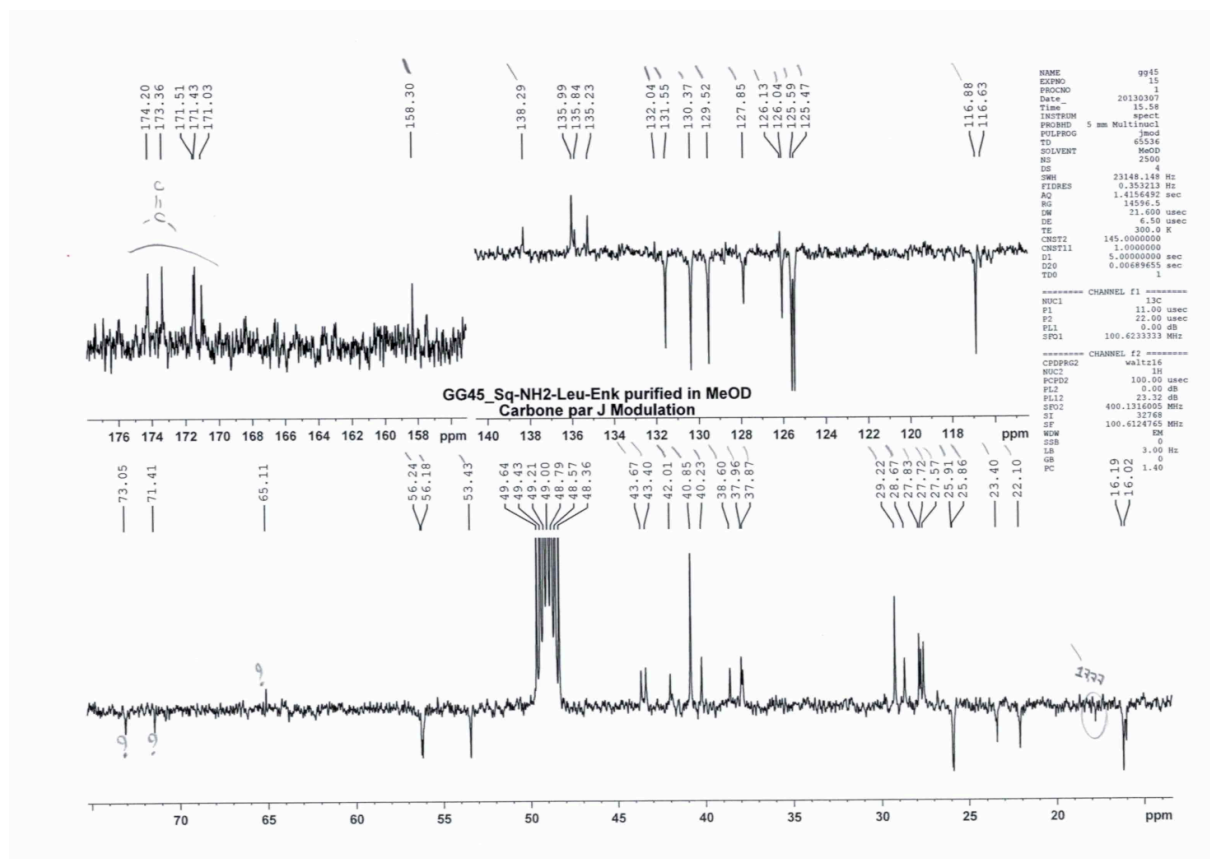
^{13}C spectrum of **SQ-Met-Enk**

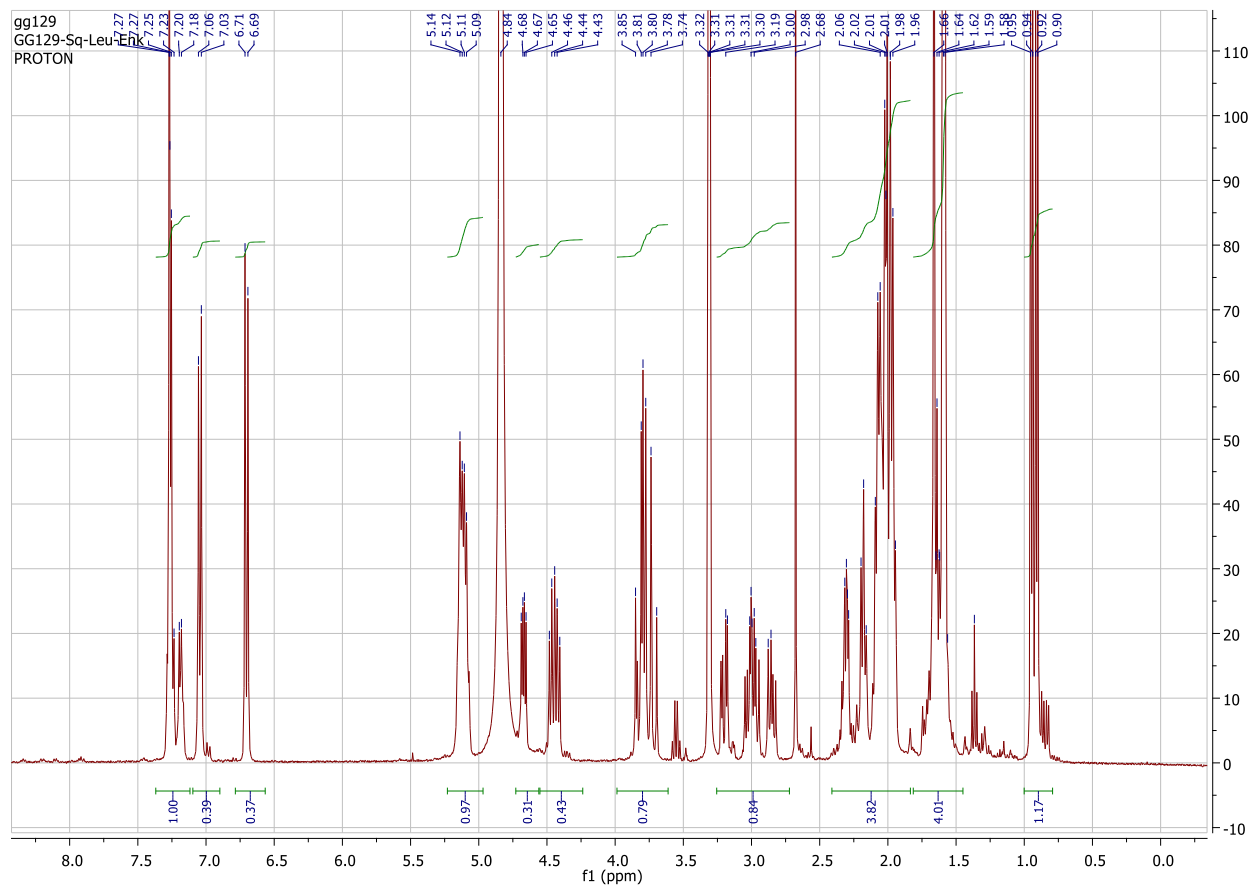


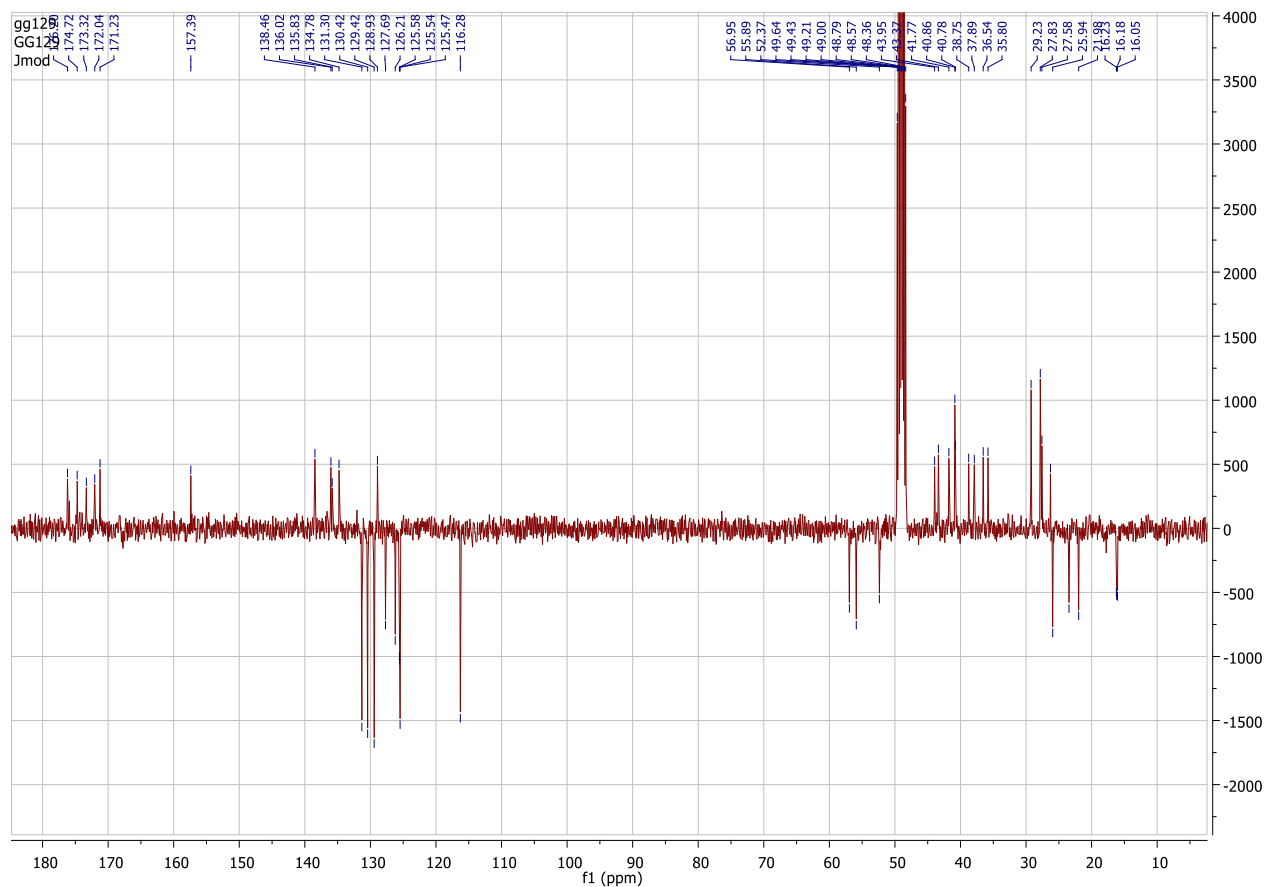
¹H spectrum of SQ-Leu-Enk

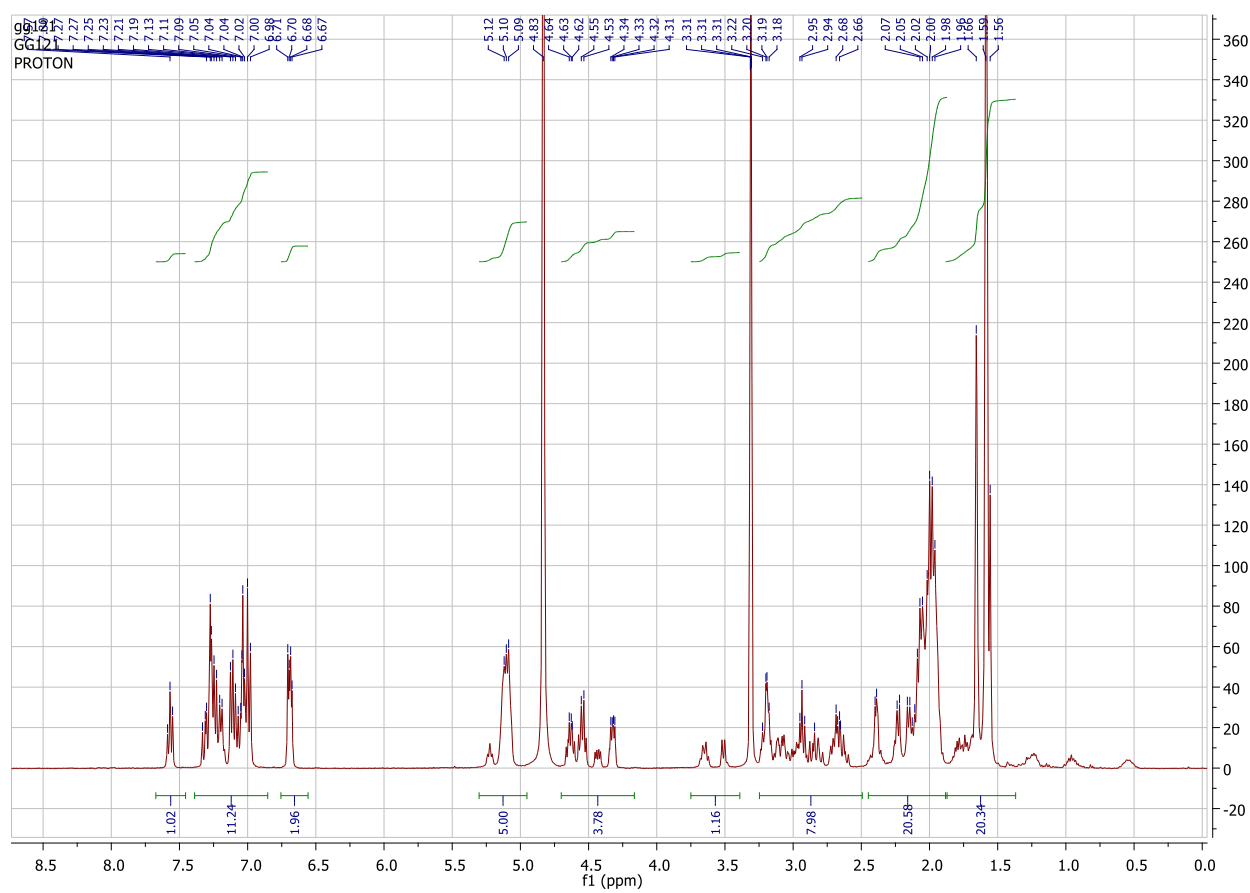


¹³C spectrum of SQ-Leu-Enk

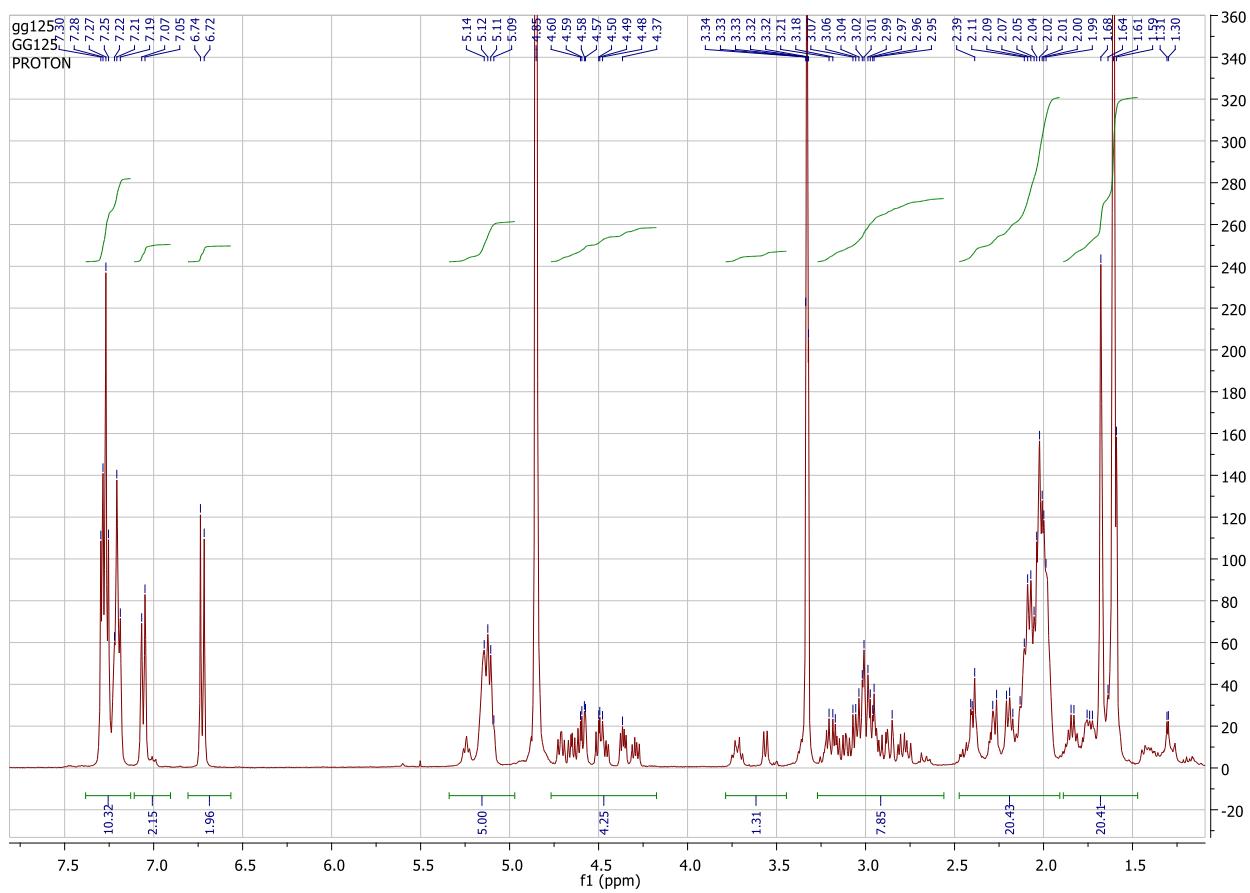


^1H spectrum of **SQ-Leu-Enk** (alternate synthesis)

^{13}C spectrum of **SQ-Leu-Enk** (alternate synthesis)

^1H spectrum of SQ-EM-1

¹H spectrum of SQ-EM-2



4. Cell proliferation studies

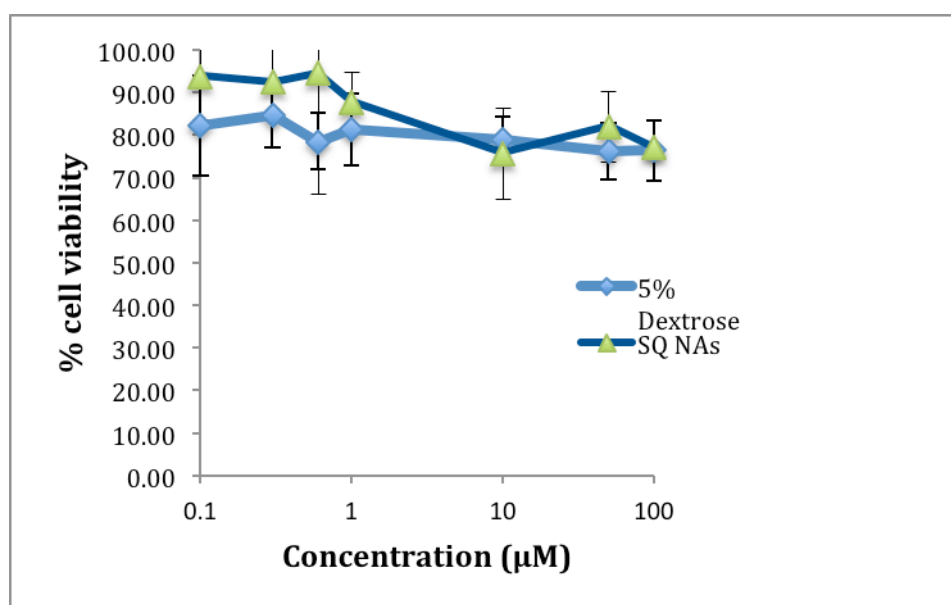


Figure SI.1. Control antiproliferation experiments. Cell viability of MiaPACA-2 cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye in standard MTT tests. Viability of Mia PACA-2 against (A) 5% dextrose and (B) SQ NAs show that they have no effect in the cell proliferation rate.

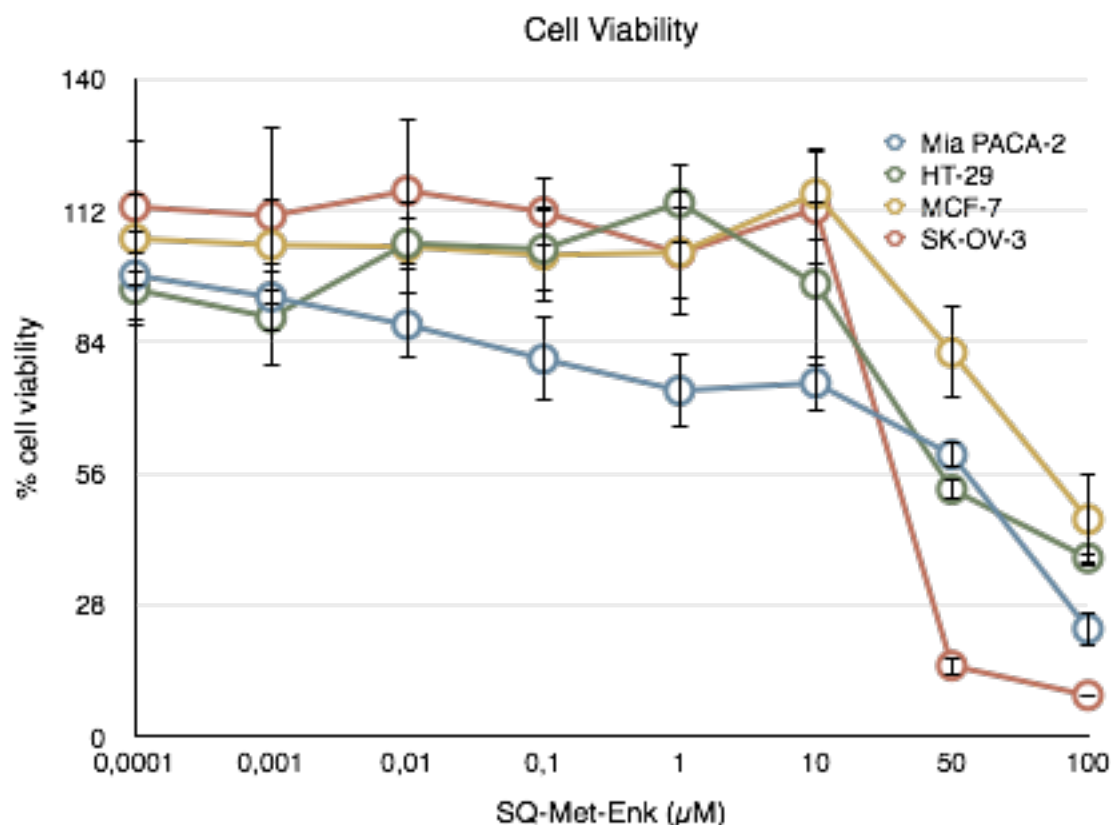


Figure SI.2. Antiproliferative activity of SAME NAs on different cell types. Cell viability of different cancer cells was determined using standard MTT tests. Viability of Mia PACA-2 (A), MCF-7 (B), HT-29 (C) and SKOV-03 (D) cells against SAME NAs.

5. *In-vivo* drug treatments

Animals. 6- to 8-week-old female athymic nude mice were purchased from Harlan Laboratory. All animals were housed in appropriate animal care facilities during the experimental period, and were handled according to the principles of laboratory animal care and legislation in force in France. All *in vivo* studies were performed in accordance with a protocol approved by the Ethical Committee of the Institut Gustave Roussy.

5.1. Systemic toxicity:

The systemic toxicity of SAME NAs and their mixture with Gem was firstly investigated and compared to those of free Gem and SQ NAs after repeated intravenous (i.v.) injections to healthy nude mice. 6- to 8-week-old female athymic nude mice were used to evaluate the toxicity of SAME NAs after repeated

administration. All groups (n = 4) received intravenous injections of (i) 7 mg/kg Gem on days 1, 4, 7 and 10, (ii) 15 mg/kg Met-Enk on days 0-4, 7 and 10, (iii) SAME NAs (15 mg/kg equiv. Met-Enk) on days 0-4, 7 and 10, (iv) 15 mg/kg Met-Enk on days 0-4, 7 and 10 and 7 mg/kg Gem on days 1, 4, 7 and 10, (v) SAME NAs (15 mg/kg equiv. Met-Enk) on days 0-4, 7 and 10 and 7 mg/kg Gem on days 1, 4, 7 and 10, and (vi) 5 mg/kg SQ NAs daily (i.e., on days 0-4, 7 and 10; equivalent of SQ in SAME NAs). By using a 10% weight loss as a standard threshold value for animal health status, no toxicity was observed for all treatments (see Figure SI.4). We therefore used this protocol to evaluate the antitumor activity of the SAME NAs against human pancreatic (Mia PACA-2) carcinoma xenograft model in nude mice.

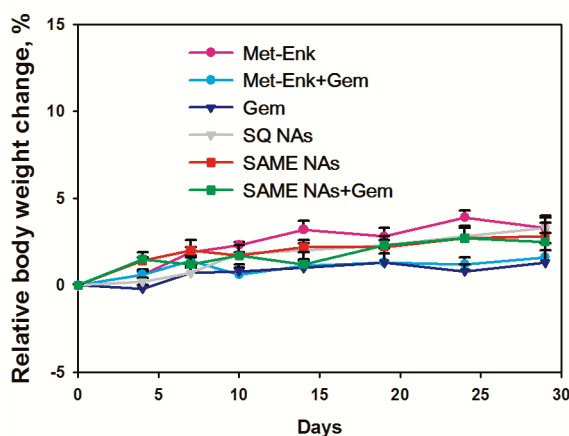


Figure SI.3. Body weight variations observed during 29 days after intravenous administration of SAME NAs, SAME NAs + Gem, Met-Enk, Met-Enk + Gem, free Gem, and SQ NAs into female nude mice. All groups (n = 4) received intravenous injections of (i) 7 mg/kg Gem on days 1, 4, 7 and 10, (ii) 15 mg/kg Met-Enk on days 0-4, 7 and 10, (iii) SAME NAs (15 mg/kg equiv. Met-Enk) on days 0-4, 7 and 10, (iv) 15 mg/kg Met-Enk on days 0-4, 7 and 10 and 7 mg/kg Gem on days 1, 4, 7 and 10, (v) SAME NAs (15 mg/kg equiv. Met-Enk) on days 0-4, 7 and 10 and 7 mg/kg Gem on days 1, 4, 7 and 10, and (vi) 5 mg/kg SQ NAs daily (i.e., on days 0-4, 7 and 10).

5.2. In vivo anticancer activity on solid tumor-bearing mice:

The antitumor efficacy of SAME NAs and their mixture with Gem was investigated on the human pancreatic carcinoma xenograft model Mia PACA-2. Briefly, 200 μ L of the Mia PACA-2 cell suspension, equivalent to 1×10^7 cells, were injected subcutaneously into nude mice toward the upper portion of the right flank, to develop a solid tumor model. Tumors were allowed to grow to a volume of $\sim 100 \text{ mm}^3$ before initiating the treatment. Tumor length and width were measured with calipers, and the

tumor volume was calculated using the following equation: $V_{\text{tumor}} = \text{length} \times \text{width}^2/2$. Tumor-bearing nude mice were randomly divided into 7 groups of 7 each and all groups received intravenous injections of (i) 0.2 mL of sterile saline daily (i.e., on days 0-4, 7 and 10), (ii) 7 mg/kg Gem on days 1, 4, 7 and 10, (iii) 15 mg/kg Met-Enk on days 0-4, 7 and 10, (iv) SAME NAs (15 mg/kg equiv. Met-Enk) on days 0-4, 7 and 10, (v) 15 mg/kg Met-Enk on days 0-4, 7 and 10 and 7 mg/kg Gem on days 1, 4, 7 and 10, (vi) SAME NAs (15 mg/kg equiv. Met-Enk) on days 0-4, 7 and 10 and 7 mg/kg Gem on days 1, 4, 7 and 10, and (vii) 5 mg/kg SQ NAs daily (i.e., on days 0-4, 7 and 10). On the days when both drugs were given together, SAME NAs or Met-Enk was administered first and immediately followed by Gem. The injected volume was 10 $\mu\text{L/g}$ of the body weight. The mice were monitored regularly for changes in tumor size and weight.