Ligation of anti-cancer drugs to self-assembling ultrashort peptides by click chemistry for localized therapy

Michael R. Reithofer, Kiat-Hwa Chan, Anupama Lakshmanan, Dang Hoang Lam, Archana Mishra, Began Gopalan, Mangesh Joshi, Shu Wang, and Charlotte A. E. Hauser**

Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos, Singapore 138669 (Singapore)
e-mail: chauser@ibn.a-star.edu.sg, mreithofer@ibn.a-star.edu.sg

Electronic Supporting Information

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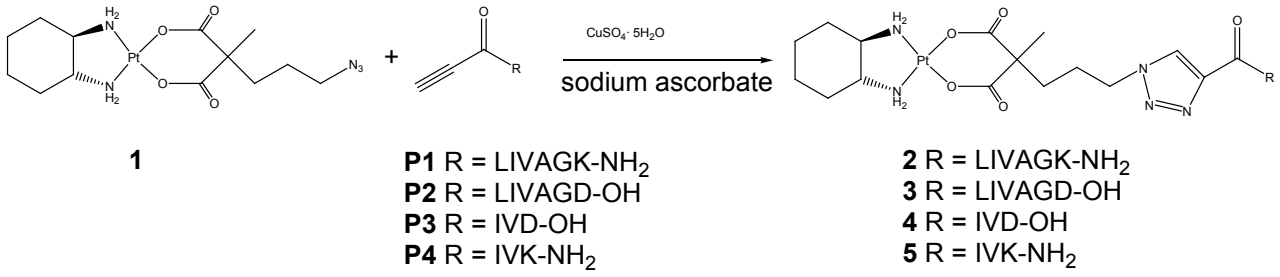
Experimental Section

General Procedure

All reagents and solvents were obtained from commercial suppliers and used as received. The starting compounds (*SP*-4-2)-(*trans*-(R,R)-1,2-diaminocyclohexane)diiodoplatinum(II), and dimethyl-2-(3-bromopropyl)-2-methylmalonate were synthesized according to standard literature procedures.[1, 2] (*SP*-4-2)-(*trans*-(R,R)-1,2-diaminocyclohexane) diiodoplatinum(II) was synthesized using a method similar to the one described for (*SP-4-2*)-diamminediiodoplatinum(II) using *1R,2R*-DACH instead of ammonia as the ligand. All peptide based compounds were purified on an Agilent 1260 Infinity preparative HPLC system equipped with a Zorbax SB-C18 column (21.2 × 150 mm 7 µM). The HPLC was coupled over an active splitter to a SQ-MS for mass triggered fraction collection. MilliQ water and HPLC grade acetonitrile, both containing 0.1% formic acid, were used as eluents, and the solvent gradient was adjusted for each compound. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-400 (400 MHz) instrument and all signals were referenced to the solvent residual peak.

The ^{1}H,^{13}C-HMBC and ^{1}H,^{13}C-HMQC NMR experiments for compound **2** and **4** (1 and 3 mM in d_7-DMF respectively) required for assigning the 'C' chemical shifts were recorded on a 800 MHz Bruker Avance^{TM} spectrometer with a triple shielded Z gradient and a cryo-probe at 25 ^{0}C. The TOCSY experiments for the ^{1}H chemical shift assignments were recorded on a 400 MHz Bruker Avance^{TM} spectrometer with a cryo-probe.



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P1 R = LIVAGK-NH_2
P2 R = LIVAGD-OH
P3 R = IVD-OH
P4 R = IVK-NH_2

2 R = LIVAGK-NH_2
3 R = LIVAGD-OH
4 R = IVD-OH
5 R = IVK-NH_2

Figure S 1: Synthesis of oxaliplatin peptide conjugates and the numbering scheme.

Peptide Synthesis

The solid phase peptide synthesis of IVD-OH and LIVAGD-OH commenced with Fmoc-Asp(O^{t}Bu)-functionalised Wang resin (GL Biochem) and the SPPS of LIVAGKK-NH_2 commenced with Fmoc-Lys(Boc)-functionalised Rink amide resin (GL Biochem) following standard peptide synthesis protocols.[3] The de-protection of Fmoc was achieved by treating the resin with piperidine in DMF. The supernatant was filtered off and the resin washed with DMF. Coupling of the appropriate Fmoc-protected amino acid to the resin was done by treating the resin with a combined solution of the amino acid (3 equivalent), TBTU (3 equivalent) and DIPEA (3 equivalent) in DMF. The filtering-cum-washing, de-protection, and coupling cycle was then repeated until all the amino acids of the peptide were linked. In the final step, coupling of an alkyne group to the N-terminus was achieved by treating the resin with a combined solution of propiolic acid (2 equivalent) and HATU (2 equivalent) in DMF without the addition of DIPEA. After coupling, the resin was washed with a 10 wt% solution of DIPEA in DMF.[4] The final coupling step was repeated until the Kaiser test showed negative. After final washing of the resin with DMF and CH_2Cl_2, the resin was dried in vacuum. Subsequently, the *N*-propiolyl-peptide was cleaved from the resin by treating it with TFA. This step removed the O^{t}Bu protecting groups on Asp and Lys as well. The *N*-propiolyl-peptide was then precipitated from TFA by

the addition of diethyl ether. After filtration, the *N*-propiolyl-peptide was further purified by HPLC.

Peptide P1: Yield: 384mg (from 2 g of K(Boc)-Rink amide resin)

^{1}H-NMR(*d*$_6$-DMSO): 8.92 (1H, d, $^{3}J_{HH}$ = 8.1 Hz), 8.16 (1H, t, $^{3}J_{HH}$ = 5.5 Hz), 8.04 (1H, d, $^{3}J_{HH}$ = 6.7 Hz), 7.97 (1H, d, $^{3}J_{HH}$ = 8.7 Hz, N*H*), 7.89 (1H, d, $^{3}J_{HH}$ = 8.1 Hz, N*H*), 7.82(1H, d, $^{3}J_{HH}$ = 8.7 Hz, N*H*), 7.37 (1H, s), 7.08 (1H, s), 4.39 – 4.31 (m, 1H), 4.29 – 4.22 (m, 1H), 4.21 – 4.10 (m, 5H), 3.72 (d, 2H, $^{3}J_{HH}$ = 5.6 Hz), 2.74 (t, 2H, $^{3}J_{HH}$ = 7.4 Hz), 2.54 (s, 1H), 2.00 – 1.90 (m, 1H), 1.77 – 1.62 (m, 3H), 1.61 – 1.33 (m, 7H), 1.32 – 1.24 (m, 2H), 1.20 (d, 3H, $^{3}J_{HH}$ = 6.9 Hz), 1.11 – 0.98 (m, 2H), 0.90 – 0.74 (m, 16H) ppm.

^{13}C-NMR(*d*$_6$-DMSO): 173.5, 172.5, 171.2, 171.0, 170.6, 168.6, 151.5, 78.2, 76.4, 57.6, 56.9, 52.1, 51.5, 48.4, 42.1, 40.4, 38.8, 36.3, 31.3, 30.4, 26.9, 24.4, 24.3, 23.0, 22.4, 21.5, 19.2, 18.1, 15.3, 11.0 ppm.

FT-IR: v = 1211 (w), 1507 (m), 1600 (s), 2104 (vw), 2944 (vw), 3277 (w) cm^{-1}.

ESI-MS: Calculated for $C_{31}H_{55}N_8O_7$ ($[M+H^+]^+$) 651.42, Found: *m/z* 651.5.

Peptide P2: Yield: 107.2 mg (from 1 g of D(Boc)-Wang resin)

^{1}H NMR (95 % H_2O/5 %D_2O): 8.94 (d, 1H, *J* = 5.8 Hz), 8.34 (d, 1H, *J* = 4.6 Hz), 8.30 – 8.17 (m, 4H), 7.96 (d, 1H, *J* = 8.0 Hz), 4.40 – 4.32 (m, 1H), 4.29 – 4.19 (m, 1H), 4.14 – 4.06 (m, 1H), 4.05 – 3.97 (m, 1H), 3.85 (d, 2H, *J* = 5.9 Hz), 3.41 (s, 1H), 2.75 (m, 2H), 1.99 – 1.89 (m, 1H), 1.79 – 1.69 (m, 1H), 1.55 – 1.44 (m, 3H), 1.43 – 1.34 (m, 1H), 1.30 (d, 3H, *J* = 7.4 Hz), 1.16 – 1.03 (m, 1H), 0.86 – 0.70 (m, 18H) ppm.

^{13}C NMR (95 % H_2O/5 %D_2O): 175.24, 173.78, 173.43, 172.90, 170.66, 167.93, 154.32, 77.37, 75.60, 59.22, 58.07, 53.03, 50.64, 49.93, 42.49, 39.57,

38.77, 36.83, 35.67, 30.28, 24.43, 24.34, 21.87, 21.07, 18.41, 17.70, 16.64, 14.69, 9.65 ppm.

FT-IR: v = 1252 (m), 1501 (m), 1524 (s), 1597 (vs), 2100 (w), 2986 (w), 3256 (m) cm^{-1}.

ESI-MS: Calculated for $C_{29}H_{47}N_6O_{10}$ ($[M+H^+]^+$) 639.33, Found: *m/z* 639.4.

Peptide P3: Yield: 103 mg (from 2 g of D(Boc)-Wang resin)
^{1}H-NMR(*d*$_6$-DMSO): 12.54 (bs, 2H, COOH), 8.85 (d, 1H, $^3J_{HH}$ = 8.6 Hz), 8.20 (d, 1H, 8.2 Hz), 7.83 (d, 1H, 8.6 Hz), 4.51 (m, 1H), 4.19 (m, 2H), 4.15 (s, 1H), 2.67 (m, 1H), 2.56 (m, 1H), 1.96 (m, 1H), 1.74 (m, 1H), 1.40 (m, 1H), 1.10 (m, 1H), 0.82 (m, 12H) ppm.

^{13}C-NMR(*d*$_6$-DMSO): 172.2, 171.5, 170.4, 170.1, 151.5, 78.1, 76.3, 57.4, 57.3, 48.5, 35.9, 35.7, 30.7, 24.5, 19.0, 17.9, 15.2, 10.6 ppm.

FT-IR: v = 1232 (w), 1401 (m), 1503 (s), 1575 (s), 1611 (m), 2102 (vw), 2977 (vw), 3261 (m) cm^{-1}.

ESI-MS: Calculated for $C_{18}H_{28}N_3O_7$ ($[M+H^+]^+$) 398.19, Found: *m/z* 398.3.

Peptide P4: Yield: 86.8 mg (from 2 g of K(Boc)-Rink amide resin)
^{1}H NMR (95 % H_2O/5 %D_2O): 8.37 (d, 1H, J = 7.2 Hz), 8.26 (d, 1H, J = 7.2 Hz), 7.49 (s, 1H), 7.01 (s, 1H), 4.22 – 4.13 (m, 1H), 4.10 – 4.05 (m, 1H), 4.00 (t, 2H, J = 8.7 Hz), 3.43 (s, 1H), 2.89 (t, 2H, J = 6.8 Hz), 1.99 – 1.88 (m, 1H), 1.78 – 1.52 (m, 5H), 1.45 – 1.24 (m, 3H), 1.15 – 1.03 (m, 1H), 0.88 – 0.72 (m, 12H) ppm.

^{13}C NMR (95 % H_2O/5 %D_2O): 176.31, 173.25, 173.08, 154.51, 77.41, 75.55, 59.69, 59.13, 53.47, 39.47, 35.99, 30.49, 30.09, 26.45, 24.75, 22.16, 18.44, 17.96, 14.72, 10.19 ppm.

FT-IR: v = 194 (m), 1224 (m), 1501 (m), 1524 (s), 1577 (s), 2100 (vw), 3256 (m) cm^{-1}.

ESI-MS: Calculated for $C_{20}H_{36}N_5O_4$ ($[M+H^+]^+$) 410.6, Found: *m/z* 410.2.

2-(3-azidopropyl)-2-methylmalonic acid

Dimethyl 2-(3-bromopropyl)-2-methylmalonate (10.6 g, 39.7 mmol) was dissolved in 80 mL of a 6:1 mixture of acetone/water and solid NaN_3 (4.4 g, 67.7 mmol) was added. The resulting suspension was stirred under reflux overnight. Subsequently, the acetone was removed under reduced pressure and the resulting solution was extracted three times with EtOAc. The combined organic layer was dried over $MgSO_4$ filtered and the solvent removed under reduced pressure. The crude product so obtained was immediately dissolved in a mixture of THF/H_2O (1:1) and 3 equivalent NaOH was added. The suspension was stirred at 40°C overnight and the resulting solution was extracted three times with DCM. Subsequently, the pH of the aqueous phase was adjusted to 1 using a 12M HCl solution. After three rounds of extraction with EtOAc, the combined organic layer was dried over $MgSO_4$ filtered and the solvent was removed under reduced pressure. The title compound was obtained in sufficient purity as a white powder after vacuum drying. Yield: 4.5 g (56 %, overall yield)
^{1}H NMR (95 % H_2O/5 %D_2O): 3.20 (t, 3H, J = 6.6 Hz, CH_2N_3), 1.80 – 1.74 (m, 2H, CH_2CH_2CH_2N_3), 1.48 – 1.39 (m, 2H, CH_2CH_2N_3), 1.27 (s, 3H) ppm.

^{13}C NMR (95 % H_2O/5 %D_2O): 177.36 (*C*O_2H), 53.45 (C(CH2)), 50.92 (*C*H_2N_3), 32.83 (*C*H_2CH_2CH_2N_3), 23.62 (*C*H_2CH_2N_3), 19.94 (*C*H_3) ppm.

ESI-MS: Calculated for $C_7H_{11}N_3O_4Na$ ($[M+Na^+]^+$) 224.06, Found: *m/z* 224.1.

(SP-4-2)-(2-(3-azidopropyl)-2-methylpropanedioato-κO1,κO3)-(trans-(R,R)-1,2-diaminocyclohexane)platinum(II)

$[PtI_2(DACH)]$ (1 g; 1.78 mmol) was suspended in 50 ml of water. After addition of $AgNO_3$ (589 mg; 3.47 mmol) the suspension was stirred overnight in the dark at room temperature. AgI was filtered off and the filtrate was added to a solution of 2-(3-azidopropyl)-2-methylmalonic acid (349 mg; 1.74 mmol) and 1.0M NaOH (3.6 ml; 3.6 mmol) in ca. 10 ml of water. The resulting solution was stirred for 1 h at room temperature. Subsequently the solvent was removed, the residual solid was suspended in a minimal amount of water and stored in the fridge overnight, the product was filtered off, washed with water (three times), and dried in vacuum. Alternatively, the pure product can be obtained by preparative HPLC of the supernatant solution.
Yield: 351 mg (40%).

^{1}H NMR (95 % H_2O/5 %D_2O): 5.75 (bs, NH_2), 3.45 (m, 2H), 3.05 (m, 1H), 2.79 (m, 1H), 2.40 (bs, 2H), 2.05 (m, 2H), 1.61 (m, 4H), 1.40 – 1.08 (m, 7H) ppm.

^{13}C NMR (95 % H_2O/5 %D_2O): 181.9, 62.8, 57.0, 51.1, 36.4, 31.8, 24.0, 20.9 ppm.

FT-IR: v = 1493 (s), 1601 (m), 2094 (w) cm^{-1}.

ESI-MS: Calculated for $C_{13}H_{24}N_5O_4Pt$ ($[M+H^+]^+$) 509.15, Found: *m/z* 509.2.

Peptide ligation reaction

General Procedure

An equimolar amount of peptide and metal compound was suspended in a 1:1 mixture of MilliQ water and *t*BuOH. To increase the solubility about 10% DMF was added. Subsequently 10 mol% of sodium ascorbate dissolved in a minimum of water was added followed by 1 mol% $CuSO_4$•$5H_2O$ dissolved water. The

resulting suspension was stirred in the dark overnight and purified directly by preparative HPLC.

Compound 2: Yield: 49 mg (29 %).

^{1}H NMR (95 % H_2O/5 %D_2O): 8.50 (d, 1H, J = 6.7 Hz), 8.39 (s, 1H), 8.34 (d, 1H, J = 5.5 Hz), 8.31 – 8.22 (m, 2H), 8.18 (d, 1H, J = 8.5 Hz). 8.09 (d, 1H, J = 7.8 Hz), 7.54 (s, 1H), 7.00 (s, 1H), 5.66 – 5.37 (m, NH_2), 4.51 – 4.35 (m, 2H), 4.24 – 4.08 (m, 2H) 4.07 – 3.88 (m, 2H), 3.79 (d, 2H, J= 5.6 Hz), 3.54 (s, 3H), 2.91 – 2.68 (m, 3H), 2.57 – 2.43 (m, 1H), 2.31 – 2.13 (m, 1H), 2.09 – 1.94 (m, 1H), 1.93 – 0.89 (m, 29H), 0.88 – 0.60 (m, 18H).

^{13}C NMR (95 % H_2O/5 %D_2O): 181.2, 181.0, 176.6, 175.4, 174.2, 173.4, 172.9, 171.3, 161.7, 141.5, 127.4, 62.8, 62.3, 59.2, 58.2, 56.4, 53.2, 52.7, 50.6, 49.9, 42.4, 40.1, 39.4, 35.7, 35.5, 31.7, 30.3, 30.1, 26.2, 25.1, 24.6, 24.3, 23.9, 23.8, 22.0, 21.8, 21.2, 20.7, 18.3, 17.7, 16.4, 14.6, 9.7 ppm.

FT-IR: v = 1395 (m), 1414 (m), 1481 (s), 1512 (s), 2951 (w), 3280 (w) cm^{-1}.

ESI-MS: Calculated for $C_{44}H_{78}N_{13}O_{11}Pt$ ([M+H$^+$]$^+$) 1159.59, Found: *m/z* 1159.6.

Compound 3: Yield: 3 mg (4.4 %).

^{1}H NMR (95 % H_2O/5 %D_2O): 8.50 (d, 1H, J = 6.3 Hz), 8.39 (s, 1H), 8.33 (d, J = 5.7 Hz), 8.29 – 8.23 (m, 2H), 8.18 (d, 1H, J = 8.4 Hz), 7.91 (d, 1H, J = 7.8 Hz), 5.60 – 5.35 (m, NH_2), 4.48 – 4.37 (m, 2H), 4.17 (t, 1H, J = 6.3 Hz), 4.04 (t, 1H, J = 8.5 Hz), 3.95 (t, 1H, J = 8.3 Hz), 3.80 (d, 1H, J = 5.8 Hz), 2.80 – 2.70 (m, 2H), 2.73 (d, 1H, J = 6.0 Hz), 2.68 (t, 1H, J = 5.7 Hz), 2.56 (s, 3H), 2.55 – 2.44 (m, 1H), 2.06 – 0.91 (m, 21H), 0.83 – 0.63 (m, 20H) ppm.

^{13}C NMR (95 % H_2O/5 %D_2O): 181.6, 181.1, 175.2, 174.1, 173.3, 172.8, 170.5, 161.7, 141.5, 127.5, 62.9, 62.3, 59.2, 58.3, 56.4, 52.7, 50.3, 50.7, 49.9, 44.8, 42.5, 40.1, 38.7, 37.0, 35.8, 35.6, 31.8, 30.2, 25.1, 24.6, 24.4, 24.0, 21.85, 21.3,

20.7, 18.4, 17.6, 16.6, 14.6, 9.80 ppm.

FT-IR: *v* = 1201 (m), 1425 (m), 1502 (m), 1611 (s) cm^{-1}.

ESI-MS: Calculated for $C_{42}H_{70}N_{11}O_{14}Pt$ ($[M+H^+]^+$) 1147, Found: *m/z* 1147.1.

Compound 4: Yield: 11 mg (24 %).
^1H NMR (95 % H_2O/5 %D_2O): 8.40 (s, 1H), 8.36 (d, 1H, J = 7.7 Hz), 8.29 (t, 2H, J = 7.7 Hz), 5.66 – 5.35 (m, N*H*$_2$), 4.47 (m, 1H), 4.24 (t, 1H, J = 7.7 Hz), 4.00 (t, 1H, J = 8.1 Hz), 2.83 – 2.66 (m, 3H), 2.59 – 2.46 (m, 1H), 2.29 – 2.11 (m, 1H), 2.07 – 1.66 (m, 7H), 1.51 – 1.33 (m, 4H), 1.27 – 0.92 (m, 8H), 0.89 – 0.62 (m, 14H) ppm.

^{13}C NMR (95 % H_2O/5 %D_2O): 181.3, 181.1, 174.8, 173.2, 172.6, 161.8, 141.5, 127.4, 62.8, 62.3, 59.5, 58.5, 56.4, 50.6, 50.0, 36.5, 36.2, 35.6, 31.7, 30.1, 25.0, 24.8, 23.9, 23.8, 20.6, 18.2, 17.8, 14.7, 10.1 ppm.

FT-IR: *v* = 1394 (m), 1411 (m), 1501 (s), 1515 (s), 2960 (w), 3270 (w) cm^{-1}. ESI-

MS: Calculated for $C_{31}H_{51}N_8O_{11}Pt$ ($[M+H^+]^+$) 906.33, Found: *m/z* 906.3.

Compound 5: Yield: 12 mg (22.1 %).
^1H NMR (95 % H_2O/5 %D_2O): 8.39 (s, 1H), 8.35 (d, 1H, J = 7.9 Hz), 8.28 (s, 1H), 7.48 (s, 1H), 6.99 (s, 1H), 5.63 – 5.38 (m, N*H*$_2$), 4.23 (t, 1H, J = 8.1 Hz), 4.16 – 4.08 (m, 1H), 3.95 (t, 1H, J = 8.1 Hz), 2.89 – 2.78 (m, 2H), 2.76 – 2.66 (m, 1H), 2.54 – 2.43 (m, 1H), 2.28 – 2.13 (m, 1H), 2.07 – 0.93 (m, 24H), 0.88 – 0.64 (m, 14H) ppm.

^{13}C NMR (95 % H_2O/5 %D_2O): 181.35, 181.09, 176.23, 173.33, 173.23, 161.94, 141.53, 127.39, 62.83, 62.33, 59.65, 58.47, 56.40, 53.37, 50.63, 39.35, 36.46, 35.56, 31.77, 30.34, 29.96, 29.57, 26.34, 25.09, 24.81, 23.91, 23.81, 22.03,

20.75, 18.24, 17.95, 14.66, 10.15 ppm.

FT-IR: ν = 1392 (m), 1413 (m), 1583 (s), 1608 (s) cm^{-1}.

ESI-MS: Calculated for $C_{33}H_{59}N_{10}O_8Pt$ ($[M+H^+]^+$) 918, Found: *m/z* 918.2.

NMR assignment for compounds 2 and 4

^1H and ^{13}C chemical shifts for compound **2** (1 mM in d_7-DMF):

Peptide moiety

	C'	Cα	Cβ	$C\gamma_1$	$C\gamma_2$	Cδ	Cε	H	Hα	Hβ	$H\gamma_1$	$H\gamma_2$	H δ
Leu	169.2	58.7	41.8	23.1		18.1		7.8	4.3	1.7, 1.8	1.4		0.9
Ile	172.6	58.2	36.9	23.9	19.3	10.8		8.3	4.4	1.9	1.4	0.9	0.8
Val	171.9	58.8	31.6	21.6				8	4.3	2.1	0.9		
Ala	173.4	49.8	17.5					8.2	4.3	1.4			
Gly	169.2	43.0						8.4	3.9, 3.8				
Lys		52.0				29.7		8.4	4.8	1.7		1.8	

1R,2R-DACH and the linker

DACH	C12	C36	C45	H12	H361	H362	H451	H452	NH1	NH2
	50.6	32	32.1	4.6	1.7	1.8	1.3	1.9	5.3	6.1
	C4	C5	C7	C_{Az1}	CO	H4	H5	H_{Az1}	H7	
linker	30.8	24.7	17.3	126.9	178.4	2.1	1.6	7.8	1.4	

^{1}H and ^{13}C chemical shifts for compound **4** (3 mM in d_7-DMF):

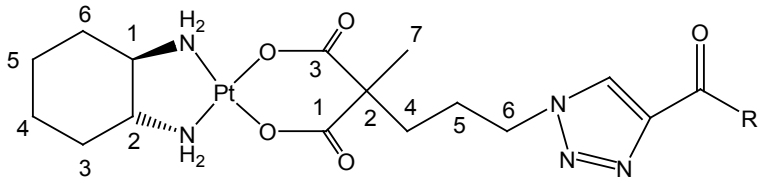
Peptide moiety

	C'	Cα	Cβ	$C\gamma_1$	$C\gamma_2$	Cδ	Cε	H	Hα	Hβ	$H\gamma_1$	$H\gamma_2$	H δ
Ile	171.1	57	38.2	24.7	15.4	10.8		8.1	4.6	1.9	1.1	0.8	0.7
Val	171.2	58.3	30.9	19	17.6			8.3	4.3	2.0	0.8	0.7	
Asp	171.3	57.1	38					8.0	4.5	2.6			

For *1R,2R*-DACH and the linker

	C12	C36	C45	H12	H361	H362	H451	H452	NH1	NH2
DACH	50.6	32.2	32.1	4.4	1.3	1.9	1.4	1.9	5.3	6.1

	C4	C5	C7	C_{Az1}	CO	H4	H5	H_{Az1}	H7
linker	35.8	24.4	17.3	126.9	178.4	2.1	1.6	7.8	1.2



2 R = LIVAGK-NH_2
4 R = IVD-OH

Figure S 2: Numbering scheme for compound **2** and **4.**

FT-IR comparison of synthesized compounds

FT-IR spectra were recorded either on a PerkinElmer Spectrum 100 IR spectrometer, fitted with a germanium-based PIKE MIRacle attenuated total reflectance (ATR) sampling accessory, or on a PerkinElmer Spectrum One FT-IR spectrometer outfitted with a diamond-based Specac Golden Gate attenuated total reflectance (ATR) sampling accessory.

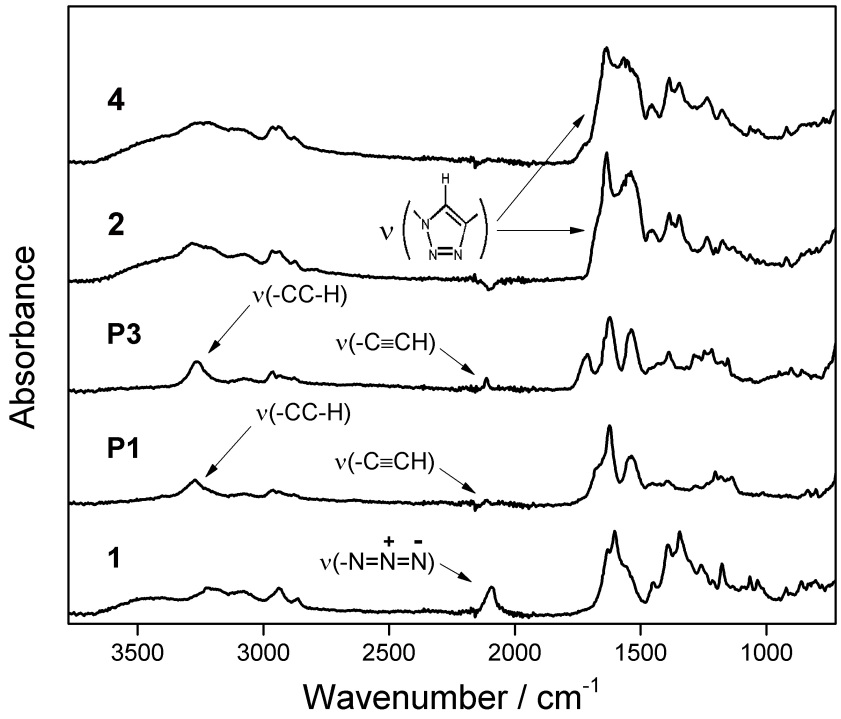


Figure S 3: FT-IR spectrum of compound **1**, as well as compound **2** and **4** in comparison to their respective peptide precursors **P1** and **P3** respectively.

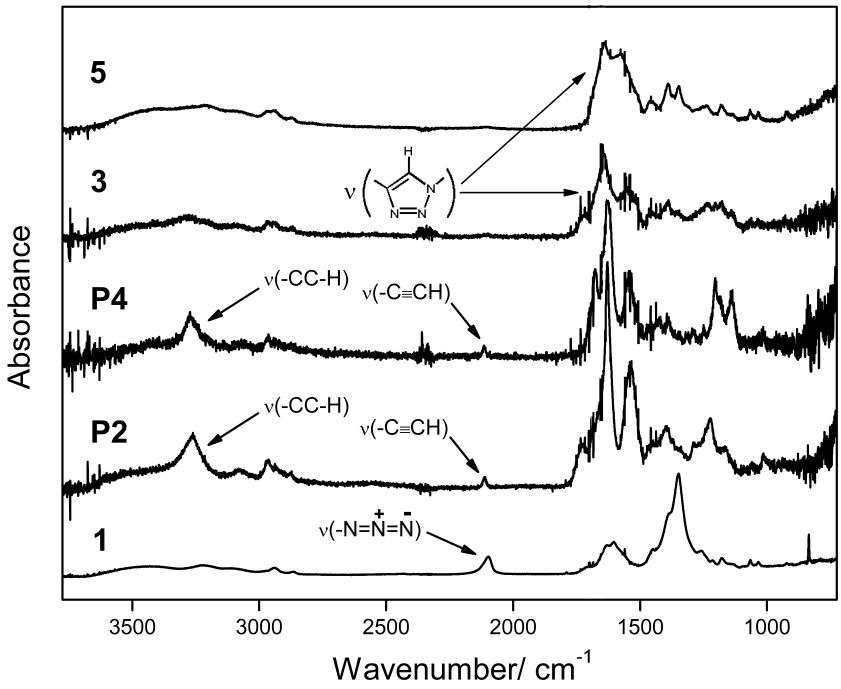


Figure S 4: FT-IR spectrum of compound **1**, as well as compound **3** and **5** in comparison to their respective peptide precursors **P2** and **P4** respectively

ESI-MS spectra for compounds 2-5

ESI-MS spectra were measured in positive mode on an Agilent 6130 Quadrupole LC/MS system equipped with an ESI spray chamber and coupled to a preparative Agilent 1260 HPLC unit. Samples were added in split mode using an active splitter (Agilent G1968 D Active Splitter) and split rate was adjusted according to the flow rate of the HPLC pumps. To achieve a reproducible ESI spray, an isocratic pump with a flow rate of 0.4 mL/min was used to transport the sample to the MS.

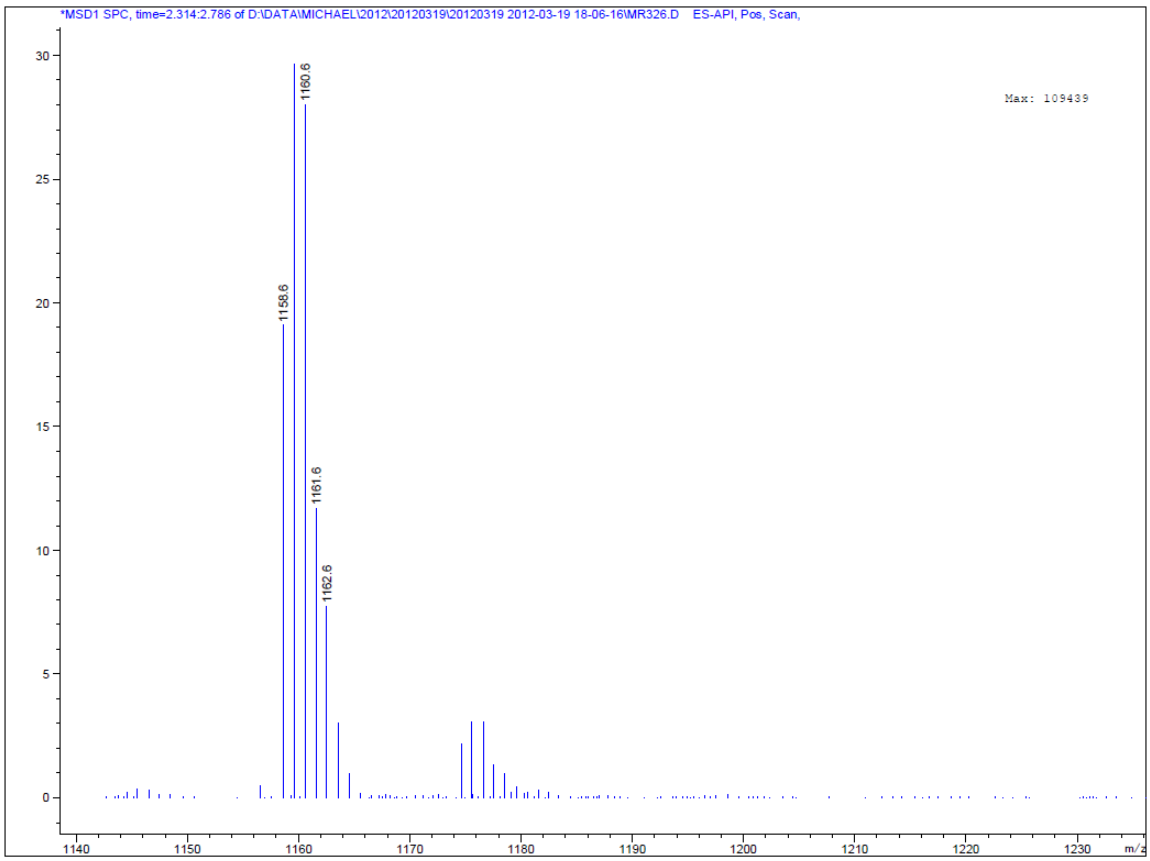


Figure S 5. ESI-MS spectrum of compound **2**.

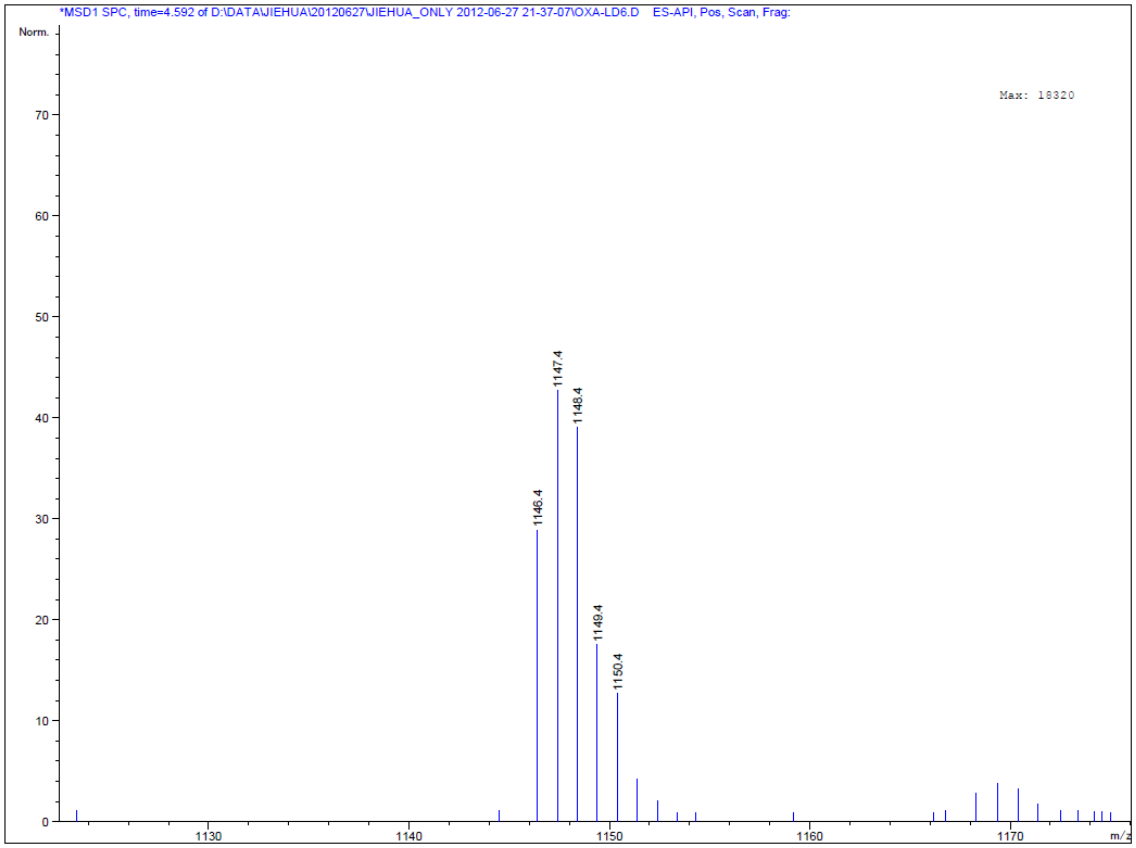


Figure S 6. ESI-MS spectrum of compound **3**.

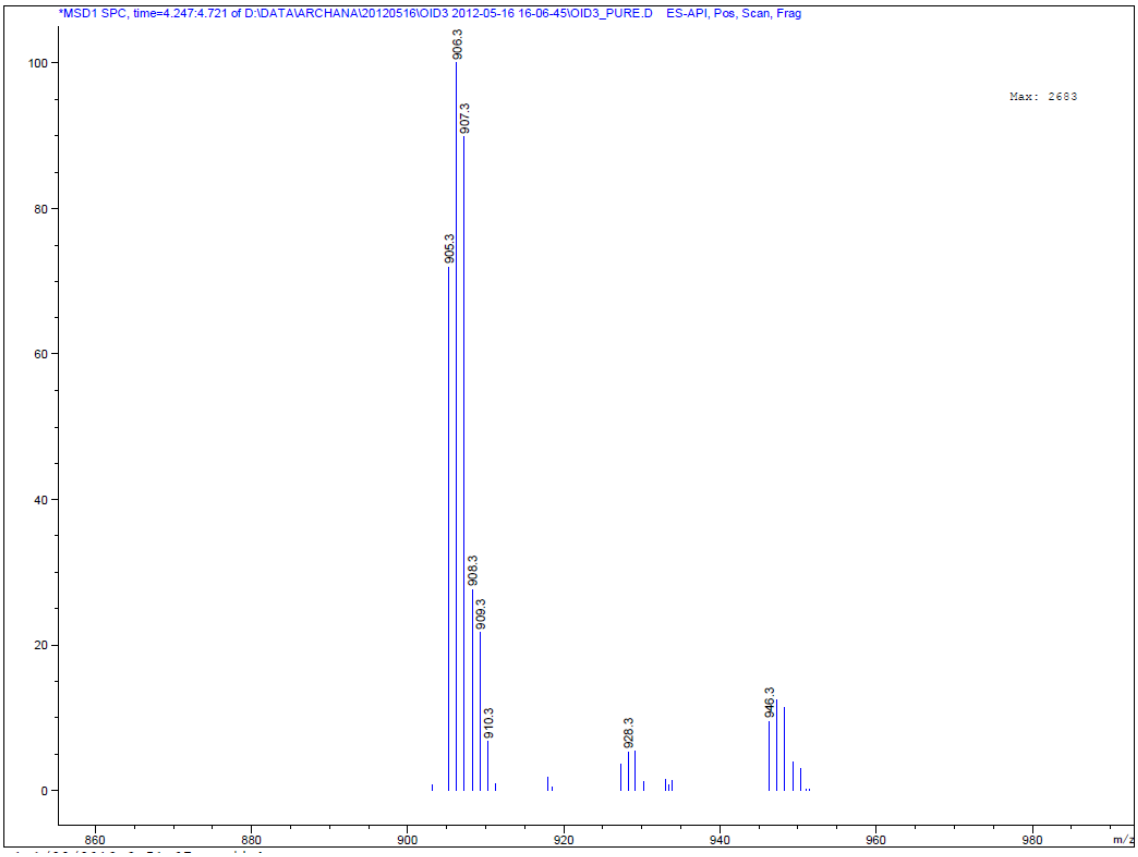


Figure S 7. ESI-MS spectrum of compound **4**.

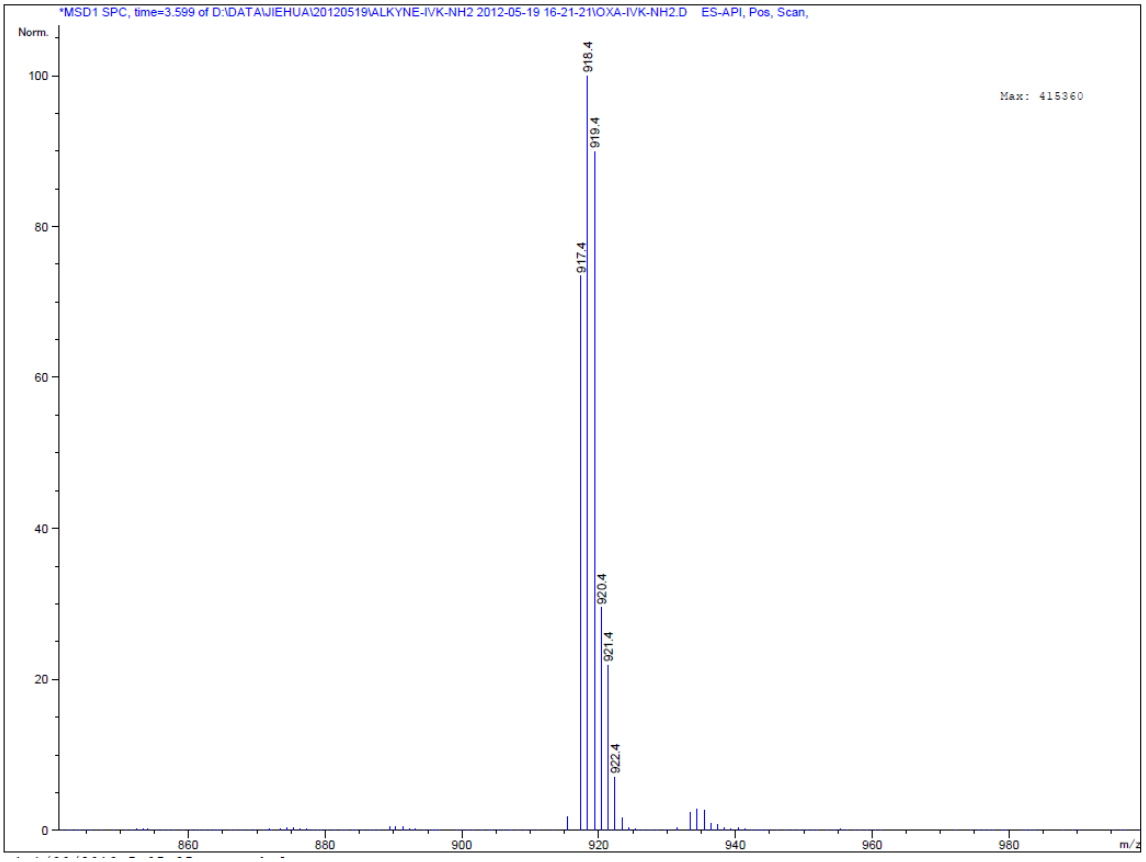


Figure S 8. ESI-MS spectrum of compound **5**.

UV-Vis spectra of selected compounds

UV-Vis spectra were recorded on a Hitachi U-2810 spectrometer between 700 nm and 190 nm using a scan rate of 200 nm/min and a slit of 1.5 nm. For the measurements, drug solutions at a concentration of 0.5 mg/mL and 0.025 mg/mL in MilliQ water were used.

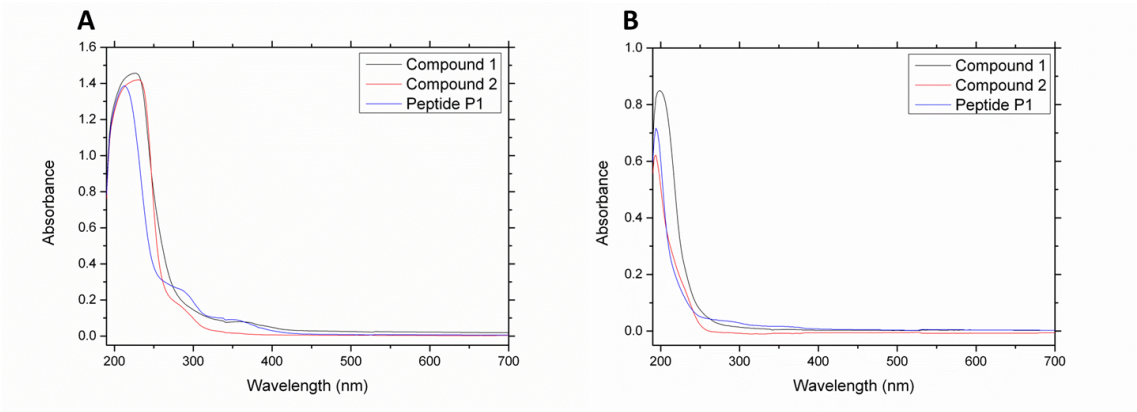


Figure S 9: Comparison of the UV-Vis absorbance of compound **1**, peptide **P1** and compound **2** at 0.5 mg/mL (**A**) and 0.025 mg/mL (**B**).

Quantification of residual copper in the final product

To determine whether significant amounts of copper were still present in the final product after HPLC purification, individual stock solutions of compound **2** (58 µg/mL) as well as compound 5 (60 µg/mL) were prepared. The copper content was determined by ICP-MS using a Perkin Elmar Elan DRC II instrument. An insignificant copper content of less than 0.25 ‰ was detected for compound **2** while for compound **4**, the copper content was below the detection limit of 10 ppb.

FESEM

Hydrogel samples were shock frozen and kept at -80°C. Frozen samples were then freeze-dried. Lyophilized samples were fixed onto a sample holder using a carbon conductive tape and sputtered with platinum from both the top and the sides in a JEOL JFC-1600 High Resolution Sputter Coater. The coating current was 20 mA and the process lasted for 50 sec. The surface of interest was then examined with a JEOL JSM-7400F Field Emission Scanning Electron Microscopy (FESEM) system using an accelerating voltage of 2 kV.

Drug release study

All drug release studies were carried out in 24-well transwell plates (Corning Transwell® 6.5 mm, 8.0 μM with a polycarbonate membrane insert) using 100 μL of gel in the transwell and 400 μL of DMEM in the peripheral well.

Drug release study in the presence of cells

HeLa cells (3500 cells/transwell) were seeded onto 24-well transwell plates in 100 μL DMEM supplemented with 10% FBS and 1% Pen-strep (growth medium). 400 μL of the growth medium was added to the peripheral well, giving a total volume of 500 μL. The cells were incubated overnight after which all the medium was removed. 100 μL of Ac-LIVAGK-NH_2 gel (15 mg/mL in 1xPBS) containing either 45 μM of compound **2**, 45 μM of oxaliplatin or no drug, was added on top of the cells in the transwell and 400 μL of the growth medium was added to the peripheral well. The release of platinum was studied by sampling 400 μL of medium at 1 h, 2 h, 4 h, 8 h, 1 d, 2 d, 3 d, and 4 d. Each time the hydrogel was untouched and only the medium surrounding the transwell was replenished. At the end of the release study, the residual gels were dissolved in 400 μL MilliQ water to determine the total drug concentration.
The concentration of released platinum was measured by inductively coupled plasma mass spectrometry (ICP-MS).

Drug release study without cells

The experiment was conducted in the same way as described above; with the exception that no cells were seeded onto the transwells and the DMEM medium did not contain FBS and pen-strep. At the end of the release study, the residual gels were dissolved in 400 μL MilliQ water to determine the total drug concentration. Three replicates were used and ICP-MS measurements were performed to determine the amount of drug released at each time point.

Long-term drug release study

Long-term drug release studies were carried out in 24-well transwell plates at 37 °C using PBS buffer as the solvent system. For this purpose, three gels containing 10% of compound **2** and 90% of Ac-LIVAGK-NH_2 (total concentration of 15 mg/mL) were independently prepared by completely dissolving compound **2** and the parent peptide in 90% of water and then adding 10x PBS, giving a 1x PBS solution. 100 µL of each gel was added to the transwell and 500 µL of PBS was added to the surrounding well. Samples were taken at different time points and the drug release was determined by inductively coupled plasma mass spectrometry (ICP-MS) analysis. At the end of the release study, the residual gels were dissolved in 500 µL PBS to determine the total drug concentration.

Inductively coupled plasma mass spectrometry (ICP-MS) analysis to determine platinum concentration

All ICP-MS analyses were performed on a Perkin Elmer Elan DRC II instrument and the ^{195}Pt isotope was used for quantification.

All samples containing growth medium were digested with nitric acid (68% suprapur, Merck) in a 1:1 ratio by volume overnight, prior to ICP- MS analysis. All nitric acid containing samples were diluted to a suitable concentration for ICP-MS analysis. All other samples were measured without nitric acid treatment, and each sample was diluted depending on the platinum concentration. Calibration was conducted using an external standard (Sigma-Aldrich) between 1-200 ppb. The concentrations of the samples were calculated based on the calibration curve plotted using Elan software, and was used to calculate the accumulative release profile as a percentage.

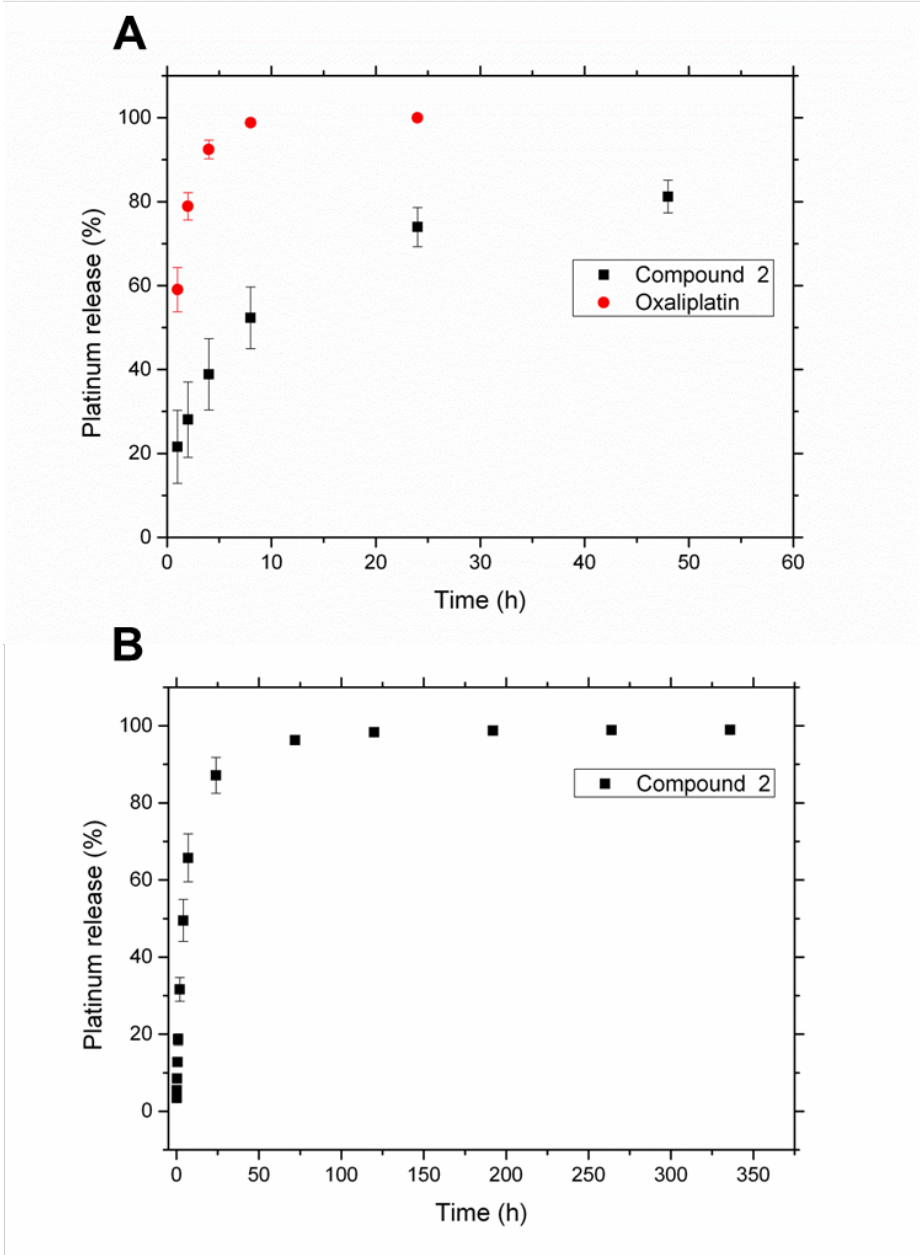


Figure S 10: Platinum release of compound **2** and oxaliplatin control over a period of two days (**A**) and of a hybrid hydrogel containing 10 wt% of compound **2** over a period of 14 days (**B**).

Characterization of released compounds

To study the release compounds, hydrogel samples containing compound **2** were incubated in a 24-well transwell plates at 37 °C, using PBS buffer for 24 h. The

compounds were then characterized by HPLC-MS. For this purpose, hydrogels containing 10% of compound **2** and 90% of Ac-LIVAGK-NH_2 (total concentration of 15 mg/mL) were prepared by completely dissolving compound **2** and the parent peptide in 90% of water, adding 10x PBS to a final 1x PBS solution. 100 µL gel material was placed into the transwell and 500 µL of PBS was added to the surrounding well. Due to platinum's light sensitivity, the resulting solution was incubated in the dark at 37 °C for 24 h. The supernatant was taken and analyzed by HPLC that was coupled to an Agilent 6130 Quadrupole mass spectrometer. In order to characterize even very low compound concentration, HPLC-MS was performed in split less mode using a continues gradient with a flow rate of 0.6 mL/min and an Agilent Eclipse Plus C18 (3.5 µM; Ø 4.6 mm x 100 mm length) column. To use it as a control, [PtI_2(DACH)] was activated in water with $AgNO_3$, filtered and diluted with PBS. The resulting solution was filtered and HPLC-MS was carried out in the same manner as described above.

Cell Culture

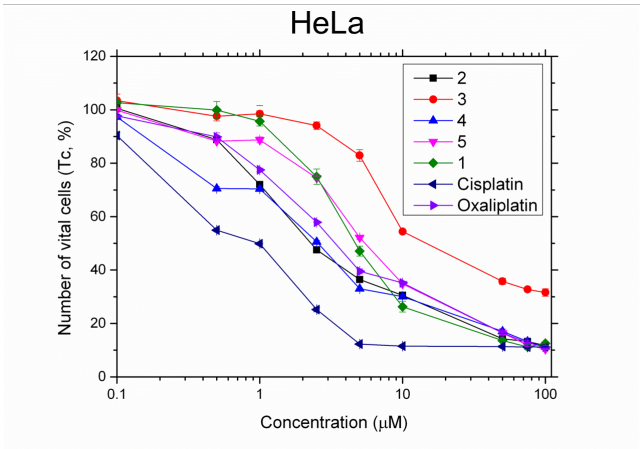
Human cervical carcinoma cell line HeLa, human colon carcinoma cell line SW480 and mouse breast cancer cell line 4T1 were purchased from the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in DME medium (Biopolis shared facilities, A*STAR, SG) supplemented with 10% heat inactivated fetal bovine serum and 1% of mixture of penicillin and streptomycin (Invitrogen, CA, U.S.A). The cells were maintained in a 75 mL cell culture flask (Nunc) at 37 °C in a humidified incubator with 95% air and 5% CO_2.

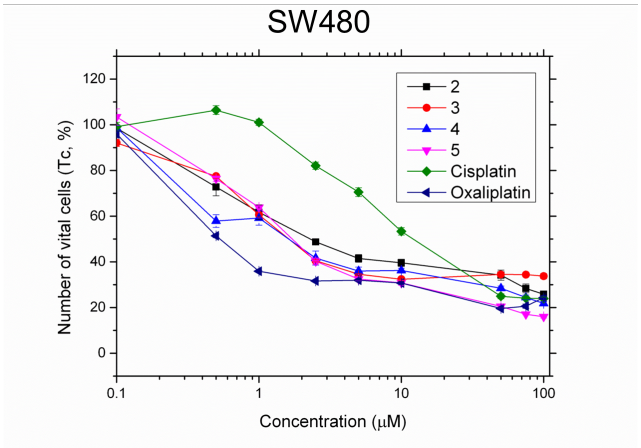
Cell proliferation assay

Cytotoxicity was determined by means of a colorimetric microculture assay (MTs assay, MTS = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) For this purpose, HeLa, SW480, and 4T1 cells were harvested from culture

flasks by trypsinization and seeded into 96-well microculture plates in cell densities of 3.5 × 10^3 cells/well. The cells were allowed to attach for 6 h. Susequently, cells were exposed to serial dilutions of the test compounds in 150 µL/well complete culture medium for 96 h. Then 25 uL of CellTiter 96®Aqueous one solution reagent was added to the cells, followed by incubation for 2 h at 37°C. After incubation, the absorbance was measured at a wavelength of 499 nm using a microplate reader. The quantity of vital cells was expressed in terms of T/C values in comparison to untreated control microcultures, and 50% inhibitory concentrations (IC_{50}) were calculated from concentration-effect curves by a nonlinear curve fit using Origin Lab 7.5.

Concentration effect curves





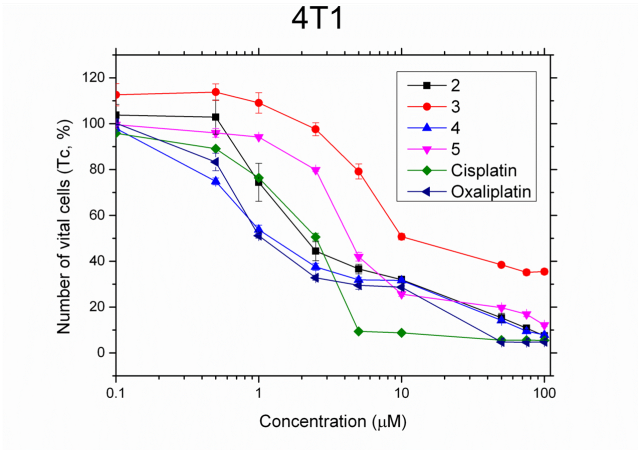
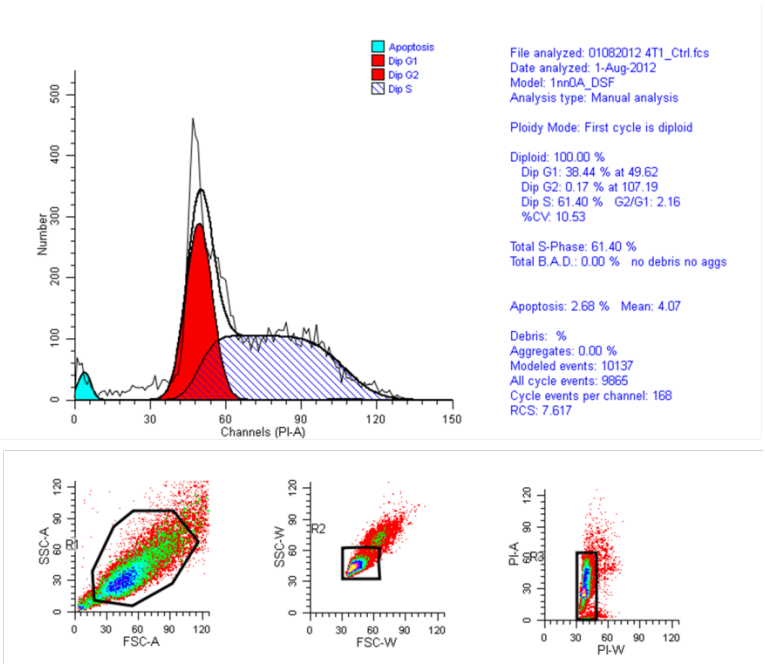


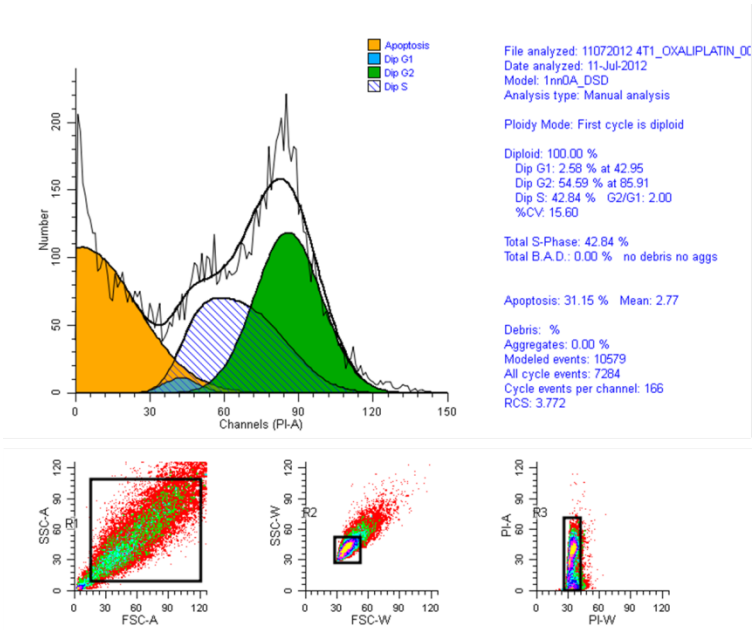
Figure S 11. Concentration effect curve of compounds **1-5** in HeLa, and **2-5** in SW480 and 4T1 cells in comparison with cisplatin and oxaliplatin.

Cell Cycle Analysis

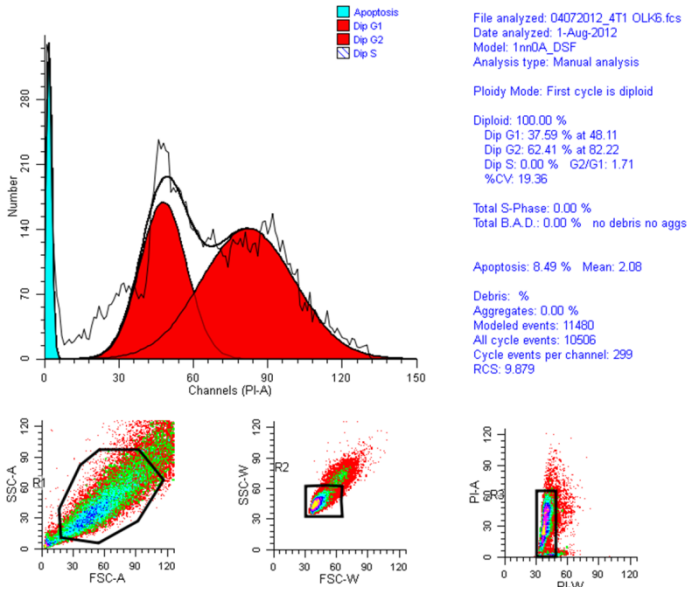
Cells (3×10^6 cells) were seeded into 10 cm petridishes and allowed to recover for 24 h. The medium was exchanged to serum free DME medium and the cells were allowed to synchronize for 24 h. The medium was later changed to DME medium containing 10% heat inactivated fetal bovine serum and 1% of mixture of penicillin and streptomycin (Invitrogen, CA, U.S.A). Cells were exposed to 10 μM of the test compounds for 96 h. Control and treated cells were collected, washed with PBS, fixed in 70% ice-cold ethanol, and stored at −20 °C. To determine cell cycle distributions, cells were transferred to a PBS solution, incubated with 10 μg/mL RNase A for 30 min at 37 °C, and treated with 5 μg/mL propidium iodide for 30 min. Fluorescence of individual cells was measured by flow cytometry, at Biopolis Shared Facilities (BSF), A*Star, Biopolis, Singapore.



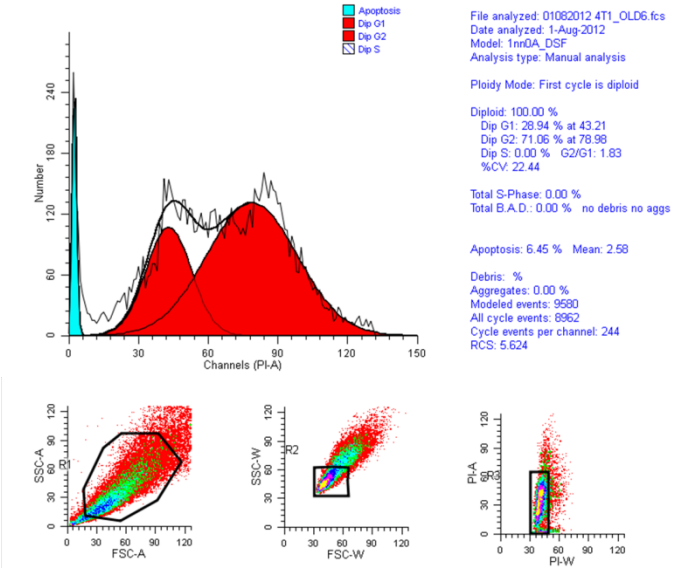
Untreated cells



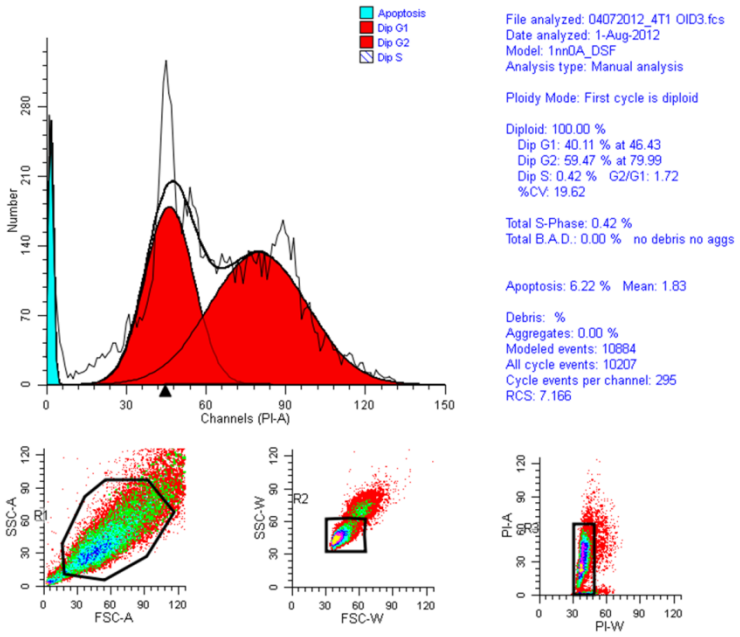
Oxaliplatin (10 μM)



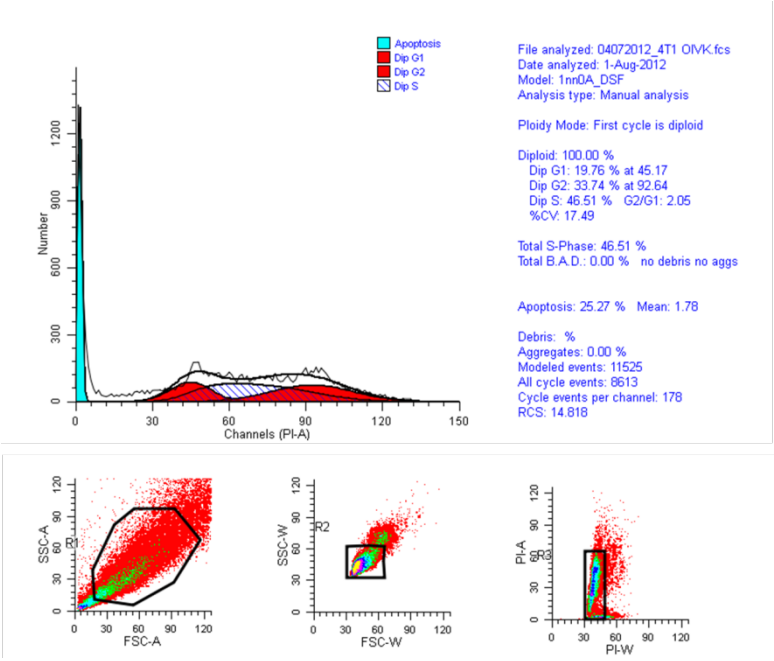
Compound **2** (10 μM)



Compound **3** (10 μM)

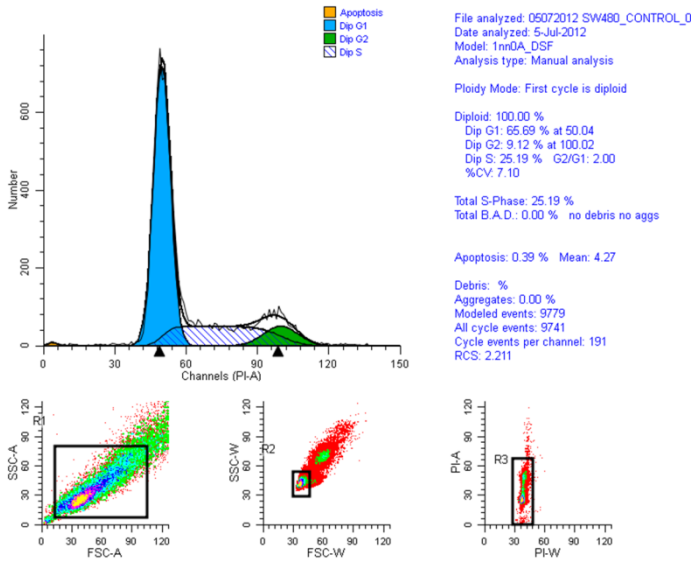


Compound **4** (10 μM)

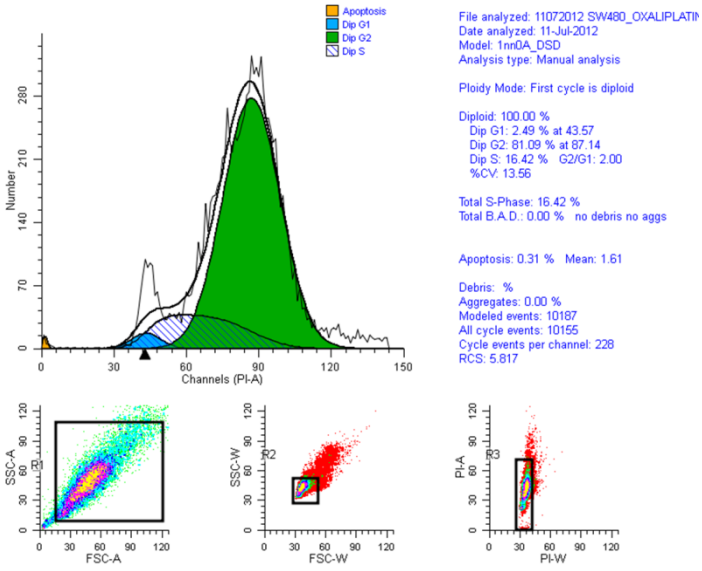


Compound **5** (10 μM)

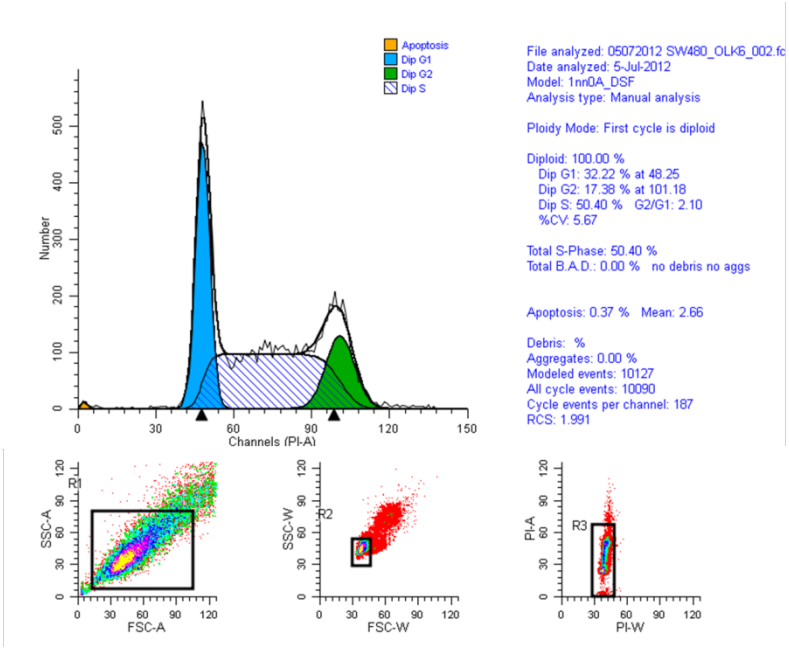
Figure S 12. Cell cycle analysis of compounds **2-5** in comparison with untreated cells (negative control) and oxaliplatin (positive control) for 4T1 cells. The concentration used was 10 μM for compounds **2-5** and oxaliplatin.



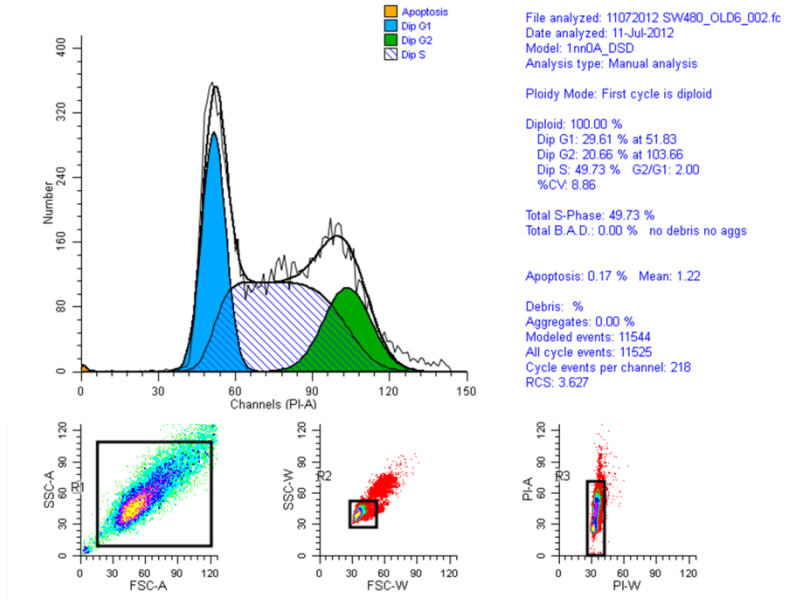
Untreated cells



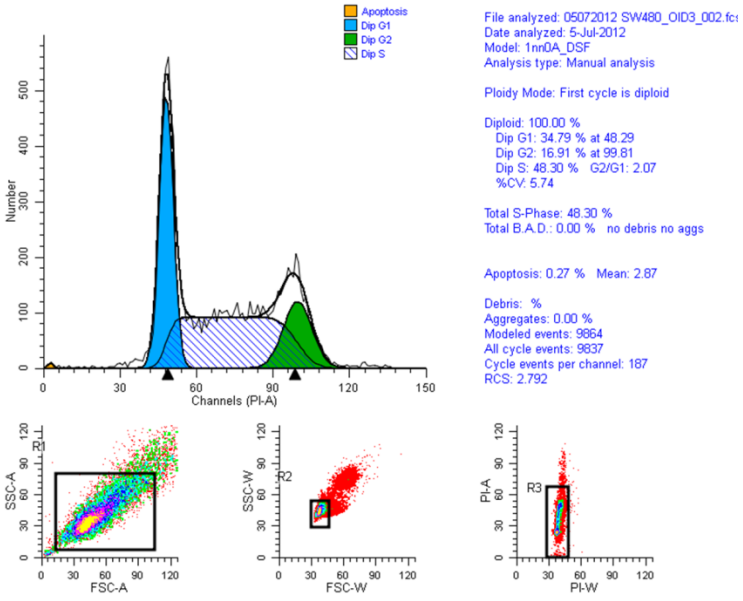
Oxaliplatin (10 μM)



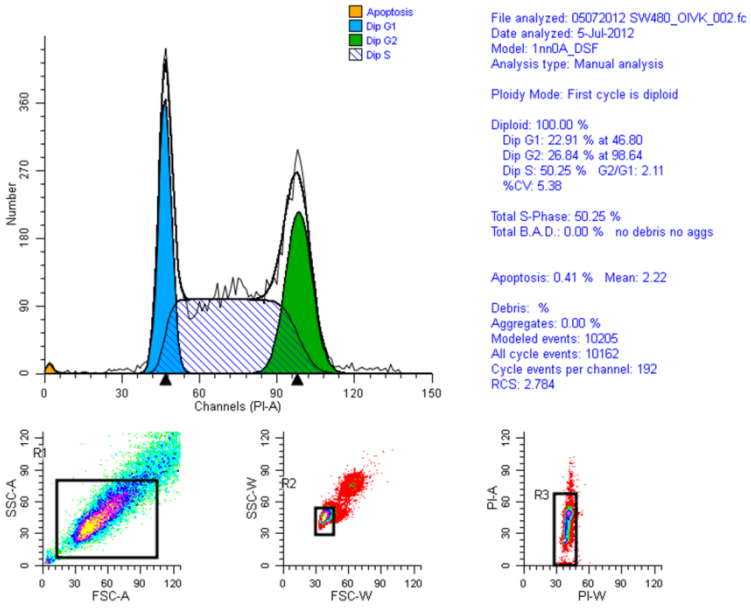
Compound **2** (10 μM)



Compound **3** (10 μM)



Compound **4** (10 μM)



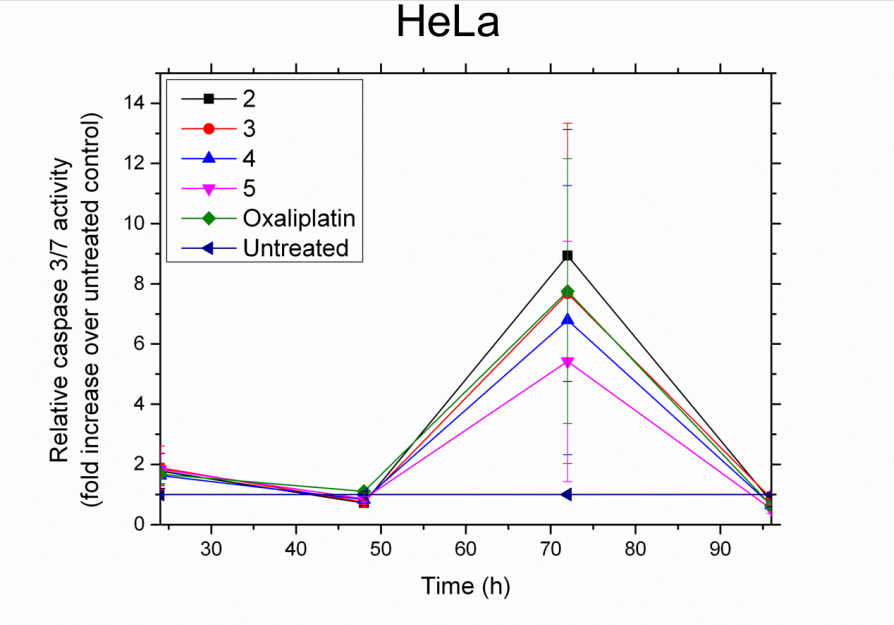
Compound **5** (10 μM)

Figure S 13. Cell cycle analysis of compound **2-5** in comparison with untreated cells (negative control) and oxaliplatin (positive control) for SW480 cells. The concentration used was 10 μM for compound **2-5** and oxaliplatin.

Caspase 3/7 Assay

Caspase 3/7 activity in HeLa, SW480, and 4T1 cells was measured using a Caspase-Glo® 3/7 assay (Promega). In short, cells were plated in a white-walled 96-well as described for the cell proliferation assay. The cells were exposed to different concentrations of the test compounds for 24, 48, 72, and 96 h. Subsequently 100 µL of fresh medium was added followed by 100 µL Caspase-Glo® 3/7 Reagent. The resulting solution was mixed and incubated for 3h. Luminescence reading was taken on a TECAN Infinite M200 96 well plate reader at 560 nm.

Caspase 3/7 activity at different time points



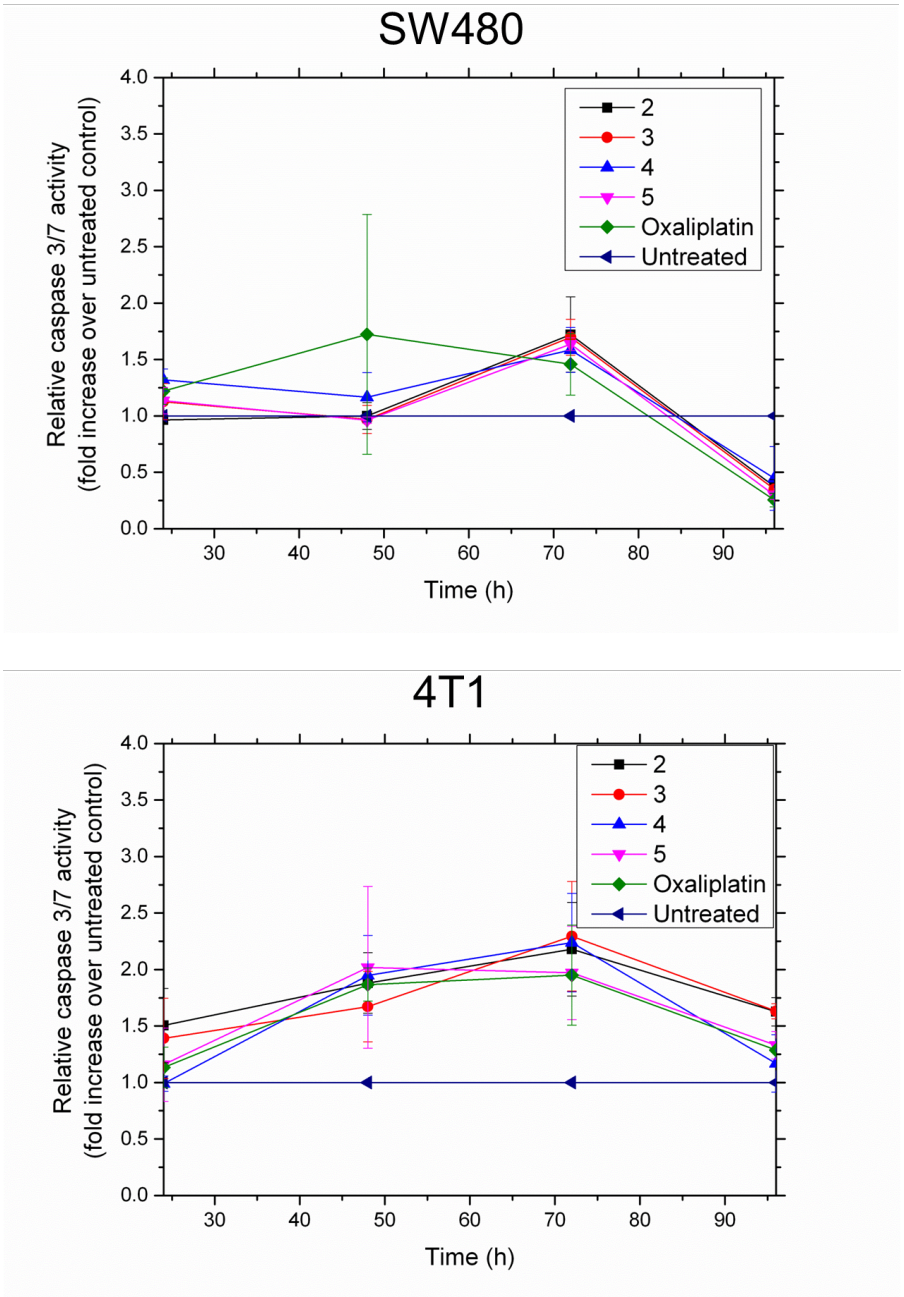


Figure S 14. Time dependence of caspase 3/7 activity in HeLa, SW480 and 4T1 cells. The concentration used for the oxaliplatin control as well as for compounds **2**-**5** was 10μM.

Concentration effect on caspase 3/7 activity in 4T1 cells

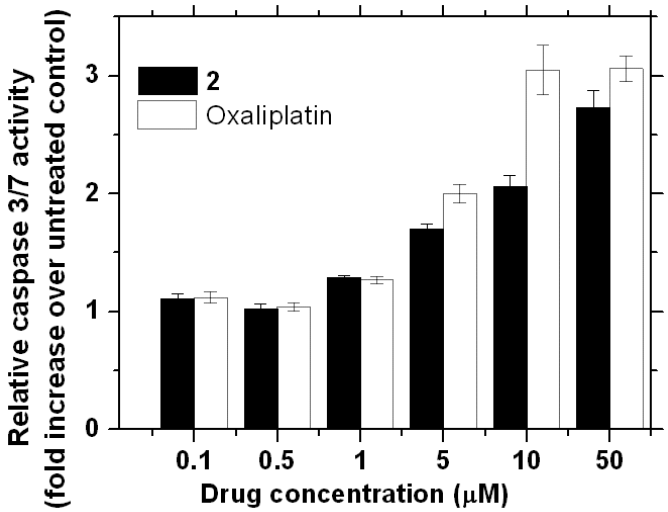


Figure S 15. Effect of concentration on caspase 3/7 activity using different concentrations of compound **2** in comparison to oxaliplatin. Luminescence reading was taken after 72 h of drug exposure.

DNA Platination

HeLa cells were grown in 100 mm tissue culture plates (Nunc) at 37 °C for 48 h to approximately 70% confluency, using DMEM medium. The cells were then exposed to 10 μM of compound **2.** As positive control 10μM oxaliplatin was used. Untreated cells served as a negative control. DNA extraction of approximately $4x10^6$ cells was performed, by an incubation step of the cell pellet that were re-suspended in 180 μl PBS, using 20 μl of proteinase K (DNeasy Blood & Tissue Kit; Qiagen, Hilden, Germany). The incubation of the cell suspension took place in a shaker at 56 °C and 800 rpm for 15 mins.

Next, 200 μl of Buffer AL was added followed, by adding 200 μl ethanol; samples were mixed vigorously and transferred into a DNeasy Mini spin column. The column was centrifuged at 8,000 rpm for 1 minute. Afterwards, the spin column

membrane was washed with 500 µl of Buffer AW1 and 500 µl of Buffer AW2. DNA was eluted from the column with 200 µl of Buffer AE and quantified, using a NanoDrop ND-1000 instrument (NanoDrop Technologies, Oxfordshire, UK). Platinum quantification was done by ICP-MS analysis. All experiments were carried out in triplicates.

In vivo study to evaluate therapeutic efficacy

For handling and care of animals, the Guidelines on the Care and Use of Animals for Scientific Purposes, issued by the National Advisory Committee for Laboratory Animal Research, Singapore, were followed. The experimental protocols of the current study were approved by the Institutional Animal Care and Use Committee (IACUC), Biological Resource Centre, the Agency for Science, Technology and Research (A*STAR), Singapore.

Adult female Balb/c mice used in this study (weight 20 g; aged 6-8 weeks) were provided by Biological Resource Center (BRC), A*Star, Biopolis, Singapore. For tumor establishment, $1x10^6$ mouse 4T1 breast cancer cells were injected subcutaneously into the flank of Balb/c mice (designated as day 0). To investigate the effect of the mixture of Oxaliplatin and hydrogel, the tumor-inoculated animals were divided into 4 groups (n = 9 per group) on day 7 post-tumor inoculation and received an intratumor injection of sample solutions: phosphate buffered saline (PBS), Ac-LIVAGK-NH_2 (12.5 mg/mL) oxaliplatin (15 mg/kg), **2** (44 mg/kg) and Ac-LIVAGK-NH_2 (12.5 mg/mL). A rather high concentration of oxaliplatin was chosen and the amount of compound **2** was adjusted to administer the same amount of platinum.[5] 100µL of each sample were injected directly into the tumor. The hydrogels were prepared prior to injection by dissolving the peptide in 1X PBS and vortexing for 1 min. The sample containing **2** was prepared in a similar manner; **2** was dissolved in 1xPBS and the resulting solution was added to the peptide and vortexed for 1 min. A solid

hydrogel was observed about 10 min after sample preparation, to allow *in situ* gelation inside the tumor.

Tumor volume was determined by measurement with an electronic digital vernier calliper. Readings were taken once a week until day 28 post-tumor inoculation. The ellipsoid volume of the tumor (mm^3) was then calculated using the formula: Volume = (Width) x (Length) x (height) x π/6.

All handling and care of animals was carried out in accordance with the Guidelines on the Care and Use of Animals for Scientific Purposes issued by the National Advisory Committee for Laboratory Animal Research, Singapore.

Data are represented as mean ± SE. The statistical significance of differences was determined by the analysis of variance (ANOVA) with replication followed by Fisher's Least Significant Difference *post hoc* analysis. A *P*-value of < 0.05 was considered to be statistically significant.

Measuring platinum content in tumor and organ samples

Ammonia solution (25% suprapur) and nitric acid (68% suprapur) were purchased from Merck. H_2O_2 (30% (w/w) and the platinum standards were purchased from Sigma-Aldrich.

Liver, kidney and tumor tissues were cut into small pieces and rinsed in ice-cold PBS. The kidney, liver and tumor sections were accurately weighed and transferred into a glass vial and treated as described previously.[6] Briefly, these tissues were digested with 0.4 ml of nitric acid (68% (v/v) and 0.8 ml of H_2O_2 (30% (v/v). The mixture was placed in an 80 °C water bath for 1 h, to get sufficient digestion. While cooling, 0.4 ml of ammonia water (25–28% (v/v) was added to neutralize the excess acid, and the mixture was diluted to a final volume of 2.0 ml. After vortexing, the mixture was filtered using a 0.2 µm filter. 200 µl of the filtered samples were diluted to a final volume of 10 ml using Milli-Q water.

The diluted samples were used for analyzing the platinum content by ICP-MS. All experiments were carried out on a Perkin Elmer Elan DRC II ICP-MS instrument.

Statistical evaluation

Tumor volume

Day 7

One Way Analysis of Variance

Data source: Day 7 in Notebook 1

Group Name	N	Missing	Mean	Std Dev	SEM
PBS	9	0	87.932	45.075	15.025
Ac-LK6-NH2	9	0	52.556	32.184	10.728
Oxaliplatin	9	0	61.938	42.224	14.075
3+Ac-LK6-NH2	9	0	84.641	45.924	15.308

Source of Variation	DF	SS	MS	F	P
Between Groups	3	8034.344	2678.115	1.539	0.223
Residual	32	55675.438	1739.857		
Total	35	63709.782			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.223).

Power of performed test with alpha = 0.050: 0.144

The power of the performed test (0.144) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

Day 14

One Way Analysis of Variance

Data source: Day 14 in Notebook 1

Group Name	N	Missing	Mean	Std Dev	SEM
PBS	9	0	388.691	241.541	80.514
Ac-LK6-NH2	9	0	236.319	90.924	30.308
Oxaliplatin	9	0	135.101	63.650	21.217
3+Ac-LK6-NH2	9	0	163.451	100.345	33.448

Source of Variation	DF	SS	MS	F	P
Between Groups	3	347887.073	115962.358	5.746	0.003
Residual	32	645838.386	20182.450		
Total	35	993725.459			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.003).

Power of performed test with alpha = 0.050: 0.867

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
PBS vs. Oxaliplatin	253.590	3.787	0.000635	0.009	Yes
PBS vs. 3+Ac-LK6-NH	225.240	3.363	0.00201	0.010	Yes
PBS vs. Ac-LK6-NH2	152.372	2.275	0.0297	0.013	No
Ac-LK6-NH2 vs. Oxali	101.218	1.511	0.141	0.017	No
Ac-LK6-NH2 vs. 3+Ac	72.868	1.088	0.285	0.025	No
3+Ac-LK6-NH2 vs. Ox	28.350	0.423	0.675	0.050	No

Day 21

One Way Analysis of Variance

Data source: Day 21 in Notebook 1

Group Name	N	Missing	Mean	Std Dev	SEM
PBS	9	0	676.963	222.409	74.136
LK6	9	0	583.982	196.922	65.641
OXAL	9	0	96.493	65.969	21.990
OLK6	9	0	435.156	180.942	60.314

Source of Variation	DF	SS	MS	F	P
Between Groups	3	1751731.523	583910.508	18.635	<0.001
Residual	32	1002688.947	31334.030		
Total	35	2754420.470			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
PBS vs. OXAL	580.470	6.956	0.0000000704	0.009	Yes
LK6 vs. OXAL	487.488	5.842	0.00000172	0.010	Yes
OLK6 vs. OXAL	338.663	4.059	0.000297	0.013	Yes
PBS vs. OKL6	241.807	2.898	0.00673	0.017	Yes
LK6 vs. OKL6	148.825	1.784	0.0840	0.025	No
PBS vs. LK6	92.982	1.114	0.273	0.050	No

Day 28

One Way Analysis of Variance

Data source: Day 28 in Notebook 1

Group Name	N	Missing	Mean	Std Dev	SEM
PBS	9	0	1106.266	446.815	148.938
Ac-LK6-NH2	9	0	1400.703	570.088	190.029
Oxaliplatin	9	0	250.650	277.625	92.542
3+Ac-LK6-NH2	9	0	700.723	378.256	126.085

Source of Variation	DF	SS	MS	F	P
Between Groups	3	6746391.610	2248797.203	12.077	<0.001
Residual	32	5958379.127	186199.348		
Total	35	12704770.737			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.999

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Ac-LK6-NH2 vs. Oxali	1150.053	5.654	0.00000296	0.009	Yes
PBS vs. Oxaliplatin	855.616	4.206	0.000196	0.010	Yes
Ac-LK6-NH2 vs. 3+Ac	699.980	3.441	0.00163	0.013	Yes
3+Ac-LK6-NH2 vs. Ox	450.073	2.213	0.0342	0.017	No
PBS vs. 3+Ac-LK6-NH	405.543	1.994	0.0548	0.025	No
Ac-LK6-NH2 vs. PBS	294.437	1.447	0.157	0.050	No

Weight measurements

Statistical evaluation

Day 20

One Way Analysis of Variance

Data source: Day 20 in Notebook 1

Group Name	N	Missing	Mean	Std Dev	SEM
PBS	9	0	22.567	1.405	0.468
Ac-LK6-NH2	9	0	22.586	1.118	0.373
Oxaliplatin	9	0	19.754	1.110	0.370
3+Ac-Lk6-NH2	9	0	22.526	1.146	0.382

Source of Variation	DF	SS	MS	F	P
Between Groups	3	53.132	17.711	12.277	<0.001
Residual	32	46.163	1.443		
Total	35	99.294			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.999

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Ac-LK6-NH2 vs. Oxali	2.831	5.000	0.0000199	0.009	Yes
PBS vs. Oxaliplatin	2.813	4.967	0.0000218	0.010	Yes
3+Ac-Lk6-NH2 vs. Oxa	2.772	4.896	0.0000269	0.013	Yes
Ac-Lk6-NH2 vs. 3+Ac-	0.0593	0.105	0.917	0.017	No
PBS vs. 3+Ac-Lk6-NH2	0.0407	0.0720	0.943	0.025	No
Ac-LK6-NH2 vs. PBS	0.0186	0.0328	0.974	0.050	No

Day 28

One Way Analysis of Variance

Data source: Day 28 in olk6 weight.SNB

Group Name	N	Missing	Mean	Std Dev	SEM
PBS	9	0	22.335	1.469	0.490
Ac-LK6-NH2	9	0	22.576	1.252	0.417
Oxaliplatin	9	0	20.593	0.894	0.298
3+Ac-LK6-NH2	9	0	22.820	1.657	0.552

Source of Variation	DF	SS	MS	F	P
Between Groups	3	27.615	9.205	5.065	0.006
Residual	32	58.152	1.817		
Total	35	85.767			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.006).

Power of performed test with alpha = 0.050: 0.801

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
3+Ac-LK6-NH2 vs. Oxa	2.227	3.504	0.00138	0.009	Yes
Ac-LK6-NH2 vs. Oxalip	1.982	3.119	0.00382	0.010	Yes
PBS vs. Oxaliplatin	1.742	2.741	0.00995	0.013	Yes
3+Ac-LK6-NH2 vs. PBS	0.485	0.763	0.451	0.017	No
3+Ac-LK6-NH2 vs. Ac-	0.244	0.385	0.703	0.025	No
Ac-LK6-NH2 vs. PBS	0.241	0.379	0.708	0.050	No

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