

MIR
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Final Tabular Report to: Sanare A.S.

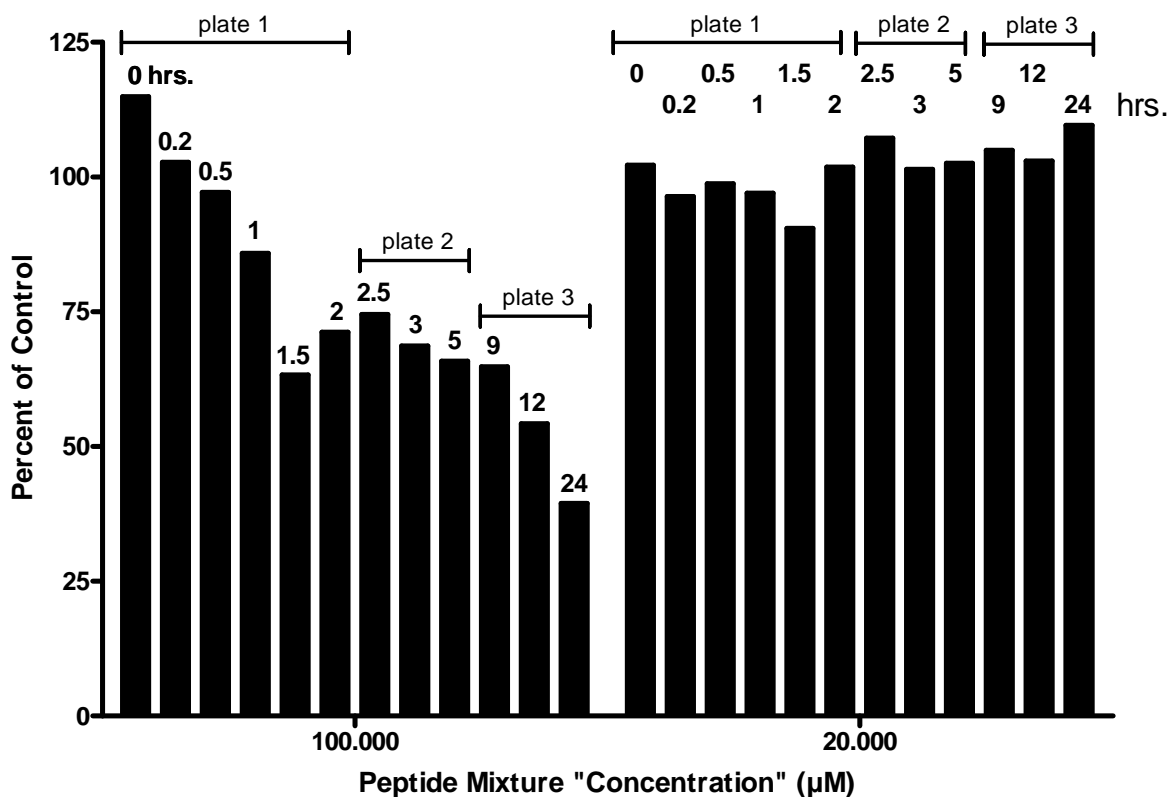
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Time Course of Peptide Mixture Effect on MCF-7 Cell Line Proliferation


charles river

24hrs. Time Course w/MCF-7 Cells
April 16, 2009



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Materials and Methods

Cell Culture

The MCF-7 human cancer cell line was purchased from the American Type Culture Collection (ATCC). Cultures were established using standard *in vitro* culture methods and ATCC recommended media (Appendix I) in 175cm² tissue culture treated flasks. Cultures were incubated in humidified 37°C, 5% CO₂, 95% air incubators. Cultures were split regularly to maintain log phase growth.

Test Agent Preparation

Peptides 13-24 (also known as peptides 37 – 49) (fine, white powders) were received from Sanare in Parafilm® sealed glass vials. Vials were stored at 4°C in a covered box to prevent exposure to light.

Peptides were dissolved in Dulbecco's phosphate-buffered saline (DPBS) as described in a previous Sanare study (SANA200805R1a/MIR1007). In MIR1007, initial 25mM stocks were made using DPBS. Seven out of the twelve peptides did not go into solution at the 25mM concentration. In an attempt to keep all of the peptides at the same concentration, another volume of DPBS was added to all peptide stocks (12.5mM; 39mg/mL for Peptide 13). Some of the peptides would still not go into solution. Additional volumes of DPBS were added, accompanied by intermittent vortexing and heating to 37°C in a water bath (5-10min), attempting to prepare clear solutions for the peptide stocks. As some of the stocks became uniform suspensions, it was determined that these stocks should not be diluted further (limiting further decreases in final dosage concentrations) but rather should be used as suspensions (assuming that solubility may improve with the final dilution as they were added to the wells of the treatment plates).

Test Agent Stock Preparation Table

Compound	M.W.	Quantity (mg)	1x DPBS Vehicle (µl)	Stocks Final Conc. (mM)	Stocks Final Conc. (mg/mL)
Peptide 13; 37-4061559	3125.5	6	154	12.5	39
Peptide 14; 49-4064221	1732.9	6	154	22.5	39
Peptide 15; 39-4061561	1910.2	6	376	8.4	15.9
Peptide 16; 40-4061562	1909.3	6	630	5.0	9.5
Peptide 17; 41-4061563	2399.0	6	802	3.1	7.5
Peptide 18; 42-4061564	1326.5	6	154	29.4	39
Peptide 19; 43-4061565	1129.3	6	154	34.5	39
Peptide 20; 44-4061566	2223.7	6	324	8.3	18.5
Peptide 21; 45-4061567	1746.2	6	412	8.3	14.6
Peptide 22; 46-4061568	2070.3	6	579	5.0	10.4
Peptide 23; 47-4061569	1570.8	6	154	24.8	39
Peptide 24; 48-4061570	1886.2	6	509	6.2	11.8

Note: The peptide concentrations were targeted for 12.5mM, however, the calculation was done in MIR1007 for Peptide 13 with the (incorrect) assumption that the molecular weights for all of the peptides were the same. Therefore, the peptides were dissolved

using the same method for this study and the actual concentrations of the individual peptides is listed in the table below in mM and mg/mL units.

60µL of each peptide was combined in a single tube and mixed well (720µL total volume). 57.6µL peptide mixture was added to 242.4µL culture media, mixed well by pipetting and then serially diluted 1:5 down six wells (transfer 60µL to 240µL culture media). 100µL serially diluted peptides were added to 100µL cells/media at various time points for an additional 2x dilution. Serial dilutions were prepared no more than 30 minutes prior to addition to the assay plate.

Cisplatin (a fine, dark yellow powder) was obtained from Sigma (M8407) and stored in a sealed amber vial at -20°C in a covered box to prevent exposure to light. A 4mM stock solution was made using 0.9% saline. The stock solution was aliquoted into microcentrifuge tubes (for one-time use) and frozen at -20°C. For this experiment, one frozen aliquot was quickly thawed in a 37°C water bath and placed on wet ice until ready to use. To prepare the treatment solution, the 4mM stock solution was diluted 1:2 using complete media to yield a 2mM (2x) working solution for use in the first well of the dilution plate. Serial 1:5 dilutions were performed prior to adding to the assay plate containing cells/media for 24 hours exposure.

Time Course Assay

In order to assess cell viability rapidly enough to detect changes between the shorter time points, Celltiter-Glo was used for this assay. The CellTiter-Glo® Luminescent Cell Viability Assay (Promega) is a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells. The homogeneous "add-mix-measure" format results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present which is directly proportional to the number of cells present in culture. The CellTiter-Glo® Assay generates a "glow-type" luminescent signal, which has a half-life generally greater than five hours.

Cells in the log phase of growth were seeded at the indicated densities listed in Appendix I into 96 well culture plates in 0.1mL of complete media in all wells except BLANK wells which was reserved for media only control (No Cells) and allowed to attach overnight at 37°C. Peptides diluted in culture media (see *Test Agent Preparation*) were added to each well in a volume of 0.1mL for a final volume of 0.2mL/well. Diluted peptides were added to the assay plates at various times (see "Dosing Schedule" below) and cell proliferation was measured using Celltiter-Glo as per the manufacturer's instructions. Briefly, 100µL culture supernatant was carefully removed and the plates equilibrated to room temperature for 10 minutes. 100µL Celltiter-Glo reagent was added to each well and the plate mixed for 2 minutes on a rotary plate shaker followed by incubation at room temperature for 10 minutes in the dark. Luminescence was measured using a Synergy 2 (Biotek) multi-mode plate reader in luminescence mode. Luminescence values were auto scaled to the Cells Only wells with a maximum RLU = 10,000. Values were converted to Percent of Control by

dividing sample well luminescence by the Cells Only luminescence and multiplying by 100. Peptide concentration was plotted against Percent of Control values to calculate IC₅₀ values, however, only the 100µM and 20µM concentrations showed any effect so these values were graphed as a bar graph (see Appendix II).

Dosing Schedule

Time Point	Plate #	Celltiter-Glo		
		Addition	Dose Time	Dose Date
0	1	3:30pm	3:30pm	4/15/2009
0.2	1	3:30pm	3:18pm	4/15/2009
0.5	1	3:30pm	3:00pm	4/15/2009
1	1	3:30pm	2:30pm	4/15/2009
1.5	1	3:30pm	2:00pm	4/15/2009
2	1	3:30pm	1:30pm	4/15/2009
2.5	2	1:15pm	10:45am	4/16/2009
3	2	1:15pm	10:15am	4/16/2009
5	2	1:15pm	8:15am	4/16/2009
9	3	5:00pm	8:00am	4/16/2009
12	3	5:00pm	5:00am	4/16/2009
24	3	5:00pm	5:00pm	4/15/2009

Celltiter-Glo added to Plate 1 on 4/15/2009 and to Plates 2 and 3 on 4/16/2009.

Data Retrieval

DIS-Ann Arbor (formerly MIR Preclinical Services) retains permanent “active” copies (on CD) of all experiments unless advised otherwise.

References

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Appendix I - Cell Culture

Cell Culture Protocol for Passaging Adherent Cells

All manipulations are carried out in a Class II HEPA filtered biosafety hood using sterile technique.

1. Aspirate and discard culture medium.
2. Add 3mL of 0.25% (w/v) Trypsin, 0.53mM EDTA solution (CellGro 25-053-CI) to each flask and ensure complete coverage of the cell monolayer by rocking gently in multiple directions.
3. Observe cells under an inverted microscope until cell layer is dispersed (usually within 5 minutes). Note: To avoid clumping, do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add an equal volume of fresh media to neutralize the trypsin. Aspirate cells by gently pipetting and rinsing the monolayer area then immediately add appropriate aliquots of the cell suspension to new culture vessels (T175 flasks) containing 30mL fresh pre-warmed media (room temperature to 37°C).
5. Incubate cultures at 37°C in a humidified 5% CO₂ incubator. Subculture and/or change media every 2 – 3 days.

Cell Line Propagation Conditions

(Supplement percentages are vol./vol.)

Cell Line: **MCF-7**
Media: RPMI1640 (CellGro 10-040-CV)
Supplements: 1% L-glutamine, 10% FBS, 1% PSG
Atmosphere: 5% CO₂, 95% air
Properties: adherent

* FBS - Fetal Bovine Serum (Gibco 10082-147; lot #1354986)

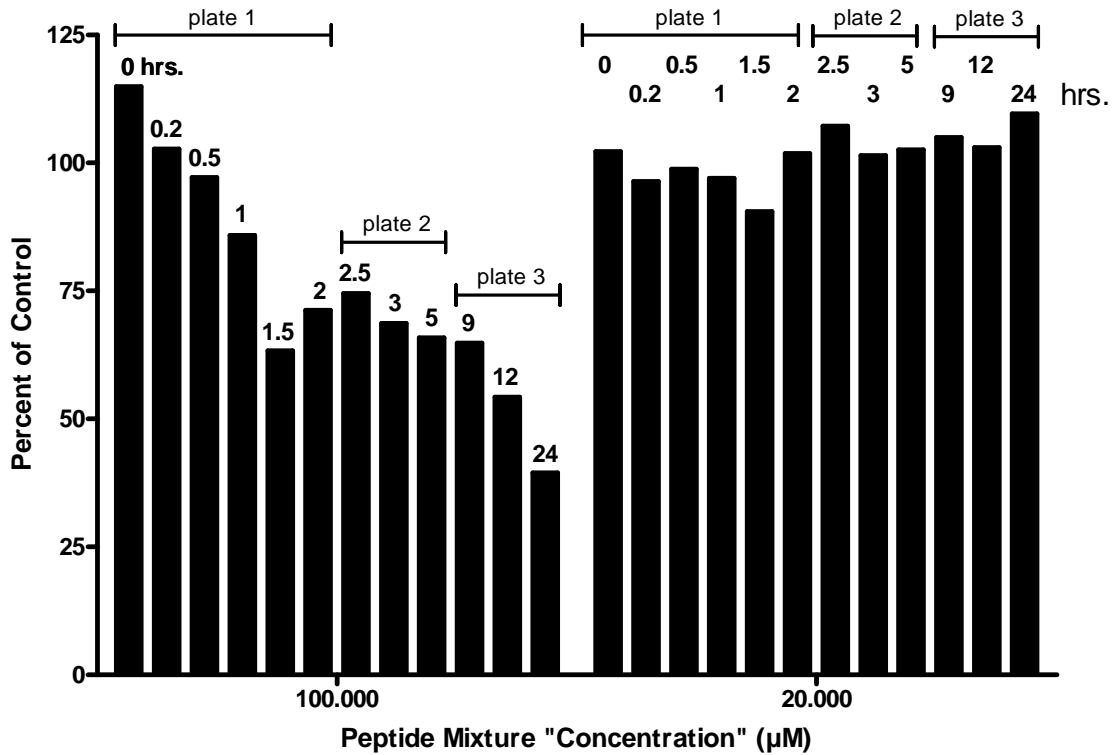
* PSG - Penicillin, Streptomycin, L-Glutamine Solution (CellGro 30-009-CI)

Seeding Densities for IC₅₀ Assays

Cell Line	cells/well (x10³)
MCF-7	6

Appendix II – Raw Data and Graphs

24hrs. Time Course w/MCF-7 Cells
April 16, 2009



Serially diluted Sanare peptide mixture was applied to MCF-7 cells for various incubation times over 24 hours. Peptide mixture effect on cell viability/proliferation was assessed by measuring ATP using Celltiter-Glo reagent (Promega). Luminescence values were converted to percent of No Drug control and initially plotted against peptide concentration to look for a dose response curve, however, only the highest concentration (100µM) showed significant changes in ATP content of the cells. Therefore, the 100 and 20µM test wells are presented here in bar graph format.

Percent of Control Values

Time (hrs.)	Conc. (μM)	
	100	20
0	114.9	102.2
0.2	102.7	96.4
0.5	97.2	98.8
1	85.9	97.0
1.5	63.3	90.5
2	71.2	101.8
2.5	74.5	107.2
3	68.7	101.4
5	65.9	102.6
9	64.8	104.9
12	54.3	103.0
24	39.5	109.6

Plate 1 Raw Data (RLU)

μM peptide	blank	0 hrs.	0.2 hrs.		0.5 hrs.	
0	57	8566	8335	9720	8614	9834
100	68	9837	8817	9727	8767	9159
20	72	8756	8460	8950	8897	9328
4	64	8474	8249	8986	8188	8061
0.8	62	8606	8335	8767	8595	8509
0.16	64	7896	7836	8587	8129	8346
0.032	56	7486	8217	8009	8179	8389
0.0064	48	7206	7379	8173	7494	7836

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Average

μM peptide	1.0 hrs.		1.5 hrs.		μM peptide	2 hrs.	
0	9606	9883	8028	9143	0	7487	7276
100	8052	8699	5212	5706	0	7494	6822
20	9741	9173	7986	7565	100	4989	5562
4	8415	9148	7255	7533	20	7664	7368
0.8	8195	8839	7403	7237	4	7428	7283
0.16	8235	8828	7039	8320	0.8	6889	7029
0.032	8473	9268	7398	7431	0.16	6503	7326
0.0064	7952	8592	8003	8271	0.032	6279	6673

Plate 2 Raw Data (RLU)

μM peptide	blank	No Drug	2.5 hrs.		3 hrs.		5 hrs.	
0	43	8426	8745	9012	8919	8388	8775	9123
100	49	9371	6975	6927	6594	6228	6354	5946
20	53	9861	9637	10326	9431	9457	9610	9493
4	51	9395	9435	10049	9218	9252	9480	8704
0.8	51	9441	9295	9124	10117	9910	8913	8795
0.16	52	9385	8589	9216	9529	8941	9817	9110
0.032	46	9403	8771	9012	9384	9520	8792	8804
0.0064	40	9214	9011	9906	8338	8953	9339	8885
	48	9312						
	Average	Average						

Plate 3 Raw Data (RLU)

μM peptide	blank	No Drug	9 hrs.		12 hrs.		24 hrs.	
0	42	8833	9718	9900	9280	10072	9877	9210
100	49	9630	6616	6136	5519	5025	3821	3769
20	55	9890	10586	9998	9686	10238	10158	10755
4	54	9672	9988	10139	10499	10058	12507	10348
0.8	51	9689	9659	9545	9714	10016	10612	10200
0.16	53	9099	9351	9566	9727	9700	9650	9987
0.032	48	9331	9021	9464	9608	9090	9621	9145
0.0064	40	9159	10180	9486	9841	9003	9871	8909
	49	9413						
	Average	Average						

RLU = Relative Luminescence Units (*or Relative Light Units*)