

**MIR**  
**Discovery and Imaging Services**  
Formerly MIR Preclinical Services

Final Tabular Report to: Sanare A.S.

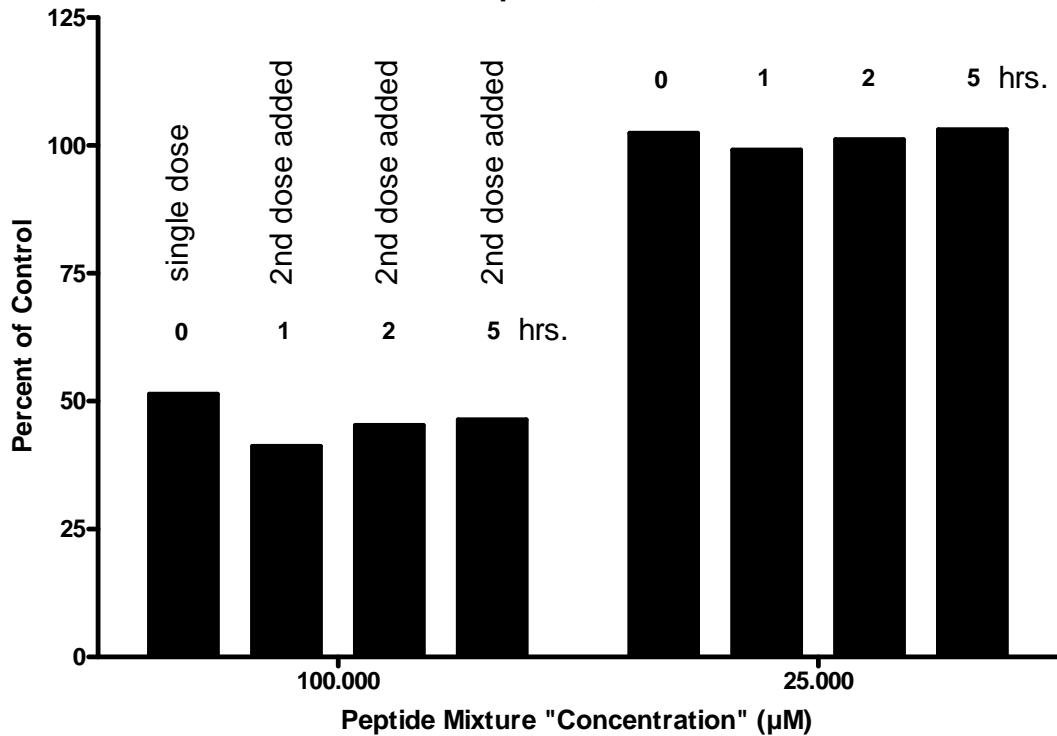
Study: SANA200904 (MIR1138)

Date: June 4, 2009

Peptide Mixture (Two Dose) Time Course Effect on MCF-7 Cell Line Proliferation

  
charles river

**2x Dose During 24hrs. Time Course w/MCF-7 Cells  
April 24, 2009**



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## Materials and Methods

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### 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 175cm<sup>2</sup> tissue culture treated flasks. Cultures were incubated in humidified 37°C, 5% CO<sub>2</sub>, 95% air incubators. Cultures were split regularly to maintain log phase growth.

### Test Agent Preparation

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 1:4 dilutions were performed prior to adding to the assay plate containing cells/media for 24 hours exposure.

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.

Peptide stock solutions from SANA200903 (MIR1114) were used for this study. Please refer to the study report for details of the preparation of the stock solutions. The table below gives some details of the concentrations of the peptide stock solutions.

**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

### Time Course Assay

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 column 12 wells which were reserved for media only control (blank) and allowed to attach overnight at 37°C in a humidified 5%CO<sub>2</sub> incubator.

35µL of each peptide was combined in a single tube and mixed well (420µL total volume). 57.6µL peptide mixture was added to 242.4µL culture media, mixed well by pipetting and then serially diluted 1:4 down eight wells (transfer 60µL to 240µL culture media). Serial dilutions were prepared no more than 10 minutes prior to addition to the assay plate. An initial 100µL of serially diluted peptides was added to 100µL cells/media at time zero to all "peptide" wells of the assay plate (see plate map below). Addition of diluted peptides to the assay plate provided an additional 2x dilution. A second dose of serially diluted peptides was added to the indicated wells at 1, 2 and 5 hours after the initial dose (T=0 wells received only a single dose). The second doses were added after 100µL of culture supernatant was removed by gentle pipetting. Incubation was continued for a total of 24 hours and cell viability was measured using Celltiter-Glo<sup>®</sup> (Promega).

	Peptide/Compound Concentration (µM)										11	12
	T=0 hours		T=1.0		T=2.0		T=5.0		Cisplatin 24hrs.			
	1	2	3	4	5	6	7	8	9	10		
A	100	100	100	100	100	100	100	100	1,000	1,000	Cells Only	No Cells
B	25	25	25	25	25	25	25	25	250	250	Cells Only	No Cells
C	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	50	50	Cells Only	No Cells
D	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	10	10	Cells Only	No Cells
E	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	2.0	2.0	Cells Only	No Cells
F	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.40	0.40	Cells Only	No Cells
G	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.1	0.1	Cells Only	No Cells
H	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.02	0.02	Cells Only	No Cells

The CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay 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<sup>®</sup> Assay generates a "glow-type" luminescent signal, which has a half-life generally greater than five hours.

Cell viability was measured for this assay using Celltiter-Glo<sup>®</sup> as per the manufacturer's instructions. Briefly, 100 $\mu$ L culture supernatant was carefully removed and the plates equilibrated to room temperature for 10 minutes. 100 $\mu$ L Celltiter-Glo<sup>®</sup> 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 multi-mode plate reader (Biotek) in luminescence mode. Luminescence values were auto scaled to the "Cells Only" wells (No Drug Control) 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 IC<sub>50</sub> values, however, only the 100 $\mu$ M and 25 $\mu$ M concentrations showed any effect so these values were graphed as a bar graph (see Appendix II).

#### *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**

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### **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% CO<sub>2</sub> 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% CO<sub>2</sub>, 95% air  
*Properties:* adherent

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

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

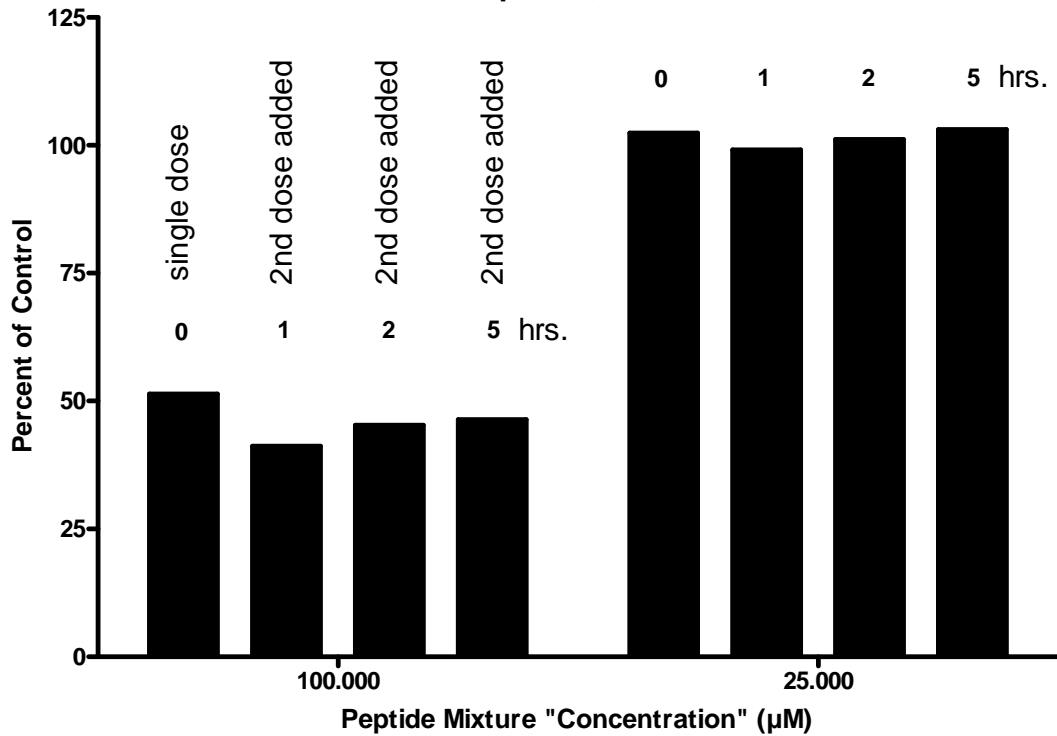
### **Seeding Densities for IC<sub>50</sub> Assays**

<b>Cell Line</b>	<b>cells/well (x10<sup>3</sup>)</b>
MCF-7	6

## **Appendix II – Raw Data and Graphs**

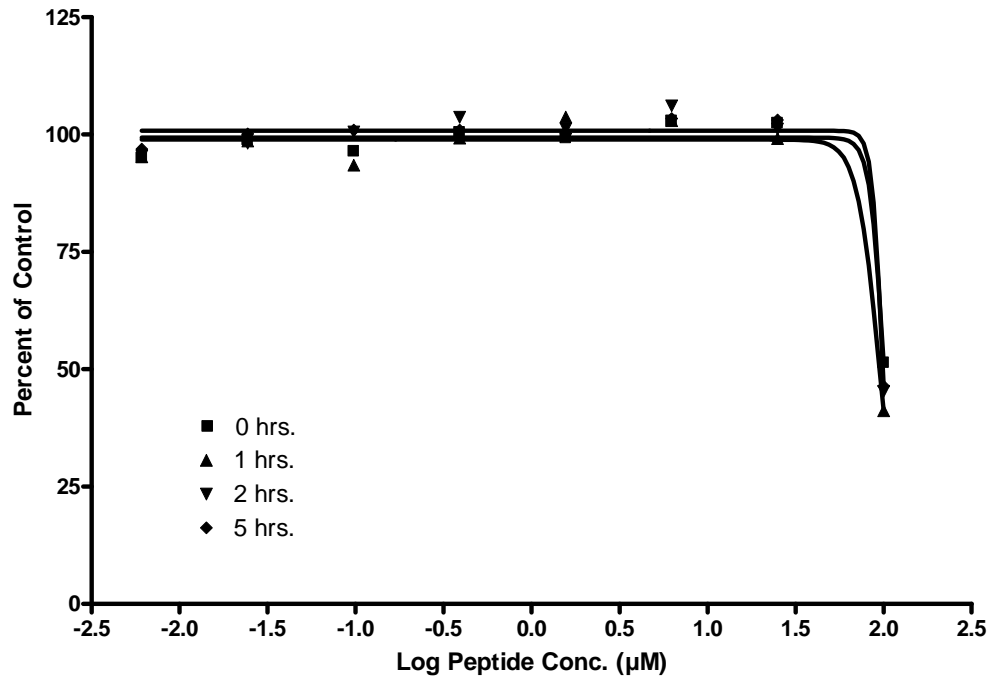
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**2x Dose During 24hrs. Time Course w/MCF-7 Cells  
April 24, 2009**



All wells were dosed at time zero and then 100µL media was removed at 1, 2 and 5 hours and an additional dose added at these time points. At 24 hours, 100µL was removed from all wells and 100µL Celltiter Glo (Promega) reagent was added to measure ATP as an indicator of cell viability. A series of eight concentrations were used in the assay (see graphs on subsequent pages), however, only the top concentrations revealed any effect and therefore are displayed here in bar graph format.

**2x Dose During 24hrs. Time Course w/MCF-7 Cells  
April 24, 2009**

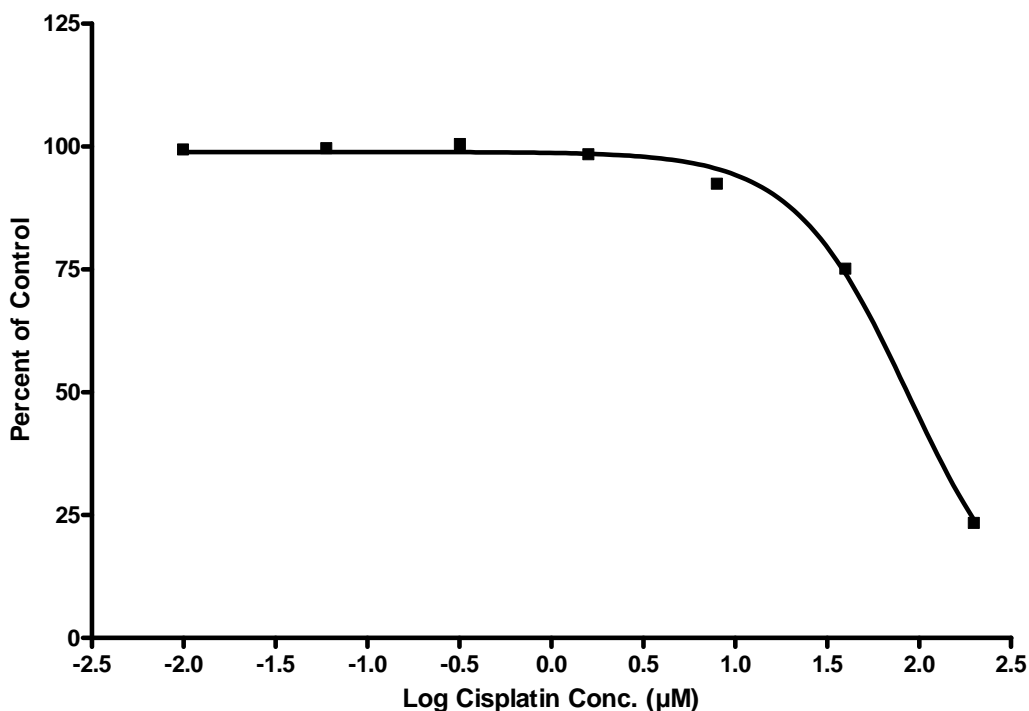


	0 hrs.	1 hrs.	2 hrs.	5 hrs.
Sigmoidal dose-response (variable slope)	Does not converge.			
Best-fit values				
BOTTOM	0.0	0.0		0.0
TOP	99.33	98.91		100.8
LOGEC50	2.003	1.978		1.995
HILLSLOPE	-10.84	-6.798		-14.42
EC50	100.6	95.16		98.90
Std. Error				
TOP	1.298	1.662		0.9647
LOGEC50	13.68	0.7442		2339
HILLSLOPE	49607	222.6		6.961e+006
95% Confidence Intervals				

Peptide Conc. (µM)	0 hrs.	1 hrs.	2 hrs.	5 hrs.
100	5168	4329	3727	3911
25	9253	9546	9130	9079
6.25	9279	9606	9693	9208
1.56	8797	9432	9532	9505
0.39	8857	9590	9014	9213
0.10	8745	8964	8462	8705
0.02	8751	9402	9000	9110
0.006	8259	9194	8918	8577

**Luminescence values (RLU)**

24 hrs. Cisplatin (single dose) w/MCF-7 Cells  
April 24, 2009



	24 hrs.
Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	98.90
LOGEC50	1.942
HILLSLOPE	-1.386
EC50	87.50
Std. Error	
TOP	0.9065
LOGEC50	0.02420
HILLSLOPE	0.08891

24 hrs.  
Cisplatin  
Conc. (µM)

1,000	3938	3977
200	2195	2179
40	6934	6877
8.0	8435	8525
1.6	9108	8943
0.32	9269	9160
0.06	9079	9200
0.01	9118	9116

← Data points excluded from graph.

High dose of cisplatin gives a higher luminescent signal than expected and is not included in the  $IC_{50}$  plot. This occurs consistently and may be due to different composition of the culture supernatant ie- large proportion of cisplatin added to get 1,000µM changes light emission (more light can be emitted).

Luminescence values (RLU)