UNIVERSITY HONORS PROGRAM

SENIOR PROJECT - APPROVAL

Name: Jennifer M Giltnane

College: Arts and Sciences Department: Microbiology

Faculty Mentor: Dr. HCR Wang, Dept of Comparative Medicine

PROJECT TITLE: Effects of a novel anticancer agent on ras- and src- transformed 10T½ cells

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I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: _____, Faculty Mentor

Date: May 13, 1999

Comments (Optional):

Effects of a novel anticancer agent on *ras*- and *src*- transformed 10T½ cells

Jena M. Giltnane
Senior Honors Project
Spring 1999
Dr. H.C.R. Wang, Mentor
Dr. T. Broadhead, Honors Program Director

Effects of a novel anticancer agent on *ras*- and *src*- transformed 10T½ cells

Table of Contents

"The goal of breast cancer prevention research is to develop readily acceptable, minimally toxic, and affordable strategies that will reduce breast cancer incidence, morbidity, and mortality without inducing increased morbidity and mortality from other conditions."*

* From the National Cancer Institute's Breast Cancer Progress Review Group
Available: http://wwwosp.nci.nih.gov/planning/prg/bprgprevention.htm

Report

Effects of a novel anticancer agent on *ras*- and *src*- transformed 10T½ cells

Jena Giltnane
Dr. Hwa-Chang R. Wang, University of Tennessee Comparative Medicine Department

I. Summary

Breast cancer is second most deadly form of cancer among American women, and oncologists regularly screen potential anti-tumor agents for unique tumor-killing activity and selectivity for cancer cells. In this study, oncogene-transformed murine cells were used as target cells to investigate the cytotoxicity of a novel naturally occurring agent, FR901228 (FR), that has been shown to possess a potent antitumor activity against human and murine tumor cells. Investigation of the cytotoxicity of FR suggested that FR may be selective in targeting cancer cells, although some growth inhibition has been observed in normal cells. Further tests will be performed to investigate the biological and molecular activities of FR901228.

II. Introduction

Breast cancer

Breast cancer and other cancers arise when certain mutations accumulate, and clonal selection begins to favor cells displaying aggressive growth rates (Fearon, 1043). Gene mutations identified in some human breast cancers include aberrations in identified genes BRCA1 and BRCA2, although these mutations are not reliable predictors of developing breast cancer (Brody, no pg.). BRCA1 and BRCA2 are both thought be involved in a protein interaction pathway which ultimately regulates the repair of some DNA damage (Fearon, 1047). In fact, there are many DNA mutations, which turn on or turn off important regulatory checkpoints in a cell's growth cycle, including errors introduced in oncogenes and tumor suppressor genes.

Oncogenes: ras and src proto-oncogenes and cancer

An understanding of the biological and genetic basis of cancer and tumor proliferation began to develop from the discovery of viral oncogenes in early studies on RNA tumor retroviruses. Just as cancer is related at the cellular level to disorders in the control of cell proliferation, differentiation, and apoptosis (programmed cell death), it is associated with oncogenes and tumor suppressor genes at the genetic level. Potential DNA targets for oncogenic mutations are proto-oncogenes, which become "oncogenes" when activated by DNA changes in gene expression.

Proto-oncongenes represent dominant mutations which result in an acceleration of cell growth promotion factors, while mutations in tumor suppressor genes are recessive, and "two hits" result in the loss of some inhibitory function of cell growth. The protein products of these genes are involved in many cell functions, including biochemical pathways for gene expression, the architecture and changes in the cell cytoskeleton, and cell metabolism. In both types of genetic damage, the result can be cells rapidly dividing with the loss of normal check-point systems—a tumor (Pusztai, 47).

In 1995, there were over one hundred oncogenes identified in human cancers, which are classified according to the how their normal proto-oncogene homologue functions in cell growth signal transduction pathways. There are currently four major classifications: abnormal signaling by a structurally abnormal growth factor, abnormal phosphorylation of proteins by altered receptors and signal transducer kinases, abnormal signaling by G proteins, and aberrant regulation of gene transcription by altered

transcription factors (Pusztai,49). The cell line used in the experiment described here was transformed to represent cancerous mutations in both the *ras* and *src* oncogene families.

Point mutations at identified codons in *ras* genes represent a classic example for mutational activation of a proto-oncogene. Genes in the *ras* family code for a major family of guanosine 5' triphosphate (GTP)-binding proteins, which are found at the inner surface of the plasma membrane and function to bind and hydrolyse GTP to GDP. They are cell messengers which intercept growth factor signals from receptors, and present them to internal secondary cell messengers (Pusztai,55).

The indication that a family of *ras* oncogenes was found to frequently contain mutations in human tumors caused one scientific group to screen novel products from bacterial fermentation broths for activity against *ras*-transformants (Ueda 1994). They found that FR901228, the compund that is tested in this experiment, would selectively reverse the phenotype of a ras transformant, and identified this compound as a part of a new class of anticancer drug targeting the components of ras-mediated signaling transduction pathways (Ueda, J. of Antibiot., I., 301).

Some membrane-associated signal transducers are proteins with tyrosine kinase activity, which are recruited to the membrane and catalytically activated via growth factor receptors. The *src* family of proto-oncogenes belongs in this classification, because activated c-src is thought to bind intracellular targets such as P13K and GAP. Although not fully understood, the activation of src genes probably starts a complex tryosine phosphorylation cascade of many other proto-oncogene transcriptional factors, cytoskeleton proteins, and intermediate and effector signal transduction molecules

(Pusztai, 53-54). *src*-transformed cells were also tested in this experiment for comparison to normal and *ras*-transformed cells.

Anti-Cancer Drug Discovery

The drive in molecular oncology research is to develop new, more effective, and less toxic anticancer agents that will target specific gene changes and cutoff rapid division of tumor cells. Rapid new technologies are speeding up research, and researchers are quickly making insightful discoveries in cancer biology, moving toward understanding fully what the subtle changes in a cell's cycle may predict cancerous growth. If scientists can harness this new information to develop drugs with specific gene targets, we may be well on the road to effective treatment.

The current drug discovery program at NCI begins with a screening of potential anticancer agents against 60 tumor cell lines, divided by tissue type (breast, pancreatic, colon, lung, etc.). From there, drugs which show promise move on to screening protocols in mice, including xenografts, in which human tumor cells are injected, grow, and are treated within immune-deficient mouse clones. Unique anticancer agents may move on to testing in larger animals, cytotoxicity tests, and human clinical trials. Only 1 drug in 10,000 screened makes it to human trials, and only 20% of those tested in humans attain FDA approval (Available *http://rex.nci.nih.gov/massmedia/pressreleases/discovery.html*).

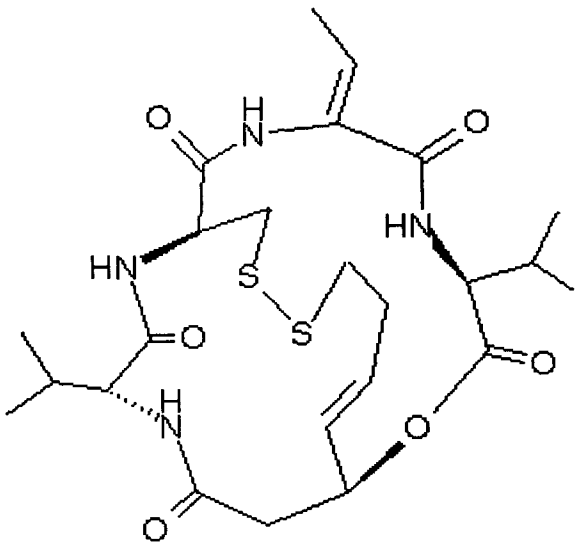
Several problems exist with using murine models for oncogenic investigations. First, several mouse cancer models do not develop the same tumors seen in humans, and sometimes develop tumors unknown in human cases. Secondly, mice with engineered

cancer susceptibility do not develop cancers corresponding to human homologues at the expected rate (Fearon, 1049). Often, murine tumors will not show significant response to drug treatments known to work in humans. In fact, when researchers at NCI tested 12 current anticancer agents, they find that 30 out of 48 tumor cell lines showed no response to drugs used in homologous tumors in humans (Gura,1041). Evidence of these shortcomings leave scientists looking for a better model.

In August 1998, the National Cancer Institute identified the following goals for future modelling in breast cancer research:

- Animal models with multiple genetic and epigenetic alterations that will more closely approximate human precancerous changes.
- Viable precancerous tissue from which to establish cell lines and complete biomarker characterization of established cell lines.
- Funding for long-term prospective human studies.
- A central distribution source for cell lines and specialized animal models with on-line information available on both cell lines and animal models. *(Available http://wwwosp.nci.nih.gov/planning/prg/bprgprevention.htm)*

FR901228: Discovery, structure and function in previous studies



FR901228 Depsipeptide {(E)-(1*S*,4*S*,10*S*,21*R*)-7-[(Z)-ethyl-idene-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo-[8,7,6]-tricos-16-ene-3,6,9,22-pentanone} (Chassaing, 170)

First identified by Fujisawa Company in 1994, FR901228 (FR) is a bicyclic depsipeptide now in preclinical and early clinical development as an anticancer therapeutic agent (Chan, 195). The agent was isolated as prisms in a broth culture of Chromobacterium violaceum (No. 968) and when tested, the agent reverted the transformed morphology of *ras*-transformed cells to normal (Ueda, J. of Antiobiot., I., 301).

Reviewing current research publications and selecting promising new agents for anti-cancer activity is part of the drug discovery program at the National Cancer Institute. In fact, NCI's Developmental Therapeutics Program has been responsible for the discovery and/or development of about one-half of chemotherapeutic drugs currently used for cancer treatment by oncologists (Press Release 1998). In 1998, NCI laboratories began to test FR in clinical trials as a potential therapeutic agent. More specifically, breast cancer is the second leading cause of cancer among women in the United States, and much time and funding has been devoted to the search for anticancer agents needed for selective therapy.

In cytotoxicity tests *in vitro*, FR showed potent activity against human lung adenocarcinoma, lung squamous cell carcinoma, stomach adenocarcinoma, mammary carcinoma, and colon adenocarcinoma, and weak to no cytotoxic activity against human endothelial cells and human and mouse fibroblast cells (Ueda, J. of Antibiot., I., 307). In *in vivo* murine tests, it prolonged the life of mice with leukemia and melanoma tumors, and inhibited the growth of both murine and human solid tumors implanted mice. (Ueda, J. of Antibiot., III., 315). Further studies in Japanese laboratories showed correlation

between treatment with FR and growth inhibition of *ras*-transformants, specifically G0/G1 cell cycle phase arrest (Ueda, Biosci. Biotechnol.Biochem., 1579).

Recently, an article in Breast Cancer Research and Treatment described the effects of FR901228 on human breast cancer cells. This study revealed that FR induced "apoptotic-like" cell death in two human breast cancer cell lines, MCF7 and MDA-MB231 (Rajgolikar, 29). The article included a protocol to test the cytotoxicity effect of FR on cancerous cell lines, which was used as a model for the study described here.

If an agent such as FR901228 is found to have cancer cell-selective therapeutic activity in the earliest stages of drug development, it will be screened by anti-cancer laboratories for intensive analysis of cytotoxic effects and molecular mechanism of function. Dr. HCR Wang, a University of Tennessee professor in the Comparative Medicine Department is currently investigating such characteristics of FR901228 in his laboratory.

III. Materials and methods

Cell Cultures and determination of cell survival for cytotoxicity of anticancer agents

Cells were grown in 12-well culture plates and treated at specific concentrations of two anticancer agents, FR901228 and staurosporin (STSP) over a period of 48 hours. After treatment, cells were rinsed with phosphate buffered saline (PBS), trypsinized for 2 minutes, and neutralized with 5% Fetal Calf Serum (FCS)-containing Dulbecco's modified Eagle's medium (DMEM). Cells were harvested and resuspended in 0.2% Trypan blue/FCS-free DMEM for staining. Cell viability was determined in a hemocytometer, according to methods described elsewhere (Ausubel, Chapter 11). Dead cells exhibited stain uptake, while live cells were identified by stain exclusion, counted, and recorded.

Statistical Analysis

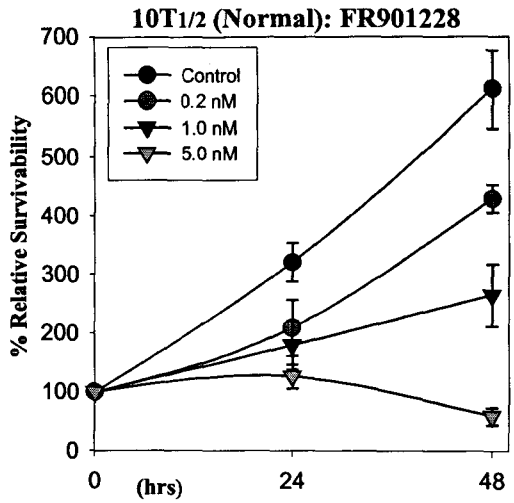
Each cell sample was counted in triplicate, and the original data sheets are included in this report (See Appendix). The average number of surviving cells was determined and compared to average number untreated cells at time zero (100%) to calculate relative survivability for each cell line and dosage amount, according to the following formula:

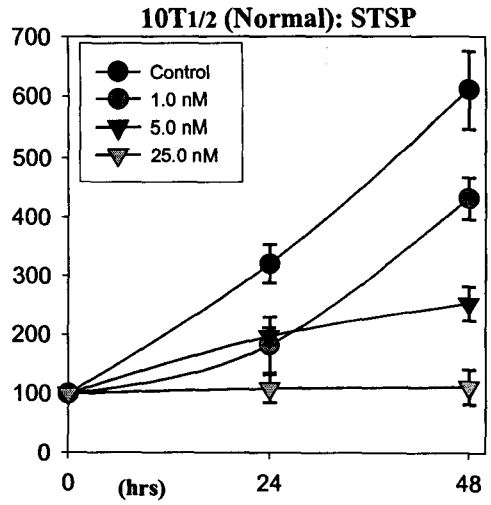
$$\frac{(E1+E2+E3)/3}{(C^o1+C^o2+C^o3)/3} \times (100\%) = \textbf{Relative Cell Survivability}$$

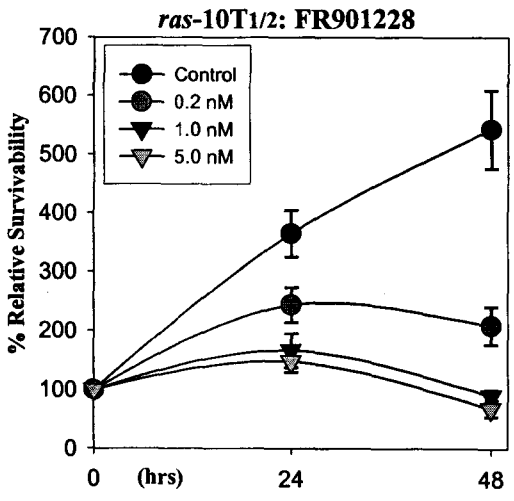
(Where E= experimental cell data and C^o= control cell data at time zero)

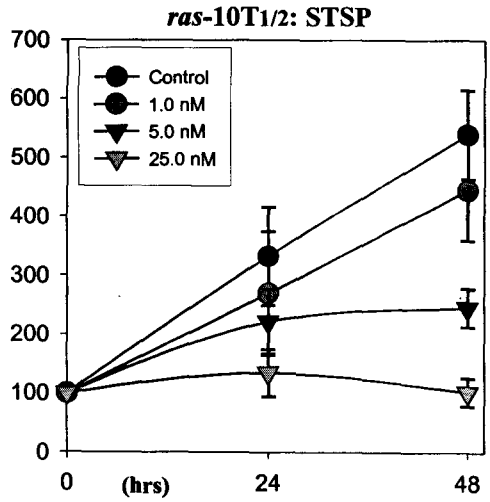
These calculations were used to generate the following comparative charts on SigmaPlot 4.0 for Windows; error bars represent standard deviation.

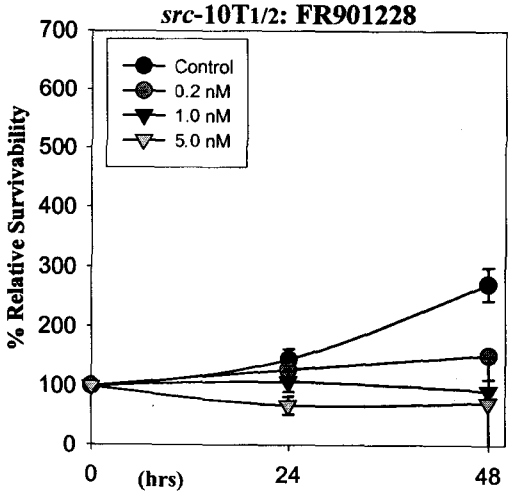
IV. Results

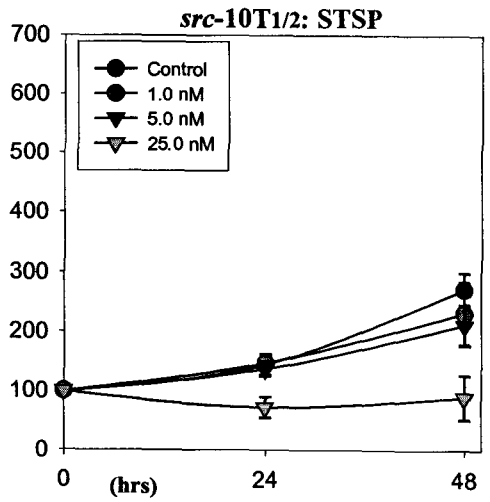

10T1/2 (Normal): FR901228
% Relative Survivability
Control
0.2 nM
1.0 nM
5.0 nM
0
100
200
300
400
500
600
700
0
(hrs)
24
48


10T1/2 (Normal): STSP
Control
1.0 nM
5.0 nM
25.0 nM
0
100
200
300
400
500
600
700
0
(hrs)
24
48


ras-10T1/2: FR901228
% Relative Survivability
Control
0.2 nM
1.0 nM
5.0 nM
0
100
200
300
400
500
600
700
0
(hrs)
24
48


ras-10T1/2: STSP
Control
1.0 nM
5.0 nM
25.0 nM
0
100
200
300
400
500
600
700
0
(hrs)
24
48


src-10T1/2: FR901228
% Relative Survivability
Control
0.2 nM
1.0 nM
5.0 nM
0
100
200
300
400
500
600
700
0
(hrs)
24
48


src-10T1/2: STSP
Control
1.0 nM
5.0 nM
25.0 nM
0
100
200
300
400
500
600
700
0
(hrs)
24
48

V. Discussion

The preceding graphs show treatment of 10T1/2, *ras*-10T1/2, and *src*-10T1/2 cell lines with low, intermediate and high dosage concentrations of FR901228 (FR) and staurosporin (STSP), an antitumor agent currently under investigation in other laboratories (Gurley 1998). Treatment with FR resulted in cell-line dependent effects, while STSP exhibited non-selective, dosage dependent growth inhibition effects.

Normal 10T1/2 cells showed dosage-dependent cell growth inhibition when treated with both FR and STSP. Low FR concentrations exhibited a slight inhibitive effect, increasing with dosage. The results of treatment with 5.0 nM suggests that cytotoxic effects may be seen in cells treated with 25.0 nM of FR901228.

Similarly, *src*-transformed 10T1/2 cells show dosage dependent growth inhibition in treatment with both agents. It is thought that *src* mutations have a widespread effect on cell functions, and so many other transcriptional and growth regulators and other proteins are affected. While FR may have some inhibitory effect on *src*-transformed cell lines, it would be extremely difficult to "rescue" *src*-transformed cell lines.

ras-transformed 10T1/2 cells displayed interesting results when treated with FR. At 24 hours, all samples exhibited slight cell growth inhibition, increasing with dosage concentration. However, after 48 hours, there is a dramatic drop in cell survival from the untreated curve to the first dosage concentration at 0.2 nM. Morphology reversion to normal and apoptotic-like cell death is observed in all three dosages (for example of apoptotic like cell death, see (Wijsman 1993)). STSP treatment results are similar to effects observed in normal 10T1/2 cells.

Further analysis of the effects of FR901228 on these cell lines is essential before many conclusions can be reached. According to the charts generated in this experiment, FR induced selective cell death in *ras*-transformed 10T1/2 cell line when compared to the normal 10T1/2 cell line. This effect is not apparent in the *src*-transformed 10T1/2 cell line. In order to confirm these findings, tests will be conducted by gel electrophoresis of DNA from treated cells, and it is expected that apoptotic death will result in fragmented DNA, readily distinguishable from cell death by necrosis. If apoptosis was indeed induced in these cell lines, it will be apparent after this analysis.

An investigator at NCI, Dr. April Robbins, is working in collaboration with other scientists to discover the mechanism of action associated with the cell-growth inhibition and apoptotic induction caused by treatment with FR. In preliminary studies, her group has found that FR blocks cell growth in early prometaphase (Robbins, no pg.).

If FR901228 continues to exhibit promising experimental results such as the anti-tumor effects displayed in this experiment in *ras*-transformed cells, perhaps we will be one step closer to understanding and unraveling the mysteries surrounding cancer treatment.

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VIII. Appendix

The following pages are copies of data entry sheets from February to April, 1999, for cell line treatment with FR901228 and represent the data from which results (see page 9) were calculated.

4/6/89

10% FCS

(1) RAS: FR/STSP

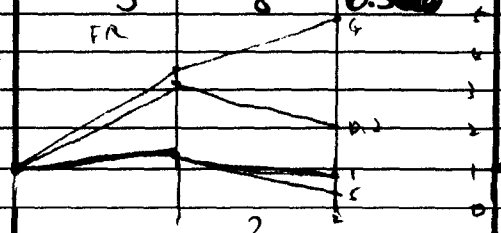
Ras: FR-nM	1 Top	1 Bottom	1 Average	2 Top	2 Bottom	2 Average	3 Top	3 Bottom	3 Average	Means
Day 0, 4/7	19	9	14	21	12	16.5	13	15	14	14.03
[redacted]										
Day 1- 4/8 0	69	71	70.0	74	86	80.0	65	73	69.0	73.0
0.2	49	41	45.0	41	53	47.0	43	43	43.0	45.0
1	29	38	43.5	28	38	33.0	34	42	38.0	34.83
5	25	33	29.0	31	25	28.0	35	24	29.5	28.83
Day 2- 0	82	87	84.5	85	90	87.5	106	99	102.5	91.5
0.2	29	38	43.5	32	27	29.5	37	38	37.5	35.167
1	11	12	11.5	11	12	11.5	11	11	11.0	11.33
5	10	16	13.0	8	13	10.5	6	14	10.0	11.167
[redacted] 0										
1										
5										
25										

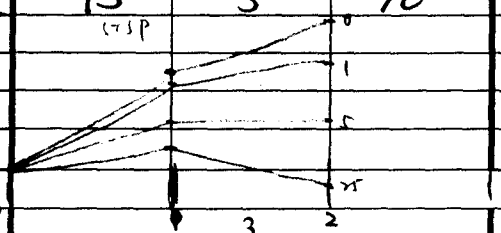
Ras: STSP-nM	1 Top	1 Bottom	1 Average	2 Top	2 Bottom	2 Average	3 Top	3 Bottom	3 Average	Means
Day 0, 4/7	19	9	14	21	12	16.5	13	15	14	14.03
[redacted]										
Day 1- 0	69	71	70.0	74	86	80.0	65	73	69.0	73.0
1	52	57	54.5	61	54	57.5	47	64	55.5	55.83
5	45	50	47.5	36	50	43.0	56	44	50.0	46.83
25	28	35	31.5	34	26	30.0	29	29	29.0	30.167
Day 2- 0	82	87	84.5	85	90	87.5	106	99	102.5	91.5
1	79	72	75.5	71	64	67.5	79	74	76.5	73.167
5	37	42	39.5	30	33	31.5	36	31	33.5	34.83
25	18	23	20.5	14	15	14.5	14	26	20.0	18.33
[redacted] - 0										
1										
5										
25										

101 TCS

(2) RAS: FR/STSP

	1			2			3			
	Top	Bottom	Average	Top	Bottom	Average	Top	Bottom	Average	Means
Ras: FR-nM										
Day 0, [redacted]	19	20	19.5	18	15	16.5	15	15	15.0	17.0
[redacted]										
Day 1- 0	63	51	57	65	67	66	62	63	61	61.83
0.2	49	47	48	57	69	63	50	38	44	51.6
1.0	25	18	21.5	22	22	22	27	20	23.5	22.3
*clumpy 5	27	24	25.5	24	32	28	17	16	16.5	23.3
Day 2- 0	68	75	71.5	81	99	90	79	95	87	82.8
0.2	44	32	38.0	29	44	36.5	25	31	27	33.8
1.0	12	16	14	15	12	13.5	19	16	17	14.8
5.0	4	8	6	5	8	6.5	15	5	10	7.5
~~Day 3-~~										





	1			2			3			
	Top	Bottom	Average	Top	Bottom	Average	Top	Bottom	Average	Means
Ras: STSP-nM										
Day 0, [redacted]			19.5			16.5			15.0	
[redacted]										
Day 1- 0	63	51	57	65	67	66	62	63	61	
1	45	36	40.5	64	56	60	65	62	63.5	54.67
5	35	37	36	44	35	39.5	29	34	31.5	35.67
25	25	22	23.5	27	25	26.0	25	24	24.5	24.67
Day 2- 0	68 [redacted]	75 [redacted]	71.5	81 [redacted]	99 [redacted]	90	79 [redacted]	95 [redacted]	87	
1	62	76	69	62	46	54	61	66	63.5	62.17
5	35	36	35.5	34	38	36	43	40	41.5	37.67
25	10	9	9.5	9	13	11	8	10	9.0	9.83
~~Day 3~~										

4/12/99 Gena. Just count the three wells labelled w/ Day 0 on Monday.

10 serum %?

40 dpL

(3) RAS: FR/STSP

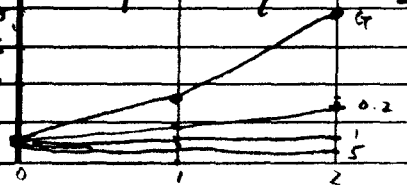
Rev FR [redacted]: [redacted] nM	1 Top	1 Bottom	1 Average	2 Top	2 Bottom	2 Average	3 Top	3 Bottom	3 Average	Means
Day 0, [redacted] 4/12	11	9	10.0	6	11	8.5	11	9	10.0	9.5
Day 0, [redacted]										
Day 1- 0	19	27	23.0	27	16	21.5	30	27	28.5	24.3
0.2 [redacted]	13	13	13.0	12	11	11.5	17	11	14.0	12.8
1 [redacted]	14	14	14.0	11	13	12.0	6	8	7.0	11.0
5 [redacted]	10	10	10.0	11	12	11.5	10	11	10.5	10.6
Day 2- [redacted] 0	51	59	55.0	46	45	45.5	51	58	54.5	51.6
0.2 [redacted]	14	26	18.0	18	15	16.5	20	16	18.0	18.167
1 [redacted]	12	10	11.0	11	7	9.0	8	14	11.0	10.3
5 [redacted]	8	5	6.5 [redacted]	7	9	8.0	10	9	9.5	8.0
Day 3- [redacted]										
[redacted]										
[redacted]										
[redacted]										

RAS [redacted] STSP nM	1 Top	1 Bottom	1 Average	2 Top	2 Bottom	2 Average	3 Top	3 Bottom	3 Average	Means
Day 0, [redacted]	11	9	10.0	6	11	8.5	11	9	10.0	9.5
Day 0, [redacted]										
Day 1- 0	19	27	23.0	27	16	21.5	30	27	28.5	24.3
1	21	19	20.0	17	18	[redacted]	28	32	27.0	21.5
5 [redacted]	19	20	19.5	14	23	18.5	21	19	19.5	19.3
25 [redacted]	12	9 [redacted]	10.5	9	9	9.0	6	11	8.5	9.3
Day 2- 0	51	59	55.0	46	45	45.5	51	57	54.5	51.6
1	50	41	45.5	56	53	54.5	43	54	48.5	49.5
5 [redacted]	31	18	24.5	24	26	25.0	17	26	21.5	23.167
25 [redacted]	12	8	10.0	18	10	14.0	13	7	10.5	11.33
Day 3- 0										
1										
10										
50										

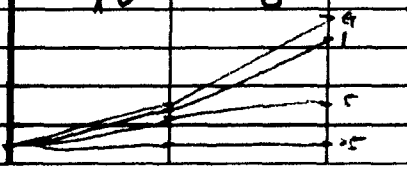
7.5%
FCS

(4) RAS: FR/STSP(7.5%FCS)

1958	1			2			3			
Ras: FR-nM	Top	Bottom	Average	Top	Bottom	Average	Top	Bottom	Average	Means
Day 0, 3/12	13	9	11.0	8	12	10.0	9	6	7.5	9.5
Day 1- 3/13 0	23	35	29	25	31	28	23	31	27	28.0
0.2	15	22	18.5	19	14	16.5	17	14	15.5	16.83
1.0	10	7	8.5	8	13	10.5	17	6	11.5	10.16
5	11	8	9.5	16	10	13.0	15	7	11.0	11.16
Day 2- 3/14 0	61	89	75	77	69	72.5	77	74	75.5	74.5
0.2	25	21	23.0	19	26	22.5	19	20	19.5	21.6
1.0	10	10	10.0	10	13	11.5	7	13	10.0	10.5
5.0	3	10	4.5	8	9	8.5	4	7	5.5	6.16
~~Day 3 0~~										
~~1~~										
~~5~~										
~~25~~										



	1			2			3			
Ras: STSP-nM	Top	Bottom	Average	Top	Bottom	Average	Top	Bottom	Average	Means
Day 0,	13	9	11.0	12	8	10.0	9	6	7.5	9.5
~~Day 0, 3~~										
Day 1- 3/13 0	23	35	29	25	31	28	23	31	27	28.0
1	31	23	27	26	24	25	27	29	28	26.6
5	18	21	19.5	15	22	18.5	16	24	20	19.3
25	17	9	13.0	13	14	13.5	9	11	10.0	12.16
Day 2- 3/14 0	61	89	75	77	69	72.5	77	74	75.5	74.5
1	57	64	60.5	61	78	69.5	47	60	53.5	61.6
5	29	31	30.0	31	31	31.0	28	27	27.5	29.5
25	7	11	9.0	6	8	7.0	10	8	9.0	8.3
~~Day 3 0~~										
~~1~~										
~~40~~										
~~50~~										



IVS: FR/STSP

4/4/99

IVS	1			2			3			
	Top	Bottom	Average	Top	Bottom	Average	Top	Bottom	Average	Means
[redacted]2: FR-nM										
Day 0, A 4/5	18	9	13.5	15	10	12.5	10	15	12.5	12.83
~~Day 0, B~~										
Day 1- 4/6 0	17	22	19.5	26	15	20.5	18	15	16.5	18.83
0.2	16	19	17.5	15	20	17.5	13	17	15.0	16.67
1	13	17	15	10	13	11.5	14	18	16	14.167
5	7	13	10.5	7	8	7.5	11	4	7.5	8.5
Day 2- 4/7 0	38	41	39.5	45	27	36	[redacted]	32	32.5	36.0
0.2	23	14	18.5	23	18	20.5	22	19	20.5	19.83
1	17	13	15.0	9	11	10.0	14	10	12.0	12.3
5	3	1	2.0	5	2	3.5	4	6	5.0	3.5
~~Day 3~~										

	1			2			3			
	Top	Bottom	Average	Top	Bottom	Average	Top	Bottom	Average	Means
IV5: STSD ~~FR~~-nM										
Day 0, A	18	[redacted]	13.5	[redacted]	15	12.5	[redacted]	15	12.5	12.83
~~Day 0, B~~										
Day 1- 0	17	22	19.5	26	15	20.5	18	15	16.5	18.83
1	17	19	18	16	23	19.5	17	24	20.5	19.33
5	20	19	19.5	14	20	17.0	19	17	18.0	18.167
25	9	7	8.0	8	16	12.0	9	9	9.0	9.67
Day 2- 0	38	41	39.5	45	27	36	33	32	32.5	36.0
1	30	25	27.5	24	29	26.5	32	43	37.5	30.5
5	31	34	32.5	26	26	26	23	28	25.5	28.0
25	10	10	10.0	18	15	16.5	6	12	9.0	11.83
[redacted]										

IV5: FR/STSP

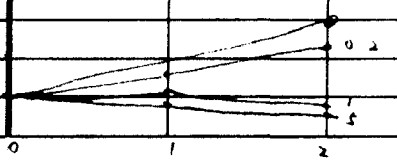
3/30/18

IV5

FR-nM

	1			2			3			
	Top	Bottom	Average	Top	Bottom	Average	Top	Bottom	Average	Means
Day 0, 3/31	5	4	4.5	8	7	7.5	5	* 13	9.0	? 7.0
Day 0,										
Day 1- 0	8	5	6.5	5	4	4.5	3	7	5.0	? 5.3
0.2	10	17	13.5	9	10	9.5	13	8	10.5	11.16
1	5	8	6.5	9	5	7.0	11	7	9.0	7.5
5	8	2	5.0	5	5	5.0	7	6	6.5	5.5
Day 2- 0	21	20	20.5	23	20	21.5	18	19	18.5	20.16
0.2	13	14	13.5	20	10	15	13	23	18	15.5
1	9	4	6.5	5	4	4.5	3	5	4	5.0
5	4	7	5.5	5	2	3.5	4	5	4.5	9.0 4.5
Day 3- 0										
1										
5										
25										

1.0
1.6
1.1
0.8
2.9
2.2
0.7
0.6



3/30/18

IV5

STSP-nM

	1			2			3			
	Top	Bottom	Average	Top	Bottom	Average	Top	Bottom	Average	Means
Day 0, 3/31	5	4	4.5	8	7	7.5	5	13	9.0	7.0
Day 0,										
Day 1- 0	8	5	6.5	5	4	4.5 9.5	3	7	5.0 10.5	6.3 11.16
1	13	15	14	11	9	10.0	10	18	14.0	12.6
5	5	11	8	8	4	6	10	10	10	8
25	7	6	6.5	5	4	4.5	3	13	8.0	6.3
Day 2- 0	21	20	20.5	23	20	21.5	18	19	18.5	20.16
1	23	22		19	21		21	24		21.6
5	15	15		17	22		14	13		16.0
25	4	6		3	9		4	8		5.6
Day 3- 0										
1										
10										
60										

1.0
1.8
1.1
0.9
2.9
3.1
2.3
1.8