Summary of Experiments

Summary of Experiments

This is a summary of a series of experiments that were carried out in my laboratory after accusations were made against Anupam Bishayee with regard to an experiment carried out on XXX 2001.

I. Multicellular Cluster, 50% Labeling, Survival & Mutation
- Ten experiments
- No survival bystander effect observed
- Apparent mutation bystander effect observed as shown in Marek Lenarczyk's compilation of experiments that had both survival and mutation data (see attachment A)

II. Multicellular Cluster, 100% Labeling, Survival & Mutation
- Two experiments
  - Exp 5 survival data on Page **B**
  - Exp 5 mutation data on Page **C**
    - Some dose-dependent induction of mutations at about the same levels observed in apparent bystanders.
  - Exp 6 survival data on Page **D**
  - Exp 6 mutation data on Page **E** (analysis from 12/3/01 email from Marek)
    - Essentially no increase in mutations with increasing dose. Why aren't there consistent mutations in labeled cells?

Discussion and Interpretation

In the ten experiments that were carried out with 50% labeling, no survival bystander effects were seen in these studies while an apparent mutation bystander was observed. To ensure the reliability of our mutation assay, we also carried out important control experiments to show that we could induce mutations when 100% of the cells were labeled (Pages B,C,D,E). One would anticipate a strong linear dose-response for the 100% labeling conditions. However, one of these experiments showed an unexpectedly weak mutation response and the second experiment showed essentially no mutation response at all. Thus, the 100% labeling mutation data cast doubts on the bystander mutation response observed in the 50% labeling case. Marek Lenarczyk and I had discussed this problem more than two or three years ago.

Furthermore, a close examination of the 50% labeling survival data shows that the survival fractions are generally well above 0.5. This implies that a substantial percentage of the labeled cells survived despite the fact that they had high uptake of radioactivity. This is highly unexpected. This also means that the observed mutations are the sum of those found in bystander cells and labeled cells. Therefore they may, or may not, represent a mutation bystander effect. While Marek and I had hoped to be able to publish on the mutation bystander effect in a peer reviewed journal, these inconsistencies have precluded this.

Inconsistencies in the mutation data aside, we still have to address why the 50% labeling data do not reproduce our published survival bystander effect. While we always strive to

maintain tight control over our experiments, one must examine which variables may have changed and which variables we know have changed. These are listed below.

- Variables that may have changed
  - Source of microfuge tubes that the clusters are maintained in. Only ultrapure tubes are free of trace elements and we have never used ultrapure tubes.
  - pH of media
  - Level of trace elements in UMDNJ deionized water from which cell culture media is prepared
  - Wetting agents on filter apparatus used to sterilize cell culture media
  - Methods used to clean bottles used to prepare and store media
  - Sodium bicarbonate used to be prepared from powder as opposed to the liquid form obtained from the manufacturer
- Variables that we know have changed
  - Different incubator (rusting Queue incubator was replaced with Napco incubator obtained from donation by private industry)
  - Fetal calf serum (FCS)
  - Flasks that cells were grown in
  - Different V79 cells used in the new experiments
    - Original stocks of cells stored at -196°C were lost due to dewar failure. Forced to use much later passage of V79 cells that were stored at -70°C.
    - Also used V79 cells from ATCC.
    - Passage numbers also not known.

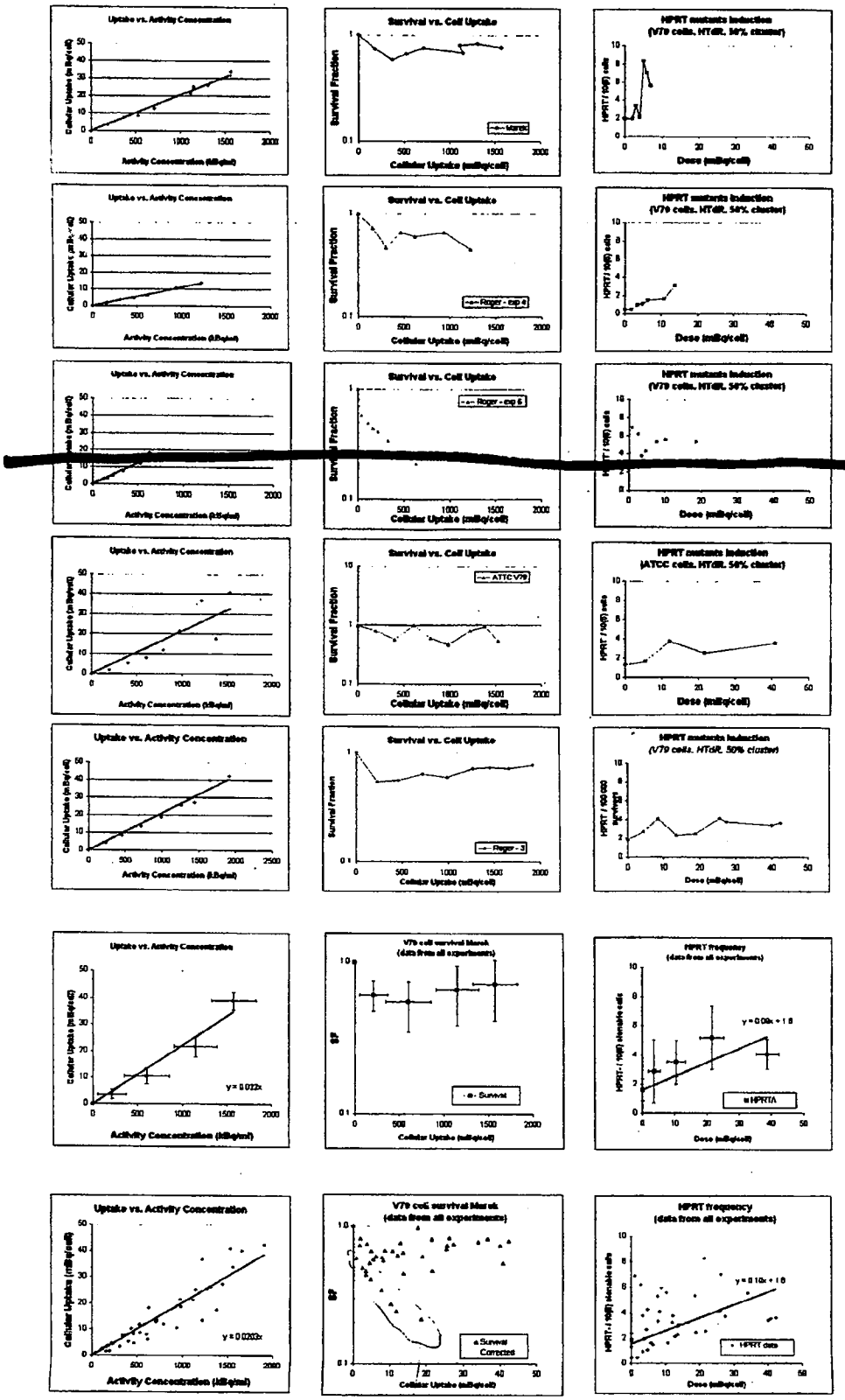
While all of these variables are important, a few of them are most notable. First is the pH of the media. Ludwig Feinendegen, one of the most influential scientists in the radiation research community, informed me that a pH change of only 0.1 units completely abrogated an important biological response reported in his laboratory. It took his staff a year of dedicated effort to track this down. Second is the possibility of changes in the levels of trace elements in the UMDNJ deionized water system. This could result from new piping installations, change of filters, etc. This could potentially have a serious impact on the capacity of labeled cells to send damaging signals to neighboring bystanders. Third, fetal calf serum is a critical component in cell culture media that is highly variable from manufacturer to manufacturer, batch to batch, and storage time and temperature. The serum used in the published experiments was obtained from Gibco and routinely stored at -70°C in our laboratory. The serum used in the experiments described above was from Hyclone and stored at -20°C. With the help of Gibco, I was eventually able to track down some of the original sera that was owned by an investigator at another institution. This serum was stored by them (and us) at -20°C and used in one of the 50% labeling experiments and no survival bystander effects were observed. Finally, of greatest importance are the V79 cells themselves. Our original stocks of V79 cells were lost when our liquid nitrogen dewar failed. Therefore, we used V79 cells that had been frozen in my lab in October 2000 as well as some V79 cells we obtained from ATCC. Furthermore, in keeping with my graduate training when I first worked with V79 cells, we had never maintained records of their passage number. Based on the date of the frozen stocks of V79 cells that were available to carry out these experiments, and the fact that we passage the cells twice a week, it is possible that as many as 100 or more passages may have elapsed. At the time these experiments were carried out, it was

brought to my attention that V79 cells are known to be genomically unstable. Therefore, many changes could have occurred in the cells that altered their survival bystander response. This also may be the reason we are seeing survival fractions in excess of 50% even when 50% of the cells are heavily labeled with radioactivity.

While we did not observe survival bystander responses in the studies described above, it is clear that the experimental conditions do not sufficiently reproduce the conditions in our published experiments. I have a great degree of confidence in our published data for the following reasons. Our experimental multicellular cluster model was conceived as a way to verify the theoretical multicellular dosimetry models that we published in 1994. We were not familiar with bystander effects at that time. As I have stated in each of my seminars about our published data, our initial experiments with ^{3}HTdR were intended to be control experiments for our multicellular cluster model which was to be used in conjunction with a variety of other radionuclides that emit radiations with different ranges in tissue. When ^{3}HTdR is loaded into cells, the extremely short-range ^{3}H beta particles irradiate only the labeled cells and cannot hit neighboring unlabeled cells. Therefore, I expected an exponential survival response when 100% of the cells were labeled and Anupam Bishayee's experiments did reveal this. I expected to observe a saturation at 50% survival when 50% of the cells were heavily labeled. This expectation was discussed with Anupam. In contrast, he found that the survival dropped well below 50% and continued to fall as the amount of radioactivity in the labeled cells was increased. I informed him that this was not possible and asked him to repeat the studies wherein he found the same response. It was only then after reading the literature that we concluded that it must be a bystander response similar to those already published. Accordingly, given that we were not looking for a bystander response, I have a high degree of confidence in our published data.

My confidence in the published data is further supported by the fact that we and others have observed ^{3}HTdR-induced bystander responses in other experimental models. In my laboratory, Bogdan Gerashchenko has observed and published (in the journal Cytometry) bystander responses in a two dimensional WB-F344 cell culture model. In a 3D model of human tissue, Massimo Pinto is observing cell cycle delays in bystander cells adjacent to cells labeled with ^{3}H. In Edouard Azzam's laboratory, a variety of ^{3}HTdR-induced bystander effects have been observed including stress response signaling, cell cycle delays, and induction of micronuclei (see abstract in folder). These data have been reported at national and international meetings. Finally, we are aware that scientists at other institutions are also in the process of publishing ^{3}HTdR induced bystander effects.

Marek Lenarczyk Analysis of Data [illegible] on 9/22/04



100% label

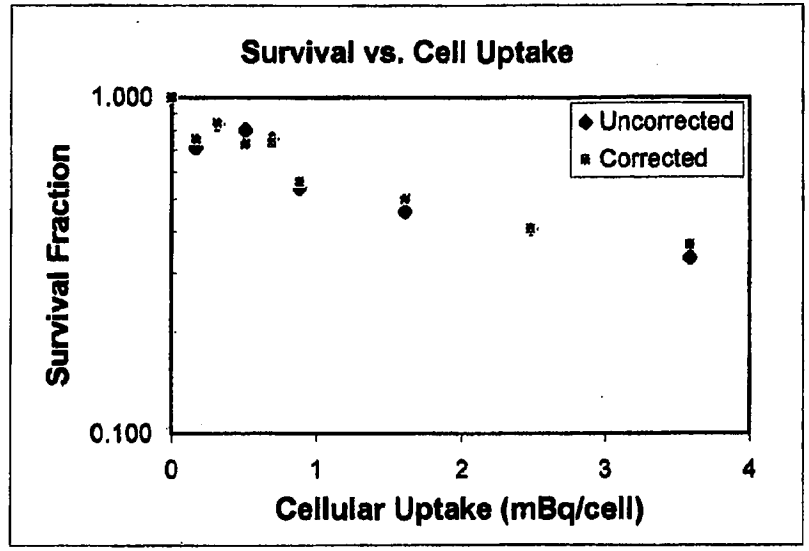
100% label

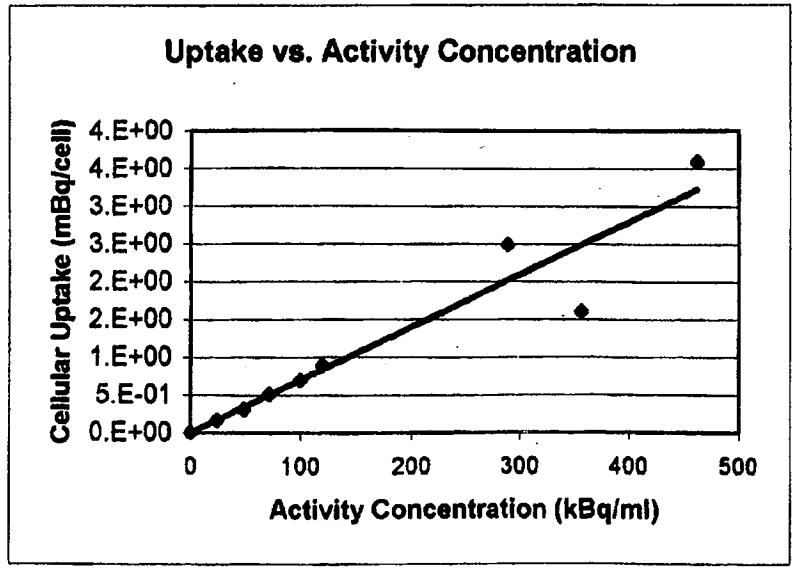
A

Exp. 5 100% Label 3HTdR

Experiment:
Date/Time: 7/16/01

Tube #	Activity Conc. (kBq/ml)	Activity/Cell (mBq/cell)	Survival Uncorrected	Survival Corrected
1	*0.000*	*0.000*	*1.0000*	*1.0000*
2	*0.000*	*0.000*		
3	23.876	0.168	0.7105	0.7527
4	48.877	0.313	0.8333	0.8428
5	71.600	0.510	0.8001	0.7289
6	99.530	0.694	0.7537	0.7363
7	119.618	0.888	0.5412	0.5639
8	288.980	2.485	0.4050	0.4049
9	357.505	1.607	0.4582	0.4998
10	462.409	3.590	0.3320	0.3623



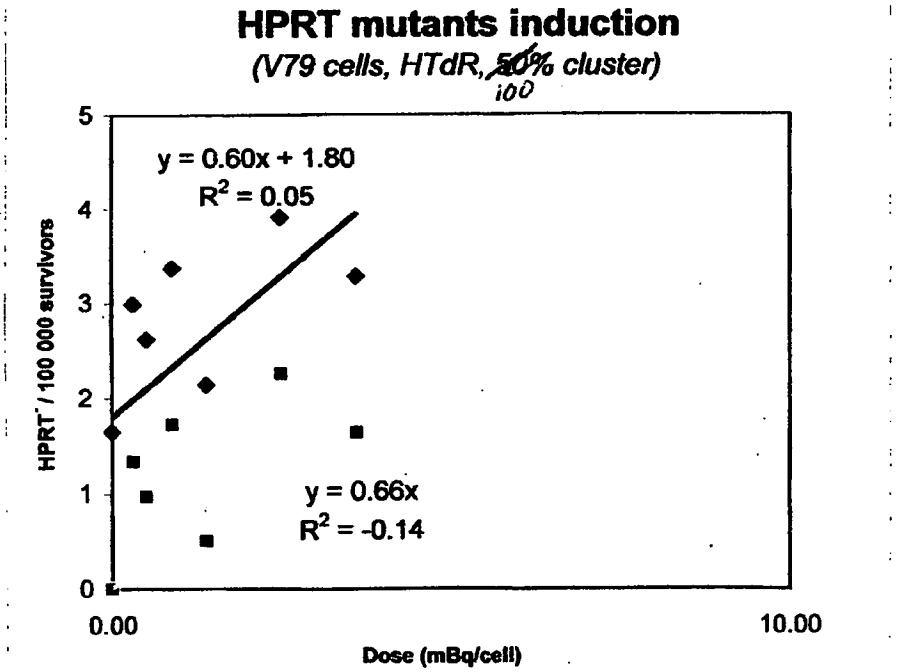


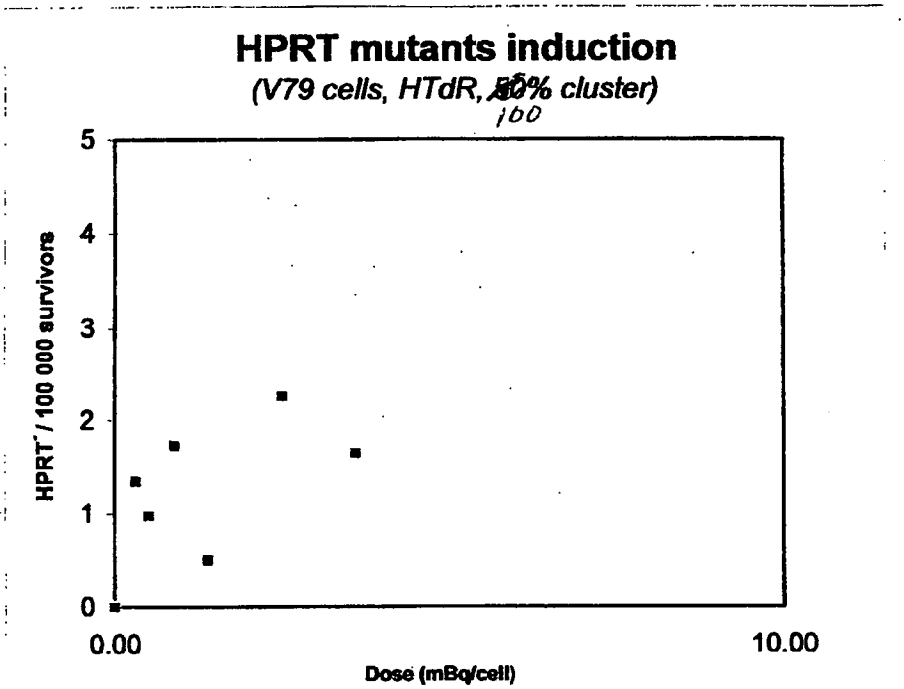
Exp 5 100% Label 3HTdR Survival Data

3

Exp. 5 100% Label ^{3}HTdR

Mutation Data



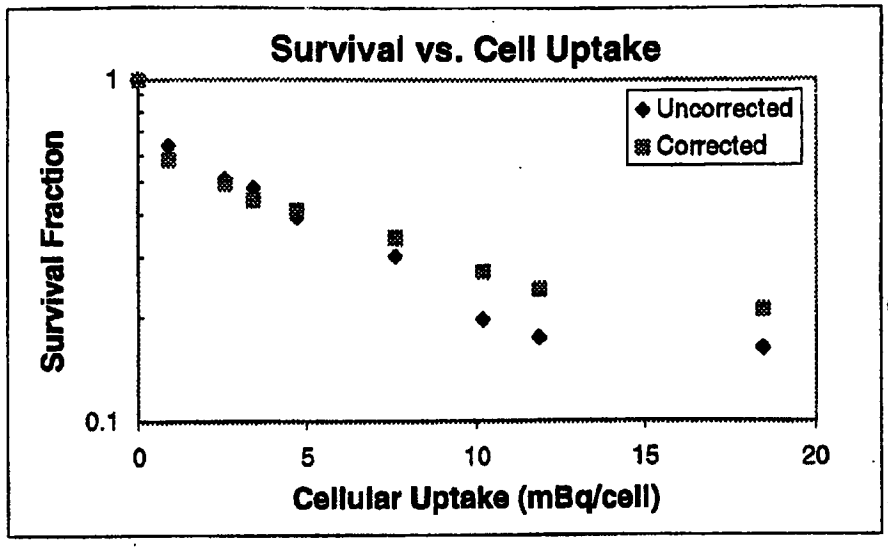


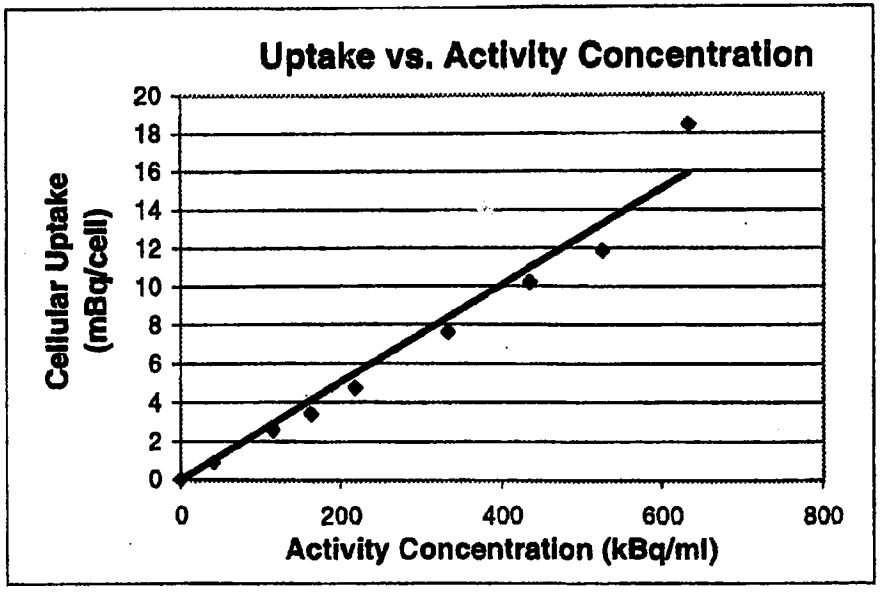
C

Summary

Experiment:
Date/Time: 9/27/2001

Tube #	Activity Conc. (kBq/ml)	Activity/Cell (mBq/cell)	Survival Uncorrected	Survival Corrected
1	*0.000*	*0.000*	*1.0000*	*1.0000*
2	*0.000*	*0.000*		
3	41.559	0.898	0.6397	0.5824
4	116.004	2.578	0.5097	0.4955
5	163.336	3.410	0.4798	0.4425
6	218.323	4.735	0.3937	0.4130
7	333.744	7.625	0.3023	0.3420
8	436.214	10.192	0.1968	0.2718
9	527.317	11.867	0.1747	0.2417
10	633.470	18.456	0.1634	0.2125



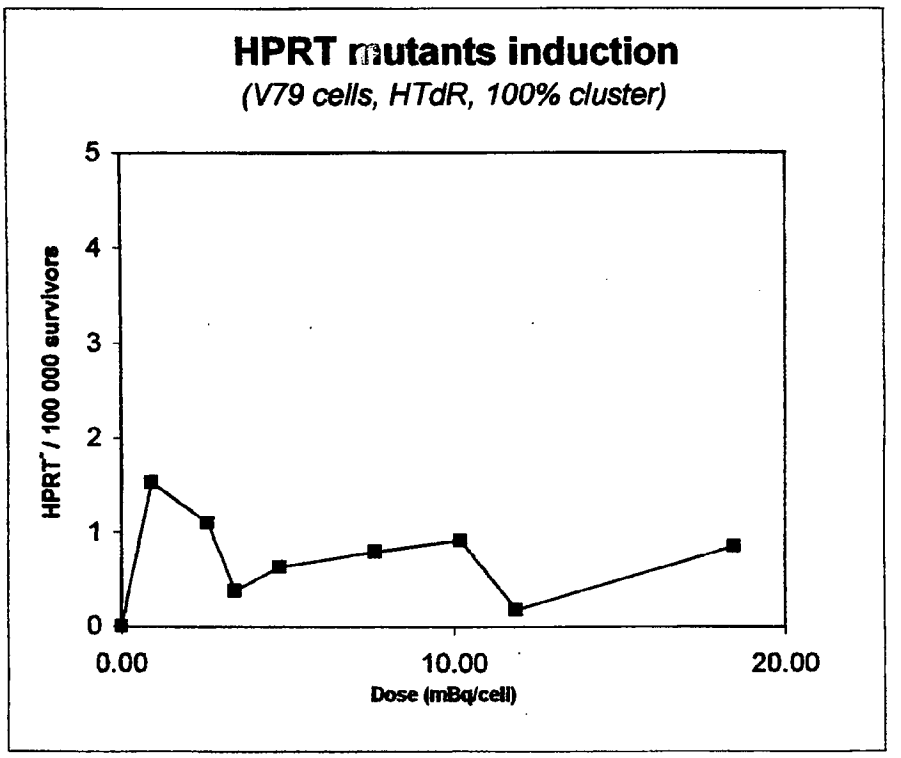


Exp. 6 100% Label ^{3}HTdR Survival Data

D

B018325

Marek's Analysis of Exp. 6 100% Label ^{3}HTdR
From 12/3/01 email



E