Expert Report

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1. Scope of the report by Dr. Robbins and incompatibility of expertise.

The report by Dr. Robbins attempts to justify the accusation of fraud regarding experimental work with various tissue culture cells, their labeling with tritiated thymidine (^{3}H-TdR), and their survival as a function of the degree of this labeling under various experimental conditions.

In order to address the issue of cell damage leading to cell death under specific culture conditions following ^{3}H-TdR labeling, certain general and specific facts on cell biology in culture, on biochemistry of DNA synthesis and especially TdR incorporation in living cells, and on the physics and the biophysical consequences of tritium decay must be taken into consideration properly.

In reading the statements made by Dr. Robbins, there is doubt that the report expresses the expertise that appears necessary for evaluating the research and for reaching the opinion that the experimental data are incorrect or falsified. The key lack of expertise lies in the fact that his report fails to demonstrate that he comprehends the biochemistry of thymidine incorporation into the cell. To do so requires detailed knowledge of the nucleotide pool of the cells and its components, of the biochemical behavior of the nucleotide pool under various external and internal cellular conditions, and of the fate of thymidine that is not incorporated into DNA of the cell. Moreover, there appear inadequacies in his capacity to assess the biological effect of tritium that is incorporated with its carrier thymidine in cellular DNA. Details on the lack of appreciation of these various facts in the experiments discussed are given below.

The statements made in Dr. Robbins' report show an expertise that does not translate to the fields he attempts to opine on in his expert report.

2. *Introduction and brief invalidation of the report by Dr. Robbins.*

a) Cell biology considerations

The report by Dr. Robbins refers basically to three reasons for claiming that the results obtained by Bishayee et al. are impossible to have been generated. These three reasons are not valid as stated in detail later in this report. A brief summary of his three reasons and why they are not valid follows:

Robbins' Reason 1. ^{3}H-TdR as such blocks the movement of cells through the various phases of the cell cycle. (See pg. 2 of Dr. Robbins' Report).

This claim totally fails to consider the fact that the amount of thymidine molecules and not ^{3}H-TdR as such may cause blocking the movement of cells through the various phases of the cell cycle. In other words, Dr. Robbins' statement fails to consider the difference between high and low specific activities of ^{3}H-TdR, i.e., the relatively large and small number of tritium atoms per unit number of thymidine molecules in the experiments by Bishayee et al.

Bishayee et al. have used high specific activity ^{3}H-TdR, as demonstrated below in more detail (pages 6-8). Stated another way, high specific activity means that a given number of tritium atoms are bound to a small amount of thymidine molecules. On the other hand, low specific activity means that the same number of tritium atoms is bound to a relatively large amount of thymidine molecules.

It follows also, that lowering the specific activity of ^{3}H-TdR in the culture medium while keeping the number of tritium atoms constant the number of thymidine molecules offered to the cells rises to eventually reach a threshold at which the system is "flooded" and limits the number of tritium atoms that are incorporated into the cell DNA.

Under the given experimental conditions, as they are described in the papers evaluated, blocking the movement of cells through the cell cycle depends on the amount of thymidine molecules that have entered the cellular nucleotide pool, i.e., the pool of DNA precursor molecules that serve as building blocks for DNA synthesis during a specifically timed

phase of the cell cycle, called S-phase, between two consecutive cell divisions. High specific activity ^{3}H-TdR permits sufficient numbers of tritium atoms to be incorporated into the DNA using only a small number of thymidine molecules. Under these conditions, ^{3}H-TdR becomes a true tracer of physiological metabolism and does not perturb the physiological nucleotide pool and does not cause secondary consequences of feed-back control mechanisms. However, low specific activity ^{3}H-TdR may not only eventually limit the number of tritium atoms that are incorporated from the culture medium into the cell DNA, it also may cause secondary consequences by perturbing the nucleotide pool. The intracellular specific pool of thymidine is rather small, as further presented below. One of the reasons for this is that thymidine after having entered the cell is rapidly phosphorylated and then incorporated into DNA during S-phase. If not incorporated, thymidine is rapidly lost from the cell by back transport into the extracellular environment or by metabolism or both.

Perturbation of the cellular nucleotide pool by thymidine may block the movement of cells through the various phases of the cell cycle. This only occurs when the pool is "flooded", which again depends on the pool size. As stated above, this pool generally is small but varies in different cell types. The pool has been analyzed for V79 cells as discussed in more detail below (pages 4; 13). Bishayee et al. used high specific activity ^{3}H-TdR, bringing to the cells an amount of thymidine far below the level needed for "flooding" the pool. Hence, there was no perturbation of the pool by thymidine in the experiments by Bishayee et al. and thus, also with certainty, no thymidine related blocking of movement of cells through the various phases of the cell cycle.

The report of Dr. Robbins does not consider the above cell biological phenomena related to thymidine incorporation into DNA and, thus, comes to an erroneous conclusion

Robbins' Reason 2. No deoxycytidine was present in the culture medium at the time the cells were exposed to ^{3}H-TdR (See pg. 2 of Dr. Robbins' Report).

Indeed there was no deoxycytidine present. Deoxycytidine is required only when the nucleotide pool of the cells has been perturbed by a relatively large amount of thymidine molecules added to the cells. The amount of thymidine that causes nucleotide pool perturbation in V79

cells is further specified in detail below (pages 4;13). It is known, as Dr. Robbins rightly states, that adding deoxycytidine to the nucleotide pool of the cells may overcome the block that is produced by a certain perturbing amount of thymidine molecules in the nucleotide pool. However, as referred to above under Reason 1, high specific activity ^{3}H-TdR provides for true tracer conditions without metabolic effects caused by the presence of thymidine. Therefore, no deoxycytidine needs to be added to the cells being exposed to ^{3}H-TdR in the experiments of Bishayee et al. Dr. Robbins' second reason, therefore, does not apply.

Robbins' Reason 3. No attempt was made to synchronize the cells into the same phase of the cell cycle prior to their treatment with ^{3}H-TdR. (See pg. 2 of Dr. Robbins' Report).

Again, as stated above, since high specific activity ^{3}H-TdR provides for true tracer conditions of the experiments and no perturbation of the nucleotide pool of the cells, there is no need for cell synchronization. In fact, such synchronization always somehow interferes with normal cellular kinetics and would at least temporarily disturb cellular metabolism to such an extent that the results eventually may be misleading. Therefore, Bishayee correctly chose not to synchronize the cells prior to labeling them with ^{3}H-TdR. Consequently, the third reason also does not apply.

Other Issues. There are also perhaps simple oversights in using biochemical nomenclature.

Regarding nomenclature in Dr. Robbins' report, one should expect consistency of terms. Thymidine is usually abbreviated by TdR, and not by dThd, or Tdr; the non-specialist may be confused by changing terminology for the same molecular compound in one text. Another example concerns the classification of thymidine. This molecule is a nucleoside and not a pyrimidine, as stated in his section "Effect of ^{3}H-TdR on the Cell Cycle". The pyrimidine base is called thymine.

b) Radiobiology considerations

In addition to the above sequels of erroneously not attending to the importance of the cellular nucleotide pool regarding the incorporation of

thymidine into cells, there appear some inconsistencies regarding radiobiological facts.

For instance, under "Effect of ^{3}H-TdR on the Cell Cycle" there is the statement "When tritium decays, it releases low-energy beta radiation in the form of an electron that will break the DNA present in the cell nucleus resulting in cell death or mutation that can lead to cancer." (See pg. 3 of Dr. Robbins' Report). This statement may lead the reader to believe that every beta particle, i.e., electron, from tritium decay in the cell nucleus causes a DNA break with the consequence of cell death or mutation leading to cancer. Relevant, however, is the probability of a DNA break to cause cell death or cancer. The probability of a DNA break per tritium decay in the cell nucleus is approximately 1: Yet, the number of such breaks to cause cell death is in the range of a thousand (10^{3}) if they occur all within a very narrow range of time, or simultaneously. The probability of such a break on average to be associated with, or cause, a mutation in the cell and ultimately develop into a lethal cancer in a mammalian organism is in the range of 1 in 10-100 trillion (10^{13} - 10^{14}) (*1*). Without the given range of probabilities, the reader may become misled into attributing to a single beta-particle effect on DNA an enormous danger. This is not correct.

One of the experiments of Bishayee et al. show 90 % of the cells to be killed by ^{3}H-TdR incorporated into the cells, when the number of decays that has accumulated per labeled cell conforms to the value of about 1 mBq of tritium per cell (see for instance Figure 2 in Dr. Robbins' report). This number of decays per cell nucleus corresponds to approximately 1 decay per 1000 seconds, i.e., about 1 decay every 16 minutes. Each beta-particle absorbed in the cell nucleus with a mass of ~ 0.5 ng causes an absorbed dose average of ~ 2 mGy to that nucleus (*2*). The average absorbed dose to the cell nucleus per ^{3}H decay has been estimated specifically for V79 cells, with a 4 μm radius cell nucleus, to be 2.6 mGy (*3*). In fact, in Bishayee's experiments, there are three critical time periods of exposure of the labeled cells to beta particles emitted by ^{3}HTdR: a) the initial period of labeling for 15 hours; b) three days of cell incubation at the temperature of 10.5°C, with no cell division, during which time the amount of tritium in the cells is constant as measured and expressed in mBq/cell; c) seven days of growth during the cell survival assay where the surviving fraction is defined as the fraction of clones that multiply to greater than 50 cells in seven days; during this time of 7 days cells either die or divide several times; the activity per surviving cell then decreases to one half with each cell division. This all has

been considered for calculating the average absorbed dose to the cells (*4, 5*). With this proper approach a D_{37} value of about 0.8 Gy results, a value that is fully consistent with the fact that the survival curves in the Bishayee experiments are logarithmically linear and do not show a so-called shoulder indicative of resistance to radiation. If one questions the accuracy of the experimental data by Bishayee et al., such considerations are helpful. The statement by Dr. Robbins, however, abstains from making such estimates which actually support the accuracy of the data.

The above considerations indicate that the statement by Dr. Robbins lacks qualification for pleading that the Bishayee et al. experiments appear falsified.

3. Specific rebuttals of the statements by Dr. Robbins.

1) Tritiated thymidine used in the experiments by Bishayee et al.

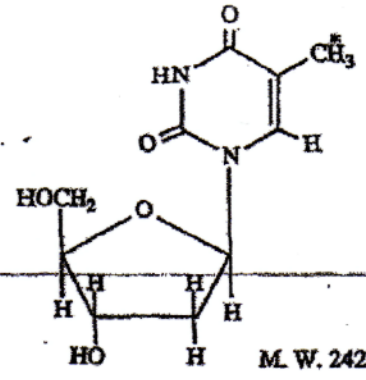
The experiments by Bishayee et al. in 1999 used thymidine labeled with tritium in the 5 position as indicated below. There is confirmation that all experiments have used high specific activities of tritiated thymidine (Catalog # NET-027Z) labeled as follows:

Caution: For laboratory use. A research chemical for research purposes only.

NET-027Z THYMIDINE, [METHYL-^{3}H]-

Catalog Number: NET027Z

Lot Number: 3106309
Specific Activity: 89.000000 Ci/mm
3322.000000 GBq/mm



M. W. 242.2

SPECIFIC ACTIVITY RANGE DESIGNATION AND PACKAGING:

NET-027: 6.7 Ci/mmol; aqueous solution, steri-packaged, at 1.0 mCi/ml (37 MBq/ml).
NET-027A: 2.0 Ci/mmol; aqueous solution, steri-packaged, at 1.0 mCi/ml (37 MBq/ml).
NET-027X: 20 Ci/mmol; aqueous solution, steri-packaged, at 1.0 mCi/ml (37 MBq/ml).
NET-027E: 20 Ci/mmol; ethanol : water solution, 7:3, at 1.0 mCi/ml (37 MBq/ml).
NET-027Z: 50-90 Ci/mmol; aqueous solution, steri-packaged, at 1.0 mCi/ml (37 MBq/ml).

2) The number of tritium atoms per molecule of thymidine can be calculated as follows

Specific activity of no carrier added ^{3}H = 1065.6 GBq/mmole = 28.8 Ci/mmole

Specific activity NEN NET-027Z tritiated thymidine = 3322.6 GBq/mmole = 89.8 Ci/mmole

$$\frac{\text{tritium atoms}}{\text{thymidine molecule}} = \frac{3322.6 \text{ GBq/mmole thymidine}}{1065.6 \text{ GBq/mmole tritium}} \approx 3$$

This implies that each of the hydrogen atoms on the methyl group have been replaced by tritium (see page 69-70 in Ref. (*6*)).

3) The question of thymidine blocking the movement of cells during the various phases of the cell cycle.

a) As stated above, Dr. Robbins fails to recognize the critical role of the specific activity of the tritiated thymidine (^{3}H-TdR) in determining its cellular uptake and toxicity in exponentially growing asynchronous V79 cells. In radiochemistry, specific activity is defined as the activity of a given radiochemical per unit mass, volume, or molarity. For radiochemicals that are inherently chemically toxic, the specific activity can determine whether the chemical itself (e.g. thymidine) plays a significant role in the biological response of the cells exposed to the radiochemical.

b) Specifications for tritiated thymidine used in Bishayee's experiments are found in B012757. All information lists high specific activities:

> NEN Life Science Products NET-027Z, thymidine [methyl-^{3}H]
> September 11, 1998, Specific activity 81.0 Ci/mmole
> February 10, 1999, Specific activity 88.5 Ci/mmole
> March 5, 1999, Specific activity 89.8 Ci/mmole

This same product was also used for many other experiments as evidenced by B000363, B000802, B001067, B001071, B001529, B003945, B003949, B003984, B013127, B019261, B019265, B019270, B019275, B019280, B019286, B006313.

As per calculations below, the thymidine concentration in culture medium

containing 10 μCi/ml of high specific activity ^{3}H-TdR is about 0.1 μM

$$\frac{10\ \frac{\mu Ci}{ml}}{81\ \frac{Ci}{mmole}} = \frac{10000\ \frac{\mu Ci}{liter}}{81\times10^{6}\ \frac{\mu Ci}{mmole}} = 1.2\times10^{-4}\ \frac{mmoles}{liter} = 0.12\ \mu M$$

c) Relatively large amounts of thymidine molecules, i.e., thymidine concentrations expressed in terms of Moles (M), are required for blocking the movement of V79 cells through the various phases of the cell cycle:

The statement of Dr. Robbins cites several articles that use excess thymidine to block various cells from proceeding through S-phase. Many of these citations are papers that describe thymidine block of human lymphocytes. Indeed, some human lymphocytes are very sensitive to a thymidine block with as little as 10 μM thymidine yielding a 50% inhibition (*7*), and 90% with 50 μM thymidine (*8*). Galavazi showed that thymidine concentrations of 2 to 7.5 mM are required for a human T-cell line isolated from the kidney (*9, 10*). Similarly high concentrations of thymidine are required to effect the block for Chinese hamster V79 cells. For example, Dr. Robbins cited Sinclair's 1967 article wherein 7.5 mM thymidine is used to block V79 cells (*11*). Hagan et al. also used 7.5 mM (*12*). He also cited Tobey et al. who used 5 mM to block Chinese hamster ovary (CHO) cells (*13*). These concentrations for blocking Chinese hamster cells are typical in the literature with most ranging from 2 to7.5 mM. These concentrations are thousands of times higher than the concentrations used in the studies by Bishayee et al. Indeed, as Dr. Robbins has stated, deoxycytidine is needed to overcome the block at these very high concentrations of thymidine (*14, 15*). He also cites references regarding the use of deoxycytidine to relieve a 60 μM thymidine block of thymidine sensitive lymphocytes (*7*).

A crucial question addresses the concentration level of thymidine that is required to cause a cell cycle block in V79 cells. The following paragraph addresses this issue.

d) The minimum concentration of thymidine in the culture medium required for blocking V79 cells in various phases of the cell cycle is about 500 times

higher than the concentrations used in the studies by Bishayee et al.

Fujikawa-Yamamoto & Odashima have studied the effect of thymidine on exponentially growing Chinese hamster V79 cells (*16*). They determined that concentrations of thymidine in the cell culture medium in excess of 50 μM are required to decrease proliferation of V79 cells. Much higher concentrations are required for blocking the cell cycle (see their Fig 1. below). The figures below correspond to Fig. 1 and Fig. 2 in their cited paper.

Dr. Bishayee's concentrations are 500 times less than the minimum concentration required to inhibit cell proliferation.

Therefore, the concentrations of thymidine in the culture medium in the studies published by Bishayee et al. are not expected to block the cells from moving through the various phases of the cell cycle. Also, as stated above, the absence of deoxycytidine in the culture medium does not prevent V79 cells from advancing into S-phase and incorporating ^{3}H-TdR into the DNA.

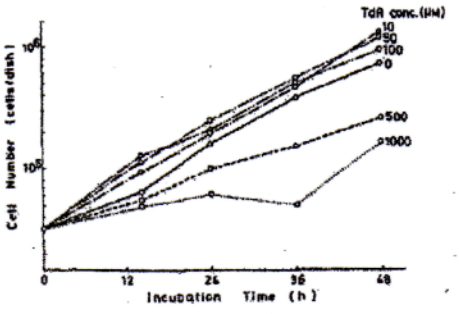


Fig. 1. Effects of TdR on growth of V79 cells. 24 h after cell seeding, the cells were exposed to TdR at final concentrations of 0 (—, control), 10 (— · —), 50 (— · · —), 100 (— · · · —), 500 (– –) and 1000 (· · ·) μM. At various times, cell density was determined. Open circles represent the mean values of duplicate measurements.

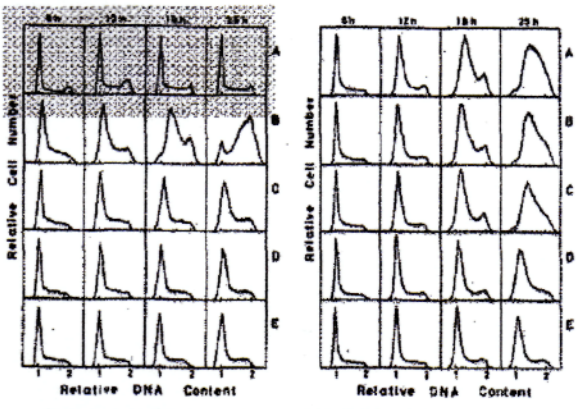


Fig. 2. Changes in DNA fluorescence histograms of V79 cells. Left hand side; 24 h after cell seeding, the cells were exposed to 50 μM TdR and HU at final concentrations of 0 (A), 0.38 (B), 0.57 (C), 0.76 (D) and 0.95 mM (E). Right hand side; 24 h after cell seeding, the cells were exposed to 0.6 mM HU and TdR at final concentrations of 0 (A), 5 (B), 10 (C), 50 (D) and 100 μM (E). At various times (each column), histograms were obtained.

e) The above statement is confirmed by Burki et al. who labeled with ^{3}H-TdR relatively radiation-resistant, exponentially growing Chinese hamster V79 cells and radiation-sensitive L5178Y cells without synchronization in a certain growth phase (*17*). The V79 cells were exposed for 15 h to 0.1 to 1 μCi/ml ^{3}H-TdR with a final TdR concentration of 10 μM in the culture medium (*17*). No deoxycytidine was added. The labeled cells were then frozen in liquid nitrogen to accumulate intracellular radioactive decays.

Subsequently, the labeled cells were thawed and analyzed for survival as shown in their figure below.

As evidenced by more than 99 % killing of V79 cells, Burki's data show that more than 99% of these cells must have passed through S-phase for becoming labeled with ^{3}H-TdR.

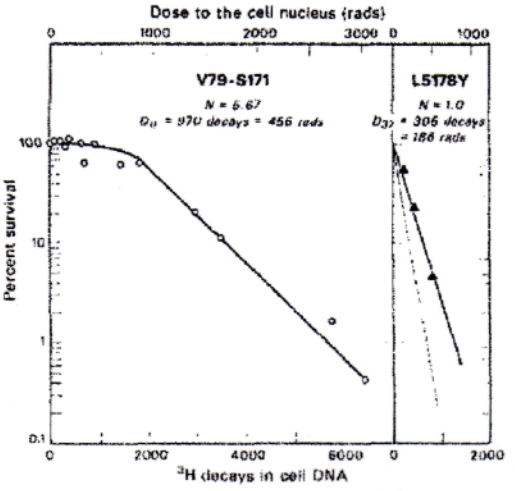


Figure 2. ^{3}H decays in mammalian cell DNA. The per cent survival of mammalian cells is given as a function of the number of disintegrations of ^{3}H accumulated in the cells at −196°C.

Left Panel: V79 S–171 (^{3}H-methyl TdR). Each point is the average of four replicate plates. Standard deviations not shown but similar to V79 data on figure 1.

Right Panel: L5178Y cells (^{3}H IUdR-labelled L5178Y cells = solid line with triangles; ^{3}H–TdR = dashed line reproduced from Burki and Okada 1970). End-points in the two L5178Y cell experiments are slightly different. [^{3}H–TdR data based on the growth curve extrapolation method; ^{3}H–IUdR data based on colony-forming ability in soft agar.] Survival curve parameters were determined as in figure 1.

f) Chinese hamster Don cells show the same pattern as Chinese hamster V79 cells.

Panter labeled Chinese hamster Don cells in exponential growth phase for 14 h with 0.2 to 5 μCi/ml ^{3}H-TdR with a final TdR concentration of 10 μM (*18*). No deoxycytidine was added to the culture medium. The labeled cells were then frozen in liquid nitrogen to accumulate intracellular radioactive decays. Subsequently, the labeled cells were thawed and seeded for colony forming ability as shown in Panter's figure below.

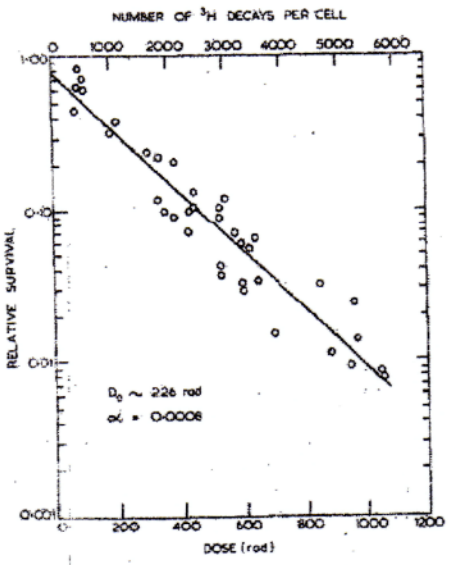


FIG. 2. The effects of decays from [³H]thymidine on survival of unifilar-labeled Don cells at −196°C.

These data provide evidence that ^{3}H-TdR can kill logarithmically more than 99% of Chinese hamster Don without the use of deoxycytidine and that more than 99% of the cells must have passed through S-phase of the cell cycle to become labeled during the 14 hours of exposure.

g) In addition, experiments in the Howell laboratory in 1992 and 1996 show that deoxycytidine is not required to kill more than 99% of the labeled V79 cells again indicative of more than 99% of cells having passed through S-phase during exposure for becoming labeled with ^{3}H-TdR.

The 1996 data on V79 cell killing by ^{3}H-TdR to about 1% survival were published in 1998 (*4*). These data are located in B013447 through B013476.

Also unpublished V79 cell survival experiments were carried out with ^{3}H-TdR by Dr. Ravi Harapanhalli in the Howell laboratory in 1992. The cells were exposed for more than 10 hours to ^{3}H-TdR in the culture medium in the absence and presence of deoxycytidine. Both culture medium formulations produced survival curves that showed more than 99 % of the cells having been killed.

These data show that, in the absence or presence of deoxycytidine, greater than 99% of the cells can be labeled with ^{3}H-TdR and that there cannot have

been a block in the movement of the cells through the various phases of the cell cycle preventing cells from entering S-phase during the time of labeling.

4) Impact of experimental work presented above on Dr. Robbins' statements:

1) The facts above question the validity of Dr. Robbins' three reasons for the impossibility of killing more than 99% of the V79 cells labeled with ^{3}H-TdR in culture medium as explained in the experimental design.

a) Regarding Dr. Robbins' Reason 1:

> 1. ^{3}H-TdR blocks the movement of cells through the various phases of the cell cycle. Thus, cells that are not in the S phase of the cell cycle during the overnight incubation with ^{3}H-TdR cannot enter S phase, will not incorporate ^{3}H-TdR into their DNA, and will not be killed by the subsequent radioactive decay of the ^{3}H.
>
> [See pg. 2 of Dr. Robbins' Report.]

Reason 1 is contradicted by data presented and discussed above. These data come from different laboratories (*17, 18*) as well as from the Howell laboratory published earlier (*4*) and from unpublished data (Harapanhalli 1992). Greater than two logs of kill were obtained in all of these experiments with exponentially growing V79 cells. More than 99% of the cells enter S-phase during the 12-14 h incubation period and incorporate lethal amounts of ^{3}H-TdR. Indeed, as pointed out by Dr. Robbins, once the ^{3}H-TdR is incorporated into the DNA during S-phase, it remains bound to DNA and can cause damage to DNA with certain probabilities of causing delays in the movement of the cells though the cell cycle subsequent to the DNA synthesis phase such as through G2-phase, mitosis and G1-phase (*19, 20*). Other toxic effects such as chromosome aberrations, cell death, and mutations also can occur with given probabilities (*21, 22*).

b) Regarding Dr. Robbins' Reason 2:

> 2. No deoxycytidine (dCyd) was present in the medium at the time the cells were exposed to ^{3}H-TdR. Previous studies have shown that the inclusion of dCyd in the medium prevents the ^{3}H-TdR from blocking cell movement through the cell cycle leading to an exponential decrease in cell survival.
>
> [See pg. 2 of Dr. Robbins' Report.]

Reason 2 is contradicted first on theoretical grounds because Bishayee et al. used high specific activity ^{3}H-TdR that delivers such small amounts of thymidine molecules that the nucleotide pool is not perturbed. Also there is experimental evidence contradicting reason 2 from other laboratories (*17, 18*) as well as from earlier published (*4*) and unpublished work (Harapanhalli 1992) from the Howell laboratory. Deoxycytidine is only required when the thymidine concentration in the culture medium is sufficiently high for perturbing the nucleotide pool; this concentration lies about 500 times above the concentration used by Bishayee et al. Deoxycytidine is not required to obtain an exponential decrease in cell survival when high specific activity ^{3}HTdR is used as in the studies by Bishayee et al.

c) Regarding Dr. Robbins' Reason 3:

> 3. No attempt was made to synchronize the cells into the same phase of the cell cycle prior to their treatment with ^{3}H-TdR. If all the cells were in the same phase of the cell cycle then there is a possibility that they would all have been in the S phase of the cell cycle at the time the ^{3}H-TdR was added. However, as will be discussed below, special experimental procedures are required to ensure that the cells are synchronized, and these were not used in the experiments performed by Bishayee.
>
> [See pg. 2 of Dr. Robbins' Report.]

Reason 3 again is contradicted by experimental evidence from other laboratories (*17, 18*) as well as from earlier published (*4*) and unpublished work (Harapanhalli 1992) from the Howell laboratory. All of these experiments were carried out with exponentially growing asynchronous cells. These cells grew in medium with ^{3}H-TdR over a period of 12 - 15 hr. All cells became exposed to toxic amounts of ^{3}H-TdR indicative that all cells passed through the S-phase. Therefore synchronization is not required to obtain greater than two logs of kill provided that the cells are incubated in the presence of ^{3}H-TdR for at least one complete cell cycle time, i.e. doubling time.

5. Hypoxia and the bystander effect

Dr. Robbins has concerns regarding the possible presence of hypoxia in the multicellular clusters (see text below).

Why did Lenarczyk and Howell fail to observe a bystander effect? It should be noted that experiments recently published by Persaud et al (2005) do indicate a bystander effect, with survival of the bystander cells appearing to be around 60% (***Figure 6***). What is the difference between these studies? There is a major difference in the protocols used by the two groups. Persaud et al used microfuges tubes with 100 µL of air present above the cells. In contrast, Lenarczyk and Howell used Helena tubes in which no air was present above the cells. In this situation the cells would have been hypoxic, a condition in which they are much more resistant to the killing effects of radiation. Data in support of this conclusion are shown in ***Figure 7***. This shows data from studies performed in the Howell lab as well as data published in the literature. In ***experiment #1, B007921***, performed by Bishayee 9/7/98, a colony forming assay was obtained using V79 cells cultured in Falcon tubes, in which the cells were aerobic and therefore radiosensitive. The cell survival curve shown in ***Figure 7*** indicates a marked decline in surviving fraction, as expected. These data are in agreement with survival data for V79 cells obtained from the literature (Cox et al, 1977; Goodhead & Thacker, 1977; Han & Elkind, 1977; Hill et al, 1985; Millar et al, 1978; Raaphorst & Kruuv, 1976; Radford & Hodgson, 1987) and shown as a star on the graph. These data show the average dose at which a surviving fraction of 0.1, or 10%, was observed. The remaining plots are from cell survival assays performed by Drs. Bishayee (***B07927/B07910-B007911, B07894***), Lenarczyk (***B019856***) and Bogdan (***B002754-B002760***) using Helena tubes and irradiating the cells as clusters or in suspension. In all of these experiments, the survival curves are much shallower, reflecting radioresistant cells due to their being hypoxic. Thus, given the experimental protocol used in the Howell lab, the cells were hypoxic when cultured in the Helena tubes, and thus would be radioresistant. Thus, the lack of a bystander effect observed by both Lenarczyk and Howell is a result of the cells being hypoxic.

Bishayee's conditions and protocols were the same as those used by Lenarczyk and Howell and thus the cells would have been hypoxic. The marked radiosensitivity of the cells on the 50% experiments indicates that his results could not have been generated without falsification of the data.

[See pg. 7 of Dr. Robbins' Report.]

The last statement of this paragraph shows that Dr. Robbins does not understand the experimental model. One can discuss the radiosensitivity of the labeled cells, however, neighboring unlabeled cells that receive a bystander effect technically should not be characterized as either radioresistant or radiosensitive since they are not being irradiated. They are either sensitive or resistant to a bystander effect imparted by neighboring cells irradiated with DNA-incorporated ^{3}H-TdR. It is not clear whether there is a correlation between radiosensitivity and susceptibility to the bystander effect.

Nevertheless, the presence of some hypoxia in the V79 cell clusters was described and discussed in Dr. Howell's 1999 NIH grant application. In fact, data from B007891 and the following statements appear on page 30 of the grant application:

> "This experiment demonstrates that, after the 72 h incubation, hypoxia is present in the clusters. However, it appears to be uniform throughout the pellet since differentially hypoxic populations could result in a two- or more component exponential response to uniform irradiation. This is an important point because differential hypoxia would make data interpretation difficult. The OER is substantially less than 2.5 to 3.0, the maximum range expected for anoxia (Ref. (*23*), pg. 135) so the clusters are not completely hypoxic. In fact,

reference to the relative radiosensitivity versus oxygen tension suggests an oxygen tension of approximately 3 mm Hg or ½% (Ref. (*23*), p. 138, Figure 8-5)."

With respect to the interpretation of the experimental data, of concern was not that the cells were somewhat hypoxic, rather, it was that cells at the center and bottom of the multicellular cluster may be more hypoxic than those at the top (differentially hypoxic) and that might lead to a two-component exponential curve. The experiments by Bishayee (B007891) and Neti (*24*) indicated that the survival curves after acute or chronic irradiation with gamma rays follow a classic 2-component linear-quadratic dose response.

The statement of Robbins declares that there cannot be a bystander effect in the 50% experiments because of the reduced radiation sensitivity of hypoxic V79 cells. Again, this concerns interpretation of experimental data. There are numerous interpretations based on various possibilities. First, while radiation sensitivity to gamma rays may be affected by the reduced oxygen concentration, bystander effects imparted by ^{3}H-TdR labeled V79 cells are not necessarily abolished. Regardless, even if the presence of hypoxia would abolish the bystander effect, the irradiation schedule in the tritium experiments is very different than the gamma ray experiments. In the gamma ray experiments, the cells are irradiated at the end of the 72 h period of incubation at 10.5°C. In contrast, well oxygenated V79 cells were labeled with ^{3}H-TdR over a 12 - 14 h period, and then mixed with well oxygenated bystander cells, gently pelleted in the Helena tubes at 4°C, and then stored at 10.5°C for 72 h to accumulate tritium decays. Given the low respiration rate at this temperature, it would take quite some time before hypoxia sets in. Thus, bystander effects may be imparted before hypoxia sets in. Indeed, the time over which hypoxia sets in most likely depends on temperature. If the cells were not kept at 10.5 C or below at all times, hypoxia could set in very quickly. Thus, there is ample room for "protocol drift" which can contribute to the different responses observed.

6. Robbins' commentary on B0180319 prepared by Dr. Howell

Several possible reasons need be considered for differences between Bishayee's published data and the data published later as considered in document B0180319, which was prepared by Dr. Howell. While experiments were not conducted to test all of the possible reasons, these reasons are possible and all possibilities must be considered.

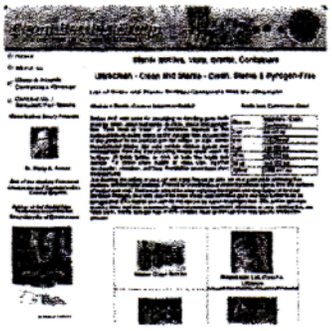
According to the statement of Robbins:

a)

- Variable source of microfuges tubes: It seems highly unlikely that "contamination" of the microtubes used with trace elements would explain the failure to replicate Bishayee's findings. No experiments were performed by Howell to substantiate this "claim."

[See pg. 8 of Dr. Robbins' Report.]

Notwithstanding Dr. Robbins' unsubstantiated conclusion, the supplier of bottles and vials specifies:



"Bottles and vials used for packaging or handling pure fluids must be clean; clean and sterile; or clean, sterile and pyrogen free. Their level of "clean" or cleanliness depends on the particulate level and/or sterile and/or pyrogen free requirement of the product to be inserted into the bottles or vials. Newly manufactured bottles and vials contain release agents and contaminants from the manufacturing factory which coats all surfaces of these bottles or vials on both the inside and outside. To obtain clean bottles or clean vials, rigorous mechanical, chemical, and heat sterilization procedures must be employed. Just blowing the bottles or vials out with clean filtered air does not do a good job in removing fine particles and biological material. Filtered air is ineffective in removing release agents from the surfaces of bottles and vials. Sterile vials should be clean vials that have gone one step further to be heat sterilized. Pyrogen free vials should be clean sterile vials that have gone one step further to be de-pyrogenated. EPA bottles and vials do not need to be sterile or pyrogen free. EPA bottles and vials must be clean and then submitted to rigorous chemical treatment. All bottles or vials, whether they are rated clean, sterile, pyrogen free or EPA certified, must go through their own specific sterilization process."

b)

- pH of media: This is not a valid concern. If the pH changed during the course of the experiment then it would be noted by the color of the phenol red in the medium; any acidification due to contamination would lead to stopping the experiment.

[See pg. 8 of Dr. Robbins' Report.]

To the contrary of Dr. Robbins' unsubstantiated conclusion, small changes in pH may not produce noticeable changes in the color of the phenol red. Dr. Feinendegen (*25*) has stated that pH changes of only 0.1 units completely abrogated some experimental responses in his own laboratory. Therefore unnoticed changes of pH cannot be ruled out to cause effects.

c)

- Level of trace elements in the water: Without any evidence to indicate that this either occurred or would have any significant impact on the data generated, this response provides no explanation.

- Wetting agents on filter apparatus: Without any evidence to indicate that this either occurred or would have any significant impact on the data generated, this response provides no explanation.

- Methods used to clean bottles: It is not clear how this might impact the studies or serve as a potential explanation for the inability to duplicate Bishayee's data.

- Sodium bicarbonate product changed: As long as the final concentration of chemical used was the same, there would be absolutely no difference in the experimental conditions.

[See pg. 8 of Dr. Robbins' Report.]

The possible reasons cited above from Dr. Howell's document B018319 are actually supported by recommendations regarding the testing of disposable laboratory supplies (http://www.hyclone.com/pdf/validation_strategies.pdf).

Moreover, other factors could also be involved. Trypsin and serum are known to affect the response of V79 cells to ionizing radiation (*26-28*).

Accordingly, sufficient scientific variables exist to rebut the unsubstantiated conclusions of Dr. Robbins.

d)

- Different V79 cells used: A review of the literature reporting cell survival for V79 cells after irradiation indicates very little difference in the radiation response of these cells over several decades and being cultured in numerous laboratories in Europe and the United States (Cox et al 1977; Goodhead and Thacker 1977; Han and Elkind 1977; Hill et al 1988; Millar et al 1978; Raaphorst and Kruuv 1976; Radford and Hodgson 1987). There are no data showing the 100-fold difference in survival of V79 cells following irradiation noted between Bishayee and Lenarczyk and Howell.

[See pg. 9 of Dr. Robbins' Report.]

V79 cells are known to be genomically unstable. As demonstrated in the literature, Zyuzuikov et al. (*29*), many investigators have shown that V79 cells can vary widely in terms of their intrinsic radiosensitivity (see figure below).

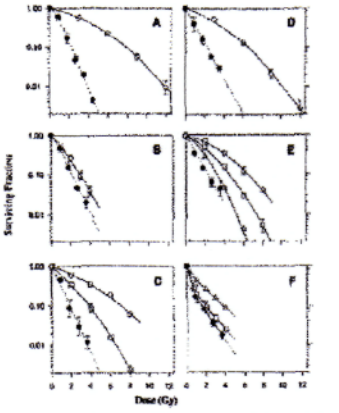


Fig. 1. Cell survival curves of V79 (*panel A*),VC-4 (*panel B*), V-H4 (*panel C*), V79B (*panel D*), CL-5VB (*panel E*) and XR-V15B cells (*panel F*) exposed to x-rays (*open symbols*) and to α-particles (*closed symbols*). Error bars are the standard errors of the mean surviving fractions from 2–4 independent experiments. In the panels describing the results for the strains V-H4 C, CL-V5B E and XR-V15B F, the squares describe experiments with x-irradiation according to method C, whereas the circle describe experiments with x-irradiation according to method A (or A and B combined); the triangles describe results with method B. In the other three cell lines, no differences were found between the methods

The data obtained by Howell's laboratory show, for external gamma rays, differences in radiosensitivities of the different V79 cells that were used.

Other changes in these cells also could have occurred and contributed to particular patterns of incorporation of ^{3}H-TdR that differ strikingly between early and later experiments in the laboratory of Howell. For instance, changes in the type of buffer used in the medium, slight alterations in membrane functions and their associated transport facilities are known to be sensitive to alterations in the cellular environment as it is presented by the

culture medium. Changes in ionic and other compositions of the culture medium, if not extremely carefully controlled, may have profound intracellular metabolic consequences (*6, 25*).

For the reasons set forth above, the statements made by Dr. Robbins regarding B0180319 are scientifically unfounded and, therefore, fail to support Dr. Robbins' allegations of fraud.

7. Conclusion.

The allegations put forward by the statement of Dr. Robbins may be viewed in two principally separate sections:

a) the data published by Bishayee et al. are impossible and thus must have been fabricated;

b) the differences in the experimental results published by Bishayee et al. and later by other scientists in the Howell laboratory are due to the falsification of the data published by Bishayee et al.

This present analysis clearly indicates that the allegations of falsification of data by Bishayee et al. cannot be maintained. These allegations are based on wrong assumptions and lack of understanding of the function of the experimental tools and protocols used by Bishayee et al., as is carefully explained here and based on a large set of published work. Therefore, the second allegation is without foundation. In fact, the second allegation relates to the question of interpretation of properly obtained experimental data and does not appear to be a topic in need of discussion in the context of the claim that the data are falsified.

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