HYPER-RADIOSENSITIVITY AND INDUCED RADIORESISTANCE (HRS/IRR).

THE EFFECT OF USING DIFFERENT DOSE-RATES FOR PRE-EXPOSURE ON THE HYPER-RADIOSENSITIVITY IN T-47D CELLS.

Cand. Scient. Thesis in Physics
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April 2003

ACKNOWLEDGEMENTS

The work presented in this thesis was carried out at the Biophysics Department at The University of Oslo, and at Centre for Education and Research in Radiation Oncology (SUFS) and Department for Medical Physics, Institute for Cancer Research at The Norwegian Radium Hospital (DNR). In addition facilities at the Department of Biophysics, Institute for Cancer Research at the Norwegian Radium Hospital was used by the courtesy of the Head of the Department Professor Harald B. Steen and Senior Scientist Kristian Berg.

First of all I wish to express my gratitude to my supervisors Professor Dr. Philos Erik O. Pettersen and Professor Dr. Philos. Dag Rune Olsen for their enthusiastic and constructive tutoring, which has been very inspiring.

I also wish to thank the following:
Charlotte Borka for her instructions to the laboratory techniques, for her technical assistance during the experiments, and for being such good company,
Torbjørn Furre for his helpful assistance and guidance as regards the irradiation procedures with the cobalt source,
Pål Graff, who did the flow cytometry and provided the DNA histograms used in this thesis,
Hans Kristian Opstad, who did all the curve fittings of the data from this study as part of his own thesis, and
My father Kristian Jeppesen for being available on the telephone for linguistic consultations.

Finally I wish to thank students and employees at the Biophysics Department at UIO for creating a stimulating and enjoyable atmosphere.

Blindern, April 2003

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SUMMARY

Irradiating T-47D cells with doses below 1 Gy revealed that cells of this line express hyper-radiosensitivity (HRS). In the present study HRS has been investigated when a challenge dose was given 6 or 24 hours after a priming dose delivered with varying dose-rates. In addition the effect of a challenge dose to T-47D cells that had been pre-irradiated for several months by incorporated tritium was investigated.

A ^{60}Co-source at the Radium Hospital was used for all irradiations. It was chosen to give a fixed priming dose of 0.3 Gy, but dose-rates were varied between 0.045 Gy/h, 0.32 Gy/h, 0.9 Gy/h or high dose-rate (HDR) of 1 Gy/min. Immediately after the priming exposure, the cells were trypsinized and seeded in small flasks (25 cm^2) and incubated for 6 or 24 hours before they received the HDR challenge doses.

DNA histograms revealed that the T-47D cells in the cell culture used in the experiments with priming exposures of 0.32 and 0.045 Gy/h and also in one of the experiments with HDR priming, had been mixed with cells of another stemline. The mixed cell culture (denoted T-47D$_{mix}$) was also used in the experiment with incorporation of tritium into T-47d cells, but in this case it was found from DNA histograms that only the control cells were mixed, but that the cultures exposed to protracted low dose-rate (LDR) irradiation consisted of only T-47d cells. Investigations were made that suggested that the cells mixed into the T-47D cells were of the line NHIK 3025.

The following was observed from the experiments:

- T-47D cells clearly express both HRS/IRR and the adaptive response in the low-dose range (below 1 Gy).

- NHIK 3025 cells do not express HRS/IRR.

- T-47D cells that had been pre-irradiated by tritium-decay electrons did not express HRS/IRR.

- T-47D cells with functional pRb adapted to the continuous irradiation with electrons from incorporated tritium, while the putative NHIK 3025 cells without functional pRb were eradicated by the same treatment.

- When priming doses were delivered with the lowest dose-rates used (i.e. 0.32 Gy/h and 0.045 Gy/h) the priming effect as measured 6 hours following the termination of the priming exposure seemed to be larger than when the priming dose was delivered with higher dose-rates (i.e. HDR or 0.9 Gy/h).

- 24 hours after the priming doses delivered with 0.32 Gy/h and 0.045 Gy/h, HRS was still absent (perhaps even further reduced), while 24 hours after a HDR priming dose HRS was partially restored.

These results led to speculations concerning:

- The influence of micro-environmental conditioning effects on the radiation response of NHIK 3025 cells that are growing in a mixed culture with T-47D cells under conditions that would normally be lethal for NHIK 3025 cells.

- An alternative theory for HRS/IRR in which it is assumed that the repair processes of the cell are permanently induced. When radiation damages are so small, that the tissue as a whole would profit from cell suicide relative to repair with the danger of mis-repair, the repair processes are subdued by apoptosis.

- The involvement of pRb in the suppression of apoptosis of the alternative theory for HRS/IRR and in the adaptation of T-47D cells to continuous irradiation by decay electrons from incorporated tritium.

- The possibility that there are two different regulation pathways for induction of the cell-protective mechanisms that reduce HRS after pre-irradiation: One that is dose dependent, instantly induced by HDR irradiation but not by LDR irradiation, and which induces short-lasting mechanisms. Another pathway that works over time and is induced by LDR and probably also by HDR irradiation. The effect and duration of the mechanisms induced by this late-responding pathway depends on the duration of exposure.

For any conclusions to be drawn, further investigations are required.

TABLE OF CONTENTS

ABBREVIATIONS AND DESIGNATIONS

Acute irradiation:	Irradiation given with a dose-rate of ~1 Gy/min or higher.
Counter-HRS effect	The abolition of low dose HRS in response to a preceding exposure.
HDR:	High dose-rate (~1 Gy/min or higher).
HRS:	Hyper-radiosensitivity. Used only in connection with response to doses below 1 Gy.
IR-model:	Induced repair model.
IRR:	Induced-radio-resistance- used only in connection with response to doses below 1 Gy.
LDR:	Low dose-rate.
LQ-model:	Linear quadratic model.
pRb:	Protein of retinoblastoma gene (tumor suppressor).
T-47D$_{mix}$	Used as designation of the T-47D cell culture that had another stem line mixed into it.

1 INTRODUCTION

Treatment of cancer using radiotherapy implies not only finding the best regime for rate and fractionation of dose delivery for killing of the cancer cells. An even greater concern is to obtain utmost sparing of the normal tissue. A precise knowledge of the biologic effect of radiation is thus necessary when treatment plans are made for the patient. During external radiation therapy not only the tumor is irradiated, the normal tissue in the penumbra will also receive a radiation dose. In brachytherapy radioactive material is implanted either directly into the tumor (interstitial therapy) or close to it inside a catheter (intercavitary therapy). In this case the normal tissue surrounding the target is irradiated at dose-rates diminishing with the distance from the source.

In the clinic doses are traditionally given as 2 Gy doses with 24 hour intervals but also regimens with smaller doses with a shorter interval between are used in order to exploit the difference in sparing between late and early responding tissues as a consequence of dose fractionation.

In many mammalian cells, a region of high sensitivity in the radiation survival response at doses below ~0.5 Gy has been observed. This phenomenon has been termed hyper-radiosensitivity (HRS). It precedes the occurrence of a relative resistance to cell killing by radiation over the dose range ~0.5-1 Gy, which has been termed increased radioresistance (IRR) (Joiner and Johns, 1988 and Joiner et al., 2001). In the HRS dose range a reverse fractionation effect may be expected if doses are administered in a way that allows the hypersensitive response to be repeated. In order to be able to exploit HRS in radiotherapy and predict the effects of a particular treatment, it is important to investigate the dependence of the response of low dose HRS to a preceding exposure (here denoted a priming dose). Both the size and dose-rate of the priming dose and the interval between this and the subsequent doses are parameters that need closer investigation.

The preliminary experiments of this study revealed a hypersensitive response to low doses of γ-radiation in T-47D cells that was not expected. In fact the original intention was to investigate effects of pulsed dose-rate in two cell lines, one with and one without hypersensitivity; V79 cells were chosen as the cell line with known HRS/IRR and T-47D cells as the cell line believed to be without HRS/IRR. Instead experiments T1-T6 and V1-V6 revealed a larger response to doses below 1 Gy for T-47D cell than for V 79 cells. This encouraged further investigations into the phenomena of hypersensitivity and adaptive effects as responses to low doses for T-47D cells.

The aim of this study became to investigate the influence of a priming dose on the radiation response of T-47D cells when the priming dose was delivered with different dose-rates. The priming dose was chosen to be 0.3 Gy. This dose was large enough to reduce HRS when cells were given a subsequent challenge dose, but so small that a complete induced IRR was not seen.

Furre et al. (2003) found that pRb was activated by radiation delivered with dose-rates of 0.3 and 0.9 Gy/h. We expected a counter-HRS effect of priming doses delivered with HDR, which we also found, and wanted to investigate how low the dose-rate had to be for the counter-HRS effect not to be induced. Experiments were performed with four different dose-rates of the priming dose: In addition to HDR, 0.9 and 0.3 Gy/h were chosen because the irradiation set-

up was established by Furre et al., and the dose-rate was further lowered to 0.045 Gy/h in the attempt to find the lowest dose-rate of the priming dose eliciting a counter-HRS effect. In all these experiments a counter-HRS effect was observed.

In experiments without priming exposure T-47D cells express HRS. This implies that the dose-rate of background irradiation is too low for the counter-HRS effect to be induced. Thus, there must exist a dose-rate lower than 0.045 Gy/h that does not induce the counter-HRS effect. When it comes to administering dose-rates lower than 0.045 Gy/h, external irradiation has limitations. The cells will need exchange of medium during irradiation and it can be a problem to have the irradiation facilities at disposal for longer periods.

A method for internal irradiation was developed by Søvik (2002), who irradiated T-47D cells by incorporation of tritium and established the dosimetry for 2 different dose-rates. The lowest dose-rate was 0.01 Gy/h to the cell nucleus. At this dose-rate the cells were found to adapt to the continuous irradiation. We wanted to investigate whether the mechanisms behind this adaptation is related to those that induce the counter-HRS effect , and experiments were performed in which cells that had been pre-irradiated by electrons from incorporated tritium for several months were challenged by HDR irradiation.

2 THEORY

2.1 CELL BIOLOGY

Chapters 2.1.1.and 2.1.2 are based upon (Alberts et al., 1994) and (Hall, 2000).

2.1.1 THE CELL CYCLE

Cells proliferate by division after duplicating their contents. This is a cyclic phenomenon known as the cell cycle.

The cell cycle is traditionally divided into interphase and mitosis (M-phase). During mitosis which in most cells takes about an hour, the nuclear envelope disintegrates and the DNA condense into visible chromosomes that align on the mitotic spindle (metaphase) after which the cromatides separate and move to the poles of the spindle (anaphase). The nuclear envelope is re-created and the cromatides decondense (telophase). The M-phase ends with cytokinesis dividing the cell into two new cells. Interphase, the interval between two consecutive mitoses, consists of the phases G_1, S, and G_2. The replication of the cell's DNA takes place during S-phase (S=synthesis), while the G_1- and G_2-phases (G=gap) provide time for cell growth and preparation for replication and mitosis, respectively. The lengths of all the phases vary to some extent between different cells under different circumstances, but the greatest variation occurs in the length of the G_1-phase. From G_1 the cell can exit the cycle and enter a quiescent state $G_{0;}$ some cells are terminally differentiated to perform their function in the organism, while other cells after a longer or shorter period in G_0 re-enter into the cycle.

2.1.2 CELL CYCLE REGULATION

Cytoplasmic proteins that are activated by the enzymatic activity (phosphorylation) of cyclin-dependent kinases (cdks) control transitions from one cell cycle phase to the next. Cdks levels remain stable during cell cycle, but each cdk must bind the appropriate cyclin in order to be activated. Each cyclin protein is synthesized at a discrete phase and is degraded after fulfilling its purpose so that cyclin levels oscillate as the cell goes through cell cycle (figure 2.1).

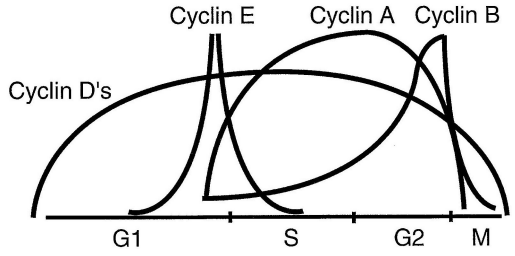

Figure 2.1: *Oscillation of cyclin levels with phase of cell cycle. Progression through the cell cycle is governed by protein kinases, activated by cyclins (Hall, 2000).*

The "decision" to proceed in the cycle is evaluated at checkpoints where feedback signals ensure that previous processes are completed before the next one is initiated. Two major checkpoints, G_{1k} and G_{2k} are located prior to the transitions into S and M. These checkpoints allow extracellular signals to effect on the control system and also retain the cells in case of DNA damage in order to provide time for repair before replication or mitosis. Radiation-induced damage can also cause a delay in G_2 at a separate point before G_{2k} (G_2-block) preventing propagation of defective DNA. In case of DNA being damaged after passing G_{1k}

the cell has mechanisms for depressing the replication until the damage is repaired, and in the metaphase of mitosis the proper alignment of the chromosomes is ensured at another restriction point.

In the rest state G_0 is also an important checkpoint, G_{0k}, where the amount of growth factor decides whether the cell should enter the cell cycle and start proliferation. The growth factor receptors are located on the cell membrane and activate, when stimulated, intracellular phosphorylation cascades that lead to expression of proliferation genes (proto-oncogenes) bringing the cell past G_{0k}. A mutation leading to over-expression of the proliferation gene will increase cell proliferation, and the gene is called an oncogene. Only mutation in one of the cell's two copies of the proliferation gene needs to be overactivated in order to result in cancer development. Excessive cell proliferation can also be caused by inactivating mutations in both copies of an antiproliferation gene (tumour suppressor gene) since the proteins from these genes act like brakes inhibiting cell division. The genes encoding the proteins p53 and pRb are important tumour suppressor genes.

When activated, p53 enhances transcription of another regulatory gene (p21WAF1/CIPI) resulting in the inhibition of G_1 cyclin/cdks, that normally would phosphorylate pRb releasing it from its growth-suppressive function (Hall, 2000), thus preventing the progression from G_1 into S. Under normal conditions the p53 concentration is kept very low by its relatively short half-life (about 20 min.) (Levine, 1997). At exposure to ionizing radiation the concentration increases rapidly, preventing the cell to enter S with damaged DNA either by retaining it at G_{1k} or if repair fails, by inducing apoptosis. Mutations of the p53 gene are present in more than 50 % of all cancer cases.

pRb is the protein of the retinoblastoma gene (Rb) and was identified through studies of an inherited predisposition to eye cancer in children. It is abundantly present in the nucleus of mammalian cells but the activation is regulated by the state of phosphorylation. When dephosphorylated, pRb is activated and binds regulatory proteins while phosphorylated pRb releases these proteins to induce gene transcription required for cell proliferation (figure 2.2). In G_0 pRb is dephosphorylated inhibiting proliferation. In proliferating cells, the phosphorylation of pRb rises late in G_1 and remains high through S and G_2 before pRb returns to a dephosphorylated form in mitosis.

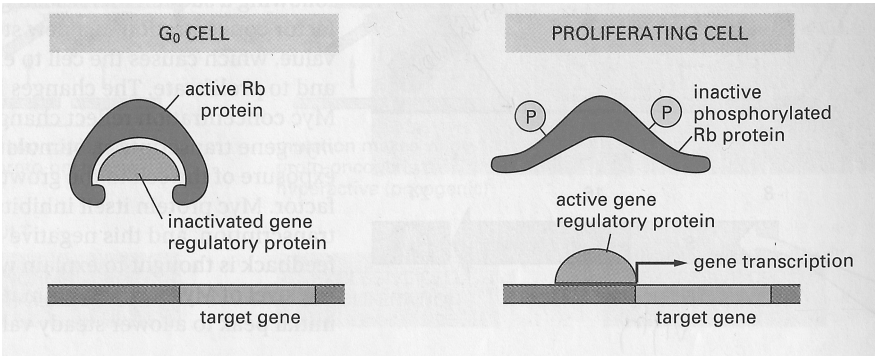

Figure 2.2: *pRb is dephosphorylated and active preventing transcription of regulatory genes from the end of mitosis until late in G_1. Phosphorylated pRb is inactive which permits the regulatory proteins to induce gene transcription required for the cell to proceed through cell cycle (Alberts et al., 1994).*

2.2 RADIOBIOLOGY

The radiation used to irradiate the cell flasks in the experiments conducted here and the radiation from incorporated tritium both originate from radioactive decay. When a radioactive nucleus disintegrates, energy is emitted as radiation that can be either particulate, electromagnetic or both. It is not possible to know when a particular atom will decay, but in a large number of atoms the proportion that will disintegrate in a given time is predictable. The number of atoms disintegrating per unit time, *dN/dt*, is proportional to the number of radioactive atoms *N*:

$$\frac{dN}{dt} = -\lambda N \tag{2.1}$$

where λ is a constant of proportionality called the decay constant. The solution to (2.1) is:

$$N = N_0 e^{-\lambda t} \tag{2.2}$$

where N_0 is the initial number of radioactive atoms.
The activity *A* is defined as the rate of decay:

$$A = -\lambda N \tag{2.3}$$

which is measured in Becquerel [Bq] or in curie [Ci].

1 Bq = 1 decay per second = 2.7×10^{-11} Ci.

The half-life, $T_{1/2}$, of a radioactive substance is defined as the time required for the number of radioactive atoms to be reduced to half of the initial number. $T_{1/2}$ is found from:

$$T_{\frac{1}{2}} = \frac{\ln 2}{\lambda} \tag{2.4}$$

2.2.1 IONIZING RADIATION

Ionizing radiation, particulate or electromagnetic, is characterized by the ability to ionize an atom of the matter with which it interacts. From this follows that it must carry energy in excess of the amount needed by a valence electron to escape an atom, which is in the order of 4-25 eV (Attix 1986). The biologic effects of radiation result principally from damage to DNA, which is the critical target (Hall 2000).

In these experiments a cobalt-60 source was used. In the cobalt-60 unit the emission of two photons, one at 1.17 MeV and the other at 1.33 MeV (Attix 1986) accompany each disintegration. At these energies the Compton process dominates (Hall 2000). The energy of the photon is so high that the binding energy of the electron of an atom in the absorbing material is negligible and the electron may be regarded as free. Part of the energy and momentum of the incoming photon is transferred to the electron and the photon is deflected from its original path with the remaining energy; the fraction lost may vary from 0 to 80%.

Thus the incoming γ-radiation can result in a large number of fast electrons able to cause biologic damage.

When the radiations interact directly with the critical targets in the cell, it is called direct action of radiation. In the cell the biomolecules are surrounded by water and the indirect action dominates; the critical target is damaged by free radicals created through interactions of the radiation with other atoms or molecules, particularly water. A free radical has an unpaired electron in the outer orbit, is highly reactive chemically, and is able to diffuse a short distance to reach the critical target. It is estimated that two thirds of the damage to DNA by x-rays is caused by the hydroxyl radical (Hall 2000) which arise from the following reactions:

$$H_2O \rightarrow H_2O^+ + e^-$$
$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH^{\cdot}$$

Changes in the bases of the nucleic acid, breakage in the strands of the double helix, and abnormal cross-links formed in the DNA or between the DNA and cellular proteins, are believed to be the lesions leading to point mutations (changes of the molecular structure of DNA) (Nias 1998).

TRITIUM

In some of the experiments conducted here, the cells had been exposed to radiation from incorporated tritium previous to the external irradiation.
Tritium disintegrates to the stable helium isotope ^{3}He emitting an electron and an anti-neutrino:

$$^3H \rightarrow {}^3He + \beta + \overline{\nu}$$

The energy released in the disintegration process is divided between the electron and the anti-neutrino resulting in a continuous energy spectrum with maximum electron energy per disintegration of 18.6 keV and mean electron energy of 5.75 keV. The recoil energy of the helium nucleus varies from 0 to about 3 eV which is too little to break molecular bonds (Feinendegen, 1967).

The range of the electrons is the expectation value of the path length that it follows until it comes to rest. In the continuous slowing down approximation (CSDA), the range in terms of the mass stopping power is defined as:

$$R_{CSDA} = \int_0^{T_0} \left(\frac{dT}{\rho dx} \right)^{-1} dT \qquad (2.5)$$

Where ρ is the density of the absorber and T_0 is the starting energy of the electron. The stopping power (dT/dx) is the expectation value of the rate of energy loss per unit path length for electrons with kinetic energy T in a given medium.

The range of the electrons in water (for dosimetric purposes, cells are considered water equivalent) is 6 μm corresponding to 18.6 keV and 1 μm corresponding to 5.75 keV (ICRP, 1983). The diameter of T-47D cells was found by Åste Søvik (2002) to be 19±1 μm, the nucleus diameter 13±1 μm.

2.2.2 CELLULAR RESPONSE TO RADIATION

The radiosensitivity of the cells varies as they go through cell cycle. In general, cells are most radiosensitive in G_2 and M, while they are most resistant in the latter part of S-phase. For cells with a relatively long G_1-phase, there is a resistant period early in G_1, followed by a sensitive period toward the end of G_1 (Hall 2000).

The radiation damage to cells can be categorized as lethal, sublethal, or potentially lethal damage (Hall 2000).

Lethal damage is irreversible and irreparable, leading to cell death.

Sublethal damage can be repaired within a few hours unless additional sublethal damage arise with which it can interact to form lethal damage. Reparation of sublethal damage is believed to be the explanation of the increase in cell survival observed when the radiation dose is split into two fractions separated by a time interval.

Potentially lethal damage will under normal conditions (cells growing exponentially at 37°C) lead to cell death, but can be repaired if the postirradiation conditions are suboptimal for growth. Also a delay in the cell cycle giving the cell time for repair before S-phase or mitosis can be provided by p53 retaining the cell at G_{1k} or by radiation induced enzymes in G_2 (G_2 block) (Alberts 1994).

The most common cell death from radiation is mitotic death, where the cells die attempting to divide, because of damaged chromosomes. This doesn't necessarily happen during the first mitosis following irradiation; the cell may go through a few cycles before succumbing to destiny. The cells swell and burst in a process called necrosis. The result is that the cytosolic contents end up in the extracellular space provoking an inflammatory response.

A way of death, more advantageous to the organism, is the programmed cell death, apoptosis: The cell undergo morphologic changes eventually separating into a number of membrane-bound fragments, that are phagocytosed by macrophages, disassembled, and reused (Hall, 2000; Alberts et al., 1994). Apoptosis after irradiation seems to be a p53-dependent process (Hall 2000).

In general, cells that have lethal DNA damage are no risk for the organism with respect to later cancer development. It is when repair processes are active, the danger of mis-repair and possible subsequent inactivation of tumour suppressor genes or activation of oncogenes, is present (Alberts et al., 1994).

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2.2.3 CELL SURVIVAL CURVES

When recording the effect of irradiation on cells, an end-point must be chosen. For cells *in vitro*, as used in these experiments, the loss of reproductive integrity is normally used. A surviving (clonogenic) cell is defined as a cell able to form a colony of 40-50 cells (5-6 generations) (Steel, 1997).

A cell-survival curve describes the relationship between the radiation dose and the fraction of cells that survive. It is usually presented in a semi-logarithmic plot with the fraction of surviving cells as the logarithmic ordinate and dose as the abscissa. For densely ionizing (high Linear Energy Transfer LET) radiation the survival approximates an exponential function of dose and the curve is a straight line. For x- or γ-rays (sparsely ionizing or low LET) the dose-response curve has an initial linear slope, followed by a shoulder where the curve bends. For very high doses the curve often straightens again (Hall, 2000) (figure 2.3).

The survival curves are best described by the linear-quadratic (LQ) model (Sinclair, 1966). It is assumed that there are two components to cell inactivation by radiation: One proportional to dose and one proportional to the square of the dose. The expression for the survival curve is

$$S = e^{-\alpha D - \beta D^2} \tag{2.6}$$

in which S is the survival fraction, D is dose, and α and β are constants. The same equation was derived by Chadwick and Leenhouts from the assumption that cell inactivation is a consequence of double-strand break in DNA (Chadwick and Leenhouts, 1973). If a single particle breaks both strands, *ln(S)* is proportional to the dose. In the case of two independent particles breaking each a strand close enough in time and space to function as a DSB, *ln(S)* is proportional to the square of the dose.

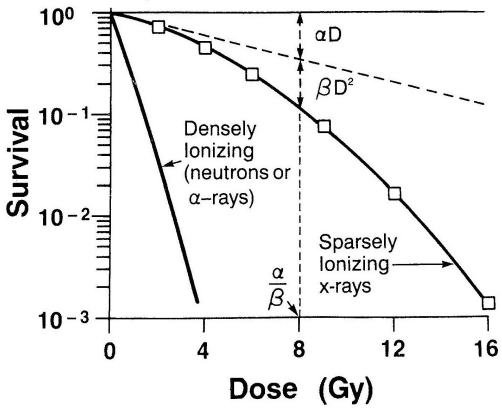


Figure 2.3: *Cell survival curves for radiation with high and low LET. Neutrons and α-rays (high LET) result in a dose response curve of a straight line. For x- and γ-rays (low LET) an initial linear slope is followed by a curved part (the shoulder) which for high doses tends to straighten out. (Hall, 2000, modified)*

The ratio α/β is the dose at which the linear and quadratic components of cell killing are equal, that is $\alpha D = \beta D^2$, and this fraction determines the size and shape of the shoulder of the cell-survival curve (figure 2.3). At least two sublethal damage sites are needed to inactivate a cell. At low doses, the cell may be able to repair a SLD before a new damage is induced which is reflected in a lower cell kill; as the SLDs accumulate, damages that are not lethal in themselves will lead to cell death in combination with the SLDs and the survival curve bends. The size of the shoulder thus reflects the amount of repair of sublethal damage (SLD); a broad shoulder corresponding to a small α/β ratio indicates a substantial amount of SLD repair and

is characteristic of late-responding tissues, while tumour- and early responding tissues have a large α/β ratio with α dominating at low doses (figure 2.4) (Hall, 2000).

The LQ-model predicts a continuously bending curve for higher doses. This is not concordant with experimental observations, but in the range of doses relevant to clinical radiotherapy the LQ-representation seems to be adequate.

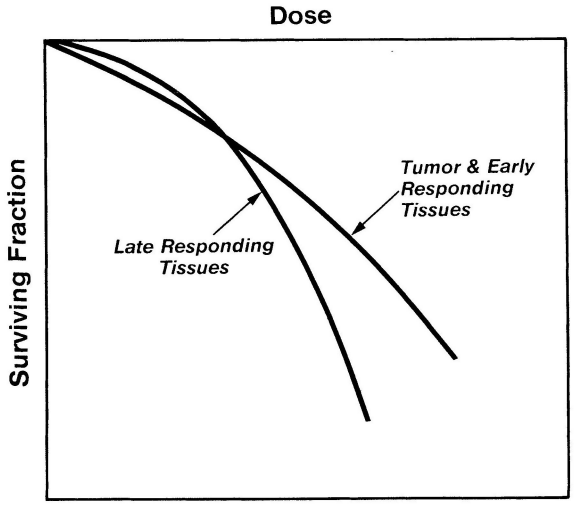


Figure 2.4: *Dose-response curves for late and early responding tissues characterized by a small and large α/β ratio, respectively (Hall, 2000).*

HYPERSENSITIVITY AND INDUCED RADIORESISTANCE

At doses smaller than 1 Gy, many cell lines show a deviation from the linear-quadratic curve, and the LQ model substantially underestimates the effect of single x- or γ-ray doses (figure 2.5). A region of hyperradiosensitivity (HRS) (doses below ~0.5 Gy) precedes the occurrence of a relative resistance per unit dose to cell killing by radiation, called increased radioresistance (IRR) (dose range ~0.5-1 Gy) (Joiner et al., 2001). Joiner and Johns (Joiner et al., 1988) proposed a modification to the linear-quadratic representation, the so-called IR (Induced Repair) model in which α decreases with increasing X-ray dose to represent the induced radioresistance. The α in equation (2.2) is replaced by:

$$\alpha = \alpha_r \left(1 + \left(\frac{\alpha_s}{\alpha_r} - 1 \right) e^{\frac{-D}{D_c}} \right) \tag{2.7}$$

where D_c represents the dose at which 63 % ($(1 - e^{-1})\%$) of induction has occurred . α_r is the initial slope of the curve using the LQ-model and α_s the corresponding slope for the IR-model (see figure 2.5) (Marples and Joiner 1993).

Low dose hypersensitivity and increased radioresistance have been observed in synchronised cell populations and this indicates that cell cycle-related variations in sensitivity do not explain these phenomena (Wouters and Skarsgard, 1997). Instead, the increased radioresistant response seems to result from the activation of a DNA repair process which is triggered by increasing levels of cellular damage. The dependence of IRR on protein synthesis is evident from experiments where cells failed to show IRR after exposure to inhibitors of DNA repair-protein transcription (Marples and Joiner, 1995 and 2000). In the part of the survival curve preceding the IRR response the amounts of damage are insufficient to trigger the mechanisms

of IRR and the result is a hypersensitive dose-response. The existence of HRS can be seen as a way to protect the organism by removing damaged cells that could represent a potential risk for carcinogenesis. As the number of damaged cells increases, a removal would threaten the integrity of the organism and IRR secures maximum recovery from the instant insult with the risk of misrepair eventually leading to cancer.

HRS/IRR has been seen in 76% of more than 45 cell lines investigated (Joiner et al., 2001).

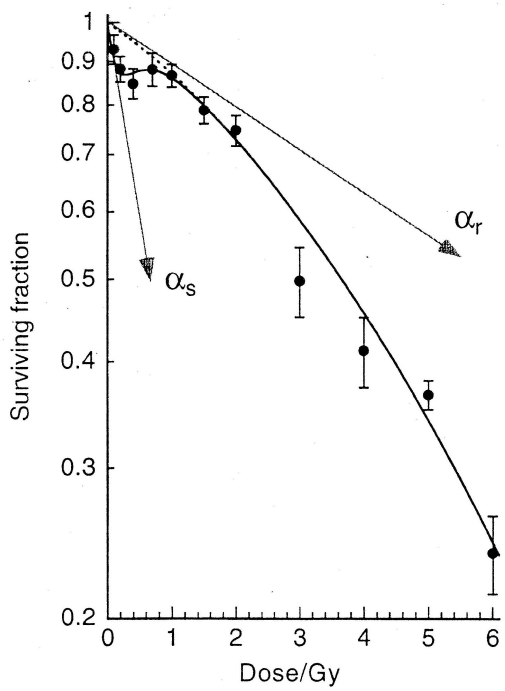


Figure 2.5: *Survival of asynchronous T98G human glioma cells irradiated with 240 kVp x-rays. The solid and dashed line show the fits of the induced repair (IR) model and linear-quadratic (LQ) model, respectively. α_r is the slope of the LQ-fit, α_s of the IR-fit (Joiner et al., 2001).*

2.2.4 ADAPTIVE RESPONSE

A small priming dose may protect against a subsequent, separate, exposure to radiation. This is called the adaptive response and seems to be related to the HRS/IRR mechanism as it also arises from the radioprotective mechanisms, or stress responses, that are upregulated in response to small doses of ionizing radiation and other DNA-damaging agents (Joiner et al., 1996). In experiments with V 79 cells, both the induction of increased radioresistance after single doses of x-rays and the adaptive response was inhibited by cycloheximide, which demonstrates the need for protein synthesis in both induced-resistance phenomena (Marples and Joiner, 1995) and this indicates that DNA repair mechanisms are likely to be involved in the adaptive response as well as in IRR. However, Wouters and Skarsgard (1997) found that a priming dose given to HT29 cells, which demonstrate HRS, abolished the hypersensitive response to small doses, but did not induce an adaptive response for higher doses. Also the interval between two doses, required to remove an adaptive response is much longer than the corresponding interval to allow HRS to recur (Mitchell and Joiner, 2002). This suggests that IRR and the adaptive response are two different mechanisms of increased radioresistance (Short et al., 2001). Induction of the adaptive response has been found to be dependent upon a number of factors including the adapting dose, dose-rate, expression time, culture conditions, and stage of the cell cycle (Cregan et al., 1999).

2.2.5 FRACTIONATED IRRADIATION

When the dose is given in fractions, the cells will have time to repair sublethal damage before the next fraction is given. This implies that the shoulder of the survival curve is repeated at each fraction resulting in a sparing effect. Since the shoulders for early responding tissues are small (corresponding to large α/β ratios), whereas the shoulders for late effects are large (small α/β ratios), the sparing effect will be much more pronounced for the late-responding tissue (figure 2.6).

When a tumour grows the required induction of blood supply is provided by angiogenesis. This new blood supply may be inadequate resulting in hypoxic areas viable at least for a time. The presence of oxygen during irradiation enhances the radiosensitivity as the oxygen fixes the damage caused by free radicals produced by radiation, which means that hypoxic areas are less sensitive to radiation (Steel, 1997). The damage to the tumour tissue may therefore be increased by fractionation because the time between fractions allow reoxygenation and also reassortment of cells into radiosensitive phases of the cell cycle. The overall treatment time is restricted by the possible proliferation of surviving tumour cells (Hall, 2000).

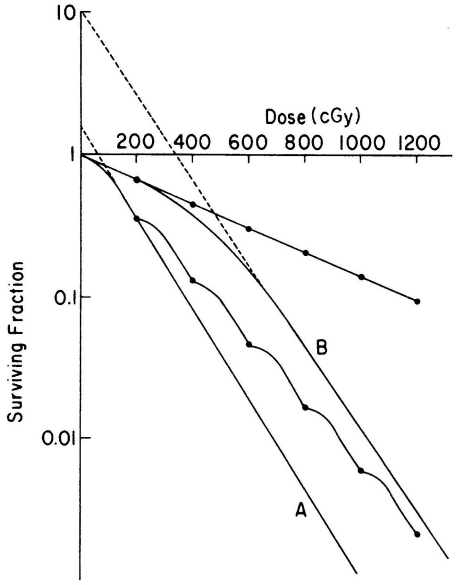


Figure 2.6: *Effect of dose fractionation on survival curves of A: early responding tissues represented by bone marrow stem cells, and B: late-responding tissues represented by cells responsible for radiation pneumonitis (Peters et al., 1979. Nias, 1998).*

2.2.6 LOW DOSE-RATE IRRADIATION

Dose-rate is one of the principal factors that determine the biologic consequences of a given absorbed dose of x- or γ-rays. As dose-rate is lowered, the time taken to deliver a particular radiation dose increases, and biological processes that take place during irradiation modify the observed radiation response. The dose-rate effect results from the repair of sublethal damage. The continuous low dose-rate irradiation may be considered to be an infinite number of infinitely small fractions, and the shoulder of the survival curve is repeated with each fraction resulting in a straight line, with the shoulder disappearing and the curve getting shallower as the dose-rate is reduced (dose-rate sparing). The dose-rate effect caused by repair of sublethal damage is most pronounced between 0.01 and 1 Gy/min (Hall, 2000). The magnitude of the dose-rate effect is related to the size of the shoulder of the survival curve for acute exposure since both are expressions of the cell's capacity to accumulate and repair sublethal damage.

Instead of a dose-rate sparing response, some cell lines demonstrate an inverse dose-rate effect at dose-rates below 1 Gy/h (Mitchell et al., 2001), in which decreasing the dose-rate results in increased cell killing per unit dose. An explanation to this phenomenon was proposed by Mitchell (Mitchell et al., 1979): Since cells move out of G_2 phase only when most of the radiation induced damage is successfully repaired, an accumulation in G_2 will take place when cell are being constantly irradiated and not are able to repair all damage. As G_2 is the most radiosensitive phase of the cell cycle, this will result in an increased cell kill. A further reduction in dose-rate will allow the cells to escape the G_2 block and proliferation may occur during irradiation leading to a reduction in biologic effect. This is summarized in fig. (2.7).

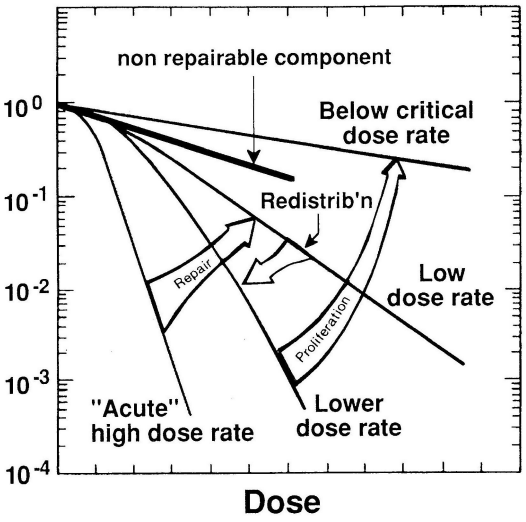


Figure 2.7: *The dose-rate effect. As dose-rate is reduced, the dose-response curve gets shallower as a result of sublethal damage repair. In some cell lines redistribution to a radiation-sensitive cell cycle phase will lead to a lower survival as the dose-rate is reduced. Further reduction of dose-rate allows escape from the G_2-block and the curve gets shallower as a result of proliferation (Hall, 2000).*

Another explanation to the inverse dose-rate effect suggested by Cao et al. (1983) is the lack of induction of repair processes at the lowest dose-rates. Experiments by Mitchell et al. (2002) confirm this relation to the hypersensitivity at low doses as they found an inverse dose-rate effect only in the cell lines known to show HRS/IRR at low acute doses. Cells of a HRS/IRR-negative cell line showed a sparing effect.

2.3 DOSIMETRY

2.3.1 DEFINITIONS

The following is based on Attix, 1986.
The energy imparted by ionizing radiation to matter of mass *m* in a finite volume *m* is defined as (ICRU, 1980):

$$\varepsilon = (R_{in})_u - (R_{out})_u + (R_{in})_c - (R_{out})_c + \sum Q \tag{2.8}$$

where $(R_{in})_u$ and $(R_{in})_c$ is the radiant energy of uncharged and charged radiation, respectively, entering *V*, and $(R_{out})_u$ and $(R_{out})_c$ is the corresponding energy leaving *V*. ΣQ is the net energy derived from rest mass in *V*.

The absorbed dose *D* at a point *P* in a finite volume *V* can be defined as:

$$D = \frac{d\varepsilon}{dm} \tag{2.9}$$

where dm is the mass in the infinitesimal volume dv and $d\varepsilon$ is the expectation value of the energy imparted in dv.
The absorbed dose is thus a measure of the radiation energy that remains in the matter where it may produce biologically significant effects.
The unit used for absorbed dose is the Gray [Gy], and is defined as: 1 Gy = 1 J/kg.

The absorbed dose-rate at a point P and time t is given by:

$$\dot{D} = \frac{dD}{dt} = \frac{d}{dt}\left(\frac{d\varepsilon}{dm}\right) \tag{2.10}$$

2.3.2 TRITIUM DOSIMETRY

The dosimetry of the incorporated tritium was estimated by Åste Søvik (2002).
Because of the very short range of the electrons emitted by tritium compared to the size of the cells, the dosimetry of cellular systems is complicated by the lack of homogeneity both in the composition of the cell and in the distribution of the radioactive tritium. The critical target for radiation is considered to be the DNA in the cell nucleus, but the tritium will be distributed also in the cytoplasm as it is built into a certain fraction of the proteins synthesized after the addition of the tritiated valine. Both electrons generated in the nucleus and in the cytoplasm can contribute to the nuclear dose but can also deposit their energy outside the nucleus or the cell. An inhomogeneous distribution of radioactivity between the cell nucleus and its surroundings would result in more energy transported out of the volume with higher activity than received from the one with lower activity. In addition the course of the particle is deflected by inelastic scattering and the stopping power of the electron will vary as they slow down and loose energy.

A model introduced by Goddu et al. (1997) was used, where the cell was regarded as two concentric spheres both with density 1 g/cm^3 and with a homogeneous distribution of radioactivity within the cytoplasm and cell nucleus, respectively. A number of factors are not included in this model. The energy transported out of the target area by delta-electrons (very fast secondary electrons) was considered to be of little importance for low-energy electrons, the dose contribution from emitters outside the cell was considered negligible, and the curvature of the electron course was ignored because of the large dimensions of the cell and cell nucleus compared to the electron range. The model will not be able to give the dose-rate given to a single cell with a particular size, cell geometry, intracellular activity, and distribution of energy deposit, but only an estimate of the average dose-rate to a population of cells. Because of the large number of decays necessary to give a significant dose when using low LET emitters like tritium, this average dose-rate is considered to be of relevance when describing the radio-biologic response of a cell population.

The activity of the cell nucleus and the cytoplasm (difference between activity of whole cell and cell nucleus) was found experimentally using a liquid scintillation counter. The results

were used both to calculate the dose-rate from the model mentioned above and to plot the cell activity as a function of time to find when the incorporation was completed.

2.3.2 THERMO LUMINESCENCE DOSIMETRY (TLD)

TLD makes use of crystals with a trace amount of impurities, which have the ability to store a minute fraction of the absorbed energy in the crystal lattice. The absorbed radiation excites electrons or positive holes into metastable energy traps created by the presence of impurities for usefully long periods of time until deliberate heating releases the energy by emission of light photons.

In order to avoid contributions from unstable low-energy traps, the crystals are preheated for 10 minutes at 100 °C. The dose information is read by exciting the crystal with preheated nitrogen gas. The emitted light is measured by a photomultiplier tube which converts light into an electrical current. The current is then amplified and measured by a recorder. Finally the crystal is heated further to release any remaining energy from deeper traps. (Attix, 1986, Khan, 1994).

3 MATERIALS AND METHODS

3.1 THE CELL LINES

An established cell line is a cell population that has escaped senescence and divide indefinitely in culture. This "immortality" reflects the presence of genetic changes that have altered their proliferative properties (Alberts et al., 1994).

Three cell lines were used, denoted T-47D, V 79, and NHIK 3025:

The **T-47D** cell line was established in 1974 from the pleural effusion of a patient with breast carcinoma. The cells exhibit epithelial morphology and form monolayers in culture (Keydar et al. 1979). Immunohistologic studies and electron microscopy confirm the mammary epithelial origin. T-47D cells have normal RB function (2.1.2) (Åmellem et al., 1998; Stokke et al., 1993), but contain only a single mutated p53 gene (Casey et al., 1991). Under normal favourable growth conditions, T-47D divide exponentially with a doubling time of 37.2±2.0 hours (Stokke et al.; 1993). T-47D cells were routinely grown in the medium RPMI (Roswell Park Memorial Institute) 1640 with an addition of 10% foetal bovine serum, 1 % penicillin (5000 IU/ml), 1% streptomycin (5000 μg/ml), 1% L-glutamine and 0.2% insulin. RPMI 1640 contains all the necessary nutrients, vitamins and inorganic salts, only fresh L-glutamine was added to the medium weekly because of its rapid decomposition. RPMI 1640 contains the pH indicator phenol red that changes colour from yellow to red when pH exceeds approximately 7.4.

V 79 cells originate from Chinese hamster embryonic lung fibroblasts and have a doubling time of 11±1 hours. P53 function is inactivated due to two point mutations in the DNA binding domain of p53. (Yu et al., 2000). V 79 cells were grown in MEM medium (Eagle's Minimum Essential Medium) supplemented with 15% foetal calf serum, 2% penicillin/streptomycin, and 1% L-glutamine.

NHIK 3025 cells were derived from cervical carcinoma in situ (Furre et al., 1999, Oftebro and Nordby 1969). This cell line express genes from the human papilloma virus 18 and the resulting oncoproteins E6 and E7 bind the proteins p53 and pRb obstructing their normal function. NHIK 3025 cells were grown in MEM medium like V 79. The doubling time is 22 hours (Furre et al., 1999).

3.2 CELL CULTIVATION

3.2.1 EQUIPMENT

The cells used in these experiments were grown in the Biophysics Cell Laboratory at the Department of Physics, University of Oslo (UIO) and irradiated with ^{60}Co-γ-radiation at the Norwegian Radium Hospital (DNR).

To ensure sterility, all work where the cells or solutions, which would come in contact with the cells, were exposed to air took place in a sterile LAF bench (LAF = Laminar Flow) that was disinfected with 70% ethanol before and after use. The LAF bench used at UIO was of the type VB 2040 from OAS. For experiments T7-T20 facilities at the Norwegian Radium

Hospital was used for seeding the cells after the priming dose and for medium change and flushing before the challenge dose. In experiments T7-T9 the laboratory at the Biophysics Department (at DNR) was used; the LAF bench was a Holten Lamin Air HV 2448. This laboratory did not have facilities for sterile flushing, so after infection in part of experiment T9, further work was postponed until a new laboratory under the auspices of SUFS (Centre for Education and Research in Radiation Oncology) adjoining the irradiation room was finished. Experiments T10–T23 and N1-N3 were performed here in a LAF bench from Odd A. Simonsen A/S, Oslo.

Only sterile equipment was used when working with the cells. Apart from polystyrene sterile pipettes (Bibby Sterilin Ltd, England) that were disposed of after use, all equipment was washed and sterilized at the laboratory at UIO. After lying in soap water (Decon 90, Decon Laboratories Limited England) it was rinsed in de-ionized water. Glass- and metal equipment was wrapped in aluminium foil and sterilized in a Termaks oven (Termaks, Bergen Norway) for approximately 2 hours at 180 °C. Rubber equipment and sheets were wrapped in sealed autoclave paper bags and autoclaved (Webco) for 20 minutes at 120 °C. The glass equipment was also burnt before use in a gas- or ethanol flame. The trypsin and medium were prepared at the laboratory (UIO) and sterilized by filtration (Millex-GP_{50} Filter Units, 0.22 µm, Millipore AS, Ireland). The medium was kept incubated at 37 °C for two days and controlled for infection to ensure sterility. The cells used in the experiments were grown in 25 cm^2 sterile plastic culture flasks (Nunclon, Denmark) and were incubated in a semi-sterile CO_2 incubator (UIO: National Heinicke Company, DNR, Biophysics department: Forma Scientific, USA, DNR, new laboratory: Thermo Forma, USA) with the lids unscrewed at 37 °C, 95% humidity and 5 % CO_2.

3.2.2 MAINTENANCE OF THE CELL LINE

In order to ensure continuous proliferation, the cells need frequent fresh supplies of nutrients and growth factors and to be relieved of their own toxic waste. It is also important to control the cell density since both too high and too low density can repress cell growth. The cells were kept in exponential growth by recultivation twice a week, Monday and Friday and change of medium every Wednesday.

Trypsin is a proteolytic enzyme that breaks the protein bonds between the cells and the surface they are attached to, and between neighbouring cells. The trypsin used for T-47D cells were added 0.2 mg/ml EDTA that bind the Ca^{2+} on which cell-cell adhesion depends (Alberts et al., 1994) and thereby enhances the effect. V 79 and NHIK 3025 cells are more fragile and require a more gentle treatment than T-47D cells; here was used trypsin puck without EDTA.

At recultivation the old medium was first removed, whereupon the cells were washed with trypsin (once for T-47D, twice for V 79 and NHIK 3025 cells) before new trypsin (3 ml in the 25 cm^2 flasks and 5 ml in the 80 cm^2 flasks) was added. This was left until the cells had detached from the bottom and a Pasteur pipette was used to pump the suspension in order to separate the cells. The separation was examined in a microscope (Nikon TMS, Japan). The cell suspension was added to a test tube containing the same amount of medium as the amount of trypsin; this stopped the effect of the trypsin because of its reaction with the serum protein in the medium. The cells were centrifuged for 5 minutes at 1100 rpm and then resuspended into fresh medium, after the trypsin and medium had been discarded. A suitable amount of cells resulting in a favourable density was then added to flasks containing fresh medium (5ml for 25 cm^2 flasks, 15 ml for 80 cm^2 flasks) and flushed with 5% CO_2 for a pH of ~7.4. The flasks were sealed before transfer to the incubator.

3.3 EXPERIMENTS

T-47D cells need at least five hours to attach to the surface. In experiments T1-T6 the seeding took place the day before the irradiation at UIO. The next day the cells were flushed and sealed and transported in a preheated car enclosed in a thermos bag to DNR. Here the flasks were irradiated before being returned to UIO where the lids were unscrewed and the cells re-incubated. The process of transport and irradiation took approximately 1 hour and 10 minutes. The experiments with V 79, V1-V6, followed the same routines except that they attach much more quickly and therefore were seeded the same day as the rest of the treatment was given.

The cells that were given a priming dose were transported to DNR and irradiated with the priming dose in a 80 cm^2 flask. After irradiation the cells were seeded at DNR following the procedures described below and incubated also at DNR until they were to receive the challenge dose. The medium was replaced with fresh flushed medium in order to discard of unattached cells, and the flasks were flushed and sealed before the challenge doses were given either 6 or 24 hours after the priming dose. In experiments T7–T9, where the laboratory at the Department for Biophysics (DNR)was used, the flasks were then transported to UIO for incubation. In T10–T22 and N1-N3, the cells were incubated at the new SUFS-laboratory, where they had also been seeded, for four to six days before they were taken back to UIO; the lids were sealed during transport.

The cells in experiments T20-T22 and N1-N3 were seeded early in the morning at DNR and irradiated 6 and 4 hours later, respectively (NHIK 3025 attach in about two hours); the medium was exchanged and the flasks flushed with CO_2 just before irradiation as in the priming dose experiments.

3.3.1 SEEDING

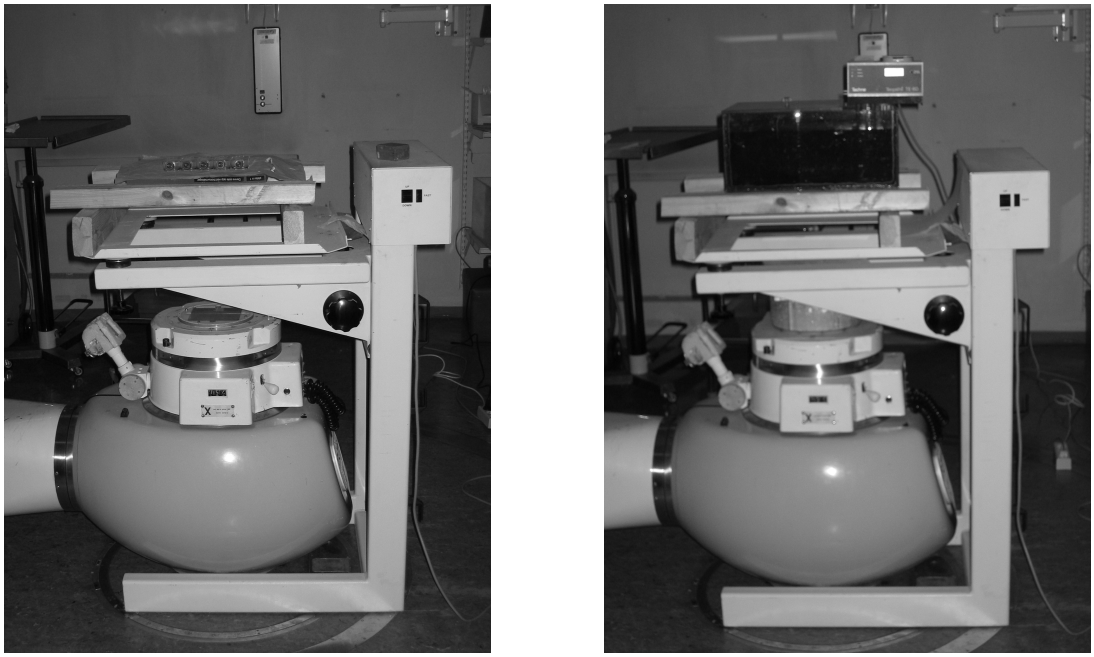
The cells used in experiments were trypsinized and centrifuged as described for recultivation. After resuspending the pellet into fresh medium, the cell suspension was diluted further before a sample was taken out to be counted in a Bürker chamber. The Bürker chamber consists of two chambers each with 3x3 squares. The number of cells was counted in 5 squares of each network, the highest and lowest values were discarded and the average from the 2 set calculated. The volume of each compartment with the cover glass put on was 10^{-4} ml; the number of cells per ml cell suspension was found by multiplying the average number with 10^4. Appropriate dilutions were made and a specified number of cells depending on the irradiation dose to follow were seeded in 25 cm^2 flasks (5 per dose) that were incubated in a CO_2 incubator, the lids unscrewed.

3.3.2 EXTERNAL IRRADIATION

The irradiations took place at the Norwegian Radium Hospital with a cobalt source (Molbatron 80; TEM Instruments, Sussex, England). The cobalt source was installed in 1996 and the inverse dose-rate was measured (SSD = 80 cm and field = 10 cm × 10 cm) at 0.5 cm depth to be 31.350 s/Gy on September 15th 1996. The half-life of ^{60}Co is 1925.1 days. The irradiation time for a particular dose was found from the inverse dose-rate corrected for the reduction in activity, field size and depth.

When irradiated with the highest dose-rate (HDR = acute irradiation) as well as with 0.9 Gy/h, the 5 flasks were placed on a 2 cm plate of water equivalent material (Perspex) and irradiated from beneath (gantry at 180°) (figure 3.1 a). The SSD (source-surface distance) used was 80 cm and the field size 25 cm x 25 cm. For HDR the time for the shutter to be open to give the

wanted dose was calculated from the monitor factor (s/Gy) (inverse dose-rate corrected for decay) which was multiplied with a field size factor (0.95) and depth dose factor (2 cm ~ 1.097 Gy). The dose-rate for acute radiation was approximately 50 Gy/h (54.6 Gy/h in the first experiments falling to 47.3 Gy/h in the last ones). To obtain the dose-rate of 0.9 Gy/h, the source was shielded by a 2 cm thick plate of Roos metal.



a) b)

Figure 3.1: *Irradiation set-up: a) For HDR irradiation and irradiation with 0.9 Gy/h, the duration of the exposure was so short that irradiation could be performed in room-temperature. The cell flasks were placed on a 2 cm Perspex-plate that rested on wooden beams to get the right height. In order to obtain the dose-rate of 0.9 Gy/h, the source was shielded with a 2 cm thick plate of Roos metal. b) During exposure with dose rates of 0.32 and 0.045 Gy/h the cell flasks were submerged in a water tank that held 37 °C. The source was shielded with blocks of Roos metal.*

In order to obtain the lowest dose-rates used for priming, the source was shielded by blocks of Roos metal (Sn 25%, Pb 25%, Bi 50%, melting point 96 °C, specific weight 9.85 g/cm^3). The thickness was 10cm for the dose-rate 0.32 Gy/h and 13cm for the dose-rate 0.045 Gy/h. In addition 1.6 cm brass was used to obtain the dose-rate 0.045 Gy/h. Because of the long irradiation time the flask was sealed within a plastic bag and submerged in an open water bath of Perspex (42×35 cm^2, height 20 cm) maintained at a temperature of 37 °C by the use of a temperature-controlled heater (Tecne Temlette TE-8D, Princeton, NJ, USA) that also kept the water circulating. A lead block was put on top to keep the bottle from floating and ensure the right placement in the field (figure 3.1 b).

DOSIMETRY

The dose-rates for the shielded cobalt source were measured by Thorbjørn Furre using thermoluminescence dosimetry. Ribbons ($3.2 \times 3.2 \times 0.9$ mm^3) made of LiF (TLD-100; Harshaw TLD Bicrom, Solon, OH, USA) were put in flasks containing 5 ml of absolute alcohol and irradiated in the set-up concerned. The readout process of the ribbons and the dose calculations followed the procedures standard at DNR.

3.3.3 TRITIUM IRRADIATION

The priming dose in experiments T 12, T 17 and T 19 was obtained from incorporated tritium. The tritium isotope ^3H was incorporated in the proteins in the cells using the amino acid Valine as a specific marked precursor. Valine is one of the essential amino acid for humans and is a required component in the growth medium given to human cells in culture. Tritiated valine (TRK533, 1.0 mCi/ml, Amersham England) was added to the medium in a concentration corresponding to a specific activity of 1.6 Ci/mol (5.9×10^{10} Bq/mol). The specific activity was kept at a constant level by use of a high concentration of valine, 1.0 mM, in the medium.

The cells were grown in medium with tritiated valine for four or five months. Six to seven hours before irradiation with the cobalt source, they were plated in medium with cold valine (no tritium), which was also used in all the following exchanges of medium. The cells were then irradiated according to the same challenge dose regime as were the other experiments involving priming doses.

3.3.4 INCUBATION AND FIXATION

The cells were incubated for a certain period depending on cell line and dose size (10-14 days for V 79 and NHIK 3025, 2–3 weeks for T-47D) and during this time medium was changed every 4–7 days. When the cell colonies were macroscopically visible, the cells were fixed. First the flasks were washed twice with medium or PBS (phosphate-buffered saline) to remove excess proteins. Then 2.5 ml ethanol was added and left for 3 minutes in order to fix the cells. Finally the cells were stained with 2.5 ml methylene blue for another 3 minutes before being gently de-stained by rinsing with water and left to dry.

3.4 CALCULATION OF CELL SURVIVAL

3.4.1 SURVIVING FRACTION

The cell colonies were counted using a counter with a simple magnifier (Gerber Instruments, Germany). Only colonies consisting of more than 40 cells were scored as survivors. In cases of doubt, a microscope (Nikon TMS, Japan) was consulted.

Each dose of radiation was given to five flasks. In addition five flasks were seeded as an unirradiated control group to measure the plating efficiency (*PE*), which is found as the fraction of cells seeded that grow into colonies:

$$PE = \frac{N(C)}{N_0(C)} \tag{3.1}$$

where $N(C)$ is the mean number of colonies in the control flasks and $N_0(C)$ is the number of cells seeded per flask in this group.
The surviving fraction for each set of five flasks given the same treatment is given by:

$$SF(B) = \frac{N(B)}{N_0(B)\times PE} \tag{3.2}$$

N(B) is the mean number of counted colonies in the five flasks given treatment *B* and $N_0(B)$ is the number of cells seeded in each of the five flasks receiving treatment *B*.
The standard error of the calculation of the mean value for each set of five flasks is given as:

$$SE(N(B)) = \sqrt{\frac{1}{n(n-1)}\left(\sum_{i=1}^{n} X_i - X_m\right)^2} \tag{3.3}$$

The survival fraction *SF(B)* is a function of two variables *N(B)* and $N_0(B)$ (equation 3.2) and the standard error of the surviving fraction is given as:

$$SE(SF(B)) = \sqrt{\left(\left(\frac{\delta SF(B)}{\delta N_0(B)} SE(N_0(B))\right)^2 + \left(\frac{\delta SF(B)}{\delta N(B)} SE(N(B))\right)^2\right)}$$

$$= \sqrt{\left(\left(\frac{1}{N_0} SE(N)\right)^2 + \left(\frac{-N}{N_0^2} SE(N_0)\right)^2\right)} \tag{3.4}$$

3.4.2 CORRECTION FOR MULTIPLICITY

Not all the cells were seeded as single cells. This is because complete cell separation would take so much time that trypsinization might harm the cells. In addition some cells will divide during the time between seeding and irradiation. This was particularly evident in the cases of 24 hours between priming- and challenge dose. Multi-cell units would imply an enhanced probability of colony formation compared to single cells, since all cells in a unit would have to be inactivated to prevent the colony from being formed. In order to make corrections for this multiplicity, extra flasks were always seeded to be fixed at the time of each irradiation. These flasks were studied under microscope and all singlets, doublets, triplets etc. were counted. The multiplicity *M* was calculated as the mean number of cells per colony-forming unit:

$$M = \sum_{i=1}^{m} x_i \cdot i \tag{3.5}$$

where x_i is the fraction of cell units consisting of i cells.

The actual fraction of surviving cells is *f* and the probability of a cell not forming a colony is *(1-f)*. The probability of a cell unit of *m* cells not forming a colony is $(1-f)^m$ which means that the observed survival probability for one cell unit of *m* cells *F* will be:

$$F = 1-(1-f)^m \tag{3.6}$$

For a population of multi cell units containing 1 to *m* cells the surviving fraction *SF* is given by:

$$SF = \sum_{i=1}^{m} x_i \left(1 - (1-f)^i\right) \tag{3.7}$$

Had there been only singlets and doublets the corrections would be as follows:

$$M = x_1 + 2x_2 \tag{3.8}$$

Where $x_1 + x_2 = 1$

x_1 and x_2 are found from these equations and inserted into equation (3.7) which is solved for *f,* the actual surviving fraction, giving it as a function of the observed surviving fraction *SF* and the mean multiplicity *M* (= mean number of cells per colony forming unit):

$$f = \frac{M - \sqrt{M^2 - 4(M-1)SF}}{2(M-1)} \tag{3.9}$$

In the present experiments the multi cell units were not restricted to singlets and doublets. In order to calculate the exact correction for multiplicity one would end up with very complicated expressions for *f.* As an approximation, *f* was found from equation (3.9) using *M* from equation (3.5). The deviations from the exact solution using this approximation was calculated for cell units containing up to three cells by Heidi Lorentzen (Lorentzen, 2001) and found to be less than 1 %.

To find a value for the uncertainty of *M,* denoted ΔM, some of the flasks were counted several times, since both the specific part of the surface chosen (as the cell distribution will never be homogeneous) as well as human interpretations of what is actually seen influence on the result. ΔM was found to be 0.03.

The standard error of *f* is a function of ΔM and *S.E.(SF)* (=ΔSF in the following):

$$SE(f) = \sqrt{\left(\frac{\delta f}{\delta SF} \cdot \Delta SF\right)^2 + \left(\frac{\delta f}{\delta M} \cdot \Delta M\right)^2}$$

$$\frac{\delta f}{\delta SF} = \frac{1}{\sqrt{M^2 - 4(M-1)SF}}$$

$$\frac{\delta f}{\delta M} = \frac{-1 + \dfrac{M + 2SF - 2SF \cdot M}{\sqrt{M^2 - 4(M-1)SF}}}{2(M-1)^2}$$

Giving:

$$SE(f) = \sqrt{\left(\frac{\Delta SF}{\sqrt{M^2 - 4(M-1)SF}}\right)^2 + \left(\frac{-1 + \dfrac{M + 2SF - 2SF \cdot M}{\sqrt{M^2 - 4(M-1)SF}}}{2(M-!)^2} \cdot \Delta M\right)^2} \qquad (3.10)$$

3.4.3 THE MEAN VALUE OF SURVIVING FRACTIONS

In the repeated experiments the mean survival fraction, f_m, was found weighting the number of flaks in each experiment, n_i:

$$f_m = \frac{\sum_{i=1}^{i=k} n_i f}{\sum_{i=1}^{i=k} n_i} \qquad (3.11)$$

where k is the number of experiments.

Since the calculations of error in each experiment were encumbered with uncertainties, the standard error was used for the mean survival fraction:

$$\Delta f_m = \sqrt{\frac{1}{k(k-1)}\left(\sum_{i=1}^{i=k} f_i - f_m\right)^2} \qquad (3.12)$$

3.4.4 PRESENTATION OF SURVIVAL DATA

The survival curves present the surviving fractions as a function of dose in a semi-logarithmic plot. The plots of the data from each experiment are included in appendix D together with the raw data and plots of mean values of surviving fraction from parallel experiments as a function of dose are shown in appendix E.

The survival curve fits presented in chapter 4 represent curve fittings done by Hans Kristian Opstad using IDL curve fit. The data were fitted by either the linear-quadratic model (LQ-model) or the induced repair model (IR-model) using the method of least–squares and weighting the errors. The details of this will be presented in the cand. Scient. thesis by Opstad.

The standard error for the mean survival of the control flasks is implicit in the errors for the other survival data through the error in the plating efficiency. Therefore, the survival of 1 at dose zero is plotted without error-bars in all dose-response curves.

The averaged data points are also replotted in the form of effect per unit dose. In this type of plot, a LQ response will follow a straight line with a y-intercept equal to α and a slope equal to β:

$$-(\ln S)/D = \alpha + \beta D \tag{4.1}$$

In the limit of $D \rightarrow 0$, $-(\ln S)/D$ approaches α.
In the IR-model (equation 2.7) α is replaced with:

$$\alpha = \alpha_r \left(1 + \left(\frac{\alpha_s}{\alpha_r} - 1\right) e^{-\frac{D}{D_c}}\right) \tag{4.2}$$

For doses much larger than D_c this α approaches α_r with increasing doses. In the limit of $D \rightarrow 0$, α approaches α_s, which means that the y-intercept from a back-extrapolation of the low dose data equals to α_s.

3.5 FLOW CYTOMETRY

Flow cytometry is a quantitative method for analysis and separation of cells in suspension. A fluorescent dye is attached to a specific component of the cells or cell nuclei and the cells in the suspension are led to flow, one at a time, through one or more laser-beams. The laser beam induces a fluorescence pulse whose intensity represents the quantity of the cellular component in question. The forward light scatter depends on the cell size and the side scatter reflects the morphology of the cell. The flow cytometry was performed by Pål Graff and is used in this study to find the distribution of cells in the various phases of the cell cycle; the relative cell number was plotted against the relative DNA content in a DNA histogram.

The cells were trypsinized and washed once with PBS, fixed in 70% methanol, and stored at -20 ºC. The subsequent steps were done at 0 ºC. The cells were washed three times in PBS prior to the DNA staining with 2 µg ml^{-1} Hoechst 22158.
Stained cells were measured in a FACStar^{PLUS} flow cytometer (Becton Dickinson) equipped with one argon and one krypton laser (Spectra Physics) tuned to 48 nm and UV, respectively. Forward light scatter (FSC) and side scatter (SSC) were measured. The data were gated on FSC versus SSC and Hoechst 33258-flourescence pulse area versus pulse width to exclude dead cells and aggregates of cells, respectively (not shown in the figures).

4 RESULTS

4.1 ACUTE IRRADIATION OF THREE CELL LINES

Cell survival experiments were conducted with three different cell lines of which two, T-47D and V79, were found to express hyper-radio-sensitivity (HRS) in the low dose region. The survival curves are shown in figure 4.1. Values for the parameters from the fittings of the LQ-model and the IR-model, respectively, are given in table 1 and 2 along with their associated errors.

For T-47D and V79 data cells from six experiments were used, three and three preponderant in the high or low dose region, respectively (experiments T1-T6 and V1-V6), and there is no doubt that the IR-model makes the best fit to data in the low-dose range from both cell lines. The survival curves for T-47D and V79 cells are almost coincident for doses larger than 2 Gy but the T-47D curve has a much more pronounced HRS at small doses.

The averaged data points from figure 4.1 are also replotted in figure 4.2 in the form of effect per unit dose (see chapter 3.4.4). In this type of plot, a LQ response (equation 2.6) will follow a straight line with a y-intercept equal to α and a slope equal to β.

Table 3 summarizes the values of the α and β values from linear regression to a selection of the data that fits the LQ-model. The transition from the hypersensitive to the more radioresistant response varies somewhat in the two cell lines. The fit of the LQ-model was done to data from 1 Gy and up for T-47D cells but from 0.75Gy and up for V79 cells. NHIK 3025 cells were irradiated with doses up to 5 Gy with emphasis on the low doses (experiments N1-N3). For NHIK 3025 cells, the LQ-model seemed to make the best fit and it was not possible to detect any signs of HRS (figure 4.2c and figure 4.1). These cells had a much lower survival for doses larger than 2 Gy than cells of the other two cell lines (two decades at 10 Gy).

Cell line	α (Gy^{-1})	β (Gy^{-2})	α/β (Gy)
T-47D	0.186 ± 0.010	0.0129 ± 0.0012	14.4 ± 1.5
NHIK 3025	0.125 ± 0.019	0.064 ± 0.004	1.88 ± 0.33
V79	0.137 ± 0.006	0.0182 ± 0.0006	7.53 ± 0.41

Table 1: *Values of the parameters from the best curve fit of the* ***LQ-model*** *to the experimental data. For T-47D and V79 cells the fitting was done only to data from doses above the HRS/IRR range and the data points were weighted according to standard error in each experiment.*

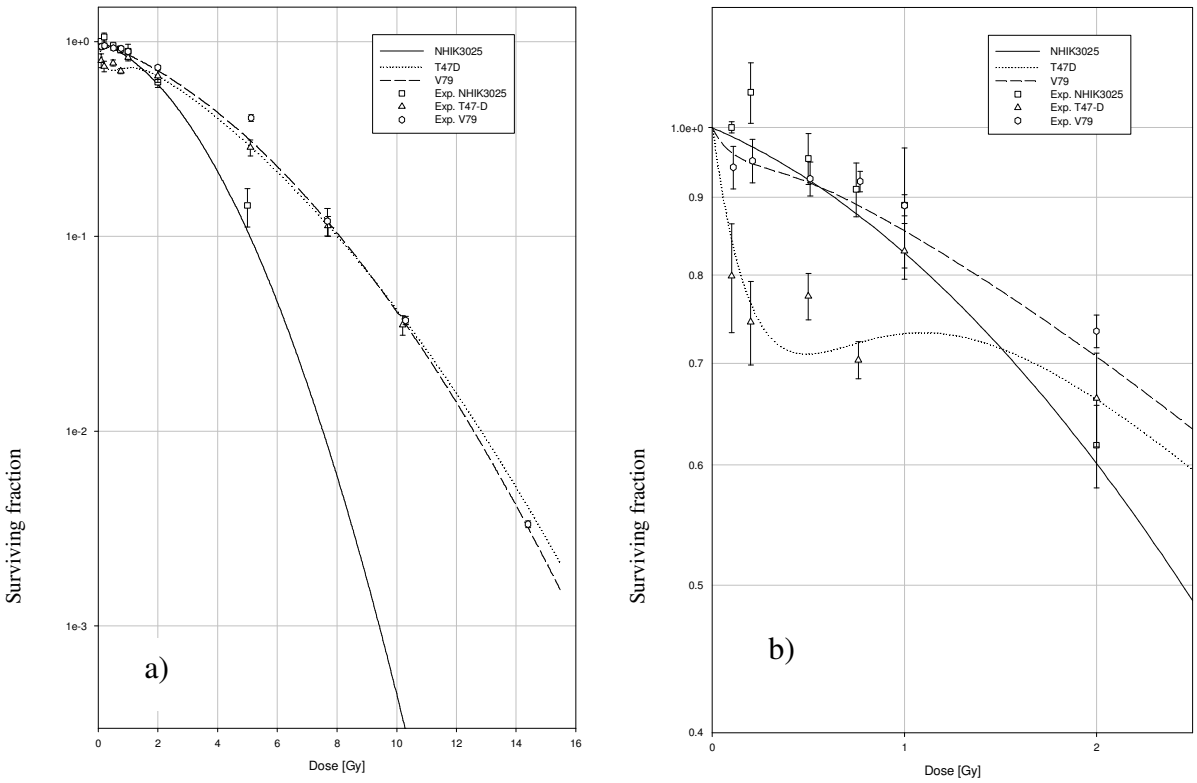


Figure 4.1: *Surviving fraction as a function of dose for T-47D cells* (Δ)*, V79 cells* (○)*, and NHIK 3025 cells* (□)*. Data points represent mean values from three independent experiments and vertical bars represent standard errors. The curves represent model fits to the data and the data points were weighted according to standard error in each experiment: The IR-model was fitted to the data for T-47D (experiments T1-T6) and V79 (experiments V1-V6) cells. Data for NHIK 3025 cells (experiments N1-N3) were fitted by the LQ-model, the values of α and β used were found from linear regression in the effect-per-unit-dose plot (figure 4.2 c). a): All data included. b): Only data up to 2 Gy included.*

Cell line	α_s (Gy^{-1})	α_r (Gy^{-1})	β (Gy^{-2})	α/β (Gy)	***Dc*** (cGy)
T-47D	2.19 ± 0.54	0.168 ± 0.011	0.0149 ± 0.0013	11.3 ± 1.2	36 ± 6
V79	0.58 ± 0.21	0.137 ± 0.002	0.0182 ± 0.0002	7.43 ± 0.14	16 ± 5

Table 2: *Values of the parameters from the best curve fit of the* ***IR-model*** *to the experimental data. The data points were weighted according to standard error in each experiment.*

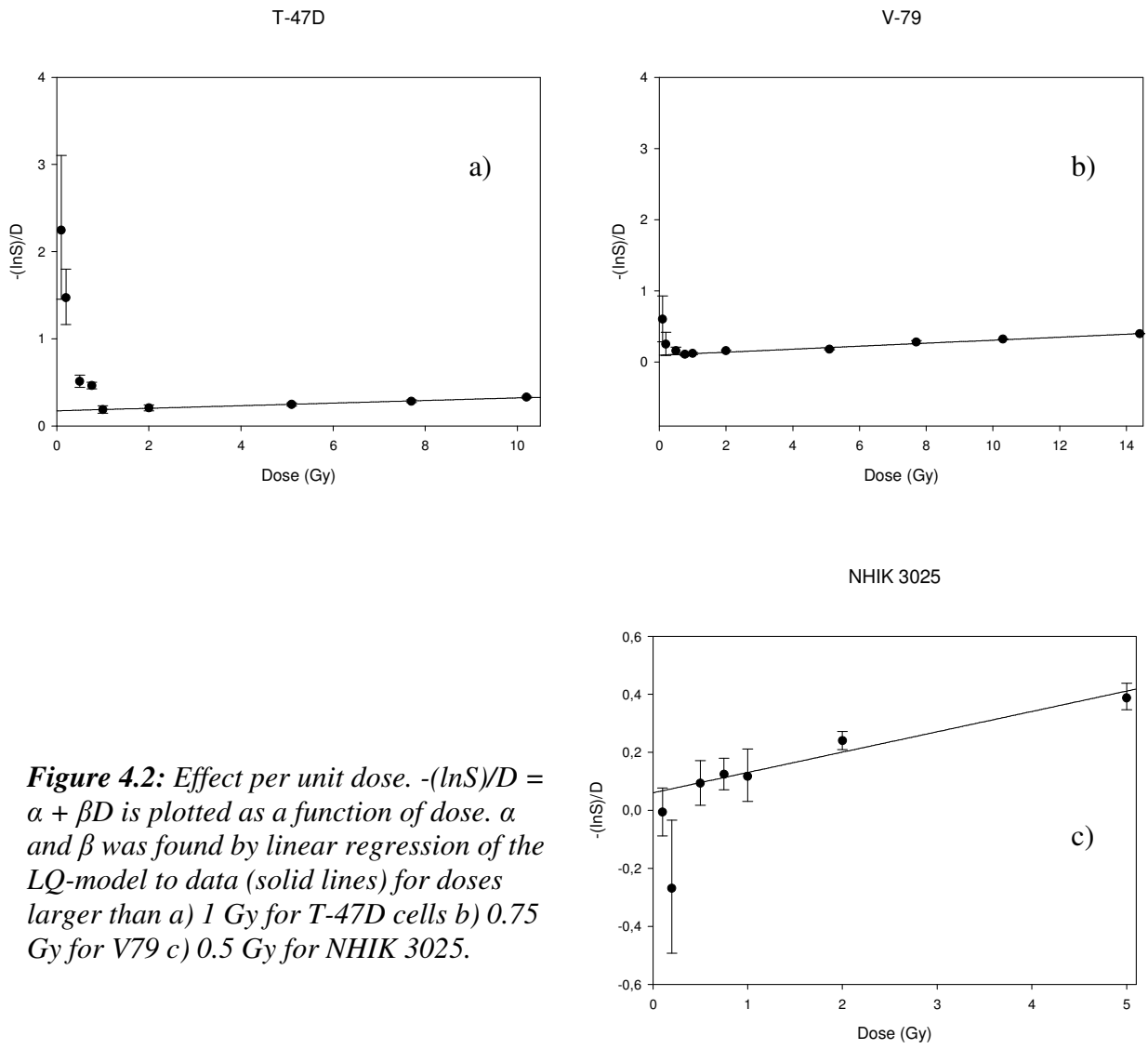


Figure 4.2: *Effect per unit dose. -(lnS)/D = α + βD is plotted as a function of dose. α and β was found by linear regression of the LQ-model to data (solid lines) for doses larger than a) 1 Gy for T-47D cells b) 0.75 Gy for V79 c) 0.5 Gy for NHIK 3025.*

Cell line	α (Gy^{-1})	β (Gy^{-2})
T-47D	0.172 ± 0.166	0.0148 ± 0.0333
NHIK 3025	0.0606 ± 0.0094	0.0701 ± 0.0807
V79	0.0969 ± 0.0668	0.0209 ± 0.0194

Table 3: *Values of the parameters found by linear regression of the LQ-model to data points in the effect-per-unit-dose versus dose plots. The data points do not include the point (D=0, f=1) and are not weighted*

4.2 ADAPTIVE RESPONSE IN T-47D CELLS

In all the experiments with acute irradiation subsequent to a priming exposure, the cells responded with a reduction of the HRS. In the fits of the IR-model to the data fixed values of the parameters α_r, β, and D_c was used, which were obtained from the fits to the data from experiments without a priming dose (table 2). It was assumed that these parameters were characteristic of the cell line as the irradiation setup was kept the same.

Figure 4.3 and figure 4.4 show the survival curves for T-47D cells given an acute priming dose of 0.3 Gy (experiments T7 and T8) 6 hours and 24 hours prior to the challenge dose, respectively. Six hours after the priming exposure the HRS was diminished as compared to that observed after one acute dose (figure 4.3) and 24 hours after the priming exposure HRS was only partially restored (figure 4.4). The radiation response six hours after a low dose-rate priming dose (0.9 Gy/h) was similar to that following a high dose-rate priming dose (figure 4.6). The data 24 hours after priming are missing because of an infection.

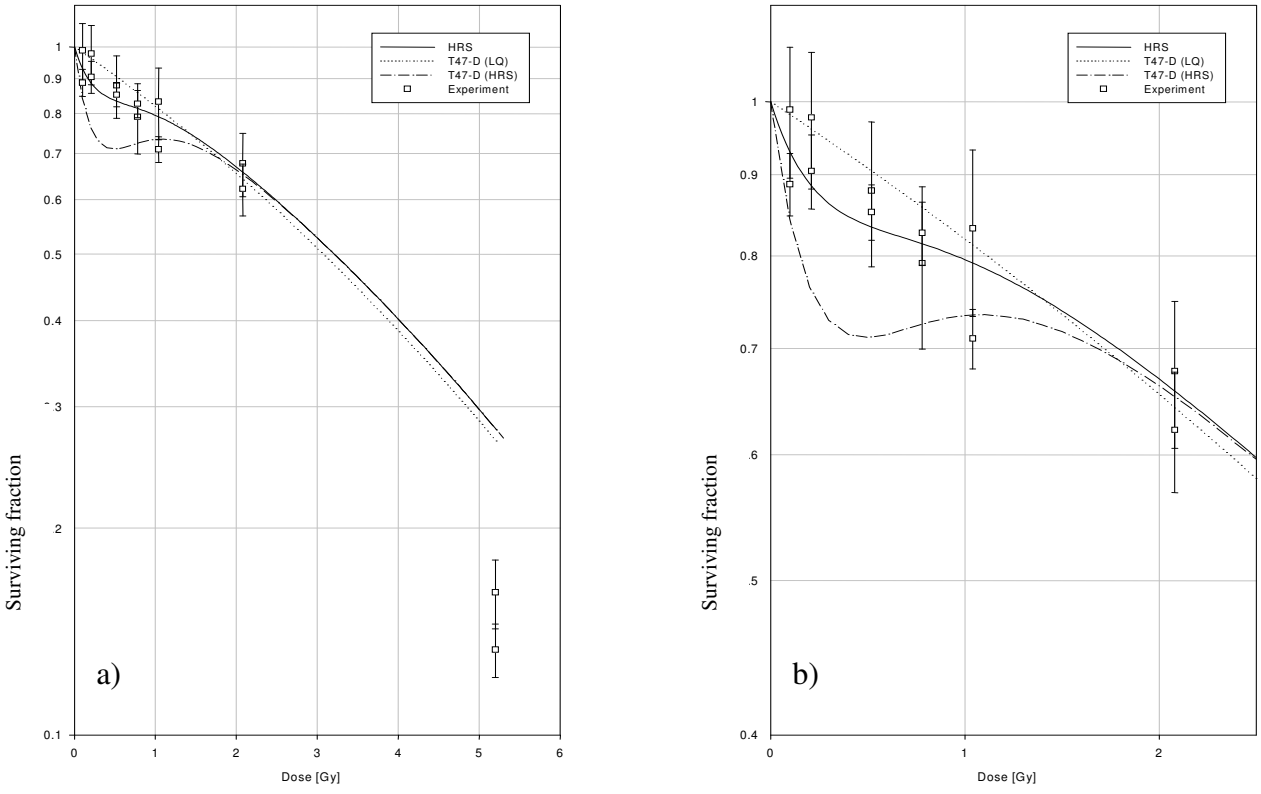


Figure 4.3: *Surviving fraction as a function of dose. Data points represent single observations of two independent experiments for T-47D cells given a HDR priming dose of 0.3 Gy 6 hours prior to the challenge doses and vertical bars represent standard errors. The curves represent model fits to the data: The IR-model was fitted to the data points shown (solid line). Also shown are the fit of the LQ-model (dotted line) and the IR-model (dashed line) to data from unprimed T-47D cells. a): All data included. b): Only data up to 2 Gy included.*

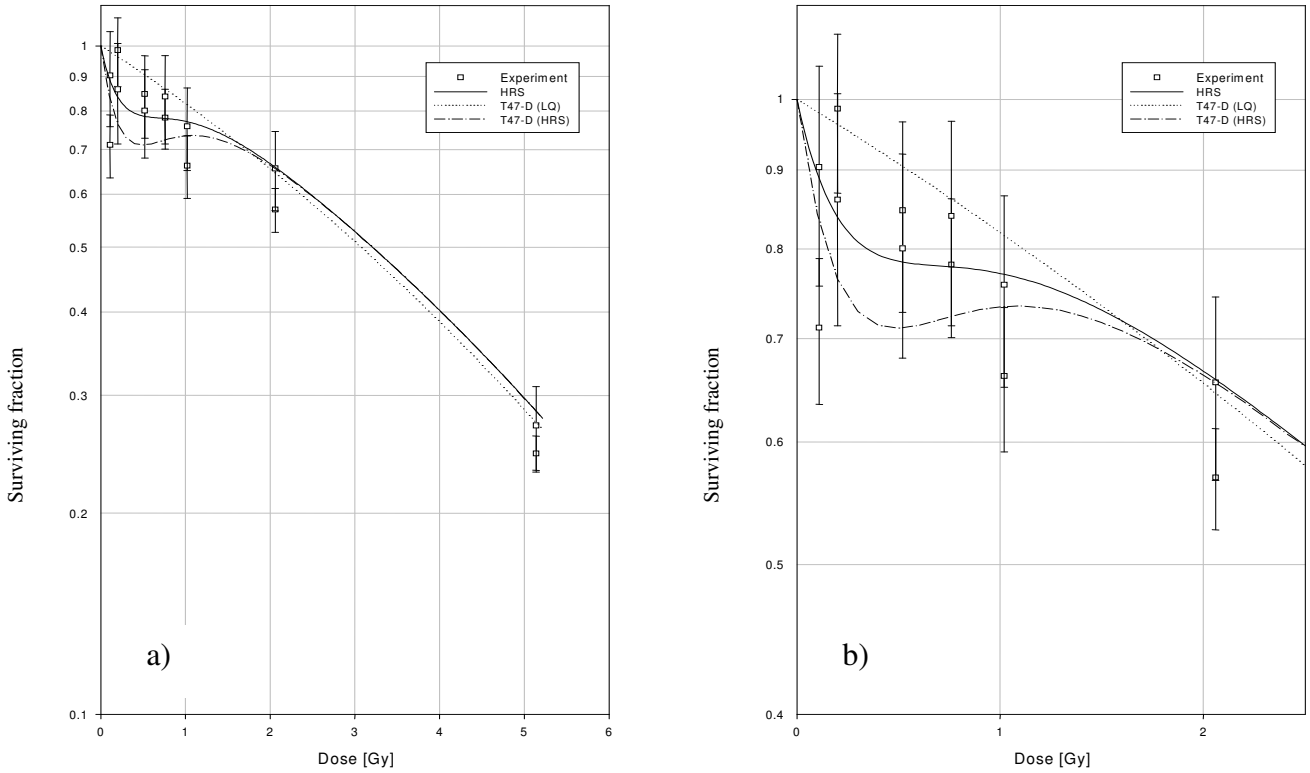


Figure 4.4: *Surviving fraction as a function of dose. Data points represent single observations of two independent experiments for T-47D cells given a HDR priming dose of 0.3 Gy 24 hours prior to the challenge doses and vertical bars represent standard errors. The curves represent model fits to the data: The IR-model was fitted to the data points shown (solid line). Also shown are the fit of the LQ-model (dotted line) and the IR-model (dashed line) to data from unprimed T-47D cells. a): All data included. b): Only data up to 2 Gy included.*

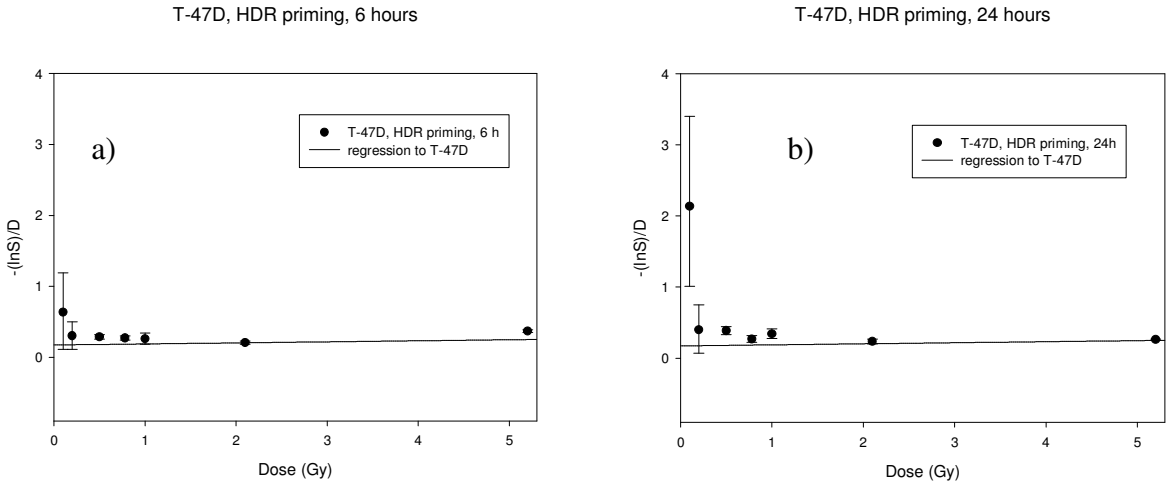


Figure 4.5: *Effect per unit dose. -(lnS)/D = α + βD is plotted as a function of dose. T-47D cells were pre-exposed to a HDR priming dose of 0.3 Gy. The solid line represents linear regression to data for unprimed T-47D cells (figure 4.2 a). The interval between priming dose and challenge dose were either a) 6 hours or b) 24 hours.*

Figure 4.5 and figure 4.7 show the effect-per-unit-dose as a function of dose. The solid line represents the best fit of the LQ-model to the data for T-47D cells without priming from figure 4.2a. It is seen to fit the data quite well, which is also the case relative to the survival curves fitted by the LQ-model to data from unprimed cells (dotted line) as seen in figures 4.3, 4.4, and 4.6, and this justifies the practice of using the fixed values of α and β in the fittings.

The α_s and α_s/α_r values from the IR-fits are shown in table 4. The degree of HRS relative to a back-extrapolation of the LQ-curve is reflected in the size of α_s/α_r. α_s/α_r dropped from 13.1 without priming to 5.4 (4.2 in experiment T9 with priming dose-rate 0.9 Gy/h) six hours after a priming dose of 0.3 Gy. After another 18 hours following the priming dose the α_s/α_r had increased to 8.3 which is approaching, but still significantly lower than, the 13.1 for the unprimed cells.

In two experiments the cells that were used had been pre-irradiated by incorporated tritium for several months at a dose-rate to the cell nucleus of 0.01 Gy/h. The total dose received by this low-dose pre-irradiation at the time of the two acute irradiation experiments was 24.7 Gy and 36.2 Gy, respectively. The survival curve of the subsequent acute irradiation is shown in figure 4.8. The HRS is totally absent and the data are best fitted by the LQ-model. Figure 4.9 shows the effect per unit dose. The solid line represents the best fit of the LQ-model to the data for T-47D without priming from figure 4.2 a.

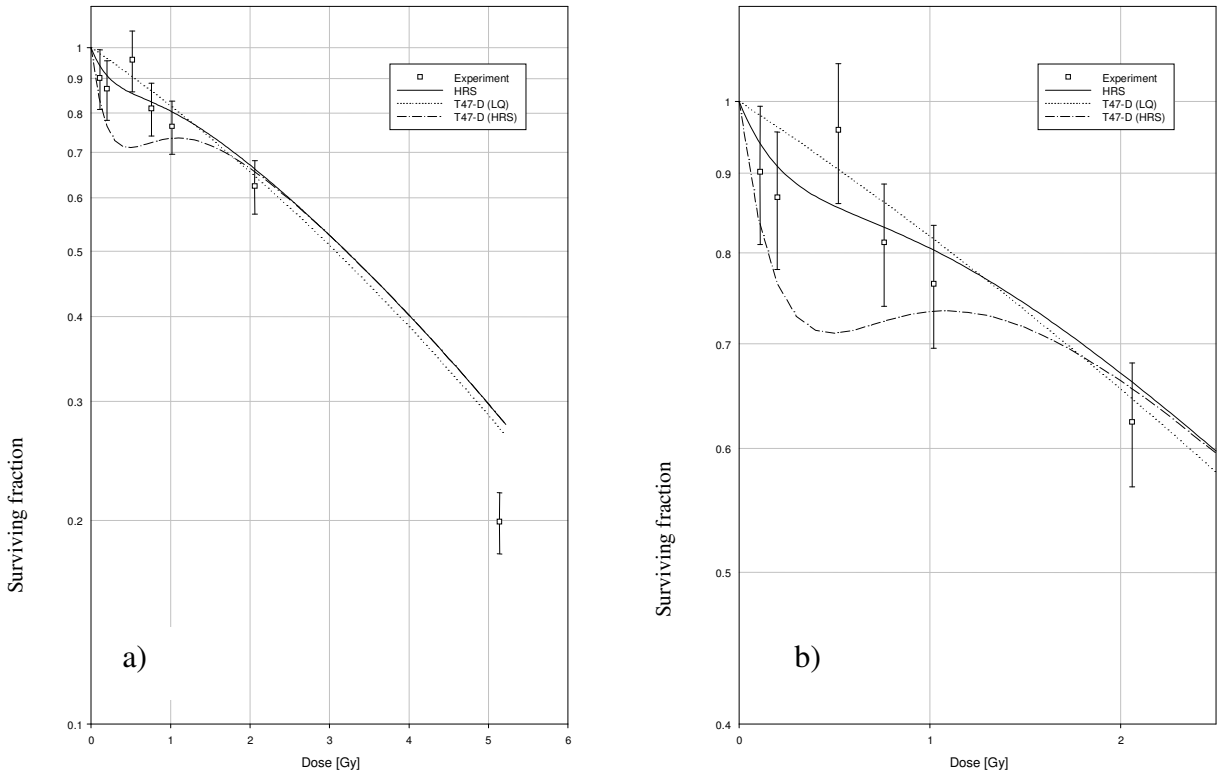


Figure 4.6: *Surviving fraction as a function of dose. Data points represent one experiment for T-47D cells given a 0.3 Gy priming dose with dose-rate 0.9 Gy/h prior to the challenge doses and vertical bars represent standard errors. The curves represent model fits to the data: The IR-model was fitted to the data points shown (solid line). Also shown are the fit of the LQ-model (dotted line) and the IR-model (dashed line) to data from unprimed T-47D cells. a): All data included. b): Only data up to 2 Gy included.*

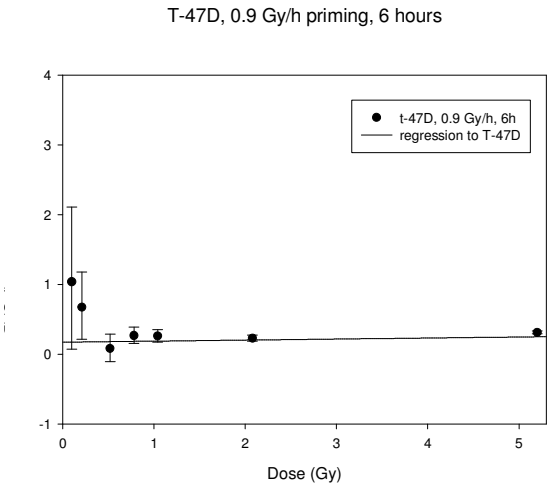


Figure 4.7: *Effect per unit dose. -(lnS)/D = α + βD is plotted as a function of dose. T-47D cells were pre- exposed to a priming dose of 0.3 Gy given with 0.9 Gy/h 6 hours before challenge dose. The solid line represents linear regression to data for unprimed T-47D cells (figure 4.2 a).*

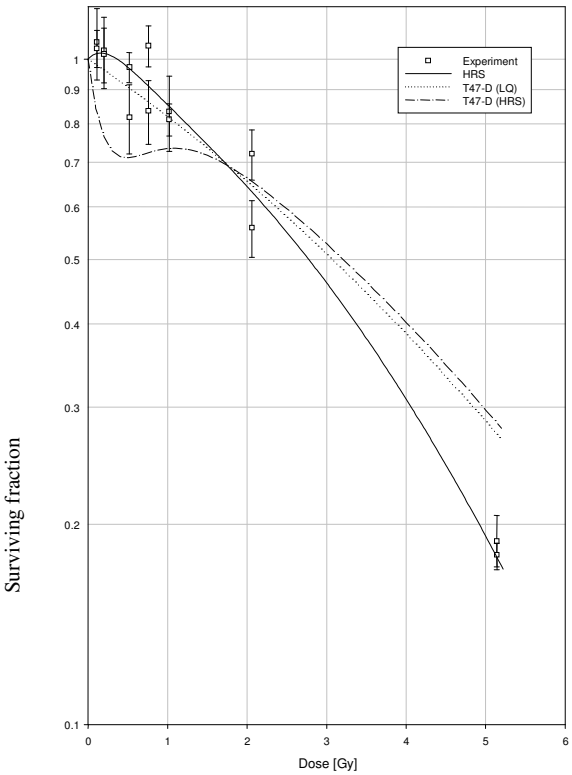


Figure 4.8: *Surviving fraction as a function of dose. Data points represent single observations of two independent experiments for T-47D cells pre-irradiated by incorporated tritium for several months at a dose-rate to the nucleus of 0.01 Gy/h. The curves represent model fits to the data: The IR-model was fitted to the data points shown (solid line). Also shown are the fit of the LQ-model (dotted line) and the IR-model (dashed line) to data from unprimed T-47D cells.*

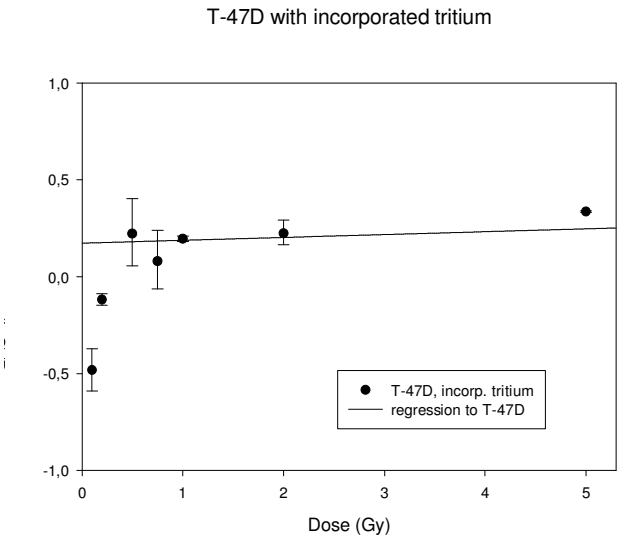


Figure 4.9: *Effect per unit dose. $-(\ln S)/D = \alpha + \beta D$ is plotted as a function of dose. T-47D cells were pre-irradiated by incorporated tritium for several months at a dose-rate to the nucleus of 0.01 Gy/h. The solid line represents linear regression to data for unprimed T-47D cells (figure 4.2 a).*

Priming dose (Gy)	**Priming dose-rate** (Gy/h)	**Time between priming and challenge doses** (h)	α_s (Gy^{-1})	α_s/α_r
0.3	HDR	6	0.9 ± 0.2	5.4 ± 1.2
0.3	HDR	24	1.4 ± 0.4	8.3 ± 2.4
0.3	0.9	6	0.7 ± 0.5	4.2 ± 3.0
~30	0.01	0	(-0.03 ± 0.3)	
none			2.2 ± 0.5	13.1 ± 3.1

Table 4: *α_s and α_s/α_r -values from the fits to the **IR-model** of data from experiments with **T-47D** cells. The size of the α_s/α_r-values reflects the degree of HRS.*

4.3 FLOW CYTOMETRY DATA

A DNA histogram normally has two peaks, one for cells with DNA content corresponding to G_1- phase and the second for cells with the double amount of DNA corresponding to G_2- and M-phase. In between the two peaks, cells in the S-phase are registered. The areas of the peaks reflect the number of cells in the particular phases. As the duration of the G_1-phase is generally considerably longer than G_2- and M-phase, the first peak (normalized to channel 200) is much larger than the second peak. T-47D cells spend 23.6±2.9 hours in G_1 and the full cell cycle lasts 37.2±2.0 hours (Stokke et al., 1993). These numbers are well in line with the DNA histogram shown in figure 4.10 a, of T-47D cells prepared in May 2002. However, when a DNA histogram was made of the T-47D cells in December 2002, a change from the usual pattern was discovered (figure 4.10 b). This DNA histogram indicates that our cell line at that time contained two different stem lines with different DNA content. The indications that there could be a mix of cells were confirmed after repeated investigations in January 2003. In addition to the normal spectrum, the histogram in figure 4.10 b shows a large peak close to channel 400 and a smaller one with twice the DNA content. Although such a histogram is usually taken to indicate the presence of another cell line, one can not exclude that there is a population of T-47D cells that for some reason has an altered DNA-ploidy.

We wanted to test whether we could have had a mix-in from another cell line cultured in our laboratory. Of the cell lines present in the laboratory, The T-47D cells adapted to hydroxyurea (HU) and MCF-7 were quickly ruled out as candidates (data shown in appendix F). The remaining possibility was NHIK 3025 cells. The DNA histogram of cells of this cell line is shown in figure 4.10 c. A comparison of panel b and c in figure 4.10 shows a convincing agreement between DNA histograms of the mixed cells and NHIK 3025 cells.

The experiment in which cells with incorporated tritium were irradiated was initiated at a time where the cells could have been of the T-47D_{mix}-type. The similarity between the DNA histograms of the control group (figure 4.11 a) and of T-47D_{mix} (figure 4.10 b) confirm that this was the case. The DNA histogram of the irradiated cells (figure 4.11 b) was on the other hand in exact agreement with the one of pure T-47D cells (figure 4.10 a).

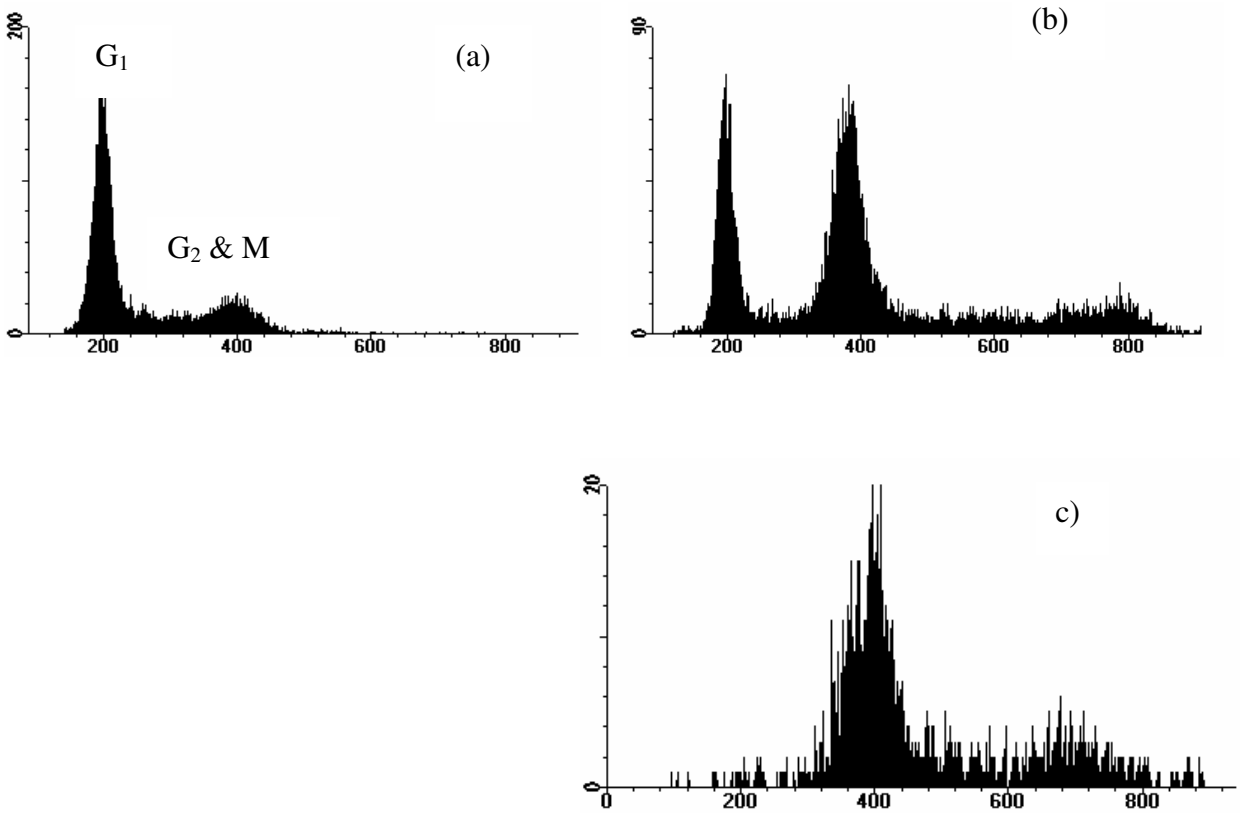


***Figure 4.10**: DNA histograms. The relative cell number is plotted against the relative DNA content given as channel numbers of the multichannel analyzer used. a): DNA histogram of T-47D cells (May 2002). The first peak contains the cells with a DNA amount corresponding to G_1-phase and the second the cells with twice the amount corresponding to G_2- and M-phase. In between the S-phase cells are registered. b): The DNA histogram for T-47D cells prepared December 2002 shows a mixture of two stem lines having different DNA content (T-47D$_{mix}$). c) DNA histogram of NHIK 3025 cells.*
Note that in panels a and b the ordinate axes are placed at channel number 80, while in panel c it is placed at channel number 0.

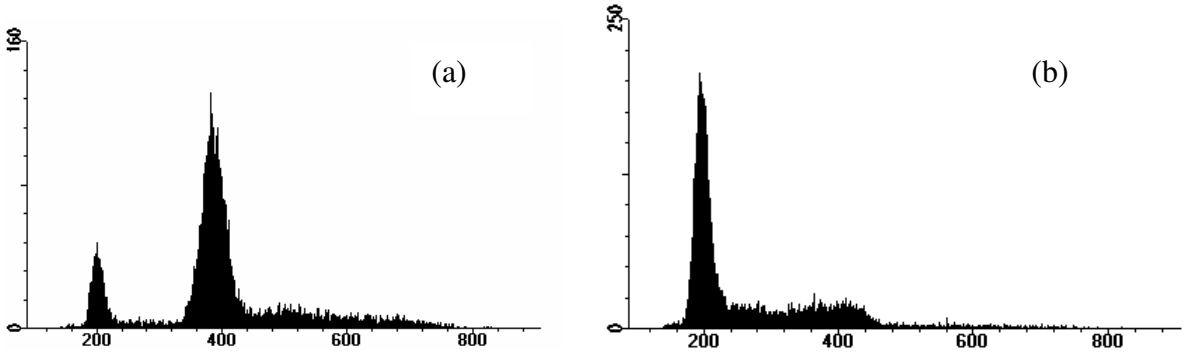


***Figure 4.11** DNA histograms. The relative cell number is plotted against the relative DNA content given as channel numbers of the multichannel analyzer used. a): The unirradiated cells from the experiment with incorporated tritium. b): Cells irradiated by incorporated tritium for several month at a dose-rate to the cell nucleus of 0.01 Gy/h. Both these cell types were cultured in a medium containing 1 mM valine, which is a higher concentration than in usual RPMI medium.*

4.4 SURVIVAL CURVES OF THE MIXED CELL POPULATION

The effect-per-unit-dose as a function of dose plot for the T-47D$_{mix}$ cells (i.e. the T-47D cells with an extra unknown stem line) (experiments T20-T22) is shown in figure 4.14. A fit of the LQ-model was done to the data from 2Gy and up by linear regression. The α and β values determined from figure 4.14 (table 5) was used in the fitting of the IR-model shown in the survival curve in figure 4.12 (dashed line with double dots), which was only done to data below 1 Gy simply in order to make the best fitting to the data. The dose response of a mixed population can probably not be expected to be described by the standard models, so certain variations were tried as to which parameters to fix and which data to weight in all the fittings to data from this cell population. Even then it is seen from the resultant survival curves (figures 4.12, 4.15, 4.17, 4.19, 4.21, 4.23, and 4.25) that the IR-model in most cases does not make convincing fittings to the data.

Figure 4.13 shows the mean surviving fraction data in addition to the respective curve fits by the IR-model for T-47D (dotted line) and T-47D$_{mix}$ cells (dashed line with double dots) and by the LQ-model for NHIK 3025 cells (solid line). Values of the parameters of the fit to data for T-47D$_{mix}$ cells are shown in table 5. The T-47D$_{mix}$ cells seem to be more sensitive to doses larger than 5 Gy than pure T-47D cells although they still have a higher survival than the NHIK 3025 cells. At doses less than 1 Gy the T-47D$_{mix}$ cells show HRS in contrast to the NHIK 3025 cells, though somewhat less than for the pure T-47D cells. Figure 4.12 and figure 4.13 also include a theoretically calculated curve for a mixed population of T-47D and NHIK 3025 cells, respectively (dashed line). The weighting of the NHIK 3025 parameters was chosen to be 55 % from an estimation of the areas of the peaks in the DNA histogram for T-47D$_{mix}$ (figure 4.10 b). In figure 4.12 it is seen that the calculated curve of this theoretical mix makes a possible fit though not the best one to the T-47D$_{mix}$ data. The fitting of the T-47D$_{mix}$ data as well as the calculated curve of the theoretical mixed population of T-47D and NHIK 3025 cells is seen to lie above the averaged data points in the hypersensitive dose range and in this dose region the cells seem to respond much more like T-47D than NHIK 3025 cells. In the high dose range the T-47D$_{mix}$ survival data show lower survival than both T-47D cells and the calculated curve for the theoretically mixed population of T-47D and NHIK 3025 cells predicts, and in this dose range the response is closer to that of NHIK 3025 cells.

α_r (Gy^{-1})	β (Gy^{-2})	α/β (Gy)	α_s (Gy^{-1})	D_c (Gy)	α_s/α_r
0.0989 ± 0.2375	0.0337 ± 0.0491	2.9	1.08 ± 0.39	45 ± 13	10.9

Table 5: *Values of parameters from two different fittings. α_r and β are found from linear regression by the LQ-model to* ***T-47D$_{mix}$*** *data as plotted in the effect per unit dose-plot (figure 4.14) and α_s and Dc are parameters of the fit by the IR-model to* ***T-47D$_{mix}$*** *data as plotted in the survival curve (figure 4.12).*

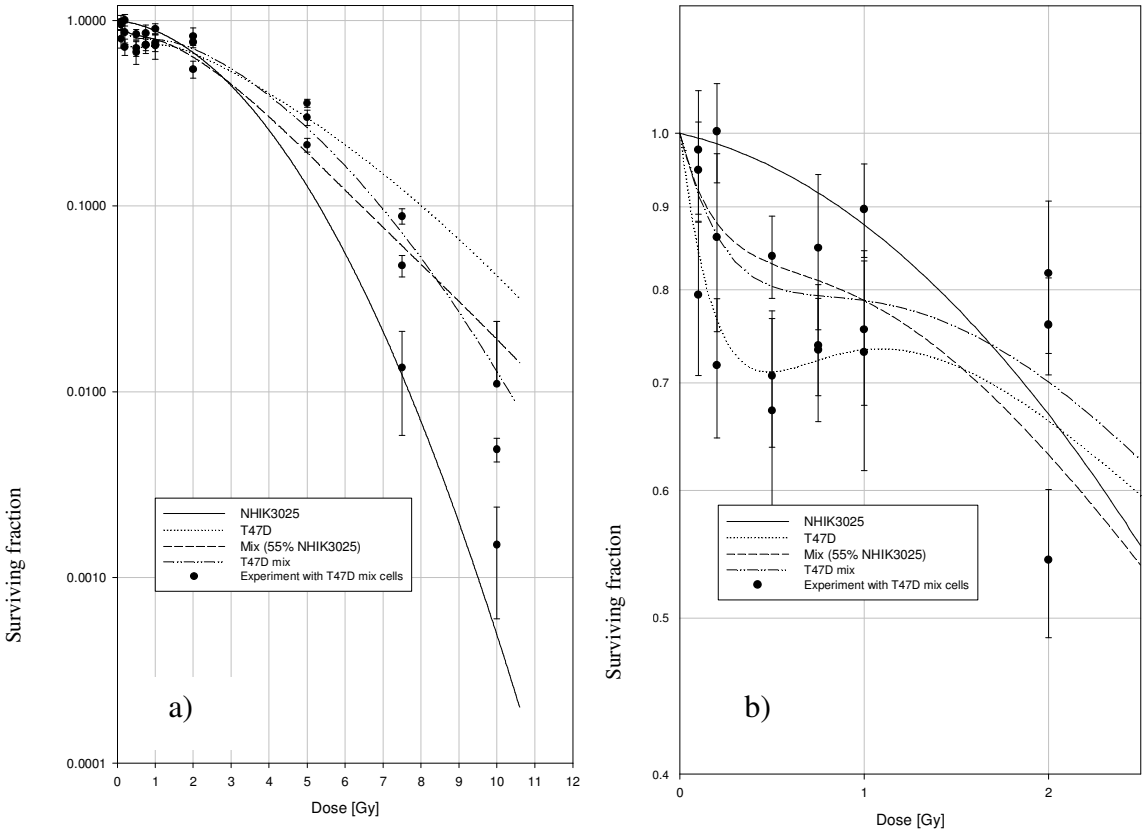


Figure 4.12: *Surviving fraction as a function of dose. Data points of T-47D$_{mix}$ cells represent single observations of three independent experiments and vertical bars represent standard errors. The curves represent model fits to the data: The IR-model was fitted to the data points below 1 Gy with fixed values of α_r and β obtained from the fit done to the data in the effect-per-unit-dose plot (figure 4.14) (dashed line with double dots). Also shown are the fit of the IR-model to data for T-47D cells (dotted line) and the LQ-model to data for NHIK 3025 cells along with the calculated curve for the theoretically mixed population of 55 % NHIK 3025 and 45 % T-47D cells. a): All data included. b): Only data up to 2 Gy included.*

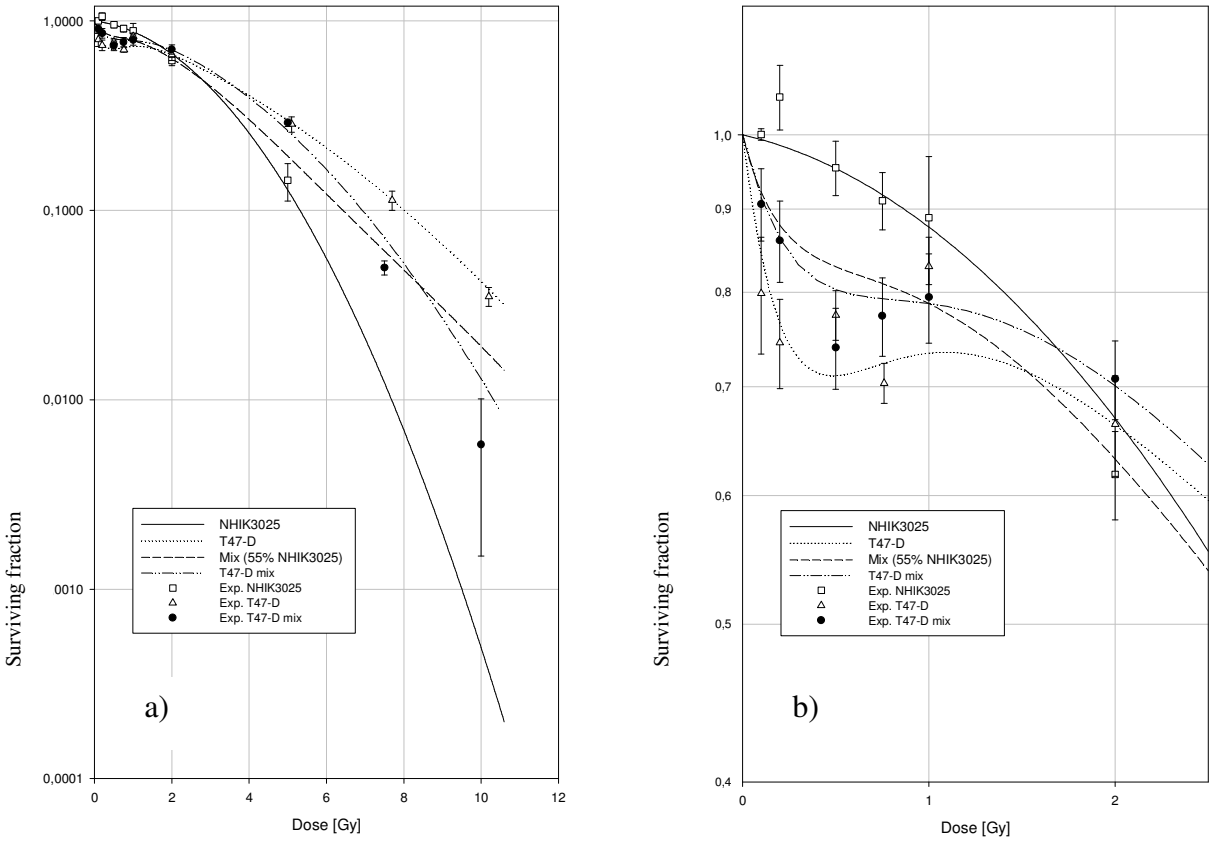


Figure 4.13: *Surviving fraction as a function of dose for NHIK 3025 cells (□), T-47D cells (Δ), and T-47D$_{mix}$ cells (●). Data points represent mean values of three independent experiments and vertical bars represent standard errors. The curves represent model fits to the data: The IR-model was fitted to the data for T-47D$_{mix}$ cells (dashed line with double dots) and to data for T-47D cells (dotted line) and the LQ-model to data for NHIK 3025 cells. Also shown is the calculated curve for the theoretically mixed population of 55 % NHIK 3025 and 45 % T-47D cells. a): All data included. b): Only data up to 2 Gy included.*

T-47D$_{mix}$

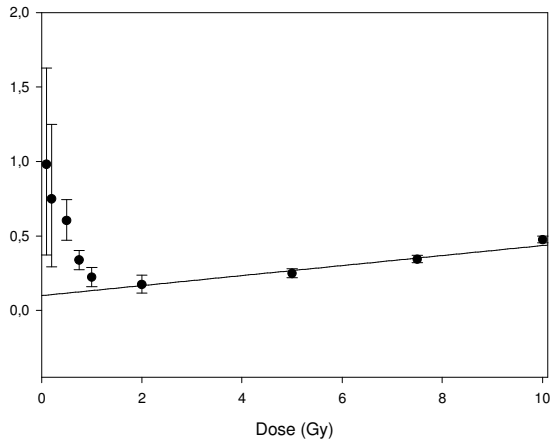


Figure 4.14: *Effect per unit dose. -(lnS)/D = α + βD is plotted as a function of dose. α and β was found by linear regression of the LQ-model to data for* ***T-47D$_{mix}$*** *cells (solid lines) for doses larger than 2 Gy.*

4.5 ADAPTIVE RESPONSE IN THE MIXED CELL POPULATION

The cells used in the experiments with the priming dose given with low dose-rates turned out to be of the mixed cell population. Also one of the experiments with HDR priming happened to be with T-47D$_{mix}$ cells. In all the fittings used the α and β values from the unprimed T-47D$_{mix}$ (table 4) were used and the fitting of the IR –model was done to the complete data sets except for the experiment with HDR priming 6 hours before the challenge dose, where only data for doses below 1 Gy was used in order to obtain a satisfying fit (figure 4.15 and figure 4.17). Since the HDR priming was done in only one single experiment the interpretation of figure 4.15 and 4.17 and the effect-per-unit-dose versus dose plots (figure 4.16 and figure 4.18) is far from conclusive. There is no doubt that the HRS has vanished 6 hours after priming and there is a tendency of recovery 24 hours after priming but not nearly as strong as for the pure T-47D cells. Six experiments were done with low-dose-rate (LDR) priming (using a priming dose of 0.3 Gy) with dose-rates of 0.32 Gy/h (figure 4.19 and figure 4.21) or 0.045 Gy/h (figure 4.23 and figure 4.25). Even these LDR priming doses was seen to reduce HRS 6 hours after the priming dose (figure 4.19 and figure 4.23). When the dose-rate of the priming dose was 0.32 Gy/h, the HRS had totally vanished after 24 hours (figure 4.21), and also with dose-rate 0.045 Gy/h there is little left if any (figure 4.25). The effect-per-unit-dose versus dose plot for the experiment with the dose-rate of 0.32 Gy/h of the priming dose is shown in figure 4.20 and figure 4.22. It is seen that the α and β values from the unprimed T-47D$_{mix}$ fitting (figure 4.11) make convincing fits to most data points of all experiments with primed cell as they would in absence of HRS (figures 4.16, 4.18, 4.20, 4.22, 4.24, and 4.26). Values of α_s, α_s/α_r, and D_c are given in table 6, but since it is questionable to use the IR-model in the case of a mixed population of cells, these values may contain systematic uncertainties.

Because of the problems with applying the IR-model to the data of the mixed population of cells, the plots of surviving fraction versus dose without any fittings are shown for experiments with T-47D$_{mix}$ cells given a priming dose of 0.3 Gy with the low dose-rates (figure 4.27). The same trends as mentioned above are seen and in these plots they appear more convincing.

Priming dose (Gy)	**Priming dose-rate** (Gy/h)	**Time between priming and challenge doses** (h)	α_s (Gy^{-1})	D_c (Gy)	α_s / α_r
0.3	HDR	6	-36 ± 52	0.027 ± 0.010	
0.3	HDR	24	0.52 ± 1.72	0.16 ± 80	5.3
0.3	0.32	6	0.310 ± 0.079	0.73 ± 0.24	3.1
0.3	0.32	24	0.23 ± 2.30	0.18 ± 0.94	2.3
0.3	0.045	6	0.32 ± 0.16	0.63 ± 0.44	3.2
0.3	0.045	24	0.32 ± 0.20	0.50 ± 0.26	3.2
None			1.09 ± 0.38	0.45 ± 0.13	11.0

Table 6: *α_s and α_s/α_r -values from the fits to the **IR-model** of data from experiments with **T-47D$_{mix}$** cells. The size of the α_s/α_r-values reflects the degree of HRS.*

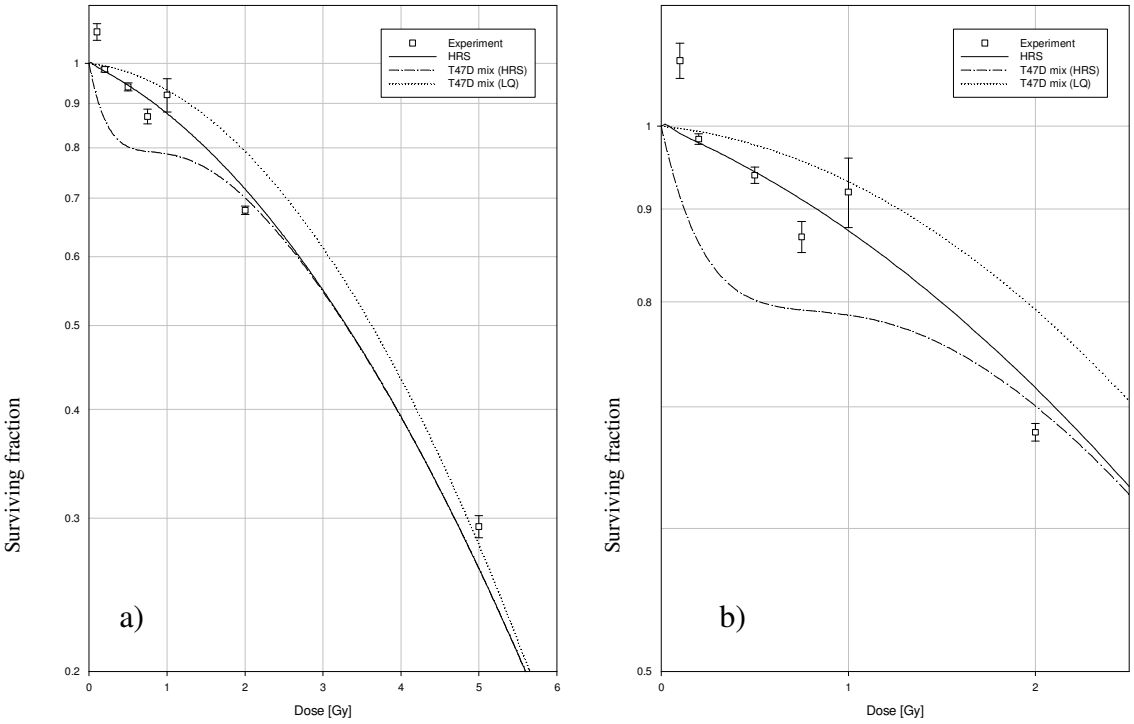


Figure 4.15: *Surviving fraction as a function of dose. Data points represent one experiment for T-47D$_{mix}$ cells given a HDR priming dose of 0.3 Gy 6 hours prior to the challenge doses and vertical bars represent standard errors. The curves represent model fits to the data: The IR-model was fitted to the data points shown (solid line). Also shown are the fit of the LQ-model (dotted line) and the IR-model (dashed line) to data from unprimed T-47D$_{mix}$ cells. a): All data included. b): Only data up to 2 Gy included.*

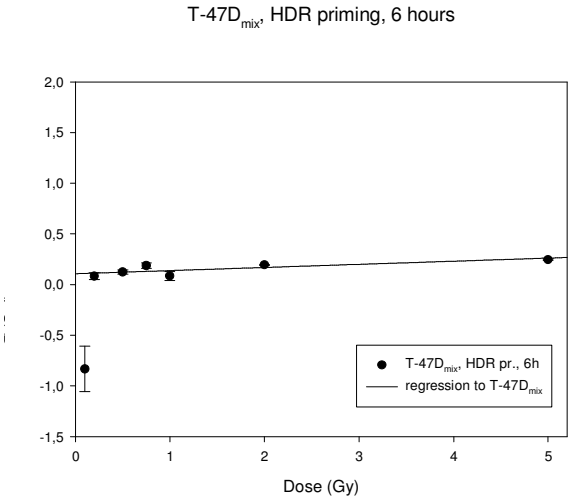


Figure 4.16: *Effect per unit dose. -(lnS)/D = α + βD is plotted as a function of dose. T-47D$_{mix}$ cells were exposed to a HDR priming dose of 0.3 Gy. The solid line represents linear regression to data for unprimed T-47D$_{mix}$ cells (figure 4.14). The interval between priming dose and challenge dose was 6 hours.*

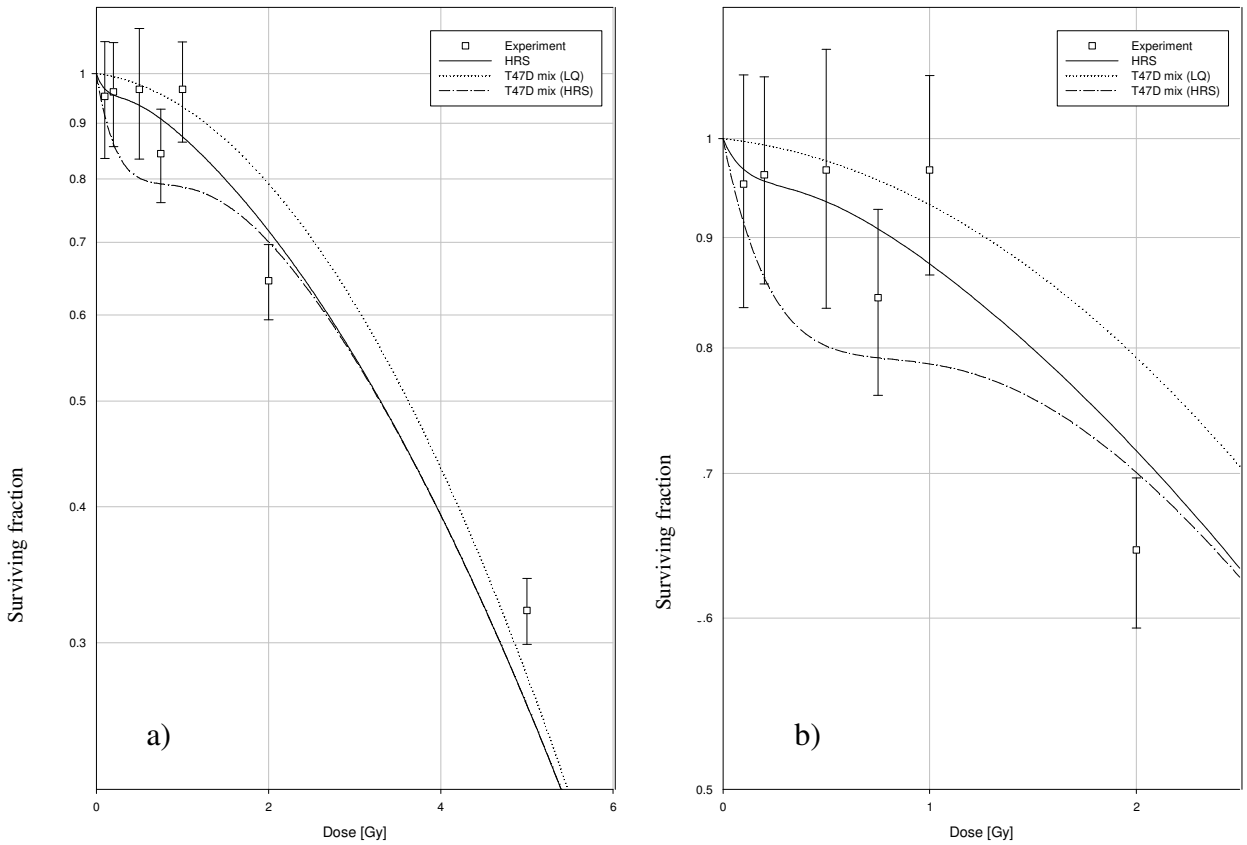


Figure 4.17: *Surviving fraction as a function of dose. Data points represent one experiment for T-47D$_{mix}$ cells given a HDR priming dose of 0.3 Gy 24 hours prior to the challenge doses and vertical bars represent standard errors. The curves represent model fits to the data: The IR-model was fitted to the data points shown (solid line). Also shown are the fit of the LQ-model (dotted line) and the IR-model (dashed line) to data from unprimed T-47D$_{mix}$ cells. a): All data included. b): Only data up to 2 Gy included.*

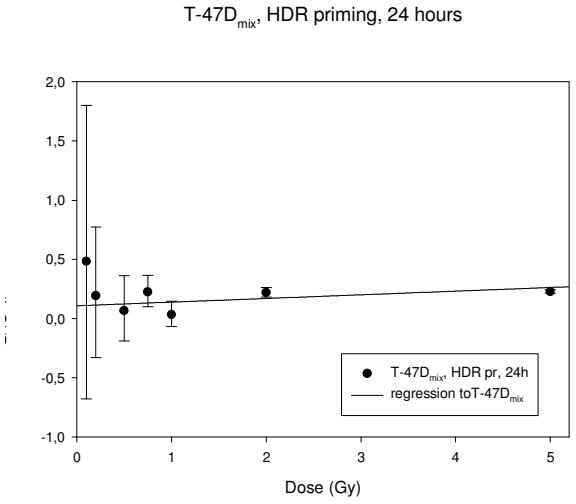


Figure 4.18: *Effect per unit dose. -(lnS)/D = α + βD is plotted as a function of dose. T-47D$_{mix}$ cells were exposed to a HDR priming dose of 0.3 Gy. The solid line represents linear regression to data for unprimed T-47D$_{mix}$ cells (figure 4.14). The interval between priming dose and challenge dose was 24 hours.*

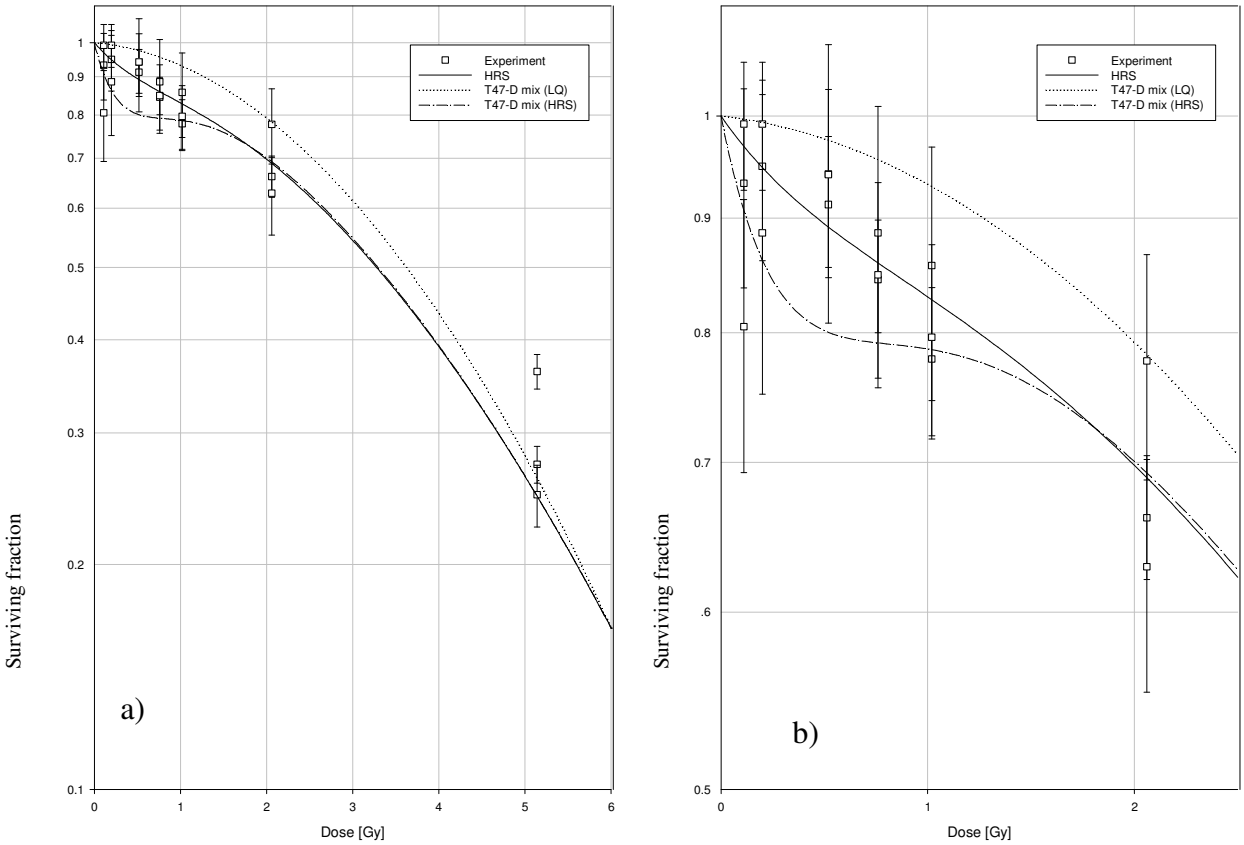


Figure 4.19: *Surviving fraction as a function of dose. Data points represent single observations of three experiments for T-47D$_{mix}$ cells given a priming dose of 0.3 Gy with dose-rate 0.32 Gy/h 6 hours prior to the challenge doses and vertical bars represent standard errors. The curves represent model fits to the data: The IR-model was fitted to the data points shown (solid line). Also shown are the fit of the LQ-model (dotted line) and the IR-model (dashed line) to data from unprimed T-47D$_{mix}$ cells. a): All data included. b): Only data up to 2 Gy included.*

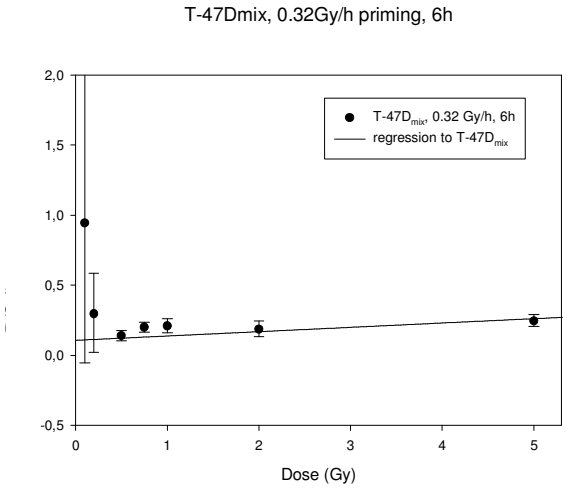


Figure 4.20: *Effect per unit dose. -(lnS)/D = α + βD is plotted as a function of dose. T-47D$_{mix}$ cells were exposed to a priming dose of 0.3 Gy with dose-rate 0.32 Gy/h. The solid line represents linear regression to data for unprimed T-47D$_{mix}$ cells (figure 4.14). The interval between priming dose and challenge dose was 6 hours.*

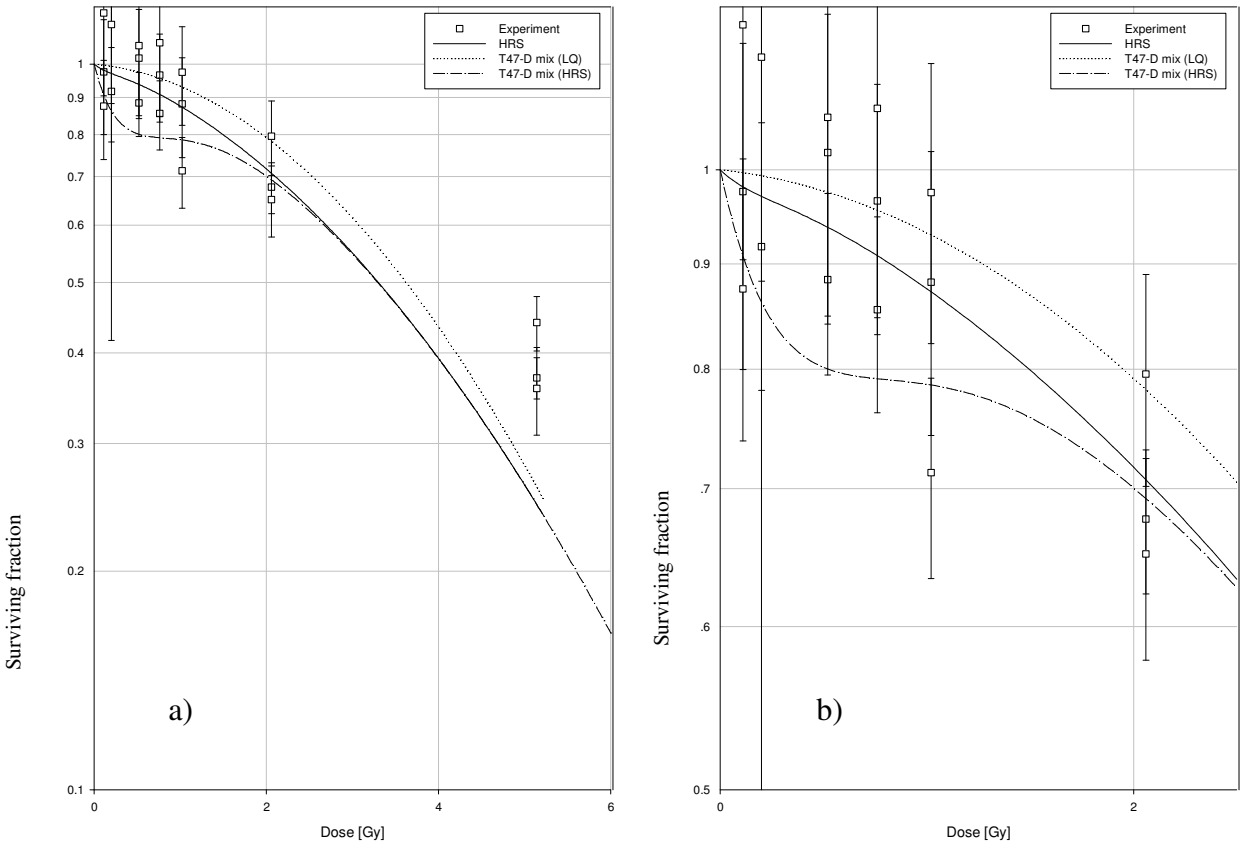


Figure 4.21: *Surviving fraction as a function of dose. Data points represent single observations of three experiments for T-47D$_{mix}$ cells given a priming dose of 0.3 Gy with dose-rate 0.32 Gy/h 24 hours prior to the challenge doses and vertical bars represent standard errors. The curves represent model fits to the data: The IR-model was fitted to the data points shown (solid line). Also shown are the fit of the LQ-model (dotted line) and the IR-model (dashed line) to data from unprimed T-47D$_{mix}$ cells. a): All data included. b): Only data up to 2 Gy included.*

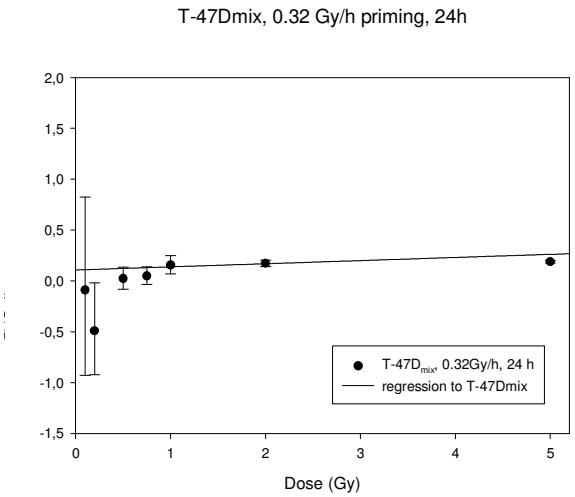


Figure 4.22: *Effect per unit dose. -(lnS)/D = α + βD is plotted as a function of dose. T-47D$_{mix}$ cells were exposed to a priming dose of 0.3 Gy with dose-rate 0.32 Gy/h. The solid line represents linear regression to data for unprimed T-47D$_{mix}$ cells (figure 4.14). The interval between priming dose and challenge dose was 24 hours.*

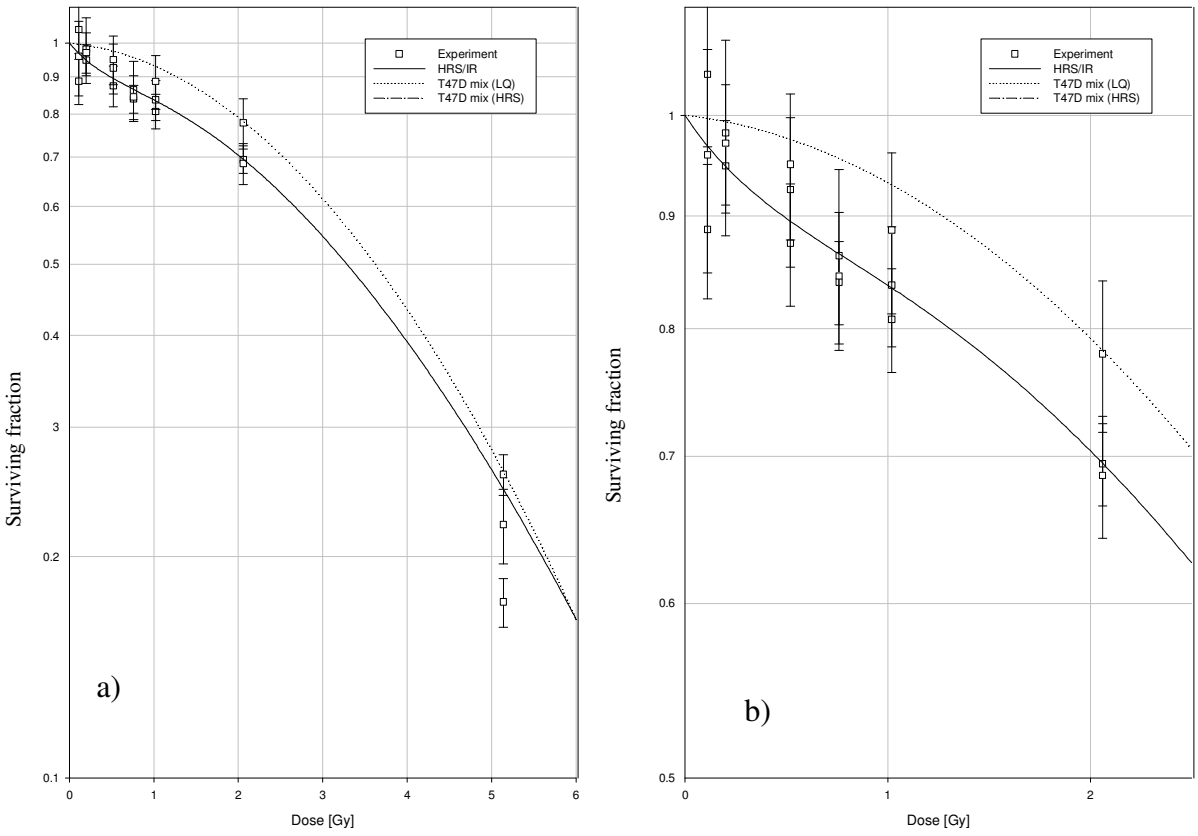


Figure 4.23: *Surviving fraction as a function of dose. Data points represent single observations of three experiments for T-47D$_{mix}$ cells given a priming dose of 0.3 Gy with dose-rate 0.045 Gy/h 6 hours prior to the challenge doses and vertical bars represent standard errors. The curves represent model fits to the data: The IR-model was fitted to the data points shown (solid line). Also shown are the fit of the LQ-model (dotted line) and the IR-model (dashed line) to data from unprimed T-47D$_{mix}$ cells. a): All data included. b): Only data up to 2 Gy included.*

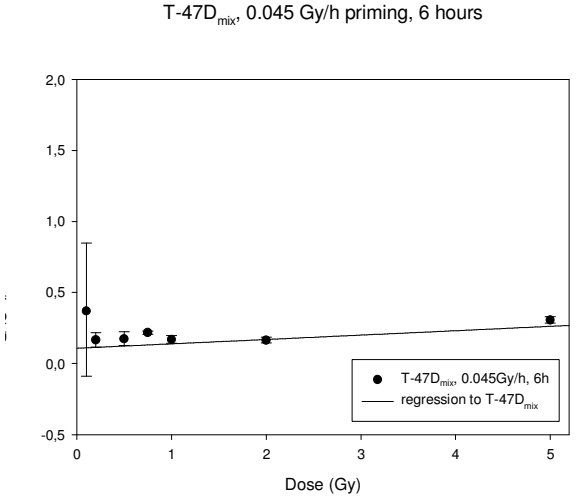


Figure 4.24: *Effect per unit dose. -(lnS)/D = α + βD is plotted as a function of dose. T-47D$_{mix}$ cells were exposed to a priming dose of 0.3 Gy with dose-rate 0.045 Gy/h. The solid line represents linear regression to data for unprimed T-47D$_{mix}$ cells (figure 4.14). The interval between priming dose and challenge dose was 6 hours.*

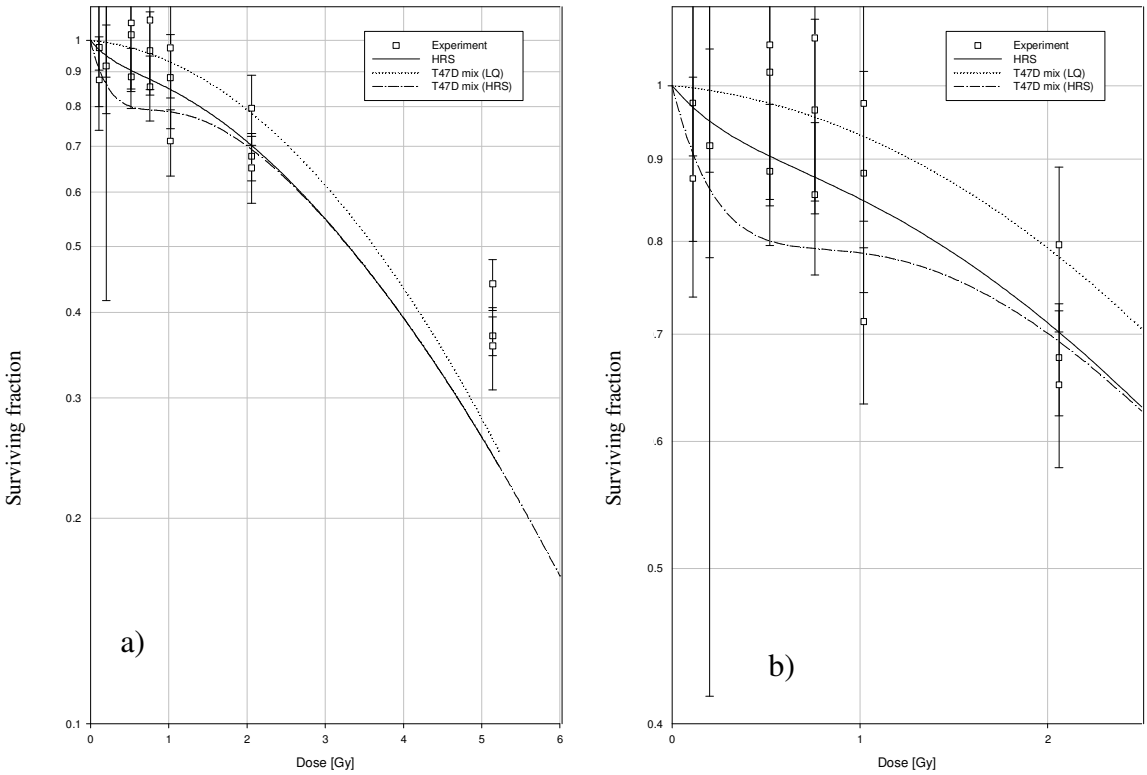


Figure 4.25: *Surviving fraction as a function of dose. Data points represent single observations of three experiments for T-47D$_{mix}$ cells given a priming dose of 0.3 Gy with dose-rate 0.045 Gy/h 24 hours prior to the challenge doses and vertical bars represent standard errors. The curves represent model fits to the data: The IR-model was fitted to the data points shown (solid line). Also shown are the fit of the LQ-model (dotted line) and the IR-model (dashed line) to data from unprimed T-47D$_{mix}$ cells. a): All data included. b): Only data up to 2 Gy included.*

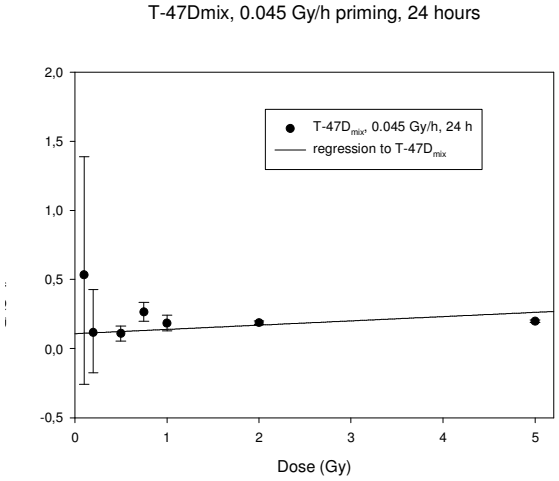
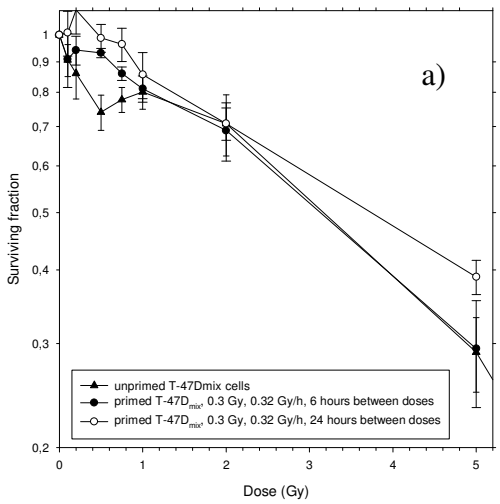
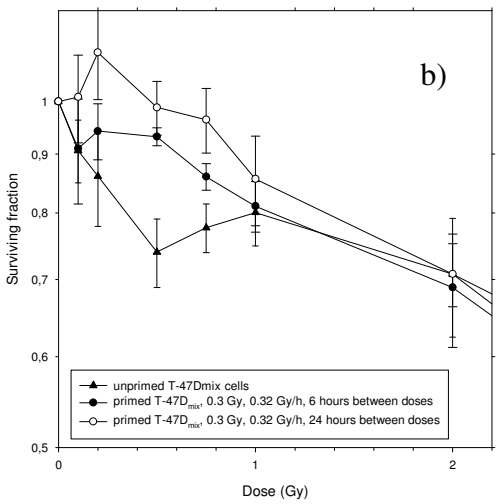


Figure 4.26: *Effect per unit dose. -(lnS)/D = α + βD is plotted as a function of dose. T-47D$_{mix}$ cells were exposed to a priming dose of 0.3 Gy with dose-rate 0.045 Gy/h. The solid line represents linear regression to data for unprimed T-47D$_{mix}$ cells (figure 4.14). The interval between priming dose and challenge dose was 24 hours.*

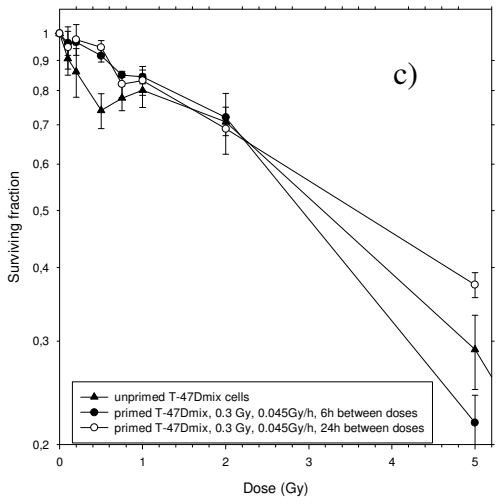
T-47D$_{mix}$ cells, unprimed and with a 0.3 Gy priming dose delivered with 0.32 Gy/h mean values of surviving fraction from three independent experiments.



T-47D$_{mix}$ cells, unprimed and with a 0.3 Gy priming dose delivered with 0.32 Gy/h mean values of surviving fraction from three independent experiments.



T-47D$_{mix}$ cells, unprimed and with a 0.3 Gy priming dose delivered with 0.045 Gy/h mean values of surviving fraction from three independent experiments.



T-47D$_{mix}$ cells, unprimed and with a 0.3 Gy priming dose delivered with 0.045 Gy/h mean values of surviving fraction from three independent experiments.

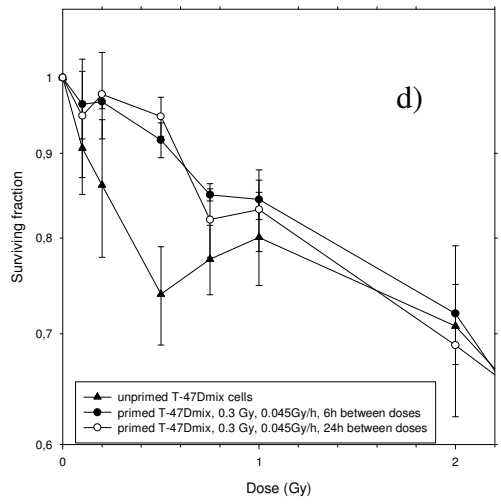


Figure 4.27: *Surviving fraction as a function of dose for T-47D$_{mix}$ cells. Data points represent mean values of three independent experiments and vertical bars represent standard errors. In all plots data points of unprimed cell are marked with ▲, data points of cells given the priming dose 6 hours before the challenge dose are marked with ●, and data points of cells given the priming dose 24 hours before the challenge dose are marked with ○. a): Dose-rate of priming dose is 0.32 Gy/h and all data are included. b): Dose-rate of priming dose is 0.32 Gy/h and only data up to 2 Gy are included. c) Dose-rate of priming dose is 0.045 Gy/h and all data are included. d) Dose-rate of priming dose is 0.045 Gy/h and only data up to 2 Gy are included.*

5 DISCUSSION

5.1 RADIATION RESPONSE FOLLOWING ACUTE IRRADIATION

Of the cell lines used in these experiments only V79 cells were known to express HRS as a response to low dose irradiation. The first experiments in this study (experiments T1-T6, acute irradiation of T-47D cells and experiments V1-V6, acute irradiation of V79 cells) not only revealed that T-47D cells do express HRS but also that it is much more pronounced than for V79 cells (figure 4.1). This is consistent, however, with a general tendency for cells of human lines, which do express HRS, relative to V79 cells, which are of Chinese hamster origin (Joiner et al., 2001). Later, cells of a third line NHIK 3025 of human origin were investigated, and in these cells no HRS was found; the cells of this cell line were also much more radiosensitive to high doses of radiation than those of the other two lines (figure 4.1).

For V79 cells the survival curve of the present study is in concordance with those published by Joiner at al. (1996) within the whole dose range examined (table 8). Several other reports show lower cell survival for cells of various V79 sublines The results of Tsoulou et al. (2001) indicate a 4-5 times lower surviving fraction than that of the present study for 10 Gy, and also the survival found in the studies of Skwarchuk et al. (1993) and Skarsgard et al. (1993) were lower at 10 Gy than the value from the present study. For T-47D cells there is a clear HRS, which had not been detected in our previous studies since these did not include doses below 1 Gy. In addition the T-47D cells used in the present study seemed to be more resistant to high radiation doses than those studied in experiments listed in table 7. At 10 Gy the present data indicated a survival 5 to 10 times higher than those of Hanish and Furre. Also the survival of NHIK 3025 cells was found to be higher in the present study than expected from previous studies. At 10 Gy the surviving fraction recorded in the present experiments was 5 times higher than in that of Furre et al. (1999) and 3 times higher than that of Pettersen and Wang (1996) for asynchronous NHIK 3025 cells.

It seems that all the cell lines were more resistant to radiation in the present experiments as compared to most earlier investigations. The cells were irradiated with 60-cobalt γ-rays in the present and with x-rays in the other studies. The exception is the experiments performed by Tsoulou et al. (2001), but their experiments were performed with semi-confluent cells and can not be directly compared with those with exponentially growing cells. However, there is no particular reason to believe that the difference in radiation modality should account for the discrepancies.

Tables 7, 8 and 9 give the α/β values from previous studies on cells of various cell lines along with the values from the present study. The values listed in table 7 (for T-47D cells) and tables 9 (for NHIK 3025 cells) from the present study are from curve fittings based on the LQ model (figure 4.1 and table 1). As regards V79 cells, the values obtained from the LQ-fit (table 1)are in almost perfect agreement with the ones found using the IR-model (table 2), whereas the values obtained from the effect-per-unit-dose plot (figure 4.2 b and table 3), deviate to some extent from the values of the two other fits. When plotted in the effect-per-unit-dose plot the data points used in the linear regression do not include the point (D=0, f=1) and are not weighted. The values from the curve fit to data for V79 cells, presented in table 8, are based on the IR-model.

In the case of T-47D cells the discrepancy between values of the LQ-fit and the IR-fit is more pronounced. Possibly, the absence of data points below a survival of 0.01 inevitably reduces the influence of the β parameter on the fitting. The highest dose of 10 Gy resulted in a surviving fraction of 3-4 %, implying a 5 to 10 times higher survival than expected from the data of Lorentzen and Furre, which were used for planning of the experiment. The fit with the IR-model presumably gives the best values for α and β and is also in better agreement with the values obtained from the effect per unit dose plot, but since the comparative studies referred in table 7 do not include data for doses below 1 Gy for T-47D cells, the values from the fit with the LQ-model are used in table 7.

How much information that is gained from a comparison of the values for the α/β-ratio is doubtful as not only the radiation quality, dose-rate, and experimental set-up but also the cell-cycle distribution varies. The most trustworthy values for α/β-ratios are probably those obtained using synchronized or confluent cell populations. In exponentially growing cell populations, the survival curve is in reality a conjunction of responses of subpopulations with different radiosensitivities.

	Cell cycle phase	**Radiation quality**	**Dose-rate** (Gy/min)	α (Gy^{-1})	β (Gy^{-2})	***Surviving fraction at 10Gy***	α/β (Gy)
Ryste Hauge, 2000	Asynch-ronous	220 kVp x-rays	0.9	0.24 ± 0.02	0.033 ± 0.006	0.003	7.3 ± 1.3
Raaphorst and Boyden, 1999	Plateau phase	250 kVp x-rays	1.42				7.2 ± 1.9
Lorentzen, 2001	Asynch-ronous	80 kVp x-rays	1.72	0.16 ± 0.04	0.035 ± 0.005	0.006	4.0 ± 1.2
Hanisch, 1998	Asynch-ronous	Linear accelerator, 4.3 MV	1	0.278 ± 0.022	0.0155 ± 0.0020	0.01	17.9 ± 2.7
Furre, (Hanisch, 1998)	Asynch-ronous	Linear accelerator, 4.3 MV	4	0.269 ± 0.060	0.029 ± 0.0055	0.004	9.3 ± 2.7
Present study (figure 4.1, table 1)	Asynch-ronous	Co-60	0.9	0.186 ± 0.010	0.0129 ± 0.0012	0.04	14.4 ± 1.5

Table 7: *Values of parameters from fits with the LQ-model to survival data for* ***T-47D*** *cells from previous studies and the present. The surviving fraction at 10 Gy is calculated from equation 2.6 by use of the values of α and β from this table.*

	Cell cycle phase	**Radiation quality**	**Dose-rate** (Gy/min)	α_s (Gy^{-1})	α_r (Gy^{-1})	β (Gy^{-2})	D_c (Gy)	***Surviving fraction at 10Gy***	α/β (Gy)
Marples and Joiner, 1993	Asynch-ronous	250 kVp x-rays	0.016 & 0.44 & 1.7 (dose dependent)					0.05*	9.2
Skwarchuk et al., 1993.	Asynch-ronous V79-WNRE cells in suspension	250 kVp x-rays	2.3		0.250	0.0260		0.006	9.6
Skarsgard et al., 1993.	Asynch-ronous V79-171 cells in suspension	250 kVp x-rays	2.3		0.225 ± 0.03	0.0181 ± 0.0038		0.017	12.4 ± 3.1
Tsoulou et al., 2001	Semi-confluent	Co-60	1	1.83 ± 1.07	0.27 ± 0.10	0.02 ± 0.02	0.44 ± 0.28	0.009	13.5
Present study (figure 4.1, table 2)	Asynch-ronous	Co-60	0.9	0.54 ± 0.18	0.137 ± 0.002	0.0183 ± 0.0002	0.17 ± 0.06	0.04	7.4 ± 0.1

Table 8: *Values of parameters from fits with the IR-model to survival data for* ***V79*** *cells from previous studies and the present. The surviving fraction at 10 Gy is calculated from equation 2.6 by use of the values of α and β from this table, except when marked with* in which case the value was read from the survival plot.*

	Cell cycle phase	**Radiation quality**	**Dose-rate** (Gy/min)	α_r (Gy^{-1})	β (Gy^{-2})	***Surviving fraction at 10Gy***	α/β (Gy)
Furre, 1999	Asynchronous	Linear accelerator, 5 MV	4	0.47 ± 0.05	0.042 ± 0.005	0.0001	11.2 ± 1.8
Pettersen and Wang., 1996	G_1	220 kVp x-rays	1.6-1.9			0.00015*	
Present study (figure 4.1, table 1)	Asynchronous	Co-60	0.8	0.12 ± 0.02	0.064 ± 0.004	0.0005	1.9± 0.3

Table 9: *Values of parameters from fits with the LQ-model to survival data for* ***NHIK 3025*** *cells from previous studies and the present. The surviving fraction at 10 Gy is calculated from equation 2.6 by use of the values of α and β from this table, except when marked with* in which case the value was read from the survival plot.*

5.2 THE MIXED CELL POPULATION

DNA histograms from flow cytometry (see section 3.5) with T-47D cells, revealed the intrusion of another cell population a little less than twice the DNA content of parent T-47D

cells (figure 4.10 b). Two possibilities were considered: Either a subpopulation of T-47D cells could have emerged, having doubled DNA-ploidy or the T-47D culture could accidentally have been mixed with cells of one of the other cell lines cultured in the laboratory.

A comparison of the DNA histograms of the mixed cells (T-47D$_{mix}$) and NHIK 3025 cells (figure 4.10 b and c) discloses very small discrepancies between the DNA-ploidy of NHIK 3025-cells and the extra stemline appearing in the population measured in figure 4.10 b, and suggests a mix-in of NHIK 3025 cells into the T-47D culture. Survival experiments were then conducted with NHIK 3025 cells for comparison to the ones carried out for T-47D$_{mix}$ cells, and a curve was constructed based on a calculation of a theoretical mix of T-47D and NHIK 3025 cells with their respective parametric values. Based on an estimation of the areas of the peaks in the DNA histogram for T-47D$_{mix}$ cells (figure 4.6 b), the calculation was done on a mixture of 55 % NHIK 3025 cells and 45 % T-47D cells.

Within the hypersensitive dose range, neither the curve fit based on the IR-model, nor the calculated curve for the theoretically mixed population represent close fits to the data, rather both curves seem to represent an over-estimation of the survival relative to the averaged data points (figure 4.12 b). At the highest doses (shown in figure 4.12 a) the cell kill of the T-47D$_{mix}$ cells exceeds what is predicted by the calculated curve of the mixed population. However, the discrepancies are not large enough to discredit the hypothesis of the mix-in with NHIK 3025 cells. It seems a paradox though, that the response of the mixed cell population is closer to that of T-47D cells in the hypersensitive dose range, but to that of NHIK 3025 cells for the high dose range, than to the calculated response for the theoretically mixed population. One may wonder if the presence of T-47D cells somehow influences on the response of NHIK 3025 cells in the hypersensitive dose range (i.e. up to 0.5 Gy), while for doses above 2 Gy NHIK 3025 cells sensitize T-47D cells. If this is the case, it could be due to micro-environmental conditioning effects.

5.2.1 MICRO-ENVIRONMENTAL CONDITIONING EFFECTS

If the T-47D$_{mix}$ culture is in fact a mix of T-47D cells and NHIK 3025 cells, the NHIK 3025 cells must have somehow adapted to a different medium and particularly to trypsinization using trypsin with EDTA, which under normal conditions is lethal to these cells. The most probable explanation is that T-47D cells have functioned as a kind of feeder cells. Feeder cells are normal cells that have been inactivated by gamma irradiation. In culture, these cells can serve as a basal layer for other cells and supply important metabolites without further growth or division of their own (ATCC, 2003). In this case the T-47D cells are proliferating themselves, and it is maybe more correct to assume that both cell lines influence each other reciprocally probably by exchange of chemical signaling.

Cells possess complex systems, including receptors, kinases, phosphatases, GTP-binding proteins and other molecules, that enable them to send or respond to signals to or from other cells. A so-called bystander effect (i.e. the induction of damage in cells that were not directly hit by radiation) has been ascribed to the high cell kill at very low doses in cell lines that express HRS as a consequence of paracrine or autocrine signaling. A technique called the ICM treatment consists in replacing the medium of non-irradiated cells with medium taken from cell cultures previously exposed to radiation. Studies applying this technique showed that this treatment can reduce clonogenic survival of unexposed cells (Ballarini et al., 2002). When medium from irradiated keratinocytes (epithelial cells) was transferred to unirradiated keratinocytes or fibroblasts the survival of the unexposed cells was reduced. When the same was done with medium from irradiated fibroblasts no effect was observed. Treatment with

medium irradiated in absence of cells had no effect on any of the cell lines (Mothersill et al., 1997). In another investigation by the same authors (Mothersill et al., 1998), it was found that the degree of cell-cell contact during irradiation was not significant, whereas the number of irradiated cells was the only relevant parameter.

With this in mind it would be reasonable to expect that a mixture of two different cell types might behave differently from two coexisting populations without mutual influence, either due to mutual exchange or one-way transmission of molecules secreted.

A puzzling aspect is that it was not possible to detect any difference in morphology of the mixed population compared to pure T-47D cultures. Normally colonies of NHIK 3025 cells grow less densely than colonies of T-47D cells and therefore look quite different. The mixed cell population was trypsinized and grown under conditions that are usually harmful to NHIK 3025 cells and one can not rule our that the morphology NHIK 3025 cell colonies is closer to that of T-47D cell colonies grown under the present conditions. The morphology of NHIK 3025 cell colonies when grown separately under the same conditions can not be tested because they will die out.

5.3 EFFECTS OF REDUCING THE DOSE-RATES OF THE PRIMING DOSE

One of the purposes of this study was to investigate how low the dose-rate of the priming dose had to be in order to prevent the induction of the counter-HRS effect. Since HRS was not found in NHIK 3025 cells, the HRS observed in the T-47D$_{mix}$ population must be due to T-47D cells (also in case it is T-47D cells with changed DNA content that is in the mix) possibly mediated to the other cells through micro-environmental conditioning effects, and one can argue that the results from the low dose-rate experiments with T-47D$_{mix}$ cells are at least indicative of possible trends. The conclusion to be drawn from the experiments with these arguments in mind is that the lowering of the dose-rate of the priming dose to 0.32 Gy/h or even 0.045 Gy/h does not prevent a reduction of HRS in response to subsequent irradiation, in fact for the lowest dose-rates the HRS seem to have been further reduced (figure 4.19 and figure 4.23)
In the case of T-47D cells given a HDR priming dose, the HRS was observed to be partially restored 24 hours after the priming dose; this was not the case for T-47D$_{mix}$ cells given the priming dose with 0.32 Gy/h or 0.045 Gy/h. Instead, the radioresistance for these cells 24 hours after priming seemed amplified relative to the response 6 hours after the priming. This trend is more clearly seen in figures with data plotted without curve fits (figure 4.27) than in those having fitted curves included (figures 4.17, 4.21, and 4.25 compared to 4.15, 4.19, and 4.23, respectively).

The cells that had grown in a medium with tritiated valine had adapted to the continuous irradiation. Every week a specimen of cells with incorporated tritium was seeded into medium with cold valine and the plating efficiency was compared to the one of a control group. In parallel experiments with higher dose-rates (higher activity) the cell population died out after a few weeks, but in cells irradiated with 0.01 Gy/h to the cell nucleus, the plating efficiency was stabilized at a level of ~50% as compared to the control cells. From the DNA histograms in Figure 4.11 a, it appears that the control flask contained the mixed cells (T-47D$_{mix}$) while from figure 4.11 b the irradiated cells ended up with DNA histograms similar to that of T-47D cells (figure 4.10 a), which means that a numerical comparison of the plating efficiencies of the control and irradiated cells is irrelevant. It also implies that cells of the cell line mixed into

the T-47D population had become extinct while the T-47D cells had adapted to a continuous irradiation at 0.01 Gy/h.

When the T-47D cells that had been pre-irradiated with 0.01 Gy by electrons from incorporated tritium for several months were challenged by HDR irradiation, the counter-HRS effect was found to be fully induced. These cells had received extremely high doses (~30Gy) over a very long period (see discussion of the impact of exposure-duration in section 5.5) and it remains to be investigated at what point the counter-HRS effect is induced.

5.4. THE LENGTH OF THE INTERVAL BETWEEN PRIMING AND CHALLENGE DOSE

If doses are administered in a way that allows HRS to be repeated, a reversed fractionation effect may be obtained, in which a number of very small dose fractions can result in a smaller surviving fraction than if the total dose was given as a single dose.

Experiments performed by Short et al. (2001) in order to elucidate the reversed fractionation effect, revealed a reduced cell survival in three radioresistant human glioma cell lines, which demonstrate HRS, after fractionated acute irradiation with three doses of 0.4 Gy relative to a single dose of 1.2 Gy. How much the cell survival was reduced by fractionation was dependent on the interval between the dose fractions, and the maximum effect was seen when the interval between doses was 3 hours for T98G cells, 4 hours for A7 cells, and about 1 hour for U87 cells. The surviving fraction as a function of the interval between the three fractions is shown for T98G cell in figure 5.1 as an example. When the interval between fractions was increased above those values, there was an increased surviving fraction, which was attributed to cell proliferation.
The minimum cell survival of T98G and U87 correlated to the predicted values assuming a full restitution of HRS after each dose but for A7 cells the survival was 50 % higher than the predicted value.

In the present study the HRS was found to be strongly reduced in T-47D cells 6 hours after the priming exposure and still not completely recovered after 24 hours. This suggests a very different timescale for recovery of HRS following a priming dose for these cells as compared to those examined by Short et al. Unfortunately, the Rb status of the cell lines used in the study of Short et al. is not known, but it is tempting to associate the prolonged timescale in T-47D cells to the presence of functional pRb, of which it is known that the activation is induced over some time (see section 5.6). This would result in a delayed and prolonged effect of a priming dose compared to that of cells that depend on the normal radiation-induced G_2 delay for protective repair (see section 2.1.2). An increase in cell survival was observed when the time between dose fractions were increased beyond the values that resulted in the strongest HRS (the intervals beyond 3 hours in figure 5.1). Short et al suggested that this increase in cell survival was due to cell proliferation. However, if the increased cell survival is not attributed to proliferation alone, an alternative explanation to the experimental results of both the present study (in which cell proliferation has been corrected for through the plating efficiency of the control group) and the one of Short et al. is tempting. This will be presented in the last paragraph of section 5.5.

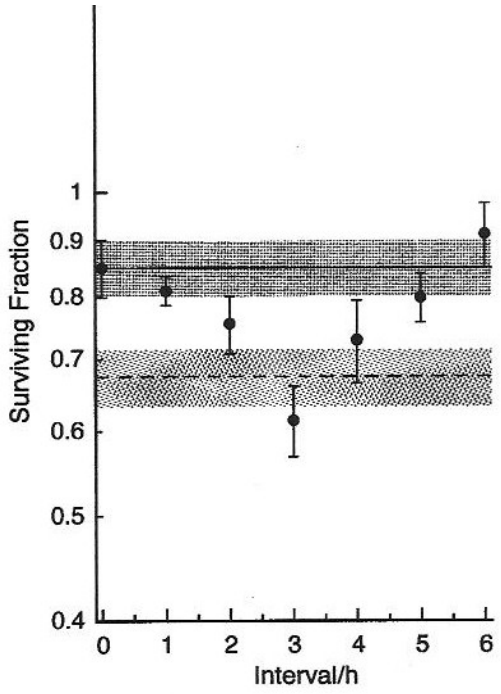


Figure 5.1: *Survival of exponentially growing T98G cells after acute irradiation with three doses of 0.4 Gy separated by the intervals shown. The solid line and shading are the measured SF after a single dose of 1.2 Gy. The broken line and stippled shading are the predicted SF assuming HRS occurs after each dose (Short et al., 2001)*

In the present study we chose the interval of 6 hours between priming and challenge doses with reference to experiments done by Raaphorst and Boyden (1999). They investigated the change of response of T-47D cells in plateau phase to a challenge dose of 4 Gy following a priming dose of 0.5 Gy, as the interval between the doses was changed, and found that most of the response was achieved in 2 hours and persisted for up to 24 hours. Raaphorst and Boyden concluded that an interval of 6 hours ensured that most of the response had been established. Raaphorst and Boyden found a strong adaptive response at challenge doses of 2 Gy and 4 Gy for priming doses of 0.5-2 Gy. When similar experiments were performed with exponentially growing T-47D cell cultured in our laboratory (Lorentzen, 2001) no such effect was observed. The experiments by Raaphorst and Boyden were done with T-47D cells brought into plateau phase by high cell-density, while the cells used in Lorentzens experiments were recultivated regularly in order for them to remain in exponential growth. The survival curves of Lorentzen's experiments (the ones for primed and unprimed cells are indistinguishable from one another) lie well above Raaphorst and Boyden's curve of unprimed cells but are coincident with their curve for primed cells. Lorentzen suggested that this difference could be caused by a permanently induced stress in the cell cultured in our laboratory possibly related to the detected presence of the stress-induced protein p53R2 (Graff, unpublished data)

A tempting speculation is that adaptive responses in the high and low dose range may be separate phenomena, and that the HRS/IRR is not related to induced repair since it is reduced or removed by a priming dose in proliferating cells, that do not express adaptive effects for high challenge doses. Instead hypersensitivity at very low doses could be a result of induced apoptosis over-running repair processes. The effect of the priming dose then could be to turn off the induction of apoptosis. It may be worthwhile to investigate if this turning off-process controlled by pRb (see discussion in section 5.6).

It follows that the time scales of the two adaptation phenomena (high and low dose range) can not be expected to be identical since they relate to different processes.

5.5 A POSSIBLE CORRELATION BETWEEN DOSE-RATE OF PRIMING DOSE AND DEGREE OF HRS

Mitchell and Joiner (2002) investigated combinations of various priming dose sizes and priming dose-rates that would affect the response of their glioma cells to an immediate acute HDR x-ray exposure. The cell line used was T98G both in asynchronous and confluent cultures but since the present study only involves exponentially growing cells, the results with the confluent cells will not be discussed here. Priming doses of 2 Gy and 5 Gy were given with dose-rates of 0.6, 0.3, 0.1, and 0.05 Gy/h with ^{60}Co γ-rays and were followed by immediate acute HDR x-ray exposure.

After a priming dose of 2 or 5 Gy, delivered with varying dose-rates, it appears that the cells retain some HRS at all dose-rates, but that the degree of HRS varies depending on the dose-rate used to deliver the priming dose. This has not been commented on by the authors themselves, but a comparison of the α_s/α_r values listed in the paper clearly show, that the dose-rate least effective in reducing HRS is 0.1 Gy/h.

The α_s/α_r values for unprimed cells were not reported by Mitchell and Joiner so a comparison of the degree of HRS of the pre-irradiated cells relative to cells not pre irradiated can not be made. This, in combination with the lack of knowledge of Rb-status in T98G cells and differences as to how the experiments were conducted, makes a direct correlation to the present results difficult.

There are, however, some observations in the present study that are comparable to those of Mitchell and Joiner: For all the examined dose-rates, a very small priming dose of 0.3 Gy was enough to reduce the HRS to subsequent challenge irradiation given 6 hours after the priming dose. However, even when it was given with HDR (figure 4.3) or 0.9 Gy/h (figure 4.6) the HRS was not completely abolished after 6 hours, but when the dose-rate was reduced to 0.32 Gy (figure 4.19 and figure 4.27 b) and even further to 0.045 Gy/h (figure 4.23 and figure 4.27 d) the HRS was practically repealed after 6 hours. The experiments with the lowest priming dose-rates were performed with the cell culture denoted T-47D$_{mix}$, which is T-47D cells with another stem line mixed into it. Since the cells mixed into the T-47D cells is most likely of the line NHIK 3025, which does not express HRS, there is reason to believe, that the same change in degree of HRS would have been found in a cell culture of only T-47D cells.

The variable effect of various priming dose-rates are interesting from a biological point of view because biological processes take time and the duration of irradiation may be important as a parameter of its own. An example of molecular effects induced over time is the results of Furre et al. (1999 and 2003). Furre et al. irradiated T-47D and NHIK 3025 cells for protracted periods with low dose-rates of 0.3 and 0.9 Gy/h and found for both T-47D and NHIK 3025 cells that the number of cells accumulated in G_2-phase increased with time independently of the dose-rate. For a certain total dose, the number of cells accumulated in G_2-phase was therefore higher for the lowest than for the highest dose-rate. The accumulation of cells in G_2-phase as a function of time after an acute HDR dose was also examined for T-47D cells and it was found to follow the same time scale as observed for the T-47D cells that were exposed to protracted low-dose irradiation.

These results of Furre et al. imply that there are molecular mechanisms induced in response to radiation that are not dependent on dose size but instead on the irradiation time and how long time that has passed after the irradiation. The observations of Mitchell and Joiner and of the present study may be viewed as indications that the duration of the exposure is important for the response to the priming exposure at the very low dose-rates. A possible explanation to the variation in the reduction of HRS as the dose-rate of the priming exposure is changed (reflected in the α_s and α_s/α_r values) that is seen in Mitchell an Joiners publication (2002) as well as in the present study, is that there may exist two different regulation-pathways when inducing mechanisms responsible for reduction of HRS; one pathway dominant for HDR in which the induction depends primarily on dose size, and another pathway for LDR, in which the induction is time dependent. The suggestion, thus, is that the protection of cells induced by a HDR priming dose is triggered by a certain amount of damage. With LDR irradiation used for the priming dose this may however be different. Not only the amount of damage may activate the process, but also the time over which new damage appear may be of importance.

The lower the dose-rate is, the longer is the exposure time required to deliver a certain dose. If the regulation-pathways responsible for the abolition of HRS during LDR need time to get induced, then very low dose-rates may provide better time than higher dose-rates for the protective mechanisms to reach their full capacity. Thus, there could be a dose-rate for the priming dose, which may have little effect in reducing HRS of the following reason: It may not be high enough to induce the type of counter-HRS effect that acute irradiation induces. However, it may still be low enough to induce the other type of counter-HRS effect, the one induced by LDR, but the time at disposal may be too short for the protective mechanisms to be fully expressed. An even lower dose-rate may be just as effective in inducing the LDR counter-HRS effect, but since irradiation lasts longer, it may allow more time for the full effect to develop, and therefore seem more effective than the higher LDR.

Figure 5.2 shows the counter-HRS effect of a HDR priming dose of 0.3 Gy delivered either 6 hours before the challenge dose (●) or immediately before the challenge dose (▲).The resulting survival curves suggest that with HDR the complete removal of HRS takes place immediately (▲) but is somewhat reduced after 6 hours (●) (see also figure 4.3). 24 hours after the priming dose HRS had partially recovered but still not to its full extend (figure 4.4). These findings speak in favor of the theory of two independent pathways for induction of the mechanisms for reducing HRS, one for HDR radiation in which the protective mechanisms are instantly induced, and another for LDR in which they are induced over time.

It should be investigated, however, if there is a recovery of HRS between 0 and 6 hours after the priming dose in T-47D cells as was found in the cell lines examined by Short et al. (2001) (see section 5.4 and figure 5.1). This would suggest that the induction pathway of the LDR counter-HRS effect is also induced by HDR in addition to the instant responding pathways. If this is true, it would imply a correlation between the present results and those of both Short et al. (2001) and Mitchell and Joiner (2002): For HDR priming the induction-pathway instantly induces mechanisms that remove HRS but the effect only last a few hours (the decrease in survival when the interval between doses increases from 0 to 3 hours in figure 5.1). At a certain time induction-pathway of the LDR counter-HRS effect, which are induced by both HDR and LDR, begin to affect the survival and reduce HRS gradually with time. At 6 hours after the HDR priming it was found, in the present experiments, that HRS was reduced but not removed and after 24 hours it had partially returned. For LDR-fractionation the duration of the exposure should be added to the time between doses, which would explain the fuller effect

observed after 6 hours. Possibly, only the LDR priming exposure provides enough time for a full induction of these mechanisms, which would explain the very long-lasting counter-HRS effect of 24 hours seen only after LDR priming doses, and not after HDR priming doses.

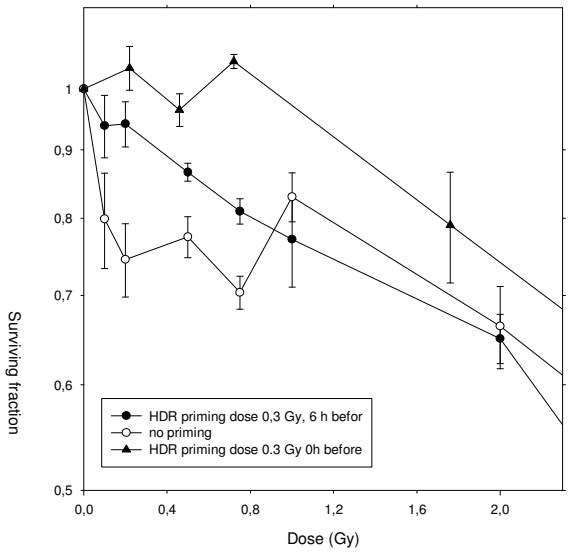


Figure 5.2: *Surviving fraction of T-47D cells as a function of challenge dose are shown.* (○): *Cells irradiation with one single dose.* (▲):*Data from the same experiments replotted as HDR challenge doses immediately subsequent to a 0.3 Gy priming dose.* (●): *A 0.3 Gy HDR priming dose was given 6 hours before the challenge dose. The values for 0 hours between priming and challenge doses* (▲) *were calculated from the values of acute irradiation (○): 0.3 Gy was deducted from the dose of the original experiment and the surviving fraction was corrected by division by a value of the surviving fraction for 0.3 Gy found by inserting the* α_r, α_s, D_c *and* β *values from table 2 and d = 0.3 Gy into equation 2.7 and 2.6.*

5.6 THE POSSIBLE INFLUENCE OF pRb

When studying effects of low dose-rates in T-47D and NHIK 3025 cells, Furre et al. (1999 and 2003) found an inverse dose-rate effect in NHIK 3025 cells but not in T-47D cells. In both cell lines accumulation of cells in the radiosensitive G_2-phase was observed, but in T-47D cells this did not result in an enhanced cell kill. The reason for this was attributed to the presence of activated pRb in G_2 that was found in T-47D cells, which was assumed to be radiation-induced since pRb is usually inactive in G_2 (see chapter 2.1.2). Activation of pRb and binding in the cell nucleus in other cell cycle phases than G_1 had previously been observed as a stress response following hypoxia (Åmellem et al., 1996, 1998). The activation of pRb is not instantaneous, but takes place during the radiation-induced G_2-delay and is supposed to provide a protection of the individual cells that prevents the enhanced radiosensitivity of the G_2-phase (Furre et al., 2003). It is not known by what mechanism pRb induces radioprotection in G_2, but it could perhaps relate to the traditional explanation of the need for radiation-induced G_2-delay in cells, providing prolonged repair-time before mitosis. NHIK 3025 cells are infected with human papilloma virus that induces transcription of oncoproteins which bind to the underphosphorylated form of pRb and prevents its normal function. In consequence of the present experimental results it is tempting to connect the presence of HRS to the presence of functional pRb.

When pRb-activation is considered as a possible determinant for HRS/IRR, this opens for an understanding of HRS/IRR, which is quite different from the traditional working hypothesis of

an induced repair causing the IRR. Since an immense amount of damage occur at all times as the DNA is replicated and as a result of environmental influence, it is more likely that repair processes are permanently activated, but that this repair is subdued by apoptosis in case of small radiation damages. Only when the radiation dose exceeds the size where a removal of all cells receiving radiation damage would endanger the function of the tissue as a whole, is the induction of apoptosis turned off, allowing the repair processes to have their effect. pRb is a slow responder. It takes time before hypoxia or radiation results in pRb activation, and once activated it takes time before the protein is deactivated. Thus, pRb could be involved in the LDR counter-HRS effect perhaps through repression of apoptosis.

The cells of the cell line mixed into the T-47D population had become extinct while the T-47D cells had adapted to a continuous irradiation at 0.01 Gy/h (figure 4.11). If the in-mixed cells are of type NHIK 3025, this is understandable since these cells are more radiosensitive than T-47D cells on a general basis and particularly so under continuous LDR irradation. From the data in figure 4.8 the LDR irradiation has obviously induced an adaptive effect in the T-47D cells since no HRS is seen. One can only speculate whether this priming effect of 0.01 Gy/h also coincides with activation of pRb. This remains to be investigated.

5.7 SOURCES OF ERROR

When low-dose responses of human cell lines are usually investigated, either a fluorescence-activated cell sorter (FACS) is used to plate an exact number of cells or the exact number of cells is identified after plating by use of microscopic scanning. The method used in the present study of estimating the concentration of cells by counting in a Bürker Chamber introduces an uncertainty in the number of cells seeded in each flask. The inaccuracy in the concentration estimate is corrected for by multiplying the number of cells seeded by the plating efficiency of the control flasks, but for very low doses, where the surviving fraction is close to 100 %, only small inaccuracies in the volume and homogeneity of the cell suspension administered to each flask is enough to obscure observations. In order to reduce these inaccuracies, dilutions of the cell suspension was adjusted so, that each flask received 1 ml of cell suspension in distinction to 0.2 ml per flask traditionally used in the laboratory, and special attention was paid to keep the cells in a homogenous suspension before administering each quantity.

Since the findings in the experiments were both significant and reproducible, it seems that this conventional assay is applicable to qualitative studies of low-dose responses but for precise quantitative experiments it is necessary to know the exact number of cells plated, and application of one of the two alternative assays mentioned above would improve the accuracy of the measurements.

The flasks were transported by car between the Radium Hospital and the University (5 km), in some cases immediately after irradiation, in other cases when the colonies had grown about a week after irradiation. Great care was taken that the cells should not be shaken and in addition to transporting the flasks in thermo boxes, the car was pre-heated.

In the irradiation process the time for the shutter to be open to administer a prescribed dose was calculated as described in chapter 3.3.2. Since the timer operated exclusively with whole seconds, a rounding off was necessary, but this would lead to deviations too small to be relevant since the doses are given with only one decimal.

The exact distance from the source to the flasks was maintained by use of laser beams from laser guns installed in the radiation room for the purpose. Because of a defect mechanism of the table used in the first experiments the distance at the irradiation time was 79 cm instead of 80 cm. This distance was large enough for the source to be considered as a point, so the corrections were calculated by use of the inverse square law.

When irradiating with the lowest dose-rate, the open air distance between the blocks and the cells was approximately 22 cm, which means that a number of secondary photons (which arise from scattering of the primary photons in the roos metal of the shielding blocks) of energies lower than those of the two cobalt lines could possibly reach the target. In that case, the dose received would originate in an energy spectrum resembling x-ray radiation, which is different from the practically monochromatic cobalt radiation. Whether this has any effect on the response is doubtful but not eliminable.

5.8 SUGGESTIONS FOR FURTHER INVESTIGATIONS

The original intention of investigating a possible change in response as the dose-rate of the priming dose was varied was impeded by the mixing of another stem-line into the T-47D cells. Instead the experiments with the mixed cells revealed a lot of other information that would be interesting to pursue in future investigations.

DNA histograms and survival curves indicate that the stemline that was mixed into the T-47D cells was NHIK 3025, but small discrepancies in the DNA content and radiation response of the mixed cell population relative to what would be theoretically expected of a cell population with coexisting T-47D and NHIK 3025 cells, prevent a definite confirmation of the presence of NHIK 3025 cells in the mixed population. However, there is no reason why the concept of two co-existing populations without mutual influence should apply; the two cell types are prone to affect each other chemically and the response as a result of this could well be as observed in these experiments. The fact that the mixed-in cell line became extinct after a few weeks of irradiation by incorporated tritium, while the T-47D cells adapted to the imposed stress, is a strong indication that the cells in the mixed population were of the radiosensitive NHIK 3025 cell line. The only observation that remains inexplicable if NHIK 3025 cells are in fact the mixed-in cell line is the resemblance in morphology of the mixed cells to pure T-47D cells.

Experiments are currently being conducted in our laboratory to confirm the presence of NHIK 3025 cells in the mixed cell population. NHIK 3025 cells with incorporated tritium have been observed to have a low and declining survival but this experiment still proceeds. Also a DNA histogram of a deliberate mix of 55 % NHIK 3025 cells and 45 % T-47D cell is planned. The attempt to get hold of an antibody that can identify the human papilloma virus has not yet been successful.

The reason why it is so important to confirm that the mixed-in cell line is NHIK 3025 is that, if this is the case, there are interesting implications. T-47D cells that had adapted to the continuous LDR irradiation by incorporated tritium were not hypersensitive to low doses, and this suggests that the same mechanisms are involved in the adaption to LDR irradiation and in HRS/IRR. This connection was confirmed by the facts that NHIK 3025 cells were found not to express HRS/IRR and that the putative NHIK 3025 cells did not adapt to the irradiation from incorporated tritium. NHIK 3025 cells do not have functional pRb but T-47D cells do.

These findings support the theory that pRb is responsible for protective mechanisms induced by stress in the form of radiation.

The T-47D cells showed an adaptive response for challenge doses below 1 Gy to priming doses of 0.3 Gy given with HDR or LDR (0.9 Gy/h) 6 hours after the priming dose. The HRS had not been completely abolished but it was significantly reduced approximately to the same level for the two different dose-rates. 24 hours after the priming dose the HRS had partially but not completely recovered. Investigations should be made first to establish the time scale for both degree of HRS and activation and inactivation of pRb. Then the minimum HDR priming dose required in order to obliterate HRS should be found and with that established, a regimen of different low dose-rates should be applied to the priming dose. The cells with incorporated tritium had been given extremely large doses (~30 Gy) but over an extended period with a very small dose-rate to the cell nucleus (0.01 Gy/h). The mechanisms that protected the T-47D cells during the continuous irradiation could obviously be the same as those responsible for the enhanced resistance relative to cells not pre-irradiated seen after subsequent HDR irradiation, so that further experiments as to when this effect occurs after the incorporation could probably contribute to a better understanding of these mechanisms. All these experiments should be correlated with an examination of the pRb status.

For further understanding of HRS/IRR and in order to test the alternative theory mentioned in section 5.6, experiments with very low doses should be conducted that are followed by a concrete identification of apoptotic cells.

It would also be interesting to examine the pRb status for the T-47D cells 24 hours after both HDR and LDR priming to see if the disappearance or lack of disappearance of HRS is related to pRb activation. Does HRS reappear later or is the effect permanent in case of LDR priming?

5.9 CLINICAL IMPLICATIONS

Detailed knowledge of the radiation response of the cancer cell stemlines in question can be a tool to customizing radiation therapy for better effect. If the cell line of the surrounding tissue that is irradiated at very low doses expresses HRS, the risk of inducing cancer is lower than current estimates (based on a back-extrapolation of the LQ-curve) suggests. When the dose is given in fractions the response could vary depending on the interval between doses in relation to the characteristics of the cell line concerned. Fractionated radiation therapy is used to spare the late-responding normal tissues but this may have unexpected consequences. If the length of the interval allows the HRS to be reestablished, the resulting reversed fractionation effect could have unwanted effects in normal tissues exposed to small doses. The same effect may also be exploited in irradiation of the tumor cells to improve the therapeutic gain, if only the tumor cells and not the surrounding tissue express HRS.

In the clinic doses are traditionally given as 2 Gy HDR doses with 24 hour intervals. The surrounding normal tissues will receive smaller doses with the same interval and it is therefore important to establish the degree of HRS after 24 hours for low doses. The present results indicate a small sparing effect when the second dose is administered.
.
If a correlation is found between pRb status and HRS/IRR as well as the adaptive response to low doses and its timescale, this could provide an important tool in radiotherapy because

cancer cells often have either a mutated Rb gene or a virus infection that prevents the function of pRb. It is possible to test the patients for these specific gene products.

If it is proved that the stem line that had been mixed into the T-47D cells was NHIK 3025, the extinction of the NHIK 3025 while the T-47D were adapted to the continuous irradiation of 0.01 Gy/h to the cell nucleus might have implications for brachytherapy to tumor cells with dysfunctional pRb. The experiment with cells with incorporated tritium of this study suggests that the tumor cells could be eradicated by a treatment with very low dose-rate, that would be lenient towards the pRb protected normal tissues.

In and around a tumor, different cell stemlines co-exist. The experimental results of this study suggest that the response of a cell population is changed by the presence of cells from other stemlines. Whether this is also true *in vivo* remains to be investigated.

The indications of the present study that the very lowest dose-rate induce a counter-HRS effect to subsequent irradiation that disappears when the dose-rate is slightly increased and re-appears for priming with higher dose-rates has implications for brachytherapy. In brachytherapy the tissues surrounding the actual target will be irradiated at dose-rates diminishing with the distance from the source which implies that at a certain distance the normal tissue will be harmed to a greater extend than normally expected.

6 CONCLUSION

The results of the present study give rise to a lot of speculations, but not many conclusions can be drawn without supplementary investigations.

The main findings are:

- T-47D cells clearly express both HRS/IRR and the adaptive response in the low-dose range (below 1 Gy).
- NHIK 3025 cells do not express HRS/IRR.
- T-47D cells that had been pre-irradiated by tritium-decay electrons did not express HRS/IRR.
- T-47D cells with functional pRb adapted to the continuous irradiation with electrons from incorporated tritium, while the putative NHIK 3025 cells without functional pRb were eradicated by the same treatment.
- When priming doses were delivered with the lowest dose-rates used (i.e. 0.32 Gy/h and 0.045 Gy/h) the priming effect as measured 6 hours following the termination of the priming exposure seemed to be larger than when the priming dose was delivered with higher dose-rates (i.e. HDR or 0.9 Gy/h).
- 24 hours after the priming doses delivered with 0.32 Gy/h and 0.045 Gy/h, HRS was still absent (perhaps even further reduced), while 24 hours after a HDR priming dose HRS was partially restored.

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APPENDIX A: List of Chemicals

Below is a list of chemicals used in this study in alphabetic order. The list states the manufacturer of each chemical.

CHEMICAL	MANUFACTURER
Methylene blue	Merck (Germany)
EDTA (Ethylenediaminetetraacetic acid)	Fluka (Switzerland
Ethanol 96%	Arcus produkter AS (Oslo)
Foetal bovine serum	Gibco (Germany)
Glucose	Merck (Germany)
Insulin	Novo Nordisk AS (Denmark)
KCl	Merck (Germany)
L-Glutamine	Sigma (USA)
MEM powder w/Hanks	Gibco (Scotland)
Milli-Q water	Millipore (USA)
NaCl	Riedel de Haën (Germany)
$NaHCO_3$	Norsk Medisinaldepot (Oslo)
Penicillin-Streptomycin (5000 IU/ml – 5000 µg/ml)	Gibco (Scotland)
Phenol red	Merck (Germany)
RPMI 1640 powder, w/L-glutamine, w/o $NaHCO_3$	Gibco (Scotland)
Trypsin powder	Boehringer Mannheim (Germany)

APPENDIX B: Recipes

RPMI 1640 medium

1 liter stem solution:

RPMI 1640 powder	10.43 g
$NaHCO_3$	2.00 g
Milli-Q H_2O	1.00 ltr

1 liter RPMI medium with serum:

RPMI 1640 stem solution	880 ml
Foetal calf serum (10%)	100 ml
Penicillin/streptomycin	10 ml
Insulin (200 units/l)	2 ml
L-Glutamine	10 ml

MEM medium

1 liter stem solution:

MEM powder	10.63 g
Milli-Q H_2O	1 ltr
$NaHCO_3$	2.4 g

1 liter MEM medium with serum:

MEM with Hanks	810 ml
Foetal calf serum (10%)	150 ml
Penicillin/streptomycin	20 ml
L-glutamine	10 ml

TRYPSIN

1 Liter trypsin stem solution:

NaCl	8.00 g
KCl	0.40 g
Glucose	1.00 g
$NaHCO_3$	0.35 g
Phenol Red	0.02 g

1 liter trypsin with EDTA:

Trypsin stem solution	1.0 ltr
EDTA	200 mg
Trypsin powder	500 mg

L-GLUTAMINE, 35 ml

L-glutamine	1.0227 g
RPMI or MEM stem solution	35 ml

COLD VALINE, 82.9 mM solution

L-Valine powder	1.00 g
RPMI stem solution	103 ml

PBS, 1 liter

NaCl	8.000 g
KCl	0.201 g
KH_2PO_4	0.204 g
$NaHPO_4 \bullet 12H_2O$	2.858 g
Milli-Q H_2O	1 ltr

APPENDIX C: List of experiments

Experiment number	Cell line	Priming dose (Gy)	Priming dose-rate (Gy/h)	Challenge doses / acute doses (Gy)	Time between priming and challenge dose (h)
V1-V3	V 79	none		0.5, 1, 2, 5, 7.5, 10, 14	
V4-V6	V 79	none		0.1, 0.2, 0.5, 0.75, 1, 2, 5	
T1-T3	T-47D	none		0.5, 1, 2, 5, 7.5, 10	
T4-T6	T-47D	none		0.1, 0.2, 0.5, 0.75, 1, 2, 5	
T7, T8, T10	T-47D (T7+T8), T-47D$_{mix}$ (T10)	0.3	HDR	0.1, 0.2, 0.5, 0.75, 1, 2, 5	6 and 24
T9	T-47D	0.3	0.9	0.1, 0.2, 0.5, 0.75, 1, 2, 5	6 (24 was infected)
T11, T14, T15	T-47D$_{mix}$	0.3	0.32	0.1, 0.2, 0.5, 0.75, 1, 2, 5	6 and 24
T13, T16, T18	T-47D$_{mix}$	0.3	0.045	0.1, 0.2, 0.5, 0.75, 1, 2, 5	6 and 24
T12, T17, T19(infected)	T-47D	24.7 (T12), 36.2 (T17)	0.01	0.1, 0.2, 0.5, 0.75, 1, 2, 5	(6)
T20-T22	T-47D$_{mix}$	None		0.1, 0.2, 0.5, 0.75, 1, 2, 5, 7.5, 10	
N1-N3	NHIK 3025	None		0.1, 0.2, 0.5, 0.75, 1, 2, 5	

APPENDIX D: Experimental raw data

In this appendix all raw data from the experiments are listed. The characteristics of each experiment are summarized in appendix C. In appendix D the raw data of experiments with similar procedures are listed together and data of each group are summarized in a plot showing surviving fraction as a function of dose for each individual experiment

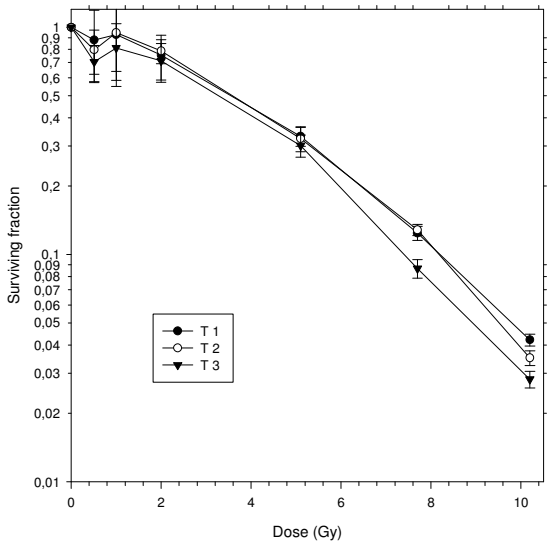
I: T-47D, acute irradiation, no priming dose

Experiment:	T1	January 9, 2002.					
Multiplicity M=	1.854	Plating efficiency PE=			0.366		
Dose (Gy)	Control	0.51	1.03	2.06	5.12	7.69	10.24
flask 1	64	63	79	113	241	329	311
flask 2	75	76	84	184	178	293	302
flask 3	81	49	82	126	183	273	282
flask 4	70	94	74	117	182	320	250
flask 5	76	73	57	128	169	374	256
Number of cells seeded per flask	200	200	200	400	1000	4000	10000
Mean number of colonies per flask: N	73.2	71.0	75.2	133.6	190.6	317.8	280.2
ΔN	2.89	7.44	4.85	12.90	12.84	17.19	12.09
Number of cells seeded multiplied with PE: N_0		73.2	73.2	146.4	366	1464	3660
ΔN_0		2.888	2.888	5.776	14.440	57.758	144.395
Surviving fraction: SF		0.970	1.027	0.913	0.521	0.217	0.077
ΔSF		0.109	0.078	0.095	0.041	0.015	0.004
Surviving fraction corrected for multiplicity: f		**0.879**	**0.928**	**0.754**	**0.331**	**0.124**	**0.042**
Δf		**0.308**	**0.289**	**0.168**	**0.032**	**0.009**	**0.003**

Experiment:	T2	January 16, 2002.					
Multiplicity M=	1.826	Plating efficiency PE=			0.43		
Dose (Gy)	Control	0.51	1.03	2.06	5.12	7.69	10.24
flask 1	102	100	68	158	272	371	265
flask 2	79	63	77	152	256	380	223
flask 3	87	74	73	169	174	374	274
flask 4	77	89	108	163	187	373	286
flask 5	85	74	99	154	196	400	307
Number of cells seeded per flask	200	200	200	400	1000	4000	10000
Mean number of colonies per flask: N	86.0	80.0	85.0	159.2	217.0	379.6	271.0
ΔN	4.40	6.49	7.82	3.09	19.67	5.32	13.91
Number of cells seeded multiplied with PE: N_0		86	86	172	430	1720	4300
ΔN_0		4.405	4.405	8.809	22.023	88.091	220.227
Surviving fraction: SF		0.930	0.988	0.926	0.505	0.221	0.063
ΔSF		0.089	0.104	0.051	0.053	0.012	0.005
Surviving fraction corrected for multiplicity: f		**0.796**	**0.947**	**0.787**	**0.324**	**0.128**	**0.035**
Δf		**0.175**	**0.397**	**0.097**	**0.041**	**0.007**	**0.003**

Experiment:	T3	January 23, 2002.					
Multiplicity M=	1.826	Plating efficiency PE=	0.691				
Dose (Gy)	Control	0.51	1.03	2.06	5.12	7.69	10.24
flask 1	105	145	129	298	409	416	361
flask 2	145	111	110	257	331	375	338
flask 3	125	108	137	248	306	423	352
flask 4	157	130	112	215	330	458	346
flask 5	159	120	166	216	310	496	416
Number of cells seeded per flask	200	200	200	400	1000	4000	10000
Mean number of colonies per flask: N	138.2	122.8	130.8	246.8	337.2	433.6	362.6
ΔN	10.27	6.75	10.17	15.31	18.65	20.43	13.87
Number of cells seeded multiplied with PE: N_0		138.2	138.2	276.4	691	2764	6910
ΔN_0		10.268	10.268	20.537	51.342	205.368	513.420
Surviving fraction: SF		0.889	0.946	0.893	0.488	0.157	0.052
ΔSF		0.082	0.102	0.086	0.045	0.014	0.004
Surviving fraction corrected for multiplicity: f		**0.723**	**0.830**	**0.730**	**0.311**	**0.090**	**0.029**
Δf		**0.130**	**0.224**	**0.140**	**0.035**	**0.008**	**0.002**

T-47D acute irradiation (large doses)

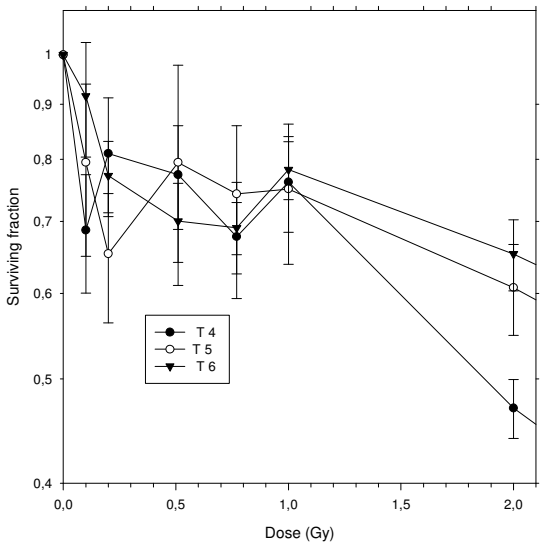


Experiment:	T4	March 7, 2002						
Multiplicity M=	1.826	Plating efficiency PE=	0.747					
Dose (Gy)	Control	0.1	0.2	0.5	0.76	1	2.1	5.1
flask 1	154	148	155	150	123	123	199	173
flask 2	157	134	122	130	142	148	196	172
flask 3	159	136	145	139	134	142	224	168
flask 4	130	102	134	145	101	129	201	115
flask 5	147	126	144	122	141	139	189	183
Number of cells seeded per flask	200	200	200	200	200	200	400	800
Mean number of colonies per flask: N	149.4	129.2	140.0	137.2	128.2	136.2	201.8	162.2
ΔN	5.26	7.66	5.59	5.05	7.60	4.51	5.91	12.06
Number of cells seeded multiplied with PE: N_0		149.4	149.4	149.4	149.4	149.4	298.8	597.6
ΔN_0		5.259	5.259	5.259	5.259	5.259	10.519	21.037
Surviving fraction: SF		0.865	0.937	0.918	0.858	0.912	0.675	0.271
ΔSF		0.060	0.050	0.047	0.059	0.044	0.031	0.022
Surviving fraction corrected for multiplicity: f		**0.687**	**0.810**	**0.774**	**0.678**	**0.762**	**0.470**	**0.160**
Δf		**0.086**	**0.102**	**0.086**	**0.084**	**0.078**	**0.030**	**0.014**

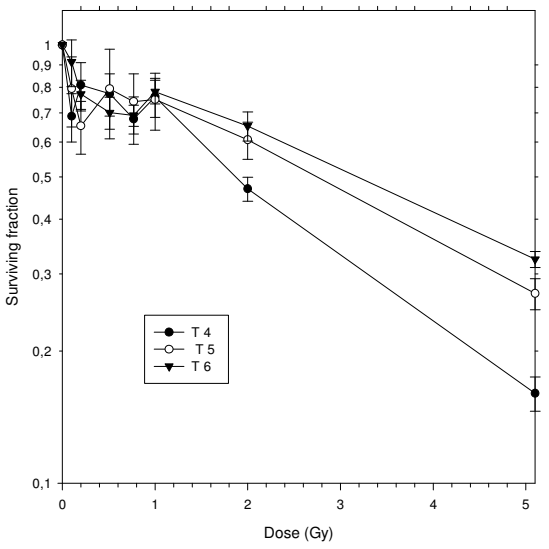
Experiment:	T5	March 13, 2002						
Multiplicity M=	1.927	Plating efficiency PE=	0.461					
Dose (Gy)	**Control**	**0.1**	**0.2**	**0.5**	**0.76**	**1**	**2.1**	**5.1**
flask 1	85	83	74	84	92	95	158	163
flask 2	81	89	87	106	71	88	157	164
flask 3	110	75	79	69	83	77	148	148
flask 4	93	92	68	93	88	82	145	191
flask 5	92	97	90	84	90	84	156	171
Number of cells seeded per flask	200	200	200	200	200	200	400	800
Mean number of colonies per flask: N	92.2	87.2	79.6	87.2	84.8	85.2	152.8	167.4
ΔN	4.97	3.80	4.06	6.08	3.76	3.02	2.63	6.99
Number of cells seeded multiplied with PE: N_0		92.2	92.2	92.2	92.2	92.2	184.4	368.8
ΔN_0		4.974	4.974	4.974	4.974	4.974	9.948	19.896
Surviving fraction: SF		0.946	0.863	0.946	0.920	0.924	0.829	0.454
ΔSF		0.066	0.064	0.083	0.064	0.060	0.047	0.031
Surviving fraction corrected for multiplicity: f		**0.794**	**0.653**	**0.794**	**0.743**	**0.751**	**0.608**	**0.271**
Δf		**0.145**	**0.090**	**0.184**	**0.117**	**0.112**	**0.059**	**0.022**

Experiment:	T6	March 20, 2002						
Multiplicity M=	1.807	Plating efficiency PE=	0.722					
Dose (Gy)	**Control**	**0.1**	**0.2**	**0.5**	**0.76**	**1**	**2.1**	**5.1**
flask 1	140	154	139	124	113	127	235	296
flask 2	149	133	137	139	130	136	208	320
flask 3	140	141	120	136	124	130	265	279
flask 4	152	129	140	111	130	128	257	272
flask 5	141	149	124	118	126	143	242	280
Number of cells seeded per flask	200	200	200	200	200	200	400	800
Mean number of colonies per flask: N	144.4	141.2	132.0	125.6	124.6	132.8	241.4	289.4
ΔN	2.54	4.69	4.16	5.30	3.12	2.99	9.89	8.60
Number of cells seeded multiplied with PE: N_0		144.4	144.4	144.4	144.4	144.4	288.8	577.6
ΔN_0		2.542	2.542	2.542	2.542	2.542	5.083	10.167
Surviving fraction: SF		0.978	0.914	0.870	0.863	0.920	0.836	0.501
ΔSF		0.037	0.033	0.040	0.026	0.026	0.037	0.017
Surviving fraction corrected for multiplicity: f		**0.915**	**0.772**	**0.701**	**0.690**	**0.782**	**0.653**	**0.324**
Δf		**0.112**	**0.059**	**0.059**	**0.038**	**0.048**	**0.050**	**0.014**

T-47D acute irradiation (small doses)



T-47D acute irradiation (small doses)

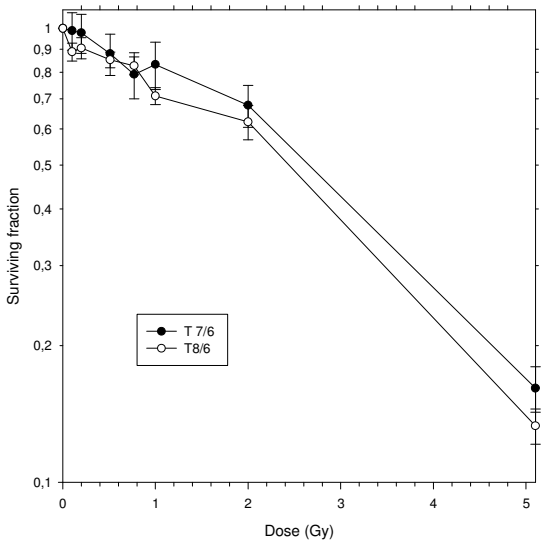


IIa: T-47D, 0.3 Gy HDR priming dose 6 hours before acute irradiation.

Experiment:	T7/6	June 6, 2002						
Multiplicity M=	1.090	Plating efficiency PE=			0.39667			
Dose (Gy)	Control	0.1	0.2	0.5	0.76	1	2.1	5.1
flask 1	121	110	100	113	75	96	90	53
flask 2	125	126	116	86	100	117	114	45
flask 3	150	123	126	105	117	86	125	65
flask 4	107	120	118	118	99	120	121	47
flask 5	92	110	123	107	89	84	103	65
Number of cells seeded per flask	300	300	300	300	300	300	400	800
Mean number of colonies per flask: N	119.0	117.8	116.6	105.8	96.0	100.6	110.6	55.0
ΔN	9.68	3.32	4.51	5.45	6.91	7.60	6.36	4.29
Number of cells seeded multiplied with PE: N_0		119	119	119	119	119	158.667	317.333
ΔN_0		9.680	9.680	9.680	9.680	9.680	12.907	25.813
Surviving fraction: SF		0.990	0.980	0.889	0.807	0.845	0.697	0.173
ΔSF		0.085	0.088	0.086	0.088	0.094	0.069	0.020
Surviving fraction corrected for multiplicity: f		**0.989**	**0.978**	**0.880**	**0.792**	**0.833**	**0.677**	**0.161**
Δf		**0.093**	**0.097**	**0.092**	**0.093**	**0.100**	**0.072**	**0.018**

Experiment:	T8/6	June 12, 2002						
Multiplicity M=	1.109	Plating efficiency PE=			0.51333			
Dose (Gy)	Control	0.1	0.2	0.5	0.76	1	2.1	5.1
flask 1	161	149	140	130	140	106	132	60
flask 2	140	134	136	143	122	112	135	59
flask 3	156	140	151	132	132	109	103	68
flask 4	147	127	153	128	121	123	166	70
flask 5	166	142	124	134	134	114	129	42
Number of cells seeded per flask	300	300	300	300	300	300	400	800
Mean number of colonies per flask: N	154.0	138.4	140.8	133.4	129.8	112.8	133.0	59.8
ΔN	4.70	3.72	5.29	2.60	3.64	2.89	10.02	4.94
Number of cells seeded multiplied with PE: N_0		154	154	154	154	154	205.333	410.667
ΔN_0		4.701	4.701	4.701	4.701	4.701	6.268	12.536
Surviving fraction: SF		0.899	0.914	0.866	0.843	0.732	0.648	0.146
ΔSF		0.037	0.044	0.031	0.035	0.029	0.053	0.013
Surviving fraction corrected for multiplicity: f		**0.888**	**0.905**	**0.853**	**0.827**	**0.710**	**0.622**	**0.133**
Δf		**0.040**	**0.049**	**0.034**	**0.038**	**0.031**	**0.054**	**0.012**

T-47D, 0.3Gy HDR primer, 6 hours interval

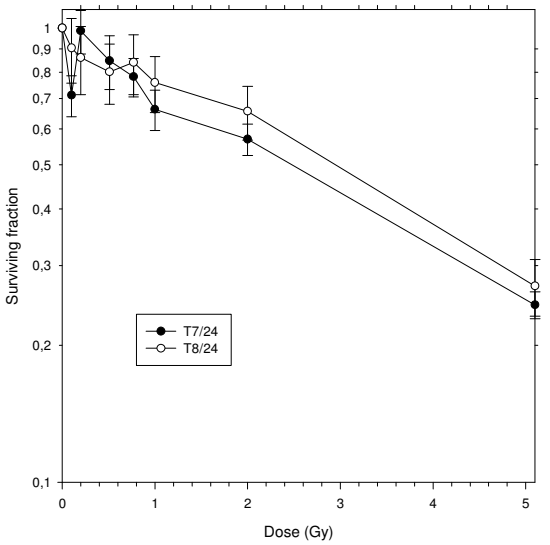


IIb: T-47D, 0.3 Gy HDR priming dose 24 hours before acute irradiation.

Experiment:	T7/24	June 6, 2002						
Multiplicity M=	1.417	Plating efficiency PE=			0.72			
Dose (Gy)	Control	0.1	0.2	0.5	0.76	1	2.1	5.1
flask 1	140	93	173	126	131	125	185	159
flask 2	159	143	139	99	120	106	213	177
flask 3	139	112	121	124	102	80	154	222
flask 4	138	117	144	131	114	120	212	179
flask 5	144	109	137	169	147	113	203	193
Number of cells seeded per flask	200	200	200	200	200	200	400	800
Mean number of colonies per flask: N	144.0	114.8	142.8	129.8	122.8	108.8	193.4	186.0
ΔN	3.89	8.11	8.48	11.26	7.65	7.88	11.06	10.50
Number of cells seeded multiplied with PE: N_0		144	144	144	144	144	288	576
ΔN_0		5.259	5.259	5.259	5.259	5.259	5.259	5.259
Surviving fraction: SF		0.797	0.992	0.901	0.853	0.756	0.672	0.323
ΔSF		0.063	0.069	0.085	0.062	0.061	0.040	0.018
Surviving fraction corrected for multiplicity: f		**0.712**	**0.986**	**0.847**	**0.782**	**0.662**	**0.569**	**0.246**
Δf		**0.077**	**0.116**	**0.119**	**0.080**	**0.071**	**0.043**	**0.015**

Experiment:	T8/24	June 12, 2002						
Multiplicity M=	1.417	Plating efficiency PE=			0.618			
Dose (Gy)	Control	0.1	0.2	0.5	0.76	1	2.1	5.1
flask 1	114	121	128	103	121	94	164	233
flask 2	112	105	121	104	107	110	187	159
flask 3	101	111	117	117	107	107	180	153
flask 4	121	115	106	96	115	103	198	154
flask 5	170	129	91	116	104	102	198	173
Number of cells seeded per flask	200	200	200	200	200	200	400	800
Mean number of colonies per flask: N	123.6	116.2	112.6	107.2	110.8	103.2	185.4	174.4
ΔN	12.04	4.13	6.47	4.04	3.14	2.71	6.35	15.08
Number of cells seeded multiplied with PE: N_0		123.6	123.6	123.6	123.6	123.6	247.2	494.4
ΔN_0		12.036	12.036	12.036	12.036	12.036	24.072	48.143
Surviving fraction: SF		0.940	0.911	0.867	0.896	0.835	0.750	0.353
ΔSF		0.097	0.103	0.091	0.091	0.084	0.077	0.046
Surviving fraction corrected for multiplicity: f		**0.904**	**0.861**	**0.801**	**0.841**	**0.759**	**0.656**	**0.270**
Δf		**0.147**	**0.147**	**0.121**	**0.127**	**0.107**	**0.089**	**0.039**

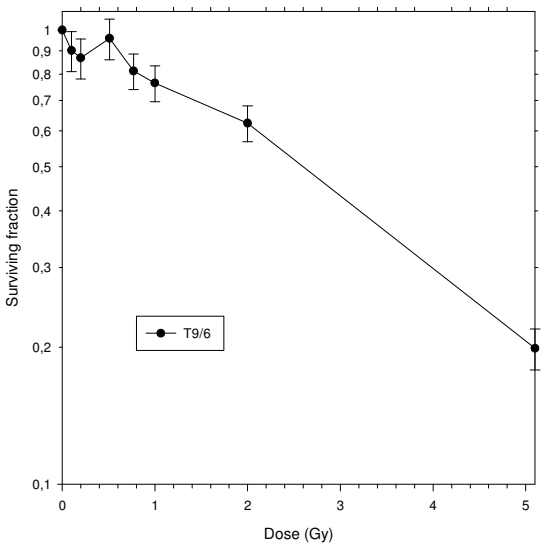
T-47D, 0.3 Gy HDR primer, 24 hours interval



III: T-47D, 0.3 Gy priming dose, dose-rate 0.9 Gy/h, 6 hours before acute irradiation.

Experiment:	T9/6	June 19, 2002						
Multiplicity M=	1.132	Plating efficiency PE=			0.31467			
Dose (Gy)	Control	0.1	0.2	0.5	0.76	1	2.1	5.1
flask 1	105	84	71	98	79	69	74	52
flask 2	75	83	80	88	80	78	85	55
flask 3	90	77	88	102	79	74	88	50
flask 4	85	88	88	81	81	76	83	69
flask 5	117	99	90	86	74	75	82	51
Number of cells seeded per flask	300	300	300	300	300	300	400	800
Mean number of colonies per flask: N	94.4	86.2	83.4	91.0	78.6	74.4	82.4	55.4
ΔN	7.44	3.65	3.54	3.90	1.21	1.50	2.34	3.50
Number of cells seeded multiplied with PE: N_0		94.4	94.4	94.4	94.4	94.4	125.867	251.733
ΔN_0		7.440	7.440	7.440	7.440	7.440	9.921	19.841
Surviving fraction: SF		0.913	0.883	0.964	0.833	0.788	0.655	0.220
ΔSF		0.082	0.079	0.086	0.067	0.064	0.055	0.022
Surviving fraction corrected for multiplicity: f		**0.901**	**0.868**	**0.959**	**0.813**	**0.764**	**0.624**	**0.199**
Δf		**0.091**	**0.088**	**0.098**	**0.073**	**0.069**	**0.057**	**0.021**

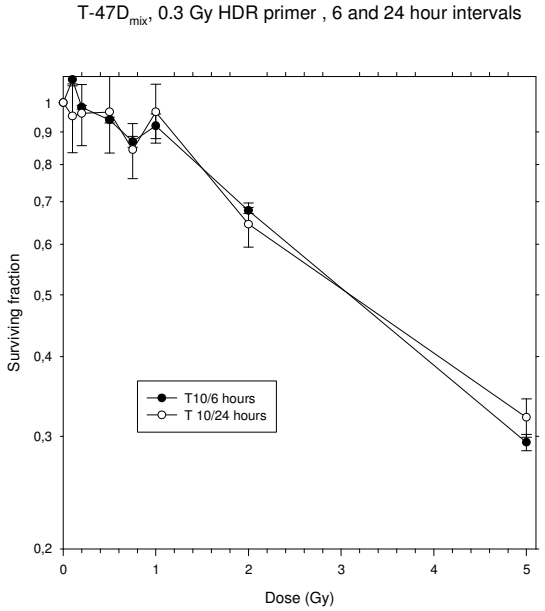
T-47D, 0.3 Gy primer with dose rate 0.9 Gy/h, 6 hours interval



IV: T-47Dmix, 0.3 Gy HDR priming dose, 6 and 24 hours before acute irradiation.

Experiment:	T10/6	November 20, 2002						
Multiplicity M=	1.161	Plating efficiency PE=	0.65					
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5
flask 1	195						188	172
flask 2	194	215	194	183				
flask 3	196	210			168	168	182	177
flask 4		202	191	188	173	188	186	
flask 5			192	184	178	189		161
Number of cells seeded per flask	300	300	300	300	300	300	400	800
Mean number of colonies per flask: N	195.0	209.0	192.3	185.0	173.0	181.7	185.3	170.0
ΔN	0.58	3.79	0.88	1.53	2.89	6.84	1.76	4.73
Number of cells seeded multiplied with PE: N_0		195	195	195	195	195	260	520
ΔN_0		0.577	0.577	0.577	0.577	0.577	0.770	1.540
Surviving fraction: SF		1.072	0.986	0.949	0.887	0.932	0.713	0.327
ΔSF		0.020	0.005	0.008	0.015	0.035	0.007	0.009
Surviving fraction corrected for multiplicity: f		**1.087**	**0.984**	**0.940**	**0.869**	**0.920**	**0.678**	**0.294**
Δf		**0.024**	**0.006**	**0.010**	**0.017**	**0.041**	**0.008**	**0.009**

Experiment:	T10/24	November 20, 2002						
Multiplicity M=	1.533	Plating efficiency PE=	0.816					
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5
flask 1	190	143	162	185	150	171	264	270
flask 2	147	156	160	165	133	167	274	318
flask 3	152	184	172	135	149	157	249	270
flask 4	166	158	143	160	166	148	232	262
flask 5	161	156	164	158	148	160	233	308
Number of cells seeded per flask	200	200	200	200	200	200	400	800
Mean number of colonies per flask: N	163.2	159.4	160.2	160.6	149.2	160.6	250.4	285.6
ΔN	7.48	6.71	4.76	7.99	5.23	4.01	8.32	11.39
Number of cells seeded multiplied with PE: N_0		163.2	163.2	163.2	163.2	163.2	326.4	652.8
ΔN_0		7.479	7.479	7.479	7.479	7.479	14.959	29.917
Surviving fraction: SF		0.977	0.982	0.984	0.914	0.984	0.767	0.438
ΔSF		0.061	0.054	0.067	0.053	0.051	0.043	0.027
Surviving fraction corrected for multiplicity: f		**0.953**	**0.962**	**0.967**	**0.844**	**0.967**	**0.645**	**0.321**
Δf		**0.117**	**0.106**	**0.133**	**0.083**	**0.102**	**0.051**	**0.022**



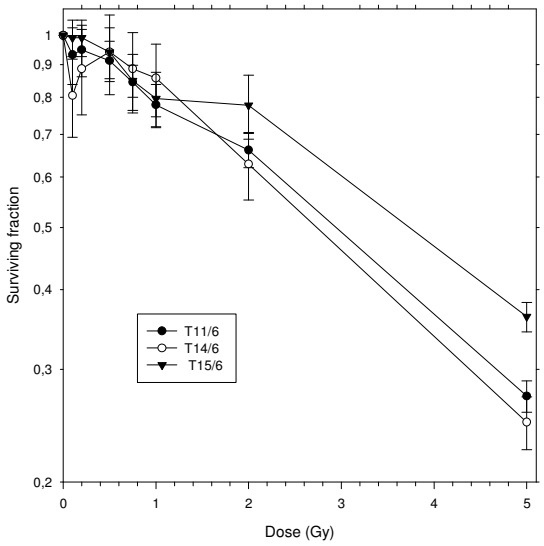
Va: T-47Dmix, 0.3 Gy priming dose, dose-rate 0.32 Gy/h, 6 hours before acute irradiation.

Experiment:	T11/6	November 26, 2002						
Multiplicity M=	1.282	Plating efficiency PE=			0.64867			
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5
flask 1	163	199	192	175	146	179	193	170
flask 2	205	191	153	194	183	166	191	182
flask 3	200	203	199	189	155	153	180	168
flask 4	220	188	200	174	166	160	187	167
flask 5	185	144	193	178	208	147	189	165
Number of cells seeded per flask	300	300	300	300	300	300	400	800
Mean number of colonies per flask: N	194.6	185.0	187.4	182.0	171.6	161.0	188.0	170.4
ΔN	9.68	10.60	8.74	4.01	10.99	5.52	2.24	3.01
Number of cells seeded multiplied with PE: N_0		194.6	194.6	194.6	194.6	194.6	259.467	518.933
ΔN_0		9.678	9.678	9.678	9.678	9.678	12.904	25.807
Surviving fraction: SF		0.951	0.963	0.935	0.882	0.827	0.725	0.328
ΔSF		0.072	0.066	0.051	0.072	0.050	0.037	0.017
Surviving fraction corrected for multiplicity: f		**0.933**	**0.949**	**0.913**	**0.845**	**0.779**	**0.661**	**0.272**
Δf		**0.095**	**0.088**	**0.066**	**0.089**	**0.059**	**0.041**	**0.015**

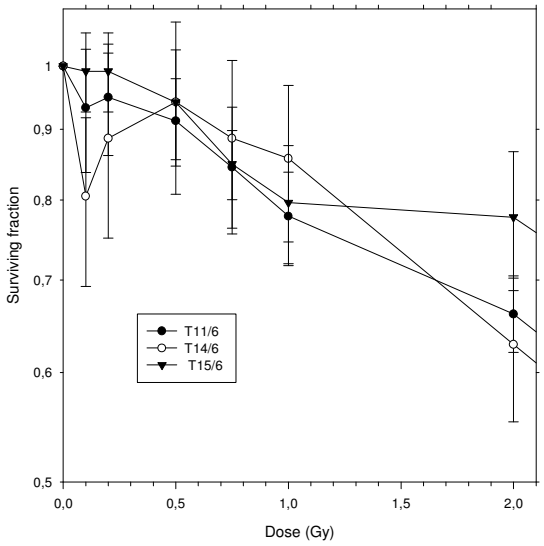
Experiment:	T14/6	December 12, 2002						
Multiplicity M=	1.294	Plating efficiency PE=			0.262			
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5
flask 1	57	36	55	42	46	50	63	51
flask 2	40	46	41	44	43	41	59	51
flask 3	62	47	41	58	41	53	47	46
flask 4	59	54	59	52	56	41	57	47
flask 5	44	40	44	55	54	49	48	43
Number of cells seeded per flask	200	200	200	200	200	200	300	600
Mean number of colonies per flask: N	52.4	44.6	48.0	50.2	48.0	46.8	54.8	47.6
ΔN	4.37	3.09	3.77	3.10	2.98	2.46	3.14	1.54
Number of cells seeded multiplied with PE: N_0		52.4	52.4	52.4	52.4	52.4	78.6	157.2
ΔN_0		4.366	4.366	4.366	4.366	4.366	6.549	13.097
Surviving fraction: SF		0.851	0.916	0.958	0.916	0.893	0.697	0.303
ΔSF		0.092	0.105	0.099	0.095	0.088	0.070	0.027
Surviving fraction corrected for multiplicity: f		**0.805**	**0.886**	**0.942**	**0.886**	**0.857**	**0.629**	**0.248**
Δf		**0.112**	**0.136**	**0.134**	**0.123**	**0.111**	**0.076**	**0.024**

Experiment:	T15/6	January 9, 2003						
Multiplicity M=	1.157	Plating efficiency PE=			0.421			
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5
flask 1	71	81	80	89	71	58	125	94
flask 2	82	92	92	79	73	59	116	101
flask 3	87	89	80	94	80	66	102	103
flask 4	90	78	89	72	69	79	71	97
flask 5	91	78	77	66	73	84	94	109
Number of cells seeded per flask	200	200	200	200	200	200	300	600
Mean number of colonies per flask: N	84.2	83.6	83.6	80.0	73.2	69.2	101.6	100.8
ΔN	3.65	2.91	2.91	5.19	1.85	5.27	9.35	2.58
Number of cells seeded multiplied with PE: N_0		84.2	84.2	84.2	84.2	84.2	126.3	252.6
ΔN_0		3.652	3.652	3.652	3.652	3.652	5.479	10.957
Surviving fraction: SF		0.993	0.993	0.950	0.869	0.822	0.804	0.399
ΔSF		0.055	0.055	0.074	0.044	0.072	0.082	0.020
Surviving fraction corrected for multiplicity: f		**0.992**	**0.992**	**0.941**	**0.849**	**0.796**	**0.777**	**0.363**
Δf		**0.065**	**0.065**	**0.086**	**0.049**	**0.079**	**0.090**	**0.019**

T-47D$_{mix}$, 0.3 Gy primer with dose rate 0.32 Gy/h, 6 hour interval



T-47D$_{mix}$, 0.3 Gy primer with dose rate 0.32 Gy/h, 6 hour interval



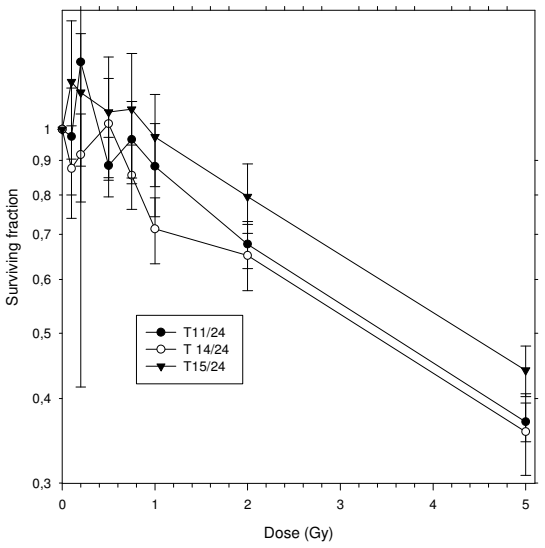
Vb: T-47Dmix, 0.3 Gy priming dose, dose-rate 0.32 Gy/h, 24 hours before acute irradiation.

Experiment:	T11/24	November 26, 2002						
Multiplicity M=	1.609	Plating efficiency PE=			0.713			
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5
flask 1	147	171	142	142	130	139	245	289
flask 2	154	127	165	133	131	142	215	291
flask 3	117	143	137	129	141	158	231	322
flask 4	142	126	165	138	157	128	225	279
flask 5	153	139	147	133	144	107	239	278
Number of cells seeded per flask	200	200	200	200	200	200	400	800
Mean number of colonies per flask: N	142.6	141.2	151.2	135.0	140.6	134.8	231.0	291.8
ΔN	6.76	8.15	5.85	2.26	4.93	8.45	5.25	7.98
Number of cells seeded multiplied with PE: N_0		142.6	142.6	142.6	142.6	142.6	285.2	570.4
ΔN_0		6.757	6.757	6.757	6.757	6.757	13.514	27.029
Surviving fraction: SF		0.990	1.060	0.947	0.986	0.945	0.810	0.512
ΔSF		0.074	0.065	0.048	0.058	0.074	0.043	0.028
Surviving fraction corrected for multiplicity: f		**0.976**	**1.258**	**0.884**	**0.966**	**0.882**	**0.677**	**0.370**
Δf		**0.176**	**0.841**	**0.089**	**0.134**	**0.139**	**0.054**	**0.024**

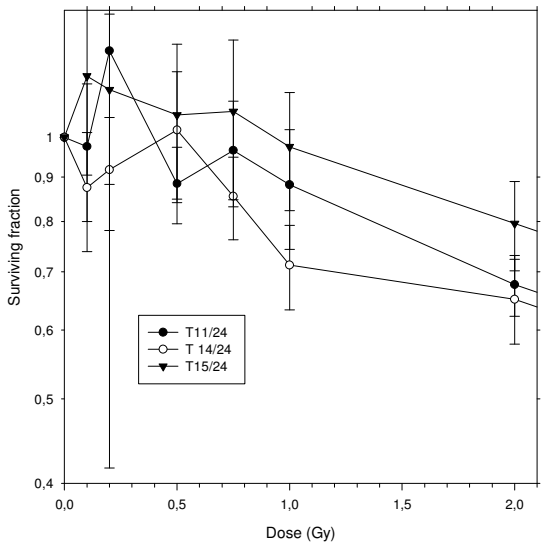
Experiment:	T14/24	December 12, 2002						
Multiplicity M=	1.498	Plating efficiency PE=	0.313					
Dose (Gy)	**Control**	**0.1**	**0.2**	**0.5**	**0.75**	**1**	**2**	**5**
flask 1	62	63	61	63	56	50	67	114
flask 2	53	44	66	66	58	54	64	102
flask 3	70	56	55	70	55	46	78	77
flask 4	73	62	67	51	57	58	78	63
flask 5	55	66	50	66	61	47		87
Number of cells seeded per flask	200	200	200	200	200	200	300	600
Mean number of colonies per flask: N	62.6	58.2	59.8	63.2	57.4	51.0	71.8	88.6
ΔN	3.96	3.90	3.25	3.25	1.03	2.24	3.66	8.99
Number of cells seeded multiplied with PE: N_0		62.6	62.6	62.6	62.6	62.6	93.9	187.8
ΔN_0		3.957	3.957	3.957	3.957	3.957	5.936	11.872
Surviving fraction: SF		0.930	0.955	1.010	0.917	0.815	0.764	0.472
ΔSF		0.086	0.080	0.082	0.060	0.063	0.062	0.056
Surviving fraction corrected for multiplicity: f		**0.875**	**0.918**	**1.019**	**0.855**	**0.713**	**0.651**	**0.357**
Δf		**0.137**	**0.136**	**0.170**	**0.093**	**0.080**	**0.073**	**0.049**

Experiment:	T15/24	January 9, 2003						
Multiplicity M=	1.549	Plating efficiency PE=	0.513					
Dose (Gy)	**Control**	**0.1**	**0.2**	**0.5**	**0.75**	**1**	**2**	**5**
flask 1	83	106	118	114	103	94	133	165
flask 2	96	118	98	105	117	91	131	163
flask 3	119	109	117	85	94	113	125	190
flask 4	102	103	105	118	94	103	138	178
flask 5	113	109	101	104	120	106	154	190
Number of cells seeded per flask	200	200	200	200	200	200	300	600
Mean number of colonies per flask: N	102.6	109.0	107.8	105.2	105.6	101.4	136.2	177.2
ΔN	6.35	2.51	4.12	5.70	5.54	4.01	4.91	5.83
Number of cells seeded multiplied with PE: N_0		102.6	102.6	102.6	102.6	102.6	153.9	307.8
ΔN_0		6.345	6.345	6.345	6.345	6.345	9.518	19.035
Surviving fraction: SF		1.062	1.051	1.025	1.029	0.988	0.885	0.576
ΔSF		0.070	0.076	0.084	0.083	0.073	0.063	0.040
Surviving fraction corrected for multiplicity: f		**1.176**	**1.134**	**1.061**	**1.071**	**0.975**	**0.796**	**0.440**
Δf		**0.272**	**0.251**	**0.219**	**0.224**	**0.151**	**0.094**	**0.038**

T-47D$_{mix}$, 0.3 Gy primer with dose rate 0.32 Gy/h, 24 hour interval



T-47D$_{mix}$, 0.3 Gy primer with dose rate 0.32 Gy/h, 24 hour interval



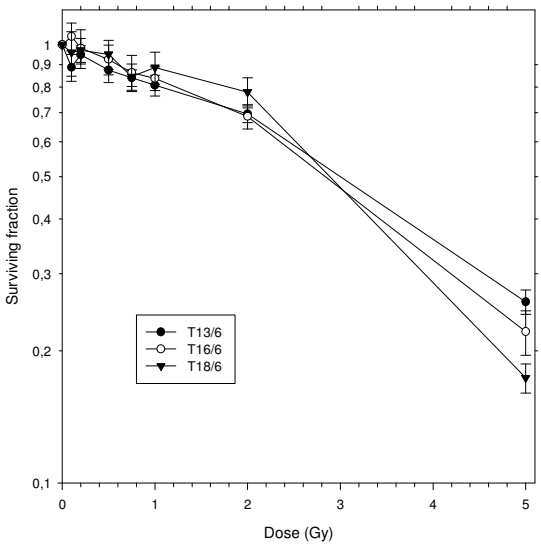
VIa: T-47Dmix, 0.3 Gy priming dose, dose-rate 0.045 Gy/h, 6 hours before acute irradiation.

Experiment:	T13/6	December 5, 2002						
Multiplicity M=	1.145	Plating efficiency PE=	0.595					
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5
flask 1	172	169	163	177	151	158	169	153
flask 2	185	153	183	162	153	156	177	113
flask 3		188	168	149	148	153	176	143
flask 4		152	173	168	157	136	165	132
flask 5		143	166	139	158	138	176	141
Number of cells seeded per flask	300	300	300	300	300	300	400	800
Mean number of colonies per flask: N	178.5	161.0	170.6	159.0	153.4	148.2	172.6	136.4
ΔN	6.50	7.94	3.50	6.76	1.86	4.65	2.38	6.73
Number of cells seeded multiplied with PE: N_0		178.5	178.5	178.5	178.5	178.5	238	476
ΔN_0		6.500	6.500	6.500	6.500	6.500	8.667	17.333
Surviving fraction: SF		0.902	0.956	0.891	0.859	0.830	0.725	0.287
ΔSF		0.055	0.040	0.050	0.033	0.040	0.028	0.018
Surviving fraction corrected for multiplicity: f		**0.888**	**0.949**	**0.875**	**0.840**	**0.808**	**0.695**	**0.259**
Δf		**0.062**	**0.046**	**0.056**	**0.037**	**0.044**	**0.030**	**0.016**

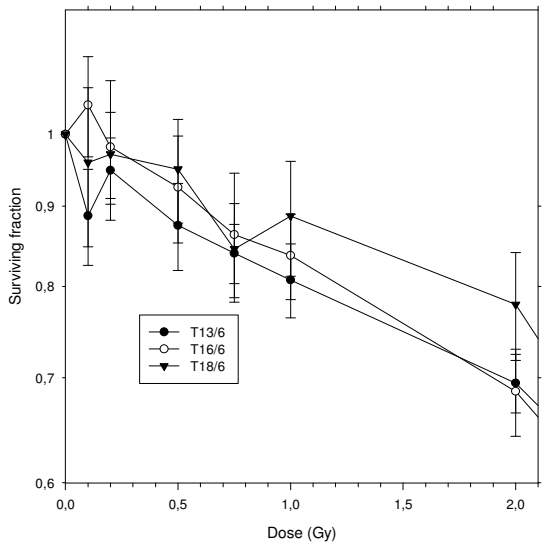
Experiment:	T16/6	January 15, 2003						
Multiplicity M=	1.156	Plating efficiency PE=	0.516					
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5
flask 1	117	117	124	83	74	91	116	82
flask 2	90	114	98	95	102	87	120	83
flask 3	102	110	113	109	108	89	116	97
flask 4	97	100	85	91	85	96	100	73
flask 5	110	94	88	105	86	80	105	49
Number of cells seeded per flask	200	200	200	200	200	200	300	600
Mean number of colonies per flask: N	103.2	107.0	101.6	96.6	91.0	88.6	111.4	76.8
ΔN	4.75	4.34	7.43	4.71	6.16	2.62	3.79	7.94
Number of cells seeded multiplied with PE: N_0		103.2	103.2	103.2	103.2	103.2	154.8	309.6
ΔN_0		4.748	4.748	4.748	4.748	4.748	7.121	14.243
Surviving fraction: SF		1.037	0.984	0.936	0.882	0.859	0.720	0.248
ΔSF		0.064	0.085	0.063	0.072	0.047	0.041	0.028
Surviving fraction corrected for multiplicity: f		**1.044**	**0.982**	**0.925**	**0.863**	**0.837**	**0.686**	**0.221**
Δf		**0.077**	**0.100**	**0.072**	**0.081**	**0.052**	**0.044**	**0.026**

Experiment:	T18/6	January 22, 2003						
Multiplicity M=	1.121	Plating efficiency PE=	0.475					
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5
flask 1	93	96	92	78	81	71	125	56
flask 2	107	70	95	97	82	81	113	54
flask 3	83	92	91	94	74	97	114	58
flask 4	85	118	90	97	89	88	123	44
flask 5	107	82	95	88	83	90	95	60
Number of cells seeded per flask	200	200	200	200	200	200	300	600
Mean number of colonies per flask: N	95.0	91.6	92.6	90.8	81.8	85.4	114.0	54.4
ΔN	5.18	7.98	1.03	3.60	2.40	4.41	5.31	2.79
Number of cells seeded multiplied with PE: N_0		95	95	95	95	95	142.5	285
ΔN_0		5.177	5.177	5.177	5.177	5.177	7.765	15.531
Surviving fraction: SF		0.964	0.975	0.956	0.861	0.899	0.800	0.191
ΔSF		0.099	0.054	0.064	0.053	0.067	0.057	0.014
Surviving fraction corrected for multiplicity: f		**0.960**	**0.971**	**0.950**	**0.845**	**0.887**	**0.779**	**0.174**
Δf		**0.111**	**0.061**	**0.072**	**0.058**	**0.074**	**0.061**	**0.013**

T-47D$_{mix}$, 0.3 Gy primer with dose rate 0.045 Gy/h, 6 hour interval



T-47D$_{mix}$, 0.3 Gy primer with dose rate 0.045 Gy/h, 6 hour interval



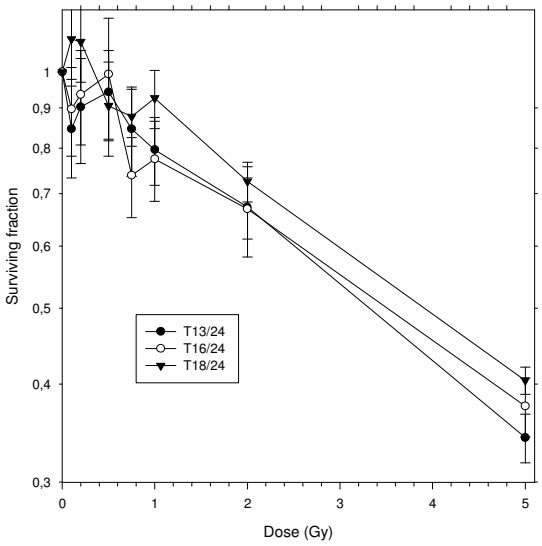
VIb: T-47Dmix, 0.3 Gy priming dose, dose-rate 0.045 Gy/h, 24 hours before acute irradiation.

Experiment:	**T13/24**	**December 5, 2002**						
Multiplicity M=	1.599	**Plating efficiency PE=**			0.647			
Dose (Gy)	**Control**	**0.1**	**0.2**	**0.5**	**0.75**	**1**	**2**	**5**
flask 1	109	121	116	118	135	113	201	267
flask 2	138	115	117	133	123	122	202	234
flask 3	145	118	138	130	120	115	225	254
flask 4	139	105	108	123	117	117	211	246
flask 5	116	139	139	127	103	111	202	233
Number of cells seeded per flask	200	200	200	200	200	200	400	800
Mean number of colonies per flask: N	129.4	119.6	123.6	126.2	119.6	115.6	208.2	246.8
ΔN	7.09	5.55	6.28	2.63	5.15	1.89	4.58	6.38
Number of cells seeded multiplied with PE: N_0		129.4	129.4	129.4	129.4	129.4	258.8	517.6
ΔN_0		7.089	7.089	7.089	7.089	7.089	14.179	28.358
Surviving fraction: SF		0.924	0.955	0.975	0.924	0.893	0.804	0.477
ΔSF		0.066	0.071	0.057	0.064	0.051	0.047	0.029
Surviving fraction corrected for multiplicity: f		**0.846**	**0.902**	**0.943**	**0.846**	**0.796**	**0.672**	**0.342**
Δf		**0.113**	**0.138**	**0.122**	**0.110**	**0.079**	**0.060**	**0.024**

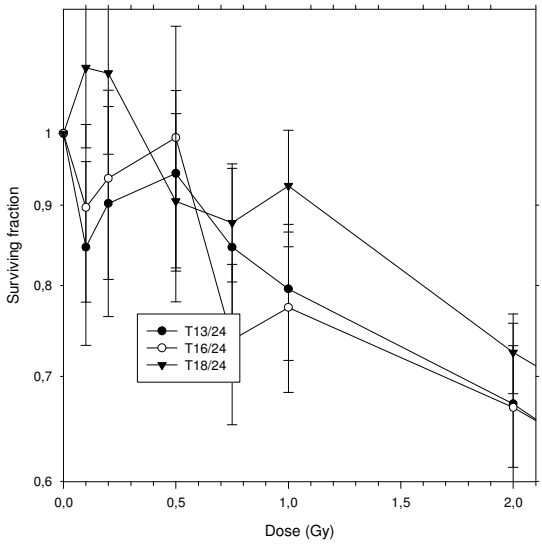
Experiment:	T16/24	January 15, 2003						
Multiplicity M=	1.530	Plating efficiency PE=	0.45667					
Dose (Gy)	**Control**	**0.1**	**0.2**	**0.5**	**0.75**	**1**	**2**	**5**
flask 1	126	130	123	130	128	131	194	218
flask 2	118	142	134	127	115	107	172	214
flask 3	171	128	131	119	116	122	142	214
flask 4	137	125	145	154	100	116	162	194
flask 5	133	123	130	153	117	118	138	186
Number of cells seeded per flask	300	300	300	300	300	300	400	800
Mean number of colonies per flask: N	137.0	129.6	132.6	136.6	115.2	118.8	161.6	205.2
ΔN	9.09	3.33	3.59	7.13	4.47	3.92	10.24	6.37
Number of cells seeded multiplied with PE: N_0		137	137	137	137	137	182.667	365.333
ΔN_0		9.094	9.094	9.094	9.094	9.094	12.125	24.251
Surviving fraction: SF		0.946	0.968	0.997	0.841	0.867	0.885	0.562
ΔSF		0.067	0.069	0.084	0.065	0.064	0.081	0.041
Surviving fraction corrected for multiplicity: f		**0.897**	**0.936**	**0.994**	**0.739**	**0.775**	**0.800**	**0.432**
Δf		**0.116**	**0.129**	**0.177**	**0.087**	**0.091**	**0.119**	**0.038**

Experiment:	T18/24	January 22, 2003						
Multiplicity M=	1.516	Plating efficiency PE=	0.595					
Dose (Gy)	**Control**	**0.1**	**0.2**	**0.5**	**0.75**	**1**	**2**	**5**
flask 1	113	124	118	90	120	126	148	194
flask 2	126	139	138	139	108	122	142	198
flask 3	128	121	129	117	104	106	144	180
flask 4	117	113	121	113	101	112	142	183
flask 5	111	124	113	106	122	106	163	189
Number of cells seeded per flask	200	200	200	200	200	200	300	600
Mean number of colonies per flask: N	119.0	124.2	123.8	113.0	111.0	114.4	147.8	188.8
ΔN	3.42	4.21	4.40	7.97	4.24	4.12	3.95	3.34
Number of cells seeded multiplied with PE: N_0		119	119	119	119	119	178.5	357
ΔN_0		3.421	3.421	3.421	3.421	3.421	5.131	10.262
Surviving fraction: SF		1.044	1.040	0.950	0.933	0.961	0.828	0.529
ΔSF		0.046	0.048	0.072	0.045	0.044	0.033	0.018
Surviving fraction corrected for multiplicity: f		**1.101**	**1.093**	**0.905**	**0.877**	**0.926**	**0.725**	**0.404**
Δf		**0.122**	**0.122**	**0.124**	**0.073**	**0.079**	**0.042**	**0.016**

T-47D$_{mix}$, 0.3 Gy primer with dose rate 0.045 Gy/h, 24 hour interval



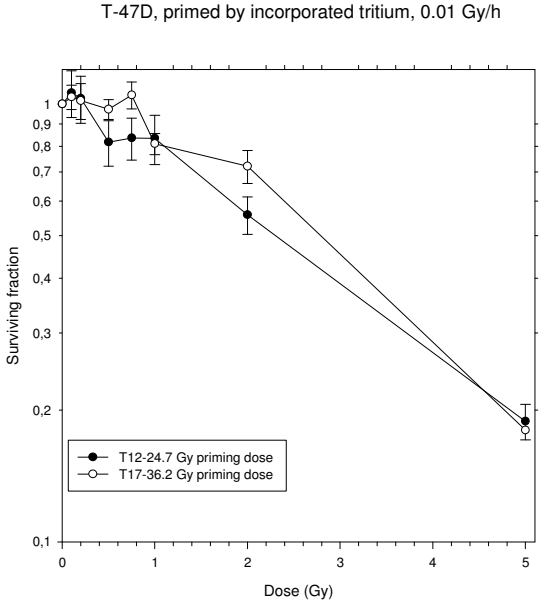
T-47D$_{mix}$, 0.3 Gy primer with dose rate 0.045 Gy/h, 24 hour interval

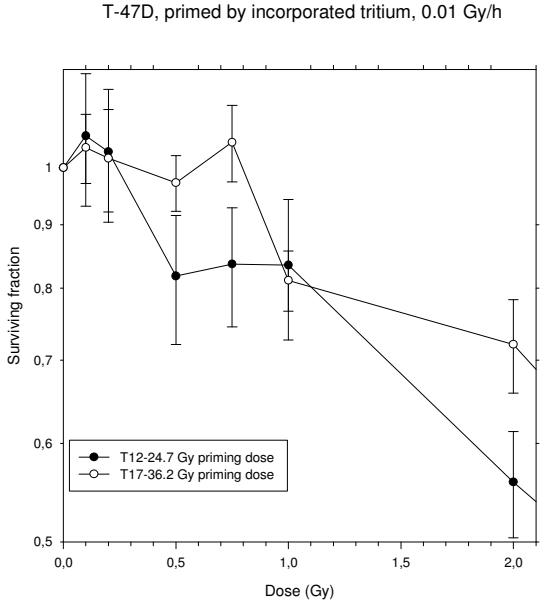


VII: T-47D, primed by incorporated tritium, 0.01 Gy/h, before acute irradiation.

Experiment:	T12	November 28, 2002						
Multiplicity M=	1.332	Plating efficiency PE=			0.381			
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5
flask 1	176	163	147	156	137	108	142	110
flask 2	152	146	156	113	127	137	132	90
flask 3	169	147	140	132	124	127	136	86
flask 4	151	175	175	135	130	161	161	88
flask 5	114	161	159	125	154	138	161	82
Number of cells seeded per flask	400	400	400	400	400	400	600	1000
Mean number of colonies per flask: N	152.4	158.4	155.4	132.2	134.4	134.2	146.4	91.2
ΔN	10.75	5.42	5.94	7.05	5.35	8.60	6.17	4.88
Number of cells seeded multiplied with PE: N_0		152.4	152.4	152.4	152.4	152.4	228.6	381
ΔN_0		10.745	10.745	10.745	10.745	10.745	16.118	26.863
Surviving fraction: SF		1.039	1.020	0.867	0.882	0.881	0.640	0.239
ΔSF		0.081	0.082	0.077	0.071	0.084	0.053	0.021
Surviving fraction corrected for multiplicity: f		**1.061**	**1.030**	**0.818**	**0.836**	**0.835**	**0.559**	**0.189**
Δf		**0.130**	**0.126**	**0.097**	**0.092**	**0.108**	**0.055**	**0.018**

Experiment:	T17	January 17, 2003						
Multiplicity M=	1.167	Plating efficiency PE=			0.2395			
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5
flask 1	105	102	80	91	95	80	107	49
flask 2	100	99	88	89	92	84	83	
flask 3	99	108	94	94	116	81	126	
flask 4	92	87	108	96	99	72	114	
flask 5	83	98	116	98	96	84	112	
Number of cells seeded per flask	400	400	400	400	400	400	600	1000
Mean number of colonies per flask: N	95.8	98.8	97.2	93.6	99.6	80.2	108.4	49.0
ΔN	3.81	3.43	6.56	1.63	4.25	2.20	7.08	
Number of cells seeded multiplied with PE: N_0		95.8	95.8	95.8	95.8	95.8	143.7	239.5
ΔN_0		3.813	3.813	3.813	3.813	3.813	5.720	9.533
Surviving fraction: SF		1.031	1.015	0.977	1.040	0.837	0.754	0.205
ΔSF		0.054	0.080	0.042	0.061	0.040	0.058	0.008
Surviving fraction corrected for multiplicity: f		**1.038**	**1.018**	**0.973**	**1.048**	**0.812**	**0.721**	**0.180**
Δf		**0.066**	**0.096**	**0.050**	**0.074**	**0.045**	**0.062**	**0.007**





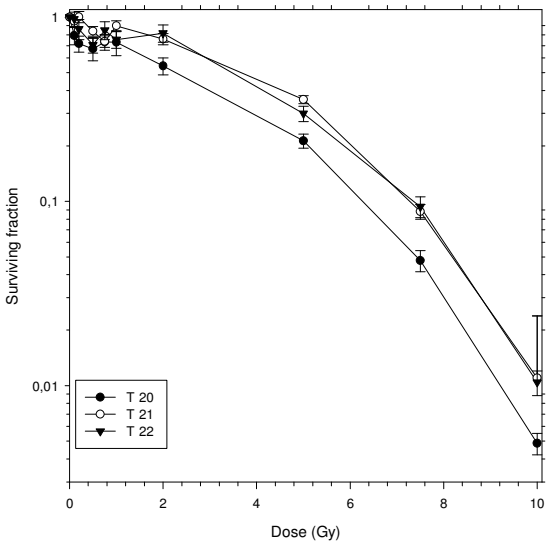
VIII: T-47Dmix, acute irradiation, no priming dose.

Experiment:	T20	January 27, 2003								
Multiplicity M=	1.447	Plating efficiency PE=	0.376							
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5	7.5	10
flask 1	71	57	59	78	65	79	69	58	29	10
flask 2	88	73	55	52	68	57	88	79	28	10
flask 3	73	70	72	53	57	72	82	57	44	16
flask 4	65	57	60	58	57	47	61	68	39	12
flask 5	79	69	58	49		53	69	63	52	18
Number of cells seeded per flask	200	200	200	200	200	200	300	600	1500	5000
Mean number of colonies per flask: N	75.2	65.2	60.8	58.0	61.8	61.6	73.8	65.0	38.4	13.2
ΔN	3.90	3.41	2.92	5.21	2.81	6.00	4.89	4.01	4.55	1.62
Number of cells seeded multiplied with PE: N_0		75.2	75.2	75.2	75.2	75.2	112.8	225.6	564	1880
ΔN_0		3.904	3.904	3.904	3.904	3.904	5.856	11.712	29.279	97.596
Surviving fraction: SF		0.867	0.809	0.771	0.821	0.819	0.654	0.288	0.068	0.007
ΔSF		0.064	0.057	0.080	0.057	0.090	0.055	0.023	0.009	0.001
Surviving fraction corrected for multiplicity: f		**0.794**	**0.718**	**0.673**	**0.734**	**0.731**	**0.543**	**0.213**	**0.048**	**0.005**
Δf		**0.087**	**0.071**	**0.095**	**0.072**	**0.114**	**0.057**	**0.018**	**0.006**	**0.001**

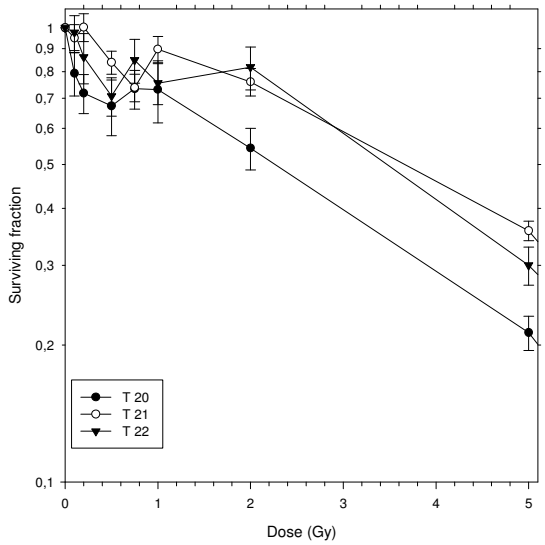
Experiment:	T21	January 30, 2003								
Multiplicity M=	1.254	Plating efficiency PE=	0.466							
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5	7.5	10
flask 1	98	104	99	73	75	83	94	109	61	106
flask 2	81	86	85	89	64	87	118	116	69	72
flask 3	93	83	84	81	69	89	124	108	85	71
flask 4	99	86	102	82	75	95	114	123	68	52
flask 5	95	89	97	82	84	75	114	125	96	89
Number of cells seeded per flask	200	200	200	200	200	200	300	600	1500	5000
Mean number of colonies per flask: N	93.2	89.6	93.4	81.4	73.4	85.8	112.8	116.2	75.8	78.0
ΔN	3.23	3.72	3.72	2.54	3.36	3.32	5.04	3.48	6.40	9.13
Number of cells seeded multiplied with PE: N_0		93.2	93.2	93.2	93.2	93.2	139.8	279.6	699	5662.65
ΔN_0		3.231	3.231	3.231	3.231	3.231	4.847	9.693	24.233	6566.265
Surviving fraction: SF		0.961	1.002	0.873	0.788	0.921	0.807	0.416	0.108	0.014
ΔSF		0.052	0.053	0.041	0.045	0.048	0.046	0.019	0.010	0.016
Surviving fraction corrected for multiplicity: f		**0.949**	**1.003**	**0.839**	**0.739**	**0.897**	**0.761**	**0.357**	**0.088**	**0.011**
Δf		**0.067**	**0.071**	**0.049**	**0.051**	**0.060**	**0.053**	**0.018**	**0.008**	**0.013**

Experiment:	T22	January 31, 2003								
Multiplicity M=	1.056	Plating efficiency PE=	0.32							
Dose (Gy)	Control	0.1	0.2	0.5	0.75	1	2	5	7.5	10
flask 1		59	73	38	66	43	95	53	54	23
flask 2	56	59	62	47	47	50	91	50	37	16
flask 3	76	68	45	42	44	54	67	69	52	15
flask 4	67	69	52	52	56		76	64	34	11
flask 5	57	58	46	51	61		68	63	59	23
Number of cells seeded per flask	200	200	200	200	200	200	300	600	1500	5000
Mean number of colonies per flask: N	64.0	62.6	55.6	46.0	54.8	49.0	79.4	59.8	47.2	17.6
ΔN	4.71	2.42	5.30	2.66	4.14	3.21	5.80	3.57	4.93	2.36
Number of cells seeded multiplied with PE: N_0		64	64	64	64	64	96	192	3313.95	11046.5
ΔN_0		4.708	4.708	4.708	4.708	4.708	7.062	14.124	1848.837	6162.791
Surviving fraction: SF		0.978	0.869	0.719	0.856	0.766	0.827	0.311	0.014	0.002
ΔSF		0.081	0.105	0.067	0.090	0.075	0.086	0.030	0.008	0.001
Surviving fraction corrected for multiplicity: f		**0.977**	**0.862**	**0.707**	**0.849**	**0.755**	**0.819**	**0.300**	**0.014**	**0.002**
Δf		**0.086**	**0.109**	**0.069**	**0.094**	**0.078**	**0.089**	**0.029**	**0.008**	**0.001**

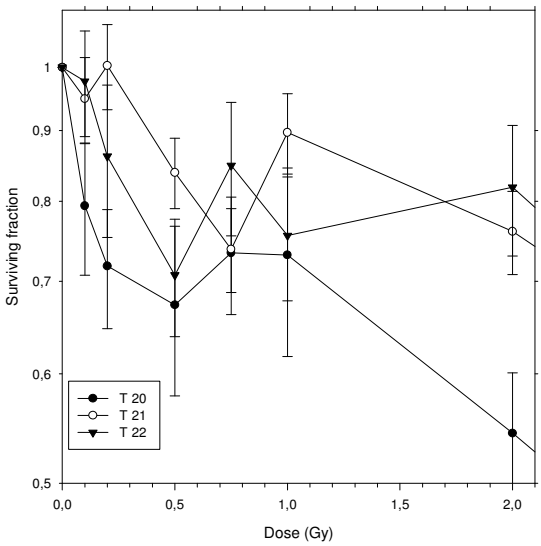
T-47D$_{mix}$, acute irradiation



T-47D$_{mix}$, acute irradiation



T-47D$_{mix}$, acute irradiation



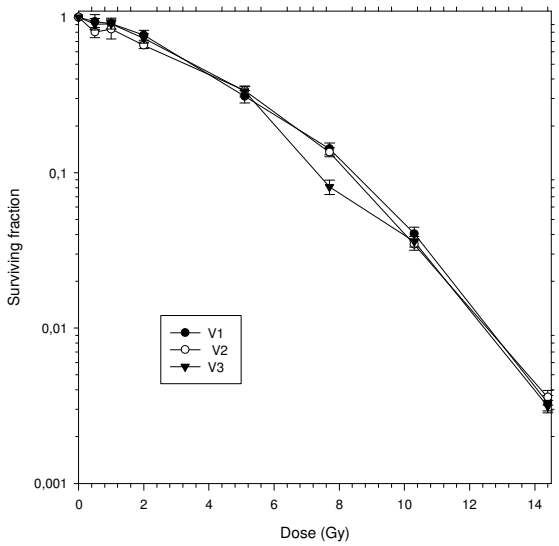
IX: V 79, acute irradiation, no priming dose.

Experiment:	V 1	February 7, 2002						
Multiplicity M=	1.052	Plating efficiency PE=	0.605					
Dose (Gy)	Control	0.5	1	2	5.1	7.7	10.2	14.4
flask 1	114	100	117	201	97	125	85	43
flask 2	144	103	108	176	115	131	99	36
flask 3	125	95	118	191	130	129	89	37
flask 4	96	128	119	189	124	167	113	37
flask 5	126	143	93	184		124	128	57
Number of cells seeded per flask	200	200	200	400	600	1500	4000	20000
Mean number of colonies per flask: N	121.0	113.8	111.0	188.2	116.5	135.2	102.8	42.0
ΔN	7.89	9.26	4.91	4.12	7.19	8.05	7.94	3.95
Number of cells seeded multiplied with PE: N_0		121	121	242	363	907.5	2420	12100
ΔN_0		7.887	7.887	15.773	23.660	59.150	157.734	788.670
Surviving fraction: SF		0.940	0.917	0.778	0.321	0.149	0.042	0.003
ΔSF		0.098	0.072	0.053	0.029	0.013	0.004	0.000
Surviving fraction corrected for multiplicity: f		**0.937**	**0.913**	**0.768**	**0.310**	**0.143**	**0.040**	**0.003**
Δf		**0.103**	**0.075**	**0.055**	**0.028**	**0.013**	**0.004**	**0.000**

Experiment:	V 2	February 14, 2002						
Multiplicity M=	1.144	Plating efficiency PE=	0.71					
Dose (Gy)	Control	0.5	1	2	5.1	7.7	10.2	14.4
flask 1	138	128	82	191	166	185	137	76
flask 2	165	134	161	190	151	143	93	56
flask 3	140	114	106	202	162	154	106	44
flask 4	136	103	118	207	134	179	123	49
flask 5	131	106	143	194	174	150	100	66
Number of cells seeded per flask	200	200	200	400	600	1500	4000	20000
Mean number of colonies per flask: N	142.0	117.0	122.0	196.8	157.4	162.2	111.8	58.2
ΔN	5.94	6.07	13.85	3.31	6.93	8.33	8.02	5.78
Number of cells seeded multiplied with PE: N_0		142	142	284	426	1065	2840	14200
ΔN_0		5.941	5.941	11.883	17.824	44.560	118.828	594.138
Surviving fraction: SF		0.824	0.859	0.693	0.369	0.152	0.039	0.004
ΔSF		0.055	0.104	0.031	0.022	0.010	0.003	0.000
Surviving fraction corrected for multiplicity: f		**0.801**	**0.840**	**0.661**	**0.337**	**0.135**	**0.035**	**0.004**
Δf		**0.060**	**0.115**	**0.033**	**0.021**	**0.009**	**0.003**	**0.000**

Experiment:	V 3	February 28, 2002						
Multiplicity M=	1.067	Plating efficiency PE=	0.907					
Dose (Gy)	Control	0.5	1	2	5.1	7.7	10.2	14.4
flask 1	205	149	162	261	214	112	150	48
flask 2	173	197	162	249	190	101	140	56
flask 3	204	141	151	294	174	131	109	69
flask 4	157	168	194	257	213	91	151	73
flask 5	168	171	160	292	160	149	146	57
Number of cells seeded per flask	200	200	200	400	600	1500	4000	20000
Mean number of colonies per flask: N	181.4	165.2	165.8	270.6	190.2	116.8	139.2	60.6
ΔN	9.78	9.75	7.34	9.35	10.63	10.43	7.79	4.57
Number of cells seeded multiplied with PE: N_0		181.4	181.4	362.8	544.2	1360.5	3628	18140
ΔN_0		9.781	9.781	19.561	29.342	73.354	195.612	978.059
Surviving fraction: SF		0.911	0.914	0.746	0.350	0.086	0.038	0.003
ΔSF		0.073	0.064	0.048	0.027	0.009	0.003	0.000
Surviving fraction corrected for multiplicity: f		**0.905**	**0.908**	**0.733**	**0.335**	**0.081**	**0.036**	**0.003**
Δf		**0.077**	**0.067**	**0.049**	**0.027**	**0.008**	**0.003**	**0.000**

V-79 acute irradiation (large doses)

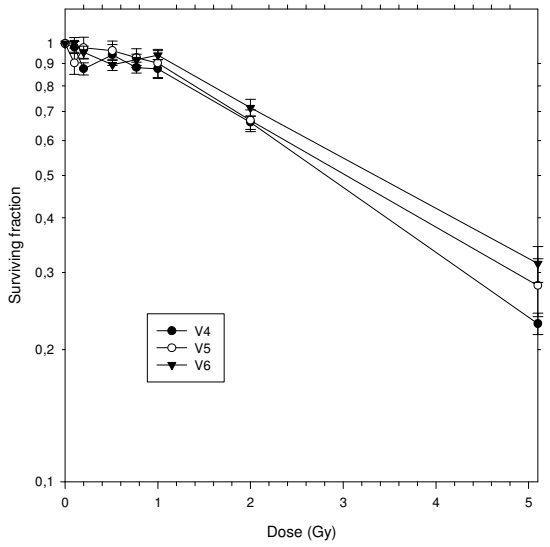


Experiment:	V 4	March 3, 2002						
Multiplicity M=	1.066	Plating efficiency PE=			0.88			
Dose (Gy)	Control	0.1	0.2	0.5	0.8	1	2	5.12
flask 1	176	184	146	192	159	140	264	162
flask 2	183	172	164	175	150	159	233	178
flask 3	164	176	163	165	165	168	233	139
flask 4	175	160	156	150	159	170	229	180
flask 5	182	172	148	151	149	140	230	190
Number of cells seeded per flask	200	200	200	200	200	200	400	800
Mean number of colonies per flask: N	176.0	172.8	155.4	166.6	156.4	155.4	237.8	169.8
ΔN	3.39	3.88	3.71	7.87	3.03	6.55	6.60	8.91
Number of cells seeded multiplied with PE: N_0		176	176	176	176	176	352	704
ΔN_0		3.391	3.391	3.391	3.391	3.391	6.782	13.565
Surviving fraction: SF		0.982	0.883	0.947	0.889	0.883	0.676	0.241
ΔSF		0.029	0.027	0.048	0.024	0.041	0.023	0.013
Surviving fraction corrected for multiplicity: f		**0.981**	**0.876**	**0.943**	**0.882**	**0.876**	**0.661**	**0.229**
Δf		**0.031**	**0.029**	**0.051**	**0.026**	**0.043**	**0.023**	**0.013**

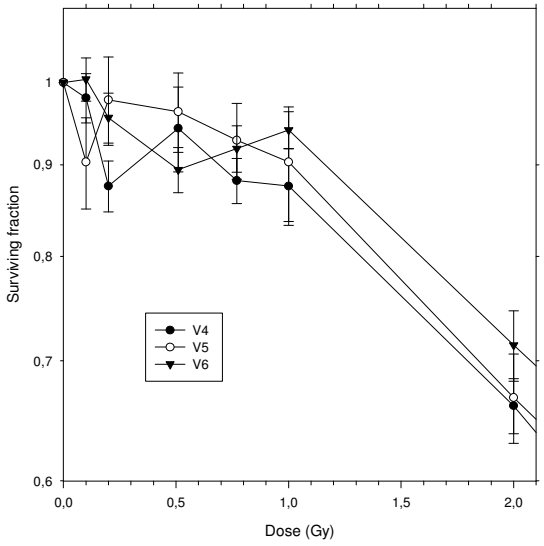
Experiment:	V 5	April 4, 2002						
Multiplicity M=	1.042	Plating efficiency PE=			0.568			
Dose (Gy)	Control	0.1	0.2	0.5	0.8	1	2	5.12
flask 1	104	122	114	122	113	99	153	101
flask 2	117	94	127	111	109	108	158	144
flask 3	106	102	113	112	108	116	170	133
flask 4	127	97	105	104	94	111	148	149
flask 5	114	100	97	99	105	81	140	129
Number of cells seeded per flask	200	200	200	200	200	200	400	800
Mean number of colonies per flask: N	113.6	103.0	111.2	109.6	105.8	103.0	153.8	131.2
ΔN	4.13	4.94	5.00	3.91	3.22	6.16	5.02	8.37
Number of cells seeded multiplied with PE: N_0		113.6	113.6	113.6	113.6	113.6	227.2	454.4
ΔN_0		4.130	4.130	4.130	4.130	4.130	8.261	16.522
Surviving fraction: SF		0.907	0.979	0.965	0.931	0.907	0.677	0.289
ΔSF		0.055	0.057	0.049	0.044	0.063	0.033	0.021
Surviving fraction corrected for multiplicity: f		**0.903**	**0.978**	**0.963**	**0.929**	**0.903**	**0.668**	**0.280**
Δf		**0.056**	**0.059**	**0.051**	**0.046**	**0.066**	**0.034**	**0.021**

Experiment:	V 6	April 10, 2002						
Multiplicity M=	1.042	Plating efficiency PE=			0.755			
Dose (Gy)	**Control**	**0.1**	**0.2**	**0.5**	**0.8**	**1**	**2**	**5.12**
flask 1	158	156	146	136	133	140	217	185
flask 2	152	151	151	141	143	143	220	188
flask 3	144	161	153	137	132	145	242	196
flask 4	144	145	141	139	141	146	215	182
flask 5	157	145	132	125	147	138	197	226
Number of cells seeded per flask	200	200	200	200	200	200	400	800
Mean number of colonies per flask: N	151.0	151.6	144.6	135.6	139.2	142.4	218.2	195.4
ΔN	3.03	3.12	3.78	2.79	2.91	1.50	7.18	8.00
Number of cells seeded multiplied with PE: N_0		151	151	151	151	151	302	604
ΔN_0		3.033	3.033	3.033	3.033	3.033	6.066	12.133
Surviving fraction: SF		1.004	0.958	0.898	0.922	0.943	0.723	0.324
ΔSF		0.029	0.032	0.026	0.027	0.021	0.028	0.015
Surviving fraction corrected for multiplicity: f		**1.004**	**0.956**	**0.894**	**0.919**	**0.941**	**0.714**	**0.315**
Δf		**0.030**	**0.033**	**0.027**	**0.028**	**0.022**	**0.028**	**0.015**

V-79 acute irradiation (small doses)



V-79 acute irradiation (small doses)

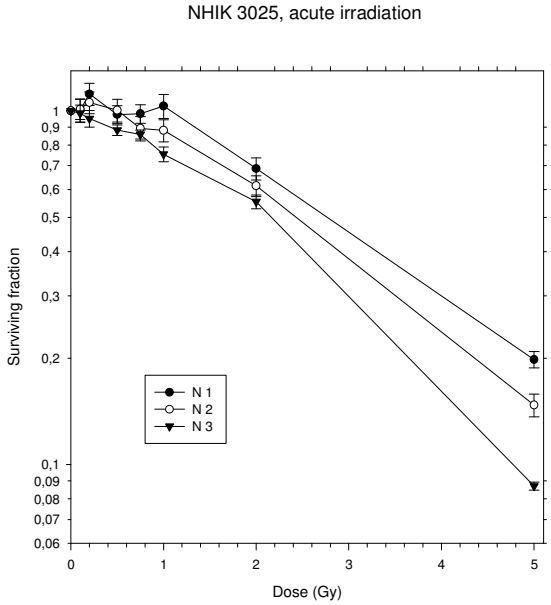


X: NHIK 3025, acute irradiation, no priming dose.

Experiment:	N1	February 11, 2003						
Multiplicity M=	1.17031	Plating efficiency PE=			0.666			
Dose (Gy)	control	0.1	0.2	0.5	0.75	1	2	5
flask 1	121	119	150	131	135	131	202	288
flask 2	124	130	161	131	138	144	173	311
flask 3	155	151	146	131	129	145	219	308
flask 4	126	137	140	134	125	148	178	284
flask 5	140	132	132	126	129	116	192	309
Number of cells seeded per flask	200	200	200	200	200	200	400	2000
Mean number of colonies per flask: N	133.2	133.8	145.8	130.6	131.2	136.8	192.8	300.0
ΔN	6.35	5.21	4.86	1.29	2.33	5.96	8.32	5.77
Number of cells seeded multiplied with PE: N_0		133.2	133.2	133.2	133.2	133.2	266.4	1332
ΔN_0		6.351	6.351	6.351	6.351	6.351	12.703	63.514
Surviving fraction: SF		1.005	1.095	0.980	0.985	1.027	0.724	0.225
ΔSF		0.062	0.064	0.048	0.050	0.066	0.047	0.012
Surviving fraction corrected for multiplicity: f		**1.005**	**1.117**	**0.977**	**0.982**	**1.033**	**0.687**	**0.198**
Δf		**0.075**	**0.081**	**0.057**	**0.060**	**0.081**	**0.050**	**0.011**

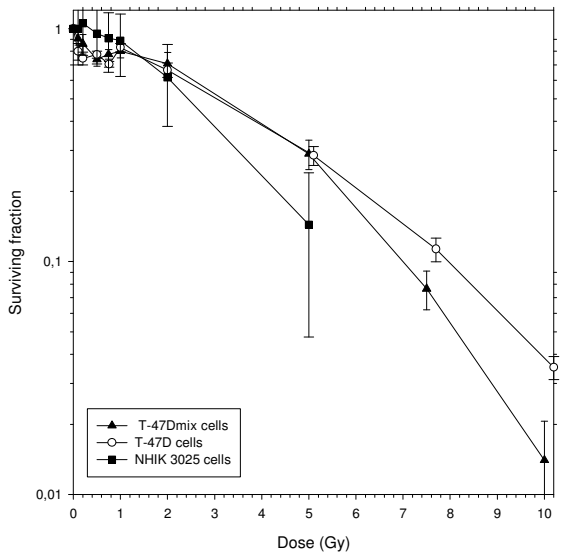
Experiment:	N2	February 12, 2003						
Multiplicity M=	1.11163	Plating efficiency PE=			0.569			
Dose (Gy)	control	0.1	0.2	0.5	0.75	1	2	5
flask 1	103	111	122	122	92	91	137	174
flask 2	103	120	121	103	105	100	158	155
flask 3	110	110	123	108	116	99	156	191
flask 4	137	117	106	114	108	109	138	197
flask 5	116	117	125	124	93	109	140	201
Number of cells seeded per flask	200	200	200	200	200	200	400	2000
Mean number of colonies per flask: N	113.8	115.0	119.4	114.2	102.8	101.6	145.8	183.6
ΔN	6.29	1.92	3.41	4.00	4.58	3.40	4.61	8.51
Number of cells seeded multiplied with PE: N_0		113.8	113.8	113.8	113.8	113.8	227.6	1138
ΔN_0		6.288	6.288	6.288	6.288	6.288	12.576	62.881
Surviving fraction: SF		1.011	1.049	1.004	0.903	0.893	0.641	0.161
ΔSF		0.058	0.065	0.066	0.064	0.058	0.041	0.012
Surviving fraction corrected for multiplicity: f		**1.012**	**1.056**	**1.004**	**0.893**	**0.881**	**0.614**	**0.147**
Δf		**0.066**	**0.075**	**0.074**	**0.070**	**0.063**	**0.042**	**0.011**

Experiment:	N3	February 14, 2003						
Multiplicity M=	1.01498	Plating efficiency PE=			0.786			
Dose (Gy)	control	0.1	0.2	0.5	0.75	1	2	5
flask 1	153	163	136	146	131	108	174	139
flask 2	151	176	157	128	137	111	197	136
flask 3	168	138	156	140	139	129	178	138
flask 4	148	137		137	137	131	169	138
flask 5	166	160		144	132	116	158	142
Number of cells seeded per flask	200	200	200	200	200	200	400	2000
Mean number of colonies per flask: N	157.2	154.8	149.7	139.0	135.2	119.0	175.2	138.6
ΔN	4.09	7.56	6.84	3.16	1.56	4.68	6.40	0.98
Number of cells seeded multiplied with PE: N_0		157.2	157.2	157.2	157.2	157.2	314.4	1572
ΔN_0		4.091	4.091	4.091	4.091	4.091	8.183	40.915
Surviving fraction: SF		0.985	0.952	0.884	0.860	0.757	0.557	0.088
ΔSF		0.054	0.050	0.031	0.024	0.036	0.025	0.002
Surviving fraction corrected for multiplicity: f		**0.985**	**0.951**	**0.883**	**0.858**	**0.754**	**0.554**	**0.087**
Δf		**0.055**	**0.051**	**0.031**	**0.025**	**0.036**	**0.025**	**0.002**

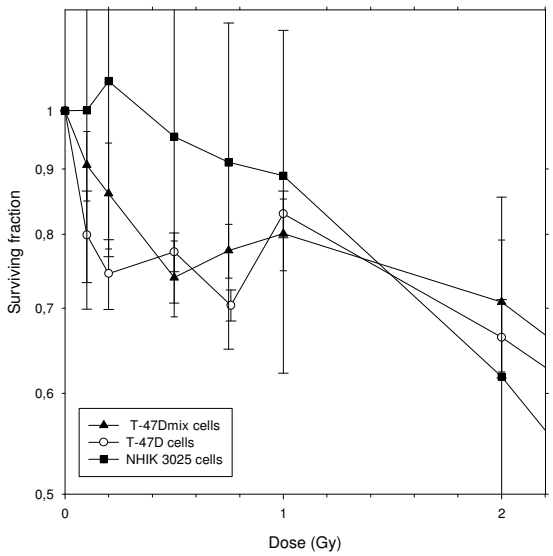

NHIK 3025, acute irradiation
Surviving fraction
Dose (Gy)
N 1
N 2
N 3
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,09
0,08
0,07
0,06
0
1
2
3
4
5

APPENDIX E: Dose-response curves with calculated mean values of survival fraction

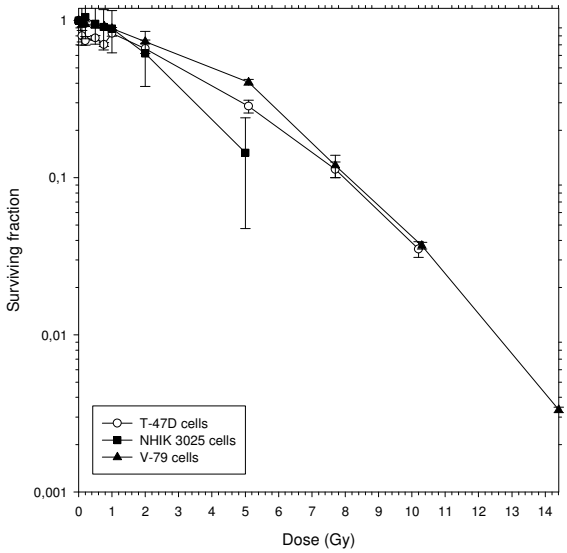
Mean surviving fraction of three independent experiments for T-47D, T-47D$_{mix}$, and NHIK 3025 cells



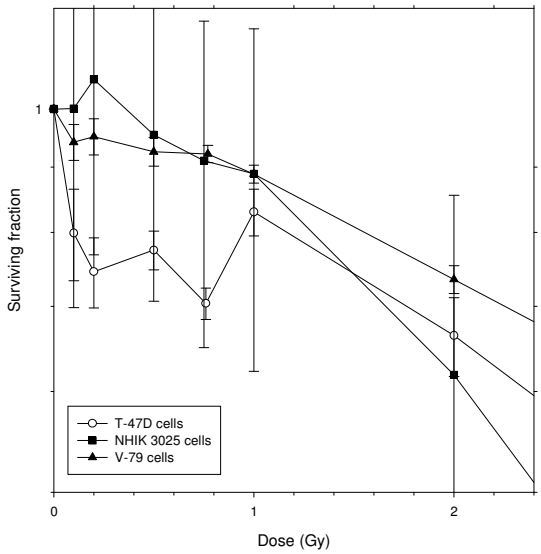
Mean surviving fraction of three independent experiments for T-47D, T-47D$_{mix}$, and NHIK 3025 cells



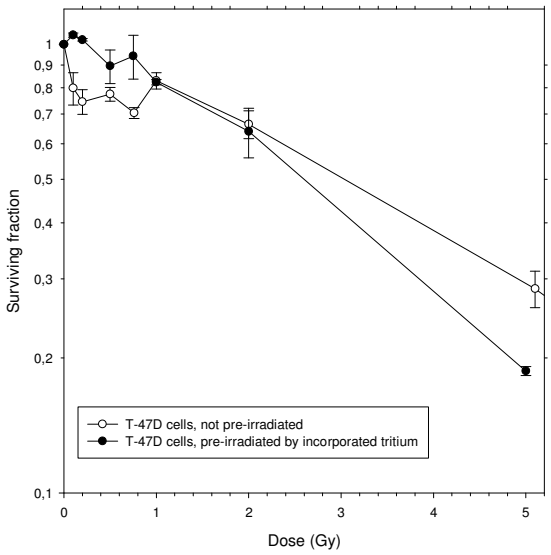
Mean surviving fraction of three independent experiments for T-47D, V-79, and NHIK 3025 cells



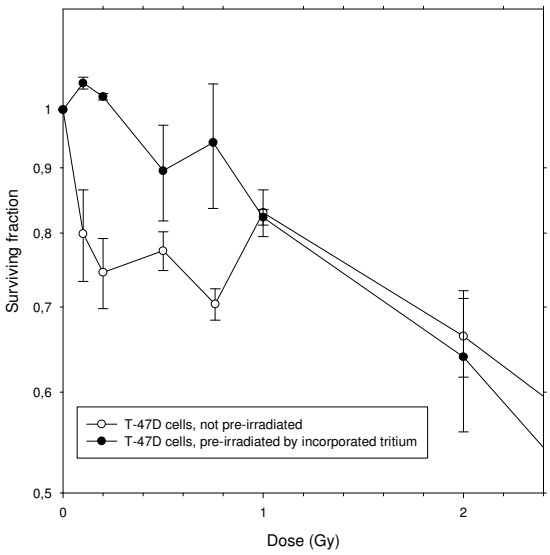
Mean surviving fraction of three independent experiments for T-47D, V-79, and NHIK 3025 cells



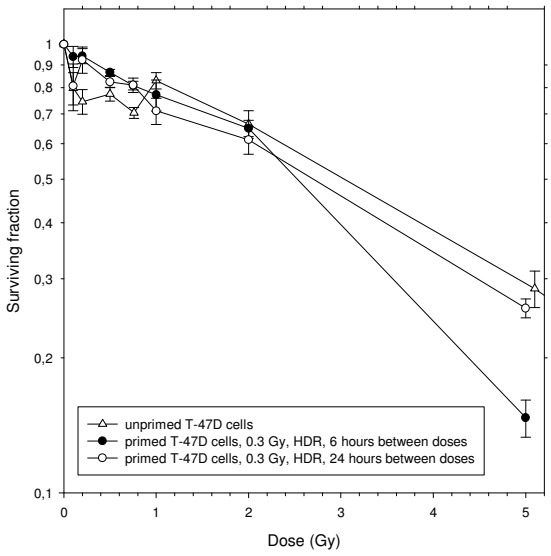
Mean values of surviving fraction
for T-47D cells, not pre-irradiated (three experiments) and
T-47D cells, pre-irradiated by incorporated tritium (two experiments)



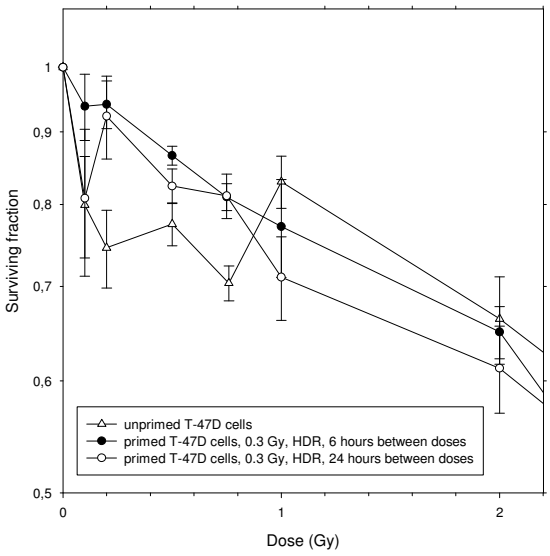
Mean values of surviving fraction
for T-47D cells, not pre-irradiated (three experiments) and
T-47D cells, pre-irradiated by incorporated tritium (two experiments)



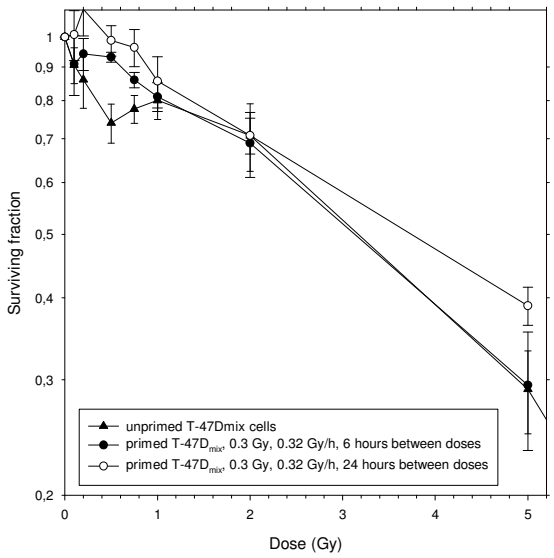
Mean values of surviving fraction
for T-47D cells, not pre-irradiated (three experiments) and
T-47D cells with HDR priming dose of 0.3 Gy (two experiments)



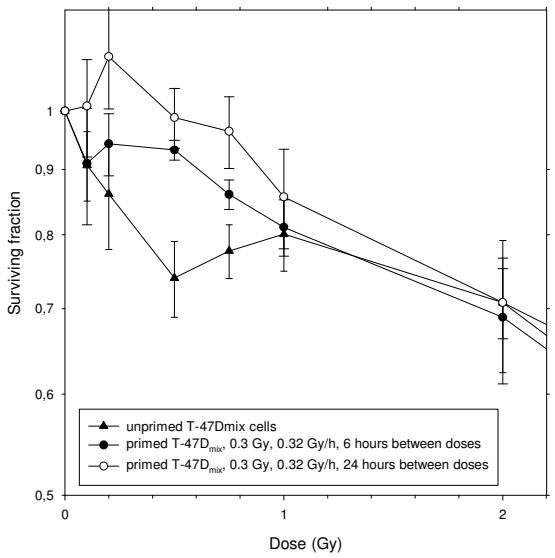
Mean values of surviving fraction
for T-47D cells, not pre-irradiated (three experiments) and
T-47D cells with HDR priming dose of 0.3 Gy (two experiments)



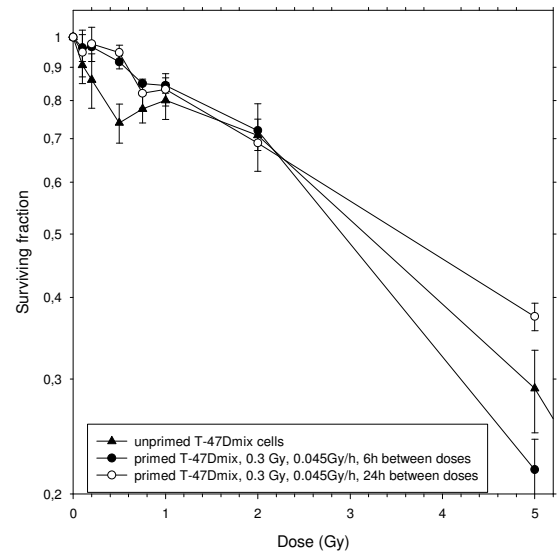
T-47D$_{mix}$ cells, unprimed and with a 0.3 Gy priming dose delivered with 0.32 Gy/h mean values of surviving fraction from three independent experiments.



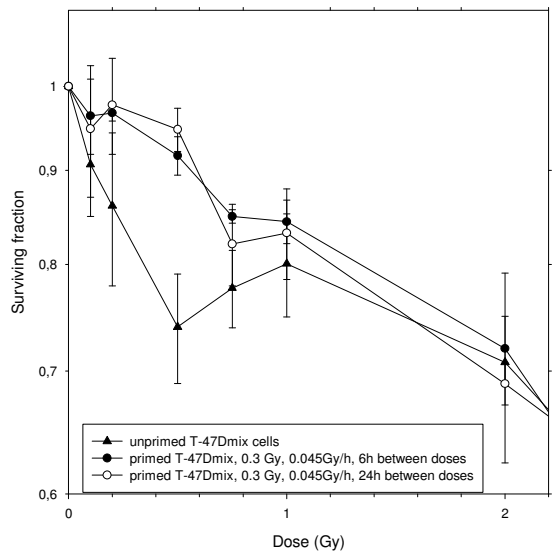
T-47D$_{mix}$ cells, unprimed and with a 0.3 Gy priming dose delivered with 0.32 Gy/h mean values of surviving fraction from three independent experiments.



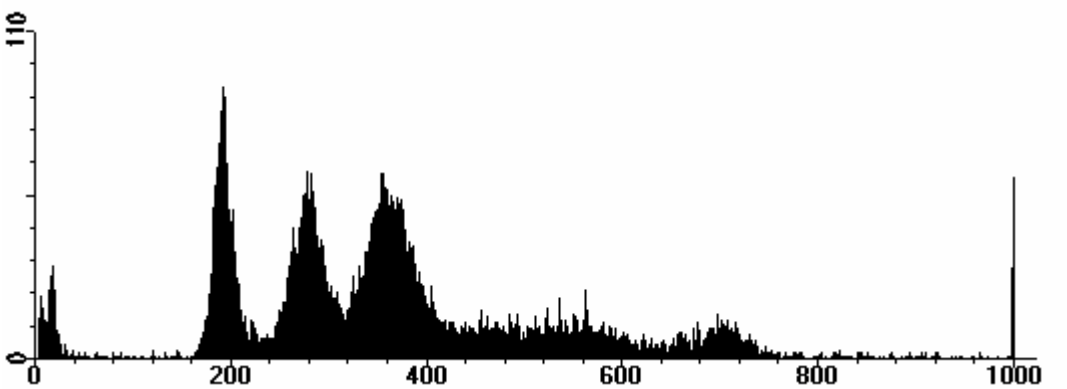
T-47D$_{mix}$ cells, unprimed and with a 0.3 Gy priming dose delivered with 0.045 Gy/h mean values of surviving fraction from three independent experiments.



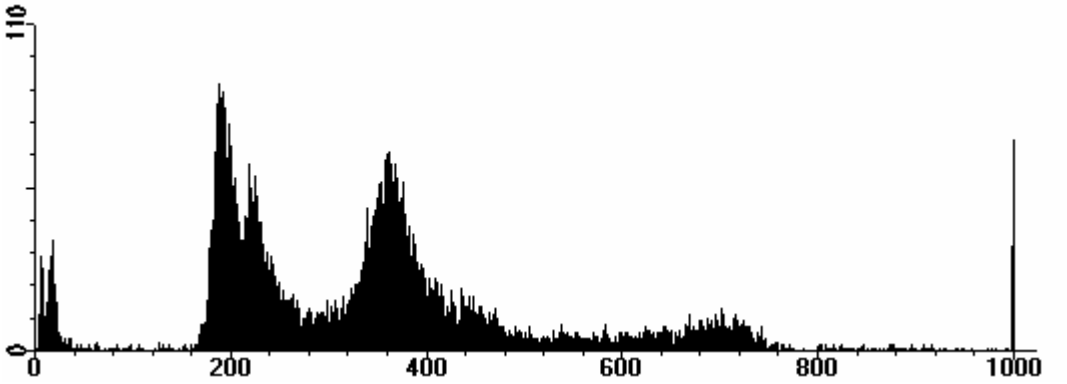
T-47D$_{mix}$ cells, unprimed and with a 0.3 Gy priming dose delivered with 0.045 Gy/h mean values of surviving fraction from three independent experiments.



APPENDIX F: Supplementary DNA histograms from flow cytometry



DNA histogram of a cell population of MCF7 cells added to the mixed cell population (T-47D$_{mix}$). Relative cell number is shown as a function of relative DNA content.



DNA histogram of a cell population of HU adapted T-47D cells added to the mixed cell population (T-47D$_{mix}$). Relative cell number is shown as a function of relative DNA content.