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Biophysical Study of the Effect of IGF1R inhibition on the breast cancer cells

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ABSTRACT

Radiation induced damage is mainly initiated by the deposition of energy directly or indirectly by the primary products of water radiolysis. These products immediately interact with the surrounding biological molecules, forming secondary organic radicals, the later can be subjected to further transformation as a result of intermolecular energy migration process or interaction with molecules that are effective energy acceptors, causing their modifications. In this paper we aimed to study the effect of changing radiation quality on the radiation- induced inactivation of breast cancer cells, MCF7 andMDA-MB-231 in IGF1R depleted and normal cells, results showed that a-particle is more effective in inducing DNA double strand break damage (DSB) and that the absence of insulin growth factor receptor type-1 (IGF1R) cause the apoptosis of breast cancer cells that was more severe in MDA-MB-231 comparing with MCF7.

Key words: Radiation, IGF1R inhibition, breast cancer cells.

Introduction

Breast cancer is the most common tumor amongst women worldwide, and the second leading cause of cancer-related death in America (Song *et al.,* 2007). Two third of breast cancer cells are known to be Estrogen receptor positive (Song *et al.,* 2007).Reports have proven higher levels of oxidatively induced stress and DNA repair defects for breast cancer cells (Francisco, Peddi *et al*. 2008). Moreover, IGF1R is highly expressed in many cancers including breast cancer cells (Clark *et al.,* 2005),(Jackson, White *et al*. 1998). MCF7 breast cancer cell line are known to over express IGF1R, several studies have proved that IGF1R is expressed more in MCF7 cell line compared to MDA-231cells (Song *et al.,* 2007).

Apoptosis is a hallmark in cancer, whereas, insulin growth factor type 1 receptor (IGF1R) plays a pivotal role in the protection of cells from apoptosis, and inducing normal and malignant cell transformation and proliferation (Xiong *et al.,* 2007). IGF1R is a membrane associated tyrosine receptor, consisting of two identical α-subunits responsible for ligand binding, two identical ß- units with transmembrane domain, an intra cellular tyrosine kinase and a COOH-terminal domain (Girnita *et al.,* 2004;Xiong *et al.,* 2007 and Trojanek *et al.,* 2003).

Ionizing radiation transfers its energy to the cell through clusters of ionization and excitation interactions. Depending on the quality of radiation, these interactions may occur directly or indirectly. Direct interaction, as the name implies, involves the direct ionization of the target molecule (e.g DNA), producing delta electrons which continue interacting with the molecules of the medium directly or indirectly. Whereas, on the other hand, indirect ionization occurs through the ionization of the water molecules surrounding the target medium producing free radicals, reactive species that ionize the target molecules (Hill, 1999 and Goodhead, 1999).

The relative biological effectiveness (RBE) is used to differentiate between different particles having the same LET (Hill, 1999). RBE is defined as the ratio of doses for equal biological effect relative to low LET hard X-ray or γ radiation as a reference.

Experimentally, it is found that the RBE increases as the LET increases up to 100KeV/µm, beyond this value the RBE falls with increasing LET. RBE peaks at this value (100 KeV/µm), since the diameter of the DNA double strand coincides with the distance separating two ionization events (2 nm or 20 Å). Radiation with this density is more likely to produce more double strand breaks which are the origin of biological damage.

The low LET radiation such X-rays produce sparse double strand break because the distance separating the events is less than the diameter of the DNA double helix. As a consequence, low LET radiation has low RBE (Goodhead, 1994; Hall and Hei, 2003;Hill, 1999) at values greater than 100 KeV/ µm (much denser ionization) the energy are wasted because the events is too close to produce DSB (Hall and Hei, 2003). This paper aimed to study the relationship between radiation quality factor and the inactivation of breast cancer cells in the presence and absence of IGF1R.

Materials and Methods

Cell lines:

Frozen MCF10A cell line where commercially purchased from ATCC and following retrieval from liquid nitrogen, the cells were re-seeded in 25cm^2 flask in Dulbecco's modified Eagle's medium (DMEM)/ ham's F-12

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supplemented with 5 % horse serum, 10 µg/ml human insulin, 10 ng/ml EGF, 100 ng/ml cholera toxin, 0.5 g/ml hydrocortisone,100 units/ml of penicillin/ streptomycin sulphate, and 0.01% L-Glutamine. The cells were left to grow to 70% confluent in humidified atmosphere of 95% air, 5% CO_2, tripsinized with 0. 5% trypsin and then transferred into 75 cm^2 falcon flasks.

Frozen MCF7 and MDA- MB-231 were obtained from Laura Philips a DPhil student in Ruth Michel's group at the Gray Institute, The University of Oxford. Cells retrieved from liquid nitrogen, were seeded in 25cm^2 flasks into DMEM medium supplemented with10% fetal bovine serum, 10 units penicillin/streptomycin and 0.01% L-Glutamine. Cells were left to grow to 70% confluent in humidified atmosphere of 95% air , 5% CO_2,tripsinized with 0.25% trypsine and then transferred into T75 flask.

Mycoplasma test:

HEK–Blue 2 cells were plated at 2x10^6 per flask for 48 h before the test to 50-70% confluence, while test cells were plated in the absence of antibody for 48h before testing. The tested cells were removed by scraping, and 500µl of supernatant was collected and placed into 1.5 ml microfuge tube. The samples were heated at 100^{o}C for 15 min, followed by cooling for few minutes at room temperature. Samples were then mixed by vigorous vortexing followed by addition of 50 µl to each of the sample/well of flat bottom 96 well plate. 50µl of 1x negative control was added to one well and a 50µl of 1xpositive control to another well. HEK-Blue 2 cells were detached by adding 1xPBS, spun Eppendorf Centrifuge (5702R), re-suspended cells in HEK-Blue2 detection medium at density of 1-3.5x10^5 cells/ml. In the mycoplasma room 200 µl of HEK-Blue 2 cells were mixed and added to each well containing sample, controls or no sample, and finally incubate at 37^{O}C for 16-24h. Plates were then visualized by naked eye, i.e., purple/blue staining or pink/light purple staining identify positive or negative cells respectively.

Irradiation facility:

Cells were irradiated with either ^{137}Cs γ–rays which is considered as low linear energy transfer (LET) ionizing radiation or ^{238}Pu α-particles, as an example of high LET.

Monolayer cells were irradiated at room temperature with ^{137}Cs γ–rays at a dose rate 1.94Gy/min, and then were transferred after irradiation onto an iced surface then medium were changed immediately. The cells were kept at 37^{o}C in incubator for a given time depending on the endpoint to be determined. Cells were irradiated with a-particles using238Pu, (LET is approximately 129 KeV/µm if a cell thickness of 5 µm is assumed). ^{238}Pu with energy 3.2 MeV is produced in reactors by bombarding uranium oxide with deuterons. Prior to irradiation, cells were seeded either onto a 0.9 µm mylar-based dish. To help MCF10A cells to attach as a monolayer population, the mylar surface was initially treated with Cell Tak cell adhesive. Before seeding, Cell Tak was added to dishes at a density of 3µg cm^{-2} of surface area in 0.1 M sodium bicarbonate buffer for 45 mint then washed three times with 1X PBS. Cells were irradiated at room temperature then dishes are transferred to an iced cold surface before changing the medium. The cells were incubated at 37^{o}C.

The mean nuclear area of MDA-MB-231 and MCF10A cells were measured before and after irradiation using confocal microscope (Zeiss LSM 710). Cells were stained with rhodamine-123 to identify the nucleus of the attached cells. Nuclear area measurements were used to calculate the average number of α- particles track traversal per nucleus using the following equation:

$$N= (DA/0.16L),$$

Where N is number of alpha particles traversals, L is linear energy transfer (LET, keV/µm), A is nuclear area (µm^2) and D is dose in Gy (Portess, Bauer *et al*. 2007).

Survival curves

Low LET survival curves:

Exponentially growing MCF10A, MCF7 and MDA-MB-231 were seeded at a cell number of 10^6 per 25cm^2 falcon flasks with five flasks for each cell line and incubated at37^{o}C for 24 h. Flasks were irradiated with Cs-137 γ-radiation with doses of 0, 1, 2, 4 and6 Gy with a dose rate 1.94Gy/min, as mentioned in the irradiation facility section.

Cells were then harvested with 0.25, 0.5 and 1% trypsin for the cell lines MCF7,MCF10A and MDA-MB-231 respectively. Cell densities were counted using hemocytometer for each dose and cell line. MCF7 and MDA-MB-231 were plated at a cell number of 600, 780, 1560, 3960, and 15600 cells/ 6 cm dish for doses of 0, 1, 2, 4,and 6 Gy respectively. Whereas, MCF10A were plated at cell numbers of 200, 260, 520,1320, and 5200 cells/6 cm dish. For each dose, cells were plated in three 6cm dishes and incubated at 37^{o}C for eight days under an atmosphere of 95% air 5% CO_2. Colonies, defined as a group of 50 or more cells originating from a single cell, were washed 3 times with 1X PBS, then stained with 0.5 % crystal violet for 30 min. Dishes were then

washed in running tap water by immersing dishes perpendicularly in a big jar. Colonies were counted using colony counter to obtain the mean number of colonies/dose in replicates.
The plating efficiency was calculated as shown below for un-irradiated samples: Plating efficiency (PE) =
The cell survival for each dose is then calculated as shown below:
Surviving fraction (SF)/dose = no of colonies at a given dose x PE/ no of cells seeded/dish

To determine cellular inactivation following high LET, exponentially growing MCF10A,and MDA-MB-231 were seeded at a cell number of $0.5X10^6$ into 0.9 µm mylar dishes, three dishes for each dose and incubated for 24 h. As mentioned the mylar was pretreated with Cell Tak before seeding MCF10A into dishes to insure the formation of a monolayer cells. Dishes were α- particle irradiated with doses of 0, 0.5, 1, 2, and 3 Gy and then transferred onto an iced cold surface before the medium was changed and then returned to the incubator to 37°C. After irradiation, a 26 mm diameter disc at the centre of the dish was cut out and put in 0.5, and 1% trypsin for cell line MCF10A and MDA-231 respectively to detach the cells. The disc was cut out using custom-made three bladed cutting tools in order to avoid areas around the edges of the dish where cells may be shielded from radiation by growing one poxy spreading out from under the glass ring. Cell densities were counted using haemocytometer for each dose and cell line. Both cell lines were plated at cell numbers of 660, 1000, 2000, 5000, and 20000 cells/ 6cm/dish for doses of 0, 0.5, 1, 2, and 3 Gy respectively. Dishes were then left at 37°C for eight days before being collected for staining process to calculate the cell survival as described above.

Viability test 10^3-10^4 cells /well were seeded in five 96 well plates for 24 h and were then treated with the following concentrations of AG538: 0, 25, 35, 45, 55, 65, 75 µM for one hour.
Subsequently, cells were irradiated with 0, 1, 2, 4 and 6 Gy. After 24 h the medium was removed and 100 µl medium containing 10 µg /ml resazurin is added per well for one hour at 37°C. The plates were then read using Perkin Elmer (Envision 2103 multilablel) 96 well plate reader. The medium containing resazurin was then removed and fresh medium containing the drug with the corresponding concentration is added and the same procedure repeated after 48 and 72 hrs.

Results:

Clonogenic assay has been performed for cells exposed to gamma radiation (low LET radiations known as sparsely ionizing radiation) which deposit its energy forming low energy secondary electrons responsible for the formation of DSB, cell inactivation and other kinds of biological effects.

Figure (1) shows the dependence of the mean surviving fraction on the dose for three experiments, each set of data being corrected to its own individual plating efficiency. The mean plating efficiency of the non-irradiated cells is 0.32±0.14, 0.18±0.066 and0.27±0.023 for MCF10A, MCF7 and MDA-MB-231 respectively.

Before performing clonogenic assay for the breast cancer cells MCF7 and MDA-MB-231and their control MCF10A a nuclear area measurement had to be performed to calculate the mean number of alpha particle track/cell resulting for one Gy irradiation. In alpha particle irradiation experiments, MCF7 cell line have not been used as they grow in clumps and not in monolayers required to obtain a homogenous distribution of radiation with no shielding of some cells due to clumping. MCF10A cells do not readily attach to the 0.9 µm mylar based rings necessary for the growth of monolayer cells for alpha irradiation. As a consequence the mylar was treated with Cell Tak to ensure the growing of cells as a monolayer (treatment of mylar with Cell Tak has been described in the material and method section). After seeding cells for 24 hours on 0.9 µm mylar based dishes, one drop of Rhodamine-123 was added immediately to the cell medium to detect the cells under a confocal microscope, Rhodamine-123 is a laser dye that binds tomitochondrial membrane and known to absorb at 506 nm and emits light at 560 nm. Three images were taken for each dish and then nuclear areas were measured for each cell using special software. After determining the nuclear areas for each cell line, figure(2) the mean nuclear areas have been used in the following equation to determine the mean number of alpha particle track per cell:
$N= (DA/0.16L)$, (1) (Hill *et al.,* 2004)

Where n is the mean number of alpha particles traversals per cell nucleus, L is linear energy transfer (LET, keV/µm), A is the mean nuclear area (μm^2) and D the dose in Gy.

The mean nuclear areas calculated for MCF10A and MDA-MB-231 are 120 and 140 µm respectively and the mean number of alpha track per 1 Gy per cell nucleus is ±6.2 and±7.2 tracks for MCF10A and MDA-MB-231 cells respectively. Clonogenic assay were performed for cells exposed to alpha particles which deposit energy in a linear heavily ionizing track in very short time scale in the order of picoseconds producing large clusters of ionization/excitation with lesser damage repair.

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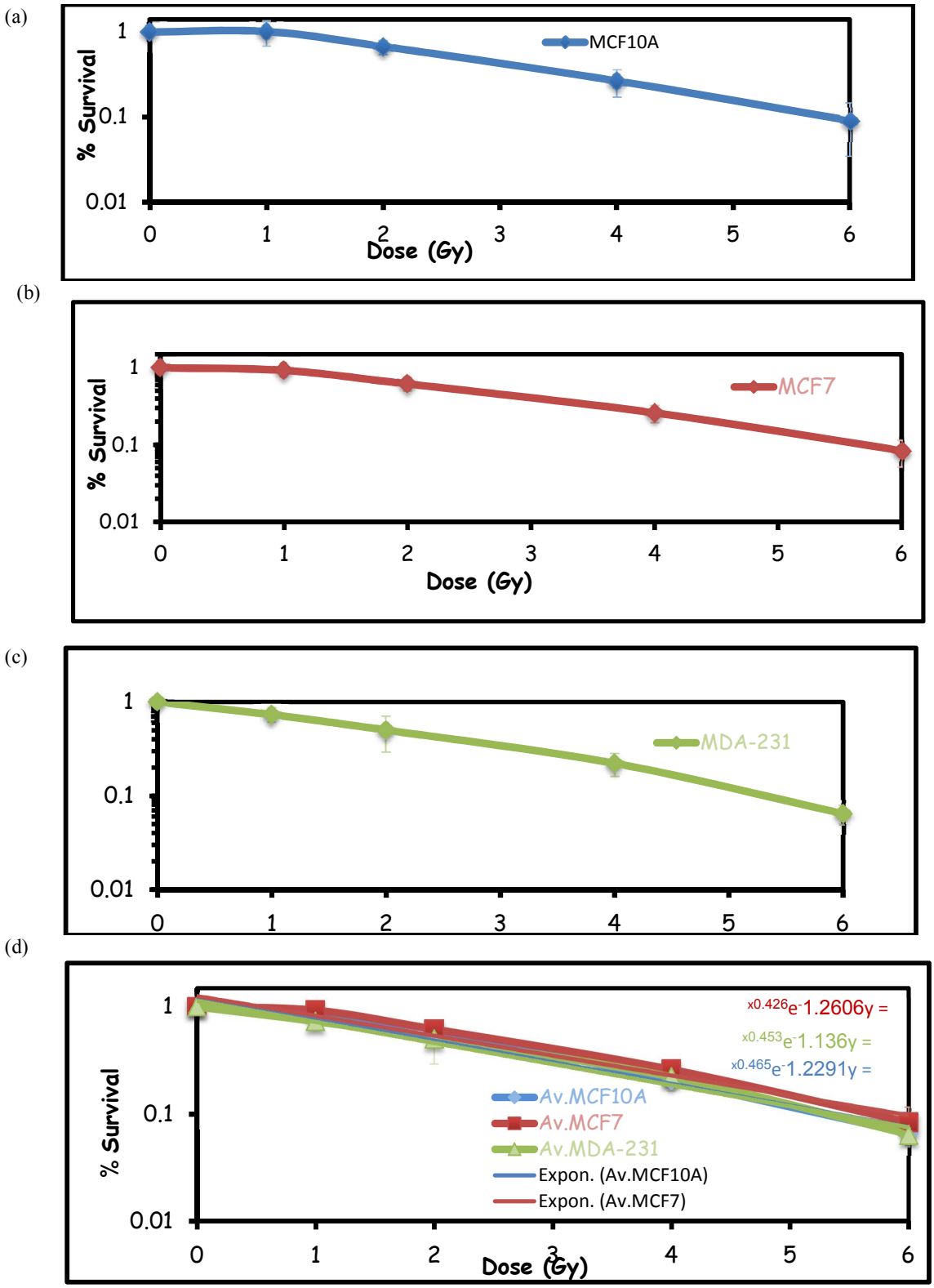

Fig. 1: The percentage survival of control breast cells line MCF10A (a), breast cancer cell line MCF7 (b) and breast cancer cell line MDA-MB-231(c) exposed to 1,2, 3, 4, and 6 Gy gamma radiation. (d) is merge between the three cell lines.

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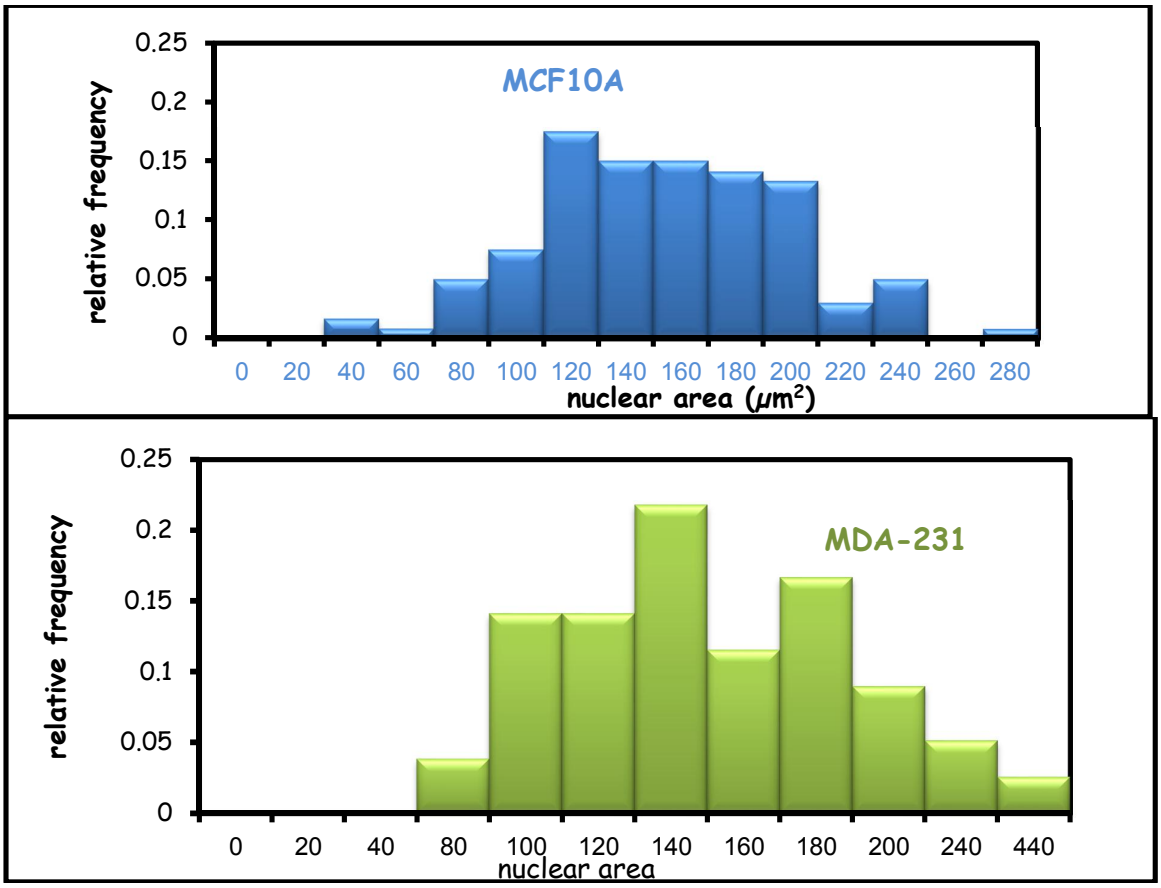

Fig. 2: The measured nuclear area distribution for (a) MCF10A control breast cell line and (b) MDA-MB-231 breast cancer cells, images were using by a confocal laser scanning microscope with x40 oil objective.

Figure (3) represents the dose dependence of the mean surviving fraction for three repeats, each set of data being corrected for its own individual plating efficiency. The mean plating efficiency of the non- irradiated cells is as following: 0.11± 0.05 and 0.482±0.21for MCF10A and MDA-MB-231 respectively. In addition, MCF10A cells after alpha particle irradiation show more relative biological effectiveness to high LET α-particle irradiation compared with MDA-MB-231 cells this difference does not reflect any differences in the mean cell morphology. Results showed that the percentage of survival of both control MCF10A and breast cancer cell line MDA-MB-231 decreases after the exposure to alpha radiation compared with gamma irradiation. The survival curve does not have a shoulder compared with that seen following γ -irradiation consistent with the findings with other cell (Hill, 1999).MDA-231 cells are also more radio resistant to alpha particles compared to the control cell line MCF10A after alpha –particles irradiation.

The viability assay is a direct measure of the metabolic competence of cell cultures Resazurin reduction which may provide a convenient index of cell proliferation following irradiation. The assay has been performed in 96 well plates using Resazurin dye which is known to convert into Resazufin in a viable cell; the later emits fluorescence at 590 nm as a result of metabolic reduction reaction. Cells were treated with 25, 35, 45, 55, 65, and 75µM of AG538 and exposed to 0, 1, 2, 4, and 6 Gy of gamma radiation. Viability test was used to measure short-term cell viability in vitro; percentage of viability was measured in relation to the control in the same plate, i.e. cells not treated with AG538.

Figure (4) illustrates the percentage viability of MCF10A, MCF7 and MDA-MB-231cells after treating them with 75 µM AG538 and different doses of radiation, plates have been read in 96 well plate reader after 24, 48, and 72 hours of treatment. Graphs show that the proliferation of cells is mainly affected at higher doses radiation (6Gy) and inhibitor (75µM) and therefore, reduction in viable cells occurs after the third day post irradiation.

It is also noticeable that MDA-MB-231 cells are the more sensitive cell line comparing with the control, MCF10A cells, and the breast cancer cells, MCF7.

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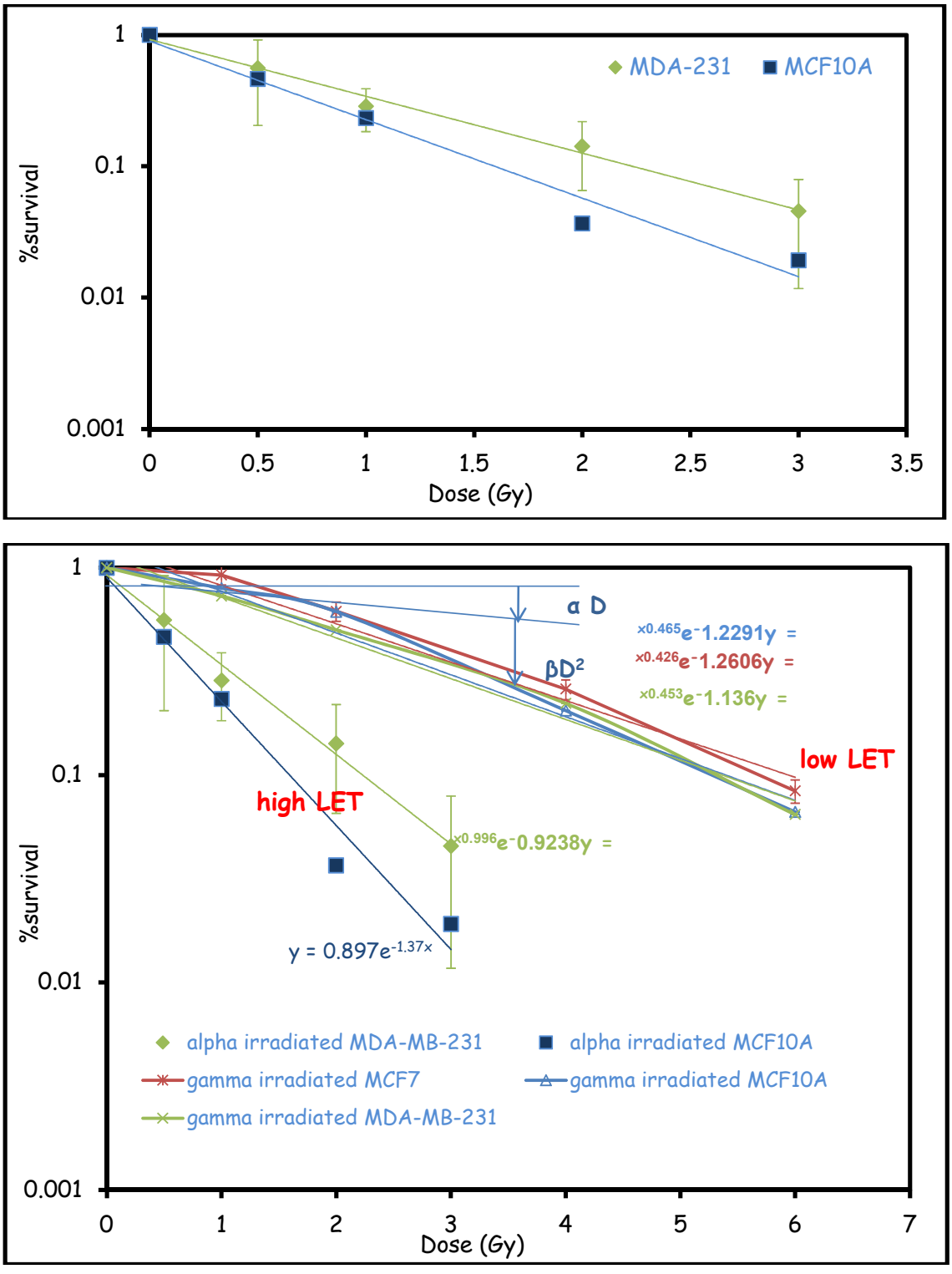

Fig. 3: The dose dependence for survival experiments of the breast cancer cell line MDA-MB-231 and its control MCF10A (a) for three replicate experiments after exposure to alpha particles with doses of: 0, 0.5, 1, 2 and 3 Gy. (c) is merge for both low and high LET survival curves of breast cancer cell lines and their control

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Table 1: The calculated values of a and ß parameters for both alpha and gamma breast cancer cell lines (MCF7 and MDA-MB-231) and their control MCF10A

Gamma		α-particles		
Cell type	α (Gy^{-1})	β (Gy^{-2})	α (Gy^{-1})	β (Gy^{-2})
MCF10A	0.045±0.05	0.07±0.03	055±0.08	0.14±0.05
MCF7	0.047±0.01	0.04±0.003		
MDA-231	0.03±0.01	0.017±0.009	0.14 ±0.04	0.045±0.02

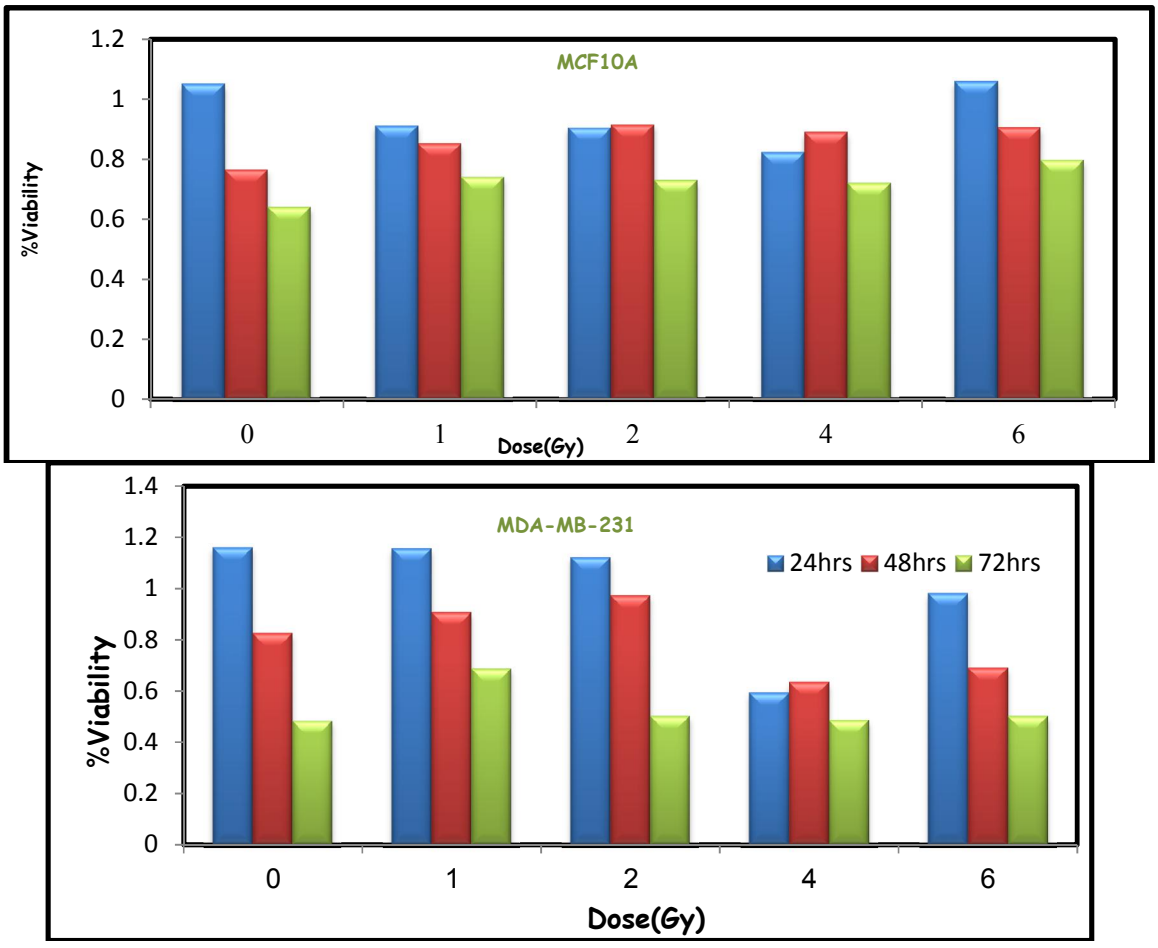

Fig. 4: The percentage viability for two replicate experiments when the breast control cell line MCF10A (a) and breast cancer cell linesMCF7(b) and MDA-MD-231(c) were exposed to0, 1, 2, 4 and 6 Gy gamma rays and treated with75 µM AG538, The cell viability was determined after 24, 48 and 72 hours of treatment using the resazurin assay.

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Discussion:

Results show that exponentially growing MDA-MB-231 and MCF7 breast cancer cell lines are similarly radiosensitive to gamma irradiation and similar to other cell lines, including the control breast cell line MCF10A. It is inferred that the DSB repair machinery for these cell lines is able to rejoin the double strand breaks induced after exposure to gamma irradiation. It is inferred that these cells are repair proficient since cells deficient in repair proteins involved in DSB repair pathways are more radiosensitive than repair proficient cells to low LET radiation (Hall J. E., 2004). Additionally gamma irradiation relative effectiveness to repair DSB is higher than those induced by densely ionising radiation, since it was hypothesised that the DSB are less complex for low LET irradiation and are therefore repaired more effectively (Goodhead 1989; Goodhead, 2006 ;Thacker *et al.,* 1993; Goodhead 1994 and Jenner *et al.,* 1993). In the mentioned studies, they showed that the levels of DSB induced by low LET and high LET radiation are similar but the majority of the DSB induced by low LET are repaired within a few hours compared with the persistence of some DSB after a few hours when induced by high LET radiation. These differences in reparability of DSB induced by different radiations are consistent with the DSB induced by low LET being less complex.

No significant differences in inactivation was observed between MDA-231 and MCF7,this result is consistent with previous findings (Yasui *et al.,* 2001). In the study by Yasui *et al.* (2001) showed that γ-irradiating MCF7 and MDA-231 cause slightly more inactivation in MDA-231, whereas, other studies have shown that MDA-231 are more radio resistance than MCF7. Both breast cancer cell lines MDA-231 and MCF7 show proliferation, although both cell lines express IGF1R, higher levels of expression have been shown in MCF7 cell line (Song *et al.,* 2007).

Results showed that the percentage of survival of both control MCF10A and breast cancer cell line MDA-231 decreases after the exposure to alpha radiation compared with gamma irradiation. The survival curve does not have a shoulder compared with that seen following γ -irradiation consistent with the findings with other cell (Hill, 1999). MDA231 cells are also more radio resistant to alpha particles compared to the control cell line MCF10A after alpha –particles irradiation.

Few studies have been reported on the effects of alpha particles irradiation on breast cancer cells, as the majority of studies focus on the damage resulting from γ-irradiation. Other work has studies the radio sensitivity of alpha particles relative to low LET radiation for repair deficient relative to repair competent cells (Hill *et al.,*2004). This work showed that repair deficient cells are similarly radiosensitive to alpha particles in contrast to the difference in radio sensitivity between repair deficient and competent cells when γ -irradiated. Our results are consistent with the findings from the latter studies that alpha particles irradiation is more biologically effective per unit absorbed dose compared with low LET radiation such as X-ray. As shown later MDA231cells are more radio resistance than control breast cell line, even though MDA-231cells when subjected to low or high LET radiation, show slow rate of DSB repair, even though both cell lines are thought to be repair competent. Whether the small differences reported by Hill *et al*, (2004), between the radio sensitivity of repair proficient and deficient cells to alpha particles is due to other factors than repair remains open to question.

Figure (3) illustrates the cell survival curve of alpha and gamma irradiated control MCF10A and breast cancer cell lines MCF7 and MDA-MB-231. Results show that the survival curve can be fit to the linear-quadratic model. The initial slope of the linear logarithmic plot gives cell killing proportional to $e^{-\alpha D}$ and the latter part given by e which bends at higher doses. With high linear energy transfer radiation ß is zero and the curve is exponentially expressed by $e^{-\alpha D}$.

In table (1) we have calculated the α and ß parameters in the linear-quadratic equation for both alpha and gamma irradiated rats using origin 6 program. A parameter may reflects the induction of clustered damage and ß may reflects the less complex lesions that become lethal. It has been hypothesis that the more complex the spectrums of damage components induced by radiation are the less repairable and therefore the more biological effectiveness. Results show that the high L *Et* alpha particles induce more complex lesions that are difficult to repair compared to low LET gamma radiation (Hill *et al.,*2004).

Viability tests with MCF7 and MDA-231 cell lines showed that a dose of 75µM AG538,which compete with the phosphorylation site in IGF1R at the molecular level (Blum, Gazit *et al*. 2000), reduce the viability of cells by 50% compared with untreated cells after three days of treatment for all doses of gamma radiation. In a previous experiment in our lab, serum starved non irradiated MCF10A, MCF7 and MDA-231 cell lines treated with: 1, 2 and 3 µM PPP; 25, 50 and 100µM AG538 and 2, 5 and 10µM AG1024 showed that the most effective inhibitor is AG538 at a concentration of 100 µM. This concentration reduced survival to less than half after 72 h of treatment for all cell lines, whereas in a previous study by Rosengren *et al*, 2006, with cells expressing IGF1R,2.5µM PPP reduce the percentage survival to 20 after 48 hrs of treatments, similar -ßD2

findings were reported for MDM2 defective cells, and in p6 cells (Vasilcanu *et al.,* 2008). In contrast, the IC50 is 1-2nM PPP in MCF7 (Vasilcanu *et al.,*2004).

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