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Alpha particles induce pan-nuclear phosphorylation of H2AX in primary human lymphocytes mediated through ATM

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Abstract

The use of high linear energy transfer radiations in the form of carbon ions in heavy ion beam lines or alpha particles in new radionuclide treatments has increased substantially over the past decade and will continue to do so due to the favorable dose distributions they can offer versus conventional therapies. Previously it has been shown that exposure to heavy ions induces pan-nuclear phosphorylation of several DNA repair proteins such as H2AX and ATM in vitro. Here we describe similar effects of alpha particles on ex vivo irradiated primary human peripheral blood lymphocytes. Following alpha particle irradiation pan-nuclear phosphorylation of H2AX and ATM, but not DNAPK and 53BP1, was observed throughout the nucleus. Inhibition of ATM, but not DNA-PK, resulted in the loss of pan-nuclear phosphorylation of H2AX in alpha particle irradiated lymphocytes. Pan-nuclear gamma-H2AX signal was rapidly lost over 24 hours at a much greater rate than foci loss. Surprisingly, pan-nuclear gamma-H2AX intensity was not dependent on the number of alpha particle induced double strand breaks, rather the number of alpha particles which had traversed the cell nucleus. This distinct fluence dependent damage signature of particle radiation is important in both the fields of radioprotection and clinical oncology in determining radionuclide biological dosimetry and may be indicative of patient response to new radionuclide cancer therapies.

Keywords

Alpha particles, complex damage, DNA double strand break, pan-nuclear intensity
**Introduction**

The phosphorylated histone variant gamma-H2AX is a sensitive and well established biomarker of exposure to ionising radiation [1, 2]. Detection of X-ray induced DNA double strand breaks (DSBs) by gamma-H2AX foci/intensity quantification has proved useful for biological dosimetry [3] and for investigating intra-cell and inter-individual responses to low linear energy transfer (LET) radiation [4]. For high LET exposures, such as those induced by heavy ions or alpha particles, slower repair of DSBs has been observed, likely due to the greater complexity and subsequent greater difficulty in resolving the break [5]. This has been demonstrated by the bi-exponential repair of low LET induced DSBs, containing both a rapid and slow component [3], whereas high LET induced DSBs are lost at a slower rate with greater numbers of residual DSBs remaining several hours post exposure compared to X-rays. While both low and high LET induced DSBs can be identified using a number of molecular techniques, distinguishing between low and high LET breaks is somewhat more difficult. In biological dosimetry, the use of the dicentric assay has proved useful in identifying high LET radiation exposures from low LET due to the formation of complex aberrations generated by high LET radiation [6, 7]. These types of aberrations are more frequent as a result of the formation of densely clustered DSBs caused by high LET particles leading to misrepair events not observed with sparsely induced DSBs from, for example, X-rays [8].

With respects to DSBs, it has been observed that *in vitro* heavy ion irradiation can induce not only phosphorylation of H2AX at DSBs, but also throughout the nucleus outside of directly induced DSBs [9]. This pan-nuclear phosphorylation of H2AX (and ATM) was not observed after X-ray exposure, suggesting it is a phenomenon associated with high LET radiation only. In clinical oncology, radionuclides are used extensively in the treatment of thyroid cancer (iodine-131), in prostate brachytherapy (iodine-125, palladium-103) and bone metastasis (strontium-90, samarium-153). For these treatments, the radionuclides are exclusively low LET radiation emitters of X- and gamma-rays and beta electrons. More recently the clinical benefit of utilising high LET radionuclides has been demonstrated in a phase III clinical trial [10]. In men with castrate resistant prostate cancer with bone metastases, those treated with the alpha particle emitter radium-223 had a median overall survival of 14.9 months vs. 11.3 in the placebo control.
group (hazard ratio 0.70). Previously treatment with beta electron emitting radionuclides had shown no overall survival benefit, only pain relief [11-13].

If pan-nuclear phosphorylation of H2AX is present in high LET irradiated primary human tissue, it may expand the paradigm that the biological effectiveness of high LET radiations in regards to cell kill and mutagenesis is due solely to induction of localised complex DNA DSBs. For the therapeutic use of alpha particles and heavy ions, the presence (or absence) of pan-nuclear gamma-H2AX in high LET irradiated tissues will help confirm that the calculated doses to tissues at risk match those actually delivered.

Materials and methods

Blood collection and lymphocyte isolation

After obtaining ethical approval from Queen’s University Belfast Medical School Ethics Board (Ref 12.13v2) and informed consent from donors, peripheral blood from healthy donors (no known previous medical radiation exposures, aged 24-31) was collected into EDTA vacutainer tubes. Lymphocytes were isolated from the blood using Histopaque-1077 solution (Sigma-Aldrich) and resuspended in RPMI-1640 (supplemented with 10% foetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin) at 37°C.

Alpha particle and X-irradiation of Lymphocytes

For alpha particle irradiation, lymphocytes were irradiated under sterile conditions with a 1 μCi americium-241 source, which decays to give ~5.45 MeV alpha particles. Lymphocytes were irradiated in 20 μl of media at a concentration of 2x10^7 cells per ml on top of the source separated by a 0.9 μm Mylar foil. Lymphocytes were irradiated for eight minutes, being aspirated every two minutes, giving a non homogenous exposure to the cell population with the majority of the cells containing zero foci, representing the partial body non-homogenous exposures to radium-223 seen clinically. For X-ray exposures lymphocytes were uniformly irradiated with 1 Gy at 225 kVp in flasks. Lymphocytes were also irradiated with X-rays on
Mylar and no difference in gamma-H2AX signal (breaks or nuclear intensity) was observed between lymphocytes irradiated in flasks and on Mylar. All controls were sham irradiated and all irradiations took place at 4°C.

**Alpha particle and X-irradiation of Fibroblasts**

For alpha particle irradiation, cultured normal human Fibroblasts (AGO1522) were grown and irradiated on 0.9 µm Mylar foil using the 1 µCi americium-241 source discussed above. AGO1522 cells were irradiated with 2 Gy of alpha particles to 50 % of the cell population (the limit of our alpha irradiation setup) producing a heterogeneous sample of irradiated and unirradiated cells, again mimicking the partial body non-homogenous exposures to radium-223 seen clinically. For X-ray exposures AGO1522 cells were uniformly irradiated with 4 Gy at 225 kVp in flasks. AGO1552 cells were also irradiated with X-rays on Mylar and no difference in gamma-H2AX signal (breaks or nuclear intensity) was observed between cells irradiated in flasks and on Mylar. All controls were sham irradiated and all irradiations took place at 4°C.

**Inhibitor treatment**

ATM inhibitor KU60019 and DNAPK inhibitor NU7441 (Tocris bioscience, UK) were used at a concentration of 10 µM. All inhibitors were dissolved in DMSO and controls were treated with DMSO only. Cells treated with inhibitors or DMSO only were treated for one hour before irradiation.

**Fixation and staining of cells**

Lymphocytes were dried onto Superfrost plus slides (VWR), fixed using 4 % formaldehyde/PBS (or 100% methanol for gamma-H2AX/p-DNAPK) for 10 minutes, washed in PBS, permeabilised using 0.5 % (v/v) Triton-X/PBS (skipped for methanol fixed cells) for 10 minutes then washed in PBS. Blocking was achieved using 1 % (w/v) bovine serum albumin (BSA) in PBS for 30 minutes. Cells were then incubated with a dual combination of either 1:2000 mouse gamma-
H2AX antibody (Millipore 05-636), 1:2000 rabbit 53BP1 antibody (Bethyl Laboratories NB100-304), 1:2000 mouse phospho-serine 1981 ATM antibody (Millipore MAB3806) and 1:1000 rabbit phospho-serine 2056 DNAPK antibody (Abcam ab18192) in 1% BSA/PBS for 1 hour at room temperature. Cells were then washed in 1% BSA/PBS, incubated in 1:2000 anti-mouse AlexaFluor 488 conjugated antibody (Invitrogen A11029) and 1:2000 anti-rabbit AlexaFluor 568 conjugated antibody (Invitrogen A11036) in 1% BSA/PBS for 1 hour at room temperature. Cells were then washed in PBS, dried, mounted with a cover slip using Prolong gold antifade with DAPI (Invitrogen P36931) and left to dry for 24 hours at room temperature in the dark. Slides were analysed with a Zeiss epifluorescence microscope using a x63 objective. For the apoptosis assay the FLICA Poly Caspase kit (Immunochemistry technologies) was used as per the manufacturer’s instructions.

**Immunoblotting**

AGO1552s were trypsinised off Mylar, lysed and protein concentration determined using the Bradford assay. Lysates were then run on 12% acrylamide gels (8% for p-ATM), transferred to a nitrocellulose membrane, blocked in 5% milk followed by incubation overnight at 4°C with anti-gamma-H2AX (see above), anti-p-ATM (see above), anti-Actin, anti-BCL-2, anti-Bax, anti-Caspase 3, anti-Caspase 7, anti-Caspase 9 and anti-p-p53 (Cell Signalling) primary antibodies. Membranes were then washed in TBS-0.1% tween20 and incubated at room temperature for 1 h with HRP-conjugated secondary antibodies (Cell Signalling). Luminata (Merck Millipore) was used for chemiluminescence and membranes were visualised on a Syngene G:BOX with exposure time set automatically to prevent saturation. AGO1552 cells were used for western blotting to as the number of alpha particles delivered to each adhering fibroblast cell could be kept far more consistent than with lymphocytes in suspension.

**Software and statistics**

Microscope images were taken using an Axiovision microscope (Zeiss) with cells identified and intensity measurements made using Volocity software (PerkinElmer). For non-foci intensity measurements, gamma-H2AX foci were removed from each cell in each image and the
remaining gamma-H2AX intensity quantified. The fold intensity change between time matched irradiated and sham-irradiated controls was then calculated. ImageJ (NIH) was used to generate cell images and measure foci size and intensity. Graphs were made using PRISM 5 (Graphpad Software) and all statistics were performed using SPSS (IBM). Foci counts were made manually by eye at x63 magnification. All error bars represent the standard error of the mean of three experiments. At least 50 cells were scored for foci counting and intensity measurements for all data points, with 200 scored for alpha particle irradiated samples to account for the low number of cells with tracks. For the track vs intensity analysis, 79 cells were used.

Results

Alpha particles, but not X-rays, induce pan-nuclear phosphorylation of H2AX and ATM in primary human lymphocytes and normal human fibroblasts

Observations that alpha particle irradiated lymphocytes displayed intense gamma-H2AX (Fig 1A) and phospho-ATM (Fig 1B) signal vs X-ray irradiated lymphocytes and controls led to a more in depth analysis. After exposure to alpha particles or X-rays, an increase in nuclear foci is observed, indicated by co-localising gamma-H2AX/53BP1, phospho-ATM/53BP1 and gamma-H2AX/p-DNAPK focus formation (Figures 2A, 2B and 2C). However alpha particles, but not X-rays, induced pan-nuclear phosphorylation of both H2AX and ATM in lymphocytes, which did not co-localise with 53BP1. This nuclear intensity was less than the individual focus intensity (average gamma-H2AX focus intensity was 84% greater than the nuclear intensity, p = 0.0077, Students t test). Gamma-H2AX intensity measurements of each irradiated nucleus (excluding intensity contribution from each focus) showed a greater than two fold increase in nuclear intensity at 1 and 4 hours post exposure to alpha particles (Figure 2D). A similar pattern was also observed with phospho-ATM, with intensity measurements significantly elevated against baseline at 1, 4 and 24 hours post exposure (Figure 2E). Alpha particle irradiation failed to induce any significant pan-nuclear phosphorylation of DNAPK (Figure 2F).
Both alpha particle induced gamma-H2AX foci and intensity reduced over time, however these two processes occurred at different rates (Figure 3A). While 24 hour foci numbers remain above 50% of the peak numbers seen at 1 hour, 24 hour intensity values dropped to below 20% of the 1 hour peak. The pan-nuclear phosphorylation of H2AX and ATM was confirmed not to be linked to apoptosis through the quantification of activated caspases which showed few apoptotic lymphocytes present <8 hours post exposure to alpha particles (Figure 3B). When comparing the size of alpha particle vs. X-ray induced gamma-H2AX foci, at both 1 and 24 hours alpha particle induced foci were more than 2 times (and significantly) larger than X-ray induced foci (Figure 3C). There was no significant difference in the size of 1 h and 24 h foci for alpha particles or X-rays. Gamma-H2AX intensity was also significantly higher at both 1 h and 24 h in alpha particle induced foci vs. X-rays (Figure 3C). However while there was no significant difference between X-ray foci intensity at 1 and 24 h, alpha particle induced foci were significantly more intense at 1 h when compared to 24 h.

As observed in lymphocytes, alpha particle irradiated normal human fibroblasts, AGO1552s, also displayed pan-nuclear phosphorylation of H2AX outside of DNA DSBs whereas X-irradiated cells did not (Figure 4A). Confirming this by western blot, both Gamma-H2AX and phospho-ATM can be detected in irradiated cell lysates at 1 hour post exposure for both and only gamma-H2AX at 24 hours (Figure 4B), with no intense bands detected outside their expected regions on the membrane. Probing for proteins involved in apoptosis, p-p53 is strongly up regulated in the X-irradiated AGOs and less so in the alpha-irradiated AGOs (Fig 4B). Perhaps unexpectedly, both the pro-apoptotic BAX and anti-apoptotic BCL-2 proteins were strongly up regulated in all irradiated samples post exposure (Figure 4B). Both cleaved caspase 7 and 9 were detected in all irradiated and unirradiated AGO samples (Figures 4D and 4E) while cleaved caspase 3, the terminal effector caspase is absent in both X- and alpha-irradiated AGO lysates at both 1 and 24 hours post exposure (Figure 4C). A DU-145 prostate cancer cell line treated with SAHA (a histone deacetylase inhibitor) was used as a positive control (Figures 4C-E).

Alpha particle track number, not DSBs induced by each track, determine pan nuclear gamma-H2AX intensity
While it was noted that cells containing greater foci numbers also had higher gamma-H2AX intensity values in alpha particle irradiated cells, we decided to determine whether this was a result of the number of DSBs induced or the number of alpha particles which had traversed the nucleus (a single alpha particle induced anywhere between 1-6 observable foci per track). Cells were classified as having one, two or three tracks per cell (Figure 5A) and intensity measurements taken. Plotting gamma-H2AX intensity vs. foci per cell for one (Figure 5B), two (Figure 5C) or three tracks (Figure 5D) apart from each other, no significant correlations were observed. However once all the data points for one, two and three tracks was analysed together, a strong, significant correlation between intensity and foci was observed (Figure 5E). This suggests that the intensity of pan-nuclear phosphorylation of H2AX is dependent on the number of alpha particles which have traversed the nucleus and caused at least one DSB. The actual number of DSBs each track induces above one appeared to not influence the intensity of pan-nuclear gamma-H2AX.

**ATM and not DNAPK drives the pan nuclear phosphorylation of H2AX after exposure to alpha particles**

Through the use of potent inhibitors of ATM and DNAPK, the kinase responsible for the activation of H2AX after exposure to alpha particles and X-rays was investigated. The use of the ATM inhibitor, KU60019 significantly reduced the pan-nuclear intensity of gamma-H2AX and p-ATM after exposure to alpha particles (Figure 6A). Recruitment of 53BP1 to DSBs was seemingly unaffected while gamma-H2AX foci intensity was also reduced. Treatment with KU60019 also abolished the phosphorylation of DNAPK at serine 2056. The DNAPK inhibitor, NU7441 failed to have significant impact of the pan-nuclear phosphorylation of either H2AX or ATM (Figure 6B). Fold gamma-H2AX (Figure 6C) and phospho-ATM (Figure 6D) intensities over time are further reduced by a combination of KU60019 and NU7441. NU7441 did not prevent the phosphorylation of DNAPK at serine 2056 or DNAPKs recruitment to DSBs, however the number of DSBs at 24 hours post X-irradiation was increased in NU7441 treated lymphocytes vs. DMSO treated controls (Figure 7) demonstrating that the inhibitor is reducing the rate of DNA repair.
Discussion

Here we have confirmed that high LET alpha particles (~135 keV/μm) induce pan-nuclear phosphorylation of H2AX and ATM previously demonstrated by Meyer et al [9], who used heavy ions ranging from 290-15000 keV/μm to irradiate cells. While not fixed, it has been suggested that the lower limits for high LET radiation is 50 keV/μm [14-15] and this phenomenon appears to be confined to radiations of high LET as neither X-rays nor 20 MeV protons [16] (2.6 keV/μm) induced pan-nuclear gamma-H2AX.

Importantly, high LET induced pan-nuclear gamma-H2AX is a cell cycle independent process, as the signal was observed in both cycling AGO1522 primary human fibroblasts and in quiescent primary human lymphocytes. Our work using fresh (taken and irradiated on the day) primary human lymphocytes further suggests that this is unlikely to be an artefact produced by cell culture.

Immunofluorescence failed to detect activated caspases in alpha particle irradiated lymphocytes while immunoblotting detected low levels of cleaved caspase 7 and 9 in all our AGO1522 samples (even unirradiated controls) whereas cleaved caspase 3 was only observed in SAHA treated DU-145 cells. In AGO1522s (a cell line well known to be apoptosis resistant), the up regulation of both pro and anti apoptotic factors likely represents separate pathways pushing towards a survive or die response, although up regulation of BCL-2 has been shown to block pro BAX apoptosis [17, 18] suggesting that the strong BCL-2 induction we observe suggests the cells are neither apoptotic or necrotic. Perhaps the single biggest indicator that at 1 hour post alpha particle irradiation cells are not dying (when gamma-H2AX and phospho-ATM signal is greatest) is that the DNA content, structure and morphology of the cells remains normal. For both lymphocytes and AGO1552s the cells do not appear in any way different from controls or X-ray irradiated cells, when it is known that hallmarks of both apoptosis and necrosis is aberrant cell morphology and DNA fragmentation [19-21].
The pan-nuclear activation of H2AX here was driven primarily through ATM, with DNAPK playing little if any role. This was in contrast to Meyer et al [9] who observed that ATM and DNAPK shared the role of triggering pan-nuclear gamma-H2AX after high LET irradiation. It is known that there is redundancy between the kinases involved in DNA repair with H2AX being a target of ATM [22], ATR [23] and DNAPK [24]. Our data suggests that at DSBs, H2AX is phosphorylated by DNAPK as in the presence of KU60019, gamma-H2AX foci were still present (albeit much reduced). Whereas pan-nuclear phosphorylation of H2AX appeared to be regulated via ATM, as cells treated with KU60019 displayed reduced pan-nuclear phosphorylation after alpha particle irradiation. This would make sense as pan-nuclear phospho-ATM is observed after high LET radiation which would be required for activation of H2AX genome wide. Phospho-DNAPK on the other hand was only present at DSBs, reinforcing the notion that it only phosphorylates H2AX at DSBs as it is not seen dispersed throughout the nucleus. It should be noted however that previous work has shown that ATM inhibition did not alter DNAPK phosphorylation at S2056 due to it being an autophosphorylation site [25]. An explanation for this difference could be that lymphocytes being non dividing cells are more reliant on ATM for DSB detection and recruitment of repair factors to the break, with inhibition of ATM strongly suppressing DNAPK autophosphorylation.

The role of ATM and DNAPK in the repair of DSBs has been well documented, with DNAPK identified as having a major role in the repair of the majority of DSBs [26, 27]. ATMs role appears to be more subtle, with suggestions that this kinase is involved in the repair of DSBs that require re-sectioning and those breaks lying within heterochromatic regions [28, 29], along with its critical role in DNA damage signalling [30] and apoptosis [31]. With ATM/DNAPK inhibition reducing the rate of DSB repair, but not altering pan-nuclear intensity at 24 hours post exposure, suggests that pan-nuclear gamma-H2AX damage (if damage at all) is not repaired by either non homologous end joining (NHEJ) or homologous recombination (HR) (lymphocytes being quiescent do not have access to HR). 53BP1 and phospho-DNAPK are known markers of DSBs and the lack of a pan-nuclear signal for either of these proteins suggest this does not represent DSBs. The evidence suggests pan-nuclear gamma-H2AX induced by high LET particles is not the result of either direct or indirect ionisations as the penetrating distance of delta electrons is in the nm range [32], with pan-nuclear signal observed throughout the nucleus up to several μm away from any DSBs.
While it first appeared that gamma-H2AX pan nuclear intensity was determined by the number of DSBs induced, this turned out to be a casual correlation. The number of alpha particle tracks which had traversed each lymphocyte determined the relative strength of the pan-nuclear gamma-H2AX signal regardless of the number of DSBs the track induced. The consequence of this could be fragmentation of the DNA over Mbps around each DSB, which when overlapping in the case of alpha particle tracks saturates the signal. Separate tracks inducing as many or fewer foci may produce a greater signal due to less overlap of fragmenting DNA [33-35].

The presence of pan-nuclear gamma-H2AX/p-ATM signal after high LET irradiation is relevant to both the fields of radioprotection and clinical oncology. Previously there have been no distinct molecular markers of exposure to high LET radiation, making biological dosimetry in radiation protection dependent on the presence of complex chromosomal rearrangements found within high LET irradiated cells, a difficult and time consuming assay [36]. The use of gamma-H2AX foci scoring for X-irradiated cells has already been proposed [3, 37] and the presence of pan-nuclear gamma-H2AX and delayed foci repair in high LET irradiated lymphocytes may aid in the differentiation between radiation exposures of differing LET. For clinical oncology, the recent approval of the alpha particle emitting radionuclide, Xofigo (previously Alpharadin, radium$^{223}$) for the treatment of bone metastases [10] has revealed the need of further research in determining patient uptake and response to treatment to new radical radionuclide therapies. Individual patient response to high LET damage is especially of interest as it has previously been shown that in women who received radiotherapy for breast cancer that those who had slower rates of DSB repair in their lymphocytes also displayed greater normal tissue toxicity [38]. Greater understanding of the molecular damage and response to high LET radiations is required to further comprehend their biological effects.

**Acknowledgements**

The authors would like to thank Dominique French for her help with the western blot protocol and Chris Armstrong for providing the DU-145 SAHA treated lysates. This work was supported by the European Commission EPIRADBIO (project No.269553), the UK Home Office/Public Health England (PN1725/107036) and Friends of the Cancer Centre.
References


Figure Legends

Figure 1. Grey scale image of Gamma-H2AX (A) and phospho-ATM (B) in primary human lymphocytes. The alpha particle irradiated samples contain a heterogeneous population of irradiated and unirradiated cells.

Figure 2. Alpha particles, but not X-rays, induce pan nuclear phosphorylation of H2AX and ATM in lymphocytes. Primary human lymphocytes were irradiated with alpha particles displayed gamma-H2AX (A) and phospho-ATM (B) signal which did not correlate with 53BP1. No phospho-DNAPK signal was observed outside of alpha particle induced DSBs (C). Non-foci intensity measurements showing fold change against controls for gamma-H2AX (D), phospho-ATM (E) and phospho-DNAPK (F). $p = <0.05^*, <0.01^{**}$ and $<0.001^{***}$, students t test.

Figure 3. (A) Gamma-H2AX loss over 24 hours following alpha particle irradiation for foci (right axis) and intensity (left axis). The number of apoptotic lymphocytes detected (represented by being FLICA positive) steadily increased between 1-24 hours post alpha particle irradiation (B). Gamma-H2AX foci size and intensity was significantly greater compared to X-rays at both 1 and 24 hours post exposure. $p = <0.05^*$ and $<0.01^{**}$, students t test.

Figure 4. Immuno-grey scale image of unirradiated or 1 Gy X- or alpha irradiated AGO1552 fibroblasts (A). (B) Western blots of radiation responsive proteins involved in DNA repair, cell cycle check points and apoptosis to 4 Gy X-rays or 2 Gy alpha particles. Western blots of caspases/cleaved caspases 3 (C), 7 (D) and 9 (E). DU-145 cells were treated with 1 µM SAHA. The region of cleaved caspases is indicated by the arrows.

Figure 5. Immunofluorescent image of lymphocytes containing one, two or three alpha particle tracks (A). Gamma-H2AX intensity plotted against gamma-H2AX foci per cell in lymphocytes containing one (B), two (C) or three (D) alpha particle tracks or all three data sets combined (E). Pearson product-moment correlation coefficient was used to determine significant correlation between intensity and foci, which was only observed in the combined data set.

Figure 6. The ATM inhibitor, KU60019, significantly reduces gamma-H2AX, phospho-ATM and phospho-DNAPK intensity in alpha particle irradiated lymphocytes (A). (B) The DNAPK inhibitor, NU7441, failed to have an effect on either gamma-H2AX, phospho-ATM or phospho-DNAPK foci or pan nuclear intensity (B). Intensity decreases after inhibitor treatment in alpha particle irradiated lymphocytes for gamma-H2AX (C) and phospho-ATM (D) $p = <0.05^*$, $<0.01^{**}$ and $<0.001^{***}$, students t test.

Figure 7. Gamma-H2AX/53BP1 foci counts in lymphocytes irradiated with 1 Gy X-rays. Cells were treated with either KU60019 (ATMi), NU7441 (DNAPKi), a combination of both or DMSO only (IR).
Figure 1

A
Gamma -H2AX
DNA

B
p-ATM
DNA

Alpha particles
X-rays
**Figure 2**

A. **Alpha particles** vs. **X-rays**

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B. **53BP1**

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C. **p-DNAPK**

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Graphs showing fold change in gamma-H2AX, p-ATM, and p-DNAPK for Alpha and X-rays treatments at 1h, 4h, and 24h.
Figure 3

A

Fold gamma-H2AX change vs. Time (h)

B

Mean foci per cell vs. Time (h)

C

Fold gamma-H2AX change vs. Foci size and Foci intensity

Legend:
- X-ray 1h
- Alpha particles 1h
- X-ray 24h
- Alpha particles 24h

Significance:
- **p < 0.01
- *p < 0.05
**Figure 4**

### A

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<th>Alpha 1 h</th>
<th>X-ray 24 h</th>
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### C

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### D

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### E

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<td>X-ray 24 h</td>
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<tr>
<td>DU-145 SAHA</td>
<td><img src="caspase-9-du145-saha.png" alt="Image" /></td>
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</table>
Figure 5

A) Single track, Two tracks, Three tracks

53BP1
Gamma-H2AX
DNA

B) Single track

Gamma-H2AX intensity (AU) vs. Mean foci per cell

Pearson's r = 0.1571
p = 0.4701

C) Two tracks

Gamma-H2AX intensity (AU) vs. Mean foci per cell

Pearson's r = 0.09846
p = 0.7069

D) Three tracks

Gamma-H2AX intensity (AU) vs. Mean foci per cell

Pearson's r = 0.3078
p = 0.3062

E) Two tracks

Gamma-H2AX intensity (AU) vs. Mean foci per cell

Pearson's r = 0.6194
p < 0.001

Legend: Single track, Two tracks, Three tracks
Figure 6

A

ATMi

Control  Alpha  Control  Alpha  Control  Alpha

53BP1  53BP1  p-DNAPK

Gamma-H2AX  p-ATM  Gamma-H2AX

DNA  DNA

B

DNAPKi

Control  Alpha  Control  Alpha  Control  Alpha

53BP1  53BP1  p-DNAPK

Gamma-H2AX  p-ATM  Gamma-H2AX

DNA  DNA

C

D

Fold gamma-H2AX change

Fold p-ATM change

1 h  4 h  24 h

IR only  ATMi  DNAPKi  ATMi+DNAPKi  IR only  ATMi  DNAPKi  ATMi+DNAPKi

**  ***  ****

**  ***