Inhibition of ataxia telangiectasia related-3 (ATR) improves therapeutic index in preclinical models of non-small cell lung cancer (NSCLC) radiotherapy

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Abstract: Background and purpose: To evaluate the impact of ATR
inhibition using AZD6738 in combination with radiotherapy on the response
of non-small cell lung cancer (NSCLC) tumour models and a murine model of
radiation induced fibrosis.
Materials and methods: AZD6738 was evaluated as a monotherapy and in
combination with radiation in vitro and in vivo using A549 and H460 NSCLC
models. Radiation induced pulmonary fibrosis was evaluated by cone beam
computed tomography (CBCT) and histological staining.
Results: AZD6738 specifically inhibits ATR kinase and enhanced
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which were less significant in A549 cells. Combination of AZD6738 with
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(p > 0.5) and histological scoring of radiation induced fibrosis (p >
0.5).
Conclusion: Inhibition of ATR with AZD6738 in combination with
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late radiation induced toxicity further underpinning translation towards
clinical evaluation in NSCLC.

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2th June 2017

Dear Sir,

I would like to submit the revised manuscript entitled 'Inhibition of ataxia telangiectasia related-3 (ATR) improves therapeutic index in preclinical models of non-small cell lung cancer (NSCLC) radiotherapy' by Dunne *et al.* to be considered for publication as an original paper in Radiotherapy and Oncology.

The manuscript has been revised in line with the constructive criticisms of the reviewers. This has added to the quality and presentation, and we hope the article meets the required standards for publication in Radiotherapy and Oncology.

I confirm that the author and co-authors currently have no papers that are under review or in press relevant to this publication.

I look forward to hearing from you.

Yours sincerely

Karl Butterworth, PhD
Assistant Professor in Translational Radiation Biology
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*Point by Point detailed Response to Reviewer comments

Radiotherapy and Oncology

If you are submitting a revised manuscript, please also:

a) outline each change made (point by point) as raised in the reviewer comments AND/OR b) provide a suitable rebuttal to each reviewer comment not addressed

Response to Reviewers' comments:

Reviewer #2: Please add p-values to table 1 (drug versus control, drug versus RT and RT+ drug versus RT alone)

P values have been added for statistical comparison of different groups in table 1.

Inhibition of ataxia telangiectasia related-3 (ATR) improves therapeutic index in preclinical models of non-small cell lung cancer (NSCLC) radiotherapy

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Running title: ATR inhibition improves therapeutic index in NSCLC models *in vivo*

Keywords: ataxia telangiectasia related-3 (ATR), AZD6738, DNA damage response, non-small cell lung cancer (NSCLC), fibrosis, small animal radiotherapy research platform (SARRP).

ABSTRACT

Background and purpose: To evaluate the impact of ATR inhibition using AZD6738 in combination with radiotherapy on the response of non-small cell lung cancer (NSCLC) tumour models and a murine model of radiation induced fibrosis.

Materials and methods: AZD6738 was evaluated as a monotherapy and in combination with radiation *in vitro* and *in vivo* using A549 and H460 NSCLC models. Radiation induced pulmonary fibrosis was evaluated by cone beam computed tomography (CBCT) and histological staining.

Results: AZD6738 specifically inhibits ATR kinase and enhanced radiobiological response in NSCLC models but not in human bronchial epithelial cells (HBECs) *in vitro*. Significant tumour growth delay was observed in cell line derived xenografts (CDXs) of H460 cells (p < 0.05) which were less significant in A549 cells. Combination of AZD6738 with radiotherapy showed no significant change in lung tissue density by CBCT (p > 0.5) and histological scoring of radiation induced fibrosis (p > 0.5).

Conclusion: Inhibition of ATR with AZD6738 in combination with radiotherapy i~~mproved~~increases tumour growth delay ~~control~~ without observable augmentation of late radiation induced toxicity further underpinning translation towards clinical evaluation in NSCLC.

Introduction

Lung cancer is the most frequently diagnosed cancer and leading cause of cancer mortality accounting for 1.8 million deaths annually (1). Radiotherapy has a major role in the management of non-small cell lung cancer (NSCLC), in particular the use of stereotactic ablative radiotherapy (SABR). SABR is now considered standard of care for inoperable patients with peripheral early-stage NSCLC (2) with 55.8% overall survival reported at 3 years with high rates of local tumour control and moderate treatment-related morbidity (3). Despite these improvements, there remains an urgent need for the development of new therapies for NSCLC.

Combining novel agents targeting the hallmarks of cancer and their biological response pathways has significant potential to improve the therapeutic index of radiotherapy (4–6). In particular, as the main biological effectiveness of radiotherapy results from DNA damage and considering the frequency of functional loss or dysregulation of key DNA damage response (DDR) proteins in tumours, agents directly targeting components of the DDR may hold significant therapeutic potential (7, 8).

Ataxia telangiectasia mutated (ATM) and Rad3-related (ATR) are critical proteins which orchestrate the DDR, acting as apical kinases in response to DNA double strand breaks (DSBs) initially detected by damage sensing proteins such as MRN (MRE11-RAD50-NSB1) or PARP1 (poly(ADP-ribose) polymerase1). Both proteins belong to the phosphotidyl-inositol 3-kinase related proteins (PIKK) family of serine/threonine kinases and crucially halt downstream effectors including cell cycle checkpoint kinases 1 (CHK1) and p53 causing cell cycle arrest at S and G2 checkpoints. ATM is the primary responder to DSBs whilst ATR is activated by a range of lesions including single strand DNA structures at resected ends of DBSs, strand cross links and after replication fork stalling (9–12).

Over the past decade, a number of selective ATR inhibitors have been reported as tools in early studies to elucidate ATR pharmacology (10, 11, 13). Of these agents, AZD6738 has been developed as a highly potent, selective and bioavailable ATR inhibitor and evaluated as a monotherapy or in combination including radio- and chemotherapy (14, 15). Cumulatively, these data have supported the translation of AZD6738 and other ATR inhibitors to early phase clinical evaluation as a mono- and combination therapy with DNA damaging and molecular targeted agents (NCT02157792, NCT01955668, NCT02223923 and NCT02264678).

Here, we evaluated AZD6738 as a monotherapy and in combination with multiple radiotherapy schedules in NSCLC models and a preclinical model of radiation induced fibrosis. Our data show significant tumour specific radiosensitization and tumour growth delay with no potentiation of radiation induced toxicity in a murine model of pulmonary underpinning future clinical evaluation in NSCLC.

Materials and Methods

Cell lines, drugs and reagents

A549, H460 and human bronchial epithelial cells (HBECs) were purchased from the American type Culture collection (ATCC) and routinely tested for mycoplasma. Cell culture conditions are described in appendix A.

AZD6738 was provided by AstraZeneca. For *in vitro* use, AZD6738 was dissolved in DMSO to a stock concentration of 10 mM and diluted in DMSO to the required working concentration. For *in vivo* use, AZD6738 was dissolved in DMSO at a concentration of 25 mg/mL and diluted 1 in 5 with propylene glycol. An equal amount of water was added to yield 2.5 mg/mL in 10% DMSO, 40% propylene glycol and 50% sterile water. AZD6738 or drug vehicle was delivered by oral gavage at a concentration of 25 mg/kg for 28 days.

Western blotting, clonogenic assay, DNA damage analysis and *in vitro* irradiation

Protein analysis was performed by Western blotting (Appendix A). Clonogenic assay was performed using the protocol of Markus and Puck (16). DNA damage analysis was performed by quantification of DSBs using immuno-cytochemical staining of 53BP1 (Appendix A). All cells were irradiated with 225 kVp X-rays using an X-Rad 225 generator (Precision X-ray Inc., North Bradford, CT) with a 2 mm copper filter at a dose rate of 0.57 Gy min^{-1}.

Animals and maintenance

Tumour response studies were performed by establishing A549 and H460 cell line derived xenografts (CDXs, Appendix A) in 8-10 week old female SCID mice obtained from Charles River Laboratories (Oxford, UK).

Radiation induced toxicity was investigated in fibrosis prone C57BL/6J mice at 8-10 weeks old obtained from Charles River Laboratories (Oxford, UK). All experimental procedures

were performed in accordance with UK Home Office approved protocols for *in vivo* experimentation. For all *in vivo* studies a minimum of 7 animals per group were used.

Preclinical study design

A schematic overview of the study design is shown in Figure 2. Tumour response was evaluated in A549 and H460 CDXs and volumes calculated from calliper measurements using the modified ellipsoidal formula ((17), Appendix 1). AZD6738 was administered 1 hour prior to irradiation. Mice were irradiated with a total dose of 12 Gy delivered as a single fraction or as three daily fractions of 4 Gy. Treatment plans were generated to give 100% tumour coverage using a parallel opposed beam geometry with a 10 x 10 mm collimator at a dose rate of 2.8 ± 0.12 Gy/min. Mice were removed from the study at a maximum tumour volume of 600-800 mm^3.

Radiation induced pulmonary fibrosis was induced by delivering a total dose of 20 Gy as a single fraction or as three daily fractions of 6.67 Gy/day across consecutive days. A parallel opposed beam geometry with a 5 x 5 mm collimator (dose rate 0.27 ± 0.16 Gy/min) was used to target an isocentre in the upper left lung for minimal displacement during breathing and to spare organs at risk.

CBCT imaging and analysis

Lung tissue density was monitored using on board CBCT imaging on SARRP. Images were acquired at 50 kVp before and at 4, 12 and 26 weeks after irradiation. Non-irradiated control animals were imaged at the same time intervals. Quantitative CBCT data was extracted from the scans by outlining the whole of the irradiated lung using 3D Slicer 4.0 (www.slicer.org). Further details are given in Appendix A.

Histological assessment of radiation induced fibrosis

Lung tissues were fixed by inter-tracheal infusion with 4% formaldehyde and processed for histological evaluation detailed in Appendix A.

Data fitting and statistical analysis

Details of statistical analysis are given in Appendix A.

RESULTS

AZD6738 inhibits ATR kinase and radiosensitizes NSCLC models but not human bronchial epithelial cells *in vitro*

The effect of AZD6738 as a single agent and in combination with radiotherapy was investigated in A549 and H460 NSCLC cells and HBECs. From Figure 1 (Panel A), at the protein level, both A549 and H460 cells show high endogenous expression of ATR which is selectively inhibited by AZD6738 at concentrations of 250 nM in A459 cells and 100 nM in H460 and HBEC cells following 2 hours treatment. Both tumour models showed high expression of ATR following exposure to 2 Gy which decreased with AZD6738 treatment. In comparison, HBEC cells have low endogenous levels of ATR which was induced in response to radiation exposure and increased with 100 nM AZD6738.

The effect of AZD6738 on cell survival was determined from un-irradiated, drug treated control samples which showed significant ($p < 0.05$) decreases compared to vehicle treated controls of 33.4%, 41.1% and 16.4% in A549, H460 and HBEC cells respectively. In combination with radiation, AZD6738 increased the radiosensitivity of A549 and H460 but not HBECs. In A549 cells, a significant radiosensitizing effect was observed only at 8 Gy at a concentration of 250 nM ($p = 0.07$). In comparison, at a concentration of 100 nM, H460 cells showed significant radiosensitization at all doses ($p < 0.038$). Dose enhancement factors were calculated as 1.03 and 1.5 in A549 and H460 cells respectively. No significant effects on the radiobiological response of HBECs were observed ($p > 0.5$), supporting the underlying tumour specificity of AZD6738.

At the DNA damage level, AZD6738 significantly increased 53BP1 foci in A549 ($p < 0.01$) but not in H460 cells ($p > 0.5$). When combined with radiation, A549 cells showed significantly increased foci numbers at 4 hours ($p = 0.03$). H460 cells showed a significant

increase in DSB induction at 2 hours (p < 0.01) which remained elevated at 4 and 8 hours (p < 0.03). In both cell lines elevated levels of DNA damage in AZD6738 treated cells had been resolved by 24 hours (p > 0.5). HBECs showed an elevated level of foci when treated as a single agent (p = 0.04) with no significant increase in combination with radiation at each time point investigated (p > 0.5).

AZD6738 with radiotherapy causes significant tumour growth delay *in vivo*

AZD6738 as a single agent and in combination with radiotherapy was investigated in CDX bearing mice ~~treated~~and the effects on tumour growth delay parameters for each of the experimental groups summarised in table 1.~~-~~25 mg/kg of AZD6738 was selected as a known well tolerated dose (AstraZeneca personal communication, (14)). 12 Gy was selected based on the radiobiological response parameters of the cell models (A549, α = 0.39 ± 0.03, β = 0.01 ± 0.01; H460, α = 0.12 ± 0.05, β = 0.05 ± 0.01). BED values for single fraction exposures in A549 and H460 cells were 15.7 Gy and 72 Gy respectively. For fractionated exposures this was calculated to be 13.2 Gy and 32 Gy in A549 and H460 cells respectively.

A549 tumours treated with AZD6738 showed no significant tumour growth delay compared to vehicle treated control animals (p < 0.5). Both single fraction and fractionated animals showed significant tumour growth delay compared to control animals (p < 0.01) from day 20 with an approximate doubling of tumour volume over the 80 days of the experiment. When combined with AZD6738, tumour growth was further delayed for approximately 4 and 10 days for single fraction and fractionated exposures respectively (p < 0.03). In comparison, H460 tumours treated with AZD6738 as a single agent showed significant tumour growth delay compared to vehicle treated control animals (p = 0.04) with a quadrupling time ~~,~~ ~~reaching a volume of~~to 400 mm^3 ~~in~~ of 19 days. Irradiated animals showed significant tumour growth delay compared to all non-irradiated treated animals, with a further delay of 20 and 23

days to reach a volume of 400 mm^3 (p < 0.002). When combined with AZD6738, tumour growth was further delayed for approximately 24 and 23 days with single fraction and fractionated exposures respectively (p < 0.04). AZD6738 was well tolerated in all animals with transient weight loss of less than 10% observed immediately following radiotherapy.

AZD6738 with radiotherapy does not cause significant changes in lung tissue density and radiation induced pulmonary fibrosis

Lung tissue density changes were determined by CBCT imaging on SARRP. As a single agent, AZD6738 showed no significant increase in average lung CT number over 26 weeks compared to drug vehicle treated animals (Appendix A, p > 0.5). Using an α/β value of 2.5 for mouse lung (18, 19), the BEDs for single fraction and fractionated deliveries were 180 and 73.4 Gy respectively.

All animals exposed to single fraction and fractionated radiotherapy showed tissue density increases as a function of time reaching a maximum at 26 week ((p < 0.01, Figure 4). Changes in lung density were localised to the irradiated region for all groups and time points. CT numbers of approximately 500 ± 44.2 at week 0 in all experimental groups increased to 750 ± 54.1 and 780 ± 60.2 for single fraction and fractionated exposures respectively. No significant changes were detected between irradiated animals and those receiving drug vehicle or combination treatment with AZD6738 (p > 0.5).

Tissue sections were scored using the modified Ashcroft scale (20) for pulmonary fibrosis. Histological evaluation of control lungs at week 0 and un-irradiated lungs at each time point showed normal lung architecture with no fibrotic burden.

Mice irradiated with a 20 Gy single fraction in combination with drug vehicle (Figure 5, panel A) or with AZD6738 (Figure 5, panel C) showed equal increases in fibrosis score at 26 weeks to less than 2 compared to week 4 (p < 0.001). Sections showed clear fibrotic changes

with unconnected knot like formations, partially enlarged alveoli with around a 3 fold increase in septal thickening. For fractionated exposures with drug vehicle (Figure 5, panel B) and AZD6738 (Figure 5, panel D), much smaller changes in fibrotic index were observed with values < 0.5 at 26 weeks ($p < 0.1$).

Discussion

ATR is a promising target in cancer treatment for which several ~~small~~ inhibitors have ~~been developed and~~ shown efficacy in combination with radiotherapy (10, 11, 15, 21–25). In this study, AZD6738 was evaluated as a monotherapy or in combination with radiotherapy in NSCLC tumour models, and in a radiation induced fibrosis model of normal tissue complication. To our knowledge this is the first systematic evaluation of ATR inhibition to simultaneously interrogate tumour and normal tissue response across multiple radiotherapy dosing schedules using small animal image guided radiotherapy.

Our data show AZD6738 to be a highly selective and potent inhibitor of ATR causing dose dependent radiosensitization in A459 and H460 but not HBEC cells *in vitro*. Using CDXs, AZD6738 was shown to have more pronounced effects on H460 cells compared to A549 cells which showed lower levels of tumour growth over the same time period. These findings are in close agreement with those of previous reports demonstrating AZD6738 as a potent sensitizer to cisplatin, gemcitabine (14) and radiation (15). We propose that the observed radiosensitization in our study is due to abrogation of the radiation induced G2 cell cycle checkpoint and inhibition of homologous recombination (15). The differential effects in A549 and H460 cells agree with observations of Vendetti *et al,* and may similarly be due to the predominance of apoptosis in H460 cells rather than senescence in A549 cells (14). Furthermore, differences in tumour growth delay agreed with BED calculations validating the empirical dose fractionation hypothesis in our experimental models.

Our data further provide a strong rationale for targeting ATR inhibitors due to its deleterious effect on cancer cells harbouring defects in homologous recombination which could potentially enhance the effectiveness of DNA damaging agents including radiotherapy. However, a major concern in combination therapy is normal tissue complication. Inhibition

of ATR could potentially impact normal tissue as ATR is an essential protein that regulates the firing at origins of replication during S phase and has been shown to be embryonically lethal in mice (26). Additionally, ATR inhibition may prevent replication and increase breaks at fragile sites in the DNA of normal cells which may be especially prone to breakage after DNA damaging treatment in combination with an inhibitor and may impact ATM function through direct interactions. Our data showed high tumour specificity of AZD6738 with no significant effects on the radiobiological response of HBEC cells *in vitro* and on lung tissue density and fibrosis in C56BL6 mice using the dosing regimens investigated. We hypothesis that these effects may be due to differences in the fidelity of HR mediated repair and P53 status.

CBCT analysis detected comparable changes at matched time points across all groups yet these changes did not correlate with fibrosis scores for animals treated with fractionated radiotherapy. For lung tissue, animal studies of late effects have an established α/β ratio of approximately 2-3 Gy (18, 19). Our data may therefore be attributed to differences in the BED which was approximately 2.5 times greater for single fraction exposures. Additionally, although C57BL6 are a known fibrosis prone strain, application of the model is confounded by the late response being dominated by large accumulation of pleural fluid detected following whole thorax irradiation with 15 Gy (28). Our data indicate a lack of fibrosis following fractionated schedules with the observed increase in tissue density being attributed to pleural effusion. The observed increase in CT numbers at 26 weeks is comparable to the trends previously reported for partial lung irradiation at the same dose (29). Although the *in vivo* toxicity profile and long term effects AZD6738 remain to be determined in the clinical setting, as conditional reduction of ATR expression to 10% of normal levels in adult mice has been shown to minimal impact on normal tissue homeostasis (30).

Our experimental approach may be improved with analysis of additional endpoints including early effects such as pneumonitis, functional assessment of radiation induced lung pathology and improvements in preclinical radiotherapy procedures such as beam gating or forced breath hold to more accurately model clinical developments (31–33). Further investigation in specific patient subgroups with known driver mutations associated with NSCLC including EGFR, MET, BRAF, and TP53 (34) should also be explore for clinical translation along with the identification of biomarkers to select patients most likely to benefit from the novel treatment combination (35).

Conclusions

This work demonstrates the therapeutic advantage of combining AZD6738 with radiotherapy in the treatment of NSCLC by ~~improving~~ increasing tumour growth~~control~~ delay with no significant increase in radiation induced toxicity. These data provide further support for AZD6738 in combination with radiotherapy to be explored in early phase clinical evaluation.

Contribution of authors

VD, MG and CBMC performed the experimental work and data analysis. DS, SW and CCT provided expertise on sample handling and lung pathology. SO and CKMcG provided expertise on CBCT data analysis and interpretation. KMP, GGH and KTB designed and supervised the studies and prepared the manuscript. All authors contributed to the drafting and final review of the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

Figure legends

Fig. 1. Impact of AZD6738 on ATR protein levels and radiobiological response of A549 and H460 non-small cell lung cancer (NSCLC) and human bronchial epithelial cells *in vitro*. (A) Protein analysis of total ATR and total AKT by Western blotting for cells treated with 100 nM AZD6738 in combination with 2 Gy; (B) Radiation dose response curves for cells treated with 100 nM AZD6738 in combination radiation; (C) DNA damage induction and repair at time intervals up to 24 hrs following irradiation by scoring of double strand breaks (DSBs) using immuno-histochemical staining of 53BP1. Probability values were classified as *** ($p < 0.005$), ** ($p < 0.05$) and * ($p < 0.1$).

Fig. 2. Schematic overview of the preclinical study design. All mice received AZD6738 (25 mg/kg delivered by oral gavage once per day, p.o.q.d) prior to radiotherapy. Tumour response studies were initiated in mice bearing cell line derived xenografts (CDXs) at a volume of 100 mm^3 and irradiated with a total dose of 12 Gy delivered as a single fraction or three daily fractions of 4 Gy. Radiation induced fibrosis was evaluated by irradiated a 5 x 5 mm sub-volume of the upper right lung with a total dose of 20 Gy delivered as a single fraction or three daily fractions of 6.67 Gy.

Fig. 3. Impact of AZD6738 on cell lines derived xenografts (CDXs) of A549 (panels A and B) and H460 (panels C and D) cells following single fraction (panels A and C) and fractionated radiotherapy exposures (panels B and D). In all panels mice were treated with either drug vehicle (■); AZD6738 (25 mg/kg per day for 28 days (▼); radiotherapy (●); radiotherapy in combination with AZD6738 (▲). Data is presented as median tumour volume for the experimental group ± standard error.

Fig. 4. Quantitative analysis of lung tissue density changes measured by cone beam computed tomography (CBCT) longitudinally for 26 weeks after irradiation. Mice were irradiated with a total dose of 20 Gy single fraction in combination with drug vehicle (Panel A) or AZD6738 (25 mg/kg per day, Panel C), and with three fractions of 6.67 Gy in combination with drug vehicle (Panel B) or AZD6738 (Panel D). Data is presented as the difference between the average CT number of the irradiated lung and the average CT number in air. Data is presented as raw numbers at all time-points. Probability values were classified as *** ($p < 0.005$), ** ($p < 0.05$) and * ($p < 0.1$).

Fig. 5. Histological staining and quantification of radiation induced pulmonary fibrosis. Stained lungs were electronically scanned and five images scored from the upper region of the left lobe. Pulmonary fibrosis was scored using the modified Ashcroft scale (0-8) at 10 x magnification by independent and blind observers. Mice were irradiated with a total dose of 20 Gy single fraction in combination with drug vehicle (Panel A) or with AZD6738 (25 mg/kg per day, Panel C), and with three fractions of 6.67 Gy in combination with drug vehicle (Panel B) or with AZD6738 (Panel D). Data is presented as the fibrosis score ± SEM. A representative image from each of the experimental groups is inset in each panel. Probability values were classified as *** ($p < 0.005$), ** ($p < 0.05$) and * ($p < 0.1$).

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Inhibition of ataxia telangiectasia related-3 (ATR) improves therapeutic index in preclinical models of non-small cell lung cancer (NSCLC) radiotherapy

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Running title: ATR inhibition improves therapeutic index in NSCLC models *in vivo*

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ABSTRACT

Background and purpose: To evaluate the impact of ATR inhibition using AZD6738 in combination with radiotherapy on the response of non-small cell lung cancer (NSCLC) tumour models and a murine model of radiation induced fibrosis.

Materials and methods: AZD6738 was evaluated as a monotherapy and in combination with radiation *in vitro* and *in vivo* using A549 and H460 NSCLC models. Radiation induced pulmonary fibrosis was evaluated by cone beam computed tomography (CBCT) and histological staining.

Results: AZD6738 specifically inhibits ATR kinase and enhanced radiobiological response in NSCLC models but not in human bronchial epithelial cells (HBECs) *in vitro*. Significant tumour growth delay was observed in cell line derived xenografts (CDXs) of H460 cells ($p < 0.05$) which were less significant in A549 cells. Combination of AZD6738 with radiotherapy showed no significant change in lung tissue density by CBCT ($p > 0.5$) and histological scoring of radiation induced fibrosis ($p > 0.5$).

Conclusion: Inhibition of ATR with AZD6738 in combination with radiotherapy increases tumour growth delay without observable augmentation of late radiation induced toxicity further underpinning translation towards clinical evaluation in NSCLC.

Introduction

Lung cancer is the most frequently diagnosed cancer and leading cause of cancer mortality accounting for 1.8 million deaths annually (1). Radiotherapy has a major role in the management of non-small cell lung cancer (NSCLC), in particular the use of stereotactic ablative radiotherapy (SABR). SABR is now considered standard of care for inoperable patients with peripheral early-stage NSCLC (2) with 55.8% overall survival reported at 3 years with high rates of local tumour control and moderate treatment-related morbidity (3). Despite these improvements, there remains an urgent need for the development of new therapies for NSCLC.

Combining novel agents targeting the hallmarks of cancer and their biological response pathways has significant potential to improve the therapeutic index of radiotherapy (4–6). In particular, as the main biological effectiveness of radiotherapy results from DNA damage and considering the frequency of functional loss or dysregulation of key DNA damage response (DDR) proteins in tumours, agents directly targeting components of the DDR may hold significant therapeutic potential (7, 8).

Ataxia telangiectasia mutated (ATM) and Rad3-related (ATR) are critical proteins which orchestrate the DDR, acting as apical kinases in response to DNA double strand breaks (DSBs) initially detected by damage sensing proteins such as MRN (MRE11-RAD50-NSB1) or PARP1 (poly(ADP-ribose) polymerase1). Both proteins belong to the phosphotidyl-inositol 3-kinase related proteins (PIKK) family of serine/threonine kinases and crucially halt downstream effectors including cell cycle checkpoint kinases 1 (CHK1) and p53 causing cell cycle arrest at S and G2 checkpoints. ATM is the primary responder to DSBs whilst ATR is activated by a range of lesions including single strand DNA structures at resected ends of DBSs, strand cross links and after replication fork stalling (9–12).

Over the past decade, a number of selective ATR inhibitors have been reported as tools in early studies to elucidate ATR pharmacology (10, 11, 13). Of these agents, AZD6738 has been developed as a highly potent, selective and bioavailable ATR inhibitor and evaluated as a monotherapy or in combination including radio- and chemotherapy (14, 15). Cumulatively, these data have supported the translation of AZD6738 and other ATR inhibitors to early phase clinical evaluation as a mono- and combination therapy with DNA damaging and molecular targeted agents (NCT02157792, NCT01955668, NCT02223923 and NCT02264678).

Here, we evaluated AZD6738 as a monotherapy and in combination with multiple radiotherapy schedules in NSCLC models and a preclinical model of radiation induced fibrosis. Our data show significant tumour specific radiosensitization and tumour growth delay with no potentiation of radiation induced toxicity in a murine model of pulmonary underpinning future clinical evaluation in NSCLC.

Materials and Methods

Cell lines, drugs and reagents

A549, H460 and human bronchial epithelial cells (HBECs) were purchased from the American type Culture collection (ATCC) and routinely tested for mycoplasma. Cell culture conditions are described in appendix A.

AZD6738 was provided by AstraZeneca. For *in vitro* use, AZD6738 was dissolved in DMSO to a stock concentration of 10 mM and diluted in DMSO to the required working concentration. For *in vivo* use, AZD6738 was dissolved in DMSO at a concentration of 25 mg/mL and diluted 1 in 5 with propylene glycol. An equal amount of water was added to yield 2.5 mg/mL in 10% DMSO, 40% propylene glycol and 50% sterile water. AZD6738 or drug vehicle was delivered by oral gavage at a concentration of 25 mg/kg for 28 days.

Western blotting, clonogenic assay, DNA damage analysis and *in vitro* irradiation

Protein analysis was performed by Western blotting (Appendix A). Clonogenic assay was performed using the protocol of Markus and Puck (16). DNA damage analysis was performed by quantification of DSBs using immuno-cytochemical staining of 53BP1 (Appendix A). All cells were irradiated with 225 kVp X-rays using an X-Rad 225 generator (Precision X-ray Inc., North Bradford, CT) with a 2 mm copper filter at a dose rate of 0.57 Gy min^{-1}.

Animals and maintenance

Tumour response studies were performed by establishing A549 and H460 cell line derived xenografts (CDXs, Appendix A) in 8-10 week old female SCID mice obtained from Charles River Laboratories (Oxford, UK).

Radiation induced toxicity was investigated in fibrosis prone C57BL/6J mice at 8-10 weeks old obtained from Charles River Laboratories (Oxford, UK). All experimental procedures

were performed in accordance with UK Home Office approved protocols for *in vivo* experimentation. For all *in vivo* studies a minimum of 7 animals per group were used.

Preclinical study design

A schematic overview of the study design is shown in Figure 2. Tumour response was evaluated in A549 and H460 CDXs and volumes calculated from calliper measurements using the modified ellipsoidal formula ((17), Appendix 1). AZD6738 was administered 1 hour prior to irradiation. Mice were irradiated with a total dose of 12 Gy delivered as a single fraction or as three daily fractions of 4 Gy. Treatment plans were generated to give 100% tumour coverage using a parallel opposed beam geometry with a 10 x 10 mm collimator at a dose rate of 2.8 ± 0.12 Gy/min. Mice were removed from the study at a maximum tumour volume of 600-800 mm^3.

Radiation induced pulmonary fibrosis was induced by delivering a total dose of 20 Gy as a single fraction or as three daily fractions of 6.67 Gy/day across consecutive days. A parallel opposed beam geometry with a 5 x 5 mm collimator (dose rate 0.27 ± 0.16 Gy/min) was used to target an isocentre in the upper left lung for minimal displacement during breathing and to spare organs at risk.

CBCT imaging and analysis

Lung tissue density was monitored using on board CBCT imaging on SARRP. Images were acquired at 50 kVp before and at 4, 12 and 26 weeks after irradiation. Non-irradiated control animals were imaged at the same time intervals. Quantitative CBCT data was extracted from the scans by outlining the whole of the irradiated lung using 3D Slicer 4.0 (www.slicer.org). Further details are given in Appendix A.

Histological assessment of radiation induced fibrosis

Lung tissues were fixed by inter-tracheal infusion with 4% formaldehyde and processed for histological evaluation detailed in Appendix A.

Data fitting and statistical analysis

Details of statistical analysis are given in Appendix A.

RESULTS

AZD6738 inhibits ATR kinase and radiosensitizes NSCLC models but not human bronchial epithelial cells *in vitro*

The effect of AZD6738 as a single agent and in combination with radiotherapy was investigated in A549 and H460 NSCLC cells and HBECs. From Figure 1 (Panel A), at the protein level, both A549 and H460 cells show high endogenous expression of ATR which is selectively inhibited by AZD6738 at concentrations of 250 nM in A459 cells and 100 nM in H460 and HBEC cells following 2 hours treatment. Both tumour models showed high expression of ATR following exposure to 2 Gy which decreased with AZD6738 treatment. In comparison, HBEC cells have low endogenous levels of ATR which was induced in response to radiation exposure and increased with 100 nM AZD6738.

The effect of AZD6738 on cell survival was determined from un-irradiated, drug treated control samples which showed significant ($p < 0.05$) decreases compared to vehicle treated controls of 33.4%, 41.1% and 16.4% in A549, H460 and HBEC cells respectively. In combination with radiation, AZD6738 increased the radiosensitivity of A549 and H460 but not HBECs. In A549 cells, a significant radiosensitizing effect was observed only at 8 Gy at a concentration of 250 nM ($p = 0.07$). In comparison, at a concentration of 100 nM, H460 cells showed significant radiosensitization at all doses ($p < 0.038$). Dose enhancement factors were calculated as 1.03 and 1.5 in A549 and H460 cells respectively. No significant effects on the radiobiological response of HBECs were observed ($p > 0.5$), supporting the underlying tumour specificity of AZD6738.

At the DNA damage level, AZD6738 significantly increased 53BP1 foci in A549 ($p < 0.01$) but not in H460 cells ($p > 0.5$). When combined with radiation, A549 cells showed significantly increased foci numbers at 4 hours ($p = 0.03$). H460 cells showed a significant

increase in DSB induction at 2 hours (p < 0.01) which remained elevated at 4 and 8 hours (p < 0.03). In both cell lines elevated levels of DNA damage in AZD6738 treated cells had been resolved by 24 hours (p > 0.5). HBECs showed an elevated level of foci when treated as a single agent (p = 0.04) with no significant increase in combination with radiation at each time point investigated (p > 0.5).

AZD6738 with radiotherapy causes significant tumour growth delay *in vivo*

AZD6738 as a single agent and in combination with radiotherapy was investigated in CDX bearing mice and the effects on tumour growth delay parameters for each of the experimental groups summarised in table 1.25 mg/kg of AZD6738 was selected as a known well tolerated dose (AstraZeneca personal communication, (14)). 12 Gy was selected based on the radiobiological response parameters of the cell models (A549, $\alpha = 0.39 \pm 0.03$, $\beta = 0.01 \pm 0.01$; H460, $\alpha = 0.12 \pm 0.05$, $\beta = 0.05 \pm 0.01$). BED values for single fraction exposures in A549 and H460 cells were 15.7 Gy and 72 Gy respectively. For fractionated exposures this was calculated to be 13.2 Gy and 32 Gy in A549 and H460 cells respectively.

A549 tumours treated with AZD6738 showed no significant tumour growth delay compared to vehicle treated control animals (p < 0.5). Both single fraction and fractionated animals showed significant tumour growth delay compared to control animals (p <0.01) from day 20 with an approximate doubling of tumour volume over the 80 days of the experiment. When combined with AZD6738, tumour growth was further delayed for approximately 4 and 10 days for single fraction and fractionated exposures respectively (p <0.03). In comparison, H460 tumours treated with AZD6738 as a single agent showed significant tumour growth delay compared to vehicle treated control animals (p = 0.04) with a quadrupling time to 400 mm^3 of 19 days. Irradiated animals showed significant tumour growth delay compared to all non-irradiated treated animals, with a further delay of 20 and 23 days to reach a volume of

400 mm^3 (p<0.002). When combined with AZD6738, tumour growth was further delayed for approximately 24 and 23 days with single fraction and fractionated exposures respectively (p<0.04). AZD6738 was well tolerated in all animals with transient weight loss of less than 10% observed immediately following radiotherapy.

AZD6738 with radiotherapy does not cause significant changes in lung tissue density and radiation induced pulmonary fibrosis

Lung tissue density changes were determined by CBCT imaging on SARRP. As a single agent, AZD6738 showed no significant increase in average lung CT number over 26 weeks compared to drug vehicle treated animals (Appendix A, p > 0.5). Using an α/β value of 2.5 for mouse lung (18, 19), the BEDs for single fraction and fractionated deliveries were 180 and 73.4 Gy respectively.

All animals exposed to single fraction and fractionated radiotherapy showed tissue density increases as a function of time reaching a maximum at 26 week ((p < 0.01, Figure 4). Changes in lung density were localised to the irradiated region for all groups and time points. CT numbers of approximately 500 ± 44.2 at week 0 in all experimental groups increased to 750 ± 54.1 and 780 ± 60.2 for single fraction and fractionated exposures respectively. No significant changes were detected between irradiated animals and those receiving drug vehicle or combination treatment with AZD6738 (p > 0.5).

Tissue sections were scored using the modified Ashcroft scale (20) for pulmonary fibrosis. Histological evaluation of control lungs at week 0 and un-irradiated lungs at each time point showed normal lung architecture with no fibrotic burden.

Mice irradiated with a 20 Gy single fraction in combination with drug vehicle (Figure 5, panel A) or with AZD6738 (Figure 5, panel C) showed equal increases in fibrosis score at 26 weeks to less than 2 compared to week 4 (p < 0.001). Sections showed clear fibrotic changes

with unconnected knot like formations, partially enlarged alveoli with around a 3 fold increase in septal thickening. For fractionated exposures with drug vehicle (Figure 5, panel B) and AZD6738 (Figure 5, panel D), much smaller changes in fibrotic index were observed with values < 0.5 at 26 weeks (p < 0.1).

Discussion

ATR is a promising target in cancer treatment for which several inhibitors have shown efficacy in combination with radiotherapy (10, 11, 15, 21–25). In this study, AZD6738 was evaluated as a monotherapy or in combination with radiotherapy in NSCLC tumour models, and in a radiation induced fibrosis model of normal tissue complication. To our knowledge this is the first systematic evaluation of ATR inhibition to simultaneously interrogate tumour and normal tissue response across multiple radiotherapy dosing schedules using small animal image guided radiotherapy.

Our data show AZD6738 to be a highly selective and potent inhibitor of ATR causing dose dependent radiosensitization in A459 and H460 but not HBEC cells *in vitro*. Using CDXs, AZD6738 was shown to have more pronounced effects on H460 cells compared to A549 cells which showed lower levels of tumour growth over the same time period. These findings are in close agreement with those of previous reports demonstrating AZD6738 as a potent sensitizer to cisplatin, gemcitabine (14) and radiation (15). We propose that the observed radiosensitization in our study is due to abrogation of the radiation induced G2 cell cycle checkpoint and inhibition of homologous recombination (15). The differential effects in A549 and H460 cells agree with observations of Vendetti *et al,* and may similarly be due to the predominance of apoptosis in H460 cells rather than senescence in A549 cells (14). Furthermore, differences in tumour growth delay agreed with BED calculations validating the empirical dose fractionation hypothesis in our experimental models.

Our data further provide a strong rationale for targeting ATR inhibitors due to its deleterious effect on cancer cells harbouring defects in homologous recombination which could potentially enhance the effectiveness of DNA damaging agents including radiotherapy. However, a major concern in combination therapy is normal tissue complication. Inhibition

of ATR could potentially impact normal tissue as ATR is an essential protein that regulates the firing at origins of replication during S phase and has been shown to be embryonically lethal in mice (26). Additionally, ATR inhibition may prevent replication and increase breaks at fragile sites in the DNA of normal cells which may be especially prone to breakage after DNA damaging treatment in combination with an inhibitor and may impact ATM function through direct interactions. Our data showed high tumour specificity of AZD6738 with no significant effects on the radiobiological response of HBEC cells *in vitro* and on lung tissue density and fibrosis in C56BL6 mice using the dosing regimens investigated. We hypothesis that these effects may be due to differences in the fidelity of HR mediated repair including P53 status.

CBCT analysis detected comparable changes at matched time points across all groups yet these changes did not correlate with fibrosis scores for animals treated with fractionated radiotherapy. For lung tissue, animal studies of late effects have an established α/β ratio of approximately 2-3 Gy (18, 19). Our data may therefore be attributed to differences in the BED which was approximately 2.5 times greater for single fraction exposures. Additionally, although C57BL6 are a known fibrosis prone strain, application of the model is confounded by the late response being dominated by large accumulation of pleural fluid detected following whole thorax irradiation with 15 Gy (28). Our data indicate a lack of fibrosis following fractionated schedules with the observed increase in tissue density being attributed to pleural effusion. The observed increase in CT numbers at 26 weeks is comparable to the trends previously reported for partial lung irradiation at the same dose (29). Although the *in vivo* toxicity profile and long term effects AZD6738 remain to be determined in the clinical setting as conditional reduction of ATR expression to 10% of normal levels in adult mice has been shown to minimal impact on normal tissue homeostasis (30).

Our experimental approach may be improved with analysis of additional endpoints including early effects such as pneumonitis, functional assessment of radiation induced lung pathology and improvements in preclinical radiotherapy procedures such as beam gating or forced breath hold to more accurately model clinical developments (31–33). Further investigation in specific patient subgroups with known driver mutations associated with NSCLC including EGFR, MET, BRAF, and TP53 (34) should also be explore for clinical translation along with the identification of biomarkers to select patients most likely to benefit from the novel treatment combination (35).

Conclusions

This work demonstrates the therapeutic advantage of combining AZD6738 with radiotherapy in the treatment of NSCLC by increasing tumour growth delay with no significant increase in radiation induced toxicity. These data provide further support for AZD6738 in combination with radiotherapy to be explored in early phase clinical evaluation.

Contribution of authors

VD, MG and CBMC performed the experimental work and data analysis. DS, SW and CCT provided expertise on sample handling and lung pathology. SO and CKMcG provided expertise on CBCT data analysis and interpretation. KMP, GGH and KTB designed and supervised the studies and prepared the manuscript. All authors contributed to the drafting and final review of the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

Figure legends

Fig. 1. Impact of AZD6738 on ATR protein levels and radiobiological response of A549 and H460 non-small cell lung cancer (NSCLC) and human bronchial epithelial cells *in vitro*. (A) Protein analysis of total ATR and total AKT by Western blotting for cells treated with 100 nM AZD6738 in combination with 2 Gy; (B) Radiation dose response curves for cells treated with 100 nM AZD6738 in combination radiation; (C) DNA damage induction and repair at time intervals up to 24 hrs following irradiation by scoring of double strand breaks (DSBs) using immuno-histochemical staining of 53BP1. Probability values were classified as *** ($p < 0.005$), ** ($p < 0.05$) and * ($p < 0.1$).

Fig. 2. Schematic overview of the preclinical study design. All mice received AZD6738 (25 mg/kg delivered by oral gavage once per day, p.o.q.d) prior to radiotherapy. Tumour response studies were initiated in mice bearing cell line derived xenografts (CDXs) at a volume of 100 mm^3 and irradiated with a total dose of 12 Gy delivered as a single fraction or three daily fractions of 4 Gy. Radiation induced fibrosis was evaluated by irradiated a 5 x 5 mm sub-volume of the upper right lung with a total dose of 20 Gy delivered as a single fraction or three daily fractions of 6.67 Gy.

Fig. 3. Impact of AZD6738 on cell lines derived xenografts (CDXs) of A549 (panels A and B) and H460 (panels C and D) cells following single fraction (panels A and C) and fractionated radiotherapy exposures (panels B and D). In all panels mice were treated with either drug vehicle (■); AZD6738 (25 mg/kg per day for 28 days (▼); radiotherapy (●); radiotherapy in combination with AZD6738 (▲). Data is presented as median tumour volume for the experimental group ± standard error.

Fig. 4. Quantitative analysis of lung tissue density changes measured by cone beam computed tomography (CBCT) longitudinally for 26 weeks after irradiation. Mice were irradiated with a total dose of 20 Gy single fraction in combination with drug vehicle (Panel A) or AZD6738 (25 mg/kg per day, Panel C), and with three fractions of 6.67 Gy in combination with drug vehicle (Panel B) or AZD6738 (Panel D). Data is presented as the difference between the average CT number of the irradiated lung and the average CT number in air. Data is presented as raw numbers at all time-points. Probability values were classified as *** ($p < 0.005$), ** ($p < 0.05$) and * ($p < 0.1$).

Fig. 5. Histological staining and quantification of radiation induced pulmonary fibrosis. Stained lungs were electronically scanned and five images scored from the upper region of the left lobe. Pulmonary fibrosis was scored using the modified Ashcroft scale (0-8) at 10 x magnification by independent and blind observers. Mice were irradiated with a total dose of 20 Gy single fraction in combination with drug vehicle (Panel A) or with AZD6738 (25 mg/kg per day, Panel C), and with three fractions of 6.67 Gy in combination with drug vehicle (Panel B) or with AZD6738 (Panel D). Data is presented as the fibrosis score ± SEM. A representative image from each of the experimental groups is inset in each panel. Probability values were classified as *** ($p < 0.005$), ** ($p < 0.05$) and * ($p < 0.1$).

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Table

Treatment Group	Cell model	
	A549 tumour doubling time (days)	H460 tumour quadrupling time (days)
1. Vehicle	38 ± 5.12	3 ± 2.53
2. Vehicle + 12 Gy	73 ± 5.94 (cf. group 1, $p = 0.004$)	25 ± 5.01 (cf. group 1, p = 0.002)
3. Vehicle + 3 x 4 Gy	80 ± 8.34 (cf. group 1, $p = 0.003$) (cf. group 2, $p = 0.016$)	40 ± 3.24 (cf. group 1, $p = 0.002$) (cf. group 2, $p = 0.013$)
4. AZD6738	49 ± 3.89 (cf. group 1, $p = 0.380$) (cf. group 2, $p = 0.035$) (cf. group 3 $p = 0.103$)	19 ± 2.53 (cf. group 1, $p = 0.039$) (cf. group 2, $p = 0.165$) (cf. group 3 $p = 0.062$)
5. AZD6738 + 12 Gy	77 ± 7.16 (cf. group 2, $p = 0.005$)	52 ± 6.07 (cf. group 2, $p = 0.019$)
6. AZD 6738 + 3 x 4 Gy	90 ± 5.12 (cf. group 3, $p = 0.030$)	63 ± 2.18 (cf. group 3, p = 0.033)

Table 1

Summary of tumour growth delay parameters with uncertainties for NSCLC cell line derived xenografts (CDXs) following treatment with AZD6738 and in combination with single fraction and fractionated radiotherapy exposures. Statistical comparisons across different groups are shown in parenthesis with calculated p values.

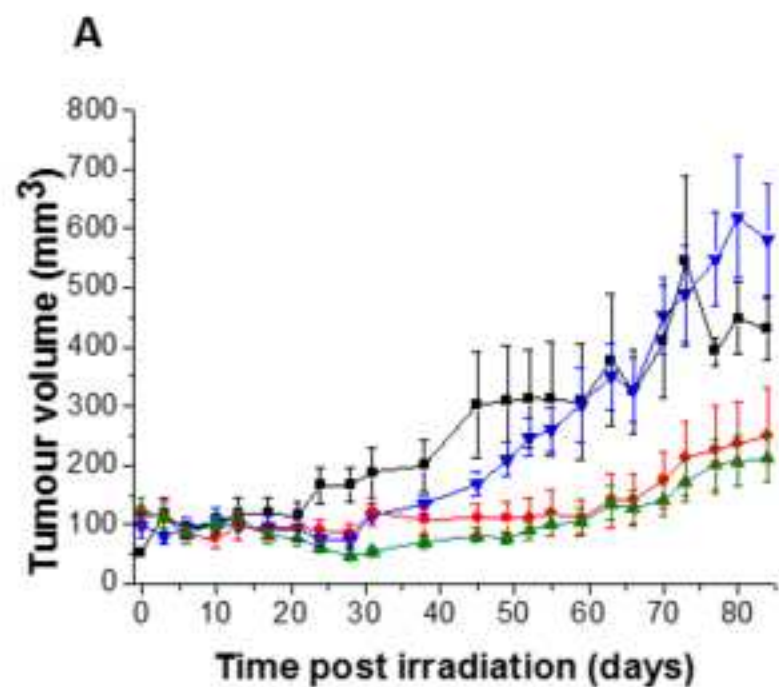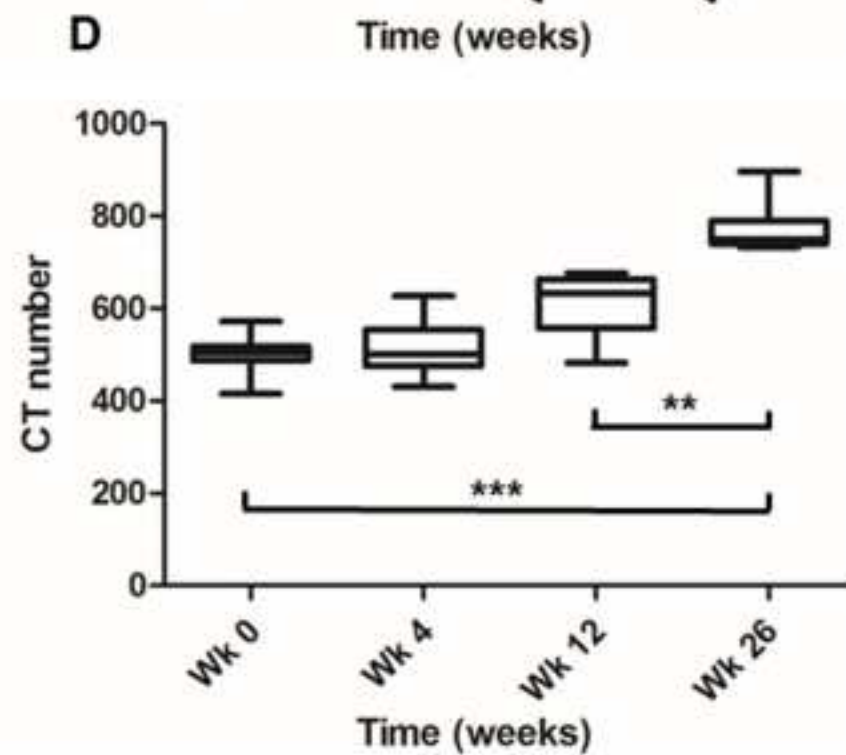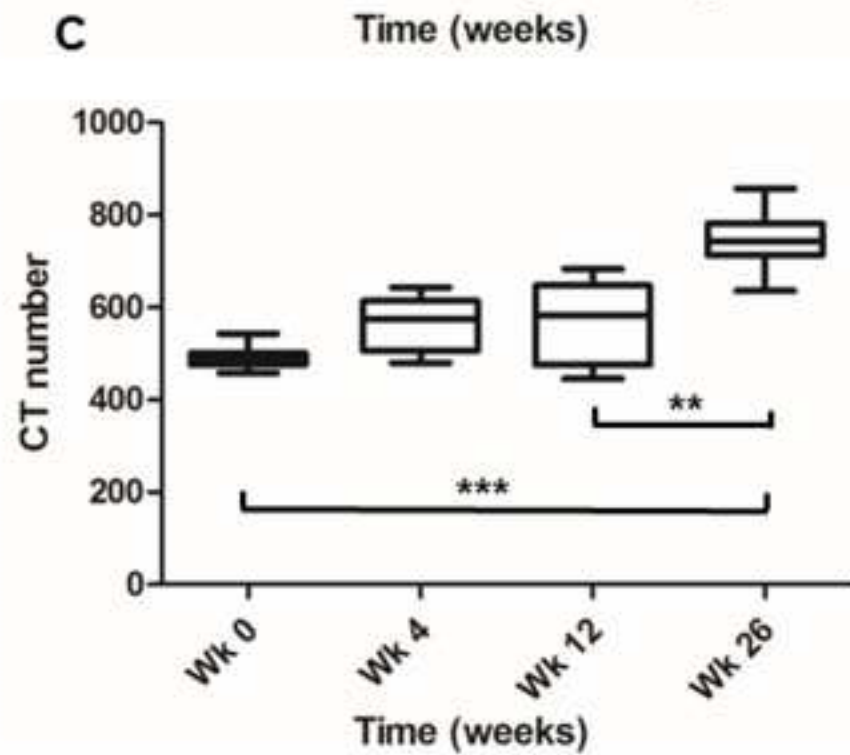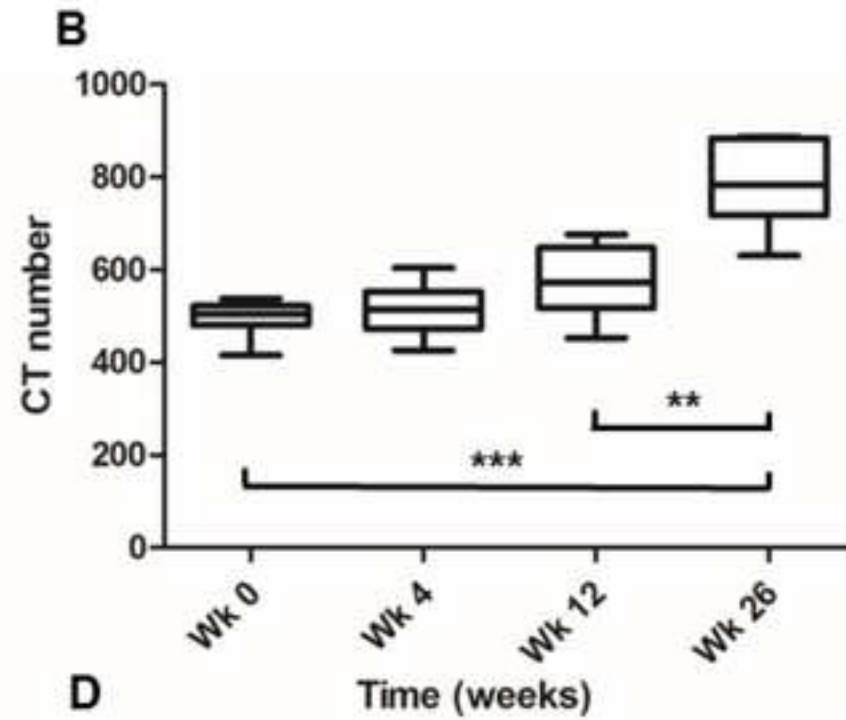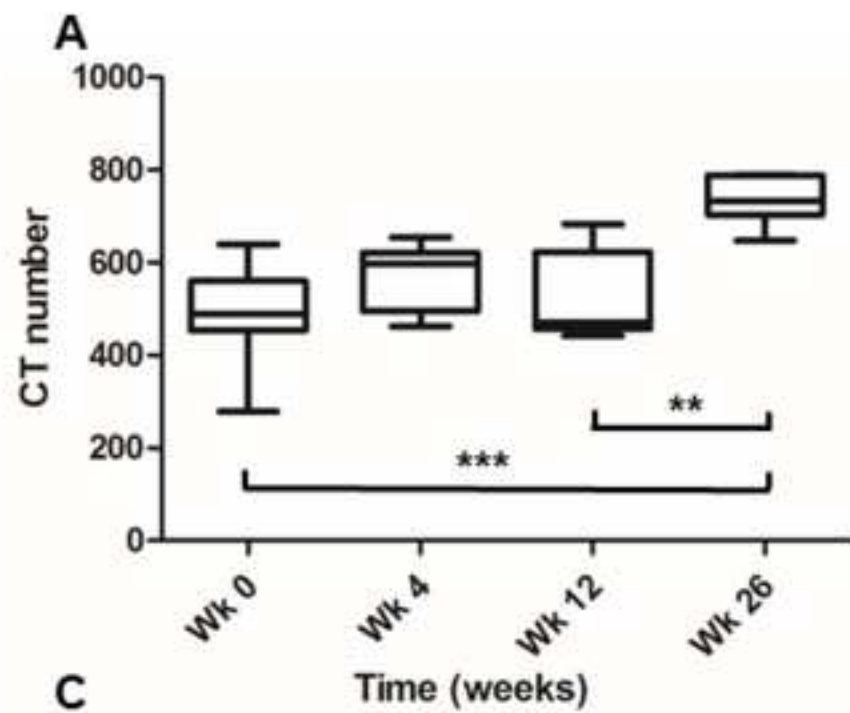**Figure 1**
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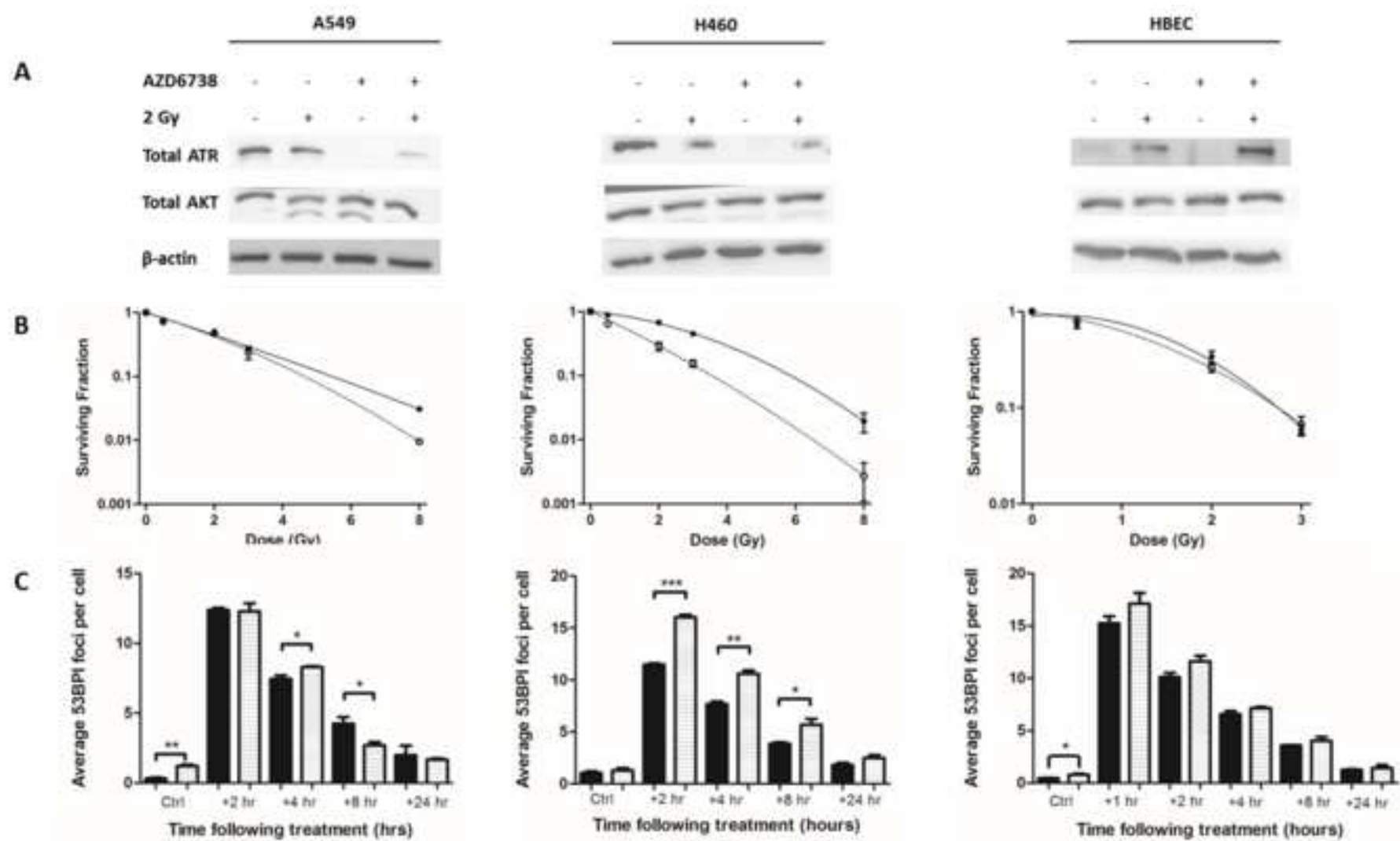
Figure 2
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12 Gy or
3 x 4 Gy

Wk 0 AZD 6738
25 mg/kg p.o q.d

20 Gy or
3 x 6.7 Gy

Tumour response
(SCID + NSCLC xenograft)

TV max

Late toxicity (fibrosis)
(C57BL/6)

Wk 4   12   26

CBCT and tissue collection
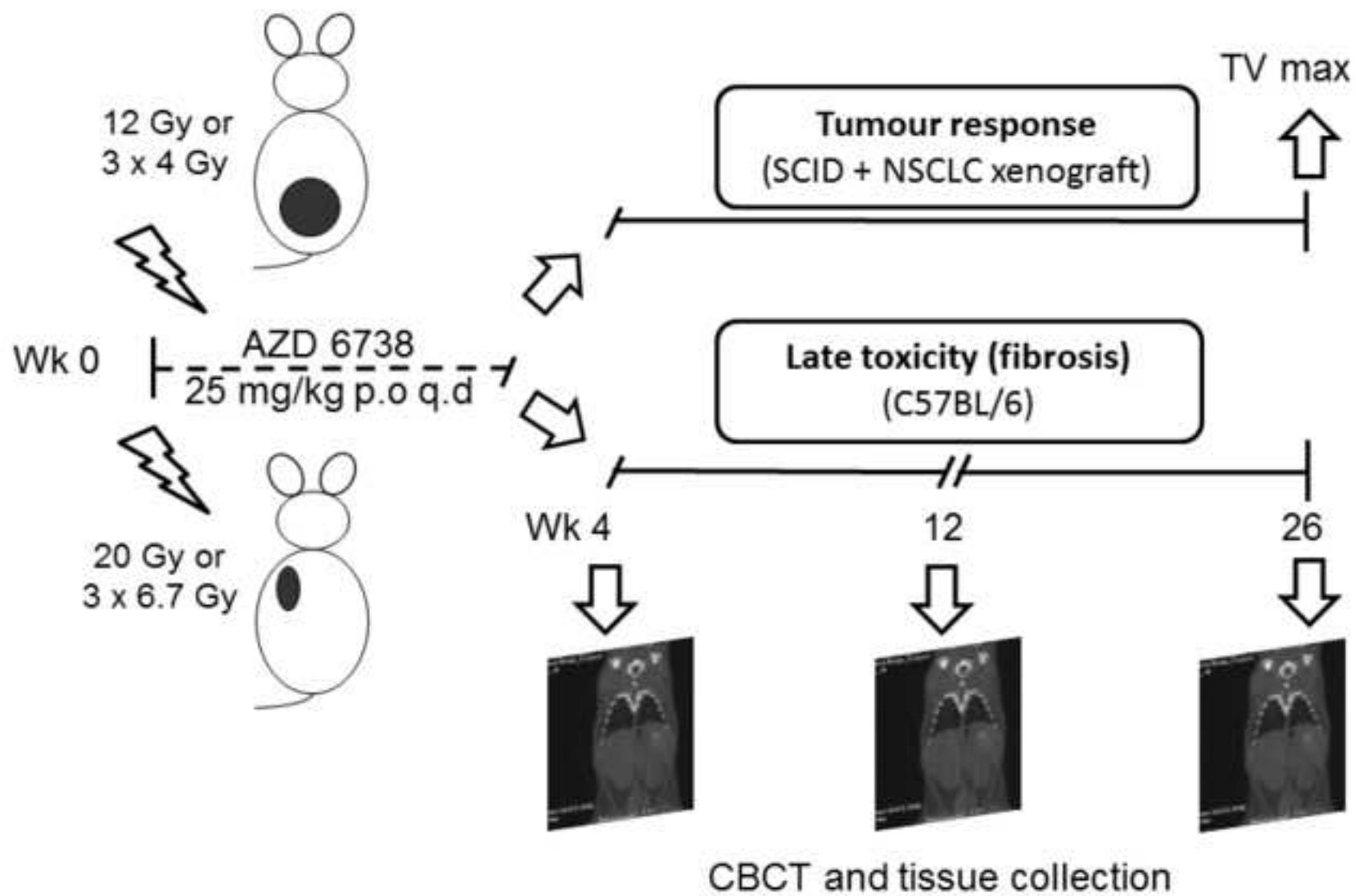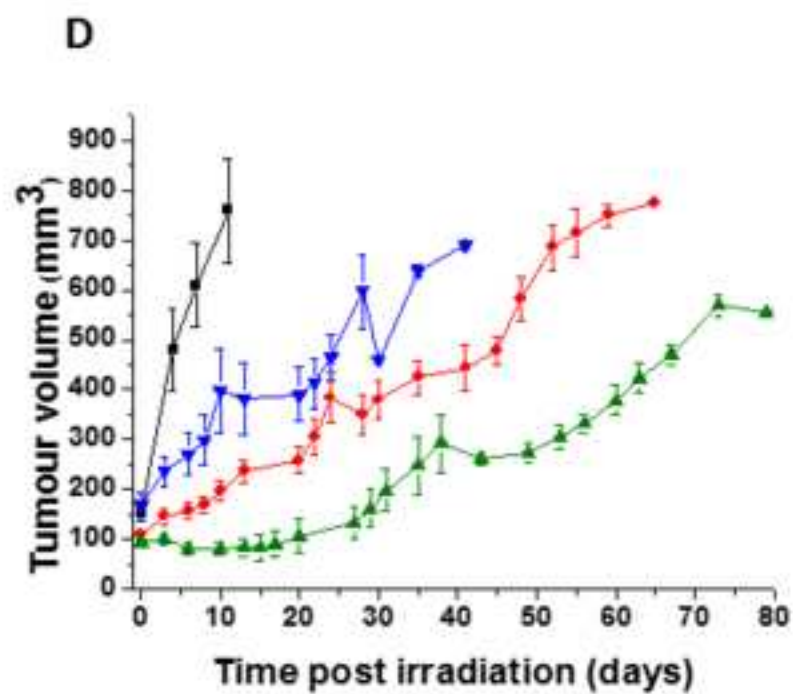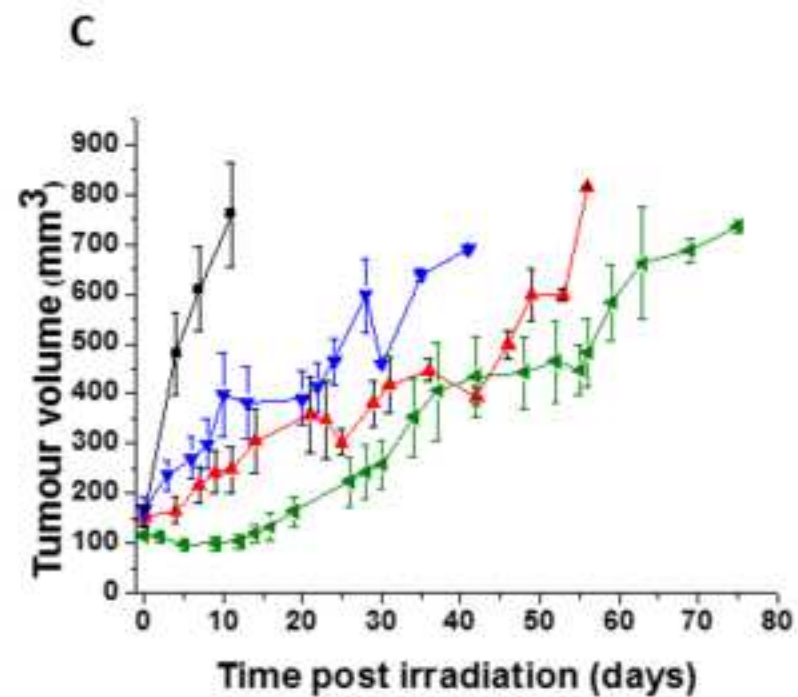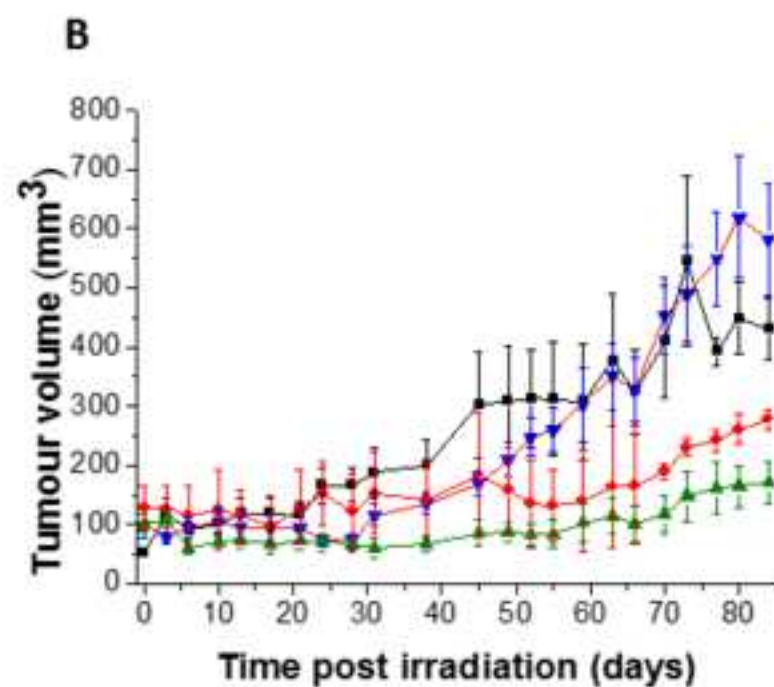
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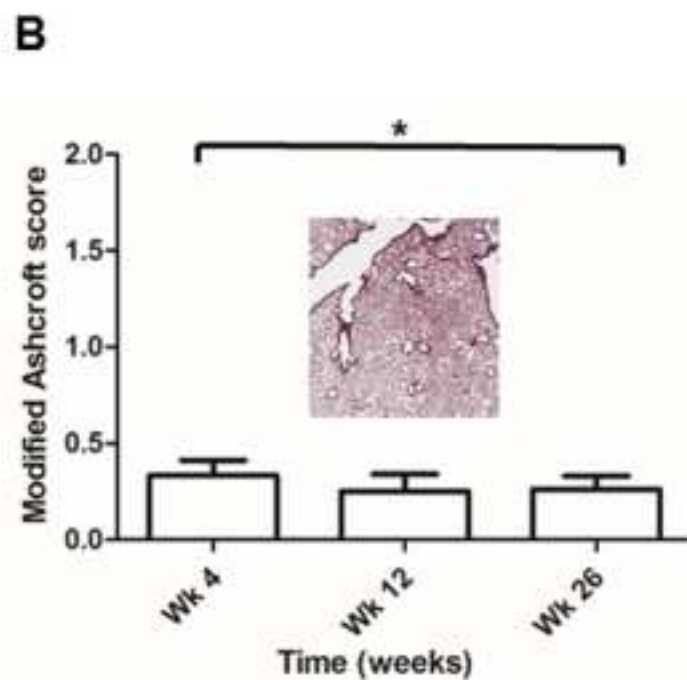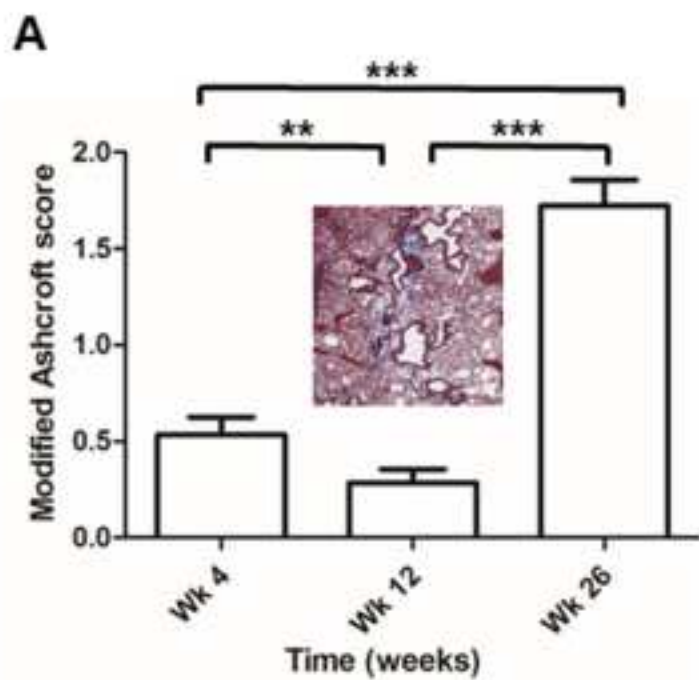Click here to download high resolution image

Figure 5
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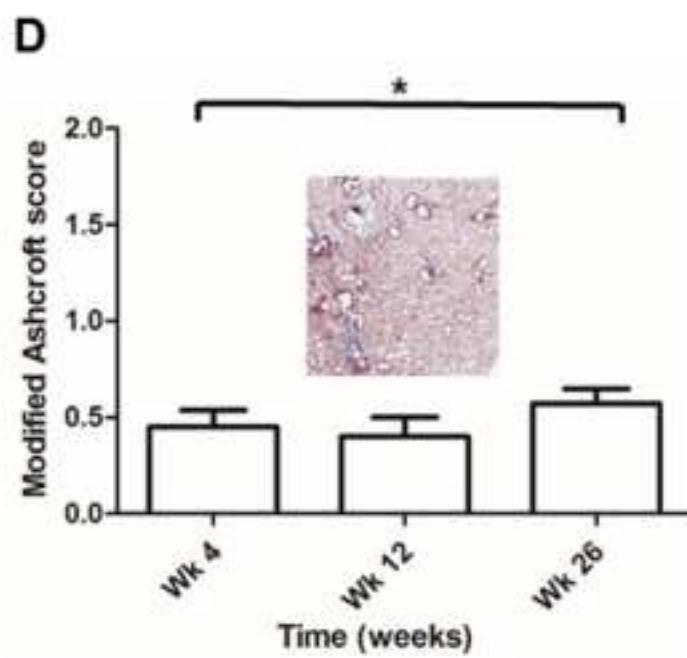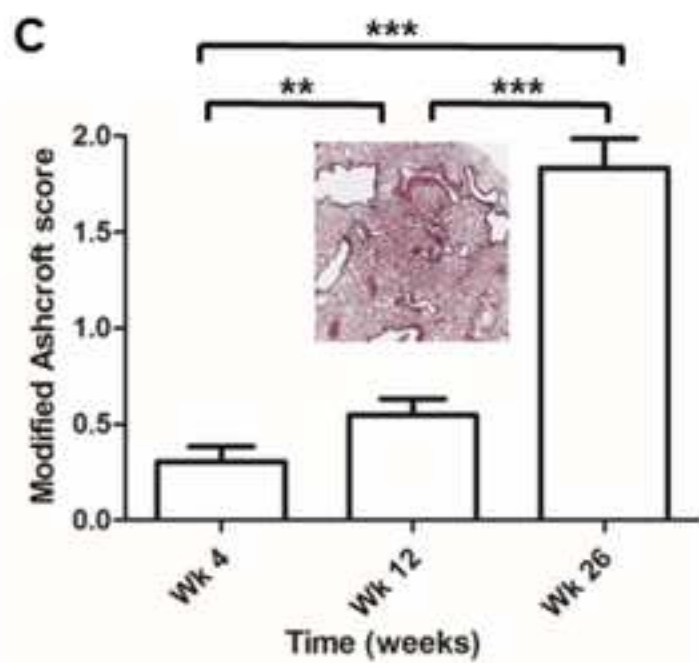
Supplementary Files
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Conflict of interest

The authors declare no conflict of interest.