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Lung Cancer Progression Using Fast Switching Multiple Ion Beam Radiation and Countermeasure Prevention

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Abstract

Most of the research in understanding space radiation-induced cancer progression and risk assessment has been performed using mono-energetic single-ion beams. However, the space radiation environment consists of a wide variety of ion species with a various range of energies. Using the fast beam switching technology developed at the NASA Space Radiation Laboratory (NSRL) at Brookhaven National Laboratory (BNL), ion species can be switched rapidly allowing investigators to use multiple ions with different energies to simulate more closely the radiation environment found in space. Here, we exposed a lung cancer susceptible mouse model (K-ras^{LA-1}) to three sequential ion beams: Proton (H) (120 MeV/n) 20 cGy, Helium (He) (250 MeV/n) 5.0 cGy, and Silicon (Si) (300 MeV/n) 5.0 cGy with a dose rate of 0.5 cGy/min. Using three ion beams we performed whole body irradiation with a total dose of 30 cGy in two different orders: **3B-1 (H→He→Si)** and **3B-2 (Si→He→H)** and used 30 cGy H single-ion beam as a reference. In this study we show that whole-body irradiation with H→He→Si increases the incidence of premalignant lesions and systemic oxidative stress in mice 100 days post-irradiation more than (Si→He→H) and H only irradiation. Additionally, we observed an increase in adenomas with atypia and adenocarcinomas in H→He→Si irradiated mice but not in (Si→He→H) or H (30 cGy) only irradiated mice. When we used the H→He→Si irradiation sequence but skipped a day before exposing the mice to Si, we did not observe the increased

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Author Contribution to Study

All authors listed on your paper must have made significant contributions to the study. To ensure clarity, you are required to enter the specific details of each author's contribution, which must substantiate the inclusion of each person on the manuscript.

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Declarations of Competing interest

Life Sciences require that the **corresponding author**, signs on behalf of all authors, a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

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incidence of cancer initiation and progression. We also found that a non-toxic anti-inflammatory, anti-oxidative radioprotector (CDDO-EA) reduced H $\rightarrow$ He $\rightarrow$ Si induced oxidative stress and cancer initiation almost back to baseline. Thus, exposure to H $\rightarrow$ He $\rightarrow$ Si elicits significant changes in lung cancer initiation that can be mitigated using CDDO-EA.

Keywords

Ionizing Radiation; GCR Simulation; Multiple Ions; CDDO-EA

Introduction

One of the major reasons for limited human long-term interplanetary travel is due to uncertainties and concerns of serious health risk to astronauts associated with radiation exposure in space (1–3). Humans on earth and astronauts on the International Space Station are partially protected from galactic cosmic radiation (GCR) exposure by the Earth's magnetic field, but astronauts would be exposed to GCR during deep space missions even with shielding. The long-term health risks due to radiation exposure range from alterations in the central nervous system, increases in cardiovascular disease, pulmonary disease and cancer risks to different organs such as the lung, colon, stomach, and breast (4). Unlike terrestrial radiation which is primarily composed of low linear energy transfer (low-LET) photons, such as x-rays and γ -rays, astronauts in space would be exposed to rare solar particle events (SPEs) which are predominantly protons, and continuous GCR which consists of energetic protons, helium nuclei, and heavier atomic nuclei with high charge and energy (HZE) such as silicon (5). Low-LET radiation is considered sparsely ionizing in contrast to high-LET radiation that is considered densely ionizing which may produce secondary radiations known as δ -rays. Such radiation-induced DNA damage from high-LET radiation is difficult to repair, often leading to mutagenic repair and induction of genomic instability potentially leading to increases in tumorigenesis compared to equivalent doses of low-LET radiation (6–8).

Previously, using different mouse models, other investigators have demonstrated that high-LET radiation compared to low LET radiation resulted in a higher risk of increased tumorigenesis in the lung, mammary gland, liver and gastrointestinal tract (9–11). The lung has a large surface area which makes it a prominent target for radiation exposure (12). The analysis from the atomic bomb survivor cohort is evidence that the lung is one of the organs with a higher solid tumor incidence (13–16), and lung cancer accounts for more cancer-related deaths than any other cancer type among both men and women (Cancer facts and Fig. 2018) (17).

While physical shielding may reduce some of the risks of cancer from space radiation, biological countermeasures may be required to ensure that the established limits of increased lifetime fatal cancer risks are not exceeded. In this study, we evaluated CDDO-EA (a synthetic triterpenoid) as a potential radiation countermeasure to a simplified GCR simulation. CDDO-EA (CDDO) is an orally available anti-inflammatory/anti-oxidant modulator drug, and previously we showed CDDO provided in lab chow prior to a lethal

dose of whole-body irradiation protected mice from acute gastrointestinal toxicity with enhanced DNA damage repair resulting in improved overall survival (18).

Most of the studies on biological effectiveness of space radiation have been performed using either terrestrial (low-LET) radiation or mono-energetic single high-LET ion type radiation which does not simulate the complex space radiation environment. To address this gap in how multiple ions might interact, we used three ions: protons (H), helium (He) and silicon (Si) to create a simplified fast switching ion beam GCR simulation comparable to particle radiation found in space. Previously we have shown that with higher total doses of protons 2.0 Gy or iron 1.0 Gy results in an increase in the carcinoma incidence in the K-ras^{LA-1} mouse model (9, 19). Using these sequential three ions beams we exposed the lung cancer susceptible mouse model (K-ras^{LA-1}) at the NSRL (Brookhaven National Laboratory) with a total dose of 30 cGy using recently developed ion fast switching capabilities (16). We predicted sequential particle radiation exposure would increase the incidence of invasive carcinoma, and CDDO would be a potential countermeasure reducing inflammation and carcinogenesis.

Material and Methods

Animals and Irradiation

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Texas Southwestern Medical Center at Dallas (UTSW) and Brookhaven National Laboratory (BNL) (Upton, NY). Mice were housed and bred following an approved husbandry protocol in ventilated micro-isolator cages within a pathogen-free facility at UTSW. Transgenic K-ras^{LA1} mice on a 129S2 background as previously described (20) were obtained from Dr. Jonathan Kurie (University of Texas M.D. Anderson Cancer Center, Houston, TX), and 129S2 K-ras^{LA1} breeding pairs were established to generate both heterozygous and wild-type littermate controls

Both male and female transgenic K-ras^{LA1} mice, ages eight to twelve weeks old were whole-body irradiated with different radiation protocols as shown in Figure 1A. Animals subjected to irradiation (Figure 1B) and unirradiated controls for both time points and survival studies (Figure 1C) were transported via World Courier (New Hyde Park, NY) overnight delivery to Brookhaven National Laboratory and returned to UT Southwestern within 1 week after irradiation.

Synthetic Triterpenoid (CDDO) Treatment

To evaluate the ethyl amide derivative of CDDO (CDDO-EA) as a potential countermeasure against multiple ion beam radiations, we fed CDDO-EA or control chow to aged-matched 129S2 transgenic K-ras^{LA1} mice at Brookhaven National Laboratory. Both males and female mice were assigned randomly into the experimental cohorts. The concentration of CDDO-EA in the diet was 400mg/kg (provided by Reata Pharmaceuticals, Irving Texas) and prepared into chow pellets by Purina Mills. Animals were fed with CDDO-EA diet or control diet three days prior to the radiation, and the diet was changed to the normal diet one day after the radiation exposure.

Lung Tumor Evaluation and Histology

For the survival studies, mice were monitored 1-year post-irradiation or sacrificed with evidence of increased morbidity. At the time of sacrifice or death, carcasses were necropsied and removed lungs were inflated by intra-tracheal infusion with 4% v/v formaldehyde solution, the trachea clamped, and the whole lung immersion-fixed overnight in 4% v/v formaldehyde solution. The lung tissues were processed, paraffin embedded, cut at 5 microns thick sections, and stained with Hematoxylin and Eosin (H&E). To evaluate the grade and quantify lesions in the lung, two coronal (frontal) sections were cut 50µm apart per mouse. Tumor grades and other histopathological characteristics were described previously (20) and included adenocarcinoma, adenoma with atypia, adenoma, hyperplasia, pneumonia, bronchial extensions, and autolysis. Premalignant lesions include hyperplastic lesions and adenomas. Hyperplastic lesions are characterized as small proliferative areas without inflammatory cells in the alveolar epithelium, and as these hyperplasias grow they are composed of a monomorphous population of airway epithelia pushing the surrounding airways and then are considered adenomas. For more advanced tumors adenomas with atypia are a putative in situ (noninvasive) precursor of adenocarcinoma. Adenocarcinomas are potentially invasive and characterized by tumor cells with high-nuclear-to cytoplasmic ratios, definite nuclear pleomorphism, with an undifferentiated appearance, and stromal interactions with the tumor along with inflammatory cells. All pathology was confirmed in a blinded fashion by a DVM/Ph.D. pathologist (J.R.) specializing in mouse pathology.

To quantify the sizes and numbers of premalignant lesions, a subset of aged-matched 129S2 mice were sacrificed one hundred days post-irradiation. Lungs tissues were removed, processed and paraffin embedded. Three coronal sections of each lung per animal were cut approximately 50 µm apart and stained with Hematoxylin and Eosin (H&E). Using a Hamamatsu Nanozoomer 2.0-HT mounted CCD camera (Whole Brain Microscopy Facility at UTSW) images were taken and analyzed using the Nanozoom Digital Pathology Software.

Malondialdehyde Assay (MDA)

Aged-matched mice blood samples were collected in 0.5cc microcentrifuge tubes with 0.5M EDTA by the submandibular blood collection method from both unirradiated control and irradiated animals 100 days post-irradiation. The blood samples were then centrifuged 700–1000× G for 10 minutes at 4° C. From the centrifuged samples, the plasma (upper clear layer) was collected and assayed on the same day or stored at –80° C. Stored plasma serum samples were subjected to MDAs assay within a month per manufacturer's instruction. To show the MDA levels, we averaged the MDA levels from different runs for the same radiation protocol (Figure S1). MDA is a byproduct of lipid peroxidation. MDA levels in plasma were measured using the TBARS Assay Kit (Cayman Chemical, 10009055) as per the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 7.00 for Windows, (GraphPad Software, La Jolla California USA). At least five independent biological samples were used per radiation dose. To make a comparison between irradiated and control groups

one-way ANOVA with Tukey correction along with pairwise comparisons for the P values was used. Some radiation protocols were carried multiple times (Figure S1), so we averaged the data obtained for average lesions and MDA concentrations from different runs as shown in Figure (2–5). Differences were considered significant at $P < 0.05$. Statistical analysis of the histopathology was performed using two-tailed Fisher exact (95% confidence interval, CI) or Chi-square test.

Results

Whole Body Irradiation Using Multiple Ion Beam

In this study, we more closely simulated the space environment which is composed of heterogeneous radiation fields predominated with low background fluences of low-LET radiation and lower fluences of high-LET radiation. We used fast switching of protons (H), helium (He) and silicon (Si) particles to deliver sequential ion species. We delivered these particles 120 MeV/n protons (LET=0.64 keV/μm), 250 MeV/n helium ions (LET=0.40 keV/μm) and 300 MeV/n silicon ions (LET=76 keV/μm) in dose proportions of 66.7%: 16.67%: 16.67% respectively where silicon was used as a surrogate for particles of Z greater than 2. The total dose of 30cGy with a dose rate of 0.5cGy/min (measured at target) was delivered in approximately 60 minutes for these experiments including the time consumed in rapid switching between particle beams. We delivered these ions in two different orders: three ions beams order 1 or 3B-1 (H→He→Si) and three ion beams order 2 or 3B-2 (Si→He→H), and we used 30 cGy H as a single beam reference (Figure 1A). Small plastic boxes were used for the housing of mice during radiation exposure, with 2–3 mice fitting comfortably in a single box. These boxes had small holes drilled for the ventilation for the period of radiation exposure. Using 60cm × 60cm beam size 8–12 weeks old mice were arranged in the center of the field to assure the best uniformity (< 2.5%) for total body irradiation (TBI) (Figure 1B). During the irradiation, none of the animals showed any observable stress. Following irradiation, we measured biological outcomes in the K-ras^{LA1} mouse model at two different time points, 100 days and 1-year post-irradiation (Figure 1C). Some of the irradiation and unirradiated control protocols were carried out in multiple NSRL runs (Figure S1), for those experiments data were pooled together and shown as an average.

Order of Particles in Multiple Ion Beam Radiation

Initially, we predicted that both 3B-1 and 3B-2 irradiation protocols would equally induce lung cancer progression in the K-ras^{LA1} mouse model compared to proton irradiation alone and unirradiated controls. However, when we performed histopathological and biochemical analyses in lung and plasma respectively of irradiated mouse and controls 100 days post-irradiation, we observed the 3B-1 (H→He→Si) irradiation protocol appeared to be more cancer promoting. To determine if multiple beam exposure affected the carcinogenic process, we compared premalignant lesions of aged-matched unirradiated control K-ras^{LA1} mouse lungs with proton irradiated mouse lungs or 3B-1 irradiation or 3B-2 (Si→He→H) irradiation 100 days post-irradiation. Compared with unirradiated, 3B-2 or proton only irradiation, K-ras^{LA1} mice irradiated with 3B-1 developed higher numbers of precancerous lesions. 3B-1 irradiated mice show significantly high incidence (average of 10 lesions per section) compared to unirradiated, 3B-2, or proton irradiated animals, which show less than

5 lesions per section (Figure 2A). While there were no significant increase in the number of lesions with 30 cGy proton radiation, our previous reports showed that exposure to higher doses of protons (200 cGy) radiation was associated with an increase in lesions as well as oxidative stress in the lung and plasma of the mice (19). On analysis of blood plasma 100 day post-irradiation for lipid peroxidation as an indicator of oxidative stress, we observed plasma collected had more than a 2.5-fold increased amount of MDA present in the 3B-1 irradiated animals compared to unirradiated controls. In comparison to 3B-1, 3B-2 and proton did not have persistent increases in the MDA level in the plasma (Figure 2B). Thus, the order of ion irradiation appeared to be biologically important.

To further determine tumor progression following radiation exposure, one-year post-irradiation K-ras^{LA1} mice lung were evaluated histologically for the appearance of adenomas with atypia and invasive carcinomas. K-ras^{LA1} mice exposed to 3B-1 (H→He→Si) exhibited a significant increase in adenomas with atypia (79%) and an increase in invasive carcinoma formation (28.6%) (Figure 2C and 2D). Unirradiated Kras^{LA1} mice possess a background level of adenomas with atypia and a carcinoma incidence of approximately 55% and 10% respectively. Mice exposed to 3B-2 (Si→He→H) and proton radiation did not display any significant increase in adenomas with atypia (54% for 3B-2 and 58% for proton irradiation) or invasive carcinomas (12.9% for 3B-2 and 16.1% for proton) one-year post-irradiation compared to the unirradiated control.

Skipping a Day for Silicon in 3B-1 (H→He→Si) Irradiation Attenuates Cancer Progression Effects

Because we observed an increase in invasive carcinoma incidence in 3B-1 after histopathological analysis, we next evaluated if skipping a day before silicon irradiation would have any rescue effect due to DNA repair of the damage induced by protons and helium. We reasoned that even though we used 0.5 cGy per min dose rate, the dose rates in space are much lower and thus there could be time for proton and helium damage repair before exposing mice to silicon. Thus, we modified the 3B-1 irradiation protocol where we delivered two particles 120 MeV/n protons (20cGy) and 250 MeV/n helium (5 cGy) ions day 1, and after approximately 24 hrs we delivered 300 MeV/n silicon ions at 5cGy and designated this modified protocol as “3B-1-1” (H→He + 24 hrs→Si). We also irradiated mice with two particles 120 MeV/n protons and 250 MeV/n helium ions only with the total dose of 25cGy as a reference for 3B-1-1. 3B-1-1 radiation exposure did not increase the number of lesions nor did it increase chronic lipid peroxidation levels in plasma as observed earlier with 3B-1 (H→He→Si) irradiation in K-ras^{LA1} mice 100 days post-irradiation (Figure 3). 3B-1-1 (H→He + 24 hrs→Si) irradiated mice had an average of 5 lesions, proton and helium only irradiated mice had an average of 4 lesions which are similar to unirradiated controls whereas 3B-1 (H→He→Si) had an average of 10 lesions per animal (Figure 3A). The level of lipid peroxidation that we observed in 3B-1-1 (H→He + 24 hrs→Si) irradiated mice was comparable to unirradiated mice and proton and helium only irradiated mice (Figure 3B). Taken together we interpret these results to suggest that not only does the order of particle beam irradiation matter in a biological context, but also all particles must be given together acutely in specific order to increase cancer risks.

CDDO-EA as a Countermeasure in Multi-ion Irradiations

To determine the radioprotective effect of the CDDO-EA, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid-ethyl amide (a synthetic triterpenoid), on 3B-1 (H→He→Si) induced tumor progression, groups of age-matched mice were fed ad libitum a diet containing CDDO-EA (400 mg/kg diet) for 3 days continuously prior to 3B-1 irradiation including one-day post-irradiation (Figure 4A). The mice were on CDDO-EA diet for a total of 4 days. Pretreatment of CDDO-EA prior to 3B-1 exposure decreased the number of premalignant lesions (Figure 4B) when examined 100 days post-irradiation. The CDDO-EA treated mice had an average of 4 lesions compared to an average of 10 lesions on 3B-1 irradiated mice with control diet and 4 lesions on unirradiated control mice (Figure 4B). Similarly, we also observed a reduction of MDA concentration (lipid peroxidation) in the blood plasma of the CDDO-EA treated mice 100 day post-irradiation which were comparable to unirradiated mice (Figure 4C). These data can be interpreted to substantiate the previous findings that CDDO-EA could be a potential space radiation lung cancer radioprotector as previously observed in a colon-cancer susceptible mouse model (18, 21).

Titration of Silicon Total Dose to Better Simulate the Space Environment

As reported above the order of particle in multiple beam irradiation given acutely affected the outcome of tumorigenesis of K-ras^{LA1} mice, and dose proportions of the proton, helium, and silicon was 66.7%, 16.67%, and 16.67% respectively. To better simulate the space environment, we made modification on the 3B-1 (H→He→Si) radiation protocol by titrating the dose of silicon and helium particles. The modification of 3B-1 changed the dose proportion of the particles. The first titration protocol designated as 3B-1-2 irradiation included 120 MeV/n protons with 20 cGy, 250 MeV/n helium with 5 cGy, and 300 MeV/n silicon ions with 2 cGy in dose proportions of 74.07%: 18.52%: 7.54% respectively with a total dose of 27 cGy. The second titration protocol designated 3B-1-3 irradiation included 120 MeV/n protons with 22 cGy, 250 MeV/n helium with 3 cGy, and 300 MeV/n silicon ions with 0.5 cGy in dose proportions of 86.3%: 13.3%: 1.96% respectively with a total dose of 25.5 cGy (Figure 5A). With the titration of high-LET silicon particle, we saw a dose-dependent effect on the incidence of cancer initiation and the level of oxidative stress in the plasma of K-ras^{LA1} mice. As the dose proportion of silicon was decreased, a decrease in the average number of lesions in the lungs as well as a decrease in the level of MDA concentration in blood plasma of K-ras^{LA1} mice 100 days post-irradiation was observed (Figure 5B and 5C). 3B-1-2 irradiated mice had an average number of 8 lesions and 3B-1-3 had an average number of 7 lesions compared to 3B-1 which had an average of 10 lesions per animal and unirradiated control mice which had an average number of 5 lesions (Figure 5B). When we titrated the dose proportion of silicon on 3B-1 radiation even further, the MDA level in plasma were at background levels with dose proportion of silicon 7.54% and 1.96% in both 3B-1-2 and 3B-1-3 respectively 100 days post-irradiation (Figure 5C).

Discussion

Galactic cosmic radiation (GCR) exposure occur at low fluence rates predominated by low background fluences of low-LET radiation with lower fluences of high-LET radiation that cannot be fully simulated on earth. It is estimated that individual cells in an astronaut would

be traversed by a proton every three days, helium nuclei every few weeks, and high atomic number (Z) and energy (HZE) nuclei about once every month (22). Using fast ion switching capabilities at the NSRL we exposed mice to proton (H), helium (He) and silicon (Si) particles to delivery sequential ion species to more closely approximate the space environment. In the present study, we demonstrated that multiple ion beam radiations given in a specific order acutely is effective in causing lung cancer initiation/progression in the K-ras^{LA1} mouse model. Previously studies have used monoenergetic single ion beam exposures with either only low-LET or high-LET radiation to understand and model the risk of radiation-induced tumorigenesis in space (9, 19, 23, 24). While mono-energetic high dose of HZE particles, such as iron, silicon, and oxygen, have been shown to induce an increased incidence of lung tumorigenesis (25), with lower dose single particles (e.g. 5 cGy silicon) that we used in this study, there was no increase in lung invasive carcinomas (data not shown). To our knowledge, this is the first study in a mouse model of whole body exposure to relatively low doses of multiple ions to simulate more closely the space radiation environment. In these studies, we evaluated the biological risk of space radiation-induced lung cancer initiation and progression.

A main biological effect due to radiation exposure results in DNA damage either directly or indirectly through the generation of ROS from radiolysis of water. Through these actions, radiation can induce base damage, single-strand breaks, double-strand breaks, and DNA protein cross-links. If irradiated cells do not repair correctly, it can induce carcinogenesis and other abnormalities (26). Additionally, it is well established that high-energy particles cause clustered more complex DNA damage, and if the damage is not repaired correctly, it can induce carcinogenesis and other abnormalities (7, 8, 26–28). In the 3B-1 (H→He→Si) irradiation protocol, when mice are irradiated with proton initially, every cell nucleus in a mouse body is predicted to be traversed by a proton ions approximately 150 times, helium ions approximately 15 times and silicon one third times respectively. It is well accepted that following radiation, chromatin undergoes conformational changes to allow access of DNA repair proteins, thus DSBs caused by secondary radiation may be impacted (29–32). Thus, in our protocol irradiating mice with a second ion or third ion (helium and silicon a high-LET ion respectively) we speculate that this may lead to inefficient DNA damage repair following proton irradiation. In contrast, irradiating mice with the 3B-2 (Si→H→He) protocol with silicon a high-LET (76 keV/μm) ion initially would be predicted to impacted only one-third of the nuclei in a mouse body, thus irradiating mice with the second or third ions (both low-LET) may not have significantly altered the DNA damage repair system similarly to 3B-1. Additionally, irradiating mice with protons and helium on day one followed by silicon irradiation after 24 hrs (H→He + 24 hrs→Si) reduced the biological impact that was previously observed using the 3B-1 irradiation protocol (Figure 3A and 3B) perhaps due to repair of DNA damage between irradiations. When we titrated the dose of HZE ion in the 3B-1 irradiation protocol, there was a dose-dependent effect of silicon ions delivered (Figure 5) and by lowering the total dose of silicon from 5 cGy to 2 cGy and finally to 0.5 cGy, progressively resulted in reducing cancer progression back to background rates. These results provide a possible mechanism that may have contributed to the observed carcinogenic effect due to multiple ion beam radiation given acutely in a specific order. Considering our findings, with previously published data, overall suggest proton irradiation

followed with HZE-particle might be more carcinogenic (33, 34). Further studies are required to clarify the role of DNA repair mechanisms *in vivo* using a mixed radiation field.

There is accumulating evidence of an adaptive response role in low-LET radiation, such that a low priming dose of radiation may result in an enhanced resistance to a second and larger dose of radiation (35, 36). This may be important in understanding the risk in the space radiation environment as it is likely that every cell in an astronaut is likely to be traversed with proton ions multiple times before any HZE ions hits a cell. The studies on the adaptive response on high-LET radiation are mixed and have depended on the model system used. In some studies, initial exposure decreased biological damage when followed by a second exposure (37) while in other studies initial exposure increased the subsequent damage following the second exposure (38, 39). With the 3B-1 irradiation protocol (H→He→Si), all the cells in the mouse body were primed with proton ions before cells were shortly thereafter irradiated with a second or third ion. In our study priming with the proton irradiation prior to second and third irradiation caused more transformations given acutely, but such transformation was reduced to background level if the priming was done 24hr apart (H→He + 24 hrs→Si) from the second radiation (Figure 2A and 3A). Similarly, the transformation was not observed when primed with silicon ions prior to helium and proton irradiation (Figure 3A). While these ideas are speculative at the present time, similar findings have been reported previously in cell culture experiments (33). As CDDO-EA has been shown to enhance DNA repair and act as a radioprotector, we predicted it would provide protection against the 3B-1 exposure (18). CDDO is an anti-oxidant and anti-inflammatory modulator that is currently in several Phase 3 pivotal clinical trials (40). Here we also demonstrated that prior treatment with CDDO-EA can protect mice and reduce tumor incidence/progression against multiple sequential ion beam exposures.

Our sequential fast switching beam studies highlight the limitations and difficulties in accurately assessing health risks due to multiple ion irradiations as outcomes are likely to depend not only on the order of ions, but also energies and doses used and importantly the biological endpoints of the experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A.

Three ion beam order 1 (3B-1) : H-He-Si (30cGy)

Ion	Energy (MeV/n)	Total Dose (cGy)	Dose Rate (cGy/min)	Estimated time (min)	66
Proton (H) (66.66 %)	120	20	0.5	40	
Switch to He				~3	
Helium (He) (16.67 %)	250	5	0.5	10	
Switch to Si				~3	
Silicon (16.67%)	300	5	0.5	10	

Three ion beam order 2 (3B-2) : Si-He-H (30cGy)

Ion	Energy (MeV/n)	Total Dose (cGy)	Dose Rate (cGy/min)	Estimated time (min)	66
Silicon (16.67%) ↓	300	5	0.5	10	
Switch to He				~3	
Helium (He) (16.67 %) ↓	250	5	0.5	10	
Switch to Si				~3	
Proton (H) (66.66 %)	120	20	0.5	40	

Three ion beam order skip a day (3B-1-1) : H-He-Si (30cGy)

Ion	Energy (MeV/n)	Total Dose (cGy)	Dose Rate (cGy/min)	Estimated time (min)	66
Proton (H) (66.66 %)	120	20	0.5	40	
Switch to He				~3	
Helium (He) (16.67 %)	250	5	0.5	10	
Switch to Si after 24 hrs				~3	
Silicon (16.67%)	300	5	0.5	10	

H (30cGy)

Ion	Energy (MeV/n)	Total Dose (cGy)	Dose Rate (cGy/min)	Estimated time (min)
Proton (H) (66.66 %)	120	30	0.5	60

B.

C.

1. 3B-1 (30cGy)
2. 3B-2 (30cGy)
3. H (30cGy)
4. Unirradiated Control

Figure 1. Experimental design for the three-beam radiation.

(A) Different approaches for sequential beam radiation using Hydrogen, Helium and Silicon ions. (B) This figure shows the experimental setup of how mice were arranged in the beamline. (C) Schematic of experimental design for total body irradiation of mice.

Figure 2. Tumorigenic effect of multiple ion radiation in K-ras^{LA1} mouse model.

(A) Quantification of an average number of premalignant lesions in age-matched K-ras^{LA1} lungs from 100 days post-irradiation with 3B-1 (30 cGy), 3B-2 (30 cGy), protons (30 cGy), and unirradiated controls (n>=6 mice per group). Differences in the number of premalignant lesions. ** P=0.0020, *** P=0.0005, **** P<0.0001. (B) Assay of MDA in the serum of MT aged matched K-ras^{LA1} mice 100 days post-irradiation with 3B-1 (30 cGy), 3B-2 (30 cGy), protons (30 cGy), and unirradiated controls (n>=6 mice per group). * P=0.0185, *** P=0.0002, *** P=0.0003. The error bars represent standard errors. (C) Representative

images of adenoma with atypia, and quantification of percent adenomas with atypia from the survival cohort. * $P=0.0299$ in contingency Chi-square test 3B-1 compared with UNIR. (D) Representative images of invasive adenocarcinoma, and quantification of percent adenocarcinoma. * $P=0.0351$ in contingency Chi-square test 3B-1 compared with UNIR.

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Figure 3. Skipping a day for silicon ion in 3B-1 total body irradiation rescues the biological damage in the mice.

(A) Quantification of an average number of premalignant lesions in age-matched K-ras^{LA1} lungs from 100 days post-irradiation with 3B-1 (30 cGy), 3B-2 (30 cGy), 3B-1-1 (30 cGy), proton and helium (25 cGy), and unirradiated controls (n≥6 mice per group). Differences in the number of premalignant lesions. * P=0.0267, * P=0.0393, *** P=0.0006. (B) Assay of MDA in the serum of MT aged matched K-ras^{LA1} mice 100 days post-irradiation with 3B-1 (30 cGy), 3B-2 (30 cGy), 3B-1-1 (30 cGy), proton and helium (25 cGy), and unirradiated controls (n≥6 mice per group). * P=0.0198, **** P<0.0001, **** P<0.0001. The error bars represent standard errors.

Figure 4. CDDO as a countermeasure against multiple beam ion radiation.

(A) Quantification of average number of premalignant lesions in age-matched K-ras^{LA1} lungs from 100 days post-irradiation with 3B-1 (30 cGy), 3B-1 + CDDO (30 cGy) and unirradiated controls ($n \geq 6$ mice per group). Differences in the number of premalignant lesions. *** $P=0.0001$, **** $P<0.0001$. (B) Assay of MDA in the serum of MT aged matched K-ras^{LA1} mice 100 days post-irradiation with 3B-1 (30 cGy), 3B-2 (30 cGy) and protons (30 cGy), and unirradiated controls ($n \geq 6$ mice per group). ** $P=0.0025$, ** $P=0.0025$. The error bars represent standard errors

A.**3B-1-2 : H-He-Si (27cGy)**

Ion	Energy (MeV/n)	Total Dose (cGy)	Dose Rate (cGy/min)	Estimated time (min)
Proton (H) (74.07 %)	120	20	0.5	40
↓ Switch to He				~3
Helium (He) (18.52 %)	250	5	0.5	10
↓ Switch to Si				~3
Silicon (7.54%)	300	2	0.5	4

Total Dose = 27 cGy
Total Time ~60 min

3B-1-3 : H-He-Si (25.5cGy)

Ion	Energy (MeV/n)	Total Dose (cGy)	Dose Rate (cGy/min)	Estimated time (min)
Proton (H) (86.3 %)	120	22	0.5	44
↓ Switch to He				~3
Helium (He) (13.3 %)	250	3	0.5	6
↓ Switch to Si				~3
Silicon (1.96%)	300	0.5	0.5	1

Total Dose = 25.5 cGy
Total Time ~57 min

B.

100 Days Post IR

C.

100 Days Post IR

Figure 5. Titration of Silicon ion dose in 3B-1 irradiation shows dose response.

(A) Illustration of mixed beam radiation using Hydrogen, Helium and Silicon ions. (B) Quantification of average number of premalignant lesions in age-matched K-rasLA1 lungs from 100 days post-irradiation with 3B-1 (30 cGy): (H (20cGy) + He (5cGy) + Si (5cGy) (30 cGy)), 3B-1-2 (27 cGy): H (20cGy) + He (5cGy) + Si (2cGy), 3B-1-3 (25.5 cGy): H (22cGy) + He (3cGy) + Si (0.5cGy) and unirradiated controls (n ≥ 6 mice per group). Differences in the number of the premalignant lesions. ** P=0.0064, *** P=0.0002. (C) Assay of MDA in the serum of MT aged matched K-rasLA1 mice 100 days post-irradiation

with 3B-1, 3B-1-2, 3B-1-3, and unirradiated controls ($n \geq 6$ mice per group). ***
 $P=0.0002$, **** $P<0.0001$. (B) The error bars represent standard errors.

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