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Evaluation of Efficacy of X-Ray Alone and in Combination with Radiosensitizer in Subcutaneous and Intracranial Tumor Models

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Abstract

In this study, 13 tumor cell line derived subcutaneous models and one intracranial tumor model were evaluated by the application of X-ray radiation. The response of radiation levels on different tumor types and different tumor models were assessed by using the device (X-RAD225, Pxi precision, USA) delivering the targeted radiation directly on the focal tumor site. Furthermore, we investigated the combined benefits of radiation and a chemotherapy medication, Gemcitabine, on the H22 murine hepatic carcinoma cells derived subcutaneous syngeneic model. The impact of radiation treatment on the NCI-H1975-luc, human non-small cell lung cancer intracranial model in combination with Human checkpoint kinase ataxia telangiectasia-mutated (ATM) kinase inhibitor AZD0156 was studied. In addition, the integrity of the blood-brain barrier and the presence of the pharmacodynamic marker pRAD50 of AZD0156 were evaluated. The study outcome demonstrated that X-ray radiation has anti-tumor effects across all studied models, and also in combination treatment with radiosensitizer, Gemcitabine or AZD0156. We believe this study demonstrates there is a lot of potential fully utilizing radiation platform identifying radiation sensitizer or chemo candidates to benefit the management of oncology society.

Keywords: X-Ray, Tumor model, Radiosensitizer, Combination therapy

Introduction

It is well known that radiation therapy is another cornerstone of anticancer treatments, either prior to, or in conjunction with surgery, chemotherapy, or certain type of cancer which could not intervene by surgery [1]. The sensitivity of numerous tumors to radiation are variant. Inquiry radiation responses in animals, many research facilities still rely on standard cabinet or cesium radiators [2]. These devices typically irradiate multiple animals simultaneously and lack precision in conforming the radiation dose to the specific target, limiting the ability of current animal research to mimic clinical scenarios accurately. Animal model studies are vital for bridging the gap between basic research and clinical application. Given that different tumor type exhibit varied sensitivity to X-ray radiation, understanding a suitable tumor model and radiation regimen is crucial for antitumor research. Advancement technology has allowed modern radiation to target only on the local tumor site, to allow researchers evaluate and recognize the sensitivity of each tumor type in pre-clinical study on small animal models which significantly benefit oncology R&D to develop sensitizer or chemo to enhance the treatment of cancer patients minimized the gap between basic research and

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clinical outcome. It could help scientists better understand the mechanism of action either and therapeutic effect with sensitizer or chemo avoid the complication of adverse effect following systemic exposure to the radiation [1].

Despite these challenges, the importance of radiotherapy cannot be understated, and in combination with chemotherapy radiotherapy is increasingly used. Anti metabolites drugs of Gemcitabine, 5-FU, and DNA damaging drug, Cisplatin as radio-sensitizing agents are commonly used in combination to enhance antitumor effects [3,4]. The increasing need for radiosensitizers [5] prompts a demand for precise local radiation application in the research of small animal models. In summary, radiation oncology provides an important open access for research scientists and clinicians who are involved in the control of disease progress and treatment for cancer patients. Understanding the effects and safety of radiation, identify a novel radio sensitizer, develop the best chemo agents for combination treatment becomes fundamental desires for researchers considering the complex of cancer pathogenesis and variety of tumor types. To address the clinical needs, in this study, we utilized a device capable of local radiation, and in combination with sensitizer or chemo compounds employed various radiation doses, and assessed the response of radiation and combination treatment conditions on various tumor models. In addition, we also explored the possible impact of high-dose radiation on the blood-brain barrier, the study outcome could further strengthen the role of radiation oncology.

Material and Methods

Cell lines and reagents

Fourteen cell lines which were purchased from ATCC or COBIOER for the purposes of oncology research (Table 1). Cells were cultured in the respective mediums at 37°C in an incubator with humidified atmosphere of 5% CO_2. The cell culture medium RPMI 1640, DMEM, Waymouth's MB 752/1, F12K, McCoy's 5a medium modified, IMDM, MEM, and FBS were purchased from Gibco.

Table 1: Cell lines and culture conditions

	Cell Line	Source	Tissue	Mouse strain	Culture Medium	Cell No./mouse
1	4T1	ATCC	Breast cancer	BALB/c	RPMI 1640+10% FBS	1×10^4
2	B16-F10	ATCC	Melanoma	C57BL/6	DMEM+10% FBS	1×10^5
3	EMT6	ATCC	Breast cancer	BALB/c	Waymouth's MB 752/1 Medium with 2mM L-glutamine+15%FBS	2×10^4
4	CT26	ATCC	Colon cancer	BALB/c	RPMI 1640+10% FBS	3×10^5
5	MC38	COBIOER	Colon cancer	C57BL/6	DMEM+10% FBS	1×10^6
6	A20	ATCC	B lymphocyte	BALB/c	RPMI 1640+10%FBS+0.05mM 2-mercaptoethanol	3×10^5
7	H22	COBIOER	Liver cancer	BALB/c	RPMI 1640+10%FBS	2×10^6
8	A549	ATCC	Lung cancer	BALB/c nude	F12K+10% FBS	5×10^6
9	HCT 116	ATCC	Colon carcinoma	BALB/c nude	McCoy's 5a medium modified +10%FBS	5×10^6
10	Capan-1	ATCC	Pancreatic cancer	BALB/c nude	IMDM+10% FBS	4×10^6
11	MIA PaCa-2	ATCC	Pancreatic cancer	BALB/c nude	DMEM+10% FBS+2.5% HoS	1×10^7
12	Fadu	ATCC	Head and neck squamous cell carcinoma	BALB/c nude	MEM+10% FBS	5×10^6
13	LN-18	ATCC	Glioblastoma	NOG	DMEM + 5%FBS	1.5×10^7
14	NCI-H1975-luc	Pharmaron (NCI-H1975 parental from ATCC)	Lung cancer (NSCLC)	BALB/c nude	RPMI 1640+10% FBS	2×10^5

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Animals

All animal studies were carried out in AAALAC accredited animal facility at Pharmaron and performed according to the guidelines approved by the IACUC and the guidance of AAALAC international's three primary standards (AAALAC Guide, Ag Guide and ETS 123). The Animal Use Protocol (AUP) for the studies were approved by Pharmaron IACUC. The General procedures for animal care and housing were in accordance with the standard of Commission on Life Sciences, National Research Council, SOPs. At the end of study, all the animals were terminated by carbon dioxide (CO_2) euthanasia, animals were put in a 12-liter chamber with 50% volume displacement per minute for 3 minutes, CO_2 flow was maintained for 1 minute after respiratory arrest to confirm the death of animal. Female mice aged 7 to 9 weeks with body weight between 18-22 g were purchased from Beijing Vital River Laboratory Animal Technology Co, Ltd. (joint venture with Charles River Laboratories) and Beijing Anikeeper Biotech Co, Ltd. Prior to the study, all the mice were quarantined for 3 days (immunocompetent mice) or 7 days (immunodeficiency mice) initially, and complete health checks were performed by an experienced veterinarian. The animals were housed in polycarbonate cages with free access to standard rodent chow and water available ad libitum, under a 12-hour light/dark cycle, with ad libitum UV-treated water and rodent diet.

Subcutaneous tumor model

Mice were inoculated subcutaneously on the right flank with single-cell suspension of the tumor cells in culture medium for tumor development. The cell number and mouse strains for each model are listed in Table 1

Intracranial tumor model

For intracranial implantation, mice were anesthetized by intramuscular injection of mixed anesthetics of Zoletil (22.8 mg/kg) and Xylazine (4.6 mg/kg). 2×10^5 luciferase-expressing NCI-H1975-luc tumor cells were injected into the right forebrain by positioning the needle at 2.0 mm lateral to the sagittal suture, 0.5-1.0 mm anterior to coronal suture with the injection depth precisely controlled at 3.0 mm. After surgery, Meloxicam (20ul/mouse, Boehringer Ingelheim) was injected into animals subcutaneously for 3 consecutive days for analgesia.

X-ray therapy

An X-ray device (X-RAD225, Precision X-ray Inc, US) was used for the experiments. Mice were anesthetized and placed on the shelf before being subjected to radiation at doses of 0.5 Gy, 1 Gy, 2 Gy or 6 Gy, delivered at a rate of approximately 200 cGy/min. The distance between the infrared (IR) source and the mouse was maintained at 50 cm, ensuring that the radiation, passing through a 1 cm aperture fixed beam collimator, was accurately focused on the tumor.

Tumor measurements

Tumor volume (TV) of subcutaneous tumors was measured via caliper twice or three times per week and calculated as $0.5 \times (\text{major axis}) \times (\text{minor axis})^2$. The TVs were used for calculation of the tumor growth inhibition (TGI), an indicator of treatment efficacy using the formula: TGI = (1-T/C) × 100%, where "T" and "C" are the mean relative volumes (% tumor growth) of the tumors in the treated and control groups, respectively, at a given day after tumor inoculation. Tumor bioluminescence of intracranial tumors was measured via In Vivo Imaging System (IVIS). Mice were injected intraperitoneally with 15 mg/mL (at 5 µL/g body weight) of D-luciferin (Perkin Elmer) and following this, mice were anesthetized via inhalation of 1-2% isoflurane. 10 minutes after the luciferin injection, bioluminescent imaging of the mice was performed using the IVIS Lumina III system (Perkin Elmer). The Living Image software (Perkin Elmer) was employed to define regions of interest (ROI) within the brain and to quantify the total bioluminescent signal within each ROI.

Investigation for blood-brain barrier (BBB) integrity

Evans blue dye was administered intravenously into the femoral vein. Approximately 15 minutes after the Evans blue injection, the mice were euthanized using CO_2, subsequently perfused with saline, the brains were collected for analysis of Evans blue distribution. The permeability of the BBB was evaluated by observing and measuring the extravasation of Evans blue dye as previously described elsewhere [6].

Western Blot

Protein samples were obtained using RIPA lysis buffer (complete ultra-Tablets, Mini, EDTA-free, easy pack, and phosphatase inhibitor cocktail, Roche, Cat No. 5892791001). Protein samples (30 µg each) were separated by NuPAGETM 4-12% Bis-Tris Gel (Novex, Cat No. NP0323BOX) and electro transferred to nitrocellulose membrane (Millipore, Cat No. HATF00010, 0.3A, 1.5h). After blocking with blocking buffer for 1 hour at room temperature, membranes were probed with RAD50 antibody (CST, Cat No. 3427, 1:1000), pRAD50 (Ser635) antibody (CST, Cat No. 14223, 1:1000) and GAPDH antibody (CST, Cat No. 97166, 1:3000) dilution with antibody dilution buffer (5% non-fat milk) overnight at 4 °C. Membranes were incubated with secondary antibodies (Li-COR, CAT NO. 926-32211& 926-68070, 1:5000) and were detected with Odyssey CLx.

Statistical analysis

The significance of differences between groups were assessed using independent-samples nonparametric tests or T-test or one-way ANOVA test, and the level of significance was set at 0.05 or $P < 0.05$.

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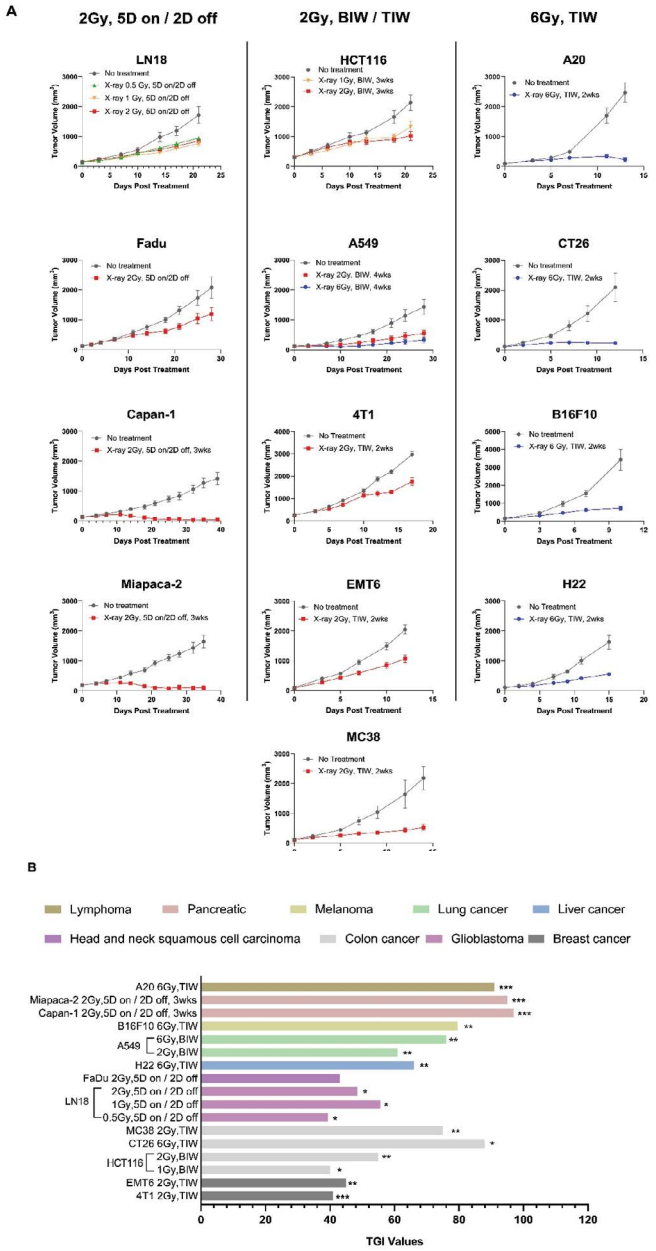
Results

The therapeutic effect of X-ray radiation in various tumor models

To verify the therapeutic effect of X-ray on different tumor models in our platform, we validated a total of 13 subcutaneous tumor models representing a variety of cancers (Table 1). The doses of X-ray were set at 0.5 Gy, 1 Gy, 2 Gy, and 6 Gy, and were delivered with varying frequency: twice per week (BIW), three times per week (TIW), or 5 days on/ 2 days off (5D on/2D off). The TGI of X-ray at different doses was confirmed (Fig. 1).

Figure 1: Tumor curves of different tumor models treated with X-ray radiation



Growth curves for 13 tumor models (LN8, FaDu, Capan-1, Miapaca-2, HCT-116, A549, 4T1, EMT6, MC38, A20, CT26, B16F10, H22) treated with X-ray radiation. Treatments started when the mean tumor volumes reached 100-300 mm^3. Radiation was delivered at doses of 0.5 Gy, 1 Gy, 2 Gy and 6 Gy with different regimens: twice a week (BIW), three times a week (TIW), or 5D on / 2D off. The data shown are mean tumor volumes ± SEM, n ≥5 mice/group (Fig 1A). The summary of tumor growth inhibition (TGI) is graphically depicted. TGI = (1-T/C) × 100%, where "T" and "C" are the mean relative volumes (% tumor growth) of tumors in the treated and control groups. n ≥ 5 mice/group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, determined by T-test or one way ANOVA in comparison to the control group (Fig 1B).

A chemotherapy agent, Gemcitabine as a radiosensitizer can significantly enhance the therapeutic activity of X-ray on subcutaneous H22 liver cancer model

With the desire to help identifying sensitizers and understand the efficacy of radiotherapy and chemotherapy in combination, we further explored the effectiveness of combining X-ray with sensitizer Gemcitabine. The subcutaneous H22 liver cancer model was utilized here. Gemcitabine in combination with X-ray significantly inhibited tumor growth compared with X-ray or Gemcitabine alone ($p < 0.01$, compared with X-ray or Gemcitabine alone) (Fig. 2).

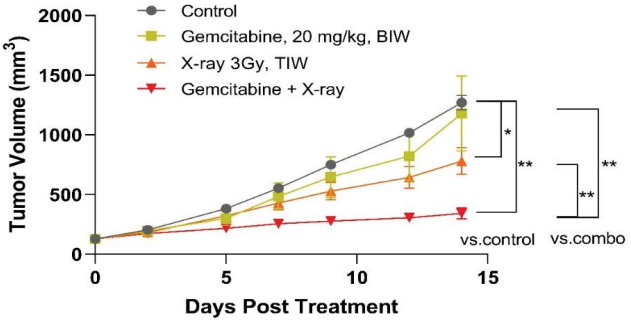


Figure 2: Combination effect of Gemcitabine and X-ray radiation in H22 tumor model

Treatment started when the mean tumor volume reached 129 mm^3, and mice were randomized into 4 groups: Control; X-ray 3Gy, TIW; Gemcitabine 20 mg/kg, BIW and the combination of Gemcitabine with X-ray. Data shown are mean tumor volumes ± SEM, n = 6 mice/group. (*, $p < 0.05$; **, $p < 0.01$, by using independent sample test.)

The effect of X-ray on blood-brain barrier permeability is negligible

To further investigate the capability of X-ray effect in tumor model, an intracranial tumor was considered as next

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model to challenge the combination therapy of X-ray and a radiosensitizer. Before the efficacy study, considering that X-rays might have potential impact on the blood-brain barrier, thereby affecting the absorption of compounds, it is necessary to explore the impact of X-rays on the blood-brain barrier. The permeability of the blood-brain barrier (BBB) was evaluated using measuring extravasation of Evans blue, a dye that binds to albumin and is consequently unable to cross an intact BBB but can penetrate a structurally compromised BBB [6]. To investigate the impact of intracranial tumor growth, we used the NCI-H1975-luc tumor model. The results demonstrated clear visibility of Evans blue in the tumor section, while it remained invisible in the unaffected brain tissue. This suggested that the BBB, also referred to as the blood-tumor barrier (BTB) in the tumor context, is compromised in the NCI-H1975-luc intracranial tumor model (Fig. 3). In a second study, we investigated the integrity of the BBB after high-dose X-ray radiation of the brain of naïve (non-tumor bearing) animals. Brain samples were collected at various time points post-radiation. Both visual examination of the brain and quantification of Evans blue staining indicated that a 12 Gy X-ray radiation resulted in only a limited influence on the BBB (Fig. 4).

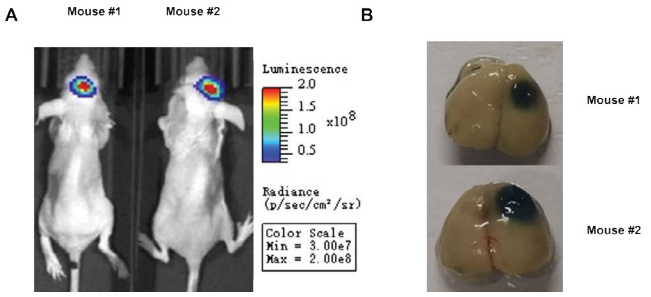

Figure 3: Integrity of the BBB in the presence of brain tumors and after X-ray radiation

Two mice were intracranially inoculated with luciferase-expressing NCI-H1975-luc tumor cells. At 27 days after tumor cell implantation, mice were imaged with the IVIS Lumina III (Perkin Elmer) to monitor the intensity of the bioluminescence of the tumor cells (Fig 3A). The photographs show the isolated brains following perfusion at 15 minutes after Evans Blue injection. The mice were not subjected to either radiation or chemotherapy (Fig 3B).

20 Naïve mice were randomized into 4 groups: a no treatment control group and three other groups that received a single targeted irradiation of 12 Gy to the entire brain. Brains were isolated at 2 h, 24 h and 48 h after irradiation, respectively. Photographs showing the perfused brains from the control group and at 2 h, 24 h and 48 h post-radiation (Fig 4A). Quantitative analysis of the final concentration of Evans blue in the brains ($P < 0.05$ were considered significant differences vs no treatment, not significant) (Fig 4A).

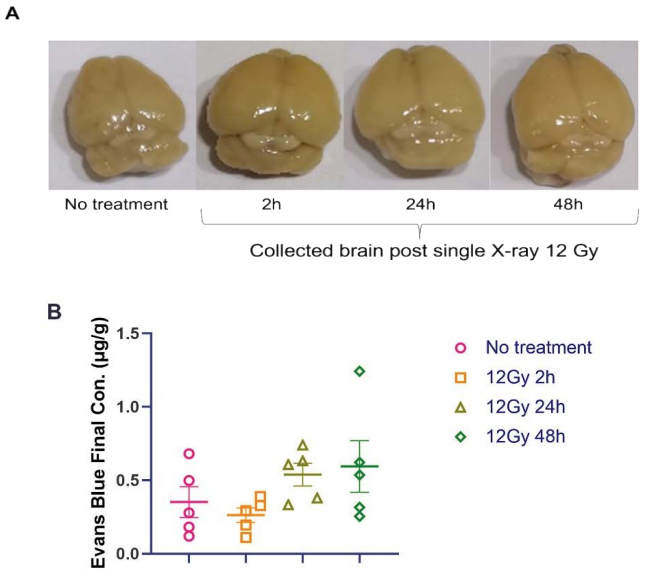

Figure 4: The impact of 12Gy X-ray radiation on the integrity of the BBB in naïve mice

ATM inhibitor AZD0156 can enhance the anti-tumor effect of X-ray therapy

The main limitation of successful radiotherapy in the treatment of cancer is the radiation resistance of cancer cells. Radiation causes DNA double-strand breaks, triggering a series of cellular DNA damage responses to repair DNA, which is a significant factor contributing to the resistance of cancer cells to radiation therapy [7]. RAD50, a protein implicated in DNA double-strand break repair and DNA damage checkpoint activation [8], has been identified in numerous studies as a predictor of resistance to radiotherapy [9-11]. ATM-dependent phosphorylation of RAD50 regulates DNA repair and cell cycle control [12], and pRAD50 expression is increased in X-ray damaged cells. To enhance the effectiveness of radiotherapy, various radiosensitizers, we decided to use AZD0156 to challenge the combination therapy. AZD0156 is a potent and selective, bioavailable inhibitor of ataxia-telangiectasia mutated (ATM) protein, a signaling kinase involved in the DNA damage response [13]. and has demonstrated efficacy as a radiosensitizer in the treatment of multiple types of cancer [12, 14, 15]. To preliminary determine the radio sensitizing effect of AZD0156, we examined pRAD50 and RAD50 as pharmacodynamic markers in a single dose PD study. At 24 days after intracranial tumor implantation, we treated the mice with a single irradiation dose either alone or in combination with AZD0156. The results showed that radiation significantly increased the presence of pRAD50 in the tumors. However, combination treatment with AZD0156 exhibited a low expression level of pRAD50 proving the effective inhibition of the DNA damage response by the

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drug (Fig. 5). We showed the ATM inhibitors (AZD0156) abrogate phosphorylation of RAD50, consistent with the expected mechanism of action for these compounds.

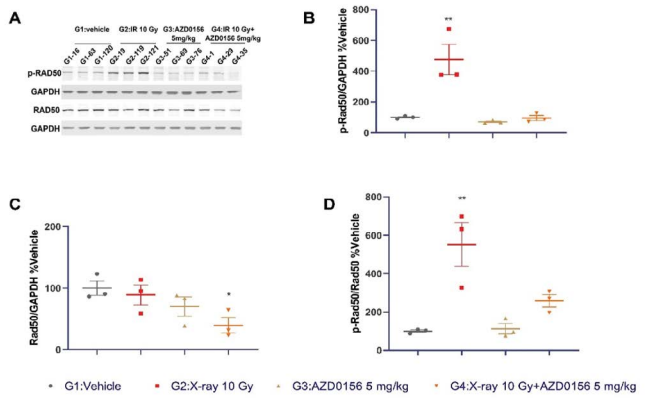

Figure 5: Radiosensitizer AZD0156 effectively inhibits tumor growth in radiated brain tumors

Mice with intracranial NCI-H1975-luc tumors were randomized into 4 groups: Control; a single dose of 10Gy radiation; AZD0156 5 mg/kg, and combination treatment of AZD0156 with radiation. Western Blot analysis showing the expression of pRAD50, RAD50 and the housekeeping protein GAPDH (Fig 5A). Quantification of the Western Blot via Image studio system (LI-COR). All values are presented as the mean ± SEM, Data was analyzed by one-way ANOVA method, ** $p<0.01$, compared with vehicle group (Fig 5B, Fig 5C and Fig 5D).

In a subsequent study, we explored whether combination therapy with AZD0156 could enhance the effectiveness of radiation on tumor growth. Utilizing the NCI-H1975-luc intracranial model, we examined the radiosensitizing properties of AZD0156. Our findings revealed that the application of AZD0156 not only improved the anti-tumor effect of radiation, but also alleviated the animal body weight loss caused by the cancer (Fig. 6).

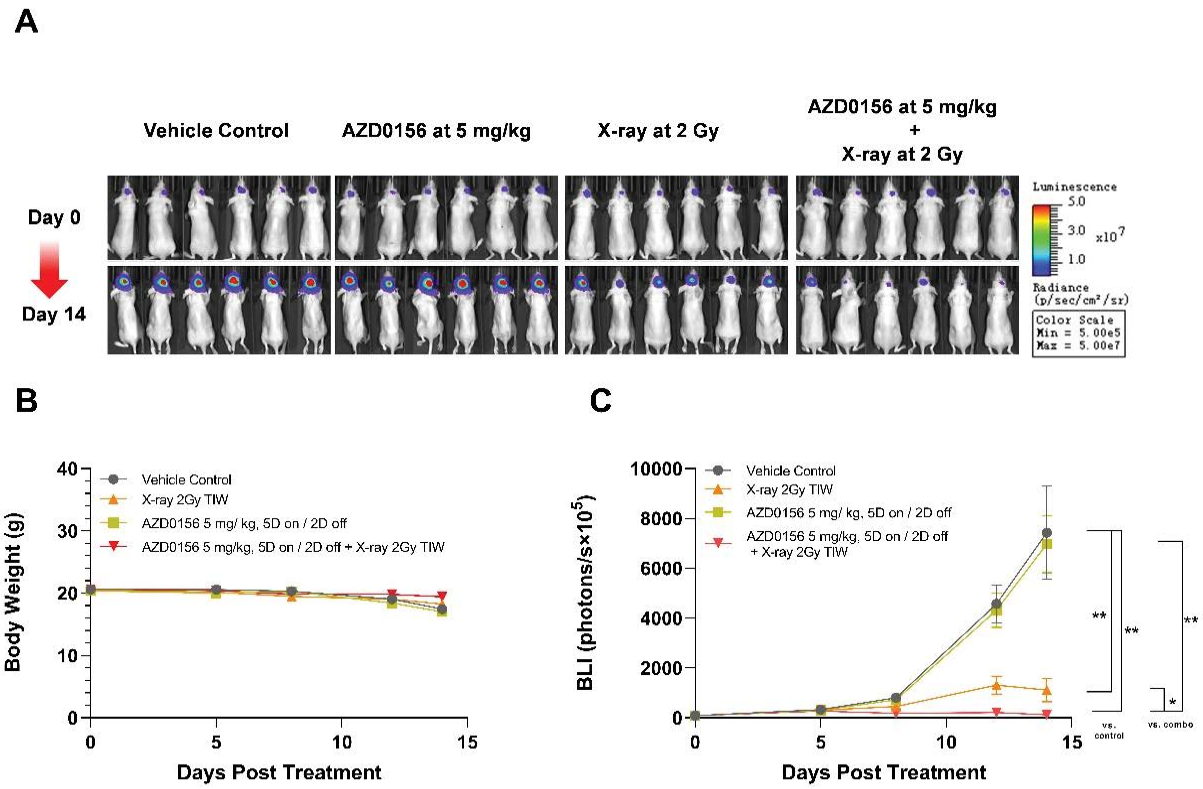

Figure 6: Radiosensitizing effects of AZD0156 on intracranially growing tumors after radiation therapy.

NCI-H1975-luc cells were intracranially implanted into mice. When the BLI value reached 85 × 10^5 photons/s, mice were randomized into 4 groups (6 animals/group): control, X-ray 2Gy TIW, AZD0156 5 mg/kg 5D on / 2D off and combination. Bioluminescence readouts of mice (Fig 6A). Mean body weights relative to starting weights (Fig 6B). Growth of NCI-H1975-luc intracranial tumors (treatment started on 6 days post cell inoculation). Values are represented as mean ± SEM (**, $P < 0.01$; *, $P < 0.05$) (Fig 6C).

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Discussion

In this study, total 14 tumor models (13 subcutaneous and 1 intracranial model) were evaluated to understand the responses of radiation treatment on different tumor types. Some of tumor models, such as Capan-1 and Miapaca-2, are very sensitive to radiation therapy with over 95% TGI. Overall, the data illustrated pending on the tumor type, the responses to the radiation treatment are different, this information is useful for scientist considering the model selection for supporting their R&D program. Furthermore, we evaluated the BBB integrity in the brain with or without radiation, and a visual assessment of the brain and quantification of Evans blue staining showed that X-ray radiation at 12 Gy resulted in limited influence on BBB in naïve animal. At the meantime, we verify that the BBB is opened in NCI-H1975-luc intracranial tumor in current study condition, this selective permeability phenomenon plays an important role in maintaining the stability of the brain environment. Before we challenge the X-ray in intracranial tumor model, it is necessary to explore the impact of X-ray on the blood-brain barrier [18]. In this paper, the results showed that the BBB in the tumor which is otherwise known as blood-tumor barrier (BTB) is opened in NCI-H1975-luc intracranial tumor, and a visual assessment of the brain and quantification of Evans blue staining showed that 12 Gy X-ray radiation could result limited influence on BBB in naïve animal, the results suggest the possibility of crossing the BTB for test compound, especially for small molecular.

In addition, we assessed two efficacy studies by combination of x-ray with Gemcitabine on H22 syngeneic subcutaneous model, and the combination of X-Ray with AZD0156 on CDX NCI-H1975_luc intracranial model. As a result, the data illustrated that X-ray radiation in combination with Gemcitabine resulted significant effect than mono radiation therapy, Gemcitabine enhanced the TGI of radiation from 40% to 73%, which is consistent with the literature [16,17]. Furthermore, AZD0156 also enhanced the radiation therapeutic effect from 85% to 98% TGI in our study. As known, radiotherapy has achieved great success in cancer treatment, but its damage to DNA double-strands is not only a side effect on healthy tissues, but also the main source of the radiation resistance of cancer cells. AZD0156 is an inhibitor of Ataxia telangiectasia mutated (ATM), which is a serine threonine kinase and plays a key role in DNA damage response (DDR) related to double-strand breaks [19-20]. This enables AZD0156 can eliminate radiation induced ATM signals and become one of a variety of radiosensitizers currently used to enhance the efficacy of radiotherapy. As a reasonable and effective combination, AZD0156 and radiotherapy have been widely discussed in many preclinical studies [21,22,11]. Meanwhile, pRAD50 can be utilized as a biomarker for the AZD0156 clinical trial [23]. Therefore, we used the NCI-H1975_luc intracranial tumor model to verify the combination of X-ray and AZD0156. The results showed that the AZD0156 alone treatment had no antitumor activity at all, but it significantly improved the therapeutic effect of radiation, In the single dose PD study, the Western Blot result showed that radiotherapy significantly increased the expression of pRAD50 in tumor, it suggested that the DNA damage reaction produced by X-ray radiation was significantly improved. The combination treatment with AZD0156 exhibited a low expression level of pRAD50 and pRAD50/RAD50 that proved the effective inhibition of the DNA damage response by the drug. These results showed that the X-ray radiation platform can successfully simulate the radiation transmission of cancer clinical treatment and provide safe and convenient data support for preclinical research of cancer treatment. An important obstacle in translating laboratory findings into radiation therapy is the research on the technical differences between the radiation methods used for human therapy and animal therapy. This paper uses a variety of models to evaluate the availability of this equipment, providing data reference for future research.

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Statements & Declarations

This work was supported by Pharmaron Beijing, Co. Ltd. Author Jingqi Huang has received research support from Pharmacology Pharmaron Beijing, Co. Ltd.

Author Contributions

Jingqi Huang is fully responsible to the study design and data quality.

Linfeng Li managed ex vivo section

Wentao Li, Xiaoyan He, Weiwei Cheng, Guannan Li, Linfeng Li, Qiuliang Li, Yongfei Wang, Xuesong Ren, Zhi Wei, Long Shi, Yiran Wei, Jing Jin, Wei Yun performed in vivo studies.

Xiaoyan He composed the manuscript.

All authors read and approved the final manuscript.

Availability of Data and Materials

The data generated during and analyzed during the current study are available from the corresponding author on reasonable request.

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Declarations

Conflict of interest

All authors are employed by Pharmaron Beijing, Co. Ltd. This study was sponsored by Pharmacology Pharmaron Corporation.

Consent for publication: Not applicable.

Ethics Approval

All in vivo experiments were conducted in Pharmaron Pharmacology with approved animal protocols. In vivo experiments were done according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Pharmaron Pharmacology in accordance with all standards of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

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