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Sensitization of cancer cells to radiation by selenadiazole derivatives by regulation of ROS-mediated DNA damage and ERK and AKT pathways

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ABSTRACT

X-ray-based radiotherapy represents one of the most effective ways in treating human cancers. However, radioresistance and side effect remain as the most challenging issue. This study describes the design and application of novel selenadiazole derivatives as radiotherapy sensitizers to enhance X-ray-induced inhibitory effects on A375 human melanoma and Hela human cervical carcinoma cells. The results showed that, pretreatment of the cells with selenadiazole derivatives dramatically enhance X-ray-induced growth inhibition and colony formation. Flow cytometry analysis indicates that the sensitization by selenadiazole derivatives was mainly caused by induction of G2/M cell cycle arrest. Results of Western blotting demonstrated that the combined treatment-induced A375 cells growth inhibition was achieved by triggering reactive oxygen species-mediated DNA damage involving inactivation of AKT and MAPKs. Further investigation revealed that selenadiazole derivative in combination with X-ray could synergistically inhibit the activity of thioredoxin reductase-1 in A375 cells. Taken together, these results suggest that selenadiazole derivatives can act as novel radiosensitizer with potential application in combating human cancers.

1. Introduction

Cancer, due to its uncontrolled and rapid proliferation properties, has become the leading cause of human death in the world. Nowadays, due to the high metastatic potential sand resistance towards chemotherapy, radiotherapy and immunotherapy, the management of melanoma represents one of the most challenging problems in clinical oncology [1]. Radiotherapy (RT) has been widely used in clinics for decades as a representative protocol for the noninvasive treatment of cancers [2]. In contrast to surgical treatment, lesser physiological and psychological burden was placed on patients by RT than surgical and chemotherapy [3]. RT can force/localize almost all radiation exclusively on tumors once precisely positioned, thus reducing possible toxicity to the surrounding normal tissues [4]. However, despite these advantages, RT may still fail to efficiently eradicate hypoxic tumors due to their insensitivity to radiation [5]. On the one hand, the high doses of radiation needed for tumor therapy may probably exceed the tolerance of normal cells, which will inevitably cause damage to normal cells at the same time it is killing cancerous cells [6]. Moreover, it can lead to the possibility of secondary tumors. The combination of many therapies, including RT, surgery, and chemotherapy (CT), has become the standard modality in the clinic for many decades in order to achieve better cancer treatment [4]. In order to enhance the sensitivity of hypoxic cells to radiation and achieve the optimal efficiency, a frequent strategy relies on the combination of CT and RT. This modality, named chemradiation therapy (CRT), is a standard treatment option for many types of solid tumors [7–9].

Selenium (Se) is an essential trace mineral required in maintaining health in humans and animals. Epidemiological, preclinical and clinical studies support the role of Se compounds as potent cancer chemopreventive agents [10,11]. Se supplementation was found effective in reducing the incidence of cancers including prostate, lung, colon and liver cancers [12]. At present, several mechanisms have been postulated to elucidate the anticancer activity of Se, which include induction of cell apoptosis or cell cycle arrest, inhibition on cell proliferation, modulation of redox state, detoxification of carcinogen, stimulation of the immune system and inhibition of angiogenesis [11]. Recent studies have demonstrated that Se shows promise as an agent that can reduce the harmful side-effects of RT, clinical evaluations using varying doses of Se

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have demonstrated a significant reduction in toxicity of CT and RT, but not compromising the effectiveness of treatments [13]. Selenadiazole derivatives have been identified as novel agents with stronger antiproliferative effect against human cancer cells through the induction of apoptosis or cell cycle arrest with the involvement of oxidative stress [11,14]. Despite this potency, selenadiazole derivatives showed less toxic to human normal cells [11]. Furthermore, we also proved that selenadiazole derivatives triggered apoptosis in cancer cells by activating AKT pathway and p53 phosphorylation [11,15]. However, the basis of synergic interaction between the radiation and the selenadiazole derivatives *in vivo* remains unclear. Therefore, in the present study, we evaluated the ability of selenadiazole derivatives to potentiate the inhibitory action of radiation against A375 human melanoma and Hela human cervical carcinoma cells, and elucidated the molecular mechanisms accounting for the synergism of the combined treatments. Collectively, the results from these studies provide a novel therapeutic strategy to use selenadiazole derivatives as radiosensitizing agents against A375 and Hela cells.

2. Materials and methods

2.1. Cell culture and MTT assay

A375 human melanoma cell line and HeLa cell line were obtained from American Type Culture Collection (ATCC, Manassas, VA), the cells were routinely grown in Dulbecco's Modified Eagle Media (DMEM) medium supplemented with fetal bovine serum (10%), penicillin (100 units/ml) and streptomycin (50 units/ml) at 37 °C in a humidified incubator with 5% CO_2, all cultures were routinely passaged twice a week. Cell viability was determined by MTT assay as previously described [16].

2.2. Clonogenic assays

A375 and Hela cells were plated (1×10^6/10-cm dish), grown for 24 h, and then treated with different concentrations of selenadiazole derivatives for an additional 6 h. Immediately after exposed to 8 Gy, the cells were trypsinized and re-seeded (500 cells/well) into 6 well dishes. One week later, cells were stained with 6.0% glutaraldehyde (vol/vol), 0.5% crystal violet (wt/vol) in water and photographed colonies of greater than 50 cells were counted. Mixture was removed and colonies formation was counted.

2.3. Flow cytometric analysis

The cell cycle distribution was analyzed by flow cytometry analysis as previously reported [16].

2.4. Measurement of ROS generation

The effects of selenadiazole derivatives or/and radiation (8 Gy) on ROS-initiated intracellular oxidation were evaluated by DCF fluorescence assay [4].

2.5. Determination of TrxR activity

Inhibition of TrxR was measured using a Thioredoxin Reductase Assay Kit (Cayman) as previously described [17].

2.6. Western blot analysis

Total cellular proteins were extracted by incubating cells in lysis buffer obtained from Cell Signaling Technology and protein concentrations were determined by BCA assay. The effects of 2b and X-ray on the expression levels of proteins associated with different signaling pathways were examined by Western blot analysis [4].

2.7. Statistical analysis

All experiments were carried out in triplicate and data were expressed as mean ± standard deviation. Differences between two groups were analyzed by two-tailed Student's test. Difference with $P < 0.05$ (*) or $P < 0.01$ (**) was considered statistically significant. One-way analysis of variance (ANOVA) was used in multiple group comparisons. Bars with different characters are statistically different at $P < 0.05$ level.

3. Results

3.1. Selenadiazole derivatives enhance radiation-induced inhibition on cancer cell growth and colony formation

In this study, selenadiazole derivatives were synthesized according to schematic route showed in Fig. S1. 2a, 2b and 2c have electronic groups, while 2d has electronic group. These compounds were used to compare the difference of the effects of electronic groups and electron-withdrawing groups on the therapeutic action of X-ray. The *in vitro* anticancer activity of selenadiazole derivatives and radiation against radiation-resistant A375 and HeLa cells lines was evaluated by means of clonogenic assays. A375 and HeLa cells were exposed to selenadiazole derivatives with or without X-ray radiation, and then subjected to MTT assay and cell clonogenic assay. As shown in Fig. 1B and C, in A375 cells, we observed that 2b alone decreased the cell colony formation by 13.7%, while 8 Gy radiation decreased the colony formation by 52.8%. However, the combined therapy almost completely abolished colony formation. A decrease in colony formation of 91.5% was observed in treated cells. Similarly, 2b and 8 Gy respectively decreased the colony formation of HeLa cells by 5.5% and 44.3%, with a significant decrease in colony formation by 89.2% observed in combined treatment. The results showed that, 2b in combination with X-ray exhibited higher inhibition on A375 cells than Hela cells. Under different doses of X-ray (2, 4, 6 and 10 Gy), their inhibitory effects on cell colony formation were all enhanced by combined treatment of 2b (Fig. 1D). Similar results are also found in cells exposed to different doses of X-ray in combination with 2a and 2c (Fig. 1E and F). However, the effect of 2d treatment combined with radiation was only little more potent than those of radiation alone (Fig. S2). These data indicates that selenadiazole derivatives could sensitize cancer cells to X-ray.

3.2. Selenadiazole derivatives enhances radiation-induced growth inhibition on A375 cells through induction of G2/M arrest

Since selenadiazole derivatives in combination with X-ray are more effective against A375 cells than HeLa cells to inhibit the colony formation, we chose A375 cells for further studies on the action mechanisms. As shown in Table S1, pretreatment of A375 cells and with selenadiazole derivatives and X-ray displayed higher antiproliferative activities than those of individual treatment. For instance, as shown in Fig. 3, treatment of A375 cells with 2b (5 μM) and radiation alone inhibited the cell proliferation by 16.0% and 7.6%, respectively. Nevertheless, pretreatment of the cells with 2b (5 μM) in combination with radiation significantly inhibited the cell proliferation by 53.2%. Therefore, the effect of 2b treatment combined with radiation was 5.5 times more potent than those of radiation alone. The effects of 2a and 2c treatment

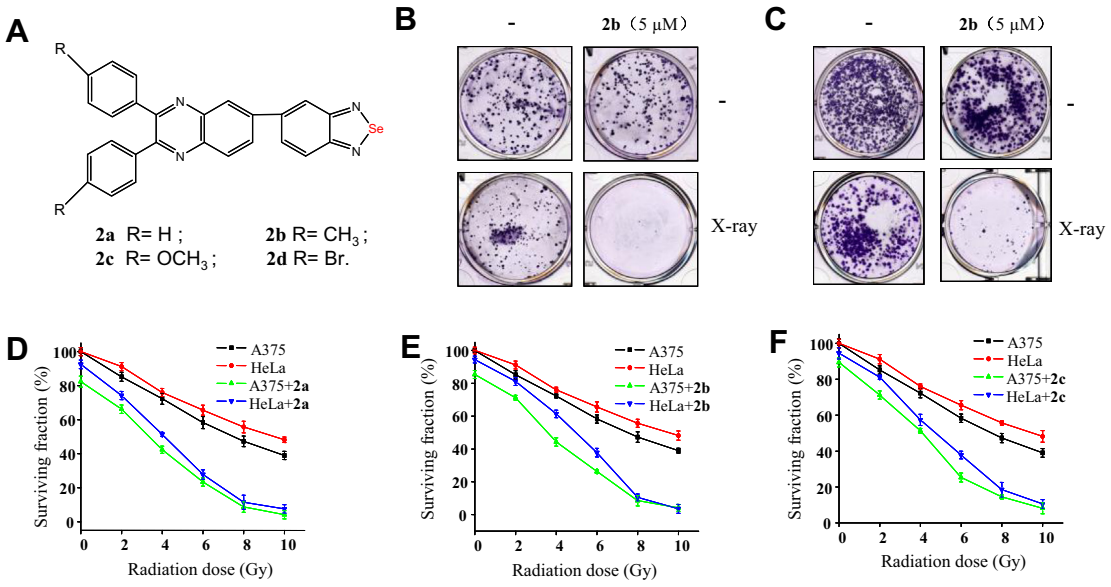

Fig. 1. Selenadiazole derivatives enhance radiation-induced inhibition on cancer cell colony formation. A375 and HeLa cells were pretreated with selenadiazole derivatives for 6 h and then exposed to 8 Gy X-ray. The cells were then cultured for 7 days. (A) Structures of Selenadiazole derivatives studied in this work .(B) and (C) Clonogenic assay performed in 6 well plates cultured up to 7 days after 2b (5 μM), radiation and combined treatment. (A) A375 cells; (B) HeLa cells. (D–F) Effects of 2a (10 μM), 2b (5 μM), 2c (10 μM) on radiosensitivity of cancer cells to different does of X-ray.

combined with radiation were 2.5 and 2.8 times more potent than those of radiation alone. However, the effect of 2d treatment combined with radiation was only slightly higher than that of radiation alone. Taken together, these results suggest that selenadiazole derivatives enhance the cell killing activity of radiation.

Moreover, propidium iodide-flow cytometric analysis was performed to determine action modes mechanisms of cell death. Fig. 2A and B show the representative DNA distribution histograms of A375 cells, which revealed that exposure of cells to 2b in combination with radiation induced growths inhibition was mainly caused by cell cycle arrest at G2/M-phase. No significant change in the cell population at G2/M phases was observed in cells exposed to 2b alone. Treatment of radiation alone resulted in increase in the population at G2/M phases cells from 10.2% (control) to 35.9%. However, significant increase in G2/M phase cells populations was observed in cells co-incubated with 2b and radiation (8 Gy). For instance, the cells exposed to 2b (10 μM) and radiation (8 Gy) displayed 54.8% of G2/M phases cells. Furthermore, A375 cells exposed to 2b in combination with radiation showed dose-dependent decrease in the expression levels of CyclinB and Cdc2 (Fig. 2C), which can form a complex with CDK to regulate the progression of cells. The down-regulation of CyclinB and Cdc2 may disturb the progression of cells at G2/M-phase. The similar results were also found in cells exposed to 2a and 2c (Fig. S4). Taken together, induction of G2/M-phase arrest by down-regulation of CyclinB and Cdc2 could be the major mechanism for cell death induced by 2b and radiation.

3.3. Selenadiazole derivatives trigger ROS generation to sensitize cancer cells to X-ray

Radiosensitization by anticancer drugs may be achieved by various molecular mechanisms, especially promotions of ROS overproduction [18]. In this study, firstly, as demonstrated from the results of H NMR (Fig. S5), the structure selenadiazole derivatives were not changed after X-ray radiation, which indicate the chemical stability of the compounds. Therefore, we continued to detect whether selenadiazole derivatives were able to synergize with radiation to trigger ROS generation by measuring the DCF fluorescence intensity. As showed in Fig. 3A, pretreatment of the cells with 2b significantly enhanced radiation-induced ROS generation a time-dependent manner. However, only slight increase in intracellular ROS generation was observed in cells exposed to radiation alone. Interestingly, intracellular ROS generation in cells exposed to 2b alone was much lower than control group. Similar results were also observed in cells treated with 2a and 2c (Fig. S6). The results suggest that selenadiazole derivatives combine with radiation to induce cancer cell growth inhibition in a ROS-dependent manner, without change in the chemical structure of the compounds.

3.4. Induction of DNA damage-mediated p53 phosphorylation

RT can cause damage to DNA, proteins, and lipid membranes in cancer cells, leading to cell death [9]. Many studies have showed that selenocompounds exhibited the potential to induce DNA damage, and thus activated p53 signaling pathway [11,15]. P53 is a major player in the apoptotic or cell cycle arrest response of cells to DNA damage as well as a transcription factor which can directly or indirectly induce cell apoptosis or cell cycle arrest [19]. To examine 2b and radiation whether induce DNA damage, Western blotting was employed to detect the changes of p53 and histone phosphorylation, two of DNA damage marker, which can be activated in response to DNA damage. As shown in Fig. 3B, 2b pretreatment strongly enhance radiation induced DNA damage, as evidenced by enhanced expression of phosphorylation levels of ATM (Ser 1981), ATR (Ser 428), chk2, chk1, p53 (Ser 15) and histone (Ser 139). The results support that, enhancement of DNA damage contributes to the synergistic effects of 2b and radiation in A375 cells.

3.5. Involvement of AKT and MAPKs pathways in the synergistic effect of 2b and radiation

AKT and MAPKs signaling pathway plays an important role in regulating cell cycle progression and proliferation through transmitting extracellular signals from the cell membrane to the nucleus [20]. Studies reported that selenocompounds exhibited the ability to induce apoptosis or to enhance the efficiency of chemotherapeutic drugs by inhibition of AKT and/or ERK phosphorylation [21]. In this study, we showed that, pretreatment of the cells with 2b significantly enhanced the radiation-induced dephosphorylation of AKT

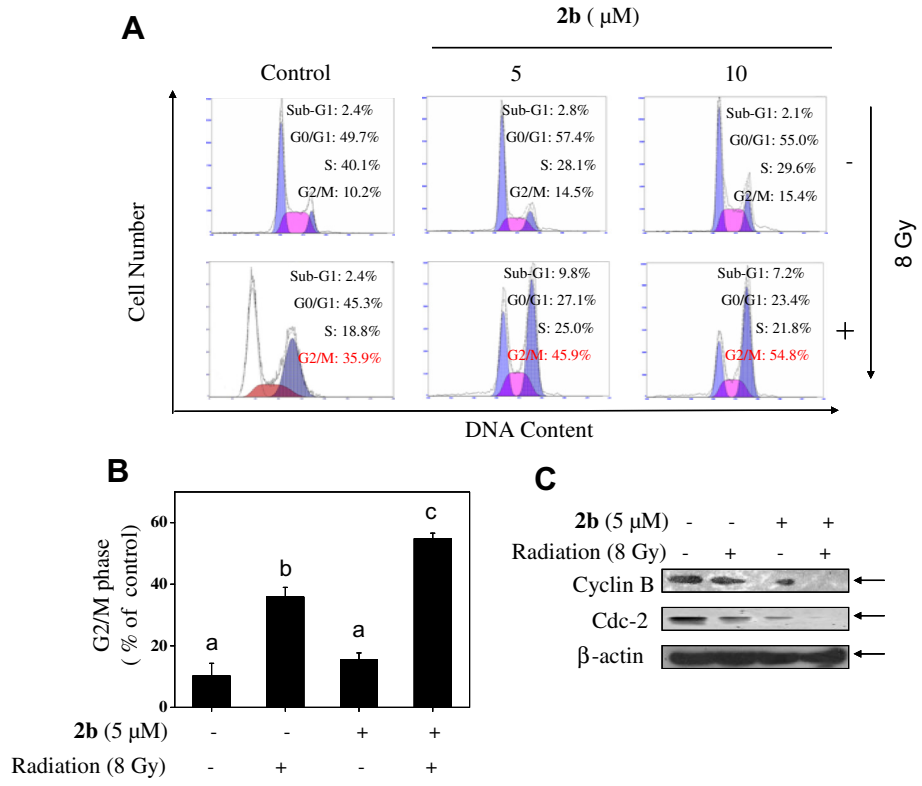

Fig. 2. 2b enhances radiation-induced G2/M cell cycle arrest in A375 cells. A375 cells were pretreated with various concentrations of 2b for 6 h and then exposed with or without X-ray (8 Gy), the cells were cultured another for 12 h. (A) 2b increased radiation-induced G2/M population accumulation. (B) Effects of G2/M phase arrest induced cells under different treatments. (C) Western blot analysis of expression levels of Cdc2 and CyclinB in A375 cells. All results were obtained from three independent experiments. Bars with different characters are statistically different at the $P < 0.05$ level.

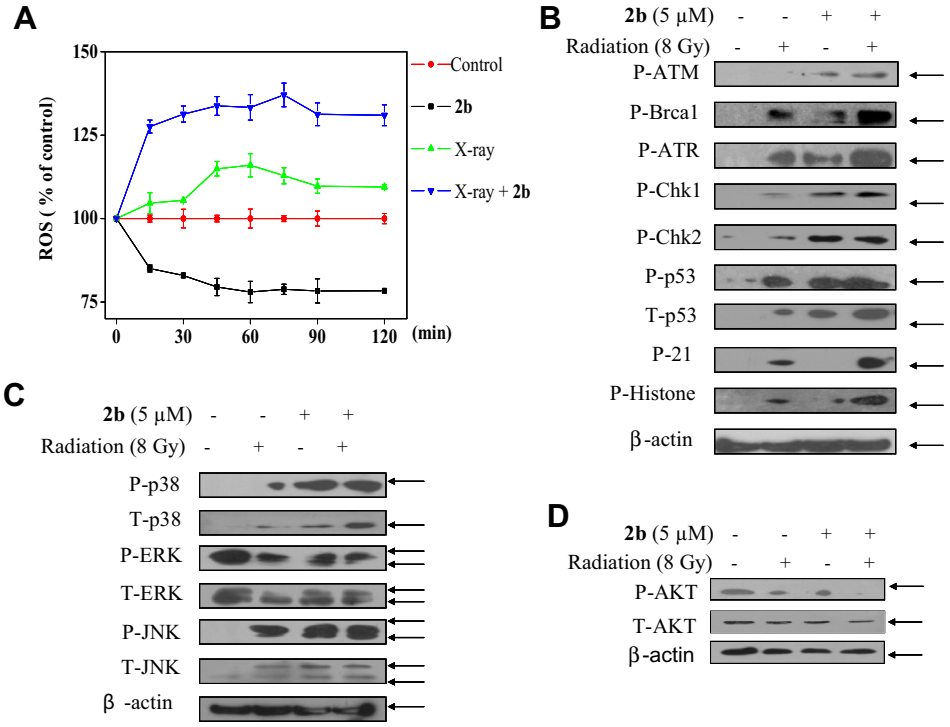

Fig. 3. 2b enhances radiation-induced DNA damage through ROS overproduction, Akt and MAPK signaling pathways. (A) A375 cells were treated with indicated concentration of 2b (5 μm), or/and radiation for different times and the levels of the intracellular ROS were analyzed. (B) 2b enhances radiation-induced DNA damage-mediated p53 phosphorylation. (C) and (D) Effects of 2b and radiation on the phosphorylation status and expression levels of MAPKs and AKT pathways.

and ERK1/2, with no obvious change in total AKT and ERK1/2 observed (Fig. 3C and D). X-ray alone exhibited no effect on the expression of phosphorylated p38 MAPK, while only a slight elevation phosphorylated p38MAPK was observed in cells exposed to 2b alone. Whereas, 2b and radiation in combination significantly induce rapid augment in the expression level of phosphorylated p38 MAPK and total expression level of phosphorylated p38 MAPK. Taken together, 2b potentiated radiation to induce growth inhibition with involvement of down-regulation of AKT and ERK1/2 phosphorylation and up-regulation of phos-phorylated p38 MAPK.

3.6. Roles of TrxR in the cooperative action of 2b and X-ray

Thioredoxin reductase (TrxR) system plays a major role in the regulation of the cellular redox state [22]. An increase TrxR activities has been correlated with evasion of apoptosis and acceleration of tumor growth TrxR are generally expressed in mammal cells, the expression of TrxR is closely correlated with the resistance of cancer cells to chemotherapeutic agents, such as cisplatin and doxorubicin. Till now, many inhibitors of TrxR have been developed for treatment of tumors [23,24]. In the present study, to investigate the effects of 2b and radiations on TrxR activity in cell lysates, the insulin reduction assay was performed. As shown in Fig. 4A and B, treatment of the cells with 2b or radiation alone resulted in slight decrease in the activities of TrxR to 83.0% (2b, 5 μM) and 86.8% (8 Gy) respectively. In contrast, significant decrease in TrxR activity (65.8%) was observed in cells cooperatively treated with 2b (5 μM) and radiation (8 Gy). Moreover, Western blot analysis was also employed to examine the expression level of TrxR and generation of Trx-reduced (Fig. 4C). The results revealed that significant down-regulation in the expression level of TrxR and Trx proteins was observed in A375cells exposed to 2b or radiation alone, and the down-regulation of Trx-reduced was synergistically enhanced by combination of 2b and radiation, which was highly consistent with the tendency of TrxR activity. Taken together, our results indicate that, 2b and radiation synergistically induced cancer cell growth inhibition through inhibition of TrxR activity.

4. Discussion

RT is an essential treatment for patients with cancers. However, many tumors are either less sensitive or resistant to radiation [5]. Therefore, the development of new radiosensitizers to improve both the survival rate of patients and the prospect of organ preservation is needed. In this study, we showed that selenadiazole derivatives could potentiate radiation-induced cell growth inhibition against A375 melanoma cells through G2/M-Phase arrest by triggering DNA damage and inactivation of AKT and ERK through ROS overproduction. Collectively, the results provide a novel therapeutic strategy to use selenadiazole derivatives as radiosensitizing agents against A375 and Hela cells.

The action of ionizing radiation, which includes both photons and particles, is initiated by the transmission of its energy to selenadiazole derivatives. When an energy higher than the binding energy of the inner shell is applied to an atom or a molecule (for instance, by photoabsorption), the system is ionized. That is, an electron is ejected and a vacancy is left in the shell (inner-shell ionization). This ionization is followed by a process of deexcitation in which an electron of an outer shell (higher energy state) drops into the lower energy shell in order to fill the vacancy. The energy difference between the two shells is thus transferred to a fluorescence photon or to another electron that is ejected from an outer shell. The latter phenomenon is called the Auger effects. On the one hand, X-ray can create charged particles (free radicals) within the cells that in turn could break down the DNA and inhibit DNA repair. On the other hand, the yields of molecular damage in an aqueous system which lead to radiobiological effects were caused by Auger effects. Previous evidence has already suggested that Se

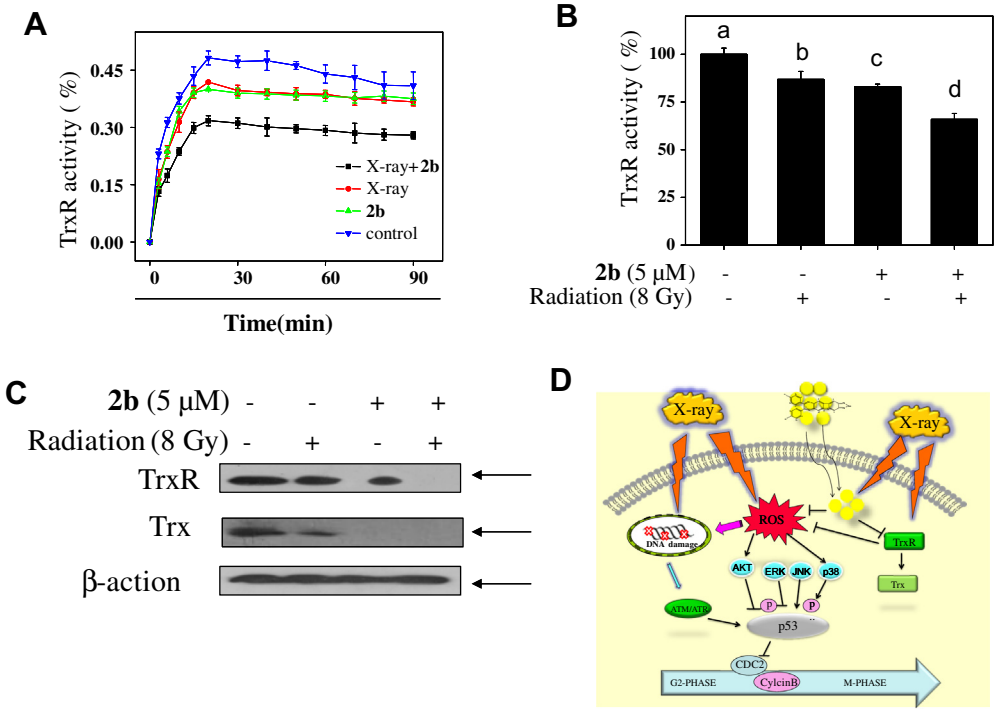

Fig. 4. Changes of antioxidant enzyme activities of TrxR after different treatments in A375. A375 cells were treated with various concentrations of 2b for 6 h and then exposed with or without X-ray (8 Gy), the cells were cultured another for 12 h. (A) and (B) inhibition of thioredoxin reductase activity by 2b or/and radiation. (C) Cell lysates were subjected to Western blot analysis for protein levels of TrxR and redox thioredoxin. (D) Proposed signaling pathways of G2/M cell cycle arrest induced by X-ray and selenadiazole derivatives.

in X-ray irradiated can cause Auger effect. A large amount of water radicals such as OH radicals are produced in the system and they can attack biological molecules, resulting in oxidized molecular changes or damage to Auger effects can induced oxidative stress by regulation of the redox system.

ROS play an important role in the biological effects induced by ionizing radiation [8]. Excess ROS could attack various components of DNA, leading to the generation of a variety of ROS-mediated modified products, including oxidized bases, DNA strand breaks, DNA intra-strand adducts, and DNA–protein crosslinks. Several protein kinases have been known to regulate cell proliferation and survival. MAPKs and AKT pathways are the major oxidative stress sensitive signal transduction pathways in most cell types [20]. For instance, AKT could transduces the signals from growth factors and oncogenes to downstream targets that control the crucial elements in tumor development. In this study, ROS overproduction and the resulting cellular redox change induced by 2b and radiation were found as part of the signal transduction pathways leading to cell cycle arrest. Specifically, we showed that 2b in combination with radiation induced rapid ROS generation, DNA damage and activation of ATM, ATR and p53 in cancer cells in time-and dose-dependent manner. We also showed that the combined treatment with 2b and radiation notably decreased ERK and AKT phosphorylation in cancer cells, significantly enhanced the combined treatment-induced cell growth inhibition, indicating that AKT and ERK pathway may contribute mainly to the combined treatment-induced cell cycle arrest.

TrxR plays important roles ranging from maintaining nucleotide pools associated with DNA replication and repair to defending against oxidative stress directly and indirectly via redox signaling [22]. As such, inhibiting TrxR activity can produce robust anticancer effects, including the growth inhibition and cell death. TrxR is overexpressed in many cancer cells and has been identified as a potential target of anticancer drugs. For example, several selenocompounds were also found be able to compete with Trx to inhibit TrxR activity [25]. In the present study, we showed that, 2b and radiation could cooperatively inhibit the TrxR and Trx activity.

In summary, this study demonstrates the novel property of selenadiazole derivatives to enhance RT-induced inhibition on cancer cell growth and colony formation. The results of mechanistic investigation revealed that, selenadiazole derivatives could dramatically enhance the radiation-induced apoptosis in A375 human melanoma cells by triggering ROS-mediated DNA damage, inactivation of AKT and MAPKs, and inhibit TrxR activity (Fig. 4D). These findings suggest that selenadiazole derivatives may be further developed as a novel tumor tissue sensitizer to increase the therapeutic index of RT for improving treatment of pancreatic cancer patients.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.151.

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