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Quercetin Iron (III) Complex Enhances Radiation-Induced Cell Death in Human Erythroleukemic Cell Lines by Increasing the Generation of Intracellular ROS

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Abstract: Quercetin iron (III) complex (IronQ) is a paramagnetic agent which exhibits several biological effects. Despite extensive studies about quercetin metal complex, the role of complexes to enhance the effect of radiation still remains unexplored. To this end, we determined whether and how IronQ sensitizes cancer cells to Ionizing Radiation (IR). Doxorubicin resistant leukemic cells (K562/Adr) and their parental cell lines (K562) were used in this study. After treated with IronQ and radiation exposure (1-6 Gy), clonogenic survival assay, apoptosis, cell cycle distribution, acidic vesicular organelle staining and intracellular Reactive Oxygen Species (ROS) generation were evaluated. Combined treatment of IronQ with radiation significantly reduced the cell survival rate in both cell lines compared to radiation exposure alone or the control group. The mechanism underlying the cell growth rate inhibition of IronQ with IR was inducing cell arrest at G2/M phase. Moreover, IronQ pretreatment enhanced radiation-induced apoptosis in both cell lines with a greater efficacy in sensitive cell. Interestingly, increased intracellular ROS and accumulation of Acridine Orange (AO) in acidic organelle compartments in response to IronQ treatment combined with IR were observed 24 h post-treatment. In the context of our present findings demonstrating that IronQ in combination with radiation potentially improves the radiotherapeutic efficacy, the obtained results serve as a new strategy in the development of theranostic medicine.

Keywords: Reactive Oxygen Species, Quercetin, Radiation, Erythroleukemic Cells, Radiosensitizer

Introduction

Reactive Oxygen Species (ROS) are generated in most cells as a byproduct of aerobic respiration and substrate oxidation in aerobic organisms. Moreover, the generation of ROS increases in response to external stimuli including drugs, xenobiotics, smoke and radiation (Pelicano *et al*., 2004). Under normal physiologic conditions, cellular redox state must be tightly regulated. Moderate levels of ROS are needed to regulate many signal transduction pathways that are essential for various biologic processes, such as cell proliferation and differentiation (Valko *et al*., 2007; Ji *et al*., 2010). However, alteration of ROS accumulation results in oxidative stress that leads to irreversible damage to biological macromolecules, resulting in cell death and various pathologies of aging diseases (Uttara *et al*., 2009; Aiken *et al*., 2011; Sohal and Orr, 2012).

ROS act as a key intermediate to tumor toxicity in radiotherapy and chemotherapy and also play an important role as a second messenger in several signaling pathways (Zhang *et al*., 2016; Holley *et al*., 2014). Up until now, radiation has been the first choice of cancer treatment; however, its efficacy can be limited by toxicity of healthy tissues in the neighborhood of the target and resistance of tumor cells to radiation causing the recurrence of tumors after treatment (Kim *et al*., 2014a). Therefore, treatment strategies that can

simultaneously increase the radiosentivity of cancer cells and radioresistance of normal tissues are needed to improve the rank in the therapeutic index for radiotherapy treatment. It is well established that most of cancer cells exhibit high levels of ROS as a consequence of their high metabolism rate (Nogueira and Hay, 2013; Tong *et al*., 2015). To protect against high ROS levels, cancer cells have developed an efficient mechanism of ROS detoxification that presents a selective advantage for its survival under stress conditions (Acharya *et al*., 2010). Thus, one of the potential approaches to achieve tumor toxicity is the induction of ROS-mediated damage in cancer cells by ROS-generating agents with safe therapeutic profiles that promote ROS generation beyond the cancer cells tolerance limit. This has been considered an effective strategy to preferentially kill cancer cells while sparing normal cells, which are characterized by having lower intracellular ROS levels (Trachootham *et al*., 2009).

Quercetin has been recognized largely as a beneficial antioxidant which possesses a wide range of biological effects including anti-cancer, anti-hypertension, anti-inflammatory and antimicrobial activities (Boots *et al*., 2008; Hämäläinen *et al*., 2007; Federico, 2012). Mounting evidence suggests that polyphenol antioxidants may have pro-oxidant activity under certain conditions such as at high doses or in the presence of metal ions (Forester and Lambert, 2011; Lee-Hilz *et al*., 2006). Like other flavonoids, quercetin has been shown to induce the production of superoxide anion, hydrogen peroxide and other species (Fonseca-Silva *et al*., 2011; Kim *et al*., 2014b). Recently, the pro-oxidant activity of quercetin and its ability to cause mitochondrial dysfunction induced apoptosis has been suggested as a possible anticancer mechanism (Chang *et al*., 2006). Metal chelation is considered to be another mechanism of the antioxidant activity of flavonoids. The interaction of flavonoids with metal ions has been shown to change the antioxidant properties and some biological effects of the flavonoids (de Souza and De Giovani, 2004). It has been reported that quercetin can form complexes with transition metal ions, such as Cu (II), Mn (II) and Fe (II). These quercetin-metal complexes exhibit broad biological activities with better antioxidation and antitumor activity than quercetin alone (Afanas'eva *et al*., 2001). Recently, Tan *et al*. (2009a) evaluated the DNA binding, apoptotic inducing activity and potential molecular mechanism of quercetin zinc (II) and copper (II) complexes. The quercetin zinc (II) complex possesses the ability to inhibit the proliferation of three cancer cell lines (HepG2, SMMC7721 and A549); its anti-tumor effect might be related to the intercalation of the complex into the DNA (Tan *et al*., 2009b). Moreover, these authors have shown the oxidative DNA cleavage activity and apoptosis-inducing activity of quercetin copper (II) complex via the process of ROS generation and also its specific interaction with DNA (Tan *et al*., 2009a). The antitumor properties of the quercetin metal complexes have been investigated by many researchers. So far, the application of the complexes as ROS-generating agents to enhance the effect of radiation on cancer therapy remains unexplored.

According to the array of pro- and antioxidant properties of quercetin metal complexes now established, in our opinion, quercetin metal complexes may increase the therapeutic efficiency of radiotherapy by enhancing radiation induced ROS production resulting in an enhanced therapeutic effect in patients. In our laboratory we established the quercetin iron (III) complex, IronQ. Previously, we reported that IronQ induced the proliferation and differentiation of progenitor cells in a fraction of peripheral blood mononuclear cells. Moreover, with the paramagnetic properties of IronQ, this complex can be used as a contrast agent for a T1-wieghted image in Magnetic Resonance Imaging (MRI), making it possible to use IronQ as a probe for cell tracking with a non-invasive MRI technique (Kantapan and Dechsupa, 2016). Thus, the benefits and safety of IronQ to peripheral blood mononuclear cells and its properties to initiate ROS production in the proper conditions, prompted us to investigate whether it can sensitize cancer cells to radiation by enhancing the generation of ROS. Accordingly, it is unknown if the radiation sensitizing effects of IronQ differs between drug-sensitive and drug–resistant cancer cells. To test this hypothesis, the drug-sensitive erythroleukemic cells (K562) and their doxorubicin-resistant P-gp overexpressing erythroleukemic cells (K562/Adr) were selected as a model. Cancer cell lines were incubated with IronQ then exposed to varying levels of radiation ranging from 1 to 6 Gy. The clonogenic survival assay, apoptosis, cell cycle distribution, presence of Acidic Vesicular Organelles (AVO) and generation of ROS were evaluated. We show here that the pro-oxidant activity of IronQ drives radiation-induced radical production in cancer cells and potentially enhances the effect of radiation on drug-sensitive and -resistant erythroleukemic cells. We also report here that drug-resistant P-gp overexpressing erythroleumic cells has no effect on radioresistance. These results identify the potential of IronQ to function as a novel radiosensitizer by enhancing the impact of radiation on ROS generation.

Materials and Methods

Cell lines and Cell Culture Conditions

Doxorubicin-sensitive erythroleukemia cells (K562) and doxorubicin-resistant erythroleukemia cells

(K562/Adr) were cultured in a RPMI 1640 medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) penicillin-streptomycin in an incubator at 37°C, in a 95% humidified atmosphere containing 5% CO_2. The culture was started at a density of 10^5 cells/ mL and cells grew exponentially to about 10^6 cells/mL in 3 days. The K562/Adr cell line was cultured in a RPMI 1640 medium in the presence of 10 nM of doxorubicin (Sigma Aldrich) for 72 h, after that the cells were grown in RPMI 1640 medium without doxorubicin for 2 weeks before the experiments. In order to get the cells to their exponential phase for the experiments, the cells were seeded at the density 5×10^5 cells/mL and cultured for 24 h after which the cells reached a density about 8×10^5 cells/mL.

Irradiation

K562 and K562/Adr were irradiated in the presence or absence of IronQ with a 6 MV linear accelerator (Primus; Siemens Healthineers, USA) at room temperature, with doses of 1, 2, 4, or 6 Gy at a dose rate of 200 cGy/min.

Survival Assay

To evaluate whether the IronQ complex may enhance the effects of radiation in vitro, the survival fraction at different doses was determined by a survival assay. K562 and K562/Adr cells were treated with IronQ for 24 h, after that they were exposed to 0, 1, 2, 4, or 6 Gy of Ionizing Radiation (IR). Then, cells were counted and seeded at a density of 10^4 cells/mL in 24 well plates. The cells were incubated for 10 days in an incubator at 37°C, in a 95% humidified atmosphere containing 5% CO_2. The number of surviving cells was assessed by trypan blue dye exclusion. The procedure was then triplicated.

Apoptosis Assay

Apoptosis levels in K562 and K562/Adr cells were determined at 0, 4, 24 and 48 h after expose to 6 Gy IR. After treatment cells were washed with ice-cold Phosphate Buffered Saline (PBS) and then 10^6 cells from each group were stained with annexin V conjugated with FITC (AnnexV-FITC; Sigma Aldrich) for 15 min in the dark, followed by propidium iodide (PI; Sigma Aldrich) staining. The stained cells were analyzed by flow cytometry (Coulter Counter, Epics). The results were expressed as percentage of living ($AnnexV^-$, PI^-) and apoptotic cells ($AnnexV^+$, PI^-/ $AnnexV^+$, PI^+). The procedures were then triplicated.

Cell Cycle Distribution

Cells were collected 24 and 48 h after treatment and washed with PBS. Then, cells were fixed in ice-cold 70% ethanol overnight at 4°C. Subsequently, cells were washed in 1 mL of PBS and resuspended in a PBS buffer containing 0.1% triton x-100, 100 μg mL^{-1} and 0.1 mg mL^{-1} PI and incubated for 30 min at 37°C in the dark. Cells were then analyzed by flow a cytometer (Coulter Counter, Epics).

Acidic Vesicular Organelles (AVO) Staining

To evaluate the development of acidic compartment organelles after 24 and 48 h post-treatment with IR alone or IronQ and IR, K562 and K562/Adr cells were stained with AO at a final concentration of 1 μg mL^{-1} for 15 min at 37°C, then cells were washed with PBS. The fluorescent intensity of AO was analyzed using a flow cytometer (Coulter Counter, Epics).

Analysis of ROS Levels

ROS production was detected using the dye 2', 7'–dichlorofluorescin diacetate (DCFH-DA; Sigma Aldrich) according to the procedure described previously (Sarkar *et al*., 2006). Briefly, cells were washed with PBS and then incubated with 10 μM DCFH-DA at 37°C for 30 min in the dark. Cells were then washed, resuspended in PBS and immediately analyzed by flow cytometer (Coulter Counter, Epics). The results were expressed as relative fluorescence intensity, normalized to the un-irradiated control.

Statistical Analysis

The results were reported as the mean ± standard deviation. The statistical analyses were determined by Student's *t*-test. Values of $p<0.05$ were considered statistically significant at the 5% significance level.

Results

Cytotoxicity of Quercetin Iron (III) Complex

To investigate the effects of IronQ on the enhancement of radiation-induced cell death *in vitro*, we chose two erythroleukemic cell lines, K562 and K562/Adr as models. We first determined the toxicity of IronQ treatment alone on these cells. We found that treatment of the cells with the complexes alone exhibit a minimal effect on the cell survival (Fig. 1a). We further examined the effect of IronQ complex to enhance the cytotoxicity of radiation using a clonogenic cell survival assay. As shown in Fig. 1b, we found for these cell lines that over expression of P-gp has no effect on radioresistance. Treatment of cells with IronQ plus IR significantly reduced the cell survival rate compared to IR alone or the control ($p<0.05$) in both cell lines at each of the radiation dose levels (Fig. 1c and d). Moreover, combined treatment of IronQ complex with IR induced greater toxicity in K562 than in K562/Adr cells. These results demonstrate that pre-treatment with IronQ sensitizes erythroleukemic cells to ionizing radiation.

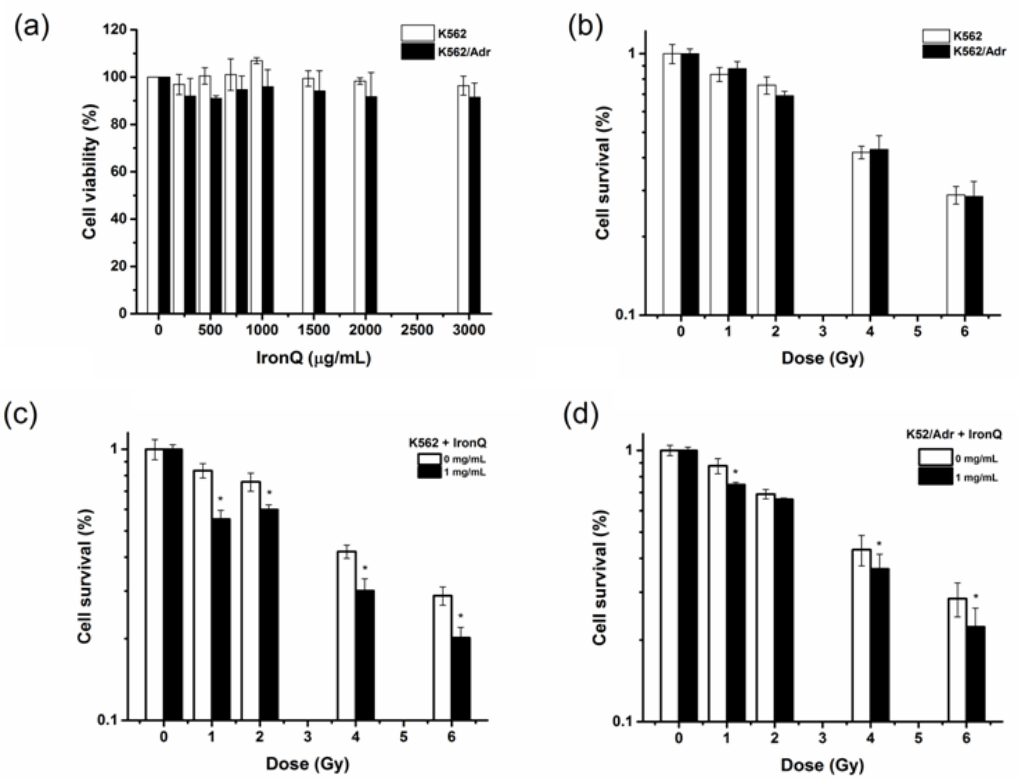

Fig. 1. IronQ sensitizes erythroleukemic cell lines to Ionizing Radiation (IR). (a) K562 and K562/Adr cells were treated with various concentration (250, 500, 750, 1000, 1,500, 2,000, or 3,000 μg mL^{-1}) of IronQ for 72 h and cell viability was assessed. (b) K562 and K562/Adr cells were exposed to IR (1-6 Gy); survival rate was assessed by trypan blue dye exclusion. (c) and (d) K562 and K562/Adr cells were treated with 1 mg mL^{-1} IronQ for 24 h followed by 1-6 Gy IR. Survival rate was assessed by trypan blue dye exclusion. Results represent the averages of three independent experiments. * p<0.05 (combined treatment Vs IR alone)

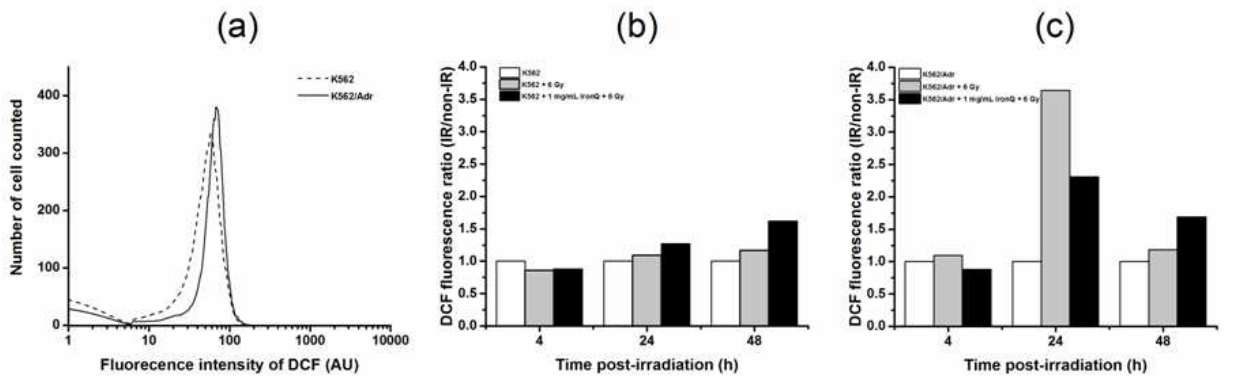

Fig. 2. Changes of levels of intracellular ROS after treatment. (a) Basal level of intracellular ROS in K562 and K562/Adr cells (b) Quantification of fluorescence intensity changes reflecting ROS levels in K562 cells pretreated with or without 1 mg mL^{-1} IronQ followed by exposure to 6 Gy IR. (c) Fluorescence intensity changes reflecting ROS levels in K562/Adr cells pretreated with or without 1 mg mL^{-1} IronQ followed by exposure to 6 Gy IR

IronQ Complex Amplified Radiation-Induced ROS Generation in Cancer Cells

To address the mechanism underlying the radio-enhancing effect of IronQ we evaluated ROS generation in tumor cells. It is well documented that under certain conditions the antioxidant agents also act as a pro-oxidants by producing ROS. We first investigated the basal level of ROS in two cancer cell lines as shown in Fig. 2a and found the ROS level in resistant cells

(K562/Adr) was slightly higher than the parental cell line K562. At 4 h after treatment, neither radiation alone nor combined treatment caused significant change in the production of ROS; the change in ROS level was only observed at 24 h. Figure 2b shows that treatment with IR alone slightly increased ROS levels as a function of time in K562 cells (up to 48 h). Moreover, the ROS generation in combined treatment of cells with IR and IronQ showed additive effect. A significant increase in ROS of 1.75 folds, compared with radiation alone (1.18 fold), was found 48 h after treatment. In contrast, radiation treated K562/Adr cells dramatically increased ROS levels to approximately 3.75 fold higher than control cells at 24 h after treatment and ROS generation decreased to a level similar to K562 cells at 48 h. In combined treatment of K562/Adr the ROS generation also increased by more than 2.3 fold compared with the control at 24 h and decreased to reach approximately 1.9 fold at 48 h. It should be noted that the decreased ROS levels in combined treatment seem to be less than in cells treated with radiation alone (3.75 to 1.18 fold Vs 2.3 to 1.9 fold in IR alone and combined treatment, respectively). From this data, we can conclude that the resistant cells were vulnerable to further oxidative stress induced by exogenous stimulation, but the fact that they had already adapted themselves to unfavorable conditions (by increasing their defense mechanism systems) led them to be resistant to excessive damage.

Oxidative Stress Triggers the Formation of Acidic Vesicular Organelles after Treatments

Autophagy is a cellular defense mechanism responding to stress conditions to either promote survival or lead to cell death, depending on the stress and cell type (Yang *et al*., 2011). We therefore ask whether autophagy was involved in the regulation of radiosensitivity under our experimental conditions. We performed an assay to evaluate the formation of AVO using AO staining (Paglin *et al*., 2001), since the formation of AVO is indicative of the induction of autophagy. K562 and K562/Adr cell lines were irradiated with 6 Gy or treated with IronQ plus IR, after 24 and 48 h of treatment the cells were stained with AO as described in the materials and methods section. To measure acidity of AVO as the cellular response after treatment, we determined the mean red/green fluorescence ratio in control and treated cells. At 24 h after irradiation, the observed increase of the red/green fluorescent ratio in treated cells of both cell lines indicated that IR and IronQ had induced the formation of AVO (Fig. 3). The highest increase in the mean value of red/green fluorescence ratio was for the combined of IronQ and IR treatment for K562/Adr, in which there was found to be a 1.5 fold increase. However, at 48 h post-treatment the mean value of red/green fluorescence ratio had decreased. The increase followed by the decrease in the value of red/green fluorescence ratio was associated with the ROS level produced (which peaked at 24 h post-treatment) and apoptosis induction that could be detected at 48 h post-treatment.

Cell Cycle Distribution and Apoptosis Induction after Treatments

The cytotoxic mechanism underlying the observed differences in cell survival after treatment with radiation and in combined treatment was investigated by analyzing the cell cycle distribution and the degree of apoptosis using flow cytometry. Figure 4 reveals the cell cycle distribution of K562 and K562/Adr cells response to radiation; both cell types respond to radiation by initiating arrest in G2 phases at 24 h post-treatment. Because of the G2 checkpoint deficit in K562 cells, at 48 h K562 cells showed a loss of G2-phase cell populations along with increased accumulation of sub-G1 phase cells (Fig. 4a, Table 1). In contrast, the percentage of the cell population in G2 phases was largely unchanged at 48 h in K562/Adr, as observed with the small amount of sub-G1 phase cells. These results suggest that the resistant K562/Adr cells maintain cell arrest at G2 phases to a large extent for longer than 48 h, but K562 cells are unable to sustain G2 phase arrest for longer than 48 h and subsequently die through apoptosis. In addition, the combined treatment showed decreased G2 phase cells and a corresponding increase in the percentage of sub-G1 cells in comparison to IR alone, while with IronQ alone, the percentage of cells distributed in different cell cycle phases was unchanged (Fig. 4b, Table 1). Therefore, our data indicates that pretreatment with IronQ before IR induced a higher percentage of cell death (sub-G1 cells) than IR alone, especially in K562 cells. We further determined the degree of apoptosis after treatment at different time points (4, 24 and 48 h), by Annexin V/PI co-staining and flow cytometer. At 4 h post-treatment, an increased percentage of apoptotic cells was observed in both IR alone and in the combined treatment for both cell lines. After 24 h the apoptotic level dramatically decreased and reached the level similar to the control (Fig. 5). The second increase in apoptosis then occurred at 48 h. A significant increase of apoptotic level was observed in K562 cells treated with IronQ in combination with IR with contrast to those treated with IR alone and control cells. At 48 h post-treatment, the percentage of apoptotic cells of K562/Adr mildly increased (Fig. 5), which corresponds to the low percentage of sub-G1 cells. Since both cell lines had a similar decrease in survival rates (Fig. 1), but the apoptotic rate was different at 48 h post-treatment, it should be speculated that an intrinsic characteristic of K562/Adr is to prolong G2 phase arrest and delay apoptosis induction.

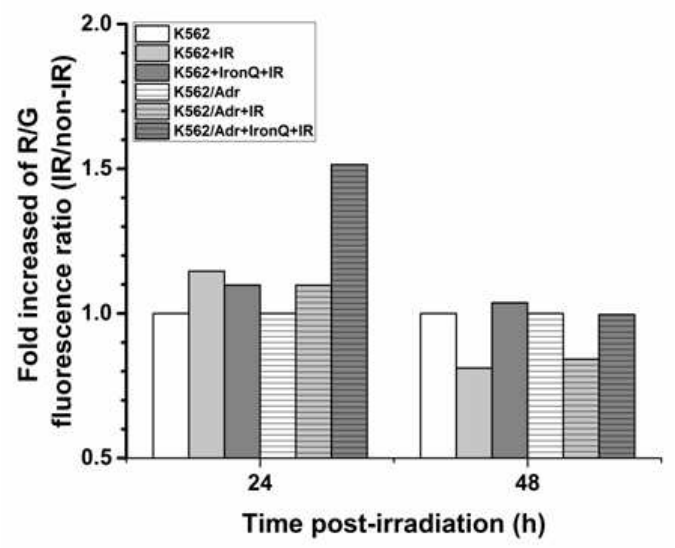

Fig. 3. The formation of Acidic Vesicular Organelles (AVO) was determined using Acridine Orange (AO) staining for both K562 and K562/Adr. The change of red/green fluorescence ratio of AO in erythroleukemic cell lines was determined at 24, 48 h post-treatm

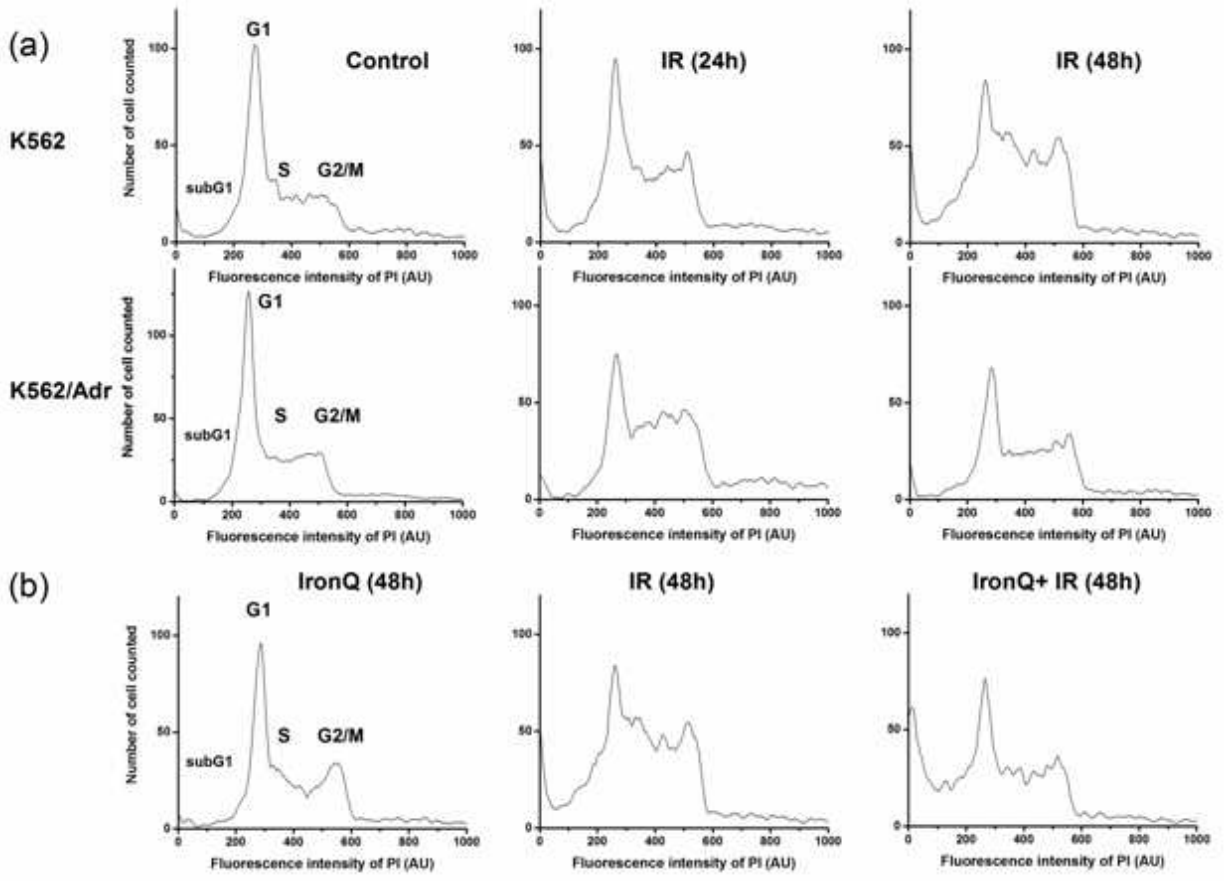

Fig. 4. Flow cytometric analysis of cell cycle distribution in K562 and K562/Adr cells. (a) K562 and K562/Adr cells treated with IR (6 Gy) alone, analysis was performed at 24, 48 h after irradiation. (b) K562 cells treated with IronQ alone (left), IR alone (middle), or a combination of IronQ followed by IR (right), analysis was perform at 48 h after irradiation. Percentage cells in the G1, S and G2/M phases of the cell cycle are reported in Table 1

Table 1. Cell cycle distribution of K562 and K562/Adr cells after treatment with IR and a combination treatment with IronQ and IR

	Phase distribution							
	24h post-irradiation				48h post-irradiation			
Treatment								
K562 cells	subG1	G1	S	G2/M	subG1	G1	S	G2/M
IR (Gy)								
0	8.5±1.9	35.8±0.9	19.5±0.4	36.2±3.2	15.7±3.3	30.5±0.7	23.4±1.3	30.4±1.2
1	7.1±0.7	32.2±2.1	21.0±0.3	39.6±1.6	17.8±2.9	33.8±0.1	20.6±0.1	27.7±3.0
2	7.5±0.9	33.8±0.9	21.9±1.3	36.8±0.5	17.5±0.9	32.6±1.1	19.3±0.5	30.7±2.5
4	6.9±1.1	28.7±2.9	22.2±2.6	42.1±0.7	18.6±4.1	33.4±0.6	18.8±2.0	29.2±1.5
6	9.2±2.2	25.8±2.0	20.2±1.2	44.7±2.9	17.5±2.8	24.8±0.6	24.0±0.4	33.7±3.1
1mg/mL IronQ + IR (Gy)								
0	12.4±2.2	29.5±2.3	19.7±0.8	38.3±0.7	26.7±0.4	27.5±1.5	20.1±0.1	25.6±2.0
1	15.4±0.4	30.8±1.3	18.5±0.7	35.3±1.6	21.6±0.3	33.0±0.4	18.0±0.1	27.4±0.6
2	13.2±1.1	25.4±1.5	21.7±0.3	39.7±0.1	21.7±1.9	26.7±0.5	18.9±0.7	32.7±0.8
4	13.1±0.5	23.6±1.5	20.9±0.3	42.4±1.8	23.4±2.4	19.1±1.0	18.0±0.6	39.4±2.9
6	16.4±1.4	21.7±6.3	18.0±2.0	43.9±6.8	27.7±0.2	24.6±0.5	17.3±1.0	30.5±1.7
K562/Adr cells	subG1	G1	S	G2/M	subG1	G1	S	G2/M
IR (Gy)								
0	5.7±1.7	31.3±2.1	20.5±0.4	42.5±0.8	5.9±1.3	32.6±1.6	23.9±0.3	37.6±1.6
1	5.1±0.8	32.2±2.7	22.6±1.9	40.1±3.8	7.8±2.6	34.2±0.4	22.5±0.6	35.5±2.4
2	5.6±1.3	29.8±1.1	21.8±0.9	42.8±1.5	7.3±1.1	34.6±1.9	22.2±0.9	35.9±2.5
4	5.2±1.2	23.7±0.7	22.4±1.6	48.7±2.6	7.2±1.1	26.5±1.4	22.0±1.3	44.2±1.6
6	4.7±0.6	20.8±1.4	21.3±0.8	53.1±1.6	6.7±0.5	26.1±1.0	21.7±0.9	45.5±0.4
1mg/mL IronQ + IR (Gy)								
0	8.7±4.4	34.9±2.2	24.3±0.8	32.1±6.3	11.3±0.1	36.5±0.3	24.5±0.7	27.7±1.1
1	7.0±1.0	32.0±0.8	23.6±0.1	37.4±0.7	10.9±0.8	35.9±1.4	23.5±0.3	29.7±1.5
2	5.8±0.4	32.1±2.2	23.2±0.4	38.9±2.6	9.8±0.7	33.3±2.1	22.3±0.7	34.6±3.2
4	8.2±0.2	32.8±1.0	24.8±0.8	34.1±0.8	10.1±0.6	33.7±2.2	23.6±0.8	32.6±3.5
6	10.2±0.2	32.2±2.9	25.5±2.4	32.1±5.4	9.0±0.3	29.2±0.3	25.2±2.0	36.6±1.9

Duplicated data represent as Mean ± SD

Discussion

In order to improve the therapeutic index of cancer therapies, strategies such as combining anticancer drugs, or combining drugs with radiation, are often employed. However, combined treatments are sometimes accompanied by severe side effects to normal tissue (Kirwan *et al*., 2003). Therefore, the search for cancer-targeting therapeutic agents that could overcome the resistance of cancer cells to radiation while sparing normal cells has attracted a lot of attention from scientists. In our laboratory we established a quercetin iron (III) complex (IronQ), which exhibited both antioxidant and pro-oxidant properties. This complex is non-toxic to normal peripheral blood cells. We also provide data here (see supplement data in Fig. 1S) that the IronQ can improve the radioresistant activity of the PBMCs by additive action to decreasing of the intracellular ROS levels and a formation of acidic vesicular organelles. On the other hand, they induce the proliferation and differentiation of progenitor cells as previously reported by our group (Kantapan and Dechsupa, 2016). Moreover, the paramagnetic properties of IronQ allow this complex to be used as a contrast agent for T1-wieghted images in magnetic resonance imaging and it is possible to track the cellular accumulation of IronQ complex through the non-invasive MRI technique. Taken together, these characteristics show the potential of IronQ to be utilized as an ROS amplification agent when combined with radiation therapy.

In this study we aim to investigate the radio-enhancing effect mediated by IronQ in doxorubicin sensitive and –resistant erythroleukemic cell lines. We compared the cell survival rate after treating cells with IR alone and IR combined with IronQ. We observed that the combined IronQ and IR treatment resulted in a significant decrease in the cell survival rate when compared to the cells treated with IR alone. However, the radio-enhancing effect of IronQ was found to be greater in K562 than K562/Adr. Interestingly, our results reveal that the over expression of P-gp in K562/Adr cells has no significant effect on radiation resistance (Fig. 1b). Our results are in an agreement with other studies in the field (Ruth and Roninson, 2000). They suggest that Pgp inhibits radiation-induced apoptosis, but this effect of Pgp provides no substantial increase in radiation resistance of the tested cell lines. Similar enhanced effects were also observed in the apoptotic sensitivity after combined treatments with IronQ

complex and IR, especially in K562 cells. Cell cycle arrest and apoptosis are commonly observed in cells treated with DNA-damaging agents, such as IR (Nowsheen and Yang, 2012). Damaged cells arrest in G1, S or G2/M phases to provide an opportunity for cells to repair DNA damage before entering the mitotic phase. Severely damaged cells that cannot be repaired undergo apoptosis (Shinomiya, 2001). Recent studies have indicated that in K562 with mutant or deleted p53, when treated with IR, the G1 phase arrest is absent, but the cells accumulate in G2/M phase (Matsui *et al*., 2001; Jeong *et al*., 2004). We analyzed the cell cycle distribution in response to IR and in combination with IronQ and IR. In both cell lines, we observed an arrest in G2/M phase after 24 h of IR without significant differences between cells treated with IR alone and cells treated IronQ plus IR. At 48 h post-treatment, cells were released from the cell arrest stage and entered the normal cycle, at which time the sub-G1 fraction was observed in K562 cells. On the other hand, K562/Adr cells appeared to undergo prolonged G2 phase arrest (up to 48 h) and had a rather slow and delayed induction of apoptosis. It seems that the prolonged G2 phase arrest may provide an opportunity for cell survival and contribute to resistance in K562/Adr cells (Reinhardt and Yaffe, 2009).

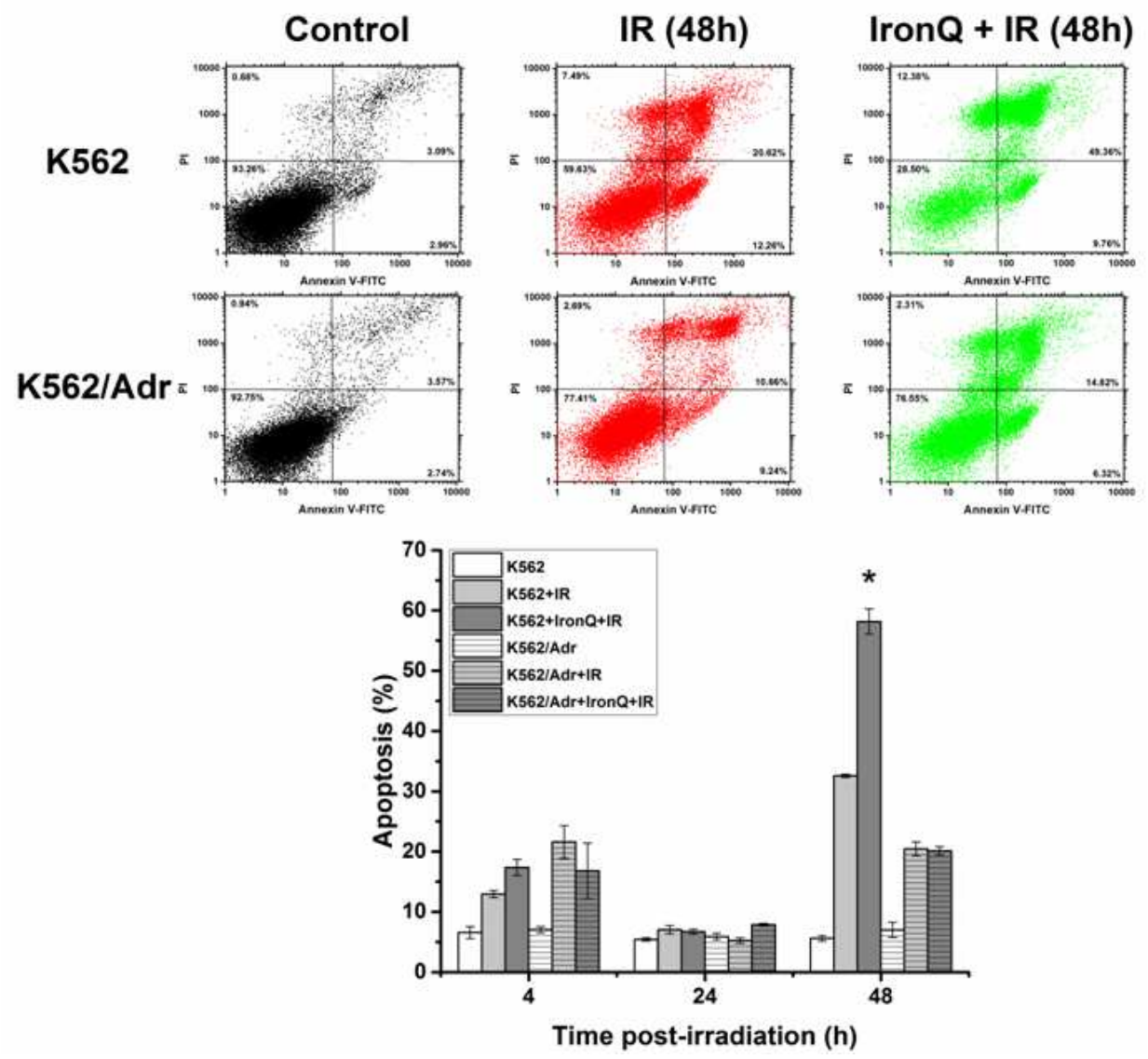

Fig. 5. Flow cytometric analysis of apoptosis in K562 and K562/Adr cells with Annexin V/PI co-staining. The figure represents apoptosis in K562 and K562/Adr cells: Early apoptotic cells (lower-right quadrant) and late apoptotic cells (upper-right quadrant). Quantification of percentage of apoptotic cells (early and late apoptotic) was analyzed at 4, 24, or 48 h post-treatment. * $p<0.05$ (combined treatment Vs IR alone)

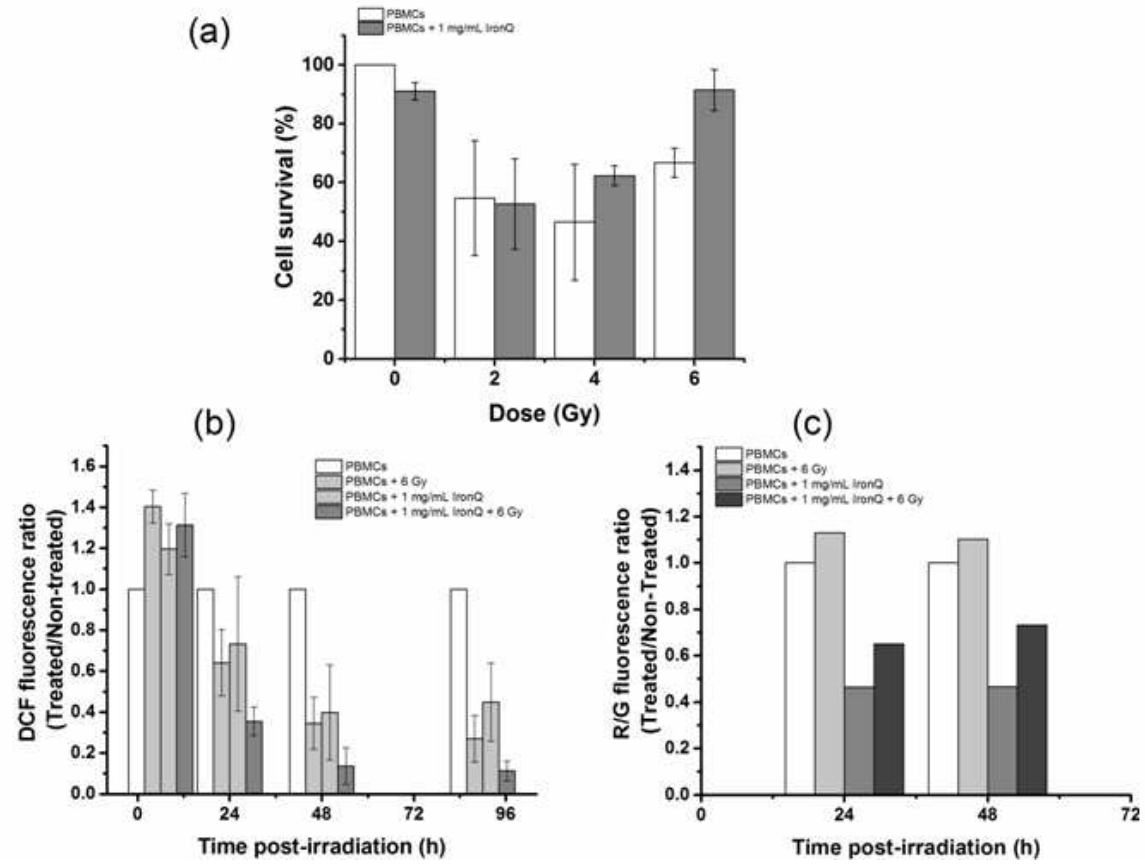

Fig. 1S. The radioprotective effect of IronQ on Peripheral Blood Mononuclear Cells (PBMCs), the ROS level and the formation of AVO in PBMCs after combined-treatment by IronQ and ionizing radiation. (a) Cell survival of PBMCs pre-treated with or without 1 mg mL^{-1} IronQ for 24h followed by exposure to 0, 2, 4 and 6 Gy IR, determined at Day5 post-irradiation. (b) Changes of levels of intracellular ROS after treatment (6, 24, 48 and 90h). Quantification of DCF fluorescence intensity changes reflecting ROS levels in PBMCs cells pre-treated with or without 1 mg mL^{-1} IronQ followed by exposure to 6 Gy IR. (c) The formation of Acidic Vesicular Organelles (AVO) was determined using Acridine Orange (AO) staining for PBMCs. The change of red/green fluorescence ratio of AO in PBMCs was determined at 24, 48 h post-treatment. (Data N = 2)

Radiation induced cytotoxicity has been associated with two distinct form of cell death including apoptotic cell death and mitotic catastrophe (Eriksson and Stigbrand, 2010). Mitotic catastrophe is the product of premature or inappropriate mitosis, this process proceeds cell death, which can occur through necrosis or apoptosis (Portugal *et al*., 2010). A recent study indicated that in p53-deficient cancer cells, the alteration in signaling lead to DNA damage causing mitotic catastrophe cell death (Fragkos and Beard, 2011). P-gp has been shown to inhibit radiation induced caspase-mediated apoptosis (Johnstone *et al*., 1999), but the expression of P-gp has no significant effect on radiation resistance because the inhibition of apoptosis is associated with a further increase in mitotic catastrophe in damaged cells (Ruth and Roninson, 2000). We found parallel results in our study of K562/Adr cells (which are P-gp overexpressing cells), as we observed a low apoptotic rate in K562/Adr cells response to radiation, but the survival rate did not differ between K562 and K562/Adr cells in the long term. It seems likely that early phase radiation induced mitotic catastrophe in K562/Adr due to the P-gp inhibiting apoptosis, however further cell death occurred 3-4 days later. Thus, we can conclude that IronQ has no effect on cell cycle or P-gp function.

We sought to quantify and understand the effect of IronQ to enhance radiation cytotoxicity. Since radiation induced cancer cytotoxicity is associated with the generation of ROS, the ROS concentration in K562 and K562/Adr cells was measured using the fluorescence intensity of DCF to determine the potency of IronQ for ROS formation. We have demonstrated that IronQ complex in combination with IR treated cells results in enhanced ROS formation leading to the enhancement of radiation treatment. These enhancements remained up to 48 h (Fig. 2). The evidence of ROS accumulation indicates the central role of ROS production in

inducing autophagy and cell death in many cancer cell types (Karna *et al*., 2010; Shrivastava *et al*., 2011). A recent report suggests that the differing levels of ROS production elicit different responses in cancer cells. While low levels of ROS were shown to induce autophagy, excessive ROS accumulation triggered both apoptosis and cell death (Zhang *et al*., 2015). In concordance with this study, we observed that the low level of ROS produced at 24 h post-treatment was shown to induce autophagy as a self-defense survival mechanism (Fig. 3), while excessive accumulation of ROS and the prolonged exposure resulted in severe damage and subsequently induced apoptotic cell death in erythroleukemic cells K562 and K562/Adr at 48 h post-treatment (Fig. 5). It is noteworthy that the radio-enhancing effect of IronQ possess a significantly smaller effect on K562/Adr than K562 cells. Thus, the strategy of using an ROS-generating agent to enhance cytotoxicity may not be generally applied to all cases. For resistant cells with a high antioxidant capacity, such strategies enhance cell survival and impair cellular responses to anticancer therapy (Suttana *et al*., 2010: Ding *et al*., 2015). Molecular event triggers in these cells require further study to better understand the mechanism underlying the effect of IronQ, although the present study provides preliminary experimental evidence of the efficiency of IronQ to sensitize cancer cells to radiotherapy.

Conclusion

In the present study, we investigated the radio-enhancing effect of quercetin iron (III) complex (IronQ) on erythroleukemic cells K562 and K562/Adr. Our results indicate that IronQ effectively enhances radiation-induced ROS resulting in excessive cellular damage, ultimately inducing apoptosis and inhibiting cancer cell growth. These paramagnetic agents act as imaging vehicles and exhibit a synergistic effect in combination with radiation against erythroleukemic cells and introduce a new possibility for their applications in theranostic medicine.

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Author's Contributions

All authors equally contributed in this work.

Ethics

The corresponding author confirms that this article is original and contains unpublished material. All of the other authors have read and approved the manuscript and no ethical issues involved.

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