

Original Article

Valproic Acid-Mediated Reduction of DNA Double-Strand Break Repair Capacity of Irradiated MCF-7 Cells

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Abstract

Introduction

Histone deacetylase inhibitors (HDIs), as radiation sensitizing agents, are considered as a novel class of anti-cancer factors, which are studied in various tumor cell-lines. Valproic acid (VPA) is an HDI, which is effectively used in the treatment of epilepsy, migraines, and some particular types of depression. In this study, we evaluated the effects of VPA and ionizing radiation separately, as well as combined, with the alterations of histone H2AX phosphorylation (γ H2AX) at Ser139, a marker of DNA damage and its repair, on MCF-7 breast cancer cell line.

Materials and Methods

Three groups of cells were selected, including 1) pretreated with VPA for 48 h followed by irradiation, 2) VPA only, and 3) irradiation only. The levels of γ H2AX expression were evaluated using Western blot.

Results

The results of our study showed that VPA significantly enhanced the expression of γ H2AX, when applied 48 h prior to irradiation compared to the IR or VPA only treated cells. We also concluded that VPA pre-treatment delayed γ H2AX dephosphorylation and dispersal for up to 12 h after irradiation, while γ H2AX dephosphorylation disappeared in just 2 h when using irradiation alone and without VPA pre-treatment.

Conclusion

Our findings are consistent with the general consensus that VPA efficiently sensitizes cancer cells to the effects of ionizing radiation and prevents DNA double-strand break repair, which leads to enhanced breast cancer cell death.

Keywords: Valproic Acid, Radiosensitizer, γ H2AX

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1. Introduction

Breast cancer (BC) is the second most prevalent cancer worldwide and is considered as one of the most important leading causes of morbidity and mortality in women [1, 2]. The main goal of radiotherapy is to increase DNA impairments, which ends in cell death by mediators of DNA damage [3]. Radiation-modified cell damage occurs by the direct effects of radiation on DNA and also its indirect effects on other cell targets, which is modulated by reactive oxygen species (ROS)[3]. Double-strand break (DSB) is most serious effects of radiation to DNA, which is the most fatal type of DNA impairment and causes cell death if not repaired [4]. Phosphorylation of histone type H2 (H2AX consists of 10% nucleosome; however, it can vary according to the type of cell) is one of the first cell responses against DSB [5]. The phosphorylation of H2AX occurs on the serine139 residue of C-terminal. This phenomenon takes place in the megabase domains of H2AX histone, around the DSB molecule, and is known as γ H2AX [6, 7].

H2AX variant is mainly defined according to its capacity and potency of accepting phosphorylation on the serine139 residue of C-terminal through the activity of PIKKs, such as ATM, DNA-PK, and ATR on the SQ [E/D] motif [8]. The accumulation of most proteins contributing to DNA ionizing radiation-induced foci (IRIF) is dependent on the presence of phosphorylated H2AX (γ H2AX) [9]. DNA damage or its reparation can be evaluated using the number of these foci.

Nowadays, radio sensitizing properties of some drugs has been accepted. These agents can improve the cytotoxicity of radiotherapy for tumor cells. The radio sensitizing potency of histone deacetylase inhibitor (HDI) is a growing field of interest and they are novel candidates of targeted anticancer therapeutics [10-12].

Many studies have shown HDIs to cause cell cycle arrest, stimulate apoptosis and differentiation. Furthermore, they might cause synergistic effects when used in combination with other cytotoxic cancer agents [13-16]. The mentioned activities of HDIs are related to the inhibition of mechanisms resulting in DNA synthesis and DSB repair [16, 17]. There are

notable differences in chemical stability, antitumor activity, and cytotoxicity of HDIs; however, they all induce histone acetylation.

Valproic acid (VPA) is a histone deacetylase inhibitor agents, which nowadays, is effectively used to treat epilepsy, migraines, and some special types of depression [18-20]. Various studies showed that VPA may cause death of tumor cells through increasing acetylation of histones, chromatin condensation, and continuing the expression of γ H2AX induced by radiation [21, 22]. Moreover, several studies suggest that radiotherapy induces cell death, which its improvement by VPA can be related to inhibition of DSB repair [19, 23].

Herein, we aimed to assess potent radiation enhancing properties of VPA on MCF-7 breast cancer cell line. In so doing, we evaluated the effect of VPA and IR in combination and alone on the changes in histone H2AX phosphorylation at Ser139, as a marker of DNA damage and its repair. Furthermore, the level of γ H2AX was measured after radiotherapy for exploring the inhibitory potency of VPA on DSB reparation.

2. Materials and Methods

Immunoblot analysis of phosphorylated histone H2AX (γ H2AX) was used for the investigation of the mechanism mediating radiosensitization by valproic acid. γ H2AX is applied as a sensitive marker of DSB following radiation, so that we can monitor the effect of cell treatment with HDIs prior to irradiation on DSB induction and repair [9, 24]. MCF-7 cells were pretreated with 2 and 4 mM of VPA for 48 h, and then irradiated with two doses of 2 and 4 Gy irradiation.

2.1. Cell Culture and Treatments

Human MCF-7 breast cancer cells (obtained from Pasteur Institute, Tehran, Iran) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 100 U/ml streptomycin, 100 U/ml penicillin in a humidified incubator at 37°C in a 5% CO₂ atmosphere. All the reagents were purchased from Gibco, Germany, unless stated otherwise. Briefly, MCF-7 cells were allowed to attach to the surface for 24

h prior to the treatment. Then, they were treated with 4 and 8 mM of VPA (Sigma Aldrich, Germany) for 48 h at 37°C. The VPA was dissolved in phosphate buffer saline (PBS) to a stock concentration of 100 mM and stored at -20°C. Subsequently, untreated MCF-7 cells and cells pretreated with VPA were irradiated using γ -rays from a Cobalt 60 source at doses of 2 Gy and 4 Gy. The cell plates were placed on the table of cobalt unit. Source to plate surface distance was 100 cm. Afterwards, cells were examined for γ H2AX status, using Western blotting.

2.2. Western Blot Analysis

Adherent and floating cells were lysed in 100 mM of Tris-HCL (pH=7.4), 1 ml of Triton X-100, 5% sodium deoxycholate, 150 mM of NaCl, 5 mM of EDTA, 5% sodium dodecyl sulfate (SDS), and 10% glycerol. The lysis buffer supplemented with phosphatase inhibitor cocktail (Sigma, p004) and protease inhibitor cocktail (Sigma, p8340). Protein concentration of lysates was determined by BCA protein assay kit (Thermo, 23227) and the lysates were adjusted with lysis buffer. The lysates containing an equal amount of protein (20 μ g) were loaded into each lane of a polyacrylamide gel (concentration: 15%).

Proteins were transferred to nitrocellulose membranes for 50 min at 110 V, using transfer buffer (15.6 Mm of tris-base, a20 Mn of glycine, 10% methanol, and pH=8.4%) (88018, Thermo Scientific) and blocked with 3% skim milk in PBS-T (0.05% Tween-20) for 2-3 h at room temperature. Thereafter, the membranes were incubated with anti-phosphorylated histone H2AX (Millipore, 05-636) at 4°C overnight. The blots were rinsed three times with BPS-T, they were then incubated with Goat anti-Mouse IgG and horseradish peroxidase-conjugated secondary antibodies (Millipore, 12-349) for 1-2 h at room temperature on a rotating platform. After incubation with secondary antibody, the membranes were washed three times and immunoreactivity was detected by enhanced chemiluminescence as described by the manufacturer (Amersham ECL Biosciences, RPN2235). ImageJ software (NIH, Bethesda, MD) was used to measure the relative optical densities of the bands. β -actin antibody was

used as the internal control. All the experiments were repeated at least three times.

2.3. Statistical Analysis

The data were analyzed using SPSS, version 16. Kolmogorov-Smirnov Shapiro-Wilk test was used to measure the normality of data distribution. In the cases of normality, One-way ANOVA was run followed by post-hoc Tukey's test. P-value less than 0.05 was considered statistically significant.

3. Results

Our results indicated that irradiation provokes a significant increase in expression of γ H2AX one hour following irradiation. The findings also showed that treatment of cells with VPA alone for 48 h promoted γ H2AX level compared to untreated cells. Although the changes in γ H2AX induced by irradiation and VPA alone are modest compared to the combined effect of pretreatment of cells with VPA followed by irradiation (Figure 1).

In addition, the level of γ H2AX expression was measured after irradiation for exploring the inhibitory potency of VPA on DSB repair. We observed γ H2AX dispersal after a 2 h irradiation, but it was delayed up to 12 h when pre-treated with VPA prior to irradiation (Figure 2).

Western blot bands were scanned and analyzed using ImageJ software (version 1.49p) in order to quantitatively evaluate in vitro irradiation. Both color intensity and the area under density profile were scored. We aimed to convert the profile areas and band intensities to numeric quantities for statistical analysis, making comparisons, and reaching a definitive conclusion (Figures 1 and 2).

γ -H2AX was employed as an indicator of DNA DSBs. MCF-7 cells were cultured with or without VPA for 48 h followed by irradiation. After 1 h, lysates were collected and total cellular proteins were analyzed by Western blotting. Each blot is a representative of three independent experiments with β -actin, used as the loading control. It was significantly different from the controls ($P < 0.001$).

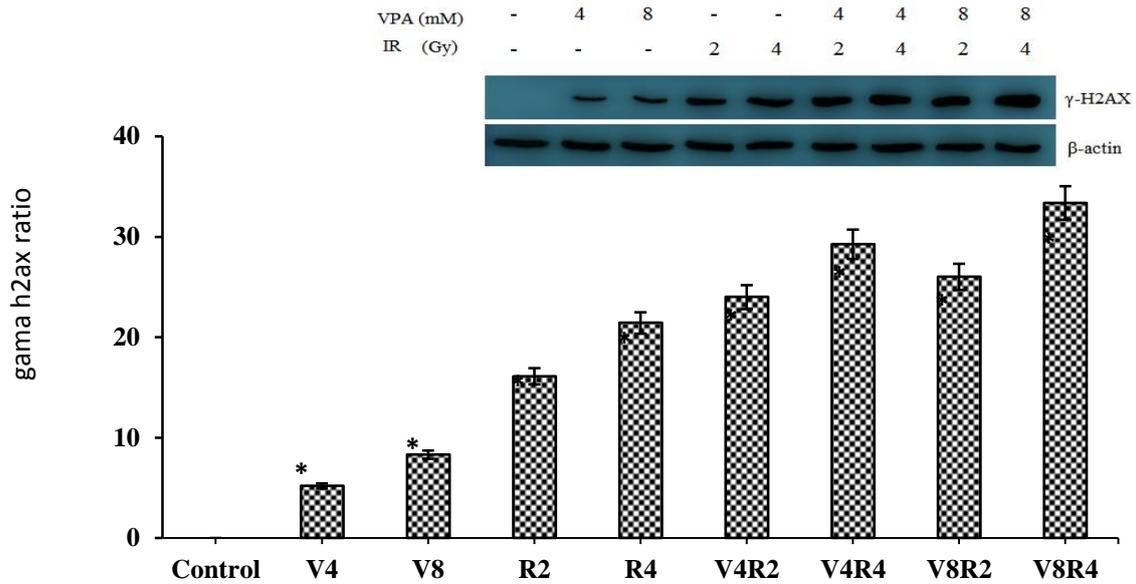


Figure 1. The effect of Valproic acid and ionizing radiation on DNA Double-strand breaks

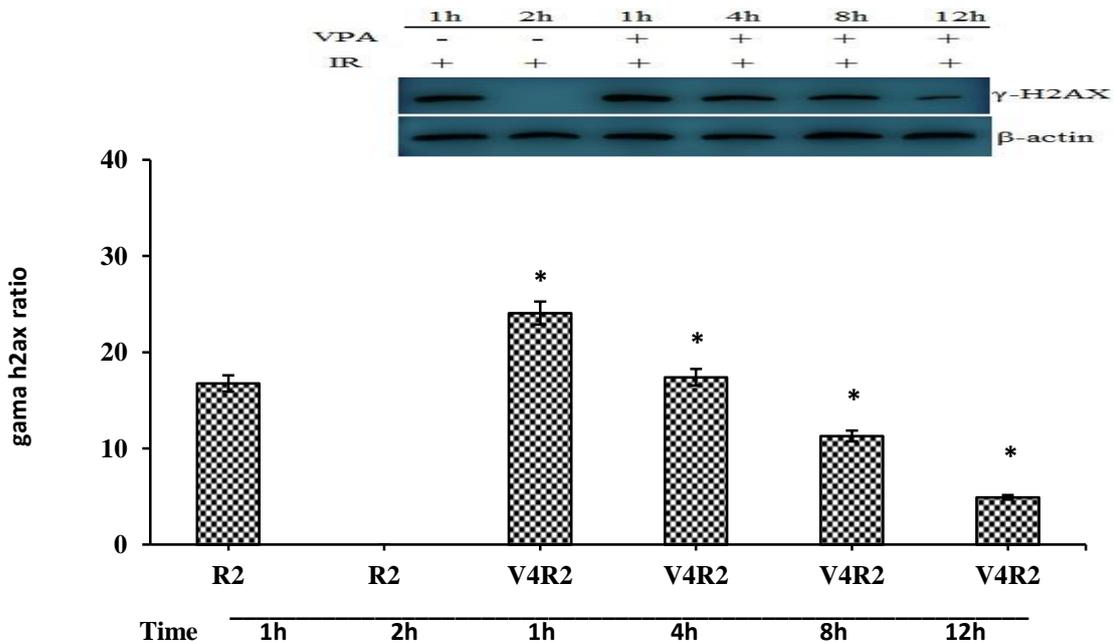


Figure 2. Effect of Valproic acid on dephosphorylation of γ H2AX

*Significantly different from the controls ($P < 0.001$)

Cells were untreated or treated with 4 mM of VPA for 48 h, and then were irradiated at a 2 Gy dose of irradiation. Western blotting analysis of the total cell lysates was performed at 1, 2, 4, 6, and 12 h after irradiation. Each blot is a representative of three independent experiments with β -actin, which was used as the loading control.

4. Discussion

Several HDIs are used as the radio sensitizing agents in the treatment of various tumors [7, 16, 24-26]. However, there are still controversies regarding the mechanisms contributing to HDI-induced radio sensitization, but defining the exact mechanism would help the possibility of

clinical utilization of HDIs as radiation sensitizers.

There were reports claiming that HDIs prevent DSB repair and prolong expression of γ H2AX, a marker for DNA DSBs, following radiation [27]. Also, a variety of HDIs can delay the dispersal of radiation-induced γ H2AX foci [28-31].

The present study analyzed the use of VPA in combination with irradiation, focusing on the effects of VPA on DNA damage in MCF7 cell-line. Our results showed that the 48 h long pre-incubation of MCF7 cells with VPA followed by irradiation leads to enhanced phosphorylation of H2AX in these cells compared to the IR or VPA only treated cells (Figure 1).

Linear correlation between the number of DSBs and γ H2AX protein under the effect of various doses of ionizing irradiations was illustrated before [32]. In fact, the researchers have demonstrated that the kinetic formation and dispersal of γ H2AX has a significant correlation with the kinetic occurrence and reparation of DSBs [33]. In fact, ionizing irradiations cause DNA DSBs and alterations in chromatin structure. These alterations seem to activate ATM protein kinase, the protein that arrests cell cycle and induces apoptosis by phosphorylating and activating BRCA1, CHK2, and H2AX [34, 35].

This phosphorylatory effect of VPA on H2AX can be exerted through the formation of ROS that results in DSBs [36]. ROS can cause macromolecular damages to cellular components such as lipids, proteins, and DNA [37]. Such findings confirm the hypothesis that relates the mechanism of VPA-mediated radiosensitization to the alterations of chromatin structure. The increase in γ H2AX expression of MCF7 cell lines with combination of VPA and IR (Figure 2) suggests the accumulation of hyperacetylated histone tails may result in an increase in the number of sites of ionizing radiation-induced DNA damage.

We also concluded that VPA pre-treatment, delayed γ H2AX dephosphorylation, and dispersal for up to 12 h after irradiation, while

it disappeared in just 2 h when using irradiation alone and without VPA pre-treatment. Overall, the dispersal of γ H2AX expression is related to the damage repair, and single-strand breaks are repaired usually within 2 h [18]. The duration of γ H2AX expression may suggest that VPA-induced radio sensitization is mediated by inhibiting DSB repair. Involving VPA-mediated hyperacetylation of histones, such as H2AX, is one of the possible mechanisms leading to persistent recruitment of DNA damage repair proteins by affecting DNA repair complex. Reduction of other factors involved in DNA repair (e.g., Rad51, Ku86, and Ku70) and the induction of Bax (a pro-apoptotic protein) expression are other suggested mechanisms; however, the exact details of VPA's effect remains unknown [16, 35, 38].

5. Conclusion

VPA sensitizes cells to the effects of ionizing radiation and prevents DNA DSB repair, which leads to enhanced breast cancer cell death. In vitro, VPA in combination with radiotherapy exerts significant antitumor effects. Thus, treatment with VPA in combination with radiotherapy could be applied for the inhibition of the growth of breast cancer cells. Although accepted as a radiosensitizer, further studies are required to explore the detailed mechanism of radiation sensitization activity of VPA. This would be helpful in the development of new classes of drugs to eliminate tumors and to exploit the deleterious characteristics of irradiation to lower its adverse effects as much as possible.

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