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Radiosensitivity enhancement of human thyroid carcinoma cells by the inhibitors of histone deacetylase sodium butyrate and valproic acid.

Marina Perona, Ph.D.\textsuperscript{1,2}, Lisa Thomasz, Ph.D.\textsuperscript{1,2}, Luciano Rossich, Ph.D.\textsuperscript{1}, Carla Rodriguez, M.Sc.\textsuperscript{1}, Mario A. Pisarev, M.D.\textsuperscript{1}, Cinthia Rosemblit, Ph.D.\textsuperscript{1,3}, Graciela A. Cremaschi, Ph.D.\textsuperscript{1,3}, María Alejandra Dagrosa, Ph.D.\textsuperscript{1,2}, Guillermo J. Juvenal, Ph.D.\textsuperscript{1,2}

\textsuperscript{1}Dept. of Radiobiology (CAC), National Atomic Energy Commission (CNEA), Av. Libertador 8250 (1429), San Martín, Buenos Aires, Argentina. \textsuperscript{2}National Scientific and Technical Research Council (CONICET), Av. Rivadavia 1917 (1033), Ciudad Autónoma de Buenos Aires, Argentina. \textsuperscript{3}Neuroimmunomodulation and Molecular Oncology Division, Institute for Biomedical Research (BIOMED), School of Medical Sciences, Pontifical Catholic University of Argentina (UCA), Buenos Aires, Argentina.

Corresponding author: Guillermo Juvenal, Ph.D., Dept. of Radiobiology, CNEA, Av. Del Libertador 8250, Buenos Aires 1429, Argentina.

Telephone # 5411 6772 7186; E mail: juvenal@cnea.gov.ar
Abstract

Radiotherapy is one of the leading treatments for clinical cancer therapy. External beam radiotherapy has been proposed as an adjuvant treatment for patients bearing differentiated thyroid cancer refractory to conventional therapy. Our purpose was to study the combined effect of HDAC inhibitors (HDACi) and ionizing irradiation in thyroid cancer cell lines (Nthy-ori 3-1, WRO, TPC-1 and 8505c). HDACi radiosensitized thyroid cancer cells as evidenced by the reduction of survival fraction, whereas they had no effect in the normal cells. HDACi enhanced radiation-induced cell death in WRO cells. Gamma-H2AX foci number increased and persisted long after ionizing exposure in the HDACi-treated cells (WRO and TPC-1). Moreover, the expression of the repair-related gene Ku80 was differentially modulated only in the cancer cells, by the compounds at the protein and/or mRNA levels. We present in vitro evidence that HDACi can enhance the radiosensitivity of human thyroid cancer cells.

Keywords: radiotherapy, histone deacetylase inhibitors, sodium butyrate, valproic acid, radiosensitivity, thyroid cancer
1. Introduction

Histone acetylation and deacetylation, a process controlled by histone acetylases (HAT) and histone deacetylases (HDACs), respectively, modifies nucleosome and chromatin structures regulating gene expression [1]. HDACs are overexpressed in different cancers, representing an attractive anticancer target [2]. This process can also alter the acetylation status of diverse non-histone targets, including proteins implicated in proliferation, cell death and DNA repair [3], that could be involved in tumor progression and resistance to treatments. Previous studies showed that HDAC inhibitors (HDACi) are effective anticancer agents acting through different mechanisms including induction of cell cycle arrest, apoptosis, mitotic and autophagic cell death [4].

Radiotherapy is one of the leading treatments for clinical cancer therapy [5]. However, tumor radioresistance and radiotherapy side effects limit its therapeutic gain and application. Combination with radiosensitizing agents could increase the therapeutic potential. Many studies show that HDACi are effective radiosensitizers in various types of malignancies [6]. However, the exact mechanism of radiosensitization has not been completely elucidated.

Valproic acid (VA) and sodium butyrate (NaB) are short-chain fatty acids classified as HDACi. VA has been used as an anti-convulsant for more than 40 years [7]. It induces the redifferentiation of different types of cancer cells in vitro and suppresses tumor growth and metastasis in vivo [8, 9]. NaB causes cell cycle arrest, differentiation, and apoptosis in human tumor cell lines [10, 11]. Both compounds enhanced the radiosensitivity of many tumor cells [10-15]. Furthermore, VA is currently part of an active clinical protocol (NCI-06-C-0112), which evaluates the efficacy of the addition
of VA to standard radiation therapy (RT) plus temozolomide (TMZ) in patients with newly diagnosed glioblastoma [16].

Thyroid carcinoma is the most frequent malignancy of the endocrine system [17]. Well-differentiated thyroid carcinoma (DTC), especially papillary and follicular variants, account for about 94% of the cases and the prognosis is favourable. However, there remains a subset of patients with advanced or recurrent disease with a poorer response to conventional therapy [17]. External beam radiotherapy (EBRT) is a treatment option especially for papillary thyroid carcinoma [18, 19]. Moreover, EBRT may also preclude further surgery in the future if the tumor recurs. A clinical need for new therapeutic approaches to improve the clinical outcome for these patients is needed.

The aim of the present study was to evaluate whether NaB and VA could sensitize poor differentiated thyroid cancer cells to radiation.

2. Materials and Methods

2.1. Cell Culture
Normal human primary thyroid follicular epithelial (Nthy-ori 3-1), human follicular (WRO) and anaplastic thyroid cancer cells (8505c) were grown in RPMI medium and 10% fetal bovine serum (FBS). Human papillary (TPC-1) thyroid cancer cells were grown in Dulbecco’s modified Eagle medium and 5% FBS. Cells were kept at 37 °C in 5% CO₂-95% air in a humidified atmosphere. Exponential growing cells were used for the experiments.
2.2. Irradiation Characteristics

Cells were gamma-irradiated with a $^{137}$Cs of 189 TBq source (IBL-437C Irradiator; CIS Bio-International, CEBIRSA, Argentina) at a dose rate of 7.7 Gy/min.

2.3. Viability Assay

Cells were seeded in 96-well plates and incubated with different concentrations of NaB or VA for 24 h. Cell viability was assessed by MTT assay. The absorbance of viable cells was measured in a Spectra Microplate Reader with a test wavelength of 570 nm.

2.4. Clonogenic Assay

Cells were divided in two groups: (1) irradiation (IR) alone and (2) incubated with the drug 24 h before IR (1 mM NaB (IR+NaB) and 1 mM VA (IR+VA)). Clonogenic assay was performed as previously described [20]. Curves were fitted according to the linear-quadratic model (Survival Fraction (SF) = $\exp^{-\alpha(D)+\beta(D)^2}$) using Origin 7.5 software (OriginLab, Northampton, MA). SF at 2 Gy (SF2) and dose modifying factor (DMF) at doses that reduce survival to 37% were calculated.

2.5. Apoptosis determination by nuclear morphology

Cells were handled as described for the clonogenic assay and irradiated at a dose of 3 Gy. Apoptosis was assessed using fluorescence staining with a MIX buffer containing Hoechst 33258 (0.59 mg/mL), 4, 5-diaminofluorescein (DAF) (0.12 mg/mL) and Propidium Iodide (PI) (0.59 mg/mL) as previously described [20].

2.6. Cell cycle
Cells were fixed in absolute ethanol at −20°C, O/N, stained with a solution of PBS containing 60 µg/mL PI and 50 µg/mL RNase and analysed with a flow cytometer (BD FACSCalibur) 6, 24 and 48 h after irradiation. Processing and analysis of flow cytometry data were performed on a PC-based computer system.

2.7. Caspase-3 activity
Caspase-3 activity was measured in the supernatant 24 and 48 h after irradiation with the Caspase-3 Colorimetric Assay Kit (CASP-3-C, Sigma, St. Louis, MO) according to the manufacturer’s instructions.

2.8. Immunofluorescence and quantification of γH2AX
30 min, 4 and 24 hours after irradiation cells were fixed, permeabilized, blocked and incubated O/N with a monoclonal anti-γH2AX antibody (1:500, Upstate, Lake Placid, NY) and detected with FITC labeled secondary antibody (Sigma). Cells were stained, mounted with 6-diamidino-2-phenylindole dihydrochloride dihydrate (DAPI) and examined in an Olympus BX51 epifluorescence microscope. The area of each γH2AX foci was quantified by using the NIH ImageJ software.

2.9. Western Blot
Proteins were extracted in lysis buffer RIPA containing protease inhibitors, electrophoresed on 10% polyacrylamide gels and transferred to polivinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat-dried milk in PBS with 0.2% Tween 20 and 5% bovine serum albumin (BSA) for 1 h at RT and incubated overnight at 4 °C with monoclonal anti-Ku80 (Cell Signaling Technology, USA) and anti-Rad51 (Santa Cruz CA, USA). Membranes were washed and incubated
1 h at RT with peroxidase-labelled secondary anti-rabbit antibody (Amersham). Densitometric analysis was performed using the NIH ImageJ Software (Wayne Rasband, NIH, USA). β-actin was used as a loading control.

2.10. RNA isolation, reverse transcription and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted by TRIzol reagent and cDNA was synthesized using the Superscript III reverse transcriptase (Invitrogen). qRT-PCR was performed with SYBR Green PCR Master Mix (Biodynamics) according to the manufacturer’s instructions. β-actin was used as a loading control. Primers were purchased from Genbiotech SRL, Argentina, and were as follows. Ku80: 5′-TGACTTCTGGATGCACTAATCGT-3′ (forward) and 5′-TTGGAGCCAATGGTCAGTCG-3′ (reverse), Rad51: 5′-CTTTGGCCCACAACCCATTTTC-3′ (forward) and 5′-ATGGCCTTTCTCCACCTCCAC-3′ (reverse); and β-actin: 5′-ACCAACTGGGACGACATGGAG-3′ (forward) and 5′-GTGAGGATCTTCATGAGGTAGTC-3′ (reverse). Expression levels of mRNAs relative to untreated controls were calculated by the ∆∆CT method.

2.11. Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM) of at least three independent experiments. Differences between SF were calculated with Student’s two tails t test. Significant changes between groups means were determined using one way ANOVA, followed by Tukey multiple comparison test. A p value of < 0.05 was considered statistically significant.

3. Results
3.1 HDACi increased the radiosensitivity

We studied the viability of the cells at different concentrations of HDACi. After 24 h, a significant reduction in survival was observed at the higher dose in WRO and TPC-1 cells (1.5 mM, p<0.05, Suppl. Fig.1). Thereby we selected the highest dose (1 mM) with no significant reduction of the viability for the following experiments.

As shown in Figure 1D-I, both HDACi diminished cell clonogenic survival in the cancer cell lines. SF2 values were decreased in WRO cells from 68.4 ± 1.4 in the controls to 42.0 ± 2.7 (p<0.001) in the NaB-treated cells and to 56.3 ± 1.6 in the VA-treated cells (p<0.01). In TPC-1 cells SF2 was reduced from 31.8 ± 0.8 in the controls to 24.1 ± 0.6 (p<0.01) and 27.9 ± 1.9 (p<0.05), respectively. DMF for WRO cells treated with NaB was 1.61 and 1.28 for VA-treated cells, whereas for TPC-1 was 1.26 and 1.08, respectively (Suppl. Table 1). Similar results were obtained in the anaplastic cell line 8505c (Suppl. Fig.2) with SF2 being reduced from 58.4 ± 2.1 to 36.2 ± 1.7 (NaB, p<0.001) and to 37.9 ± 2.6 (VA, p<0.001). DMF were 1.51 for NaB- and 1.44 for VA-treated cells. Normal cells were not radiosensitized after treatment with the same concentration of both HDACi (Fig.1A-C).

3.2 Cell cycle arrest induction

As shown in Figure 2, treatment with both HDACi did not induce significant changes compared to the control cells. A significant G2/M-phase block was observed in response to IR alone and combined with the drugs (p<0.05 and p<0.01) 6 h after irradiation with a concomitant reduction in the G1-phase cells in both cell lines (p<0.001 for WRO cells at 6 h and p<0.05 for TPC-1 at 24 h) (Fig. 2A, D), respectively. The G2/M-phase block persisted in the treated cells compared to the control cells at 24 h after IR (p<0.05 for IR+NaB and p<0.01 for IR+VA, Fig.2B, E) while at 48 h it was significant only for the VA-treated group in the TPC-1 cells.
(p<0.05, Fig. 2F). The combination treatment did not result in a synergistic effect on cell cycle arrest.

3.3 HDACi increase cell death in irradiated cells

A decrease in cell viability and clonogenic survival of NaB and VA treatment indicated an increment of cell death after irradiation. Irradiation alone increased the percentage of apoptotic and necrotic cells in all the groups (Fig. 3A-D). Pretreatment with HDACi enhanced significantly radiation-induced apoptosis in WRO as evidenced by morphological features of programmed cell death such as pyknosis, karyorrhexis, cell shrinkage and cell blebbing (Fig. 3E-G and Suppl. Fig. 3). The predominant mode of cell death at all times was necrosis with statistically significant differences observed in all irradiated groups. An increase was observed with the addition of NaB in WRO cells (p<0.01) and VA (p<0.001) 24 h after irradiation. The activity of the downstream apoptosis effector caspase-3 was enhanced in the irradiated cells at 24 h. Pretreatment with HDACi induced a significant increase in the activity in WRO cells 24 h after irradiation (p<0.01 for NaB and p<0.05 for VA vs. IR) (Fig. 3H). As previously described, there was an increase in apoptosis in TPC-1 cells, although not statistically significant.

3.4. HDACi increased DNA damage and prolong the expression of γ-H2AX foci

To evaluate DNA double-strand breaks (DSB) formation and repair, IR-induced γ-H2AX foci was quantified at different times. Representative images are shown in Figures 4A and B. Average number of γ-H2AX foci per cell in all the irradiated groups was significantly greater than control groups at 30 min and 4 h (Fig. 4C, D, p<0.001). HDACi increased the number of foci per cell (p<0.001 vs. IR) in both cell lines (Fig.
4C, D). Treatment with HDACi alone had a non-significant slight effect on γ-H2AX foci. To determine whether HDACi affected γ-H2AX dispersal, a quantitative analysis of foci size was performed. Foci size histograms (Fig. 4E, F) show that average foci areas per cell were significantly higher than control groups in all the irradiated cells. Pretreatment with both HDACi increased foci size compared to irradiated alone cells (p<0.01 IR+NaB vs. IR in WRO cells at 24 h). Although numerous homogenously situated γ-H2AX foci were found in the early interval, we observed a few separated large foci in the irradiated groups pretreated with both HDACi at later times. Thereby we plotted the frequency of foci larger than 1.0 µm^2. We observed a pronounced persistence of large foci in WRO NaB and VA-pretreated cells at 24 h after IR (p<0.001) and the same effect in TPC-1 cells at 4 h and 24 h after IR (p<0.01 IR+HDACi vs. IR 4 h, and p<0.05 IR+VA vs. IR) (Fig. 4G, H, respectively), even though the frequency was higher in WRO cells. The DNA damage repair could be related with persisting damage being not successfully repaired in the cells pretreated with the HDACi, which in turn could be related to the previous observed radiosensitizing effect.

3.5. Modulation of DNA repair gene expression by HDACi

Mammalian cells can repair DNA DSBs mainly by homologous recombination (HR) and non-homologous end joining (NHEJ). Ku80 protein levels increased in the irradiated only cells (WRO: p<0.05; TPC-1: p<0.001). HDACi decreased protein levels compared to the irradiated only cells (WRO: p<0.001 and TPC-1: p<0.001 IR+VA vs. IR (Fig. 5A, B, respectively). Levels of Rad51 (involved in HR repair) were reduced in all irradiated cells compared to non irradiated cells (WRO: p<0.001 IR+NaB vs. Control; TPC-1: p<0.01 vs. Control, Fig. 5C, D, respectively).
The genes that encode these proteins were studied. We observed a reduction in Ku80 mRNA levels following treatment with both compounds compared to irradiated only cells (WRO: p<0.001 IR+NaB and IR+VA vs. IR; TPC-1: p<0.001 IR+VA vs. IR, Fig. 5E and 5F, respectively). Rad51 mRNA levels were significantly diminished in all irradiated WRO cells (p<0.001, Fig. 5G) whereas in TPC-1 cells the reduction was significant for the IR+VA group (p<0.05, Fig. 5H). We observed no change in the protein and mRNA levels of Ku80 and Rad51 in the normal cells, Nthy-ori 3-1 (Fig. 6A, C, G, E). In the anaplastic cell line, both HDACi diminished the protein level of Ku80 (p<0.05, Fig. 6B).

4. Discussion

Many studies have demonstrated that HDACi can enhance the cellular response to ionizing radiation showing promising properties [6, 10-15, 21, 22]. We observed that the HDACi radiosensitized human thyroid cancer cell lines based on a clonogenic cell survival assay but not the normal thyroid follicular epithelial cells. Data from the survival curves also demonstrate that NaB was more effective in enhancing clonogenic cell death than VA. Interestingly, WRO cells were more radioresistant than TPC-1 and 8505c cells. In agreement with our observation, it was shown that thyroid papillary carcinoma cells were relatively more sensitive to radiation effects than normal thyroid, follicular thyroid cancer and hyperthyroid cells [23].

HDACi have multiple mechanisms to induce cell death such as cell cycle regulation. The radiosensitizing effect seemed not to be related to changes in cell cycle distribution since we observed a similar G2/M delay in all irradiated groups. Furthermore, we observed different behaviors between cell lines. WRO cells showed an activation of the
G2/M checkpoint without G1/S checkpoint activation, which is predictable in cells with a mutant copy of p53 [24], while in TPC-1 cells it was observed an increase in the G2/M delay with a concomitant decrease in cell number in the S phase at 24 h. This difference could be related with TPC-1 cells maintaining a functional copy of p53 [25]. These results are in agreement with those described by Namba et al. [26] and Abou-El-Ardat et al. [27] in irradiated thyroid carcinoma cells.

When we evaluated cell death the percentage of apoptotic and necrotic cells increased in all irradiated groups in a time-dependent manner. Combination with NaB or VA enhanced cell death in WRO cells especially at later times. Caspase-3 activity was enhanced in all irradiated cells. Significant differences were found with the addition of both HDACi in WRO cells. Therefore, the radiosensitizing effect could be associated with the induction of apoptosis and necrosis like other studies have demonstrated [10, 14, 15, 22]. Irradiated TPC-1 exhibited an irreversible senescent-like profile that was associated with the failure to repair DNA damage and could explain the lower level of apoptosis [27].

Unrepaired or misrepaired DNA damage is observed in irradiated cells [28]. Thus, we studied whether cellular DNA repair mechanisms could be inhibited by the combined treatment. Double-strand breaks (DSBs) are the major lethal lesions induced by ionizing radiation. After a DSB, the histone H2AX is rapidly phosphorylated (γ-H2AX) representing a well described in situ DSB marker [29]. Dephosphorylation and dispersal of γ-H2AX foci correlates DNA DSBs’s repair [30] and cellular radiosensitivity [31]. We observed that the number of foci per nucleus was significantly increased with the combined treatment compared to radiation alone. Moreover, foci increased in size in all
irradiated cells. At earlier times, we detected diffuse foci in the cell nucleus while at 24 hours after irradiation, we found irregular and larger foci compared to the irradiated only cells. HDACi significantly increased the percentage of foci larger than 1.00 µm² in the irradiated cells 4 and 24 hours after irradiation. Persisting foci are a signal of unrepaired lesions [30]. This prolongation of γ-H2AX foci levels following the combined treatment suggests that NaB or VA mediated radiosensitization involves an inhibition of the repair of DNA damage in agreement with other studies [14, 22, 32].

Mammalian cells repair DSBs by homologous recombination (HR) and non-homologous end-joining (NHEJ). NHEJ is particularly important for repairing radiation-induced DSBs that cause the loss of clonogenic survival [33, 34]. Many studies show that inhibition of DSB repair is one of the mechanisms for increased radiosensitivity with HDACi [35]. NaB enhanced synchronized HeLa cell's radiosensitivity by the reduction of NHEJ and HR rate [31] and decreased the expression of Ku70 enhancing radiosensitivity of glioblastoma cells [14]. In esophageal squamous cell carcinoma, VA decreased the expression of Rad51 [15]. We found that the protein/gene expression of Ku80 was statistically downregulated in the IR+VA WRO and TPC-1 cells and after in the IR+NaB WRO cells. Both HDACi diminished Ku80 protein levels in the anaplastic cell line, 8505c. On the other hand, Rad51 expression was lower than the controls in all the irradiated cancer cells. No effect was observed in the normal thyroid follicular epithelial cells. These results observed in the normal thyroid cells and the lack of effect of both HDACi on the radiosensitivity, are in agreement with previous reports [15, 36].

5. Conclusion
Our report provides *in vitro* evidence that NaB and VA radiosensitize human thyroid tumor cells mainly by impairing DNA damage repair processes. Therefore, HDAC inhibition could function as a general strategy for enhancing tumor cell radiosensitivity. These findings may have a significant clinical implication for management of those patients bearing well-differentiated thyroid carcinoma who have a high risk of local recurrence and fail to respond to standard therapeutic modality.

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Figures legends

Fig. 1. **Effects on cellular post-irradiation survival.** Survival curves and survival fractions of Nthy-ori 3-1 (A-C), WRO (D-F) and TPC-1 (G-I) at different radiation doses. Results are expressed as the mean ± SEM of each group. °p<0.05, °°p<0.01 and °°°p<0.001 vs. irradiated only cells.

Fig. 2. **Effect of NaB and VA on cell cycle distribution.** WRO (A-C) and TPC-1 (D-F) cells were treated with NaB or VA for 24 hours, irradiated and cell cycle was measured at 6 (A, D), 24 (B,E) and 48 hours (C,F) by flow cytometry. Results are expressed as the mean ± SEM of each group. *p<0.05, **p<0.01 and ***p<0.001 vs. control cells.

Fig. 3. **HDACI effect on radiation-induced cell death.** WRO and TPC-1 cells with or without NaB or VA were irradiated and apoptosis was evaluated after 6, 24, 48 hours by fluorescence microscopy. Percentage of apoptotic cells in WRO (A) and TPC-1 cells (C), or necrotic cells (B) and (D), respectively. E-G Representative images from stained cells 24 hours after irradiation. Apoptotic nuclei labelled with Hoechst 33258 (arrowheads in red) exhibited peripheral chromatin clumping, blebbing and fragmentation (E), cytoplasm of living cells was labelled with DAF (F) and necrotic cells were labelled with IP (G). Enlargement of the boxes showed in Suppl. Fig. 3. Caspase-3 activity was measured in the supernatant of WRO (H) and TPC-1 (I) cells at 24 and 48 hours after irradiation. Results are expressed as the mean ± SEM of each group. *p<0.05, **p<0.01 and ***p<0.001 vs. control cells. °p<0.05, °°p<0.01 and °°°p<0.001 vs. irradiated only cells. x p<0.05, xxx p<0.001 vs. IR+VA.
**Fig. 4.** Quantification of nuclear $\gamma$-H2AX foci, representative images and determination of nuclear $\gamma$-H2AX foci size. WRO and TPC-1 cells were incubated with NaB or VA, irradiated and $\gamma$-H2AX foci were quantified at 30 min, 4 and 24 hours after irradiation. A-B, Representative images from WRO and TPC-1 stained cells 30 min and 24 hours after irradiation. DAPI: staining of nuclear DNA. $\gamma$-H2AX: FITC staining of $\gamma$-H2AX foci. C-D: Average foci number per cell in WRO and TPC-1 cells, respectively. Histograms of the average foci size and foci with size $> 1.1 \mu m^2$ (%) in WRO (E, F) and TPC-1 (G, H). Results are expressed as the mean ± SEM of each group. ***p<0.001 vs. control cells. °°p<0.01 and °°°p<0.001 vs. irradiated only cells. xxxp<0.001 vs. IR+VA.

**Fig. 5.** Changes in the expression of DNA repair proteins in WRO and TPC-1 cells treated with NaB or VA 4 hours after irradiation. Expression of $\beta$-actin was taken as internal control. Representative Western blot of three (n=3) independent experiments for Ku80 (A, B) and Rad51 (C, D) and bar diagram representing the mean optical density of bands obtained in A-D normalized to that of $\beta$-actin. Normalized fold changes of Ku80 (E, F) and Rad51 (G, H) mRNA levels of three independent real time PCR experiments. $\beta$-actin was taken as internal control. Results are expressed as the mean ± SEM of each group. *p<0.05, **p<0.01 and ***p<0.001 vs. control cells. °°°p<0.001 vs. irradiated only cells.

**Fig. 6.** Changes in the expression of DNA repair proteins in Nthy-ori 3-1 and 8505c cells treated with NaB or VA 4 hours after irradiation. Expression of $\beta$-actin was taken as internal control. Representative Western blot of three (n=3) independent experiments for Ku80 (A, B) and Rad51 (C, D) and bar diagram representing the mean optical
density of bands obtained in A-D normalized to that of β-actin. Normalized fold changes of Ku80 (E, F) and Rad51 (G, H) mRNA levels of three independent real time PCR experiments. β-actin was taken as internal control. Results are expressed as the mean ± SEM of each group. °p<0.05 vs. irradiated only cells.

Supplementary Figure 1. Effect of NaB and VA on cell growth. Relative cell viabilities of Nthy-ori 3-1 (A, B), WRO (C, D), TPC-1 (E, F) and 8505c (G, H) incubated with the HDAC inhibitors for 24 hours. Cellular viability was measured by MTT assay. Results are expressed as the mean ± SEM of each group. *p<0.01 vs. control cells.

Supplementary Figure 2. Effects on cellular post-irradiation survival. Survival curves and survival fractions of 8505c (A-C) at different radiation doses. Results are expressed as the mean ± SEM of each group. °p<0.05 and °°°p<0.001 vs. irradiated only cells.

Supplementary Figure 3. HDACI effect on radiation-induced cell death. Enlargements of the boxed sections in the representative images from stained cells 24 hours after irradiation shown in Figure 3 E-G. Apoptotic nuclei labelled with Hoechst 33258 (arrowheads in red) exhibited peripheral chromatin clumping, blebbing and fragmentation (A), cytoplasm of living cells was labelled with DAF (B) and necrotic cells were labelled with IP (C).
A

**Apoptotic cells (%)**

| Control | NaB | VA | IR | IR+NaB | IR+VA |
|---------|-----|----|----|--------|-------|
| 0       | 2   | 4  | 6  | 24 h   | 48 h  |

***, ○, □, ***

B

**Dead cells (%)**

| Control | NaB | VA | IR | IR+NaB | IR+VA |
|---------|-----|----|----|--------|-------|
| 0       | 2   | 4  | 6  | 24 h   | 48 h  |

***, ○, □, ***

C

**Apoptotic cells (%)**

| Control | NaB | VA | IR | IR+NaB | IR+VA |
|---------|-----|----|----|--------|-------|
| 0       | 2   | 4  | 6  | 24 h   | 48 h  |

***, ○, □, ***

D

**Dead cells (%)**

| Control | NaB | VA | IR | IR+NaB | IR+VA |
|---------|-----|----|----|--------|-------|
| 0       | 2   | 4  | 6  | 24 h   | 48 h  |

***, ○, □, ***

E

WRO

F

TPC-1

G

H

**Caspase-3 activity**

| Control | NaB | VA | IR | IR+NaB | IR+VA |
|---------|-----|----|----|--------|-------|
| 0       | 2   | 4  | 6  | 24 h   | 48 h  |

***, ○, □, ***

I

**Caspase-3 activity**

| Control | NaB | VA | IR | IR+NaB | IR+VA |
|---------|-----|----|----|--------|-------|
| 0       | 2   | 4  | 6  | 24 h   | 48 h  |
A. 

**WRO**

- IR (30 min)
- + IR (30 min)

**TPC-1**

- IR (24 h)
- + IR (24 h)

**NaB**

**VA**

B. 

**C.**

![Graph showing average foci/cell for WRO cells with various conditions and time points.](image)

**D.**

![Graph showing average foci/cell for TPC-1 cells with various conditions and time points.](image)

**E.**

![Graph showing average foci size (µm²) for WRO cells with various conditions and time points.](image)

**F.**

![Graph showing average foci size > 1 µm² for WRO cells with various conditions and time points.](image)

**G.**

![Graph showing average foci size (µm²) for TPC-1 cells with various conditions and time points.](image)

**H.**

![Graph showing average foci size > 1 µm² for TPC-1 cells with various conditions and time points.](image)
WRO

|       | C    | NaB | VA | IR  | IR+NaB | IR+VA |
|-------|------|-----|----|-----|--------|-------|
| Control | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| IR    | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| IR+NaB| ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) |
| IR+VA | ![Image](image19.png) | ![Image](image20.png) | ![Image](image21.png) | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) |

\[\text{Relative KU80 O.D.}\]

TPC-1

|       | C    | NaB | VA | IR  | IR+NaB | IR+VA |
|-------|------|-----|----|-----|--------|-------|
| Control | ![Image](image25.png) | ![Image](image26.png) | ![Image](image27.png) | ![Image](image28.png) | ![Image](image29.png) | ![Image](image30.png) |
| IR    | ![Image](image31.png) | ![Image](image32.png) | ![Image](image33.png) | ![Image](image34.png) | ![Image](image35.png) | ![Image](image36.png) |
| IR+NaB| ![Image](image37.png) | ![Image](image38.png) | ![Image](image39.png) | ![Image](image40.png) | ![Image](image41.png) | ![Image](image42.png) |
| IR+VA | ![Image](image43.png) | ![Image](image44.png) | ![Image](image45.png) | ![Image](image46.png) | ![Image](image47.png) | ![Image](image48.png) |

\[\text{Relative Rad51 O.D.}\]
A. Nthy-ori 3-1

B. 8505c

C. Nthy-ori 3-1

D. 8505c

E. Nthy-ori 3-1

F. 8505c

G. Nthy-ori 3-1

H. 8505c

Ku80

β-actin

Rad51

β-actin

Relative expression of Ku80 and Rad51 in Nthy-ori 3-1 and 8505c cells under different treatments (Control, IR, IR+NaB, IR+VA).

Note: The images show the expression levels of Ku80 and Rad51 proteins, with β-actin used as a loading control. The data represent the mean ± SEM of three independent experiments.
• HDAC inhibitors (HDACi) are effective anticancer agents acting through different mechanisms

• Sodium butyrate and valproic acid (HDACi), enhance radiosensitivity in thyroid cancer cells

• The effect was mainly mediated by affecting the ability to repair radiation-induced DNA damage