SPANDIDOS PUBLICATIONS

Scriptaid, a novel histone deacetylase inhibitor, enhances the response of human tumor cells to radiation

TAKESHI KURIBAYASHI, MAKI OHARA, SAKURA SORA and NOBUO KUBOTA

Department of Radiological Sciences, Ibaraki Prefectural University of Health Sciences, 4669-2 Ami, Ami-machi, Inashiki-gun, Ibaraki 300-0394, Japan

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Abstract. A group of histone deacetylase (HDAC) inhibitors has been shown to suppress the growth of a variety of human tumor lines *in vitro* and *in vivo* and they are among the most promising candidates for anti-cancer therapeutic agents. We investigated the ability of scriptaid, a novel HDAC inhibitor and trichostatin A (TSA) to enhance cell killing by radiation in radioresistant SQ-20B cells derived from human head and neck squamous carcinoma. SQ-20B cells were treated with scriptaid or TSA in combination with radiation. Cell survival was determined by a colony formation assay and protein levels were examined by Western blotting. DNA double strand breaks were measured by a γ-H2AX focus assay. Radiosensitization was observed for SQ-20B cells incubated with scriptaid at 5 μM or TSA at 0.1 μM for 24 h. Radiosensitization by scriptaid was accompanied by a prolonged retention of γ-H2AX foci, suggesting that the enhancement of radiation cell killing by scriptaid involved inhibition of DNA double strand break repair. In addition, treatment with scriptaid suppressed expression of Ku80, but not Ku70. Scriptaid may be a useful radiosensitizer in the treatment of radioresistant human carcinomas.

Introduction

Chromatin remodeling plays an important role in transcriptional regulation. In eukaryotic cells, DNA is packaged into chromatin and dynamic processes are required to alter gene expression. Acetylation/deacetylation of histones is one such mechanism facilitated by histone acetyltransferases and histone deacetylases (HDAC), which are emerging as important molecules in transcriptional regulation (1). Acetylation of lysine residues in histones results in more open chromatin structure and activation of transcription. Hypoacetylation of histones results in condensed chromatin structure and repression of gene transcription. Aberrant regulation of histone acetylation is among the epigenetic modifications that contribute to inappropriate gene expression in cancer cells. HDACs participate in this mode of gene regulation and are recognized as promising targets for cancer therapy (2,3). Trichostatin A (TSA) has been used in studies investigating the role of HDAC inhibition on gene expression. However, TSA is of limited therapeutic use due to poor stability *in vivo* and high toxicity. Structurally varied HDAC inhibitors have been discovered, including hydroximates, cyclic peptides, aliphatic acids and benzamides (4). HDAC inhibitors induce differentiation, growth arrest and apoptosis in tumor cells *in vitro* and inhibit tumor growth in animals *in vivo* (5-9). Several such drugs are now in clinical trials.

The HDAC inhibitors have shown synergistic anti-tumor effects with a wide range of chemotherapeutic agents (10). In addition, several studies have shown that HDAC inhibitors, including TSA (11), MS-275 (12), sodium butyrate (13,14), suberoylanilide hydroxamic acid (SAHA) (15) and PCI-24781 (16), sensitize human tumor cells to ionizing radiation.

Using a high-throughput system based on a stably integrated transcriptional reporter to screen a library of compounds, a novel HDAC inhibitor, scriptaid, was identified. Scriptaid belongs to an existing class of hydroxamic acid-containing HDAC inhibitors and is less toxic than TSA (17). It has been shown previously that scriptaid and TSA induce growth inhibition, cell cycle arrest and apoptosis in human cells, suggesting recovery of tumor suppressor function in response to histone deacetylase (18,19). These data indicate that scriptaid is a promising candidate for further study in cancer therapy. In this study, we investigated the effect of scriptaid on the radiosensitivity of human tumor cells. The data presented indicate that scriptaid enhances the response of human tumor cells to radiation and the radiosensitizing effect is associated with a decreased capacity of cells to repair DNA double strand breaks.

Materials and methods

Cell culture and chemicals. The human squamous cell carcinoma cell line SQ-20B was maintained in α-minimal essential medium (α-MEM) supplemented with 20 mM 4-(2-hydroxyethyl) pipderazineethane sulphonic acid (HEPES), 8 mM $NaHCO_3$, streptomycin, penicillin and 10% fetal bovine serum. Cells were cultured in a humidified incubator at 37˚C

Correspondence to: Dr Nobuo Kubota, Department of Radiological Sciences, Ibaraki Prefectural University of Health Sciences, 4669-2 Ami, Ami-machi, Inashiki-gun, Ibaraki 300-0394, Japan
E-mail: kubota@ipu.ac.jp

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with a mixture of 98% air and 2% CO_2. Scriptaid (6-(1,3-dioxo-*1H*,*3H*-0benzo[*de*]isoquinolin-2-yl)-hexanoic acid hydroxyamide) and TSA were purchased from Sigma (St. Louis, MO, USA).

Irradiation. Cells were irradiated with 10 MV X-rays from a linear accelerator (Mitsubishi Medical Linac, Mitsubishi Electric, Tokyo, Japan) at a dose rate of ~4 Gy/min. Doses were measured using an Innax Dosemaster (NE Technology, Berks, UK) before irradiation.

Clonogenic assay. Cell survival was measured by a colony formation assay (20). Briefly, cells were seeded in 24-cm^2 flasks and incubated for ~24 h before treatment with scriptaid or TSA. Cells were exposed to scriptaid or TSA for 16 h, irradiated with X-rays and incubated in the presence of the drug for a further 8 h. Cells were washed with Dulbecco's phosphate-buffered saline (PBS) and dispersed with 0.05% trypsin containing 0.02% EDTA. Single cells were counted and seeded in 60-mm dishes at various cell densities. The dishes were incubated in a CO_2 incubator for 12 days. Colonies were stained with crystal violet dissolved in 20% methanol. Colonies of >50 cells were counted as survivors.

Assessment of apoptosis. Induction of apoptosis was assayed by detecting apoptotic bodies. Cells were exposed to scriptaid for 16 h and irradiated with X-rays (6 Gy). The cells were incubated in the presence of scriptaid for a further 8 h. The medium was then removed and replaced with fresh medium free of the drug. Twenty-four hours after X-irradiation, both floating and attached cells were collected by centrifugation, resuspended in a fixative solution containing 3% para-formaldehyde in PBS and stained with the Hoechst 33258 fluorescent DNA stain (Sigma). The cells were placed on microscope slides and covered with glass cover slips. The number of apoptotic cells was counted and cells were photographed using a fluorescence microscope.

Western blot analysis. Cells were treated with scriptaid for 24 h. In the case of a combination of scriptaid and X-irradiation, cells were treated with scriptaid for 16 h, irradiated with X-rays and incubated in the presence of the drug for a further 8 h. Cells were lysed in lysis buffer (Cell Signaling Technology, Beverly, MA, USA) and then frozen and thawed three times. The lysates were centrifuged at 15,000 rpm to remove any cellular debris. Protein levels were quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins were resolved by electrophoresis on 7.5% poly-acrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (20). Target protein levels were assessed using antibodies to Bcl-2, Apaf-1, Ku70, Ku80 (Cell Signaling Technology) and actin (Chemicon International Inc., Temecula, CA, USA). Primary antibodies were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ, USA).

Immunofluorescent staining for γ-H2AX. Cells were grown on glass slides placed in 60-mm dishes and exposed to scriptaid at 5 μM for 16 h, irradiated with X-rays of 1 Gy and incubated in the presence of the drug for a further 8 h. At specified times, medium was removed, and cells were fixed with cold methanol for 20 min followed by acetone for 5-10 sec. Cells were blocked with 10% bovine serum albumin in PBS for 20 min, washed twice with PBS and incubated with anti-γ-H2AX antibody (Upstate Biotechnology, Charlottesville, VA, USA) for 1 h. Cells were again washed twice with PBS before incubating in the dark with a FITC-labeled secondary antibody for 1 h. Cells were washed twice with PBS, incubated in the dark with 4',6-diamidino-2-phenylindole (DAPI) in PBS for 5 min and again washed twice. Cover slips were mounted and slides were examined with a fluorescence microscope (Olympus, Japan). Images were captured by a CCD camera and γ-H2AX foci were counted in at least 50 cells.

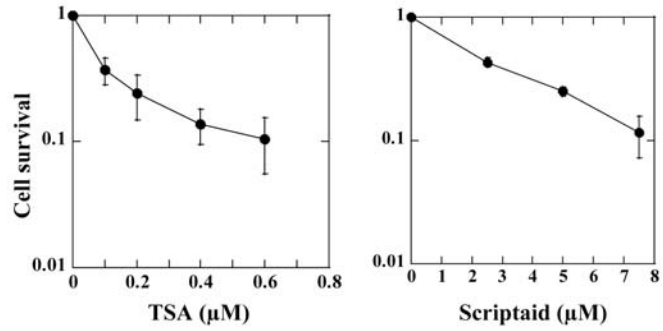

Figure 1. Effect of TSA or scriptaid on survival of SQ-20B cells. Cells were exposed to either drug for 24 h at 37˚C and cell survival was measured by colony formation assay. Data points and bars represent the average and standard errors, respectively, from two or three separate experiments.

Results

Radiosensitization by scriptaid. First, we examined the effect of scriptaid and TSA on survival of SQ-20B cells by a colony formation assay. Cells were treated with various concentrations of the drugs for 24 h. Dose-dependent curves of cell survival of SQ-20B cells treated with scriptaid or TSA are shown in Fig. 1. To assess the effects of scriptaid on the radiosensitivity of SQ-20B cells, we chose the concentration of 5 μM of scriptaid and 0.1 μM of TSA, respectively, which allowed cell survival at a rate of 0.3-0.4. We constructed cell survival curves for SB-20B cells after X-irradiation in the presence or absence of scriptaid and TSA. The cells were exposed to scriptaid or TSA for 16 h at 37˚C, irradiated with X-rays and incubated for an additional 8 h. SQ-20B cells showed a significant increase in radiation-induced cell death when treated with scriptaid or TSA (Fig. 2). The radiosensitivity enhancement ratios measured at a survival level of 10% were 1.4 and 1.5, respectively.

Effect of scriptaid on apoptosis. Fig. 3a shows the percentage of apoptotic cells 24 h after X-irradiation. SQ-20B cells exhibit a relatively low basal level of apoptosis. After exposure of cells to X-rays with and without scriptaid, no significant increase in apoptosis was observed. The protein level of Bcl-2 remained essentially unchanged after drug treatment, X-irradiation or both (Fig. 3b). In addition, the expression of Apaf-1, which regulates the activation of caspase-9, was

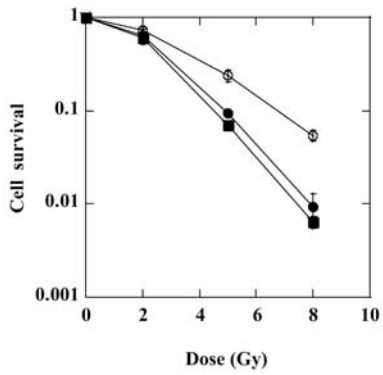

Figure 2. Radiosensitization of SQ-20B cells by scriptaid or TSA. Cells were incubated with DMSO (○), 5 μM scriptaid (●) or 0.1 μM TSA (■) for 16 h, irradiated with X-rays and then incubated for a further 8 h. Thereafter, cells were trypsinized, counted and plated in 60-mm dishes. Data points and bars represent the average and standard errors, respectively, from two or three separate experiments.

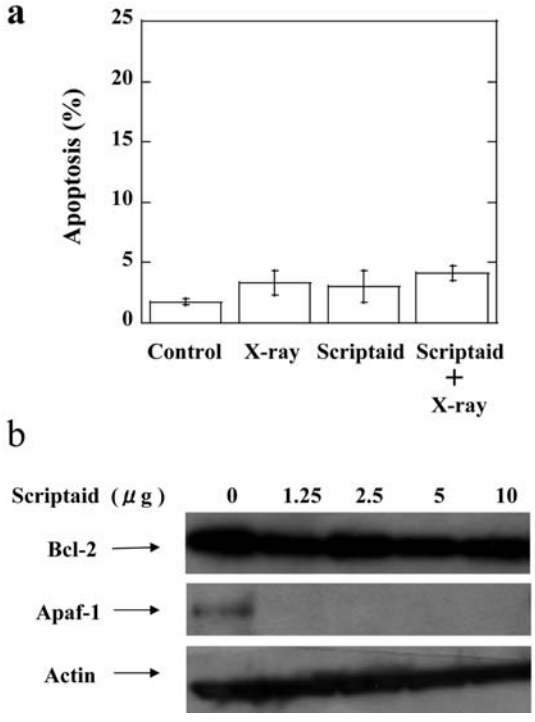

Figure 3. (a) Apoptosis in SQ-20B cells following X-irradiation (6 Gy), scriptaid treatment (5 μM) or both. Apoptotic cells were measured 24 h after X-irradiation. Error bars represent standard errors from three separate experiments. (b) Effect of scriptaid on the expression of apoptosis-related proteins in SQ-20B cells. Whole lysates were subjected to Western blotting for Bcl-2 and Apaf-1. Western blots for actin are shown as loading controls.

downregulated by scriptaid. These data suggest that SQ-20B cells are radiosensitized without a significant effect on apoptotic cell death.

Effect of scriptaid on the retention of radiation-induced γ-H2AX foci and expression of DNA repair-related proteins. We measured the effect of scriptaid on the retention of radiation-induced γ-H2AX foci, an indicator of DNA double strand breaks, in SQ-20B cells. The number of γ-H2AX foci per cell was counted and the results indicate that more foci formed in cells irradiated with X-rays and incubated with scriptaid 4 to 24 h after irradiation than with X-rays alone (Fig. 4). To investigate the mechanism responsible for scriptaid-mediated prolongation of γ-H2AX foci after irradiation, we examined the effect of scriptaid on the expression of DNA repair-related proteins Ku70 and Ku80. Cells were exposed to scriptaid at various concentrations for 24 h and then were collected and lysed for immunoblot analysis. As shown in Fig. 5, scriptaid downregulated expression of Ku80, but not Ku70, in SQ-20B cells. The decrease in Ku80 expression was evident at a 1.25 μM concentration of scriptaid. In addition, there was a decrease in the level of Ku80 protein after scriptaid treatment in X-irradiated cells. These data suggest that Ku80 down-regulation induced by scriptaid seems to play an important role in inhibiting the rejoining of radiation-induced DNA double strand breaks.

Discussion

It is known that HDAC inhibitors induce cell cycle arrest, terminal differentiation, mitotic cell death and inhibition of angiogenesis in many human tumor cell lines and *in vivo* tumors. In addition, they activate extrinsic and intrinsic apoptosis pathways (4,21). In comparison, normal cells are more resistant to HDAC inhibitor-induced cell death (9). Thus, HDAC inhibitors that induce differentiation and/or death of tumor cells may provide an alternative or additional approach to the treatment of cancers.

Sun *et al* identified a novel HDAC inhibitor, scriptaid, using a high-throughput system based on a stably integrated transcriptional reporter to screen the entire library consisting of 16,320 compounds (17). Scriptaid belongs to an existing class of hydroxamic acid-containing HDAC inhibitors, which includes TSA. Scriptaid and TSA possess the same hydroxamic acid group, an aliphatic chain and an aromatic cap at the other end and both have a five-carbon link between a bulky end group and the hydroxamic acid moiety. Scriptaid has been reported to increase the levels of acetylated histones H3 and H4 in cancer cells and to be highly effective in suppressing the growth of human endometrial and ovarian cancer cells with little effect on normal human endometrial epithelial cells (19). These findings suggest that scriptaid may be a useful agent with relatively low toxicity for the treatment of carcinoma.

Radiotherapy is an important component in the treatment of cancers. However, radiotherapy alone often fails to suppress tumor growth in advanced stages. It is generally considered that the presence of inherently radioresistant tumor cells is one of the reasons for radiation therapy failure. Thus, radio-sensitivity of tumor cells is a major determinant of local control (22). The HDAC inhibitors have shown synergistic cell killing for human tumor cell lines with chemotherapeutic drugs (23,24). In addition, several HDAC inhibitors have been reported to enhance cell killing by ionizing radiation in human tumor cells (8,12-14,16). In this study, we demonstrated that the HDAC inhibitor scriptaid also sensitized human tumor cells to radiation. It has been reported that HDAC inhibitors are potent inducers of apoptotic cell death (9). After exposure of cells to X-rays with and without scriptaid, no significant increase of apoptosis was observed, indicating the mechanisms underlying scriptaid- or TSA-induced radiosensitization of

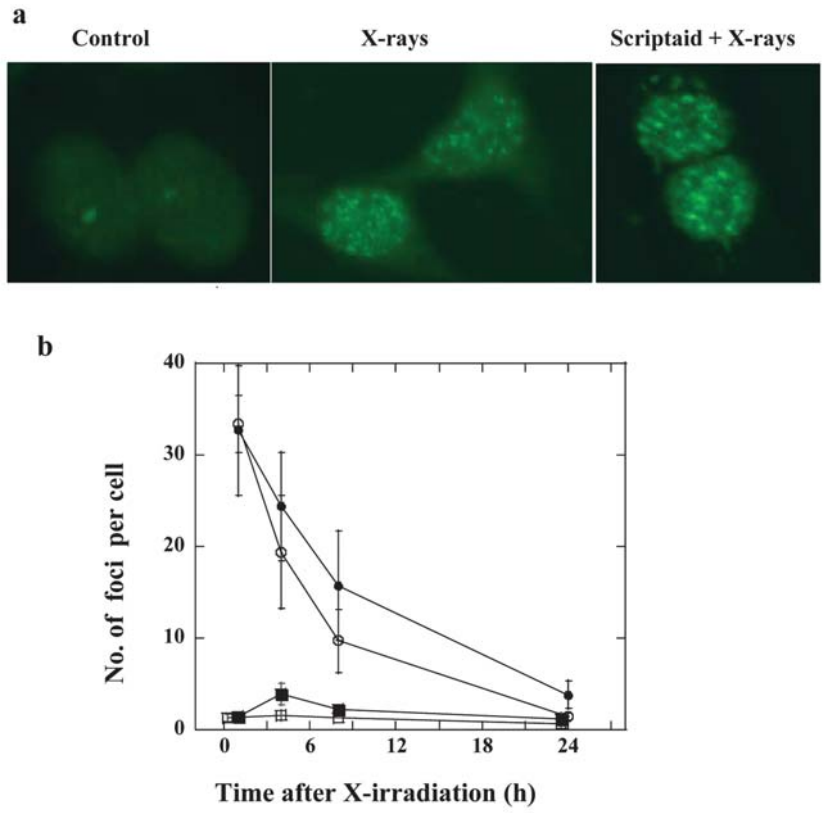

Figure 4. (a) Micrographs of γ-H2AX foci from cells taken 1 h after exposure to X-rays alone or X-rays in combination with scriptaid. (b) Quantitative analysis of γ-H2AX foci/cell following X-irradiation, incubation with 5 μM scriptaid or both in SQ-20B cells. Cells were exposed to scriptaid for 16 h, irradiated with X-rays (1 Gy) and incubated in the presence of the drug for a further 8 h. Then cells were changed to fresh medium without the drug. Control (□), scriptaid alone (■), X-ray alone (○), scriptaid + X-ray (●). Error bars represent standard errors from two or three separate experiments.

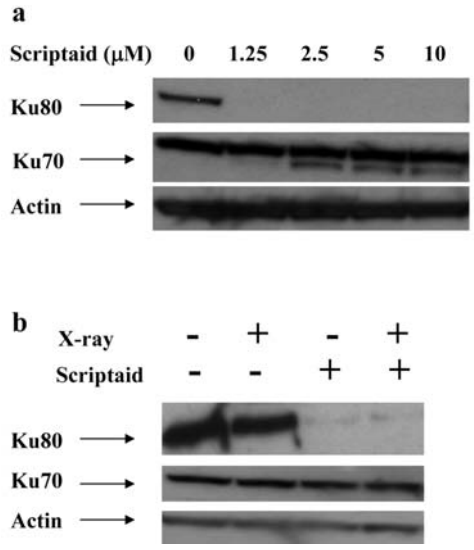

Figure 5. Effect of scriptaid on the expression of Ku70 and Ku80 in SQ-20B cells. (a) Cells were treated with scriptaid at various concentrations for 24 h. (b) Cells were treated with scriptaid (5 μM) for 16 h, irradiated with X-rays and incubated in the presence of the drug for a further 8 h. Whole cell lysates were subjected to Western blotting for Ku70 and Ku80. Western blots for actin are shown as loading controls.

SQ-20B cells do not involve an increase in apoptosis. Similar results were reported in TSA-mediated radiosensitization in SQ-20B cells (11).

Recently, γ-H2AX expression has been established as a sensitive indicator of DNA double strand breaks induced by low doses of radiation. The histone H2AX is phosphorylated (γ-H2AX) at sites of radiation-induced DNA double strand breaks and readily forms γ-H2AX foci, which are visible by immunofluorescence staining (25). In several studies on HDAC-mediated radiosensitization in human tumor cells, the prolonged retention of radiation-induced γ-H2AX foci, a hallmark of DNA double strand breaks, was observed in tumor cells after treatment with a combination of radiation and HDAC inhibitors when compared to radiation alone (12,15). These findings indicate that the mechanism responsible for HDAC-mediated radiosensitization is the inhibition of DNA double strand breaks. Recently, it has been reported that sodium butyrate (NaB) and SAHA (vorinostat) suppressed the levels of Ku70, Ku80 and Rad50 proteins (14,15), which play a role in the non-homologous end-joining pathway for DNA double strand break repair (26). In the present study, the number of radiation-induced γ-H2AX foci was higher in cells exposed to X-rays in combination with scriptaid 4-24 h after X-irradiation than in cells exposed to X-rays alone. We also examined the effect of scriptaid on the levels of Ku70 and Ku80 in SQ-20B cells. Treatment with scriptaid suppressed the expression of Ku80, but not Ku70 (Fig. 5), suggesting that Ku80 is the target of radiosensitization by scriptaid in SQ-20B cells. Ku80 is an important component of the DNA double strand repair system (27) and cells deficient in the Ku80 protein are hypersensitive to radiation (28,29). It is well known

genic mutations in HRAS, which frequently occur in es of cancers, could contribute to radiation resistance of tumor cells (30,31). It has been shown that Ku80 contributes to the oncogenic HRAS-mediated increase in the capacity of NIH3T3 cells to repair DNA double strand breaks and to afford protection against ionizing radiation (32). Taken together, it is considered that scriptaid sensitizes SQ-20B cells to radiation by reducing the expression of Ku80 and subsequently inhibiting DNA double strand break repair by a non-homologous end-joining pathway.

In summary, the present study indicates that the molecular mechanism underlying radiosensitization by scriptaid is due, at least in part, to the downregulation of the Ku80 protein. Our results show that scriptaid is a promising agent that may be useful in combination with radiotherapy against radio-resistant carcinoma.

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