

A novel resveratrol analogue HS-1793 treatment overcomes the resistance conferred by Bcl-2 and is associated with the formation of mature PML nuclear bodies in renal clear cell carcinoma Caki-1 cells

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Abstract. Bcl-2 protects cancer cells from the apoptotic effects of various chemotherapeutic agents. Inhibition or down-regulation of Bcl-2 represents a new therapeutic approach to bypass chemoresistance in cancer cells. Previously we designed and synthesized the resveratrol analogue HS-1793 displaying stronger antitumor efficacy than resveratrol and further demonstrated the HS-1793 resistance conferred by Bcl-2 in human leukemic U937 cells. We undertook this study to determine if HS-1793 treatment can bypass the anti-apoptotic effects of Bcl-2 in human renal cancer cells, with a specific focus on the involvement of promyelocytic leukemia nuclear bodies (PML-NBs). Experiments were conducted with Bcl-2-overexpressing human renal clear cell carcinoma Caki-1 cells. Various apoptosis assessment assays demonstrated that HS-1793 overcomes the resistance conferred by Bcl-2 in Caki-1 cells by inducing apoptosis. We elucidated that HS-1793-induced formation of mature promyelocytic leukemia (PML) nuclear bodies (NBs) correlates with overcoming the anti-apoptotic effects of Bcl-2 in Caki-1 cells. Our findings show that the resveratrol analogue HS-1793 might provide a novel promising strategy for overcoming the resistance conferred by Bcl-2 via PML protein and the formation of mature PML-NBs.

Introduction

Drug resistance remains one of the primary causes of sub-optimal outcomes in cancer chemotherapy. The study of the mechanisms by which cancers elude treatment has yielded a wealth of information on how to circumvent drug resistance in cancer cells and/or design agents that are not subject to the usual means of resistance (1). Anti-apoptotic factors, including Bcl-2, protect the tumor cells from the apoptotic effects of various antineoplastic agents and thus impair the ability to achieve remission and cure with chemotherapy (2,3). Renal clear cell carcinoma expresses high levels of Bcl-2, which is one of the early discovered and thoroughly studied inhibitors of apoptosis (4). Therefore, bypassing resistance conferred by Bcl-2 represents an attractive therapeutic strategy against renal clear carcinoma cells.

Since the discovery of Bcl-2 as an anti-apoptotic protein, several theories explaining anti-apoptotic mechanism exerted by Bcl-2 have been proposed (5). Bcl-2 has been demonstrated to block activation of the intrinsic mitochondrial apoptotic pathway by preventing the release of proapoptotic mitochondrial proteins such as cytochrome c into the cytosol. This prevents the activation of caspase-9 and its downstream caspases (4,6). Bcl-2 can regulate intracellular Ca²⁺ levels (7).

Previous studies showed that increased expression of Bcl-2 has been associated with poor response to chemotherapy in various malignancies (8). In some preclinical systems, Bcl-2 overexpression has been shown to attenuate apoptosis or to restore the clonogenic potential of malignant progenitor cells (9,10). In addition, a previous study showed that overexpression of Bcl-2 attenuates resveratrol-induced apoptosis through the inhibition of caspase-3 activity and sustained expression of the inhibitor of apoptosis protein (IAP) caspase inhibitors (10).

Resveratrol, a naturally occurring phytoalexin (3,4',5-trihydroxystilbene) contained in medicinal plants, grape skin,

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peanuts and red wine (11,12), acts on the process of carcinogenesis by affecting the three phases (tumor initiation, promotion and progression) and is also able to activate apoptosis (10,13-15). The chemopreventive and chemotherapeutic properties associated with resveratrol offer promise for the design of new chemotherapeutic agents. However, resveratrol is not a potent cytotoxic compound when compared with other chemotherapeutic drugs. Thus, exposure to high doses of resveratrol is required to induce apoptosis in cancer cells (10,16-19). Moreover, resveratrol's biological activity is limited by its photosensitivity and metabolic instability. Thus, several studies were undertaken to obtain synthetic analogues of resveratrol with potent activity. We previously demonstrated that a novel resveratrol analogue HS-1793 induces apoptosis and overcomes the resistance conferred by Bcl-2 in U937 cells through 14-3-3 (20).

The promyelocytic leukemia protein (PML) is a tumor suppressor that distinctly localizes to a nuclear substructure called the PML nuclear body (PML-NB). Numerous proteins reside in PML-NBs. Consequently, PML-NBs are implicated in the regulation of various cellular functions, such as the induction of apoptosis and cellular senescence (21).

This study was undertaken to determine if HS-1793 treatment can bypass the anti-apoptotic effects of Bcl-2 in renal clear cell carcinoma, with a specific focus on the involvement of PML-NBs. For this study, we employed Bcl-2-overexpressing renal clear cell carcinoma Caki-1 (Caki-1/Bcl-2) cells. As will be shown, a novel resveratrol analogue HS-1793 treatment overcomes the resistance conferred by Bcl-2 and is associated with the formation of mature PML-NBs in Caki-1/Bcl-2 cells.

Materials and methods

Reagents. Rabbit polyclonal anti-human p53 (full length), Sp100, PML, CBP, SUMO-1, Daxx and mouse monoclonal anti-human PML and CBP antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit and horse anti-mouse IgG antibodies were obtained from Vector (Burlingame, CA). Texas Red-conjugated horse anti-mouse and goat anti-rabbit IgG antibodies were obtained from Molecular Probes (Eugene, OR, USA). JC-1 was obtained from Molecular Probes (Eugene). TO-PRO3 was obtained from Molecular Probes (Eugene). Neomycin sulfate (G418) was from Calbiochem (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Gaithersburg, MD). Mouse monoclonal anti-human SC35 antibody, resveratrol and propidium iodide (PI) were obtained from Sigma (St. Louis, MO).

Preparation of resveratrol analogues. To obtain analogue with improved antitumor activity, we substituted the stilbene double bond present in resveratrol with a naphthalene ring. Detailed procedure to synthesize HS-1793 was described in a previous study (20).

Cell culture and establishment of Bcl-2-overexpressing Caki-1 cells. Renal clear cell carcinoma Caki-1 cells were obtained from the American Type Culture Collection (ATCC; Rockville,

MD). The culture medium used throughout these experiments was DMEM medium, containing 10% fetal bovine serum (FBS) and 100 μ g/ml penicillin-streptomycin. Bcl-2-overexpressing Caki-1 cells were generated using a pMAX vector containing the human Bcl-2 gene (provided by Dr Rakesh Srivastava, NIH/NIA). The Caki-1 cells were transfected in a stable manner with the pMAX Bcl-2 plasmid, or control plasmid pMAX vector using Lipofectamine as prescribed by the manufacturer (Invitrogen). After 48-h incubation, transfected cells were selected in primary cell culture medium containing 700 μ g/ml G418 (Invitrogen). After 2 or 3 weeks, to rule out the possibility of clonal differences between the generated stable cell lines, the pooled Caki-1/pcDNA 3.1 and Caki-1/Bcl-2 clones were tested for Bcl-2 expression by immunoblotting and were used in this study.

Resveratrol and HS-1793 treatment and assessment of cell viability. Resveratrol (100 mM) in EtOH was prepared and stored at -80°C until use, and 10 mM of HS-1793 in EtOH was prepared and stored at -80°C until use. Cells were treated with resveratrol at 0-80 mM or HS-1793 at 0-25 mM. Cells were harvested 24 h after treatment, and cell viability was determined with the Vi-Cell cell counter (Beckman Counter, Fullerton, CA), which performs an automated trypan blue exclusion assay.

Flow cytometric analysis. Ice-cold 95% ethanol with 0.5% Tween-20 was added to the cell suspension to a final concentration of 70% ethanol. Fixed cells were pelleted and washed in a 1% BSA-PBS solution. Cells were resuspended in 1 ml PBS containing 11 K U/ml RNase (Sigma), incubated at 4°C for 30 min, washed once with BSA-PBS, and resuspended in PI (50 μ g/ml). Cells were incubated at 4°C for 30 min in the dark and washed with PBS. DNA content was then measured with an Epics XL flow cytometer (Beckman Coulter, Miami, FL). Data were analyzed using Multicycle software, which can perform simultaneous estimation of cell cycle parameters and apoptosis.

Nuclear morphology analysis of apoptosis. Twenty-four hours after treatment, the cell suspension was cytospun onto a clean fat-free glass slide. Cytoentrifuged samples were fixed for 10 min in 4% paraformaldehyde and stained with 4 μ g/ml Hoechst 33342 (Sigma) for 30 min at 4°C. The total cell number (300 cells from each experiment) was counted using differential interference contrast (DIC) optics, and the number of cells showing condensed or fragmented nuclei, as visualized by Hoechst staining, was calculated using epifluorescence optics by an observer in a blinded manner regarding the experimental group.

Assay of mitochondrial membrane potential (MMP). JC-1, which is a cationic dye labeling mitochondria, was added directly to the cell culture medium (1 μ M final concentration) and incubated for 15 min. The medium was then replaced with PBS, and cells were resuspended in 70% methanol and incubated at 37°C for 30 min. MMP was measured by flow cytometry with an Epics XL flow cytometer (Beckman Coulter, Miami, FL). Data were acquired and analyzed using EXPO32 ADC XL 4 color software. The analyzer threshold

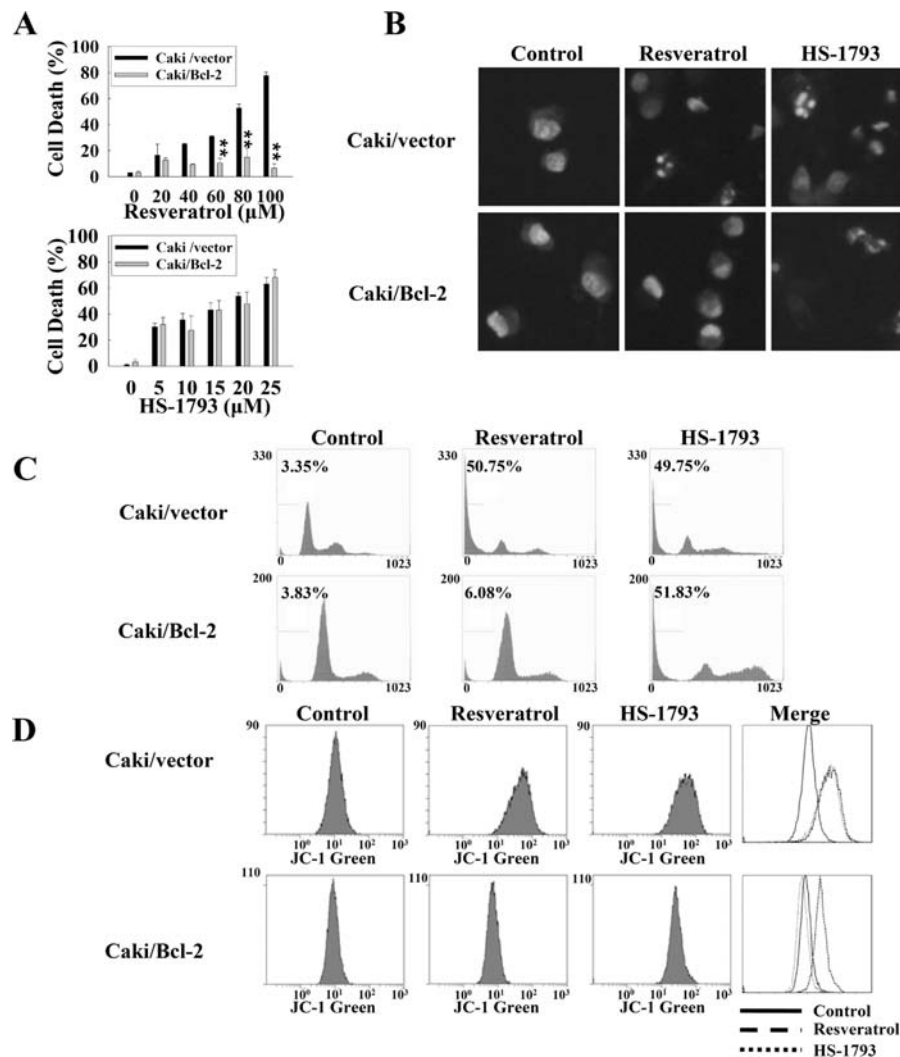


Figure 1. Antitumor activity of synthetic resveratrol analogues HS-1793 and resveratrol in Caki-1 and Caki-1/Bcl-2. HS-1793 overcomes resistance conferred by Bcl-2 in renal clear cell carcinoma Caki-1 cells via inducing apoptosis. (A) Viability assay. Cells were harvested 24 h after treatment with HS-1793 or resveratrol, and viability was determined by automated trypan blue exclusion assay with a cell counter (Vi-Cell cell counter; Beckman Counter). Resveratrol and HS-1793 reduced viability to a similar extent in both Caki-1/vector and Caki-1/Bcl-2 cells. While overexpression of Bcl-2 significantly attenuated resveratrol-induced reduction of viability. Statistical significance: ** $P < 0.01$. Data are presented as mean \pm SD ($n = 4$). (B) Hoechst staining of cells harvested 24 h after treatment. Numbers represent the percentage of apoptotic cells having condensed nuclei. Nuclear condensation or fragmentation was observed in HS-1793 or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells. Magnification $\times 800$. (C) Flow cytometric analysis. Representative histograms show cell cycle progression and induction of apoptosis. Percentage of apoptotic cells was increased in HS-1793 or resveratrol-treated Caki-1 and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells. (D) MMP assay. Flow cytometry data showing the reduction of MMP in cells undergoing apoptosis. Mitochondrial retention of JC-1 decreased in HS-1793 or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells.

was adjusted on the forward scatter channel (FSC) to exclude noise and most of the subcellular debris.

Immunofluorescent staining and confocal microscopy. A cell suspension was cytospun onto a clean fat-free glass slide. Cells were incubated with each primary antibody for 2 h at 37°C, washed 3 times for 5 min each and then incubated with FITC-conjugated or Texas Red-conjugated secondary antibodies for 1 h at room temperature. Fluorescent images were visualized and analyzed with a Zeiss LSM 510 laser-scanning confocal microscope (Zeiss, Goettingen, Germany). Cells were costained with TO-PRO3 for 10 min at 37°C to observe nuclear morphology.

Statistical analysis. Four independent *in vitro* experiments were completed. Statistical results are expressed as the mean

\pm standard deviation of the means obtained from triplicates of each independent experiment. Statistical significance was determined by the paired Kruskal-Wallis non-parametric test.

Results

HS-1793 reduces cell viability to a similar extent in both Caki-1/vector and Caki-1/Bcl-2 cells. Treatment with 20–100 μ M resveratrol efficiently reduced the viability of Caki-1/vector cells in a dose-dependent manner, while overexpression of Bcl-2 significantly attenuated resveratrol-induced cell death. In contrast, treatment with 5–25 μ M HS-1793 reduced cell viability to a similar extent in both Caki-1/vector and Caki-1/Bcl-2 cells (Fig. 1A). These data suggest that HS-1793 overcomes the resistance conferred by Bcl-2 in renal clear cell carcinoma Caki-1 cells.

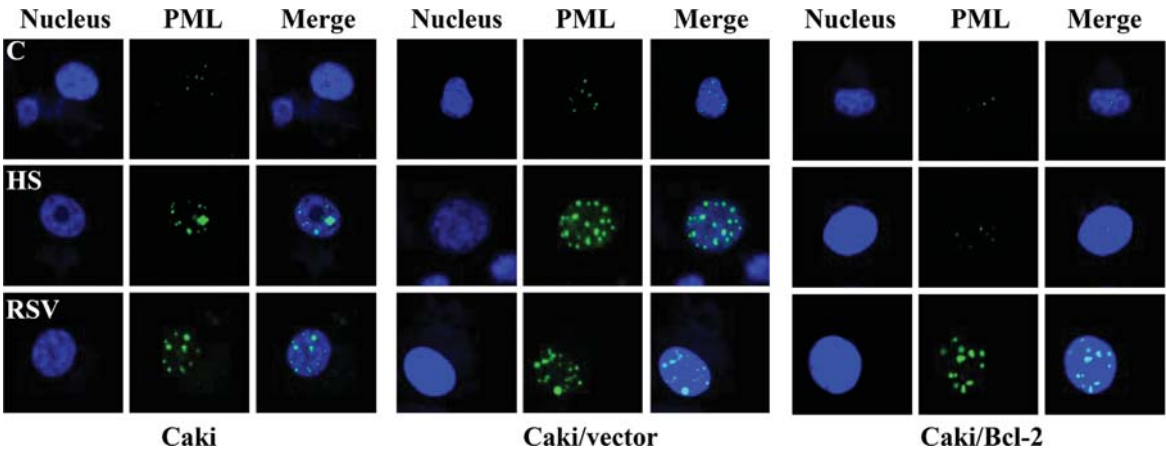


Figure 2. Aggregated or mature PML-NBs are formed in Caki-1 cells undergoing apoptosis. Confocal microscopy images show the morphology of PML-NBs after a 24-h treatment period. Nucleus was stained with TO-PRO3 (blue) which is highly specific for DNA. Microspeckled PML-NBs are demonstrated in HS-1793 or resveratrol-treated Caki-1 and Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells. C, control; RSV, resveratrol; HS, HS-1793.

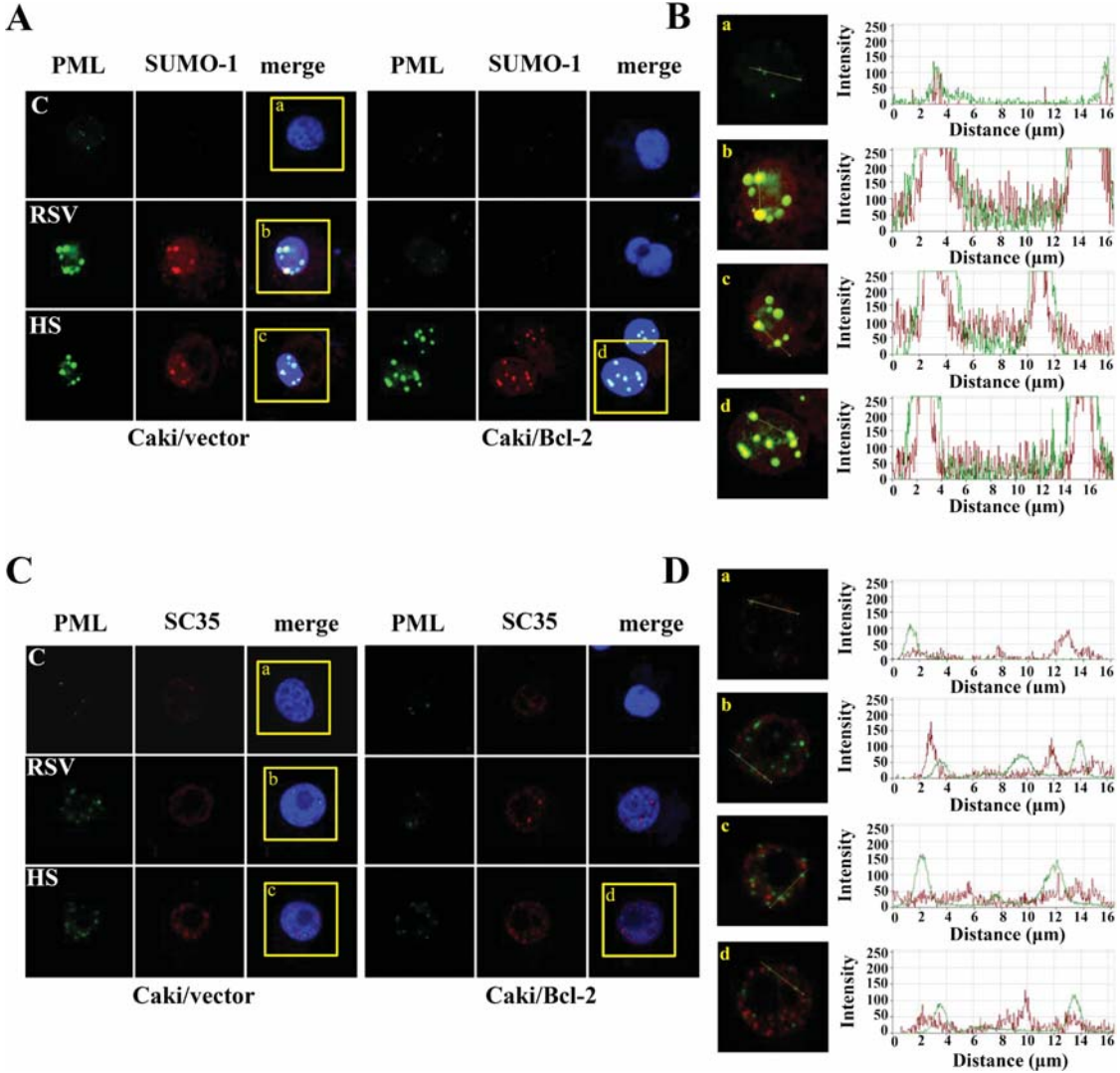


Figure 3. HS-1793 induces the colocalization between PML and SUMO-1 aggregated PML-NBs of Caki-1/Bcl-2 cells. Confocal microscopy images show the colocalization of PML and SUMO-1. (A) The enhanced colocalization is demonstrated in HS-1793 or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells. (B) The profile of SUMO-1 and PML fluorescence intensity is depicted. The intensity of SUMO-1 is shown in red, and that of PML is shown in green. In contrast to untreated Caki-1/vector cells, SUMO-1 protein is concentrated within PML-NBs in HS-1793 or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells. (C) Confocal microscopy images show that SC35 does not associate with PML. (D) The profile of SC35 and PML fluorescence intensity is depicted. The intensity of SC35 is shown in red, and that of PML is shown in green. SC35 protein is not concentrated within PML-NBs. C, control; RSV, resveratrol; HS, HS-1793.

HS-1793 overcomes the resistance conferred by Bcl-2 in renal clear cell carcinoma Caki-1 cells by inducing apoptosis. Since the dose required for half-maximal inhibition of viability in Caki-1/vector cells was 20 μ M for HS-1793 and 80 μ M for resveratrol, these concentrations were utilized for further study. To prove that HS-1793 reduced the viability of Caki-1/Bcl-2 cells by inducing apoptosis, cells were examined for the presence of DNA fragmentation, abnormalities in cell cycle status, and atypical nuclear morphology, all of which can indicate that the cells are apoptotic. Hoechst staining showed that both Caki-1/vector and Caki-1/Bcl-2 cells treated with HS-1793 had fragmented atypical nuclei (Fig. 1B). A simultaneous estimation of cell cycle parameters and apoptosis by flow cytometry demonstrated that HS-1793 increased the proportion of sub-G1 hypoploid cells in both HS-1793-treated Caki-1/vector and Caki-1/Bcl-2 cells (Fig. 1C). Flow cytometry revealed an increase in the portion of cells with depolarized MMP in both HS-1793-treated Caki-1/vector and Caki-1/Bcl-2 cells (Fig. 1D). Resveratrol also induced these apoptotic manifestations in Caki-1/vector cells, but they were substantially attenuated in resveratrol-treated Caki-1/Bcl-2 cells.

Aggregated or mature PML-NBs are formed in Caki-1 cells undergoing apoptosis. We next asked whether PML-NBs are involved in the resistance to apoptosis conferred by Bcl-2 in renal clear cell carcinoma Caki-1 cells. Visualization of the PML-NBs by confocal microscopy after HS-1793 treatment demonstrated morphological alterations in the nuclear bodies. Whereas PML-NBs were microspeckled in untreated Caki-1, Caki-1/vector or Caki-1/Bcl-2 cells, HS-1793 induced the formation of mature or aggregated PML-NBs in all three cell types. Although the aggregation of PML-NBs was also observed in resveratrol-treated Caki-1 or Caki-1/vector cells, this was not observed in resveratrol-treated Caki-1/Bcl-2 cells (Fig. 2).

HS-1793 induces the colocalization between PML and SUMO-1, Daxx, or Sp100 in the aggregated PML-NBs of Caki-1/Bcl-2 cells. Confocal images showed that Small Ubiquitin-like Modifier (SUMO)-1, Daxx and Sp100 displayed a diffuse nuclear localization pattern in the control cells, although they also colocalized with PML in discrete microspeckled PML-NBs. However, enhanced colocalization of PML with SUMO-1, Daxx and Sp100 was observed in the aggregated PML-NBs of both HS-1793 treated Caki-1/vector and Caki-1/Bcl-2 cells. Although this enhanced colocalization was also observed in resveratrol-treated Caki-1/vector cells, it was not observed in resveratrol-treated Caki-1/Bcl-2 cells (Figs. 3A, 4A and C). The profile of PML and PML-NB components showed that SUMO-1, Daxx, and Sp100 proteins are concentrated within PML-NBs in HS-1793- or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells, but not in resveratrol-treated Caki-1/Bcl-2 cells (Figs. 3B, 4B and D). As a control, we examined whether SC35 protein, which is known to be a constituent not of PML-NB but of another nuclear body SC35 speckle, is associated with mature PML-NBs. Confocal images and the profile of PML and SC35 showed that SC35 protein was not concentrated on PML-NBs (Fig. 3C and D).

HS-1793 induces the colocalization of PML with p53 and CBP in the aggregated PML NBs of Caki-1/Bcl-2 cells. We next asked whether the interaction of PML with p53 or CBP is involved in HS-1793-induced apoptosis. Confocal images of PML, p53, and CBP showed that p53 and CBP display a diffuse nuclear localization pattern in the control cells. However, neither p53 nor CBP colocalized with PML. Noticeably, both p53 and CBP colocalized with PML in the aggregated PML-NBs of HS-1793-treated Caki-1/Bcl-2 as well as in Caki-1/vector cells (Fig. 5).

Discussion

The present study demonstrates that HS-1793 at 5-25 μ M induces apoptosis to a similar extent in both Caki-1 and Caki-1/Bcl-2 cells. Our data indicate that HS-1793 at the usual dose overcomes the resistance to apoptosis conferred by Bcl-2 in Caki-1 cells. Although the information provided by the present study is still fragmentary and contradictory, our data, along with the previous findings mentioned above (20), raise the possibility that HS-1793 could be an effective strategy against Bcl-2-overexpressing cancer cells that have acquired resistance to standard chemotherapeutic agents. Since the mechanism by which HS-1793 bypasses the anti-apoptotic effects of Bcl-2 in renal clear cell carcinoma cells remains to be determined, deciphering the molecular mechanism of their actions is an important and challenging task. The most intriguing finding of our study is the formation of the mature PML-NBs in HS-1793-treated Bcl-2-overexpressing Caki-1 cells.

PML-NBs, which are macromolecular nuclear domains present in virtually every mammalian cell, are proteinaceous structures that are found predominantly in the nucleus (22,23). Under normal growth conditions, each cell usually harbors several dozen PML-NBs (24). PML protein is a major constituent of PML-NBs. PML protein was originally identified in leukemic blasts from patients suffering from acute promyelocytic leukemia (APL) (25). In APL blasts, the expression of the oncogenic PML-retinoic acid receptor- α (RAR α) fusion protein, the product of a reciprocal chromosomal translocation t(15;17), disrupts the structural integrity of PML-NBs (26). The formation of PML-NBs is known to be associated with a large number of fundamental cellular processes (27).

The assembly of PML into PML-NBs is frequently accompanied by dynamic changes in the structure of PML-NBs and in PML gene expression. PML-NB formation is also associated with modifications of PML protein and changes in the association of PML with other proteins (28). Numerous proteins have been reported to be present in, or association with, PML-NBs. One group of proteins is constitutively present in PML NBs and includes PML itself, Sp100, and members of the small ubiquitin-like modifier (SUMO) family. Other group of proteins may be readily detected in, or associated with, PML NBs only after specific treatments, which includes p53 (28-30).

Various dynamic changes in the molecular structures of PML-NB constituents and their interactions are associated with the regulation of apoptosis. SUMO is an important factor to control PML-NB assembly. The assembly of PML

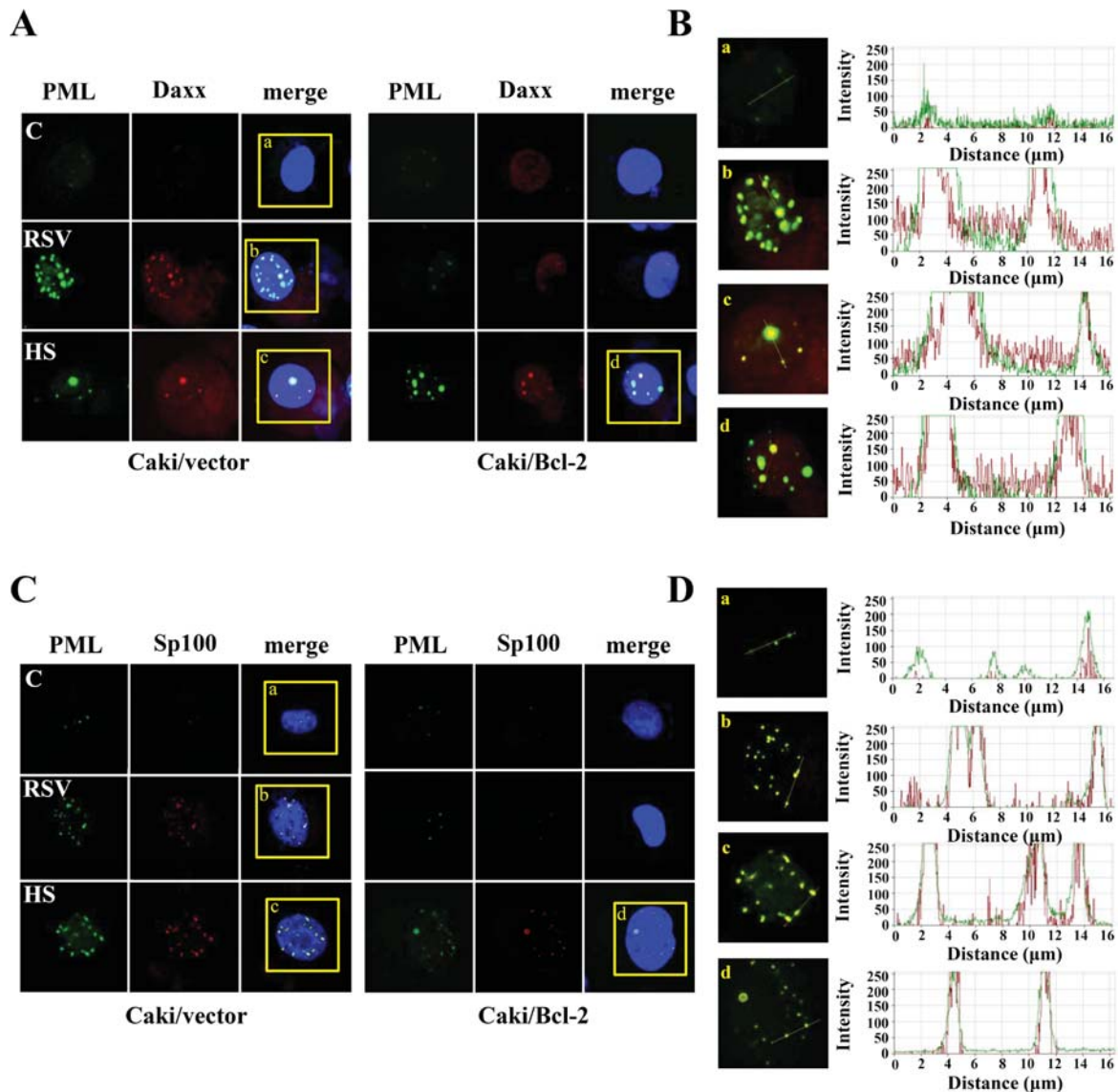


Figure 4. HS-1793 induces the colocalization between PML and Daxx or Sp100. (A) Confocal microscopy images show the colocalization of PML and Daxx. The enhanced colocalization is demonstrated in HS-1793 or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells. (B) The profiles of Daxx and PML fluorescence intensity are depicted. The intensity of Daxx is shown in red, and that of PML is shown in green. In contrast to untreated Caki-1/vector cells, Daxx proteins are concentrated within PML-NBs in HS-1793 or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells. (C) Confocal microscopy images show the colocalization of PML and Sp100. The enhanced colocalization is demonstrated in HS-1793 or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells. (D) The profiles of Sp100 and PML fluorescence intensity are depicted. The intensity of Sp100 is shown in red, and that of PML is shown in green. In contrast to untreated Caki-1/vector cells, Sp100 proteins are concentrated within PML-NBs in HS-1793 or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells.

into macromolecular PML-NBs depends on its covalent post-translational modification with SUMO-1. Sumoylation pathways also play an important role in the assembly of PML-NBs in cells undergoing apoptosis. The covalent post-translational modification of PML with SUMO-1 regulates the interaction between PML and other PML-NB components such as Sp100, Daxx, p53 and CBP (31). SUMO-conjugated PML recruits Daxx from the chromatin into the PML-NBs (32). Daxx participates in other apoptosis pathways as a transcriptional corepressor or coactivator (33). The interaction between PML and p53 in PML-NBs is also biologically important. PML directly interacts with the DNA-binding domain of p53 and recruits p53 to PML-NBs, which leads to

p53 transcriptional activation and apoptosis (34,35). PML is also involved in the phosphorylation and acetylation of p53 (36,37). Besides p53 itself, multiple factors that regulate p53, including CBP, are found within, or in association with, PML-NBs (37). Acetylated p53 that is localized to PML-NBs has been shown to form a trimeric p53-PML-CBP complex (37).

Although previous descriptions of the morphology of PML-NBs are not consistent and morphological characteristics are presumed to be cell specific, PML-NBs are mostly microspeckled in control cells and aggregated in cells undergoing apoptosis. An elaborate study demonstrated that dephosphorylation targets PML to the nuclear matrix which

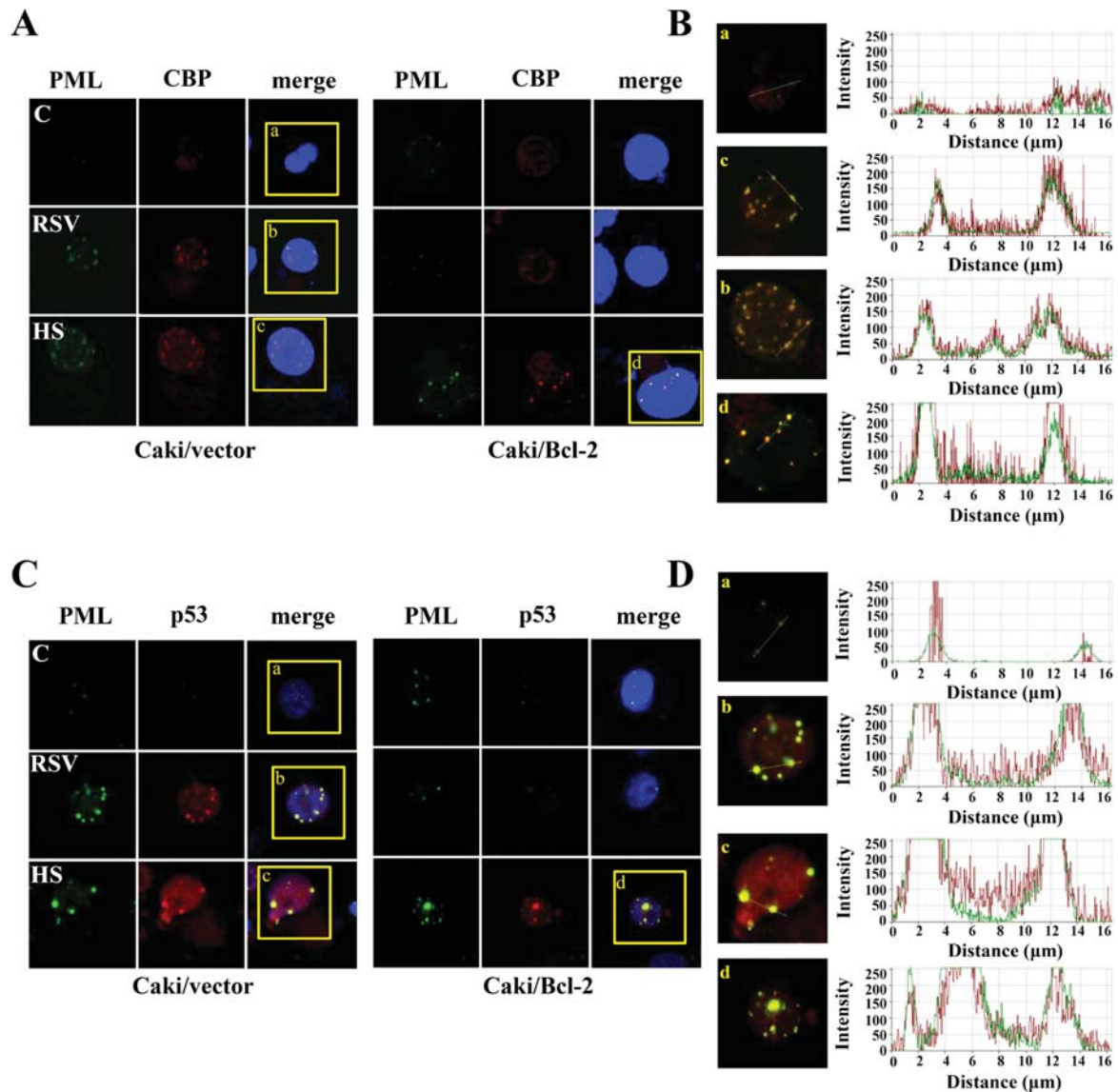


Figure 5. HS-1793 induces the colocalization between PML and CBP or p53. (A) Confocal microscopy images show the colocalization of PML and CBP. The enhanced colocalization is demonstrated in HS-1793 or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells. (B) The profiles of CBP and PML fluorescence intensity are depicted. The intensity of CBP is shown in red, and that of PML is shown in green. In contrast to untreated Caki-1/vector cells, CBP proteins are concentrated within PML-NBs in HS-1793 or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells. (C) Confocal microscopy images show the colocalization of PML and p53. The enhanced colocalization is demonstrated in HS-1793 or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells. (D) The profiles of p53 and PML fluorescence intensity are depicted. The intensity of p53 is shown in red, and that of PML is shown in green. In contrast to untreated Caki-1/vector cells, p53 proteins are concentrated within PML-NBs in HS-1793 or resveratrol-treated Caki-1/vector and HS-1793-treated Caki-1/Bcl-2 cells but not in resveratrol-treated Caki-1/Bcl-2 cells.

results in the formation of primary PML-NBs. Sumoylation then induces the maturation to secondary, or mature, PML-NBs. In mature PML-NBs, PML forms the outer shell of the structure, and many of the proteins described above are found within the electron clear core of PML (38,39). The presence of these factors in mature PML-NBs seems to affect cellular sensitivity to apoptosis. Furthermore, SUMO-dependent recruitment of proteasome components into mature PML-NBs was shown to coincide with PML degradation, which plays a key role in the induction of apoptosis (38,40).

In the study presented here, we observed the formation of mature PML-NBs in all experimental groups of cells undergoing apoptosis after HS-1793 or resveratrol treatment. Importantly, our data show that HS-1793-induced formation

of mature PML-NBs correlates with overcoming the anti-apoptotic effects of Bcl-2 overexpressed Caki-1 cells. These findings suggest that PML and the formation of mature PML-NBs could represent therapeutic targets that could overcome the resistance conferred by Bcl-2. However, the mechanism by which PML bypasses the anti-apoptotic effects of Bcl-2 still remains to be determined. Although some previous studies have shown that proteins localized within the nuclear bodies are implicated in the regulation of transcription, only a few published sources provide information on the regulation of the Bcl-2 gene by PML-NB constituents. One study showed that Daxx protein that is localized to PML-NBs can downregulate Bcl-2 (41). Based on these studies, we propose that HS-1793 induces the localization of

PML-NB protein constituents to the PML-NBs and that the interactions between these proteins affect Bcl-2 expression. We believe that future investigations may provide important information for understating the underlying mechanism.

In conclusion, HS-1793-induced apoptosis in overcoming the resistance conferred by Bcl-2 is associated with the formation of mature PML nuclear bodies in renal clear cell carcinoma Caki-1 cells. The exact molecular mechanism by which PML regulates Bcl-2 remains an open question. It may be anticipated that the answer to the question will yield further insights into the mechanism of the resistance conferred by Bcl-2 and the development of therapeutic strategies to overcome this resistance.

Acknowledgments

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