Calpain and Caspase Orchestrated Death Signal to Accomplish Apoptosis Induced by Resveratrol and Its Novel Analog Hydroxstilbene-1 in Cancer Cells

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ABSTRACT

Stomach ulceration is a major side effect of most chemopreventive drugs. We have established that although resveratrol is a promising chemopreventive compound, it delays the ulcer healing process. However, its analog hydroxstilbene-1 (HST-1) was devoid of such an ulcerogenic side effect. Consequently, here we tried to explore the chemopreventive efficacy of HST-1 compared with resveratrol in different cancer cell lines and identified the probable signaling pathways responsible for cell death. Our cell viability study established that HST-1, compared with resveratrol, showed better chemopreventive potential in all of the cell lines tested, with U937 and MCF-7 being the cells most affected. Furthermore, in U937 and MCF-7 cell lines, terminal deoxynucleotidyl transferase dUTP nick end labeling assay, cell cycle analysis, and nuclear fragmentation by confocal microscopy established that both HST-1 and resveratrol switched on the apoptotic death cascade to execute cell death. The initiator signal was Fas-independent but synchronized in terms of cytosolic Ca²⁺ influx, dissipation of mitochondrial membrane potential, and oxidative burst. It is noteworthy that the executioner signal was cell-specific as in U937 cells; HST-1 and resveratrol treatment induced mitochondrial permeabilization followed by cardiolipin depletion and cytochrome c release, which eventually activated downstream caspases 9 and 3 to execute the death process. In contrast, in MCF-7 cells the death process was executed in a caspase-independent but calpain-dependent manner as calpain activation induced cleavage of cytosolic α-fodrin, stimulated mitochondrial release of apoptotic inducing factor and endonuclease G, and thus harmonized cytosolic and mitochondrial death signals to accomplish apoptosis.

Resveratrol (Resv), a naturally occurring dietary compound, had already established its tremendous anticancer potential on different types of cancers by interfering with different cellular events associated with initiation, promotion, and progression of multistage carcinogenesis (Jang et al., 1997). We have previously reported that the use of Resv as an anticancer drug was severely constrained because of its tendency to prolong preexisting gastric ulceration (Guha et al., 2009). Furthermore, we showed that...
HST-1 (3,5,3',5'-tetrahydroxy stilbene), a novel congener of Resv, was devoid of such an ulcerogenic side effect (Guha et al., 2009). Therefore, for the first time, we have attempted to explore the chemopreventive efficacy of HST-1 compared with Resv and enumerate the activation of the probable signaling cascade to delineate the mode of action of HST-1.

**Materials and Methods**

**Chemicals and Reagents.** RPMI medium 1640, Dulbecco’s modified Eagle’s medium, fetal bovine serum, antibiotics (penicillin-G, streptomycin, Gentamicyn), Hoechst, Alexa Fluor 488-AnnexinV/PI kit, CM-H2DCFH-DA, 10-Nonyl-acridin orange, a MitoProbe transition pore assay kit, fluo-4-acetoxymethyl ester (Fluo-4 AM), and calcium calibration buffer kits were from Invitrogen (Carlsbad, CA). Primary antibodies (PARP, α-fodrin, AIF, Endo G, β-actin, and cytochrome c oxidase subunit IV) and polyclonal secondary antibody were obtained from Cell Signaling Technology (Danvers, MA). A Cycle Test Plus DNA reagent kit and JC-1 kit were from BD Biosciences (San Jose, CA). Apo-direct TUNEL assay kit, caspase protease assay kit (caspases 3, 8, and 9), and an anticytochrome c ELISA kit were from Millipore Bioscience Research Reagents (Temecula, CA). BAPTA-AM, thapsigargin, (2,5-dihydroxybenzoic acid) 2-sulfanylpropanoic acid (PD150606), and (μ)[HCO3]N,N,N,N,N-(Ru1)OC3 (Ru360) were from Calbiochem (San Diego, CA). Trans-reseveratrol, a mammalian cell lysis kit, protease inhibitor cocktail, BSA, Tween 20, Tri-HCl, DSMO, caspase inhibitors Z-YAD-FMK (Pan caspase inhibitor), Z-DQMD-FMK (caspase 3-specific inhibitor), Z-LEHD-FMK (caspase 9-specific inhibitor), Z-IETD-FMK (caspase 8-specific Inhibitor), NAC, PEG-SOD, and PEG-catalase, and BSA were procured from Sigma-Aldrich (St. Louis, MO). One-step NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) was from Pierce Biotechnology (Rockford, IL). A mitochondria/cytosol fractionation kit and calapin activation assay kit were from BioVision (Mountain View, CA), and a CellTiter-Glo luminescent cell viability assay kit was from Promega (Madison, WI).

**Culture of Cell Lines.** Cell lines studied included U937 (human leukemic monocyte lymphoma), K562 (human myelogenous leukemia), HepG2 (human hepatocellular carcinoma), MCF-7 (human breast cancer), and NHEK (normal human embryonic kidney) that were obtained from the National Centre for Cell Science, Pune, India. Human peripheral blood mononuclear cells (PBMCs) were harvested from healthy donors. MCF-7, HepG2, and NHEK cells were cultured in Dulbecco’s modified Eagle’s medium, pH 7.4, supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin-G, 100 μg/ml-streptomycin, and 6 μg/ml Gentamicyn). RPMI 1640 medium was used for U937, K562, and PBMCs. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

**CellTiter-Glo Luminescent Cell Viability Assay.** The CellTiter-Glo luminescent cell viability assay (Promega) was used in a homogeneous method to determine the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells. In brief, the cells (5 x 104 cells in 100 μl of medium/well) were plated in 0.02 to 0.07% DMSO in media as control in 96-well plates. The cells as such or in the presence of Resv or HST-1 (dissolved in 0.02–0.07% DMSO in media) were incubated for 48 h. At the end of the treatments, each well was treated with a volume of CellTiter-Glo reagent equal to the volume of cell culture medium present in each well (e.g., 100 μl of reagent added to 100 μl of medium containing cells for a 96-well plate). Then contents were mixed for 2 min on an orbital shaker to induce cell lysis, and the plate was incubated at room temperature for 10 min in the dark to stabilize the luminescent signal. At the end cellular luminescence was recorded in a luminometer (FLX800; BioTek Instruments, Winooski, VT).

**Cell Cycle Analysis.** U937 and MCF-7 cells (1 x 106) were incubated for 48 h with HST-1 and Resv after which cell cycle distribution was studied with a Cycle Test Plus DNA reagent kit and acquired on a linear scale in a flow cytometer (FACS Calibur; BD Biosciences, San Diego, CA) equipped with a fluorescence detector (488-nm argon laser light source and 623-nm band pass filter), and analyzed by using CellQuest software (BD Biosciences). A total of 10,000 events were acquired and analyzed.

**Cellular and Nuclear Morphology Analysis by Confocal Microscopy.** A TCS-SP2 confocal microscope (Leica, Wetzlar, Germany) was used for all microscopic imaging for nuclear morphology with Hoechst staining, as described previously (Guha et al., 2009).

**TUNEL Assay.** To confirm the nature of tumor killing by different concentrations of HST-1 and Resv on U937 and MCF-7 cells, the cells were fixed, permeabilized, and incubated with terminal deoxynucleotidyl transferase enzyme and fluorescein isothiocyanate-UTP. Cells were washed, incubated with RNase solution, and analyzed by FACS (BD Biosciences). The fragmented DNA of apoptotic cells was labeled by using an Apo-Direct TUNEL assay kit (Millipore Corporation, Billerica, MA). The cells were then analyzed by a FACS cytometer (equipped with a 488-nm argon laser light source; 515-nm band pass filter, FL1-H; and 623-nm band pass filter, FL2-H) using CellQuest software. A total of 10,000 events were acquired and analyzed.

**Measurement of Mitochondrial Membrane Potential.** MMP was measured with a JC-1 kit following the protocol submitted by the supplier. The mitochondrial membrane potential (ΔΨm) detection kit used a unique fluorescent cationic dye, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethyl-benzimidazolylcarbocyanine iodide) (excitation, 488 nm and emission, 525 nm), to signal the loss of MMP. Cells were harvested after HST-1 and Resv treatment. Then mitochondrial permeability transition was determined by staining the cells with JC-1 as described. In brief, equal numbers of cells (1 x 106) were incubated with JC-1 at 2.5 μg/ml in 1 ml of PBS for 30 min at 37°C with moderate shaking. Cells were then centrifuged at 1000 g at 4°C for 5 min, washed twice with ice-cold PBS, and resuspended in 200 μl of PBS. Mitochondrial permeability transition was subsequently quantified on a spectrofluorimeter (FP6300; Jasco, Tokyo, Japan). Data are given in a ratio of 590/530. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as a mitochondrial uncoupler.

**Measurement of Mitochondrial Cardiolipin Depletion.** The binding of 10-Nonyl-acridin orange to mitochondria-specific cardiolipin was measured spectrofluorimetrically at an excitation of 485 nm and an emission of 530 nm as described previously (Asumendi et al., 2002).

**Flow Cytometric Measurement of the Mitochondrial Permeability Transition Pore.** The MitoProbe transition pore assay kit (Invitrogen) was used to study the opening of the mitochondrial transition pore (MPTP) in HST-1- and Resv-treated cells. This kit provides a more direct method of measuring mitochondrial permeability transition pore opening than assays relying on mitochondrial membrane potential alone. In brief, 1 x 106 cells were loaded with the acetoxymethyl ester of calcein dye, calcein AM, which passively diffused into the cells and accumulated in cytosolic compartments, including the mitochondria. Once inside cells, intracellular esterases cleaved the acetoxymethyl esters to liberate the very polar fluorescent dye calcein. The fluorescence from cytosolic calcein was quenched by the addition of CoCl2 (cobalt chloride), while the fluorescence from the mitochondrial calcein was maintained. However, opening the MPTP instigated the release of mitochondrial calcein to the cytosol where CoCl2 was quenched, leading to the dramatic reduction of calcein fluorescence.

**Determination of Cytosolic Release of Mitochondrial Cytochrome c.** MCF-7 and U937 cells were harvested after treatment with HST-1 and Resv for 24 and 48 h. Isolation of a highly enriched mitochondrial fraction and cytosolic fraction of cells was performed by using a mitochondria/cytosol fractionation kit (BioVision, Mountain
tain View, CA). In brief, cells (5 × 10⁵) were centrifuged at 600g for 5 min at 4°C, resuspended in ice-cold PBS, and centrifuged at 600g for 5 min at 4°C. Then the cells were resuspended in 1.0 ml of cytosol extraction buffer mix containing diithiothreitol and protease inhibitors and incubated on ice for 10 min. The cells were homogenized on ice. The homogenate was centrifuged at 700g for 10 min at 4°C, and the supernatant was collected and centrifuged at 10,000g for 30 min at 4°C. Then the supernatant was collected as the cytosolic fraction. The pellet was resuspended in 0.1 ml of mitochondrial extraction buffer mix containing diithiothreitol and protease inhibitors, vortexed for 10 s, and saved as the mitochondrial fraction. These isolated cytosolic and mitochondrial fractions were subjected to colorimetric ELISA for cytochrome c (cyt c).

Activity of Caspases. Caspases 3, 9, and 8 were assayed by using a caspase fluorimetric assay kit (Millipore Corporation, Billerica, MA) per the manufacturer’s instructions.

Western Blot Analysis. Cell lysates were prepared by using a mammalian cell lysis kit; in brief, cells were immersed in freshly prepared lysis buffer with protease inhibitor cocktail, sonicated, and centrifuged (12,000 × g for 10 min), and the protein concentration of the supernatant was measured (Bradford, 1976). The proteins (50 µg) were resolved by 10% nonreducing SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked for 2 h at room temperature in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.02% Tween 20 containing 3% BSA followed by overnight incubation at 4°C in 1:500 dilution of the respective antibodies for PARP, α-fodrin, AIF, and Endo G in 3% BSA. The membrane was washed three times with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.02% Tween 20 containing 3% BSA and incubated with alkaline phosphatase-conjugated secondary antibody. The bands were visualized by using a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate.

Intracellular Ca²⁺ Measurement. Intracellular Ca²⁺ was measured by using the fluorescent probe Fluo 4-AM spectrally at an excitation of 488 nm and an emission of 516 nm. In brief, the cells were preloaded with Fluo 4-AM in loading media for 30 min at 37°C after which the cells were washed in loading media devoid of Fluo 4-AM. The cells were resuspended in the same media, and spectrally the cellular analysis was carried out followed by HST-1 and Resv treatment. Response calibration was carried out with calcium calibration buffer kits. The following equation was used to determine cytosolic calcium concentration:

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[F_{\text{Ca}^{2+}}]_{\text{free}} = \frac{K_d(F - F_{\text{min}})}{(F_{\text{max}} - F)}
\]

where \(F_{\text{min}}\) is the fluorescence intensity of the indicator in the absence of calcium, \(F_{\text{max}}\) is the fluorescence of the calcium-saturated indicator (after 1 µM tetracycline treatment), and \(F\) is the fluorescence at intermediate calcium levels in the cell. The \(K_d\) value of Fluo 4-AM was 345 nM.

Calpain Activation Assay. Activation of calpain is involved in many forms of physiological and pathologic processes (e.g., apoptosis). A calpain activation assay kit (BioVision) was used for measuring intracellular calpain activity quantitatively. This fluorometric assay was based on the detection of cleavage of the calpain substrate Ac-LLY-AFC. Ac-LLY-AFC emits blue light (\(A_{\text{max}} = 400 \text{ nm}\)) upon cleavage of the substrate by calpain, and free AFC emits a yellow-green fluorescence (\(A_{\text{max}} = 505 \text{ nm}\)), which was quantified by using a fluorometer. In brief, 1 to 2 × 10⁶ cells were suspended in 100 µl of extraction buffer (provided in the kit), and samples were incubated on ice for 20 min. The samples were gently mixed by tapping several times during incubation. They were centrifuged for 1 min in a microcentrifuge (10,000g), and supernatant was transferred to a fresh tube and put on ice. Then protein concentration was assayed, and the cell lysate (50–200 µg) was diluted to 85 µl of extraction buffer. For positive control, 1 to 2 µl of active calpain (provided in the kit) was added to 85 µl of extraction buffer. Then, 10 µl of 10X reaction buffer (provided in the kit) was added followed by 5 µl of calpain substrate to each assay. The reaction mixture was incubated at 37°C for 1 h in the dark. The samples were read in a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter. The changes in calpain activity were expressed as relative fluorescent unit (RFU) per milligram protein of each sample.

To explore the probable effects of calpain on HST-1 and Resv-induced cell death the cells were pretreated for 3 h with the calpain-specific inhibitor PD150606 at a dose of 50 µM.

Detection of Reactive Oxygen Species. Reactive oxygen species (ROS) was measured by CM-H2DCFDA (excitation 490 nm; emission 527 nm). The cells were preloaded with these dyes, and their reactivity with ROS was analyzed spectrophotometrically.

Statistical Analysis. Data are expressed as mean ± S.D. unless otherwise stated. Comparisons were made between different treatments (analysis of variance) by using the software InStat (GraphPad Software Inc., San Diego, CA), where an error protecting the multiple comparison procedure, namely Tukey-Kramer multiple comparison tests, was applied for the analysis of significance of all of the data.

Results

Effects of Resveratrol and HST-1 on Cancer Cell Lines. The antiproliferative effects of Resv and HST-1 (Fig. 1, A and B) were examined and evaluated on K562, U937, HepG2, and MCF-7 cell lines by using the CellTiter-Glo luminescent cell viability assay kit. Our study revealed that a significant (p < 0.001) reduction in cell viability was evident at 48 h of HST-1 and Resv treatment at their varying concentrations. Treatment with HST-1 showed comparatively better antiproliferative effects than Resv in all of the tested cell lines (Fig. 1, C–F). The comparative IC₅₀ values enumerated that HST-1 and Resv at their minimum doses exerted profound cytotoxic effects on U937 cells (HST-1, 25 µM; Resv, 50 µM) and MCF-7 cells (HST-1, 100 µM; Resv, 125 µM) compared with K562 cells (HST-1, 50 µM; Resv, 100 µM) and HepG2 cells (HST-1, 150 µM; Resv, 175 µM). The cytotoxicity of HST-1 and Resv was also assessed on normal human PBMCs and NHK, and the IC₅₀ of Resv and HST-1 for PBMCs was 100 and 125 µM, respectively, whereas in NHK it was higher at 160 and 175 µM, respectively. However, DMSO, the vehicle control at the concentration of 0.07% present in the drugs could affect suspension and adherent cell lines because drugs could affect suspension and adherent cultures differently (Kimura et al., 2004). In suspension culture in U937 cells IC₅₀ values were low compared with values in K562 cells (25 µM in HST-1 and 50 µM in Resv-treated cells). However, in adherent culture the IC₅₀ value of HST-1 and Resv on MCF-7 cells was 100 and 125 µM, respectively, which was comparatively lower than the IC₅₀ values of these compounds on HepG2 (HST-1, 150 µM; Resv, 175 µM). Therefore, for the detailed experiments we choose U937 as a representative of suspension culture and MCF-7 as a representative of adherent culture.

HST-1 and Resv Treatment Induced Apoptotic Cell Death in U937 and MCF-7 Cell Lines. To study the nature of cell death, TUNEL assay was performed after 48 h of HST-1 and Resv treatment (U937 25 µM; MCF-7 100 µM). Flow cytometric data revealed that in comparison with vehicle-treated control HST-1 and Resv treatment showed significant (p < 0.001) TUNEL positivity in both cell lines (Fig. 2,
A and B), indicating DNA fragmentation. In unison, confocal imaging clearly exhibited punctated and brightly stained nuclei, indicating chromosomal condensation and apoptosis 48 h after treatment with HST-1 and Resv in U937 and MCF-7 cells (Fig. 2, C and D). After treatment with HST-1 and Resv, an increased sub-G₀ phase (hypoploid DNA) population was evident (Fig. 2, E and F), providing further supportive evidence of degradation of cellular DNA resulting in apoptosis.

HST-1 and Resv Treatment Failed to Activate the Fas-Mediated Death Cascade but Predominantly Disrupted the Mitochondrial Membrane Potential and Triggered an Oxidative Burst. To enumerate whether HST-1 and Resv instigated a Fas-dependent death signal, U937 and MCF-7 cells were pretreated for 2 h with Fas/Fas ligand antagonist (Kp7-6) (1 mM), but they failed to prevent HST-1- and Resv-induced cell death (Fig. 3, A and B), indicating that HST-1- and Resv-driven cell death was largely Fas-independent in U937 and MCF-7 cell lines.

Dissipation of mitochondrial transmembrane potential ($\Delta \Psi_m$) is another hallmark of apoptotic initiator signal (Kroemer et al., 1998). To determine the changes in the $\Delta \Psi_m$, we used JC-1. To confirm the sensitivity of the reagent to the change in mitochondrial membrane potential, U937 and MCF-7 cells were initially treated with the mitochondrial-uncoupling agent CCCP (1 $\mu$M). Treatment with HST-1 and Resv dissipated $\Delta \Psi_m$ in both cell lines, and comparative analysis revealed that after 24 h of treatment HST-1 significantly dissipated $\Delta \Psi_m$ (24.73% in U937 and 23.48% in MCF7; $p < 0.001$) compared with Resv (Fig. 3, C and D).

Because ROS generation is an intimate event triggering the mitochondrial death signal, cells were stained with an oxidation-sensitive probe, CM-H$_2$DCFDA, and analyzed spectrofluorimetrically after HST-1 and Resv treatment. Fluorimetric data showed that CM-H$_2$DCFDA fluorescence increased with a peak approximately 6 h after treatment, in both the U937 and MCF-7 cell lines (Fig. 3, E and F). Moreover, pretreatment of U937 and MCF-7 cells with cell-permeable ROS scavengers PEG-SOD (400 U/ml), PEG-catalase (2000 U/ml), and NAC (10 mM) protected the cells profoundly ($p < 0.001$) (Fig. 3, G and H) and significantly reduced the fluorescence signal of CM-H$_2$DCFDA (Fig. 3, E and F).

HST-1 and Resveratrol Stimulated Cytosolic Calcium (Ca$^{2+}$) Influx, which Modulated $\Delta \Psi_m$ and ROS Generation. Intracellular Ca$^{2+}$ influx is an important phenomenon for activation of the intrinsic mitochondria-mediated death cascade (Orrenius et al., 2003). Treatment with HST-1 and Resv resulted in a significant increase of intracellular Ca$^{2+}$ load in both the U937 and MCF-7 cell lines, as...
Fig. 2. A–D, detection of apoptosis was carried out flow cytometrically by TUNEL assay in U937 cells (A), and MCF-7 cells (B), followed by confocal microscopy of U937 cells (C) and MCF-7 cells (D) to study chromosomal condensation and fragmentation. White arrows indicate nuclear fragmentation. E and F, apoptotic cell death was reconfirmed by cell cycle distribution by FACS in U937 cells (E) and MCF-7 cells (F). M1, M2, and M3 represent the number of cells at the sub-G0 phase. All data are representative of three different experiments.
studied by increased fluorescence of the intracellular Ca\textsuperscript{2+} indicator Fluo-4 AM (Fig. 4, A and B). The initial rise of Ca\textsuperscript{2+} (\sim 1.3-fold of basal fluorescence of U937, i.e., 110 \pm 10 nM; \( n = 6 \)) was evident almost 30 min after HST-1 and Resv treatment and peaked at 3 h in HST-1 (385 \pm 28 nM) and Resv (354 \pm 21 nM). However, in MCF-7, an initial rise of Ca\textsuperscript{2+} (\sim 1.7-fold of basal fluorescence of MCF-7; i.e., 139 \pm 14 nM; \( n = 6 \)) was evident within 15 min of HST-1 and Resv treatment and peaked after 4 h after treatment with HST-1 (462 \pm 26 nM) and Resv (449 \pm 29 nM). Pretreatment with 1 \( \mu \)M BAPTA-AM, an intracellular calcium chelator, significantly (\( p < 0.001 \)) reduced the fluorescence of Fluo-4 AM and simultaneously protected the cells significantly after HST-1 and Resv treatment (Supplemental Fig. 6), thus validating involvement of intracellular calcium influx (Fig. 4, A and B).

Because the endoplasmic reticulum (ER) is a major intracellular reservoir of Ca\textsuperscript{2+}, we tested whether the Ca\textsuperscript{2+} signal evoked by HST-1 and Resv originated from the ER. Accordingly, we used TG (5 \( \mu \)M), a known specific mobilizer of the ER Ca\textsuperscript{2+} stores that caused rapid and transient increase in [Ca\textsuperscript{2+}], in both the U937 and MCF-7 cell lines. If HST-1 and Resv exposure increased release of Ca\textsuperscript{2+} stored in the ER,
then TG administration would not cause additional release of Ca$^{2+}$. TG after 1 min in the U937 and MCF-7 cell lines evoked a typical Ca$^{2+}$ concentration of 436 ± 22 nM ($n = 6$) and 503 ± 20 nM ($n = 6$), respectively. However, after the addition of TG after 3 and 4 h of Resv and HST-1 treatment in U937 and MCF-7 cells (Fig. 4, C and D), none of the cells that initially responded to Resv and HST-1 exhibited a significant rise in intracellular Ca$^{2+}$ levels after TG administration (Fig. 4, C and D insets). Moreover, treatment with EGTA also did not result in any significant effect on intracellular Ca$^{2+}$ influx (Supplemental Fig. 5). Hence, we clearly established that HST-1- and Resv-mobilized intracellular Ca$^{2+}$ store ER for the influx of [Ca$^{2+}$]$\text{_{i}}$.

Furthermore, pretreatment with Ru360, an inhibitor of mitochondrial Ca$^{2+}$ uniporter (5 μM), for 1 h before treatment with Resv or HST-1 significantly ($p < 0.001$) prevented the loss of mitochondrial membrane potential and intracellular ROS generation (Fig. 4, E-H). Taken together, it was confirmed that calcium was an important molecular switch that worked upstream in the initiator death signal induced by HST-1 and Resv.

**HST-1 and Resv Treatment Preceded Cyt c Release by Cardiolipin Depletion and Mitochondrial Permeability Transition Pore Opening.** The overall mitochondrial permeability transition pore opening in HST-1- and Resv-treated cells was analyzed with a MitoProbe transition pore assay kit (Fig. 5, A and B) by flow cytometry. In response to HST-1 and Resv, a sharp drop in fluorescence of calcein-AM was evident compared with vehicle-treated control cells, which signified the opening of mitochondrial permeability transition pores. Conversely, cyt c is bound to the inner mitochondrial membrane (IMM) by its association with the anionic phospholipid cardiolipin (Kroemer et al., 1998). Spectrofluorimetric data demonstrated that treatment with HST-1 and Resv first showed significant cardiolipin depletion after 12 h of drug treatment and further reduced progressively in a time-dependent manner in both cell lines (Fig. 5, C and D). However, cardiolipin depletion was considerably ($p < 0.001$) prevented by Ru360 and NAC pretreatment (Fig. 5, E and F), which confirmed the potential involvement of Ca$^{2+}$ and ROS in cardiolipin depletion.
Fig. 5. HST-1 and Resv treatment preceded cyt c release by cardiolipin depletion and mitochondrial permeability transition pore opening. A and B, flow cytometric data revealed opening of the MPTP in U937 (A) and MCF-7 (B) cells. The flow cytometric data are representative of three different experiments. Further peroxidation of cardiolipin caused by HST-1 and Resv treatment were analyzed by 10-Nonyl-acridin orange dye. C and D, spectrofluorimetric data revealed depletion of mitochondrial cardiolipin occurred in treated U937 (C) and MCF-7 (D) cells in a time-dependent manner.
Because cardiolipin depletion could precede the release of mitochondrial cyt c, we explored the status of cyt c after HST-1 and Resv treatment. Treatment with HST-1 and Resv induced a significant (p < 0.001) increase in the release of mitochondrial cyt c within 24 h in MCF-7 cells (HST-1, 6.91-fold; Resv, 6.46-fold) and U937 cells (HST-1, 6.19-fold; Resv, 5.52-fold) compared with their respective vehicle control (Fig. 5, G and H). The release of cyt c was efficiently perturbed by pretreatment with both a MPTP blocker cyclosporine A (U937: HST-1, 55.69%; Resv, 60%; MCF-7: HST-1, 51.35%; Resv, 42.66%) and Ru360, an inhibitor of mitochondrial uniporter (U937: HST-1, 58.37%; Resv, 56.67%; MCF-7: HST-1 47.76%; Resv 40.56%) (Fig. 5 G and H). Thus, we confirmed that HST-1 and Resv treatment preceded cyt c release by cardiolipin depletion and mitochondrial membrane permeabilization.

**Apoptosis Induced by Resv and HST-1 Was a Caspase-Dependent Phenomenon in U937 Cells.** Pretreatment of U937 cells with a pan caspase inhibitor (Z-VAD-FMK) at 50 μM prevented HST-1- and Resv-induced death signal, thus establishing that in U937 cells the death signal was caspase-dependent (Fig. 6A). However, activation of effector caspases 9 and 3 was validated by inhibitor study (Fig. 6, B and C). Because activation of caspase 8 was not evident even after 48 h of treatment (Fig. 6D), pretreatment of U937 cells with a caspase 8-specific inhibitor (Z-IETD-FMK) at 50 μM failed to provide any significant protection (Fig. 6D). Moreover, cleavage of PARP protein by Western blotting confirmed caspase-3 activation (Fig. 6F) because PARP was a substrate of caspase 3 (Boulares et al., 1999).

MCF-7 cells were caspase 3-deficient and largely independent of caspase activation (Sareen et al., 2007). It was expected that HST-1 and Resv would not be able to induce caspase activation, which was collaborated by the pan-caspase inhibitor study (Fig. 6E), and also fail to impart any activation of specific caspases (caspases 3, 9, and 8) (Supplemental Fig. 2).

**After HST-1 and Resv Treatment Calpain Drove Cell Death in the MCF-7Cell Line.** Calcium-dependent calpain activation can induce cell death in a caspase-independent manner (Sareen et al., 2007), and accordingly, to evaluate intracellular calpain activation, we performed the calpain activity assay in MCF-7 and U937 cells by using a fluorimetric calpain assay kit.

Time-dependent study revealed that significant calpain activity in HST-1- and Resv-treated MCF-7 cells was early and significantly higher (p < 0.001) compared with basal levels (Fig. 7A) but was insignificant in U937 cells (Supplemental Fig. 3). As expected, in MCF-7 cells pretreatment with the intracellular Ca2+ chelator BAPTA-AM and calpain inhibitor PD150606 substantially inhibited calpain activity after treatment with HST-1 (39.85 and 54.04%, respectively) and Resv (33.40 and 45.59%, respectively), but the pan caspase inhibitor was ineffective on calpain activation (Fig. 7B). This showed that the calpain activation in this assay depended on an intracellular surge of cytosolic Ca2+ but was independent of caspase activity. Calpain inhibitors provided significant (p < 0.001) protection in MCF-7 cells after treatment with HST-1 (43.76%) and Resv (54.63%) (Fig. 7C) but failed to do so in U937 cells (Supplemental Fig. 4).

**Calpain Orchestrated Death Signal in Cytosolic and Mitochondrial Ways.** To study the mode of action of calpain in HST-1- and Resv-treated cells, further analysis was carried out with the MCF-7 cell line. Treatment with HST-1 and Resv induced cleavage of α-fodrin, a cytosolic substrate of calpain evident after 48 h of treatment, and was completely inhibited by pretreatment of the calpain inhibitor PD150606 (50 μM) (Fig. 8A). Moreover, Western blot analysis demonstrated that HST-1 and Resv treatment instigated cytosolic release of mitochondrial AIF and Endo G after 48 h of treatment (Fig. 8, B and C), which was substantially inhibited by pretreatment with PD150606 (50 μM).

**Discussion**

Aggressiveness and multidrug resistance of cancer cells along with the diverse contraindicative effects of present anticancer drugs demand the synthesis of new compounds to enrich the existing chemotherapeutic regimen. Although Resv has established itself as a promising anticancer drug (Dörrie et al., 2001) and currently is in clinical trials, its ulceroergic contraindicative affects severely constrain its therapeutic use. Hence we tried to explore the chemopreventive potential of HST-1, a nonulceroergic novel congener of Resv (Guha et al., 2009), to potentiate the chemotherapeutic family of Resv.

Our cell viability study demonstrated that the 3′-5′-hydroxylated congener of Resv, (E)-HST-1, synthesized in multigram scale (Supplemental Fig. 1), demonstrated significantly better chemotherapeutic potential compared with Resv on different cancer cell lines (Fig. 1, C–F). After 48 h of treatment cell proliferation was inhibited approximately 50% compared with vehicle control. Hence data of cell viability were represented only at 48 h. The comparative IC50 values enumerated that HST-1 and Resv at their minimum doses exerted profound cytotoxic effects on U937 and MCF-7 cell lines compared with other tested cell lines (K562 and HepG2) (Fig. 1, C–F). Hence, to explore the plausible mechanism of action of HST-1 and Resv detailed experiments were carried out mainly on these two cell lines at the IC50 dose of HST-1 (U937, 25 μM; MCF-7, 100 μM).

HST-1 and Resv showed cytotoxic effects on normal cell lines (PBMC and NHEK) above the dose 120 μM. However, after HST-1 and Resv treatment the IC50 value exceeded 120 μM in HepG2. Therefore, it could elicit adversative effects on normal cells when used to treat breast cancer or liver cancers in vivo. Hence, further chemical modification of HST-1 would be a positive step through reducing its cytotoxic effects on normal cells but targeting the neoplastic cells specifically.

Different reports have stated that Resv-induced cell death was executed by an apoptotic process (Huang et al., 1999). In agreement with this we found that treatment with Resv and HST-1 induced apoptosis in U937 and MCF-7 cells, as evident by TUNEL assay, confocal imaging of chromosomal

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E and F, however, pretreatment of U937 (E) and MCF-7 cells (F) with NAC and Ru360 prevented the loss of cardiolipin. Loss of cardiolipin instigated cytosolic release of mitochondrial cyt c as analyzed colorimetrically by ELISA. G and H, in HST-1- and Resv-treated U937 (G) and MCF-7 cells (H) cytosolic release of mitochondrial cyt c was evident. The data are represented as mean ± S.D. (n = 3). **+, p < 0.001 versus HST-1-treated group; ###, P < 0.001 versus Resv-treated group. ns, nonsignificant.
condensation, and increased amount of cells with hypoploid DNA (sub-G₀ phase) after 48 h of treatment (Fig. 2, A–F).

Apoptosis is a very complex event because it can be executed by diverse signals. The linkage activation of Fas receptor and mitochondria could accomplish apoptosis or independently either Fas or mitochondria could be able to elicit apoptotic signals (Larbi et al., 2006). Figure 3, A and B clearly depicts that pretreatment of U937 and MCF-7 cells with Fas/Fas ligand antagonist (Kp7-6) failed to impart any significant protection in cell viability compared with the com-

Fig. 6. Apoptosis induced by Resv and HST-1 was a caspase-dependent phenomenon in U937 cells, but in MCF-7 cells calpain drove away the cell death. A and E, pan caspase inhibitor treatment (Z-VAD-FMK) revealed that after HST-1 and Resv treatment caspase activation was prominent in U937 cells (A) but absent in MCF-7 cells (E). B–D, further spectrofluorimetric study and cell viability assay illustrated the activation of caspases 9 and 3, but activation of caspase 8 was absent in U937 cells. F, the cleavage of PARP protein signified the activation of caspase 3 in U937 cells. G and H. caspase 3-dependent death cascade was also involved in K562 (G) and HepG2 (H) cell lines.
pound-treated cells. However, treatment of cells with HST-1 and Resv sharply dissipated $\Delta \Psi_m$ compared with the DMSO control (Fig. 3, C and D). Thus, it could be suggested that HST-1- and Resv-induced apoptosis was largely Fas-independent but worked out through the mitochondrial way.

Imbalance of cellular redox state is intimately related to the stability of $\Delta \Psi_m$ (Hampton and Orrenius, 1998). Spectrofluorimetric data indicated that within 6 h of HST-1 and Resv treatment ROS generation increased up to the peak level (Fig. 3, E and F). However, to examine whether ROS generation was an important event of the apoptotic cascade or a stress-induced additional consequence, the cells were pretreated with PEG-SOD, PEG-catalase, and NAC. Fig. 3, G and H clearly illustrates that pretreatment of cell-permeable free radical scavengers efficiently reduced ROS generation and exerted significant ($p < 0.001$) protection to HST-1- and Resv-treated cells. Thus it has been confirmed that in the case of HST-1- and Resv-treated apoptotic cell death ROS generation was a mainstream event.

It had become clear that perturbation of intracellular Ca$^{2+}$ compartmentalization was interwoven with the mainstream apoptosis executioners (Orrenius et al., 2003). Thus we explored the potential participation of Ca$^{2+}$ in HST-1- and Resv-treated U937 and MCF-7 cells. Spectrofluorimetric

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**Fig. 7.** HST-1 and Resv treatment triggered calpain activation in MCF-7 cells. A and C, time-dependent study enumerated calpain activation in MCF-7 cells (A), which was validated by cell viability study by pretreatment of MCF-7 cells with PD150606 (calpain inhibitor) (C). B, Ca$^{2+}$-dependent calpain activation in MCF-7 cells was validated by inhibitor study. The data are represented as mean ± S.D. ($n = 3$). ***, $P < 0.001$ versus HST-1-treated group; ###, $P < 0.001$ versus Resv-treated group; ns, nonsignificant.
data showed that significant cytosolic Ca\(^{2+}\) flux was evident at 3 h in U937 cells and 4 h in MCF-7 cells followed by HST-1 and Resv treatment (Fig. 4, A and B). However, pretreatment with 1 \(\mu\)M BAPTA-AM, the intracellular Ca\(^{2+}\) chelator, efficiently perturbed the Ca\(^{2+}\) influx (Fig. 4, A and B). Moreover, pretreatment with BAPTA-AM significantly inhibited drug-induced cell death (Supplemental Fig. 6), proving the importance of Ca\(^{2+}\) influx in HST-1- and Resv-induced cell death. Recent findings indicated that interfering with the sequestration of Ca\(^{2+}\) into intracellular pools, the ER, could be sufficient to trigger apoptosis as part of a stress response (Sareen et al., 2007). We tested whether the Ca\(^{2+}\) signal evoked by HST-1 and Resv originated from this organelle by using TG. TG alone evoked a significant [Ca\(^{2+}\)]\(_i\) flux; however, when TG was added to cells after HST-1 and Resv, none of the cells that initially responded to HST-1 and Resv exhibited a rise in intracellular Ca\(^{2+}\) levels (Fig. 4, C and D). Thus it clearly suggested that HST-1 and Resv mobilized ER to sequester Ca\(^{2+}\) that stimulated intracellular Ca\(^{2+}\) influx, leading to the cytotoxicity.

Furthermore, Fig. 4, E–H clearly shows that pretreatment of cells with Ru360 significantly prevented the dissipation of \(\Delta\Psi_m\) and reduced intracellular ROS generation in HST-1- and Resv-treated cells, thus confirming the major role of Ca\(^{2+}\) in the perturbation of mitochondrial function.

In both cell line experiments with the MitoProbe transion pore assay kit clearly delineated the opening of MPTP (Fig. 5, A and B), which was followed by a significant amount of intramitochondrial cardiolipin depletion (Fig. 5, C and D), which could eventually facilitate the dissociation of cyt c from the IMM (Orrenius et al., 2003) followed by the cytosolic release of cyt c (Fig. 5, G and H) through MPTP. However, pretreatment with Ru360 and NAC strongly prevented \(p < 0.001\) the cardiolipin depletion (Fig. 5, E and F), thus reducing cyt c release. These data signified that Ca\(^{2+}\) and ROS played a crucial role in the opening of MPTP and dissociation of cyt c from the IMM because of significant depletion of cardiolipin in both cell lines.

In both cell lines initiator signals for apoptosis by HST-1 and Resv were quite alike, but the executioner signals were branched off in different ways. Spectrofluorimetric data and cell viability study after inhibitor treatment clearly demonstrated that caspase 9 and 3 activation was significantly evident in HST-1- and Resv-treated U937 cells (Fig. 6, B and C) that was further validated by the cleavage of PARP (Fig. 6F); however, caspase 8 activation was absent in U937 cells (Fig. 6D). HST-1 and Resv were unable to induce caspase activation in the MCF-7 cell line (Fig. 6E). Therefore, it could be delineated that in MCF-7 cells cell death was driven out in a caspase-independent manner.

Although recent studies have focused on caspases, there is considerable evidence that members of the calpain family of Ca\(^{2+}\)-activated cysteine proteases participate in apoptosis. Recently, Sareen et al., 2007 demonstrated that Resv treatment could elicit calpain activation in MCF-7 cells. We investigated whether in MCF-7 cells HST-1 also could induce calpain activation. Our spectrofluorimetric data showed that, like Resv, HST-1 stimulated calpain activation in a time-dependent manner, and the activation of calpain was significantly controlled by intracellular Ca\(^{2+}\) flux (Fig. 7).

However, experimental data showed that intracellular Ca\(^{2+}\) influx was evident in U937 cells but calpain activation was not (Supplemental Fig. 3). Thus it proved that in MCF-7 cells cell death was solely calpain-dependent but in U937 cells it was caspase-mediated.

Therefore it could be delineated that in U937 cells HST-1 and Resv treatment triggered executioner death signal in a mitochondria-dependent way where mitochondrial cyt c release activated a downstream caspase cascade to accomplish apoptotic process. However, the actual mode of action of calpain in the execution of death process in MCF-7 cells was not unveiled.

To enumerate the actual mode of action of calpain further experiments were carried out on MCF-7 cells. Mainly active calpain resides at cytosol, and it also has cytosolic targets, including a-fodrin. Our Western blot data enumerated that HST-1 and Resv treatment induced cleavage of a-fodrin after 48 h of treatment in the MCF-7 cell line, and it was completely inhibited by pretreatment of calpain inhibitor, thus proving its cytosolic mode of action (Fig. 8A). Conversely, Norberg et al., 2008 have reported that activation of mitochondrial calpain could process mitochondrial proapoptotic protein AIF and in turn facilitate cytosolic release of AIF. In contrast, mitochondrial proapoptotic proteins AIF and Endo G could accomplish caspase-independent apoptosis (Lorenzo and Susin, 2004). Fig. 8, B and C illustrates that HST-1 and Resv treatment induced the release of AIF and Endo G from mitochondria to cytosol but at the same time pretreatment of cells with PD150606 confirmed that cytosolic release of AIF and Endo G were predominantly caspase-dependent. So from these experiments it could be demonstrated that in the MCF-7 cell line the apoptotic executioner signal was calpain-dependent where activation of calpain harmonized cytosolic and mitochondrial signals to execute the death process (Fig. 9A).
Finally, it could be delineated that HST-1 was devoid of ulcerogenic contraindicative effects of Resv but did not compromise its chemopreventive potential, rather it was comparatively more effective than Resv and could execute death signal in both a caspase-independent and -dependent manner (Fig. 9). Therefore, more extensive work in the near future could establish HST-1 as a plausible substitute of Resv in cancer chemoprevention.

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References


Fig. 9. Mechanism of HST-1- and Resv-induced cell death. A, in MCF-7 cells HST-1 and Resv treatment induced caspase independent cell death. HST-1 and Resv triggered mitochondrial death signal that was facilitated by intracellular Ca\textsuperscript{2+} influx. Sequestration of Ca\textsuperscript{2+} from ER activated calpain, which substantially induced cytosolic and mitochondrial death cascade. It cleaved the cytosolic substrate α-fodrin, thus destabilizing the membrane stability. However, activation of mitochondrial calpain instigated the mitochondrial release of AIF and Endo G into cytosol followed by caspase-independent cell death. B, conversely, in U937 cells the phenomenon occurred in a caspase-dependent manner. HST-1 and Resv treatment mobilized ER calcium and facilitated dissipation of mitochondrial membrane potential followed by intramitochondrial ROS generation, peroxidation of cardiolipin, and cytosolic release of cytochrome c. Cytosolic release of cytochrome c in turn activated downstream caspase cascade (caspase 9 followed by caspase 3) and induced apoptosis in a caspase-dependent, but calpain-independent, way.

References


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