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Synthetic Chenodeoxycholic Acid Derivative HS-1200-induced Apoptosis of Human Melanoma Cells

Chul-Jung Baek, Ji-Hak Min, Seong-Hyeok Moon, In-Ryoung Kim,
Seung-Eun Lee, Duk-Han Kim, Gyoo-Cheon Kim,
Hyun-Ho Kwak, Bong-Soo Park

Department of Oral Anatomy, College of Dentistry, Pusan National University

Abstract : Bile acids and their synthetic derivatives induced apoptosis in various kinds of cancer cells and anticancer effects. It has been reported that the synthetic chenodeoxycholic acid (CDCA) derivatives showed apoptosis-inducing activity on various cancer cells *in vitro*. It wasn't discovered those materials have apoptosis-inducing effects on G361 human melanoma cells. The present study was done to examine the synthetic bile acid derivatives, HS-1199 and HS-1200, induced apoptosis on G361 cells and such these apoptosis events.

The viability of G361 cells was assessed by the MTT assay. Induction of apoptosis was confirmed by DNA electrophoresis and Hoechst staining. Westen blot analysis and immunofluorescent staining were performed to study the alterations in expression level and translocation of apoptosis-related proteins. Proteasome activity and mitochondrial membrane potential (MMP) change were also assayed.

Tested G361 cells showed several lines of apoptotic manifestation such as activation of caspase-3, DFF and PARP, DNA degradation (HS-1200 only), nuclear condensation, inhibition of proteasome activity, reduction of mitochondrial membrane potential, and the release of cytochrome c and AIF to cytosol. Between two synthetic derivatives, HS-1200 showed stronger apoptosis-inducing effect than HS-1199 did.

Taken collectively, we here demonstrated for the first time that synthetic CDCA dedrivatives induce apoptosis of human melanoma cells through the proteasome, mitochondria and caspase pathway. Therefore our data provide the possibility that HS-1200 could be considered as a novel therapeutic strategy for human melanoma cells from its powerful apoptosis-inducing activity.

Key words : Synthetic chenodeoxycholic acid (CDCA) derivatives, HS-1200, Apoptosis, Human melanoma cells

Introduction

Bile acids are polar derivatives of cholesterol essential for the absorption of dietary lipids and regulate the transcription of genes that control cholesterol homeostasis. Different bile acids exhibit distinct biological effects. Importantly, natural bile salts were reported to inhibit cell proliferation and induce apoptosis in various cells (Blake et al. 1988, Martinez et al. 1998). Im et al. (1999, 2001) developed several ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) derivatives, and it have been reported that they had apoptosis-inducing effect in various cancer cells (Choi et al. 2003a, Jeong et al. 2003, Seo et al. 2003, Park et

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Correspondence to : Bong-Soo Park (Department of Oral Anatomy, College of Dentistry, Pusan National University)
E-mail : parkbs@pusan.ac.kr

al. 2004, Im et al. 2005, Kim et al. 2006).

Apoptosis is also implicated in a wide range of pathological conditions, including immunological diseases, allergy and cancer (Carson and Ribeiro 1993, Ohta and Yamashita 1999). During apoptosis, cells undergo specific morphological and biochemical changes, including cell shrinkage, chromatin condensation, and internucleosomal cleavage of genomic DNA (Williams 1991). Multiple lines of evidence indicate that apoptosis can be triggered by the activation of caspase (Thornberry et al. 1997). In addition, mitochondria are known to central death regulators in response to several apoptotic stimuli (Green and Reed 1998).

Malignant melanoma is the most important cutaneous malignancy because it accounts for the majority of mortality from skin disease and malignant melanoma patients have increased in number in recent years. Furthermore malignant melanoma cells have been reported to be highly resistant to chemotherapeutic agents (Shibuya et al. 2003).

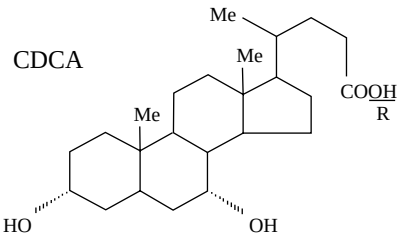
Although synthetic bile acid derivatives have been demonstrated to induce apoptosis of various cancer cells, there have been no report of their effect on human melanoma cells. This study was conducted in order to investigate the effect of synthetic bile acid derivatives on human melanoma cells (G361 cells) proliferation and demise as it pertains to both its basic drug mechanism and the potential therapeutics.

In this study, we analyzed whether synthetic CDCA derivatives, HS-1199 and HS-1200, have apoptotic effects on human melanoma cells. As will be shown, a synthetic CDCA derivative HS-1200 induces strong caspase-dependent apoptosis via mitochondrial and proteasome pathway in human melanoma cells *in vitro*.

Materials and Methods

1. Reagents

CDCA was obtained from Dae-Woong Pharmaceutical Co. (Seoul, Korea) and Aldrich (Milwaukee, WI, USA). The synthetic bile acid derivatives, HS-1199 and HS-1200 were kindly provided by Professor Young-Hyun Yoo (Department of Anatomy, College of Medicine, Dong-A University, Busan, Korea). The structure and methods of the synthesis of the synthetic bile acid derivatives were described (Im et al. 2001). HS-1199 is a conjugate form of CDCA with L-phenyl alanine benzy l ester (*N*-[(3α, 5β, 7α)-3, 7-dihydroxyl-24-oxocholan-yl] L-phenyl alanine benzyl ester). HS-1200 is a conjugate form of CDCA with β-alanine benzyl ester (*N*-[(3α, 5β, 7α)-3, 7-dihydroxyl-24-oxocholan-yl] β-alanine benzyl ester). CDCA and its derivatives were dissolved in absolute ethanol, and dilutions were made in culture medium. The final concentration of ethanol in the medium was less than 0.1% (vol/vol) in the treatment range (10～100 μM) and showed no influence on cell growth (data not shown). The structures of CDCA and its conjugate forms (HS-1199 and HS-1200) are shown in Fig. 1.



R1: $NHCH(CH_2C_6H_5)COOC_4H_9$ (HS-1199)
R2: $NH(CH_2)_2COOC_4H_9$ (HS-1200)

Fig. 1. Chemical structures of CDCA and its derivatives.

The following reagents were obtained commercially: Rabbit polyclonal anti-human caspase-3 and anti-horse cytochrome c, and anti-human DNA fragmentation factor (DFF), and goat polyclonal anti-mouse AIF antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Mouse polyclonal anti-human poly (ADP-ribose) polymerase (PARP) antibody was from Oncogene (Cambridge, USA); FITC-conjugated

goat anti-rabbit and horse anti-mouse IgGs were from Vector (Burlingame, USA); HRP-conjugated donkey anti-rabbit and sheep anti-mouse IgGs were from Amersham Pharmacia Biotech (Piscataway, NJ). USA 5, 5′, 6 ,6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazol carbocyanine iodide (JC-1) was from Molecular Probes (Eugene, USA). Suc-LLVY-AMC was from Calbiochem (EMD Biosciences, Germany). Dulbecco's modified Eagle's medium (DMEM) and FBS were from Gibco (Gaithersburg, USA). Dimethyl sulfoxide (DMSO), Hoechst 33342, RNase A, proteinase K, aprotinin, leupeptin, PMSF and thiazolyl blue tetrazolium bromide were from Sigma (St. Louis, USA); SuperSignal West Pico enhanced chemilumin, Gescence Western blotting detection reagent was from Pierce (Rockford, USA).

2. Cell culture

The G361 human melanoma cell line was purchased from ATCC (Rockville, USA). Cells were maintained at 37°C with 5% CO_2 in air atmosphere in minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 μg/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, and supplemented with 10% FBS. Cells were matained in Dulbecco's modified Eagle's medium with 10% FBS.

3. MTT assay

Cells were placed in a 96-well plate and incubated 24 h. Then cells treated with 10, 25, 50, 100 μM of CDCA, HS-1199, and HS-1200 for 3 h. And then cells were treated with 500 μg/mL of thiazolyl blue tetrazolium bromide (MTT solution). Cells were incubated at 37°C with 5% CO_2 for 4 h. And then the medium was aspirated and formed formazan crystals were dissolved in the mixture solution of 75 μL DMSO and 75 μL absolute ethanol. Cell viability was measured by a ELISA reader (Sunrise Remote Control, Tecan, Austria) at 570 nm excitatory emission wavelength.

4. Hoechst staining

Cells were harvested and cell suspension was centrifuged onto a clean, fat-free glass slide with a cytocentrifuge. The samples were stained in 4 μg/mL Hoechst 33342 for 30 min at 37°C and fixed for 10 min in 4% paraformaldehyde.

5. DNA electrophoresis

2×10^6 cells were resuspended in 1.5 mL of lysis buffer [10 mM Tris (pH 7.5), 10 mM EDTA (pH 8.0), 10 mM NaCl and 0.5% SDS] into which proteinase K (200 μg/mL) was added. After samples were incubated overnight at 48°C, 200 μL of ice cold 5 M NaCl was added and the supernatant containing fragmented DNA was collected after centrifugation. The DNA was then precipitated overnight at −20°C in 50% isopropanol and Rnase A-treated for 1 h at 37°C. The DNA from 10^6 cells (15 μL) was equally loaded on each lane of 2% agarose gels in Tris-acetic acid/EDTA buffer containing 0.5 μg/mL ethidium bromide at 50 mA for 1.5 h.

6. Proteasome activity

After treatment with 50 μM of CDCA, HS-1199, and HS-1200 for 3 h, cells were lysed in proteasome buffer [10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM ATP, 20% glycerol, and 4 mM dithiothreitol (DTT)], sonicated, and then centrifuged at 13,000 g at 4°C for 10 min. The supernatant (20 μg of protein) were incubated with proteasome activity buffer [0.05 M Tris-HCl, pH 8.0, 0.5 mM EDTA, 50 μM Suc-LLVY-AMC] for 1 h at 37°C. The intensity of fluorescence of each solution was measured by a modular fluorimetric system (Spex Edison, USA) at 380 nm excitatory and 460 nm emission wavelengths. All readings were standardized using the fluorescence intensity of an equal

volume of free AMC solution (50 μM).

7. Western blot analysis

Cells (2×10^6) treated with CDCA, HS-1199 and HS-1200 were washed twice with ice-cold PBS, resuspended in 200 μL ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% TritonX-100, 2 mM PMSF, 2 μL/mL aprotinin and 2 μL/mL leupeptin] and incubated at 4°C for 30 min. The lysates were centrifuged at 14,000 revolutions per min for 15 min at 4°C. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, USA) and 50 μg of proteins were loaded onto 7.5 ~15% SDS/PAGE. The gels were transferred to Nitrocellulose membrane (Amersham Pharmacia Biotech, UK) and reacted with rabbit polyclonal anti-human caspase-3 (1 : 1,000) and anti-human DNA fragmentation factor (DFF) (1 : 1,000) antibodies, mouse polyclonal anti-human poly (ADP-ribose) polymerase (PARP) antibody (1 : 1,000), and goat polyclonal anti-mouse AIF antibody (1 : 500). Immunostaining with antibodies was performed using SuperSignal West Pico enhanced chemiluminescence substrate and detected with Alpha Imager HP (Alpha Innotech, USA).

8. Immunofluorescent staining

Cells were cytocentrifuged and fixed for 10 min in 4% paraformaldehyde, incubated with each primary antibody for 1 h, washed 3 each for 5 min, and then incubated with FITC-conjugated secondary antibody for 1 h at room temperature. Cells were mounted with PBS. Fluorescent images were observed and analyzed under Zeiss LSM 510 laser-scanning confocal microscope (Göettingen, Germany).

9. Assay of mitochondrial membrane potential (MMP)

JC-1 was added directly to the cell culture medium (1 μM final concentration) and incubated for 15 min. The medium was then replaced with PBS, and cells were resuspended in 10 μg/mL of methanol and incubated at 37°C for 30 min. Flow cytometry to measure MMP was performed on a Epics XL (Beckman Coulter, FL, USA). Data were acquired and analyzed using EXPO32 ADC XL 4 color software. The analyzer threshold was adjusted on the FSC channel to exclude noise and most of the subcellular debris.

10. Statistical analysis

Three independent experiments were performed for each experimental group and each experiment was performed in triplicate. The results of the experimental and control groups were compared for statistical significance ($p < 0.01$ or 0.05) using Student's *t*-test for summary data.

Results

1. Synthetic CDCA derivatives reduced viability in G361 cells.

As determined by MTT, synthetic CDCA derivatives

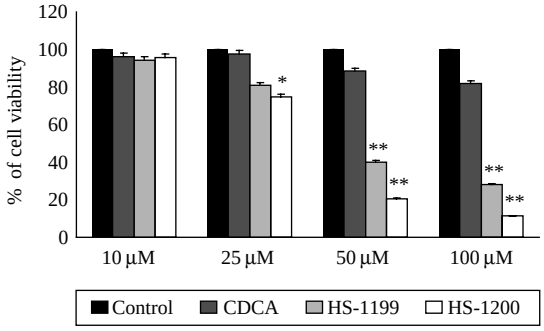


Fig. 2. G361 cells treated with synthetic CDCA derivatives, HS-1199 and HS-1200, at 10 μM, 25 μM, 50 μM and 100 μM for 3 h show the reduction of viability in a dose-dependent manner. Result is expressed as percentage of the control ±SD of three separate experiments. *, $p < 0.05$, **, $p < 0.01$.

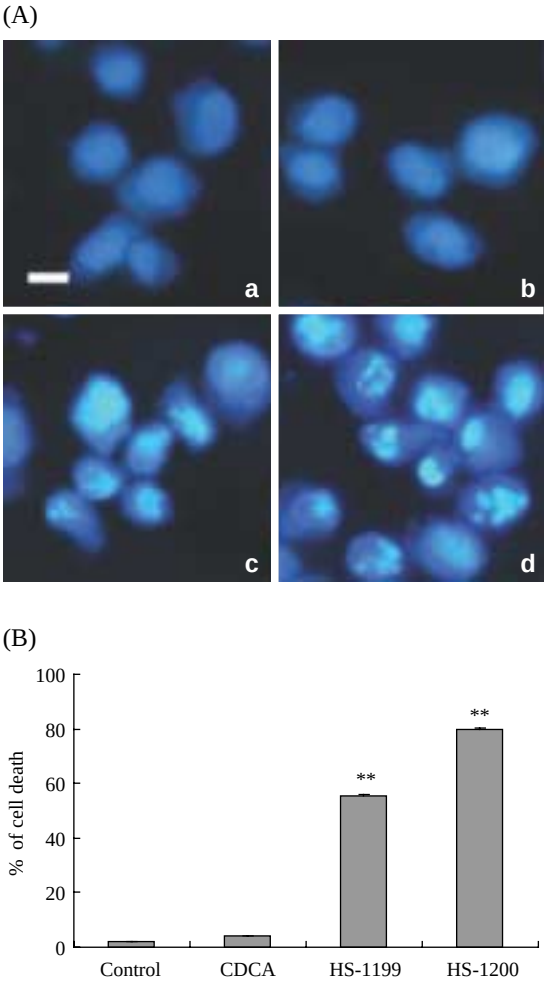


Fig. 3. G361 cells treated with synthetic CDCA derivatives, HS-1199 and HS-1200, at 50 μM for 3 h show the nuclear condensation or fragmentation compared to the negative control or the CDCA-treated group. (A) Hoechst staining. scale bar, 10 μm. a, control cells; b, CDCA treated cells; c, HS-1119 treated cells; d, HS-1200 treated cells. (B) Quantification of the nuclear condensation determined by Hoechst staining. Result is expressed as percentage of the control ± SD of three separate experiments. *, $p < 0.05$, **, $p < 0.01$.

at 50 μM for 3 h significantly reduced viability of cells. But CDCA at 50 μM for 3 h did not. Both synthetic CDCA derivatives decreased the viability of G361 cells in a dose-dependent manner (Fig. 2).

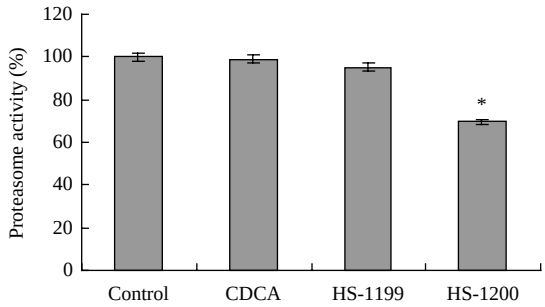


Fig. 4. G361 cells treated for 3 h with synthetic CDCA derivative, HS-1200, at 50 μM show the reduction of proteasome activity compared to the CDCA and HS-1199 treated group. Data are presented as the percent of the control. *, $p < 0.05$, **, $p < 0.01$.

2. Synthetic CDCA derivatives induced nuclear condensation and fragmentation in G361 cells

Hoechst staining proved that the reduction in viability resulted from apoptosis. G361 cells treated with synthetic CDCA derivatives, HS-1199 and HS-1200, at 50 μM for 3 h show the nuclear condensation or fragmentation compared to the negative control or the CDCA-treated group. Especially HS-1200 showed stronger cytotoxic effect than HS-1199 at the same dose (Fig. 3A and 3B).

3. Synthetic CDCA derivative HS-1200 inhibited proteasome activity in G361 cells

The synthetic CDCA derivative HS-1200 caused decrease in proteasome activity of G361 cells. But HS-1119 treated cells show slight decrease compared to control cells and CDCA treated cells (Fig. 4).

4. Nuclear events were demonstrated in G361 cells after synthetic CDCA derivatives treatment

Western blot assay showed degradation and cleavage of caspases-3, DFF and PARP in the treatment of HS-1200. In the treatment of HS-1199 at same dose, the

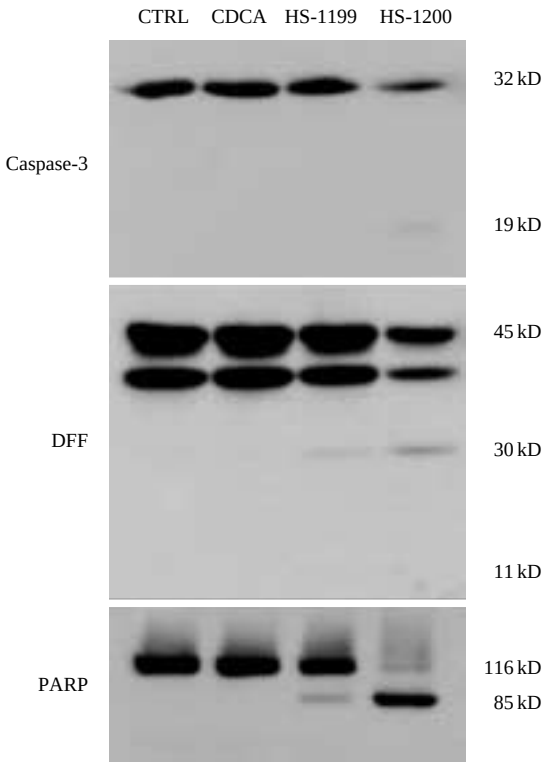


Fig. 5. G361 cells treated for 3 h with synthetic CDCA derivatives, HS-1199 and HS-1200, at 50 μM produce nuclear events. Western blot analyses showing degradation of caspase-3, DFF and PARP. HS-1200 induced caspase-3, DFF and PARP degradation, and produced 19 kd cleavage of caspase-3, 30 kD cleavage of DFF and 85 kd cleavage of PARP. HS-1119 produced 30 kD cleavage of DFF and 85 kd cleavage of PARP.

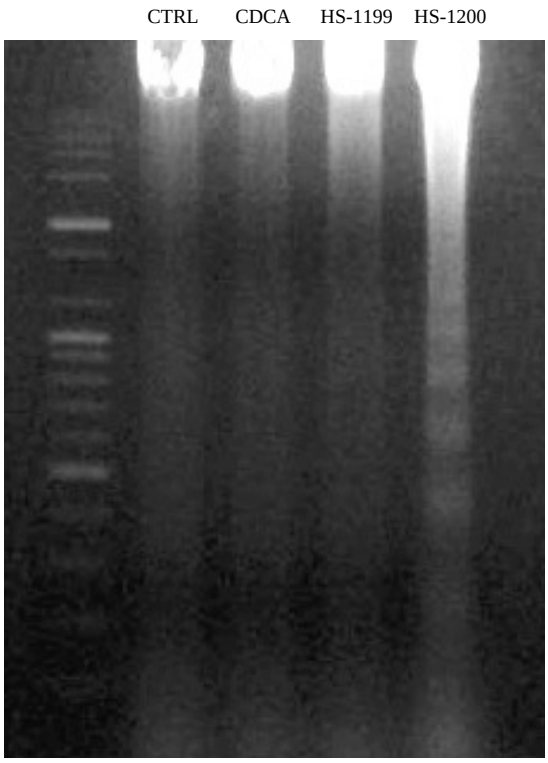


Fig. 6. DNA electrophoresis demonstration of G361 cells treated for 3 h with synthetic CDCA derivatives, HS-1199 and HS-1200, at 50 μM. DNA electrophoresis evidently showed DNA ladder in G361 cells treated with HS-1200.

cleavage of DFF and PARP showed whereas the cleavage of caspase-3 did not (Fig. 5). DNA electrophoresis showed a ladder pattern of DNA fragments in the treatment of HS-1200 whereas did not show a ladder pattern of DNA fragments in the treatment of CDCA and HS-1199 (Fig. 6).

5. Synthetic CDCA derivatives induced apoptosis in G361 cells via mitochondrial pathway.

Mitochondrial membrane potential (MMP) was reduced after treatment with HS-1199 and HS-1200 but was not reduced after treatment with CDCA. In G361 cells treated with HS-1200, MMP was significantly and remarkably reduced (Fig. 7A). Immunofluorescent study showed that synthetic CDCA derivatives led to the release of cytochrome c from mitochondria into the cytosol (Fig. 7B).

Western blot assay and confocal microscopy were conducted to examine whether another mitochondrial apoptogenic factor AIF is involved or not. Expression level of this protein did not increase after treatment of HS-1199 and HS-1200 (Fig. 8A). AIF was shown to release from mitochondria, and tranlocation onto nuclei was evident after treatment of HS-1199 and HS-

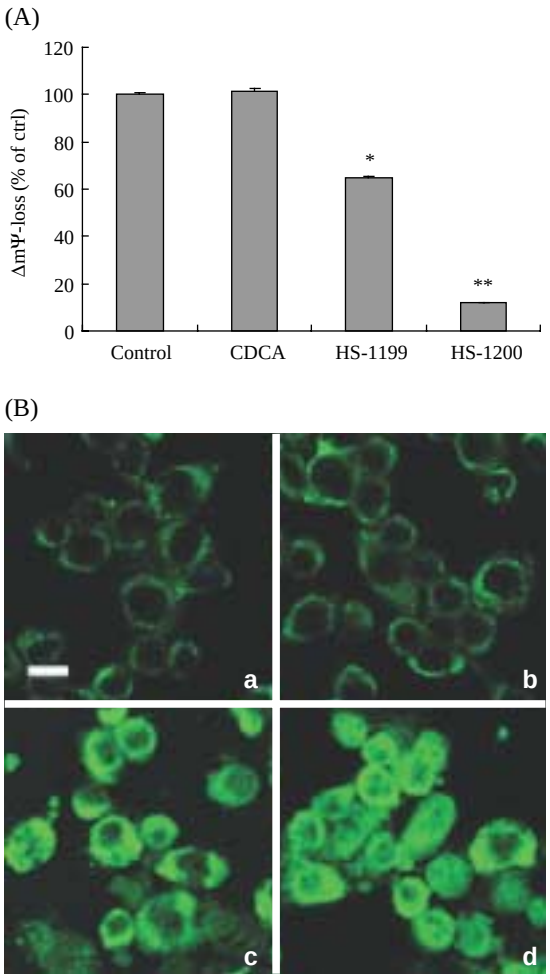


Fig. 7. G361 cells treated for 3 h with synthetic CDCA derivatives, HS-1199 and HS-1200, at 50 μM induce mitochondrial events. (A) Loss of mitochondrial membrane potential (MMP) was showed after treatment with HS-1199 and HS-1200 but was not showed after treatment with CDCA. *, $p < 0.05$, **, $p < 0.01$. (B) Confocal microscopy showing the release of cytochrome c from mitochondria into the cytosol after treatment with HS-1119 and HS-1200. a, control cells; b, CDCA treated cells; c, HS-1119 treated cells; d, HS-1200 treated cells. scale bar, 10 μm.

1200 (Fig. 8B).

These data support that HS-1199 and HS-1200 induce apoptosis via mitochondrial pathway in G361 cells.

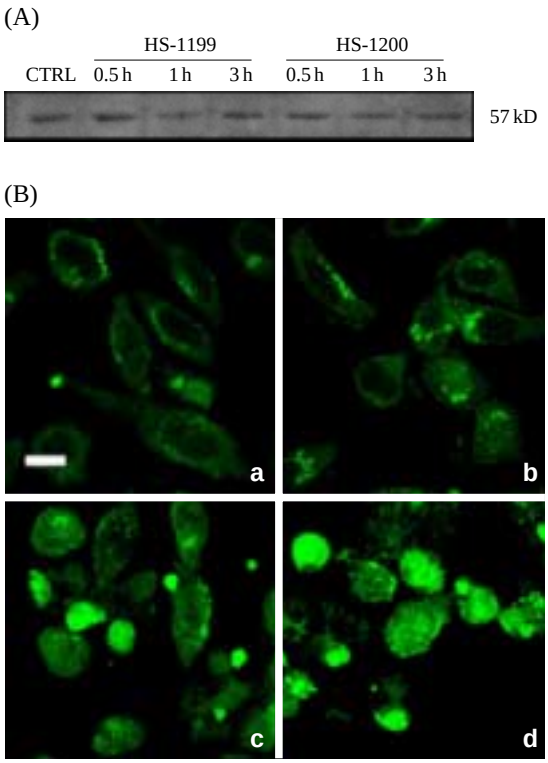


Fig. 8. AIF involves apoptosis in G361 cells treated with synthetic CDCA derivatives, HS-1199 and HS-1200, at 50 μM. (A) Western blot analysis was undertaken at various time points. Expression level of this protein did not increase compared to the control. (B) Confocal microscopy showing that AIF was released from mitochondria, and that translocation onto nuclei was evident in HS-1119 and HS -1200 treated cells for 3 h. a, control cells; b, CDCA treated cells; c, HS-1199 treated cells; d, HS-1200 treated cells. scale bar, 10 μm.

Discussion

Numerous studies have focused on the targeted induction of apoptosis in order to control the unlimited growth of cancer cells. Moreover, induction of apoptosis of the activated cell may promote therapeutic efficiency. Therefore inducing human melanoma cells (G361 cells) may contribute as a potential therapeutics managing the malignant melanoma.

It has been reported the antiproliferative efficacy of

synthetic CDCA derivatives in various cancer cells by inducing apoptosis. Those studies demonstrated the decrease of proteasome activity, mitochondrial events, and nuclear condensation (Choi et al. 2001, Yoon et al. 2001, Choi et al. 2003b, Jeong et al. 2003, Seo et al. 2003) in synthetic CDCA derivatives-inducing apoptosis. And they also showed that they produced apoptosis via p53 independent pathway (Im et al. 2001).

Proteasome is a fundamental non-lysosomal tool that cells use to process or degrade a variety of short-lived proteins. Proteolysis mediated by the ubiquitin-proteasome system has been reported to be implicated in the regulation of apoptosis (Drexler et al. 2000). The proteasome pathway is mostly known to work upstream of the mitochondrial alterations and caspase activation (Orlowski 1999) and can involve in different systems including NF-kB, Bax and Bcl-2 (Grimm et al. 1996, Sadoul et al. 1996, Orlowski 1999, Li and Dou 2000). Proteasome inhibitors, as single or combined with other anticancer agents, are suggested as a new class of potential anticancer agents (Chandra et al. 1998, Delic et al. 1998, Orlowski et al. 1998, Adams et al. 1999, Fanelli et al. 1999, Golab et al. 2000, Li and Dou 2000). Also in our recent study a proteasome inhibitor, lactacystin augmented genistein-induced apoptosis of p815 mastocytoma cells (Park et al. 2002). HS-1200 not only produced decrease of proteasome activity, but also induced augmented apoptotic effect in the combination therapy of HS-1200 and lactacystin at low concentration (Seo et al. 2003). In this study, synthetic CDCA derivative, HS-1200 also produced the reduction of proteasome acitivity in G361 human melanoma cells.

Mitochondria plays an important role in apoptosis and induction of the mitochondrial permeability transition play a key part in the regulation of apoptosis (Kroemer et al. 1997, Green and Reed 1998, Susin et al. 1999). Permeabilization of the outer mitochondrial membrane (OMM) is modulated by members of the Bcl-2 family of proteins. Anti-apoptotic members, such as Bcl-2 and Bcl-XL, inhibit protein release, whereas pro-apoptotic members, such as Bax and Bak, stimulate this release (Orrenius 2004). OMM becomes permeable to intermembrane space proteins such as cytochrome *c* (Golab et al. 2000) and AIF (apoptosis inducing factor) during apoptosis. Once released, cytochrome *c* promotes the activation of pro-caspase-9 directly within the apoptosome complex (Li et al. 1997). Cytochrome *c* release and disruption of MMP are in fact known features in apoptosis triggered by proteasome inhibition (Wagenknecht et al. 2000, Marshansky et al. 2001). On induction of apoptosis, AIF translocates to the nucleus, resulting in chromatin condensation and large-scale DNA fragmentation (Daugas et al. 2000). This study was evidently demonstrated that these representative mitochondrial events are involved in synthetic CDCA derivatives-inducing apoptosis of G361 cells.

Common final event of apoptosis is nuclear condensation, and this event is controled by caspase, DFF, and PARP. DFF triggers both DNA fragmentation and chromatin condensation during apoptosis (Liu et al. 1998). In this study cleavages of caspase-3, DFF, and PARP were shown in CDCA derivative HS-1200 treated G361 cells.

It remains an open question through which exact molecular mechanism synthetic CDCA derivatives exert apoptotic activity. Furthermore, identification of the targets of synthetic CDCA derivatives in cancer cell apoptosis is needed. Future studies may provide important information for understanding the mechanism underlying synthetic CDCA derivatives-inducing apoptosis and their clinical application.

In conclusion, although many important issues for their therapeutic application remain to be elucidated, we here demonstrated for the first time that synthetic CDCA dedrivatives induce apoptosis of human melanoma cells through the proteasome, caspase and mitochondrial pathway. Between two synthetic derivatives,

HS-1200 showed stronger apoptosis-inducing effect than HS-1199 did. Therefore synthetic CDCA derivative HS-1200 was demonstrated to have the most efficient apoptotic effect. Therefore our data provide the possibility that synthetic CDCA derivative HS-1200 could be considered as a novel therapeutic strategy for human melanoma cells from its powerful apoptosis-inducing activity.

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합성 chenodeoxycholic acid 유도체 HS-1200이 유도한 사람흑색종세포 세포자멸사 연구

백철중, 민지학, 문성혁, 김인령, 이승은
김덕한, 김규천, 곽현호, 박봉수
부산대학교 치과대학 구강해부학교실

간추림 : 담즙산과 합성담즙산유도체가 여러 종류의 암세포에 세포자멸사(apoptosis)를 유도하며, 항암효과가 있다고 알려져 있다. 또한 합성 chenodeoxycholic acid (CDCA) 유도체가 여러 가지 암세포에 유도한 세포자멸사 연구들이 보고되어 왔다. 하지만 아직까지 사람흑색종세포에 합성 CDCA 유도체가 유도한 세포자멸사 연구는 보고되지 않았다. 그래서 본 연구는 합성 CDCA 유도체인 HS-1199와 HS-1200이 사람흑색종세포(G361 세포)에 세포자멸사 효과와 세포자멸사 기작을 밝혀내기 위해서 수행되었다.

합성 CDCA에 처리된 G361 세포의 생존율을 확인하기 위해서 MTT 방법을 사용하였고, 세포자멸사 유도 검증은 DNA 전기영동법과 Hoechst 염색을 이용하였다. 세포자멸사에 관계하는 단백질의 발현 변화와 세포 내에서 이동을 밝혀내기 위해서 Western bot 분석과 면역형광염색법을 수행하였다. 더 나아가서 proteasome 활성도와 사립체막 전위 변화를 측정하였다.

합성 CDCA 유도체로 처리된 G361 세포에서 caspase-3, DFF, PARP의 파괴, caspase-3 (HS-1200 only), PARP, DFF의 분절화, DNA 조각남 (HS-1200 only), 핵 응축, proteasome 활성화의 감소, 사립체막전위 (MMP)의 감소, 그리고 cytochrome *c*와 AIF의 사립체에서 세포질로의 유리와 같은 다양한 세포자멸사의 증거를 보였다. 두 개의 합성 CDCA 유도체 중에서 HS-1200이 HS-1199보다 더욱 강한 세포자멸사 효과를 보였다.

본 연구는 CDCA 유도체인 HS-1200이 사람흑색종세포에서 proteasome, 사립체 그리고 caspase 경로을 통해서 세포자멸사를 유도하는 것을 증명하였다. 이러한 결과는 HS-1200이 사람흑색종의 새로운 치료적 전략으로 응용될 수 있다고 생각한다.

찾아보기 낱말 : 합성 chenodeoxycholic acid (CDCA) 유도체, HS-1200, 세포자멸사, 사람흑색종세포

교신저자 : 박봉수 (부산대학교 치과대학 구강해부학교실)
전자우편 : parkbs@pusan.ac.kr