J. Microbiol. Biotechnol. (2008), **18**(11), 1862–1867
doi: 10.4014/jmb.0800.124
First published online 4 June 2008

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Streptochlorin Isolated from *Streptomyces* sp. Induces Apoptosis in Human Hepatocarcinoma Cells Through a Reactive Oxygen Species-Mediated Mitochondrial Pathway

Shin, Dong Yeok[1,2], Hee Jae Shin[3], Gi-Young Kim[4], JaeHun Cheong[2], Il-Whan Choi[5], Se-Kwon Kim[6], Sung-Kwon Moon[7], Ho Sung Kang[2], and Yung Hyun Choi[1,7]*

[1]*Department of Biochemistry, Dongeui University College of Oriental Medicine and Department of Biomaterial Control (BK21 Program), Dongeui University Graduate School, Busan 614-052, Korea*
[2]*Department of Molecular Biology, College of Natural Sciences, Pusan National University, Busan 609-735, Korea*
[3]*Marine Natural Product Chemistry Laboratory, Ocean Research and Development Institute, Ansan 425-600, Korea*
[4]*Faculty of Applied Marine Science, Cheju National University, Jeju 690-756, Korea*
[5]*Department of Microbiology, College of Medicine and Center for Viral Disease Research, Inje University, Busan 614-735, Korea*
[6]*Department of Chemistry and Marine Bioprocess Research Center, Pukyong National University, Busan 608-737, Korea*
[7]*Department of Food and Biotechnology, Chungju National University, Chungju 380-702, Korea*

Received: February 11, 2008 / Accepted: April 2, 2008

Streptochlorin is a small molecule isolated from marine *Streptomyces* sp. that is known to have antiangiogenic and anticancer properties. In this study, we examined the effects of this compound on reactive oxygen species (ROS) production and the association of these effects with apoptotic tumor cell death, using a human hepatocarcinoma Hep3B cell line. The results of this study demonstrated that streptochlorin mediates ROS production, and that this mediation is followed by a decrease in the mitochondrial membrane potential (MMP, $\Delta\Psi_m$), activation of caspase-3, and downregulation of antiapoptotic Bcl-2 protein. The quenching of ROS generation by *N*-acetyl-L-cysteine administration, a scavenger of ROS, reversed the streptochlorin-induced apoptosis effects *via* inhibition of ROS production, MMP collapse, and the subsequent activation of caspase-3. These observations clearly indicate that ROS are involved in the early molecular events in the streptochlorin-induced apoptotic pathway. Taken together, our data imply that streptochlorin-induced ROS is a key mediator of MMP collapse, which leads to the caspase-3 activation, culminating in apoptosis.

Keywords: Streptochlorin, Hep3B, apoptosis, ROS, MMP

Apoptosis, a programmed cell death, is an essential biochemical process needed to control cell numbers in many developmental and physiological settings. In general, apoptosis can be initiated by either an extrinsic or an intrinsic pathway [4]. The extrinsic pathway is initiated by cell surface receptors, whereas the intrinsic pathway is initiated by the mitochondria [6, 7]. Mitochondria are both the source and target of reactive oxygen species (ROS) generation, and damaged mitochondria can release more ROS [1]. ROS are thought to participate in a wide variety of cellular functions, including cell proliferation, differentiation and apoptosis [9]. Previous observations have suggested that ROS and mitochondria might mediate apoptosis induction under both physiologic and pathologic conditions. ROS can cause mitochondrial membrane potential (MMP, $\Delta\Psi_m$) loss by activating mitochondrial permeability transition, and induce apoptosis by releasing apoptogenic protein such as cytochrome *c* to the cytosol [6, 7]. In the cytosol, cytochrome *c* can activate caspase-9, and activated caspase-9 in turn cleaves and activates executioner caspase-3. After caspase-3 activation, some specific substrates for caspase-3 such as poly(ADP-ribose) polymerase (PARP) are cleaved, and eventually lead to apoptosis [10]. Consistent with the role of mitochondria in the control of cell death, survival or apoptotic factors such as Bcl-2 and Bax act on the organelle to prevent or facilitate the release of apoptogenic factors such as cytochrome *c* [12, 13, 18].

Recent studies indicated that marine microorganisms are proving to be a novel and rich source of bioactive compounds owing to their potential pharmacological activities. Despite decades of research, all evidences suggest that there must remain many interesting natural molecules with potential therapeutic application yet to be discovered. Among them, marine bacteria have been used as a main source for this

*Corresponding author
Phone: 82-51-850-7413; Fax: 82-51-853-4036;
E-mail: choiyh@deu.ac.kr

study of hitherto unknown biological activities of natural marine products [15, 17]. Investigations into components of marine microorganisms have proven that many are not general cytotoxic agents but rather are targeted towards specific cellular or biochemical events and therefore hold strong potential as antimicrobial, anticancer, or anti-inflammatory agents [14, 17]. Through the screening of marine natural products that inhibit cancer cell proliferation, we previously reported that streptochlorin isolated from *Streptomyces* sp. (strain 04DH110) exhibited selective cytotoxicity against several cancer cell lines [3, 16]; however, the molecular mechanisms of its antiproliferative action on malignant cell growth are not completely known.

To further explore the mechanism of its anticancer activity, in the present study, we hypothesized that streptochlorin would induce functional changes in the mitochondria in association with ROS generation in the course of apoptosis induction of human cancer cells *in vitro*. To test this hypothesis, we evaluated the effect of streptochlorin on MMP, ROS, and apoptosis using the human hepatocarcinoma Hep3B cell line, and our results indicated the requirement of ROS generation in apoptosis induced by streptochlorin in Hep3B cells.

Materials and Methods

Cell Culture and Viability Assay

Human hepatocarcinoma Hep3B cells were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 units/ml penicillin/streptomycin and supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, U.S.A.) at 37°C and 5% CO_2. Streptochlorin (Fig. 1A) was prepared as described previously [16] and dissolved in dimethyl sulfoxide (DMSO) as a stock solution at 1 mg/ml concentration, and stored in aliquots at −20°C. Measurement of cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, U.S.A.) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzyme.

Nuclear Staining with DAPI

After treating the cells with streptochlorin for 48 h, the cells were harvested, washed in ice-cold phosphate-buffered saline (PBS), and fixed with 3.7% paraformaldehyde (Sigma) in PBS for 10 min at room temperature. The fixed cells were washed with PBS and stained with a 4,6-diamidino-2-phenylindole (DAPI; Sigma) solution for 10 min at room temperature. The nuclear morphology of the cells was examined by fluorescent microscopy (Carl Zeiss, Germany).

DNA Fragmentation Assay

The cells were treated with different streptochlorin concentrations for 48 h and lysed on ice in a buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min. The lysates were vortexed and cleared by centrifugation at 10,000 ×*g* for 20 min. The fragmented DNA in the supernatant was extracted using an equal volume of neutral phenol:chloroform: isoamylalcohol (25:24:1, v/v/v) and analyzed electrophoretically on 1.0% agarose gel containing ethidium bromide (EtBr; Sigma).

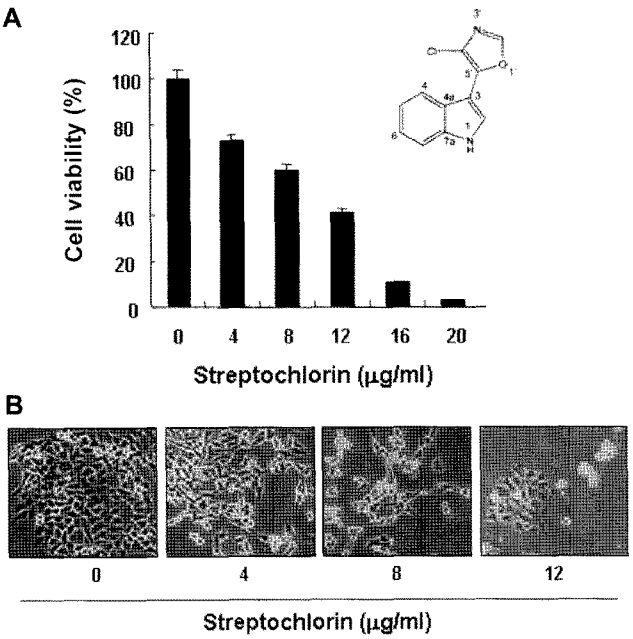

Fig. 1. Inhibition of cell viability by streptochlorin treatment in human hepatocarcinoma Hep3B cells.
A. Hep3B cells were plated at a concentration of 1×10^5 cells per 60-mm plate and then incubated for 24 h. The cells were treated with various concentrations of streptochlorin for 48 h and the cell viability was measured using a MTT assay. The data shown represent the mean±SD of three independent experiments. **B.** After 48 h incubation with streptochlorin, the cells were sampled and examined by inverted microscopy. Magnification, ×200.

Flow Cytometry Analysis for Measurement of Sub-G1 Phase

The cells were harvested and washed once with PBS, fixed in ice-cold 70% ethanol, and stored at 4°C. Prior to analysis, the cells were washed once again with PBS, suspended in 1 ml of a cold propidium iodide (PI; Sigma) solution containing 100 μg/ml RNase A, 50 μg/ml PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40, and further incubated on ice for 30 min in the dark. Flow cytometric analyses were carried out using a flow cytometer (FACS Caliber, Becton Dikinson, San Jose, CA, U.S.A.) and CellQuest software was used to determine the relative DNA content based on the presence of a red fluorescence.

Measurement of Intracellular ROS and MMP

ROS production was monitored using the stable nonpolar dye 2,7 dichlorofluorescein diacetate (DCFH-DA) that readily diffuses into cells [2]. The cells were seeded in 24-well plates and incubated in the absence or presence of streptochlorin for different periods of time. Later, the cells were incubated with 10 μM DCFH-DA for 30 min. The ROS production in cells was monitored by a flow cytometer using CellQuest software. To measure the MMP, a dual-emission potential-sensitive probe, 5,5 V, 6,6 V-tetrachloro-1,1 V,3,3 V-tetraethyl-imidacarbocyanine iodide (JC-1, Sigma), was used. After treatment

with streptochlorin, 5×10^5 cells were collected, stained with 2 mg/l JC-1 at 37°C for 20 min, and then analyzed using a flow cytometer.

Protein Extraction and Western Blotting

The cells were harvested and lysed. The protein concentrations were measured using a BioRad protein assay (BioRad Lab., Hercules, CA, U.S.A.) according to the manufacturer's instructions. For Western blot analysis, an equal amount of protein was subjected to electrophoresis on SDS-polyacrylamide gel and transferred by electroblotting to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.). The blots were probed with the desired antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibody, and visualized by enhanced chemiluminescence (ECL) according to the recommended procedure (Amersham Corp., Arlington Heights, IL, U.S.A.). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.) and Cell Signaling Technology, Inc. (Boston, MA, U.S.A.). The peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham Corp.

***In Vitro* Caspase-3 Activity Assay**

The caspase-3 activity was determined by a colorimetric assay using a caspase-3 activation kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, U.S.A.). Briefly, the cells were lysed in a lysis buffer for 30 min on an ice bath. The supernatants were collected and incubated at 37°C with the reaction buffer supplied, which contained dithiothreitol and Asp-Glu-Val-Asp (DEVD)-p-nitroaniline (pNA) as substrates. The optical density of the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

Statistical Analysis

The data are expressed as a mean±SD. A statistical comparison was performed using one-way ANOVA followed by a Fisher test. The significant differences between the groups were determined using an unpaired Student's *t*-test. A p value <0.05 was considered significant.

Results and Discussion

Inhibition of Cell Viability and Induction of Apoptosis by Streptochlorin

In order to determine if streptochlorin decreases the cell viability, Hep3B cells were stimulated with various concentrations of streptochlorin for 48 h, and the cell viability was measured by a MTT assay. As shown in Fig. 1A, the streptochlorin treatment significantly inhibited the cell viability in a concentration-dependent manner. After a 48 h treatment, 12 μg/ml and 16 μg/ml streptochlorin inhibited the cell viability by approximately 59% and 90% compared with the controls, respectively. Direct observations by inverted microscopy demonstrated that Hep3B cells treated with streptochlorin showed many morphological changes compared with the control cells (Fig. 1B). In particular,

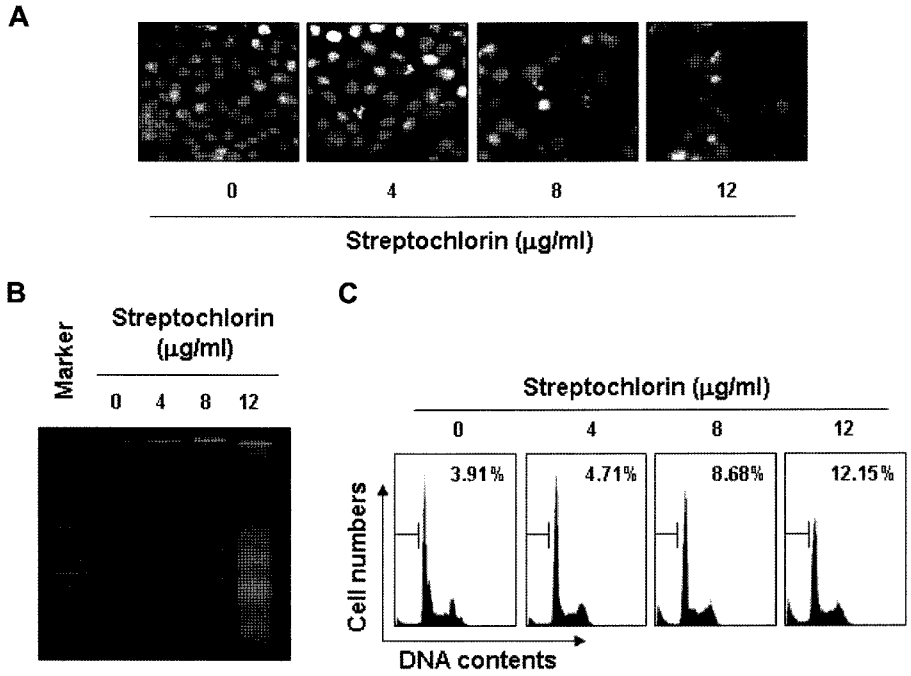

Fig. 2. Induction of apoptosis by streptochlorin treatment in human hepatocarcinoma Hep3B cells.
A. After being treated with streptochlorin for 48 h, the cells were fixed, stained with DAPI, and the nuclear morphology was then photographed under fluorescence using a blue filter. Magnification, ×400. **B**. For the analysis of DNA fragmentation, the genomic DNA was extracted and then electrophoresed on a 1.0% agarose gel. **C**. To quantify the degree of apoptosis induced by streptochlorin, the cells were evaluated for sub-G1 DNA content, which represents the fractions undergoing apoptotic DNA degradation, using a flow cytometer. Each point represents the mean of representative experiments performed at least three times.

cell shrinkage, cytoplasm condensation, and formation of cytoplasmic filaments appeared. Further experiments using fluorescent microscopy, agarose gel electrophoresis, and flow cytometry analyses were carried out to determine if the inhibitory effect of streptochlorin on the cell viability is the result of apoptotic cell death. Morphological analysis with DAPI staining showed nuclei with chromatin condensation and the formation of apoptotic bodies in the cells cultured with streptochlorin in a concentration-dependent manner. On the other hand, very few were observed in the control culture (Fig. 2A). In addition, agarose gel electrophoresis indicated that streptochlorin treatment induced the progressive accumulation of fragmented DNA, which appeared as a typical ladder pattern of DNA fragmentation due to internucleosomal cleavage associated with apoptosis (Fig. 2B) [12]. Therefore, the degree of apoptosis was determined by analyzing the amount of sub-G1 DNA that was in the cells treated with streptochlorin using a flow cytometer [5]. As shown in Fig. 2C, the addition of streptochlorin resulted in the increased accumulation of cells in the sub-G1 phase, which was similar to the results observed when the streptochlorin-induced loss of cell viability, formation of apoptotic bodies, and accumulation of extranuclear fragmented DNA were evaluated. This suggests that Hep3B cells may undergo apoptosis after exposure to streptochlorin, and indicates that there is a good correlation between the extent of apoptosis and the inhibition of cell viability.

Loss of MMP, Downregulation of Bcl-2, and Activation of Caspase-3 by Streptochlorin

The role of the mitochondria in streptochlorin-induced apoptosis of Hep3B cells was further investigated by examining the effect of streptochlorin on Hep3B MMP as well as the levels of Bcl-2 family and caspase-3 activity. Exposure of Hep3B cells to various concentrations of streptochlorin for 48 h led to a significant reduction in the MMP level in a dose-dependent manner (Fig. 3A). Western immunoblotting showed that the levels of antiapoptotic Bcl-2 expression were markedly downregulated in the streptochlorin-treated cells, whereas the levels of pro-apoptotic Bax expression remained unchanged (Fig. 3B). Furthermore,

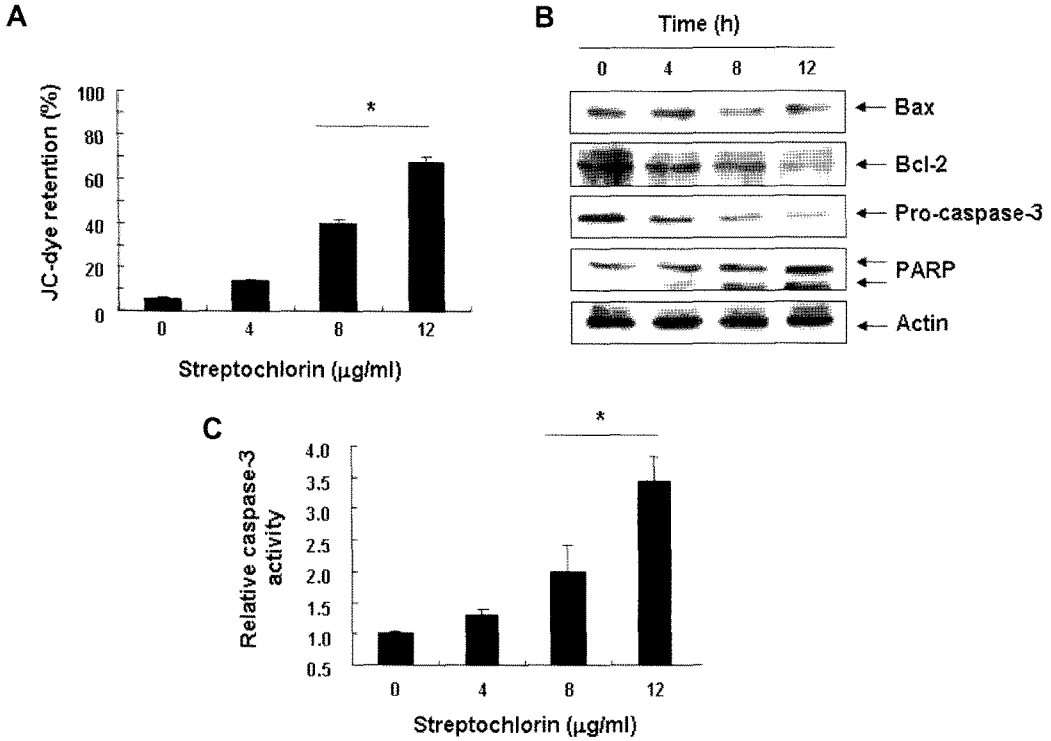

Fig. 3. Loss of MMP and activation of caspase-3 by streptochlorin treatment in human hepatocarcinoma Hep3B cells.
A. The cells were treated with the indicated concentrations of streptochlorin for 48 h, stained with JC-1, and incubated at 37°C for 20 min. The mean JC-1 fluorescence intensity was detected using a flow cytometer. Data represent the mean±SD of representative experiments performed at least three times. The significance was determined by a Student's *t*-test (*, $p<0.05$ *vs.* untreated control). **B.** After being treated with streptochlorin for 48 h, equal amounts of the cell lysates (30 μg) were resolved by SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with the indicated antibodies. Proteins were then visualized using an ECL detection system. Actin was used as the internal control. **C.** The cell lysates obtained from cells grown under the same conditions as (**A**) were assayed for *in vitro* caspase-3 activity using DEVD-*p*NA as substrates. The concentrations of the fluorescent products released were then measured. The results are expressed as the mean±SD of three independent experiments. The significance was determined by a Student's *t*-test (*, $p<0.05$ *vs.* untreated control).

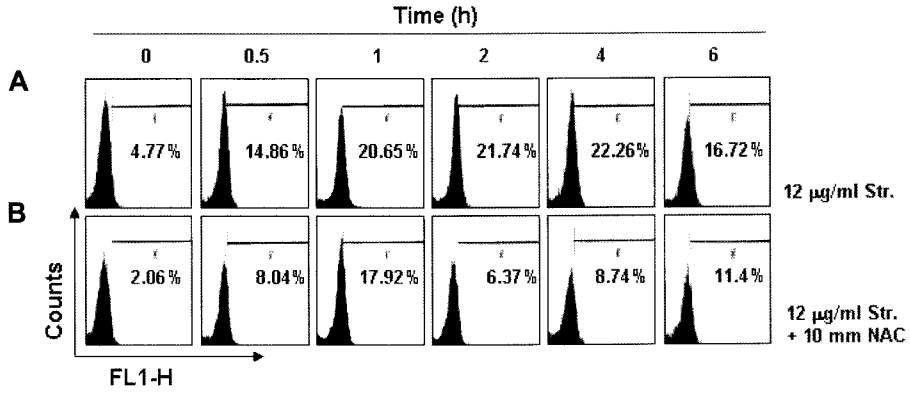

Fig. 4. ROS generation by streptochlorin treatment in human hepatocarcinoma Hep3B cells.
Cells were treated with 12 μg/ml streptochlorin for the indicated times without (**A**) or with ROS scavenger NAC (10 mM, **B**), and then stained with DCFH-DA and incubated at 37°C for 30 min. At each time point, the fluorescent intensity was measured using a flow cytometer. Data represent the means of two representative experiments.

treatment with 12 μg/ml of streptochlorin for more than 4 h significantly inhibited the levels of pro-caspase-3 expression and induced the cleavage of PARP, a caspase-3 target substrate protein [10], in a time-dependent manner (Fig. 3B). Next, cell lysates containing equal amounts of total protein from cells treated with streptochlorin were assayed for *in vitro* caspase-3 activity [8]. As shown in Fig. 3C, treatment with streptochlorin significantly increased the activity of caspase-3 in a dose-dependent fashion. These results indicate that streptochlorin treatment induces apoptotic death in Hep3B cells, at least in part through a mitochondria-dependent pathway.

Streptochlorin-Induced Apoptosis is Associated with the Generation of Intracellular ROS

The events involved in streptochlorin-induced apoptosis were evaluated by kinetic studies that were designed to determine the amount of intracellular ROS production induced by streptochlorin. To accomplish this, ROS production was measured using a cell-permeant, oxidation-sensitive dye, DCFH-DA [2]. The results of these experiments indicated that extending the time of streptochlorin treatment to 0.5 h and 4 h increased the ROS production to greater than 3.1 and 4.8 times that of the control, respectively (Fig. 4A). These results led to the assumption that if ROS

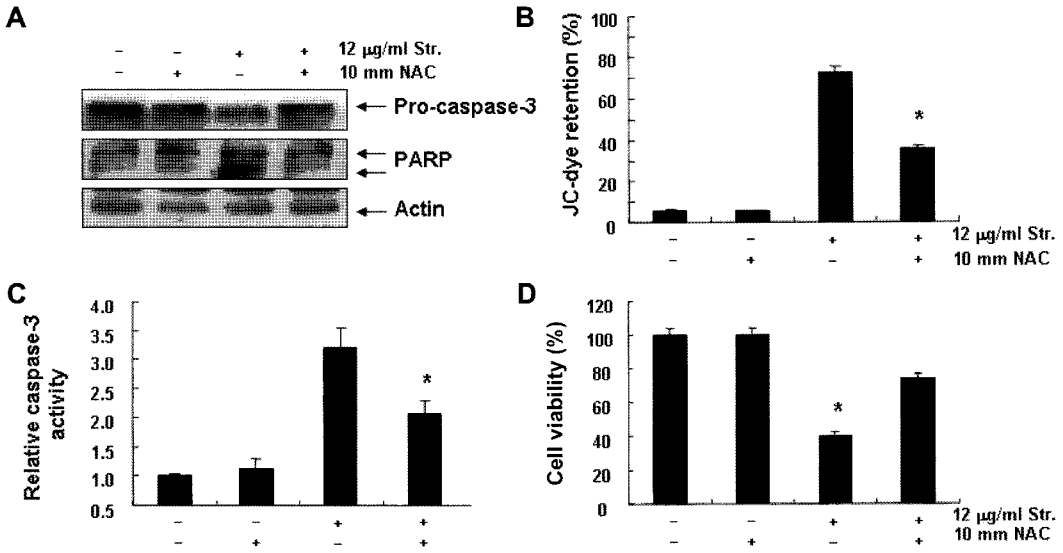

Fig. 5. Streptochlorin-induced apoptosis is associated with ROS generation in human hepatocarcinoma Hep3B cells.
A. Cells were treated with or without NAC for 1 h before challenge with 12 μg/ml streptochlorin for 48 h and then the subcellular fractions were prepared as described in Materials and Methods. The proteins were separated using SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes, which were probed with anti-caspase-3 and anti-PARP antibodies. Proteins were then visualized using an ECL detection system. Actin was used as the internal control. **B–D.** The cells under the same conditions as (**A**) were evaluated for caspase-3 activity (**B**), MMP (**C**), and cell viability (**D**) as described in Materials and Methods. Results represent the mean±SD of triplicate determinations. The significance was determined by a Student's *t*-test (*, $p<0.05$ *vs.* untreated control).

were a crucial factor in the induction of apoptosis, a ROS scavenger must abrogate apoptosis. Therefore, Hep3B cells were pretreated with 10 mM *N*-acetyl-L-cysteine (NAC), a commonly used reactive oxygen intermediate scavenger [19], for 1 h, and then treated with streptochlorin. As shown in Fig. 4B, streptochlorin-induced ROS generation in Hep3B cells that were co-cultured with NAC was effectively blocked, indicating that NAC could be used to determine if ROS is essential for streptochlorin-induced apoptosis. In order to demonstrate that the generation of ROS is a key step in the streptochlorin-induced apoptotic pathway, the cells were pretreated with NAC for 1 h, followed by treatment with streptochlorin for 48 h. As shown in Fig. 5A, NAC alone had no effect on the pro-caspase-3 expression and PARP cleavage at a concentration of 10 mM; however, the presence of 10 mM NAC almost completely suppressed the streptochlorin-induced down-regulation of caspase-3 and degradation of PARP. Furthermore, blocking of the generation of ROS by pretreatment of the cells with NAC significantly prevented the streptochlorin-induced caspase-3 activation as well as loss of cell viability and MMP (Figs. 5B–5D). Taken together, these findings suggest that an increase in ROS is required for streptochlorin-induced apoptosis in Hep3B cells to occur.

In summary, the present study demonstrates that Hep3B human hepatocarcinoma cells undergo apoptosis in response to treatment with streptochlorin through a mitochondria-mediated pathway that requires ROS generation upstream of disruption of MMP, modulation of Bcl-2 family members, and activation of caspase-3. Our data emphasize the key role of ROS in apoptosis induced by streptochlorin in Hep3B cells, and indicate that a positive correlation exists between ROS and mitochondrial events leading to apoptosis. Although in this paper we only roughly assessed whether ROS was involved in the apoptosis induced by streptochlorin, the present results may help to understand the mechanisms for the anticancer activity of streptochlorin.

Acknowledgment

This work was supported by a grant from MarineBio21, Ministry of Maritime Affairs and Fisheries, Korea.

References

1. Boneh, A. 2006. Regulation of mitochondrial oxidative phosphorylation by second messenger-mediated signal transduction mechanisms. *Cell. Mol. Life Sci.* **63:** 1236–1248.
2. Cathcart, R., E. Schwiers, and B. N. Ames. 1984. Detection of picomole levels of lipid hydroperoxides using a dichlorofluorescein fluorescent assay. *Methods Enzymol.* **105:** 352–358.
3. Choi, I. K., H. J. Shin, H. S. Lee, and H. J. Kwon. 2007. Streptochlorin, a marine natural product, inhibits NF-κB activation and suppresses angiogenesis *in vitro*. *J. Microbiol. Biotechnol.* **17:** 1338–1343.
4. Chowdhury, I., B. Tharakan, and G. K. Bhat. 2006. Current concepts in apoptosis: The physiological suicide program revisited. *Cell. Mol. Biol. Lett.* **11:** 506–525.
5. Darzynkiewicz, Z., E. Bedner, T. Traganos, and T. Murakami. 1998. Critical aspects in the analysis of apoptosis and necrosis. *Hum. Cell* **11:** 3–12.
6. Fulda, S. and K. M. Debatin. 2006. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* **25:** 4798–4811.
7. Ghobrial, I. M., T. E. Witzig, and A. A. Adjei. 2005. Targeting apoptosis pathways in cancer therapy. *CA Cancer J. Clin.* **55:** 178–194.
8. Gurtu, V., S. R. Kain, and G. Zhang. 1997. Fluorometric and colorimetric detection of caspase activity associated with apoptosis. *Anal. Biochem.* **251:** 98–102.
9. Kroemer, G., L. Galluzzi, and C. Brenner. 2007. Mitochondrial membrane permeabilization in cell death. *Physiol. Rev.* **87:** 99–163.
10. Lazebnik, Y. A., S. H. Kaufmann, S. Desnoyers, G. G. Poirier, and W. C. Earnshaw. 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371:** 346–347.
11. Mohamad, N., A. Gutierrez, M. Nunez, C. Cocca, G. Martin, G. Cricco, V. Medina, E. Rivera, and R. Bergoc. 2005. Mitochondrial apoptotic pathways. *Biocell* **29:** 149–161.
12. Montague, J. W. and J. A. Cidlowski. 1996. Cellular catabolism in apoptosis: DNA degradation and endonuclease activation. *Experientia* **52:** 957–962.
13. Orrenius, S., V. Gogvadze, and B. Zhivotovsky. 2007. Mitochondrial oxidative stress: Implications for cell death. *Annu. Rev. Pharmacol. Toxicol.* **47:** 143–183.
14. Proksch, P., R. A. Edrada, and R. Ebel. 2002. Drugs from the seas – current status and microbiological implications. *Appl. Microbiol. Biotechnol.* **59:** 125–134.
15. Schweder, T., U. Lindequist, and M. Lalk. 2005. Screening for new metabolites from marine microorganisms. *Adv. Biochem. Eng. Biotechnol.* **96:** 1–48.
16. Shin, H. J., H. S. Jeong, H. S. Lee, S. K. Park, H. M. Kim, and H. J. Kwon. 2007. Isolation and structure determination of streptochlorin, an antiproliferative agent from a marine-derived *Streptomyces* sp. 04DH110. *J. Microbiol. Biotechnol.* **17:** 1403–1406.
17. Singh, S. B. and F. Pelaez. 2008. Biodiversity, chemical diversity and drug discovery. *Prog. Drug Res.* **65:** 143–174.
18. Yin, X. M. 2000. Signal transduction mediated by Bid, a pro-death Bcl-2 family proteins, connects the death receptor and mitochondria apoptosis pathways. *Cell Res.* **10:** 161–167.
19. Zafarullah, M., W. Q. Li, J. Sylvester, and M. Ahmad. 2003. Molecular mechanisms of *N*-acetylcysteine actions. *Cell. Mol. Life Sci.* **60:** 6–20.