Ecteinascidin 770, a Tetrahydroisoquinoline Alkaloid, Sensitizes Human Lung Cancer Cells to Anoikis

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Abstract. *Background: The strategies for achieving anti-metastasis have received increased research interest and clinical attention. The anoikis-sensitizing effect of ecteinascidin 770 (ET-770) was investigated in the present study in non-small cell lung cancer cells. Materials and Methods: ET-770 isolated from Ecteinascidia thurstoni was tested for its anoikis-sensitizing effect on H23 and H460 human lung cancer cells by 2,3-b-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) assay. The levels of proteins being involved in anoikis of cells were determined by western blot analysis. Results: ET-770 was shown to enhance anoikis response of human lung cancer H23 cells in a dose-dependent manner. The underlying mechanism was investigated and it was found that ET-770 sensitized the cells by activating the p53 protein, which in turn down-regulated anti-apoptotic myeloid cell leukemia sequence-1 (MCL1) and up-regulated BCL2–associated X protein (BAX) proteins. However, B-cell lymphoma-2 (BCL2) proteins were not significantly affected by ET-770. Further, the anoikis sensitization of ET-770 was observed in H460 lung cancer cells. Conclusion: The present results reveal for the first time that ET-770 can sensitize anoikis through the p53 pathway and further development of this compound for therapeutic use is warranted.*

Metastasis in patients with lung cancer has become an important hallmark of difficulty in treatment and poor prognosis (1, 2), since metastatic tumors cannot be completely cured by surgery and frequently exhibit resistance to anticancer drugs (2-6). For metastasis to occur, cancer cells have to escape detachment-induced apoptosis, termed anoikis (7), thereby spreading to secondary sites (1, 8). The intrinsic apoptosis or mitochondrial apoptosis pathway seems to be the primary machinery which is responsible for cell anoikis (8, 9) and most evidence indicates proteins in the B-cell lymphoma-2 (BCL2) family as being key players (8-11). Numerous studies have shown that resistance to anoikis in certain types of cancer, including lung cancer, is due to the increase of antiapoptotic proteins (11-13). Among various antiapoptotic proteins in the BCL2 family, myeloid cell leukemia sequence-1 (MCL1) has garnered most attention and is believed to have a dominant effect on anoikis resistance of lung cancer cells (12-14). Recently, we have shown that overexpression of MCL1 suppressed the anoikis response in H460 human lung cancer cells, while shRNA-mediated down-regulation of this protein had an anoikis-sensitizing effect (13). Defects in p53 activation, as well as a decrease of cellular pro-apoptotic protein, including BCL2–associated X protein (BAX), were shown to be involved in anoikis resistance (11, 15-22). Moreover, caveolin–1 (CAV1), a major structural protein of caveolae in plasma membrane, was shown to regulate drug resistance, confer poor prognosis and anoikis resistance (13, 22-25).

Marine organism-derived compounds have been accepted for cancer treatment. Ecteinascidin 743 (ET-743, Yondelis™, Trabectedin (Zeltia and Johnson & Johnson)), a tetrahydroisoquinoline marine product, has been approved by the European Union for use in humans with soft tissue sarcoma (26). Renieramycin M, another tetrahydroisoquinoline isolated from a blue sponge, *Xestispongia* sp., was also shown to possess an ability to overcome anoikis resistance (27). As ongoing research for testing potential compounds from marine organisms for anticancer approaches, ecteinascidin 770 (ET-770), an ecteinascidin derivative that was isolated from Thai tunicate *Ecteinascidia thurstoni*, was investigated for its anoikis-sensitizing effect in the present study.

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Materials and Methods

Ecteinascidin 770. ET-770 (Figure 1) was isolated from the Thai tunicate *Ecteinascidia thurstoni* as previously described (28, 29) and was dissolved in dimethyl sulfoxide (DMSO) and distilled water to achieve the indicated concentrations containing less than 0.1% DMSO.

Cells and reagents. Human non-small cell lung cancer H23 and H460 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 medium containing 5% fetal bovine serums (FBS), 2 mM L-glutamine and 100 units/ml penicillin/streptomycin in a 5% CO_2 environment at 37°C (Gibco, Gaithersburg, MA, USA). Hoechst 33342, propidium iodide (PI), 2,3-*b*-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide salt (XTT), and DMSO were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Antibody for MCL1, BCL2, and CAV1 were obtained from Abcam Ltd. (Cambridge, MA, USA). Antibodies against p53, β-Actin, and BAX were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell viability. Cells were seeded into 96-well plates at 1×10^5 cell/ml for 24 h and then treated with different concentrations of ET-770 for 24 h. Cells were then incubated with 20 μM of XTT reagent for a further 4 h at 37°C. The intensity of the formazan product was measured at 450 nm using a microplate reader. All analyses were established in at least three independent replicate cultures. The cell viability was calculated from the optical density (OD) ratio of treated to non-treated control cells and is presented as a percentage to that of the non-treated controls.

Apoptosis and necrosis assays. Hoechst 33342 and propidium ioide (PI) staining assay: Cells were stained with 10 μM Hoechst 33342 and 5 μg/ml PI. Cells that had intensely condensed and/or fragment nuclei stained by Hoechst 33342 were considered as apoptotic cells, those staining only with PI were considered as necrotic cells. The fluorescent dye stained in cells was visualized under a fluorescence microscope (Olympus IX51with DP70 camera, Tokyo, Japan).

Anoikis assay. H23 and H460 cells in the culture plate were detached and made into a single-cell suspension in RPMI serum-free medium and then seeded into an ultra-low attachment plate (Cornig, Acton, MA, USA) at a density of 1.5×10^5 cell/ml. Cells were then harvested at 0, 3, 6, 9, 12, and 24 h. Cell viability was measured by the XTT assay as mentioned above.

Western blotting. To determine proteins, after specific treatment, cells were incubated with lysis buffer containg 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) for 30 min on ice. The protein concentration was measured by the BCA protein assay kit (Pierce, Rockford, IL, USA). Each sample was denaturized by heating at 95°C for 5 min with Laemmli loading buffer. Subsequently, samples were loaded on sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) at equal amounts of proteins (40 μg/lane) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h in 5% skimmed
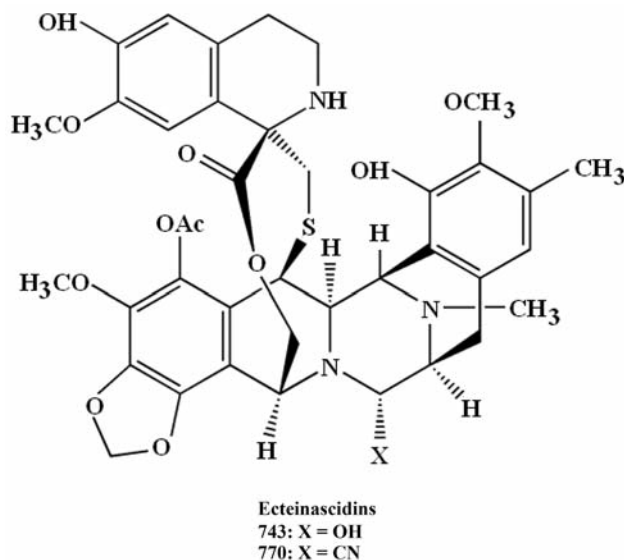

Figure 1. *Structures of ecteinascidins 743 and 770.*

milk in TBST [25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween 20] and incubated with MCL1, BCL2, p53, β-Actin, BAX, or CAV1 (Abcam, Cambridge, MA,USA) at 4°C overnight. Membranes were washed three times with TBST for 8 min, followed by incubation with horseradish peroxidase-conjugated specific secondary (Cell Signaling Technology, Beverly, MA, USA) for 2 h at room temperature. Detection was performed by chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL, USA) and quantification by image densitometry using analyst/PC densitometry software (Bio-Rad). Mean densitometric data from independent experiments were normalized to the β-Actin protein.

Cell-cycle analysis. Apoptotic DNA fragmentation was determined by cell-cycle analysis of the sub-G_0 fraction. Detached cells in the presence or absence of ET-770 were harvested, re-suspended, and incubated with PI buffer at 37°C for 30 min. Cell-cycle profiles were analyzed by a FACScan flow cytometer using a 488 nm excitation beam and a 630-nm band-pass filter with the CellQuest software (Becton Dickinson, Rutherford, NJ, USA).

Statistical analysis. The data are presented as the mean±standard deviation (S.D.). Mean data from at least three independent experiments were normalized to give the non-treated control. Values were analyzed by one-way ANOVA and significance levels were determined by post-hoc analysis with Tukey's test. Significance was set at 0.05.

Results

ET-770 induces apoptosis of H23 human non-small cell lung cancer cells. The cytotoxic effect of ET-770 was first characterized. H23 lung cancer cells were incubated with different concentrations of ET-770 (0-50 nM) for 24 h, and cell viability was analyzed. Figure 2A shows that ET-770 at
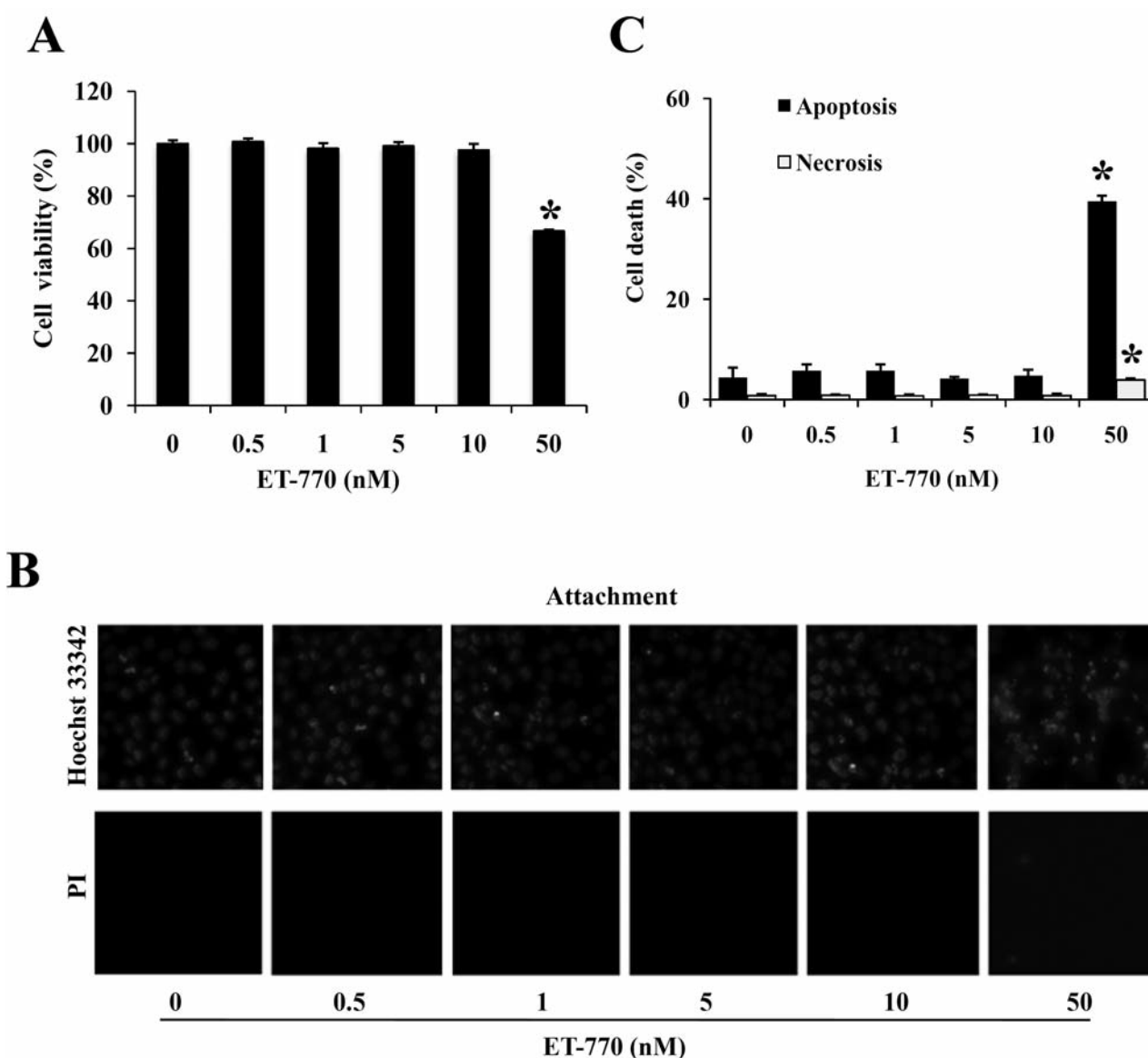
Figure 2. *Cytotoxic effect of ecteinascidin 770 (ET-770) on H23 human lung cancer cells. H23 cells were treated with different concentrations of ET-770 (0–50 nM) for 24 h. A: Cell viability was determined by 2,3-b-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) assay. Values are means±S.D. (n=5), *p<0.05 versus non-treated controls. B: Nuclear morphology of Hoechst33342/PI staining of cells was captured under fluorescence microscopy. C: Percentages of cell apoptosis and necrosis was obtained from Hoechst 33342/PI assays.*

the concentration of 50 nM significantly reduced the viability of H23 cells, while at lower concentrations (1-10 nM) it exhibited neither toxic nor proliferative effects. Therefore, concentrations of 1-10 nM were used for anoikis experiments.

Furthermore, Hoechst 33342/PI nuclear staining was performed to elucidate the mode of cell death induced by ET-770. The results revealed that only a minimal number of cells exhibited apoptotic (~5%) and necrotic (<1%) characteristics when the cells were treated with 1-10 nM of ET-770 (Figure 2B and C). As shown in Figure 2A, ET-770

at 50 nM significantly reduced cell viability to ~66%. Consistently, such a concentration of ET-770 induced approximately 40% apoptosis and 3.9% necrosis of H23 human lung cancer cells (Figure 2B and C).

ET-770 sensitizes H23 human non-small cell lung cancer cells to anoikis. To study the effect of ET-770 on cell anoikis, cells were detached and suspended in the presence or absence of ET-770 (0-50 nM) and cell viability was analyzed at various times (0-24 h). Figure 3A shows that ET-770 at concentrations
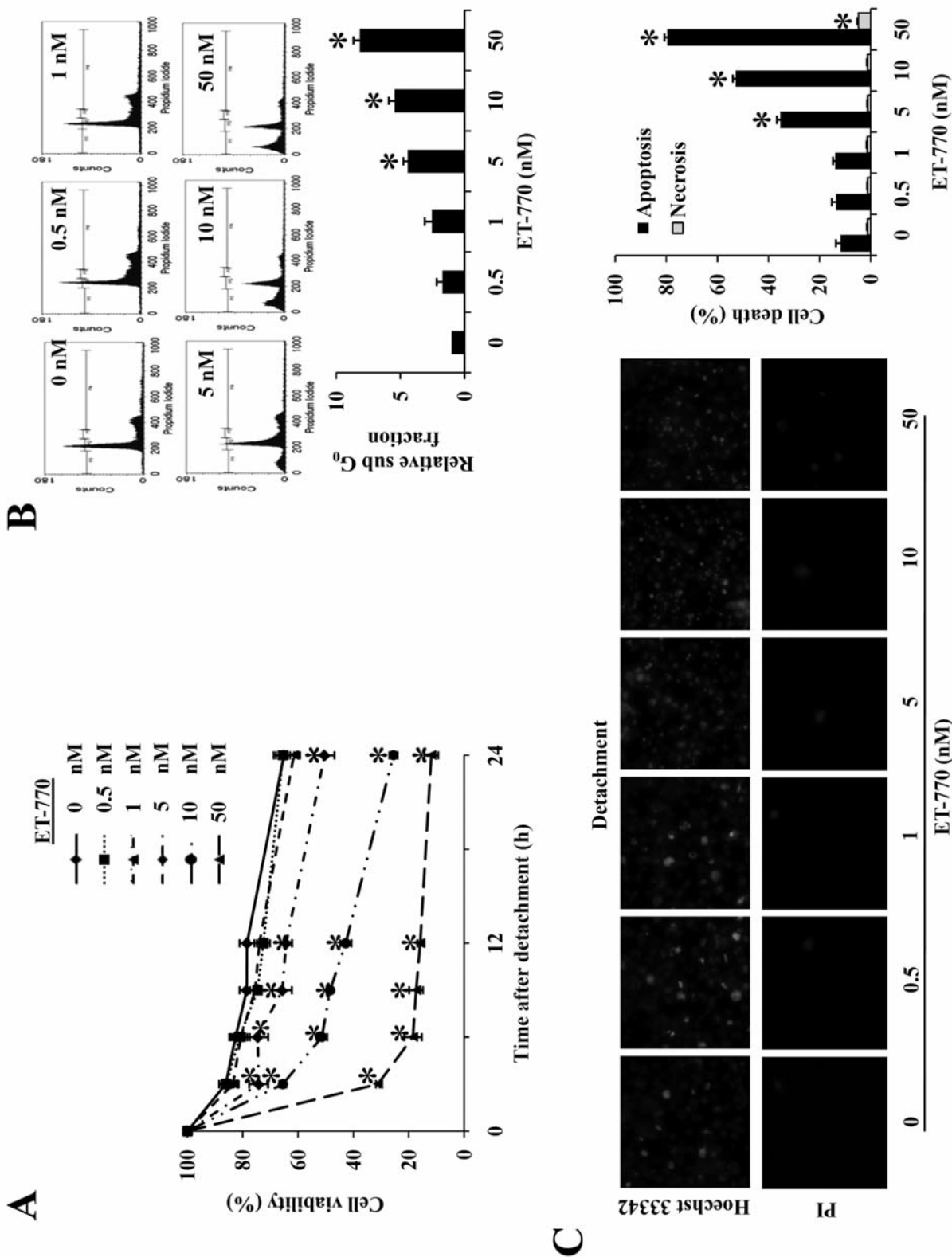
Figure 3. Ecteinascidin 770 (ET-770) sensitizes H23 lung cancer cells to anoikis. A: Detached cells were treated with 0–50 nM of ET-770 and cell viability was determined by 2,3-b-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) assay at the indicated times. Values are means±S.D. (n=5), *p<0.05 versus non-treated controls. B: Sub G_0 fraction of the cells was evaluated by flow cytometry and propidium iodide. C: Apoptosis and necrosis were detected by Hoechst 33342/PI staining assay. The percentages of cell apoptosis and necrosis were obtained. Values are means±S.D. (n=3), *p<0.05 versus non-treated controls at 12 h.
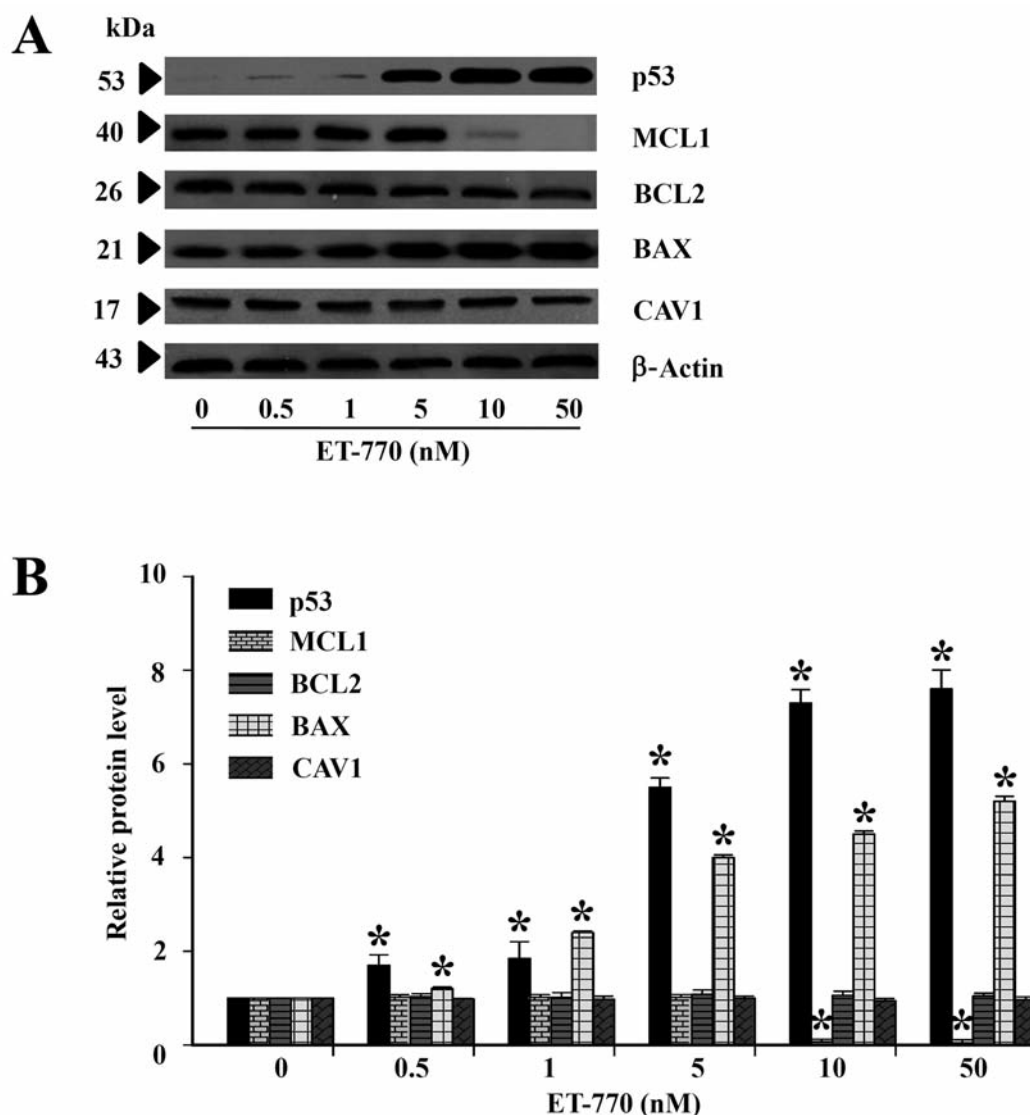
Figure 4. *Ecteinascidin 770 (ET-770) sensitizes H23 cell to anoikis through a p53-dependent mechanism. Cells were detached and incubated with 0–50 nM of ET-770 for 12 h. A: Caveolin-1 (CAV1), p53, myeloid cell leukemia sequence-1 (MCL1), B-cell lymphoma-2 (BCL2) and BCL2-associated X protein (BAX) expressions were determined by western blotting. Blots were also carried out with β-Actin antibody to confirm equal loading of the samples. B: Plot shows values relative to controls at 12 h. Values are means±S.D. (n=3), *p<0.05 versus non-treated controls.*

of 5-50 nM significantly reduced cell viability of the detached cells as compared to non-treated controls. A significant decrease in cell viability was observed as early as 3 h after cell detachment, with approximately 75%, 65% and 30% of cells remaining viable in response to 5, 10 and 50 nM of ET-770, respectively. To determine whether the observed reduction in cell viability was due to apoptotic cell death, we analyzed the sub-G_0 fraction by flow cytometry and the profile of nuclear morphology of the cells by Hoechst 33342/PI staining assay. Cell-cycle analysis showed that the sub-G_0 fraction was significantly increased in the ET-770-treated cells, suggesting

that apoptosis was indeed the main mechanism of death under these conditions (Figure 3B). Hoechst 33342/PI staining assay also indicated condensed and/or fragmented nuclear morphology of apoptotic cells in response to ET-770, while PI-positive cells were only detected in cells treated with 50 nM of ET-770 (Figure 3C).

ET-770 sensitizes H23 cell anoikis by inducing cell death through a p53-dependent mechanism. In order to clarify the mechanism behind ET-770 in sensitizing anoikis, effects of ET-770 on anoikis regulatory proteins p53, MCL1, BCL2,
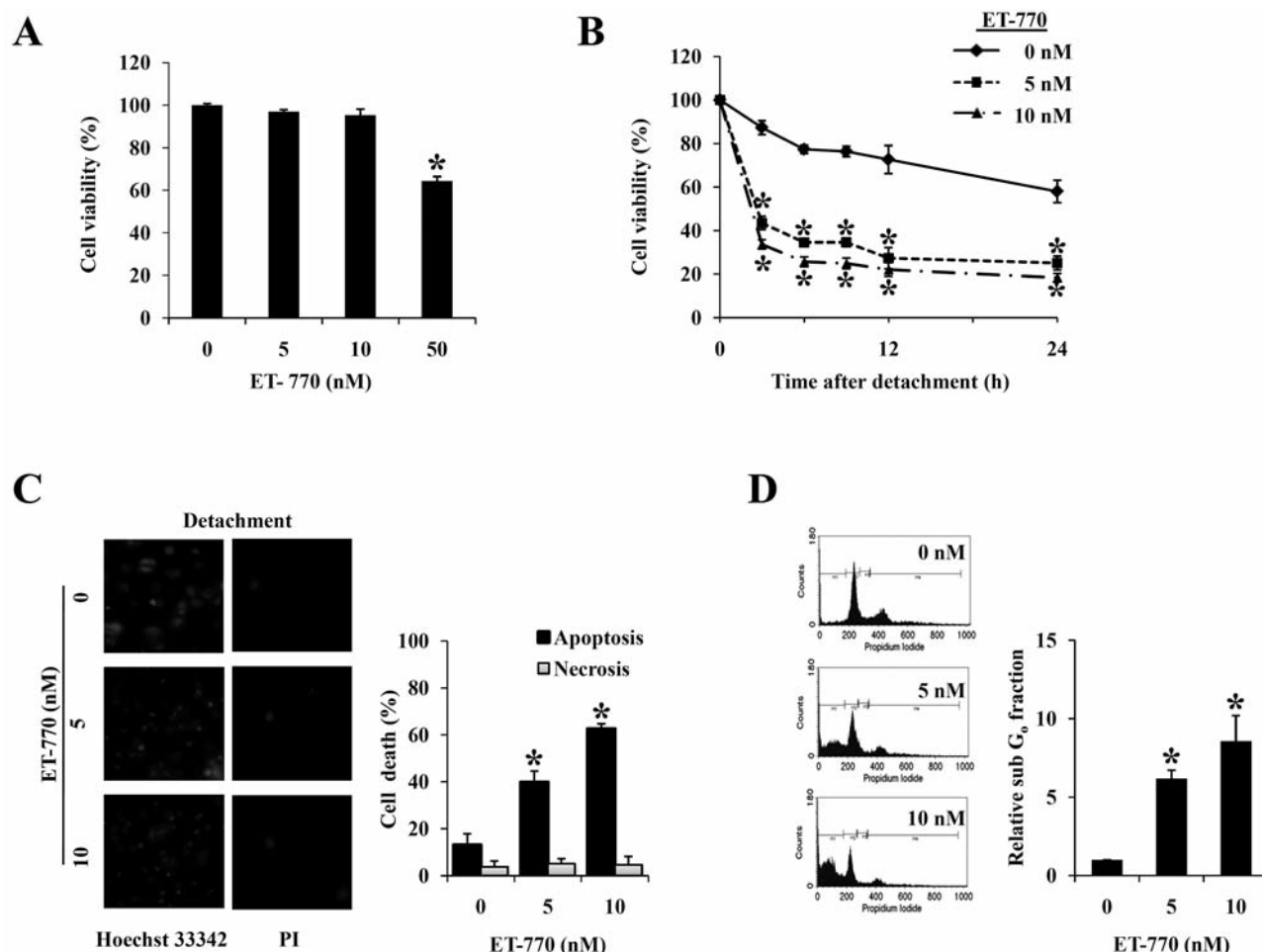
Figure 5. *Ecteinascidin 770 (ET-770) induces anoikis sensitization of H460 human lung cancer cells. H460 cells were treated with 0–50 nM of ET-770 for 24 h. A: Cell viability was determined by 2,3-b-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) assay. Values are means±S.D. (n=3), *p<0.05 versus non-treated controls. B: Detached cells were treated with 5 and 10 nM of ET-770 and cell viability was determined by 2,3-b-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) assay at the indicated times. Values are means±S.D. (n=4), *p<0.05 versus non-treated controls. C: Apoptosis and necrosis were detected by Hoechst 33342/PI staining assay at 12 h. D: Cells undergoing anoikis were evaluated in terms of the sub-G_0 fraction. Values are means±S.D. (n=3), *p<0.05 versus non-treated controls at 12 h.*

BAX, and CAV1 were evaluated by western blot analysis. Detached H23 cells were treated with different concentrations of ET-770 (0-50 nM) for 12 h and the expression of the indicated proteins was analyzed. Figures 4A and B show that ET-770 significantly increased p53 and BAX levels, while reducing that of MCL1. However, no significant alteration of BCL2 and CAV1 was observed in response to ET-770. Taken together, these results revealed that ET-770 sensitizes H23 cells to anoikis through a p53-dependent mechanism.

Anoikis-sensitizing effect of ET-770 on H460 cells. Having shown that ET-770 sensitized H23 cells to anoikis, we provided further information of such a sensitizing effect on other lung cancer cells. Sub-toxic concentrations of ET-770

were determined and used for anoikis assay of H460 human lung cancer cells. Cytotoxic evaluation indicated that ET-770 at 5 and 10 nM caused no significant toxic effects on H460 cells (Figure 5A). Anoikis of H460 cells in response to ET-770 was similarly determined, as described above. Consistent with H23 cells, H460 exhibited anoikis resistance, with approximately 86% and 60% of the cells remaining viable after 3-h and 24-h detachment, respectively (Figure 5B). Figure 5B shows that a significant decrease in cell viability was observed as early as 3 h, with approximately 43% and 33% of cells remaining viable in response to 5 and 10 nM of ET-770, respectively. Moreover, we analyzed the sub-G_0 fraction by flow cytometry and the profile of chromosomal DNA of the cells by Hoechst 33342/PI staining assay at 12 h

after detachment. Hoechst 33342/PI staining assay indicated an intense signal of nuclear fluorescence, chromatin condensation and DNA fragmentation of apoptotic cells in response to ET-770 in a dose-dependent manner (Figure 5C). Flow cytometric analysis of the sub-G_0 fraction also indicated a similar increase of the sub-G_0 fraction in response to ET-770, as found in the experiment for H23 cells (Figure 5D). Taken together, our findings indicate that ET-770 sensitizes lung cancer cells to anoikis.

Discussion

So far, it is well-accepted that metastasis is the dominant cause of death in many types of cancer, including lung cancer (1-6). Among multiple processes of cancer metastasis, the survival of cancer cells after detachment has dramatically gained attention in cancer research (8-11, 30-33). Cell loss of attachment or inappropriate contact with the extracellular matrix or surrounding cells induces apoptosis termed anoikis, which is the primary biological process that prevents cancer metastasis (7-9). As a hallmark of successful metastasis, anoikis resistance is considered an important capability of cancer cells required for spreading (1, 8-11, 31). Therefore, anoikis sensitization is interesting for cancer prevention as well as therapy.

ET-770, an ecteinascidin derivative isolated from Thai tunicate *E. thurstoni,* was demonstrated herein, to our knowledge for the first time to sensitize anoikis in human non-small lung cancer cells. We provided insight of the molecular mechanism indicating that ET-770 sensitized H23 cell anoikis through a p53-dependent pathway. The main machinery of anoikis involves intrinsic apoptosis signaling pathway in which the interaction between anti-apoptotic and pro-apoptotic proteins plays an essential role (8-11). Among anti-apoptotic proteins in such a pathway, MCL1 has been shown to be the major protein in inhibition of anoikis in many types of cancer (34-38), and to be an important cause of anoikis resistance (8-12, 14, 34-38).

Loss of p53 function can lead to the genomic instability and tends to render cells resistant to apoptosis by altering the expression of BCL2 family members which are required in the development of malignancy (15-18). In terms of anoikis, cancer cell suppression of p53 activation was shown to render cells resistant to anoikis (20-22). Our study found that ET-770 increased the p53 level, which in turn down-regulated anti-apoptotic MCL1, and increased BAX protein expression.

Interestingly, H23 cells used in the present study exhibited characteristics of anoikis-resistance, with more than 60% of cells surviving after 24-h detachment. This response of the cells may be explained by the low level of p53 in cells under the detached condition (Figure 4A). ET-770 at non-toxic concentrations was able to increase its expression when used

at concentrations of 5-10 nM (Figure 4A) and such an increase of p53 was accompanied by the increase of BAX and the decrease of MCL1, resulting in anoikis of these cells. With a similar anoikis-enhancing effect in H460 lung cancer cells, ET-770 may be a good candidate for anti-metastasis therapy. These findings may be beneficial for the development of new approaches of cancer therapy for the improvment a patient's quality of life.

Acknowledgements

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