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Recombinant bacterial-protein Colicin N causes cytotoxicity and oxidative stress in human lung cancer cells

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Abstract

Due to high cancer mortality rate and current therapeutic limitations like chemoresistance and non-selective toxicity, there is a persisting demand for novel anti-cancer agents. Increasing evidences suggest that bacteria-produced antimicrobial peptides and proteins called bacteriocins are promising candidates for investigation. Some bacteriocins like the colicins have displayed selective toxicity towards various cell lines and tumor xenografts. Colicin N, being the smallest pore-forming colicin retaining all characteristic domains, is a good model for understanding the other pore-forming colicins which have been previously noted to have toxicity against cancer cells. C-terminal hexa-histidine tagged Colicin N (ColN) was expressed and then purified by immobilized metal ion affinity chromatography facilitated by Fast Protein Liquid Chromatography (FPLC). Protein identity and function was confirmed by SDS-PAGE and antibiotic testing using broth microdilution. As measured by MTT assay, ColN in the concentration range of 1-15 µM, exhibits selective, significant, dose-dependent cytotoxicity towards human lung cancer cell line (H460) as compared to normal human dermal papilla (DP) cells. Hoechst 33342: propidium iodide co-staining confirmed apoptosis induction in treated H460 cells. Oxidative stress is known to be linked to apoptosis and this study also shows that ColN caused a dramatic increase in intracellular reactive oxygen species (ROS) as detected by 2',7'-dichlorofluorescein diacetate (DCFH-DA) probe. These data support the future biomedical applications of colicins by revealing the novel finding that ColN causes oxidative stress and selectively induces apoptosis in cancer cells.

Introduction

Lung cancer is the primary cause of cancer mortality in the world and non-small cell carcinoma (NSCLC) accounts for 80% of the cases.[1] Standard chemotherapy regimens are inadequate for lung cancer due to low drug response and non-selective toxicity.[2] Chemotherapy relies on inducing cancer cells to undergo apoptosis which is an organized suicide program that causes minimal damage to surrounding tissues.[3] The search for new cancer-killing agents has revealed the potential of bacteriocins, which are bacteria-produced proteins or peptides that target related species to decrease competition.[4] There is a wide range of bacteriocins that have shown selective cytotoxicity toward cancer cells, making bacteriocins interesting candidates

for novel cancer therapy.[5] A primary example is nisin, a bacteriocin that is used as a food preservative but is also capable of inducing apoptosis in cultured colon cancer cells by Lewies and colleagues (2017) and in melanoma cells by Ahmadi *et al.* (2018).[6,7] Colicins are among the bacteriocins considered to have cancer therapeutic potential.[8] Chumchalova and Smarda revealed in 2003 that human tumor cells are selectively inhibited by colicins, especially pore-forming colicins, in a cell-specific and colicin-specific manner.[9] Colicin N (ColN) is the smallest (~42 kDa) representative that contains all three characteristic colicin domains, making ColN a good model for understanding the pore-forming colicins better.[4,9] Moreover, the anti-cancer activity of ColN is previously unreported, and the mechanism by which colicins inhibit eukaryotic cells is unknown. This study details an optimized procedure to express and purify ColN in the laboratory, followed by testing the acquired ColN possesses selective cytotoxicity towards human cancer cells. To this end, it is necessary to determine the ability of ColN to induce programmed cell death in human lung cancer cells. Furthermore, ColN effect on intracellular ROS level is measured because oxidative stress is correlated with cell death.[10]

Methodology

Chemical Reagents

For protein expression, arabinose was purchased from Merck (Billerica, MA, USA). Monobasic sodium phosphate was purchased from Vivantis Technologies (Selangor Darul Ehsan, MY, USA), Imidazole for buffer solutions, DNase I and Ampicillin Sodium Salt were acquired from PanReac Applichem (Darmstadt,DE, USA). Luria-Bertani broth and agar are acquired from Hardy Diagnostics (Santa Maria, CA, USA). AccuPrep® Nano-Plus Plasmid Mini Extraction Kit is sourced from Bioneer Inc. (Alameda, CA, USA). Pierce™ protease inhibitor tablets and bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Scientific (Waltham, MA, USA). For cell culture of cell lines, Roswell Park Memorial Institute (RPMI) 1640 medium, phosphate-buffered saline (PBS) pH 7.4, trypsin, L-glutamine, fetal bovine serum (FBS) and penicillin/streptomycin solution were obtained from Gibco (Gaithersburg, MA, USA) while Prigrow III medium for human dermal papilla (DP) cells was purchased from Applied Biological Materials Inc. (Richmond, CA, USA). Cell experiment reagents including trypsin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst33342, propidium iodide (PI), DMSO (dimethysulfoxide), 2',7'-dichlorofluorescein diacetate (DCFH-DA) were procured from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Protocol

Plasmid transformation and cloning

Transformation mixture consists of pET3a plasmids encoding full-length ColN with C-terminal histidine tag and Oneshot® Mach1™-T1R (Invitrogen) chemically competent *E. coli*. The vial with such mixture was placed in ice for 30 min. The cells were then subjected to heat-shock by placing the vials in 42˚ C water bath for 30 seconds. After addition of 250 µL of Lysogeny Broth (LB) medium, the vial was placed in a shaking incubator at 37˚C for 1 hour at 225 rpm. The transformation mixture was added to LB agar plates containing 100 µg/mL of ampicillin to select for transformed bacteria. After overnight incubation, colonies were inoculated again in tubes of LB broth and placed in a shaking incubator set at 37˚C overnight. The *E. coli* cells were collected by centrifugation at >8,000 rpm for 2 min. Purification of the plasmids was performed using the AccuPrep® Nano-Plus Plasmid Mini Extraction Kit according to manufacturer instructions.

Recombinant Protein expression and purification

BL21-AI™ One Shot® chemically competent *E. coli* (Invitrogen) were transformed with the plasmids encoding ColN and the transformants were selected using LB agar plate containing 100 μg/ml ampicillin at 37 °C. At OD_{600} of 0.6, colicin production was induced by the addition of arabinose at a final concentration of 0.2% (w/v). Three hours after induction, the cells were collected by centrifugation for 10 min at 8,000 g at 4˚C then resuspended in the binding buffer containing 50mM sodium phosphate buffer, pH 8.0, 300 mM NaCl and 10 mM imidazole. Protease inhibitor RNase, and DNase I were added to the suspension and cell lysis was carried out through pulse sonication for 15 min on ice. After centrifugation at 12,000 g, 4˚C for 10 minutes, ColN was purified from the supernatant by affinity chromatography utilizing a nickel-sepharose HisTrap™ HP affinity column (GE Healthcare). Purification was carried out through fast protein liquid chromatography (FPLC) using an ÄKTA Start machine (GE Healthcare). The supernatant was loaded by sample pump onto the column pre-equilibrated with the binding buffer. After washing the column with the binding buffer, ColN was eluted with a buffer containing 50 mM sodium phosphate buffer, pH 8.0 300 NaCl and 250 mM imidazole. Real-time UV absorbance of the machine allowed collection of fractions with the eluted proteins, which were then analyzed by SDS-PAGE for confirmation. Pooled protein was lyophilized, resuspended in PBS and the protein concentration was measured by BCA assay. Protein activity was tested by broth microdilution assay in microplate format, with LB media for the growth of *E. coli* 8739™ (ATCC, Manassas, VA).

Cell Culture

Human lung cancer H460 (ATCC, Manassas, VA) were cultured in RPMI 1640 medium while human dermal papilla cells (Applied Biological Materials Inc., Richmond, Canada) were cultured in Prigrow III medium. The culture media are supplemented with 2 mM L-glutamine, 10% FBS and 100 units/mL of penicillin/streptomycin and the cell lines are subcultured regularly, and maintained in optimum conditions of 5% CO_2 at 37°C. Cells are grown to 70–80% confluence in plates for each experiment.

Cytotoxicity Assay

Cells were seeded onto a 96-well plate at a density of 1×10^4 cells/well. The cells were treated with 0–15 μM purified ColN for 24 h, then incubated with 0.4 mg/mL of MTT at 37 °C for 4 h. The formazan product was dissolved with DMSO and the resulting color intensity was examined by microplate reader (Anthros, Durham, NC) at 570 nm. The cell viability presented is the absorbance of the treated cells relative to that of non-treated control.

Detection on mode of cell death

Apoptotic and necrotic cell death determination was performed by co-staining of Hoechst 33342 and propidium iodide (PI) and visualization under fluorescence microscope (Olympus IX51 with DP70). Cells were seeded at a density of 1×10^4 cells/well in a 96-well plate, treated with ColN (0–15 μM) for 24h and incubated with 10 μM of Hoechst33342 and 5 μg/mL PI for 30 min at 37 °C.

Intracellular ROS detection

Cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and incubated overnight. The media was removed and 10 μM of DCF in PBS is added to the wells. The probe was allowed to permeate the cell for 30 minutes at a cold temperature, protected from light. Following the removal of DCF solution, the cells were then incubated either with ColN (15

μM) or a general antioxidant, NAC (5mM). The fluorescence intensity was measured by microplate reader and measured at 30 min, 1 h and 3 h.

Statistical Analysis

Mean data were averaged from three independent replicates. Statistical analysis was performed on SPSS Statistics 22 version (Armonk, NY, USA) using one-way ANOVA and Tukey post hoc test. A *p*-value of ≤0.05 was considered as statistically significant.

Results and Discussion:

Recombinant ColN functions like native ColN

The chromatogram presented in figure 1A reflects the efficiency of immobilized metal affinity chromatography in isolating pure ColN from crude sample. After equilibration of the column and sample application, unbound proteins were washed with binding buffer and represents the first peak in chromatogram (fig.1A) and the flow-through (FT) or lane 3 in the SDS-PAGE gel (fig.1B). The addition of the elution buffer with increased concentration of the competitive ligand, imidazole, corresponds to increased gradient concentration and a sharp peak of eluted fraction (EF) in the chromatogram and a single band in lane 4 of the gel. ColN construct is known to be 42.87 kDa and the EF protein band was found to be ~40 kDa, as indicated by the arrow.

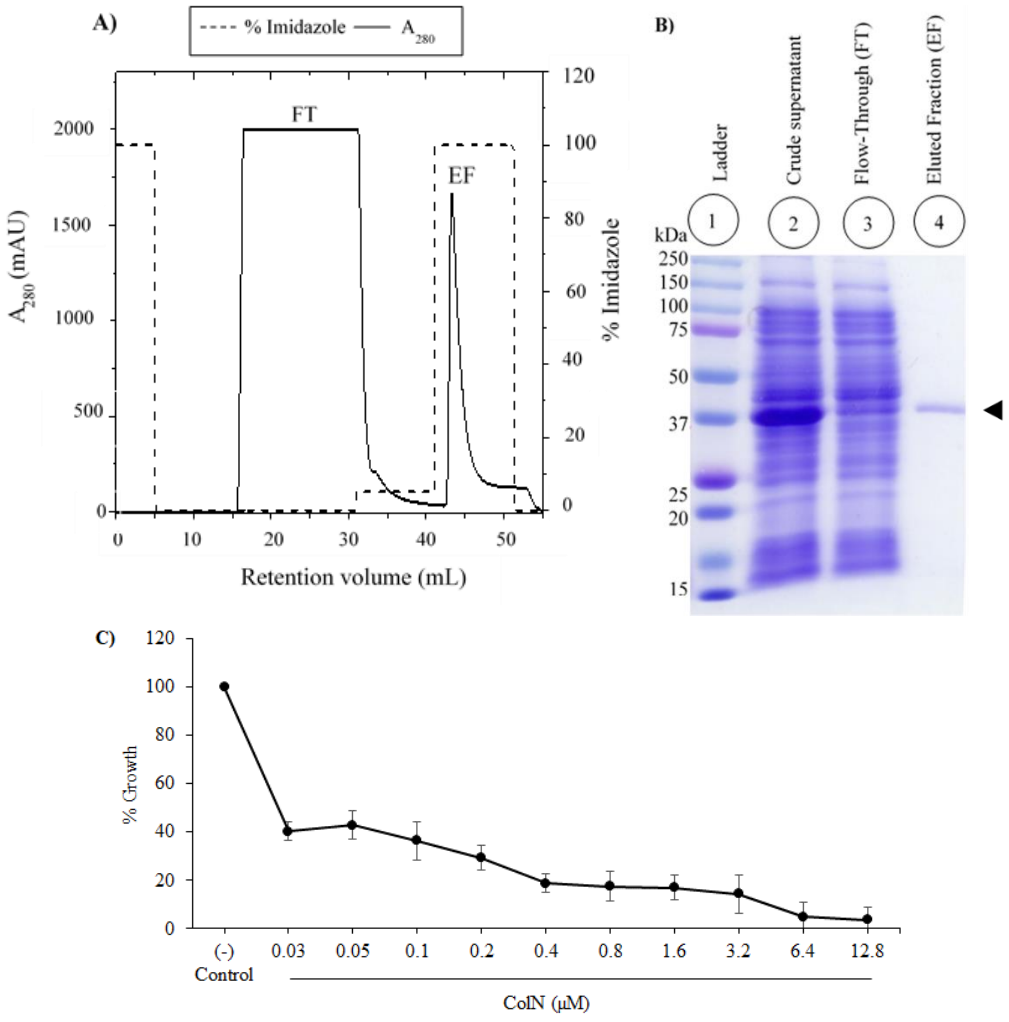

Figure 1. Purification of recombinant colicin N (ColN) with C-terminal histidine-tag using affinity chromatography. **A)** Chromatogram of affinity chromatography by a HisTrap™ HP affinity column. **B)** SDS-PAGE analysis of protein containing fractions from affinity chromatography. **C)** ColN retains growth inhibitory effect on *E. coli* ATCC® 8379™

Histidine-tagged colicin N was previously shown to have similar biophysical characteristics, protein folding and overall structure as the native colicins.[11,12] As quality control for the protein purification, broth microdilution assay was performed following the protocol for antimicrobial peptides that was established by Wiegand and colleagues in 2008.[13] Following the protocol, the serial dilution starts from 12.8 µM of ColN and % growth of the test *E. coli* (ATCC 8379™) increased as the concentration of ColN decreased. Against 100% growth from negative control containing *E. coli* ATCC 8379™ only, all ColN-treated wells displayed growth inhibition. Thus, biological activity of this study's produced ColN was intact (fig.1C). Growth (%) presented in the graph was based on measured optical density of treatment relative to untreated control presented. Minimum Inhibitory Concentration (MIC) is the lowest concentration of the antimicrobial substance that inhibits growth of the test bacteria. As determined by microbroth dilution assay with appropriate sterility control and negative control, MIC for ColN in *E. coli* ATCC® 8379™ is 4.4 µM.

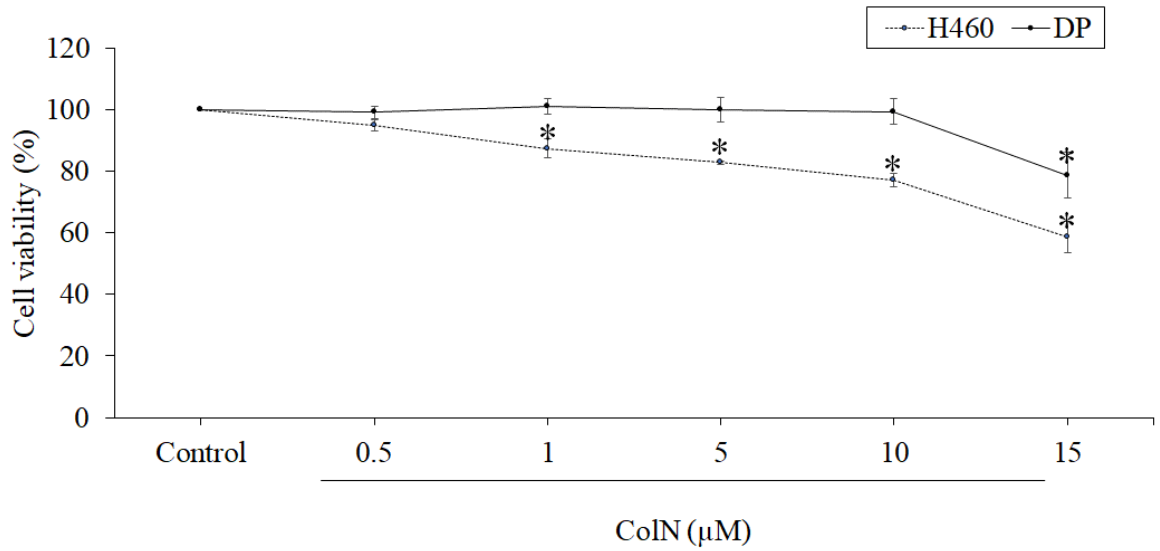

Figure 2. Cytotoxicity of Colicin N (ColN) in H460 and DP cells. Values are means of the independent triplicate experiments ± SD. * $p \leq 0.05$ versus non-treated control.

ColN exhibits selective toxicity towards human lung cancer cells

Cisplatin remains to be the standard therapy for NSCLC patients, and it is often included in regimens.[14] However, cisplatin typically causes inadvertent cytotoxicity to rapidly dividing, normal cells like those from the dermal papilla. This toxicity results in hair loss which is an additional factor decreasing quality of life.[15] A 24 h of incubation with 0.5-10 µM of ColN caused no significant decrease in cell viability in DP cells. In H460 cancer cells, a statistically significant decrease in cell viability was noted at 1-15 µM of ColN. At 15 µM, the mean cell viability observed in DP cells is at 80%, while the viability of H460 lung cancer cells was reduced to 58% with treatment of 15 µM of ColN. This indicated that ColN seemed to be more selective towards the cancer cell line used, rather than DP cells (fig. 2).

ColN induces apoptosis in human lung cancer cells

Nuclear co-staining using Hoechst 33342 and propidium iodide was used to determine % apoptosis induction by ColN (5-15 µM) for 24 h (fig. 3A). At the specified dose range, there was statistically significant apoptosis induction in treated H460 compared to non-treated

control. ColN treatment in H460 cells triggered apoptosis (fig. 3B), characterized by cell shrinking, plasma membrane blebbing, organelle integrity, and DNA fragmentation. Hoechst 33342 staining of ColN-treated population at 24h visualized the characteristic apoptotic cell fragments that stained bright blue. Necrotic cells are characterized by a red color as their loss of membrane integrity makes them permeable to propidium iodide.[16] Thus, according to characteristic morphology, ColN induced apoptosis but not necrosis in the specified dose range of 5-15 µM. This is a desirable trait in cancer therapy, as cell apoptosis triggers phagocyte clearance, accounting for a non-inflammatory event and lesser toxicity to surrounding normal cells.[17]

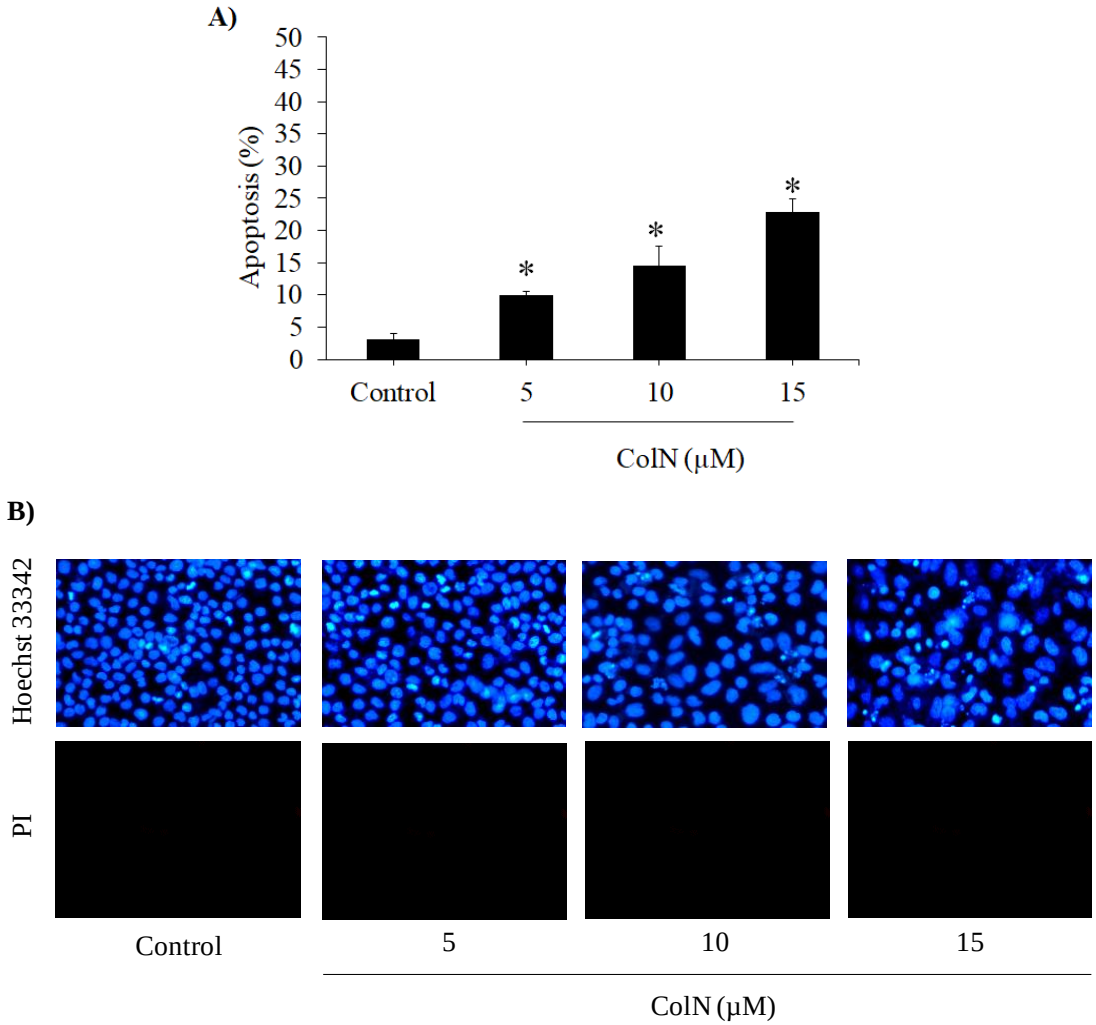

Figure 3. A) ColN increased apoptosis in human lung cancer H460 cells **B)** Apoptosis cell death detection by nuclear co-staining**.** Values are means of the independent triplicate experiments ± SD. * $p \leq 0.05$ versus non-treated control.

ColN-induced oxidative stress in human lung cancer cell

Redox regulation is vital in modulating death of cells, and this is often used in therapy for cancer.[18] In the context of antimicrobial peptides (AMPs), pardaxin induces caspase-dependent, ROS-mediated apoptosis in human fibrosarcoma cell line[19]. Similarly, the AMP, Nisin Z, which selectively induces apoptosis in cultured melanoma cells, also generates ROS dramatically.[6] The measurement of ROS in this study relies on fluorescence released by oxidation of dichlorodihydrofluorescein (DCF) by several ROS, making the probe an excellent tool to assess oxidative stress.[20]

H460 cells were treated with 15 µM ColN for 0.5-3 h, and ROS production was analyzed by DCFH-DA probe (fig. 4). Using the representative toxic dose of 15 µM, ColN treatment increases intracellular ROS significantly, as compared to untreated cells and the general antioxidant NAC at a non-toxic 5 mM dose. This points to the likelihood that the cytotoxic activity of ColN could be linked to ROS modulation in cancer cells.

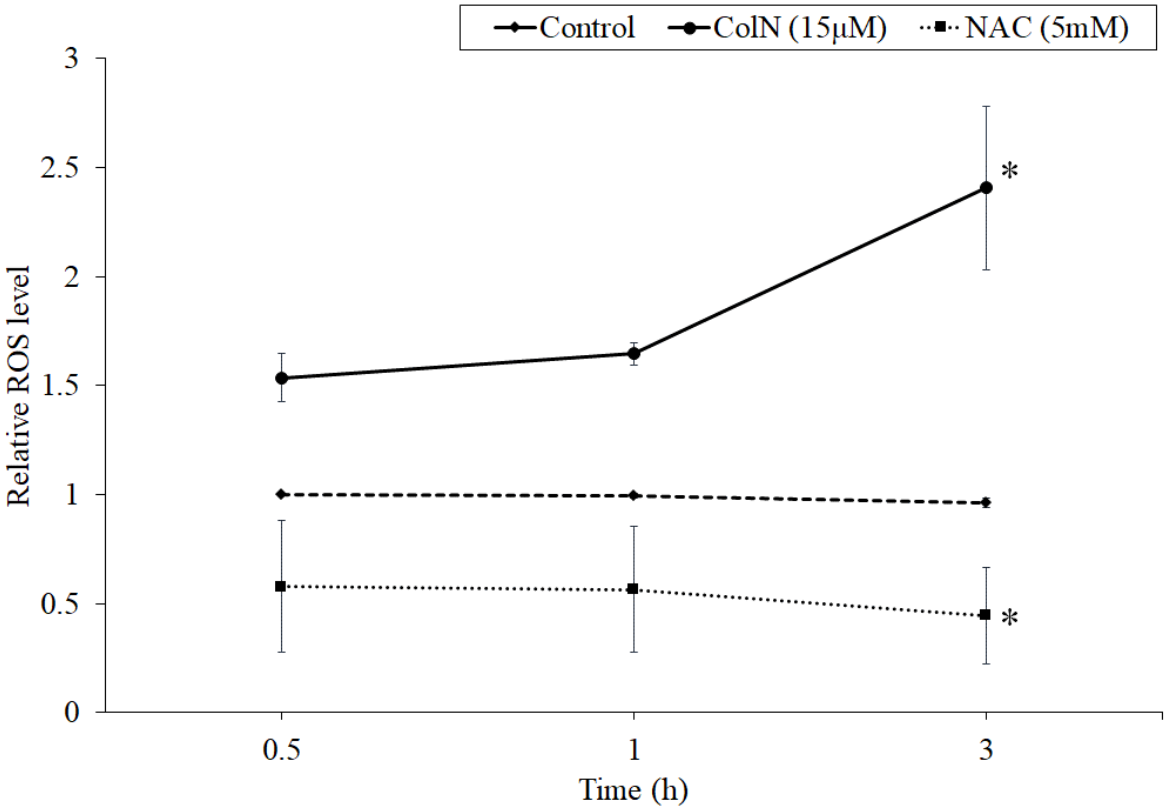

Figure 4. ColN increases intracellular ROS in H460. Values are means of the independent triplicate experiments ± SD. * $p \leq 0.05$ versus non-treated control.

Conclusion

The purified ColN produced for this experiment retains its biological function as an antibiotic and also displays selective cytotoxicity towards the non-small cell lung cancer cell line, H460. ColN induced apoptosis, and generated oxidative stress in the human cancer cell line, and the ROS modulation may be among the mechanisms involved in the cytotoxicity observed. The study paves the way for more mechanistic studies and potential biomedical applications of the colicins aside from antibiotic activity.

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