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Possible Anticancer Effect of Bioactive Extract Isolated from *Agrocybe Chaxingu* on Human Bladder Cancer Cells

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Abstract To seek for a more effective way to treat bladder cancer with the poor outcomes, we have been working on natural products with anticancer activity for a decade. Recently, we came across the bioactive extract of *Agrocybe chaxingu* mushroom, CHX, which had been shown to have anticancer activity with few side/adverse effects. Accordingly, we then investigated if CHX might have anticancer effect on the relatively less aggressive human bladder cancer 5637 cells (grade 2) *in vitro*. Cells were treated with varying concentrations of CHX (0-500 μg/ml) for 72 h, and cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay to assess anticancer effect. To explore the anticancer mechanism of CHX, we also examined induction of oxidative stress (OXS), cell cycle, and apoptosis. We first found a significant (~50%) cell viability reduction in 5637 cells with 350 μg/ml of CHX, indicating its anticancer effect. Lipid peroxidation (LPO) assay revealed the significantly (~2.2-fold) increased oxidative stress (OXS) level as well. CHX also led to a G_1 cell cycle arrest with a ~37% *increase* in G_1-phase cell number and a ~44% *decrease* in S-phase cell number, compared to those in controls. This was further confirmed by the up-regulation of two G_1-specific cell cycle regulators, $p21^{WAF1}$ and $p27^{Kip1}$, with CHX treatment. Consequently, 5637 cells were found to undergo apoptosis, indicated by the down-regulation of anti-apoptotic bcl-2 concomitant with the up-regulation of pro-apoptotic Bax with CHX. In conclusion, CHX has anticancer effect on human bladder cancer 5637 cells, significantly reducing their cell viability. This is presumably attributed to elevated OXS, a G_1 cell cycle arrest, and ultimate apoptosis. Therefore, it is plausible that CHX may offer an alternative therapeutic option for low-grade bladder cancer cases.

Keywords: *bladder cancer, Agrocybe chaxingu, anticancer, oxidative stress, cell cycle arrest, apoptosis*

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1. Introduction

Bladder cancer is the second most common urologic malignancy next to prostate cancer in the United States [1]. The majority of bladder cancers present as superficial (80%) with 15% presenting as invasive cancer and 5% as metastatic disease [2]. Currently, urothelial cell carcinoma (UCC) is the most prevalent primary bladder tumor: 82,000 new cases and 17,000 deaths were estimated in 2022 [1]. Although endoscopic transurethral resection (TUR) has been often performed as a primary therapy, 60%-70% of patients would recur in 5 years and ~25% could progress to muscle invasive disease [3]. Hence, the outcomes are yet unsatisfactory today. However, it is also true that the various pathological stages of bladder cancer will complicate a choice of appropriate therapeutic interventions.

To properly and optimally manage bladder cancer, the two major classifications have been adopted based on the tumor-node-metastasis (TNM) classification system [4]: non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC) [5]. NMIBC, comprising Ta, T1, and CIS (carcinoma *in situ*), is also considered as a low-grade, non-metastatic tumor and does not generally threaten the lives of patients [5]. However, it has a high recurrence rate and could yet develop to MIBC in some of those patients [5,6]. MIBC is an invasive, high-grade, metastatic tumor with a high mortality rate, and unfortunately no effective therapies would be available at this moment [6]. Hence, the immediate therapeutic aims are to effectively prevent multiple recurrences in NMIBC and its progression to a more advanced MIBC. Currently, intravesical immunotherapy with bacillus Calmette-Guerin (BCG), an attenuated strain of *Mycobacterium bovis*, is the most effective immunotherapy available for recurrent NMIBC (superficial tumor and CIS) as well as high-grade

bladder cancer [7]. Unfortunately, potential severe side effects of BCG therapy, including cystitis, hematuria, allergic reactions, sepsis etc., are limiting its use in clinical practice. Due to these drawbacks, the outcomes are only slowly getting better every year, thereby urgently demanding a prompt improvement.

We have been seeking for *natural products*, which could be used to treat bladder cancer with different cancer stages/grades, and recently came across the bioactive extract of *Agrocybe chaxingu* mushroom [8], CHX, with potential anticancer activity. A number of scientific/medical studies have also revealed medicinal/pharmacological properties of CHX, including anticancer, antioxidant, hypoglycemic, osteoclastic as well as Cox-1/2 inhibitory activities etc. [9-13]. It was tempting for us to examine if CHX might have such anticancer activity against bladder cancer cells *in vitro*. Bladder cancer 5637 cells were established from a 68-year-old male patient with a grade 2 bladder cancer in 1974 [14], which was known to be less aggressive in a metastatic potential. Actually, such grade 2 bladder carcinoma is the relatively manageable case that requires pharmacological treatments following surgical resection to eliminate potential residual cancer cells and prevent future relapses [14]. It is thus significant that 5637 cells will be a suitable experimental model for studying a grade 2 tumor to find a better and safer therapeutic option.

Accordingly, we investigated if CHX would have anticancer effect against 5637 cells and also explored its anticancer mechanism, focusing on induction of oxidative stress (OXS), cell cycle, and apoptosis. More details are described and the interesting findings are also discussed herein.

2. Materials and Methods

2.1. Cell Culture

Human bladder cancer 5637 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). They were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). CHX was a generous gift from the manufacturer (Mushroom Wisdom, Inc., East Rutherford, NJ) and its anticancer effect was assessed by cell viability (MTT) assay as described below.

2.2. MTT (Cell Viability) Assay

5637 cells seeded in the 6-well plate were treated with varying concentrations of CHX (0-500 μg/ml) for 72 h. Cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay following the vendor's protocol (Sigma-Aldrich, St. Louis, MO). MTT reagent (1 mg/ml) was added to each well in the plate, which was then incubated for 3 h in an incubator. After discarding MTT, dimethyl sulfoxide was added to the plate and absorbance of samples was read in a microplate reader. Cell viability was expressed by the % of the readings of optical density (OD) relative to the control reading (100%).

2.3. Lipid Peroxidation (LPO) Assay

Severity of oxidative stress (OXS) induced with CHX was assessed by LPO assay, measuring the amount of malondialdehyde (MDA) formed in the plasma membrane due to oxidative stress [15]: *the more MDA formed, the greater OXS*. The detailed procedures were described in the vendor's protocol (ABCAM, Waltham, MA). Briefly, cells exposed to CHX for indicated times were lysed to obtain cell extracts. The reaction was then initiated by mixing cell extracts with thiobarbituric acid (TBA) solution and incubated in a boiling water bath (~100 °C) for 1 h. Samples were read at A_{532} on a microplate reader, and the amount of MDA formed was calculated from the MDA standards and expressed by μM.

2.4. Cell Cycle Analysis

5637 cells treated with CHX for 72 h were harvested and subjected to cell cycle analysis. Cells (~1 x 10^6 cells) were first resuspended in propidium iodide solution, followed by a 1-h incubation at room temperature. Approximately 10,000 nuclei from each sample were then analyzed on a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ), equipped with a double discrimination module. CellFit software was used to quantify cell cycle compartments to estimate the % of cells distributed in the different cell cycle phases (G_1, S, and G_2/M).

2.5. Western Blot Analysis

Briefly, an equal amount of proteins (10 μg) from control and CHX-treated cell lysates was resolved by 10% SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and transferred to a nitrocellulose membrane (blot). The blot was first incubated for 90 min with the primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) against $p21^{WAF1}$ and $p27^{Kip1}$ (cell cycle regulators) or bcl-2 and Bax (apoptotic regulators), followed by incubation with the appropriate secondary antibody conjugates (Santa Cruz Biotechnology) for 30 min. After discarding antibodies, the specific immunoreactive proteins were then detected by chemiluminescence (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) on an X-ray film (autoradiography).

2.6. Statistical Analysis

All data are presented as the mean ± SD (standard deviation), and statistical differences between groups are assessed with either one-way analysis of variance (ANOVA) or the unpaired Student's t test. Values of $p < 0.05$ are considered to indicate statistical significance.

3. Results

3.1. Effect of CHX on 5637 Cell Viability

To assess anticancer effect of CHX, bladder cancer 5637 cells were cultured with varying concentrations of CHX (0-500 μg/ml) and cell viability was determined in 72 h by MTT assay. A significant cell viability reduction was seen at ≥300 μg/ml of CHX with IC_{50} of ~350 μg/ml

(Figure 1). These results suggest that CHX appears to have anticancer effect and its concentration of 350 μg/ml was adequate and used in the rest of our study.

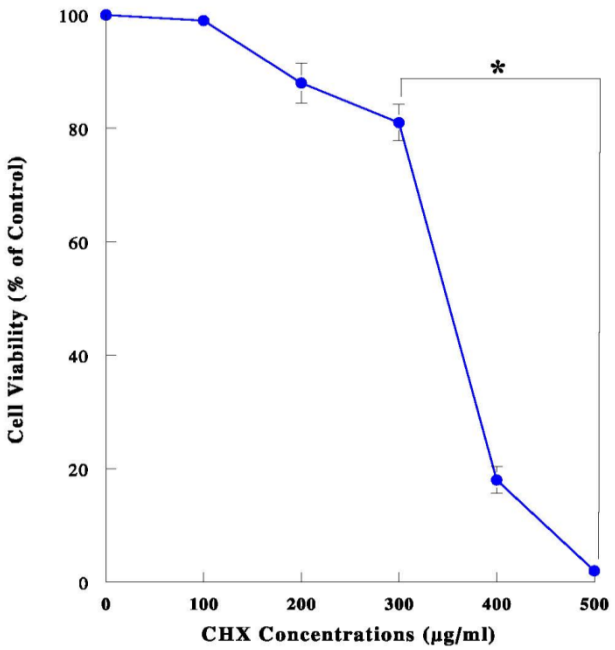

Figure 1. Effect of CHX on 5637 cell viability. Cells were treated with varying concentrations of CHX (0-500 μg/ml) for 72 h and cell viability was determined by MTT assay. Cell viability was expressed by the % of viable cell numbers in CHX-treated cells relative to that in control cells (100%). All data represent mean ± SD (standard deviation) from three independent experiments (*p <0.05 compared with control).

3.2. Exertion of CHX-Induced OXS on 5637 Cells

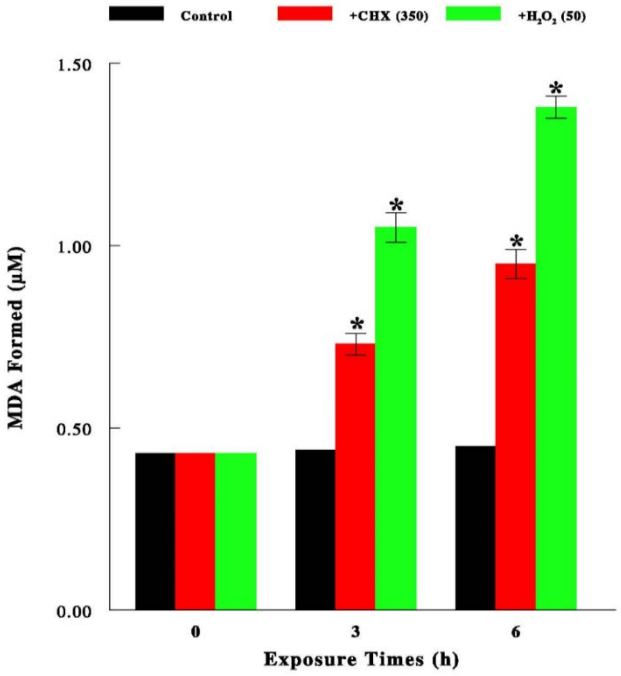

Figure 2. CHX-induced OXS. 5637 cells exposed to CHX (350 μg/ml) for 3 or 6 h were subjected to LPO assay. Severity of OXS is determined by the amount of MDA formed (μM), and the data are mean ± SD from three separate experiments (*p <0.05 compared with control).

To explore the anticancer mechanism of CHX, possible induction of OXS with CHX was first examined. After cells were briefly exposed to CHX (350 μg/ml) for 3 or 6 h, they were subjected to lipid peroxidation (LPO) assay to asses any potential OXS. Such assay revealed that OXS was significantly (~2.2-fold) increased with CHX, indicated by a ~2.2-fold increase in MDA formation (Figure 2). Hydrogen peroxide (H_2O_2, 50 μM) was also used as a positive control for exerting OXS, and it indeed resulted in a ~3.2-fold increased OXS. This finding implies that CHX is capable of inducing severe OXS in 5637 cells, subsequently leading to the cell viability reduction or even cell death. It is thus plausible that CHX could act as a prooxidant.

3.3. Cell Cycle Arrest with CHX

Adverse cellular stimulus such as OXS is also known to affect cell cycle, which critically regulates cell proliferation. This possibility was then tested. Cells treated with CHX (350 μg/ml) for 72 h were subjected to cell cycle analysis using a flow cytometer. Analysis revealed that CHX led to a ~37% *increase* in G_1-phase cell number/population concomitant with a ~44% *decrease* in S-phase cell number, compared to those in controls (Figure 3A). This cell accumulation in the G_1 phase is known as a *G_1 cell cycle arrest* [16].

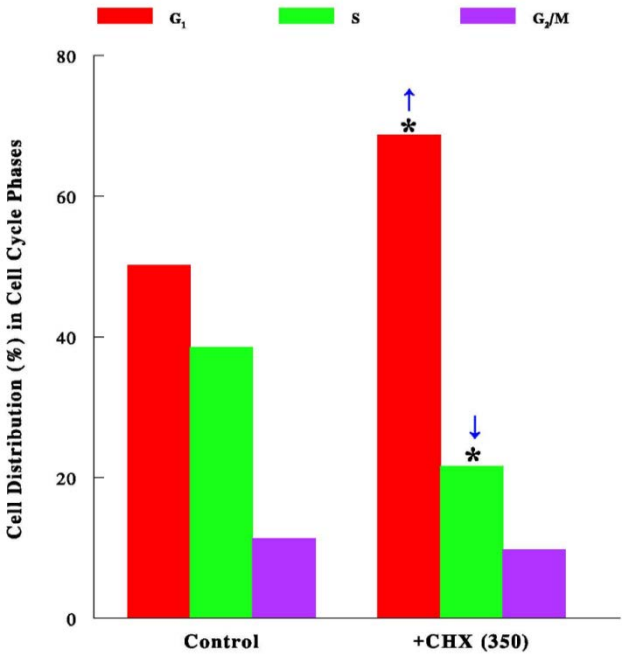

Figure 3A. CHX-induced G_1 cell cycle arrest. Cells treated with CHX (350 μg/ml) for 72 h were subjected to cell cycle analysis. The cell distribution (%) at each cell cycle phase in control and CHX-treated cells is shown. The data are mean ± SD from three separate experiments but only mean values (no error bars) are shown (*p < 0.05 compared with control).

CHX-induced cell cycle arrest was further confirmed by analyzing the G_1-specific cell cycle regulators, $p21^{WAF1}$ and $p27^{Kip1}$ [17]. Western blots revealed that compared to controls, the expressions of both $p21^{WAF1}$ and $p27^{Kip1}$ were significantly enhanced or up-regulated with CHX treatment (Figure 3B), confirming a G_1 cell cycle arrest. Thus, CHX-treated cells will eventually result in growth cessation and cell viability reduction.

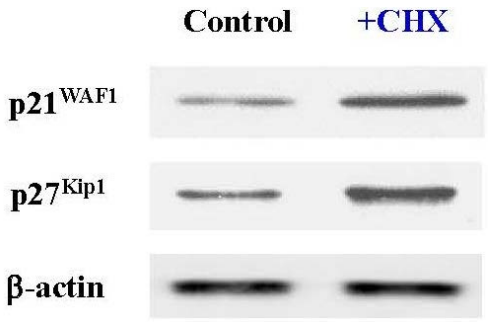

Figure 3B. CHX-induced G_1 cell cycle arrest. Expressions of two G_1-specific cell cycle regulators, $p21^{WAF1}$ and $p27^{Kip1}$, were analyzed by Western blots. Autoradiographs of these regulators in control and CHX-treated cells are shown for comparison. Beta-actin is also shown as an internal loading control.

3.4. Induction of Apoptosis with CHX

Lastly, the fate of CHX-treated cells, whether they would undergo apoptosis, was examined. 5637 cells treated with CHX (350 μg/ml) for 72 h were assayed for apoptosis using Western blots. The results revealed that bc-2 (anti-apoptotic) was down-regulated (with reduced protein expression) while Bax (pro-apoptotic) was up-regulated (with enhanced expression) following CHX treatment (Figure 4). Since the protein profile of down-regulated bcl-2 concomitant with up-regulated Bax [18] is rather indicative of apoptosis, CHX appears to ultimately induce apoptosis in 5637 cells. It is thus possible that CHX could act as an apoptosis inducer.

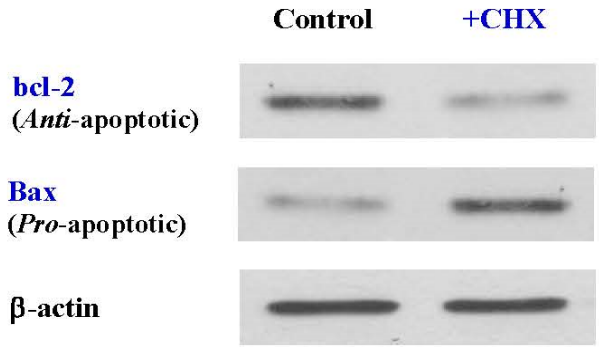

Figure 4. CHX-induced apoptosis. Cells treated with or without CHX (350 μg/ml) for 72 h were analyzed for two apoptotic regulators, bcl-2 and Bax, using Western blots. Autoradiographs of these regulators in control and CHX-treated cells are shown for comparison. Beta-actin is also shown as a loading control.

4. Discussion

Due to the unsatisfactory outcomes following current therapeutic modalities for bladder cancer, an alternative and improved option has been demanded over the decades. We can see some gradual improvements but no break-through has been yet made. To at least find a better way to treat bladder cancer, we have been working on natural products for nearly 15 years. As we recently found the bioactive extract of *Agrocybe chaxingu* mushroom [8], CHX, with possible anticancer activity, we investigated if it might certainly have anticancer effect against bladder cancer 5637 cells (grade 2) [14] *in vitro*.

A dose-dependent study showed that CHX (IC_{50} of 350 μg/ml) demonstrated its significant anticancer effect on 5637 cells (Figure 1). We then explored to have a better understanding of its anticancer mechanism, focusing on OXS, cell cycle, and apoptosis.

Whether CHX is capable of exerting OXS on 5637 cells was first examined. It should be noted that our pilot study indicated that relatively short exposure times (to CHX) were sufficient to properly monitor the severity of OXS. Accordingly, a 6-h exposure of cells to CHX showed a ~2.2-fold higher OXS level (than that of control). Hence, it is possible that such elevated OXS could more likely damage or kill 5637 cells, and it is known as prooxidant effect. In fact, OXS has been lately considered as one of anticancer strategies by taking advantage of cancer cells being *more* vulnerable to OXS than normal counterparts [19]. The successful outcomes using OXS have been indeed reported in several cancer cases [20,21,22]. Although the exact reason for such high vulnerability of cancer cells (to OXS) remains uncertain, it is at least believed to be the inherent difference in inactivated or lack of antioxidant enzymes in cancer cells. Hence, relatively weak/low OXS could be strong enough to kill cancer cells lacking adequate antioxidant system (enzymes), whereas normal cells with strong antioxidant system may remain intact. It is conceivable that CHX-induced OXS is severe enough to injure or kill 5637 cells, resulting in the significant cell viability reduction that is primarily attributed to prooxidant effect.

In addition, it is known that OXS could have adverse effects on cell cycle, and we found that CHX indeed induced a G_1 cell cycle arrest [16]. A cell cycle progression was blocked and cells were unable to enter the next S phase, accumulating at a G_1 phase. This CHX-induced cell cycle arrest was also confirmed by the up-regulation of $p21^{WAF1}$ and $p27^{Kip1}$, indicating a G_1-specific cell cycle arrest [17]. Due to this G_1 cell cycle arrest, cell cycle cannot be completed, eventually resulting in the growth cessation or cell death, which would account for the reduction in 5637 cell viability (i.e., anticancer effect).

We were tempted to address the fate of cells following CHX treatment and found the down-regulation (reduced expression) of bcl-2 while the up-regulation (enhanced expression) of Bax in CHX-treated cells. Since bcl-2 is anti-apoptotic and Bax is pro-apoptotic [18], the pattern of down-regulated bcl-2 with up-regulated Bax apparently indicates induction of apoptosis. Thus, these results suggest that CHX-treated cells appear to ultimately follow the apoptotic death pathway.

Nevertheless, the same question on why induction of apoptosis by various agents, biologicals, drugs etc. would be significant is often raised. Actually, it is significant in terms of clinical application/utility. Apoptosis is considered as “cell suicide”, as opposed to necrosis or “cell murder” caused typically by chemotherapy [23]. In short, apoptosis is a highly organized biochemical death process *without* causing secondary injury or inflammation to the surrounding cells/tissues (i.e., side effects) [24]. In contrast, necrosis resulted from chemotherapy, randomly killing cancer as well as normal cells, will cause severe side effects, due to secondary inflammation [25]. Hence, it is significant that any regimens ultimately inducing apoptosis (in cancer cells) may have little side effects and

are safer and more suitable to be given to cancer patients. As shown here, CHX has not only anticancer activity but also apoptosis-inducing capability, presumably having its clinical implications.

5. Conclusion

In the present study, the bioactive mushroom extract, CHX, demonstrated anticancer effect on human bladder cancer 5637 cells. Such an anticancer mechanism appears to be attributed to exertion of oxidative stress, a G_1 cell cycle arrest, and ultimate apoptosis. Therefore, it is rather plausible that CHX may have clinical implications as a viable option for patients with low-grade bladder cancer. Further studies are warranted.

Conflict of Interest

The authors have no competing interest.

Acknowledgements

We thank Donna Noonan (Mushroom Wisdom, Inc.) for providing us with CHX and are also indebted to financial support from the Seize the Ribbon in this study.

Abbreviations

CHX: extract of Agrocybe chaxingu mushroom
MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
OXS: oxidative stress
LPO: lipid peroxidation
UCC: urothelial cell carcinoma
TUR: transurethral resection
TNM: tumor-node-metastasis
NMIBC: non-muscle invasive bladder cancer
MIBC: muscle invasive bladder cancer
CIS: carcinoma in situ
BCG: bacillus Calmette-Guerin
OD: optical density
MDA: malondialdehyde
TBA: thiobarbituric acid
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SD: standard deviation
ANOVA: analysis of variance
IC_{50}: the half-maximal inhibitory concentration

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