Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 66 (2006) 455–462
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Cell-cycle arrest and apoptosis induction in human breast carcinoma MCF-7 cells by carboxymethylated β-glucan from the mushroom sclerotia of *Pleurotus tuber-regium*

Mei Zhang, Peter C.-K. Cheung *, Lawrence C.-M. Chiu, Elaine Y.-L. Wong, Vincent E.-C. Ooi

Department of Biology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

Received 7 February 2006; received in revised form 24 March 2006; accepted 31 March 2006
Available online 24 May 2006

Abstract

The mechanism for the anti-tumor activity of a water-soluble carboxymethylated β-glucan (CMPTR), partially synthesized from an insoluble native glucan isolated from the sclerotia of *Pleurotus tuber-regium*, was studied using human breast carcinoma MCF-7 breast cancer cells *in vitro*. CMPTR-induced anti-proliferative activity dose-dependently, with an IC_{50} of 204 μg/ml. CMPTR inhibited the cell proliferation of MCF-7 by arresting the G_1 phase of its cell cycle after 48 h of incubation as shown by flow cytometry. Such G_1 phase arrest was associated with the down-regulation of cyclin D1 and cyclin E expressions in the breast cancer cells. In addition, the CMPTR-treated MCF-7 cancer cells were associated with decreased expression of anti-apoptotic Bcl-2 protein and increased expression of Bax/Bcl-2 ratio. This study shows that CMPTR can inhibit the proliferation of MCF-7 by cell-cycle arrest and apoptosis induction. The potential development of this mushroom polysaccharide as a water-soluble anti-tumor agent requires further investigation.

Keywords: *Pleurotus tuber-regium*; Sclerotia; Carboxymethylation; β-Glucan; Cell-cycle arrest; Apoptosis; Mushroom sclerotia; MCF-7 cells

1. Introduction

It has been shown that mushroom polysaccharides exhibited direct inhibitory effects on cancer cell growth by modulating cell-cycle progression and inducing apoptosis (Wang et al., 2002). A polysaccharide-peptide complex (PSP) extracted from *Trametes versicolor* significantly reduced proliferation of MAD-MB-231 breast cancer cells by increasing p21 expression and decreasing cell-cycle protein cyclin D1 expression (Chow, Lo, Loo, Hu, & Sham, 2003). A protein bound polysaccharide (PBP) isolated from *Phellinus linteus* had an anti-proliferative effect on SW480 human colon cancer cells mediated by inducing apoptosis and G_2/M cell-cycle arrest with a decrease of Bcl-2 expression, an increase of cytochrome *c* release and reduced cyclin B1 expression (Li, Kim, Kim, & Park, 2004). In addition, a water-soluble β-glucan isolated from *Poria cocos* was shown to have growth-inhibitory effects on human breast carcinoma MCF-7 cells mediated by cell-cycle arrest and apoptosis induction (Zhang, Chiu, Cheung, & Ooi, 2006). Therefore, anti-tumor activities of mushroom polysaccharides are not only mediated by the immunopotentiation (Zaidman, Yassin, Mahajna, & Wasser, 2005), but can also be resulted from a direct inhibition on the tumor cells. However, structure of mushroom polysaccharides are very diverse in terms of their monosaccharide composition, linkages in the main chain, degree of branching, percentage of non-carbohydrate components such as protein or peptide percentage as well as functional group modification. Thus, the correlation of the chemical structure of mushroom polysaccharides and the mechanisms of their anti-proliferation activities is still unclear.

* Corresponding author. Tel.: +852 2609 6144; fax: +852 2603 5646.
E-mail address: petercheung@cuhk.edu.hk (P.C.-K. Cheung).

0144-8617/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2006.03.031

The sclerotia of *Pleurotus tuber-regium* were firstly discovered in Africa and are used as functional food to promote health and longevity (Zoberi, 1973). In China, these sclerotia are mainly distributed in Yunnan province (Southwestern China) and are used by local people as folk medicine to treat asthma, stroke, and breast cancer as well as to promote the development of fetus (Huang, Guo, & Huang, 1996). The sclerotia of *P. tuber-regium* are edible and extremely rich in non-starch polysaccharides that are mainly composed of β-glucan (Cheung & Lee, 1998). A native water-insoluble β-glucan (PTR) isolated from the sclerotia of *P. tuber-regium* was characterized by our laboratories to be a $1 \rightarrow 3$ linked β-glucan, with one $1 \rightarrow 6$ branches at every third glycosidic residues (Zhang, Zhang, & Cheung, 2001). PTR was demonstrated to have both immunomodulatory and direct cytocidal anti-tumor activities (Zhang, Cheung, & Zhang, 2001). We have recently introduced carboxymethylated groups to the native glucan, producing a novel water-soluble CMPTR with enhanced anti-tumor activity (Zhang, Cheung, & Zhang, 2004). Our previous study showed that the administration of CMPTR to the BALB/c mice bearing Sarcoma S-180 cells enhanced the production of TNF-α and might thereby inhibit the tumor growth *in vivo*. Compared to PTR, CMPTR had generally higher *in vitro* antiproliferation of cancer cell lines including HL-60 and HepG2. Therefore, CMPTR has very good potential in the development as an anti-tumor agent because of its higher water solubility and enhanced immunomodulatory and anti-tumor activities. However, the anti-proliferative effects of CMPTR on other cell lines and their mechanisms are still not clear. In this project, a mechanistic study of the anti-proliferative activity of CMPTR on the human breast cancinoma MCF-7 cells *in vitro* was investigated.

2. Materials and methods

2.1. Chemicals and antibodies

Samples of carboxymethylated β-glucan (CMPTR) were obtained from the sclerotia of *P. tuber-regium* by methods described previously (Zhang et al., 2004). Mouse anti-human cyclin D1 monoclonal antibody (Cat. No. 554181), cyclin E monoclonal antibody (Cat. No. 551160), and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig specific polyclonal antibody (multiple adsorption) (Cat. No. 554001) were purchased from BD Pharmingen. Mouse anti-human Bcl-2 monoclonal antibody (Cat. No. 610538) and Bax monoclonal antibody (Cat. No. 610982) were provided by BD Transduction Laboratories. Goat anti-mouse IgG antibodies conjugated with alkaline phosphatase were obtained from Bio-Rad (Cat. No. 1705010); Unless otherwise stated, all the other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Cell lines and cell culture

Human breast carcinoma MCF-7 cells were obtained from American Type Culture Collection (ATCC). The cells were grown in RPMI-1640 medium supplemented with 10% FBS. The cell cultures were incubated at fully humidified atmosphere of 95% room air and 5% CO_2 at 37 °C, and were passed three times a week.

2.3. In vitro proliferation and cytotoxicity assay

The breast cancer cells were incubated with CMPTR at the concentrations of 12.5, 25, 50, 100, 200, 400 μg/ml. The metabolic activity of the living cells, indicating their proliferation and viability at the end of incubation period was determined by colorimetric method based on a tetrazolium salt (MTT) (Mosmann, 1983). The treatment groups were compared with control groups in the absence of CMPTR; results were expressed as an inhibition ratio of the control cell metabolic activity calculated as $[(A - B)/A]$, where A and B are metabolic activities of the control and the treatment cells, respectively. The value of IC_{50}, which is the concentration of CMPTR required to inhibit the breast cancer cells by 50% of the control level, was estimated from the plot.

2.4. Cell-cycle analysis

The breast cancer cells were incubated with the IC_{50} of CMPTR for 24, 48, and 72 h, respectively. After treatment, the cells were washed with PBS twice and fixed by 70% ice ethanol at −20 °C for at least 2 h. Prior to the analysis, the fixed cells were again washed with PBS and stained with 50 μg/ml of propidium iodide (PI) solution. The stained cells were then transferred to flow tubes by passing through nylon mesh with pore size of 40 μm. Flow cytometric analysis was performed on a flow cytometer (Beckman Coulter, Epics XL MCL). Apoptotic cells were determined by their hypochromic sub-diploid staining profiles. The distribution of cells in the different cell-cycle phases was analyzed from the DNA histogram using Multicycle software (Phoenix Flow Systems, San Diego, CA).

2.5. Western blot analysis

Western blot was conducted to analyse the effects of CMPTR on the expressions of cell-cycle regulators cyclin D1 and cyclin E as well as apoptosis regulators Bcl-2 and Bax in the breast cancer cells. Equal amounts of proteins (30 μg/lane) were diluted by loading dye and subjected to 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was then transferred to a nitrocellulose membrane (Amersham). The membrane was then blocked in the blocking solution containing 0.2% Aurora@ blocking reagent (ICN Biochemical) and 0.1% Tween in PBS solution at room temperature (r.t.) for at least 2 h, which was subsequently incubated with

an appropriate dilution of corresponding primary antibody (mouse anti-human β-actin, cyclin D1, cyclin E, Bcl-2, and Bax) with continuous agitation at r.t. overnight. The membrane was then washed twice with the blocking buffer and incubated with the secondary antibody (AP-conjugated goat anti-mouse Igs) with continuous agitation for 1 h at r.t. Finally, membrane was washed twice by the blocking buffer and the antibody-bound protein was visualized with the enhanced chemiluminescence detection system (Bio-Rad).

2.6. Flow cytometric analysis of cyclin D1 and cyclin E expressions in different cell-cycle phases

The breast cancer cells were incubated with or without CMPTR for 24, 48, and 72 h, respectively. The cell pellet was collected by centrifugation, and then washed twice with PBS and fixed in 70% ethanol at 4 °C for at least 2 h. After the fixation, the ethanol was removed and the cells were washed with 1% BSA solution. The primary antibody at concentration of 10 μg/ml was added to recognize the cyclins. For isotype control, mouse IgG1 was used instead of the primary antibody. The cells were incubated for 1.5 h, with vortex mixing at every 30 min. The excess antibody was removed by washing with 1% BSA solution. The cells were then incubated with the FITC-conjugated goat anti-mouse Igs secondary antibodies in dark for 1 h, with vortex mixing at every 30 min. The excess antibodies were removed by washing with 1% BSA solution and the cells were re-suspended in the PI solution. The stained cells were then passed through the nylon mesh with 40 μm pore size before being subjected to analysis by flow cytometer (Eptcs XL, Beckman Coulter, Miami, FL). The FITC-stained cells containing the cyclin were determined using EXPO32.ADC XL4color software (Beckman Coulter). The cell was considered as expressing the cyclin if its FITC level was above the mean plus three times the standard deviation of its isotype control at the corresponding cell-cycle probe.

2.7. Statistical analysis

Difference in means between the control and the experimental groups were compared by Student's *t*-test. The Western blot and flow cytometric analyses were repeated twice to confirm the findings.

3. Results

3.1. Effect of CMPTR on the growth of MCF-7 cells

MTT assay determines the metabolic activity of the cells and reflects its growth potential which is a balance between proliferation and death. The breast cancer cells were incubated with 12.5, 25, 50, 100, 200, and 400 μg/ml CMPTR for 72 h. The results showed that CMPTR inhibited the cancer cell growth in a dose-dependent manner (Fig. 1).

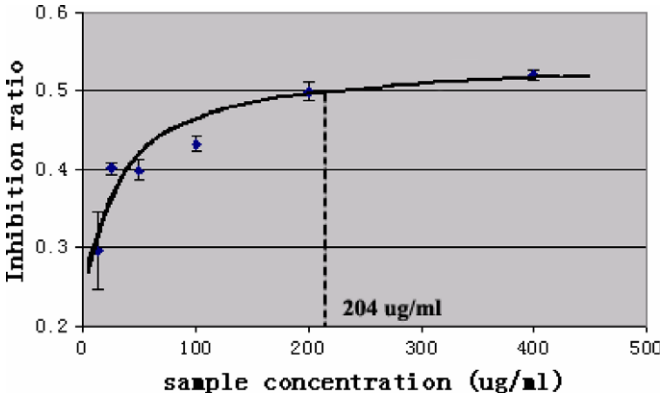

Fig. 1. Effect of CMPTR on the growth of MCF-7 cells. The breast cancer cells were incubated with CMPTR at concentrations of 12.5, 25, 50, 100, 200, and 400 μg/ml for 72 h. Results are expressed as means ± *SD* ($n = 3$).

The concentration of CMPTR with 50% inhibition on the cancer cell growth, i.e. IC_{50}, was found to be 204 μg/ml. The mechanism of action for the growth inhibition was further investigated after the breast cancer cells had been incubated with CMPTR at the value of IC_{50} for 24, 48, and 72, respectively.

3.2. CMPTR arrests cell-cycle progression and induces apoptosis

The CMPTR-treated and untreated MCF-7 cells were stained with PI, which were then analyzed by flow cytometer to determine the effect of CMPTR on their cell-cycle progression. CMPTR did not change the proportion of cells in different cell-cycle phases at 24 h of incubation [Fig. 2(a) and (b)]. However, at 48 h of incubation, G_0/G_1 cells increased from 43.5% in the control to 61.7% in the CMPTR-treated sample, while the G_2/M cells decreased from 38.5% to 26.7% [Fig. 2(a) and (b)]. At 72 h of incubation, on the other hand, the G_0/G_1 cells elevated from the control 42% to 79.1% and the G_2/M cells decreased from 41.5% to 0.9% after the CMPTR treatment [Fig. 2(c) and (d)]. Meanwhile, the subG_1 or the apoptotic cells elevated by 8.1-fold at 48 h and by 131-fold after 72 h of incubation with CMPTR [Fig. 2(c) and (d)]. All these results suggested that CMPTR retards the growth of MCF-7 cells by arresting cell-cycle progression and apoptosis induction.

3.3. CMPTR depletes cyclin D1 and E expressions

Cyclins modulate cell-cycle progression through the different checkpoints and it is known that cyclin D1 and cyclin E control the progression through G_1 phase. Since CMPTR was found to arrest the breast cancer cells at G_0/G_1 phase, the functions of cyclin D1 and cyclin E in the cell-cycle arrest were determined by flow cytometry. Furthermore, in order to investigate any cell-cycle phase specific effect of CMPTR on the cyclin expressions, the cells were distinguished into those of G_1, S, G_2/M phases

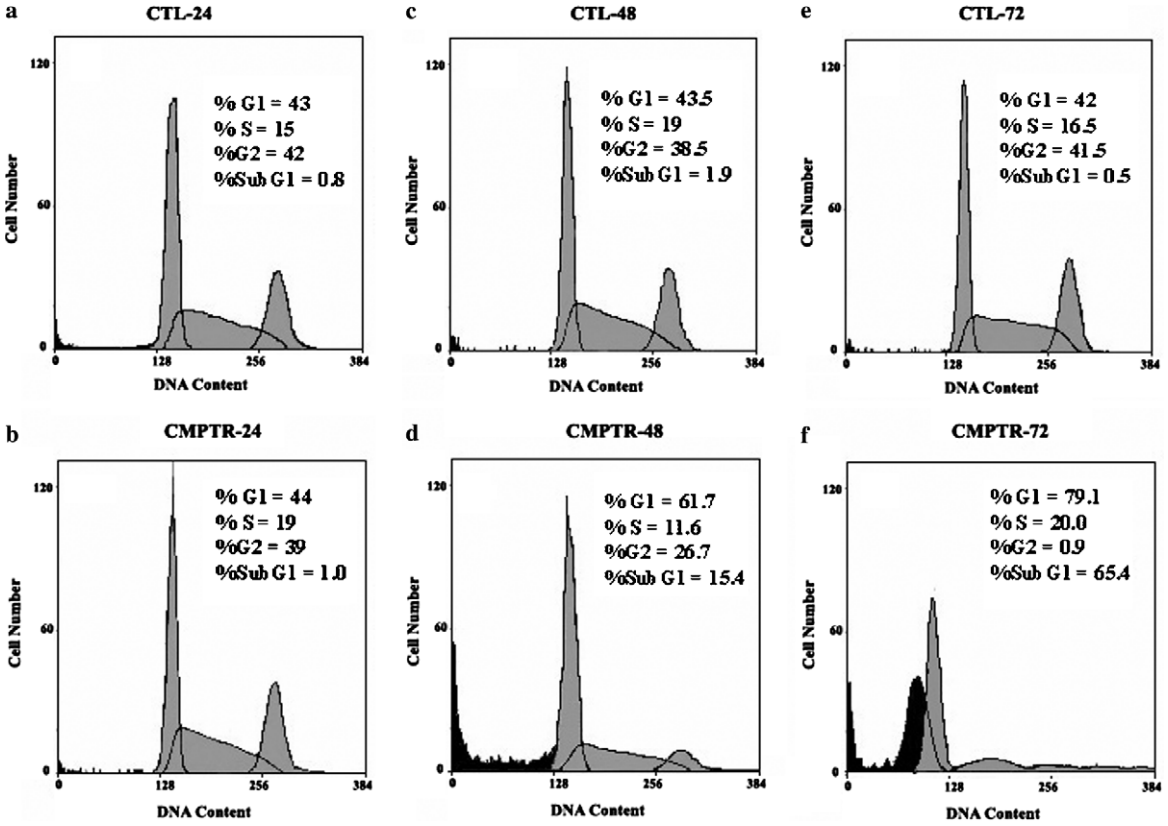

Fig. 2. DNA histograms showing the effect of CMPTR on the cell cycle of MCF-7 cells. The breast cancer cells were incubated with 200 μg/ml CMPTR for (b) 24 h, (d) 48 h, and (f) 72 h, respectively, comparing to their controls (CTL) (a, c, and e). Numeric data (mean value of triplicates) showing the proportions of cells in different cell-cycle phases are also listed.

depending on their DNA contents. In Figs. 3 and 4, those cells that were inside the rectangles were the cells expressing the cyclins. CMPTR depleted cyclin D1 expression time-dependently. At 48 h incubation with 200 μg/ml of CMPTR, when G_1 arrest occurred, the cyclin D1-positive cells at G_1 phase decreased from 9.8% to 0.9% [Fig. 3(c) and (d)]. Meanwhile, the cells expressing cyclin D1 at S and G_2/M phase were also decreased from 8.7% to 0.8% and from 5.6% to 0.5%, respectively. At 72 h of treatment, in association with the G_1 arrest, the cyclin D1-positive G_1 cells decreased from 11.3 to 0.5% and the cyclin D1-positive S and G_2/M cells decreased from 9.3% to 0.5% and from 8.5% to 0.4%, respectively. The CMPTR-induced cyclin D1 depletion seemed to happen in all the cell-cycle phases. Instead of only in late G_1 and early S phases in the normal cells, cyclin E expression was found in all the cell-cycle phases of MCF-7 cells (Fig. 3).

Similar to the effect on cyclin D1 expression, CMPTR also depleted cyclin E in all the cell-cycle phases in a time-dependent manner. At 48 h of treatment, when G_1 arrest was observed, the cyclin E-positive G1 cells decreased from 10.5% to 5.6% and the cyclin E-positive S and G_2/M cells decreased from 5.3% to 2.2% and from 9.8% to 4.4%, respectively. At 72 h of incubation, on the other hand, the cyclin E-positive G_1 cells decreased from 8.6% to 0.9% and the cyclin E-positive S and G_2/M cells decreased from 3.7% to 0.4% and from 5.7% to 0.5%, respectively. All the results suggested that CMPTR depletes both cyclin D1 and cyclin E expressions to induce the G_1 arrest in the breast cancer cells.

3.4. CMPTR depletes Bcl-2 expression

The above results clearly indicated that CMPTR-induced apoptosis to MCF-7 cells after 48 h of incubation. Apoptosis is modulating by different pro-apoptotic and anti-apoptotic factors. In this study, the functions of well characterized pro-apoptotic Bax and anti-apoptotic Bcl-2 in the CMPTR-induced apoptosis were investigated by Western blotting technique (Fig. 5). At 48 h of incubation with 204 μg/ml CMPTR, when apoptosis was observed, the protein level of Bcl-2 decreased while that of Bax remained unchanged as compared with that of the control [Fig. 5(a)], resulting in an increase in the pro-apoptotic Bax/Bcl-2 ratio [Fig. 5(b)]. At 72 h of incubation, when apoptosis occurred at a larger extent, there was more prominent decrease of Bcl-2 protein and elevation of Bax/Bcl-2 ratio, suggesting that Bcl-2 plays an important role in the CMPTR-induced apoptosis in MCF-7 cells.

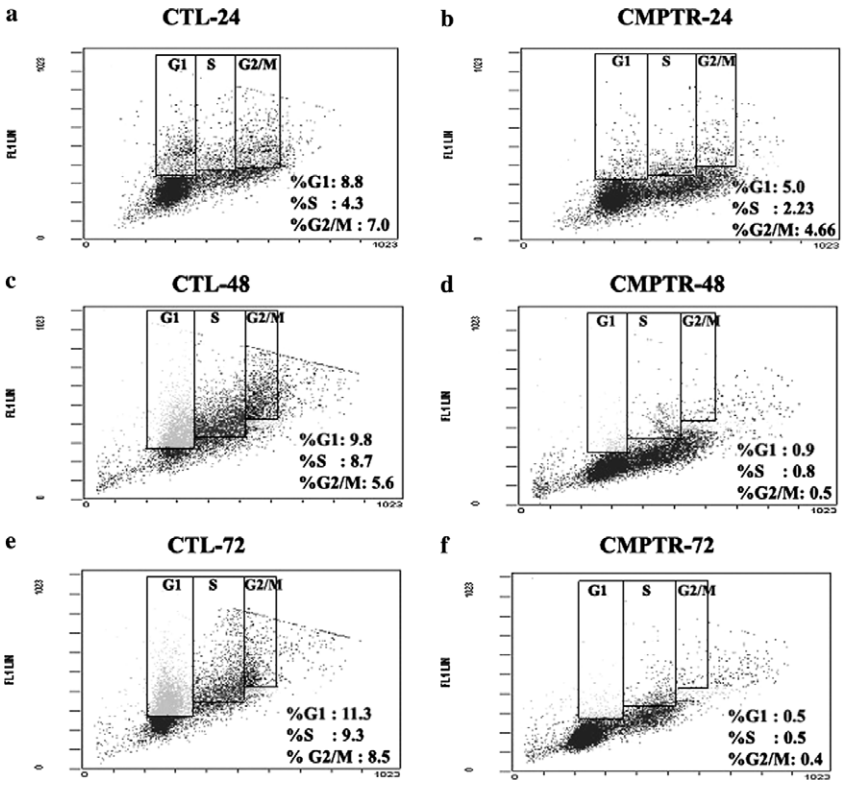

Fig. 3. Effect of CMPTR on cyclin D1 expression in MCF-7 cells. The breast cancer cells were incubated with CMPTR for (b) 24 h, (d) 48 h, and (f) 72 h, respectively, comparing with their controls (CTL) (a, c, and e). Numeric data (mean value of triplicates) showing the cyclin D1-positive cells in different cell-cycle phases are also listed.

4. Discussion

Treatment of CMPTR with MCF-7 tumor cells resulted in G_1 arrest and apoptosis induction which were time dependently as mentioned earlier. In mid-to-late G_1 phase, cyclin D1 associates with cyclin-dependent kinases phosphorylate Rb and Rb-like proteins p107 and p130 (Borgne & Golsteyn, 2003). Once phosphorylated, pRb and p130 released the bound transcription factors including the E2F family (Nevins, 2000). The transcription factors activate the expression of cyclin E, which is necessary for the S phase entry (King & Cidlowski, 1988). Over-expression of cyclin D1 and cyclin E can be found in many human cancer cells. When analyzing the cyclin D1 and cyclin E expressions by flow cytometry, our results showed that both proteins were down-regulated after the CMPTR treatment. It has been reported that mutations resulting in continual up-regulated expression of cyclin D1 contribute to the development of a variety of human cancers including breast cancers (Baldin, Lukas, Marcote, Pagano, & Draetta, 1993). Normally, cyclin D1 expressed in mid-to-late G_1 phase and started to degrade at the entry of S phase (Klein, 2004). We have shown in this study that cyclin D1 was expressed in all the cell-cycle phases in MCF-7 cells. After treatment of CMPTR, cyclin D1 dramatically decreased in G_1, S, and G_2/M phases, indicating that CMPTR not only down-regulated the cyclin D1 expression in G_1 phase to prevent the phosphorylation of Rb and the subsequent release of the bound transcription factors, but it also down-regulated the abnormal cyclin D1 expressions in the S and G_2/M phases as well. Similar observations were also made on the cyclin E expression in MCF-7 cells. CMPTR treatment down-regulated the cyclin E expression in G_1 phase to prevent the S phase entry as well as to induce G_1 phase arrest. Combining the analyses of cell cycle and cyclin D1 and cyclin E expressions, our results revealed that CMPTR down-regulated cyclin D1 and cyclin E expression to induce G_1 arrest and depleted the abnormal cyclin expression in the other cell-cycle phases of the MCF-7 cells.

Apoptosis is a form of cell death that is tightly regulated by a number of gene products that either promote or block cell death at different stages of the cell cycle. In mammals, the Bcl-2 gene family in mammals contains a number of anti-apoptotic proteins including Bcl-2 and others (Choi et al., 2002). On the other hand, the Bax and BH-3 gene families consist of pro-apoptotic proteins including Bax and others that can promote cell death. Therefore, cell apoptosis largely depends on the balance between the pro-apoptotic proteins, such as Bax, and anti-apoptotic proteins such as Bcl-2 (Ma et al., 1998). The Bax/Bcl-2 ratio has been shown to be critical in determining the susceptibility of cells to induced apoptosis (Raisova, 2001). Bax complexes *in vivo* with Bcl-2 protein and down-regulation of Bcl-2 leads to the formation of Bax homo-dimer that increases the rate of apoptosis (Solomons, 1997).

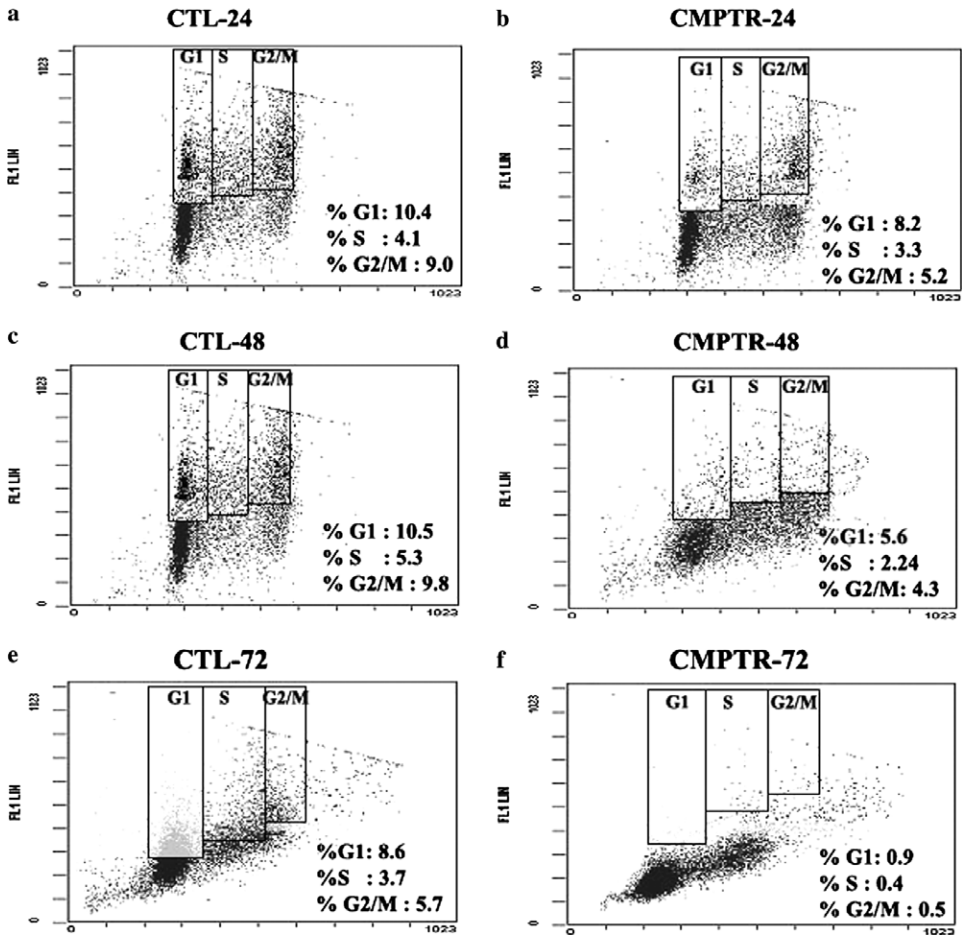

Fig. 4. Effect of CMPTR on cyclin E expression in MCF-7 cells. The breast cancer cells were incubated with CMPTR for (b) 24 h, (d) 48 h, and (f) 72 h, respectively, comparing with their controls (CTL) (a, c, and e). Numeric data (mean value of triplicates) showing the cyclin E-positive cells in different cell-cycle phases are also listed.

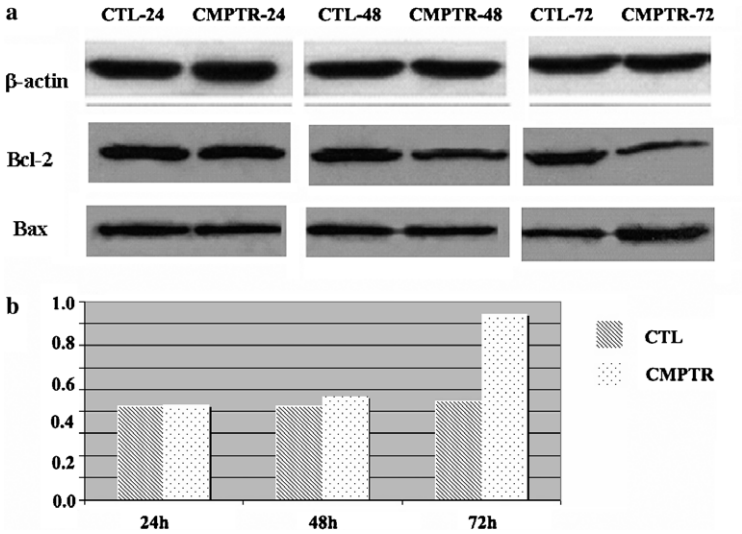

Fig. 5. Effects of CMPTR on Bcl-2 and Bax expressions in MCF-7 cells determined by western blotting analysis. (a) The breast cancer cells were incubated with 200 μg/ml of CMPTR for 24, 48, and 72 h, respectively, comparing to their controls (CTL). The protein levels of Bcl-2 and Bax in the cell lysates were measured. (b) The ratio of Bax to Bcl-2 in different treatments was calculated by dividing the densitometer reading of the Bax protein band to that of the Bcl-2 protein band.

Subsequently Bax affects the mitochondrial membrane permeability that results in the release of cytochrome *c*, apoptosis-inducing factor (AIF), and other pro-apoptotic molecules from mitochondria into the cytoplasm, which eventually lead to apoptosis through the activation of caspases (Kroemer, 2000; Marzo, 1998). The down-regulation of anti-apoptotic Bcl-2 caused an increase in the pro-apoptotic Bax/Bcl-2 ratio in the MCF-7 cells after the CMPTR treatment, suggesting that Bcl-2 participated in the CMPTR-induced apoptosis in the breast cancer cells. The findings of this investigation clearly demonstrated that CMPTR induce apoptosis in MCF-7 cells, which might account for its anti-proliferative activities.

Compared to protein and peptide, polysaccharides have many types of sugar linkages involving different kinds of monosaccharides and different degrees of branching and conformations, giving them a multitude of three-dimensional shapes. CMPTR is a modified β-glucan with carboxymethylated groups distributing along its main chain, showing the property of polyanions in aqueous solution with an extended flexible chain (Zhang, Zhang, & Cheung, 2003). CMPTR was characterized in our previous research to be a polymer with molecular mass of around 2×10^5 (Zhang et al., 2004), which would be too large to enter the cell. Hence, it might only interact with the cell surface proteins to trigger the change of signal transduction within the cells. In fact, it has been reported that an anionic sulfated polysaccharide with anti-tumor activity could bind to positively charged DNA-binding locus of enzymes via electrostatic interaction on the cell surface of human cancer cells, inhibiting its binding to DNA (Umemura et al., 2003). It is proposed that carboxymethylated polysaccharides would probably bind non-specifically to the DNA-interacting enzymes in a way similar to sulfates. Although the non-specific binding by individual carboxymethylated groups might be weaker than the specific binding by DNA, the multivalent nature of carboxymethylated polysaccharides might enable the large polysaccharidic molecules to cover the locus of the enzymes, blocking their reaction with the DNA molecules.

Our results showed that CMPTR having an enhanced anti-proliferative activity than its native counterpart (Zhang et al., 2004), retarded the growth of MCF-7 cells by arresting cell-cycle progression at G_1 phase and inducing apoptosis. The cell-cycle arrest was associated with depletions of cyclin D1 and cyclin E expressions. The pro-apoptosis Bax/Bcl-2 ratio was also elevated after the CMPTR treatment, suggesting that the polysaccharide may induce apoptosis at least partially via the mitochondrial pathway. Further studies will be focused on the effect of CMPTR on the more detailed signal transduction pathways within the MCF-7 cells.

Acknowledgements

This project was supported by the Center of Area of Excellence (AoE) in Plant and Fungal Biotechnology in The Chinese University of Hong Kong and the Research Grants Council (RGC) Earmarked Grant (CUHK4255/02M) of the HKSAR Government.

References

Baldin, V., Lukas, J., Marcote, M. J., Pagano, M., & Draetta, G. (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G_1. *Genes Development, 7*, 812–821.

Borgne, A., & Golsteyn, R. M. (2003). The role of cyclin-dependent kinases in apoptosis. *Progress in Cell Cycle Research, 5*, 453–459.

Cheung, P. C. K., & Lee, M. Y. (1998). Comparative chemical analysis of fiber material prepared by enzymatic and chemical methods from two mushrooms (*Pleurotus sajor-caju* and *Pleurotus tuber-regium*). *Journal of Agricultural and Food Chemistry, 46*, 4854–4857.

Choi, J., Inrad, C. C., Malakowsky, C. C., Talent, J. M., Yuan, C. S., & Gracy, R. W. (2002). Flavones from *Scutellaria baicalensis* Georgi attenuate apoptosis and protein oxidation in neuronal cell lines. *Biochemical and Biophysical Acta, 1571*, 201–210.

Chow, L. W., Lo, C. S., Loo, W., Hu, X., & Sham, J. (2003). Polysaccharide peptide mediates apoptosis by up-regulating p21 gene and down-regulating cyclin D1 gene. *American Journal of Chinese Medicine, 31*, 1–9.

Huang, N., Guo, M., & Huang, L. (1996). *Pleurotus tuber-regium* and its cultivation. *Edible Fungi in China, 16*, 13–14.

King, K. L., & Cidlowski, J. A. (1988). Cell cycle regulation and apoptosis. *Annual Review in Physiology, 60*, 601–617.

Klein, G. (2004). Cancer, apoptosis, and non-immune surveillance. *Cell Death Differentiation, 11*, 13–17.

Kroemer, G. (2000). Mitochondrial control of cell death. *National Medicine, 6*, 513–519.

Li, G., Kim, D., Kim, T., & Park, B. (2004). Protein-bound polysaccharide from *Phellinus linteus* induces G_2/M phase arrest and apoptosis in SW480 human colon cancer cells. *Cancer Letters, 216*, 175–181.

Ma, Y., Cao, L., Kawabata, T., Yoshino, B., Yang, B. B., & Okada, S. (1998). Cupric nitrilotriacetate induces oxidative DNA damage and apoptosis in human leukaemia HL-60 cells. *Free Radical and Biological Medicine, 25*, 568–575.

Marzo, I. (1998). Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science, 281*, 2027–2031.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. *Journal of Immunological Methods, 63*, 55–63.

Nevins, J. R. (2000). The Rb/E2F pathway and cancer. *Human Molecular Genetics, 10*, 699–703.

Raisova, M. (2001). The Bax/Bcl-2 ratio determines the susceptibility of human melanoma cells to CD95/Fas-mediated apoptosis. *Journal of Investigation of Dermatology, 117*, 333–340.

Solomons, G. S. (1997). The Baxα:Bcl-2 ratio modulates the response to dexamethasone in leukemia cells and is highly variable in childhood acute leukemia. *International Journal of Cancer, 71*, 959–965.

Umemura, K., Yanase, K., Suzuki, M., Okutani, K., Yamori, T., & Andoh, T. (2003). Inhibition of DNA topoisomerases I and II, and growth inhibition of human cancer cell lines by a microalgal polysaccharide. *Biochemical Pharmacology, 66*, 481–487.

Wang, Y. Y., Khoo, K. H., Chen, S. T., Lin, C. C., Wong, C. H., & Lin, C. H. (2002). Studies on the immuno-modulating and anti-tumor activities of *Ganoderma lucidum* (Reishi) polysaccharides: Functional and proteomic analyses of a fucose-containing glycoprotein fraction responsible for the activities. *Bioorganic and Medicinal Chemistry, 10*, 1057–1062.

Zaidman, B. Z., Yassin, M., Mahajna, J., & Wasser, S. P. (2005). Medicinal mushroom is modulators of molecular targets for cancer therapeutics. *Applied Microbiology and Biotechnology, 2*, 1–24.

Zhang, L., Zhang, M., & Cheung, P. C. K. (2001). Chemical structure and chain conformation of the water-insoluble glucan isolated from *Pleurotus tuber-regium*. *Biopolymers, 59*, 457–464.

Zhang, M., Cheung, P. C. K., & Zhang, L. (2001). Evaluation of mushroom dietary fiber (non-starch polysaccharides) from sclerotia of *Pleurotus tuber-regium* (Fries) singer as a potential anti-tumor agent. *Journal of Agricultural and Food Chemistry, 49*, 5059–5062.

Zhang, M., Cheung, P. C. K., & Zhang, L. (2004). Carboxymethylated β-glucans from mushroom sclerotium of *Pleurotus tuber-regium* as novel water-soluble anti-tumor agent. *Carbohydrate Polymers, 57*, 319–325.

Zhang, M., Chiu, L. C.-M., Cheung, P. C. K., & Ooi, V. E. C. (2006). Growth-inhibitory effects of a β-glucan from the mycelium of *Poria cocos* on human breast carcinoma MCF-7 cells: Cell cycle arrest and apoptosis induction. *Oncology Reports, 15*, 637–643.

Zhang, M., Zhang, L., & Cheung, P. C. K. (2003). Molecular mass and chain conformation of carboxymethylated derivatives of β-glucan from scleortia of *Pleurotus tuber-regium*. *Biopolymers, 68*, 150–159.

Zoberi, M. H. (1973). Some edible mushroom from Nigeria. *Nigerian Field, 38*, 81–90.