

Investigation of Boldine as a Potential Telomerase Inhibitor by Downregulation of hTERT/hTERC in HCT 116 Human Colon Carcinoma Cells

(Kajian Boldina sebagai Perencat Telomerase Berpotensi oleh Pengawalan Rendah hTERT/hTERC dalam HCT 116 Karsinoma Sel Kolon Manusia)

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ABSTRACT

Telomerase, a ribonucleoprotein (RNP) complex, is a type of RNA-dependent DNA polymerase that synthesises telomeric DNA repeats (TTAGGG) at the 3' end of chromosomes. Most of the cancer cells express high level of telomerase which results in cellular immortality. The high telomerase activity in cancer cells can be detected via expression of human TERT (hTERT), the catalytic protein subunit of telomerase, and expression of human TERC (hTERC), the RNA component in telomerase. Boldine, a natural alkaloid compound, was shown to have anticancer properties on various types of cancer cells, but the anti-telomerase property was poorly understood. This study was carried out to investigate the ability of boldine in targeting telomerase on the human colon cancer cell line, HCT 116, by analyzing the expression of hTERT and hTERC. Boldine was shown to have a time- and dose-dependent cytotoxic effect on HCT 116 cell line in SRB assay. The protein expression of hTERT was assessed through Western blot where it was observed to be down-regulated upon boldine treatment compared to control. The cells treated with boldine also exhibited a down-regulation of mRNA expression for both hTERT and hTERC in Real Time PCR (qRT-PCR). The down-regulation of hTERT protein expression correlated with the reduced hTERC mRNA expression in qRT-PCR. The observations on the down-regulation of protein and mRNA expressions of telomerase related genes, hTERT and hTERC, in this study suggested that boldine might become a significant candidate for telomerase-targeted anti-cancer therapy.

Keywords: Boldine; cancer; hTERC; hTERT; telomerase

ABSTRAK

Telomerase, iaitu satu kompleks ribonukleoprotein (RNP) merupakan sejenis DNA polimerase bersandar RNA yang mensintesis telomerik DNA berulang (TTAGGG) pada hujung kromosom. Kebanyakan sel kanser mengekspres telomerase pada tahap yang tinggi dan akan mengakibatkan sel mencapai keabadian. Aktiviti telomerase yang tinggi dalam sel kanser dapat dikesan melalui pengekspresan TERT manusia (hTERT) iaitu subunit protein katalitik telomerase dan pengekspresan TERC manusia (hTERC) iaitu komponen RNA dalam telomerase. Boldina merupakan sebatian alkaloid semula jadi yang mempunyai sifat antikanser terhadap pelbagai jenis sel kanser, namun begitu sifat anti-telomerase masih kurang difahami. Kajian ini dijalankan untuk mengkaji keupayaan boldina untuk menasaskan telomerase dalam titisan sel kanser kolon manusia, HCT 116 dengan menganalisis pengekspresan hTERT dan hTERC. Boldina dikatakan mempunyai kesan sitotoksik yang bersandar kepada masa and dos terhadap titisan sel HCT 116 dalam asai SRB. Pengekspresan protein hTERT telah dinilai melalui Western blot dengan pengawalaturannya menurun selepas rawatan dengan boldina berbanding kawalan. Sel yang dirawat dengan boldina juga menunjukkan pengawalaturan yang menurun bagi pengekspresan mRNA untuk kedua-dua hTERT dan hTERC melalui PCR masa sebenar (qRT-PCR). Pengawalaturan yang menurun bagi pengekspresan protein hTERT didapati berkorelasi dengan pengurangan pengekspresan mRNA hTERC melalui qRT-PCR. Pemerhatian terhadap pengawalaturan yang menurun bagi pengekspresan protein dan mRNA gen berkait telomerase, hTERT dan hTERC dalam kajian ini mencadangkan bahawa boldina berkemungkinan menjadi calon penting untuk terapi antikanser yang menasaskan telomerase.

Kata kunci: Boldina; hTERC; hTERT; kanser; telomerase

INTRODUCTION

Telomerase is an enzyme which synthesises telomeric DNA repeats in order to maintain telomere length. Telomerase was found to be highly expressed in various cancer cells (Fernández-Marcelo et al. 2015; Jafri et al. 2016). Telomerase is crucial in the immortalization of cancer cells to reverse the loss of telomere in every round of cell

division (Cong et al. 2002; Xu et al. 2016). High telomerase activity was observed in colon cancer cells, suggesting that colon cancer cells required telomerase to proliferate indefinitely and escape from replicative senescence (Aljarbou et al. 2018; Ayiomamitis et al. 2014; Bertorelle et al. 2014; Chadeneau et al. 1995). The high telomerase activity in cancer cells can be detected via the expression

of hTERT, the protein catalytic subunit of telomerase, and hTERC, the RNA component in telomerase (Beattie et al. 1998; Herbert et al. 1999; Ishikawa 1997; Weinrich et al. 1997). Cancer cells achieved proliferative immortality usually by up-regulating the hTERT and hTERC gene expression in telomerase (Akincilar et al. 2015; Cristofari et al. 2006; Leão et al. 2018; Xi et al. 2014). Thus, targeting the hTERT and hTERC in telomerase could be a promising approach for telomerase-based cancer therapies.

Boldine, a natural compound obtained from Boldo tree, was shown to inhibit telomerase activity mainly by down-regulating the expression of hTERT in various cancer cells. For instance, boldine suppressed the proliferation of two telomerase-positive breast cancer cells, MCF-7 and MDA-MB-231, by inhibiting the telomerase activity in both cancer cell lines. The telomerase inhibition was proven to occur via down-regulation of hTERT (Noureini et al. 2015). Boldine also showed cytotoxic effect in HepG-2 cells where boldine suppressed the immortality of HepG-2 cells through telomerase inhibition (Noureini et al. 2015a). Although boldine has been used to test on several cancer cells type such as embryonic kidney cells HEK293, human hepatocellular carcinoma cell line HepG-2 and breast cancer MCF-7, there is still no study reported on the effect of boldine on human colon cancer cell line HCT 116. Besides, boldine showed to have no toxic effect on non-cancer cells when used at the same concentrations as those used on cancer cells, and thus may be a promising compound as an anti-cancer agent in treatment of human cancer. However, limited work has been carried out on the ability of boldine to inhibit telomerase activity in human colon cancer cells as well as down-regulation of telomerase related genes especially hTERC gene.

In the present study, evidence of telomerase-based cancer therapeutic potential of boldine as a cytotoxic agent in the human colon cancer cell line, HCT 116 was investigated. Accordingly, the cytotoxicity of boldine on HCT 116 cell line was identified in this study. The effect of boldine on hTERT protein expression in HCT 116 cells was determined. qRT-PCR was also carried out to assess mRNA expression of hTERT and hTERC genes in HCT 116 cell line.

MATERIALS AND METHODS

CELL CULTURE AND COMPOUND

Human Colon Carcinoma (HCT 116) cell line was purchased from the American Type Culture Collection (ATCC, USA). HCT 116 was cultured as monolayers in RPMI 1640 (Nacalai Tesque, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, USA), 1% penicillin/streptomycin (Nacalai Tesque, Japan), and 1% amphotericin B (250 µg/mL; Sigma, USA). The cells were maintained in a humidified incubator (ESCO) with 95% air and 5% of CO₂ at 37°C. Boldine 99% was obtained from Sigma (Singapore) and the compound was dissolved in DMSO (Nacalai Tesque, Japan).

CYTOTOXIC ASSAY

Cells were seeded (5000 cells/well) into sterile 96-well micro titer plates in growth medium. The cells were allowed to proliferate in a 5% CO₂ incubator at 37°C and were cultured overnight to allow cell attachment. After overnight incubation, serial dilution of boldine was carried out to obtain different boldine concentrations (0, 3.13, 6.25, 12.5, 25, 50 and 100 µg/mL). The cells were treated with boldine for three different time points at 24, 48 and 72 h. Untreated cells were referred to the well with only the cells and the media, which served as a control.

Sulphorhodamine B (SRB) assay was carried out to measure the viability of the cells upon boldine treatment. Cell viability was assessed based on the measurement of the total protein content of viable cells (Houghton et al. 2007). Upon each incubation period (24, 48 and 72 h), 50 µL of 40% ice-cold trichloroacetic acid (TCA; Merck, Germany) was added to each of the well to fix the cells, and the plate was incubated for an hour at 4°C. The cells were then washed, followed by the addition of 0.4% SRB dye (Sigma-Aldrich, USA) to each of the well. The plate was incubated for 30 min at room temperature for staining process. After incubation period, they were then washed with 100 µL of 1% acetic acid (Merck, Germany) to remove any unbound dye. In order to solubilize the bound SRB stain, 100 µL of 10 mM Tris buffer with pH10.5 (Merck, Germany) was added and the plate was left to shake at 500 rpm for 5 min using plate shaker (Thermo Fisher, Latvia). Absorbance of the dye was measured at 492 nm using a plate reader (BioTek, USA). All experiments were performed in triplicates. The results were presented as means ± SD.

The IC₅₀ values of boldine at 24, 48, and 72 h were determined from the dose-response curves. The percentage of cell viability of each of the cells treated with various concentrations of boldine was calculated accordingly using the following formula:

$$\text{Percentage cell viability (\%)} = \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100\%$$

with OD control is the absorbance of negative control; and OD sample with the absorbance of sample

The lowest IC₅₀ value of boldine against HCT 116 cell line was chosen for subsequent experiment. Cells were then plated in six T25 flasks and allowed to adhere overnight. The next day, three flasks containing cultured cells were treated with the identified concentration of boldine (in SRB assay) while another three flasks were left untreated to serve as control. These cells were kept in a 5% CO₂ incubator at 37°C for the time period that give the lowest IC₅₀ value in SRB assay, which is 72 h.

WESTERN BLOT ANALYSIS

Cells seeded at 2×10⁶ cells per T25 flask were treated with the identified concentration of boldine initially

for 72 h upon overnight incubation. At the end of the treatment, the cells were collected. Cell scraper was used to scrap all the cells in the flask, and the detached cells were collected into a falcon tube. The cells were washed with 1× phosphate buffer saline (PBS; Nacalai Tesque, Japan) twice. After centrifuged, the supernatant in the falcon tube was removed. Cells pellet were then added with Radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, USA) containing 1% of protease inhibitor (Sigma-Aldrich, USA) and was incubated in ice for 30 min. Upon incubation period, the cell lysate was centrifuged at 14,000 rpm for 30 min at 4°C (Thermo Scientific™ Heraeus™ Pico™ 17 Microcentrifuge). The supernatant (protein) was transferred into Protein LoBind tube (Eppendorf) and protein concentration was estimated using Bradford assay (Bio-Rad, United States).

For Western blotting, each sample was prepared by mixing the protein samples with loading buffer in 3:1 ratio. All the protein samples were then boiled on a heating block (QBD2-Grant, England) at 70°C for 10 min. Western blotting was done using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% acrylamide gel, which is suitable for protein range at 10-200 kDa as the hTERT protein is about 126 kDa. Pre-stained protein ladder (GeneDireX, USA) was used as a marker. A total of 50 µg of protein samples were loaded in each well accordingly for electrophoresis. The gels were then electro-transferred onto nitrocellulose membrane using the transfer apparatus, Mini-PROTEAN® Tetra System (Bio-Rad, USA) for 2 h at 90 V. The nitrocellulose membrane was blocked with Blocking One (Nacalai Tesque, USA). For detection of housekeeping protein, a primary anti β-Actin antibody from rabbit (Cell Signalling Technology, USA) was used. In detecting hTERT protein, the primary anti-hTERT antibody from rabbit (Sigma-Aldrich, USA) was used. Both primary antibodies were diluted in 1:1000 ratio of primary antibody: blocking buffer. The membranes were incubated with primary antibody overnight at 4°C. After incubation, the membranes were washed five times with Tris buffered saline containing 0.1% of Tween 20 (TBS-T; Sigma, USA) for 5 min each. The membranes were then incubated with secondary antibody for 1 h at room temperature and washed five times with TBS-T for 5 min each. The secondary antibody used for both hTERT and β-Actin was anti-rabbit antibody (Cell Signaling Technology, USA) conjugated with reporter enzyme-horseradish peroxidase (HRP) which was prepared in 1:10000 ratio of secondary antibody: blocking buffer. The results were detected using an enhanced chemiluminescence reagent (luminol and stable peroxide solution with ratio 1:1) from Western Bright™ ECL (Advansta, USA) under gel documentation camera. The intensity of bands was quantified using ImageJ program.

REAL-TIME POLYMERASE CHAIN REACTION (qRT-PCR)

Total RNA from both boldine-treated and untreated cells were extracted using the ReliaPrep RNA Cell Miniprep System (Promega, USA) following the manufacturer's protocol. Initially, both boldine-treated and untreated cells were collected and the cells were centrifuged at 300 g for 5 min and the supernatant was removed. Cell pellet was washed with ice-cold, sterile 1× PBS and was pelleted again. Lysis buffer was then added to the cell pellet in order to lyse the cells. Upon transferring cell lysate to Minicolumn and centrifuged at high speed, DNase treatment was performed to remove any residual DNA in the sample. Then, the columns were washed repeatedly and lastly, nuclease free water was added to elute the RNA. Samples containing RNA were stored at -80°C.

The concentration of RNA samples was quantified using Take 3 Micro-Volume Plate (BioTek, USA). The values of the A_{260}/A_{280} and A_{260}/A_{230} ratios of RNA samples were identified to ensure the purity of RNA. To assess the integrity of RNA, a total of 500 ng of the RNA samples containing RNA loading dye were run beside 2 µL RiboRuler™ High Range RNA Ladder (Fermentas, Lithuania) on a 1% agarose gel stained with ethidium bromide (EtBr) at 60 V for 45 min. The gel was viewed under gel documentation camera.

Complementary DNA was synthesized with GoScript™ Reverse Transcription System (Promega, USA). An aliquot of 2 µg of total RNA was reverse-transcribed by GoScript™ Reverse Transcriptase using Oligo(dT)₁₅ primers and random primers according to the manufacturer's protocol. The real-time PCR for individual gene expression analysis was performed using Applied Biosystems StepOnePlus™ Real-Time PCR System. Relative expression levels of hTERT and hTERC were measured using TaqMan® Gene Expression Master Mix (Applied Biosystems, USA) and specific Taqman probes (Applied Biosystems, USA). The reaction conditions for all amplicons were as follows: 95°C for 20 s, followed by 40 cycles at 95°C for 1 s, 60°C for 20 s. Data were then collected and analysed using StepOne™ Software v2.2.2. Ct values were normalized to the housekeeping gene, β-Actin. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ formula.

STATISTICAL ANALYSIS

Data were expressed as mean ± SD of triplicates. Graph was plotted using three technical replicates, $n=3$ for the IC₅₀ value of boldine on HCT 116 with standard deviation (SD). The significant values (compared to control) were determined by one-way ANOVA using SPSS Statistics 22. Data of Western Blot and qRT-PCR were analyzed for significant difference using unpaired student's T-test. The number of replicates (n) was 3 for each of the experiments.

Differences among groups were considered significant at $p < 0.05$. The following notion was used: * indicated $p < 0.05$ compared to the non-treated group.

RESULTS

BOLDINE INHIBITS VIABILITY OF HCT 116 CELLS

The viability of HCT116 cells treated with various concentrations of boldine (0 to 100 $\mu\text{g}/\text{mL}$) for 24, 48 and 72 h were examined using the SRB assay which showed that boldine significantly inhibited the growth of HCT 116 cells in a time- and dose- dependent manner ($p < 0.01$) (Figure 1). In particular, exposure to boldine treatment for 24 h significantly reduced the percentage of viable cells, ranging from 84.2% to 47.3% (Figure 1(A)). Boldine showed the maximum effect against HCT 116 cells by increasing percentage of inhibition to 81.5% after 48 h and to 94.4% after 72 h, respectively, compared to untreated cells (Figures 1(B) and 1(C)). The percentage of cell viability was reduced as the concentration of boldine used to treat the cells increased. Higher concentrations of boldine gave a stronger inhibitory effect on the cell proliferation.

Boldine was shown to exhibit stronger inhibitory effect on HCT 116 cells when the duration of treatment was longer (Figure 1(D)). The graph showed that there has

been a marked decrease in the IC_{50} value of boldine across the period of incubation time. The lowest IC_{50} value of boldine on HCT 116, which was $22.4 \pm 1.2 \mu\text{g}/\text{mL}$ at 72 h, was chosen for subsequent experiments. Thus, for the subsequent experiments, HCT 116 cells were treated with $22.4 \mu\text{g}/\text{mL}$ of boldine for 72 h.

BOLDINE CAUSES DOWN-REGULATION OF hTERT PROTEIN EXPRESSION IN HCT 116 CELLS

In order to determine the ability of boldine to down-regulate hTERT protein expression in HCT 116 cells, cells were incubated in the absence and presence of boldine ($22.4 \mu\text{g}/\text{mL}$) for 72 h. Protein extracted from both treated and untreated cells were subjected to Western blotting.

As illustrated in Figure 2(A), the expression of β -Actin showed equal amounts of protein loaded in each lane. Thinner bands were observed in boldine treated cells compared to the control, indicating a pronounced reduction of hTERT protein in boldine-treated cells. The relative band intensities of control and boldine treated cells were quantified using ImageJ program. The average relative quantifications of control and boldine treated cells were 1.19 and 0.44, respectively, upon normalization with β -Actin. From this observation, exposure of HCT 116 cells to boldine decreased significantly the expression of hTERT proteins compared to the control (Figure 2(B)).

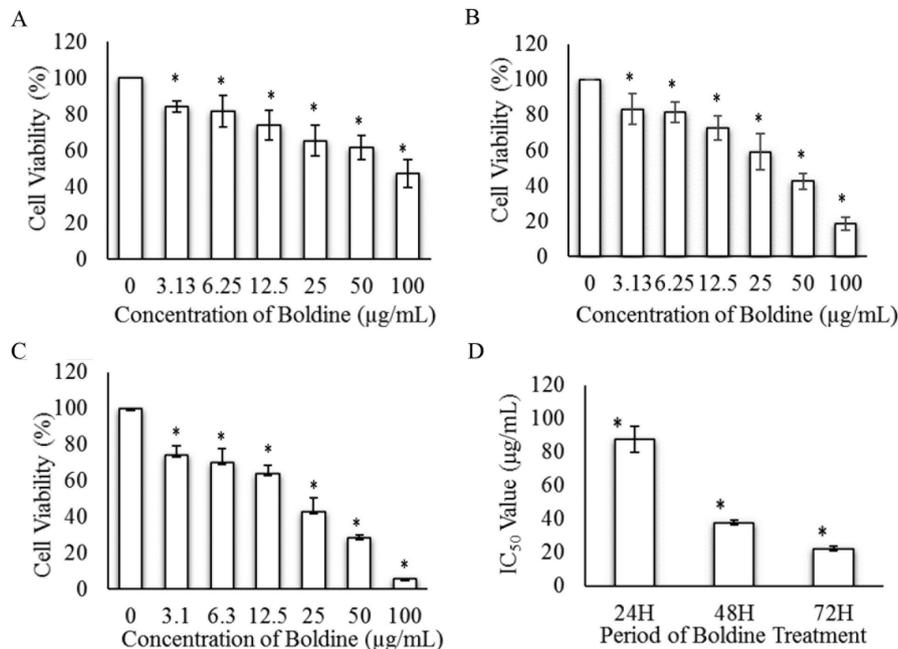


FIGURE 1. Percentage of cell viability and IC_{50} value of boldine against HCT 116 cells. Cells were seeded at 5×10^3 cells per well in 96-well plates for 24 h and the cells were treated with various concentration of boldine for (A) 24, (B) 48, and (C) 72 h. Results were expressed as percentage of cell viability as determined by SRB assay. All data shown are the means \pm standard deviation (SD) of triplicates. Cell viability in boldine-treated cells was expressed as percentage of viable cells compared to control cells. Asterisk (*) indicated significant difference in treatments compared to control with $p < 0.01$ (D) HCT 116 cells (5×10^3 cells/well) were seeded in 96 well for 24 h and then treated with boldine (3.1-100 $\mu\text{g}/\text{mL}$) at 3 different time points (24, 48 and 72 h). IC_{50} value was extrapolated from the graph of the percentage of inhibition against concentration of test sample. The IC_{50} value was presented as mean viability \pm SD.

Asterisk (*) indicated significant difference in each treatment $p < 0.05$

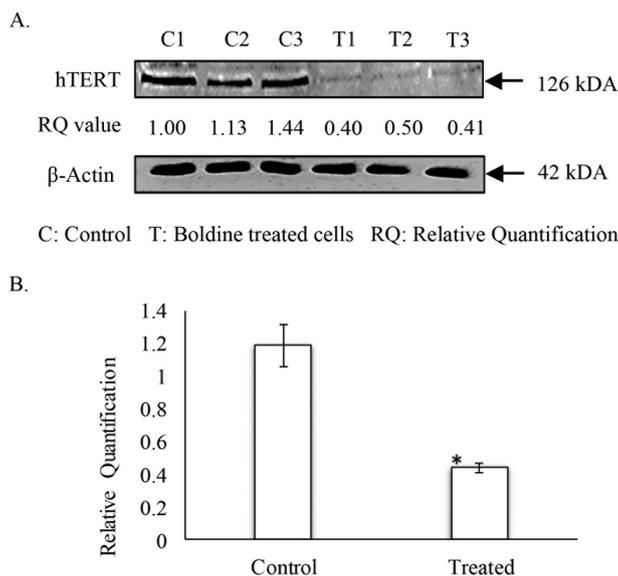


FIGURE 2. Effects of boldine on the levels of hTERT protein in HCT 116 cells. HCT 116 cells were treated with boldine (22.4 $\mu\text{g}/\text{mL}$) for 72 hours, and followed by protein extraction and Western blot analysis, (A) Blot for both control and boldine treated cells was shown and equivalent protein loading was confirmed by probing stripped blots for β -Actin, and (B) Average quantification of relative band intensities of control and boldine treated cells was shown after normalization to β -Actin signals. Images were quantified using ImageJ program. Data were presented as mean \pm SD. Asterisk indicated significant difference compared to the control with $p < 0.05$

TABLE 1. RNA purity and concentration, extracted from both control and boldine treated cells

Sample	A_{260}/A_{280}	A_{260}/A_{230}	Concentration ($\text{ng}/\mu\text{L}$)
C1	2.106	2.117	263.381
C2	2.106	1.824	221.046
C3	2.090	2.214	99.797
T1	2.063	2.266	462.118
T2	2.122	2.251	618.017
T3	2.110	2.095	270.085

HCT 116 cells were treated with 22.4 $\mu\text{g}/\text{mL}$ of boldine for 72 h, followed by RNA extraction as described in the Materials and Methods. C: Control, T: Boldine treated cells

BOLDINE REDUCED hTERT AND hTERC mRNA EXPRESSION IN HCT 116 CELLS

The concentration, purity and integrity of RNA were quantified before qRT-PCR was carried out. In this experiment, it could be seen from the data in Table 1 that the extracted RNA from both treated and untreated cells were considered as pure RNA without protein and guanidine salts contamination. As can be observed in Figure 3, two clear bands were visible on the gel, where the first band represented 28S and second band represented 18S rRNA, which indicated that no degradation occurred and high quality of RNA was obtained in each RNA sample.

The expression of hTERT and hTERC in control and treated samples were estimated in triplicates using qRT-PCR (Figure 4). Expression levels of hTERT and hTERC were normalized against β -Actin. The relative expression for both hTERT and hTERC were calculated using comparative Ct analysis. From the chart, qRT-PCR analysis showed that hTERT and hTERC mRNA

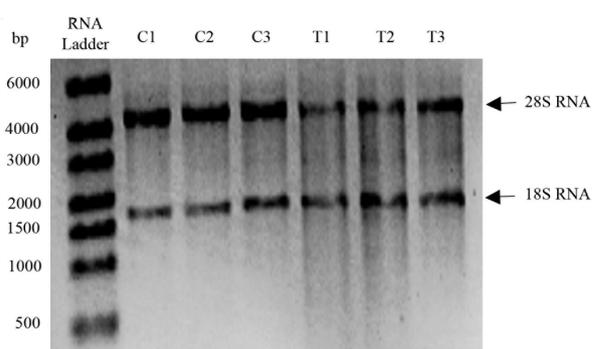


FIGURE 3. The integrity of RNA extracted from both control and boldine treated cells. Equal amount of RNA for control cells (C) and cells treated with 22.4 $\mu\text{g}/\text{mL}$ of boldine for 72 h (T), were analysed using 1% native agarose gel stained with ethidium bromide

expression was down-regulated upon boldine treatment. The relative expressions of hTERT and hTERC on

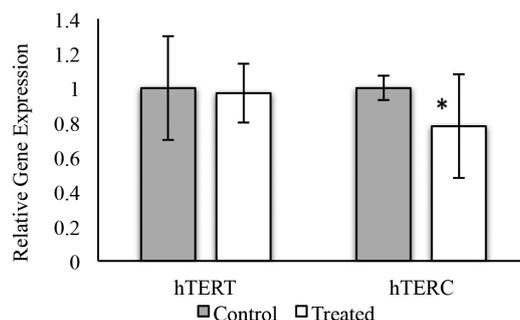


FIGURE 4. Effects of boldine on the levels of hTERT and hTERC mRNA expression in HCT 116 cells treated with boldine (22.4 $\mu\text{g}/\text{mL}$) for 72 h. Graph represented the relative mRNA expression of hTERT and hTERC in both control and boldine treated cells upon normalisation with endogenous control, β -Actin. The data were presented as mean \pm SD. Asterisk indicated significant difference in treatments compared to controls with $p < 0.05$

boldine-treated cells were 0.97 and 0.78, respectively, compared to the control. Interestingly, the mRNA expression of hTERC was shown to be down-regulated significantly. In contrast, the down-regulation of hTERT mRNA was not significant compared to control upon boldine inhibition.

DISCUSSION

Several studies proposed that cancer cells exhibit higher telomerase activity while the telomerase activity in normal cells is barely detectable (Boscolo-Rizzo et al. 2016; Makki 2015; Nakamura et al. 2010). Telomerase in cancer cells play an important role in lengthening the telomere in each round of cell division, allowing the cancer cells to escape senescence and achieve immortalization (Herbert et al. 1999; Xu et al. 2016). Thus, inhibiting telomerase expression in cancer cells is a promising approach in anticancer therapeutics. hTERT and hTERC, the subunit of telomerase are the rate-limiting components of telomerase catalytic activity (Cong et al. 2002). High level of hTERT and hTERC gene expression can be detected in a variety of human cancers. In most of the studies, inhibition of telomerase expression can be shown by the down-regulation of hTERT and hTERC genes expression.

Nowadays, many natural compounds were tested and used to fight cancer. Boldine which is a natural alkaloid compound was shown to have anticancer properties, however, its anti-telomerase property was poorly understood. Recently, one of the boldine derivatives was found to have a strong ability in inhibiting telomerase by binding to the active site of TERT (Noureini et al. 2018). Furthermore, the ability of boldine to inhibit telomerase activity by down-regulating the expression of hTERT was shown in several studies (Noureini et al. 2015a, 2015b). However, until recently, limited work was done in identifying the gene expression of hTERC in boldine treated cells, especially in HCT 116 cell line.

In this study, 37.8 ± 1.5 $\mu\text{g}/\text{mL}$ of boldine was needed to reduce 50% of cell viability of HCT 116 in 48 h. Based

on previous studies, 110 ± 4.7 μM , 150 ± 52 μM , and 160 ± 4.5 μM of boldine were needed to reduce 50% of the cell viability of HEK293, MDA-MB-231 and MCF-7 in 48 h, respectively (Noureini et al. 2015b). Boldine was showed to be cytotoxic to HCT 116 cell line, and the cytotoxic effect was almost similar to HEK293 cell line. Boldine also showed a time- and dose-dependent cytotoxicity on HCT 116 cell line. This finding was in agreement with Noureini and Wink's findings, which showed that boldine have a time- and dose-dependent inhibition of the proliferation of HepG-2 cells (Noureini et al. 2015a).

The ability of boldine to down-regulate hTERT expression in this study was explored using Western blot technique and qRT-PCR. hTERT plays an important role in performing reverse transcription activity in telomerase (Shimojima et al. 2004). The hTERT gene expression was proposed to be regulated mainly at the transcriptional level (Günes et al. 2000). Therefore, besides hTERT protein expression, mRNA expression of hTERT was analyzed to further identify at which level of hTERT gene regulation was targeted by boldine in HCT 116 cell line. In the current study, it was shown that the transcript level of hTERT was slightly down-regulated, and the protein expression of hTERT was significantly reduced upon boldine treatment. This might be because of the involvement of other aspects of telomerase regulation, including alternative splicing (Noureini et al. 2015a). hTERT mRNA can often be spliced into non-functional isoforms, and only a smaller proportion of full length transcripts will be translated into functional hTERT protein (Wong et al. 2014). Since the probes used in this study were the probe that spans an exon junction specific for amplification of cDNA, it was believed that the hTERT isoform was amplified during real time PCR. Thus, the amplified hTERT isoform in this study represented a greater level of hTERT mRNA than would be needed to produce the observed hTERT protein in Western blotting. The reduction in hTERT expression due to boldine treatment was further supported by Noureini and Tanavar's experiment. They suggested that boldine inhibits telomerase activity mainly by reducing the mRNA expression of hTERT in MCF-7 cells (Noureini et al.

2015b). Other studies also claimed that the reduction of telomerase activity resulted from the down-regulation of hTERT mRNA levels in U937 cells and HepG-2 cells (Günes et al. 2000; Noureini et al. 2015b). As these studies proposed that the repression of telomerase activity was shown to be associated with the down-regulation of hTERT expression, the reduction of hTERT expression upon treatment with boldine was able to affect the telomerase expression in HCT 116 cell line.

On the other hand, several studies suggested that the down-regulation of hTERT expression induced cellular apoptosis in various cancer cell types (Celeghin et al. 2016; Chen et al. 2017). Telomerase inhibition through the mutant hTERT in human acute leukemia cells were able to induce apoptosis in the cells (Nakajima et al. 2003). Thus, the down-regulation of hTERT expression in this study might cause telomerase inhibition and apoptosis, hence reducing the proliferation of HCT 116 cells.

Besides hTERT, hTERC is also a potential target for the down-regulation of telomerase activity as it functions as the template for elongation of telomeric repeat units (Cayuela et al. 2005). hTERC was ubiquitously expressed in cells, but the expression was five-fold higher in cancer cells (Avilion et al. 1996). In this present study, qRT-PCR analysis of hTERC mRNA expression was down-regulated upon boldine treatment. Although the level of hTERC was not significantly related with the telomerase activity, hTERC was indeed an important prerequisite for telomerase activity. It has been shown that there was no telomerase activity detected in the absence of hTERC (Feng et al. 1995). Furthermore, other studies showed that the reduction of hTERC expression resulted in reduced growth rate, cell cycle arrest and cell death in different types of cancer cell lines (Baena-del Valle et al. 2018; Natarajan et al. 2004). From these studies, it was suggested that the down-regulation of hTERC expression was able to affect the telomerase expression, reduced cell proliferation and induced cell death in boldine-treated cells. As inhibition of hTERC expression was shown to be a powerful approach to inhibit the telomerase activity, it can be suggested that boldine could be a potential anti-telomerase based cancer therapy that can be further utilized in the future.

More research is required to determine the efficacy of boldine towards cancer cell treatment. It would be interesting to assess the effects of boldine on telomerase activity in HCT 116 cells to correlate with the hTERT and hTERC gene expression assay. Apart from that, the details of regulation of telomerase splicing are still unknown. As the protein expression of hTERT was greatly reduced compared to the slightly down-regulated hTERT mRNA, boldine might affect the alternative splicing events. The identification of alternatively spliced isoform and detection of fusion transcript can also be done by performing next generation sequencing. Thus, further study can be conducted in order to investigate how gene regulation of hTERT has been affected by boldine in order to further validate the ability of boldine in reducing proliferation by targeting telomerase, especially in HCT 116 cells.

CONCLUSION

Boldine was shown to have a time- and dose- dependent cytotoxic effect on HCT 116 cells in this study. In addition, exposure to boldine induced down-regulation of hTERT and hTERC genes expression, as well as hTERT protein expression in HCT 116 cell line. From these findings, boldine might become a promising anti-telomerase compound towards HCT 116 cells. However, more research on this compound needs to be undertaken before the ability of boldine in targeting telomerase is more clearly understood and can be used as a therapeutic agent for the treatment of human colon carcinoma.

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