·论著·

Application of single and combination therapy of clarithromycin and tamoxifen to suppress breast cancer cell proliferation and metabolism

Wings T. Y. Loo, Louis W. C. Chow, Adrian Y. S Yip and Mary N. B. Cheung

【Abstract】 **Objective** This study compares the anti-tumor effects of single and combination use of clarithromycin and tamoxifen on estrogen receptor (ER) positive breast cancer cell lines, BT-483 and MCF-7 as well as triple negative cell line, MBA-MD-231, which acts as a negative control. The effect of solid breast tumor inhibition by clarithromycin is also studied. **Method** BT-483, MCF-7 and MBA-MD-231 were cultured in 6-well plates in a 37 ℃ humidified incubator without CO_2 for 24 h prior to the addition of the test drugs. The test groups were clarithromycin (Group 1), tamoxifen (Group 2), clarithromycin and tamoxifen (Group 3), and control (Group 4). Group 3 was prepared in 1 to 1 ratio at a concentration of 1.5 mmol/L clarithromycin and 25 μmol/L tamoxifen. On the other hand, 1 mm^3 solid breast tumors were submerged into various groups as above for 24 h. On the harvest day, the proliferation of cancer cells and solid breast tumor samples were measured by WST-1 proliferation reagent while ATP bioluminescence assay was employed to measure the metabolic rate of the three cell lines. **Results** The proliferation of BT-483 and MCF-7 was suppressed most by combination use of clarithromycin and tamoxifen with statistical significance. The two drugs did not have an inhibitory effect on the hormonal negative cancer cells. For solid breast tumor samples, all the test groups showed reduced metabolic rate as compared with the control group ($P<0.05$). **Conclusion** Combination use of tamoxifen and clarithromycin are effective in suppressing cell proliferation and metabolism rate of breast cancer cells while single use of clarithromycin effectively inhibits the proliferation of solid breast tumor.

【Key words】 clarithromycin, tamoxifen, breast neoplasms, metabolism, cell proliferation

1 Introduction

Antibiotics that can help repress the growth of cancer cells have been largely realized recently. Clarithromycin, an anti-inflammatory antibiotic[1], was originally used to treat infection particularly by gram negative bacteria such as *Legionella pneumophila*[2]. This 14-membered ring macrolide is semi-synthetic and is chemically related to erythromycin[3-5]. It inhibits bacterial growth by actively binding to the 50S subunit of the 70S rRNA and results in blocking translation for

DOI:10.3877/cma.j.issn.1674-0807.2012.01.007

Authors' address: UNIMED Medical Institute, Hong Kong (Wings T. Y. Loo, Louis W. C. Chow and Mary N. B. Cheung); Organization for Oncology and Translational Research, Hong Kong (Wings T. Y. Loo, Louis W. C. Chow and Adrian Y. S Yip); School of Chinese Medicine, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong (Wings T. Y. Loo); Clinical Trial Center, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong (Louis W. C. Chow)

Corresponding author: Louis W. C. Chow, E-mail: lwcchow@unimed.hk

protein synthesis, which is essential for bacterial growth. Surprisingly, recent researches have proved that clarithromycin has also an anti-angiogensis effect that reduces dense capillary networks on tumor cells[5-6], and induction effects of cell death via apoptosis[7]. Having anti-angiogenic and anti-tumor effects, clarithromycin is used to reduce tumor growth in the treatment of some cancers, especially stomach cancer.

Tamoxifen is now used for the treatment of early and advanced ER+ breast cancer in pre- and post-menopausal women[8]. It is a non-steroidal agent with potent anti-estrogenic properties that binds to estrogen receptors on tumors and other tissue targets competitively, producing a nuclear complex that decreases DNA synthesis and inhibits estrogen effects. Cells remain in the G_0 and G_1 phases of the cell cycle under the influence of tamoxifen which prevents pre-cancerous cells from dividing but does not cause cell death, so it is cytostatic rather than cytocidal[9-10]. Tamoxifen itself is a prodrug with relatively little affinity for its target protein, the estrogen receptor. It is metabolized in the liver by the cytochrome P450 isoform CYP2D6 and CYP3A4 into active metabolites such as 4-hydroxytamoxifen (see Afimoxifene) and N-desmethyl-4-hydroxytamoxifen (endoxifen)[11] which have 30–100 times more affinity with the estrogen receptor than tamoxifen itself. These active metabolites compete with estrogen in the body for binding to the estrogen receptor. 4-hydroxytamoxifen acts as an estrogen receptor antagonist in the breast tissues, so that transcription of estrogen-responsive genes is inhibited[12]. Tamoxifen binds to ER, which in turn interacts with DNA. The ER/tamoxifen complex recruits other proteins including NCoR and SMRT to stop genes being switched on by estrogen[13]. The function of tamoxifen can be regulated by different variables including growth factors[14]. Tamoxifen needs to block growth factor proteins such as ErbB2/HER-2[15] because high levels of ErbB2 have been shown to occur in tamoxifen resistant cancers[16].

As these two chemicals have different perspectives on the repression on tumor cells, it is possible that combining the two chemicals, clarithromycin and tamoxifen, will have additional beneficial effects on the repression of breast tumor cells. There has been a research worked on the combination administration of tamoxifen and Flaxseed, which shows that the combination of these two drugs had an enhancing effect on the repression on breast tumor growth[17]. However, there are no reports on the effect of using clarithromycin on the repression of breast tumor cell.

In many of our previous studies, WST-1 cell proliferation and adenosine triphosphate (ATP) bioluminescence assay have been shown to be reproducible, practicable and promising for predicting and assessing the response of breast tumor cells to cytotoxic drugs that can also be used for pre-therapeutic drug testing[18-20]. They are able to overcome major technical limitations of the older assays like the

human tumor clonogenic assay (HTCA). Testing with drug combination chemotherapy and fresh human breast cancer tumor testing are warranted and ongoing[18-20]. The number of viable cells is quantified by measuring mitochondrial activities (WST-1) or using an enzyme cocktail to link the ATP with luciferase, so that the light emission is proportional to the optical density or ATP concentration. Sonication is performed in order to lyse the cells rapidly and release the ATP[18-21]. ATP rapidly degrades in dead cells upon the action of ATPases and therefore is suitable for the sensitive measurement of biomass by luminescence[18-21].

In this study, therefore, the effects of single and combination administration of chemotherapeutic drugs, clarithromycin and tamoxifen, on the proliferation and metabolism of breast tumor cells were compared using cell proliferation assay and ATP bioluminescence assay. Also, the effect of tumor inhibition by clarithromycin was firstly tested on solid breast tumor cells.

2 Materials and Methods

2.1 Preparation of chemotherapeutic drugs

Clarithromycin (Abbott Laboratory, Illinois, USA) was dissolved in 0.9% sodium chloride. Tamoxifen was provided by Sigma Company (St. Louis, USA) and was dissolved in methanol and diluted by Hank's balanced salt solution (Invitrogen, USA) before use.

2.2 Breast cancer cell line cultivation

Estrogen-positive breast cancer cell lines, MCF-7 and BT-483, and a triple negative breast cancer cell line, MBA-MD-231 (ATCC, USA) were used for cultivation[22]. They were cultured in 6-well plates and incubated in RPMI-1640 medium for MCF-7 and BT-483, L-15 medium for MBA-MD-231 with 10% fetal bovine serum (FBS), 15 mmol/L HEPES buffer, L-glutamine, pyridoxine hydrochloride, penicillin (100 U/ml), streptomycin (100 μg/ml) and insulin (4 μg/ml) (Invitrogen Corporation, California, USA) at 37 °C without CO_2 for 24 h. After trials, the optimal and minimal concentration which provided a statistical significance between the control group and the test groups was 1.5 mmol/L clarithromycin and 25 μmol/L tamoxifen. The three test groups and the control group were as follows: clarithromycin (Group 1), tamoxifen (Group 2), clarithromycin and tamoxifen (Group 3) and control (Group 4). Group 3 was prepared in 1 to 1 ratio at a concentration of 1.5 mmol/L clarithromycin and 25 μmol/L tamoxifen.

2.3 Preparation of solid breast tumors

Sixty-four patients with histologically confirmed ductal breast cancers, aged from 29 to 84 years with mean age of 59.1 years, were recruited for this study from UNIMED Medical Institute, Hong Kong (Table 1). Written consent form was obtained

from all patients. The study protocols were approved by the Ethics Committee, Faculty of Medicine, University of Hong Kong. The breast cancer tissues were collected in sterilized bottle containing 0.9% normal saline.

Table 1 Clinical characteristics of 64 patients whose tissue had been used in this study

Clinical characteristics	*n*
Menopausal status	
Premenapausal	28
Postmenapausal	36
Histological nodal status	
Negative	40
Positive	24
Tumor size	
<2 cm (T_1)	26
2—5 cm (T_2)	30
>5 cm (T_3)	8
Histology	
Ductal	58
Lobular	6
Grading	
G_1	2
G_2	51
G_3	11
ER status	
Negative	1
Positive	63
PR status	
Negative	3
Positive	61
HER-2/neu status	
Negative	48
Positive	16

2.4 ATP bioluminescence assay applied on cell lines and human solid tumor

ATP bioluminescence assay (Roche, Germany) was used to measure the metabolic rate of both the cell lines and the human solid breast tumors. The cell lines were cultured in 100 mm culture dishes (Nunclon, Danmark). On the harvest day, the cells were centrifuged and transferred to eppendorf tubes, and 0.3 ml cell lysis reagent was added. For human solid breast tumor, the tissues were

minced into 1 mm^3 and were cultured in 24-well culture plates with medium consisting of D-MEM (Dulbecco's modified eagle medium) with 15% fetal bovine serum (FBS), 15 mmol/L HEPES buffer, L-glutamine, pyridoxine hydrochloride, penicillin (100 U/ml), streptomycin (100 μg/ml) and insulin (4 μg/ml) (Invitrogen Corporation, California, USA) for 24 h. This part consisted of two groups, the control and the clarithromycin group. Tissues were submerged in the culture medium for the control group while 15 mmol/L clarithromycin was added for the test groups. All breast tumor samples were cultured for another 24 h. The tissues were then transferred to eppendorf tubes and 0.3 ml cell lysis reagent was added.

In preparation for the luciferase reagent, 10 ml dilution buffer was added to the luciferase reagent supplied by the kit. Cell lysis was accelerated by a sonicator (Sonics & Materials Inc., Danbury, CT, USA) at a pulse of 30 per minute with 5% amplitude to enhance the release of ATP from cells. A 10 s binding period was proceeded by amalgamation of 50 μl of both samples and luciferase reagent. The absorbance values of the samples were read by TD-20/20 luminometer (Turner Designs, CA. USA) at 420 nm wavelength. This kit provides a standard ATP for conversion of samples' optical densities to bioluminescence (Relative Light Units, RLU).

2.5 Cell proliferation rate measured by WST-1 for clarithromycin and tamoxifen

The proliferation rates of BT-483, MCF-7 and MBA-MD-231 in various groups were assessed by cell proliferation reagent WST-1 (Roche, Mannheim, Germany). Cells were inoculated at a density of 4000 cells per well into 96-well plates (Nuclon, Danmark) and were then incubated at 37 ℃ for 24 h. After the treatment of clarithromycin, tamoxifen or both, plain medium and WST-1 were added and incubated according to the instructions from the manufacturers. The optical density was read at 450 nm by the Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

2.6 Statistical analysis

Statistical analysis was performed using SPSS 16.0 for Windows (SPSS Inc., USA). The measurement of the proliferation and metabolism of breast tumor cells and solid tumor were repeated three times to obtain an average value before they were evaluated by One-Way ANOVA and independent *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

3 RESULTS

3.1 ATP bioluminescence assay and proliferation rate of BT-483, MCF-7 and MBA-MD-231

From the results of the ATP bioluminescence assay (Table 2), the combination

use of clarithromycin and tamoxifen significantly lowered the metabolic rate of BT-483 and MCF-7 after 24 h of incubation. Sole administration of clarithromycin or tamoxifen partially reduced the metabolic rate of BT-483 and MCF-7 with statistically significant level ($P<0.05$) when compared with the control group (Table 2), but when the two drugs used alone showed significant difference in BT-483 cell when compared with the combination drug group (Table 2). WST-1 reagent assay also demonstrated a significantly lower proliferation rate of BT-483 and MCF-7 when compared with the control group, but among clarithromycin, tamoxifen and combination groups, no significant difference was observed (Table 3). However, single use of clarithromycin or tamoxifen was not as effective as combination use (Table 3). Both assays indicated that tamoxifen inhibited tumor growth better than clarithromycin (Tables 2 and 3). MBA-MD-231 did not show any significant inhibition effect for all test groups (Tables 2 and 3).

Table 2 The effect of single and combination use of clarithromycin and tamoxifen on metabolic rate ATP (RLU) (Mean±SD) of three breast cancer cell lines at 24 h

Groups	Mean of ATP		
	BT-483	MCF-7	MB-MD-231
control	234.43±27.06	178.05±11.4	363.52±72.78
clarithromycin	188.04±7.25[a]	63.86±9.12[a]	351.85±11.48
tamoxifen	166.53±2.78[a]	68.30±9.12[a]	377.31±23.30
clarithromycin + tamoxifen	67.32±10.52[a]	34.72±6.71[a]	309.95±28.24

a: $P<0.05$, compared with the control group

Table 3 The effects of single and combination use of clarithromycin and tamoxifen on the proliferation rate (WST-1) (Mean±SD) of three breast cancer cell line at 24 h

Groups	Mean optical dersity of proliferation rate		
	BT-483	MCF-7	MB-MD-231
control	1.53±0.10	0.88±0.25	0.73±0.13
clarithromycin	0.96±0.11[a]	0.41±0.52[a]	0.50±0.02
tamoxifen	0.92±0.07[a]	0.42±0.51[a]	0.49±0.40
clarithromycin + tamoxifen	0.55±0.05[a]	0.53±0.45[a]	0.53±0.45

a: $P<0.05$, compared with the control group

3.2 Solid breast tumor inhibitions by clarithromycin

The solid breast tumor samples showed a lower metabolic rate in the three test groups than in the control (Table 4). There was a significant difference ($P<0.05$) among the groups. This indicated that clarithromycin significantly inhibited the activity of breast tumor cells as a single use and the best effect was observed when clarithromycin and tamoxifen were used together.

Table 4 The ATP level of solid breast tumor 24 h after being submerged in culture medium with or without addition of clarithromycin or tamoxifen

Groups	ATP level (Mean±SD)
control	36720±444.86
clarithromycin	20156±289.95[a]
tamoxifen	22613±365.24[a]
clarithromycin + tamoxifen	16473±279.51[a]

a: $P<0.05$, compared with the control group

4 Discussion

The results from our study indicated that combination administration of clarithromycin and tamoxifen had a better repression on breast tumor cells by reducing the metabolic rate and cell proliferation than single administration of clarithromycin or tamoxifen in *in vitro* system. As clarithromycin delays cell growth by arresting cells in G_1 phase[23] and induces cell death through apoptosis[7] while tamoxifen inhibits cell proliferation[24], combining both drugs can therefore significantly reduce hormone responsive breast tumor growth by altering cell cycle and inducing cell death via apoptosis. It might be used in treating ER+ breast cancer patients *in vivo* to further repress tumor cells.

ATP bioluminescence assay and WST-1 are the two commonest, simplest and most accurate ways in measuring breast tumor cell growth clinically. As the results of these two assays indicate the two most essential factors for tumor growth, which are metabolic rate, in terms of ATP released, and cell proliferation, in terms of optical density, they are widely used in estimating tumor growth. Using this two assay methods, this experiment gave a perspective on the combination administration of drugs on cancer treatments.

Clarithromycin, which is mainly used to treat stomach cancer, showed some reduction on the repression of BT-483 and MCF-7. Tamoxifen also showed a repression on BT-483 and MCF-7 growth as proved previously in other researches[24-25]. Moreover, tamoxifen had a better repression than clarithromycin on hormone responsive breast tumor growth. However, these single administrations showed unstable, fluctuating results on both ATP bioluminescence assay and WST-1 reagent assay, and the trial results are not shown. In contrast, the combination administration of clarithromycin and tamoxifen showed a much greater repression on BT-483 and MCF-7 than single administration of clarithromycin or tamoxifen, which are being used nowadays. Besides, the effect of repression on tumor cells was more stable during trials in the experiment. This experiment provides a clue to the ongoing researches that the combination administration of drugs that have different mechanisms in repressing cancer might help enhance the repression on hormone responsive tumor cell growth *in vivo*. More works on the effects of anti-tumor drugs combination has on mammals and the mechanisms behind should be

further investigated.

Clarithromycin's inhibitory effect was obvious in endocrine dependent breast cancers, as first indicated by our experiment. It could inhibit estrogen synthesis within metastatic breast cancer tissue[26]. It could be used as a chemotherapeutic drug to suppress the growth of breast tumor by its ability to inhibit estrogen synthesis.

Application of clarithromycin and tamoxifen alone or combination use to treat triple negative breast cancer cell line MBA-MD-231 did not demonstrate an obvious suppression effect, indicating that these drugs may not be applied to this type of cancer cells clinically in the future.

Combination administration of clarithromycin and tamoxifen effectively reduced cells metabolic and cell proliferation rate of breast tumor cell line BT-483 and MCF-7, when compared with the control group. Single administration of clarithromycin or tamoxifen, in contrast, reduced tumor cells, metabolic rate, and tumor cells proliferation slightly when compared with the control group. Furthermore, clarithromycin had shown a significant inhibiting effect on solid breast tumor. In conclusion, combining both clarithromycin and tamoxifen had a better repression on tumor cell growth than single administration of clarithromycin or tamoxifen *in vitro*, and clarithromycin could be used in inhibiting the activity of solid breast tumor.

References

[1] Tkalcević VI, Bosnjak B, Pasalić I, et al. The anti-inflammatory activity of clarithromycin inhibits TNFalpha production and prolongs survival following lipopolysaccharide administration in mice[J]. Int J Antimicrob Agents, 2008, 32(2): 195-196.

[2] Grau S, Antonio JM, Ribes E, et al. Impact of rifampicin addition to clarithromycin in legionella pneumophila pneumonia [J]. Int J Antimicrob Agents, 2006, 28(3): 249-252.

[3] Tsuda T, Ishikawa C, Konishi H, et al. Effect of 14-membered-ring macrolides on production of interleukin-8 mediated by protease-activated receptor 2 in human keratinocytes. Antimicrob Agents Chemother, 2008, 52(4):1538-1541.

[4] Yatsunami J, Hayashi S. Fourteen-membered ring macrolides as anti-angiogenic compounds[J]. Anticancer Res, 2001, 21(6B): 4253-4258.

[5] Yatsunami J, Fukuno Y, Nagata M, et al. Antiangiogenic and antitumor effects of 14-membered ring macrolides on mouse B16 melanoma cells[J]. Clin Exp Metastasis, 1999, 17(4): 361-367.

[6] Yatsunami J, Turuta N, Wakamatsu K, et al. Clarithromycin is a potent inhibitor of tumor-induced angiogenesis[J]. Res Exp Med (Berl), 1997, 197(4): 189-197.

[7] Qiao AM, Ikejima T, Tashiro S, et al. Involvement of mitochondria and caspase pathways in N-demethyl-clarithromycin-induced apoptosis in human cervical cancer HeLa cell[J]. Acta Pharmacol Sin, 2006, 27(12): 1622-1629.

[8] The BIG 1-98. Collaborative Group. Letrozole therapy alone or in sequence with tamoxifen in women with breast cancer [J]. N Engl J Med, 2009, 361(20):766-776.

[9] Jordan VC. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer[J]. Br J Pharmacol, 2006, 147 (Suppl 1): S269-S276.

[10] Jordan VC. Fourteenth Gaddum Memorial Lecture. A current view of tamoxifen for the treatment and prevention of breast cancer[J]. Br J Pharmacol, 1993, 110 (2): 507-517.

[11] Desta Z, WardBA, Soukhova NV, et al. Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: prominent roles for CYP3A and CYP2D6[J]. J Pharmacol Exp Ther, 2004, 310 (3): 1062-1075.

[12] Wang DY, Fulthorpe R, Liss SN, et al. Identification of estrogen-responsive genes by complementary deoxyribonucleic acid microarray and characterization of a novel early estrogen-induced gene: EEIG1[J]. Mol Endocrinol, 2004, 18(2): 402-411.
[13] Shang Y, Hu X, DiRenzo J, et al. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription[J]. Cell, 2000, 103(6): 843-852.
[14] Massarweh S, Osborne CK, Creighton CJ, et al. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function[J]. Cancer Res, 2008, 68(3): 826-833.
[15] Hurtado A, Holmes KA, Geistlinger TR, et al. Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen[J]. Nature, 2008, 456(7222): 663-666.
[16] Osborne CK, Bardou V, Hopp TA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer[J]. J Natl Cancer Inst, 2003, 95(5): 353-361.
[17] Chen J, Thompson LU. Lignans and tamoxifen, alone or in combination, reduce human breast cancer cell adhesion, invasion and migration in vitro[J]. Breast Cancer Res Treat, 2003, 80(2):163-170.
[18] Loo WT, Sasano H, Chow LW. Evaluation of therapeutic efficacy of capecitabine on human breast carcinoma tissues and cell lines in vitro[J]. Biomed Pharmacother, 2007, 61(9):553-537.
[19] Liu Q, Loo WT, Sze SC, et al. Curcumin inhibits cell proliferation of MDA-MB-231 and BT-483 breast cancer cells mediated by down-regulation of NFkappaB, cyclinD and MMP-1 transcription[J]. Phytomedicine, 2009, 16(10):916-922.
[20] Loo WT, Chow LW, Suzuki T, et al. Expression of thymidine phosphorylase and dihydropyrimidine dehydrogenase in human breast carcinoma cells and tissues[J]. Anticancer Res, 2009, 29(7):2525-2530.
[21] Lau GI, Loo WT, Chow LW. Neoadjuvant chemotherapy for breast cancer determined by chemosensitivity assay achieves better tumor response[J]. Biomed Pharmacother, 2007, 61(9):562-565.
[22] Lasfargues EY, Coutinho WG, Redfield ES. Isolation of two human tumor epithelial cell lines from solid breast carcinomas [J]. J Natl Cancer Inst, 1978, 61(4): 967-978.
[23] Shinkai M, Tamaoki J, Kobayashi H, et al. Clarithromycin delays progression of bronchial epithelial cells from G1 phase to S phase and delays cell growth via extracellular signal-regulated protein kinase suppression[J]. Antimicrob Agents Chemother, 2006, 50(5): 1738-1744.
[24] Goetz MP, Kamal A, Ames MM. Tamoxifen pharmacogenomics: the role of CYP2D6 as a predictor of drug response[J]. Clin Pharmacol Ther, 2008, 83(1): 160-166.
[25] Chen J, Power KA, Mann J, et al. Flaxseed alone or in combination with tamoxifen inhibits MCF-7 breast tumor growth in ovariectomized athymic mice with high circulating levels of estrogen[J]. Exp Biol Med (Maywood), 2007, 232(8): 1071-1080.
[26] Brodie A, Lu Q, Nakamura J. Aromatase in the normal breast and breast cancer[J]. J Steroid Biochem Mol Biol, 1997, 61(3-6): 281-286.

(Received:2011-09-30)
(Edited by:Liu Jun-lan)

Loo WT, Chow LW, Yip AY, et al. Application of single or/and combination therapy of clarithromycin and tamoxifen to suppress breast cancer cell proliferation and metabolism[J/CD]. 中华乳腺病杂志:电子版,2012,6(1):45-53.