行政院國家科學委員會專題研究計畫　期中進度報告

口服抗黴菌藥物抑制腫瘤生長之分子機制研究(2/3)

計畫類別： 個別型計畫
計畫編號： NSC91-2314-B-038-005-
執行期間： 91 年 08 月 01 日至 92 年 07 月 31 日
執行單位： 臺北醫學大學醫事技術學系

計畫主持人： 何元順

計畫參與人員： 王應然, 何衛律,周&#26241；旻,陳元孝

報告類型： 精簡報告

處理方式： 本計畫可公開查詢

中　華　民　國 92 年 6 月 16 日

行政院國家科學委員會補助專題研究計畫成果報告

※

※　市售口服抗黴菌藥物抑制人類癌細胞生長之分子機制研究

※

計畫類別：　個別型計畫　　　整合型計畫

計畫編號：NSC　89－2314－B－038－036－

執行期間：　89 年 08 月 01　日至 90　年 07　月 31　日

計畫主持人：何元順

本成果報告包括以下應繳交之附件：

　　赴國外出差或研習心得報告一份

　　赴大陸地區出差或研習心得報告一份

　　出席國際學術會議心得報告及發表之論文各一份

　　國際合作研究計畫國外研究報告書一份

執行單位：台北醫學大學 醫學院 生物醫學技術研究所

中　華　民　國　　89　年　10　月　24　日

行政院國家科學委員會專題研究計畫成果報告

計畫編號：NSC91-2314-B-038-005
執行期限：91 年 08 月 01 日至 92 年 07 月 31 日
主持人：何元順
執行機構及單位名稱：台北醫學大學 醫學院 生物醫學技術研究所

一、中文摘要

TB®是目前已知有潛力的抗黴菌藥物，我們首度證實 TB 具有使抑制癌細胞週期停滯的作用，作用位置為誘發 G0/G1 phase arres 誘發 S phase arrest.TB 在低劑量(10 μg/mL)時有大部分細胞停滯於 S phase。我們已經證實 TB 造成人類癌細胞週期 G0/G1 週期停滯。已經證實細胞內基因變化為 p53, p21/Cip1 活化。我們亦證實 TB 有抑制裸鼠腫瘤生長的能力，由於參與細胞週期的基因調控目前已經相當清楚，因此本計劃以有系統的分析方式，逐步探討藥物處理後細胞週期之基因變化情形。

關鍵詞：抗黴菌藥、細胞週期、細胞凋亡

Abstract

In this study, we demonstrated that Lamisil (Terbinafine, TB) dose-dependently arrested various human cancer cells at the G0/G1 phase of the cell cycle. The protein levels of p53, p21/Cip1, and p27/Kip1 were significantly elevated by TB treatment in COLO-205 cells. Electrophoretic mobility gel shift assays (EMSA) showed that the nuclear extracts of the TB-treated COLO-205 cells exerted a significant binding between wild type p53 and its consensus-binding site present in the p21/Cip1 promoter. These results suggested that the p53-associated signaling pathway is involved in the regulation of TB-induced cancer cell growth arrest. By immunoblot analysis, we demonstrated that cyclin D3 and cyclin-dependent kinase-4 (CDK4) protein levels were inhibited by TB-treatment in the cancer cells. Significant therapeutic effect was further demonstrated in vivo by treating nude TBe bearing COLO-205 tumor xenografts with TB (50 mg/kg, i.p.). The protein expression of p53 was significantly increased in TB-treated tumor tissues by immunohistocheTBal staining technique. DNA fragmentation and TUNEL assay were performed and demonstrated that apoptosis occurred in tumor tissues treated with TB. Our study provides the novel mechanisms of antitumor effects of TB and such results may have significant applications for cancer chemotherapy.

Keywords: Oral antifungal agents, apoptosis, cell cycle

二、緣由與目的

The discovery of antifungal activity of azole compounds represented an important therapeutic advance. TBonazole (TB), ketoconazole (KT), itraconazole, and fluconazole are currently commercially available (Bodey, 1992). Among its disadvantage are limited absorption in the absence of gastric acid and its potential for drug-drug interactions; many clinicians believe that topical TB is a relatively effective agent for the treatment of most mycotic infections (Diehl, 1996). Because of its limited activity and toxicity, the TB has now been replaced by newer agents (such as terbinafine) (Leenutaphong *et al.*, 1999; McClellan *et al.*, 1999). TB, KT, bifonazole, clotrimazole, econazole, isoconazole and tioconazole are known inhibitors of cytochrome p-450 dependent steroidogenic enzymes (Ayub and Levell, 1989). Another study indicated that KT and TB inhibits cholesteryl ester formation in macrophages by blocking the intracellular transport of endocytosed cholesterol from lysosomes to the endoplasTB reticulum (Aikawa *et al.*, 1999). These antifungal imidazoles TB and KT are known to inhibit synthesis of essential cell membrane components. Furthermore, TB can exert direct physiocheTBal cell membrane damage at relatively high levels, but KT cannot (Beggs, 1984).

The antitumor effects of anti-fungal agents (such as KT) were investigated in several other laboratories (Blagosklonny *et al.*, 2000; Bok and Small, 1999; Heyns *et al.*, 1985; Mahler and Denis, 1992; Trachtenberg, 1984a; Trachtenberg, 1984b; Trachtenberg and Pont, 1984). In this study, we further demonstrated that TB induced growth inhibition in various human cancer cells through G0/G1 cell cycle arrest. The therapeutic efficacy was further examined in vivo by treating athyTB TBe bearing COLO-205 tumor xenografts with TB (50 mg/Kg, i.p.). This study provides further evidences that the antifungal agent, TB, might also have significant applications for cancer chemotherapy.

三、結果與討論

TB Induces G0/G1 Cell Cycle Arrest in Various Human Cancer Cells with Different p53 Status

As shown in figure 1, TB (10-50 μM) induced a dose-dependent inhibition of cell growth in various human cancer cells. As compared to human cancer cells, the TB-induced growth arrest of human normal keratinocytes (#76 KhGH) was less profound (Figure 1). Figure 2A showed a representative fluorescence-activated cell sorter (FACS) analysis of DNA content at various times after release from quiescence by incubation in culture media supplemented with 10% FCS and 0.1% DMSO.

Figure 2B showed that TB (20 μM) induced a significant accumulation (>85 %) of cells in G0/G1 phase of the cell cycle, suggesting that the observed growth inhibitory effect of TB in the figure 1 was due to an arrest of DNA replication in the cell cycle.

Dose-dependent Response of Cells to TB-induced G0/G1 arrest

As shown in the figure 3, significant apoptosis was induced in cells treated with higher dose of TB (> 40 ì M). However, G0/G1 arrest was observed in cells exposed to lower concentration of TB (< 30 ì M). Our recent report indicated that p53 was involved in KT-induced G0/G1 arrest and apoptosis in COLO 205 cells (Chen *et al.*, 2000; Ho *et al.*, 1998). The present study further demonstrated that G0/G1 cell cycle arrest and apoptosis were easily induced in the cells with wild type p53 (COLO 205 and Hep G2) by TB treatment. Such results suggesting that p53 might be involved in TB-induced G0/G1 arrest and apoptosis.

TB-Induced Cancer Cells Apoptosis through Caspase-3 Activation

Figure 3 shows that the sub-G1 peak was observed in cells treated with higher doses of TB (> 40 ì M). Such results revealed that apoptotic cells were presented in TB treated group. We further demonstrated that COLO 205 and HT 29 cells treated with TB (20-50ì M) exhibited morphological changes were accompanied by progressive internucleosomal degradation of DNA to yield a ladder of DNA fragments (Figure 4A). The apparent DNA ladder appeared at 24 hr after 30 ì M of TB treatment in the COLO 205 cells (Figure 4A). Figure 4B shows that the caspase-3 was activated in COLO 205 cells at 24 hr after TB (30ì M) exposure. Previous report demonstrated that the substrate of caspase-3 is the poly-ADP ribose polymerase (PARP) (Tewari *et al.*, 1995). Western blotting analysis revealed that the Mr. 116,000 PARP molecule was degraded to a relatively stable Mr. ~85,000 fragment at 24 hr after TB (10-35ì M) treatment (Figure 3C). Our study demonstrated that TB-induced cancer cells apoptosis was at least through caspase-3 pahway.

The p53 and p21/CIP1 Were the Key Regulators in TB-induced G0/G1 Arrest

Based on the FACS analysis presented in the figure 2A showed that 0, 15, 18 and 24 h represents the G0/G1, S, G2/M and 2nd G0/G1 phase. Accordingly, this time point (15 h) was selected for studying the dose-dependent effect of TB and the changes of p53 proteins for induction of G0/G1 arrest was determined by western blotting analysis (Figure 5). Our data demonstrated that the activated p53 was more significantly induced in the COLO 205 cells (with wild type p53) (Figure 5 A). As shown in the Figure 5 B, the TB-treated cells showed that the up-regulation of p21/Cip1 protein expression was observed initially at 6 h after TB treatment and persisted for at least 24 h (Figure 5B). In contrast, in the DMSO-treated control group, the expression of p21/Cip1 in the cell was up-regulated at 6 h after cells were challenged with 10% FCS and then rapidly down regulated at 15 h after treatment.

To further demonstrate the p53 protein in cells was activated by TB treatment, Electrophoretic mobility gel shift assay (EMSA) was conducted in both of the COLO 205 and HT 29 cells. The EMSA results showed that the nuclear extracts of the TB-treated COLO 205 cells exerted a significant binding between wild type p53 protein and its consensus-binding site in the p21/Cip1 promoter region (Figure 5C).

TB Induces Elevation of p21/Cip1, p27/Kip1 and Inhibition of Cyclin D3 and CDK4 Protein Expression

As shown in figure 5 and 6, the protein levels of both p53 and p21/Cip1 were induced and the CDK4 protein expression was inhibited in the TB-treated COLO 205 cells (with wild type p53). Interestingly, the other cell cycle negative regulator, p27/Kip1, protein expression were more significantly induced in the p53-null (HL 60), p53-deleted (Hep 3B) and the p53 His^{273} mutant (HT 29) cells. Such results implied that p27/Kip1 may be involved in the TB-induced G0/G1 cell cycle arrest through a p53-independent pathway in these cells.

As shown in the figure 6, the protein levels of cyclin D3, and CDK4 in the TB-treated cells were down-regulated after treatment with TB while the cyclin D1 and PCNA were not changed significantly. In this study, the faster migration form of cyclin A2 (58 kDa) and cyclin B, which promote cells entry from G0/G1 into S and from S into G2/M phase respectively, were also down regulated dose-dependently in TB-treated cancer cells (Figure 6). The protein level of CDK2 was not significantly changed in TB-treated cells. We further determined the CDK2-associated protein, cyclin E, protein expression and demonstrated that the cyclin E protein was slightly inhibited in TB-treated cells (Figure 6).

TB-induced G0/G1 Arrest Was Through Inhibit of CDK4 Kinase Activity

Our results revealed that the decreased CDK4 kinase activity was concomitant with increased expression of p21/Cip1 and p27/Kip1 in cells treated with TB (Figure 7). These results implied that the G0/G1 arrest induced by TB was due to decrease the kinase activity of CDK2 and CDK4 mediated by an increase of p21/Cip1 (or p27/Kip1)-CDKs association.

TB Causes Tumor Regression in vivo

We further examined the therapeutic efficacy of TB in vivo by treating athyTB TBe bearing COLO-205 tumor xenografts, using concentrations of TB (50 mg/Kg). After establishment of palpable tumors (mean tumor volume, 200 mm^3), animal received intraperitoneal injections of TB three times per week, as well as DMSO for a negative control. After 6 weeks, tumor volume in TB was significant inhibited in comparison with DMSO-treated controls (Figure 8 A and B). In TBe receiving these treatment regimens, no gross signs of toxicity were observed (body weight, visible inspection of general appearance and TBroscopic examination of individual organs)

(Figure 8 C). However, the tumor weight and the tumor/body weight ratio were strongly inhibited in the TB-treated group (Figure 8 D and E). Our results provide further evidences that such observations may have significance of application for cancer chemotherapeutic purposes.

四、參考文獻

Aikawa, K., Sato, Y., Furuchi, T., Ikemoto, M., Fujimoto, Y., Arai, H. and Inoue, K. (1999) Inhibition of cholesteryl ester formation in macrophages by azole antimycotics. *BiocheTBal Pharmacology*, 58, 447-53.

Ayub, M. and Levell, M.J. (1989) Inhibition of human adrenal steroidogenic enzymes in vitro by imidazole drugs including ketoconazole. *Journal of Steroid Biochemistry*, 32, 515-24.

Beggs, W.H. (1984) Growth phase in relation to ketoconazole and TBonazole susceptibilities of Candida albicans. *AntiTBrobial Agents & Chemotherapy*, 25, 316-8.

Blagosklonny, M.V., Dixon, S.C. and Figg, W.D. (2000) Efficacy of TBrotubule-active drugs followed by ketoconazole in human metastatic prostate cancer cell lines. *Journal of Urology*, 163, 1022-6.

Bodey, G.P. (1992) Azole antifungal agents. *Clinical Infectious Diseases*, 14, S161-9.

Bok, R.A. and Small, E.J. (1999) The treatment of advanced prostate cancer with ketoconazole: safety issues. *Drug Safety*, 20, 451-8.

Chen, R.J., Lee, W.S., Liang, Y.C., Lin, J.K., Wang, Y.J., Lin, C.H., Hsieh, J.Y., Chaing, C.C. and Ho, Y.S. (2000) Ketoconazole induces G0/G1 arrest in human colorectal and hepatocellular carcinoma cell lines. *Toxicology & Applied Pharmacology*, 169, 132-41.

Diehl, K.B. (1996) Topical antifungal agents: an update. *American Family Physician*, 54, 1687-92.

Heyns, W., Drochmans, A., van der Schueren, E. and Verhoeven, G. (1985) Endocrine effects of high-dose ketoconazole therapy in advanced prostatic cancer. *Acta Endocrinologica*, 110, 276-83.

Ho, Y.S., Tsai, P.W., Yu, C.F., Liu, H.L., Chen, R.J. and Lin, J.K. (1998) Ketoconazole-induced apoptosis through P53-dependent pathway in human colorectal and hepatocellular carcinoma cell lines. *Toxicology & Applied Pharmacology*, 153, 39-47.

Leenutaphong, V., Niumpradit, N., Tangwiwat, S., Sritaveesuwan, R. and Muanprasat, C. (1999) Double-blind study of the efficacy of 1 week topical terbinafine cream compared to 4 weeks TBonazole cream in patients with tinea pedis. *Journal of the Medical Association of Thailand*, 82, 1006-10.

Mahler, C. and Denis, L. (1992) Management of relapsing disease in prostate cancer. *Cancer*, 70, 329-34.

McClellan, K.J., Wiseman, L.R. and Markham, A. (1999) Terbinafine. An update of its use in superficial mycoses. *Drugs*, 58, 179-202.

Tewari, M., Quan, L.T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D.R., Poirier, G.G., Salvesen, G.S. and Dixit, V.M. (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell*, 81, 801-9.

Trachtenberg, J. (1984a) The effects of ketoconazole on testosterone production and normal and malignant androgen dependent tissues of the adult rat. *Journal of Urology*, 132, 599-601.

Trachtenberg, J. (1984b) Ketoconazole therapy in advanced prostatic cancer. *Journal of Urology*, 132, 61-3.

Trachtenberg, J. and Pont, A. (1984) Ketoconazole therapy for advanced prostate cancer. *Lancet*, 2, 433-5.