Anti-Cancer Drugs 1999, **10**, pp. 895–901

Preclinical report

Synergistic effect of paclitaxel and 4-hydroxytamoxifen on estrogen receptor-negative colon cancer and lung cancer cell lines

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Antiestrogen tamoxifen (Tam) is the most prescribed drug for the treatment of estrogen receptor (ER)-positive breast cancers. It is also used in long-term clinical trials with encouraging preliminary results as a chemopreventive agent for breast cancer. The effect of Tam on ER-negative cancers, however, is unclear. Here we reported that paclitaxel and 4-hydroxytamoxifen (4-HT) have a synergistic cytotoxic effect on the ER-negative colon cancer cell line HCT15, which is refractory to paclitaxel alone. Our results showed that 4-HT at submicromolar concentrations effectively enhanced the antiproliferative effect of paclitaxel. In addition, at 1/10 of the paclitaxel concentrations used for HCT15, 4-HT and paclitaxel also showed synergistic effect on NCI H460, an ER-negative lung cancer cell line. For both cell lines, the effective concentration for paclitaxel to inhibit cell growth was 1 log lower in the combination treatment than the concentration used in the single treatment. Cell cycle analysis showed that the combination of paclitaxel and 4-HT increased the G_2/M population and resulted in the increase of apoptosis in both cell lines. Enhanced early release of cytochrome *c* from mitochondria may be the apoptotic pathway activated in the combination treatment in HCT15 cells. [© **1999 Lippincott Williams & Wilkins.**]

Key words: Apoptosis, cancer, combination treatment, cell cycle, paclitaxel, tamoxifen.

Introduction

Paclitaxel, a diterpene derived from the bark of the western yew *Taxus brevifolia*, is a potent growth inhibitor of a variety of cancer cells. Unlike vinca alkaloids that destabilized microtubules, paclitaxel and its analog docetaxel stabilize microtubules and arrest cells at mitosis, which eventually leads to programmed cell death.[1–4] Over the last two decades, paclitaxel has demonstrated its impressive efficacy as a single agent for the treatment of a number of solid tumors including cancers of the breast. Among patients with breast cancer, paclitaxel achieved a response rate of more than 50% when it was used as first-line therapy for primary cancer and 40% as second-line therapy for metastatic cancer.[5] In combination regimens, paclitaxel also showed advantages in the combination treatment for several types of cancers including ovarian. The median survival for advanced ovarian cancer patients treated with the combination of cisplatin and paclitaxel was 13 months longer than those treated with the combination of cisplatin and cyclophosphamide.[6] Despite these successes, clinical benefits of taxanes are limited largely due to the apparent drawbacks related to solubility, dose-limiting toxicity and development of drug resistance.[7]

In order to minimize the side effects caused by the cytotoxic agents, new generations of combination therapy employing cytostatic agents such as farnesyl transferase inhibitors (FTI)[8] is emerging and showing promises in pre-clinical studies. Experimental FTI has been shown to act synergistically with paclitaxel to increase the sensitivity of breast cancer cells to paclitaxel-induced cell cycle arrest at G_2/M phase.[9]

Currently, the most widely prescribed cytostatic anti-cancer drug is tamoxifen (Tam), a non-steroidal anti-estrogenic agent used to treat breast cancers of all stages.[10] A large number of clinical data recorded over more than two decades show that Tam is a remarkably safe drug with predictable and generally well tolerated side effects. It produced a favorable response in more than 60% of patients with estrogen receptor (ER)- and progesterone receptor (PR)-positive breast cancer.[11] The observation that Tam greatly reduced the incidence of developing contralateral breast cancer[12]

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has led to clinical trials for the prophylactic use of the agent in healthy women who are at high risk of developing breast cancer.[13] The mechanism of its favorable role in some ER- and PR-negative breast cancer cells is less well understood.[14,15] Nevertheless, Tam has been used in clinical trials with non-microtubule binding drugs in breast cancer[16] and other types of cancers including melanoma with beneficial effects.[17] A recent report showed that combination of Tam and paclitaxel synergistically inhibited breast cancer cell growth independent of ER expression.[18]

The purpose of this study was to evaluate if there was a synergistic effect of paclitaxel in combination with 4-hydroxytamoxifen (4-HT), a potent anti-estrogen and metabolite of Tam, on ER-negative non-breast cancer cell lines HCT15 and NCI H460. The results here showed that combined treatment of paclitaxel and 4-HT synergistically increased the antiproliferative activity of paclitaxel on the colorectal adenocarcinoma cell line HCT15 and the NCI H460 cell line derived from large cell carcinoma of the lung. The addition of 4-HT also enhanced paclitaxel induced G_2/M arrest and apoptosis through the cytochrome *c* pathway.

Materials and methods

Cell culture

Both HCT15 colon cancer and NCI H460 lung cancer cell lines were obtained from ATCC (Rockville, MD). The HCT15 cells were maintained in RPMI 1640 medium containing 1% of antibiotic-antimycotic (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS). NCI H460 cells were grown in the same medium with 10% FBS. The cell lines were maintained at 37 C in a humidified chamber with 5% CO_2. Both paclitaxel and 4-HT were purchased from Sigma (St Louis, MO) and kept in 100% ethanol at various concentrations at −20 C. The compounds were diluted in the media at 1:1000 to achieve the final concentrations. The control samples always contained the same dilution of the vehicle.

Cell proliferation

The cells were seeded at 5000 cells/well in a 96-well culture plate (Costar, Cambridge, MA) and allowed to grow for 24 h before drug treatments. Treatments with vehicles, 4-HT, paclitaxel and 4-HT combined with paclitaxel were outlined for individual experiments at the indicated concentrations and duration. Cell proliferation was determined using the alamarBlue cell proliferation detection kit according to the manufacturer's procedure (Alamar BioSciences, Sacramento, CA). Fluorescence intensity was measured with a Cytofluo 2300 fluorescence plate reader (PerSeptive Biosystem, Framingham, MA).

Cell cycle analysis and detection of apoptosis

Approximately 1×10^6 HCT15 and NCI H460 cells treated with the drugs for 24 h were trypsinized, washed with PBS and fixed with methanol (−20 C) for 1 h. The cells were then re-suspended in PBS with 180 U/ml of ribonuclease A and incubated at 37 C for 20 min. After washes with PBS, cells were stained with propidium iodide at 50 μg/ml in PBS at 4 C in the dark. DNA content was analyzed by flow cytometry using a FACSCalibur system (Becton Dickinson, San Jose, CA).

Cells treated with the drugs for 12 h were monitored for cell death using cell death detection ELISA^{PLUS} assay kit according to the protocol described by the manufacturer (Roche Diagnostics, Indianapolis, IN). The signals were measured in a SpectraMax 340 Plate Reader (Molecular Devices, Sunnyvale, CA).

Western blot analysis for cytochrome *c*

Cytosolic cell extracts free of mitochondria were prepared as described previously.[19] Briefly, cells were harvested and washed once with ice-cold PBS. The cell pellet was re-suspended in five volumes of buffer A containing 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 250 mM sucrose. Cells were homogenized in a Teflon homogenizer for 10 strokes and centrifuged twice at 750 *g* for 10 min at 4 C. The supernatants were centrifuged at 10 000 *g* for 15 min at 4 C followed by an additional centrifugation at 100 000 *g* for 1 h at 4 C. The resulting supernatants (S-100) were stored in small aliquots at −80 C after measurement of the protein concentration using the BioRad Protein Assay kit (BioRad, Hercules, CA).

The release of cytochrome *c* from mitochondria was detected in the S-100 fraction of the cell extracts by Western blot analysis. Protein samples were resolved by SDS-PAGE in a 4–20% gradient minigel (Novex, San Diego, CA) and transferred to nitrocellulose membranes (Schleicher & Schuell Optitran BA-S 83). Membranes were probed with a mouse anti-cyto-

chrome *c* monoclonal primary antibody (PharMingen, San Diego, CA) and incubated with a horseradish peroxidase-conjugated secondary antibody from sheep (Amersham, Arlington Heights, IL). The 15 kDa cytochrome *c* protein band was detected by enhanced chemiluminescence using the ECL kit (Amersham) according to the manufacturer's instructions.

Results

Anti-proliferation activity of paclitaxel and 4-HT

As shown in Figure 1(A and B), single treatment with 4-HT alone had no effect on the proliferation of either HCT15 or NCI H460 at concentrations up to 3 μM. In order to select the effective combination dose for paclitaxel and 4-HT, various doses of 4-HT were combined with paclitaxel at the indicated concentrations. At all doses tested, combination treatment had much more effective inhibition than treatment with paclitaxel alone. The synergistic effect was especially pronounced at low doses of paclitaxel in combination with 4-HT (Figure 1C and D). NCI H460 is known to be sensitive to paclitaxel because of the absence of the multidrug-resistant (MDR) gene product such as P-glycoprotein in this cell line. HCT15, on the other hand, expresses P-glycoprotein and shows a paclitaxel-resistant phenotype as shown in Figure 1(C). The observation that both cell lines were sensitized by 4-HT in these experiments strongly suggested that 4-HT might work beyond MDR-based mechanisms.

The combined effect of 4-HT and paclitaxel was evident in both the 24 and 48 h treatments for both cell lines (Figure 2). The effect of combination treatment was synergistic based on the Isobole method.[20] The results showed that 4-HT increased

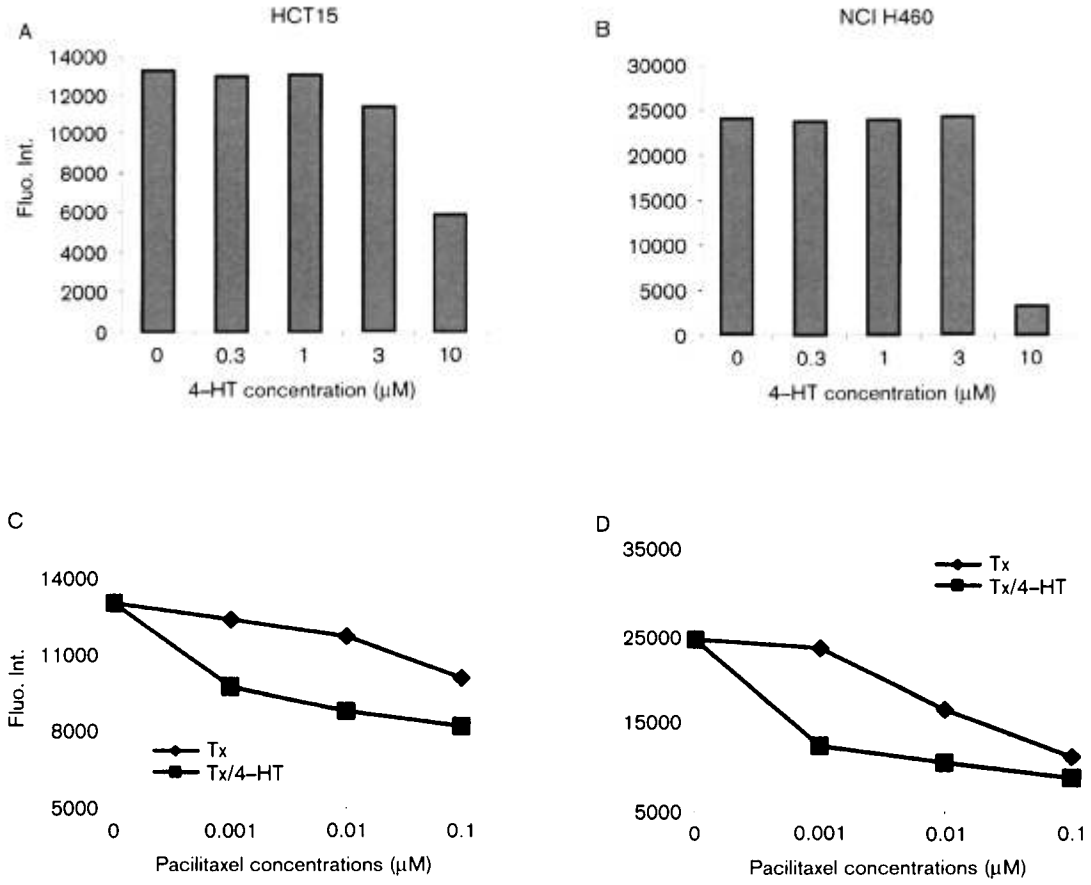

Figure 1. Inhibition of cell proliferation by 4-HT, paclitaxel (Tx) and Tx/4-HT. Single treatment of 4-HT in HCT15 (A) and NCI H460 (B) cell lines showed little inhibitory effect on proliferation of the cells up to 3 μM. Tx alone at the indicated concentration had minimal effect on HCT15 cells (C) and a dose-dependent effect on NCI H460 cells (D). Effective combined doses were tested for Tx/4-HT at 0.001/0.1, 0.01/0.3 and 0.1/1 μM, respectively (C and D). The results indicated that the combined treatment was more effective at inhibiting cell proliferation than Tx alone at all dose combinations for both cell lines. Fluorescence intensity (Fluo. Int.) was recorded as a measure for cell proliferation.

the effective concentration of paclitaxel by about 10-fold for both cell lines. Paclitaxel at 0.1 μM with 4-HT achieved the same extent of growth inhibition as single paclitaxel treatment at 1 μM in HCT15 cells (Figure 2B). The same extent of enhancement was also apparent in the NCI H460 cells (Figure 2D).

Cell cycle analysis

Paclitaxel is known to arrest cells at G_2/M phase that eventually leads to programmed cell death. In order to examine the effect of combination treatment on cell cycle regulation, we treated cells at different paclitaxel concentrations in combination with 4-HT. The data demonstrated that combination treatment increased the population of cells arrested at G_2/M phase in a dose-dependent manner for both cell lines (Figure 3). Once again combination treatment more significantly arrested cells in G_2/M phase of the cell cycle than the single treatments with paclitaxel or 4-HT alone at all doses.

Apoptosis analysis

To examine the effect of combination treatment on apoptosis, we performed ELISA apoptosis assays for both cell lines. Our results showed a slight increase of apoptosis in cells treated with a low dose of paclitaxel and little increase of apoptosis in 4-HT-treated cells for HCT15 (Figure 4A) and NCI H460 (Figure 4B). In contrast, combination treatment resulted in a large increase of the number of apoptotic cells in both cell lines. The enhancement of paclitaxel-induced apoptosis by 4-HT was obvious at all concentrations except at 1 and 0.1 μM for HCT15 and NCI H460, respectively, at which paclitaxel alone was sufficient to induce apoptosis alone (Figure 4C and D).

Cytochrome *c* release from mitochondria

Release of cytochrome *c* from mitochondria is one of the major pathways activated in many drug-induced apoptosis events. Cytochrome *c* release is known to

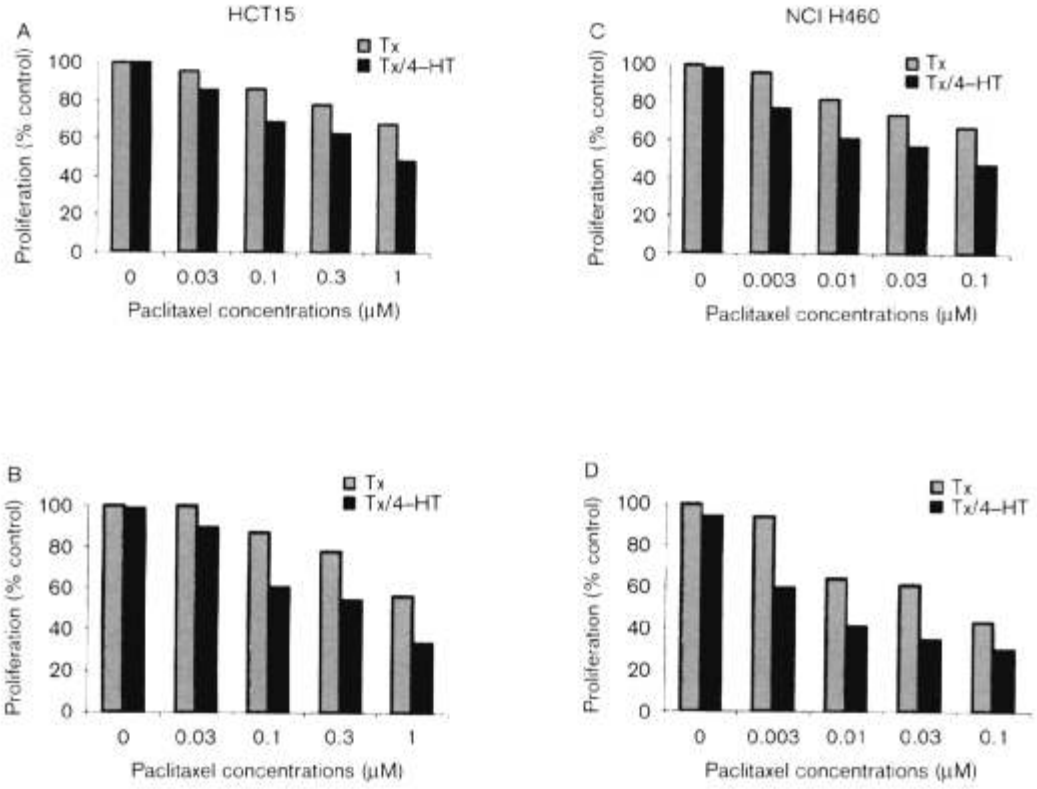

Figure 2. Dose- and time-dependent effects of combination treatment on cell proliferation. HCT15 cells were treated with paclitaxel (Tx) or Tx in combination with 0.3 μM of 4-HT (Tx/4-HT) for 24 (A) and 48 (B) h. The anti-proliferation effect of Tx at concentrations from 0.03 to 1 μM was consistently strengthened by 4-HT. The enhanced anti-proliferation effect by 4-HT at 0.3 μM was also observed in NCI H460 cell line treated with the compounds for 24 (C) and 48 (D) h at concentrations for Tx from 0.003 to 0.1 μM.

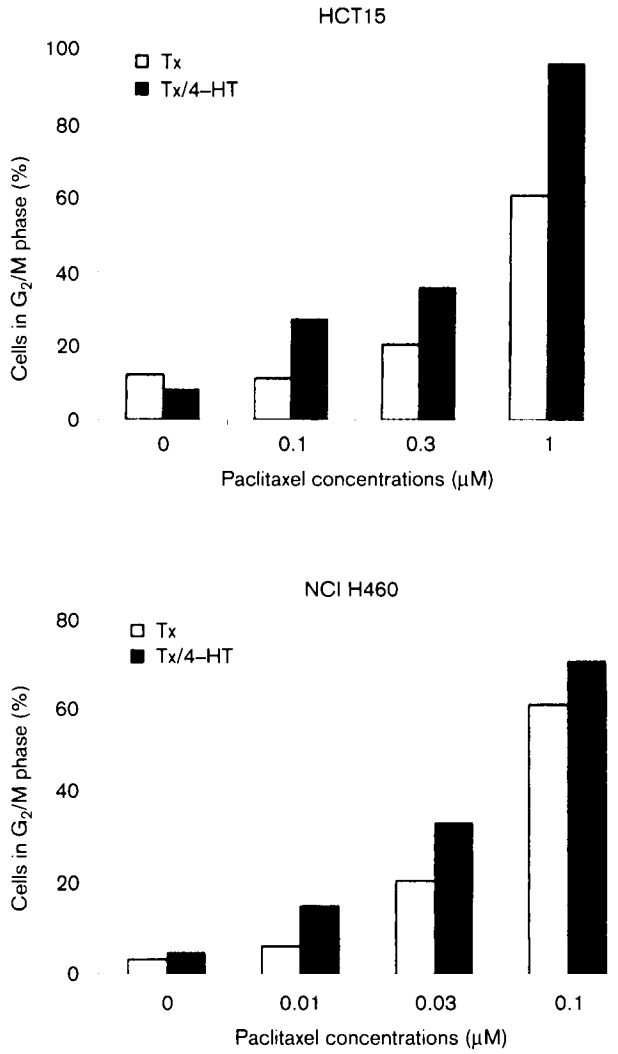

Figure 3. Enhanced cell cycle arrest at G_2/M in the combination treatment. For both HCT15 and NCI H460 cell lines, combination treatment (Tx/4-HT) significantly increased the population of cells arrested in G_2/M phase of the cell cycle after 24 h treatment at the indicated concentrations for Tx and 0.3 μM of 4-HT.

activate the apoptotic program through Apaf-1 and the subsequent activation of the caspase cascade. We tested cytochrome *c* release in the drug-treated cells to determine whether the cytochrome *c* pathway occurred in combination treatment. The results showed a marked increase in cytochrome *c* release into the cytosolic fraction in the combined treatment compared to paclitaxel alone (Figure 5). Cells started to undergo apoptosis as early as 3 h after combination treatment and continued to release cytochrome *c* from mitochondria 6 h after the treatment. Single-agent treatment with paclitaxel did not show any apparent increase in cytochrome *c* release 6 h after treatment. This suggested that 4-HT enhanced paclitaxel-induced cell death by promoting cytochrome *c* release from the mitochondria in HCT15 cells.

Discussion

The results from this study suggest that combination treatment with paclitaxel and 4-HT could be effective against ER-negative non-breast cancer cells that were refractory to paclitaxel treatment alone. The compound 4-HT, an active metabolite of non-steroidal antiestrogen Tam, had no *in vitro* anti-proliferation activity at 3 μM on both cancer cell lines. When combined with paclitaxel, 4-HT at as low as 0.3 μM for 48 h greatly increased the potency of paclitaxel to inhibit cell growth. The results were consistent with a previous report showing the effect of combination treatment on breast cancer cells.[18]

Like the previous report, we also observed that combination treatment greatly increased the population of cells blocked at G_2/M phase of the cell cycle in a dose-dependent manner for both cell lines. Alteration of the G_2/M phase of the cell cycle eventually led to an increase in apoptosis in the cells treated with paclitaxel and 4-HT. These results might explain the decrease of cell numbers observed in the proliferation assays. The release of cytochrome *c* from mitochondria to the cytosol is a hallmark of cells committed to apoptosis.[21] This study provided evidence which suggested that combination treatment induced apoptosis through the cytochrome *c* pathway. Unlike paclitaxel treatment alone, combination treatment sensitized the cells for apoptosis as early as 3 h after the treatment.

The mode of action for the synergistic activity of paclitaxel and 4-HT is not clear. Tam was previously reported to be a MDR reversing agent.[15] Paclitaxel is a known substrate of P-glycoprotein that was overexpressed in HCT15 cells (data not shown). However, combination of paclitaxel and 4-HT also significantly inhibited proliferation of NCI H460, an MDR-negative cell line, at 1/10 of the dose used for HCT15 cells. This suggested that the synergistic effect observed here might not depend on the modulation of the activity of P-glycoprotein. It was noteworthy that this experiment used a metabolite of Tam, 4-HT, which was about 10 times more potent than Tam in inhibition of ER-mediated breast cancer cell growth. Nevertheless, the effect of 4-HT to enhance paclitaxel-mediated inhibition of cell growth and proliferation was comparable with the results reported for Tam in

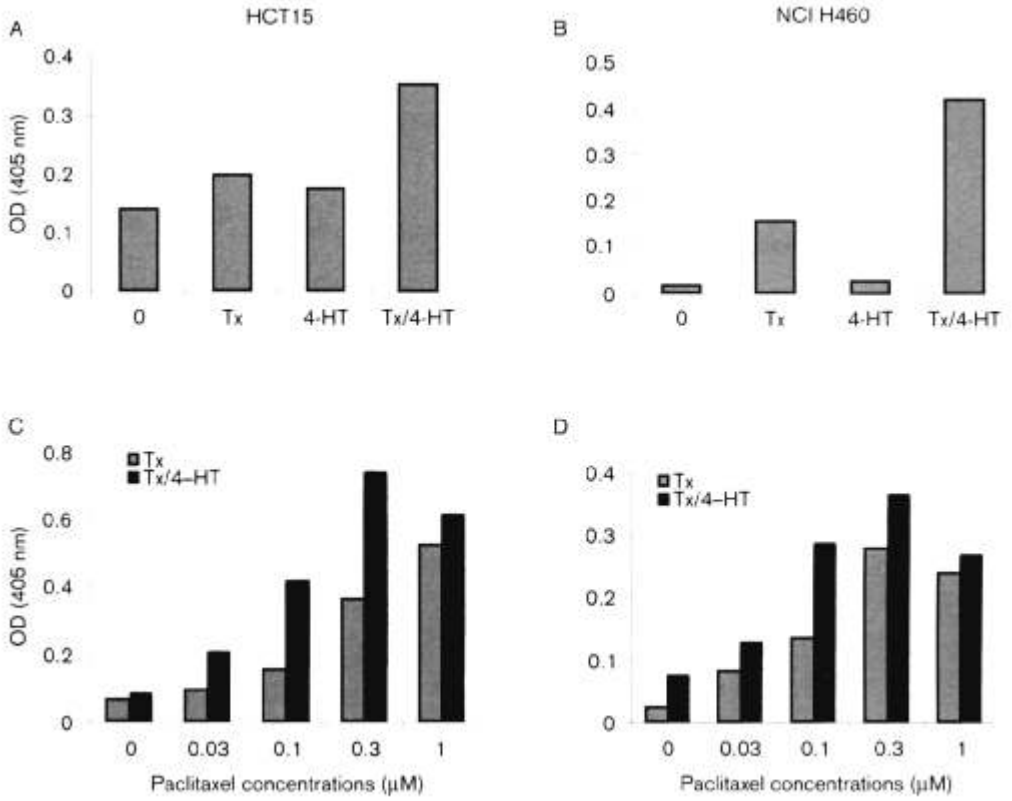

Figure 4. Increased apoptosis among the cells treated with paclitaxel (Tx) and 4-HT. (A) ELISA apoptosis analysis showed that Tx alone at 0.1 μM or 4-HT alone at 3 μM resulted in little increase of the number of apoptotic cells while the two compounds together greatly increased the number of apoptotic cells at the combined single doses. (B) Tx at 0.01 μM was sufficient to induce apoptosis while 4-HT at 3 μM had no effect. Nevertheless, combination of the two single doses greatly enhanced Tx-induced apoptosis in the NCI H460 cells. The enhancement was also apparent at 3 μM 4-HT and a wide range of Tx concentration in HCT15 (C) and NCI H460 (D) cells.

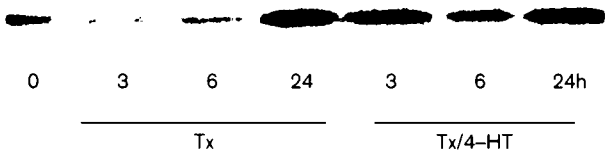

Figure 5. Western blot analysis for the release of cytochrome *c* from mitochondria to cytosol. HCT15 cells were treated with 0.1 μM of paclitaxel (Tx) in the absence or presence of 3 μM of 4-HT for the indicated time. The results showed that cytochrome *c* started to release into the cytosol 3 h after the combination treatment. The single treatment with Tx alone did not release cytochrome *c* until after 6 h.

ER-negative breast cancer cells.[18] This observation supported the notion that 4-HT-mediated enhancement of cytotoxic drugs did not work through the ER-dependent signal transduction pathway and that the application of a combination of cytostatic Tam and paclitaxel might extend beyond the arena of breast cancer to other types of cancers.

Conclusion

In contrast to monotherapy, the combination of paclitaxel and 4-HT significantly increased the growth inhibitory effect on ER-negative non-breast cancer cell lines HCT15 and NCI H460, especially at lower doses. The mechanism of this synergistic effect may be attributed in part to the enhancement of cell cycle arrest at G_2/M and induction of apoptosis through the cytochrome *c*/Apaf-1 pathway. Our results support the use of low-dose paclitaxel in combination with Tam in clinical trials for various types of cancers.

Acknowledgment

The authors would like to acknowledge Dr David Frost for his helpful discussions related to this work.

References

1. Crommentuyn KML, Schellens JHM, Berg VD, Beijnen JH. *In-vitro* metabolism of anti-cancer drugs, methods and applications: paclitaxel, docetaxel, tamoxifen and ifosfamide. *Cancer Treat Rev* 1998; **24**: 345-66.
2. Horwitz SB. Mechanism of action of paclitaxel. *Trends Pharmac Sci* 1992; **13**: 134-5.
3. Gelman K. Taxoids: paclitaxel and docetaxel. *Lancet* 1994; **344**: 1271.
4. Kaye SB. Taxoids. *Eur J Cancer* 1995; **31A**: 823-6.
5. Marty M, Extra JM, Cottu PH, Espie M. Prospects with docepaclitaxel in the treatment of patients with breast cancer. *Eur J Cancer* 1997; **suppl 7**: S26-9.
6. McGuire WP, Hoskins WJ, Brady MF, *et al.* Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. *N Engl J Med* 1996; **334**: 1-6.
7. Huizing MT, Sewberath Misser VH, Pieters RC, *et al.* Taxanes: a new class of antitumor agents. *Cancer Invest* 1995; **13**: 381-404.
8. Gu WZ, Tahir SK, Wang YC, *et al.* Effect of novel CAAX peptidomimetic farnesyltransferase inhibitor on angiogenesis *in-vitro* and *in-vivo*. *Eur J Cancer* 1999; **35**: 1394-1401.
9. Moasser MM, Sepp-Lorenzino L, Kohl NE, *et al.* Farnesyl transferase inhibitors cause enhanced mitotic sensitivity to paclitaxel and epothilones. *Proc Natl Acad Sci USA* 1998; **95**: 1369-74.
10. Jordan VC. Current approved uses of tamoxifen and recommendations in the United States. In: Jordan VC, ed. *Tamoxifen, a guide for clinicians and patients*. Huntington, NY: PRR 1996: 37-40.
11. Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res* 1990; **50**: 4177-89.
12. Cuzick J, Baum M. Tamoxifen and contralateral breast cancer. *Lancet* 1985; **ii**: 282.
13. Fisher B. Highlights from recent National Surgical Adjuvant Breast and Bowel Project studies in the treatment and prevention of breast cancer. *CA: Cancer J Clin* 1999; **49**: 159-77.
14. Kang Y, Cortina R, Perry RR. Role of c-*myc* in tamoxifen-induced apoptosis in estrogen-independent breast cancer cells. *J Natl Cancer Inst* 1996; **88**: 279-84.
15. Kirk J, Syed SK, Harris AL, *et al.* Reversal of P-glycoprotein-mediated multidrug resistance by pure antioestrogens and novel tamoxifen derivatives. *Biochem Pharmacol* 1994; **48**: 277-85.
16. Henderson C. Overviews of adjuvant tamoxifen therapy. In: Jordan VC, ed. *Tamoxifen, a guide for clinicians and patients*. Huntington, NY: PRR 1996: 41-63.
17. Creagan ET, Suman VJ, Dalton RJ, *et al.* Phase III clinical trial of the combination of cisplatin, dacarbazine, and carmustine with or without tamoxifen in patients with advanced malignant melanoma. *J Clin Oncol* 1999; **17**: 1884-90.
18. Ferlini C, Scambia G, Distefano M, *et al.* Synergistic antiproliferative activity of tamoxifen and docetaxel on three oestrogen receptor-negative cancer cell lines is mediated by the induction of apoptosis. *Br J Cancer* 1997; **75**: 884-91.
19. Yang J, Liu XS, Bhalla K, *et al.* Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science* 1997; **275**: 1129-32.
20. Berenbaum MC. Criteria for analyzing interaction between biologically active agents. *Adv Cancer Res* 1981; **35**: 288-335.
21. Liu XS, Kim CN, Yang J, Jemmerson R, Wang XD. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell* 1996; **86**: 147-57.

(Received 10 August 1999; revised form accepted 17 August 1999)