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Inhibition of the Membrane Translocation and Activation of Protein Kinase C, and Potentiation of Doxorubicin-Induced Apoptosis of Hepatocellular Carcinoma Cells by Tamoxifen

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ABSTRACT. Hepatocellular carcinoma (HCC) is characterized by high drug resistance to currently available chemotherapeutic agents. In a prospective clinical study, we have demonstrated that high-dose tamoxifen significantly enhanced the therapeutic efficacy of doxorubicin in patients with far-advanced HCC. In a search for a possible mechanism, we found that tamoxifen at a clinically achievable concentration (2.5 μM) significantly enhanced doxorubicin-induced cytotoxicity and apoptosis of Hep-3B cells, a multidrug resistance (MDR)-1 expressing HCC cell line. This synergistic cytotoxic effect of tamoxifen, at this concentration, however, was not mediated by MDR inhibition. Instead, as evidenced by both western blot and immunofluorescence studies, tamoxifen inhibited the cytoplasmic-membrane translocation of protein kinase C (PKC)-α. 12-O-Tetradecanoylphorbol-13-acetate (TPA) restored the membrane translocation of PKC-α and abrogated the synergistic cytotoxicity of tamoxifen. We also showed that tamoxifen, at this concentration, did not directly affect the enzyme activity of PKC. Further, membrane translocation of other membrane-bound proteins, such as Ras protein, was similarly inhibited by tamoxifen, but could not be restored by the addition of TPA. Together, these data suggested that tamoxifen may act on the cytoplasmic membrane, and thereby inhibit PKC-α translocation to the membrane where it is activated. We hypothesize that high-dose tamoxifen may be an effective modulator of doxorubicin in the treatment of HCC, and suggest that biochemical modulation of PKC as a measure to improve systemic chemotherapy for HCC deserves further investigation. BIOCHEM PHARMACOL 55;4:523–531, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. hepatocellular carcinoma; protein kinase C; tamoxifen; apoptosis; chemotherapy

HCC** is one of the most common cancers in the world. Except for a minority of patients who are amenable to curative surgical resection, most HCC patients die from advanced disease within a relatively short period of time [1]. To date, systemic chemotherapy has played only a minor role in the treatment of HCC. Doxorubicin remains the most effective single agent, with a tumor response rate of approximately 10–15%; combination chemotherapy has not conferred any additional benefit [1, 2]. Although new anti-cancer drugs have been tested in HCC, doxorubicin remains the most effective agent. Therefore, it may be fruitful to explore established drugs in new combinations in which their therapeutic efficacy may be improved by biochemical modulation.

Since the majority of HCC cells express the gene *MDR-1* [3], agents with MDR-inhibiting potential have been screened regularly for this purpose. Tamoxifen, a triphenylethylene with an MDR-1 protein inhibitory effect, has been shown previously to reduce drug resistance due to MDR [4–6]. In a prospective clinical study of 36 patients with inoperable HCC, we demonstrated that high-dose tamoxifen (160 mg/m^2/day, days 1–7) plus doxorubicin (60 mg/m^2, day 4), repeated every 3 weeks, yielded good partial remission in 12 patients [7]. If extrapolated from a phase I study conducted by Trump *et al.* [8], the 160 mg/m^2/day dose of tamoxifen that was used in our clinical study could be expected to result in a combined serum concentration of approximately 3.0 μM tamoxifen and 3.0 μM *N*-desmethyltamoxifen, its major metabolite. Results of a previous

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** *Abbreviations:* HCC, hepatocellular carcinoma; MDR, multi-drug resistance/resistant; *MDR-1*, multi-drug resistance-1 gene; Pgp, P-glycoprotein; PKC, protein kinase C; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; and TPA, 12-O-tetradecanoylphorbol-13-acetate.

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controlled clinical trial using doxorubicin and tamoxifen (10 mg, p.o., b.i.d.) versus doxorubicin alone in the treatment of HCC suggested that lower doses of tamoxifen may not be effective in enhancing the cytotoxicity of doxorubicin [9]. Therefore, we were particularly interested in examining the biologic activities of tamoxifen at the concentration range of 1–5 μM.

The current *in vitro* study was conducted for this purpose. We were surprised to find that tamoxifen at this concentration range had no effect on MDR-1/Pgp inhibition. Instead, its synergistic cytotoxicity with doxorubicin appeared to be related to another mode of biochemical modulation, i.e. inhibition of PKC activity [10]. We have specifically demonstrated that tamoxifen inhibited the membrane translocation of PKC-α. Associated with this inhibition of PKC-α translocation, tamoxifen exposure led to the potentiation of doxorubicin-induced apoptosis in HCC cells.

MATERIALS AND METHODS

Cell Culture and Cytotoxicity Assay

Hep-3B, an *MDR-1*-expressing human HCC cell line [11], was maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco Co.). The expression of Pgp and MDR mRNA in Hep-3B cells was confirmed in our laboratory by northern and western blot analyses. The growth inhibitory effects of the drugs were determined by a tetrazolium-based semi-automated colorimetric assay (MTT assay) as previously described [12]. Briefly, cells were plated in 96-well plates at 5×10^3 cells/well. After an overnight incubation, various concentrations of drugs were added in triplicate samples for each concentration. Cells were exposed to drugs continuously. After 3–4 days of culture, when cells in drug-free wells reached 90% confluency, cell numbers were evaluated using the MTT method with an ELISA reader at O.D. 492. The experiments were repeated at least twice. The data were reported as the mean ± SEM for all values obtained.

The combination effect of tamoxifen (Sigma Co.) and doxorubicin (Farmitalia Carlo Erba) was analyzed further by a computerized program based on a median effect equation, as described previously [13].

Apoptosis Assay by DNA Fragmentation and Cell Cycle (sub-G1) Analysis

Apoptotic DNA fragments were isolated as previously described [14]. Briefly, control and drug-treated Hep-3B cells were harvested from a T150 flask, washed with PBS, and collected by centrifugation. Then the cell pellet was treated with 100 μL of lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris–HCl, pH 7.5) for 10 sec. After centrifugation at 1600 *g* for 5 min, the apoptotic DNA-containing supernatant was collected. To the supernatant, SDS was added to a final concentration of 1%; then the supernatant was treated with RNase A (final concentration 5 μg/μL) at 56° for 2 hr followed by proteinase K digestion (final concentration 2.5 μg/μL) at 37° for at least 2 hr. The DNA was then ethanol precipitated and dissolved in 50 μL TE buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8.0). One-third of the DNA was loaded in a 1.7% agarose gel for electrophoretic separation. For the "sub-G_1" analysis, a published method was followed with modification [15]. Briefly, control and drug-treated Hep-3B cells were harvested and fixed by suspending in 70% ethanol and incubating at −20° overnight. Then the fixed cells were collected by centrifugation and resuspended in Hanks' buffered salt solution diluted 1:3 with 0.2 M Na_2HPO_4/0.1 M citric acid buffer (pH 8.0) containing 0.1% Triton X-100, and incubated for 30 min at 25°. This treatment removed low molecular weight DNA fragments from the apoptotic cells and allowed for analysis of the sub-G_1 peak during subsequent cell cycle studies. After this treatment, cells were collected by centrifugation and resuspended and incubated in 1 mL of PI buffer (propidium iodide at 50 μg/mL, RNase A at 100 μg/mL in PBS) for 30 min at room temperature. The fluorescence (DNA content) of individual cells was measured with a FACScan™ flow cytometer (Becton Dickinson) and analyzed with CellQuest™ software (Becton Dickinson).

Rhodamine 123 Dye Uptake and Efflux Assay

Flow cytometric analyses of rhodamine-123 (Sigma) uptake and efflux were performed according to the methods described previously [16]. Cells at a density of 1×10^6/mL in exponential growth were used for each test. In the retention study, cells were incubated in the presence or absence of various concentrations of tamoxifen in culture medium at 37° for 1 hr before 200 ng/mL of rhodamine dye was added. Verapamil, 10 μM, was used as a positive control for an MDR-inhibitory agent. In the efflux study, cells were first incubated with medium containing 200 ng/mL of rhodamine at 37° for 1 hr, washed three times with rhodamine-free medium, and then incubated with or without verapamil or tamoxifen. The intensity of rhodamine fluorescence was measured with the FL1 channel of the FACScan, and 1×10^4 cells were measured every 30 min for up to 3 hr. Three separate experiments were carried out for each treatment.

In Vitro *Enzyme Assay of Protein Kinase* C

A micellar assay, using whole rat brain PKC and histone IIIs as substrate, was employed as previously described [10, 17]. The standard reaction mixture (0.12 mL) contained 200 mM Tris–HCl at pH 7.5, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 1 mM EGTA), phosphatidylinositol (10 μg/mL) (or no phospholipid), 70 μM [γ-^{32}P]ATP (250–400 cpm/pmol), histone IIIs (0.67 mg/mL) or protamine sulfate (0.67 mg/mL), 1–4 μg rat brain PKC, and tamoxifen at the indicated concentrations. Reactions were initiated by the addition of enzyme and

incubated at 30° for 5 min. Reactions were terminated by pipetting a 40-μL aliquot onto phosphocellulose paper. The papers were rinsed with three 1-L volumes of water and counted in Aquasol. All assays were done in triplicate.

Analysis of Membrane Translocation of PKC and Ras by Western Blot

Analysis of the membrane portion of PKC-α and other PKC isoenzymes by western blot was done by methods previously described with slight modification [18–20]. Approximately 1×10^7 Hep-3B cells in a T150 flask were scraped with a policeman into a 14-mL centrifugation tube, washed once with PBS, resuspended in 1 mL of buffer A (2 mM EDTA, 4 mM EGTA, 10 μg/mL leupeptin, 25 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride, 0.25 M sucrose, 20 mM Tris–HCl, pH 7.5), transferred to a 1.5-mL Eppendorf tube, and homogenized by ultrasonication for 30 strokes (output control 3–4, duty cycle 30–40%; Sonifer 250, Branson Ultrasonics Corp.) on an ice-water bath. The particulate (membrane) fraction was collected by centrifugation at 14,000 *g* for 30 min at 4°, washed once with buffer A, and finally resuspended in 0.5 mL of buffer B (1% SDS and 10% glycerol, made in buffer A). The protein concentration was determined with the Bio-Rad DC protein assay kit (Bio-Rad Laboratories) using bovine serum albumin as a standard. Amounts of the membrane fraction corresponding to 40 μg protein were resolved by 10% SDS–PAGE. Proteins were electro-transferred onto a 50-cm^2 PVDF membrane at a constant 400 mA for 1 hr in Towbin buffer (192 mM glycine, 0.1% SDS, 25 mM Tris base, pH 8.3). The membrane was first incubated with 5 mL blocking solution (0.1% Tween-20, 1% non-fat dry milk, made in PBS) at 4° overnight, then with 5 mL fresh blocking solution containing 1 μL (0.1 μg) mouse monoclonal antibody to either v-H-Ras or various human PKC isoenzymes (IgG, Transduction Laboratories) for 45 min at room temperature. The membrane was washed with the blocking solution three times (15 min each). To detect the antibody-bound target protein band, the Western Exposure Chemiluminescent Detection System (Clontech Laboratories, Inc.) kit was employed. Briefly, the washed membrane was transferred to a sealed bag and incubated in 5 mL blocking solution containing 0.5 μL alkaline phosphatase-conjugated secondary antibody (goat anti-mouse IgG) at 25° for 45 min with gentle agitation. The membrane was then washed with blocking solution three times (45 min each), incubated in 5 mL assay buffer (0.1 M diethanolamine, 1 mM $MgCl_2$, pH 10) containing 3% enhancer solution for 5 min at room temperature, and washed twice with assay buffer. The membrane was finally incubated in 2 mL of assay buffer containing a 25 mM concentration of the chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (CSPD; Boehringer Mannheim Co.). After 5 min of incubation at room temperature, excessive solution was removed from the membrane by briefly blotting on a 3 MM paper. Then the damp membrane was wrapped and exposed to an X-ray film for 5–30 min.

Immunofluorescence Staining for the Cellular Localization of PKC-α

Cells (1×10^5) in the exponential phase of growth were treated with different concentrations of tamoxifen in the presence or absence of 200 nM TPA (Sigma). After 30 min of drug treatment, the cells were harvested, serum was removed, and the cells were washed with PBS, fixed in cold acetone:methanol (1:1, v/v) for 5 min, and then cytospun onto glass slides by centrifugation at 100 *g* for 5 min. After being washed in PBS buffer, the smears were incubated with monoclonal anti-PKC-α antibodies (host, mouse; isotype, IgG2b; Transduction Laboratories). The antibodies were diluted 500-fold in PBS buffer, and incubation was carried out at room temperature for 1 hr. After an interval of 5 min for washing with PBS buffer, the smears were incubated with fluorescein isothiocyanate (FITC) conjugated sheep anti-mouse IgG antibodies (polyclonal, The Binding Site Ltd.) at room temperature for 1 hr. After washing in PBS three times, the smears were mounted and the signals were visualized under a Nikon Microphot-FXA microscope system.

RESULTS

Synergistic Cytotoxicity of Tamoxifen and Doxorubicin

As shown in Fig. 1, at a non-toxic concentration of 2.5 μM, tamoxifen decreased the IC_{50} of doxorubicin from 0.9 to 0.1 μM in Hep-3B cells. This synergistic cytotoxic effect of tamoxifen and doxorubicin was confirmed further by the results of median effect analysis, in which the combination index at the fractional inhibition of 0.4 to 1.0 was around 0.2 to 0.5 (Fig. 2) [14].

TPA abrogated this effect of tamoxifen (Fig. 1).

Effect of Tamoxifen on Doxorubicin-Induced Apoptosis

As shown in Fig. 3, incubation of Hep-3B cells with 2.5 μM tamoxifen was associated with a significant potentiation of doxorubicin-induced internucleosomal DNA fragmentation. This effect was inhibited by co-treatment of the cells with TPA.

The time–course of drug-induced apoptosis was examined further by flow cytometric analysis of the sub-G_1 cell cycle phase. As illustrated in Fig. 4, tamoxifen potentiated doxorubicin-induced apoptosis as shown in the increased sub-G_1 phase cells, and this effect could also be inhibited by TPA. The flow cytograms are not displayed in detail. In general, doxorubicin results in an initial G_2/M block, which resulted in elevated S-phase. When apoptosis ensued, the sub-G_1 phase increased, and the G_0/G_1 peak, S phase, and G_2/M peak gradually decreased. Addition of tamoxifen to doxorubicin did not alter the general pattern of the flow cytogram as induced by doxorubicin alone.

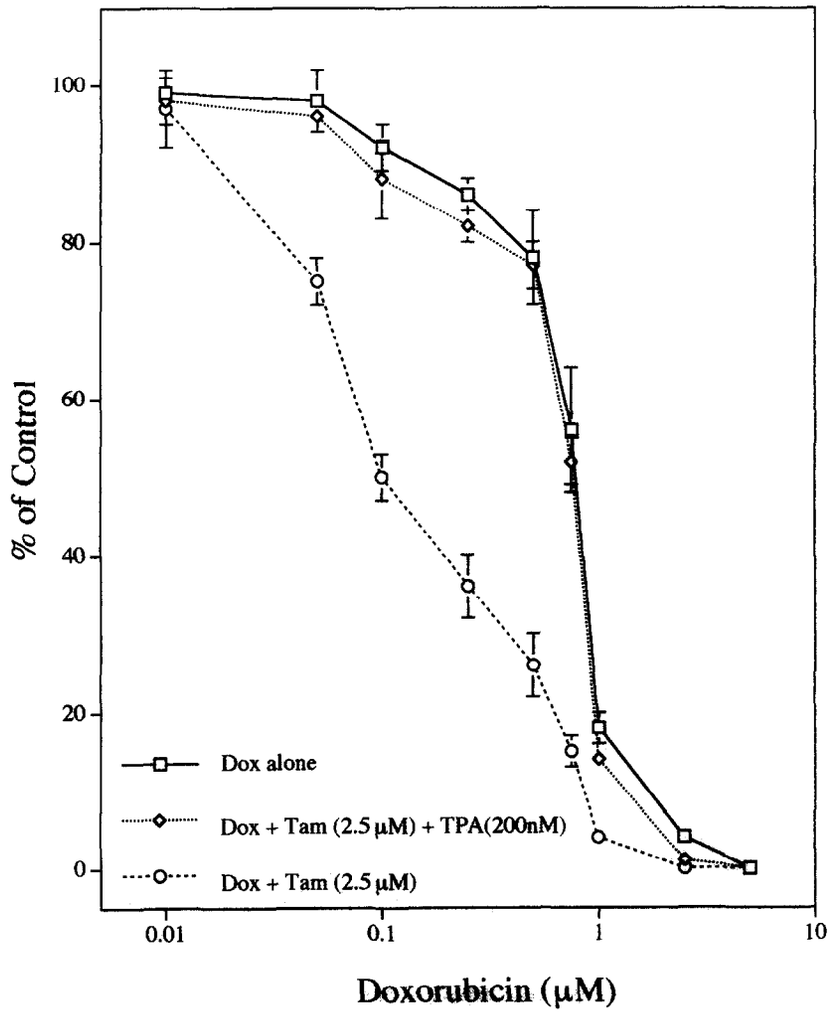

FIG. 1. Growth inhibition of Hep-3B cells by doxorubicin alone, doxorubicin plus tamoxifen (2.5 μM), and doxorubicin plus tamoxifen (2.5 μM) and TPA (200 nM). Tamoxifen (2.5 μM) and TPA (200 nM) alone were not inhibitory on the cell growth of Hep-3B cells. Abbreviations: Tam, tamoxifen; Dox, doxorubicin; and TPA, 12-O-tetradecanoylphorbol-13-acetate. All values represent means ± SD of 3 experiments. The beginning numbers of cells were 5000 per well (200 μL).

Rhodamine 123 Dye Uptake and Efflux Assay

Results of the effect of tamoxifen on the multidrug-exportation function of the MDR-1 protein are shown in Fig. 5. Verapamil had a significant effect on the uptake and efflux of rhodamine, indicating the existence of a functional multidrug exporter in Hep-3B cells. Although at higher concentrations tamoxifen partially inhibited the efflux of rhodamine 123, a negligible effect was noted at a concentration of 2.5 μM. Tamoxifen, up to 10 μM, had no effect on rhodamine 123 dye uptake. These results indicated that the *in vitro* synergistic cytotoxicity of tamoxifen with doxorubicin may not be related to the MDR-inhibitory effect.

Effect of Tamoxifen on PKC Enzyme Activity

As shown in Table 1, the *in vitro* concentrations of tamoxifen needed to inhibit the activity of the total mixture, as well as specific PKC isoenzymes, were all over 100 μM.

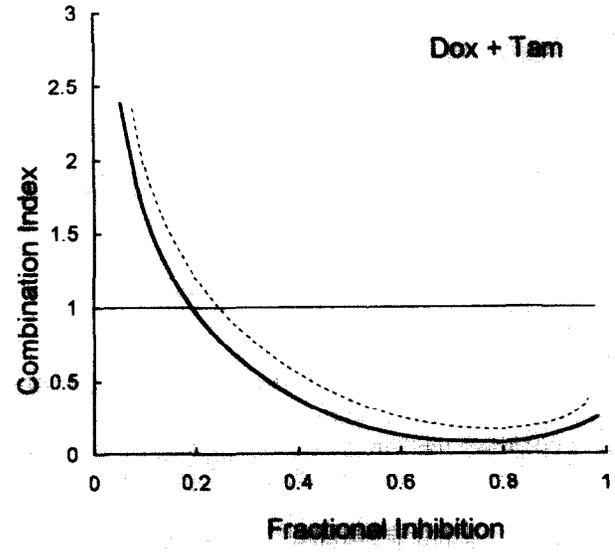

FIG. 2. Median effect analysis of the combination effect of doxorubicin and tamoxifen. The analysis was under the assumption of mutual exclusiveness (solid line) or mutual non-exclusiveness (dotted line) of the mechanism of drug action. Hep-3B cells were exposed continuously to a fixed ratio (1:10) of doxorubicin and tamoxifen.

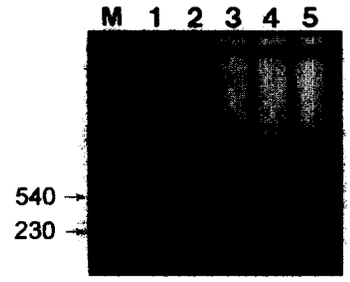

FIG. 3. Analysis of the internucleosomal DNA fragmentation after 48 hr of exposure to (1) 0.1% ethanol (solvent control), (2) tamoxifen (2.5 μM), (3) doxorubicin (1.0 μM), (4) doxorubicin (1.0 μM) + tamoxifen (2.5 μM), and (5) doxorubicin (1.0 μM) + tamoxifen (2.5 μM) + TPA (200 nM). M = markers.

Effect of Tamoxifen on Membrane Translocation of Various PKC Isoenzymes and V-H-Ras

The PKC isoenzymes, including PKC-α, PKC-γ, PKC-δ, PKC-ε, PKC-θ, PKC-λ, PKC-ξ, and PKC-μ, could be demonstrated in Hep-3B cells by western blot analysis. Since PKC-α was the most abundant and consistently expressed isoenzyme, it was studied in more detail. A concentration-dependent inhibition of the membrane translocation of PKC-α by tamoxifen is shown in Fig. 6. It was noteworthy that the effect of tamoxifen took place at a much lower concentration (≤5 μM) than that necessay to inhibit *in vitro* enzyme activity of PKC, as shown in Table 1. This effect of tamoxifen could be reversed by the co-treatment of the cells with TPA (Fig. 7). A similar inhibitory effect of tamoxifen, but with concentrations of more than 5 μM, on the membrane translocation of PKC-γ, PKC-δ, and PKC-ε was also demonstrated. The results of PKC-γ are shown in Fig. 8.

This translocation of PKC-α, as well as its inhibition by tamoxifen, could also be visualized by immunofluorescence staining of the cells (Fig. 9).

Other categories of membrane-bound proteins, such as V-H-Ras, were similarly affected by tamoxifen, but the addition of TPA did not restore its membrane translocation (Fig. 10).

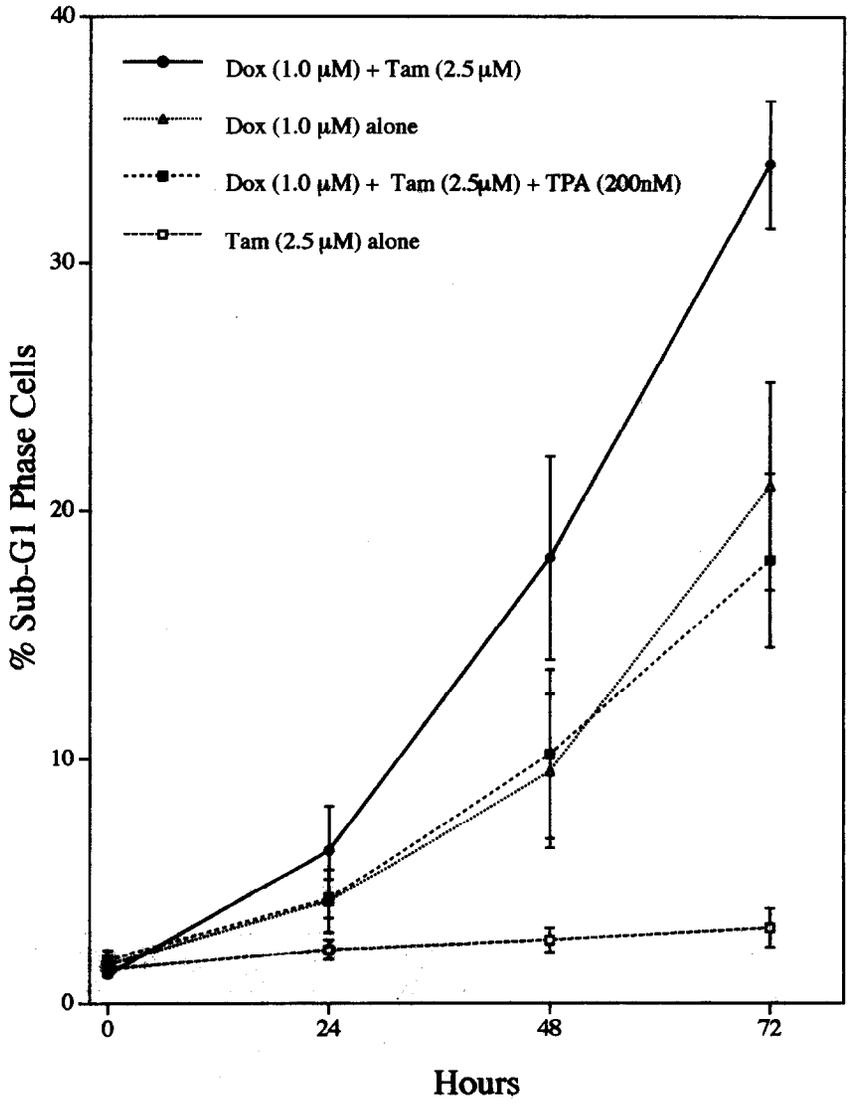

FIG. 4. Flow cytometric analysis of the Hep-3B sub-G_1-phase cells after exposure to tamoxifen (2.5 μM), doxorubicin (1.0 μM), doxorubicin (1.0 μM) + tamoxifen (2.5 μM), and doxorubicin (1.0 μM) + tamoxifen (2.5 μM) + TPA (200 nM). Abbreviations: Tam, tamoxifen; Dox, doxorubicin; TPA, 12-O-tetradecanoylphorbol-13-acetate. All values represent means ± SD of 3 experiments.

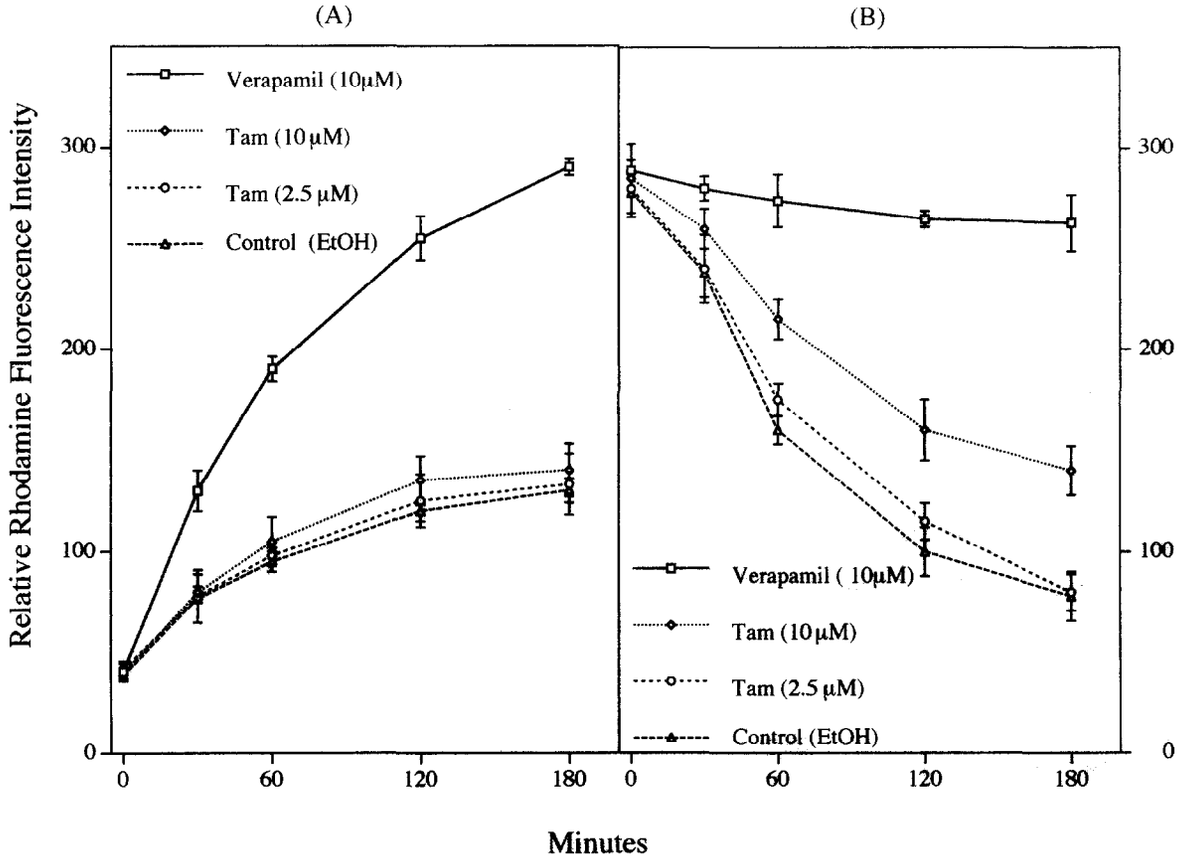

FIG. 5. Effects of verapamil and tamoxifen (Tam) on the uptake (A) and efflux (B) of rhodamine dye. All values represent means ± SD of 3 experiments.

DISCUSSION

Tamoxifen, a triphenylethylene, has been demonstrated to have biologic and pharmacologic activities beyond its traditional role as an anti-estrogenic agent. Among these are inhibition of MDR [4–6], PKC [10, 11], calmodulin [21], insulin growth factor [22], and transforming growth factor-α [23]. In addition, tamoxifen has been associated with transforming growth factor-β_1 induction [24], immune reaction modulation [25], apoptosis induction [26, 27], and reduction of fluidity of the cytoplasmic membrane [28]. It has been speculated that some of these activities might be responsible for the unexpected therapeutic effect of tamoxifen, alone or in combination with other anti-cancer drugs, in various cancers including malignant melanoma [29, 30], brain glioma [31], and lymphoma [32]. The potential synergistic cytotoxic effect between tamoxifen and chemotherapeutic agents in estrogen-independent solid tumors has been further studied and documented recently [27, 33].

Although tamoxifen has been recognized as an MDR-inhibitory agent, our *in vitro* data, as well as the data of others [4–6], suggest that a relatively high concentration (>10 μM) was needed for this effect [4–6]. A phase I clinical study showed that the intracellular concentration of daunomycin did not change with the use of high-dose tamoxifen (up to 700 mg/day, p.o.), which resulted in peak plasma tamoxifen levels of approximately 7 μM [34]. Therefore, other mechanisms of action may explain the *in vitro* chemosensitization effect that occurred at low concentrations of tamoxifen. Since Hep-3B cells do not express estrogen receptors, a hormonal effect of tamoxifen is unlikely [35].

TABLE 1. PKC inhibition by tamoxifen in micelle assay

Form	IC_{50} (μM)
PKC-α	116 ± 14
PKC-βI/PKC-βII	142 ± 18
PKC-γ	130 ± 10
Mixture	121 ± 13

Values are means ± SD of three experiments.

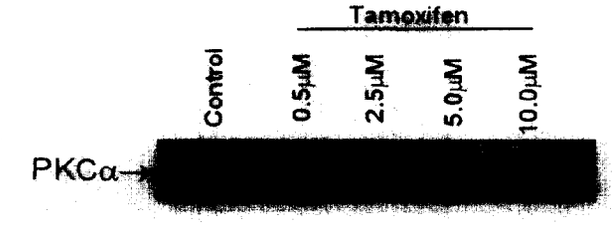

FIG. 6. Concentration-dependent inhibition of membrane translocation of PKC-α. Hep-3B cells were exposed to various concentrations of tamoxifen for 30 min before harvesting.

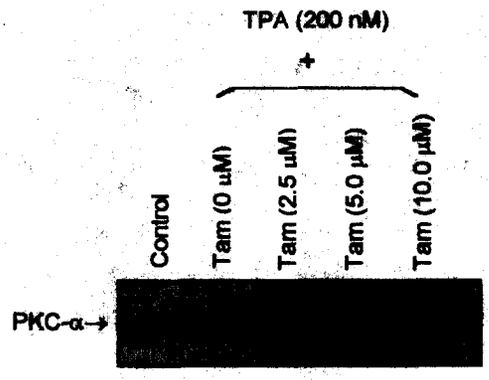

FIG. 7. Reversal of the effect of tamoxifen by TPA. TPA, 200 nM, overcame the inhibitory effect of tamoxifen upon membrane translocation of PKC-α in Hep-3B cells.

Tamoxifen is a PKC inhibitor that presumably disrupts the hydrophobic interaction between the regulatory domain of PKC and phospholipid [36]. Previous studies, most of which had utilized crude extracts of murine brain in the enzyme assay, indicated that a high concentration of tamoxifen (10–100 μM) was needed for this effect [10, 11]. This observation was confirmed in our study. However, we have demonstrated that, in living cells, a much lower and clinically achievable concentration of tamoxifen was sufficient to inhibit the membrane translocation of PKC in Hep-3B cells. This activity of tamoxifen may be mediated by its nonspecific effect on cytoplasmic membranes, since other membrane-bound proteins such as Ras are similarly affected. This direct membrane effect of tamoxifen may be related to its chemical properties. Tamoxifen is lipophilic, and is expected to partition into hydrophobic domains in the fluid mosaic structure of cell membranes. Clarke *et al.* [28] have demonstrated previously that tamoxifen, at concentrations greater than 1 μM, significantly decreased the fluidity of the plasma membrane of estrogen receptor (ER)-negative breast cancer cells, and may contribute to its non-ER-mediated cytotoxicity. One possible consequence of this decreased fluidity of the cell membrane is an altered binding capacity to the membrane-bound proteins, and thus possibly inhibition of the activity of PKC, which upon activation translocates to the cell membrane to function as a key signal transduction element. It was speculated that the ability of tamoxifen (≤4 μM) to reverse the mitogenic properties of epidermal growth factor and insulin in MCF-7 cells growing in the absence of estrogen may reflect an

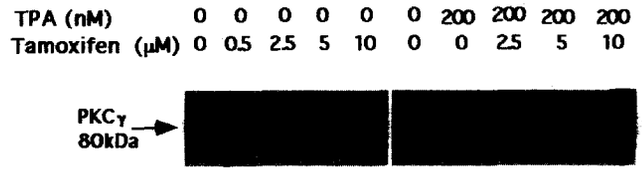

FIG. 8. Concentration-dependent inhibition of membrane translocation of PKC-γ. Tamoxifen (10 μM) inhibited membrane translocation of PKC-γ in Hep-3B cells (left panel). TPA (12-o-tetradecanoylphorbol-13-acetate) restored and overshot the membrane translocation of PKC-γ (right panel).

inhibition of this signal transduction pathway [37]. Inhibition of PKC-α translocation may be closely related to the enhancement of doxorubicin-induced apoptosis of Hep-3B cells, since TPA, a PKC activator, restored the membrane translocation of PKC-α and abrogated the cytotoxicity and apoptosis-potentiating effect of tamoxifen. Although other PKC isoenzymes were similarly affected, a higher concentration, which exceeded the clinically achievable ones, was usually needed.

Recently, similar interactions between chemotherapeutic agents and other PKC modulators have been described. For example, staurosporine and safingol, two PKC inhibitors, were demonstrated to potentiate the apoptosis induced by daunorubicin and mitomycin-C in leukemic cells and gastric cancer cells, respectively [38–40]. In another study, PKC inhibitors regulated the taxol-induced apoptosis of leukemic cells possessing increased levels of anti-apoptotic Bcl-2 protein [41]. Interestingly, the PKC activator and inhibitor, bryostatin 1, and other pharmacological activators of PKC were found to potentiate cytosine arabinoside-induced apoptosis in HL-60 cells [42]. These effects of PKC modulators, either inhibitors or activators, on the regulation of apoptosis demonstrates the complexity and the cell-dependent nature of PKC modulation as a strategy for anti-cancer treatment. A PKC modulator may be stimulatory of apoptosis in one cell type while protective against apoptosis in another [43, 44]. Whether HCC represents a type of cancer for which PKC inhibition leads to chemosensitization remains to be confirmed by more studies.

Apoptosis-related genes can be regulated by PKC modulators [43, 44]. In the case of tamoxifen, a recent study has found a synergistic effect with cisplatin in the human melanoma cell line T289 in up-regulating the expression of Bax mRNA and protein, as well as the binding of Bax to Bax to form homodimers [45]. *Bax* is the major apoptosis-promoting gene and, hence, may explain, in part, the synergistic cytotoxicity of tamoxifen with alkylators like cisplatin in the treatment of malignant melanoma. In another study, c-*Myc* gene expression was increased significantly in tamoxifen-induced apoptosis in the estrogen-independent breast cancer cell line MDA 231 [33]. We have examined the effects of tamoxifen for *in vitro* regulation of a panel of apoptosis-related genes including *p53*, *Rb*, *Bcl-2*, *Bax*, *Fas*, *H-ras*, *N-ras*, and c-Myc. The mRNA expression of all these genes was not altered significantly by tamoxifen in the Hep-3B cell line (data not shown). Roles of other apoptosis-related genes, particularly members of the expanding family of *Bcl-2*, need to be further addressed.

In summary, results of both our *in vitro* and clinical studies have suggested that high-dose tamoxifen may potentiate doxorubicin-induced apoptosis of HCC cells. This effect of tamoxifen is associated with its inhibition on the membrane translocation of PKC-α. Biochemical modulation of PKC as a measure to improve systemic chemotherapy for HCC deserves further investigation.

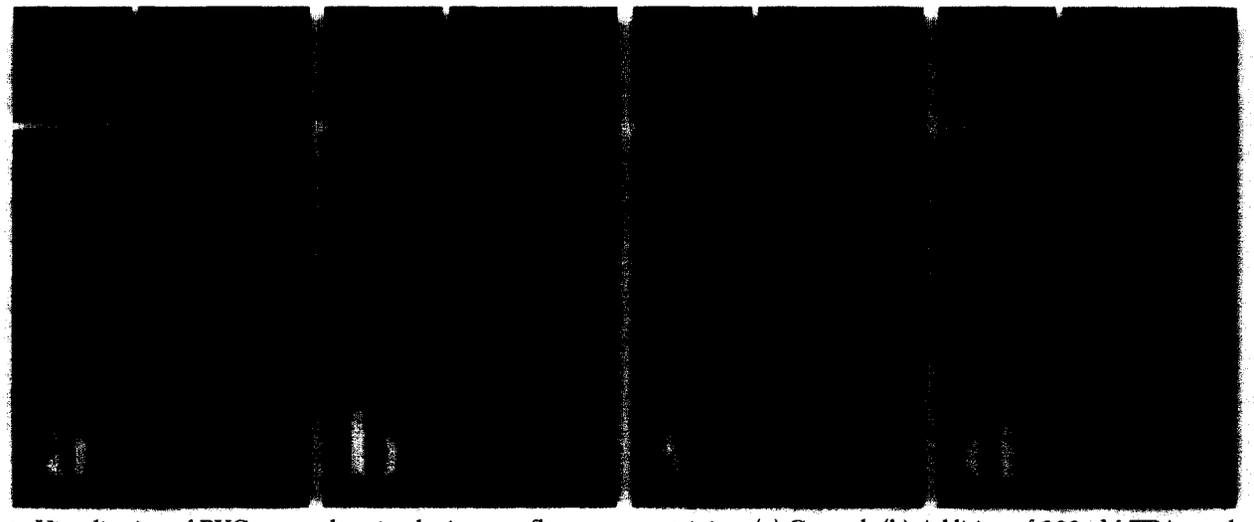

FIG. 9. Visualization of PKC-α translocation by immunofluorescence staining. (a) Control. (b) Addition of 200 nM TPA resulted in activation and membrane translocation of PKC-α. Further addition of 5 μM tamoxifen (c) and 10 μM tamoxifen (d) resulted in decreased membranous fluorescence, suggesting inhibition of PKC-α translocation to the cytoplasmic membrane. Representative cells are shown in the insets at the left upper corner of each panel.

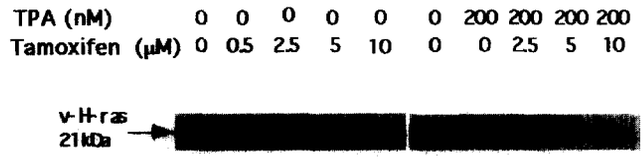

FIG. 10. Concentration-dependent inhibition of membrane translocation of V-H-Ras. (Experimental procedures were the same as those in Fig. 6, except that a V-H-Ras overexpressing Hep-3B cell clone was used.)

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