Combined Use of Tamoxifen, Cyclosporin A, and Verapamil for Modulating Multidrug Resistance in Human Hepatocellular Carcinoma Cell Lines

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The intensive use of chemotherapeutic agents for the treatment of cancer has resulted in the cure or improved survival of many patients. But unfortunately, many cancers including human hepatocellular carcinoma (HCC) don't respond to chemotherapy. One of the major mechanisms for the drug resistance in the HCC is an elevated MDR1 RNA expression which makes cells become multidrug resistant. To overcome the multidrug resistance (MDR) phenotype, a high dose of verapamil is required both clinically and experimentally. Accordingly we have examined the MDR modulating effects with combinations of tamoxifen, cyclosporin A, and verapamil in vitro with the physiologically achievable concentrations of each agent, i.e., 2.0 μM/L for tamoxifen, 1.6 μM/L for cyclosporin A, and 2.5 μM/L for verapamil respectively in HCC lines. As expected, verapamil alone with the physiologically achievable concentration at which we tested didn't enhance the doxorubicin cytotoxicity in the HCC lines. Furthermore, any verapamil combination with cyclosporin A or tamoxifen was not effective in overcoming the doxorubicin resistance in the high MDR1 expressor (Hep-G2) line. However tamoxifen reduced the IC50 of doxorubicin by a factor of 1.9 in the low MDR1 expressor (SK-Hep1) and 1.1 in the high MDR1 expressor line (p<10^-3 respectively). Of interest, combinations of tamoxifen and cyclosporin A showed a significant reduction in the IC50 of doxorubicin in both HCC lines. The IC50 of doxorubicin was reduced by a factor of 3.9 and 1.3, i.e., from 0.023943 μg/ml to 0.006157 μg/ml (p<10^-3) in the SK-Hep1 cell line, and 0.068819 μg/ml to 0.052442 μg/ml (p<10^-4) in Hep-G2 respectively when tamoxifen and cyclosporin A were administered together. Both the estrogen and progesterone receptors in the SK-Hep1 and Hep-G2 lines were less than 0.01 fmol/mg of cytosol protein, respectively. It is therefore suggested that the reversal of doxorubicin resistance is unrelated to their anti-estrogenic activity in the HCC lines. Three modulator combinations of tamoxifen, cyclosporin A, and verapamil were not more effective than the combination of tamoxifen and cyclosporin A on the sensitivity to doxorubicin. MDR modulators of tamoxifen, cyclosporin A, and verapamil didn't reduce the IC50 of cisplatin to the clinically achievable concentration range in HCC lines. In summary, the combination of tamoxifen and cyclosporin A at the concentrations normally seen after clinical administration of these modulators showed significant synergism on the sensitivity to doxorubicin in both low and high MDR1 expressor HCC lines. These data indicate the need for in vivo trials.

Key Words: Hepatocellular carcinoma, Multidrug resistance, IC50, Modulators
Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world especially in portions of Asia and Africa (Cook et al. 1985). The treatment of HCC remains unsatisfactory; especially, unresectable HCC which shows dismal prognosis with poor response to cytotoxic drugs. One of the major mechanisms for the drug resistance in this HCC is an elevated MDR1 RNA expression which makes cells become multidrug resistant (Guild et al. 1988; Goldstein et al. 1989).

The cellular mechanisms underlying the development of drug resistance are poorly understood. So far it has been shown that cells accumulate less drugs due to an increase in drug efflux in cell lines expressing the classical multidrug resistance (MDR) phenotype (Kessel and Wilberding 1985), defined by the presence of the transmembrane protein p-glycoprotein (Kartner et al. 1983). Verapamil, a calcium channel blocker, partially reverses the resistance of numerous MDR cell lines by decreasing drug efflux and increasing intracellular drug accumulation (Tsuro et al. 1982). Nevertheless, if intrinsic drug resistance in HCC can be attributed to the MDR mechanism, clinical studies incorporating modulators such as verapamil could be considered appropriate.

In such clinical studies, a particular difficulty lies in achieving plasma levels of the modulator which might have the desired effect on tumor cell drug transport (Kaye 1988). The dosage of verapamil required to test these in vitro experimental data resulted in an unacceptable level of cardiac toxicities in clinical studies (Ozols et al. 1987; Pennock et al. 1991).

Nevertheless, a range of other membrane active compounds have been identified as possessing similar MDR modulating capacities. Tamoxifen increased the sensitivity of doxorubicin-resistant murine leukemia subline (P388/ADR) (Ramu et al. 1984; Kessel 1986). Reversal of doxorubicin-acquired resistance by the tamoxifen is unrelated to its estrogenic or antiestrogenic activities (Ramu et al. 1984). Tamoxifen might exert its effects by different classes of membrane interactions to verapamil (Kessel 1986). Cyclosporin A has also been shown to modulate the expression of the MDR phenotype (Slater et al. 1986; Hu et al. 1990). Its mechanism of action is less well known than that of verapamil, but may relate to calmodulin inhibition (Slater et al. 1986). Using radiolabelled daunorubicin and flow cytometry, cyclosporin A increased drug accumulation in daunorubicin-resistant p388 murine leukemia cells, and MDR variants of T-cell lymphoblastic leukemia cell lines (Nooter et al. 1989; Hu et al. 1990).

We now report on the MDR modulating activity of a different group of modulators such as tamoxifen, cyclosporin A, and verapamil with clinically achievable concentrations in human HCC cell lines.

MATERIALS AND METHODS

Cell lines

Three human HCC cell lines, SK-Hepl, Hep-G2, and Hep-3B were obtained from the American Type Culture Collection. All cell lines were grown in RPMI 1640 (Grand Island Biological Co., Grand Island, NY, U.S.A.) supplemented with 10% fetal calf serum (Grand Island Biological Co.) and were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO2 and 95% air.

Drugs

All drugs were obtained from commercial sources: doxorubicin (Adriamycin; Adria Lab., Columbus, OH, U.S.A.); cisplatin (Ben Venue Lab., Bedford, OH, U.S.A.); tamoxifen (Sigma Chemical Co., St. Louis, MO, U.S.A.); cyclosporin A (Sandoz, East Hanover, NJ, U.S.A.); and verapamil (Searle Laboratories, Chicago, IL, U.S.A.).

Tamoxifen was dissolved with ethanol for a 10 mM/L stock solution and then diluted with normal saline for use. All other drugs were dissolved with normal saline.

Slot blot analysis

Methods for preparing RNA and slot blot analysis were performed as previously described (Davis et al. 1986). All RNA samples were loaded at serial threefold dilutions and probed with the MDR1 and β-actin probes (Goldstein et al. 1989). RNAs from the drug-sensitive KB-3-1 line and the drug-resistant KB-8-5 line were included in slot blot as in-
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ternal controls. Samples expressing ≥30 units of MDR1 RNA were scored as high expressers and those with values < 2 units were scored as low expressers (Park et al. 1990).

**MTT assay**

Single cell suspensions were obtained by trypsinization of monolayer cultures, and cell counts were performed using a hemocytometer. The MTT assay was performed as previously described (Carmichael et al. 1987); the number of cells plated into 96 wells was determined after preliminary cell growth studies using the MTT assay so that untreated cells were in the exponential phase at the time of initial harvest and at the end of the 4-day incubation. An equal number of cells were inoculated into each well in 0.18 ml of R10 medium, to which 0.02 ml of 10× concentration drug or normal saline was added. For both doxorubicin and cisplatin, 3-fold serial dilution was used with the highest doxorubicin concentration of 0.4 μg/ml and cisplatin concentration of 10 μg/ml covering clinically achievable concentration ranges (Alberts and Chen 1989).

The impact of MDR modulator was analyzed with the fixed concentration of 2.0 μM/L for tamoxifen, 1.6 μM/L for cyclosporin A, and 2.5 μM/L for verapamil which were all in the clinically achievable concentrarion range (Ozols et al. 1987; Willingham et al. 1986; Lien et al. 1991). Each single modulator as well as modulator combinations was tested for MTT assay with and without cytotoxic drugs. The IC50 (drug concentration which causes a 50% inhibition of the growth of the cell lines) value was defined as the drug concentration which produced a 50% reduction of absorbance at 540 nm.

All assays were performed in triplicate under sterile conditions. All data points represent the mean of a minimum six wells.

**Measurement of estrogen and progesterone receptors**

The tumor cells were rapidly frozen at −70°C after harvest using the rubber policeman until assayed. All assays were carried out on a cytosol fraction prepared by ultracentrifugation of the sonicated homogenates at 100,000g for one hour. The estrogen and progesterone receptors were measured by a solid phase enzyme immunoassay based on the sandwich principle using ER-EIA and PgR-EIA monoclonal antibodies’ kit by the manufacture’s protocol (Abbott Laboratories, North Chicago, IL, U.S.A.).

**Statistical analysis**

The interactions of cytotoxic drugs and MDR modulators were determined employing the previously described fractional product method of Webb as outlined by Valeriote and Lin (1975). Assume that drug A and B reduce the surviving fractions of cell lines to 10⁻³ and 10⁻⁴, respectively. The effects of the two drug combination is defined according to the surviving fraction: “antagonistic” if the surviving fraction is greater than 10⁻³; “interference” if it is between 10⁻³ and 10⁻⁴; “synergistic” if it is less than 10⁻⁴.

We considered probit, logit, one hit and Weibull models for fitting a dose-response curve (Finney 1971) and chose the model with the smallest chi-square value. IC50 is then obtained from the chosen model and its variance was obtained by the delta method (Bishop et al. 1975). To investigate cytotoxic interactions of two or more drugs, we employed a log-linear model (Wahrendorf et al. 1981; Piegrsch et al. 1988) and to estimate the model parameters we used GLIM macro (Baker and Nelder 1978; Waxcholder 1986) in the frame of a generalized linear model (McCullagh and Nelder 1983).

**RESULTS**

**Correlation between MDR1 expression level and sensitivity to cytotoxic drugs**

Levels of MDR1 RNA was high in Hep-G2, moderate in Hep-3B, and low in SK-Hepl (Fig. 1).

The half-lives of doxorubicin and cisplatin are 29.0 and 18.5 h respectively and clinically achievable area under the curves (AUCs) for doxorubicin and cisplatin are 2.9 and 1.9, respectively (Park et al. 1987). Clinically achievable drug concentrations of doxorubicin and cisplatin were calculated by the following formula: Clinically achievable AUC = clinically achievable drug concentration \( \times t_{\text{50}} \).
\[ t_{1/2} \] is the in vitro half-life of the drug at 37°C. By the calculation, the clinically achievable drug concentration was 0.053264 \( \mu \text{g/ml} \) for doxorubicin and 0.073332 \( \mu \text{g/ml} \) for cisplatin.

In each drug, if the IC50 value is greater than the clinically achievable drug concentration in a cell line, we defined it as a drug resistant line to that particular drug. By that definition, SK-Hep1, the low expressor of MDR1 RNA, was sensitive to doxorubicin. In contrast, Hep-G2 and Hep-3B, high and moderate expressors of MDR1 RNA, respectively, were resistant to doxorubicin (Table 1).

The IC50 values for cisplatin had no correlation to the MDR1 expression level and were higher than the clinically achievable drug concentrations in all 3 HCC lines that we tested.

Effect of MDR modulators on doxorubicin or cisplatin cytotoxicity

For the evaluation of the MDR modulating activity, we selected 2 cell lines of SK-Hep1 and Hep-G2 because they were low and high expressors of MDR1 RNA, respectively. Doxorubicin and cisplatin were used as positive and negative controls, respectively, to evaluate the MDR modulating activities of tamoxifen, cyclosporin A, and verapamil.

Tamoxifen showed the synergistic effect on doxorubicin cytotoxicity in both HCC lines, for high and low levels of MDR1 expression.
Table 1. IC50 (μg/ml) and its standard error (SE) of doxorubicin and cisplatin for each cell line exposed to physiologically achievable concentrations of tamoxifen, cyclosporin A, and verapamil

<table>
<thead>
<tr>
<th>Drug Combination</th>
<th>SK-Hepl (SE)</th>
<th>Hep-G2 (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX*</td>
<td>0.023943 (0.0002727)</td>
<td>0.068819 (0.0004472)</td>
</tr>
<tr>
<td>DDP*</td>
<td>1.222000 (0.0167486)</td>
<td>0.791366 (0.0068264)</td>
</tr>
<tr>
<td>DOX + Tm*</td>
<td>0.012352 (0.0001174)</td>
<td>0.063077 (0.0003162)</td>
</tr>
<tr>
<td>DDP + Tm</td>
<td>1.323800 (0.0139392)</td>
<td>0.427617 (0.0043630)</td>
</tr>
<tr>
<td>DOX + CsA*</td>
<td>0.045307 (0.00010832)</td>
<td>0.295759 (0.0054688)</td>
</tr>
<tr>
<td>DDP + CsA</td>
<td>20894900 (0.0276332)</td>
<td>1.288900 (0.0111159)</td>
</tr>
<tr>
<td>DOX + Vp*</td>
<td>0.024169 (0.0006814)</td>
<td>0.215226 (0.004733)</td>
</tr>
<tr>
<td>DDP + Vp</td>
<td>1.501740 (0.0120278)</td>
<td>0.877205 (0.0235331)</td>
</tr>
<tr>
<td>DOX + Tm + CsA</td>
<td>0.006157 (0.0001285)</td>
<td>0.052442 (0.0003162)</td>
</tr>
<tr>
<td>DDP + Tm + CsA</td>
<td>1.453110 (0.0155016)</td>
<td>1.369170 (0.0118124)</td>
</tr>
<tr>
<td>DOX + Tm + Vp</td>
<td>0.008868 (0.0001634)</td>
<td>0.92758 (0.0019148)</td>
</tr>
<tr>
<td>DDP + Tm + Vp</td>
<td>2.722980 (0.0265255)</td>
<td>0.599676 (0.0064884)</td>
</tr>
<tr>
<td>DOX + CsA + Vp</td>
<td>0.040454 (0.0009882)</td>
<td>0.077627 (0.0019308)</td>
</tr>
<tr>
<td>DDP + CsA + Vp</td>
<td>2.467250 (0.0237814)</td>
<td>1.364180 (0.0138923)</td>
</tr>
<tr>
<td>DOX + Tm + CsA + Vp</td>
<td>0.012377 (0.0001739)</td>
<td>0.150195 (0.0033353)</td>
</tr>
<tr>
<td>DDP + Tm + CsA + Vp</td>
<td>4.220110 (0.0765542)</td>
<td>4.020880 (0.0479307)</td>
</tr>
</tbody>
</table>

*drug concentration which causes 50% inhibition of the growth of the cell lines, *doxorubicin, *cisplatin, *tamoxifen, *cyclosporin A, *verapamil

\[ \text{The IC50 of doxorubicin was changed from } 0.023943 \mu g/ml \text{ to } 0.012352 \mu g/ml (p<10^{-5}) \text{ by a factor of 1.9 in SK-Hepl, and from 0.068819 \mu g/ml to 0.0630766 } \mu g/ml (p<10^{-5}) \text{ by a factor of 1.1 in the presence of tamoxifen alone. However, verapamil or cyclosporin A alone didn't} \]
show any synergism with the dose of 2.5 \( \mu M \)/L and 1.6 \( \mu M \)/L, respectively, on doxorubicin cytotoxicity in either HCC cell line (Table 1, Fig. 2, 3).

What is of special interest is that tamoxifen and cyclosporin A combinations showed universal synergism on doxorubicin cytotoxicity regardless of MDR1 expression level in both HCC cell lines, but not on cisplatin toxicity (Table 1, Fig. 3, 4). The IC50 of doxorubicin was reduced by a factor of 3.9 and 1.3, i.e., from 0.023943 \( \mu g/\text{ml} \) to 0.006157 \( \mu g/\text{ml} \) \((p<10^{-5})\) in SK-Hepl, and 0.068819 \( \mu g/\text{ml} \) to 0.052442 \( \mu g/\text{ml} \) \((p<10^{-5})\) in Hep-G2 cell lines, respectively, when tamoxifen and cyclosporin A were administered together. The tamoxifen and verapamil combination showed the synergistic effect on the doxorubicin cytotoxicity in SK-Hepl but not in Hep-G2. The IC50 of doxorubicin in SK-Hepl was reduced to 0.008698 \( \mu g/\text{ml} \) \((p<10^{-5})\) by a factor of 1.3 after combined administration of tamoxifen and verapamil. However, the modulator combination of verapamil and cyclosporin A didn’t show any synergism on doxorubicin or cisplatin cytotoxicity in either cell line.

The 3 modulator combination of tamoxifen, cyclosporin A, and verapamil were not more effective on doxorubicin cytotoxicity than the 2 modulator combination of tamoxifen and cyclosporin A (Table 1). The 3 modulator combination of tamoxifen, cyclosporin A, and verapamil showed the synergism on the doxorubicin cytotoxicity in SK-Hepl. However, the IC50 of doxorubicin was reduced to 0.0123766 \( \mu g/\text{ml} \) \((p<10^{-5})\) by a factor of 1.9 in SK-Hepl but reduced to 0.006157 by a factor of 3.9 with the two modulator combination of tamoxifen and cyclosporin A. No single modulator or modulator combination showed synergism on cisplatin cytotoxicity in both cell lines except the combination of tamoxifen and verapamil in Hep-G2 (Table 1). In the Hep-G2 cell line, the IC50 of cisplatin was reduced by a factor of 1.8, i.e., from 0.791366 \( \mu g/\text{ml} \) to 0.599676 \( \mu g/\text{ml} \) \((p<10^{-5})\) in the presence of tamoxifen and verapamil; it is, however, still resistant to cisplatin according to our definition of drug resistance.

**Estrogen and progesterone receptors**

Tamoxifen shows the synergistic effect on
doxorubicin cytotoxicity. Therefore we measured the estrogen and progesterone receptors to evaluate the relationship between the synergism and receptor status. The concentration of estrogen receptor was nil in Hep-G2 and below 0.01 fmole/mg cytosol protein in SK-Hep1. Progesterone receptor activities were below 0.01 fmole/mg of cytosol protein in both SK-Hep1 and Hep-G2 cell lines.

**DISCUSSION**

Tsuruo et al reported that verapamil increased the accumulation of vincristine in vitro in a vincristine resistant p388 murine leukemia cell line by decreasing drug efflux and also that it enhanced the cytotoxic effects of vincristine both in vitro and in vivo (Tsuruo et al. 1981; Tsuruo et al. 1982). In addition, evidence suggests that this multidrug resistance reversal is dose related, such that greater MDR reversal can be achieved with higher concentrations of verapamil (Dalton et al. 1989). In a recent pilot study, plasma levels of verapamil approaching drug concentrations needed to reverse drug resistance in vitro resulted in unacceptable cardiac toxicity (Ozols et al. 1987; Pennock et al. 1991). Therefore chemosensitizers or modulator combinations with the acceptable ranges of toxicities are needed to reverse MDR in cancer in vivo. To overcome the MDR phenotype, the high doses of verapamil are required both clinically and experimentally. Accordingly we have examined the MDR modulators of tamoxifen, cyclosporin A, and verapamil in vitro to determine whether an interaction between these compounds would allow physiologically achievable concentrations of each agent that can be used clinically to modulate drug resistance.

There was no apparent relationship between the level of MDR1 expression and adriamycin sensitivity in HCC lines. The IC50 value of doxorubicin was higher in the moderate MDR1 expressor cell line (Hep-3B) than in the high MDR1 expressor (Hep-G2) (data not shown).

However, among these 3 HCC lines the IC50 of doxorubicin was lowest in the low MDR1 expressor, cell line (SK-Hep1) being 2.9 fold lower than that of Hep-G2 and also in the clinically achievable concentration range. Dalton et al. (1989) demonstrated a close relationship between the level of resistance to doxorubicin and the amount of p-glycoprotein in the human myeloma cell line. However, Lai et al. (1989) described that there was a lack of correlation between MDR1 RNA levels and sensitivity to etoposide in lung cancer cell lines. These results including the current data also suggest that several other biochemical mechanisms including glutathione s-transferase or topoisomerase systems can be involved in MDR (Moscow and Cowan 1988).

As expected, verapamil alone with the physiologically achievable concentration at which we tested didn’t enhance the doxorubicin cytotoxicity in the HCC lines. There are many reports that verapamil alone is effective in circumventing the MDR in various tumor cell lines. They used higher concentrations of verapamil than we used in the current study (Tsuruo et al. 1983; Twentyman et al. 1986). Furthermore, no verapamil combination with tamoxifen or cyclosporin A was effective enough to overcome the doxorubicin resistance in the high MDR1 expressor line.

Both tamoxifen and cyclosporin A alone are effective for reversal of MDR in the leukemic cell lines (Ramu et al. 1984; Kessel 1986; Slater et al. 1986). In this study, however, cyclosporin A alone at the concentration that we tested was not synergistic on doxorubicin cytotoxicity but antagonistic in both the low and high MDR1 expressor HCC line. The mechanism of antagonistic interaction between cyclosporine A at the clinically achievable concentration on the doxorubicin cytotoxicity in the hepatoma cell line should be further explored. Meanwhile tamoxifen reduced the IC50 of doxorubicin by a factor of 1.9 in the low MDR1 expressor but the reduction of IC50 was made by a factor of 1.1 in the high MDR1 expressor line. Combinations of tamoxifen and cyclosporin A showed synergism on doxorubicin cytotoxicity in both HCC lines, 3.8 fold in the low MDR1 expressor and 1.3 fold in the high expressor in terms of IC50. Because tamoxifen shows the synergistic effect on doxorubicin cytotoxicity, we measured the estrogen and progesterone receptor in the HCC lines. Both the estrogen and progesterone receptor in the SK-Hep1
and Hep-G2 lines were less than 0.01 fmole/mg of cytosol protein, respectively. It is therefore suggested that the reversal of doxorubicin resistance by the tamoxifen is unrelated to their anti-estrogenic activity. Ramu et al. (1984) showed the reversal of acquired resistance to doxorubicin sensitivity of P388/ADR cells could not be reversed by 17 β-estradiol. Estrogen receptor could not be demonstrated in that cell line.

Hu et al. (1990) reported that cyclosporin A and verapamil showed significant synergism on the cytotoxicity of doxorubicin when tested in combination in the moderately and highly drug resistant T-cell leukemia cell line (CEM/VLB100 and CEM/VLB1000 respectively) at concentrations normally seen after clinical administration of these modulators. However, in the highly resistant variant, higher concentrations of both biochemical modulators for the synergistic interaction on doxorubicin cytotoxicity are required than in the moderately resistant variant line. In the current study, there was no synergistic interaction with cyclosporin A or verapamil on the doxorubicin cytotoxicity. Furthermore, the combination of cyclosporin A and verapamil didn’t show any synergism on doxorubicin cytotoxicity in the hepatoma cell lines. If we put these data together, the interactions of biochemical modulators on doxorubicin may be different by the type of tumor cell lines and the degree of resistance.

Three modulator combinations of tamoxifen, cyclosporin A, and verapamil were not more effective than the combination of tamoxifen and cyclosporin A on the sensitivity to doxorubicin. Probably verapamil at the concentration that we tested had the negative effect on the doxorubicin cytotoxicity in the HCC lines. Three HCC lines that we tested were resistant to cisplatin. These data confirm the in vivo result (Ravry et al. 1986). Only the combination of tamoxifen and verapamil reduced the IC50 of cisplatin in the Hep-G2 line by a factor of 1.3 but still the IC50 is much higher than that of the clinically achievable cisplatin concentration. By the definition of the drug resistance described before, no HCC line was sensitive to cisplatin even with the administration of biochemical modulators.

In summary, the combination of tamoxifen and cyclosporin A at concentrations normally seen after the clinical administration of these modulators showed significant synergism on the sensitivity to doxorubicin in both low and high MDR1 expressor HCC lines. These data indicate the need for in vivo trials.

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