Yonsei Medical Journal
Vol. 34, No. 1, 1993

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

Joo-Hang Kim, Jae Bok Chung, In Suh Park, Byung Soo Kim[1]
Nae Chun Yoo, Jin Hyuk Choi, Jae Kyung Roh, Hyon-Suk Kim
Oh Hun Kwon, Kyong Sik Lee and Byung Soo Kim[2]

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 ($p<10^{-5}$ respectively). Of interest, combinations of tamoxifen and cyclosporin A showed a significiant 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 ($p<10^{-5}$) in the SK-Hep1 cell line, and 0.068819 μg/ml to 0.052442 μg/ml ($p<10^{-5}$) 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 modualtor 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

Received December 17, 1992
Accepted February 10, 1993

Yonsei Cancer Center, Institute for Cancer Research [J.-H.K., N.C.Y., J.H.C., J.K.R., K.S.L., B.S.K.[2]], Departments of Internal Medicine [J.-H.K., J.B.C., I.S.P., N.C. Y., J.H.C., J.K.R.], Clinical Pathology [H.-S.K., O.H.K.] Surgery [K.S.L.], Yonsei University College of Medicine, Applied Statistics [B.S.K.[1]], Yonsei University, Seoul, Korea

This study was supported by a faculty research grant (1991) of Yonsei University College of Medicine

Address reprint requests to Dr. J-H Kim,. Yonsei Cancer Center, Department of Internal Medicine, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul, 120-749, Korea

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 the 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 incorperating 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-Hep1, 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℃ in an atomsphere of 5% CO_2 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-

ternal controls. Samples expressing≥30 units of MDR1 RNA were scored as high expressors and those with values < 2 units were scored as low expressors (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 1980).

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℃ 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).

Statistial 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^{-1} and 10^{-2}, respectively. The effects of the two drug combination is defined according to the surviving fraction: "antagonistic" if the surviving fraction is greater than 10^{-1}; "interference" if it is between 10^{-1} and 10^{-2}; "synergistic" if it is less than 10^{-3}.

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; Piegorsch *et al.* 1988), and to estimate the model parameters we used GLIM macro (Baker and Nelder 1978; Wacholder 1986) in the frame of a generalized linear model (McCullagh and Nelder 1983).

RESULTS

Correlation betwwen 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-Hep1 (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 2.0 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 $\times t_{1/2}$

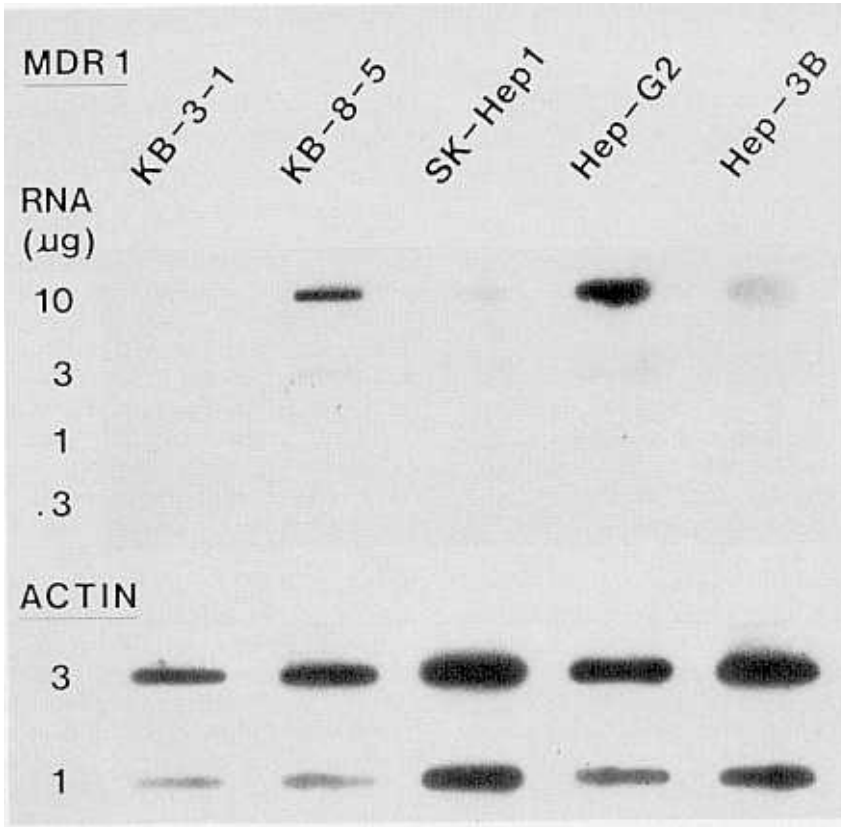

Fig. 1. *Slot blot analysis of MDR1 expression in hepatocellular carcinoma cell lines. Serial dilution of 10, 3, 1, and 0.3 μg of total RNA were applied to each well. Hybridization of β-actin probe demonstrated comparable amounts of RNA loaded in all wells. KB-3-1 is drug sensitive parental KB cell line; KB-8-5 is multidrug resistant KB subline.*

$\times 1.44 \times [1 - e^{-(.693)(96)T1/2}]$ where $t_{1/2}$ is the *in vitro* half-life of the drug at 37℃. By the calculation, the clinically achievable drug concentration was 0.053264 μg/ml for doxorubicin and 0.073332 μg/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[a] (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

Drug Combination	SK-Hep1 (SE)	Hep-G2 (SE)
DOX[b]	0.023943 (0.0002727)	0.068819 (0.0004472)
DDP[c]	1.222000 (0.0167486)	0.791366 (0.0068264)
DOX+Tm[d]	0.012352 (0.0001174)	0.063077 (0.0003162)
DDP+Tm	1.323800 (0.0139392)	0.427617 (0.0043630)
DOX+CsA[e]	0.045307 (0.0010832)	0.295759 (0.0054688)
DDP+CsA	20894900 (0.0276332)	1.288900 (0.0111159)
DOX+Vp[f]	0.024169 (0.0006814)	0.215226 (0.0044733)
DDP+Vp	1.501740 (0.0120278)	0.877205 (0.0235331)
DOX+Tm+CsA	0.006157 (0.0001285)	0.052442 (0.0003162)
DDP+Tm+CsA	1.453110 (0.0155016)	1.369170 (0.0118124)
DOX+Tm+Vp	0.008689 (0.0001634)	0.92758 (0.0019148)
DDP+Tm+Vp	2.722980 (0.0265255)	0.599676 (0.0064884)
DOX+CsA+Vp	0.040454 (0.0009882)	0.077627 (0.0019308)
DDP+CsA+Vp	2.467250 (0.0237814)	1.364180 (0.0138923)
DOX+Tm+CsA+Vp	0.012377 (0.0001739)	0.150195 (0.0033353)
DDP+Tm+CsA+Vp	4.220110 (0.0765542)	4.020880 (0.0479307)

[a]drug concentration which causes 50% inhibition of the growth of the cell lines, [b]doxorubicin, [c]cisplatin, [d]tamoxifen, [e]cyclosporin A, [f]verapamil

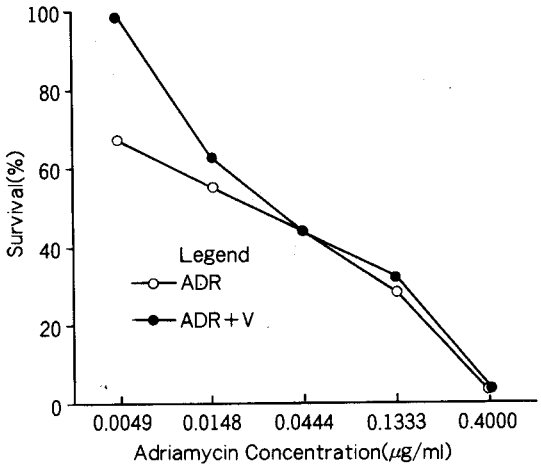

Fig. 2. *Effects of verapamil upon growth-inhibitory action of doxorubicin in SK-Hep1 cell line. Cells were treated with a fixed concentration of verapamil (2.5 μM/L), and 3 fold serial dilution of doxorubicin with the highest concentration of 0.4 μg/ml.*

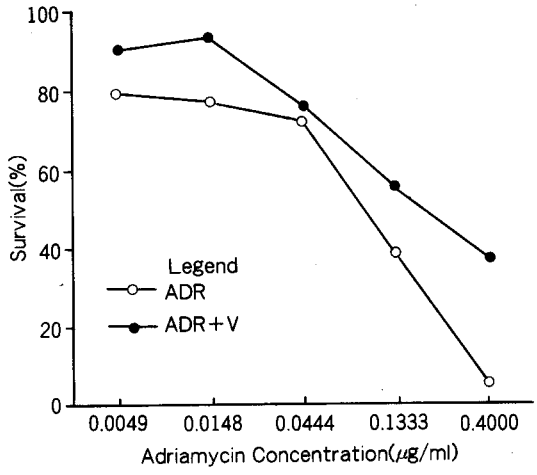

Fig. 3. *Effects of verapamil upon growth-inhibitory action of doxorubicin in Hep-G2 cell line. Cells were treated with a fixed concentration of verapamil (2.5 μM/L), and 3 fold serial dilution of doxorubicin with the highest concentration of 0.4 μg/ml.*

The IC50 of doxorubicin was changed from 0.023943 μg/ml to 0.012352 μg/ml ($p<10^{-5}$) by a factor of 1.9 in SK-Hep1, and from 0.068819 μg/ml to 0.0630766 μg/ml ($p<10^{-5}$) by a factor of 1.1 in the presence of tamoxifen alone. However, verapamil or cyclosporin A alone didn't

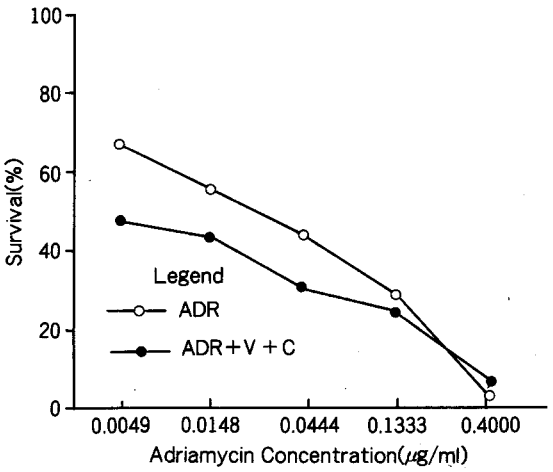

Fig. 4. *Synergistic interaction of tamoxifen and cyclosporin A combination on doxorubicin cytotoxicity in SK-Hepl cell line. Cells were treated with a fixed concentration of tamoxifen (2.0 μM/L) and cyclosporin A (1.6 μM/L), and 3 fold serial dilution of doxorubicin with the highest concentration of 0.4 μg/ml.*

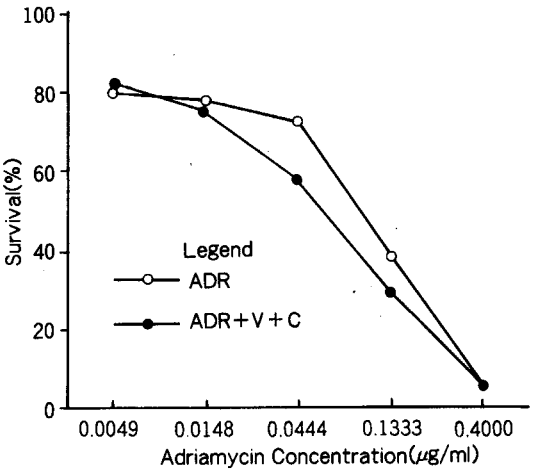

Fig. 5. *Synergistic interaction of tamoxifen and cyclosporin A combination on doxorubicin cytotoxicity in Hep-G2 cell line. Cells were treated with a fixed concentration of tamoxifen (2.0 μM/L) and cyclosporin A (1.6 μM/L), and 3 fold serial dilution of doxorubicin with the highest concentration of 0.4 μg/ml.*

show any synergism with the dose of 2.5 μM/L and 1.6 μM/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 μg/ml to 0.006157 μg/ml ($p<10^{-5}$) in SK-Hepl, and 0.068819 μg/ml to 0.052442 μg/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 0.0086898 μg/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 μg/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 μg/ml to 0.599676 μg/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

Tamoxofen 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-Hepl. Progesterone receptor activities were below 0.01 fmole/mg of cytosol protein in both SK-Hepl and Hep-G2 cell lines.

DISCUSSION

Tsuruo *et al* reported that verapamil increased the accumulation of vincristine *in vitro* in a vincristein 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; Tsuro *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-Hepl) 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 cel 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 concentratqsions 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-Hepl

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.

REFERENCES

Albert DS, Chen H-SG: *Tabular summary of pharmacokinetic parameters relevant to in vitro drug assay.* In Salmon SE, ed. *Cloning of human tumor stem cells, pp. 351-359.* New York: Alan R. Liss., *1980*

Baker RJ, Nelder JA: *The GLIM system, release 3, manual.* Oxford: Numerical Algorithm Group, *1978*

Bishop YMM, Fienberg SF, Holland PW: Discrete multivariate analysis, The MIT press, Cambridge, *1975*

Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB: Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res 47: 936-942, 1987*

Cook GC, Moosa B: Hepatocellular carcinoma: One of the world's most common malignancies. *Am J Med 233: 705-708, 1985*

Dalton WS, Grogan TM, Meltzer PS, Scheper RJ, Durie BGM, Taylor CW, Miller TP, Salmon SE: Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: Detection of p-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. *J Clin Oncol 7: 415-424, 1989*

Dalton WS, Grogan TM, Rybski JA, Scheper RJ, Richter L, Kailey J, Broxterman HJ, Pineido HM, Salmon SE: Immunohistochemical detection and quantitation of p-glycoprotein in multiple drug-resistant human myeloma cells: Association with level of drug resistance and drug accumulation. *Blood 73: 747-752, 1989*

Davis LG, Dibner MD, Battey JF: *Basic methods in molecular biology.* Amsterdam, Elsevier/North Holland, *1986*

Finney DJ: *Probit analysis,* 3rd ed, Cambridge University Press, Cambridge, *1971*

Goldstein LJ, Galski H, Fojo A, Willingham M, Lai S-L, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM, Lieber M, Cossman J, Gottesman MM, Pastan I: Expression of a multidrug resistance gene in human cancers. *J Natl Cancer Inst 81: 116-124, 1989*

Guild BC, Mulligan RC, Gros P, Housman DE: Retroviral transfer of a murine cDNA for multidrug resistance confers pleiotropic drug resistance to cell without prior drug selection. *Proc Natl Acad Sci USA 85: 1595-1599, 1988*

Hu XF, deLuise M, Martin TJ, Zalcberg JR: Effect of cyclosporin and verapamil on the cellular kinetics of daunorubicin. *Eur J Cancer 7: 814-817, 1990*

Hu XF, Martin TJ, Bell DR, deLuise M, Zalcgerg JR: Combined use of cyclosporin A and verapamil in modulating multidrug resistance in human leukemia cell lines. *Cancer Res 50: 2953-2957, 1990*

Kartner N, Riordan JR, Ling V: Cell-surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. *Science (Wash DC)221; 1285-1288, 1983*

Kaye SB: The multidrug resistance phenotype. *Br J Cancer 58: 691-694, 1988*

Kessel D: Interactions among membrane transport system: Anthracyclines, calcium antagonists and anti-estrogens. *Biochem Pharmacol 35: 2825-2826, 1986*

Kessel D, Wilberding C: Anthracyline resistance in P388 murine leukemia and its circumvention by calcium antagonists. *Cancer Res 45: 1687-1691, 1985*

Lai S-L, Goldstein LJ, Gottesman MM, Pastan I, Tsai C-M, Johnson BE, Mulshine JL, Ihde DC, Kayser K, Gazdar AF: MDR1 gene expression in lung cancer. *J Natl Cancer Inst 81: 1144-1150, 1989*

Lien EA, Solheim E, Ueland PM: Distribution of tamoxifen and its metabolites in rat and human tissues during steady-state treatment. *Cancer Res 51: 4837-4844, 1991*

McCullagh P, Nelder JA: *Generalized linear models.* London. Chapaman and Hall, *1983*

Moscow JA, Cowan KH: Multidrug resistance. *J Natl Cancer Inst 80: 14-20, 1988*

Nooter K, Oostrum R, Jonker R, Dekken H, Stokdijk W, Engh G: Effect of cyclosporin A on daunorubicin accumulation in multidrug-resistant P388 leukemia cells measured by real-time flow cytometry. *Cancer Chemother Pharmacol 23: 296-300, 1989*

Ozols RF, Cunnion RE, Klecker RW, Hamilton TC, Ostchega Y, Parrillo JE, Young RC: Verapamil and adriamycin in the treatment of drug-resistant ovarian cancer patients. *J Clin Oncol 5: 641-647, 1987*

Park J-G, Kramer BS, Steinberg SM, Carmichael J, Collins JM, Minna JD, Gazdar AF: Chemisensitivity testing of human colorectal carcinoma cell lines using a tetrazolium-based colorimetric assay. *Cancer Res 47: 5875-5879, 1987*

Park J-G, Kramer BS, Lai S-L, Goldstein LJ, Gazdar AF: Chemosensitivity patterns and expression of human multidrug resistance-associated MDR1 gene by human gastric and colorectal carcinoma cell lines. *J Natl Cancer Inst 82: 193-198, 1990*

Pennock GD, Dalton WS, Roeske WR, Appleton CP, Mosley K, Plezia P, Miller TP, Salmon SE: Systemic toxic effects associated with high-dose verapamil infusion and chemotherapy administration. *J Clin Oncol 83: 105-110, 1991*

Piegorsch WW, Weinberg CR, Margolin BH: Exploring simple independent action in multi-factor tables of proportions. *Biometrics 44: 595-603, 1988*

Ramu A, Glaubiger D, Fucks Z: Reversal of acquired resistance to doxorubicin in P388 murine leukemia cells by tamoxifen and other triparanol analogues. *Cancer Res 44: 4392-4395, 1984*

Ravry MJR, Omura GA, Bartolucci AA, Einhorn L, Kramer B, Davila E: Phase II evaluation of cisplatin in advanced hepatocellular carcinoma and cholangiocarcinoma: A Southwestern Cancer Study Group Trial. *Cancer Treat Res 70: 311-312, 1986*

Slater LM, Sweet P, Stupecky M, Gupta S: Cyclosporin A reverses vincristine and daunorubicin resistance in acute lymphatic leukemia in vitro. *J Clin Invest 77: 1405-1408, 1986*

Tsuro T, Iida H, Tsukagoshi S, Sakurai Y: Increased accumulation of vincristine and adriamycin in drug-resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. *Cancer Res 42: 4730-4733, 1982*

Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y: Potentiation of vincristine and adriamycin effects in human hemopoietic tumor cell lines by calcium antagonists and calmodulin inhibitors. *Cancer Res 43: 2267-2272, 1983*

Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y: Overcoming of vincristine resistance in P388 leukemia, in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res 41: 1967-1972, 1981*

Twentyman PR, Fox NE, Bleehen NM: Drug resistance in human lung cancer cell lines: Cross resistance studies and effects on the calcium transport blocker, verapamil. *Int J Radiat Oncol Biol Phys 12: 1355-1358, 1986*

Valeriote F, Lin H: Synergistic interaction of anticancer agents: A cellular perspective. *Can-*

cer Chemother Rep 59: 895-900, 1975

Wacholder S: Binomial regression for GLIM: Estimating risk ratios and risk differences. *Am J Epidemiol 123: 174-184, 1986*

Wahrendorf J, Zentgraf R, Brown CC: Optimal design for the analysis of interactive effects of two carcinogens or other toxicants. *Biometrics 37: 45-54, 1981*

Willingham MC, Cornwell MM, Cardarelli CO, Gottesman MM, Pastan I: Single cell analysis of daunomycin uptake and efflux in multidrug-resistant and sensitive KB cells: Effects of verapamil and other drugs. *Cancer Res 46: 5941-5946, 1986*