Arch. Pharm. Res. Vol.19, No.5, pp. 342-347, 1996

Establishment of Doxorubicin-resistant Subline Derived from HCT15 Human Colorectal Cancer Cells

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(Received July 29, 1996)

Doxorubicin, one of the clinically most useful anticancer agents, is used alone or in combination with other drugs against a wide variety of tumors, recently. But cancer cells developed resistance to this agent in many ways. This resistance is an important limiting factor of doxorubicin for anticancer drug. We newly established doxorubicin-resistant HCT15/CL02 subline from parental HCT15 human adenocarcinoma colon cancer cells. HCT15/CL02 revealed resistance to doxorubicin about 85-fold of its parental cells, and it also revealed cross-resistance to actinomycin D, etoposide and vinblastine but not to cisplatin and tamoxifen. And verapamil, a reversal agent of multidrug-resistance (MDR) by P-glycoprotein, elevated the cytotoxicity of doxorubicin against both HCT15 and HCT15/CL02 cells. But the relative resistant rate was not reduced. Verapamil had no effects on the toxicity of cisplatin to the both cell lines. These results indicate that HCT15/CL02 cells have some functionally complex mechanisms for MDR.

Key words : Doxorubicin-resistance, Multidrug-resistance, Human colon cancer, P-glycoprotein, Verapamil

INTRODUCTION

Doxorubicin is one of the most extensively used anti-cancer agents, and it exhibits a broad spectrum of antitumor activity not only against leukemia but also against solid tumors (Weiss *et al.*, 1986). Its major mechanism of antitumoral action is DNA-intercalation to interfere with the reaction of topoisomerase II, an ubiquitous enzyme that can alter the topological state of DNA and untangle intertwined DNA helices (Chen and Liu, 1994; Dimanche-Boitrel *et al.*, 1992; Lock and Ross, 1987; Wang, 1985; Zunino and Caporamco, 1990). Doxorubicin is also converted to a semiquinone free radical resulting in binding of the drug to DNA and proteins or production of the superoxide anion radical, hydrogen peroxide and ultimately hydroxyl radicals (Turner *et al.*, 1990).

Unfortunately, cancer cells developed resistance to antineoplastic drugs in many ways, and it is one of the major obstacles in cancer chemotherapy. The gastrointestinal carcinomas are known to be largely unresponsive to chemotherapy. Resistance of colorectal cancers to chemotherapy, particularly the natural products, is not surprising, since this malignancy arises from cells which are constantly exposed to naturally occuring toxins (Critchfield *et al.*, 1994; Klohs and Steinkampf, 1988). Therefore, human colon carcinoma represents a complex model in which to study multidrug resistance (MDR, Lai *et al.*, 1991a).

One of the human adenocarcinoma cell line, HCT 15 cells were established from a colorectal cancer after surgical resection before chemotherapeutic treatment (Tompkins *et al.*, 1974). HCT15 cells are exhibited naturally resistant to doxorubicin, and the resistance is considered to be mainly attributable to an overexpression of P-glycoprotein (PGP) which is a 170 kDa membrane phosphoglycoprotein (Iwahashi *et al.*, 1991; Mickley *et al.*, 1989). PGP is encoded by the mdr1 gene, and it appears to act as an ATP-dependent membrane efflux pump in MDR cells, resulting in decreased intracellular accumulation of many structurally unrelated lipophilic drugs which have differing modes of action (Damle and Desai, 1994; Fojo *et al.*, 1987; Juranka *et al.*, 1989). On the other hand, it has been already reported that verapamil and some other agents acting on membrane can reverse the MDR phenotype *in vitro* (Cornwell *et al.*, 1986; Tsuruo, 1983). Verapamil, a Ca^{++} channel blocker, increases intracellular drug levels by inhibiting the efflux of the drug from tumor cells (Tsuruo *et al.*, 1981).

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Verapamil and the anti-cancer drugs bind to PGP competitively and are believed to be transported out of the cells by a common mechanism (Naito and Tsuruo, 1989). So reversal of decreased drug accumulation by verapamil in drug-resistant cells can be a hallmark of PGP-mediated drug resistance (Lai *et al.*, 1991a).

In the present study, we have newly established a doxorubicin-resistant cell line derived from HCT15 cells by stepwise increase of doxorubicin in its concentration. And then, we have tested this subline for the cross-resistance to some other anticancer drugs and have also tested the effect of verapamil on the drug resistance of both parental HCT15 and doxorubicin-resistant HCT15/CL02 cells.

MATERIALS AND METHODS

Chemicals

Anticancer agents such as doxorubicin, vinblastin, cisplatin, tamoxifen, etoposide, actinomycin D and MDR reversal agent, verapamil were purchased from Sigma Chemical Co (St. Louis, MO). The RPMI 1640 medium and fetal bovine serum were purchased from Gibco Ltd (Grand Island, N.Y.), and other cell culture agents such as sodium bicarbonate, gentamycin, amphotericin and 1,2-cyclohexanediaminetetraacetic acid (CDTA) were obtained from Sigma Chemical Co (St. Louis, MO).

Selection of doxorubicin-resistant subline

We have established a doxorubicin-resistant subline by stepwise and continuous exposure of parental HCT 15 cells to increasing concentrations of the drug. We have started HCT15 cell cultures with 1 M doxorubicin and maintained the cells for 4 weeks in this concentration. After this, growing HCT15 cells were isolated and cultured with 3 μM doxorubicin. We also isolated growing HCT15 cells in 3 μM after 10 weeks, and the growing cells were cultured with increasing concentration of doxorubicin step by step upto 100 μM about for 2 years. Finally we selected colonies which survived at the concentration of 100 μM of the drug. These survival colonies were grown in fresh medium without doxorubicin for additional 3 months, and we tested doxorubicin-resistance of cells derived from each colony. Cells from colony-02 have revealed considerable resistance to the agent, and finally we have selected the cells as doxorubicin-resistant subline (HCT15/CL02). The summarized procedure for isolation of HCT15/CL02 was shown in Fig. 1.

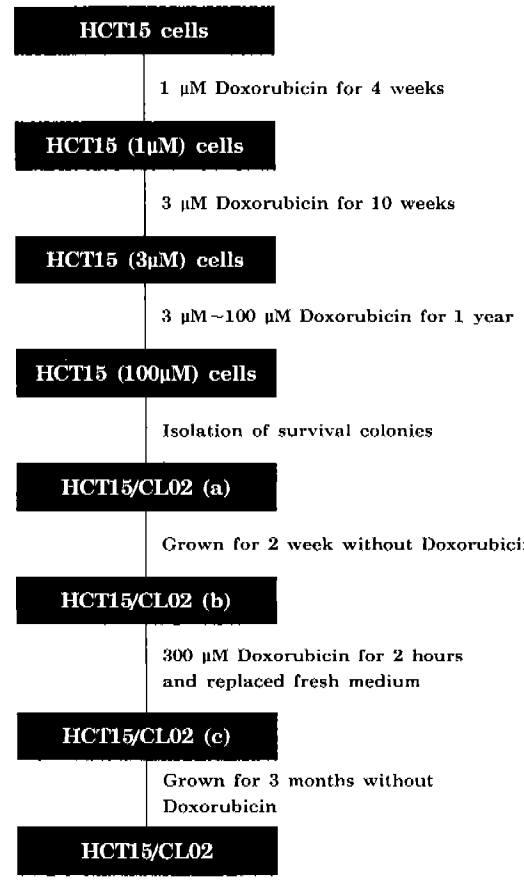

Fig. 1. Summarized scheme of isolation of doxorubicin resistant HCT15/CL02 subline from HCT15 human colorectal cancer cells

Cell culture

Parental HCT15 human colorectal tumor cells and isolated HCT15/CL02 subline cells were grown in T-25 (Falcon) flasks containing 10 ml of RPMI 1640 medium with 300 mg/l glutamine, 2.0 g/l sodium bicarbonate, 40 mg/l gentamycin, 2.5 mg/l amphotericin and 5% fetal bovine serum. The cells were dissociated with 0.25% trypsin and 3 mM CDTA in PBS in case of transfer or dispense before experiment. The cells were maintained at 37°C incubator in a humidified atmosphere of 5% CO_2 in air continuously except when adding drugs.

Cytotoxicity assay *in vitro*

All experimental procedures were followed the NCI (USA)'s protocol based on the Sulforhodamine B (SRB) method as described previously (Ryu *et al.*, 1992; Skehan *et al.*, 1990). Briefly, tumor cells were inoculated over a series of standard 96-well flat bottom microtiter plates on day 0. These cells were then preincubated for 24 hours for attachment on the microtiter plate. The compounds such as doxorubicin, cisplatin, tamoxifen, vinblastin, actinomycin D and etoposide were added to the wells in serial dilutions starting from the highest concentrations. At the ter-

mination of incubation with each drug for 72 hours, the culture medium in each well was removed, and the cells were fixed with cold 10% trichloroacetic acid. The microplates were washed and dried after incubation at 4°C for 1 hour. And then, 0.4% SRB solution was added and incubated at room temperature for 30 minutes. The cells were washed again, and the bound stain was solubilized with 10 mM unbuffered Tris base solution (pH 10.5). The absorbances were measured spectrophotometrically at 520 nm and 690 nm in a microtiter plate reader (Molecular Devices E-max, Sunnyvale, CA). The absorbance measured at 690 nm was substracted from the absorbance at 520 nm so as to eliminate the effects of non-specific absorbance.

For the study of the effects of verapamil on the cytotoxicities of anti-cancer drugs, attached cells were incubated with serial dilutions of anticaner drugs in the presence or absence of 5 μg/ml verapamil. After 72 hours of continuous drug-exposed time, the survival fractions were measured by the same method to the previous cytotoxicity test.

The data were transferred and transformed into Lotus-123 format and survival fractions were calculated by comparing the drug treated with controls. All data represented the average values for a minimum of four distinct experiments.

RESULTS AND DISCUSSION

Several mechanisms of drug resistance have been suggested. Among them, multidrug resistance by PGP has been most extensively studied and characterized (Bradley *et al.*, 1988; Chen *et al.*, 1994; Park *et al.*, 1994; Ueda *et al.*, 1987). The MDR by PGP is characterized by cross-resistance to several structurally unrelated drugs including vinca alkaloides, doxorubicin, mitomycin C and other natural products (Damle and Desai, 1994; Gheuens *et al.*, 1993; Kartner *et al.*, 1983; Ueda *et al.*, 1987).

We have established doxorubicin-resistant HCT15/CL02 subline from parental HCT15 human colorectal cell line, and it has been shown relative resistant rate 86.7 to the agent in comparison with its parental cells. And the HCT15/CL02 cells have also revealed cross-resistance to vinblastin, actinomycin D and e-toposide with 17.4, 75.0, 28.0 of the relative resistant rate, respectively. But these cells had no corss-resistance to cisplatin and tamoxifen (Fig. 2 and Fig. 3). These results were corresponding to the typical phenotype of PGP-mediated MDR of anticaner drugs (Damle and Desai, 1994; Gheuens *et al.*, 1993; Kartner *et al.*, 1983). The effective doses of 50% cell growth inhibition of each drug against both cell lines were summarized in Table I.

We have also tested the effects of verapamil, one of the MDR reversal agent, on the cytotoxicities of cancer drugs, and the responses of parental HCT15 and the doxorubicin-resistant HCT15/CL02 cells to doxorubicin and cisplatin with or without verapamil were shown in Fig. 2. In the case of cisplatin, verapamil has no considerable effects on the both cell lines. But verapamil significantly enhanced the cytotoxicity of doxorubicin against both HCT15 and

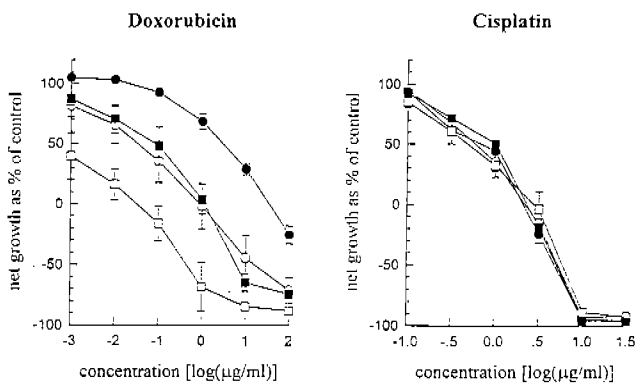

Fig. 2. Cytotoxicities of doxorubicin and cisplatin against parental HCT15 and doxorubicin-resistant HCT15/CL02 cells and the effects of verapamil *in vitro*. Each point represents mean±S.E. of at least four distinct exprements. Key: HCT15 without verapamil (○), HCT15 with verapamil (□), HCT15/CL 02 without verapamil (●), HCT15/CL02 with verapamil (■)

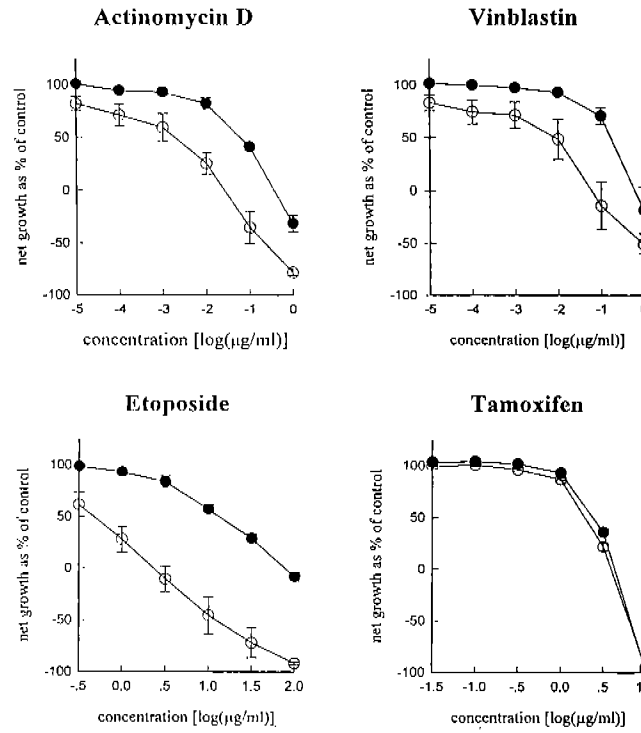

Fig. 3. Cytotoxicities of some standard anticancer drugs against parental HCT15 and doxorubicin-resistant HCT15/CL 02 cells *in vitro*. Each point represents mean±S.E. of four distinct exprements. Key: HCT15 (○), HCT15/CL02 (●)

Table I. Cytotoxicity of some anticancer drugs against HCT 15 and HCT15/DR002 *in vitro*

DRUGS	ED50[a](μg/ml)		Relative Resistant
	HCT15(a)	HCT15/CL02(b)	Rate (b/a)
Doxorubicin	0.032±0.008	2.775±0.056	86.7[c]
Doxorubicin+Ver[b]	<0.001	0.052±0.007	>52.0[c]
Cisplatin	0.584±0.014	0.593±0.034	1.0
Cisplatin+Ver[b]	0.564±0.022	0.713±0.126	1.3
Tamoxifen	1.781±0.008	1.967±0.011	1.1
Vinblastin	0.007±0.003	0.122±0.013	17.4[c]
Actinomycin D	0.001±0.000	0.075±0.003	75.0[c]
Etoposide	0.472±0.047	13.219±0.078	28.0[c]

[a]Effective dose of 50% cell growth inhibition. All data are represented mean±S.E. of at least four distinct experiments.
[b]Added verapamil 5.0 μg/ml in the medium
[c]$P<0.001$ compared ED50 against HCT15/CL02 to ED_{50} against HCT15 cells of each standard drug by t-test.

HCT15/CL02 cells. We also tested the effects of another MDR reversal agent, cyclosporin A on the resistance of both cell lines to cancer drugs, and we attained similar results from those of verapamil (data not shown). From these results, we can conclude that parental HCT15 and doxorubicin-resistant HCT15/CL 02 cells have PGP, and the PGP has effects on the resistance of both cell lines to some anticancer drugs. However, the cytotoxicity of doxorubicin in HCT15/ CL02 subline was not reversed to the level in the parental HCT15 cells by verapamil. And the relative resistant rate, (HCT15/CL02):(HCT15) to doxorubicin was not reduced by verapamil in comparison with the case of absence of this MDR reversal agent (Table I and Fig. 2). These results were shown in common to other tested drugs (unpublished data). Therefore we cannot excluded the possibility that other factors or mechanisms may be contributing the MDR in HCT15/ CL02 cells, and it suggests multiple mechanisms of MDR.

In fact, despite the high prevaling reports of PGP in MDR cells, other factors have also been reported associated with MDR such as membrane change (Callaghan *et al.*, 1993), alteration in glutathione metabolism (Awashi *et al.*, 1994; Kramer *et al.*, 1988; Lai *et al.*, 1991b), changes in topoisomerase II (Eijdems *et al.*, 1995; Fry *et al.*, 1991; Nitiss *et al.*, 1994), alteration in nuclear protein kinase C (Lee *et al.*, 1992) and appearence of membrane associated proteins distinct from PGP (Abe *et al.*, 1994; Cole *et al.*, 1992; Doyle *et al.*, 1995).

It is well known that verapamil can reverse drug resistance *in vitro*, however, verapamil has failed to reduce the drug resistance completely in this study. Therefore, we could suppose of involvement of mechanisms other than PGP in MDR of HCT15/CL02 cells. Thus both parental HCT15 and doxorubicin resistant HCT15/CL02 cell lines could be a good model not only to study the complex mechanisms of MDR but also to find new MDR-reversal agents of analogues or anticancer drugs to overcome MDR.

ACKNOWLEDGEMENT

This study was supported by a grant of G7 project, evaluation of special pharmacological activity in drug screening, from Ministry of Science and Technology, Korea.

REFERENCES CITED

Abe, T., Hasegawa, S., Taniguchi, K., Yokomizo, A., Kuwano, T., Ono, M., Mori, T., Hori, S., Kohno, K. and Kuwano, M., Possible involvement of multidrug-resistance-associated protein (MRP) gene expression in spontaneous drug resistance to vincristine, etoposide and adriamycin in human glioma cells. *Int. J. Cancer*, 58, 860-864, (1994).

Awashi, S., Singhai, S. S., Srivastava, S. K., Zimniak, P., Bajpai, K. K., Saxena, M., Sharma, R., Ziller III, S. A., Frenkel, E. P., Singh, S. V., He, N. G. and Awashi, Y. C., Adenosin triphosphate-dependent transport of doxorubicin, daunomycin and vinblastine in human tissues by a mechanism distinct from the P-glycoprotein. *J. Clin. Invest.*, 93, 958-965 (1994).

Bradley, G., Juranka, P. F. and Ling, V., Mechanism of multidrug resistance. *Biochim. Biophys. Acta*, 948, 87-128 (1988).

Callaghan, R., Zakher, G. and Epand, R., Increased accumulation of drugs in a multidrug resistant cell line by alteration of membrane biophysical properties. *Biochim. Biophys. Acta*, 1175, 277-282 (1993).

Chen, G., Jaffr zou, J. P., Fleming, W. H., Dur n, G. E. and Sikic, B. I., Prevalence of multidrug resistance related to activation of the *mdr*1 gene in human sarcoma mutants derived by single-step doxorubicin selection. *Cancer Res.*, 54, 4980-4987 (1994).

Chen, G. L. and Liu, L. F., DNA topoisomerases: essential enzymes and lethal targets. *Annu. Rev. Pharmacol. Toxicol.*, 34, 191-218 (1994).

Cole, S. P. C., Pinkoski, M. J., Bhardwaj, G. and Deeley, R. G., Elevated expression of Annexin II (Lipocortin II, p36) in a multidrug resistant small cell lung cancer cell line. *Br. J. Cancer*, 65, 498-502 (1992).

Cornwell, M. M., Gottesman, M. M. and Pastan, I. H., Increased vinblastine binding to membrane vesicles from multidrug-resistant KB cells. *J. Biol. Chem.*, 261, 7921-7928 (1986).

Critchfield, J. W., Welsh, C. J., Phang, J. M. and Yeh, G. C., Modulation of adriamycin and efflux by fla-

vonoids in HCT15 colon cells. *Biochem. Pharmacol.*, 48, 1437-1445 (1994).

Damle, B. and Desai, P., Dipyridamole reverses the resistance to topoisomerase II inhibitors but not to antimicrotubule agents in multidrug-resistant melanoma cells. *Oncol. Res.*, 6, 49-57 (1994).

Dimanche-Boitrel, M. T., Pelletier, H., Genne, P., Petit, J. M., Le Grimellec, C., Canal. P., Ardiet, C., Bastian, G. and Chauffert, B., Confluence-dependent resistance in human colon cancer cells; role of reduced drug accumulation and low intrinsic chemosensitivity of resting cells. *Int. J. Cancer*, 50, 667-682 (1992).

Doyle, L. A., Ross, D. D., Sridhara, R., Fojo, A. T., Kaufmann, S. H., Lee, E. J. and Schiffer, C. A., Expression of a 95 kDa membrane protein is associated with low daunorubicin accumulation in leukaemic blast cells. *Br. J. Cancer*, 71, 52-58 (1995).

Eijdems, E. W. H. M., de Haas, M., Timmerman, A. J., Van der Sohans, G. P., Kamst, E., de Nooij, J., Astaldi Ricotti, G. C. B., Borst, P. and Baas, F., Reduced topoisomerase II activity in multidrug-resistant human non-small cell lung cancer cell lines. *Br. J. Cancer*, 71, 40-47 (1995).

Fojo, A. T., Ueda, K., Slaman, D. J., Poplack, D. G., Gottesman, M. M. and Pastan, I., Expression of a multidrug-resistance gene in human tumors and tissues. *Proc. Natl. Acad. Sci. USA*, 84, 265-269 (1987).

Fry, A., Chrest, C., Davies, S., Walker, C., Harris, A., Hartley, J., Masters, J. and Hickson, I., Relationship between topoisomerase II level and chemosensitivity in human tumor cell lines. *Cancer Res.*, 51, 6592-6595 (1991).

Gheuens, E., van der Heyden, S., Elst, H., Eggermont, A., Oosterom, A. V. and De Bruijn E., Multidrug resistance in rat colon carcinoma cell lines CC531, $CC531^{mdr+}$ and $CC531^{rev}$. *Jpn. J. Cancer Res.*, 84, 1201-1208 (1993).

Iwahashi, T., Okochi, E., Ono, K., Sugawara, I., Tsuruo, T. and Mori, S., Establishment of multidrug resistant human colorectal carcinoma HCT15 cell lines and their properties. *Anticancer Res.*, 11, 1309-1312 (1991).

Juranka, P. F., Zastawny, R. L. and Ling, V., P-glycoprotein: multidrug-resistance and a superfamily of membrane-associated transport proteins. *FASEB J.*, 3, 2583-2592 (1989).

Kartner, N., Riordan, J. R. and Ling, V., Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. *Science*, 221, 1285-1288 (1983).

Klohs, W.D. and Steinkampf, R. W., Possible link between the intrinsic drug resistance of colon tumors and a detoxification mechanism of interstinal cells. *Cancer Res.*, 48, 3025-3030 (1988).

Kramer, R., Zakher, G. and Kim, J., Role of the glutathione redox cycle in acquired and *de novo* multidrug resistance. *Science*, 241, 694-697 (1988).

Lai, G. M., Chen, Y. N., Mickley, L. A., Fojo, A. T. and Bates, S. E., P-glycoprotein expression and schedule dependence of adriamycin cytotoxicity in human colon carcinoma. *Int. J. Cancer*, 49, 696-703 (1991a).

Lai, G. M., Moscow, J. A., Alvarez, M. G., Fojo, A. T. and Bates, S. E., Contribution of glutathione and glutathione-dependent enzymes in the reversal of adriamycin resistance in colon carcinoma cell lines. *Int. J. Cancer*, 49, 688-695 (1991b).

Lee, S. A., Karaszkiewicz, J. W. and Anderson, W. B., Elevated level of nuclear protein kinase C in multidrug-resistant MCF-7 human breast carcinoma cells. *Cancer Res.*, 52, 3750-3759 (1992).

Lock, R. and Ross, W., DNA topoisomerases in cancer therapy. Anticancer *Drug Des.*, 2, 151-164 (1987).

Mickley, L. A., Bates, S. E., Richert, N. D., Currier, S., Tanaka, S., Foss, F., Rosen, N. and Fojo, A. T., Modulation of the expression of a multidrug resistance gene (*mdr*-1/Pglycoprotein) by differentiating agents. *J. Biol. Chem.*, 264, 18031-18040 (1989).

Naito, M. and Tsuruo, T., Competitive inhibition by verapamil of ATP-dependent high-affinity vincristine binding to the plasma membrane of multidrug-resistant K562 cells without calcium-ion involvement. *Cancer Res.*, 49, 1452-1455 (1989).

Nitiss, J. L., Vilalta, P. M., Wu, H. and McMahon, J., Mutations in the gyrB domain of eukaryotic topoisomerase II can lead to partially dominant resistance to etoposide and amsacrine. *Mol. Pharmacol.*, 46, 773-777 (1994).

Park, J. G., Lee, S. K., Hong, I. G., Kim, H. S., Lim, K. H., Choe, K. J., Kim, W. H., Kim, Y. I., Tsuruo, T. and Gottesman, M. M., MDR1 gene expression: its effects on drug resistance to doxorubicin in human hepatocellular carcinoma cell lines. *J. Natl. Cancer Inst.*, 86, 700-705 (1994).

Ryu, S. Y., Choi, S. U., Lee, C. O. and Zee, O. P., Anticancer activity of *Psoralea corylifolia*. *Arch. Pharm. Res.*, 15, 356-359 (1992).

Skehan, P., Streng, R., SCudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S. and Boyd, M.R., New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.*, 82, 1107-1112 (1990).

Tompkins, W. A. F., Watrach, A. M., Schmale, J. D., Schultz, R. M. and Harris, J. A., Cultural and antigenic properties of newly established cell strains derived from adenocarcinomas of the human colon and rectum. *J. Natl. Cancer Inst.*, 52, 1101-1110 (1974).

Tsuruo, T., Reversal of acquired resistance to vinca alkaloids and anthracycline antibiotics. *Cancer Treat. Rep.*, 67, 889-894 (1983).

Tsuruo, T., Iida, H., Tsukayoshi, S. and Sukarai, 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).

Turner, M., Everman, D., Ellington, S. and Fields, C., Detection of free radicals during the cellular metabolism of adriamycin. *Free Rad. Biol. Med.*, 9, 415-421 (1990).

Ueda, K., Cardarelli, C., Gottesman, M. M. and Pastan, I., Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin and vinblastin. *Proc. Natl. Acad. Sci. USA*, 84, 3004-3008 (1987).

Wang, J. C., DNA topoisomerases. *Annu. Rev. Biochem.*, 54, 665-697 (1985).

Weiss, R., Sarory, G., Clagett-Carr, K., Russo, M. and Leyland-Jones, B., Anthracycline analogues: the past, present, and future. *Cancer Chemother. Pharmacol.*, 18, 185-197 (1986).

Zunino, F. and Capramco, G., DNA topoisomerase II as the primary target of anti-tumor anthracyclines. *Anticancer Drug Des.*, 5, 30-52 (1990).