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Indomethacin Enhances the Cytotoxicity of VCR and ADR in Human Pulmonary Adenocarcinoma Cells

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KOBAYASHI, S., OKADA, S., YOSHIDA, H. and FUJIMURA, S. *Indomethacin Enhances the Cytotoxicity of VCR and ADR in Human Pulmonary Adenocarcinoma Cells.* Tohoku J. Exp. Med., 1997, **181** (3), 361-370 —— The ability of anti-inflammatory agents to modulate cellular sensitivity to anticancer drugs was investigated for pulmonary carcinoma cells in vitro. We examined the drug sensitivity of two pulmonary adenocarcinoma cell lines (76-2, 77-4) in the presence of two drugs, an anticancer drug and an anti-inflammatory agent, for 72 hr by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with 96 well plates. Anticancer drugs used for screening test were cyclophosphamide (CPM), mitomycin C (MMC), adriamycin (ADR), 5-fluorouracil (5FU), vindesine (VDS), cisplatin (CDDP), cytarabine (Ara C), methotrexate (MTX), etoposide (VP-16), and vincristine (VCR). Anti-inflammatory agents examined as modulators to anticancer drugs were aspirin, mefenamic acid, ibuprofen, sulindac, piroxicam, phenacetin, dicrofenac, ketoprofen, tolmetin and indomethacin. Screening tests showed indomethacin to be the most effective modulator, resulting in more than a 3-fold increase in cytotoxicity of VCR as compared with that produced by VCR alone. Study of each of the ten anticancer drugs in combination with indomethacin showed VCR to be the most effective anticancer drug in this combination. In 76-2 cells, the concentration of VCR producing 50% growth inhibition (IC_{50}) for VCR alone and VCR in combination with 2 μg/ml indomethacin were 1.58 ± 0.16 and 0.52 ± 0.1 ng/ml respectively, which represents a 3-fold decrease. In 77-4 cells, the IC_{50} for VCR alone and VCR in combination with 2 μg/ml indomethacin were 2.86 ± 0.2 and 0.52 ± 0.11 ng/ml respectively, which represents a 3.8-fold decrease. Our studies indicate that clinically achievable concentrations of indomethacin may be useful in modulating VCR resistance in human pulmonary adenocarcinoma cells, so that combined use of VCR and indomethacin may be of potential clinical significance in the treatment of lung cancer. —— biochemical modulator; indomethacin; VCR; lung cancer; cell line

Although small cell lung cancer (SCLC) is highly responsive to chemotherapy, the tumour almost invariably relapse and become clinically resistant to chemotherapy (Sehested et al. 1986; Brambilla et al. 1991), with less than 15% of patients surviving more than 2 years (Souhami et al. 1994; Loehrer et al. 1995).

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Non-SCLC are also usually clinically resistant to chemotherapy at present. This intrinsic or acquired resistance of carcinoma cells to multiple anticancer drugs remains a major problem in the current chemotherapy for human lung cancer.

Use of a biochemical modulator combined with several anticancer drugs has been considered useful as an auxiliary means for combined modality therapy in patients with advanced cancers, as a means of reducing the adverse reaction to administration of massive anticancer drugs and of enhancing the synergistic therapeutic effect against cancers. Several anti-inflammatory agents, such as inhibitors of prostaglandin synthesis, have been shown to inhibit the growth of experimentally induced tumors (Sato et al. 1983). However, in the literature, the number of basic studies that have examined the combined effect of anticancer drugs in vitro using cultured human carcinoma cells is small (Bennett et al. 1987; Maca 1991). To the best of our knowledge, no detailed study has been published regarding human lung cancer cell lines.

We have been conducting a series of studies pertaining to the cultivation of pulmonary carcinoma cells and its clinical application (Kobayashi et al. 1989, 1993; Kobayashi and Fujimura 1992). Within the framework of basic studies using a number of pulmonary carcinoma cell lines established in our laboratory, the present study was undertaken in an attempt to identify useful biochemical modulators which could be used in chemotherapy against lung cancer.

In the present report, we describe the marked enhancement of vincristine (VCR) sensitivity by the administration of indomethacin in human pulmonary adenocarcinoma cells in vitro.

Materials and Methods

Cell lines. Two human pulmonary adenocarcinoma cell lines, 76-2 and 77-4 were derived from resected primary tumors in our laboratory and kept in continuous culture in Ham F12 medium with 10% fetal bovine serum, at 37°C in a humidified atmosphere containing 5% CO_2 (Kobayashi et al. 1994).

Drug sensitivity test. We examined the drug sensitivity of these cancer cell lines after exposure to anticancer drugs in combination with anti-inflammatory agents for 72 hr, by a simple screening test using Terasaki microplates and the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with 96-well flat-bottomed microplates. Anticancer drugs used for screening test were cyclophosphamide (CPM), mitomycin C (MMC), adriamycin (ADR), 5-fluorouracil (5FU), vindesine (VDS), cisplatin (CDDP), cytarabine (Ara C), methotrexate (MTX), etoposide (VP-16), and VCR. Anti-inflammatory agents examined as modulators of anticancer drugs were aspirin, mefenamic acid, ibuprofen, sulindac, piroxicam, phenacetin, dicrofenac, ketoprofen, tolmetin and indomethacin. The screening test with Terasaki microplates was done as described in our previous report (Kobayashi et al. 1993). The MTT assay with 96-well microplates was done using the method described in previous papers with a slight modification

(Carmichael et al. 1987). Cell suspensions (180 μl) were placed in the individual wells of a 96-well microplate, 20 μl of drug solution was added, and left in an incubator at 37°C. After a 72 hr of incubation, 20 μl of MTT reagent (MTT 4 mg/ml+0.1 M sodium succinil acid) was added, and incubation was performed for 4 hr at 37°C. Then the formazan had formed was extracted with 150 μl of dimethyl sulfoxide. Absorbance values were measured at a wavelength of 595 nm with a microplate spectrophotometer (Model 550; Bio-Rad Laboratories, Hercules, CA, USA), and surviving fraction was calculated as follows: Surviving fraction = OD_{595} of experiment/OD_{595} of control. All determinations were carried out in triplicate.

Results

The efficacy of the drug combination using screening assay with Terasaki microplates

Fig. 1 shows plates of an actual screening assay using Terasaki microplates. The effect of a combination of indomethacin and 2 anticancer drugs, i.e. VCR and ADR, are investigated in 76-2 adenocarcinoma cell lines (Fig. 1A). The upper 3 rows of the plate indicate the effect of the combined use of indomethacin plus VCR. The lower 3 rows indicate the effects of the combined use of indomethacin plus ADR. Indomethacin was serially diluted 10-fold down the plate and anticancer drugs were added to each well of the right row and serially diluted 2-fold to left. Fig. 1B shows the effect of a combination of ibuprofen plus each of the two anticancer drugs. We macroscopically determined that indomethacin had a marked combined effect to VCR and a potent combined effect to ADR in 76-2 pulmonary adenocarcinoma cells, whereas that ibuprofen had no combined effect

Table 1. *Combined effects of each of 10 anti-inflammatory agents and VCR*

Agents	VCR	ADR
Indomethacin	Marked	Potent
Aspirin	NCE[a]	NCE
Mefenamic acid	Potent	Slightly
Ibuprofen	NCE	NCE
Sulindac	Potent	Slightly
Piroxicam	NCE	NCE
Phenacetin	NCE	NCE
Diclofenac	NCE	NCE
Ketoprofen	NCE	NCE
Tolmetin	Slightly	NCE

[a]NCE, no combined effect.

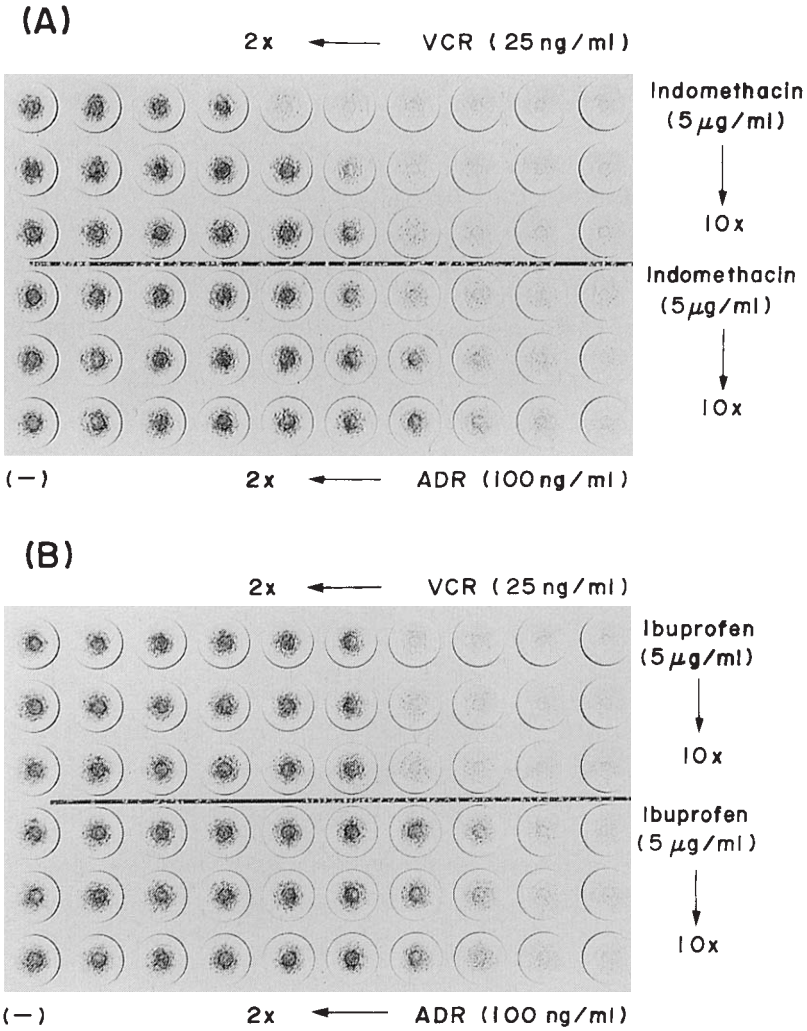

Fig. 1. The efficacy of the drug combination using screening assay with Terasaki microplates. The effect of a combination of indomethacin and 2 anticancer drugs are investigated in 76-2 adenocarcinoma cell lines (Fig. 1A). The upper 3 rows of the plate indicate the effect of the combined use of indomethacin plus vincristine (VCR). The lower 3 rows indicate the effects of the combined use of indomethacin plus adriamycin (ADR). Indomethacin was serially diluted 10-fold down the plate and anticancer drugs were added to each well of the right row and serially diluted 2-fold to left. Fig. 1B shows the effect of a combination of ibuprofen plus each of the two anticancer drugs.

to each of the drugs.

Fig. 2 shows a plate of more precise screening assay using Terasaki microplates. The effect of a combination of indomethacin and VCR was investigated in 76-2 adenocarcinoma cell line. Indomethacin was serially diluted 2-fold down the plate and 50 ng/ml of VCR solution was added to each well of the right row and serially diluted 2-fold to left. We can reveal that indomethacin interact with VCR in a dose-dependent manner.

Similar screening tests with Terasaki microplates were made for 10 anti-inflammatory agents in combination with VCR and ADR in 76-2 cells (Table 1). Indomethacin was the most effective modulator, resulting in more than a 2-fold

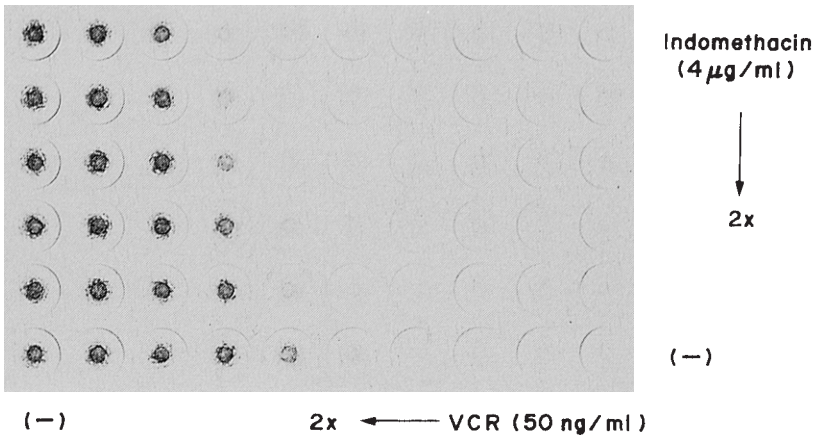

Fig. 2. The efficacy of the drug combination using screening assay with Terasaki microplates. The actual plate of more precise screening assay was shown. The effect of a combination of indomethacin and VCR are investigated in 76-2 adenocarcinoma cell lines. Indomethacin was serially diluted 2-fold down the plate and 50 ng/ml of VCR solution were added to each well of the right row and serially diluted 2-fold to left.

TABLE 2. *Combined effects of each of 10 anticancer drugs and indomethacin*

Anticancer drugs	76-2	77-4
CPM	NCE[a]	NCE
MMC	NCE	NCE
MTX	Potent	
CDDP	NCE	NCE
5FU	NCE	NCE
LDS	NCE	NCE
ADR	Potent	Slightly
VP-16	Potent	
Ara C	NCE	NCE
VCR	Marked	Marked

[a]NCE, no combined effect.

increase in cytotoxicity of VCR as compared with that produced by VCR alone. A study of each of the 10 anticancer drugs in combination with indomethacin in 76-2 cells and 77-4 cells showed VCR to be the most effective anticancer drug in this combination (Table 2).

The efficacy of the drug combination using the MTT assay

The above results suggested that indomethacin would be the potent biochemical modulator for ADR, MTX, VP-16 and VCR in lung cancer cells. Therefore, we next examined the combination effect of indomethacin on the cytotoxicity of these anticancer drugs in 76-2 cells and 77-4 cells using the MTT assay. Fig. 3

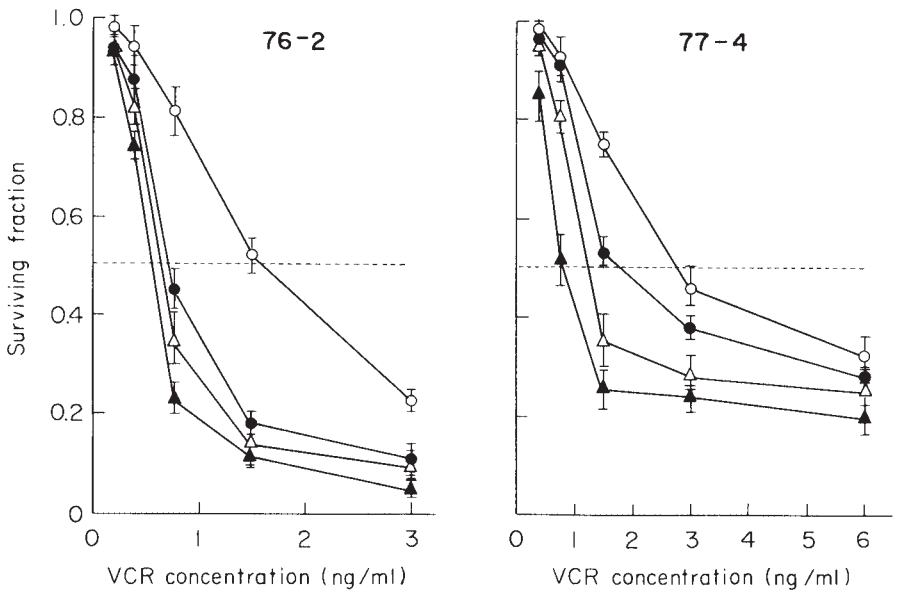

Fig. 3. The effect of indomethacin on the sensitivity of 76-2 cells and 77-4 cells to VCR. The effect of VCR on each cell line in the absence (○) or presence of 0.5 μg/ml (●), 1 μg/ml (△) and 2 μg/ml (▲) of indomethacin examined by MTT assay. Each point represents the mean of three experiments; bars, S.E.

illustrates the ability of indomethacin to enhance the cytotoxicity of VCR. In these experiments, 77-4 cells are relatively resistant to VCR to 76-2 cells. In the pulmonary cancer cell line 76-2, the IC_{50} for single agent VCR is 1.58 ± 0.16 (S.E.) ng/ml. When 2 μg/ml of indomethacin is added to the assay, the IC_{50} for VCR decreased to 0.52 ± 0.1 (S.E.) ng/ml which represents a 3-fold decrease. In 77-4 cells, the IC_{50} for VCR alone and VCR in combination with 2 μg/ml indometh-

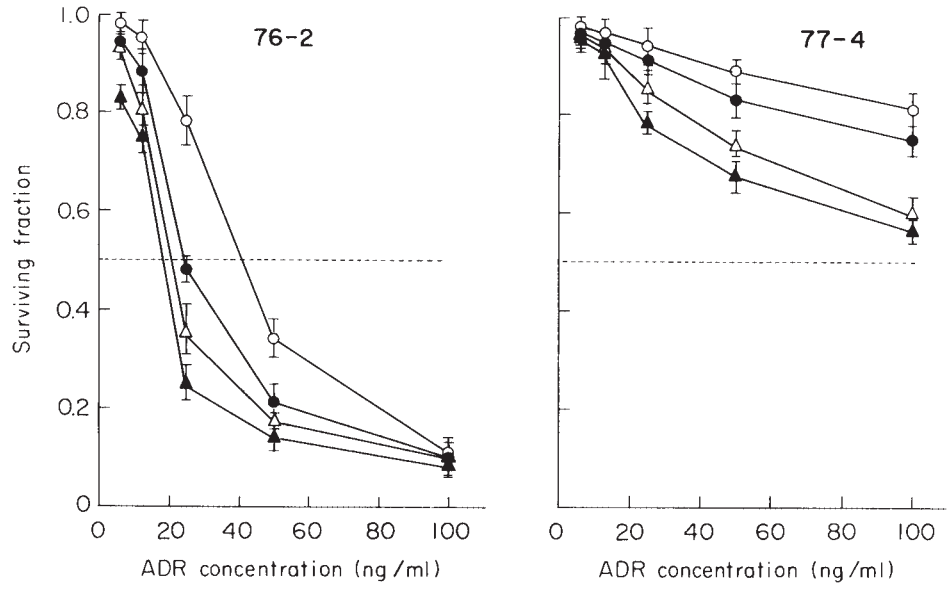

Fig. 4. The effect of indomethacin on the sensitivity of 76-2 cells and 77-4 cells to ADR. The effect of ADR on each cell line in the absence (○) or presence of 0.5 μg/ml (●), 1 μg/ml (△) and 2 μg/ml (▲) of indomethacin examined by MTT assay. Each point represents the mean of three experiments; bars, S.E.

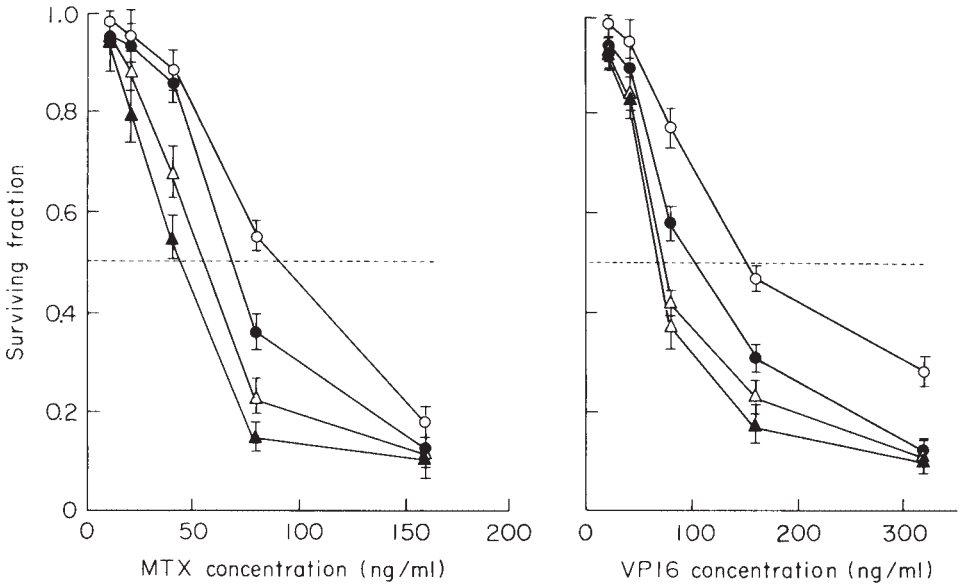

Fig. 5. Effects of methotrexate (MTX) and etoposide (VP-16) with and without indomethacin on cell proliferation in 76-2 cells. Surviving fraction of 76-2 cells after 3 days of continuous treatment with 0.5 μg/ml (●), 1 μg/ml (△) and 2 μg/ml (▲) of indomethacin in combination with various concentrations of MTX and VP-16. (○), treatment with MTX or VP-16 alone. Points show mean of three experiments; bars, S.E.

acin were 2.86±0.2 and 0.76±0.11 (S.E.) ng/ml respectively, which represents a 3.8-fold decrease.

Fig. 4 shows the dose response curves for ADR and indomethacin in 76-2 cells and 77-4 cells. In 76-2 cells, the IC_{50} for ADR alone and ADR in combination with 2 μg/ml indomethacin were 41±2 and 18±1 (S.E.) ng/ml respectively, which represents a 2.3-fold decrease. In 77-4 cells, the survival fraction for ADR at 50 ng/ml dose alone and ADR in combination with 2 μg/ml indomethacin were 0.89±0.02 and 0.67±0.04 (S.E.), respectively.

Fig. 5 shows the effect of Indomethacin on the sensitivity of 76-2 cells to MTX and VP-16. The administration of 2 μg/ml of indomethacin increased the sensitivity to MTX and VP-16 by 2-fold and 2.2-fold, respectively.

Discussion

Although chemotherapy often has some impact on the treatment of advanced lung cancer, cure is rare, since initial response is followed by relapse and resistance to further chemotherapy (Sehested et al. 1986; Brambilla et al. 1991). These tumor cell resistance to many anticancer drugs is thought to be a major cause of failure in current chemotherapy.

A variety of agents have been investigated for biochemical modulators of anticancer drugs in resistant cells in vitro (Zijlstra et al. 1987; Bellamy et al. 1988; Kramer et al. 1988). However, many of such modulators were disappointing in clinical trials. The reasons for the failure include the toxicity of the modulators

related to their own pharmacological action and the inability to achieve optimal serum concentrations. For example, verapamil caused severe cardiotoxicity at concentrations required for the combined effect in vitro (Miller et al. 1991). There remains a need to identify safer and more potent modulators.

Inhibitors of prostaglandin synthesis, such as indomethacin have been shown to inhibit the growth of animal experimental tumors (Sato et al. 1983; Lala et al. 1986). However, only a few studies have examined the role of indomethacin in enhancing the sensitivity of anticancer drugs. Maca (1991) reported that indomethacin enhanced the sensitivity of VP-16 and MTX in mouse tumor cells and human leukemia cell lines. In addition, indomethacin have been also found to increase the sensitivity of MTX in mouse tumor cells and human breast cancer cells in vitro (Bennett et al. 1987). These findings for the enhancement of MTX and VP-16 sensitivity by indomethacin were consistent with those of our observation as shown in Fig. 5. However, to the best of our knowledge, the enhancement of VCR and ADR sensitivity by indomethacin in vitro was not yet reported in both animal experimental tumors and human tumors.

The enhancing mechanism of indomethacin is still not well understood, though some authors have been reported to explain its action; inhibition of the endogenous synthesis of prostaglandins (Lala et al. 1986); acting as an immune-potentiator (Tilden and Balch 1982); relating to its antagonism to gastrin/cholecystokinin family of peptides and/or to its agonistic activity to thromboxane A2 and melatonin (Szkudlinski 1992). The exact mechanism by which indomethacin enhances the cytotoxicity of drugs is not known. Maca (1991) reported that indomethacin is not augmenting VP-16 cytotoxicity by inhibiting cyclooxygenase activity and prostaglandin production, and he proposed one possible mechanism that indomethacin modulates the sensitivity of anticancer drugs by decreasing the cellular efflux of these drugs by P-glycoprotein system and resulting in augmentation of the drug accumulation.

In the present study, we clearly demonstrate that indomethacin significantly potentiates the cytotoxic activity of VCR and ADR in human pulmonary adenocarcinoma cells. Especially, VCR is a most effective drug in combination with indomethacin in these anticancer drugs. As shown in Fig. 3, indomethacin at several concentrations interact with VCR in 76-2 cells and 77-4 cells in a dose-dependent manner. For instance, while the concentration of indomethacin is 0.5, 1 and 2 μg/ml, the survival ratio of 77-4 cells at 1.5 ng/ml concentration of VCR is 0.54, 0.35 and 0.25, respectively.

In this regard, indomethacin and its analogs, i.e. sulindac, appear to be useful modulators of anticancer drug activity in clinical use. Indomethacin has been known as an anti-inflammatory agent with safe clinical use. The concentration of indomethacin required for the potentiation of combined anticancer effect with VCR is usually in range of 0.5 to 2 μg/ml, which can be safely achieved in patients. When indomethacin has been used at ordinary doses (30 mg/m^2/8 hr),

the plasma level of indomethacin achieved was 2 μg/ml (Adams et al. 1982). The concentration of indomethacin revealed more than a 3-fold cytotoxic efficacy on 76-2 cells and 77-4 cells by combined use with VCR compared with only use of VCR in vitro.

Thus, indomethacin in combination with several anticancer drugs; VCR, ADR, MTX and VP-16 should be considered for use in cancer therapy, although the mechanism by which indomethacin enhances the cytotoxicity of these anticancer drugs remains uncertain. Especially, an obvious possibility is the use of indomethacin with VCR, ADR, VP-16 and MTX in the treatment of patients with SCLC, since ordinary chemotherapy in combination with these anticancer drugs is currently of large therapeutic value in SCLC (Ettinger et al. 1990; Wampler et al. 1991). In addition, we have preliminary data that indomethacin increases the cytotoxicity of VCR to VCR-resistant human SCLC cell lines in vitro, and have a case with advanced SCLC resistant to, and recurred after several cycles of ordinary chemotherapy achieved almost complete remission (CR) after one cycle of chemotherapy with these four drugs in combination with indomethacin (unpublished).

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