

## Schedule-dependent Synergism and Antagonism between Raltitrexed (“Tomudex”) and Methotrexate in Human Colon Cancer Cell Lines *in vitro*

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The folate-dependent enzymes are attractive targets for cancer chemotherapy. Methotrexate (MTX), which inhibits dihydrofolate reductase, has been widely used for the treatment of solid tumors and hematological cancers. Raltitrexed (“Tomudex”), which inhibits thymidylate synthase, is a novel anticancer agent active against colorectal cancer and some other solid tumors. We studied the optimal schedule of raltitrexed and MTX in combination against four human colon cancer cell lines Colo201, Colo320, LoVo, and WiDr. These cells were simultaneously exposed to raltitrexed and MTX for 24 h, or sequentially exposed to raltitrexed for 24 h followed by MTX for 24 h, or *vice versa*. Cell growth inhibition after 5 days was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The effects of drug combinations at the concentrations of drug that produced 80% and 50% cell growth inhibition (IC<sub>80</sub> and IC<sub>50</sub>) were analyzed by the isobologram method (Steel and Peckham, 1979). Cytotoxic interactions between raltitrexed and MTX were schedule-dependent. The simultaneous exposure to raltitrexed and MTX showed additive effects in Colo201, LoVo and WiDr cells and antagonistic effects in Colo320 cells. The sequential exposure to raltitrexed followed by MTX produced additive effects in all four cell lines. The sequential exposure to MTX followed by raltitrexed produced synergistic effects in Colo201, LoVo and WiDr cells and additive effects in Colo320 cells. These findings suggest that the sequential administration of MTX followed by raltitrexed produces more than the expected cytotoxicity and may be the optimal schedule at the cellular level. Further *in vivo* and clinical studies will be necessary to determine the toxicity and to test the antitumor effects of sequential administration of MTX followed by raltitrexed proposed on the basis of the *in vitro* synergism.

Key words: Raltitrexed — Methotrexate — Synergism — Isobologram — Colon cancer

The folate-dependent enzymes are attractive targets for cancer chemotherapy because of their critical role in the synthesis of the nucleotide precursors of DNA. Methotrexate (MTX), the classical antifolate, is one of the oldest and still most commonly used anti-cancer agents.<sup>1)</sup> Although the precise cytotoxic mechanism of MTX remains controversial, the main target of MTX is considered to be dihydrofolate reductase. The inhibition of this enzyme results in a lack of tetrahydrofolate coenzyme, which is required for the *de novo* synthesis of thymidylate, purines, and methionine. Thymidylate is required for the synthesis and repair of DNA, and inhibition of thymidylate synthesis is considered to be the major cytotoxic mechanism of MTX.

Currently, several new folate analogs with unique biochemical properties are being tested for clinical application.<sup>2)</sup> Raltitrexed (“Tomudex”) is a promising new agent that targets thymidylate synthase, the enzyme responsible for the final step of thymidylate synthesis.<sup>2,3)</sup> Like MTX,

this agent relies on the reduced folate carrier for cellular entry and is converted to the active form by polyglutamation. Raltitrexed is commonly administered as a 15 min i.v. infusion and the  $\beta$ - and  $\gamma$ -half-lives are 2 and >10 h.<sup>4,5)</sup> Clinical studies have shown that the dose-limiting toxicities of raltitrexed involve myelosuppression, malaise and gastrointestinal toxicity.<sup>4,6)</sup> Raltitrexed has promising therapeutic activity against colon cancer and other solid tumors.<sup>7-9)</sup> Because treatment with raltitrexed alone is not likely to be curative, there is considerable clinical interest in its combination with other anticancer agents. Clinical studies of the combination of raltitrexed with 5-fluorouracil, irinotecan or oxaliplatin are in progress.

Raltitrexed and MTX inhibit different enzymes that are important for maintaining active folate coenzymes. Due to their non-overlapping toxicity, their different mechanisms of action, and the clinical success of the combination of MTX and the indirect thymidylate synthase inhibitor ‘5-fluorouracil,’<sup>10,11)</sup> the combination of raltitrexed and MTX is an attractive subject for clinical study. Sequential blocks of folate-maintaining enzymes may produce synergistic or

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antagonistic effects. Raltitrexed and MTX are cell cycle-specific agents and the disturbance of the cell cycle produced by one of these agents may influence the cytotoxic effects of the other agent, and thus the drug schedule may be an important determinant of the activity.

The present study was aimed at elucidating the cytotoxic effects of various schedules of raltitrexed and MTX in combination on four human colon cancer cell lines. The data obtained were analyzed by the isobologram method of Steel and Peckham.<sup>12)</sup> We observed definite schedule-dependent synergism and antagonism between raltitrexed and MTX.

## MATERIALS AND METHODS

**Cell lines** Experiments were conducted with four human colon cancer cell lines, Colo201, Colo320, LoVo, and WiDr cells. Colo201, Colo320 and LoVo cells were obtained from the Health Science Research Resources Bank (Osaka). WiDr cells were obtained from the American Type Culture Collection (Rockville, MD). These cell lines were maintained in 75-cm<sup>2</sup> plastic tissue culture flasks containing RPMI1640 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Grand Island Biological Co.), 100 U/ml penicillin and 0.1 mg/ml streptomycin. The cell lines were kept in an atmosphere of 5% carbon dioxide in air at 37°C. The doubling times of Colo201, Colo320, LoVo and WiDr cells under our experimental conditions were 18–24 h.

**Drugs** Raltitrexed and MTX were obtained from Zeneca Japan Co. (Tokyo), and Lederle Japan Co. (Tokyo), respectively. Raltitrexed was dissolved in 0.15 mM NaHCO<sub>3</sub> at a concentration of 1 mM and MTX was dissolved in RPMI1640 medium at a concentration of 1 mM. The drugs were diluted with RPMI1640 containing 10% FBS. The drug concentrations used were within the ranges of *in vivo* protein-unbound drug concentrations achievable in patients.

**Cell growth inhibition by combination of raltitrexed and MTX** On day 0, exponentially growing cells were harvested with trypsin:EDTA (0.05%:0.02%) and resuspended to final concentrations of  $2.0 \times 10^4$  cells/ml for Colo201, Colo320, and LoVo cells, and  $5.0 \times 10^3$  cells/ml for WiDr cells in fresh medium containing 10% FBS and antibiotics. Cell suspensions (100  $\mu$ l) were dispensed into the individual wells of a 96-well tissue culture plate with a lid (Falcon, Oxnard, CA). Each plate had one 8-well control column containing medium alone and one 8-well control column containing cells but no drug. Eight plates were prepared for each drug combination schedule in each cell line. The cells were reincubated overnight to allow for attachment.

**Simultaneous exposure to raltitrexed and MTX** After overnight incubation, solutions of raltitrexed and MTX (50

$\mu$ l) at different concentrations were added to individual wells. The plates were incubated under the same conditions for 24 h. Cells were washed twice with culture medium, then fresh medium (200  $\mu$ l) was added and incubation was continued for a further 4 days.

**Sequential exposure to raltitrexed and MTX** After overnight incubation, medium (50  $\mu$ l) and solutions of raltitrexed (or MTX) (50  $\mu$ l) at different concentrations were added to individual wells. The plates were then incubated under the same conditions described above. After 24 h, cells were washed twice and fresh medium (150  $\mu$ l) was added, followed by the addition of solutions of MTX (or raltitrexed) (50  $\mu$ l) at various concentrations. The plates were incubated again under the same conditions. After 24 h, the cells were washed twice, fresh medium (200  $\mu$ l) was added, and incubation was continued for a further 3 days.

Since cytotoxic levels of raltitrexed and MTX in clinical medicine are generally maintained for more than 10 h, 24-h exposure to raltitrexed and MTX was used in the present experiments.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay** Viable cell growth was determined by the MTT assay as described previously.<sup>13)</sup> For controls, for each drug or drug combination, the four intermediate data values among the eight data values were used for the analysis and the two highest data and two lowest data values were discarded.

**Isobologram method of Steel and Peckham** In this study, dose-response interactions between raltitrexed and MTX at the points of 80% and 50% cell growth inhibitions ( $IC_{80}$  and  $IC_{50}$ ) were evaluated using the isobologram method of Steel and Peckham.<sup>12)</sup> The theoretical basis of the isobologram method and the procedure for making isobolograms have been described in detail.<sup>14)</sup> Based upon the dose-response curves of raltitrexed and MTX, three isoeffect curves were constructed (Fig. 1). If the agents are acting additively by independent mechanisms, combined data points will lie near the Mode I line (hetero-addition). If the agents are acting additively by similar mechanisms, combined data points will lie near the Mode II lines (iso-addition). Since we cannot know in advance whether the combined effects of two agents will be hetero-additive, iso-additive, or an effect intermediate between these extremes, all possibilities should be considered. Thus, when the data points of a drug combination fell within the area surrounded by the three lines (envelope of additivity), the combination was regarded as additive. The envelope of additivity should not be considered as a reliable definition of additivity. The expression of uncertainty is an important concept in the isobologram method of Steel and Peckham.

We used this envelope not only to evaluate combinations in which cells were simultaneously exposed to raltitrexed and MTX, but also to evaluate combinations in

which the cells were sequentially exposed to these agents, since the cytotoxicity of the first agent could be modulated by the second agent under our experimental conditions.

When the data points fell to the left of the envelope (i.e., the combined effect was caused by lower doses of the two agents than was predicted), we regarded the drug combination as having a supra-additive effect (synergism). When the points fell to the right of the envelope (i.e., the combined effect was caused by higher doses of the two agents than was predicted), but within the square or on the line of the square, we regarded the combination as having

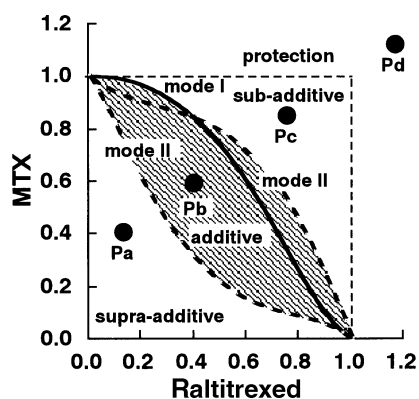


Fig. 1. Schematic representation of an isobologram.<sup>12)</sup> The envelope of additivity, surrounded by Mode I (solid line) and Mode II (dotted lines) isobologram lines, was constructed from the dose-response curves of raltitrexed and MTX. The concentrations, which produced 80% cell growth inhibition ( $IC_{80}$ ), were expressed as 1 on the ordinate and the abscissa of isobolograms. Combined data points Pa, Pb, Pc, and Pd show supra-additive, additive, sub-additive, and protective effects, respectively.

a sub-additive effect, i.e., the combination was superior or equal to a single agent but was less than additive. When the data points were outside the square, the combination was regarded as having a protective effect, i.e., the combination was inferior in cytotoxic action to a single agent. Both sub-additive and protective interactions were regarded as antagonism. Simultaneous exposure and sequential exposure to raltitrexed or MTX alone produced additive effects (data not shown). Self-synergism and self-antagonism were not produced.

**Statistical analysis** Statistical analysis was performed as described previously.<sup>15)</sup> When the observed data points for a combination fell mainly within the envelope of additivity, the combination was considered as having an additive effect. The mean value of the observed data was compared with that of the predicted minimum values and that of the predicted maximum values for an additive effect, which were on the borderline (Mode I or Mode II lines) between the additive and supra-additive areas, or between the additive and the sub-additive (or protective) areas, respectively (Table I). If the mean value of the observed data was equal to or smaller than that of the predicted maximum values and equal to or larger than that of the predicted minimum values, the combination was regarded as having an additive effect. If the mean value of the observed data was smaller than that of the predicted minimum values or larger than that of the predicted maximum values (i.e., the observed data points for drug combinations fell mainly in the area of supra-additivity or in the areas of sub-additivity and protection), the combinations were considered to have a synergistic or antagonistic effect, respectively. To determine whether the condition of synergism (or antagonism) truly existed, the Wilcoxon signed-rank test was per-

Table I. The Mean Values of Observed Data and Predicted Minimum and Predicted Maximum Values, and the Outcome for the Combination of Raltitrexed (R) and Methotrexate (M) at the  $IC_{80}$  Level

Schedule	Cell line	n	Observed data <sup>a)</sup>	Predicted min. <sup>b)</sup>	Predicted max. <sup>c)</sup>	Outcome
R+M	Colo201	8	0.63	0.49	0.64	Additive
	Colo320	11	0.90	0.65	0.74	Antagonism ( $P<0.01$ )
	LoVo	8	0.64	0.42	0.83	Additive
	WiDr	5	0.67	0.45	0.76	Additive
R→M	Colo201	11	0.42	0.42	0.86	Additive
	Colo320	12	0.76	0.68	0.85	Additive
	LoVo	9	0.69	0.35	0.73	Additive
	WiDr	4	0.77	0.52	0.77	Additive
M→R	Colo201	8	0.24	0.40	0.77	Synergism ( $P<0.02$ )
	Colo320	9	0.50	0.44	0.77	Additive
	LoVo	9	0.58	0.67	0.79	Synergism ( $P<0.01$ )
	WiDr	7	0.42	0.46	0.66	Synergism ( $P<0.05$ )

a) Mean value of observed data.

b) Mean value of the predicted minimum values for an additive effect.

c) Mean value of the predicted maximum values for an additive effect.

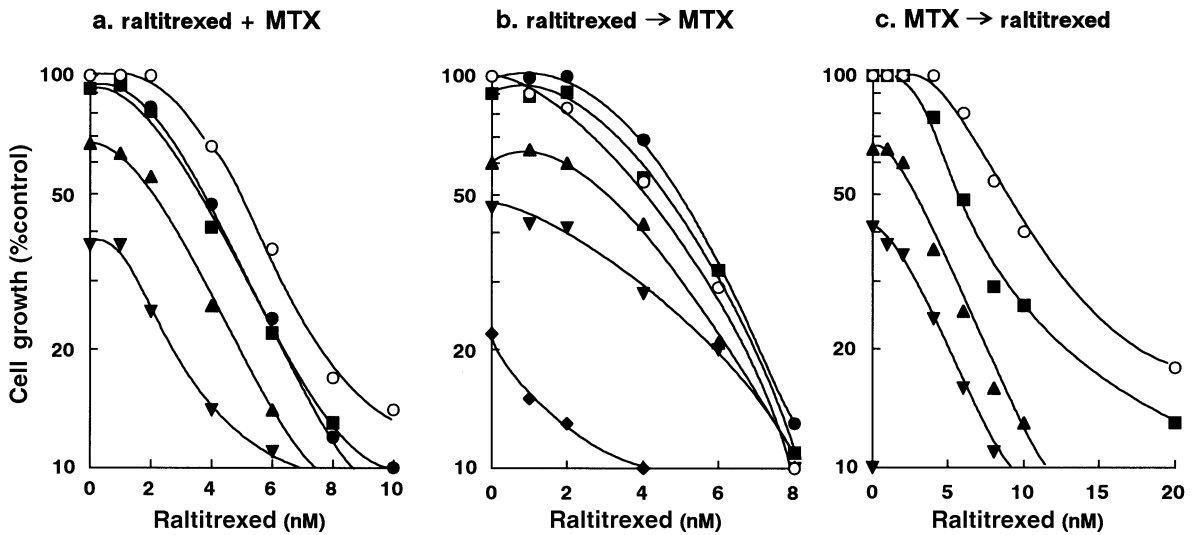


Fig. 2. Dose-response curves for raltitrexed alone, MTX alone, and their combinations in LoVo cells. Cells were exposed to drugs simultaneously for 24 h (a), raltitrexed first for 24 h followed by MTX for 24 h (b), or the reverse sequence (c). After 5 days, the cell number was determined by MTT assay and was plotted as a percentage of the control (cells not exposed to drugs). Raltitrexed concentrations are shown on the abscissa. The concentrations of MTX were 0 (○), 10 (●), 20 (■), 50 (▲), 100 (▼) and 200 (◆) nM. Each point represents the mean value for three independent experiments; SE was <25%. The dose-response curves with MTX concentrations on the abscissa were made using the same dose-response data (not shown).

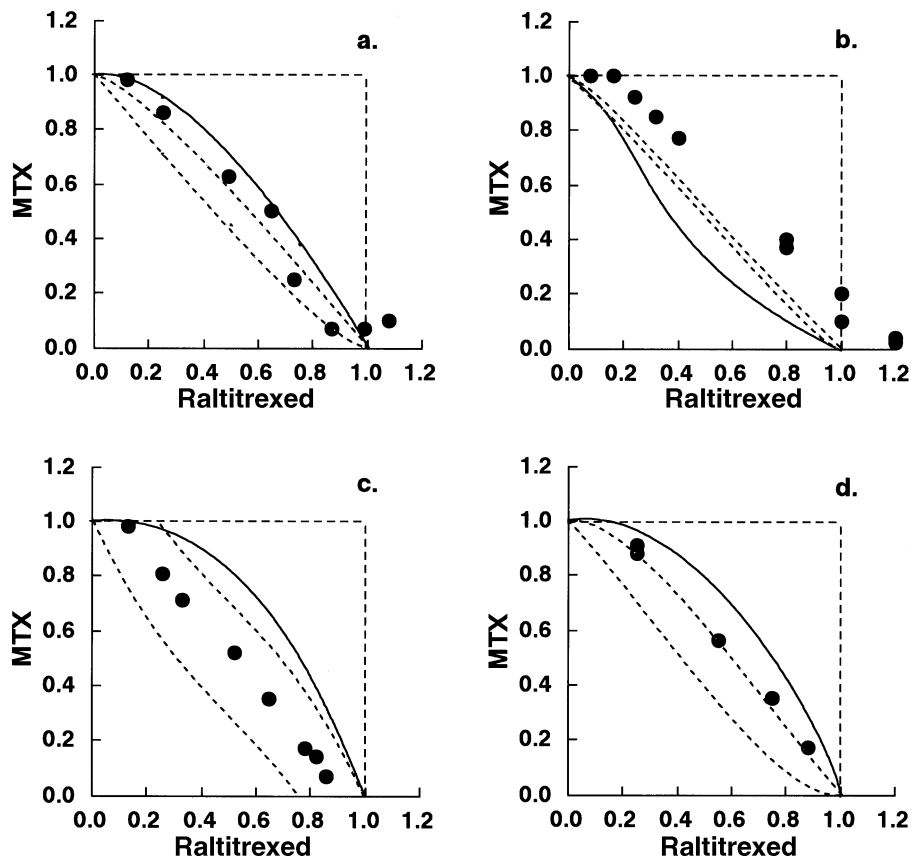


Fig. 3. Isobolograms of simultaneous exposure to raltitrexed and MTX in Colo201 (a), Colo320 (b), LoVo (c) and WiDr (d) cells at the  $IC_{80}$  level. Data are presented as mean values for three independent experiments; SE was <20%. All or most of the data points for the combination fell within the envelope of additivity for Colo201, LoVo, and WiDr cells, while all data points fell in the areas of sub-additivity and protection for Colo320 cells.

formed for comparing the observed data with the predicted minimum (or maximum) values for an additive effect which were closest to the observed data (i. e., the data on the boundary (Mode I or Mode II lines) between the additive area and supra-additive area (or sub-additive and protective areas). Probability ( $P$ ) values  $\leq 0.05$  were considered significant. Combinations with  $P > 0.05$  were regarded as having an additive to synergistic (or additive to antagonistic) effect. All statistical analyses were performed using the Stat View 4.01 software program (Abacus Concepts, Berkeley, CA).

### RESULTS

Fig. 2 shows the dose-response curves of LoVo cells to the 24 h exposure to raltitrexed and MTX on various schedules: simultaneous exposure to drugs, sequential exposure to raltitrexed followed by MTX, and sequential exposure to MTX followed by raltitrexed. Although the raltitrexed concentrations are shown on the abscissa in these figures, dose-response curves in which the MTX concentrations are shown on the abscissa can be made based on the same data (figure not shown). Each isobologram was generated based on such dose-response curves.

**Simultaneous exposure to raltitrexed and MTX** Fig. 3 shows isobolograms of the Colo201, Colo320, LoVo and WiDr cells after simultaneous exposure to raltitrexed and to MTX at the  $IC_{80}$  level. In the Colo201, LoVo and WiDr cells, most or all of the combined data points fell in the envelope of additivity. The mean values of the observed data (0.63, 0.64, and 0.67, respectively) were larger than those of the predicted minimum values (0.49, 0.42, and 0.45, respectively), and smaller than those of the predicted maximum values (0.64, 0.83, and 0.76, respectively), suggesting additive effects (Table I). In the Colo320 cells, the combined data points fell in the areas of sub-additivity and protection. The mean values of the observed data (0.90) were larger than those of the predicted maximum values (0.74), and the  $P$  values were smaller than 0.01, suggesting antagonistic effects (Table I).

**Sequential exposure to raltitrexed followed by MTX**

Fig. 4 shows isobolograms of the Colo201, Colo320, LoVo, and WiDr cells exposed first to raltitrexed and then to MTX at the  $IC_{80}$  level. In all four cell lines, all or most of the combined data points fell within the envelope of additivity. The mean values of the observed data were equal to or smaller than those of the predicted maximum values and equal to or larger than those of the predicted minimum

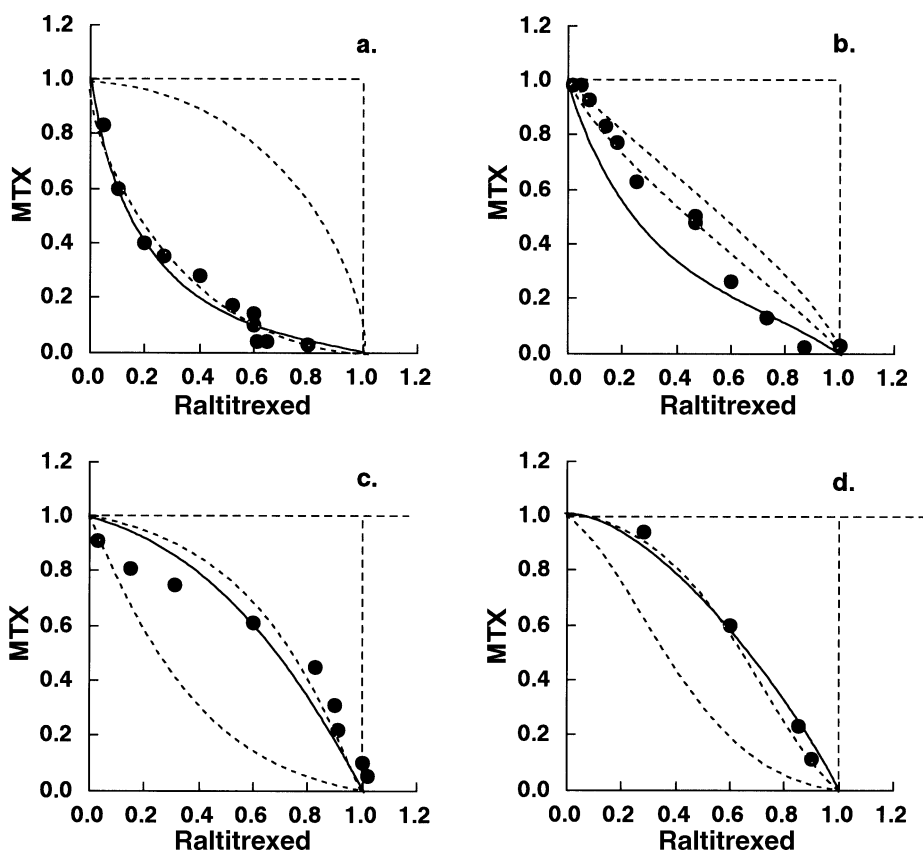


Fig. 4. Isobolograms of sequential exposure to raltitrexed followed by MTX in Colo201 (a), Colo320 (b), LoVo (c) and WiDr (d) cells at the  $IC_{80}$  level. Data are mean values for three independent experiments; SE was  $< 25\%$ . Most of the data points for the combination fell within the envelope of additivity for all four cell lines.

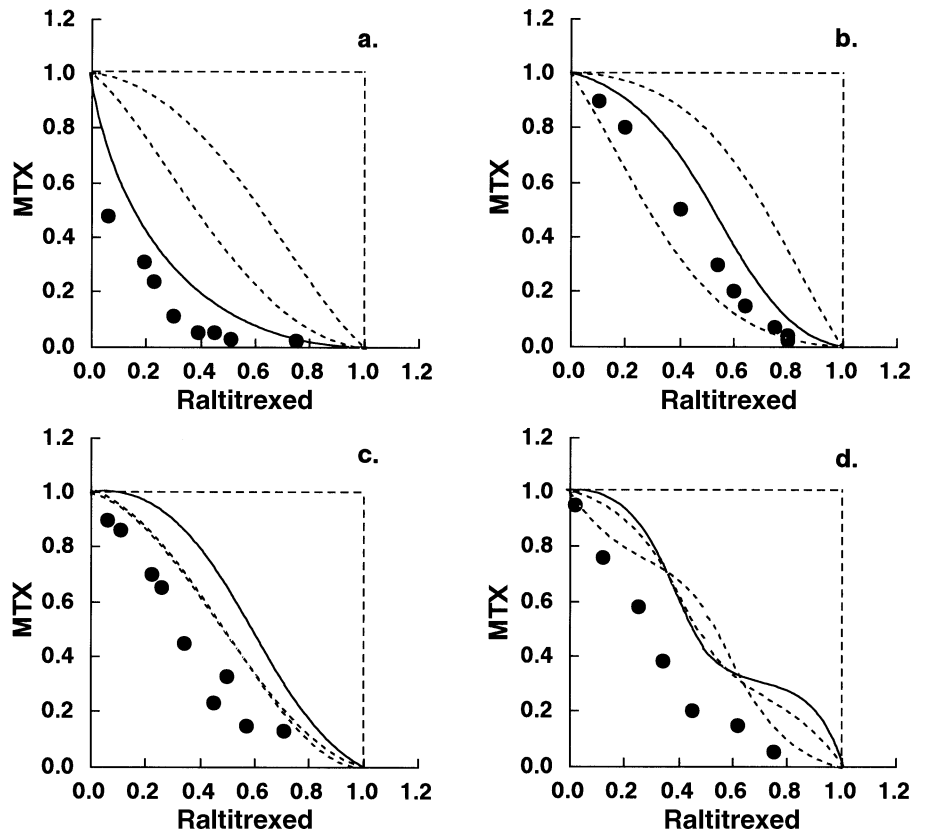


Fig. 5. Isobolograms of sequential exposure to MTX followed by raltitrexed in Colo201 (a), Colo320 (b), LoVo (c) and WiDr (d) cells at the IC<sub>80</sub> level. Data are mean values for three independent experiments; SE was <25%. All or most of the data points for the combination fell mainly in the area of supra-additivity for Colo201, LoVo and WiDr cells, while all data points fell within the envelope of additivity for Colo320 cells.

Table II. The Mean Values of Observed Data and Predicted Minimum and Predicted Maximum Values, and the Outcome for the Combination of Raltitrexed (R) and Methotrexate (M) at the IC<sub>50</sub> Level

Schedule	Cell line	<i>n</i>	Observed data <sup>a)</sup>	Predicted min. <sup>b)</sup>	Predicted max. <sup>c)</sup>	Outcome
R+M	Colo201	5	0.58	0.50	0.74	Additive
	Colo320	8	0.70	0.46	0.72	Additive
	LoVo	6	0.73	0.52	0.85	Additive
	WiDr	4	0.57	0.27	0.70	Additive
R→M	Colo201	6	0.46	0.46	0.63	Additive
	Colo320	10	0.67	0.58	0.68	Additive
	LoVo	6	0.61	0.28	0.61	Additive
	WiDr	3	0.69	0.30	0.70	Additive
M→R	Colo201	5	0.33	0.44	0.64	Additive/synergism
	Colo320	9	0.56	0.45	0.90	Additive
	LoVo	7	0.41	0.44	0.64	Additive/synergism
	WiDr	4	0.44	0.45	0.59	Additive/synergism

a) Mean value of observed data.

b) Mean value of the predicted minimum values for an additive effect.

c) Mean value of the predicted maximum values for an additive effect.

values (Table I), suggesting that the sequential exposure to raltitrexed followed by MTX produced additive effects.

**Sequential exposure to MTX followed by raltitrexed**

Fig. 5 shows isobolograms of the four cell lines treated

with the reverse sequence (MTX, then raltitrexed) at the IC<sub>80</sub> level. In the Colo201, LoVo, and WiDr cells, all or most of the combined data points fell in the area of supra-additivity. The mean values of the observed data were

smaller than those of the predicted minimum values (Table I). The *P* values were less than 0.05 ( $P < 0.02$ ,  $< 0.01$ , and  $< 0.05$ , respectively). These results suggest that the sequential exposure to MTX followed by raltitrexed produced synergistic effects in these cell lines. In Colo320 cells, the combined data points fell within the envelope of additivity and the mean value of the observed data was between those of the predicted minimum and maximum values (Table I), suggesting an additive effect of this schedule.

Similar schedule dependency was observed for the cytotoxic effects of the combination at the  $IC_{50}$  level (isobologram not shown) (Table II).

## DISCUSSION

To investigate the optimal schedule of the combination of raltitrexed and MTX, the present study compared the cytotoxic activity of simultaneous and sequential exposure to raltitrexed and MTX in four human colon cancer cell lines Colo201, Colo320, LoVo and WiDr, in culture. The analysis of the effects of drug-drug interaction was carried out by the isobologram method of Steel and Peckham.<sup>12)</sup>

We demonstrated that cytotoxic interaction between raltitrexed and MTX was definitely schedule-dependent. The simultaneous exposure to raltitrexed and MTX and the sequential exposure to raltitrexed followed by MTX produced mainly additive effects. The sequential exposure to MTX followed by raltitrexed produced mainly synergistic effects. These data suggest that the optimal schedule of this combination is MTX followed by raltitrexed.

It is noteworthy that MTX followed by raltitrexed produced synergistic effects using the isobologram method of Steel and Peckham. As already described, this method is stricter for synergism and antagonism, and we found no synergistic effects with any schedule or in any cell line for raltitrexed in combination with cisplatin,<sup>16)</sup> 5-fluorouracil,<sup>17)</sup> or SN-38 (unpublished data). Some investigators have reported synergistic effects of the combination of raltitrexed with 5-fluorouracil, SN-38, and cisplatin.<sup>18–20)</sup> The different results might be due mainly to differences in the analytical methods used to evaluate the drug combinations. The isobologram method of Steel and Peckham is generally stricter regarding synergism and antagonism than other methods. Furthermore, the experimental conditions, such as cell lines used, exposure time, and assay method, differed in each study and might also contribute to the different results.

Sequential treatment with MTX followed by the indirect thymidylate synthase inhibitor, 5-fluorouracil, has been used for the treatment of cancer,<sup>10,11)</sup> based upon synergistic interaction found in experimental studies.<sup>21,22)</sup> 5-Fluorouracil is believed to have two mechanisms of action responsible for cytotoxicity. The mechanism of synergistic

effects in the MTX-5-fluorouracil sequence is considered to be the elevation of intracellular phosphoribosyl pyrophosphate by MTX, which results in increased 5-fluorouracil nucleotide formation.<sup>22)</sup> This mechanism does not operate in the MTX-raltitrexed sequence.

Although the biochemical basis for synergistic interaction in sequential exposure to MTX followed by raltitrexed is obscure, several biochemical interactions are possible. MTX inhibits dihydrofolate reductase, resulting in a decreased 5,10-methylenetetrahydrofolate pool and increased uridylate pool. The treatment with raltitrexed may enhance the formation of a ternary complex of thymidylate synthase with raltitrexed and uridylate. Secondly, raltitrexed and MTX exert their cytotoxic effects by blocking cells in the S phase.<sup>1,23,24)</sup> Thus, MTX might enhance the cytotoxicity of raltitrexed by blocking cells in the S-phase, in which the cells are most sensitive to raltitrexed.

MTX-raltitrexed may be the optimal schedule at the cellular level, but there are a number of difficulties in the translation of results from *in vitro* or animal models to clinical therapy. The biochemistry of the cells may be quite different, although the mechanisms of the cytotoxicity are generally thought to be similar. Secondly, pharmacokinetic, cell kinetics and other parameters may be significantly different between them. Thirdly, toxic effects of the combination can not be measured by an *in vitro* system. Moderate or high-dose MTX requires leucovorin to prevent MTX toxicity in clinical therapy.<sup>1)</sup> Leucovorin rescue is generally started 24–48 h after the beginning of MTX. When the sequential administration of moderate or high-dose MTX followed by raltitrexed is employed, the administration schedules of MTX, raltitrexed and leucovorin must be carefully controlled. If leucovorin were administered simultaneously with raltitrexed or slightly after raltitrexed, the cytotoxicity of raltitrexed would be diminished, whereas if leucovorin rescue were too late or too weak, severe toxicity might occur. Low-dose MTX followed by raltitrexed without leucovorin rescue may be a reasonable choice, since synergistic effects were observed even with a low concentration of MTX in this study (Fig. 5).

To examine the usefulness and limitations of our work, we analyzed the relationship between the results of our previous studies and the clinical outcome of paclitaxel in combinations. The optimal schedules of paclitaxel with cisplatin, doxorubicin, 5-fluorouracil, etoposide and vinorelbine proposed in our studies were essentially the same as commonly used schedules of these combinations in clinics. With the paclitaxel-doxorubicin combination, we observed that simultaneous exposure to paclitaxel and doxorubicin and sequential exposure to doxorubicin followed by paclitaxel produced antagonistic effects, while the paclitaxel-doxorubicin sequence produced additive effects, suggesting that the paclitaxel-doxorubicin

sequence would be appropriate.<sup>25)</sup> Pharmacokinetic study, however, showed that the paclitaxel-doxorubicin sequence decreases doxorubicin clearance, resulting in higher toxicity than the reverse sequence<sup>26)</sup> and it is still unclear which sequence is appropriate for this combination in clinics.<sup>27)</sup>

In conclusion, the present findings suggest that the drug schedule may be an important determinant of the antitumor activity of MTX and raltitrexed in combination. The sequential administration of MTX followed by raltitrexed produced synergistic effects and may have clinical poten-

tial, while the simultaneous administration of raltitrexed and MTX and the sequential administration of raltitrexed followed by MTX produced additive effects. Further *in vivo* and clinical studies will be necessary to determine the toxicity and to test the antitumor effects of sequential administration of MTX followed by raltitrexed proposed on the basis of the *in vitro* synergism.

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