Jpn. J. Cancer Res. **91**, 424–432, April 2000

Schedule-dependent Interactions between Raltitrexed and Cisplatin in Human Carcinoma Cell Lines *in vitro*

Yasuhiko Kano,[1, 4] Miyuki Akutsu,[1] Kenichi Suzuki,[2] Yasuo Yazawa[3] and Saburo Tsunoda[1]

[1]Divisions of Medical Oncology, [2]Laboratory Medicine and [3]Orthopedic Oncology, Tochigi Cancer Center, 4-9-13 Yonan, Utsunomiya, Tochigi 320-0388

Raltitrexed ('Tomudex') is a new anticancer agent which inhibits thymidylate synthase. To provide a rational basis for clinical trial design of the combination of raltitrexed and cisplatin, we studied the cytotoxic effects of this combination using various schedules *in vitro* and four human colon cancer cell lines, Colo201, Colo320, LoVo, and WiDr. Cell growth inhibition after 5 days was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The effects of drug combinations at the concentration producing 80% cell growth inhibition (IC_{80}) level were analyzed by the isobologram method. Simultaneous exposure to raltitrexed and cisplatin for 24 h, and sequential exposure to raltitrexed followed by cisplatin produced additive effects in the Colo201, Colo320, and LoVo cells, and additive and synergistic effects in WiDr cells. Sequential exposure to cisplatin followed by raltitrexed produced additive effects in the Colo201 cells and antagonistic effects in other three cell lines. Simultaneous and continuous exposure to both agents for 5 days produced additive effects in all four cell lines. These findings suggest that the simultaneous administration of raltitrexed and cisplatin, or the sequential administration of raltitrexed followed by cisplatin, generally produce the expected cytotoxicity at the cellular level and are optimal schedules, while the sequential administration of cisplatin followed by raltitrexed produces antagonistic effects and is inappropriate for this combination. Further *in vivo* and clinical studies will be necessary to determine the toxicity and antitumor effects of this schedule.

Key words: Raltitrexed — Cisplatin — Drug combination — Isobologram

Thymidylate synthase, which catalyzes the reductive methylation of uridylate to thymidylate, plays an essential role in DNA synthesis and DNA repair. Raltitrexed ('Tomudex') is a folate analogue which inhibits thymidylate synthase by competitive binding to the binding site of the natural co-factor 5,10-methylenetetrahydrofolate.[1)] This agent is taken up via the reduced-folate carrier and requires polyglutamation for optimal inhibition of cell growth, as do reduced folates and methotrexate, an inhibitor of dihydrofolate reductase. Preclinical studies of raltitrexed have shown significant activity against a variety of tumor cell lines.[1, 2)]

Phase I study showed a maximum tolerated dose of 3.5 mg/m^2 by 15-min infusion once every three weeks, with dose-limiting toxicities involving malaise, gastrointestinal toxicity, and myelotoxicity.[3)] Pharmacokinetic studies have shown triphasic clearance with β- and γ-half-lives of 2 and >10 h.[3, 4)] Clinical studies have demonstrated significant activity in colon cancer and breast cancer[5–7)] and clinical studies for some other cancers are in progress.[8–11)] Phase III trials comparing raltitrexed with standard 5-fluorouracil/leucovorin combination in patients with advanced colorectal cancer have been completed.[7, 12, 13)] These trials showed that raltitrexed has activity comparable to that of 5-fluorouracil/leucovorin but shows less toxicity with respect to leucopenia and mucositis. A logical step in the development of raltitrexed for use against solid tumors is its evaluation in potentially synergistic or at least additive combination regimens.

Cisplatin has been widely used for the treatment of solid tumors. The dose-limiting toxicity of cisplatin involves nausea, vomiting, and nephrotoxicity. Myelotoxicity is mild. The use of cisplatin is frequently limited by the rapid development of resistance. Cisplatin acts by binding to DNA to form DNA adducts.[14)] The most common adduct involves binding of platinum to two adjacent guanines on the same DNA strand. In addition, adducts of platinum with guanine and an adjacent adenine of the same strand, with two guanines on opposite DNA strands, and with one guanine are also found. Cisplatin-DNA adducts are considered to introduce a distortion in the DNA that is large enough to stop the division of the cells

[4] To whom requests for reprints should be addressed.
E-mail: ykano@tcc.pref.tochigi.jp

The abbreviations used are: FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC_{80}, concentration producing 80% cell growth inhibition; FdUMP, fluorodeoxyuridine 5′-monophosphate; FUTP, 5-fluorouridine 5′-triphosphate.

without being recognized rapidly, and thus removed efficiently by repair enzymes. Repair replication induced by cisplatin is enhanced,[15] or intrastrand adducts are removed more rapidly in resistant cells.[16]

The rationale for the combination of raltitrexed with cisplatin is that raltitrexed and cisplatin have different mechanisms of action, different toxicity profiles, and no cross-resistance.[17] Furthermore, through inactivation of thymidylate synthase, raltitrexed inhibits DNA repair, which is considered to be the major mechanism of cisplatin resistance. The combination of 5-fluorouracil, an indirect thymidylate synthase inhibitor, and cisplatin has been widely used for the treatment of solid tumors. Clinical studies of the combination of raltitrexed and a platinum derivative are in progress.[18] Information on the experimental antitumor efficacy of the combination of raltitrexed with platinum derivatives is limited[17, 19–21] and the optimal schedule of this combination is unknown.

The present study was aimed at elucidating the cytotoxic effects of combinations of raltitrexed and cisplatin on various schedules in four human carcinoma cell lines. The data obtained were analyzed by the isobologram method of Steel and Peckham.[22]

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^2 plastic tissue culture flasks containing RPMI1640 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated 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 cisplatin were obtained from Zeneca Japan Co. (Tokyo), and Nihon Kayaku Co. (Tokyo), respectively. Raltitrexed was dissolved in 0.15 m*M* $NaHCO_3$ at a concentration of 1 m*M* and cisplatin was dissolved in RPMI1640 medium at a concentration of 1 m*M*. The drugs were diluted with RPMI1640 plus 10% FBS.

Cell growth inhibition by the combination of raltitrexed and cisplatin On day 0, exponentially growing cells were harvested with trypsin:EDTA (0.05%:0.02%) and resuspended to final concentrations of 2.0×10^4 cells/ml for Colo201, Colo320, and LoVo cells, and 5.0×10^3 cells/ml for WiDr cells in fresh medium containing 10% FBS, penicillin G and streptomycin. Cell suspensions (100 μl) 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 and continuous exposure to raltitrexed and cisplatin for 5 days After 20- to 24-h incubation, solutions of raltitrexed and cisplatin (50 μl each) at different concentrations were added to individual wells containing cell preparations. The plates were then incubated under the same conditions for 5 days.

Simultaneous exposure to raltitrexed and cisplatin for 24 h After 20- to 24-h incubation, solutions of raltitrexed and cisplatin (50 μl each) at different concentrations were added to individual wells containing cell suspensions (raltitrexed preceding cisplatin by about 10 min). The plates were then incubated under the conditions described above for 24 h. After treatment, the cells were washed twice with culture medium containing 1% FBS, then fresh medium containing 10% FBS (200 μl) was added and the cells were incubated again for 4 days.

Sequential exposure to raltitrexed for 24 h followed by cisplatin for 24 h or *vice versa* After 20- to 24-h incubation, medium containing 10% FBS (50 μl) and solutions of raltitrexed (or cisplatin) (50 μl) at different concentrations were added to individual wells containing the cell suspensions. The plates were then incubated under the same conditions for 24 h. The cells were washed twice with culture medium containing 1% FBS, and then fresh medium containing 10% FBS (150 μl) and solutions of cisplatin (or raltitrexed) (50 μl) at different concentrations were added. The plates were incubated again under the same conditions for 24 h. After treatment, the cells were washed twice, fresh medium was added and the cells were incubated again for 3 days.

MTT assay Viable cell growth was determined by MTT reduction assay as described previously.[23] For the background control, control (no drug), each drug, or drug combination, four intermediate data values of eight data values were used for the analysis and the two highest and the two lowest data values were omitted. For all four cell lines examined, we established a linear relation between the MTT assay value and the cell number within the range shown.

Isobolograms The dose-response interactions between raltitrexed and cisplatin at the point of IC_{80} were evaluated by the isobologram method of Steel and Peckham.[22] The IC_{80} was defined as the concentration of drug that produced 80% cell growth inhibition, i.e., an 80% reduction of absorbance. Recently, we have been using IC_{80} instead of the more common IC_{50}, since IC_{80} would be more important than IC_{50} for cancer chemotherapy. Although the

drug interaction at IC_{90} or more would be more important than IC_{50} or IC_{80}, it is difficult to get reliable data at the IC_{90} or IC_{99} level using insensitive MTT assay.

When the dose-response curves are far from linear, as is usually the case in cancer chemotherapy and was the case in this study, the nature of an additive response is controversial.[24–27] We use the isobologram method of Steel and Peckham because this method can be applied for agents with unclear cytotoxic mechanisms and a variety of dose-response curves. There is an area of uncertainty, the magnitude of which depends upon the non-linearity of the responses. The extent of the uncertainty is best judged by the use of this isobologram, which is an iso-effect plot indicating the separate doses of two agents that in combination give the iso-effects.

Fig. 1 shows a schematic representation of the isobologram. A French curve model fit to the data was used to make dose-response curves and the isobolograms. The procedure for making the isobologram has been described in detail previously.[28, 29]

If the two agents act additively by independent mechanisms, the combined data points will lie near the mode I line (hetero-addition). If the agents act additively by similar mechanisms, the 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 the drug combination fell within the area surrounded by mode I and/or mode II lines (i.e., within the envelope of additivity), the combination was described as additive. The envelope of additivity should not be regarded as a reliable definition of additivity. It is an expression of the uncertainty of this method, and the concept of uncertainty is important in the use of Steel and Peckham isobolograms. The Steel and Peckham isobologram is generally stricter regarding synergism and antagonism than other methods.

We used this envelope not only to evaluate the simultaneous exposure combinations of raltitrexed and cisplatin, but also to evaluate the sequential exposure combinations, since the second agent under our experimental conditions might modulate the cytotoxicity of the first agent.

A combination that gives data points to the left of the envelope of additivity (i.e., the combined effect is caused by lower doses of the two agents than is predicted) can confidently be described as supra-additive (synergistic). A combination that gives data points to the right of the envelope of additivity, but within the square or on the line of the square can be described as sub-additive (i.e., the combination is superior or equal to a single agent but is less than additive). A combination that gives data points outside the square can be described as protective (i.e., the combination is inferior in cytotoxic action to a single agent).

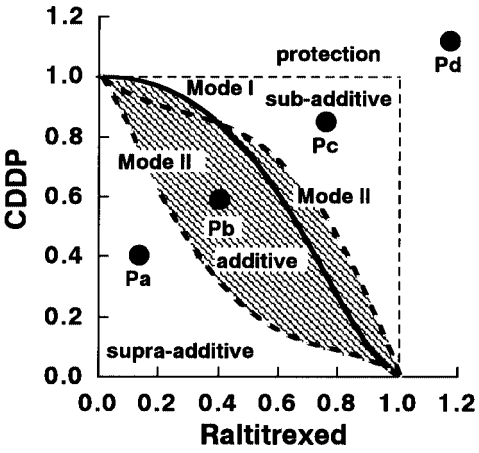

Fig. 1. Schematic representation of an isobologram (Steel and Peckham). The envelope of additivity, surrounded by mode I (solid line) and mode II (dotted lines) isobologram lines, was constructed from the dose-response curves of raltitrexed and cisplatin (CDDP). The concentrations, which produced 80% cell growth inhibition, were expressed as 1.0 in the ordinate and the abscissa of all isobolograms. Combined data points Pa, Pb, Pc, and Pd show supra-additive, additive, sub-additive, and protective effects, respectively.

A combination with either sub-additive and/or protective interactions can confidently be described as antagonistic.

Data analysis Data were analyzed as described previously.[30] When the observed data points in 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 those of the predicted maximum values and the predicted minimum values for an additive effect. 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.

When the observed data points from combinations fell mainly in the area of supra-additivity or in the areas of subadditivity and protection, i.e., 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, the combinations were considered to have a synergistic or an antagonistic effect, respectively. To determine whether the condition of synergism (or antagonism) truly existed, a Wilcoxon signed-rank test was performed to compare the observed data with the predicted minimum (or maximum) values for an additive effect. Probability values (P)$\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

Experiments were repeated three or four times in order to evaluate the validity of the assay system. Each point represents the mean value for the experiment.

Fig. 2 shows the dose-response curves obtained by the simultaneous exposure and the sequential exposure to raltitrexed and cisplatin for LoVo cells. The dose-response curves were plotted on a semilog scale as a percentage of the control, the cell number of which was obtained from the samples not exposed to the drugs administered simultaneously. The raltitrexed concentrations are shown on the abscissa. Dose-response curves in which the cisplatin concentrations are shown on the abscissa can be made based on the same data (figure not shown). Based upon the dose-response curves of raltitrexed alone and cisplatin alone, three isoeffect curves (mode I and mode II lines) were constructed.

Simultaneous exposure to raltitrexed and cisplatin for 5 days Fig. 3 shows isobolograms of simultaneous and continuous exposure to raltitrexed and cisplatin for 5 days. For Colo201, LoVo, and WiDr cells, most of the data points fell within the envelope of additivity. The mean values of the observed data (0.73, 0.75, and 0.65, respectively) were larger than those of the predicted minimum values (0.57, 0.41, and 0.36, respectively), and smaller than those of the predicted maximum values (0.84, 0.83, and 0.81, respectively), suggesting additive effects (Table I). For Colo320 cells, the combined data points fell within the envelope of additivity and in the area of sub-additivity. The mean value of the observed data (0.81) was equal to that of the predicted maximum values (0.81), suggesting additive effects.

Simultaneous exposure to raltitrexed and cisplatin for 24 h Fig. 4 shows isobolograms of the Colo201, Colo320, LoVo, and WiDr cells after simultaneous exposure to raltitrexed and to cisplatin. For Colo201, Colo320, and LoVo cells, most of the combined data points fell within the envelope of additivity. The mean values of the observed data (0.72, 0.75, and 0.79, respectively) were larger than those of the predicted minimum values (0.51, 0.55, and 0.51, respectively), and smaller than those of the predicted maximum values (0.75, 0.82, and 0.81, respectively), suggesting additive effects (Table I). For WiDr cells, the combined data points fell within the envelope of additivity and in the area of supra-additivity. The mean value of the observed data (0.58) was smaller than that of the predicted minimum values (0.59), but the *P* values were larger than 0.05, suggesting additive and synergistic effects.

Sequential exposure to raltitrexed for 24 h followed by cisplatin for 24 h Fig. 5 shows isobolograms of the Colo201, Colo320, LoVo, and WiDr cells exposed first to raltitrexed and then cisplatin. For Colo201, Colo320, and LoVo cells, most of the combined data points fell within

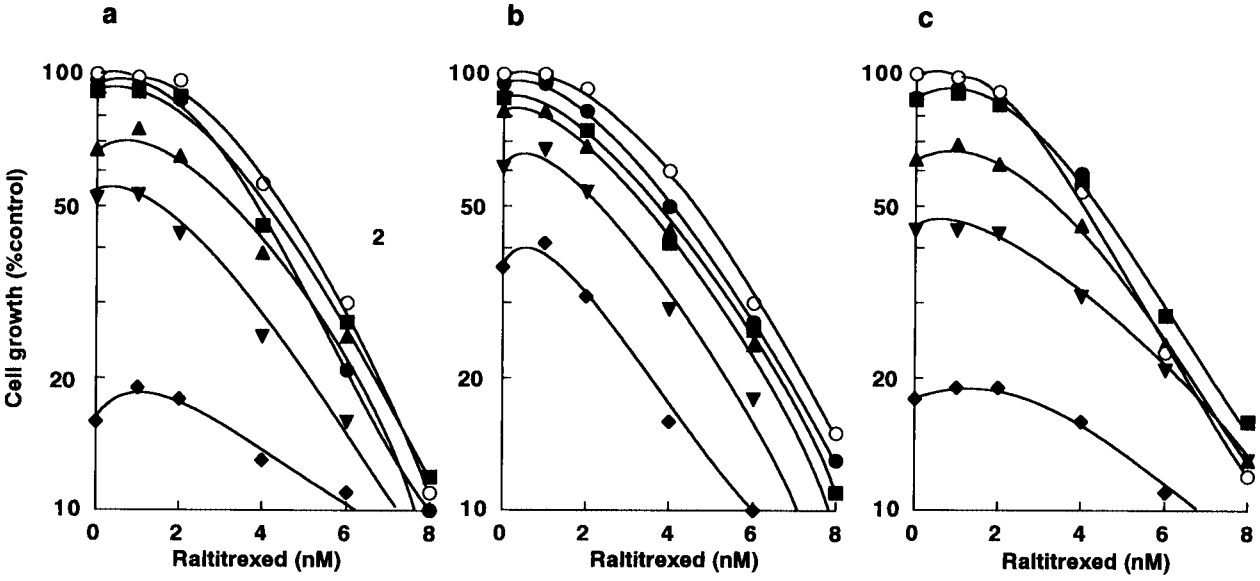

Fig. 2. Schedule dependence of the interaction between raltitrexed and cisplatin in LoVo cells. Cells were exposed to these two drugs simultaneously for 24 h (a), raltitrexed first for 24 h followed by cisplatin for 24 h (b), or the reverse sequence (c). The cell number after 5 days was measured using the MTT assay and was plotted as a percentage of the control (cells not exposed to drugs). The concentations of raltitrexed are shown on the abscissa. The concentrations of cisplatin were 0 (○), 0.1 (●), 0.2 (■), 0.5 (▲), 1.0 (▼) and 2.0 (◆) μM. Data are mean values for three independent experiments; SE was <20%.

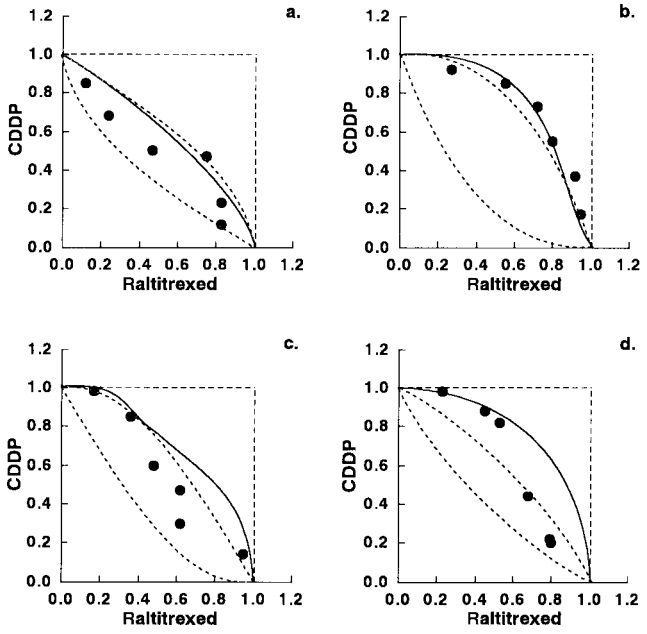

Fig. 3. Isobolograms of simultaneous and continuous exposure to raltitrexed and cisplatin for 5 days for Colo201 (a), Colo320 (b), LoVo (c), and WiDr (d) cells. Data are mean values for at least three independent experiments; SE was <15%. For Colo201, LoVo, and WiDr cells, most of the data points fell within the envelope of additivity. For Colo320 cells, the combined data points fell within the envelope of additivity and in the area of sub-additivity.

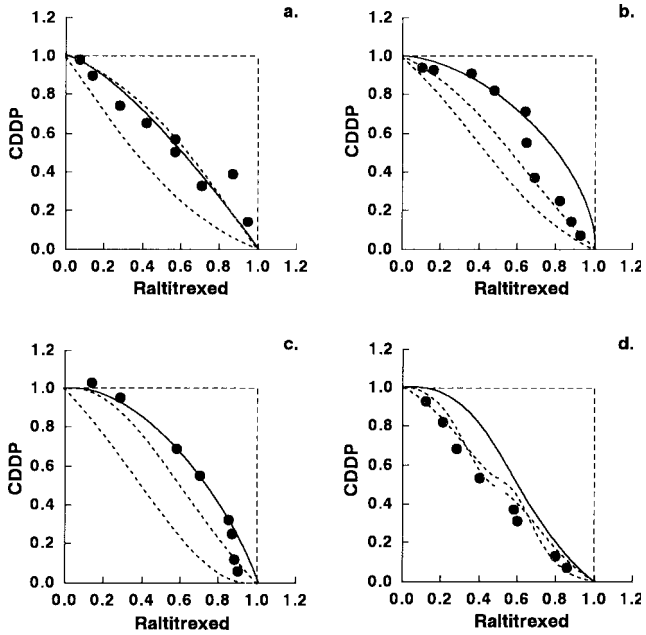

Fig. 4. Isobolograms of simultaneous exposure to raltitrexed and cisplatin for 24 h for Colo201 (a), Colo320 (b), LoVo (c), and WiDr (d) cells. Data are mean values for at least three independent experiments; SE was <20%. For Colo201, Colo320, and LoVo cells, most of the combined data points fell within the envelope of additivity. For WiDr cells, the combined data points fell within the envelope of additivity and in the area of supra-additivity.

Table I. The Mean Values of Observed Data, Predicted Minimum, and Predicted Maximum, Values, and the Outcome for the Combination of Raltitrexed (R) and Cisplatin (C)

Schedule	Cell line	*n*	Observed data[a)]	Predicted min.[b)]	Predicted max.[c)]	Outcome
R+C (5d)	CoLo201	6	0.73	0.57	0.84	additive
	CoLo320	6	0.81	0.23	0.81	additive
	LoVo	6	0.75	0.41	0.83	additive
	WiDr	6	0.65	0.36	0.81	additive
R+C (24h)	CoLo201	9	0.72	0.51	0.75	additive
	CoLo320	10	0.75	0.55	0.82	additive
	LoVo	8	0.79	0.51	0.81	additive
	WiDr	8	0.58	0.59	0.71	additive/synergism (P=NS)
R (24h)→C (24h)	CoLo201	10	0.43	0.42	0.87	additive
	CoLo320	11	0.69	0.62	0.82	additive
	LoVo	9	0.69	0.41	0.78	additive
	WiDr	10	0.56	0.57	0.72	additive/synergism (P=NS)
C (24h)→R (24h)	CoLo201	11	0.73	0.55	0.84	additive
	CoLo320	9	0.82	0.61	0.75	antagonism (P<0.05)
	LoVo	8	0.94	0.45	0.76	antagonism (P<0.02)
	WiDr	11	>0.75	0.32	0.62	antagonism (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 predicted maximum values for an additive effect.

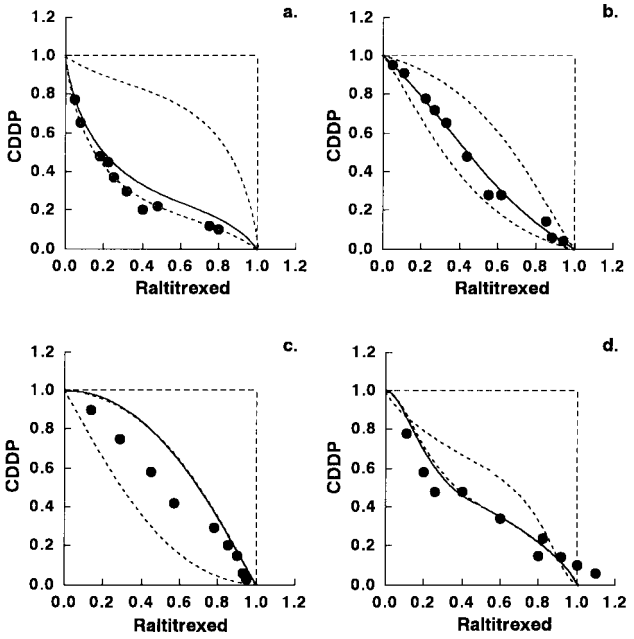

Fig. 5. Isobolograms of sequential exposure to raltitrexed (24 h) followed by cisplatin (24 h) in Colo201 (a), Colo320 (b), LoVo (c), and WiDr (d) cells. Data are mean values for at least three independent experiments; SE was <20%. For Colo201, Colo320, and LoVo cells, most of the combined data points fell within the envelope of additivity. For WiDr cells, the combined data points fell within the envelope of additivity and in the areas of supra-additivity, sub-additivity and protection.

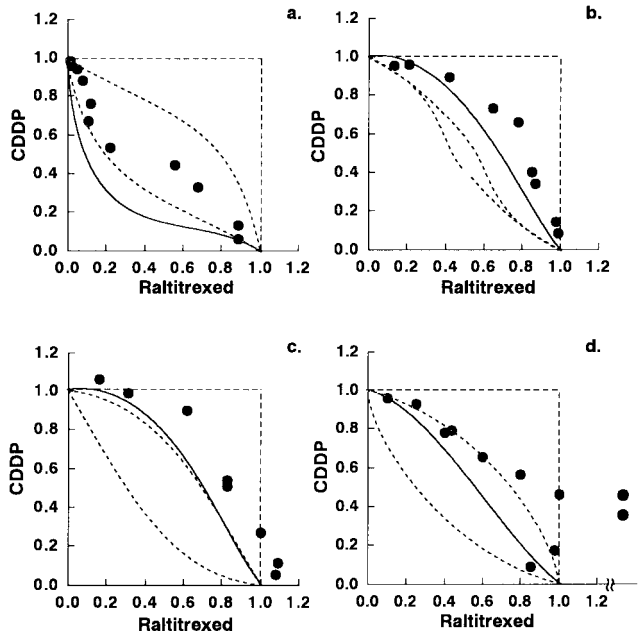

Fig. 6. Isobolograms of sequential exposure to cisplatin (24 h) followed by raltitrexed (24 h) in Colo201 (a), Colo320 (b), LoVo (c), and WiDr (d) cells. Data are mean values for at least three independent experiments; SE was <25%. For Colo320, LoVo, and WiDr cells, all or most of the combined data points fell in the areas of sub-additivity and protection. For Colo201 cells, all data points fell within the envelope of additivity.

the envelope of additivity. The mean values of the observed data were larger than those of the predicted minimum values, and smaller than those of the predicted maximum values, suggesting additive effects (Table I). For WiDr cells, the combined data points fell within the envelope of additivity and in the areas of supra-additivity, sub-additivity and protection. The mean value of the observed data (0.56) was smaller than that of the predicted minimum values (0.57), but the *P* values were larger than 0.05, suggesting additive and synergistic effects.

Sequential exposure to cisplatin for 24 h followed by raltitrexed for 24 h Fig. 6 shows isobolograms of the four cell lines treated with the reverse sequence (cisplatin, then raltitrexed). For Colo320, LoVo, and WiDr cells, all or most of the combined data points fell in the areas of sub-additivity and protection. The mean values of the observed data were larger than those of the predicted maximum values (Table I). The *P* values were less than 0.05 (<0.05, <0.02, and <0.05, respectively). These results suggest that the sequential exposure to cisplatin first followed by raltitrexed produced antagonistic effects in these cell lines. For Colo201 cells, the combined data points fell within the envelope of additivity and the mean value of the observed data was between the predicted minimum and maximum values (Table I), suggesting an additive effect of this schedule.

DISCUSSION

Raltitrexed has been studied extensively as monotherapy (particularly for colorectal cancer) over the past few years. We are now moving into a phase of clinical evaluation involving combination therapy with other cytotoxic agents for a variety of cancers, and with radiotherapy for colorectal cancer. The drug is also being evaluated as adjuvant therapy for colorectal cancer.

We studied the cytotoxic activity of simultaneous and sequential exposure to raltitrexed and cisplatin at the IC_{80} level in four human carcinoma cell lines in culture to determine the optimal schedule of the combination of raltitrexed and cisplatin. The analysis of the effects of drug-drug interaction was carried out by the isobologram method of Steel and Peckham.[22)]

We demonstrated that cytotoxic interaction between raltitrexed and cisplatin was schedule-dependent. Simultaneous exposure to raltitrexed and cisplatin for 24 h

showed additive effects in three of four cell lines, and additive/synergistic effects in one of four cell lines, while simultaneous exposure for 5 days showed additive effects in all four cell lines. Ackland *et al.*[19] and Kelland *et al.*[17] observed that simultaneous and continuous exposure to raltitrexed and cisplatin for 72 h produced additive to synergistic effects and additive effects, respectively, against human ovarian carcinoma cell lines. On the other hand, Jackman *et al*. observed that simultaneous exposure to raltitrexed and cisplatin and sequential exposure to raltitrexed followed by cisplatin or *vice versa* produced antagonistic effects in two human carcinoma cell lines.[21] Our data are consistent with the findings of Ackland *et al*. and Kelland *et al*.

Sequential exposure to raltitrexed for 24 h followed by cisplatin for 24 h showed additive effects in three cell lines, and additive/synergistic effects in one cell line. Sequential exposure to cisplatin for 24 h followed by raltitrexed for 24 h without an interval showed antagonistic effects in three of the four cell lines studied. No definite synergistic effect was found with any schedule of this combination.

Our data suggest that simultaneous exposure to raltitrexed and cisplatin and sequential exposure to raltitrexed followed by cisplatin generally produced additive effects, while sequential exposure to cisplatin followed by raltitrexed produced antagonistic effects. Schedules that produce additive or synergistic interactions, but not those that produce antagonistic interactions, are suitable for combination treatments. Therefore, the simultaneous administration of raltitrexed and cisplatin, or the sequential administration of raltitrexed followed by cisplatin, may be optimal for this combination. The sequential administration of cisplatin followed by raltitrexed should be avoided. The mechanism of the antagonistic effect of sequential exposure to cisplatin followed by raltitrexed is unknown. Cisplatin blocks the cells at the G_2 phase,[31] while S-phase cells are most sensitive to raltitrexed.[32] The disturbance of the cell cycle by cisplatin may weaken the cytotoxic effect of raltitrexed. This result is different from that of sequential exposure to cisplatin followed by 5-fluorouracil, which produced additive effects in our test system (data not shown). 5-Fluorouracil is believed to have two mechanisms of action responsible for cytotoxicity: 5-fluorouracil is converted to FdUMP and FUTP. FdUMP binds to thymidylate synthase and inhibits the formation of thymidylate, and then DNA synthesis, while FUTP is incorporated into RNA and interferes with RNA synthesis.[33] Therefore, the action of 5-fluorouracil is not strictly S-phase-specific. The differences of cytotoxic mechanism and cell cycle dependency between raltitrexed and 5-fluorouracil might contribute to the difference of results of cytotoxicity between the cisplatin/raltitrexed sequence and cisplatin/5-fluorouracil sequence.

The fact that there was no synergistic effect in any schedule of the combination of raltitrexed and cisplatin in our study does not negate the usefulness of this combination. In general, the isobologram of Steel and Peckham is stricter for synergism and antagonism than other methods for evaluating the effects of drug combinations. Drug combinations do not always require synergistic effects to achieve success in clinical regimens. Since the two drugs have different prevailing toxicities, this additivity will imply a clinical synergy if the two drugs can be combined without reducing their dose levels to a greater extent.

It must be noted that *in vitro* study represents antitumor effects only for a constant drug exposure against rapidly growing cancer cell lines and can not evaluate toxic and pharmacokinetic interactions of the combination. *In vivo*, additional factors such as drug penetration, drug metabolism, and heterogeneities of cancer cells and cell cycles must be considered. Further preclinical and clinical studies will be required to determine which schedule is optimal for the combination of raltitrexed and cisplatin.

In conclusion, our findings suggest that the cytotoxic effects of raltitrexed and cisplatin are schedule-dependent. The simultaneous administration of raltitrexed and cisplatin, and the sequential administration of raltitrexed followed by cisplatin produced the expected cytotoxicity and may be the optimal schedule at the cellular level, while the sequential administration of cisplatin followed by raltitrexed produced antagonistic effects and may be inappropriate for this combination. These findings should be helpful in designing chemotherapeutic regimens to test the efficacy of raltitrexed in combination with cisplatin in animal and clinical studies.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Cancer Research (11-8) from the Ministry of Health and Welfare, Japan.

(Received October 18, 1999/Revised January 12, 2000/Accepted January 19, 2000)

REFERENCES

1) Jackman, A. L., Taylor, G. A., O'Connor, B. M., Bishop, J. A., Moran, R. G. and Calvert, A. H. Activity of the thymidylate synthase inhibitor 2-desamino-N^{10}-propargyl-5,8-dideazafolic acid and related compounds in murine (L1210) and human (W1L2) systems *in vitro* and in L1210 *in vivo*. *Cancer Res.*, **50**, 5212–5218 (1990).
2) Jackman, A. L., Taylor, G. A., Gibson, W., Kimbell, R., Brown, M., Calvert, A. H., Judson, I. R. and Hughes, L. R.

ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth *in vitro* and *in vivo*: a new agent for clinical study. *Cancer Res.*, **51**, 5579–5586 (1991).

3) Clarke, S. J., Hanwell, J., de Boer, M., Planting, A., Verweij, J., Walker, M., Smith, R., Jackman, A. L., Hughes, L. R., Harrap, K. R., Kennealey, G. T. and Judson, I. R. Phase I trial of ZD1694, a new folate-based thymidylate synthase inhibitor, in patients with solid tumors. *J. Clin. Oncol.*, **14**, 1495–1503 (1996).
4) Beale, P., Judson, I., Hanwell, J., Berry, C., Aherne, W., Hickish, T., Martin, P. and Walker, M. Metabolism, excretion and pharmacokinetics of a single dose of [^{14}C]raltitrexed in cancer patients. *Cancer Chemother. Pharmacol.*, **42**, 71–76 (1998).
5) Blackledge, G. New developments in cancer treatment with the novel thymidylate synthase inhibitor raltitrexed ('Tomudex'). *Br. J. Cancer*, **77** (Suppl. 2), 29–37 (1998).
6) Smith, I., Jones, A., Spielmann, M., Namer, M., Green, M. D., Bonneterre, J., Wander, H. E., Hatschek, T., Wilking, N., Zalcberg, J., Spiers, J. and Seymour, L. A phase II study in advanced breast cancer: ZD1694 ('Tomudex') a novel direct and specific thymidylate synthase inhibitor. *Br. J. Cancer*, **74**, 479–481 (1996).
7) Cunningham, D. Mature results from three large controlled studies with raltitrexed ('Tomudex'). *Br. J. Cancer*, **77** (Suppl. 2), 15–21 (1998).
8) Meropol, N. J., Pazdur, R., Vincent, M., Willson, J. K., Kelsen, D. P. and Douglass, H. O., Jr. Phase II study of ZD1694 in patients with advanced gastric cancer. *Am. J. Clin. Oncol.*, **19**, 628–630 (1996).
9) Pazdur, R., Meropol, N. J., Casper, E. S., Fuchs, C., Douglass, H. O., Jr., Vincent, M. and Abbruzzese, J. L. Phase II trial of ZD1694 (Tomudex) in patients with advanced pancreatic cancer. *Invest. New Drugs*, **13**, 355–358 (1996).
10) Smith, I., Jones, A., Spielmann, M., Namer, M., Green, M. D., Bonneterre, J., Wander, H. E., Hatschek, T., Wilking, N., Zalcberg, J., Spiers, J. and Seymour, L. A phase II study in advanced breast cancer: ZD1694 ('Tomudex') a novel direct and specific thymidylate synthase inhibitor. *Br. J. Cancer*, **74**, 479–481 (1996).
11) Rougier, P., Ducreux, M., Kerr, D., Carr, B. I., Francois, E., Adenis, A. and Seymour, L. A phase II study of raltitrexed ('Tomudex') in patients with hepatocellular carcinoma. *Ann. Oncol.*, **8**, 500–502 (1997).
12) Harper, P. Advanced colorectal cancer (ACC): results from the latest Tomudex (raltitrexed) comparative study. *Proc. Am. Soc. Clin. Oncol.*, **16**, 228a (1997).
13) Pazdur, R. and Vincent, M. Raltitrexed ('Tomudex') versus 5-fluorouracil and leucovorin (5-FU+LV) in patients with advanced colorectal cancer (ACC): results of a randomized, multicenter North American trial. *Proc. Am. Soc. Clin. Oncol.*, **16**, 228a (1997).
14) Pinto, A. and Lippard, S. J. Binding of the antitumor drug (II) (cisplatin) to DNA. *Biochim. Biophys. Acta*, **780**, 167–180 (1985).
15) Masuda, H., Ozols, R. F., Lai, G. M., Foji, A., Rothenberg, M. and Hamilton, T. C. Increased DNA repair as a mechanism of acquired resistance to *cis*-diamminedichloroplatinum(II) in ovarian cancer cell lines. *Cancer Res.*, **48**, 5713–5716 (1988).
16) Bedford, P., Fichtinger-Schepman, A. M. J., Shellard, S. A., Walker, M. C., Masters, J. R. W. and Hill, B. T. Differential repair of platinum-DNA adducts in human bladder and testicular tumor continuous cell lines. *Cancer Res.*, **48**, 3019–3024 (1998).
17) Kelland, L. R., Kimbell, R., Hardcastle, A., Aherne, G. W. and Jackman, A. L. Relationships between resistance to cisplatin and antifolates in sensitive and resistant tumor cell lines. *Eur. J. Cancer*, **31A**, 981–986 (1995).
18) Fizazi, K., Soria, J. C., Bonny, M., Ruffie, P., Ducreux, M., Le Chevalier, T., Couturas, O., Poterre, M. and Armand, J. P. Phase I/II dose-finding and pharmacokinetic study of Tomudex in combination with oxaliplatin in advanced solid tumor. *Proc. Am. Soc. Clin. Oncol.*, **17**, 20a (1998).
19) Ackland, S., Kuiper, C. M., Carg, M., Bergman, A. M., Smid, K. and Peters, G. J. Variable effects of the combination of Tomudex (ZD1694) and cisplatin in ovarian cancer cell lines. *Ann. Oncol.*, **7**, 7 (1996).
20) Raymond, E., Djelloul, S., Buquest-Fagot, C., Goldwasser, F., Mester, J., Cvitkovic, E., Louvet, C. and Gespach, C. Oxaliplatin and cisplatin in combination with 5FU, specific thymidylate synthase inhibitors (AG337, ZD1694) and topoisomerase I inhibitors (SN-38, CPT-11) in human colonic, ovarian and breast cancers. *Proc. Am. Assoc. Cancer Res.*, **37**, 291a (1996).
21) Jackman, A. L., Kimbell, R. and Ford, H. E. R. Combination of raltitrexed with other cytotoxic agents: rationale and preclinical observations. *Eur. J. Cancer*, **35** (Suppl. 1), S3–S8 (1999).
22) Steel, G. G. and Peckham, M. J. Exploitable mechanisms in combined radiotherapy-chemotherapy: the concept of additivity. *Int. J. Radiat. Oncol. Biol. Phys.*, **5**, 85–91 (1979).
23) Kano, Y., Sakamoto, S., Kasahara, T., Akutsu, M., Inoue, Y. and Miura, Y. *In vitro* effects of amsacrine in combination with other anticancer agents. *Leuk. Res.*, **15**, 1059–1064 (1991).
24) Rideout, D. C. and Chou, T. C. Synergism, antagonism, and potentiation of chemotherapy. *In* "Synergism and Antagonism in Chemotherapy," ed. T. C. Chou and D. C. Rideout, pp. 3–60 (1991). Academic Press, San Diego.
25) Berenbaum, M. C. What is synergy? *Pharmacol. Rev.*, **41**, 93–141 (1988).
26) Greco, W. R., Bravo, G. and Parsons, J. C. The search for synergy: a critical review from a response surface perspective. *Pharmacol. Rev.*, **47**, 331–385 (1995).
27) Frey, C. M. Role of modelling in joint action studies. *J. Natl. Cancer Inst.*, **86**, 1493–1495 (1994).
28) Kano, Y., Ohnuma, T., Okano, T. and Holland, J. F. Effects of vincristine in combination with methotrexate and

other antitumor agents in human acute lymphoblastic leukemia cells in culture. *Cancer Res.*, **48**, 351–356 (1988).
29) Kano, Y., Suzuki, K., Akutsu, M., Suda, K., Inoue, Y., Yoshida, M., Sakamoto, S. and Miura, Y. Effects of CPT-11 in combination with other anticancer agents in culture. *Int. J. Cancer*, **50**, 604–610 (1992).
30) Kano, Y., Akutsu, M., Tsunoda, S., Suzuki, K. and Adachi, K. *In vitro* schedule-dependent interaction between raltitrexed and SN-38 (the active metabolite of irinotecan) in human carcinoma cell lines. *Cancer Chemother. Pharmacol.*, **42**, 91–98 (1998).
31) Sorenson, C. M. and Eastman, A. Mechanism of *cis*-diamminedichloroplatinum(II)-induced cytotoxicity: role of G2 arrest and DNA double-strand breaks. *Cancer Res.*, **48**, 4484–4488 (1988).
32) Jackmann, R. C. Biological effects of folic acid antagonists with antineoplastic activity. *Pharmacol. Ther.*, **25**, 61–82 (1984).
33) Inaba, M., Mitsuhashi, J. and Ozawa, S. Kinetic analysis of 5-fluorouracil action against various cancer cells. *Jpn. J. Cancer Res.*, **81**, 1039–1044 (1990).