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

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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 (IC80) 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/m² 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 cancer5–7) and clinical studies for some other cancers are in progress.8–10) 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.

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The abbreviations used are: FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC80, concentration producing 80% cell growth inhibition; FdUMP, fluorodeoxyuridine 5'-monophosphate; FUTP, 5-fluorouridine 5'-triphosphate.
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without being recognized rapidly, and thus removed efficiently by repair enzymes. Repair replication induced by cisplatin is enhanced, or intrastrand adducts are removed more rapidly in resistant cells.

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. 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. Information on the experimental antitumor efficacy of the combination of raltitrexed and cisplatin for Colo201, Colo320, and LoVo cells, and 5.0 \times 10^3 \text{ cells/ml}

Raltitrexed and cisplatin were obtained from Zeneca Japan Co. (Tokyo), and Nihon Kayaku Co. (Tokyo), respectively. Raltitrexed was dissolved in 0.15 mM NaHCO_3 at a concentration of 1 mM and cisplatin was dissolved in RPMI1640 medium at a concentration of 1 mM. 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 \times 10^5 \text{ cells/ml}

for Colo201, Colo320, and LoVo cells, and 5.0 \times 10^4 \text{ cells/ml}

for WiDr cells in fresh medium containing 10% FBS, and then fresh medium containing 1% FBS, then fresh medium containing 10% FBS (200 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 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 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, 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. 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_{50} were evaluated by the isobologram method of Steel and Peckham. The IC_{50} 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\textsubscript{90} or more would be more important than IC\textsubscript{50} or IC\textsubscript{80}, it is difficult to get reliable data at the IC\textsubscript{90} or IC\textsubscript{80} 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\textsuperscript{[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\textsuperscript{[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).
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 the envelope of additivity and in the area of sub-additivity. The mean value of the observed data (0.79) was larger than that of the predicted minimum values (0.75), but the P values were larger than 0.05, suggesting additive 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 the envelope of additivity and in the area of sub-additivity. The mean value of the observed data (0.79) was larger than that of the predicted minimum values (0.75), but the P values were larger than 0.05, suggesting additive effects.
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 | Predicted min. | Predicted max. | 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.
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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. 5 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 minimum 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.20 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 G2 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 pharmacokinetik 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.

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