Search for the Optimal Schedule for the Oxaliplatin/5-Fluorouracil Association Modulated or Not by Folinic Acid: Preclinical Data

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ABSTRACT

The combination of oxaliplatin (LOHP)-5-fluorouracil (FU)-folic acid (FA) has provided high response rates in pretreated patients with advanced colorectal cancer that is resistant to FU-FA. However, the choice of the optimal schedule between LOHP, FU, and FA remains open. The purpose of the present study was to compare, at equivalent drug area under the curve, different schedules for the LOHP-FU ± FA combinations on four human colorectal cancer cell lines. FU ± FA was tested as a 2-h short exposure ("bolus"), a 118-h continuous exposure ("infusion"), or a 22-h mixed exposure ("De Gramont protocol"). LOHP was administered for 2 h before, during, or after FU ± FA exposure. Isobologram analyses revealed that LOHP associated with FU ± FA resulted in synergistic cytotoxic effects whatever the tested schedules (in ≥75% of cases). For the FU-LOHP combination, cytotoxicity was significantly different according to the FU exposure type (short > mixed > continuous) and was independent of the LOHP position. In contrast, for the FU-FA-LOHP combination, neither the FU exposure type nor the LOHP position significantly influenced cytotoxicity. The presence of FA significantly enhanced the cytotoxicity of FU-LOHP (P < 0.001); this potentiation was independent of the FU exposure type and was significantly influenced by the LOHP position (LOHP after FU-FA > LOHP during FU-FA > LOHP before FU-FA). In conclusion, in contrast with the recognized superiority of continuous FU exposure over short exposure when the drug is given alone, the FU-LOHP combination is more cytotoxic when FU is given as a short exposure. This suggests the potential interest of such a schedule in the clinical setting.

INTRODUCTION

Cisplatin combination with FU2 has proven its clinical efficacy during induction chemotherapy of head and neck cancer (1, 2) and also for the treatment of metastatic disease in colorectal cancer (3). We previously investigated (4), at an experimental level, the importance of the sequence when combining FU and cisplatin and showed that better cytotoxic efficacy was obtained when cisplatin was applied before FU. These observations were in agreement with those of Scanlon et al. (5). Moreover, these authors demonstrated that cell exposure to cisplatin led to a cellular increase in 5–10 methylenetetrahydrofolate, a cofactor that favors the binding of 5-fluoro-dUMP to the target enzyme, TS (6). This biochemical explanation for the synergistic action between cisplatin and FU was later confirmed in animal models by Shirasaka et al. (7) and by Omura et al. (8).

On account of the relative frequency of significant side effects induced by the cisplatin-FU combination (9), the association FU-FA has been combined with a less toxic platinum derivative, LOHP (10, 11), which is devoid of nephrotoxicity and moderately hematotoxic (12, 13). Interestingly, it has been shown that the combination of LOHP-FU-FA produces a high response rate in pretreated patients with advanced colorectal cancer resistant to FU-FA (14). However, the choice of the optimal combination schedule between LOHP, FU, and FA remains open. A recent work by Raymond et al. (15) has shown that concomitant exposure to FU and LOHP for 48 h produced synergistic cytotoxicity in vitro, which was confirmed in vivo. The purpose of the present experimental study was to compare different schedules for the LOHP-FU ± FA combination. FU ± FA was tested according to three clinically relevant schedules: (a) short exposure (bolus type); (b) continuous exposure (infusion type); and (c) mixed exposure ("De Gramont" protocol type; Ref. 14). LOHP was applied for 2 h before, during, or after FU ± FA exposure. This study was undertaken on four human colorectal cancer cell lines that expressed spontaneous sensitivity to both drugs.

MATERIALS AND METHODS

Chemicals. All of the chemicals including MTT, L-ascorbic acid, and dL-5-methyltetrahydrofolate were obtained from Sigma Chemical Co. (St Quentin Fallavier, France) and were of the highest purity available. Folic acid-free DMEM was obtained from Life Technologies, Inc. (Paisley, Scotland). Regular DMEM and glutamine were obtained from Whittaker (Verviers, Belgium) and fetal bovine serum from Dutscher.
(Brumath, France). Penicillin and streptomycin were obtained from Merieux (Lyon, France). FU and LOHP were the pharmaceutical forms obtained from Roche (Neuilly-sur-Seine, France) and Sanofi Winthrop (Gentilly, France), respectively. FA (racemic mixture) was from Sigma Chemical Co.

**Cell Lines.** Four colorectal cancer cell lines of human origin were used (Table 1). Cells were routinely cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 50,000 units/liter penicillin, and 80 μg/mL streptomycin in a humidified incubator (Sanyo, Japan) at 37°C with an atmosphere containing 8% CO2. One week before starting the experiments, the cells were grown in a folate-controlled medium (folate acid-free DMEM supplemented with 40 nM of ΙL-5-methyltetrahydrofolate and 0.1 mM of L-ascorbic acid) to simulate as closely as possible the physiological situation encountered in humans (16). The above folate-controlled medium was used until the end of the experiments.

**Evaluation of Cytotoxicity.** Cells were plated in 96-well microtiteration plates (100 μL/well) to obtain exponential growth for the whole duration of the experiment (initial cell density was 3500, 4000, 7000 and 2500 cells/well for COLO 205, SW 620, CAL 14, and WIDR, respectively). Twenty-four h later, cells were exposed to the drugs. FU ± FA was administered according to three different schedules tested at equivalent FU AUC (AUC = concentration × time). The range of FU AUC tested was different according to the cell line sensitivity (from 1.18-118,000 μM × h, 11 tested values/cell line). FA was always tested at 5 μM (D,L-racemic mixture) and did not exhibit any effect on cell proliferation when tested alone. Schedules were: (a) short exposure (2 h); (b) continuous exposure (118 h); and (c) mixed exposure (2 h followed by 20 h at a 7-fold lower concentration). Whatever the schedule, LOHP was applied for 2 h before, during, or after the FU ± FA exposure (see Fig. 1 for details). The range of LOHP concentration tested was different according to the cell line sensitivity (between 0.2 and 4000 μM, 11 concentrations tested/cell line). When combined, FU and LOHP were tested at a constant FU/LOHP AUC ratio for a given cell line (three for COLO 205, 30 for SW 620, 7 for CAL 14, and 14 for WIDR); the choice of the ratio was dictated by the drug sensitivity and taking into account the different schedules. Experimental conditions were tested in sextuplicate, and experiments were performed twice. Whatever the tested schedule, growth inhibition was assessed by the MTT test (17) 120 h after the start of drug exposure. Results were expressed as the relative percentage of absorbance compared with controls without drug. The dose-effect curves were analyzed on GraphPad Software (Institute for Scientific Information, Philadelphia, PA), and the drug AUC50 as compared with controls was calculated.

The cytotoxic effects obtained when LOHP was combined with FU ± FA were analyzed according to the Chou and Talalay method (18) on Calcusyn software (Biosoft, Cambridge, United Kingdom). For that purpose, the interaction between drugs was assessed by means of an automatically computed combination index. Combination indexes were determined at 50% cell survival. The combination index indicated synergism when smaller than 0.80, antagonism when greater than 1.20, and nearly additive cytotoxic effects when located between 0.80 and 1.20.

**Statistics.** The influence of the tested schedules (either comparison of the nine tested schedules, or comparison between FU exposure types, or comparison between LOHP positions tested) was analyzed on the whole cell lines panel, by means of a nonparametric ANOVA matched for cell line and experiment (Friedman test). Comparison of FU-LOHP AUC50 in the presence of FA versus FU-LOHP AUC50 in the absence of FA was done by means of the nonparametric Wilcoxon signed rank test after merging the nine tested combination schedules. Statistics were drawn up on SPSS software (Chicago, IL).

### RESULTS

**Description of Cell Line Sensitivity.** The intrinsic cell line sensitivity to FU and LOHP tested alone is presented on Table 1 in terms of drug AUC50. For LOHP, AUC50 ranged from 20 μM × h (COLO 205) to 480 μM × h (WIDR). For FU, statistical analysis demonstrated that AUC50 were significantly different according to the exposure type (P = 0.030), with continuous exposure being the more effective schedule (median FU AUC50 = 3330 μM × h) and short exposure being the least (median FU AUC50 = 7000 μM × h). A similar influence of the FU exposure type was observed in the presence of FA (P = 0.005; median FU AUC50 = 1170 and 2820 μM × h for continuous and short exposure, respectively).

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**Table 1** Cell line characteristics

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin*</th>
<th>FU AUC50&lt;sup&gt;b&lt;/sup&gt; (mean values, μM × h)</th>
<th>LOHP AUC50&lt;sup&gt;c&lt;/sup&gt; (mean values, μM × h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLO 205</td>
<td>ATCC (CCL222)</td>
<td>2,170</td>
<td>570</td>
</tr>
<tr>
<td>SW 620</td>
<td>ATCC (CCL227)</td>
<td>15,700</td>
<td>6,190</td>
</tr>
<tr>
<td>CAL 14</td>
<td>CAL</td>
<td>492,600</td>
<td>8,900</td>
</tr>
<tr>
<td>WIDR</td>
<td>EORTC</td>
<td>2,530</td>
<td>890</td>
</tr>
</tbody>
</table>

*ATCC, American Type Culture Collection; CAL, Centre Antoine-Lacassagne, Nice, France; EORTC, European Organization for Research and Treatment of Cancer.

<sup>a</sup> See the “Material and Methods” section for details on FU exposure conditions.

<sup>b</sup> LOHP was applied for 2 h and MTT test was performed 120 h after the start of drug exposure. AUC50 values for FU were significantly different according to the schedule with short exposure > mixed exposure > continuous exposure (Friedman’s rank test, P = 0.03).
**Table 2** Analysis of the interaction between LOHP and FU ± FA including a description of the combination indexes

Combination indexes were computed at 50% cell survival from isobologram analyses performed on Calcusyn software. A combination index smaller than 0.80 indicates synergism, greater than 1.20 indicates antagonism, and between 0.80 and 1.20 indicates nearly additive cytotoxic effects. The results of isobologram analyses when LOHP was tested in association with FU ± FA are depicted in Table 2. The analysis of the combination indexes revealed that the FU-LOHP combination resulted in synergistic cytotoxic effects in 78% of the tested situations; antagonism was observed in 8% of cases. For the FU-FA-LOHP combination, synergistic effects were observed in 75% of cases (antagonist effects in 14%). Statistic analyses revealed that neither the FU exposure type nor the LOHP position had a significant influence on the combination indexes.

**Analysis of the Cytotoxicity of the Combination Schedules.** Fig. 2 shows, for each cell line, the cytotoxic effects (expressed as FU AUC50) of FU or FU-FA tested alone or in combination with LOHP. Because the FU/LOHP AUC tested was constant for a given cell line, these figures allow the cytotoxic effects of FU-LOHP ± FA to be compared between...
Fig. 2  a. COLO 205—description of drug AUC50 (mean ± SD) expressed as FU AUC50 (FU/LOHP AUC ratio = 3); b, SW 620—description of drug AUC50 (mean ± SD) expressed as FU AUC50 (FU/LOHP AUC ratio = 30); c, CAL 14—description of drug AUC50 (mean ± SD) expressed as FU AUC50 (FU/LOHP AUC ratio = 7); d, WIDR—description of drug AUC50 (mean ± SD) expressed as FU AUC50 (FU/LOHP AUC ratio = 14). FU was tested alone (solid bars), along with FA (unfilled bars), or in combination with LOHP ± FA (cross-hatched bars).

the 18 tested combination schedules. For the FU-LOHP combination, the statistical analysis demonstrated that drug AUC50s were significantly different between the nine tested schedules ($P = 0.01$). The smallest drug AUC50 (i.e., greatest cytotoxicity) was observed with short FU exposure, whatever the LOHP position (median FU AUC50s were 430, 500, and 690 μM × h for LOHP before, during, and after FU, respectively); the weakest cytotoxicity was observed with FU continuous exposure and LOHP administered after FU (median FU AUC50: 1660 μM × h). In contrast, when the FU-LOHP combination was modulated by FA, cytotoxicity was not significantly influenced by the tested schedules ($P = 0.67$), with FU AUC50 median values ranging between 300 and 780 μM × h.
**Table 3** Analysis of the potentiation of FU-LOHP by FA with a description of the potentiation factor

<table>
<thead>
<tr>
<th>Combination</th>
<th>FU-LOHP AUC50</th>
<th>FU-FA-LOHP AUC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOHP before FU</td>
<td>FU during FU</td>
<td>LOHP after FU</td>
</tr>
<tr>
<td>Short exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COLO 205</td>
<td>1.81 ± 0.65</td>
<td>1.20 ± 0.13</td>
</tr>
<tr>
<td>SW 620</td>
<td>2.02 ± 0.99</td>
<td>1.29 ± 0.16</td>
</tr>
<tr>
<td>CAL 14</td>
<td>1.19 ± 0.09</td>
<td>1.40 ± 0.45</td>
</tr>
<tr>
<td>WIDR</td>
<td>0.87 ± 0.03</td>
<td>1.19 ± 0.33</td>
</tr>
<tr>
<td>Continuous exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COLO 205</td>
<td>1.88 ± 0.08</td>
<td>2.20 ± 0.48</td>
</tr>
<tr>
<td>SW 620</td>
<td>1.70 ± 0.26</td>
<td>1.71 ± 0.23</td>
</tr>
<tr>
<td>CAL 14</td>
<td>1.31 ± 0.11</td>
<td>1.63 ± 0.24</td>
</tr>
<tr>
<td>WIDR</td>
<td>1.20 ± 0.17</td>
<td>2.69</td>
</tr>
<tr>
<td>Mixed exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COLO 205</td>
<td>1.64 ± 0.19</td>
<td>1.13 ± 0.22</td>
</tr>
<tr>
<td>SW 620</td>
<td>1.16 ± 0.06</td>
<td>1.10</td>
</tr>
<tr>
<td>CAL 14</td>
<td>1.69 ± 0.27</td>
<td>1.85 ± 0.14</td>
</tr>
<tr>
<td>WIDR</td>
<td>0.91 ± 0.48</td>
<td>1.27 ± 0.27</td>
</tr>
</tbody>
</table>

* Mean ± SE from two independent experiments.
* One experiment available.

**Analysis of the LOHP Contribution.** We analyzed the specific contribution of LOHP in the final cytotoxicity of the combination by calculating the drug AUC ratio defined as FU (± FA) AUC50 without LOHP divided by FU (± FA) AUC50 in the presence of LOHP. For the FU-LOHP combination, the drug AUC ratio was significantly different according to the FU exposure type, whatever the LOHP position (P = 0.0046); median drug AUC ratio was 20.6 for short exposure, 10.9 for mixed exposure and 3.8 for continuous exposure. In contrast, the LOHP position did not influence the drug AUC ratio. Similar results were obtained for the FU-FA-LOHP combination, with a median drug AUC ratio at 15.3 for short exposure, 8.8 for mixed exposure and 2.2 for continuous exposure (P = 0.030).

**Analysis of the FA Contribution.** The presence of FA significantly enhanced FU-LOHP cytotoxicity, as demonstrated by the significantly higher FU-LOHP AUC50 as compared with the FU-LOHP AUC50 in the presence of FA (P < 0.001). Moreover, we calculated the potentiation factor defined as FU-LOHP AUC50 divided by FU-LOHP AUC50 in the presence of FA (Table 3). When all of the tested situations were taken into account, the potentiation factor ranged from 0.67 to 6.24 (mean 1.57). The potentiation factor was significantly different according to the LOHP position, whatever the exposure type (P = 0.030), with a median value at 2.13 when LOHP was given after FU, at 1.42 when LOHP was given during FU, and at 1.39 when LOHP was given before FU.

**DISCUSSION**

The first purpose of the present study was to investigate the type of interaction (additive or not) between LOHP and FU ± FA on four human colorectal cancers expressing spontaneous sensitivity to FU and LOHP. Above all, the present data point to the synergistic effects resulting from this drug combination, whatever the tested schedule. The analysis of combination indexes revealed that the amplitude of the interaction was not different according to the tested schedules. These results confirm and extend a previous in vitro investigation by Raymond et al. (15), who exposed cells to FU and LOHP concomitantly for 48 h and concluded in favor of a synergistic antiproliferative effect resulting from this drug association. The molecular origin of this drug interaction may be questioned. Similarities with cisplatin-FU should be expected regarding the mechanisms that are at the origin of the synergy between LOHP and FU. From experiments on tumor cell lines, other investigators (5) and ourselves (4) previously reported more than additive effects when applying cisplatin before FU. In a paper by Tsai et al. (19), when the drugs were applied simultaneously, the association between cisplatin and FU resulted in the majority of cases in additive effects, and synergism was observed at the highest cytotoxic effects only. It has been shown that cisplatin can inhibit methionine uptake into tumor cells (20), resulting in increased methionine synthesis and subsequent reduced folate pool expansion (21). In the presence of FU, these biochemical events lead to greater stabilization of the ternary complex formed between 5-fluoro-dUMP-TSand 5-10 methylenetetrahydrofolate (5). One can thus hypothesize that similar biochemical mechanisms may occur with LOHP. Such a mechanism may explain, at least in part, the synergistic interaction presently observed between LOHP and FU. This remains to be proven at the experimental level. Interestingly, Raymond et al. (15) demonstrated synergistic interactions not only with FU but also with another TS inhibitor, AG337, a folate analogue that does not require reduced folates. Thus, a mechanism other than reduced-folate pool expansion could be implicated in the synergism observed between TS inhibitors and platinum compounds. For instance, recent pharmacokinetic investigations have suggested that LOHP can alter FU clearance (22). LOHP may thus inhibit DPD, the rate-controlling enzyme of FU catabolism (23). In fact, it has been shown that the variability of DPD activity in tumors is related to FU cytotoxicity efficacy both in vitro (24) and in vivo (25), and DPD inhibition has been shown to increase FU efficacy (26).

Conversely, FU may also influence LOHP cytotoxic effects. Esaki et al. (27) studied the synergistic mechanism of cisplatin-FU cytotoxicity when cisplatin was administered after FU on a squamous carcinoma cell line. They analyzed the kinetics of DNA interstrand cross-links removal and found a significant reduction of DNA cross-links removal in the cells exposed to FU-cisplatin as compared with cells not receiving FU. It is thus possible that FU may induce similar molecular effects when combined with LOHP.

Our main purpose was to compare different schedules for the LOHP-FU ± FA combination to mimic the situations encountered in the clinical setting. It is very apparent from the present study that, when FU was tested in association with LOHP, cytotoxicity was significantly influenced by the FU exposure type, the greatest cytotoxicity being observed with short FU exposure, whatever the LOHP position (Fig. 2). This result contrasts with the clinical superiority of continuous exposure over short exposure (28), presently confirmed in vitro for FU tested alone (Table 1). As discussed above, the possible mechanisms responsible for the interac-
tion between FU and LOHP lie on the DNA target. It is widely accepted that FU acts primarily on DNA synthesis via TS inhibition but, depending on the FU exposure type, FU may also be cytotoxic through its incorporation into RNA. This latter effect becomes predominant over DNA damage in the presence of high FU concentrations obtained with bolus administration (29). Interestingly, data showing that cisplatin may inhibit RNA translation have been reported (30). Similar effects could be expected with LOHP and may explain why FU short exposure at high concentrations combined with LOHP leads to the highest cytotoxicity.

In contrast, when FU-LOHP was combined with FA, cytotoxicity was not influenced by the FU exposure type nor by the LOHP position. This observation indicates that optimal efficacy of FU-FA is reached regardless of the FU exposure type. The presence of FA may suggest that such cytotoxicity results from maximal TS inhibition (6). Moreover, the presence of FA significantly enhanced the cytotoxicity of the LOHP-FU combination whatever the FU exposure type (Table 3).

In conclusion, LOHP and FU demonstrated a synergistic interaction whatever the tested combination schedule. Interestingly, in contrast with the widely recognized superiority of continuous FU exposure over short exposure with FU given alone, the FU-LOHP combination is more cytotoxic when FU is administered as a short exposure. This clinically relevant information would suggest that the convenient administration of FU as an IV bolus may be at least as effective as continuous infusion when FU is administered along with LOHP.

REFERENCES


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