Pharmacologic and clinical aspects of isolated hepatic perfusion (IHP) of liver metastases of solid tumours

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CHAPTER 6

In vitro schedule-dependent interaction between melphalan and oxaliplatin in human colorectal cancer cell lines

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Abstract

In order to determine the applicability of oxaliplatin in isolated liver perfusion, we identified the interaction between combinations of oxaliplatin and melphalan in 13 human colorectal cancer cell lines.

Cytotoxic activity was determined by the MTT-assay. Three different administration schedules of the two drugs were compared and median effect isobologram analysis was applied to the results to determine the presence of synergism, additive effects or antagonism as described by Chou and Talalay.

Resistance to melphalan did not correspond to resistance to oxaliplatin. All combinations of melphalan and oxaliplatin showed synergistic or additive interaction in the majority of the cell lines. One hour of oxaliplatin followed by 1 hour of melphalan showed the lowest percentage of cell viability, with synergy in 10 out of 13 cell lines at 50% cell viability. Simultaneous treatment showed the highest cell viability, with antagonism in 6 cell lines, additivity in 2 cell lines, synergism in 5 cell lines at 50% cell viability. One hour of melphalan followed by 1 hour of oxaliplatin showed synergy in 6 cell lines, antagonism in another 6 and additivity in 1 cell line.

Our findings suggest a schedule-dependent synergistic interaction between melphalan and oxaliplatin. Therefore, oxaliplatin should be considered as a new potentially valuable additional agent to the currently commonly used melphalan in isolated hepatic perfusion in colorectal cancer patients.

Introduction

Liver metastases are diagnosed in 10-25% of patients [1] at the time of resection of the primary colorectal tumor. Eventually up to 70 % of patients with colorectal cancer develop liver metastases. In approximately 30% of the patients the liver is the only site of metastatic disease [2, 3]. Surgical resection is considered the standard treatment since complete resection can lead to curation in 25-45% of cases. However resection is only possible in the minority of patients due to the number, location or size of the metastases [4-6]. Isolated hepatic perfusion (IHP) is a therapeutic option for irresectable liver-only metastatic disease although randomized trials versus systemic therapy are lacking. The theoretical advantage of IHP versus systemic therapy is that IHP allows the use of high therapeutic dosages that would cause fatal complications if delivered systemically. Several drugs have been applied in IHP including 5-FU [2, 3], mitomycin C [9, 10], cisplatin [7] and melphalan [7, 10-12], but in the past 10 years melphalan has been the only drug used in major clinical studies [4, 5].

In the past few years various new agents such as irinotecan, oxaliplatin, panitumumab/ cetuximab and bevacizumab, have been introduced in the systemic treatment of colorectal cancer, improving response rates, disease free survival and overall survival [14-21]. To improve the current standard of IHP, some of the newly developed drugs for systemic treatment of colorectal cancer metastases should be considered to be used. For successful application in IHP such a drug has to fulfill several conditions. Firstly, as IHP is a regional treatment, the drug should be in the active form or can be transformed to its active agent in the liver. Secondly, increased concentrations of the drug, as compared to systemic treatment, should lead to an increased tumor response. Thirdly, as IHP is a short treatment with usually a 1 hour treatment time, the administered drug should cause rapid irreversible tumor cell cytoxicity. Finally, liver toxicity should be minimal. Based on these assumptions we considered all registered drugs for colorectal cancer. First, irinotecan is not an ideal candidate for IHP, since it is a prodrug and the bioactivation to its active metabolite SN-38 is slow[6]. The monoclonal antibody bevacizumab may not be suitable either, considering it is not directly cytotoxic and has been shown to impair wound healing[23]. Similar to bevacizumab, cetuximab/panitumumab are not directly cytotoxic.

Oxaliplatin was selected as the most promising new candidate for IHP based on the following observations Oxaliplatin is rapidly absorbed by cells and transformed by non-enzymatic pathways to its biologically active species. Substantial dose-dependent DNA adduct formation occurs within one hour[24, 25]. Previous studies have shown that in systemic administration of oxaliplatin, neurotoxicity, hematological toxicity and

nephrotoxicity are dose limiting, while hepatoxicity is rarely mentioned[26]. Phase III trials have shown the inferiority of oxaliplatin monotherapy versus oxaliplatin combination therapy[27, 28], suggesting a role for the possible application of a combination of oxaliplatin and melphalan in IHP.

Therefore, we investigated the interaction between melphalan and oxaliplatin using 13 human colorectal cancer cell lines.

Materials and methods

Cell culture

The human colon cancer cell lines Caco-2, Colo320, CO115, DLD-1, HCT81, HT29, Lovo, Ls180, Ls411n, SW480, SW48 and T84 were cultured in Hepes-buffered RPMI-1640 culture medium supplemented with glutamine (2mN), penicillin (50U/ml), streptomycin (50µg/ml) and 10% (v/v) fetal calf serum (all Gibco/BRL, Paisley, UK).

Drugs

Oxaliplatin (L-OHP) was a gift from Sanofi-Aventis (Gouda, The Netherlands). Stock solutions of oxaliplatin were prepared by dissolving 50mg of oxaliplatin in 10ml fetal calf serum-free RPMI. Melphalan (L-PAM) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A melphalan stock solution was prepared by dissolving 50mg melphalan in 10ml distilled water with 0.09% hydrochloric acid.

Cytotoxicity assay

Drug concentrations that inhibit 50% of cell growth (IC_{50}) were determined using the MTT-assay, an assay designed for the spectophotometric quantification of cell growth and cell viability [7]. The cells were seeded in a 96-well microtiter-plate (Greiner, Alphen a/d Rijn, The Netherlands) in 200 µl culture medium at different densities per cell line, depending on adhesion and growth qualities (Caco-2 4000 cells/well, Colo320 2000 cells/well , CO115 1000 cells/well , DLD-1 750 cells/well , HCT81 500 cells/well, HT29 500 cells/well, Lovo 3000 cells/well, Ls180 3000 cells/well , Ls411n 2000 cells/well, RKO 2000 cells/well, SW480 4000 cells/well, SW48 4000 cells/well and T84 3000 cells/well). After 96 hours, cells were treated with 100µl of nine graded concentrations of oxaliplatin (3.75-960µM, 5.9-1500µM and 5.3-1360µM) and/or melphalan (2.3-600µM, 3.1-800µM and 4.7-1200µM) for 1 hour, based on drug sensitivity as found in preliminary experiments

Schedule A		L-PAM	L-OHP	
Schedule B		L-OHP + L-PAM		
Schedule C		L-OHP	L-PAM	
	-96h Plating	0-1h Start treatment	1-2h	72 h MTT-assay

Figure 1 The three combination schedules of treatment of the cell lines with oxaliplatin (L-OHP) and melphalan (L-PAM) using a MTT assay.

(unpublished data). The three combined treatment schedules of both drugs consisted of simultaneous, 1 hour oxaliplatin followed by 1 hour melphalan and 1 hour melphalan followed by 1 hour oxaliplatin drug exposure (figure 1). When combined, the drugs were tested at a constant concentration ratio for a given cell line. Combination ratios were determined by the IC_{25} of each drug and then grouped according to the sensitivity spectrum into 6 groups. The following ratios were used 0.88 for Caco-2, HT29, SW480, HCT81and Ls180; 0.27 for CO115 and RKO; 0.59 for Ls411n and T84; 0.63 for DLD-1; 0.20 for Colo320; 0.83 for Lovo and SW48. After drug exposure cells were washed twice with 100µl medium and 200µl fresh culture medium was added. Cells were left to grow for 72 hours, after which culture medium was removed and cells were incubated for 4 hours with 100µl fresh medium and 10 µl MTT (5 mg/ml)(Sigma-Aldrich, St. Louis, MO, USA) labeling agent. Subsequently, 100µl solubilization solution (10% v/v in 0.01 M HCL) (Bio-Rad, Hemel Hempstead, UK) was added and cells were left overnight for incubation. The absorbance at 590 nm was measured by microtiter-plate reader (BioRad Laboratories B.V., Veenendaal, The Netherlands). Wells containing untreated cells of the respective cell lines were used as controls. Each experiment was performed using three replicate wells. Results were expressed as the relative percentage of absorbance compared with controls without drug. The results were based on at least 3 independent experiments.

Analysis of combination effects

On the basis of the growth inhibition curve for each single drug, we analyzed the effects of different treatment schedules according to the method as described by Chou and Talalay [8] , using the Calcusyn software program for automated analysis (Biosoft, Cambridge, UK). The effect of combining the two drugs was evaluated by comparing the results of the sequential assays with the assays involving simultaneous oxaliplatin and melphalan treatment. The combination effect was evaluated by determination of the respective combination indexes. The combination index (CI) can be extrapolated from the various concentrations (C) and is defined as follows: $CI= C_{\text{oxaliplatin in combination}} / C_{\text{oxaliplatin}} + C_{\text{melphalan in combination}} / C_{\text{melphalan}} + \alpha [C_{\text{oxaliplatin in combination}} X C_{\text{melphalan in combination}} / C_{\text{oxaliplatin}} X C_{\text{melphalan}}$, for the same effect (where α is the parameter with value 0 when both drugs are mutually

exclusive and 1 when both drugs are mutually non-exclusive. The CI indicated synergism if <1.0, antagonism if >1.0 and additivity if 1.0.

Results

Single-agent experiments

The cytotoxicity of melphalan and oxaliplatin was tested individually on all 13 cell lines. The cells were exposed to each drug for 1 hour. The IC_{50} values (+/- SD) are summarized in Table 1. For melphalan, SW48 cells were most sensitive (41 μM) and Caco-2 cells were most resistant (806 μM). SW48 cells were also the most sensitive to oxaliplatin (36 μM), CO115 cells were the least sensitive (3119 μM). Resistance to melphalan did not necessarily imply resistance to oxaliplatin, as shown by Ls411n cells.

Combination experiments

Melphalan and oxaliplatin were tested in different combination schedules to determine the most effective schedule. Three different schedules were tested as shown in fig. 1. The combination indexes (CI) at 50 % and 25 % cell viability, approximating 50 % and 75% cell death are given in table 2 for all treatment schedules. Simultaneous treatment with the two drugs resulted in antagonistic interaction in 6 cell lines, additivity in 2 cell lines

Table 1 Cell line characteristics

Cell line	Melphalan IC_{50} (mean value, μM)	Oxaliplatin IC_{50} (mean value, μM)
Caco-2	806 +/- 290	562 +/- 185
CO115	592 +/- 180	3119 +/- 1777
Ls411n	576 +/- 313	298 +/- 133
HT29	316 +/- 166	548 +/- 144
SW480	190 +/- 177	947 +/-547
T84	171 +/- 85	241 +/- 92
RKO	132 +/- 65	1381 +/- 667
DLD-1	95 +/- 40	245 +/- 86
HCT81	65 +/- 20	256 +/- 121
Ls180	61 +/- 32	200 +/- 143
Colo320	61 +/- 18	541 +/- 203
Lovo	53 +/- 28	51 +/- 18
SW48	41 +/- 33	36 +/- 39

The IC_{50} values of melphalan and oxaliplatin are the means +/- SD of at least three independent experiments.

Table 2 Combination indexes for the different drug combinations

Cell line	Schedule A CI (+/-SD)	Schedule B CI (+/-SD)	Schedule C CI (+/-SD)
Caco-2			
- 50% cell viability	1.2 +/- 0.74	0.52 +/- 0.17†	0.77 +/- 0.29†
- 25% cell viability	1.3 +/- 0.47	0.64 +/- 0.13†	0.75 +/- 0.21†
Colo320			
- 50% cell viability	0.71 +/- 0.49†	0.85 +/- 0.24†	0.65 +/- 0.37†
- 25% cell viability	0.60 +/- 0.28†	0.84 +/- 0.38†	0.65 +/- 0.27†
CO115			
- 50% cell viability	0.23 +/- 0.25†	0.49 +/- 0.37†	0.30 +/- 0.22†
- 25% cell viability	0.38 +/- 0.15†	0.44 +/- 0.33†	0.34 +/- 0.16†
DLD-1			
- 50% cell viability	0.65 +/- 0.77†	1.81 +/- 1.65	0.72 +/- 0.40†
- 25% cell viability	0.49 +/- 0.47†	1.06 +/- 0.63	0.61 +/- 0.20†
HCT81			
- 50% cell viability	1.17 +/- 0.80	1.01 +/- 0.50	0.96 +/- 0.54†
- 25% cell viability	0.82 +/- 0.23†	1.09 +/- 0.34	0.69 +/- 0.28†
HT29			
- 50% cell viability	1.25 +/- 0.84	2.51 +/- 1.86	0.68 +/- 0.50†
- 25% cell viability	0.99 +/- 0.54†	2.67 +/- 1.30	0.64 +/- 0.40†
Lovo			
- 50% cell viability	1.75 +/- 1.45	1.03 +/- 0.35	1.42 +/- 1.35
- 25% cell viability	1.62 +/- 1.19	1.20 +/- 0.35	1.47 +/- 0.98
Ls180			
- 50% cell viability	1.79 +/- 2.18	3.18 +/- 3.22	0.68 +/- 0.36†
- 25% cell viability	1.35 +/- 1.37	2.18 +/- 1.90	0.65 +/- 0.38†
Ls411n			
- 50% cell viability	0.88 +/- 0.53†	1.33 +/- 0.95	0.58 +/- 0.28†
- 25% cell viability	0.84 +/- 0.60†	1.36 +/- 0.96	0.53 +/- 0.23†
RKO			
- 50% cell viability	0.98 +/- 0.73†	1.35 +/- 0.69	0.80 +/- 0.23†
- 25% cell viability	0.98 +/- 072†	1.28 +/- 0.73	0.71 +/- 0.22†
SW48			
- 50% cell viability	2.74 +/- 3.51	1.28 +/- 0.79	1.14 +/- 0.79
- 25% cell viability	1.37 +/- 0.94	1.15 +/- 0.57	0.78 +/- 0.27†
SW480			
- 50% cell viability	0.91 +/- 0.36†	0.66 +/- 0.25†	1.10 +/- 0.60
- 25% cell viability	1.18 +/- 0.24	0.59 +/- 0.08†	1.38 +/- 0.58
T84			
- 50% cell viability	1.01 +/- 0.41	0.95 +/- 0.70†	0.89 +/- 0.40†
- 25% cell viability	0.93 +/- 0.33†	0.91 +/- 0.42†	0.86 +/- 0.33†

Values are mean combination indexes (CI) of at least three independent experiments. Treatment A is 1 hour melphalan followed by 1 hour oxaliplatin.Treatment B is melphalan and oxaliplatin simultaneously. Treatment C is 1 hour oxaliplatin followed by 1 hour melphalan. The CI indicates synergism if <1.0, antagonism if >1.0 and additivity if 1.0. †Correspond to synergistic interactions.

and synergistic interaction in 5 cell lines at 50% cell viability. Sequential treatment with oxaliplatin followed by melphalan resulted in synergistic interaction in 10 cell lines and antagonistic interaction in the other three cell lines in nearly all ranges of cell kill fraction. Sequential treatment with melphalan followed by oxaliplatin resulted in antagonistic interaction in 6 cell lines, synergism in 6 cell lines and additive interaction in 1 cell line at 50% cell viability. Typical examples for CI/fractional effect curves are given in figure 2.

Discussion

Oxaliplatin has been successfully introduced in the treatment of metastatic colorectal cancer. Although oxaliplatin monotherapy has shown limited activity, the combination with 5-FU/leucovorin resulted in tumor responses in 50% of patients and a median time to progression of 9.0 months [16]. IHP has proven a suitable treatment option for patients with liver-only colorectal metastases, who are not eligible for other locoregional treatment options [12, 13]. Over the past 10 years melphalan (with or without TNF) has been the major drug applied in IHP. To our knowledge the addition of new, modern agents to melphalan in IHP has not been investigated. Recently Herbert *et al* published a phase I study of hyperthermic isolated hepatic perfusion with oxaliplatin in the treatment of unresectable liver metastases [9]. In our opinion a major drawback of this study is the application of oxaliplatin monotherapy, since oxaliplatin monotherapy has shown only limited efficacy in the systemic treatment of colorectal cancer patients.

We examined the interaction between oxaliplatin and melphalan in a panel of 13 colorectal cancer cell lines *in vitro*. The drug sensitivity spectrum of our cell lines showing resistance to melphalan did not necessarily correspond to resistance to oxaliplatin, suggesting different mechanisms of resistance for both agents. IHP melphalan monotherapy experience at our institution in 154 colorectal cancer patients showed an overall response rate of 50% on CT examinations[10], suggesting resistance to melphalan in 50% patients. We hypothesized that this percentage can be reduced through the addition of oxaliplatin.

Addition of oxaliplatin to melphalan resulted in synergistic or additive interaction in the majority of our cell lines for all the treatment schedules. Various other in vitro studies have shown a schedule-dependent interaction between oxaliplatin and other cytostatic agents [33-36]. Our experiments showed synergy especially when the cell lines were treated sequentially when compared to simultaneous treatment. A possible explanation is the competitive uptake of both agents. The uptake of melphalan is dependent on active carrier-mediated transport [11]. In myeloma cell lines down regulation of CD98

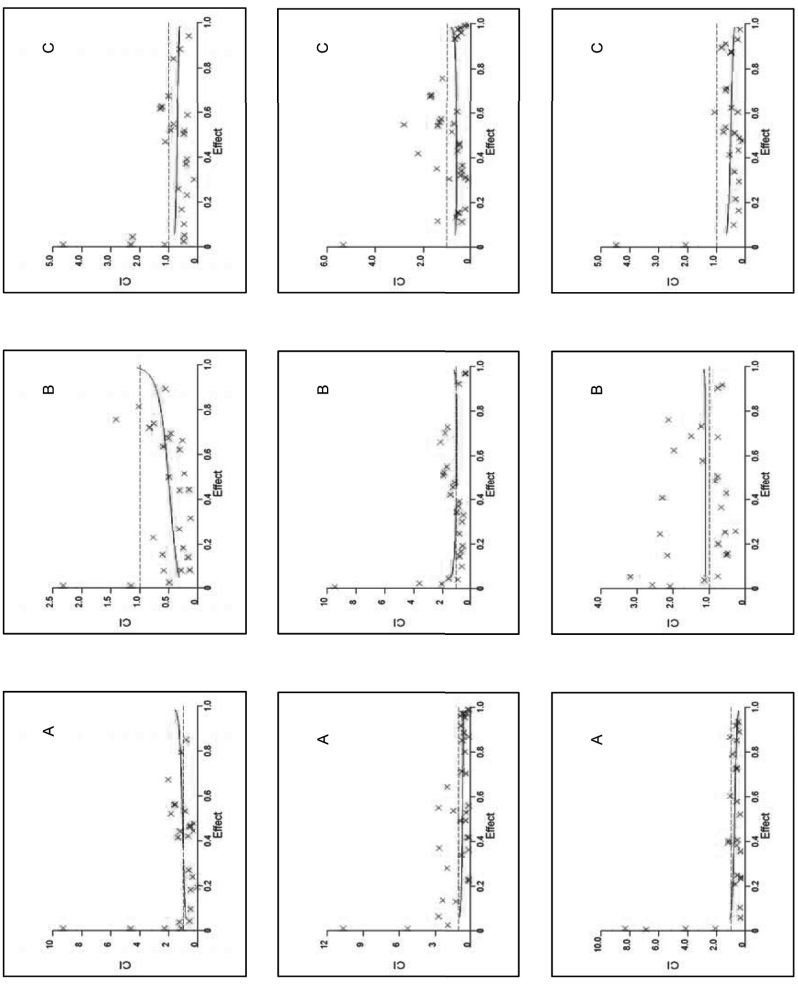

Figure 2 Typical combination index/fractional effect curve as derived from the Chou and Talalay model for three cell lines Caco-2, Colo320 and Ls411n. Each point represents the CwI at a dose effect as determined in our experiments. The dotted line indicates additivity, the area under the dotted line synergy and the area above the dotted line antagonism. Cells were treated with (A) melphalan followed by oxaliplatin, (B) melphalan and oxaliplatin simultaneously and (C) oxaliplatin followed by melphalan.

(L-phenylalanine transporter) was associated with increased resistance to and reduced uptake of melphalan [38]. Little is known about the transport mechanisms of oxaliplatin. Some studies suggest the role of organic copper-transporters in the uptake of oxaliplatin [12-14] As simultaneous treatment with both drugs resulted in the highest cell viability corresponding to the least cell death, melphalan and oxaliplatin may share a common (competitive) transporter.

Our results also suggest that treatment with oxaliplatin, followed by treatment with melphalan is superior to treatment with melphalan followed by treatment with oxaliplatin. It is possible that the schedule-dependent synergistic interaction of oxaliplatin and melphalan would be due to inhibition by melphalan of the oxaliplatin-induced Pt-adducts repair mechanisms. Further experiments are necessary to identify these mechanisms underlying the interaction between melphalan and oxaliplatin.

In conclusion, a synergistic interaction was observed between melphalan and oxaliplatin. All treatment schedules showed synergistic interaction, but the best results were obtained if oxaliplatin treatment was followed by melphalan treatment, although the mechanisms of interaction remain unknown. These *in vitro* findings provide an important basis for a future clinical trial of the combination of oxaliplatin and melphalan in isolated hepatic perfusion. At our institution we are currently performing a phase I/II trial with IHP with a combination of oxaliplatin directly followed by melphalan.

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