

**POTENTIATION OF 5-FLUOROURACIL INDUCED INHIBITION OF
THYMIDYLATE SYNTHASE IN HUMAN COLON TUMORS BY
LEUCOVORIN IS DOSE DEPENDENT.**

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INTRODUCTION

The schedule and dose dependency of leucovorin (LV) administration in order to potentiate the antitumor activity of 5-fluorouracil (5FU) is still a unclear issue.^{1,2,3} Traditionally two schedules for single agent of 5FU were used; weekly administration at 500 mg/m² and daily (x 5) administration at 300-500 mg/m², which was repeated every 4 weeks. These two schedules of 5FU have been combined with a variety of LV schedules, which can roughly be divided in high dose (2 hr infusion of LV at 500 mg/m² with 5FU injected mid-infusion),⁴ intermediate dose (2 hr infusion of LV at 200 mg/m²)^{5,6} and low-dose LV (bolus injection of LV at 20-25 mg/m² injected just before 5FU).^{6,7} LV has also been administered at similar doses but in different schedules. The response rates at the high and intermediate doses was almost always higher (about 30%) than for single agent 5FU, but for the low dose (as well as for oral LV) a larger variation in response rates was observed.^{6,7} At a recent symposium on modulation of 5FU⁸ a consensus was achieved that exposure to LV should be 2 hrs or longer in order to facilitate accumulation of polyglutamates of the reduced folate cofactor. The dose of LV remains an unresolved issue, although it was clear from preclinical studies that at least a concentration of 1 μ M should be present in the culture medium in order to achieve modulation of 5FU.⁹ Although both the intermediate and the high dose of LV result in plasma concentrations of *l*-LV above this levels¹⁰ this does not provide information on the intra-tumoral concentration of LV and the reduced folate cofactor 5,10-methylene-tetrahydrofolate (CH₂-THF). Only limited information on the CH₂-THF concentration in

the tumor after administration of a high-dose of LV is available.¹¹ No information is available on intratumoral concentration after administration of a low dose. Recently we completed a study on the inhibition of thymidylate synthase (TS) in tumors of patients after administration of 5FU or 5FU in combination with high-dose LV.^{11,13} From this study it was concluded that inhibition of TS was maintained for at least 48 hr after 5FU administration, while high-dose LV clearly potentiated this inhibition. This study was extended to the question whether low-dose LV would also be able to potentiate the inhibition of TS.

PATIENTS AND METHODOLOGY

Biopsy specimens of primary tumors or metastases were obtained during laparotomy from patients with histologically proven colorectal cancer. Patients received one i.v. bolus injection of 5FU (500 mg/m²) or LV/5FU prior to surgery, as described previously.¹²⁻¹⁴ All tumor samples were taken between 40 and 50 hr after drug administration. Sixteen patients received 5FU alone; 11 patients received high-dose LV (HD-LV; 500 mg/m² as a 2-hr i.v. infusion; with the i.v. bolus injection of 5FU midway infusion); and 8 patients received low-dose LV (LD-LV; 25 mg/m² as a bolus just before 5FU). Biopsy specimens were immediately frozen in liquid nitrogen and subsequently stored at -80 °C.

Frozen tissue samples were pulverized using a micro-dismembrator and processed as described previously.^{11,13,14} In the tissue extract we measured the activity of TS with both the FdUMP binding assay (with [6-³H]-FdUMP giving the number of FdUMP binding sites) and the tritium release assay (with [5-³H]-dUMP giving the conversion of dUMP to dTMP) at a substrate concentration of 1 μM dUMP. The inhibition of TS was evaluated by comparison of the FdUMP binding and the catalytic activity in tumor samples with (total activity; TS-*tot* and TS-*total*, respectively) and without dissociation (inhibited activity; *i.e.* the number of free binding sites and the residual activity; TS-*free* and TS-*res*, respectively) of FdUMP from the ternary complex between FdUMP-TS-CH₂-THF. The number of FdUMP binding sites and the catalytic activity was measured as described previously.^{11,14}

RESULTS

For all patients a large variation was found in both the number of total FdUMP binding sites (0-383 fmol/mg protein, median 70; 35 patients) and the total catalytic activity (0-178 pmol/hr/mg protein, median 35; 35 patients). A significant inhibition of TS was observed in tumor samples of patients who received 5FU as a bolus injection (Table 1). In patients receiving HD-LV a clear potentiation of the inhibition of TS was observed, as could be evaluated using both assays. However, in patients receiving the LD-LV the inhibition of TS was clearly less than with HD-LV, although the inhibition as evaluated using the FdUMP binding assay was more pronounced than with 5FU alone. However, when using the catalytic assay for evaluation of the results, the effect of LD-LV is lower than that of HD-LV and an even slightly higher TS activity was observed than for 5FU alone. It should be noted that the TS activity was measured at a low non-saturating dUMP concentration of 1 μM; however, at a substrate concentration of 10 μM dUMP, which is saturating, a similar relative inhibition was observed as at 1 μM dUMP. The inhibition of TS was selective for the tumor tissue,

since the inhibition of TS in normal liver and normal mucosa in these patients was not affected by LV (both LD-LV and HD-LV; data not shown). The number of free FdUMP binding sites in normal liver was 70-80% for all three protocols. For the catalytic activity (at 1 μ M) these values ranged from 69 to 106%.

Table 1. Inhibition of TS in biopsy specimens of colon tumors after administration of a test dose of 5FU or 5FU combined with HD-LV or LD-LV

Treatment	Inhibition of TS evaluated with:	
	the FdUMP binding assay	the catalytic assay
5-FU alone	49.0 (14)	74.0 (18)
HD-LV + 5-FU	24.0 (12)*	49.0 (12)*
LD-LV + 5-FU	32.0 (9)	94.7 (9)

Values (calculated as ratios x 100%, between TS-free/TS-tot or TS-res/TS-total, respectively) represent means from the number of samples indicated within parentheses. The means were calculated from the separate samples.

Significantly different from 5FU alone at the level; significantly different from 5FU at the level; *, 0.002 < p < 0.02; (Mann Whitney U test).

DISCUSSION

In this paper we demonstrate that the 5FU induced inhibition of TS in patients can be enhanced significantly by HD-LV, but not or only to a minor extent by LD-LV. The inhibition by 5FU was selective, only a minor inhibition of TS was observed in normal tissues.

Dosing of LV is a major clinical problem. From preclinical studies^{8,9} it is clear that a prolonged exposure at concentrations of at least 0.1 μ M LV is required to achieve the desired potentiating effect. However, for most cell lines a higher LV concentration is required, even higher than 1 μ M. Other cell lines can however even not be modulated at these high LV concentrations.¹⁵ Since the requirement for each tumor is not known and can not be determined in a routine clinical setting, the most optimal LV schedule and dose should be used.

Besides the concentration dependence the *in vitro* studies also demonstrated a schedule dependence of LV modulation. After intracellular uptake LV has to be metabolized to CH₂-THF, the cofactor for TS. The binding of FdUMP to TS will be enhanced after folate polyglutamylation,¹⁶ thus it is of advantage that accumulation of CH₂-THF-polyglutamates is maximal. It has been observed that polyglutamylation is a time-dependent process, and polyglutamates accumulate in time. Thus, a longer exposure at higher concentrations will result in an optimal polyglutamylation. Also *in vivo* data indicate that a prolonged exposure to LV is advantageous compared to a short exposure.^{14,17}

Up to now no clinical biochemical data were available supporting either a LD-LV or a HD-LV. The clinical observations on the antitumor activity of LD-LV + 5FU were contradictory for colon cancer,^{6,7} although this difference might also be related to scheduling. However, when using the 25 mg/m² dose of LV the potentiation of 5FU induced TS inhibition was lower than for the HD-LV. This

effect was even more pronounced for the catalytic assay. It should be noted that the catalytic assay represents the actual biochemical process to be inhibited in the tumor. Although up to now a limited number of patients could be entered in the LD-LV arm, these preliminary data suggest that LD-LV is not sufficient for most patients to achieve the desired potentiation of TS inhibition. Pending confirmation of this pattern in more patients it seems desirable to treat patients at a relatively high dose of LV.

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