**COMPARISON OF CONTINUOUS INFUSIONS AND BOLUS INJECTIONS
OF 5-FLUOROURACIL WITH OR WITHOUT LEUCOVORIN:
IMPLICATIONS FOR INHIBITION OF THYMIDYLATE SYNTHASE**

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INTRODUCTION

Although 5-fluorouracil (5FU) is in clinical use for more than three decades, it is not clear what is the most efficacious schedule of 5FU administration [1, 2]. For systemic treatment with 5FU a number of different schedules are in clinical use (Table 1). The two major schedules for bolus injections do not show significant differences in either toxicity or antitumor activity. For the prolonged administration schedules, however, the pattern of toxicity changes. For weekly bolus injections, myelotoxicity (mainly leukopenia) is usually dose-limiting; however, at the prolonged administration schedules the pattern of toxicity changes with mucositis and diarrhea as serious site-effects for continuous infusions. In addition, the hand-foot syndrome is frequently observed at protracted infusions of several weeks, as well as other skin lesions (see ref. in Table 1). Interestingly the addition of LV to both the bolus injections and the continuous infusions, caused a marked increase in gastrointestinal toxicity. In several schedules this increase of toxicity was the only result of the modulatory agent, with no apparent effect on the antitumor activity.

Most studies on protracted infusion have been performed as Phase II trials. In a large randomized trial Lokich et al [24] compared bolus injections (daily times 5) of 5FU with protracted infusion for 10 weeks. A significantly higher response rate (30%) was observed in the infusional arm compared with the bolus arm (7%), with no significant differences in the survival. A similar pattern has been observed for the comparison of bolus 5FU with LV-5FU; increased response for LV-5FU but no significant effect on survival [2, 4]. The addition of LV to the protracted infusion did however in most studies only result in an increased toxicity [18,19,25,27], as well as that of interferon- α [26]. An additional administration of interferon- α to the

Table 1: Doses and schedules used for systemic administration of single agent 5FU in various malignancies

Injections:	1.	Weekly i.v. push at 500-800 mg/m ² [1-3]
	2.	Daily times 5 repeated every 3-4 weeks at 300-500 mg/m ² /day [1, 2, 4]
Infusions:	3.	24 hr infusion at high dose (2400; tested from 750-3400 mg/m ²) [5,6,21,22]
	4.	3-5 day infusion at 1000 (varying from 185-3600) mg/m ² (7-11, 20, 23]
	5.	Protracted infusions varying from 1 week to 10 weeks at 200-750 mg/m ² /day, depending on length of infusion period [12-19, 24- 27]

Many of these schedules are being used in combination with single modulators, such as LV [2, 18, 19], dypiridamol [10, 20], interferon- α [26], PALA [5, 6], 6-methylmercaptapurine riboside [7, 22], uridine [3], cisplatinum [11, 23], irradiation [8] or multiple modulators. A large number of different schedules was used for the modulators. For combinations with *e.g.* LV and/or interferon the dose of 5FU in the continuous infusions had to be reduced.

continuous infusion schedule with LV did not indicate a substantial improvement in treatment results [28].

A scientific rationale for an improved effect of 5FU administered as continuous infusion was already formulated several years ago. Several investigators have pointed out that continuous *in vitro* exposure of cells to 5FU is much more effective than short 1-hr exposure periods followed by culture in drug-free medium [29, 30]. For some of our human colon cancer cell lines we have observed a similar pattern; a 50% growth inhibition (IC₅₀) for the 1-hr exposure was observed at about 300 μ M [32], while for a continuous exposure of 24-72 hr an IC₅₀ of 2-10 μ M was observed [31-33]. During continuous exposure to 5FU a constant high level of the active metabolite of 5FU, FdUMP, would be present in order to facilitate maximum inhibition of thymidylate synthase (TS), the target for FdUMP. TS was inhibited almost completely during exposure to 5FU, but recovered after removal of the drug [34].

The study of prolonged infusion of cytostatics in animal model systems has been hampered by the lack of reliable infusion systems. Short term infusions (24 hr) can be carried out by catheterisation of the tail vein, while for longer periods (3-7 days) osmotic pumps can be applied. For very long infusion periods one has to rely on slow-release forms of 5FU. We compared weekly i.p. bolus injections of 5FU with s.c. continuous infusions for three weeks in mice bearing subcutaneously implanted colon tumors. Both schedules were given at their maximum tolerated doses (MTD), at which the toxicity profile, 5FU plasma pharmacokinetics, and thymidylate synthase (TS) inhibition in the tumors were evaluated.

MATERIALS AND METHODS

Materials

5FU for bolus treatment of animals was obtained from Hoffmann-La Roche, Mijdrecht, the Netherlands, and was formulated as a 10 mg/ml solution. Slow-release pellets containing 5FU and intended for continuous infusions were obtained from Innovative Research of America, Toledo, OH, USA. [6-³H]-dUMP was from Radiochemical Center Amersham, England and [6-³H]-FdUMP from Moravek, Brea, CA, USA. All other chemicals were of analytical grade.

Treatment of mice and blood sampling

All mice were kept in an area with standardized light-dark cycle for at least 10-14 days prior to the beginning of an experiment. Mice had access to food and water *ad libitum*. For each treatment dose limiting toxicity was determined in healthy mice (female Balb/c and C57Bl/6 mice) before applying the schedule to tumor-bearing animals. As parameters for the MTD we used a weight loss not exceeding 15% and/or a lethality of less than 10%. Investigations on toxicity have been performed essentially as previously described [35-38]. For determination of the antitumor activity female Balb/c and C57Bl/6 mice were transplanted with two murine colon adenocarcinomas, Colon 26 and Colon 38, respectively.

For weekly bolus treatment, mice received intra-peritoneal bolus injections of 5FU. For continuous infusions slow release pellets containing 5FU (5, 10, 15, 20 mg) were implanted subcutaneously in ether anesthetized mice. After three weeks the pellets were deteriorated. Blood sampling in these mice was done at the same time point of the day and was performed by retro-orbital bleeding under slight ether anesthesia with heparinized hematocrit capillaries; plasma samples were frozen and 5FU was measured with gas-chromatography coupled to mass spectrometry, as described [39, 40]. Plasma pharmacokinetics of 5FU after bolus injections and continuous infusions have been studied in normal C57Bl/6 mice, essentially as has been described previously [40]. Because of the sensitive analytical procedure only small blood samples were required for a reliable analysis.

Enzyme assays

The activity of thymidylate synthase was measured after treatment with 5FU using two assays, the FdUMP ligand binding assay and the catalytic assay (conversion of dUMP to dTMP), as previously described [37]. Mice were killed by cervical dislocation, tumors were excised immediately and directly frozen in liquid nitrogen. The frozen tumors were pulverized using a micro-dismembrator and supernatants were prepared as described [37]. For measurement of the total TS activity in tumors from treated mice, the ternary complex consisting of FdUMP, TS and 5,10-methylene-tetrahydrofolate ($\text{CH}_2\text{-THF}$), was dissociated followed by a neutral charcoal wash.

RESULTS

Pharmacokinetics

The MTD for 5FU administered as weekly bolus injections was comparable to that reported previously [35-37], 100 mg/kg. The MTD for continuous infusions (administered as subcutaneously implanted pellets) was 10 mg/21 days per mouse, equivalent to 23.8 mg/kg/day assuming an initial mouse weight of 20 g. In order to determine whether the pellets indeed have a constant release of 5FU during the period indicated by the manufacturer, we measured the plasma 5FU concentrations during the infusion period. Plasma 5FU concentrations have been measured in the same mice sampled repeatedly during 21 days at the same time point of the day. The plasma 5FU concentrations varied between 0.1 and 1.0 μM , much lower than the peak plasma 5FU concentrations in mice after bolus injections (Fig. 1). Plasma concentrations dropped below detectable levels at 22 days after implantation of the pellets, indicating that release of 5FU was completed. The total area under the

Figure 1. Plasma 5FU concentrations in mice after administration of bolus injections of 5FU at 100 mg/kg (●) to C57Bl/6 and Balb/c mice, or implantation of slow-release pellets containing 10 mg 5FU (---) in C57Bl/6 mice [40,47]. For the bolus injections no difference was observed between the two mouse strains. Values are means of at least 6 mice. SD was less than 30%. For the bolus injections it was assumed that repeated administration did not affect the plasma pharmacokinetics [39] and the same curve was plotted three times.

plasma concentration vs time (AUC) curve was 37 $\mu\text{mol}\cdot\text{hr}/\text{l}$ for the continuous infusions, that for one bolus injection was 285 $\mu\text{mol}\cdot\text{hr}/\text{l}$ [40]. For three weekly bolus injections this would have been about 855 $\mu\text{mol}\cdot\text{hr}/\text{l}$, assuming no effect of repeated administration on plasma pharmacokinetics [39].

Toxicity

Continuous infusions caused a delayed, but considerable weight loss leading to death at the higher doses; at the 10 mg dose a maximum of 15% was observed after 11 days. After bolus injections the maximal weight loss was observed at the first days after administration of 5FU and did not exceed 10% [35-37]. In addition to the weight loss we also determined the myeloid toxicity of continuous infusions of 5FU (Fig. 2). For mice treated with saline or implanted with the carrier material of the pellets, no significant effect on blood cell count was observed. Bolus injections of 5FU caused a severe leukopenia in contrast to the decrease in leucocyte count observed in mice treated with continuous infusions. In both treatment modalities these decreases were followed by a rebound in leucocytes which was more pronounced in mice treated with a continuous infusion. The other parameters measured, thrombocytes and hematocrit, decreased to 43 and 31% of the pretreatment values.

Antitumor activity

The antitumor activity of continuous infusions was determined both in mice bearing Colon 26 (Fig. 3) and compared in the same experiment with the antitumor effect of bolus injections. In the 5FU-insensitive Colon 26 continuous infusion with 5FU resulted in a significant growth arrest in the first week after implantation of the pellet, bolus injections of 5FU only resulted in a small growth delay. After 8 days, however, the tumors resumed growth in the animals treated with a continuous

Figure 2. Comparison of the myelotoxicity of weekly bolus injections of 5FU (●-) at 100 mg/kg and continuous infusion of 5FU (-+-) at a total dose of 10 mg per mouse. Leucocyte counts relative to the value before treatment are given. Values are means $\pm$ SD of at least 6 mice and are from [35,36,38,47].

infusion; the ultimate life-span of mice treated with a continuous infusion was comparable to that in mice treated with a bolus injection. Against Colon 38, a 5FU-sensitive tumor, continuous infusions resulted in unmeasurable tumor load in several mice. However, in these mice tumors also resumed growth after 8 days and there was no difference in the overall antitumor effect of continuous infusions compared to bolus injections (data not shown).

Since LV was able to potentiate the antitumor activity of 5FU both in Colon 26 and Colon 38, we combined LV with the continuous infusion of 5FU in Colon 26. Since no clear advantage for any of the LV schedules has become apparent, we compared various schedules and doses of LV administration, weekly (d 1, 7 and 14) bolus injections at 100 mg/kg, daily (d 1-5, 8-12, 15-19) bolus injections of 5 mg/kg and three times weekly (days 1,3,5 and 8,10,12 and 15,17,19) of 10 mg/kg. However, neither of these schedules was able to enhance the antitumor activity. In contrast these schedules appeared to be more toxic than the continuous infusion of 5FU alone, as was manifested by an increased weight loss and lethality.

Activity of thymidylate synthase

For bolus injections of 5FU we obtained evidence that the antitumor activity of 5FU was related to the extent and long-term duration of TS inhibition. A resumed tumor growth was associated with an increase in the total activity of TS in these tumors [37]. This effect was more pronounced for the catalytic activity of TS than for the FdUMP binding of TS. In mice treated with a continuous infusion of 5FU we determined the activity of TS in Colon 26 tumors (Fig. 4). The extent of TS inhibition was comparable with that observed after a bolus injection. After 11 days, however, the activity of TS in the tumors recovered and exceeded that of tumors from untreated mice. The total increase in enzyme activity was comparable to that observed after treatment of mice with weekly bolus injections of 5FU, whereas this increase was not present in tumors of mice treated with LV and bolus 5FU [37].

Figure 3. Comparison of the antitumor effect of bolus injections of 5FU at weekly doses of 100 mg/kg (—●—) and continuous infusion (---+) of 5FU administered as slow-release pellets containing 10 mg 5FU. Growth of control tumors is depicted as -o-. Values are means $\pm$ SE of 6 mice and were calculated relative to the tumor volume at the first day of treatment. Implantation of the carrier material did not affect tumor growth. The maximal T/C value (ratio between tumor size from treated and control mice) for continuous infusions was 0.35 compared to 0.7 for bolus treatments (means of at least 4 separate experiments). The overall survival for both groups was comparable.

DISCUSSION

Continuous infusions in mice; effect of leucovorin

Continuous exposure of tumor cells to 5FU has shown to be more effective than short-term treatment, based on IC₅₀ values. In this study we demonstrate that continuous infusion of 5FU to mice can indeed enhance the antitumor activity of 5FU when evaluation is based on tumor size, criteria comparable to that used clinically for complete and partial response. However, when the long term effects, such as survival, are being considered this difference is not present anymore.

Continuous administration of 5FU to mice using slow-release pellets appeared to be a feasible treatment modality. The pellets released 5FU at a constant rate since the plasma 5FU concentrations were in the same range during the whole treatment period of 21 days. These plasma levels of 5FU were comparable with levels observed in patients during protracted infusion measured in this laboratory [25] and in other studies [41-43] and with that in rats [44]. 5FU plasma concentrations measured during relatively short-term infusions (3-7 days) [10, 41, 43] tended to be higher than that measured during longer infusion periods. However, this may well be related to the higher dose which can be applied at the shorter infusion periods and the fact that a number of investigators used 5FU in combination with a modulator [10, 43]. In these studies a clear relationship between dose and steady-state 5FU concentrations was observed. However, at a dose (370 mg/m²/day) comparable with our studies [10, 25], similar 5FU concentrations were observed. Considering the plasma pharmacokinetics, our murine model seems to have a number of similarities compared to continuous infusion schedules applied in the clinic.

Figure 4. Comparison of the inhibition of TS after administration of 5FU as a weekly bolus injection (-●-) at 100 mg/kg [37] and as a continuous infusion of 5FU (-+-) at a total dose of 10 mg per mouse. The absolute residual TS activity is depicted. For the bolus injections the line between days 7 and 15 is discontinued because no measurements were performed, while treatment of mice was continued at days 7 and 14. Values are means $\pm$ SD of 4-6 separate tumors.

The overall dose of 5FU administered to mice during the continuous administration was 10 mg. When compared with the weekly bolus treatment this is about twice the amount of 5FU administered as bolus injection; for a weekly bolus injection of 5FU at 100 mg/kg this would have been 6 mg of 5FU for a mouse of 20 g. This higher amount of 5FU which can be administered as a continuous infusion is consistent with the clinical protocol in which enormous amounts of the drug can be delivered using protracted infusion (about 7000 mg in 4 weeks when a dose of 300 mg/m²/day is given compared to 2 x 1000 mg for two weekly bolus injections). It has been suggested from clinical studies that dose-intensity is related with the response of 5FU [45, 46]. However, the higher dose intensity of the continuous infusion did not result in a higher exposure based on the AUC; at repeated administration of 5FU the AUC was higher than for the continuous infusion (Fig. 1). Values for the AUC based on plasma levels measured during a two week continuous infusion of 300 mg/m²/day [25] were compared with the AUC of bolus injections [39], and no advantage for the continuous infusion in view of the AUC could be demonstrated. However, measurement of plasma levels has limited value considering the relation with antitumor activity, although a relation with hematological side effects has been demonstrated [39].

Administration of 5FU as a continuous infusion was able to inhibit the TS activity in tumor to a similar extent as a bolus injection. These data support the observation of others [48] that for a maximal inhibition of TS a relatively low dose is sufficient, which would provide FdUMP levels above a threshold required to inhibit the enzyme. Recently Chen & Erlichman [49] demonstrated that in a biochemical modulation schedule higher concentrations of FdUMP did not enhance the inhibition of TS. Continued exposure of the tumor to 5FU did, however, not support a prolonged inhibition of TS, despite the presence of 5FU in the tumor at concentrations (about 1 μ mol/kg) sufficient to support TS inhibition *in vitro*. The

most likely explanation for the decreased enzyme inhibition is an enhanced enzyme synthesis. Addition of LV to the weekly bolus treatment with 5FU seemed to abrogate such an enzyme synthesis *in vivo* [37] explaining the increase in therapeutic efficacy of the LV-5FU schedule.

Administration of LV did not enhance the antitumor effect of continuous infusion of 5FU. Several schedules mimicking either a continuous exposure to LV or peak exposure were used. The only modulating effect, however, was an increase in toxicity manifested as increased weight loss due to diarrhea. This phenomenon is comparable to clinical studies in which only minimal amounts of LV (as low as 5 mg daily given orally for two weeks) increased toxicity [25], as well as other schedules of LV [18,19]. This increased toxicity is possibly due to damage to normal mucosal tissues affecting the physiological function of gut mucosa such as reabsorption of nutrients and fluids. Co-administration of LV is very likely to enhance the inhibition of TS in normal mucosa, affecting normal functioning of this tissue. Folate pools in these tissues are very low and only minimal amounts of additional folate might be responsible for such an enhanced inhibition.

Continuous infusions or bolus injections of 5FU?

The *in vitro* evidence of a enhanced efficacy of continuous exposure has led to a number of clinical studies in which patients have been treated with protracted infusions of 5FU varying from one week to several months (see Table 1). Despite a number of Phase I and II trials in which 5FU was given as a continuous infusion convincing evidence for a better antitumor activity was not present [1]. Only recently Lokich et al [24] reported an increased response rate of 30% for the protracted infusion compared to 7% for the daily administration ($\times 5$) of bolus 5FU treatment. However, this treatment was not more efficacious considering survival, which was similar in both treatment arms. These results are comparable with the present data in mice, in which the initial better anti-tumor effect was not associated with an increase in life span. These overall clinical results are comparable to that observed for the combination of 5FU with LV (Table 2), in which a higher response rate was observed consistently for a number of randomized trials irrespective of the 5FU schedule [2, 4, 28]. However, the effect of the combination regimen of LV-5FU on survival is negligible. This has led to the question which schedule would be preferable from a therapeutic point of view; protracted continuous infusions with 5FU or biochemical modulation of 5FU with LV?

There seems to be no preference for any of these two schedules, when the available data are being considered of large randomized trials in which one of these treatment regimens was included (Table 2). It should, however, be noted that not only criteria such as response and survival, but also quality of life, costs and feasibility of the treatment should be taken in consideration [13]. Generally, continuous infusion will require hospitalization for implantation of the pump; subsequent treatment can be performed on a out-patient basis. However, both the LV-5FU combinations (5FU given as a bolus) and the protracted infusions have a limited value with regard to the overall survival of the patients and most patients do not respond. The use of multiple modulators [2] such as the combination of 5FU with PALA, LV, interferon and/or uridine may be another step forward. It is, however, remarkable that these modulators have shown their efficacy mainly when 5FU was given as a bolus injection: or a short-term infusion at a high dose. A flat rate continuous infusion of 5FU may not prove to be the first choice of systemic treatment for colorectal cancer but have certainly shown their value in the treatment of other malignancies [13] especially in combination with cisplatin in

Table 2. Comparison of the therapeutic efficacy of bolus schedules and protracted infusions against advanced colorectal cancer

Treatment modality	Response rate		Dose-limiting toxicity	Ref.
Bolus 5FU*	12%	(44/366)	leukopenia	2,4,28
Bolus 5FU with LV*	34%	(155/457)	gastro-intestinal	2,4,28
PALA with high dose 5FU**	36%	(40/110)	myelosuppression, diarrhea	5,6,50
Interferon- α with 5FU@	39%	(44/112)	myelosuppression, mucositis neurological	51,52,53
Protracted infusion#	40%	(160/399)	mucositis, HFS, diarrhea	54
Protracted infusion with LV##	30%	(16/48)	mucositis, HFS, diarrhea	18,19

*Summary of controlled randomized trials comparing 5FU with LV-5FU using various schedules.

**, 5FU given as a bolus or a high dose at 24 hr infusion.

@, 5FU was given as a loading dose of 5 days continuous infusion (750 mg/m²) followed by weekly bolus at 750 mg/m², with IFN α .

#, compilation of Phase II trials and one randomized trial [24] as summarized by Ahlgren [54]. Various lengths and intensities of the 5FU schedules were used. HFS, hand-foot syndrome.

##, compilation of Phase I and II trials, using different length and dosages of 5FU infusion as well as that of LV. Generally the dose of 5FU was decreased in the presence of LV, since the grade of toxicity was enhanced in the presence of LV.

head and neck cancer. However, other treatment modalities might find their application in the treatment of colorectal cancer. It has for instance been shown that a continuous exposure to 5FU is essential in combination with radiation [55]. Also chronomodulation of 5FU administration might enable to reduce the toxicity and enhance the antitumor effect [56]. However, only a randomized comparison of the possibly active schedules can give the answer.

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