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Original Paper

Antitumour Activity, Toxicity and Inhibition of Thymidylate Synthase of Prolonged Administration of 5-Fluorouracil in Mice

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Continuous infusions of 5-fluorouracil (5-FU) are increasingly used in the treatment of cancer. Their optimal use, however, has still to be determined since the availability of suitable animal models is limited. We studied continuous infusions in mice using subcutaneously implanted pellets that release 5-FU over a period of 3 weeks. At the maximum tolerated dose (MTD) (based on the systemic toxicity in healthy animals) we assessed the antitumour activity, haematological toxicity, inhibition of thymidylate synthase (TS) in tumours and the concentration of 5-FU in plasma during the 3-week period. We also studied the addition of leucovorin in different schedules. The dose-limiting toxicity was weight loss, and at the MTD of 10 mg of 5-FU released in 21 days per mouse myelosuppression was tolerable (nadir for leucocytes and thrombocytes was approximately 40% of pretreatment levels). In several independent experiments using the 5-FU-resistant Colon 26 tumour, a good antitumour acitivity was observed during the first part of the infusion, but thereafter the growth of the tumours resumed; the overall effect of continuous infusions was thus comparable to that of bolus injections. Co-administration of leucovorin did not enhance the therapeutic results; depending on the schedule used, it proved ineffective or only increased toxicity. Similar results were obtained with head and neck squamous cell carcinomas and with the 5-FU-sensitive tumour Colon 38. In Colon 26 tumours the TS activity (FdUMP-binding assay) initially decreased to 20–30% of controls and returned to normal after 11 days. In the catalytic TS assay a slight inhibition was observed for the continuous infusion, followed after 11 days by a marked (4-fold) increase in activity. 5-FU plasma levels varied from 0.1 to 1 μM following a circadian rhythm (with a peak at 6 h after light onset), and were maintained during the entire period. Subcutaneously implanted pellets represent a suitable model to study prolonged administration of 5-FU in mice and to evaluate the effect of modulating agents in laboratory animals before transferring data obtained *in vitro* to the clinic.

Key words: continuous infusion, 5-fluorouracil, thymidylate synthase
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INTRODUCTION

5-FLUOROURACIL (5-FU) has been used for more than 30 years for the treatment of cancer, but even though this is one of the most widely employed drugs in clinical oncology, the most effective schedule has yet to be established [1–5]. A daily bolus for 5 consecutive days, one weekly injection, and prolonged infusions have been employed, but with no clear differences in antitumour acitivity for the different modalities of administration [6]. The pattern of toxicity, however, varies for the different schedules. The dose-limiting toxicity for bolus injections is myelosuppression, while for continuous infusion gastrointestinal toxicity is prominent; mucositis and diarrhoea can be severe, and at times life-threatening, while the hand–foot syndrome is almost exclusively observed with prolonged infusions [7–9].

The potential use of 5-FU continuous infusion has been known for many years: pharmacokinetic and *in vitro* data support the concept that a constant exposure is more effective than bolus administration even if plasma concentrations with continuous infusion are lower. When cells in culture are treated for a

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prolonged period of time the drug shows more activity than in a 1 h exposure followed by culture in drug-free medium [10–14]. In cell lines the 50% growth inhibiting concentration (IC_{50}) of 5-FU for a 1 h exposure is approximately 300 μM, while for 24–72 h exposure it is 2–10 μM. Continuous exposure leads to the constant presence of the active metabolite FdUMP in relatively high concentrations [3] so that the inhibition of thymidylate synthase (TS), the target of FdUMP, is facilitated. During 5-FU exposure the enzyme was inhibited but its activity recovered when the drug was removed [15, 16]. Previously we obtained convincing evidence that in a murine model for colon carcinoma, Colon 26, the relatively poor inhibition of TS and its retention was associated with the limited antitumour effect of 5-FU but that modulation by leucovorin (LV) enhanced TS inhibition and the antitumour activity [17]. *In vitro* experiments showed that, during prolonged exposure of cells to 5-FU, cytotoxicity was due to inhibition of TS while the effect on RNA was prominent during short-term exposure to high concentrations of the drug [16].

The efficacy of 5-FU during continuous infusion may be influenced by the circadian behaviour of levels of plasma 5-FU [18–20], which are related to the diurnal variations in the levels of the 5-FU-degrading enzyme, dihydropyrimidine dehydrogenase (DPD) [19, 21]. In mice, rats and humans evidence is accumulating that various enzymes involved in 5-FU metabolism, such as DPD, uridine phosphorylase and thymidine kinase have a circadian rhythm [21–24]. Such variations in enzyme activity have not been related to plasma 5-FU concentrations during continuous infusion of 5-FU alone.

Optimisation of continuous infusion of 5-FU requires the availability of suitable animal models. Although continuous exposure of cells to 5-FU *in vitro* is more effective than pulse exposure, *in vitro* models lack the possibility of investigating the pharmacodynamics of bolus and continuous infusion of 5-FU; e.g. the selectivity of both protocols cannot be evaluated. Other factors which cannot be studied *in vitro* are the possible effects of circadian variations and accumulation of the drug in tissues. Recently, we demonstrated that plasma 5-FU levels do not reflect tissue levels [25]. Prolonged continuous infusions are complex to perform in small animals: a venous access is difficult to maintain even for a relatively short time and other devices for continuous infusions including implantable osmotic pumps are only effective for a few days [26, 27]. Long-term infusions in small animals can best be carried out by means of slow-release forms of 5-FU.

In the experiments described in this paper we have used pellets containing 5-FU that are implanted subcutaneously and release the drug for several weeks. We have characterised the maximum tolerated dose (MTD), plasma levels, toxicity, antitumour activity and effect on the target enzyme of 5-FU given as a continuous administration using subcutaneous pellets. We have also examined the possibility of modulating 5-FU activity with LV given in different doses and schedules.

MATERIALS AND METHODS

Chemicals

5-FU for bolus injections was obtained from ABIC (Netanya, Israel). 5-FU pellets were purchased from Innovative Research of America (Toledo, Ohio, U.S.A.). Leucovorin was provided by the Pharmacy department of the Free University Hospital. 6-[^{3}H]-FdUMP (specific activity 20 Ci/mmol) was purchased from Moravek Biochemical Inc. (Brea, California, U.S.A.) and 5-[^{3}H]-dUMP (specific activity 10.9 Ci/mmol) from Amersham International (Buckinghamshire, U.K.). dl-Tetrahydrofolate (Sigma Chemicals Co., St Louis, Missouri, U.S.A.) was converted into 5, 10-methylenetetrahydrofolate by addition of formaldehyde [12]. All other chemicals were of analytical grade and are commercially available.

In vivo *studies*

Female, Balb/c, C57/B1 and athymic nude mice (NMRI) of 8–10 weeks were obtained from Harlan/Cpb (Zeist, The Netherlands) and were maintained in the Clinical Animal Laboratory of the Free University. All experiments were performed in accordance with the rules for animal welfare established by this institution. Toxicity studies were carried out in healthy female C57/B1 and Balb/c mice. Parameters used to define the MTD were weight loss not greater than 15% and/or lethality less than 10%. Investigations on haematological toxicity were performed as previously described in detail [28–30]. Blood sampling was performed by retro-orbital puncture with heparinised capillaries after slight ether anaesthesia [25].

Antitumour activity was studied on the murine colon carcinomas Colon 26 maintained in female Balb/c mice, Colon 38 maintained in C57/B1 mice and on xenografts of the human squamous cell carcinomas of the head and neck, HNX-14C and HNX-OE, maintained in nude mice. Tumours were transplanted subcutaneously in the thoracic region on each flank of the animal [28–31]. Tumour size was measured sequentially twice weekly using a calliper, and the volume was calculated as length × width × height × 0.5. The evaluation of antitumour activity was based on the following parameters: the ratio of the mean volume of treated tumours and the mean volume of control tumours (T/C); the increase in the life span (ILS) defined as the ratio (×100%) of the median life span (MLS) of treated mice divided by the MLS of controls (MLS is measured starting from the first day of treatment); the growth delay factor (GDF) calculated from the median tumour doubling time (TD) of the tumours of treated mice and control mice according the following formula:

$$GDF = (TD_{treated} - TD_{controls})/TD_{controls}.$$

$TD_{controls}$ is about 3 days for Colon 26, 5 days for Colon 38, 4 days for HNX-OE and 8 days for HNX-14C. For toxicity and antitumour activity studies, 5-FU pellets containing different amounts of drug (5, 10, 15, 20 mg) were implanted subcutaneously on the back of mice after ether anaesthesia. Care was taken to avoid any direct contact of the pellet with the tumour even when this had grown to its maximum size. Bolus 5-FU was administered i.p. at the MTD: 100 mg/kg i.p. for Balb/c and C57/B1 mice and 125 mg/kg for nude mice. Leucovorin was injected intraperitoneally (i.p.) according to the doses and schedules indicated in the experiments. Treatment was started when the tumours had reached a size of 50–150 mm^3 (approximately 10 days for Colon 26, 18 days for Colon 38, 16 and 24 days for HNX-14C and HNX-OE, respectively).

Enzyme assays

Tumours were removed at different time points after pellet implantation, or from untreated controls, and were immediately frozen in liquid nitrogen. TS assays, FdUMP binding assay and ^{3}H-release assay, were performed as described [17]. The frozen tissues were pulverised by means of a microdismembrator (Braun, Melsungen, Germany) and the frozen powder was

suspended in a Tris–HCl buffer (200 mM) containing 20 mM β-mercaptoethanol to increase the stability of disulphate bonds present in the TS molecule and 100 mM NaF and 15 mM CMP to inhibit the breakdown of nucleotides. Temperature was maintained at 4°C and pH at 7.4. The suspension was centrifuged at 4000 *g* and the supernatant subsequently at 7000 *g*. The enzyme-containing suspension was split in several parts and used for different assays. Details have been described previously [17].

Briefly, the first assay was a ligand-binding assay with 6-[^{3}H]-FdUMP as the substrate, used to determine the free FdUMP binding sites of TS. Enzyme suspension (50 μl) was mixed with 50 μl 6.5 mM CH_2-THF, 135 μl Tris–HCl buffer and the reaction was intitiated by addition of 15 μl 570 nM 6-[^{3}H]-FdUMP; the mixture was incubated at 37°C for 1 h, stopped by addition of 500 μl 10% neutral charcoal, vigorously mixed, chilled on ice and centrifuged. Radioactivity was estimated by liquid scintillation counting of 250 μl supernatant.

The second assay determines the catalytic activity of TS at a final concentration of 1 and 10 μM 5-[^{3}H]-dUMP by means of the ^{3}H-release. The TS catalytic activity assay in control tumours consisted of: 25 μl enzyme suspension (at different dilutions), 5 μl 6.5 mM CH_2-THF, 10 μl Tris-HCl or 10 μl 0.05 μM FdUMP to measure the potential inhibition of TS in control samples. The assay was initiated by addition of 10 μl 5-[^{3}H]-dUMP (1 or 10 μM final concentration) and was incubated for 30 min at 37°C, stopped by addition of 50 μl ice cold 35% trichloroacetic acid and 250 μl 10% neutral activated charcoal. After centrifugation, 150 μl of the supernant was counted by liquid scintillation [17].

Before the assays were performed, endogenous FdUMP and other nucleotides were removed with activated neutral charcoal in samples from treated animals. This charcoal wash was also performed on control samples and did not change the results of the assays. In a number of samples both the total amount of TS and the residual TS activity were measured. For this purpose, assays were only performed after a dissociation step. One part of the enzyme suspension was used for dissociation of FdUMP from TS, by incubation of equal volumes of the enzyme suspension and dissociation buffer (0.75 M NH_4CO_4, 100 mM NaF, 20 mM β-mercaptoethanol, 15 mM CMP, pH 7.8) and 1/20 of the total volume of 1.6 mM dUMP for 3 h at 30°C. The high concentration of dUMP facilitated dissociation, prevented reassociation of FdUMP and stabilised TS. Addition of 10% neutral charcoal (same volume as the total dissociation volume) was used to remove the nucleotides after dissociation. An equal volume of the enzyme suspension was frozen at −70°C and thawed after 3 h. This part of the enzyme suspension was also treated with 10% neutral charcoal. Both assays of TS were performed in the dissociated and non-dissociated samples, enabling determination of the total and free number of FdUMP binding sites, respectively, and the total and residual catalytic activity, respectively. Protein content of the tumours was assayed using the BioRad protein assay.

Analysis of 5-FU and FdUMP levels

Plasma concentrations of 5FU were measured in healthy female C57/Bl mice: blood was drawn retro-orbitally between 9.00 a.m. and 10.00 a.m., 3–4 h after light onset (HALO), in order to avoid circadian variability. Differences in concentrations at various time points were studied on day 10, when 5 animals were sampled at six time points from 3 HALO until 15 HALO (8 h dark, 16 h light). Blood was centrifuged and plasma samples were immediately frozen. 5-FU concentration was measured by gas chromatography coupled to mass-spectrometry (GC—MS) as previously described [25, 32]. The sensitivity and selectivity of this analytical procedure enables the use of very small samples (10 μl): in this way every animal could be sampled repeatedly during the 3 weeks and a complete pharmacokinetic profile was obtained for each animal. In order to exclude the possibility that pellets were exhausted after a few days from implantation, some pellets removed from several mice after 10 days were implanted in different mice and plasma concentration was measured during the following period. 5-FU and FdUMP concentrations were also measured in Colon 26 tumours during the infusion at different times after implantation of the pellet. Tissues were removed during anaesthesia and immediately frozen in liquid nitrogen. 5-FU and FdUMP concentrations were measured as described previously [17, 25].

RESULTS

Toxicity

The extent of systemic toxicity was expressed as percentage of weight loss following the implantation of the pellet. Pellets containing different amounts of 5-FU were studied in healthy mice; larger pellets (15 and 20 mg) caused an important loss of weight (approximately 25% at day 4) and proved rapidly fatal, while weight loss was 11% at day 11 following implantation of 10 mg pellets (Figure 1). The pattern of systemic toxicity differed from that of bolus injections [28]: weight loss was not seen in the first days after the beginning of treatment, but after 10 days, and the maximum weight loss during continuous infusions was higher than that observed after bolus injection. Similar data on toxicity were obtained in tumour-bearing animals (Table 1).

Pellets containing 10 mg 5-FU, released over 3 weeks were considered to be the MTD for this form of therapy, and these pellets have been used for all the following studies unless otherwise indicated. The additional effect of LV was investigated at various doses and schedules. A weekly bolus injection of 100 mg/kg had no effect, but small daily doses of 5 mg/kg resulted in severe diarrhoea, major weight loss and increased mortality (Table 1).

The pattern of haematological toxicity of 5-FU pellets has been studied in healthy mice at the MTD of 10 mg per mouse (Figure 2a–c). Haematocrit (Figure 2a) reached a nadir of 70%

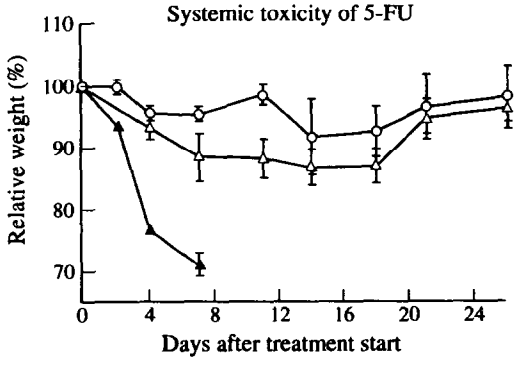

Figure 1. Systemic toxicity of 5-FU given as an i.p. bolus injection (given on days 0 and 7) or as a continuous infusion. The weight is expressed as a percentage of the initial value. The doses and schedules of 5-FU were 100 mg/kg i.p. bolus (-○-), 10 mg pellets (-△-), 15 mg pellets (-▲-). The curve for 20 mg pellets is superimposed on that of the 15 mg pellets. Values are means ± S.E. of 3–5 mice.

Table 1. Antitumour activity of 5-FU bolus and continuous infusions

5-FU	Dose	LV	Schedule@	Max. weight loss	T/C	GDF†	Median lifespan‡ (days)	Increase of lifespan‡ (%)
Colon 26								
	100 mg/kg	-	1	6.0 ± 2.0 (9)§	0.51 ± 0.14 (9)§	0.9 ± 0.4 (9)§	25 (9)§	169 ± 57 (9)§
	5 mg	-	2	6.2	0.39	2.2	23	143
	10 mg	-	2	3.8 ± 2.1 (6)	0.34 ± 0.09 (6)**	2.1 ± 0.7 (5)***	24 (6)	161 ± 32 (6)
	100 mg/kg	100 mg/kg	3	6.2 ± 1.7 (7)	0.42 ± 0.11 (7)	2.2 ± 0.7 (7)***	26 (7)	171 ± 66 (7)
	10 mg	100 mg/kg	4	5.4; 5.9	0.28; 0.35	2.2; 2.7	18; 27	120; 93
	10 mg	5 mg/kg	5	3.7	0.21	n.e.†	17	100
	10 mg	10 mg/kg	5	8.7	0.44	n.e.†	20	67
	10 mg	20 mg/kg	5	5.6	0.40	n.e.†	20	67
	10 mg	10 mg/kg	6	5.3	0.34	2.7	19	150
Colon 38								
	100 mg/kg	-	1	4.7 ± 1.9 (5)	0.13 ± 0.02 (5)	2.5 ± 0.9 (5)	> 50 (5)	n.e.‖
	10 mg	-	2	20.6	0.01*	3.5	> 50	n.e.‖
HNX-OE								
	10 mg	-	2	10	0.42	2.9	> 46	n.e.¶
HNX-14C								
	125 mg/kg	-	7	11	0.59	1.7	47	n.e.¶
	10 mg	-	2	7	0.62	1.0	44	n.e.¶

@Schedules: (1) Weekly i.p. bolus injection 5-FU, 4 ×; (2) Continuous infusion 5-FU with pellet during 21 days; (3) 5-FU as in (1), LV, 2 split doses of 50 mg/kg i.p. given 1 h before and together with 5-FU, weekly 4 ×; (4) 5-FU as in (2), LV, weekly i.p. bolus injection, 4 ×; (5) 5-FU as in (2), LV, daily i.p. bolus injection 5 days a week for 3 weeks; (6) 5-FU as in (2), LV, every other day i.p. bolus injection (3 days a week for 3 weeks; (7) Weekly i.p. bolus injection 5-FU, 2 ×; †n.e. not evaluable because no tumour doubling was reached; ‡the median lifespan was calculated starting from the day of tumour implantation; the median lifespan of control mice (starting from the day of first treatment) is set at 100%; §The number in the parentheses is the number of experiments with at least 6 mice. The data where no *n* is mentioned are from one experiment with at least 6 animals. T/C is given for day 7 for Colon 26 and Colon 38, and day 10 for HNX-OE and HNX-14C; ‖Lifespan is not assessed for mice bearing this tumour, they were sacrificed when tumour load exceeded 2000 mm^3; ¶Lifespan was not assessed for mice bearing this tumour, they were sacrificed when tumour load exceeded 1500 mm^3. Significantly different from the bolus injections (test for unpaired samples) at the level of: $0.02 < P < 0.05$ (*); $0.01 < P < 0.02$ (**) and $P < 0.01$ (***). Maximum weight loss is expressed as a percentage of the initial weight.

of initial values after 7 days and it returned to normal levels after 20 days, coinciding with the end of the infusion. The effect on granulocytes was characterised by a 60% decrease after 4 days compared with pretreatment levels (Figure 2b). However, this was followed by a marked rebound after about 20 days, and by the return to normal levels after discontinuation of the 5-FU release. The initial effect on thrombocytes (Figure 2c) was comparable to that on total white blood cells, but only a moderate rebound was observed. Animals in which only the carrier material of the pellet was implanted and untreated controls showed no significant changes in white blood cell counts or in haematocrit. A late effect of sampling was observed on the thrombocyte counts; however, the effect of 5-FU treatment was immediate and more pronounced, and cannot, therefore, be attributed to sampling.

Antitumour activity of continuous infusion 5-FU with and without leucovorin

The therapeutic effect of pellets at the MTD was compared with that of "standard" 5-FU treatment, consisting of weekly bolus injections i.p. at the MTD of 100 mg/kg. Figure 3 shows a typical growth curve for Colon 26. A bolus injection of 5-FU induced a reduction in growth rate but continuous infusion actually blocked tumour development for about 1 week. However, thereafter, the tumour started to grow at the same rate as the untreated controls. The 10 days growth inhibition followed by regrowth was also observed in Colon 38, HNX-14C and HNX-OE. The overall results in terms of survival and growth delay factor of bolus injections and continuous infusion were similar, both for Colon 26 and for the other tumours (Table 1). Using a lower dose (5 mg pellets) the tumour growth was blocked for only 3 days and then resumed.

Previously we demonstrated that the antitumour activity of bolus 5-FU against Colon 26 could be potentiated by LV [17, 30]. Therefore, we combined continuous infusion 5-FU with LV in different schedules. A single weekly bolus injection had no effect at all, nor did administration of LV in different schedules, three times a week and daily administration. The overall therapeutic efficacy of the combination of continuous infusion 5-FU and LV was not different from 5-FU continuous infusion alone. Tumour volumes exhibited a plateau phase of about 7 days followed by rapid regrowth (Figure 3).

Analysis of plasma concentrations

In order to assess whether 5-FU was released at a constant rate we determined the plasma concentrations over 3 weeks. The 10 mg dose delivered by the pellets corresponds to a daily dose of 23.8 mg/kg 5-FU (assuming a mouse of 20 g) which results in a higher cumulative dose than with weekly bolus injections (166 versus 100 mg/week). Plasma 5-FU concentrations at 3–4 HALO were in the range of 0.1–1 μM and were maintained for 3 weeks (Figure 4a). Plasma levels tended to decrease after 16 days, but 5-FU concentrations remained above the detection limit of the assay for 21 days. In the following period the drug was undetectable in plasma, which indicated that release was completed. Similar plasma concentrations were found in mice that were implanted with pellets removed from another mouse (Figure 4a).

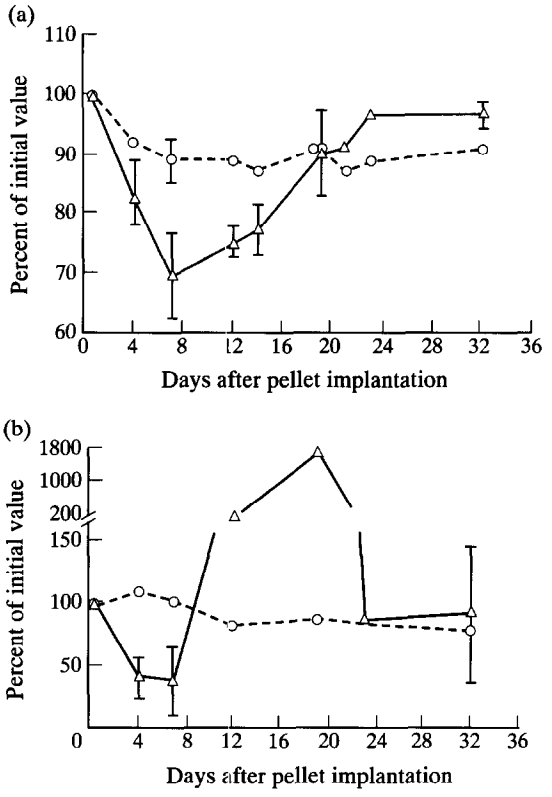

Figure 2. Haematological toxicity of 10 mg 5-FU pellets in C57/Bl mice: (a) haematocrit, (b) leucocytes, (c) platelets. Values for controls (-○-) and pellet treatment (-△-) were expressed as a percentage of initial values calculated per individual mouse. Initial values are means ±(S.D.) of 20 mice: haematocrit, 47.5% (± 3.8); leucocytes, 3.9×10^6 (± 1.1×10^6) cells/ml; platelets, 1413×10^6 (± 550×10^6) cells/ml. Values at other time points are means ± S.D. of at least 3 mice. Student's *t*-test for paired data: (a) haematocrit of treated mice is significantly lower than before treatment at days 4–14 ($P < 0.01$). (b) After 4–7 days the number of leucocytes of treated mice was significantly lower than before treatment ($P < 0.05$). At day 18 the number was significantly higher than before treatment ($P < 0.01$). (c) The number of platelets of treated mice was significantly lower than before treatment at day 4 ($P < 0.05$). At day 12 it was significantly higher than before treatment ($P < 0.05$).

We also measured plasma concentrations at different time points during the day after 10 days from the implantation of pellets. A 10-fold difference between the plasma concentration at 3 HALO and the peak value at 6 HALO was consistently observed in each animal. At 12 HALO and later, plasma 5-FU concentrations were generally undetectable (Figure 4b). Peak and trough values were significantly different from the calculated means.

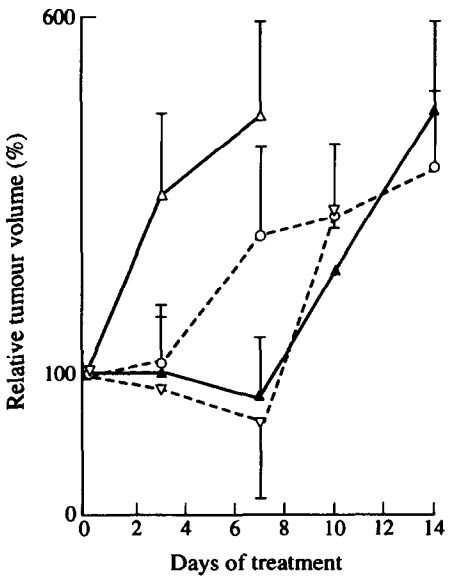

Figure 3. Relative antitumour activity on Colon 26 of 100 mg/kg bolus 5-FU (-○-) 10 mg 5-FU pellets (-▲-), 10 mg 5-FU pellets combined with 5 mg/kg LV given as 5 daily bolus injections (-▽-) compared with controls (-△-). Values are means ± S.D. of 3–6 mice (6–12 tumours).

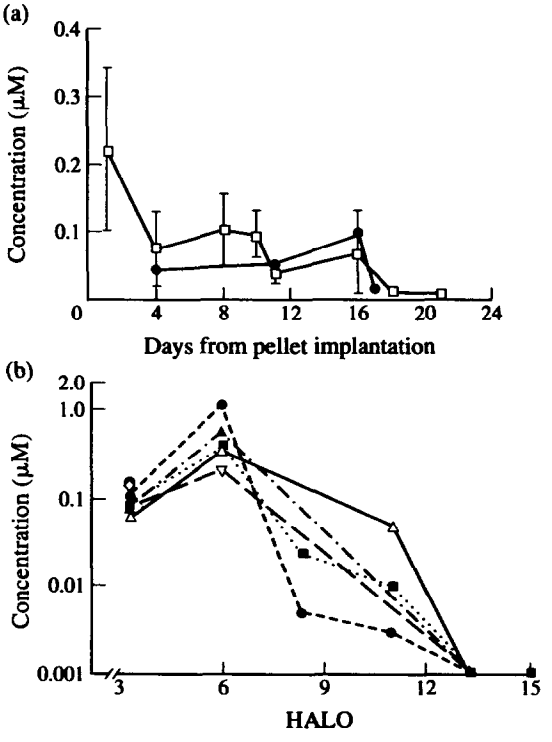

Figure 4. (a) 5-FU plasma concentration in C57/Bl mice at 3–4 HALO (mean values ± S.D. of at least three values). (-□-); values from one of the pellets that was implanted in one mouse and after 10 days was moved to another (-●-). (b) Circadian variation studied on day 10 from pellet implantation, data are shown for each separate animal. Time is measured as hours after light onset (HALO): 3 HALO corresponds to 9.00 a.m. Student's *t*-test for paired data: plasma concentration at 12.00 (6 HALO) was significantly different from all other time points ($P < 0.01$).

During the infusion we also measured 5-FU concentration in tumours (Figure 5). The highest concentrations were observed at the beginning of the infusion. At all time points 5-FU concentrations in the tumour were at least 2-fold higher than the corresponding levels in plasma.

FdUMP concentrations and effect on TS

The concentrations of FdUMP were measured in tumours at various time points after implantation of the pellets. However, at all time points the FdUMP concentrations were below the detection limit (12 pmol/g wet weight) of the assay. Despite the low levels of FdUMP, the activity of TS was inhibited significantly during the first week (Figure 6a, b). The number of free FdUMP binding sites was reduced significantly at days 4 and 7 after the start of treatment. However, after 11 days a partial recovery was observed, followed by a complete recovery to normal levels after 2 weeks. The catalytic activity of TS showed a somewhat different pattern with a small decrease in the residual TS activity after 4 and 7 days and a comparable total TS catalytic activity. After 11 days a 3-fold increase in TS residual catalytic activity was accompanied by an almost 5-fold increase in the total catalytic activity. Thereafter, the total catalytic activity decreased and was comparable on the residual activity after 14 days.

DISCUSSION

Continuous infusion of 5-FU has a considerable interest in the clinic. In this paper we show that it is feasible to administer 5-FU continuously over 3 weeks to mice attaining plasma levels that are comparable to those observed during continuous infusions in patients [33–38] and in rats [39], although in the latter species, and in some of the studies in patients, infusions were shorter. During limited infusion periods (3–7 days), plasma concentrations tended to be higher than in longer infusions (21 days) possibly due to the higher dose which could be administered. The overall results, both antitumour activity and toxicity, are also in agreement with the clinical data [7, 8], showing the validity of the murine model system. Clinical studies report a higher response rate for continuous infusion than for bolus injections, but the overall results in terms of survival are not different. The plasma and tumour concentrations in mice are in the range of the concentrations which cause cell killing *in vitro* [11]. In a cell line derived from Colon 26, C26-10, the IC_{50} for 5-FU, 72 h exposure, was 0.6 μM, at which a significant TS inhibition was observed. It should be noted that at longer

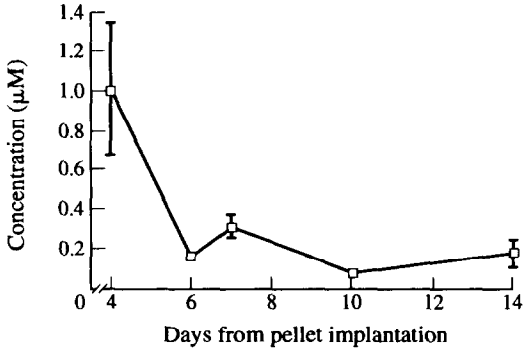

Figure 5. 5-FU concentration in Colon 26 tumour tissue (mean values ± S.D. of 3–5 tumours), 1 g of tissue was considered to be equivalent to 1 ml. Student's *t*-test for paired data: 5-FU concentration in tumours was significantly higher than plasma concentration on days 4 and 8 ($P < 0.01$).

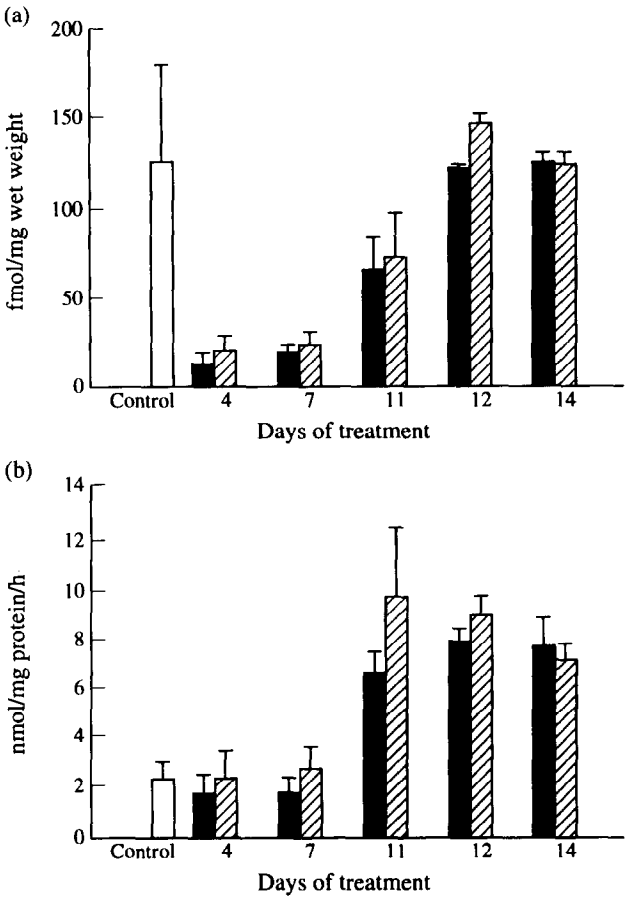

Figure 6. (a) FdUMP binding to TS in Colon 26 tumours at different time points after pellet (10 mg 5-FU) implantation. Control (open bars), free FdUMP binding sites after treatment, measured without dissociation (closed bars), total FdUMP binding sites after treatment measured after dissociation of samples (hatched bars). Values are means of at least 3 tumours ± S.D. Student's *t*-test unpaired data: at 4, 7 ($P < 0.001$) and 11 days ($P < 0.05$) after the start of 5-FU treatment the total FdUMP binding was significantly lower than control. A significant difference between the number of free binding sites and total binding sites was only observed at day 12 ($P < 0.01$). (b) TS catalytic activity of Colon 26 tumours at different time points after pellet (10 mg 5-FU) implantation. Control (open bars), residual TS catalytic activity after treatment, measured without dissociation (closed bars), total catalytic activity after treatment measured after dissociation of samples (hatched bars). Values are means of at least 3 tumours ± S.D. Student's *t*-test unpaired data: no significant differences were observed between residual and total TS catalytic activity, but the total TS catalytic acticity on days 11, 12 and 14 was significantly higher than the control value ($P < 0.001$).

incubations *in vitro* the IC_{50} values for 5-FU decreased. Our *in vivo* results demonstrate that continuous infusion was more effective than bolus treatment in the initial inhibition of tumour growth, but this did not lead to a longer survival of the animals.

From the concentration versus time plot it appeared that the profile of 5-FU concentrations in plasma was comparable to that observed in patients, but very different from that of bolus injections in which peak concentrations between 500 μM and 1 mM are observed. The somewhat higher concentration just after the implantation of the pellet (0.2 μM compared with 0.05–0.1 μM at later time points) was possibly due to the process by which pellets start to release the drug before reaching a stable rate of delivery. Only after 16 days did we observe a significant decrease in plasma levels. The increase in FdUMP binding sites in tumours, the rebound in blood cells and the increased rate of

tumour growth were already observed on day 7, well before the decrease in plasma and tissue levels of 5-FU. The small trough apparently present after 10 days was not significant. Exhaustion of the pellet is unlikely at that time point since animals that received pellets that had been implanted in different mice for 10 days showed similar plasma concentrations of 5-FU (Figure 4a). Variations in plasma concentration could also be due to changes in metabolism. These may consist of an increase in the activity of catabolising enzymes with time resulting in decreased plasma 5-FU levels despite a regular delivery from the pellet. These changes in 5-FU plasma concentrations, however, were smaller than the variations observed during continuous infusion using pumps in patients [40]. Owing to these variables we did not include a pharmacokinetic evaluation. More extensive sampling would be required for a proper pharmacokinetic evaluation, and this would be impossible in the same mouse both for ethical an methodological reasons.

Plasma 5-FU concentrations measured at 3 HALO corresponded to the values seen in the animals sampled over the entire 3 weeks. Plasma samples obtained at later points of the day show that 5-FU concentrations increased to reach a peak at 6 HALO, followed by a decline. These data are in agreement with the circadian rhythm of DPD observed in rat liver [21] which is the main tissue responsible for 5-FU elimination [1, 2]. The variation in enzyme activities is possibly related to the different antitumour activity of 5-FU when the drug is administered at different time points [41] and to the presence of varying 5-FU plasma concentrations during continuous infusion in patients [18]. These concepts are currently being explored in the clinic and "chronomodulated" delivery of 5-FU and of other drugs is becoming increasingly popular [24, 42–44].

In terms of dose delivered, continuous infusions offered a higher dose intensity than bolus injections. The total amount of drug administered was 10 mg versus 6 mg. This was comparable with the higher dose of 5-FU that can be given to patients with continuous infusion using schedules varying from 3 days to several weeks [7, 8, 33–38].

The pattern of haematological toxicity induced by continuous infusion was quite different from what was observed with bolus injections. Nadir values observed after bolus injections were lower in the same strain of mice (20%) [28, 29], but were reached later, while the rebound was lower and only observed after discontinuation of the treatment. In continuous infusion the rebound was even observed during infusion which would indicate that the low concentration of 5-FU might affect differentiation/proliferation of primitive stem cells. Evidence has indeed been obtained that low 5-FU concentrations can be immunostimulating [45] and the analysis of blood smears indicated that granulocytes were more affected than lymphocytes (data not shown). Different effects were also observed for the haematocrit (more severe after bolus injections). The data correspond to the observed lack of myeloid toxicity in the clinic with protracted infusion of 5-FU [7–9].

In contrast to the haematological toxicity, weight loss at the MTD was maximal 11 days after pellet implant, much later than what was observed with bolus injections [28]. This difference in the time course of systemic toxicity was particularly evident when, during the determination of the MTD, mice were treated with doses of 5-FU that proved lethal. When an excessive dose was given as an infusion, weight loss was rapid and death occurred within 1 week (Figure 1). This is consistent with experimental and clinical observations on the different toxicity seen after bolus or infusional administration of 5-FU: mainly haematological for bolus treatment, gastrointestinal for infusions. We have observed that a bolus injection of 5-FU can inhibit TS in mucosal tissue [46] and the prolonged presence of 5-FU might be particularly toxic to gastrointestinal tissues. LV administration enhanced the gastrointestinal toxicity of infusional 5-FU, suggesting that TS inhibition was involved. The continuous, repeated administration of LV possibly provided enough folates in these normal tissues in order to enhance this inhibition, despite the low activity of folylpolyglutamate synthetase in these tissues [47]. The importance of this enzyme in resistance to the continuous exposure to 5-FU has also been reported for tumour cells [48]. Results on gastrointestinal toxicity are consistent with the clinical observations: patients receiving LV, even at very low daily doses (5 mg/day p.o.) [33, 49–52] have a marked increase of toxicity during continuous infusion of 5-FU. In addition, in several clinical trials modulation of continuous infusion 5-FU with LV did not increase the antitumour activity [33] similar to our murine study, in which no schedule of LV administration improved the therapeutic effect.

The pattern of antitumour activity of continuous infusion was different from that obtained with bolus injections even if overall results were similar. While bolus injections only reduced the growth rate, continuous infusion actually blocked tumour enlargement for Colon 26 (for approximately 1 week) and after this time tumour growth resumed at the same rate as untreated controls. This time course was seen in all the experiments performed with continuous infusion, with or without LV, and could also be seen when a smaller pellet of 5 mg was implanted. In this last case, however, tumour growth was blocked for only 3 days. For Colon 38, a significant reduction in tumour size was observed. These results are consistent with clinical observations in which continuous infusion is reported to be associated with a higher response rate, but not with an increased survival [7, 8] compared with bolus.

Interestingly, a low FdUMP was associated with an almost total inhibition of TS activity, similarly to what has been observed in patients [53]. The growth of tumours went along with an increase in TS activity that occurred despite the sufficiently high concentration of 5-FU in plasma and in tissues to inhibit cell growth and TS activity *in vitro* [15]. The increase in TS indicates that this enzyme is a key target for the activity of continuous infusion 5-FU, in agreement with *in vitro* results [16]. An increase in TS was also an important mechanism of resistance to bolus injections of 5-FU in the same model [17], and a similar increase in TS activity has been observed after *in vitro* exposure to 5-FU [54]. Current strategies are directed at preventing the increase in TS; we have recently demonstrated that this effect can be obtained in Colon 26 with LV administration [17]. Whether this feedback regulation is similar to that reported for interferon γ [54] remains to be proven. Unfortunately, in our system the antitumour activity of continuous infusion was not improved by any combination with LV, which is consistent with *in vitro* observations that LV only marginally increased the duration of TS inhibition during continuous exposure of cells to 5-FU [16]. This modulation was also hampered by the increase in gastrointestinal toxicity. The current model seems to be useful to study the activity of 5-FU given as a continuous infusion in mice and to investigate the association with modulating agents such as interferons.

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