

ORIGINAL ARTICLE

Low-dose continuous 5-fluorouracil infusion stimulates VEGF-A-mediated angiogenesis

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Abstract

Background. Tumor growth is angiogenesis-dependent. Animal studies have demonstrated that frequent administration of chemotherapeutics may have marked antiangiogenic effects and improved antitumor effects, with less severe toxic side-effects than intermittent maximum tolerated dose chemotherapy. Currently, research focused on low-dose antiangiogenic chemotherapy is increasing. We have recently reported that certain chemotherapeutics, including 5-fluorouracil (5-FU), may in fact stimulate angiogenesis in the tumor-free rat mesenteric window assay. The aim of the present study was to extend the investigation of the angiogenesis-modulating effects of 5-FU by prolonging the continuous infusion treatment time. **Method.** Angiogenesis was induced in the mesenteric test tissue in adult male Sprague-Dawley rats by i.p. injection of VEGF-A, which is a key angiogenic factor in most tumors. During the subsequent angiogenesis, 5-FU was delivered continuously for 14 days by an osmotic pump implanted subcutaneously. The angiogenic response was analyzed by morphometry in the mesenteric windows. **Results.** The 14-days continuous infusion of 5-FU significantly stimulated angiogenesis. Thus the possibility that the previously reported surprising proangiogenic effect of 5-FU reflected an insufficiently long treatment period can be ruled out. **Conclusion.** The finding that continuously infused 5-FU is able to stimulate angiogenesis in the present rat model of angiogenesis warrants investigation of the mechanisms behind this unexpected finding. It may further have implications for the choice of antiangiogenic chemotherapeutic schedule used for cancer patients.

The fluoropyrimidine 5-fluorouracil (5-FU) is one of the most widely prescribed chemotherapeutics for cancer as it shows efficiency in many malignancies and presents a tolerable side-effect profile. The cytotoxic effects of 5-FU are mainly ascribed to the inhibition of the nucleotide synthetic enzyme thymidylate synthase and the aberrant incorporation of its fluoronucleotide metabolites into RNA and DNA [1]. 5-FU represents the classical chemotherapeutic drug that is administered as continuous infusion, which allows increases in tolerated dose and dose intensity compared to bolus-based schedules [2].

It is now widely accepted that tumors are angiogenesis-dependent. Evidence indicates that low-dose continuous or frequent dosing of lower doses of chemotherapy, can exert marked angiogenesis-suppressive effects and improve antitumor effects, with no or fewer severe toxic side-effects, as compared to

conventional maximum tolerated dose (MTD) chemotherapy [3–5]. MTD chemotherapy targets primarily rapidly proliferating neoplastic cells, while the primary target of antiangiogenic chemotherapy is the angiogenically activated and genomically stable microvessel endothelial cells. These normal endothelial cells display, in contrast to neoplastic cells, a low rate of mutation, which reduces the risk of drug resistance development. Moreover, a lower drug concentration can significantly influence the proliferating microvessel endothelial cells, which are directly exposed to the drug and more sensitive to the drug than neoplastic cells [6].

It is difficult to study in a precise manner the effects on tumor angiogenesis of a given antitumor treatment. We therefore use a tumor-free *in vivo* model that enables truly quantitative analysis of angiogenesis-modulating effects following chemotherapy,

described in detail elsewhere [7]. The present assay does, however, not take into account all aspects of tumor-induced angiogenesis, as discussed in [8,9]. Nevertheless, this VEGF-A-mediated angiogenesis assay closely reflects the indirectly assessed antiangiogenic (and antitumoral) effects of paclitaxel administered by s.c. continuous infusion in a syngeneic prostate cancer model [10].

We previously reported that chemotherapeutics administered in bolus dose demonstrate drug-specific and approximately linear dose-dependent antiangiogenic effects on VEGF-A-induced angiogenesis [8,11]. Antiangiogenic chemotherapy requires that the active drug be administered as frequently as possible, as in metronomic therapy [3,4]. Continuous infusion is not equal to metronomic chemotherapy, but may be viewed as an intensive form thereof. In our low-dose continuous infusion experiments cyclophosphamide, paclitaxel, and vinblastine exert dose-related antiangiogenic effects, whereas doxorubicin exert no significant effect on VEGF-A-induced angiogenesis [5,9]. Surprisingly and paradoxically, with continuous infusion chemotherapy, 5-FU and cisplatin stimulated VEGF-A-mediated angiogenesis [5]. To exclude the possibility that the observed proangiogenic effect of 5-FU therapy depended on an overly short duration of treatment, we here report that prolonged, 14 days continuous infusion of 5-FU stimulates VEGF-A-induced angiogenesis significantly. For clarity, all experiments performed in this model with low-dose continuous and bolus 5-FU treatments are reviewed.

Material and methods

Animals

Outbred, adult, male Sprague-Dawley rats (B&K Universal, Sollentuna, Sweden) were acclimatized to a standardized environment for a minimum of 7 days, fed *ad libitum* and randomly allocated to weight-matched groups with two animals per cage [8]. At the start of the angiogenesis experiment, the body weight (BW) was 210.8 ± 0.4 g (mean $\pm$ SEM). BW was monitored twice weekly. The controls increased in weight by a mean of 104.6 g during a 14-day period. Chemotherapy-related retardation of physiologic BW gain was regarded as a surrogate evaluation of toxicity, systemic well-being, anorexia and failure to thrive. The Animal Ethics Committee in Göteborg approved the present study. All procedures followed the standards set by the UKCCCR guidelines [12].

Angiogenesis initiation

As previously described [5,9], recombinant rat VEGF₁₆₄ (564-RV/CF; R&D Systems, Ltd., Oxon, UK), which is the predominant angiogenic isoform of VEGF-A in rats, was diluted to 96 pmole/ml, frozen, thawed, and injected i.p. in a volume of 5 ml twice daily for 4.5 days (Days 1-5). Injected VEGF-A rapidly reaches the mesenteric test tissue and its microvascular endothelial cells [7], thereby initiating microvessel network proliferation, which peaks at around Day 21. It was within this post-treatment time-frame of microvessel network expansion that chemotherapy was administered (see below).

Chemotherapy

Initial dose-finding experiments. In order to define non-toxic doses that only marginally affected the physiologic BW gain of the animals, we performed a dose-finding experiment. Based on previous experiments involving bolus doses [8] and 7-day continuous infusion [5], six animals per group were continuously infused for 16 days with 5-FU at 37.5, 75 and 150 mg/kg/week (corresponding to 5.35, 10.7 and 21.4 mg/kg/day, respectively). It has been claimed that the dose per body surface area (mg/m²) is useful in comparing drug toxicity between species (*i.e.* between laboratory animals and humans), although at least for some chemotherapeutics this type of direct comparison may be problematic [9]. For a rat that weighs 250 g, a dose calculated in mg/kg $\times$ 7 yields an approximate human dose in mg/m² [13]. Thus, the doses administered in the present study correspond to 262.5, 525 and 1 050 mg/m² per week in humans, respectively. The intention was to use doses that would retard weight gain by not more than 15% at sacrifice (the end of treatment), as compared to rapidly growing vehicle-administered control animals, while not reducing the BW in absolute terms.

Chemotherapeutic and vehicle control. The commercially available formulation of 5-fluorouracil (Fluracetyl®; Nycomed AB, Lidingö, Sweden) was used and diluted in physiological sodium chloride (0.9% NaCl; w/v). The vehicle controls also received 0.9% NaCl (pH adjusted to 8.9 with NaOH, as in the 5-fluorouracil formulation). On Day 6 after the start of the i.p. angiogenic treatment, Alzet® osmotic minipumps (Model 2ML2; Alzet® Osmotic Pump, Mountain View, CA, USA) were completely filled with 5-FU under sterile conditions. These pumps deliver a constant dose over the entire treatment period. The pumps for the controls were filled with

the vehicle. One day later, on Day 7, after being stored in sterile 0.9% NaCl (w/v) saline overnight at 37°C, the pumps were surgically implanted s.c. on the backs of rats that had been anesthetized with inhaled isoflurane (Forene®; Abbott). The skin incision was immediately sutured after pump implantation. The animals were sacrificed on Day 21. No animal died during the surgical procedure or post-surgical period.

Angiogenesis quantification

Four membranous ("window-like") sections of the mesentery from the most distal part of the mesentery, adjacent to the ileocecal valve, were examined after being spread in intact form onto objective slides [7]. Normally, in avascular regions, this tissue has a thickness of only 5–10 µm and forms a uniform, almost translucent membrane. The surrounding fatty tissue distinctly delineates each window. The central part of each window is often avascular in untreated animals. The entire vasculature of each intact mesenteric window was visualized immunohistochemically using a monoclonal antibody directed against the rat endothelium [5].

Microscopic morphometry and computerized image analysis were employed in a blinded fashion. Several variables were measured objectively in the intact thin membranous tissue, as described elsewhere [14]. Initially, the total area of each mesenteric window was measured. The vascularized area (VA), which is a measurement of the spatial extension of the microvasculature, was then assessed as a percentage of the total window area. Subsequently, the microvascular length (MVL), which is a composite measurement of microvessel density within vascularized areas, was measured. The total microvascular length (TMVL) was calculated as the VA × the mean MVL per treatment group. In addition, the following objective variables were measured within the microvessel network: (1) the lengths of individual microvessel segments (Le. MS), i.e. the true distance between two successive branching points. The Le. MS values for each treatment group were pooled and ranked in order of size. For statistical comparisons of the treatment groups, the 0–10 and 90–100 percentiles of the Le. MS were used; (2) the number of microvessel segments (No. MS), which represents the number of segments per unit tissue area (or tissue volume); (3) the number of microvessel branching points per unit tissue area (No. BP); and (4) the index of intersection (In. IS), which reflects the number of microvessel intersections per unit tissue area.

Although some of the variables are interrelated, it is fair to say that: (i) the VA, MVL and TMVL are

primarily measurements of microvessel proliferation; (ii) the No. MS and No. BP values reflect both microvessel proliferation and pattern formation; (iii) the Le. MS measures the actual microvessel segment length; and (iv) the MVT, In. IS and In. LF primarily relate to microvessel pattern formation.

Statistics

The nonparametric Mann-Whitney *U*-test for unpaired (two-tailed) observations was used. A mean of four windows per animal was used as independent data for each variable, with the exception of Le. MS, in which case a percentile of all the Le. MS values in each treatment group (5-FU or vehicle) was used, i.e. approximately 250–260 individual Le. MS values. The criterion for statistical significance was $p \leq 0.05$.

Results

Effect of continuous 5-FU infusion treatment on physiologic body weight gain

In the initial dose-finding experiment, the following doses were continuously infused for 16 consecutive days and the body weight at sacrifice as a percentage of the vehicle controls is given in parentheses: 37.5 mg/kg/w (98%), 75 mg/kg/w (91%) and 150 mg/kg/w (83%). For the highest dose, the animals were sacrificed after 11 days of treatment because their BW values were more than 15% lower than the fast-growing controls, which was the self-imposed arbitrary limit of BW retardation in the present study. In no animal did the treatment reduce the BW in absolute terms. The controls and test animals that received the two lower doses behaved normally. It was decided to use the 75 mg/kg/wk dose in the following angiogenesis experiment.

Effects of 14-day continuous 5-FU infusion treatment on angiogenesis

Chemotherapy with 5-FU did not affect the size/area of the individual mesenteric windows analyzed (data not shown). Thus, a direct comparison between the control and test groups in terms of the variables listed in Tables I and II is possible. The effects on angiogenesis of 5-FU were assessed in terms of VA, MVL, TMVL, In. IS, Le. MS 0–10 percentile and Le. MS 90–100 percentile (Table I). The BW at the end of the 14-day treatment period was 94% compared to the vehicle treated animals. The animals behaved normally and there were no signs of diarrhea or stomatitis. Statistically significant increases in VA (192% of the control value; $p \leq 0.025$) and in TMVL (226% of the control

Table I. Comparison of the effects of 14-day continuous 5-FU infusion and vehicle saline on VEGF-A-mediated angiogenesis in rats.

Variable	Treatment	
	Saline n = 14	5-FU, 75 mg/kg/w n = 12
Body weight, g*	387.4 ± 6.1	356.7 ± 6.8
VA	14.8 ± 2.4	28.4 ± 4.7 (p ≤ 0.025)
MVL	0.920 ± 0.069	1.079 ± 0.102
TMVL	13.6 ± 2.2	30.6 ± 5.0 (p ≤ 0.02)
In. IS	10.91 ± 0.83	14.83 ± 1.10 (p ≤ 0.02)
Le. MS, 0-10 percentile	n = 264	n = 252
range, µm	6 – 17	4 – 20 (p ≤ 0.0001)
Le. MS, 90-100 percentile	n = 264	n = 252
range, µm	195 – 544	208 – 693 (p ≤ 0.01)

*Body weight at sacrifice, i.e., the end of the treatment period. Values shown are mean ± SEM.

The Le. MS values for both the 0-10 and the 90-100 percentiles were significantly elongated following 5-FU treatment.

value; $p \leq 0.02$) were identified. Furthermore, the In. IS was elevated to 136% of the control value ($p \leq 0.02$). Both the shortest and longest percentile cohorts of the microvessel segments were lengthened significantly by the 5-FU treatment (for Le. MS of the 0-10 percentile, $p \leq 0.0001$; and for Le. MS of the 90-100 percentile, $p \leq 0.01$). The median Le. MS was 67.3 µm (n = 2 640) in the controls and 71.9 µm (n = 2 520) in the 5-FU-treated group.

Discussion

The present report demonstrates significant stimulation of VEGF-A-mediated angiogenesis following 14 days of low-dose continuous infusion of 5-FU in the tumor-free rat mesenteric window assay (Table I). These results are presented along with all our data (Figure 1, Table II) on the effects of 5-FU on angiogenesis, including those from a previous report in which we described the occasional occurrence of proangiogenic effects after 7 days of continuous infusion of both 5-FU and cisplatin [5]. This was, to the best of our knowledge, the first report of chemotherapeutics being able to stimulate mammalian angiogenesis *in vivo*. In the continuous infusion experiments, co-treatment with ROS scavenger N-acetylcysteine (NAC) and 5-FU infusion completely reversed the proangiogenic response produced by 5-FU alone into a statistically significant antiangiogenic effect. Monotherapy with NAC did not affect microvessel proliferation [5]. This finding supports the claim that ROS, induced in this case by the cytotoxic agent, play a crucial role in angiogenesis [15].

It is important to note that even though not all the previous individual experiments with 7-day continuous infusion of 5-FU showed statistically significant effects, the collective data points favor the stimulation of angiogenesis (*i.e.* all data points > 100% of the control animals, as expressed

in Figure 1 and Table II). In fact, the entire set of data does not reveal any indication of antiangiogenic effects by continuous 5-FU infusion in the dose range of 20–336 mg/kg/wk. In contrast, the results demonstrate that the 5-FU treatment enhances the VEGF-A-mediated angiogenesis. Similarly, for bolus administration of 5-FU [8], the accumulated data points indicate stimulation of VEGF-A-mediated angiogenesis (Table II).

More or less in agreement with our results, tumor vessel density in a murine colon cancer model was unaffected by a 2-week continuous 5-FU infusion [16]. Our results may, however, appear to conflict with those of other studies, using various angiogenesis models, in which antiangiogenic responses to 5-FU have been detected [4,17,18]. These apparent distinctions relates most probably to differences in models and experimental designs used. Divergent outcomes from different *in vivo* angiogenesis assays are not totally unexpected considering their individual features and differences in experimental design as discussed elsewhere [7,19]. Data from *in vitro* culture systems with vascular endothelial cells have further demonstrated endothelial cytotoxic effects of 5-FU. Obviously, any comparisons between *in vivo* and *in vitro* data are hampered by the large differences in experimental complexity, as it is difficult to replicate *in vitro* the *in vivo* influences of drug metabolism and the interaction between various cell types, such as platelets. It is noteworthy that platelets accumulate angiogenesis-regulatory proteins in two sets of alpha granules, one set with positive regulators and the other set with negative regulators, which may be released separately [20], hypothetically depending upon the type of therapeutic molecules used. Furthermore, the redox status of microvascular endothelial cells probably differs significantly between *in vivo* and *in vitro* compartments.

The present data, which at first glance may appear paradoxical, can probably to a large extent be

Table II.

Chemotherapy (Experiment number)	Microvessel proliferation			Pattern formation and proliferation			Microvessel pattern formation		
	VA	MVL	TMVL	No. MS	No. BP	Le. MS	MVT	In. IS	In. LF
5-FU (1)									
Bolus 10 mg/kg/wk	↑↑	↑	↑↑↑	↑↑	↑↑↑↑	0	0	0	0(+6%)
Bolus 30 mg/kg/wk	0	0	0	0	↑	↑*	0	0	0
Bolus 50 mg/kg/wk	0	0	↑	0	0	0	0	0	0
5- FU (2)									
C.I (7 days) 20 mg/kg/wk	0	0	0						
5- FU (3)									
C.I. (7 days) 168 mg/kg/wk	↑↑↑↑↑	↑↑	↑↑↑↑↑						
C.I. (7 days) 336 mg/kg/wk	↑↑	↑	↑↑↑						
5- FU (4)									
C.I. (7 days) 168 mg/kg/wk	↑↑	0	↑↑						
5- FU (5)									
C.I. (7 days) 168 mg/kg/wk	↑↑↑↑↑	↑↑	↑↑↑↑↑						
5- FU (6)									
C.I. (7 days) 168 mg/kg/wk	0	0	↑	↑↑	↑	0			
C.I. (7 days) 168 mg/kg/wk +NAC 192 mg/kg/wk	↓	↓	↓↓↓	↓	↓	↑*			
5- FU (7)									
C.I. (14 days) 75 mg/kg/wk	↑↑↑↑↑	↑	↑↑↑↑↑			↑↑**		↑↑↑	

Drug-specific effects on angiogenesis of bolus and metronomic chemotherapy with 5-FU. C.I., continuous infusion for 7 or 14 days. The fields highlighted in gray indicate statistically significant differences compared to vehicle control. The value of 0 indicates $\pm 9\%$ of the value for the vehicle control. One arrow ($\downarrow$ or $\uparrow$) indicates 10-19% difference from the control; two arrows ($\downarrow\downarrow$ or $\uparrow\uparrow$), 21-30% difference from the control; three arrows ($\downarrow\downarrow\downarrow$ or $\uparrow\uparrow\uparrow$), 31-40% difference from the control; four arrows ($\downarrow\downarrow\downarrow\downarrow$ or $\uparrow\uparrow\uparrow\uparrow$), 41-50% difference from the control; and five arrows ($\downarrow\downarrow\downarrow\downarrow\downarrow$ or $\uparrow\uparrow\uparrow\uparrow\uparrow$), >50% difference from the control. A single asterisk (*) indicates significant elongation of the 90-100 percentile cohort, and two asterisks (**) indicate significant elongation of the 0-10 and 90-100 percentiles. The data for experiment 1 are from [8] and the data for experiments 2-6 are from [5]. Experiment 7 is described in the current report.

VA, vascularized area; MVL, microvascular length; TMVL, total microvascular length; No. MS, number of microvessel segments; No. BP, number of microvessel branching points; In. IS, frequency of microvessel intersections. These parameters are described in detail in the *Material and Methods* section. The index of loop formation (In. LF), which corresponds to the frequency of interconnecting loops per unit tissue area, and the microvessel tortuosity (MVT) were assessed as described in [14] and compared to the corresponding values for the vehicle control.

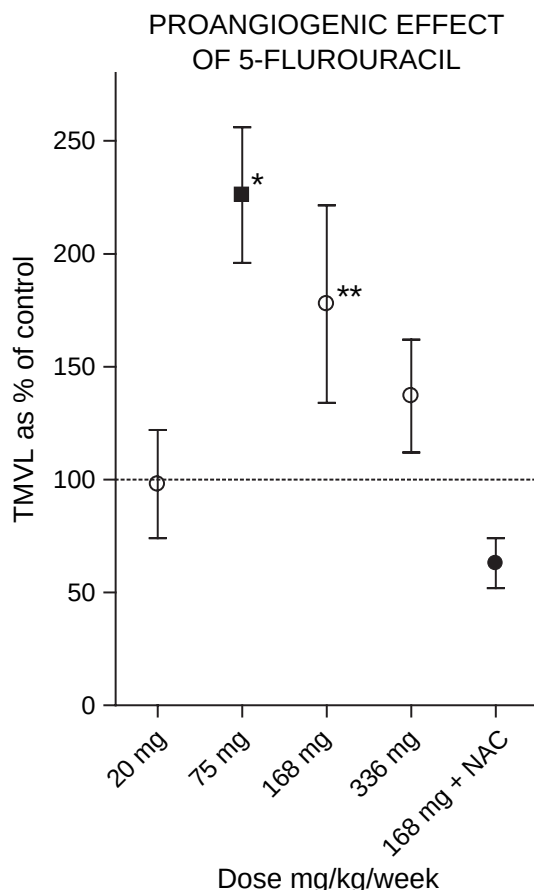


Figure 1. Graph composed of all experiments with continuous 5-FU infusion, including data from [5], in terms of total microvascular length (TMVL) presented as percent of respective experiment vehicle control $\pm$ SEM. *The 75 mg/kg/week data point (filled square) is from the here reported 14-day infusion experiment, all other are from 7 days infusion experiments (circles). **The data point for 168 mg is composed of the arithmetic mean of 4 separate experiments, for details see Table II.

explained by the fact that during 5-FU treatment the intracellular enzyme thymidine phosphorylase (TP), which is identical to platelet-derived endothelial cell growth factor, is formed. From TP, 2-deoxy-D-ribose (dRIB) is produced as a degradation product [21–24], and both TP and dRIB are angiogenic in various tumor and non-tumor tissues *in vivo* [21,25–27]. Interestingly, dRIB leads to the generation of ROS, which activates several genes and increases production of proangiogenic molecules such as VEGF-A, TP, IL-8 and matrix metalloproteinase-1 [22]. While it is highly expressed in platelets and the placenta, TP is often found to be over-expressed in a variety of neoplastic cells and may also be present in tumor stroma cells, including macrophages [23,24]. Moreover, TP is involved in the activation of 5-FU and can locally activate anticancer nucleoside compounds [1].

Thus, the role of TP in 5-FU based therapy is doubled-edged, since TP on the one hand promotes 5-FU cytotoxicity and TP-derived dRIB (as well as TP) may on the other hand stimulate tumor angiogenesis [1,28]. For these reasons, our data in the tumor-free model used must be interpreted with prudence, as discussed elsewhere [8]. However, for systemically administered metronomic paclitaxel, the current model closely reflects the indirectly assessed antiangiogenic effects seen in a syngeneic prostate cancer model [10]. It is plausible that the harmful effects of any chemotherapeutic agent on the neoplastic cell population in a tumor diminish the angiogenic reaction that is controlled directly by these cells. This implies that the antiangiogenic response to an individual cytotoxic agent is—in principle—tumor- or tumor-type-specific. This belief is supported in a renal cell carcinoma xenograft model, in which the estimated antiangiogenic effects of several chemotherapeutics, including 5-FU, were dependent upon the cell line used to induce tumor angiogenesis [29]. Although the full effect of 5-FU therapy on tumor angiogenesis cannot be captured accurately by any tumor-free assay, or any tumor model for that matter, we believe that it is important to know which metronomically administered chemotherapeutics are capable of being either antiangiogenic or proangiogenic *per se*. The model used here seems exceptionally well suited to elucidate this subject.

The connection between different free-radical scavengers and their influences on 5-FU pharmacokinetics and dynamics is particularly interesting. In preclinical studies, the antioxidant xanthine oxidase inhibitor allopurinol significantly modified the toxicity and antitumor activity of 5-FU [30], which has been confirmed in human studies, with decreased toxicity and enhanced maximum dose tolerability [31]. Furthermore, the lipid-regulating compound probucol, which is also a strong antioxidant, prevents 5-FU-induced endothelial damage in rabbits [32]. As recently demonstrated, NAC and mangafodipir (a magnetic resonance imaging contrast agent with catalase and glutathione reductase activities) protect leucocytes against the harmful effects of chemotherapeutics, including 5-FU [33]. Thus, detoxifying ROS by adding antioxidants to 5-FU-based chemotherapy regimens seems to decrease the incidence of toxic side-effects and enhance the overall antitumor effects by increasing the maximum dose tolerability. In addition, we have shown that inclusion of the antioxidant NAC in continuous infusion regimens of 5-FU can reverse the direct proangiogenic effect of 5-FU into a significant advantageous antiangiogenic effect [5].

In this context, it may be relevant to relate to the clinical discussion on how to best deliver 5-FU to patients with colorectal cancer. A meta-analysis [34] shows that 5-FU given as continuous infusion is more efficacious and less toxic than when administered as a bolus intermittently. This view is, however, not generally accepted. In fact, a recent Nordic phase III randomized comparative trial of 5-FU given as bolus or infusion in combination with irinotecan found no differences in its primary endpoint, *i.e.* time to progression [35]. Interestingly, most clinical benefit with the anti-VEGF antibody bevacizumab is achieved when given concurrently with 5-FU infusion+irinotecan as compared with the iv. bolus 5-FU+irinotecan and bevacizumab combination [36]. It must here be kept in mind that the proangiogenic effect of 5-FU, as reported here, is reversed to an antiangiogenic effect when co administration with the antioxidant (NAC) [5]. Consequently, clinical studies designed to elucidate whether antioxidants influence the outcome of chemotherapies given to cancer patients need to also include some control of the patients' redox-status, *e.g.* food composition and nutritional supplements.

We report that low-dose continuous infusion of 5-FU stimulates VEGF-A-induced angiogenesis in the adult rat mesenteric window assay. In the light of the recent development of *per oral* antitumor 5-FU pro-drugs, it is important to remember that, while not refuting the antineoplastic activities of 5-FU, it seems unlikely that its antitumor effect is mediated or enhanced by a *direct* antiangiogenic property. Clearly, frequent dosing of chemotherapeutics may be described as metronomic, although not all metronomically administered chemotherapeutics are antiangiogenic *per se*, as recently reported and discussed by us [5,9]. In contrast, the low-dose continuous infusion chemotherapy used in the current experimental setting strongly suggests drug-specific and dose-dependent effects on VEGF-A-mediated angiogenesis [5,8,9,11], with 5-FU being one of the cytotoxic agents that is prone to promote angiogenesis.

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