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Combretastatin A-4 phosphate enhances CPT-11 activity independently of the administration sequence

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Abstract

We evaluated the effect of different intervals and sequences of the vascular targeting agent combretastatin A-4 disodium phosphate (CA4DP) and CPT-11 administration on tumour growth delay and intratumoral uptake of CPT-11 using a syngeneic rhabdomyosarcoma tumour model. Irrespective of the administration sequence, the combination of CA4DP and CPT-11 significantly increases tumour growth delay in comparison with both drugs alone ($P<0.001$). Intratumoral CPT-11 concentration generally decreased (up to 5-fold) in the combination groups, while SN-38, the active metabolite of CPT-11, increased up to 9-fold. However, the increased amount of intratumoral SN-38 trapping after CA4DP injection did not correlate with the observed tumour growth delay. In conclusion, CA4DP significantly enhances the antitumour effect of CPT-11, which is not greatly influenced by the administration sequence, and which lacks a correlation with the intratumoral trapping of CPT-11 or SN-38. Mechanisms other than trapping are likely to be involved in the chemosensitising capacity of CA4DP.

Keywords: Combretastatin A-4 phosphate; CPT-11; Tumour uptake; Timing

1. Introduction

The maintenance and improvement of the vasculature of solid tumours is critical for their continued growth. Blood vessels therefore represent an interesting target for potential new anticancer therapies. In contrast to antiangiogenic therapy, which inhibits the outgrowth of new blood vessels, vascular targeting treatments selectively attack the existing tumour vasculature. They take advantage of the weaknesses of established tumour endothelial cells and their supporting structures, and induce collapse, thrombosis or haemorrhage. The tubulin binding vascular targeting agent, Combretastatin A-4 disodium phosphate (CA4DP), derived from the South African tree *Combretum caffrum*, induces vascular collapse leading to the onset of necrosis in many tumour models [1], and clinical trials are ongoing [2]. Although CA4DP has been shown to induce large areas of necrosis in tumours, some tumour cells tend to survive at the outer rim adjacent to normal tissue [3,4]. These cells contribute to tumour regrowth despite the extensive initial tumour insult. Thus, to realise the potential of these agents fully, it would appear necessary to combine them with conventional anticancer therapies.

CPT-11 (irinotecan) has become established in the treatment of colon carcinoma, and also has a significant antitumour effect on other tumours such as rhabdomyosarcoma [5,6]. CPT-11 is a topo-isomerase I inhibitor with a biological half-life in rodents of approximately 1 h [7,8]. A prodrug, it is converted by decarboxylation in the liver into the active metabolite, SN-38, which is much more cytotoxic than the parent compound. One of the main obstacles to optimum cancer treatment today remains the poor delivery of 'active' anticancer drugs like CPT-11 into the tumour. Tumoral blood vessels are necessary for the transport of oxygen and nutrients into the tumour, allowing further tumour growth. Their chaotic structure and function contributes to the inefficient intratumoral delivery of anticancer drugs, and various strategies are being explored to

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increase their delivery [9], including the concept of trapping cytostatic drugs with vascular targeting agents [10,11]. A few studies have investigated the potential chemosensitising effect of CA4DP. 5-Fluorouracil (5-FU) administered 20 min before CA4DP in a murine colon adenocarcinoma, led to a significant growth delay of 8.8 days in comparison with a placebo, while both drugs alone were ineffective. Intratumoral 5-FU concentrations were similar up to 4 h after administration, with even more rapid elimination of 5-FU after 24 h in the combination group, suggesting that trapping is ineffective at this time interval [12]. In a clonogenic cell survival assay in KHT sarcoma, effective enhancement of tumour cell killing, compared with that of cisplatin alone, was obtained when CA4DP was given between 1 and 8 h postchemotherapy, but not when it was given 1 h before cisplatin. Trapping was not investigated in this study [4]. The concept that CA4DP could trap drugs is ambiguous. On the one hand, one would expect that after vascular collapse classical anticancer drugs no longer reach the tumour, leading to impaired intratumoral availability. On the other hand, optimal timing could lead to a maintained intratumoral entry of the chemotherapeutic drug, followed by vascular collapse, resulting in the trapping of drug in the tumour. The aim of our study was to find the optimal interval and sequence of combination therapy with CA4DP, in terms of antitumour activity and effect on the intratumoral uptake of co-administered CPT-11.

2. Materials and methods

2.1. Materials

CA4DP (OXiGENE Inc., Lund, Sweden) was dissolved in 0.9% saline immediately before use. A dose of 25 mg/kg was injected intraperitoneally (i.p.) in a volume of 0.5 ml. CPT-11 was a gift from Aventis Pharma Belgium (Brussels, Belgium). A 20 mg/ml solution was available, and CPT-11 was administered at a dose of 45 mg/kg. The control rats received an i.p. injection of 0.5 ml saline only.

2.2. Tumour model and treatment

We used the highly reproducible experimental syngeneic rhabdomyosarcoma tumour model in WAG/Rij rats [13]. Tumour pieces of approximately 1 mm^3 were transplanted subcutaneously (s.c.) into the lower flank of adult rats (260–300 g). Three orthogonal diameters were measured with vernier calipers and used to calculate the volume of the tumour with the formula: $a \times b \times c \times \pi/6$, expressed in our study as cm^3. A correction for skin thickness of 1 mm was applied to all the measured diameters.

When tumour volume reached approximately 1 cm^3, the rats were randomised into different groups in the tumour growth delay studies, with eight tumours per group. CPT-11 and CA4DP were administered at different time intervals and sequences, and control groups were added (see Fig. 1). The effects of therapy were determined from the difference in the tumour volume-doubling time. The rats were sacrificed when the tumour size reached 10 cm^3.

In the second part of the study, investigating the effect of CA4DP on the intratumoral CPT-11 uptake, with seven tumours per group, several intervals between CA4DP and CPT-11 were also chosen to find the optimum interval for CPT-11 trapping. Animals were sacrificed 1 or 5 h after CPT-11 administration. All procedures were conducted in adherence with the Principles of Laboratory Animal Care and with the approval of the local animal ethics committee.

2.3. CPT-11 and SN-38 measurements in tumour tissue and plasma

One or 5 h after CPT-11 administration, the rats were sacrificed by ether inhalation. A 1-ml blood sample, obtained by intracardiac puncture, was collected in an ethylene diamine tetra acetic acid (EDTA) tube, and the tumours were resected. Each tumour was halved and snap-frozen in liquid nitrogen for the later measurement of tumour CPT-11 concentrations. The blood was centrifuged at 1500*g* for 10 min, and 100 μl supernatant plasma was removed and used to measure plasma concentrations of CPT-11 and SN-38. A validated high-performance liquid chromatographic (HPLC) method was used to determine CPT-11 and SN-38 in human plasma and tumour tissue. This has been fully described in our previous work in Ref. [14].

2.4. Statistical analysis

'Statistica 6.0' was used for statistical analysis. ANOVA was used to compare the different treatment groups, with the LSD test as a *post-hoc* comparison test. Data not distributed normally, such as the intratumoral CPT-11 and SN-38 concentrations in the CA4DP-treated animals, were compared with control animals with a Mann–Whitney U-test. The significance level was 0.05.

3. Results

3.1. Effect of the timing of CA4DP and CPT-11 combination therapy on tumour growth inhibition

CA4DP and CPT-11 combination therapy clearly demonstrated an increased tumour growth delay compared with both drugs alone that seemed more than additive (Fig. 1 and Table 1). Compared to placebo, the

tumour volume doubling time was prolonged by 2.42 and 2.53 days for CA4DP and CPT-11 monotherapy, respectively, and by 8.22 days for CA4DP + CPT-11. The timing and sequence of CA4DP and CPT-11 did not greatly influence tumour growth delay, although CA4DP + CPT-11 was slightly more efficient than CPT-11- > CA4DP ($P = 0.04$).

3.2. Effect of the timing of CA4DP and CPT-11 combination therapy on the intratumoral availability of CPT-11 and SN-38

Intratumoral uptake of CPT-11 and SN-38 was initially studied 1 h after CPT-11 administration, and a comparison was made between CPT-11 alone and different combinations of CA4DP and CPT-11 (Fig. 2a). CA4DP was administered before or together with CPT-11 in order to observe any trapping effect of CA4DP. Concomitant administration of CA4DP and CPT-11 (*CA4DP + CPT-11*) led to a clear decrease in intratumoral CPT-11, although two tumours trapped large amounts of CPT-11. In contrast, the median SN-38 intratumoral concentrations were slightly higher in this group. When CA4DP was administered before CPT-11 (*CA4DP* →30 min or 1 h *CPT-11*), median CPT-11 intratumoral uptake dramatically decreased (approximately 5-fold) in comparison with CPT-11 alone, whereas SN-38 levels remained in the same range.

The study was expanded to a 5-h interval between CPT-11 administration and CPT-11/SN-38 measurement

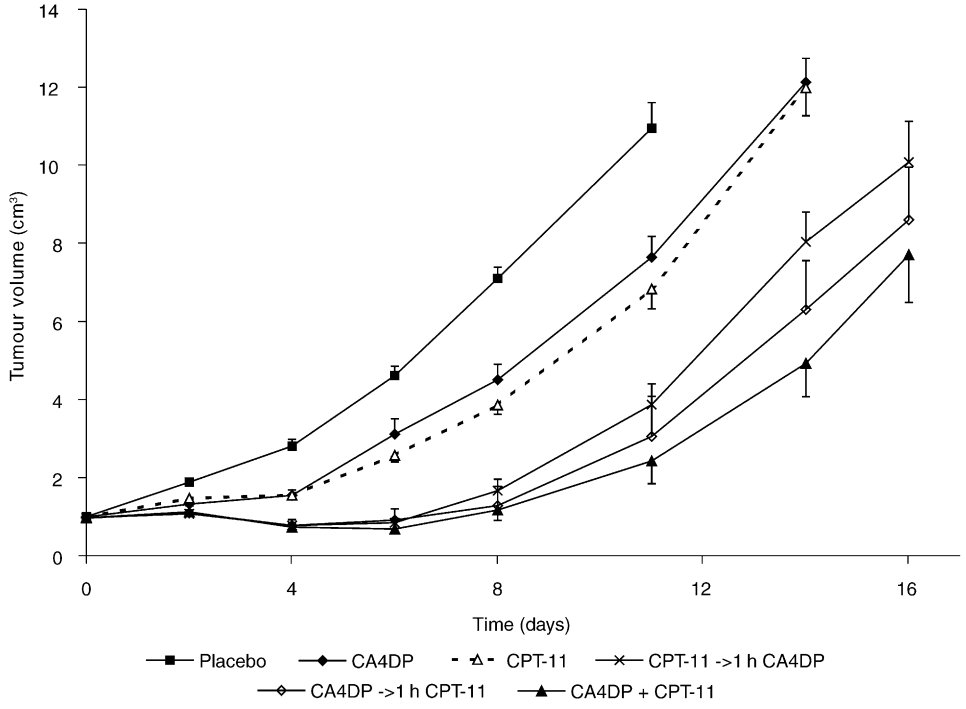

Fig. 1. Effect of combretastatin A-4 disodium phosphate (CA4DP), CPT-11, and their combination in different timing schemes on the growth of rhabdomyosarcoma tumours in rats ($n = 8$ per group). CPT-11- > 1 h CA4DP indicates that CPT-11 was administered first, followed 1 h later by CA4DP; CA4DP- > 1 h CPT-11 indicates that CA4DP was administered first, followed 1 h later by CPT-11; CA4DP + CPT-11 indicates that both drugs were administered simultaneously. Data points indicate means ± standard error of the mean (SEM).

Table 1
Tumour growth delay with CA4DP, CPT-11 and their combinations

	Tumour doubling time[a] (days)	ANOVA Fisher LSD *post-hoc* comparison[b]				
		Placebo	CA4DP	CPT-11	CPT-11- > 1 h CA4DP	CA4DP- > 1 h CPT-11
Placebo	2.38 ± 0.88					
CA4DP	4.80 ± 1.03	*0.01*				
CPT-11	4.91 ± 0.63	*0.008*	0.90			
CPT-11- > 1 h CA4DP	8.64 ± 1.68	*< 0.0001*	*< 0.0001*	*0.0002*		
CA4DP- > 1 h CPT-11	9.94 ± 2.42	*< 0.0001*	*< 0.0001*	*< 0.0001*	0.16	
CA4DP + CPT-11	10.60 ± 2.93	*< 0.0001*	*< 0.0001*	*< 0.0001*	*0.04*	0.47

[a] Indicates time (days) to reach twice the volume at treatment start (mean + standard deviation (S.D.); $n = 8$ per group).
[b] P value in italics (< 0.05) was considered significant.

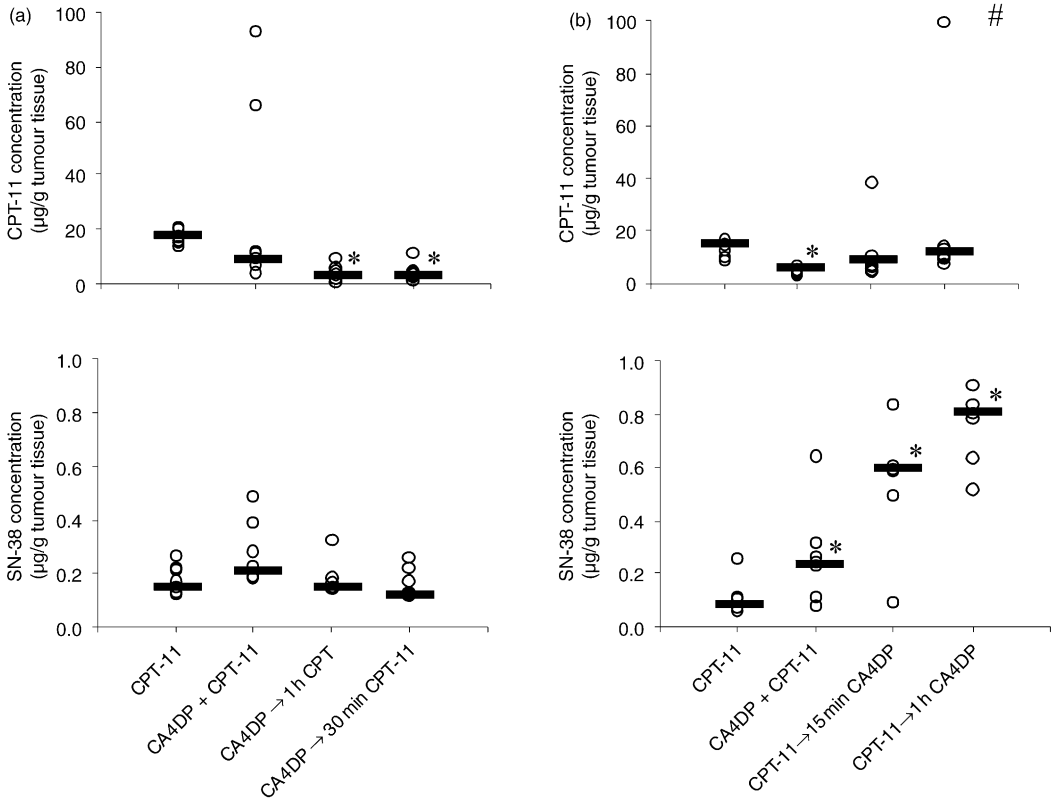

Fig. 2. Tumour CPT-11 and SN-38 concentrations measured by high-performance liquid chromatography (HPLC) (a) 1 h and (b) 5 h after CPT-11 administration ($n=7$ per group). CA4DP + CPT-11 indicates that both drugs were administered simultaneously; CA4DP→1 h CPT-11 and CA4DP→30 min CPT-11 indicate that CPT-11 was administered 1 h and 30 min after CA4DP, respectively; CPT-11→15 min CA4DP and CPT-11→1 h CA4DP indicate that CA4DP was administered 15 min and 1 h after CPT-11, respectively. Each point indicates a single measurement. ═ = median value; ∗ indicates a significant difference between the CA4DP-treated groups and control groups (CPT-11 alone) with the Mann–Whitney U-test; # the data point value was 193 µg, but was noted as 100 µg for the clarity of the figure.

for two reasons: firstly, it is interesting to have a second time point for the (*CA4DP + CPT-11*) group to see whether the observed alterations are reproduced. Secondly, the unexpected decrease of CPT-11 concentration after CA4DP administration in the 1-h interval suggested that the vessels were already damaged/closed by the time CPT-11 reached the tumour, not allowing its entrance. We hypothesised that a reversed order, namely first CPT-11 and then CA4DP, might allow intratumoral penetration of CPT-11/SN-38, and that subsequent CA4DP exposure might then damage/close the vessels and trap CPT-11/SN-38. CPT-11 before CA4DP in the 1-h group would lead to a short exposure time to CA4DP before the tumours are excised and measured, and it seemed preferable to us to study this reversed order only in the 5-h interval group (see Fig. 2b). Concomitant administration of CA4DP and CPT-11 (*CA4DP + CPT-11*) again led to a clear decrease in the intratumoral CPT-11 concentration, while the median SN-38 intratumoral concentrations significantly increased. When CPT-11 was administered before CA4DP (*CPT-11* →15 min or 1 h *CA4DP*), the median intratumoral CPT-11 concentration was somewhat lower, but again with two extreme outliers. A significant trapping of intratumoral SN-38 was observed, up to 9-fold for the (*CPT-11* →1 h *CA4DP*) group where CPT-11 circulated 1 h before the CA4DP was given.

Plasma CPT-11 and SN-38 concentrations had an approximate normal distribution, unlike those in tumour, and are therefore presented as the means + S.D. (Table 2). Since some rats had bilateral tumours, only five to six points per group were available. No significant differences in the levels of these compounds were observed between the groups combining CPT-11 and CA4DP, and the control groups with CPT-11 alone.

4. Discussion

In the present work, we tried to identify the optimal interval and sequence of combination therapy with CA4DP, with regard to antitumour efficiency and intratumoral uptake. We observed an important increase in the antitumour effect of CPT-11 with CA4DP, which unexpectedly was hardly influenced by the administration sequence. The potentiation in activity

Table 2
Plasma CPT-11 and SN-38 levels 1 and 5 h after CPT-11 administration

	Plasma CPT-11 concentration (μg/ml; means + S.D.)	Plasma SN-38 concentration (μg/ml; means + S.D.)
At 1 h		
CPT-11	9.08 ± 2.05	1.61 ± 0.33
CA4DP + CPT-11	10.52 ± 2.55	1.58 ± 0.28
CA4DP- > 1 h CPT-11	10.02 ± 2.35	1.48 ± 0.40
CA4DP- > 30 min CPT-11	10.60 ± 3.93	1.79 ± 0.51
At 5 h		
CPT-11	2.64 ± 0.55	0.77 ± 0.33
CA4DP + CPT-11	4.41 ± 1.86	0.92 ± 0.47
CPT-11- > 15 min CA4DP	3.65 ± 1.04	0.60 ± 0.26
CPT-11- > 1 h CA4DP	2.60 ± 0.41	0.47 ± 0.09

$n = 5$–6 per group. No significant differences with one-way ANOVA.

occurred with no discernible influence on toxicity. At least four potential mechanisms can be considered to play a role; (1) a trapping phenomenon, (2) complementary targets of CA4DP and CPT-11, (3) true synergism, and (4) altered pharmacokinetics.

4.1. Trapping of chemotherapy drugs

It is remarkable that tumour growth delay was less in the CPT-11 →1 h CA4DP group than the CA4DP + CPT-11 (8.6 versus 10.6 days, $P = 0.04$), whereas median intratumoral levels of the active metabolite, SN-38, were approximately 3 times higher in the former compared with the latter group (see Figs. 1 and 2b). Hence, changes in intratumoral delivery as a whole do not correlate with changes in antitumour efficacy, and trapping cannot completely explain the observed tumour growth delay results. It is possible that in our CA4DP sensitive sarcoma model, most cells affected by the CA4DP induced shut-down in blood perfusion died, while CPT-11/SN-38 killed those cells which were unaffected by CA4DP and were well oxygenated. In tumours sensitive to CA4DP, trapping may thus not be as relevant since CA4DP can perform the killing work of the sensitive cells alone, but it could be more important in less sensitive tumours, or with other vascular targeting agents such as 5,6-dimethyl-xanthenone-4-acetic acid (DMXAA). For instance, in MDAH-Mca-4 mouse mammary tumours, a 33% increase in intratumoral melphalan likely contributes to the chemosensitising effect of DMXAA [15]. DMXAA also enhances antibody-directed enzyme prodrug therapy, at least in part, by increasing retention of the antibody in tumour xenografts [10]. The therapeutic window was small, with no significant enhancement of prodrug retention when DMXAA was given at either earlier or later time points. Conversely, a clear synergistic effect was seen with DMXAA and paclitaxel in a broad range of administration intervals, with similar activity when paclitaxel was administered between 4 h before and 1 h after DMXAA. In this tumour model, synergism of paclitaxel and DMXAA was striking, whereas trapping of paclitaxel did not occur [11].

While intratumoral CPT-11 concentrations generally decrease after CA4DP and CPT-11 combination therapy, extreme 'trapping' was observed in a few tumours when CA4DP was given concurrently or after CPT-11 (see Fig. 2), suggesting that CPT-11 needs sufficient time to enter the tumour. This study cannot correlate intratumoral concentration and efficacy on an individual tumour level, i.e. it is not known whether tumours exhibiting this extreme trapping would have displayed the largest growth delay. Nevertheless, in the tumour growth delay study, no extreme outliers were observed. As previously mentioned, this trapping might not be very relevant in this CA4DP sensitive model, as the majority of cells affected by CA4DP induced vascular shut-down may have been killed regardless of a high intratumoral CPT-11 or SN-38 level. Since the trapping phenomenon was only rarely and rather randomly observed in this tumour model, it might also be unreliable in clinical practice. However it suggests that trapping of anticancer drugs with CA4DP is intrinsically possible, and we are the first to report this. CA4DP has been shown previously to sensitise tumours to chemotherapy drugs [4,12], but a trapping phenomenon has not yet been described.

4.2. Complementary targets of CA4DP and CPT-11

The lack of effect of a change in sequence is unexpected. In the CA4DP→1 h CPT-11 group particularly, one would expect the tumour vasculature to have been largely affected by the time CPT-11 is administered, hindering the penetration of CPT-11 into the tumour. CA4DP can destroy large central parts of tumours, but is generally ineffective against more mature peripheral tumoral vessels, leaving behind a rim of viable tumour tissue from which regrowth can occur. The outer tumoral rim is mainly vascularised with a more mature

and efficient vascular network, and the delivery of anticancer drugs to this part of the tumour should be less problematic than to the central, less efficiently vascularised parts. A likely explanation for the lack of a treatment schedule dependency in our sarcoma model is that most cells affected by the CA4DP induced shutdown in blood perfusion will ultimately die, while CPT-11/SN-38 kill those cells which are unaffected by CA4DP and are well oxygenated. Sarcomas are apparently very sensitive to CA4DP. In addition, in combination with radiotherapy, CA4DP significantly improves local tumour control in the KHT sarcoma model, whether it is given 1 h before, concomitantly, or one hour after radiotherapy [16]. However, in the same study, this was not true for the C3H mammacarcinoma model, where adding CA4DP to radiotherapy was only effective when it was administered concomitantly or 30 min afterwards, but not 1 h before radiotherapy. The authors hypothesised that, in the less sensitive C3H model, some parts of the tumour experience a reduction in blood perfusion after CA4DP administration, which is not long enough to cause cell death. These cells are hypoxic at the time of irradiation, and thus potentially radioresistant, but eventually reoxygenate later and survive. Given the relatively small differences between the sequence regimens, it is likely that a great part of the clear superiority of the combination therapy in our tumour model is due to the different targets of both drugs, CA4DP targeting the central badly perfused hypoxic areas, and CPT-11/SN-38 killing those cells which are unaffected by CA4DP and are well oxygenated. The same mechanism is also thought to be the main mechanism of the striking synergism between paclitaxel and DMXAA [11].

4.3. True synergism

The fact that the concomitant combination in our study was slightly superior to the CPT-11 →1 h CA4DP group ($P = 0.04$, see Fig. 1 and Table 1), and the more than additive effect on the tumour doubling time of the combination compared with both drugs alone, suggests that an intrinsic true synergistic effect might also exist. It is possible that after CA4DP treatment, surviving tumour cells are more vulnerable to CPT-11/SN-38 due to CA4DP induced microenvironmental changes (e.g. in tumour oxygenation or pH). When CPT-11 is given 1 h before CA4DP, CPT-11 and SN-38 may encounter a less favourable tumour microenvironment for effective tumour cell kill than when CA4DP has first been able to modify the microenvironment. Both acidosis and hypoxia appear to contribute to the enhancement of melphalan activity by DMXAA [15]. True synergism has also been observed in the MAC 29 colon cancer model using 5-FU and CA4DP, when 5-FU was only given 20 min before CA4DP [12]. CA4DP after chemotherapy compared with before chemotherapy was less effective in our study with CPT-11 (non-significant trend), but much more effective in the study of Siemann using cisplatin [4]. Apparently, CA4DP displays only clear synergism when cisplatin has had sufficient time to enter the tumour, while the opposite seems to be the case for CPT-11 in our tumour model. Furthermore, paclitaxel displays a striking synergy with DMXAA over a broad temporal range (DMXAA administered 4 h before to 1 h after DMXAA) that is not related to paclitaxel trapping, whereas no synergism is observed with CA4DP [11]. As with cisplatin, the combination of paclitaxel and DMXAA is much less effective when paclitaxel is administered 4 h after DMXAA than within 4 h before and 1 h afterwards, indicating that paclitaxel also needs to be able to penetrate the tumour in sufficient concentrations to display clear synergism. Unlike cisplatin and paclitaxel, our data suggest giving CA4DP concomitantly rather than after CPT-11 in future studies. There is probably not a uniform mechanism for synergism between antivascular targeting agents and chemotherapy drugs. Apart from alterations in the tumour microenvironment, other unknown mechanisms might be involved. The mechanisms of true synergism remain poorly understood, and are a very interesting field for future research.

Another intriguing interaction between CA4DP and CPT-11 is the clear discrepancy between intratumoral CPT-11 and SN-38 uptake. It has been demonstrated that CPT-11 can be metabolised to SN-38 in the liver, but also in the tumour tissue itself [17]. Although CA4DP does not seem to influence the hepatic conversion of CPT-11 to SN-38 (see Section 4.4 below), it is possible that it influences the intratumoral metabolisation of CPT-11 and SN-38. This could be a direct effect, but many indirectly related mechanisms could be involved, such as changes in oxygenation or intratumoral pH. The fact that intratumoral SN-38 concentrations increase from approximately 0.2 μg/g after 1 h up to 0.8 μg/g tissue after 5 h, and the increased absolute intratumoral SN-38 differences after 5 h compared with 1 h (see Fig. 2), are consistent with the hypothesis of intratumoral SN-38 generation due to CA4DP exposition. Decreased intratumoral SN-38 degradation/elimination might also play a role, but does not explain the marked absolute increase after 5 h. It is interesting to note that 5-FU metabolism is also seemingly increased by CA4DP or changes it induces [12]. CA4DP, or the microenvironmental changes it induces, might thus influence the intratumoral metabolism and activity of anticancer drugs.

4.4. Altered pharmacokinetics

The increased activity of the combination was probably not a result of altered plasma pharmacokinetics of

CPT-11. A complete pharmacokinetic analysis was not performed, but plasma concentrations of CPT-11 1 and 5 h after administration were not significantly altered by CA4DP, indicating that no major pharmacokinetic interactions exist. An analysis of plasma concentrations is important, since certain vasoactive agents (e.g. hydralazine) used to manipulate tumour vasculature and potentiate the antitumour properties of drugs, can cause major systemic changes in the distribution and elimination of co-administered anticancer agents. In these cases, the resulting increase in antitumour activity is probably due, at least partly, to these pharmacokinetic alterations, rather than any specific improvement in antitumour activity *per se* [18,19].

In conclusion, CA4DP significantly enhanced the response to CPT-11. This effect was independent of the sequence of administration, and also of the intratumoral trapping of CPT-11 and SN-38. This suggests that both drugs have considerable activity against separate targets; CA4DP against the hypoxic central tumour cells that become anoxic and die, and CPT-11 against the tumour components with more mature vessels, which are not responsive to CA4DP, but enable adequate CPT-11 delivery and activity. However, the data suggest that a small true synergistic effect may be present. Moreover, there may be an effect on the intratumoral metabolism of CPT-11 to the active metabolite SN-38. Generally, the trapping of CPT-11 with CA4DP induced vascular shut-down was low, but occurred to a high degree in some tumours, suggesting that the theoretical concept of trapping is possible, but rather unreliable *in vivo*. The potential effects of CA4DP on the efficacy, intratumoral uptake and metabolism of CPT-11 are likely to be complex, and warrant further studies. The combination of CA4DP with classical anticancer drugs remains appealing.

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