Development and assessment of novel all-in-one parenteral formulations with integrated anticoagulant properties for the concomitant delivery of 5-fluorouracil and calcium folinate

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5-Fluorouracil in combination with its biomodulator folinic acid maintains a pivotal position in current anticancer treatment regimens. However, limitations in clinical management persist with the administration of these drugs. These limitations are associated with the use of a high pH to maintain 5-fluorouracil in solution, resulting in high rates of phlebitis and catheter blockages. Herein, we describe and compare initial studies on novel all-in-one formulations of 5-fluorouracil and folinic acid incorporating either sulfated or hydroxypropyl β-cyclodextrins at physiological pH that potentially address these issues. All formulations markedly improved the stability of supersaturated solutions of 5-fluorouracil in the presence of folinic acid. In-vitro evaluation of the PC-3, HCT-116, MDA-MB-231, PC-14, and COLO-201 human carcinoma cell lines showed that all formulations exhibited equivalent or better cytotoxicity compared with cells exposed to 5-fluorouracil and folinic acid. Thus, these cyclodextrins do not compromise the cytotoxicity of 5-fluorouracil. Preliminary in-vivo dose tolerance profiles of the formulations were also equivalent to 5-fluorouracil and folinic acid administered separately. Furthermore, given the association between thrombosis and cancer, the potentially beneficial anticoagulant activity of the sulfated cyclodextrin-based formulations was also confirmed *in vitro*. Extended activated partial thromboplastin times and prothrombin times were observed for the sulfated cyclodextrins in human plasma both as individual compounds and as components of the formulations. In conclusion, these novel all-in-one formulations maintain the in-vitro potency while overcoming the accepted incompatibility of 5-fluorouracil and folinic acid, and represent improved injectable forms of 5-fluorouracil that may reduce phlebitis, catheter blockages, and thromboembolic events. *Anti-Cancer Drugs* 20:822–831 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

5-Fluorouracil (5FU) was invented in the mid 1950s and is the earliest example of a rationally designed, targeted anticancer drug [1]. Increased understanding of the pharmacology of 5FU, particularly over the last 20 years has led to it now being a cornerstone of cancer therapies and an essential component of current combination treatment regimens, such as FOLFOX and FOLFIRI [2]. It has now been established that one of the key mechanisms for the cytotoxic action of 5FU is the prevention of DNA synthesis by the inhibition of thymidylate synthase, the enzyme that provides the only de-novo source of thymine [3]. The antitumor activity of 5FU is enhanced when it is used in combination with its biomodulator folinic acid (leucovorin or calcium folinate or FA) [4–6]. The administration of FA increases the intracellular level of the reduced folate 5,10-methylene tetrahydrofolate, which modulates the effect of 5FU by specifically enhancing its capacity to inhibit thymidylate synthase. Clinically, this results in a two-fold improvement in response rates and a small but significant survival outcome [4,5].

Clinically, the use of 5FU presents side effects, which are typically associated with cytotoxic chemotherapeutics. A number of limitations linked to the 5FU formulation are also observed during the administration of 5FU in the clinic, both as a single preparation and when given in combination with calcium folinate [3]. Various methods are routinely used to increase and/or maintain the solubility of pharmaceuticals. For injectable formulations, these techniques include the use of cosolvents, surfactants and complexing agents, as well as salt formation and pH adjustment [7]. In current clinically used

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injectable solutions, the solubility of 5FU is increased through the use of either buffered or strong alkaline (approximately pH 9) solutions using, for example, NaOH to generate the more soluble sodium salt. As a result, intravenous delivery of 5FU typically produces severe and painful vein damage (phlebitis) in the patient. The occurrence of phlebitis in a patient can result in the cessation of 5FU administration through peripheral veins and consequently the placement of a central line is often required [8].

Owing to the widely accepted incompatibility of 5FU and FA solutions, which results in precipitation of 5FU and/or calcium carbonate ($CaCO_3$) [9–12], the clinical administration of 5FU and FA is performed in two separate injections. Normally, this involves injection of FA followed by administration of 5FU by either intravenous bolus and/or infusion methods [13]. The incompatibility issues of 5FU + FA dosing are not fully resolved by the use of sequential administration through central ports, as blockages are observed in these lines after repeated treatment cycles. In addition, the sequential administration of FA followed by infusional 5FU may result in the actual benefit of the coadministration of these components not being realized because of the short plasma half-life of FA [14].

Reformulation of 5FU for concomitant administration with FA at a physiological pH may overcome many of the current accepted clinical limitations of 5FU therapy. An alternative to the use of extreme pH for improving solubility is the use of cyclodextrins (CDs) [15,16]. Inclusion complexing of 5FU with β-CD has been reported earlier [17], while CD inclusion compounds of FA and salts have also been described [18]. Chemical modification of the parent CDs, particularly β-CD, has generated derivatives with decreased in-vivo activity and consequently more favorable toxicity profiles [19,20]. CDs with improved pharmaceutical excipient properties for parenteral formulations include the sulfoalkylether-β-CD and hydroxyalkyl-β-CD derivatives, which are currently used in several marketed injectables [15,16,20]. The use of aqueous CD solutions for the delivery of an injectable formulation is attractive, because generally the intrinsic pharmacokinetics of drugs is unaffected and the drug is usually both rapidly and quantitatively released from the CD–drug complex upon injection [21].

In this instance, significant benefits may be gained through the use of polysulfated CDs (7–14 sulfate groups; sodium salts) rather than alternative hydrophilic derivatives. Polysulfated CDs have shown heparin-like anticoagulant activity [22,23]. Although this property has previously caused complications during in-vivo administration [24] in the case of chemotherapy formulations, the anticoagulant activity of the sulfated CDs may be beneficially exploited to at least partially diminish thrombus formation, a known complication of cancer [25]. Cancer patients have an increased risk of thrombosis as a result of the disease process (especially metastasis), postoperative complications, nonsurgical treatments including radiotherapy or chemotherapy, and through the use of central venous catheters. Venous thromboembolic events are the second highest cause of death in cancer patients [25]. In attempts to reduce this impact, prophylactic treatment with heparin is often given before surgery or during chemotherapy [26]. Consequently, a β-CD derivative that mimics the anticoagulant behavior of heparin may constitute a useful ingredient in an anticancer formulation, although the strength of the CD anticoagulant activity would require characterization.

Although the development of new drug entities for the treatment of cancer are required, often small changes in the method, timing, and combination of existing therapies can make significant improvements in response rates and survival outcomes. 5FU is a prime candidate for reformulation because (i) it has been in clinical use for decades but still has intrinsic clinical limitations and (ii) 5FU is so frequently integrated into combination therapies for a broad range of cancers and therefore any improvement can have widespread benefits. Our primary objective was to combine 5FU and FA into CD-enabled formulations with a physiological pH without reducing, but potentially enhancing, the effectiveness. These 5FU formulations would provide improved patient comfort and tolerability by simplifying administration of 5FU and FA. The flow on benefits of reduced treatment time and associated hospital costs are similar to those sought by the development of oral 5FU prodrugs [27], without the bioavailability, patient compliance, and over compliance issues. To this end, we have developed three novel formulations that combine 5FU and FA, which address the incompatibility of these components and allow their concomitant delivery. Here, we present an assessment of these formulations developed with polysulfated and hydroxypropyl β-CD derivatives in terms of their stability, toxicity, and anticoagulant properties in comparison with 5FU + FA.

Materials and methods

Materials

Pharmaceutical grade 5FU (5-Fluorouracil Injection BP, 50 mg/ml) and FA (Leucovorin Calcium Injection USP, 10 mg/ml) were from Mayne Pharma Pty Ltd., Mulgrave, Victoria, Australia. Unfractionated (UF) heparin (Heparin Sodium Injection USP, 1000 IU/ml) was purchased from Pfizer Pty Ltd., West Ryde, New South Wales, Australia. Solid 5FU (99%), heptakis(6-*O*-sulfo)-β-cyclodextrin heptasodium salt [CD(7S), $\geq$ 98% purity], sulfated

β-cyclodextrin sodium salt [CD(S), approximately 10 mol sulfate groups per mol of β-cyclodextrin], and (2-hydroxypropyl)-β-cyclodextrin [CD(HP), average molecular weight approximately 1540 g/mol], were purchased from Sigma-Aldrich (West Ryde, New South Wales, Australia). All CDs were dried at 30°C under high vacuum and stored under anhydrous conditions. Investigation of the mass distribution of CD(S) using electrospray mass spectrometry showed that this product was mainly composed of a mixture of β-CDs bearing 9–12 sulfate groups (data not shown). Analytical grade standard HCl solution (1.000 mol/l) was obtained from APS (Seven Hills, New South Wales, Australia).

Phase-solubility analysis

Phase-solubility assays were conducted according to the method of Higuchi and Connors [28]. These studies were not carried out using CD(7S) because the cost was prohibitive. Aqueous solutions of CD(S) or CD(HP) were adjusted to pH 7.0 and solutions of increasing concentration (0–45%) were added to excess amounts of finely ground solid 5FU. The samples were agitated for 5 days and then allowed to settle for 2 days at 17 ± 2°C. The supernatants were decanted and filtered through 0.22 μm syringe driven filter units (Millipore, North Ryde, New South Wales, Australia). The equilibrium pH of each solution was measured (pH cube, TPS, Springwood, Queensland, Australia). The filtrates were appropriately diluted using 0.1 mol/l HCl and the absorbance at 260 nm was measured in 10 mm quartz cells using a Cary 500 UV–Vis spectrophotometer (Varian Inc., Mulgrave, Australia). The presence of either CD(S) or CD(HP) did not interfere with the spectrophotometric assay of 5FU. The 5FU concentration values shown are the mean ± SEM of two independent concentration determinations from duplicate samples. All statistical analysis was carried out using the GraphPad Prism 5.0 software package [29]. The apparent stability constant (K_c) of the 5FU : CD complex was calculated from the slope of the linear portion of the phase-solubility curves and the intrinsic solubility (S_0) of 5FU in water according to the method of Higuchi and Connors [28]; assuming a 1 : 1 ratio of complex formation:

$$K_c = \text{slope}/S_0(1 - \text{slope})$$

the complexation efficiency (CE) was also calculated from the slope of the linear portion of the phase-solubility curves using the method of Loftsson *et al.* [30]:

$$\text{CE} = \text{slope}/(1 - \text{slope})$$

Preparation of the formulations

All multicomponent 5FU-based formulations (termed FD) have final concentrations of 15 mg/ml 5FU and 1 mg/ml FA. FD(HP) was made using the CD derivative CD(HP) (100 mg/ml final concentration in formulation); FD(7S) was prepared using heptasulfated derivative CD(7S) (50 mg/ml); and FD(S) was prepared using polysulfated CD(S) (45 mg/ml). The concentrations of the CDs in the formulations could be varied; however, the above-mentioned concentrations of CD derivates were the minimum amounts required for the production of a stable formulation.

To produce 1 ml of each FD, using pharmaceutical grade 5FU, the required mass of β-CD derivative was dissolved in water (approximately 525 μl) and then 5FU solution (300 μl × 50 mg/ml) was added. The volume of water used was varied to account for the volume occupied by the CD. The pH was adjusted to 7.4 ± 0.1 units using a standard HCl solution (approximately 75 μl × 1.000 mol/l). 5FU that precipitated on addition of the HCl was subsequently dissolved by sonication of the solution at 40°C. FA solution (100 μl × 10 mg/ml) was added and the mixture agitated until a homogeneous solution formed. Using a similar method, FD(S) could also be prepared using solid 5FU. Briefly, to produce 1 ml of FD(S), finely ground solid 5FU (15 mg), CD(S) (45 mg), and water (880 μl) were warmed to 60°C until the 5FU had dissolved. The pH was adjusted to 7.4 ± 0.1 using NaOH solution (20 μl × 0.902 mol/l) and then FA solution (100 μl × 10 mg/ml) was added and the mixture was agitated to produce a homogeneous formulation. All formulations were filtered through 0.45 μm membranes (Millipore, Australia) and stored at room temperature, protected from ambient light. The concentration of each formulation is expressed as the concentration of 5FU in the formulation unless otherwise stated. For the stability assessment, the formulations were monitored for changes in physical appearance (change of color and presence of a precipitate), UV–Vis spectra (Cary-500 Scan UV–Vis–NIR spectrophotometer; Varian Inc.), and pH for a minimum period of 3 months.

Evaluation of anticoagulant properties

There are two main screening tests of the clotting system: the activated partial thromboplastin time (aPTT) and the prothrombin time (PT). The endpoint in both of these tests is the formation of a clot from citrated plasma [31]. The relative anticoagulant properties of CD(S), CD(7S), CD(HP), FD(S), FD(7S), and FD(HP) were assessed by measuring PT and aPTT in human plasma. Human blood (2.7 ml) was drawn into sterile citrated blood collection tubes (BD Vacutainer, North Ryde, New South Wales, Australia) and 100 μl of the appropriately diluted solution of the CD, FD, or UF heparin was added to give a final volume of 2.8 ml. All human blood collection was approved by the University of Wollongong, Human Ethics Committee. Samples

were kept on ice until analysis. The time to clot formation in the citrated plasma samples was measured on a Sysmex CA-1500 System Coagulometer (Dade Behring, Lane Cove, New South Wales, Australia) at Southern Pathology IML (Wollongong, Australia). The PT test was carried out using Thromborel S reagent (human thromboplastin plus calcium; Dade Behring). The aPTT assay used Platelin L Reagent and Platelin L $CaCl_2$ from bioMérieux (Baulkham Hills, New South Wales, Australia). Samples were exposed to the coagulometer detector for a maximum time of 180 s. The normal reference ranges quoted for the aPTT and PT assays for human plasma were determined from the mean values from 20 healthy individuals/volunteers; upper and lower limits of the reference range are ± 2 standard deviations from the mean. Equivalent heparin activities of the CDs (IU/ml) were interpolated from a standard curve with the equation $y = 28.179e^{5.1244x}$, generated using GraphPad Prism5.0.

Cell lines

The human colorectal (HCT-116 and COLO-201), breast (MDA-MB-231), lung (PC-14), and prostate (PC-3) carcinoma cell lines, were routinely cultured in RPMI 1640 (Invitrogen, Mount Waverley, Victoria, Australia) medium supplemented with 5% fetal calf serum (Thermo Electron, Melbourne, Australia) and maintained at 37°C, 5% CO_2. Cells were subcultured using 0.25% Trypsin/5 mmol/l EDTA (Sigma-Aldrich) as required to maintain subconfluence and viability, as described earlier [32].

Cell proliferation assay

The cytotoxicity of five different 5FU treatments [5FU, 5FU + FA, FD(S), FD(HP), and FD(7S)] as well as individual and combinations of individual components was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS; Promega, Sydney, New South Wales, Australia), as described earlier [33]. Cells were seeded into 96-well plates (10^4 cells/well) and grown for 24 h before treatment. Cells were incubated with serial dilutions of each treatment in a final volume of 100 µl for 72 h, in quadruplicate. In the absence of cells, no treatment or assayed component interacted with the MTS substrate. The absorbance at 490 nm was measured using a Spectromax 250 UV plate reader using Softmax Pro software (Molecular Devices, Sunnyvale, California, USA). Data were analyzed using a logarithmic sigmoidal dose–response curve using the variable slope parameter to determine IC_{50} (GraphPad Prism 5.0). The IC_{50} is defined as the concentration of each treatment causing 50% of maximal growth inhibition. Unless otherwise stated, an incubation period of 72 h was selected for these assays, as this was adequate to cause irreversible damage to the cells while maintaining experiment reliability and repeatability. All statistical analyses were accomplished using GraphPad Prism 5.0 [29].

Dose tolerance comparison of all-in-one formulations

Preliminary toxicity effects of administration of a multiple (fractionated) dose of the formulations (40 mg/kg five times, within 14 days) in mice were compared with 5FU + FA treatment and saline controls. This dosage schedule is representative of an established clinical protocol [34], taking into account shortened murine life expectancy. Female Balb/c mice were obtained from the Animal Resources Centre (Canning Vale, Western Australia, Australia). 5FU + FA treatments were administered by two separate sequential intraperitoneal injections on alternate sides of the midline, with FA being administered immediately before 5FU. Dose-limiting toxicity endpoints were defined as sustained (≥ 24 h) 15% loss of body weight (compared with the first day of treatment), or distressed behavior (i.e. loss of appetite, activity, and/or hunched posture). All experimentation was approved by the University of Wollongong, Animal Ethics Committee.

Results and discussion

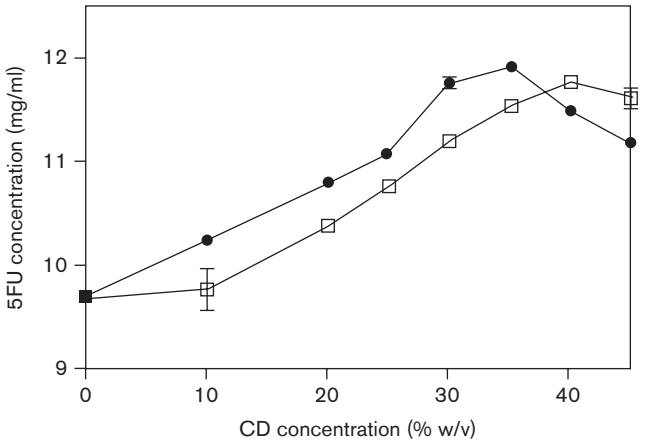
Formulation development

FD formulations were produced with varying ratios of 5FU : FA (2 : 1, 3 : 1, and 15 : 1) that corresponded to high-dose and low-dose FA as used in clinical settings [34]. Preliminary cytotoxicity assessment of these formulations showed that there was no advantage *in vitro* of using the higher ratios of FA (data not shown), which is supported by clinical findings [35]. Thus, 5FU : FA at 15 : 1 (w/w) ratios were routinely used in the FD formulations development and assessment studies. As a consequence, the primary challenge in the development of this formulation was increasing the solubility of 5FU at physiological pH.

As 5FU is the major component of the formulations, the effect of CD(HP) and CD(S) on the aqueous solubility of 5FU was evaluated using the method of Higuchi and Connors [28]. Phase-solubility profiles do not confirm the formation of inclusion complexes, but rather describe how the increasing CD concentration influences drug solubility [30], and as such may be used to determine the optimum concentration of CD to use in the formulation.

For injectable formulations, the quantity of CD used should be minimized to produce the best possible balance between the increase in drug solubility and formulation isotonicity. Figure 1 shows the phase-solubility profiles for 5FU in the presence of both the CD(HP) and CD(S) at 17°C. In the presence of both of these CDs, 5FU solubility initially increased as a linear function of CD concentration. However, negative deviations from ideal behavior were observed at higher CD concentrations ($> 35\%$), and as a result 5FU displays a nonlinear A_N-type phase-solubility profile [28] in the

Fig. 1



The phase-solubility profiles for 5-fluorouracil (5FU) in the presence of increasing concentrations of aqueous CD(S) (□) and CD(HP) (●) at 17 ± 2°C and neutral pH. The values shown are the mean ± SEM for duplicate 5FU concentration determinations from two independent experiments ($n = 4$).

Table 1 Parameters describing the solubility of 5FU in CD(S) and CD(HP) solutions at 17 ± 2°C and neutral pH

β-CD	Slope[a]	S_0 (mmol/l)	K_c (l/mol)[b]	CE[c]
CD(HP)	0.076 ± 0.003	75 ± 0.1	1.1	0.08
CD(S)	0.091 ± 0.005	75 ± 0.1	1.3	0.10

CD, cyclodextrin; CD(S), sulfated β-cyclodextrin sodium salt; CD(HP), (2-hydroxypropyl)-β-cyclodextrin; 5FU, 5-fluorouracil; S_0, intrinsic solubility.
[a]The slope determined for the linear portion of the phase-solubility curve.
[b]Apparent stability constant (K_c) for a 1 : 1 5FU : CD complex.
[c]Complexation efficiency (CE); calculated from the slope of the linear portion of the phase solubility curve.

presence of both CDs. In the case of the CD(HP), the decrease in solubility of 5FU may be a result of self-association of the CD at high concentrations, which would affect the apparent degree of complexation. At concentrations greater than 30%, the viscosity of the aqueous CD(HP) solution is observably increased; bulk properties of the solution may impact the solubility. For the CD(S), increasing the concentration results in higher ionic strength and this may be having a negative effect on the apparent solubility of 5FU. The reported intrinsic solubility (S_0) of 5FU is quite good at 11 mg/ml (22°C) [36]. At 17°C, we found S_0 for 5FU in water to be 9.7 mg/ml (75 ± 0.1 mmol/l). Marginally, better solubilization of 5FU was achieved with CD(HP) than the CD(S); the maximum solubility of 5FU observed was found to be 11.9 and 11.8 mg/ml in the presence of 35% (w/v) of CD(HP) and 40% (w/v) of CD(S), respectively. Despite these differences, such values suggest that the CDs alone do not significantly improve the solubility of 5FU. It was also possible to calculate the apparent stability constant K_c [28] and CE [37] from the linear portion of the phase-solubility curves (Table 1). The low K_c and CE values observed suggested that complexation between 5FU and CD is limited and, in our case, the improved solubility of 5FU is probably because of a combination of pH adjustment together with the action of the CD. Improved solubility of drugs by using a combination of pH adjustment and CDs is well documented [38]. As the pKa values for the two acidic groups of 5FU are 8.00 and 13.0 [39], at pH 7.4 a proportion of the 5FU will be ionized, which contributes to the enhanced solubility observed for the FD formulations.

As observed earlier for other drugs, the CE of 5FU by CDs is low, and thus relatively large amounts of CD were required to produce the small improvements in solubility observed in the FD formulations [40]. Nevertheless, at higher concentrations it has been shown that CDs form aggregates, which are able to act as solubilizers in a micellar-like manner [30]. Furthermore, hydrophilic CDs have been found to be able to solubilize compounds through stabilization of supersaturated drug solutions presumably by inhibition of nucleation and arresting crystal growth [41]. Given the low complexation values [17], our data indicate that aggregation and stabilization effects are most likely the main avenues by which the CDs solubilize the 5FU in the FD formulations.

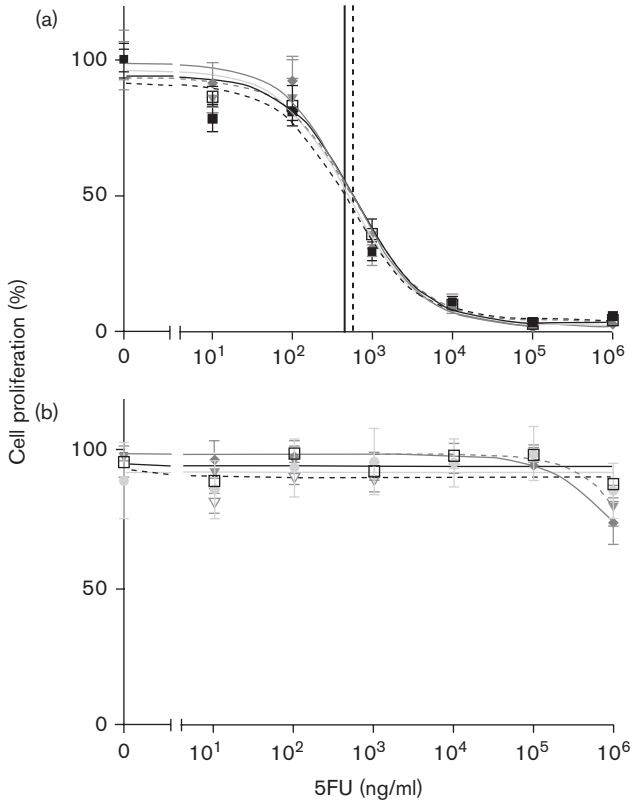
Formulation stability

The stability of the FD formulations was monitored over 12 months and compared with solutions with equivalent concentrations of 5FU + FA, but no CD, as controls. During this time, no change was observed in the physical appearance, pH, or UV–Vis absorbance spectrum (200–400 nm) of the FD formulations. However, the 5FU + FA mixtures were not stable at pH 7.4 (at room temperature), as a 5FU precipitate was noted within 24 h from the time of manufacture. In addition, no physical change or difference in the UV–Vis spectra or pH was observed upon examination of FD(S) [containing 45 mg/ml CD(S)] or FD(HP) after 12 months of storage at room temperature while protected from light (data not shown).

In-vitro toxicity assessment

To ascertain that the presence of the CDs did not compromise the cytotoxicity of the 5FU + FA combination in the multicomponent formulations, in-vitro assays were performed. Earlier cytotoxicity studies used 5FU exposure lengths ranging from 2 h [42] to 22 days [43] with the majority of studies using 48–72 h exposure periods [44,45]. In this study, a 72-h exposure period was sufficient to produce irreversible cell death while not requiring intervention for supply of fresh media, and was therefore used in these studies. Treatment of five different carcinoma cell lines representing 5FU responsive cancers, with 5FU, 5FU + FA, or the FD formulations, resulted in a dose-dependent inhibition of cell proliferation after 72 h of exposure (Fig. 2a). The IC_{50} values for these cell

Fig. 2



Dose–response curves for HCT-116 cells treated for 72 h with increasing concentrations of various 5-fluorouracil (5FU)-containing formulations (a) or with various non-5FU components of several formulations (b). Cell proliferation data were normalized to controls (100% proliferation). The *x*-axis is representative of 5FU concentrations in equivalent FD formulations to simplify comparisons between components. For curve (a) □ 5FU, ■ 5FU + FA, ◆ FD(S), ▼ FD(7S), ▽ FD(HP). For graph (b) □ NaOH (pH 9) diluted as for 50 mg/ml 5FU stock, ■ FA, ◆ CD(S), ▼ CD(7S), ▽ CD(HP). Lines represent the IC_{50} for 5FU (solid) and 5FU + FA (dashed) calculated using GraphPad Prism 5.0. Values shown are mean ± SD ($n = 4$). FA, folinic acid.

Table 2 In-vitro cytotoxicity[a] results for various 5FU-based formulations across five different human carcinoma cell lines

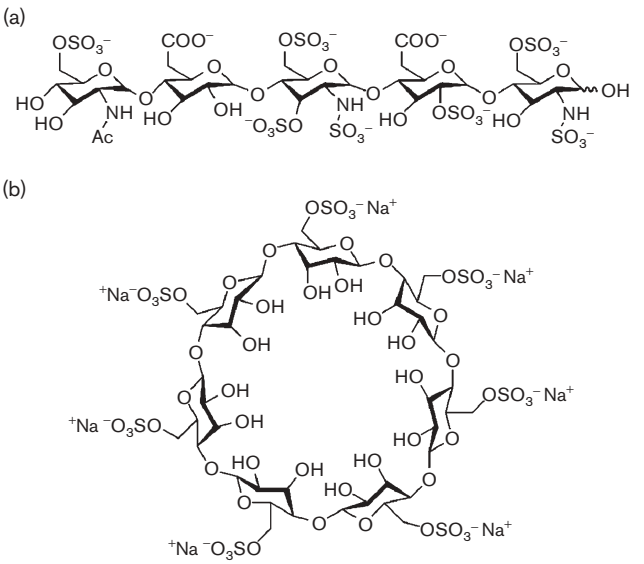
	IC_{50}^{b} (μmol/l)				
Cell line	5FU	5FU + FA	FD(S)	FD(7S)	FD(HP)
PC-3	1.4 ± 0.8	0.6 ± 0.4	0.8 ± 0.4	1.1 ± 0.8	0.6 ± 0.1
HCT-116	4.0 ± 1.3	2.5 ± 1.3	3.4 ± 1.5	2.9 ± 1.1	2.0 ± 1.3
MDA-MB-231	9.1 ± 1.4	9.1 ± 4.8	7.8 ± 2.3	9.6 ± 3.3	5.2 ± 1.5
PC-14	33.4 ± 12.2	16.2 ± 12.5	17.3 ± 7.2	20.2 ± 7.4	20.3 ± 1.5
COLO-201	39.3 ± 13.9	23.5 ± 11.1	20.8 ± 6.4	26.4 ± 6.3	26.8 ± 12.1

FA, folinic acid; 5FU, 5-fluorouracil.
[a]Cell proliferation was determined using the MTS assay.
[b]The concentration of 5FU in each treatment causing 50% of maximum growth inhibition (IC_{50}) after 72 h exposure; calculated from dose–response curves using GraphPad Prism 5.0. Values shown are mean IC_{50} ± SD from at least three independent experiments across all cell lines and treatments.

lines are presented in Table 2. All formulations showed in-vitro cytotoxicity equivalent to or better than 5FU + FA ($P > 0.05$ by Bonferroni's post-hoc tests comparing all treatments to 5FU + FA) across all cell lines, and the β-CDs and FA were not cytotoxic at levels corresponding to those present in the formulation (Fig. 2b). Thus, the inclusion of β-CDs as excipients in FD formulations does not compromise the cytotoxicity of 5FU. Overall, sensitivity to 5FU was significantly different between cell lines (two-way analysis of variance on IC_{50} values, $P < 0.0001$) with PC-3 cells being the most sensitive, followed by HCT-116, MDA-MB-231, PC-14, and COLO-201 cells (Table 2). Furthermore, although 5FU was significantly less effective than 5FU + FA or FD ($P = 0.0084$) in the COLO-201 and PC-14 cells, this was not evident for the other cell lines. Whether FA biomodulation is observed *in vitro* seems to depend heavily on the cell line, dose, and schedule used, and folate concentration in the cell culture media, with a large variation of synergy or lack thereof reported between these compounds [6,45,46].

The cytotoxicity effects of the FD formulations on the cell cycle were further determined by flow cytometry as described earlier [47]. 5FU is known to affect cell division at specific cell cycle checkpoints and can have multiple effects if given in different treatment schedules [48]. Across three cell lines tested (MDA-MB-231, HCT-116, and PC-3) similar G_1/S phase arrest was observed with 5FU + FA, FD(S), FD(7S), and FD(HP) in concentrations at or above the IC_{50} concentration of 5FU after 24 h (data not shown). This was observed as a reduction in G_2/M populations. Therefore, the response of the cells to the presence of 5FU, as either a single

Fig. 3



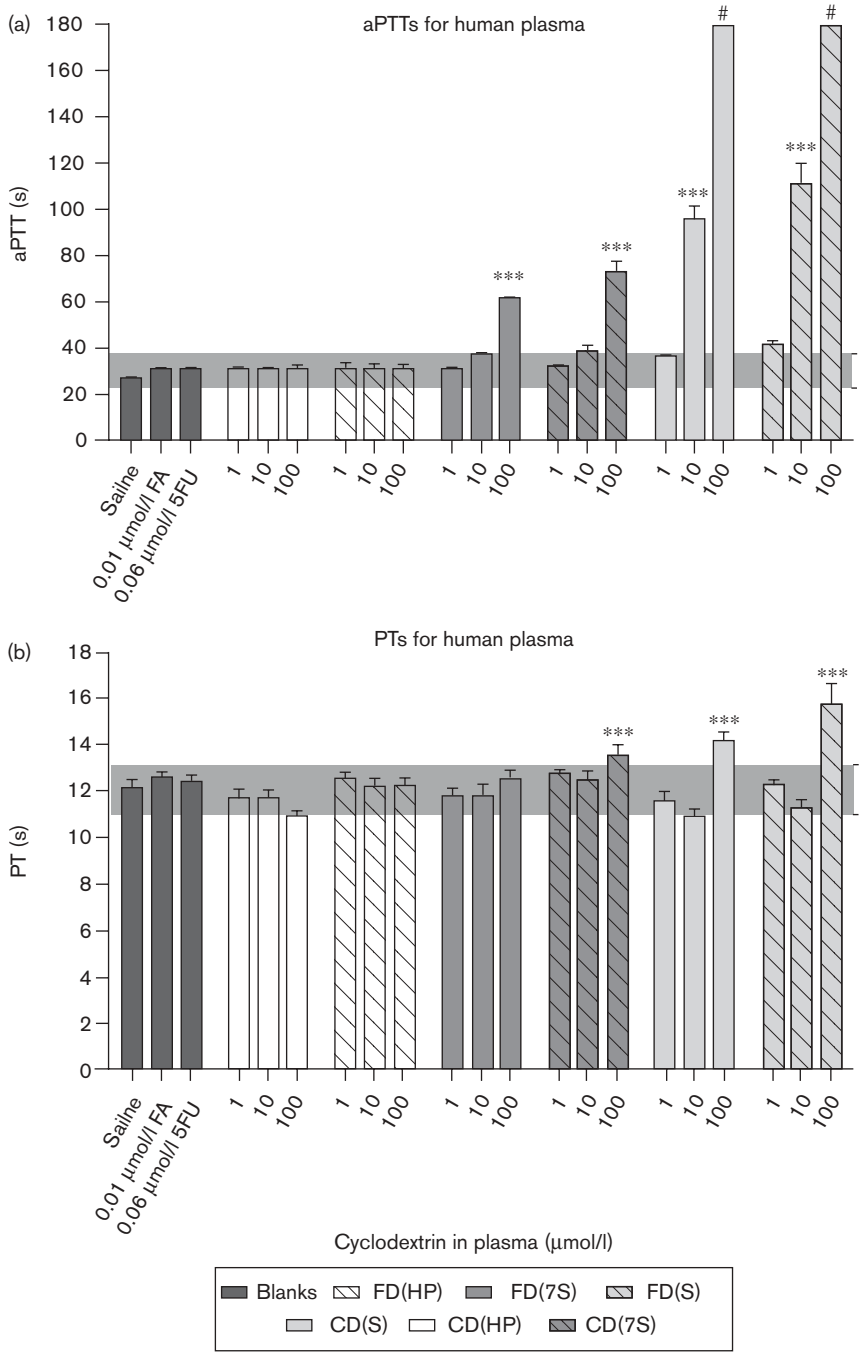
(a) The partial structure of a heparin polymer and (b) the structure of heptakis(6-*O*-sulfo)-β-cyclodextrin heptasodium salt [CD(7S)].

agent or when formulated with any of the CDs did not alter.

Assessment of formulation antithrombotic activity

The pathogenesis of hypercoagulability in cancer is not entirely understood but is routinely managed using heparin [26,49]. Heparin is effective in reducing the frequency of thromboembolic complications in patients by activating the coagulation inhibitor antithrombin, which degrades many of the serine proteases involved in the coagulation cascade [50]. Heparin can also influence malignant cell growth in cancer patients

Fig. 4



(a) Activated partial thromboplastin time (aPTT) and (b) prothrombin time (PT) assay results for the three formulations [FD(HP), FD(7S), and FD(S)] and the β-cyclodextrins [CD(HP), CD(7S), and CD(S)] in human plasma; values shown are the mean ± SEM ($n = 6$). The normal reference ranges for the aPTT and PT assays for human plasma are represented as gray shaded areas. Samples were exposed to the coagulometer detector for a maximum time of 180 s; #no clot detected after 180 s. FA, folinic acid.

through other different, interrelated mechanisms resulting in the inhibition of metastases and tumor angiogenesis (reviewed in Refs [50,51]). Of relevance to this study, some sulfated CDs have been found to exhibit anticoagulant activity [22,23] akin to heparin because of their structural similarities (Fig. 3) [52]. For example, heparin polymer strands form helices with a hydrophobic inner surface and a hydrophilic charged outer surface [53]. This is echoed by the sulfated CDs, which are also torus shaped and have a hydrophobic inner core and a negatively charged outer surface [52]. With a view of identifying a β-CD that mimics the anticoagulant behavior of heparin and could potentially be exploited by clinicians to reduce thrombus formation in cancer patients, we undertook an assessment of the antithrombotic activity of the all-in-one FD formulations and individual CDs.

In-vitro investigation of the anticoagulant properties using the aPTT test showed that FD(HP) and CD(HP) alone did not increase the aPTT values significantly above that of the controls (26–32 s) at concentrations up to 100 μmol/l (Fig. 4a). Similar clotting times were also observed for FD(7S) and CD(7S) at 1 and 10 μmol/l, respectively. However, at 100 μmol/l, aPTT values for both FD(7S) and CD(7S) increased two-fold ($P < 0.0001$). Results for the FD(S) and corresponding CD(S) at a final plasma concentration of 10 μmol/l showed that, relative to the control values, aPTTs significantly increased four-fold and three-fold, respectively ($P < 0.0001$), and at the highest concentration no clot was detected after 180 s.

PT assays were also used to provide a global assessment of coagulation function. In the case of FD(7S), FD(S), and CD(S), a concentration of 100 μmol/l was required to marginally, but significantly, increase the PT above that of the controls ($P < 0.0001$, Fig. 4b), albeit not at therapeutically relevant concentrations. However, no other FDs or CDs significantly increased the time to clot formation above control times (11–13 s) even at the highest concentration. This, together with the above aPTT data, suggests that the FD formulations containing polysulfated CDs [CD(S) and CD(7S)], mimic heparin, a contact activation (intrinsic) coagulation pathway inhibitor, as they result in an abnormally prolonged aPTTs, but relatively normal PTs. Furthermore, it is these CDs alone that are responsible for the antithrobitic effects, as both FA and FU (at equivalent concentrations found in the FDs) did not increase the aPTT or PT values when compared with saline controls (Fig. 4).

Finally, administration of UF heparin to prolong aPTT values to 1.5–2.5 times that of the control was once considered adequate to prevent or reduce thrombus formation *in vivo* [54]. This range, however, is not appropriate for all reagents and instruments used for detection [55] and should either be standardized or used in combination with other assays, such as protamine titration or anti-factor Xa [56]. The evaluation of heparin plasma concentrations by protamine titration has established a range of 0.2–0.4 units/ml that is correlated with good clinical safety and efficacy [57]. Similarly, a range of 0.3–0.7 units/ml has been reported using the anti-factor Xa assay [58]. A concentration of 10 μmol/l of CD(S) in our anticoagulation study was found to increase aPTT values to 96.0 s, approximately three times above that of normal healthy individuals. This is equivalent to a

Table 3 The relative anticoagulant properties and equivalent heparin activities of the β-cyclodextrins CD(HP), CD(7S), and CD(S)

Cyclodextrin	Concentration of cyclodextrin (μmol/l)[a]	aPTT ± SEM[b] (s)	Equivalent heparin activity (IU/ml)[c]
CD(HP)	1	30.4 ± 1.1	0.015
	10	32.3 ± 1.3	0.027
	100	30.6 ± 18	0.017
CD(7S)	1	31.2 ± 0.9	0.020
	10	37.2 ± 1.2	0.054
	100	62.4 ± 1.4	0.155
CD(S)	1	36.6 ± 1.6	0.052
	10	96.0 ± 5.1	0.239
	100	>180 (NCD)	>0.361

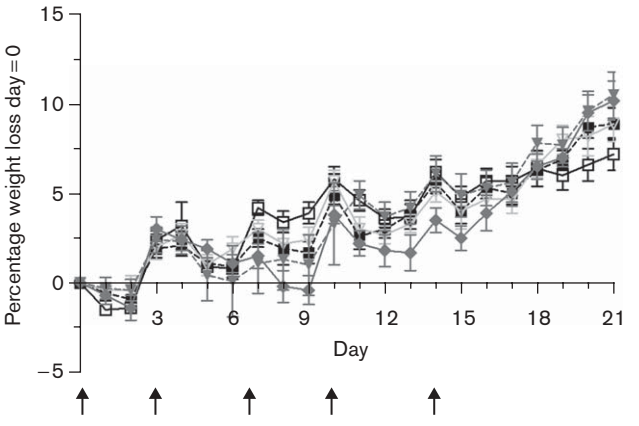
CD(HP), (2-hydroxypropyl)-β-cyclodextrin; CD(S), sulfated β-cyclodextrin sodium salt; CD(7S), heptakis(6-O-sulfo)-β-cyclodextrin heptasodium salt; NCD, no clot detected after 180 s.

[a]The final concentration of the cyclodextrin in the plasma.

[b]The quoted activated partial thromboplastin times (aPTT) are the mean ± SEM of $n = 6$ determinations.

[c]Equivalent heparin activities were interpolated from a heparin standard curve using the equation $y = 28.179e^{5.1244x}$.

Fig. 5



Toxicity effects after intraperitoneal administration of FD formulations compared with 5-fluorouracil (5FU) + folinic acid (FA) (15 : 1) in mice expressed in terms of weight loss. Animals were administered 40 mg/kg equivalent 5FU five times, over 14 days (indicated by arrows). Saline control (□, black line, $n = 13$), 5FU + FA (■, black dashed line), FD(S) (◆FD, dark gray line), FD(7S) (▼, dark gray dashed line) and FD(HP) (▽, gray line). Values shown are mean ± SEM of percent weight loss from the first day (day = 0) of administration ($n \geq 4$).

heparin concentration of 0.239 IU/ml (Table 3) and falls within the range that is clinically acceptable. Administration of a therapeutic dose of FD(S) in which the CD(S) plasma levels do not exceed this concentration may therefore help reduce some of the problems that are associated with the parenteral administration of sulfated CDs [23,59] and ultimately decrease thromboembolic complications in cancer patients in the future.

Preliminary in-vivo toxicity comparison of all-in-one formulations

Fractionated doses (40 mg/kg given five times, within 14 days) of 5FU either alone (with FA) or within the various formulations did not result in toxicity endpoints being reached by day 21 of observation, as less than 5% weight loss was noted from the first treatment in all cohorts (Fig. 5). In addition, there were no statistical differences between the FD-treated mice and the 5FU + FA or saline control cohorts indicating that this dose of 5FU is well tolerated as expected and that the toxicity of 5FU is not enhanced when part of a FD formulation containing any of the CDs.

Conclusion

Novel all-in-one and stable aqueous solution formulations (FD) of 5FU and FA at pH 7 and clinically relevant concentrations have been developed. These formulations include either sulfated-β-CDs or hydroxypropyl-β-CDs as the key solution stabilizing agents. Preliminary assessment of these formulations shows that they maintain comparable in-vitro cytotoxicities to mixtures of 5FU and FA against a range of cancer cell lines. Formulations based on the sulfated CDs [FD(S) and FD(7S)] show in-vitro anticoagulant activity as well as solution stabilizing property. The anticoagulant activity may have the added benefit of addressing the issue of thrombosis that is commonly associated with cancer leading to enhanced mortality rates [25]. In contrast, FD(HP) may have uses when anticoagulant activity is not desired. Thus, there are extensive proposed benefits of FD over current 5FU therapy. Primarily, FD overcomes problems of dual injection of a pH 9 solution (5FU) and its solubility-incompatible biomodulator (FA), resulting in streamlined administration. This should also result in reduced injection pain and side effects (phlebitis) for patients. It should also afford reductions in nursing time and level of patient intervention by reducing the occurrence of catheter blockage and their invasive and expensive replacement [60]. Furthermore, detailed preclinical studies on all the FD formulations are warranted and will be reported in due course.

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