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The antiangiogenic properties of sulfated β-cyclodextrins in anticancer formulations incorporating 5-fluorouracil

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Abstract

Sulfated β-cyclodextrins (S-β-CDs) are useful excipients for improving the solubility of drugs. One such formulation incorporating 5-fluorouracil (5-FU), termed FD(S), showed improved efficacy over 5-FU alone in orthotopic carcinoma xenograft models. S-β-CDs have heparin-like anticoagulant properties, which may have contributed toward the improved antitumor effect of FD(S). S-β-CDs have also been reported to modify a number of processes involved in angiogenesis. Although the anticoagulant nature of S-β-CDs was established, the antiangiogenic properties of S-β-CDs within FD(S) were unknown. The effect of S-β-CD and FD(S) on the proliferation and migration of endothelial cells in live-cell kinetic assays, and the reorganization of human umbilical vein endothelial cells into tubule structures in vitro was assessed. The effects of S-β-CD on angiogenesis in vitro were validated ex vivo using the rat aorta ring assay and the chick embryo chorioallantoic membrane assay. S-β-CD does not alter proliferative endothelial cell sensitivity to 5-FU cytotoxicity. S-β-CD alone and within FD(S) significantly inhibited angiogenesis by impeding endothelial cell migration, resulting in the inhibition of tubule formation and hence new vasculature. In addition to the cytotoxic action of the drug 5-FU, therapeutic inhibition of angiogenesis by S-β-CDs within FD(S) could potentially limit local invasion and metastases. This has important implications for the exploitation of S-β-CDs for drug formulation improvements or for drug delivery of anticancer biologics.

Keywords

fluorouracil, properties, sulfated, cyclodextrins, anticancer, formulations, incorporating, 5, antiangiogenic, CMMB

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The anti-angiogenic properties of sulfated β-cyclodextrins in anti-cancer formulations incorporating 5-fluorouracil

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ABSTRACT

Objective: Sulfated β-cyclodextrins (S-β-CDs) are useful excipients for improving the solubility of drugs. One such formulation incorporating 5-fluorouracil (5-FU), termed FD(S), showed improved efficacy over 5-FU alone in orthotopic carcinoma xenograft models [1]. S-β-CDs have heparin-like anti-coagulant properties, which may have contributed to the improved anti-tumour effect of FD(S). S-β-CDs have also been reported to modify a number of processes involved in angiogenesis. While the anti-coagulant nature of S-β-CDs was established, the anti-angiogenic properties of S-β-CDs within FD(S) were unknown.

Methods: The effect of S-β-CD and FD(S) proliferation and migration of endothelial cells in live-cell kinetic assays, and the reorganisation of human umbilical vein endothelial cells into tubule structures *in vitro* was assessed. Confirmation of the anti-angiogenic effect of S-β-CD was demonstrated using an *ex ovo* chick embryo chorioallantoic membrane (CAM) assay.

Results: S-β-CD does not alter proliferative endothelial cell sensitivity to 5-FU cytotoxicity. S-β-CD alone and within FD(S) significantly inhibited angiogenesis by impeding endothelial cell migration, resulting in the inhibition of tubule formation and hence new vasculature.

Conclusions: In addition to the cytotoxic action of the drug 5-FU, therapeutic inhibition of angiogenesis by S-β-CDs within FD(S) could potentially limit local invasion and metastases. This has important implications for the exploitation of S-β-CDs for drug formulation improvements or for drug delivery of anti-cancer biologics.

KEYWORDS

Angiogenesis, β-cyclodextrin, 5-fluorouracil, endothelial cells, heparin, cancer.

1. Introduction

Injectable formulations of 5-fluorouracil (5-FU) and its synergistic biomodulator Leucovorin™ (LV; calcium folinate) form the cornerstone of many current colorectal cancer (CRC) chemotherapy regimens [2-3]. We previously reported the development of all-in-one injectable pH neutral formulations of 5-FU and LV, which overcome the physical/chemical incompatibilities between 5-FU and LV and otherwise prevent a single solution intravenous administration. This was achieved by incorporating either polysulfated β-cyclodextrin (S-β-CD; 7–14 sulfate groups) or a hydroxypropyl β-cyclodextrin (HP-β-CD) as excipients [4]. These formulations signficantly reduce phlebitis, thus improving the tolerability of current intravenous 5-FU regimens whilst maintaining all cytotoxic properties *in vitro* and in animal tumour models [1].

In addition to their solubilising properties, other significant benefits may also be gained through the use of S-β-CDs in chemotherapy formulations as they are known to exhibit heparin-like anti-coagulant activity [4-7]. Heparins are routinely used for the treatment of thromboembolic complications in cancer patients, which are a result of the hyperactivation of the coagulation cascade [8-9]. When used in conjunction with chemotherapeutics, heparins have been reported to improve the survival outcomes for cancer patients in some [10-12] but not all trials which may be related to stage and/or type of cancer [13]. This may be due to the fact that cancer metastasis process requires

coagulation [14-15]. The heparins may also influence a number of cellular processes such as tumour cell growth via inhibition of heparin binding growth factors, and angiogenesis, and thereby ultimately affect cancer progression [8, 10].

Despite the potential benefits, a summary of animal model studies by Neirs *et al.* [12] concluded that high doses of heparin administered at length are required to significantly reduce metastasis while low doses of heparins given as part of short administration regimens were ineffective. Long-term administration of high dose heparin is greatly limited as patients often experience hemorrhagic complications due to the systemic inhibition of coagulation [16-19]. As S-β-CDs are associated with reduced systemic anti-coagulant activity [6], these excipients not only provide a valuable alternative to heparins but, when incorporated into chemotherapeutic drug formulations, may also improve the survival outcomes for cancer patients.

We have previously reported that a 5-FU formulation incorporating 172 mg/mL of S-β-CD (termed FD(S)) not only retained anti-coagulant properties [4] but was also shown to reduce tumour growth more effectively than 5-FU/LV in an orthotopic human xenograft tumour model in mice [1]. It is possible that the improved efficacy of FD(S) is due in part to the anti-coagulation properties of the S-β-CD component of the formulation [4]. However, S-β-CDs have also been reported to modify a number of processes involved in angiogenesis in a concentration-dependent manner [5, 20-21]. As the maximum tolerated dose (MTD) of FD(S) represents a systemic concentration of S-β-CD of ~ 2 mM per dose at which no bleeding problems were observed [1], it is conceivable that the improved anti-cancer effect of FD(S) compared to 5-FU/LV at the MTD is in part also due to the anti-angiogenesis properties of S-β-CD.

Angiogenesis is a complex process, dependent on the interactions of various host and tumour cells that involves endothelial cell (EC) adhesion, proliferation, migration and differentiation into tubule structures [22-23]. As metastasis is the major cause of treatment failure in cancer patients [14-15], the therapeutic inhibition of angiogenesis could therefore prove critical to the prevention of regrowth of tumour masses treated with cytotoxic drug regimens and to limiting metastases [24]. To this end, FD(S) may induce a synergistic effect, whereby the S-β-CD component inhibits angiogenesis and thus metastasis in addition to the cytotoxic action of 5-FU/LV on tumour cells. The purpose of the current study was thus to determine the effect of S-β-CD and FD(S) on the process of angiogenesis at a cellular level, which involved analysis of the proliferation, migration and reorganisation of ECs into tubule structures. Confirmation of this effect was also ascertained using an *ex ovo* chick embryo chorioallantoic membrane (CAM) assay. As angiogenesis is a prognostic factor for metastatic cancer [25], results demonstrating an anti-angiogenic effect of S-β-CD in FD(S) could inspire further development of cytotoxic or other anti-cancer drugs incorporating this compound in their formulations.

2. Materials and methods

2.1 Formulation preparation

Stock solutions of FD(S), S-β-CD and 2-(hydroxypropyl)-β-cyclodextrin (HP-β-CD) were prepared as described previously [4]. The components of each formulation were dissolved in endotoxin-free water (Sigma-Aldrich), and confirmed to be at pH 7.4 ± 0.1 before being passed

through a 0.22 μM filter (Millipore) to sterilise. Pharmaceutical grade 5-FU solution (ONCO-TAIN® 5-Fluorouracil Injection, 50 mg/mL) was obtained from Mayne Pharma Pty. Ltd. (Salisbury South, Australia). Leucovorin™ (LV) solution (Leucovorin Calcium Injection USP, 10 mg/mL) was obtained from Hospira Australia Pty. Ltd. (Mulgrave, Australia). For 5-FU/LV, the components were prepared immediately prior to use as described by Locke *et al.* [4]. Sulfated-β-cyclodextrin sodium salt (S-β-CD, 2206.5 g/mol, with approximately 9-12 sulfate groups per cyclodextrin molecule) and 2-(hydroxypropyl)-β-cyclodextrin (HP-β-CD, 1540 g/mol) were obtained from Sigma-Aldrich.

2.2 Cell lines and culture conditions

The SV40 transformed EC (SVEC4-10) line and primary human vascular endothelial (HUV-EC-C) line were purchased from American Type Culture Collection, distributed by Cryosite, Australia, and cultivated as recommended by the supplier. SVEC4-10 cells retain the morphological and functional characteristics of primary ECs but proliferate indefinitely without the specific growth factor requirements of primary ECs [26]. Cells were routinely cultured in cell line specific media at 37 °C in a 95% humidified atmosphere, containing 5% CO_2. SVEC4-10 cells were grown in DMEM (Invitrogen, USA) supplemented with 5% (v/v) heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich). The HUV-EC-C cells were grown in HAMS/F12K nutrient mixture (Kaighn's modification) containing 0.1 mg/mL unfractionated heparin (Sigma-Aldrich), 0.03 mg/mL endothelial cell growth supplement (ECGS) (Sigma-Aldrich), 2.5 g/L sodium bicarbonate and supplemented with 10% (v/v) heat-inactivated FCS (Sigma-Aldrich). All tissue culture flasks and cell assay plates used for the routine culture of HUV-EC-C cells (passage 3 – 6) were coated overnight with a 1% (v/v) gelatin solution (Sigma-Aldrich).

2.3 xCELLigence endothelial cell proliferation assays

The effect of 5-FU, FD(S) and S-β-CD on EC proliferation was determined using the xCELLigence System (Roche Applied Science) in specialised 16-well E-16 plates. Following the addition of 100 μL of cell culture media to the wells, the cell lines were seeded at 5000 cells/well in a volume of 80 μL. The cells were allowed to settle for 30 min at room temperature before being incubated at 37 °C for 24 h. Varying concentrations of the compounds in 20 μL sterile water were then added and the plate was again incubated at 37 °C for 72 h. Comparisons were made to untreated cells which received 20 μL of sterile water. Background wells containing media without cells were included. The adhesion, spreading and proliferation of cells was measured as a dimensionless parameter termed cell index (CI) in real-time. CI is derived from the relative change in measured electrical impedance, whereby electrical impedance represents the presence and spread of adherent cells. Real-time data was accumulated every 15 min and graphed using the RTCA-DP Software (version 1.2.1), supplied with the xCELLigence System.

2.4 xCELLigence endothelial cell invasion/migration assays

The effect of S-β-CDs and FD(S) on SVEC4-10 and HUV-EC-C cell invasion/migration was analysed with the xCELLigence System using specialised two-layer Cell Invasion and Migration plates (CIM-plates), in which a porous membrane (pore size 8 μm) separates the upper and lower wells. Cell culture media (160 μL) was added to the lower wells of the CIM-plates, omitting the test compounds. The lower wells contained the chemotactic control of FCS-containing media or serum-free (SF) media

for basal level invasion/migration. Following assembly of the CIM-plate, 25 µL of SF media was dispensed into the upper wells and the plate was incubated at 37 °C for 1 h to allow for equilibration of the membrane. For HUV-EC-C cells, the upper CIM-plate wells were pre-coated with 25 µL Geltrex Basement Membrane Matrix gel (Invitrogen, USA) in order to allow cell adhesion and spreading. During this time the cells (HUV-EC-C cells had been deprived of serum for the previous 12 h) were harvested and resuspended in SF media, and then seeded into the upper wells at an optimised count of 40,000 cells/well in a volume of 90 µL. Varying concentrations of S-β-CDs and FD(S) that were deemed non-toxic by cell proliferation assays, in 10 µL SF media were added into the upper wells containing cell suspensions. Untreated cells received 10 µL SF media alone. The plate was incubated at 37 °C and the migration of cells across the membrane into the lower wells was measured as CI in real-time over 12 h. Real-time data was accumulated and graphed as described in Section 2.3.

2.5 In vitro angiogenesis inhibition assays

The effect of S-β-CD, alone and in the FD(S) formulation, on the degree of EC tubule formation was examined using an *in vitro* Angiogenesis Assay Kit (Cayman Chemical Company, USA) in flat-bottomed 24-well microplates coated with Geltrex Basement Membrane Matrix gel (Invitrogen, USA), as a supportive matrix. Prior to cell seeding, a grid was drawn on the underside of the wells using a fine marker pen to divide the area into quadrants and the wells were coated with 95 µL Geltrex. To solidify the gel, the plate was warmed to 37 °C for 30 min. Cell suspensions were prepared in culture medium in the absence or presence of 0.3 µM JNJ-10198409 angiogenesis inhibitor as a negative control or S-β-CD or FD(S) at varying concentrations. Cells were seeded at an optimised density of 42,000 cells/cm² (80,000 cells/well) in a volume of 400 µL. Plates were incubated at 37 °C for 7 h to allow tubule formation.

At the conclusion of incubation, cell morphology and the degree of tubule formation were visualised using an inverted light microscope and inverted epifluorescence microscope with images captured using a mounted digital camera (Nikon, Instruments Inc., USA) and mounted Leica DC500 camera (Leica Microsystems, Germany), respectively. Four photographs (4 × magnification) were taken in each quadrant of duplicate treatment wells using an inverted light microscope, for manual scoring of tubule formation, followed by four epifluorescence photographs (10 ×), for visualisation. For manual scoring, multiple scorers blinded to the allocated treatment counted the number of fully enclosed vacuoles formed by tubule branches in eight photographs per treatment, four from each duplicated well; vacuoles that breached the edge of the photograph were not counted. Results were analysed using a student's unpaired t-test with $p < 0.05$ considered significant. Cell and tubule network morphology was also scored using a numerical ranking system from 0 to 3, adapted from the Millicell® µ-Angiogenesis Inhibition Assay Protocol (Millipore, USA). Briefly, a score of 0 = mostly undifferentiated cells, no/little tubule network or sprouting cells noted; 1 = sprouting of new tubules visible with large pockets of undifferentiated cells between them; 2 = sprouting of new tubules visible with small pockets of undifferentiated cells between them; 3 = expansive tubule networks with consistently elongated cells. For fluorescent visualisation, a calcein (1000 ×) AM staining solution was diluted 1:100 and added to the wells at a volume 40 µL/well. Cells were incubated at 37 °C for 30 min to allow the calcein stain to develop.

2.6 Ex ovo chick embryo chorioallantoic membrane (CAM) collagen onplant assay

To further examine the effect of S-β-CD on angiogenesis, an *ex ovo* chick embryo CAM collagen onplant assay protocol was conducted, which utilises the highly vascularised CAM as an experimental model capable of investigating angiogenesis [27]. Fertile Barter Brown Crossbreed chicken eggs were obtained from Barter and Sons Hatchery (Luddenham, Australia). To initiate embryogenesis, the eggs were placed into a rotary incubator at 37.8 °C and 60% relative humidity for 3 days. At embryonic day 3, the CAM was 'dropped' from the inside of the shell, as described in Storgard *et al.* (2004) [27]. The shell was carefully cut open under sterile conditions using an engraving power tool, allowing for the entire contents of the eggs to be transferred to pre-sterilised plastic weigh boats with slanted walls (Lomb Scientific, Australia). The contents were covered with square Petri dish lids and the containers were placed into a stationary incubator at 37.8 °C and 90% relative humidity for another 3 days to allow the CAM to develop. At embryonic day 6, collagen onplants were prepared using autoclaved 4 × 4 mm nylon mesh squares with 180 μm grid size (Sefar Inc., Australia). Onplants were assembled by overlaying two mesh squares in Petri dishes, which were lined with parafilm and sterilised with ethanol. Test compounds were diluted from stock solutions in 10 × PBS and added to neutralised 3 mg/mL collagen solutions prepared from rat-tail collagen (Sigma-Aldrich) as described in Deryugina *et al.* (2008) [28]. The final collagen solutions contained either PBS alone (control), 0.01 μM JNJ-10198409 or 0.3 mM S-β-CD, diluted from a 200 mM stock solution. This concentration of S-β-CD was chosen because it falls within the range of concentrations that inhibit EC migration and is an achievable systemic concentration after a MTD dose administration in mice [1]. The final collagen solutions (30 μL) were placed on top of the mesh squares and the onplants allowed to polymerise at 37 °C for 1 h and then solidify at room temperature. The onplants were topically positioned on the CAM in areas containing fine vascular networks. Individual embryos received four onplants, containing one of each of the four treatments, in different quadrants of the embryo. Onplant-bearing embryos were incubated for an additional 96 h at 37.8 °C and 90% relative humidity in a HERAcell stationary incubator. At this time, the extent of vascularisation within the onplant was determined using a stereomicroscope, with a Moticam 1000 live imaging camera attached (Motic, Canada).

3. Results

3.1 S-β-CD does not alter EC proliferation or sensitivity to 5-FU cytotoxicity in vitro

During angiogenesis ECs proliferate to provide additional cells that will constitute the new vessel. At this time, ECs have been activated into a proliferative state by the presence of growth factors. For this reason, transformed SVEC4-10 cells were used as a model cell line for ECs under angiogenic conditions.

To determine the effect of S-β-CD both alone and within FD(S) on EC proliferation over time and to establish non-cytotoxic concentrations for subsequent assays, real-time live-cell kinetic assays were used. In the absence of S-β-CD, SVEC4-10 cells grew exponentially up to 40 h after cell seeding then reached a plateau (Fig. 1A, untreated). The addition of 20 mM S-β-CD 24 h after seeding caused an initial steep, then continued decrease in CI values to zero impedance relative to the control within 48 h of treatment (Fig. 1A). This indicates a complete loss of cells, which is suggestive of either a potent

cytotoxic effect and/or loss of cell adhesion. At concentrations ≤ 4 mM, CI values were essentially similar to that obtained with untreated cells (Fig. 1A), indicating that SVEC4-10 EC proliferation was unaffected by S-β-CD at these concentrations. In other experiments, within 24 h after the addition of 10 mM S-β-CD, CI levels were partially reduced but recovered to that of untreated cells thereafter (data not shown).

SVEC4-10 ECs treated with FD(S) encompassing a concentration range of 5-FU previously shown to be cytotoxic after 72 h in a variety of cancer cell lines [4], displayed time-dependent cytotoxic effects (Fig. 1B). S-β-CD concentrations within the FD(S) formulation corresponded to < 1 mM indicating that cytotoxic effects seen were due to the 5-FU component of the formulation. As expected for 5-FU mediated cytotoxicity, dose-dependent cytotoxic effects were apparent 48 h after compound addition with maximal effects obvious by 72 h post FD(S) treatment (Fig. 1B). An IC_{50} value of ~ 20 μM was calculated by the RTCA-DP Software using the sigmoidal dose response curve (r^2 = 0.985). Graphpad Prism analysis of the data taken at 72 h post treatment from either the xCELLigence system or from a standard endpoint assay routinely used for determination of cytotoxic effects [4] revealed IC_{50} values of similar magnitude (i.e. 15 μM; Supplementary Fig. 1). That 5-FU alone gave a similar IC_{50} value to FD(S) (14 vs 15.8 μM, Supplementary Fig. 1B), further confirms that the S-β-CD component did not contribute to the cytotoxic action of FD(S).

The long population doubling time of HUV-EC-C cells (6 – 7 days) precluded testing the potential cytotoxic effect of FD(S) as the cells did not proliferate under the time-frame and conditions of these experiments (data not shown). S-β-CD over the concentration range 2.5 mM – 0.3 mM had no stimulatory effect on HUV-EC-C cell proliferation (data not shown).

3.2 S-β-CD alone and within FD(S) inhibits EC migration

The migratory function of ECs is essential for angiogenesis. The effect of S-β-CD on SVEC4-10 EC migration was thus determined at concentrations that were not anti-proliferative/cytotoxic (≤ 4 mM). S-β-CD over the concentration range (4 mM - 1.25 μM), inhibited the migration of SVEC4-10 ECs towards FCS as the chemoattractant in the lower chambers (Fig. 2A, control). At the highest concentration of S-β-CD CI values were essentially equivalent to basal CI values (serum-free media in the lower chambers). The inhibitory effect of S-β-CDs on SVEC4-10 migration was slightly reduced at the lower concentrations (0.8 mM – 1.28 μM), with no apparent dose response in this range (Fig. 2A). The reason for this lack of dose-dependency is unclear. SVEC4-10 EC migration was mildly stimulated (~ 12% above the level of the positive control) at very low-range concentrations of S-β-CDs (≤ 0.5 μM) (data not shown). The inhibitory effect of S-β-CD on SVEC4-10 cell migration was uncompromised within FD(S) (equivalent S-β-CD concentration range 4 mM – 1.25 μM) (Fig. 3B). The FD(S) has no cytotoxic effect within the timeframe of the migration assays (12 h) (refer to Fig. 1B) and so this effect is attributed to the S-β-CD component.

At 20 μM, 200 μM and 2 mM, S-β-CD alone completely inhibited the migration of serum-deprived HUV-EC-C cells towards FCS as the chemoattractant as the observed CI values were equivalent to basal CI values (Fig. 3A). HUV-EC-C cell migration was also inhibited by FD(S) containing equivalent concentrations of S-β-CD (Fig. 3B), though 20 and 200 μM S-β-CD in FD(S) was not as efficient as 20 and 200 μM S-β-CD alone. At 2 mM S-β-CD in FD(S), migration was

completely inhibited (Fig. 3B). Thus, incorporation of S-β-CD in FD(S) did not compromise the inhibition of HUV-EC-C cell invasion and migration. The potential requirement for an invasion step through the GELTREX coating on membranes necessary for HUV-EC-C cell attachment and spreading in the assay chambers (not required for SVEC4-10 ECs), prior to migration may have accounted for the small concentration effect difference between the HUV-EC-C and SVEC4-10 ECs.

3.3 S-β-CD inhibits spontaneous tubule formation by HUV-EC-C cells at therapeutic doses of FD(S)

The culture of ECs on a supportive matrix with growth factors results in morphological changes and the formation of tubule structures that replicate vasculature, a phenomenon known as spontaneous angiogenesis [29]. This was demonstrated by the formation of expansive tubule networks (morphology score = 3) when HUV-EC-C cells were grown on Geltrex with growth factor containing media (Fig. 4A; media control). Incubation of HUV-EC-C cells in the presence of the angiogenisis inhibitor control JNJ-10198409 reduced the formation of tubule networks with small pockets of undifferentiated cells observed between tubule branches (morphology score = 2) (Fig. 4B).

At 15.6 μM S-β-CD alone (Fig. 4C) and in FD(S) (Fig. 4D), HUV-EC-C cell morphology was similar to that after JNJ-10198409 treatment, with pockets of undifferentiated cells between tubule branches observed, and were both scored a value of 2 for tubule network morphology. Manual scoring of fully enclosed vacuoles formed by tubule branches however, found no significant differences ($p > 0.1$) compared to media control. Cells remained undifferentiated with no tubule network formation evident (morphology score = 0) after treatment with 1.56 mM S-β-CD alone (Fig. 5E) and in FD(S) (Fig. 5F). This represented significant inhibition of spontaneous angiogenesis as vacuoles formation was reduced by 96.1% ($p < 0.0001$) and 97.9% ($p < 0.0001$), respectively, compared to the media control as determined by manual scoring. This inhibitory effect was due to the S-β-CD component of FD(S) since an equivalent concentration of non-sulfated HP-β-CD, either alone or incorporated into a separate 5-FU formulation termed FD(HP) [4], did not affect the degree of tubule formation relative to the media control (Supplementary Fig. 2). Within the timeframe of these experiments (7 h), changes in cell numbers due to treatment with 1.56 mM S-β-CD were not expected (see above).

3.4 S-β-CD prevents the formation of new vasculature ex ovo

The *ex ovo* chick embryo CAM collagen onplant assay was used to confirm the inhibitory effect of S-β-CD on angiogenesis. PBS-containing control onplants did not induce the advancement of fine vessels towards the onplant after 4 days as expected (Fig. 5A), though fine vessels branching from pre-existing major vessels within the onplant site were observed. Onplants containing the inhibitory control JNJ-10198409 prevented the formation of new fine vasculature over the 4 day treatment period and a fine vessel avascular zone was evident within the onplant area (Fig. 5B). Onplants containing 0.3 mM S-β-CDs also prevented the formation of new vasculature over the 96 h treatment period (Fig. 5C). Observations at higher magnification revealed a 2-3 mm avascular region free of fine blood vessels within the onplant area (Supplementary Fig. 3).

4. Discussion

The results of the current study confirm the inhibitory potential of S-β-CD alone on angiogenesis *in vitro* and *ex ovo* and, importantly, that these anti-angiogenesis properties are maintained in FD(S). At and below concentrations of S-β-CD that are achievable after systemic administration of FD(S) doses that reduce tumour growth (< 2mM S-β-CD) [1], FD(S) dramatically inhibited spontaneous tubule formation of HUV-EC-C cells *in vitro* in a dose-dependent manner. This effect appears mediated via inhibition of invasion/migration of differentiated ECs. The lack of effect of a non-sulfated β-CD on *in vitro* angiogenesis as well as EC migration (data not shown) confirmed the necessity of sulfate groups for the anti-angiogenic activity of S-β-CDs [5, 21]. Water soluble sulfated cyclodextrins show low systemic toxicity in animals [6], and high concentrations of S-β-CD (up to 1 mM) are not cytotoxic against a variety of human carcinoma cell lines [4] or human vascular smooth muscle cells [20]. This was confirmed for the transformed EC cell line SVEC4-10 at concentrations of S-β-CD ≤ 4 mM. As reported for carcinoma cell lines [4], FD(S) encompassing these non-toxic S-β-CD concentrations was cytotoxic agaisnt SVEC4-10 ECs, reflective of the antimetabolite activity of 5-FU achieved in proliferative cells [4]. Thus, together with the potential anti-coagulant effects of S-β-CD in the metastatic process, we propose that the beneficial effect of FD(S) over 5-FU alone in tumour models was due to the synergistic action of: a) 5-FU toxicity against proliferating tumour cells as well as ECs stimulated to proliferate during the angiogenic process, and b) the immediate anti-invasive/migratory effect of S-β-CD on both differentiated and undifferentiated ECs. A detailed study to examine all of these effects of FD(S) in tumour models is now warranted.

Angiogenesis involves the rearrangement of localised ECs to form tubule structures [30], following the recruitment and migration of ECs by chemotaxis [31-32]. The inhibitory effect on *in vitro* EC tubule formation and migration of S-β-CDs is possibly linked to the ability of S-β-CDs to interact with and inhibit the activity of several key growth factors present in the FCS [33], including bFGF, PDGF and TGF-β [20, 34]. Low molecular weight heparins and S-β-CDs alike bind with high affinity to bFGF [20, 35-36] and, despite exhibiting no cytotoxic potential, S-β-CDs are able to inhibit smooth muscle cell proliferation induced by bFGF [36]. It is therefore proposed that the cellular processes mediated by these growth factors, in particular EC migration and differentiation are affected by S-β-CD and these are not substantially diminished by FD(S). In this manner, S-β-CD appears to mimic low molecular weight heparins, which inhibit bFGF and VEGF mediated angiogenesis by preventing these growth factors from binding to their respective receptors on ECs [9, 12, 37].

We also examined the effect of S-β-CDs on angiogenesis in an *ex ovo* chick embryo CAM model as there has been conflicting data in the literature regarding the pro/anti-angiogenic effects in various animal models. For example, in an embryonic chick CAM model, β-CD tetradecasulfate administered in conjunction with a steroid was found to inhibit angiogenesis at 100 to 1000 times the effectiveness of a heparin and steroid combination on a per weight basis [5]. However in a rabbit cornea model, β-CD tetradecasulfate, when administered alone at very low concentrations (15 $\mu g/mm^{3;}$ ~ 6 μM), stimulated blood vessel growth and density [5]. Using a subcutaneous plastic sponge model in mice, Strauss *et al.* [21] demonstrated a dose dependent effect after intraperitoneal administration of S-β-CDs alone on capillary density, with very low doses (200 ng/mouse; ~0.06 μM) apparently promoting

angiogenesis while “high” doses (20 μg/mouse; ~6 μM) reduced vessel index in the sponges. We found that collagen onplants in chick embryo CAMs containing S-β-CDs prevented the formation of new vasculature within the onplant site, creating an avascular regression zone free of blood vessels. Ribatti *et al.* [38], who examined angiogenesis on the CAM in response to an anti-bFGF antibody, defined inhibition to be a region around the onplant with no vascular reaction or, where pre-existing vasculature is present, it remains unresponsive to treatments. The latter was observed with onplants containing S-β-CD and JNJ-10198409 (Fig. 5). Yadav *et al.* [39], who investigated the anti-angiogenic effect of curcumin in complexation with nonsulfated β-CDs, defined angiogenesis inhibition as a zone of avascularity, which can be 2-6 mm in diameter, as a result of vessel occlusion or regression. Zones of avascularity observed in response to S-β-CDs and JNJ-10198409 were approximately 2-3 mm in diameter within the central area of the onplant.

Prolonged IV administration of S-β-CDs was found to be necessary to produce sustained inhibition of intimal hyperplasia [34], which is a vascular remodelling and repair process in response to the injury of vascular walls (Wu *et al.* 2009). Intimal hyperplasia is similar to the metastatic cascade as it involves stimulation of the coagulation cascade, EC and SMC migration and proliferation, and is dependent upon angiogenic growth factors and selectin interactions [34, 40]. The requirement of prolonged IV administration of S-β-CDs may be related to the fact that IV-administered β-CDs are eliminated rapidly from circulation [41]. Continuous administration of S-β-CDs may therefore be required and, favourably, S-β-CDS possess a reduced anti-coagulant activity as compared to heparins [6], allowing them to be administered at higher dosages [20, 42]. Importantly, FD(S) allows for the simultaneous IV-administration of all components, 5-FU, LV and S-β-CDs, and may improve the outcome for cancer patients by ensuring the continual administration of S-β-CDs at high, yet tolerable concentrations.

Finally, the inclusion of therapeutic doses of S-β-CDs in anti-cancer drug formulations, such as FD(S), offers the potential to limit local invasion and metastases, through the inhibition of angiogenesis, as well as to reduce tumour growth. Together with its useful anti-coagulant properties, S-β-CD as a component in anti-cancer drug formulations should be exploited for the development of a new generation of dual action therapeutics with potential for improved survival outcomes for cancer patients.

List of Figures

Fig. 1 The effect of **(a)** S-β-CD and **(b)** FD(S) on SVEC4-10 cell proliferation. Cells were cultivated for 24 h before the addition of S-β-CD or FD(S) at the concentrations shown, or media only (untreated controls; orange line) and monitored over 72 h in real-time using an xCELLigence RTCA DP System. The electronic readout of cell sensor impedance is displayed continuously as the Cell Index (CI) normalised to the compound addition timepoint (indicated by vertical dotted line). Values shown are means ± SD of duplicate treatments of a representative experiment.

Fig. 2 The effect of **(a)** S-β-CD and **(b)** FD(S) on the migration kinetics of SVEC4-10 ECs by continuous monitoring of live-cell migration. Cell suspensions were prepared in SF media and placed in the upper wells of CIM-plates in the absence (control/untreated; orange line) or presence of S-β-CD alone **(a)** or S-β-CD and 5-FU in FD(S) **(b)** at the concentrations shown and allowed to migrate towards 5% FCS in the lower wells over 12 h. Controls containing SF media in the lower wells (basal; green line) confirm the negligible levels of cell migration in the absence of a chemotatic stimulus. The electronic readout of cell sensor impedance, which indicates the presence of cells on the underside of the membrane through which the cells migrated, is displayed continuously as the Cell Index (CI). Values shown are means ± SD of duplicate treatments of a representative experiment.

Fig. 3 The effect of **(a)** S-β-CD and **(b)** FD(S) on the invasion/migration kinetics of HUV-EC-C cells by continuous monitoring of live-cell migration. Serum-starved cells were resuspended in SF media and placed in the upper wells of Geltrex-coated CIM-plates in the absence (control/untreated; orange line) or presence of S-β-CD alone **(a)** or S-β-CD (mM) and 5-FU (μg/mL) in FD(S) **(b)** at the concentrations shown and allowed to migrate towards 10% FCS in the lower wells over 12 h. Controls containing SF media in the lower wells (basal; green line) confirm the negligible levels of cell migration in the absence of a chemotatic stimulus. The electronic readout of cell sensor impedance is displayed continuously as the Cell Index (CI). Values shown are means ± SD of duplicate treatments of a representative experiment.

Fig. 4 HUV-EC-C cell *in vitro* angiogenesis inhibition assays. Cells were incubated on Geltrex for 7 h at 37 °C with the addition of **(a)** media only, **(b)** 0.3 μM JNJ-10198409 angiogenic inhibitor, **(c)** 15.6 μM S-β-CD, **(d)** 15.6 μM S-β-CD in FD(S) (equivalent to 3 μg/mL or 23 μM 5-FU), **(e)** 1.56 mM S-β-CD and **(f)** 1.56 mM S-β-CD in FD(S) (equivalent to 300 μg/mL or 2.3 mM 5-FU). Fluorescent images of cells stained with calcein AM staining solution are shown as viewed under the microscope at 100X magnification. Bright field images (not shown) were used only for manual scoring and morphology scoring.

Fig. 5 Ex ovo chick embryo chorioallantoic membrane (CAM) collagen onplant assay Representative macroscopic observations of the vascular response of *ex ovo* chick embryo CAMs to collagen onplants containing **(a)** PBS only, **(b)** 0.01 μM JNJ-10198409 and **(c)** 0.3 mM S-β-CD. Solidified onplants (boxed) were placed on the embryonic CAM in areas of fine vessel networks at

embryonic day 6 (corresponds to treatment Day 0), with one onplant of each treatment positioned in different quadrants per embryo. For each onplant, stereomicroscope images (8X magnification) were taken at Day 0 and 96 h (Day 4) later.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Ethical Standards

The authors declare that all experiments reported within were conducted in compliance with the current laws regarding scientific research in Australia.

Acknowledgements

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Electronic Supplementary Information

The anti-angiogenic properties of sulfated β-cyclodextrins in anti-cancer formulations incorporating 5-fluorouracil

Clare A. Watson, Kara L. Vine, Julie M. Locke and Marie Ranson

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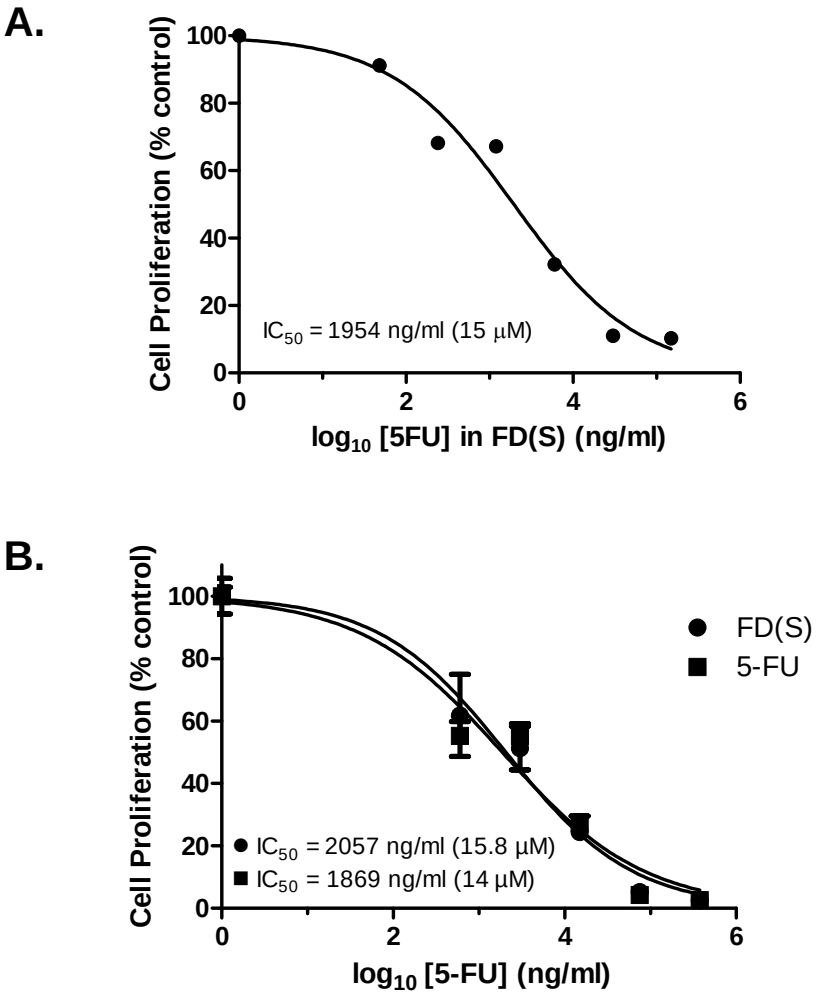
Supplementary Figure 1



Supplementary Figure 1 Legend:

A. Data taken from xCELLigence output (Fig 1B) at 72 h post FD(S) addition for SVEC4-10 ECS. Values shown are mean, n = 2, of a representative experiment. The relative IC_{50}, defined as the concentration of 5-FU causing 50% inhibition of cell proliferation, was calculated to be 1954 ng/ml (15 μM).

B. Comparison of cytotoxic effect of 5-FU versus FD(S) on SVEC4-10 ECs 72 h after treatment.

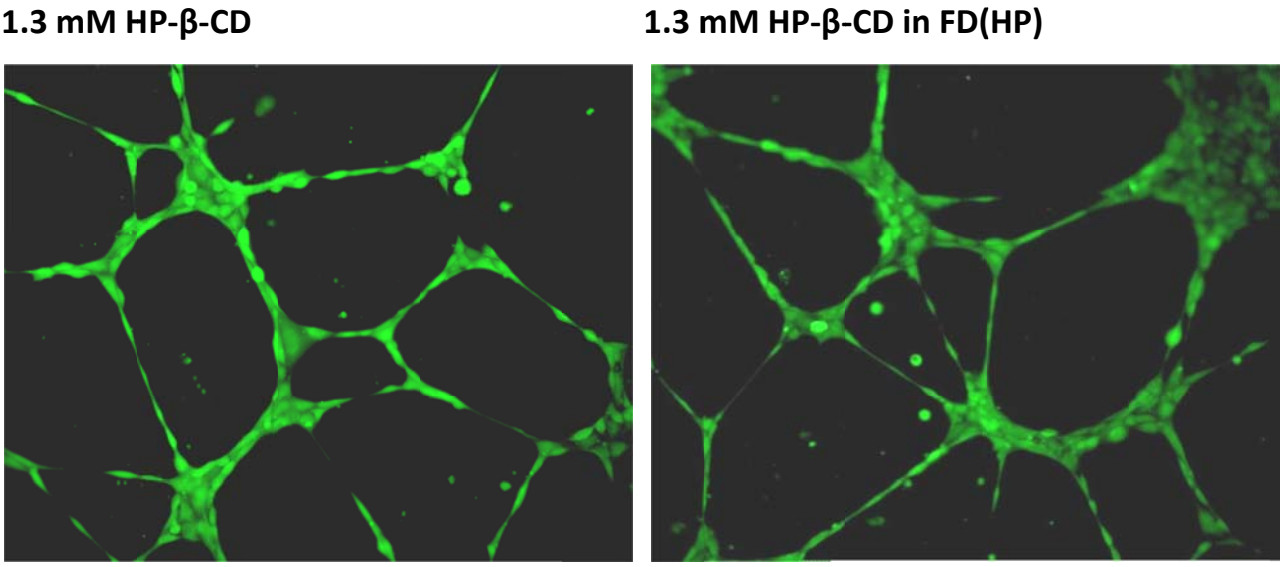
Determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS, Promega), as described previously.[1] Cells were seeded into 96-well plates (10^4 cells/well) and grown for 24 h prior to treatment with serial dilutions of FD(S) or 5-FU for 72 h. The absorbance at 490 nm was measured using a Spectromax® 250 UV plate reader employing Softmax Pro® software (Molecular Devices, USA). Values shown are mean +/-SEM, n = 3, of a representative experiment.

For both **A** and **B**: data was analysed using a sigmoidal dose-response using the variable slope parameter to determine IC_{50} values (GraphPad Prism, Version 5.0). Data was normalised to control (no drug - denoted as equivalent to 1 ng/mL) = 100% proliferation.

The relative IC_{50}, defined as the concentration of 5-FU causing 50% inhibition of SVEC4-10 EC proliferation, was calculated to be 1869 ng/mL (14 μM) for 5-FU and 2057 ng/mL (15.8 μM) for 5-FU in FD(S).

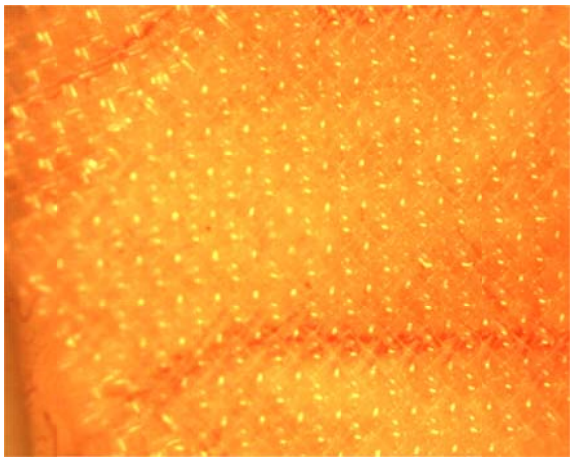
[1] Locke, J.M., et al., *Development and assessment of novel all-in-one parenteral formulations with integrated anticoagulant properties for the concomitant delivery of 5-fluorouracil and calcium folinate.* Anti-Cancer Drugs, 2009. **20**: p. 822-831.

Supplementary Figure 2



Supplementary Figure 2: HUV-EC-C cell *in vitro* angiogenesis inhibition assays. Cells were incubated on Geltrex, for 7 h at 37 ºC with the addition of either 1.3 mM hydroxypropyl-β-cyclodextrin (HP-β-CD) or Fluorodex formulation containing 1.3 mM HP-β-CD (FD(HP)) (equivalent to 300 mg/mL or 2.3 mM of 5-FU)[1]. Fluorescent images (10×) of cells stained with calcein AM staining solution (data shown is from a representative experiment). Both treatments were scored a value of 2 for tubule network morphology.

Supplementary Figure 3



Supplementary Figure 3: A representative macroscopic observation of the vascular response of an *ex ovo* chick embryo to a collagen onplant containing 0.3 mM S-β-CD. The solidified onplant was placed on the embryonic CAM in an area of fine vessel networks at embryonic day 6. This stereomicroscope image (2× magnification) was taken 96 h after placement of the onplant. Note the avascular region within the onplant site; the blood vessel seen in this area was a pre-existing vessel.