Preclinical evaluation of novel, all-in-one formulations of 5-fluorouracil and folinic acid with reduced toxicity profiles

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5-Fluorouracil (5-FU) in combination with its synergistic biomodulator folinic acid maintains a pivotal position in cancer chemotherapy. However, clinical limitations such as phlebitis and catheter blockages persist with the administration of these drugs in combination, and are associated with reduced efficacy and/or quality of life for patients. We have reported earlier on the novel, all-in-one, pH neutral, parenteral 5-FU and folinic acid formulations (termed Fluorodex) incorporating β-cyclodextrins. Fluorodex maintains potency while overcoming the accepted incompatibility of 5-FU and folinic acid. We carried out toxicological, pharmacokinetic and biodistribution, and efficacy evaluations of Fluorodex compared with 5-FU:folinic acid using several administration routes and schedules in two rodent models. These were compared with the dose-matched sequential administration of 5-FU:folinic acid. Fluorodex showed bioequivalence to 5-FU:folinic acid as assessed by the tissue distribution and pharmacokinetic studies of 5-FU, but was generally better tolerated as determined by weight loss, hematological, and other clinical parameters. Compared with 5-FU:folinic acid, Fluorodex was also associated with reduced phlebitis using a rabbit ear vein model. Furthermore, using human carcinoma tumor models in mice, Fluorodex resulted in equivalent or improved efficacy profiles compared with 5-FU:folinic acid. In conclusion, these novel, all-in-one formulations represent a superior injectable form of 5-FU that allows codelivery of folinic acid. This should translate into improved patient tolerability with potential for enhanced efficacy. *Anti-Cancer Drugs* 22:24–34 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

5-Fluorouracil (5-FU) is the fundamental component of many efficacious colorectal carcinoma (CRC) chemotherapy regimens (such as de Gramont, Mayo, FOLFOX and FOLFIRI) [1–5], and protocols that exclude 5-FU (such as IROX) have so far been clinically unsuccessful [6]. The antitumor activity of 5-FU in these regimens is enhanced by the concurrent administration of its biomodulator folinic acid (FA; leucovorin, calcium folinate) [7,8]. The use of FA increases intracellular levels of reduced folate cofactors that are essential in many reactions, including the inhibition of thymidylate synthase by 5-FU [9]. Clinically, the use of FA translates into a 2-fold improvement in 5-FU chemotherapy response rates and a small but significant increase in survival outcome [8].

Despite the long history of 5-FU use, there is still no universally recognized standard treatment regimen, and each regimen seems to be associated with differing toxicity profiles for 5-FU. These administration and scheduling variants range from bolus injection to short and long infusions (hours to days), with and without the sequential coadministration of FA, and oral prodrug forms, such as capecitabine [9–11]. The different modes of administration and range of intravenous (i.v.) schedules used for 5-FU have arisen not only because of the schedule-dependent side effects typically associated with cytotoxic drugs, but also because of a number of other formulation-related and administration-related complications, including phlebitis and catheter blockages [12–17]. To improve aqueous solubility, 5-FU is formulated as a highly alkaline solution that is associated with pain upon injection and in many cases results in phlebitis at the injection site and tracking along the vein [13,17–19]. To minimize the occurrence of phlebitis, 5-FU often necessitates delivery through a central line. The insertion of a catheter is an expensive, painful, and problematic procedure, and catheter complications (such as infections, bleeding and venous thrombosis) have been observed in up to 30% of the patients [18,20–22]. In addition, catheter blockage caused by 5-FU precipitation can occur during infusional administration [16]. Premixed solutions of 5-FU and FA can be problematic because of 5-FU precipitation (at high concentrations) [23] or calcium

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carbonate precipitation on continuous infusion [12,23]. Furthermore, repeated cycles of FA and 5-FU administration result in the formation of these deposits causing catheter blockage, and consequently lead to increased patient discomfort and potential cessation of treatment [14]. Dual catheters have been used to overcome this problem, but these are expensive and can be difficult to maintain [24]. In addition, the sequential administration of FA followed by infusional 5-FU may reduce the potential benefit of coadministering these components because of the short plasma half-life of FA [25].

Given the current inability to simultaneously administer 5-FU and FA, and the problems associated with the clinical management of current parenteral 5-FU regimens, improved formulations of these compounds that have equivalent or superior therapeutic effects, coupled with either reduced toxicity or administration complications, would be of significant clinical benefit and, importantly, improve the patient's quality of life. We have reported earlier on the development and initial assessment of the novel, all-in-one parenteral formulations of 5-FU and FA termed Fluorodex (FD) [26]. The FD formulations can be prepared with varying concentrations of FA, which are all stable at physiological pH and thus simplify administration of 5-FU and FA. These formulations incorporate either polysulfated or hydroxypropyl β-cyclodextrins (β-CD) (termed FD(S) or FD(HP), respectively) as solubilisers/stabilisers [26]. In-vitro evaluation in several human carcinoma cell lines showed that the formulations exhibited enhanced cytotoxicity equivalent to 5-FU:FA [26]. Furthermore, preliminary in-vivo dose–tolerance profiles of the formulations were also equivalent to 5-FU:FA [26]. Thus, the incorporation of the β-CDs does not compromise the in-vitro efficacy of 5-FU:FA nor do they adversely affect the tolerability of 5-FU [26]. Here we report a more detailed, preclinical toxicological and efficacy evaluation of FD(S) and FD(HP) with additional bioequivalence and phlebitis assessments performed for the latter. The data reported here supports the progression of FD(HP) towards human trials.

Materials and methods

Fluorodex formulations

All FD formulations have final concentrations of 5-FU (15 mg/ml) and FA (1 mg/ml). When administered *in vivo*, this ratio of FA to 5-FU corresponds to low-dose FA. Our earlier assessment showed no differences in the in-vitro cytotoxicity using higher ratios of FA [26], and thus the 15 : 1 ratio was chosen for the lead formulations. FD(HP) was made using (2-hydroxypropyl)-β-CD (100 mg/ml final concentration in formulation); FD(S) was prepared using polysulfated β-CD sodium salt (45–172 mg/ml) as described in detail [26]. The concentration of FD is expressed in terms of the concentration of 5-FU in the formulation.

Cell lines and animals

The human colorectal (HCT-116, HT-29) and breast (MDA-MB-231) carcinoma cell lines were obtained from the American Type Culture Collection (USA) or the European Collection of Cell Cultures (UK) and routinely cultured as described earlier [27]. The cells were routinely tested for the absence of mycoplasma contamination. SPF-bred Balb/c mice, Balb/c *nu/nu* mice, Sprague–Dawley rats, and New Zealand White rabbits were obtained from the Animal Resources Centre (Canning Vale, Western Australia, Australia) or from the University of Adelaide (Urrbrae, South Australia, Australia). All the experiments were approved by the University of Wollongong Animal Ethics Committee, the University of Queensland Animal Experimentation Committee, or the Animal Ethics Committee of the University of Adelaide.

Doses administered to animals are stated as mg/m^2 to simplify comparison with human doses. These were converted to mg/kg doses using body surface area calculation as described [28]. In brief, to convert mg/m^2 to mg/kg for mice, rats, and rabbits, divide by 3, 5.9 and 12, respectively.

Dose range finding and toxicological evaluation

Intraperitoneal bolus assessment

In brief, single or multiple (fractionated) intraperitoneal bolus (i.p.b.) doses of the formulations were compared with the sequential 5-FU:FA treatment and phosphate buffered saline (PBS, 150 mmol/l NaCl solution, pH 7.4) controls in female Balb/c mice. These dose schedules are based on established clinical protocols [3] and take into account shorter murine life expectancy. 5-FU:FA treatments were administered by two separate sequential i.p.b. injections on alternate sides of the midline, with FA being administered immediately before 5-FU. Dose-limiting toxicity endpoints were defined as 15% loss of body weight (compared with the first day of treatment and sustained for > 24 h), or clinical signs of morbidity (i.e. loss of appetite, activity and/or hunched posture, piloerection, and changes in gait).

For some experiments, excised livers, kidneys, and spleens were weighed before fixation and sectioning for haematoxylin and eosin staining and blinded histopathological evaluation. In addition, a proportion of the livers were submitted for oil red-O stains, which is used to identify exogenous or endogenous lipoid deposits.

Intravenous bolus and infusional assessment

Single intravenous bolus (i.v.b.) doses of FD were administered to female rats through the peripheral tail vein at dose levels of 425, 475 or 525 mg/m^2. The positive control group was administered a single i.v.b. dose of FA (35 mg/m^2) followed by 5-FU (525 mg/m^2) within 20 min. Both the treatment groups were then monitored over a

7-day recovery period. In other experiments using both male and female rats, i.v.b. doses of 400 mg/m^2 FD were followed immediately by continuous intravenous infusion (i.v.i.) of either 600, 1200, or 2400 mg/m^2 of FD over 48 h with a subsequent 7-day recovery period. The positive control group was matched to the highest tolerated FD dose, except that this group also received a single i.v.b. dose of FA (26.7 mg/m^2, i.e. 1/15 of the single bolus 5-FU dose) immediately before 5-FU administration. Other controls included volume-matched administration of β-CD vehicle (pH 7.3) and PBS at pH 7.3 and pH 9 (as a 5-FU pH-matched control).

Clinical signs, systolic blood pressure, proteinuria, and body weights were recorded periodically during the treatment and recovery period. Animals were then euthanised and necropsy performed in all the groups in a blinded manner by a veterinary pathologist. Major organs were also weighed and where indicated, blood was taken to assess hematology. Dose-limiting toxicity endpoints were defined as described above.

Efficacy evaluation

Tumor growth delay evaluations were conducted using either orthotopic breast carcinoma (MDA-MB-231) or subcutaneous colon carcinoma (HCT-116 or HT-29) xenograft models in female Balb/c *nu/nu* mice essentially as described earlier [27,29]. Once the tumor volumes reached approximately 100 mm^3, the animals were administered saline, FD, or 5-FU:FA either by fractionated i.p.b. injection or by i.v.b. injection as described above except that the treatments were given once per week for 4 weeks. Animals were weighed, clinical signs monitored, and tumor dimensions (length and diameter) measured regularly for calculation of tumor volume as described earlier [27]. Endpoints were as outlined above with the addition of tumor size greater than 15 × 15 mm or that impeded movement.

Pharmacokinetic and biodistribution analysis

Intraperitoneal bolus assessment

Female Balb/c mice were administered a single i.p.b. subtoxic dose of 450 mg/m^2 of either 5-FU or FD(HP) containing 2.5 μCi of 5-FU [6–^{3}H] (Moravek, Brea, California, USA), and then killed at 5, 10, 20, 30, 60, 180, 360, and 1440 min after the injection. Blood was immediately collected in anticoagulant tubes and plasma samples were removed after centrifugation. Major organs (i.e. liver, spleen, and kidneys) were collected, rinsed in PBS and weighed before taking samples for whole tissue solubilisation (Solvable, Perkin-Elmer) and β-scintillation counting using the Ultima-Gold scintillation fluid (Perkin-Elmer, Waltham, Massachusetts, USA) and a Packard TriCarb Liquid scintillation counter. All samples were bleached with hydrogen peroxide to reduce coloration and hence quenching.

Intravenous bolus assessment

Female New Zealand White rabbits were administered a single i.v.b. dose of either 400 mg/m^2 of FD(HP) or 5-FU:FA containing 50 μCi 5-FU [6–^{3}H]. For each rabbit, both ears were shaved to expose ears for injection or blood sample collection. One ear was assigned as the administration ear and received a single i.v.b. dose. The other ear was used to collect serial blood samples (500 μl) in anticoagulant tubes, approximately 24 h before administration of the dose and at various timepoints after administration. After centrifugation, the plasma samples were taken for radioactivity counting as described above. After the final blood sampling, which was taken 24 h after administration of the dose, the administration ear was assessed for phlebitis as outlined below.

For both i.p.b. and i.v.b. assessments, pharmacokinetic parameters were calculated using standard noncompartmental methods based on radioactivity measurements in the plasma samples.

Assessment of phlebitis

The injection site of the administration rabbit ear described above was examined for the degree of erythema from 0 (no erythema) to 4 (severe erythema) after which the ears were photographed. The rabbits were then euthanised and tissue specimens located at 3–10 mm from the injection site were removed. Specimens were fixed in 10% formalin, blocked, and 5-μm sections were prepared. Tissue sections were examined for histopathological changes and were graded for the following: loss of venous endothelial cells (degree 1–3, 0 = no loss), vascular thrombosis (degree 1–3, 0 = no thrombosis), perivascular inflammation (degree 1–3, 0 = no inflammation), oedema near the vein (degree 1–3, 0 = no oedema), epidermal degeneration near the vein (degree 1–3, 0 = no degeneration). All examinations were performed in a blinded manner by an independent veterinary histopathologist.

Statistical analysis

All graphs were created and statistical analyses performed using the GraphPad Prism software (Version 5.1, San Diego, CA, USA). Fisher's exact test was applied to compare all the macroscopic findings of the treatment groups relative to the positive control groups. Tumor volumes and weight changes over time were compared using a one-way analysis of variance with the Tukey posttest. Tumor weights were compared using a Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

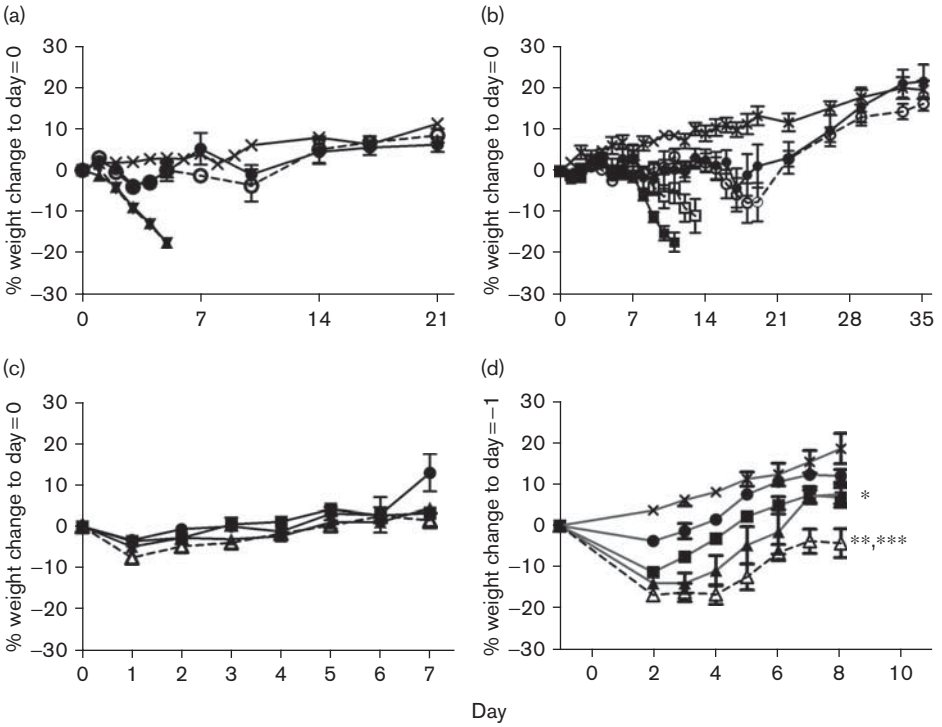
Results

Dose–tolerance evaluation of Fluorodex

Intraperitoneal bolus assessment in mice

A dose-dependent increase in toxicity was only observed in animals administered with FD or 5-FU:FA (15 : 1) as

Fig. 1



Dose–tolerance relationship for escalating-dose administration of FD compared with 5-fluorouracil (5-FU):folinic acid (FA). (a) Single intraperitoneal bolus (i.p.b.) dose administration in mice. Pooled saline control (×, black line, $n=16$), 600 mg/m^2 FD(S) (●, black line, $n=3$) and 5-FU:FA (○, black dashed line, $n=3$), 675 mg/m^2 FD(S) (■, black line, $n=6$) and 5-FU:FA (□, black dashed line, $n=6$). (b) Multiple/fractionated i.p.b dose administration in mice ($n=6$ for all cohorts). Saline control (×, black line), 120 mg/m^2 × 5 FD(S) (●, black line) and 5-FU:FA (○, black dashed line), 180 mg/m^2 × 5 FD(S) (■, black line) and 5-FU:FA (□, black dashed line). (c) Single intravenous bolus (i.v.b.) dose administration in rats ($n=6$ for all cohorts). 425 mg/m^2 (●, black line), 475 mg/m^2 (■, black line) and 525 mg/m^2 (▲, black line) FD(HP), and 525 mg/m^2 5-FU:FA (△, black dashed line). (d) i.v.b. plus intravenous infusion (over 48 h) dose administration in rats ($n=8$ for all cohorts, equal numbers of males and females). Saline control (×, black line), 400 + 600 mg/m^2 FD(HP) (●, black line), 400 + 1200 mg/m^2 FD(HP) (■, black line), 400 + 2400 mg/m^2 (▲, black line) FD(HP), and 400 + 2400 mg/m^2 5-FU:FA (△, black dashed line). All values shown are mean ± SEM. *$P<0.05$ for saline control versus FD(HP) at the two highest doses. **$P<0.01$ for saline control versus 5-FU:FA. ***$P<0.05$ for FD(HP) at all doses versus 5-FU:FA.

either a single i.p.b. dose of greater than 600 mg/m^2 or as a fractionated i.p.b. dose of greater than 120 mg/m^2 × 5 (within 14 days) compared with the saline controls (Fig. 1a and b, data shown for FD(S) only). No damage to the livers, kidneys, or spleens at 7, 14, and 21 days after i.p.b. administration of ≤ 600 mg/m^2 FD or 5-FU:FA was noted by histopathological analysis of hematoxylin and eosin-stained and oil red-O-stained sections (data not shown) confirming the tolerability and hence No observed adverse effect level of FD at these doses by this route of administration. Five of the six FD-treated and six of the six 5-FU:FA-treated mice reached toxicity endpoints by day 5 of observation after a single i.p.b. administration of 675 mg/m^2 dose (Fig. 1a). FD and 5-FU:FA at 180 and 240 mg/m^2 × 5 i.p.b. resulted in at least three of the six animals in each cohort reaching toxicity endpoints before administration of the ultimate (i.e. only 4 × 180 mg/m^2 achieved) and penultimate (i.e. 3 × 240 mg/m^2 achieved) doses, respectively. Therefore, the maximum tolerated dose of 5-FU administered either as 5-FU:FA or within FD lies between 600 and 675 mg/m^2 of 5-FU given either as single or fractionated i.p.b. doses over 2 weeks.

Intravenous assessment in rats

Given the above findings and that the human tolerability of i.v.b. administration of 5-FU is approximately 500 mg/mg^2 [4,5,30], escalating-dose i.v.b. administration of FD(HP) was compared with the high dose-matched 5-FU:FA positive controls in rats in this range. No overt signs of toxicity, as indicated by weight changes and other clinical signs, were associated with the administration of single i.v.b. doses of 425, 475, and 525 mg/mg^2 FD or with 525 mg/mg^2 5-FU (after i.v.b. administration of 35 mg/mg^2 FA) (Fig. 1c). Although the macroscopic tissue analyses found that the 5-FU group exhibited a significantly higher ($P < 0.05$, Fisher exact test) frequency of liver enlargement, cardiac hypertrophy, mesenteric lymph enlargement, and colon/cecum distension when compared with the animals administered FD at all the doses tested, no abnormal histological changes were observed. Thus, as FD (and 5-FU) at 525 mg/m^2 did not cause toxicity in this study, the NOAEL for FD is 525 mg/m^2 when administered as a single i.v.b. dose.

Next, FD was administered to male and female rats as a single i.v.b. dose of 400 mg/m^2 followed by continuous

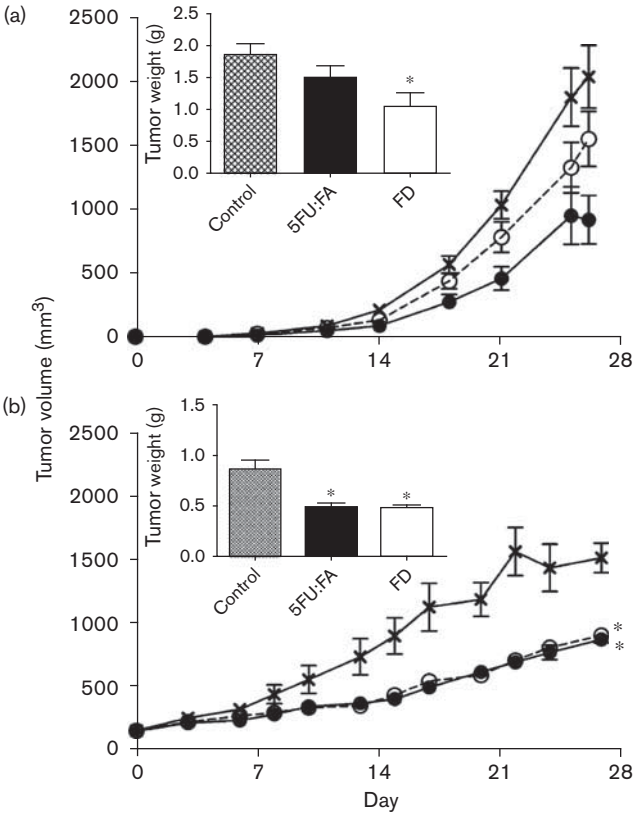
i.v.i. over 48 h at doses ranging from 600 to 2400 mg/m^2 to mimic a commonly used clinical schedule of 5-FU administration [3]. A dose-dependent increase in toxicity was observed in both the male and female rats who were administered FD, which manifested as a significant reduction in body weight compared with the controls (Fig. 1d). This was accompanied by an increase in the moderate-to-severe clinical signs and a number of changes in urine parameters during infusion, most of which normalised after infusion, except that persistent proteinuria was most evident in the male rats given the highest dose of FD. Otherwise, no sex differences were noted. Changes in hematology and organ weights indicated that FD at the highest dose induced toxicity in the bone marrow and possibly spleen, manifested particularly by reductions in the white cell and platelet counts (see Supplementary data, Tables S1 and S2 *http://ro.uow.edu.au/scipapers/177/*). Several volume-matched controls were used in this study apart from the controls with saline buffered to the exact pH of the FD batch used (i.e. pH 7.3) including controls with saline buffered to pH 9 (to match the pH of the 5-FU solution) and hydroxypropyl β-CD (pH 7.3). All controls gave similar results confirming that these components of the formulation did not contribute to toxicity (data not shown). Thus, on the basis of all of these parameters, the NOAEL for FD with this regimen was an i.v.b. dose of 400 mg/m^2 followed by an i.v.i dose of 1200 mg/m^2 over approximately 2 days.

As a comparator, an i.v.b. dose of FA (26.7 mg/m^2) followed by an i.v.b. dose of 5-FU (400 mg/m^2) and subsequent i.v.i. of 5-FU (2400 mg/m^2 over 2 days) was assessed. The rats in this treatment cohort lost significantly more weight than all the other FD cohorts (Fig. 1d). In general, the majority of parameters measured showed that FD given at this maximum tolerated dose produced less severe general toxicity, hematological, and urinary changes than 5-FU when administered under a similar dosing regimen.

In-vivo efficacy of Fluorodex compared with 5-fluorouracil:folinic acid

Initial studies using sub-maximum tolerated dose (i.e. < 600 mg/m^2 given as a fractionated dose) i.p.b. administration of drugs in both an orthotopic breast carcinoma and ectopic CRC xenograft model established using cell lines with known responsiveness to 5-FU [26] showed low-to-negligible (Fig. 2a) (data not shown) responsiveness to 5-FU:FA control treatments. The low responsiveness of the these tumor models to 5-FU:FA may be because of the dose schedule and route of administration used. Nevertheless, in the breast carcinoma model, a reduction in tumor growth was observed in the FD(S)-treated cohort as compared with the saline control cohort from day 7 until death at day 26 (Fig. 2a). This difference was reflected in the final tumor weights at the end of the experiment, which were significantly lower than the tumors excised from the saline control cohort (Fig. 2a inset). There was no significant difference in the tumor growth rates between the saline control and the 5-FU:FA cohorts (Fig. 2a). A similar trend, though not significant, was seen in this model using the same dosing regimen of FD(HP) and 5-FU:FA compared with the saline control cohort (data not shown).

Fig. 2



Efficacy assessment of Fluorodex (FD) compared with 5-fluorouracil (5-FU):folinic acid (FA) in mice (a) bearing orthotopic human breast carcinomas and (b) bearing subcutaneous human colorectal carcinomas. (a) MDA-MB-231 cells were injected on day = −10, 120 mg/m^2 was given intraperitoneal bolus on days 0, 4, 7, 11, and 14 (i.e. to mimic the maximum tolerated dose fractionated i.p.b. dose schedule). Tumor volume of saline control (×, black line), 5-FU:FA (○, black dashed line) and FD(S) (●, black line) cohorts. Inset, weight of tumors after excision at the end of the experiment. Values shown are mean ± SEM, n = 10, (from a representative experiment) *P < 0.05 for control versus FD. (b) HT-29 cells were injected on day = −12, 225 mg/m^2 was given intravenous bolus on days 0, 7, 14, and 21. Tumor volume of saline control (×, black line), 5-FU:FA (○, black dashed line) and FD(HP) (●, black line) cohorts. Inset, weight of tumours after excision at the end of the experiment. Values shown are mean ± SEM, n = number commencing study/number surviving at the end of the observation period, were saline control = 6/10, 5-FU:FA = 15/20 and FD 19/20. *P < 0.05 for control versus FD and control versus 5-FU:FA.

In contrast to the i.p.b. dosing regimen, in another ectopic CRC xenograft model there was significant inhibition of tumor growth by i.v.b. doses of 225 mg/m^2 FD(HP) and 5-FU:FA treatments administered once per week for 4 weeks compared with the saline control, as indicated by a reduction in both the tumor growth rate

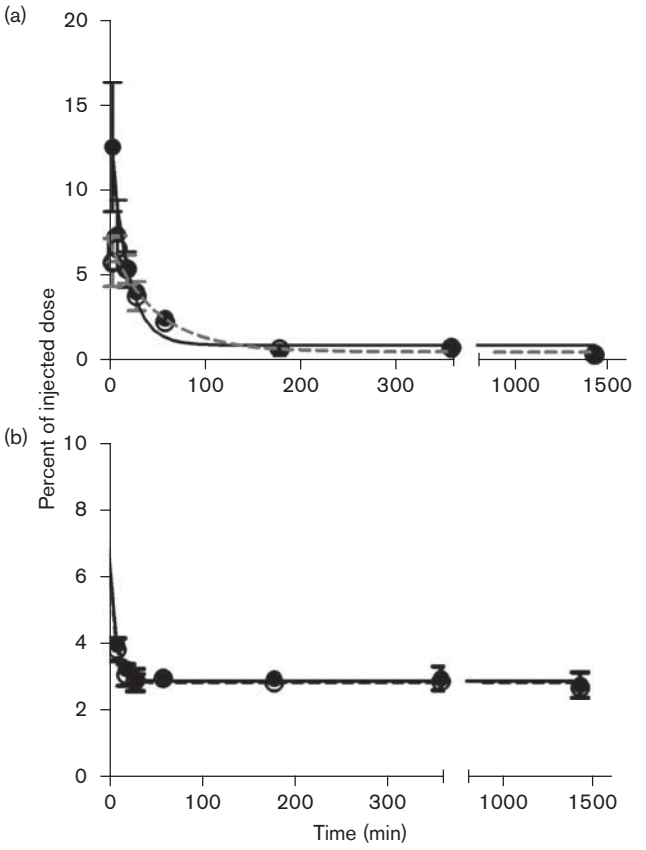
and tumor weight excised at the end of the observation period (Fig. 2b). There was no significant difference between either the tumor growth rates or excised tumor weights between the FD(HP) and 5-FU:FA treatment groups (Fig. 2b). However, 5-FU:FA seemed to be less well tolerated than FD(HP) using this dosage schedule, as only 75 versus 95%, respectively, of the animals survived till the end of the observation period as a result of reaching toxicity endpoints (excessive weight loss) rather than tumor size endpoints. Furthermore, all the mice in the 5-FU:FA treatment groups presented with transient loss of skin elasticity (indicating dehydration) and decreased body condition, 3–5 days after the initial treatment, which was resolved up to 9 days later.

Biodistribution and pharmacokinetics – comparison of route of administration

Bioequivalence analyses were carried out using FD(HP) and 5-FU:FA containing 5-FU [6-^{3}H]. The radioactivity versus time profiles obtained from the plasma samples after i.p.b. administration in mice (Fig. 3a) or i.v.b. administration in rabbits (Fig. 3b) were virtually superimposable for FD(HP) and 5-FU over the 10–1440 min sampling period, with both the treatment groups exhibiting biphasic pharmacokinetic profiles (i.e. an initial decay phase of rapid drug distribution followed by a slow decay phase of drug clearance). Although the majority of radioactivity was eliminated by 180 min in the mice after i.p.b. administration (Fig. 3a), radioactivity in both the 5-FU and FD(HP) treatment groups was still measurable in rabbit plasma 24 h after i.v.b. dosing (Fig. 3b). The reason for the latter phenomenon is unclear. Thus, pharmacokinetic parameters were calculated for the distribution phase only (Fig. 3c), as no meaningful values could be calculated for the elimination phase because of the radioactivity measurements remaining relatively unchanged over time.

In mice, the maximum concentration (C_{max}) of radioactivity in the plasma of the FD(HP) group was higher and reached maximum earlier as compared with the 5-FU group ($T_{max} = 0.083$, vs. 0.167 h, respectively) (Table 1).

Fig. 3



Pharmacokinetic profiles of Fluorodex [FD(HP)] versus 5-fluorouracil (5-FU):folinic acid (FA) in (a) mice and (b) rabbits. Mice and rabbits were given 400–450 mg/m^2 FD(HP) (●, black line) or 5-FU:FA (○, black dashed line) spiked with 5-FU [6–^{3}H] by intraperitoneal bolus or intravenous bolus administration, respectively. Animals were killed at the timepoints shown and plasma samples collected for analysis of radioactivity. All values are corrected for sample size, total plasma volume, and amount of injected dose and are presented as mean ± SEM percent of injected dose [$n = 4$ (mice), $n = 3$ (rabbits) per timepoint].

Table 1 Comparison of pharmacokinetic parameters for 5-FU and FD(HP) in the plasma of mice and rabbits after i.p.b. and i.v.b. dosing, respectively

	Mice (i.p.b.)		Rabbits (i.v.b.)	
Pharmacokinetic parameters	5-FU/FA	FD(HP)	5-FU/FA	FD(HP)
$T_{1/2}$ (h)[a]	0.42 (±0.05)	0.44 (±0.09)	0.86 (±0.05)	0.69 (±0.07)
K_{elim} (per hour)	1.72 (±0.19)	1.75 (±0.35)	0.82 (±0.08)	1.02 (±0.19)
Correlation coefficient	0.949	0.951	0.940	0.992
AUC (dpm h/ml)[b]	667 099 (±19804)	762 705 (±52206)	742 630 (±14468)	672 320 (±14518)
T_{max} (h)	0.167	0.083	0[c]	0
C_{max} (dpm)[d]	246 565 (±14492)	540 527 (±82252)	–	–

Values were calculated from data shown in Fig. 3.
Values shown are mean ± SEM.
FA, folinic acid; FD, Fluorodex; 5-FU, 5-fluorouracil; i.p.b, intraperitoneal bolus; i.v.b, intravenous bolus.
[a]$T_{1/2}$ (elimination half-life) and K_{elim} (elimination rate constant) values were calculated for the distribution phase only (i.e. from 10 to 30 min).
[b]AUC (area under the curve) was calculated over the entire sampling period: 5–1440 min for mice and 10–1440 min for rabbits.
[c]T_{max} (the time required to reach C_{max})=0 for intravenous administration.
[d]C_{max} (maximum plasma concentration).

From 0.167 h (10 min) onwards, however, there was no significant difference in the plasma radioactivity levels between the groups. The difference in T_{max} and C_{max} after i.p.b. (extravascular) administration in mice suggests faster absorption of 5-FU [6-^{3}H] from the peritoneal cavity when part of the FD(HP) formulation. Indeed, there was a lack of a measurable absorption phase observed in the FD(HP) group, even though the first blood sample was taken 5 min after administration. It is possible that as 5-FU is administered as a highly alkaline solution, it may partly crystallise within the i.p. site because of the large pH drop when injected into an area of physiological pH, whereas 5-FU within FD(HP) should resist precipitation as it is formulated to physiological pH [26].

The plasma radioactivity half-lives for 5-FU or FD(HP) were not significantly different after either route of administration and were in the range of 0.4–0.8 h (Table 1). In humans, 5-FU has a relatively rapid half-life, between 0.2 and 0.3 h after intravenous administration [31,32]. Other pharmacokinetic parameters (Table 1) showed no significant differences between 5-FU [6-^{3}H] when administered alone or as part of the FD(HP) formulation.

Very similar profiles were also obtained for 5-FU and FD(HP) in the kidneys and spleen within the first 60 min after i.p.b. administration (Fig. 4a and c). Although liver uptake of 5-FU [6-^{3}H] within FD(HP) seemed to be slower than for 5-FU alone (Fig. 4b) in the first 30 min after administration, these apparent differences were not statistically significant.

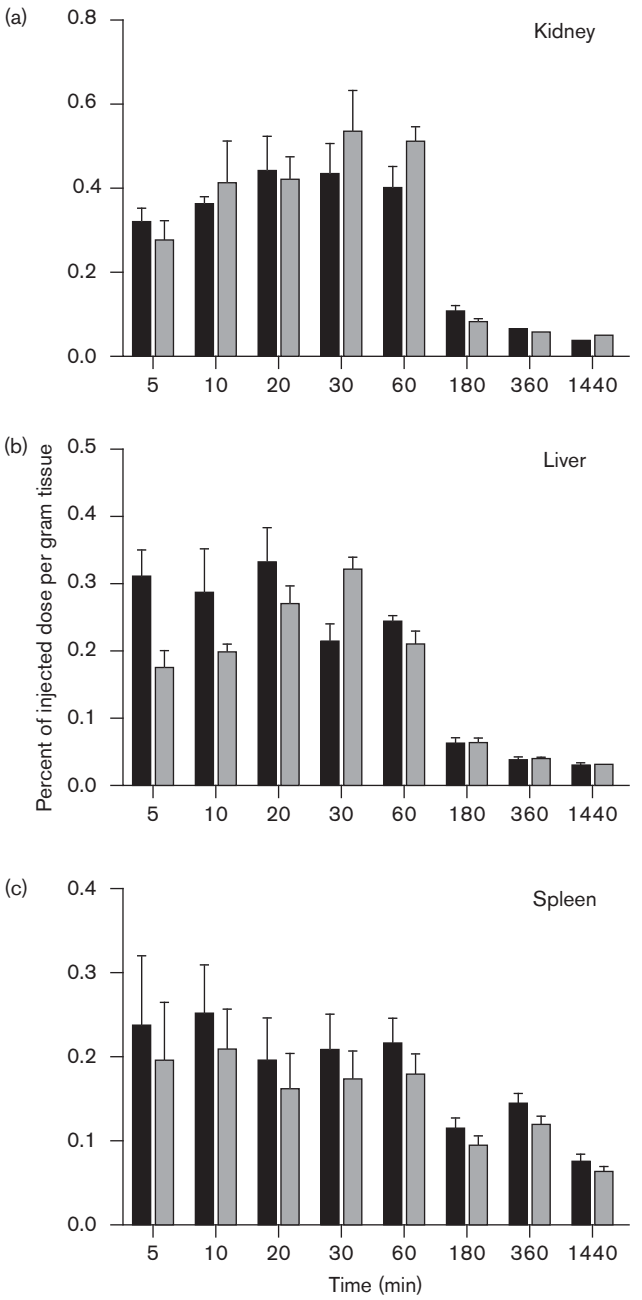
To determine whether there were any differences in tumor uptake of 5-FU [6–^{3}H] between 5-FU and FD(HP), one cohort of mice ($n = 4$) bearing human breast carcinoma xenografts were killed 1 h after i.p.b. administration of 450 mg/m^2 5-FU or FD containing 2.5 μCi 5-FU [6–^{3}H]. The mean amount of radioactivity in the tumors ranged from 0.7 to 1% of the injected dose/g with no significant differences observed between these cohorts (data not shown).

In summary, these studies suggest that plasma clearance, organ biodistribution, and tumor uptake profiles of 5-FU either in FD(HP) or alone, are essentially superimposable.

Fluorodex reduces symptoms of phlebitis

After i.v.b. administration of 400 mg/m^2 FD(HP) into veins of rabbit ear, there was less severe erythema around the injection site relative to that produced by the same dose of 5-FU:FA. This observation is highlighted by the striking macroscopic difference in the ear vein after administration of 5-FU:FA (Fig. 5a) compared with FD(HP) (Fig. 5b). When graded for the degree of erythema, all three rabbits in the 5-FU cohort suffered mild-to-moderately severe (grade 1–3) erythema, whereas all three rabbits in the FD(HP) cohort experienced mild-to-no erythema (grade 0–1).

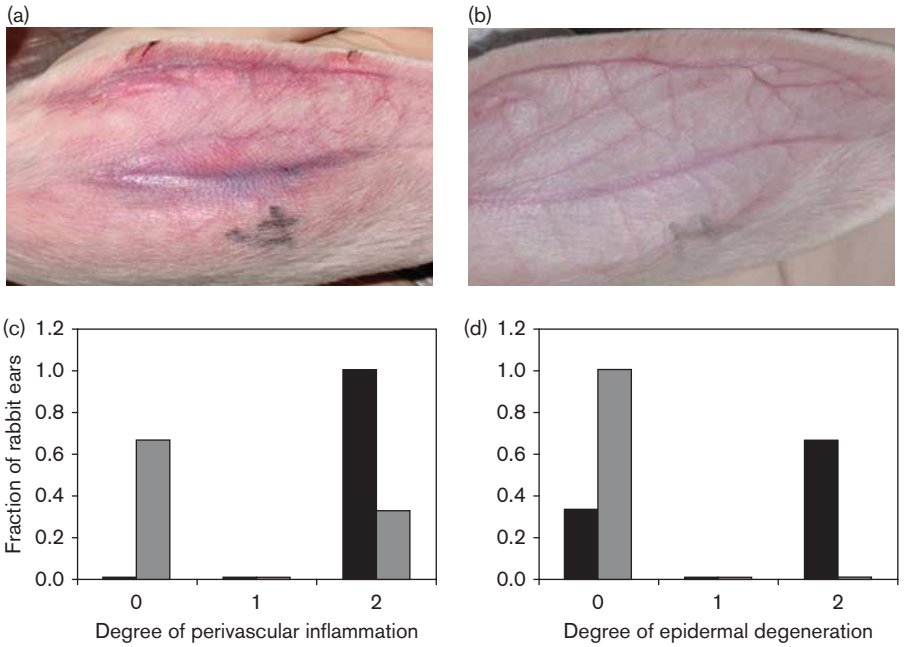
Fig. 4



Tissue distribution of Fluorodex [FD(HP)] versus 5-fluorouracil (5-FU):folinic acid (FA) over time. Mice were administered 450 mg/m^2 5-FU:FA (black bars) or FD(HP) (gray bars) spiked with 2.5 μCi 5-FU [6-^{3}H] by a single intraperitoneal bolus injection. Animals were killed at the timepoints shown and the tissues indicated were immediately collected and radioactivity counted after processing. All values are corrected to activity per gram of tissue and are presented as mean ± SEM percent of injected dose ($n = 4$ per time point).

Overall, perivascular inflammation in the ear veins of the FD(HP)-treated rabbits seemed to be less severe than for the corresponding 5-FU:FA-treated rabbit ear veins as determined by histopathological examination (Fig. 5c).

Fig. 5



Fluorodex [FD(HP)] reduces phlebitis in rabbit ear veins. Photographs of the representative ear veins of the rabbits that were administered i.v.b. doses of (a) 5-fluorouracil (5-FU):folinic acid (FA)or (b) FD(HP) at 450 mg/m^2. (c) and (d) Graphical representation summarizing the observed changes in the stated histopathological indicators of sections of ear veins taken proximal to the injection site taken from the 5-FU:FA (black bars) or FD(HP) (gray bars) treatment groups. Scoring reflects absence (grade=0) to marked (grade=2) changes in indicators. None of the rabbits exhibited extreme changes (grade=3) in these indicators. No differences in edema or superficial dermal inflammation were observed between the 5-FU and FD(HP) cohorts, which were graded as minimal to moderate in both the cohorts. No loss of venous endothelial cells or vascular thrombosis was noted for either treatment group.

Epidermal degeneration was not present in the ear veins from the FD(HP)-treated rabbits, but it was present in two of the three rabbit ear veins administered with 5-FU:FA (Fig. 5d). Thus, FD(HP) does not seem to induce the associated macroscopic or histopathological changes at the site of administration that were produced by 5-FU:FA.

Discussion

5-FU is one of the most commonly used anticancer agents in the world today. Although many advances in treatment have been made incorporating 5-FU (including modifications in administration routes, treatment regimens, and cotherapies), these improvements are hindered by the problematic administration of 5-FU. We have developed a solution to this longstanding clinical problem by exploiting the properties of β-CDs to produce novel, all-in-one formulations at physiological pH that deliver the synergistic components of 5-FU and FA. Both FD(S) and FD(HP) produced, using either polysulfated or hydroxypropyl β-CD derivatives, respectively, result in decreased toxicity profiles while maintaining or improving antitumor efficacy. As hydroxypropyl β-CD lacks heparin-like anticoagulant properties, unlike the polysulfated β-CD [26], and is a US Food and Drug Administration-approved excipient [33], FD(HP) was chosen as the formulation to take forward through additional safety and bioequivalence evaluations as required for future phase 1 trials as it thus incorporates three already-approved entities. Our studies also confirmed that FD(HP) is bioequivalent in terms of pharmacokinetics and biodistribution profiles to 5-FU. This is a favorable property for gaining regulatory acceptance of reformulated drugs. Given the association between thrombosis and cancer [34], however, the potentially beneficial anticoagulant activity of the polysulfated β-CD-based formulations FD(S) may constitute a useful ingredient in an anticancer formulation, and warrants further characterization.

The lead FDs were formulated to contain low-dose FA as several studies have shown no clinical advantage of using standard high (200 or 500 mg/m^2) or standard low (20 mg/m^2) FA [30,35–37]. However, as reported earlier, the amount of FA in FD can be varied to match the various dose ratios that are used in the clinic without adversely affecting the stability or effectiveness of the formulation [26]. Nevertheless, although infusion of 500 mg/m^2 FA was found to significantly enhance the cellular reduced folate levels in the primary CRC and liver metastases samples removed 90 min after infusion compared with infusion of 20 or 200 mg/m^2 FA [38], a recent survey of Australian physicians treating CRC found that equal numbers choose low versus high FA versus a fixed 50 mg bolus dose of FA [39].

As noted earlier, there are varied methods of 5-FU:FA administration, most of them involving a combination of 5-FU i.v.b. and infusion after a bolus or short infusion (2 h) of FA. Free folates, after FA administration, reach peak serum levels within 10 min of injection and are cleared within 6.2 h [40]. The circulating 5-FU half-life is around 8–20 min [32]. This means that most of the folates would be cleared before 5-FU administration is complete, especially in the case of protracted (12 + h) infusion. The ability to continuously co-deliver 5-FU and FA during extended infusion may thus result in increased efficacy. Indeed, simultaneous administration using a single-port catheter of high-dose 5-FU and FA has been reported to increase the response rate by 58% in CRC given as a first-line treatment using a weekly 24 h i.v.i. [41]. However, 11 of the 22 patients studied had catheter blockages because of calcium carbonate formation and could not complete their treatment [12]. This underscores the potential for enhanced efficacy in the clinic with the FDs, as catheter blockages should be negated. It is also conceivable that FDs with higher concentrations of FA may improve efficacy compared with 5-FU in a clinical setting, although altering the 5-FU:FA ratio made little difference to cytotoxicity measured *in vitro* [26].

There is no consistency in the literature regarding tumor models to successfully test new 5-FU regimens [7,42] with many reports showing low-to-no responsiveness to 5-FU in animal models. There are numerous factors that may contribute to this, including cell line selection, period of tumor cell inoculation, and route of administration or dosing schedules [43,44]. Indeed, although the tumor xenografts were equally and effectively responsive to both 5-FU:FA and FD by i.v.b. administration, they were relatively unresponsive to 5-FU:FA by i.p.b. administration, with the breast carcinoma xenografts showing some responsiveness whereas the CRC xenografts were nonresponsive. This was not because of acquired 5-FU resistance *in vivo*, as the tumor cell lines established from the tumors passaged through mice were still sensitive to 5-FU (and thus FD) *in vitro* (data not shown). Reassessment of efficacy against colorectal xenografts established from the tumor cell lines passaged through mice, using higher doses (i.e. 120 mg/m^2 × 5), still showed no difference in tumor growth between the 5-FU:FA, FD and control cohorts. However, FD generated a significant improvement in efficacy against the breast carcinoma xenografts using this route of administration compared with 5-FU:FA, suggesting some differential sensitivity.

An underrecognized problem of 5-FU administration is the significant level of 5-FU related cardiotoxicity observed. This seems to be dosage and schedule dependent, occurring in 1.6–3.0 versus 7.6–18.0% of patients after bolus or continuous infusion, respectively [45]. There is thus a fine balance between toxicity and efficacy outcomes when comparing treatment schedules, as evidence suggests continuous infusion is more efficacious [3]. Although FD could potentially be administered using both the methods in the clinic, used as a bolus treatment, this all-in-one formulation may overcome the associated harshness of commercial 5-FU solutions. FD may therefore reinvigorate bolus clinical usage of 5-FU [46], and hence decrease the occurrence of cardiotoxicity. In addition, earlier reports [47] linked 5-FU cardiotoxicity to the presence of fluoroacetaldehyde impurities that form under the basic (∼pH 9) conditions of commercial 5-FU formulations. After administration, fluoroactealdehyde is metabolized to fluoroacetate, a highly cardiotoxic compound. The formation of fluoroacetaldehyde is less likely in the neutral pH conditions of the FD formulations, thereby eliminating this potential cause of 5-FU-induced cardiotoxic events. Although no direct analysis of FD for fluoroacetaldehyde has been undertaken as yet, long-term stability studies (12 months) on FD have indicated excellent 5-FU stability [26].

FD formulations overcome the problems of delivery of admixtures of 5-FU and FA, allowing streamlined administration and reduced incidence and severity of phlebitis. This should also translate to reduced injection pain and phlebitis for patients, and minimize the nursing time and level of patient intervention, as the occurrence of catheter blockage and their invasive and expensive replacement would also be reduced [48]. FDs could also improve efficacy outcomes by virtue of the fact that patients stop treatment because of these administration side effects. By improving the conditions for i.v. use, FD also provides clinicians with a potential alternative to oral 5-FU, which has associated problems including variable bioavailability, patient compliance and overcompliance [49], and increased occurrence of the hand–foot syndrome [22,49,50]. Furthermore, pharmacokinetically guided dose adjustment with infusional 5-FU based on area under the curve determination has been shown in large multicenter trials to improve the therapeutic index of 5-FU:FA compared with the body surface area-guided dosing while reducing drug-related toxicity [10,46]. It has been suggested that this method of dose adjustment may be as effective clinically as the more expensive options FOLFOX or FOLFORI, and thus infusional 5-FU:FA may be preferred over the oral forms [10]. Given that very similar area under the curve values were obtained for 5-FU either alone or within FD by both intraperitoneal and intravenous administration, this advantage is not lost by such reformulation of 5-FU. It should also be noted that any advantages of oral 5-FU (such as ease of administration or economic advantages) are lost if it is prescribed with drugs that require parenteral administration [11].

In conclusion, we believe that the FD formulations represent a viable, superior injectable form of 5-FU:FA. To the best of our knowledge, FD will be a 'first in class' anticancer drug-biomodulator as 'all-in-one' treatments are not currently available nor are any known to be in development.

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