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RESEARCH PAPER

Fasting protects against the side effects of irinotecan treatment but does not affect anti-tumour activity in mice

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BACKGROUND
The main limitation to the use of irinotecan in the treatment of colorectal cancer is the severity of side effects, including neutropaenia and diarrhoea. Here, we explored the effects of 3 days of fasting on irinotecan-induced toxicities, on plasma, liver and tumour pharmacokinetics and on anti-tumour activity in mice.

EXPERIMENTAL APPROACH
Male BALB/c mice received C26 colon carcinoma cells subcutaneously. They were randomized 1:1 into equally sized *ad libitum* fed and fasted groups after which they were treated with irinotecan. Weight and adverse side effects were recorded daily. At the end of the experiment, tumours were resected and weighed, and concentrations of irinotecan and its active metabolite SN-38 were determined in plasma and tumour.

KEY RESULTS
Fasting prevented the diarrhoea and visible signs of discomfort induced by irinotecan. *Ad libitum* fed animals developed leucopenia compared with untreated controls, whereas fasted mice did not. Irinotecan suppressed tumour growth equally in both treated groups, compared with untreated controls. Levels of the active irinotecan metabolite SN-38 9 (calculated as AUC values) were significantly lower in fasted mice in both plasma and liver, but not in tumour tissue.

CONCLUSIONS AND IMPLICATIONS
Fasting protected against irinotecan-induced side effects without interfering with its anti-tumour efficacy. Fasting induced a lower systemic exposure to SN-38, which may explain the absence of adverse side effects, while tumour levels of SN-38 remained unchanged. These data offer important new approaches to improve treatment with irinotecan in patients.

Abbreviations
CES, carboxylesterase; DR, dietary restriction; IGF-1, insulin-like growth factor 1; PK, pharmacokinetic; SN-38, 7-ethyl-10-hydroxycamptothecin (active metabolite of irinotecan); SN-38G, SN-38 glucuronide; UGT1A, uridine diphosphate glucuronosyltransferase 1A

Tables of Links

TARGETS
Enzymes
Topoisomerase 1

LIGANDS
Irinotecan
SN-38

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

Colorectal cancer is the second most prevalent cancer, with more than 1.2 million new cancer cases and over 600 000 deaths per year (Jemal *et al.*, 2011). About 15–20% of patients at initial presentation have liver metastases, and an additional 45% are diagnosed with metastases in follow-up after resection of the primary tumour (Adam, 2007). A potent anti-cancer agent in first-line and second-line treatment for unresectable and metastatic disease is irinotecan (CPT-11), at present the most widely used topoisomerase-I inhibitor. Irinotecan can produce severe and unpredictable haematological, intestinal and systemic toxicities, including profound myelosuppression, massive diarrhoea, vomiting, fatigue and in some cases even death as a complication of the other side effects (Rowinsky *et al.* 1994; Rothenberg 1998; Kim and Innocenti 2007; Ramchandani *et al.* 2007). It is generally accepted that this toxicity is mediated by the active metabolite of irinotecan, 7-ethyl-10-hydroxycamptothecin (also known as SN-38) (Mathijssen *et al.* 2001). Importantly, irinotecan is one of the chemotherapeutic drugs that has been reported to generate high levels of oxidative stress (Conklin 2004), leading to failure of normal cellular functions and may contribute to the toxicities already discussed (Chen *et al.* 2007).

Fasting is a powerful means to increase resistance against acute stressors such as oxidative stress. We have previously shown that preoperative fasting and dietary restriction (DR) protected against acute oxidative damage induced by ischaemia–reperfusion injury in both kidney and liver (van Ginhoven *et al.* 2009; Mitchell *et al.* 2010; Van Ginhoven *et al.* 2010b; Verweij *et al.* 2011). In addition, preoperative DR reduced hepatic tumour load and the hepatic expression of the endothelial cell specific adhesion molecule, E-selectin (van Ginhoven *et al.* 2010a). It has been suggested that fasting protects normal cells by rearrangement of energy into maintenance pathways instead of reproduction and growth (Raffaghello *et al.* 2008; Lee *et al.* 2012). In this way, DR could protect against toxic side effects of anti-cancer drugs, but how this affects pharmacokinetics and anti-tumour activity is still largely unknown.

Therefore, in this study, we have examined the effects of fasting prior to administration of a high dose of the chemotherapeutic agent irinotecan on the occurrence of side effects and anti-tumour activity in mice bearing the C26 colon carcinoma-. In addition, to elucidate the mechanism involved, we have examined the pharmacokinetics of irinotecan in plasma, liver and tumour of mice, following 3 days of fasting.

Methods

Animals

All animal care and experimental protocols complied with the 1986 directive 86/609/EC of the Council of Europe and were approved by the Animal Experiments Committee, under the Dutch National Experiments on Animals Act. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.* 2010; McGrath *et al.* 2010).

Male inbred strains of mice (BALB/c) of 6–8 weeks old – weighing approximately 25 g – were obtained from Charles River, Maastricht, The Netherlands. Upon arrival, animals were housed at random in individually ventilated cages (n = 3–4 animals per cage) in a licensed biomedical facility at Erasmus University Medical Center, Rotterdam, The Netherlands. Standard laboratory conditions were maintained, with temperature ~ 22 °C, humidity ~50% and a 12 h light/12 h dark cycle. All mice had free access to watr and food (Special Diet Services, Witham, UK) unless mentioned otherwise. Animals were allowed to acclimatize for 1 week before the start of the experiments.

Fasting protocol

Mice in the *ad libitum* fed groups were allowed unrestricted access to food. Before the start of the experiment, all mice were transferred to a clean cage and, in the fasting groups, food was withheld for 3 days starting at 16:00 h. All animals were given continuous access to water.

C26 colon carcinoma cells

The murine colon carcinoma cell line C26 was kindly provided by Dr. R. Schiffelers (Utrecht Medical Centre, The Netherlands). The C26 cell line originally derived from the BALB/c mouse and was cultured in DMEM (Sigma Aldrich, St. Louis, MO, USA), supplemented with 10% foetal calf serum (Lonza, Verviers, Belgium), penicillin (100 units mL^{-1}) and streptomycin (100 units mL^{-1}) (Invitrogen, Auckland, New Zealand) at 37 °C in a 5% carbon dioxide environment (Sato *et al.* 1981). Cells were collected by brief trypsinization (0.05% trypsin in 0.02% EDTA). For s.c. injection, cells were collected and, after centrifugation, single-cell suspensions were prepared in PBS to a final concentration of 5.0×10^5 cells per 100 μL. Cell viability was determined by Trypan blue staining and was always ≥90%.

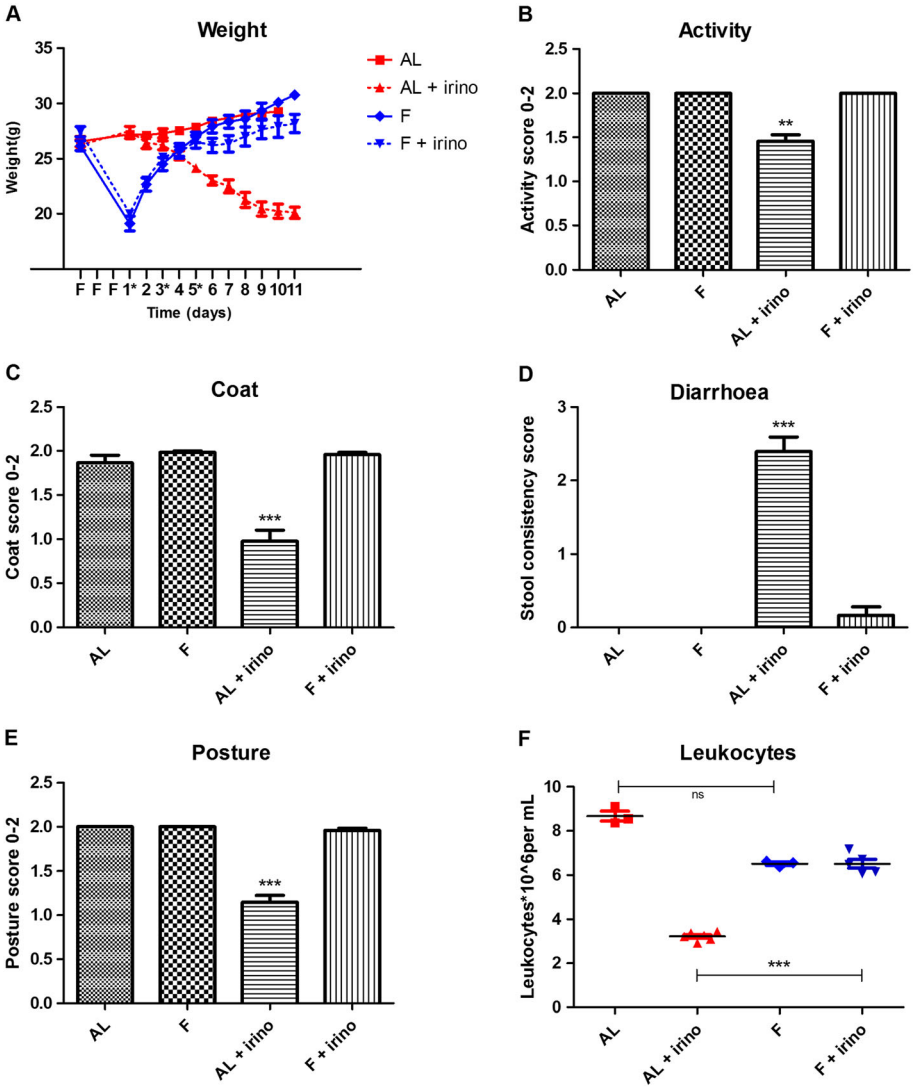

Figure 1

Protective effects of fasting against irinotecan toxicity. A total of 24 mice, six mice per group, were observed for adverse side effects for 10 days after the first irinotecan injection; irinotecan was given i.p. on days 1, 3 and 5 after fasting (shown by *). (A) Effect of fasting on body weight. Fasted (F) mice lost about 20% of their body weight during 3 days' fasting. During irinotecan treatment, *ad libitum* (AL) fed mice showed 20% weight loss, while fasted mice gained weight and reached their starting weight at day 5. (B–E) Effects of fasting on activity (B); coat (C); diarrhoea (D) and the posture of the mice (E). Data shown are means ± SEM ($n = 6$). **$P = 0.003$, *** $P < 0.001$, significantly different from (F + irino) group; one-way ANOVA with Bonferroni's correction. (F) Effect of fasting on circulating leukocyte numbers, measured on day 8 after the first irinotecan injection. Fasting alone did not affect leukocyte levels. The mice fed *ad libitum* and treated with irinotecan (AL+ irino) showed leukopenia compared with fasted mice treated with irinotecan (F + irino). *** $P < 0.001$, significantly different as indicated; one-way ANOVA with Bonferroni's correction.

Chemotherapy

Irinotecan, HCl trihydrate 20 mg mL^{-1} (Hospira Benelux, Antwerp, Belgium) was used for *in vivo* experiments. The irinotecan was diluted in sodium chloride 0.9% (Braun, Melsungen, Germany) to a final volume of 200 μL per injection and was given i.p. Optimal drug doses were determined in a pilot experiment (Supporting Information Fig. S1).

Effect of fasting on irinotecan-induced side effects

A total of 24 mice were anaesthetized with isoflurane inhalation (2%). Body temperature was maintained by placing the mice on heating pads. A lateral incision on the right flank was made to implant a tumour cube, derived from mice carrying C26 tumours subcutaneously. All mice received 0.05 mg kg^{-1} buprenorphine (Temgesic, Schering Plough, Houten, The Netherlands) via s.c. injection before the implantation of tumours. Tumour cubes measured approximately 15 mm^3 and were implanted at least 4 mm away from the incision site. Mice were randomly divided into four groups ($n = 6$ per group): group 1, *ad libitum* fed group receiving vehicle treatment; group 2, fasting group receiving vehicle treatment; group 3, *ad libitum* fed group receiving irinotecan; and group 4, fasting group receiving irinotecan treatment. The irinotecan treatment was given i.p., after

3 days of fasting or *ad libitum* food, on days 1, 3 and 5, as three equal injections amounting to a cumulative, weight-adjusted dose of 400 mg kg^{-1} (see Fig. 1A). The control groups received vehicle treatment (sodium chloride 0.9%). From the first irinotecan injection, mice were weighed and inspected daily for adverse side effects using a mouse well-being score protocol adapted from the Guidelines for welfare of animals in experimental neoplasia research (UK Co.-ordinating Committee on Cancer Research, 1988). Assessing well-being by one researcher took approximately 10 min per cage with four mice. Side effects were scored independently by two experienced researchers. Mouse cages were removed from racks and placed on a bench to facilitate visualization of the mice, but cages were not opened at any point during the scoring process, except for the determination of the stool consistency at the end of the visual examination. Mouse activity level was scored according to the amount each mouse moved in its cage. A score of 2 indicates that an animal moved around the cage normally. A score of 1 indicates that an animal was moving slowly or less frequently and with an altered gait. A score of 0 indicated that an animal was not moving and was taking no more than five steps. The appearance of the coat was scored according to the smoothness. A score of 2 indicated a healthy, smooth uninterrupted coat. A score of 1 indicated a slightly fluffy coat. A score of 0 indicated a severely fluffy coat with obvious gaps with visible skin. Severity of diarrhoea was assessed according to the stool consistency score (0: normal, 1: loose stool, 2: loose/some diarrhoea, 3: diarrhoea and 4: severe watery diarrhoea) (Fitzpatrick *et al.* 2011). Before every stool consistency measurement, clean white tissues were placed at the bottom of the cage to allow determination of the consistency of the stool. Posture was scored as follows. A score of 2 indicated a normal body posture. A score of 1 indicated a moderately hunched posture. A score of 0 indicated a severely hunched posture. Results are expressed as mean ± SEM. Leukocyte numbers were determined on day 8 after the first irinotecan injection with a Z series Coulter Counter (Beckman Coulter, Woerden, The Netherlands). Ten days after the first irinotecan injection, mice were killed by exsanguination. Tumours were resected and weighed.

Effects of fasting on irinotecan pharmacokinetics

A total of 54 mice were anaesthetized with isoflurane inhalation (2%). The right lateral flank was shaved for precise injection. A suspension of C26 cells (5.0×10^5 cells in a volume of 100 μL) were injected, using a 21 G needle. Growth kinetics following C26 tumour cube implantation were similar to growth after injection of C26 cells (Supporting Information Fig. S2). Tumours were allowed to grow for 14 days before the start of the experiment. The mice were randomly divided into three groups (n = 18 per group). Two groups were fasted for 3 days, and one group was fed *ad libitum*. After the fasting period, mice were fed *ad libitum* again. The *ad libitum* fed group and one group of fasted animals were treated with a single weight-adjusted dose of 100 mg kg^{-1} (±2.5 and ±2.0 mg, respectively) of irinotecan i.p. The other fasted group received a flat-fixed dose. This dose contained the same concentration as in *ad libitum* fed mice (2.5 mg), which were approximately 20% heavier compared with the fasted mice. The flat-fixed-dose group was included to provide an alternative dosing to correct for bodyweight loss in fasted animals, especially during the first irinotecan injection. At 1, 4, 8, 12, 24 and 48 h after irinotecan injection, mice were killed by exsanguination, and 100 μL plasma, 100 μg liver and 100 μg tumour were collected. Each time point included three mice per group.

Tissue pretreatment

Tumour and liver samples were obtained and kept frozen at −70 °C until used. Tissues were diluted in human plasma (1:4 *w*/*v*) into a 2 mL Eppendorf tube. Hereafter, a 5 mm stainless steel bead (Qiagen, Venlo, The Netherlands) was added, and the samples were homogenized with a Tissuelyser (Qiagen, Venlo, The Netherlands) and processed for 4 min at 40 Hz. Beads were removed, and homogenized samples were stored at −70 °C until analyses. Homogenized tissue samples were further processed as plasma samples.

Determination of irinotecan, SN-38 and SN-38 glucuronide concentrations

Total irinotecan and total SN-38 were quantified using a validated method involving reversed-phase HPLC with fluorescence detection as described earlier (de Bruijn *et al.* 1997; Sparreboom *et al.* 1998; de Bruijn *et al.* 2004). All frozen samples were thawed at 4 °C and were homogenized by vortex mixing. A 250 aliquot of plasma was mixed for 10 min with 500 μL 5% perchloric acid–methanol (1:1, *v*/*v*) in 1.5 mL polypropylene tubes (Eppendorf, Hamburg, Germany). After centrifugation for 5 min at 18 000 × *g*, 250 μL of the clear supernatant was mixed with 250 μL phosphate buffer. Hereafter, 100 μL was injected into the HPLC system. Chromatographic analysis was performed using an Agilent® HPLC system 1100 series (Agilent Technologies, Santa Clara, CA). Chemstation software was used for data monitoring and analysis. Separation of the compounds was achieved using an Inertsil ODS 80A (4.6 mm × 150 mm, 5 μm particle size) analytical column. The analytical column was maintained at a temperature of 50 °C. A gradient at a flow rate of 1.00 mL min^{-1} was achieved with mobile phase A, composed of 0.1 M ammonium acetate containing 0.01 M tetra-butylammonium sulphate and mobile phase B composed of methanol. A linear gradient was used, with 67–50% mobile phase A, from 0 to 25 min, followed by 50–10% mobile phase A for 5 min. This was succeed by a linear gradient back to 67% mobile phase A from 30 to 31 min, which was held for 2 min to re-equilibrate. The column eluent was monitored fluorometrically at an excitation wavelength of 375 nm and an emission wavelength of 460 nm for detection of irinotecan and an excitation wavelength of 380 nm and an emission wavelength of 532 nm for the detection of SN-38. Calibration curves were computed using the ratio of the peak height of irinotecan or SN-38 versus the nominal concentration, with a lower limit of detection of 5.00 ng mL^{-1} for irinotecan and 0.500 ng mL^{-1} for SN-38. Uridine diphosphate glucuronosyltransferase 1A (UGT1A) inactivates SN-38 by conversion to its glucuronide form, SN-38 glucuronide (SN-38G). SN-38G in plasma samples was quantified by the amount of SN-38 released following treatment for 3 h at 37 °C with 100 U of *Escherichia coli* β-glucuronidase.

Concentrations from 48 h samples were below the detection limit, and therefore, we report data only from 0 to 24 h sampling points.

Real-time quantitative reverse-transcription PCR

RNAwas isolated from liver and tumour tissues collected at 1, 8 and 12 h after irinotecan injection from the *ad libitum* fed and fasted groups (flat-fixed dose) using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To avoid genomic DNA contamination, RNA was purified by a DNAse treatment (RQ1 RNase-Free DNase; Promega, Madison, WI, USA). RNA was then reverse transcribed into cDNA using random primers (Invitrogen) and Superscript II RT (Invitrogen). Gene expression was analysed by real-time quantitative PCR using an Applied Biosystems 7700 PCR machine (Foster City, CA, USA). Reverse-transcription PCR was performed for carboxylesterase 2 (CES2) forward, 5-ggccatgtgtctgcaaaatc-3, and reverse, 5-caccatcacaggcaggttag-3. GAPDH was used as a housekeeping gene. Each sample was tested in triplicate. ΔCt values were normalized to the average ΔCt of the *ad libitum* fed group. The fold change was calculated using the Pfaffl equation, $2 - \Delta\Delta Ct$.

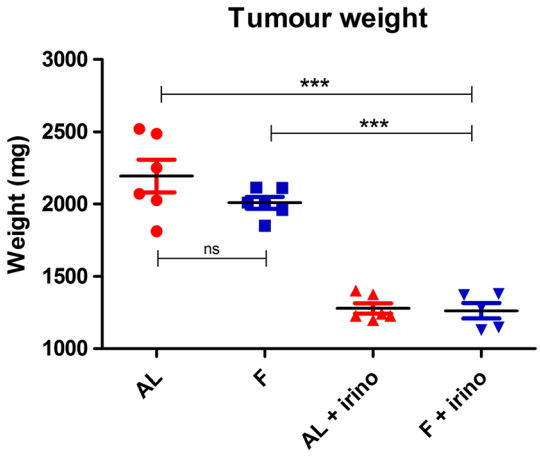

Figure 2

Effect of irinotecan treatment on tumour growth in fasted and *ad libitum* fed groups. At the end of the experiment, irinotecan-treated tumours were significantly smaller in both the fasted (F) and *ad libitum* (AL) fed groups compared with untreated controls. Data shown are means ± SEM ($n = 6$). ***$P < 0.001$, significantly different as indicated; one-way ANOVA with Bonferroni's correction. Fasting alone had no significant effect on tumour weight.

Data analysis

For each set of variables, means ± SEM were computed. All standard statistical tests were performed using SPSS version 21 for Windows software (Statistical Package for Social Sciences, Chicago, IL, USA), and $P < 0.05$ was considered to show significant differences between means. One-way ANOVA was used to assess whether fasting significantly altered side effects with the Bonferroni correction applied to correct for multiple testing. To analyse the pharmacokinetic (PK) data, Phoenix WinNonlin version 6.1 (Certara USA Inc., St. Louis, MO, USA) software was used. A plasma concentration-time curve for 0-24 h was generated by joining up the highest values at each time and the AUC for that curve calculated. Similarly, the lowest values for each time provided the lowest curve with its corresponding AUC. The remaining (intermediate) values then generated an intermediate curve with its derived AUC. Student's *t*-test was used to determine statistical differences.

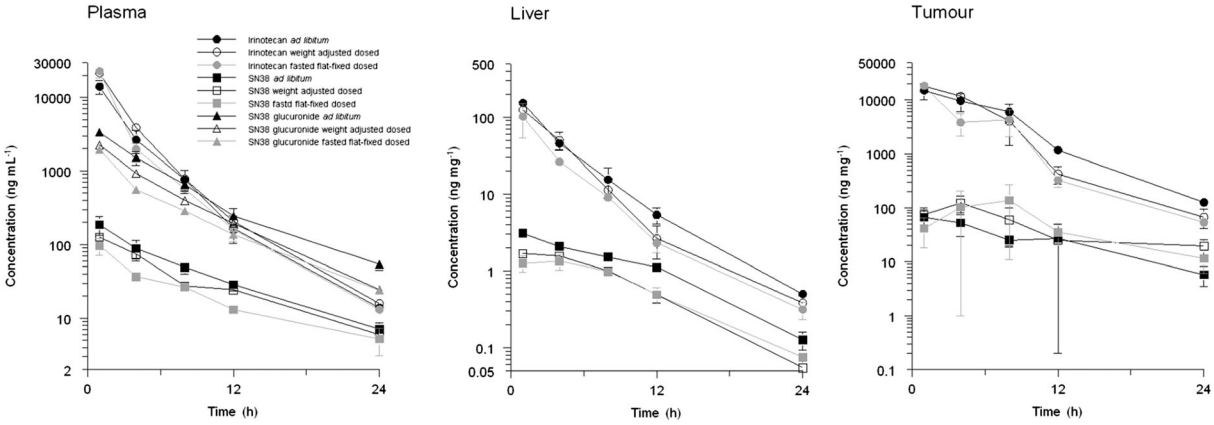

Figure 3

Pharmacokinetics of irinotecan (circles) and SN-38 glucuronide (triangles) and SN-38 (squares) in plasma, tumor and liver. Concentration-Time curves are presented for ad libitum fed groups (black symbols), fasted weight-adjusted dosed (white circles) and fasted flat-fixed dosed (grey squares). Fasted weight-adjusted dosed and fasted flat-fixed dosed animals show a trend towards higher plasma irinotecan AUC values, while irinotecan AUC values in the liver and tumor are lower in fasted animals. SN-38 AUC values are lower in both fasted groups in plasma and liver, and SN-38G AUC values are lower in plasma. SN-38 AUC values in tumor tissue show large variation.

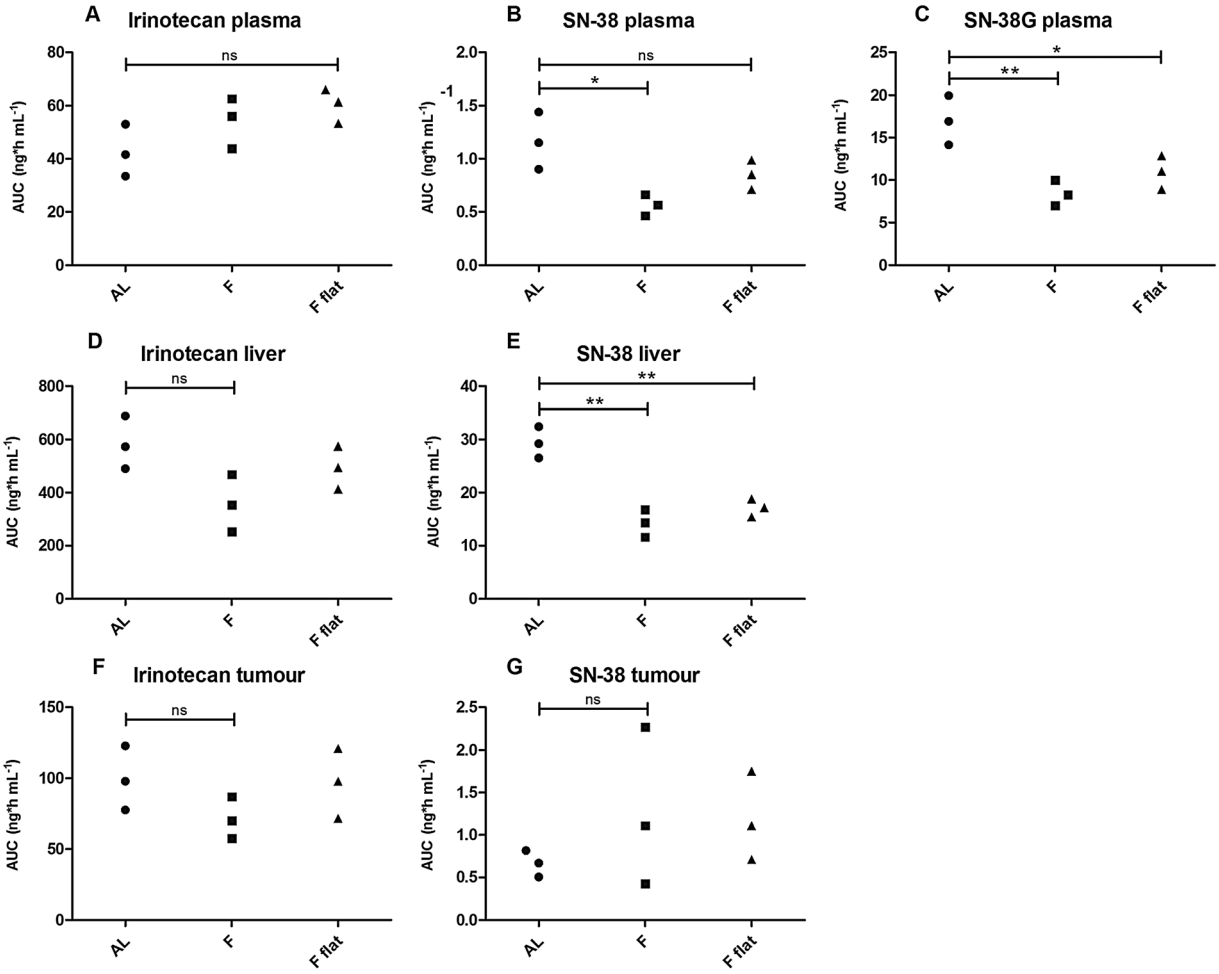

Figure 4

Statistical validation of pharmacokinetic differences. Data shown are the AUC values for each experimental group. No significant differences were found for irinotecan AUC values between *ad libitum* (AL) fed, weight-adjusted dosed fasted (F) and flat-fixed dosed fasted (F flat) groups in plasma (A + B), liver (D + E) and tumour (F + G). SN-38 AUC values in plasma (B) were significantly lower in weight-adjusted dosed fasted animals (**P* = 0.02), and SN-38G AUC values in plasma (C) were significantly lower in weight-adjusted dosed fasted animals and fasted flat-fixed dosed animals **P* = 0.04, ***P* = 0.01. SN-38 AUC values in the liver (E) were significantly lower in both fasted groups ***P* = 0.003. No significant differences were seen in SN-38 AUC values for tumour tissues; *P* = 0.27. *n* = 3; differences as indicated; Student's *t* test.

Results

To study the effect of fasting on irinotecan toxicity, we first treated *ad libitum* fed mice with different doses of irinotecan. Using cumulative doses of 500, 600 and 800 mg kg^{-1}, given in three doses over 5 days all mice died within 11 days from the first injection (Supporting Information Fig. S1). Using a cumulative dose of 400 mg kg^{-1}, also given over 5 days, mice experienced serious adverse effects like diarrhoea, hair loss and a ruffled coat but survived and recovered within the 15 day observation period. Therefore, we decided to use the cumulative dose of 400 mg kg^{-1} in our first experiments.

Fasting protects against the adverse side effects of irinotecan and does not affect anti-tumour activity

To examine the effects of fasting before irinotecan administration on the occurrence of irinotecan-induced side effects and anti-tumour effect in C26 colon carcinoma-bearing mice, we compared 3 day fasted with *ad libitum* fed mice. In the *ad libitum* fed group, mice showed weight loss from the first irinotecan injection, while the fasted mice gained weight during the treatment and observation period (Figure 1A).

From day 4 after the first irinotecan injection, other side effects were observed in the *ad libitum* fed group. They were less active ($P = 0.003$), had a ruffled coat ($P < 0.001$), suffered more from diarrhoea ($P < 0.001$) and showed a hunchback posture ($P < 0.001$) (Figure 1B-E). The fasted mice showed none of these visible side effects. On day 8 after the first irinotecan injection, the fasted group had a significantly higher blood leukocyte count than that from the *ad libitum* group ($P < 0.001$) (Figure 1f). No animals died during the experimental period (15 days) in irinotecan treated groups. At the end of the experiment, all tumours were resected and weighed. Irinotecan-treated tumours were significantly smaller in both fasted and *ad libitum* fed groups, compared with untreated controls ($P < 0.001$). Fasting alone had no significant effect on tumour growth (Figure 2).

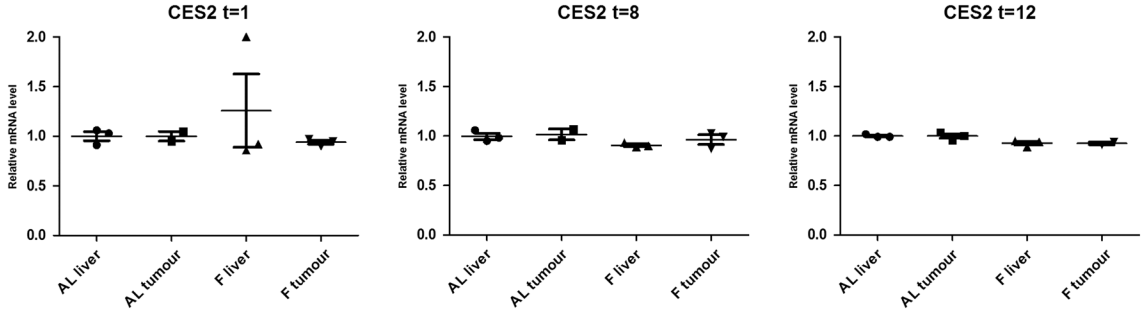

Figure 5

CES2 mRNA expression in liver and tumour tissue from *ad libitum* fed and fasted mice. CES2 expression at 1 (t = 1), 8 (t = 8) and 12 h (t = 12) after irinotecan injection in liver and tumour tissue from *ad libitum* (AL) fed and fasted (F) animals did not reveal any significant differences.

Taken together, these results show that 3 days of fasting prior to treatment with a high dose of irinotecan prevented many of the side effects but did not modify its anti-tumour effects.

Fasting reduces the systemic exposure to SN-38

To elucidate the mechanism of fasting-induced resistance against its side effects, we examined the pharmacokinetics of total irinotecan and its active metabolite SN-38 in plasma, liver and tumour tissue of 3 day fasted and *ad libitum* fed mice. Furthermore, SN-38G concentrations were measured in plasma to indicate the activity of UGT1A, the major metabolizing enzyme for SN-38 (Figure 3).

To express differences between AUCs more clearly and to perform statistics on these curves, figures were computed where the highest, intermediate and lowest AUC values were plotted (Figure 4A-G). Note that because the levels of SN-38 and SN-38G at 48 h were too low for reliable measurements, the AUC for these substrates is reported as $AUC_{0\text{-}24}$. The AUC values of the active metabolite SN-38 were significantly lower in plasma and liver in fasted animals compared with that from *ad libitum* fed animals, while in the tumour, SN-38 AUC values did not differ between fasted and *ad libitum* fed animals.

Plasma AUC values of irinotecan were not significantly different between the three groups, although there was a trend towards higher AUC values in the fasted groups (Figure 4A). For SN-38, the AUC values were reduced, by 51%, in the weight-adjusted dosed fasting group (mean $AUC_{0\text{-}24}$ 0.56 vs. 1.15 mg*h mL^{-1}) but not significantly in the flat-dosed fasting group, compared with *ad libitum* fed controls (Figure 4B). However, the AUC for SN-38G was reduced, by 51% and 35%, in the weight-adjusted dosed fasting group and the flat-dosed fasting group, respectively, compared with *ad libitum* fed controls (mean $AUC_{0\text{–}24}$ 8.3 vs. 16.9 μg * h mL^{-1}, P = 0.01, and $AUC_{0\text{–}24}$ 11.0 vs. 16.9 μg * h mL^{-1}, P = 0.04, Figure 4C).

In the liver, there were no differences between the AUC values for irinotecan, from the three experimental groups (Figure 4D) but for the active metabolite SN-38, both the weight-adjusted dosed fasting group (mean $AUC_{0\text{–}24}$ 15.5 vs. 29.2 ng * h mg^{-1}, P = 0.003; 47% reduction) and the flat-dosed fasting group (mean $AUC_{0\text{–}24}$ 17.1 vs. 29.2 ng * h mg^{-1}, P = 0.003, 41% reduction), showed lower AUC values than those from the *ad libitum* fed group (Figure 4E).

In the tumour tissue samples, no differences were found between AUCs for irinotecan in the *ad libitum* fed controls and the two fasted groups (Figure 4F). Interestingly, SN-38 AUC values in tumour tissue, in contrast to those in plasma and liver, showed no significant changes in the fasted groups, compared with the *ad libitum* fed controls (Figure 4G), only a trend towards increased values. Although these increases were not significant, our results imply that the intratumour SN-38 concentrations did not decrease after fasting.

These data demonstrated that, although irinotecan AUC values were not changed in fasted animals, the AUC values for SN-38 and its glucuronide were significantly lower in plasma and liver tissue from fasted animals, indicating that 3 days of fasting prior to irinotecan administration induced an important change in its metabolism and lowered the systemic exposure to the cytotoxic metabolite SN-38.

Carboxylesterase-2 (CES2) in liver and tumour

Conversion of irinotecan to SN-38 occurs mainly in the liver and is catalysed by the CES enzymes. Fasting is known to affect liver function and size and, therefore, hepatic CES and its metabolism of irinotecan may be changed during fasting. We measured levels of CES2 by its mRNA expression at 1, 8 and 12 h, after irinotecan injection in liver and tumour tissue from *ad libitum* fed and fasted animals. There were no significant differences in CES2 expression levels between *ad libitum* fed and fasted groups in liver and tumour tissue (Figure 5).

Discussion

The experiments presented in the current study showed that 3 days of fasting prior to treatment with a high dose of irinotecan prevented the occurrence of drug-related side effects while anti-tumour efficacy was not affected. To elucidate the mechanisms behind the fasting-induced protection from irinotecan-induced side effects, we studied the pharmacokinetics of this drug in fasted and fed mice. We found no differences in irinotecan AUC values between fasted and *ad libitum* fed animals and demonstrated that the AUC of the active and

toxic metabolite of irinotecan, SN-38, was lower in plasma and in liver tissue from fasted animals. Furthermore, AUC values for the SN-38 metabolite, SN-38G, were significantly lower in plasma from fasted animals. The AUC values for SN-38 in tumour samples in fasted mice remained unchanged. These data indicated that fasting induced important changes in irinotecan metabolism and lowered the systemic exposure to irinotecan and SN-38.

A remarkable finding in this study was that the 3 day fasted BALB/c mice did not show any signs of toxicity in response to a high dose of irinotecan. In contrast, animals regained the weight that was lost during the fasting period, approximately 20%, during the three days of high-dose irinotecan treatment, while control animals lost approximately 20% of their bodyweight during both irinotecan exposure and the subsequent observation period. It is currently thought that fasting induces a hormetic response. This is a common biological phenomenon in which exposure to a low-intensity stressor induces a general adaptive response that has beneficial effects, including protection against different types of stress (Mattson 2008a, 2008b). We observed relevant differences in irinotecan pharmacokinetics between *ad libitum* fed and fasted mice. In plasma from fasted mice, there was a trend towards higher AUC values of irinotecan compared with *ad libitum* fed mice, while SN-38 AUC values in plasma from fasted mice were clearly lower compared with those in *ad libitum* fed groups. In the liver, there was a trend towards lower AUC values of irinotecan in fasted mice, and AUC values of SN-38 were significantly lower in fasted groups compared with those in *ad libitum* groups. SN-38 is approximately 100–1000-fold more cytotoxic than its pro-drug, irinotecan (Mathijssen *et al.* 2001). Irinotecan is converted to SN-38 primarily in the liver. Catalysed by CES enzymes, but in patients, this conversion is rather inefficient (only 2–5%) when irinotecan is injected intravenously (Cao *et al.* 2006; de Jong *et al.* 2006). Therefore, it is thought that intratumour *CES* expression is responsible for the anti-tumour effect in patients (Xu *et al.* 2002). We found reduced levels of SN-38 in liver and plasma of fasted mice, but *CES* expression in liver and tumour tissue was not altered in these mice. Tumour cells have the same if not higher levels of SN-38, but *CES* expression was not higher in tumour cells of fasted mice. This may indicate that tumour cells of fasted mice are taking up more SN-38 rather than that metabolism by CES is higher in fasted mice. The glucuronide synthase UGT1A is capable of inactivating SN-38 by conversion to its glucuronide form SN-38G, leading to its excretion from the circulation (Di Paolo *et al.* 2006). We showed significantly lower SN-38G AUC values in plasma from fasted mice, indicating that the lower amount of SN-38 in plasma was not due to increased UGT1A activity.

In an attempt to explain the differential effects of fasting on the systemic level versus tumour tissue, the term 'differential stress resistance' has been coined (Raffaghello *et al.* 2008; Longo and Mattson 2014). Drug metabolism can be largely divided into three phases: phase I (redox and hydrolysis), phase II (conjugation) and phase III (transport). Diet and nutrient signalling pathways have important effects on phase I enzymes (Mathijssen *et al.* 2014). These enzymes consist primarily of cytochrome P450 superfamily proteins (Yang *et al.* 1992) and phase II proteins including enzymes regulated by Keap1-Nrf2 (Kohle and Bock 2007). During fasting, significant modulation of these pathways occurs in healthy tissue, but not in tumour tissue. We have previously shown that 3 days of fasting up-regulates canonical stress resistance pathways such as Nrf2, 'xenobiotic metabolism' and effector genes such as haem-oxygenase I, superoxide dismutase and glutathione-*S*-transferase (Mitchell *et al.* 2010; Jongbloed *et al.* 2014). These data may partly explain the concept of differential stress resistance, which predicts that these pathways are differentially regulated by fasting, in healthy compared with tumour tissue. How this affects pharmacokinetics and anti-tumour efficacy has remained unknown. We hypothesized that during fasting, cells in the liver (among others) up-regulate stress resistance pathways, which decreases conversion of irinotecan, whereas in the tumour, these pathways are unaffected. This supposition is strengthened by the fact that SN-38 AUC values remained unchanged in tumours of fasted mice.

Fasting is known to down-regulate insulin-like growth factor-1 (IGF-1) levels (Mitchell *et al.* 2010), which has a plethora of downstream effects that are likely to be linked to the protective effect induced by fasting. Many intrinsic factors may influence the PK of irinotecan (Mathijssen *et al.* 2001) and IGF-1 has been related to the outcome of combined treatment with cetuximab + irinotecan in humans (Scartozzi *et al.* 2010). However, to the best of our knowledge, IGF-1 has never been related to the PK of irinotecan *in vivo* and potential interactions between IGF-1 and irinotecan PK cannot be determined from the results presented here.

In earlier studies, protective effects against toxic agents have also been observed when animals were subjected to long-term restriction of food intake (DR), rather than fasting, which is the total absence of food. Mice subjected to 5–8 months 40% DR were significantly more resistant against paraquat toxicity than *ad libitum* fed mice (Sun *et al.* 2001). Rats allowed to eat only 65% of their normal intake for 3 weeks showed increased resistance to thioacetamide due to increased liver tissue repair mechanisms (Apte *et al.* 2003). Moreover, rats that were 40% calorically restricted for 4 weeks showed increased resistance against bleomycin-induced DNA damage (Aidoo *et al.* 1999). We have shown that the beneficial effect of DR on acute stress resistance can be induced rapidly, as 3 days of fasting protected against oxidative damage induced by renal ischaemic injury, similar in magnitude to 2–4 weeks of 30% DR (Mitchell *et al.* 2010). On a systemic level, we found an overlapping transcriptional response, indicating that short-term fasting and long-term DR induce potentially overlapping mechanisms. Furthermore, an *ad libitum* diet deficient in protein or amino acids protected mice in a surgical ischaemia–reperfusion injury model (Peng *et al.* 2012). More recently, a study in dogs with lymphoma that were treated with doxorubicin showed that 24 h of fasting immediately before administration resulted in significantly lower incidence of vomiting (Withers *et al.* 2014).

Implementation of DR and fasting in the clinic is still laborious. We have shown that preoperative DR and fasting are feasible and safe in surgical patients (van Ginhoven *et al.* 2009, 2011). Furthermore, women at risk for breast cancer showed reduced inflammation and oxidative stress during a diet of 2 days fasting per week (Harvie *et al.* 2011). Asthma patients maintained for 8 weeks on an alternate day fasting diet exhibited less clinical symptoms, pulmonary functions were improved and oxidative stress and inflammation declined during the diet (Johnson *et al.* 2007). Based on the existing evidence from

animal and human studies, there is great potential in translating these results to clinical cancer patients. In a self-reported study of patients with a variety of malignancies, a voluntarily fasting regimen prior to (48–140 h) and/or following (5–56 h) chemotherapy resulted in a decrease of common side effects and increased subjective well-being (Safdie *et al.* 2009). These data suggest that fasting is safe and feasible and might offer important new opportunities to improve chemotherapy treatment in cancer patients. However, development and completion of randomized controlled clinical trials are essential in fulfilling its clinical potential.

It is known that ectopic and orthotopic tumour environments can respond differently to a variety of treatments. However, in a recent study using FabplCre;Apc15lox/+ mice, which spontaneously develop intestinal tumours, fasting reduced tumour growth, as it did in *ad libitum* fed mice and protected against the side effects of irinotecan (Huisman *et al.* 2015). However, the results from this orthotopic tumour model do not directly prove that differential alteration of PK is the primary mechanism underlying protection from side effects and this possibility will need further investigation. We have shown here that fasting protected against the side effects of one cycle of irinotecan treatment. It remains unclear if cancer cells are sensitized or not by multiple cycles of fasting/irinotecan treatment in this experimental model. However, a recent study suggested that multiple cycles of fasting induced differential stress sensitization in a range of tumours (Lee *et al.* 2012).

In summary, the present study showed that short-term fasting protected against the adverse side effects of irinotecan in C26 colorectal carcinoma-bearing mice without interfering with anti-tumour activity. This reduction of side effects may have been the result of the lower systemic exposure to SN-38 in fasted, compared with *ad libitum* fed mice. When confirmed in clinical trials, fasting before irinotecan treatment may offer important new opportunities to improve this treatment for patients with colorectal carcinoma.

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Author contributions

S.A.H., P.dB., and I.M.G.M-H. performed the research, and collected the data. S.A.H.,J.N.M.I.J., E.A.W., R.H.J.M. and R.W.F.dB. designed the study and analysed and interpreted the data. S.A.H., R.H.J.M. and R.W.F.dB. wrote the paper.

Conflict of interest

The authors declare no conflicts of interest. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agency.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Survival of *ad libitum* fed mice treated with different doses of irinotecan. *Ad libitum* fed mice (n = 6 per group)

were treated on days 1, 3 and 5 after fasting with a cumulative dose of 400, 500, 600 or 800 mg kg^{-1} irinotecan (shown by *). Using 500, 600 and 800 mg kg^{-1} irinotecan, all animals had to be killed within 14 days, because of the severity of side effects induced by the irinotecan treatment. With a dose of 400 mg kg^{-1}, all mice survived and recovered from the side effects of irinotecan.

Figure S2 Tumour surface of *ad libitum* fed mice implanted with tumour cubes or injected with tumour cells. *Ad libitum* fed mice (n = 6 per group) were implanted with C26 tumour cubes (approximately 15 mm^3) or injected with 5.0×10^5 C26 tumour cells, on day 0. On day 5 after injection, tumours were macroscopically visible in the group injected with cells and showed growth kinetics, similar to those of the tumour cubes, over the subsequent period of implantation. On the day of harvesting the tumours (days 21 or 25), both groups had a similar tumour surface ($P > 0.05$).