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ORIGINAL ARTICLE

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Novel fluoro-substituted camptothecins: in vivo antitumor activity, reduced gastrointestinal toxicity and pharmacokinetic characterization

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Abstract *Purpose*: The novel fluoro-substituted camptothecin analog, BMS-286309, and its prodrug, BMS-422461, were evaluated for their pharmacologic, toxicologic, metabolic and pharmacokinetic developmental potential. *Methods*: In vitro and in vivo assays were used to assess the compounds for topoisomerase I activity, antitumor activity, gastrointestinal (GI) toxicity, and pharmacokinetic parameters. *Results*: BMS-286309-induced topoisomerase I-mediated DNA breaks in vitro and was similar in potency to camptothecin. Both BMS-286309 and −422461 were comparable to irinotecan regarding preclinical antitumor activity assessed in mice bearing distal site murine and human tumors. BMS-422461 was also found to be orally active. Both analogs were >100-fold more potent in vivo than irinotecan and both were superior to irinotecan with respect to toxicological assessment of GI injury in mice. The generation of parent compound from BMS-422461 was qualitatively similar in mouse, rat and human blood and liver S9 fractions. The percentage of BMS-286309 remaining as the active lactone form at equilibrium was comparable in mouse and human plasma. The pharmacokinetic profile in rat blood demonstrated that BMS-422461 was rapidly cleaved to BMS-286309. *Conclusions*: The favorable in vivo metabolic activation of BMS-422461, and the pharmacokinetic characteristics of BMS-286309, suggest that the good efficacy of BMS-422461 is derived from robust in vivo release of BMS–286309 in rodents and the likelihood that this biotransformation will be preserved in humans. The comparable antitumor activity of BMS-422461 to irinotecan, as well as reduced preclinical GI toxicity, make this novel camptothecin analog attractive for clinical development.

Keywords Camptothecin · Antitumor · Xenograft · Gastrointestinal toxicity · Pharmacokinetics

Abbreviations AUC: Area under the blood concentration-time curve · BDC: Bile duct cannulated · Irinotecan: 7-Ethyl-10[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin · DMSO: Dimethylsulfoxide · EC50: Concentration of a compound required to induce topoisomerase I-mediated single-strand breaks in 50% of the DNA substrate · GI: Gastrointestinal · HSA: Human serum albumin · HPLC: High-pressure liquid chromatography · IC50: Concentration of a compound required to inhibit cell growth by 50% relative to an untreated control · i.a.: Intraarterial · i.v.: Intravenous · LCK: Gross log cell kill · MSA: Mouse serum albumin · MTD: Maximum tolerated dose · PBS: Phosphate buffered saline · p.o.: Per oral · s.c.: Subcutaneous · L: Liters · Vss: Volume of distribution at steady-state · SD: Standard deviation

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Introduction

Camptothecin is a natural product isolated in 1966 from extracts of *Camptotheca acuminata*, a tree native to China [19]. Camptothecins are DNA topoisomerase

I-targeting agents that have emerged as a prominent class of anticancer agents [18].

Irinotecan is one of the two camptothecin analogs to have been approved thus far for clinical application. It is a prodrug that requires activation to the active metabolite, 7-ethyl-10-hydroxy camptothecin (SN-38), which is approximately 100- to 1,000-fold more potent than irinotecan [8]. Bioactivation of irinotecan by microsomal carboxylesterases occurs primarily in the liver [5], and has been studied previously using purified human carboxylesterase. In addition to the carboxylesterase-mediated conversion to the active SN-38, the pharmacological profile of irinotecan is further complicated by a host of enzymes and transporters involved in its metabolic transformation and hepatobiliary secretion [12].

In the clinical use of irinotecan, neutropenia and diarrhea are the most common toxicities encountered, with diarrhea occurring in two forms. The most common form of diarrhea, a delayed-onset diarrhea, occurs usually after the second or third dose of irinotecan. The other form is a dose-related toxicity occurring during the treatment period, which is usually associated with abdominal cramping, vomiting, flushing, visual disturbances, lacrimation, salivation, bradycardia and diaphoresis [11]. Occasionally, the severe watery diarrhea produced by irinotecan has been characterized as life-threatening [14]. The host of enzymes and transporter proteins involved in the disposition of irinotecan add significant inter-patient variability in the plasma concentrations and this variability may be a critical determinant of irinotecan toxicity and activity [4].

Camptothecins are known to exist in two distinguishable forms, an active α-hydroxy-δ-lactone closed form and an inactive open carboxylate form, between which a pH-dependent equilibrium exists. It is thought that preferential binding of the carboxylate form over the lactone to human serum albumin (HSA) makes the former more stable in the bloodstream [20]. Literature reports indicate that 9-aminocamptothecin and camptothecin display extremely poor stability in human blood due to the high affinity, noncovalent binding interactions of their carboxylate forms with HSA [1]. Both topotecan (the other camptothecin analog approved for clinical usage) and irinotecan contain structural features, which reduce the binding of their carboxylate forms to HSA, and as a result they display improved stability in human blood and plasma relative to camptothecin and 9-aminocamptothecin [1].

The development of a camptothecin analog with improved therapeutic potential would include not only the obvious enhancement of efficacy, but also the attenuation of severe toxicities if achieved without compromising activity. After many years of analog synthesis and testing for compounds possessing the desired attributes, the characterization of in vitro and in vivo efficacy, toxicity and pharmacokinetics of a novel fluoro-substituted camptothecin derivative, BMS-286309, and its water soluble prodrug, BMS-422461 are described now. (While a detailed description of the chemical rationale for these and similar analogs, including the basis for the moiety used for prodrug development, and their structure–activity relationships, will be presented in a separate chemistry-oriented manuscript, the main thrust of the chemistry modifications centered around replacement of the methylenedioxy core with a di-fluoromethylene moiety in an attempt to improve metabolic stability.) For comparison, the preclinical efficacy and toxicity of irinotecan were assessed. Also described are in vitro stability studies of BMS–286309 and BMS-422461 in whole blood and liver S9 fractions from various species. These novel camptothecin analogs retain the activity level of irinotecan, but with a reduced propensity for gastrointestinal (GI) toxicity. BMS-422461 is being actively considered for clinical development.

Materials and methods

Compounds

Irinotecan, topotecan, camptothecin (BMS-193664) and three camptothecin analogs, BMS-286309, BMS-422461, and BMS-200785, were synthesized at Research Triangle Institute and/or (for irinotecan) obtained commercially. The structures of the first two of these new camptothecin analogs are shown in Fig. 1. BMS-200785, 9-amino-10,11-methylendioxy-20(*S*)-camptothecin, was used solely as a chromatographic reference compound.

For in vitro studies, BMS-286309 and camptothecin were initially dissolved in dimethylsulfoxide (DMSO) and diluted with water (or tissue culture medium) to a

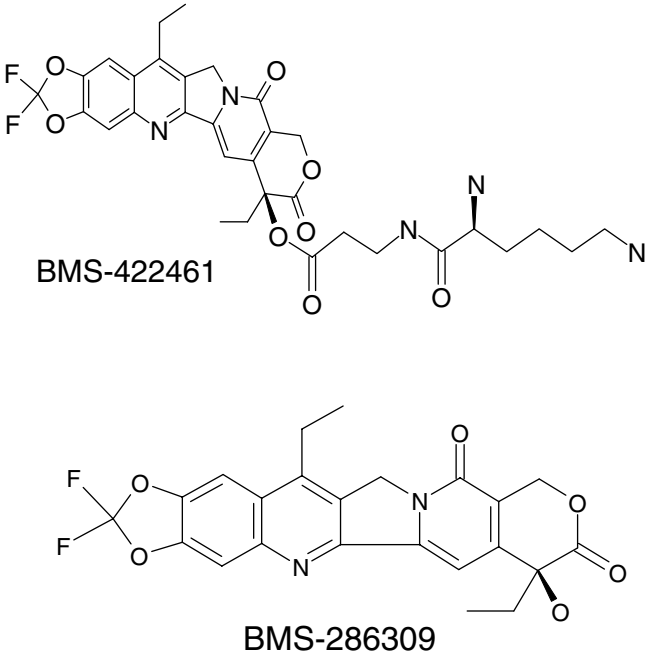

Fig. 1 Structure of BMS-286309 and BMS-422461

final DMSO concentration of less than 0.2% (or less than 1% for lactone/carboxylate equilibrium experiments). All other compounds were dissolved in water. For in vivo efficacy and toxicity studies, BMS–286309 was dissolved in DMSO and diluted with water to a final vehicle consisting of 10% DMSO in water. BMS-422461 and irinotecan were dissolved in water and topotecan was dissolved in 0.9% NaCl. For pharmacokinetic studies, BMS-286309 was administered in cremophor:ethanol:water (1:1:8) while BMS-422461 was administered in water.

In vitro cytotoxicity

Tissue culture cells were maintained in RPMI 1640 Medium, 5 mM HEPES buffer, and 10% fetal bovine serum. Adult bovine aortic endothelial cells were grown in RPMI 1640 media containing 2.0 ng/ml basic fibroblast growth factor. The cell line panel [10] is made up of human ovarian carcinoma cell lines (A2780/DDPS, A2780/DDPR, A2780/TAXS, A2780/TAXR), human breast carcinoma cell lines (MCF-7, SKBR-3), a human prostate carcinoma cell line (PC-3), human colon carcinoma cell lines (HCT-116, HCT-116/VM46, HCT-116/VP35, Caco-2, LS 174T, MIP, HT-29), human lung carcinoma cell lines (A549, LX-1), human squamous cell carcinoma cell line (A431), human leukemia cell lines (CCRF-CEM, HL-60, K562), human fibroblast cell line (HS27), a mouse lung carcinoma cell line (M109), and a mouse lung fibroblast cell line (MLF). The HCT-116/VM46 cell line is a multidrug-resistant variant of the parental HCT-116 cells, and over expresses *P*-glycoprotein. The HCT-116/VP35 cell line is resistant to etoposide (VP-16) and has been shown to have low levels of topoisomerase II. The A2780/DDPR cell line is resistant to cisplatin relative to the parental A2780/DDPS cells. A tubulin mutation is present in the A2780/TAXR cells, which are resistant to paclitaxel relative to the parental A2780/TAXS cells.

The in vitro cytotoxicity of BMS-286309 was assessed in tissue culture cells using a tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium, inner salt) assay. Cells were plated and 24 h later drugs were added and diluted serially. The cells were incubated at 37°C for 72 h at which time the tetrazolium dye, in combination with phenazine methosulfate, was added. The absorbency measured at 492 nm is proportional to the number of viable cells. The results are expressed as IC50 values, which are drug concentrations yielding 50% inhibition of cell growth relative to untreated control growth.

The IC50 values for a given test sample can be expressed visually as a mean bar graph. To do so, the logarithm of each IC50 value is calculated and a mean of the log IC50 values is determined. The difference between the mean log IC50 value and each individual log IC50 value is calculated and plotted as a bar in the mean bar graph. Bars that project to the right represent cells that are more sensitive than the mean IC50 for all of the cell lines. Bars that project to the left represent cell lines that are less sensitive (more resistant) than the mean [15].

Induction of human topoisomerase I-mediated breaks in covalently closed phage DNA in vitro

Single-strand DNA breaks were quantified by incubation of 100 ng of supercoiled, circular, covalently closed PM2 phage DNA with purified human topoisomerase I (TopoGen, Columbus, OH, USA) in the presence of different concentrations of camptothecin or BMS-286309 (10^{-11} to 10^{-4} M) at 37°C for 1 h, after which the reaction was stopped and the extracted DNA was subjected to agarose gel electrophoresis to separate the topological DNA forms. The amount of single-strand broken DNA (Form II) relative to total DNA of each sample, determined by ethidium bromide staining and scanning fluorescence quantification, was corrected for single-strand broken DNA induced by topoisomerase I alone, and plotted versus concentration. The data are presented in terms of the concentrations of compound needed to induce strand breaks in 50% of the substrate DNA (EC50) under these assay conditions.

In vivo antitumor efficacy

Conventional and athymic (“nude“) mice, 5–6 weeks of age, purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA), were quarantined for approximately 3 weeks prior to their use for tumor propagation and drug efficacy testing. They were fed food and water ad libitum. All studies involving these animals were conducted in accordance with NIH and Bristol-Myers Squibb Company (BMS) animal care and use guidelines.

The following tumors were passaged in the indicated host strain of mouse: murine C3H mammary 16/C carcinoma in C3H mice; human A2780 ovarian and HCT-116 and HT-29 colon carcinomas in nude mice. Efficacy testing was conducted in the same strains and types of mice used for tumor maintenance (passage). All tumor implants were subcutaneous (s.c.).

BMS-286309, BMS-422461, and irinotecan were administered by the intravenous (i.v.) route using various dosing regimens. BMS-422461 was also administered orally (p.o.).

The methods used to assess antitumor activity have been published extensively [17]. Antitumor effects were expressed as gross log cell kill (LCK). Activity is defined as ≥1 LCK. Efficacy data are provided for treatments that do not exceed the maximum tolerated dose (MTD), defined as not more than one death in a treatment group; any exceptions to this general rule are clearly described. Results that differ by less than 1 LCK are described as comparable.

Gastrointestinal (GI) toxicity assay

Groups of C3H mice ($n = 8$–9/group) were administered BMS-422461, BMS-286309, or irinotecan across a range of 3–4 doses to ensure an MTD was achieved for the experiment. Mice were administered the compounds by the s.c. route, daily, for five consecutive days. Three days following the last injection, a pre-selected subset of mice ($n = 3$–4) were euthanized and GI tissues processed for histopathological evaluation. The remaining five mice were observed for lethality during a post-treatment observation period to assure that an accurate MTD was determined for each experiment. Comparisons were made between compounds at their respective MTDs, which was defined as the highest dose, which failed to kill more than one of the five mice in the life span group (except, if one of the five mice died in the life span group, then any mice dying in the pathology subgroup by day 8 would disqualify that dose as an MTD and the next lower dose was deemed the MTD). Two to four separate, individual GI toxicity experiments were conducted with BMS-422461, BMS-286309, and irinotecan.

At necropsy, the entire GI tract was removed consisting of the stomach, small intestine (duodenum, jejunum, ileum), cecum, colon, and rectum. The tissues were flushed with neutral buffered formalin and immersed in the same fixative. Following fixation, multiple cross-sections were made for each segment of the small intestine and colon. In addition, longitudinal sections ($n = 2$/organ) were made of the stomach and cecum. Tissues were processed by routine methods, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A total of 15–25 sections of GI tract were evaluated by light microscopy for each animal.

Histopathological assessment for all studies was performed by one pathologist in a coded manner (slides blinded as to treatment group). Sections of GI tract were semi-quantitatively graded by light microscopy as to severity of injury based on the following criteria: grade 0, non-remarkable (similar to control); grade 1, minimal increase in the degree of apoptotic enterocytes as compared to controls; grade 2, mild increase in the degree of apoptotic enterocytes as compared to controls and mild crypt injury (e.g., dilatation, crypt drop-out); grade 3, moderate crypt injury (e.g., dilatation, crypt drop-out) and mucosal atrophy; grade 4, marked villous shortening and/or loss (mucosal atrophy) often associated with focal mucosal erosions or ulcerations. These injuries are shown in representative light microscopic photomicrographs in Fig. 2. Severity grades were averaged for each group of mice ($n = 3$–4).

Assessment of species differences in lactone-carboxylate equilibrium

BMS–286309 (20 μM added in DMSO, final solvent ≤1%, v/v) was incubated in phosphate buffered saline (PBS), in the presence of mouse or human serum albumin (30 mg/ml) and in buffered mouse or human plasma (pH 7.4, 37°C; buffered with 10% 0.28 M PBS). At various times aliquots were taken and precipitated with an equal volume of methanol chilled on a dry-ice/acetone bath. Following centrifugation, supernatants were analyzed by high-pressure liquid chromatography (HPLC) using a Waters Alliance 2690 separation module with a mobile phase of 10 mM ammonium acetate (adjusted to pH 5.5 with glacial acetic acid)/acetonitrile (gradient 95/5% to 5/95% over 20 min) at 1 ml/min through a Waters Nova-Pak C-18 column (3.9×150 mm^2, 4 μm particle size; ambient temperature). Ultraviolet absorption was monitored with a Waters 996 PDA detector. Percent lactone (relative to total) versus time was fitted to $y = a + be^{(-kt)}$, where a is percent lactone at equilibrium, $a + b$ is initial percent lactone, and k is rate constant for equilibrium.

Comparative release of parent from prodrug and intermediate in rodent versus human blood and liver S9 fractions

BMS-422461 (20 μM added in DMSO, final solvent ≤1%, v/v) was incubated in fresh human, mouse, and rat blood (buffered with 10% 0.28 M PBS, 37°C) or liver S9 fractions (1 mg protein/ml in 56 mM phosphate buffer, pH 7.4, 37°C). At various times aliquots were taken and protein precipitated with a onefold to twofold volume acetonitrile chilled on a dry-ice/acetone bath. Following centrifugation, supernatants were analyzed by HPLC as described previously.

Metabolite profile of BMS-422461 in bile duct-cannulated (BDC) rats

The metabolic profile of BMS-422461 was investigated in bile and urine obtained from BDC rats ($n = 2$) following a 0.5 mg/kg (0.8 μmol/kg) intra-arterial (i.a.) infusion (10 min constant rate). Bile samples were collected at 0–4 and 4–8 h time intervals and urine collected 0–8 h. Samples were treated with two volumes of acetonitrile followed by centrifugation to remove precipitated protein. The supernatant was evaporated and reconstituted in mobile phase prior to analysis by LC/MS using a Waters Alliance 2690 separations module interfaced to a Finnigan TSQ-7000 triple quadrupole mass spectrometer using positive ion electrospray. The column was a YMC-ODS AQ (2×150 mm^2, 3 μm), mobile phase A = 95/5, B = 5/95 10 mM ammonium acetate (0.1% acetic acid)/acetonitrile with 0% B for 2 min, 0–90% B in 32 min and held at 90% B for 3 min at a flow rate of 0.3 ml/min.

Pharmacokinetic evaluation in rats

The i.a. and p.o. pharmacokinetics and p.o. bioavailability of BMS-422461 and BMS-286309 were evaluated

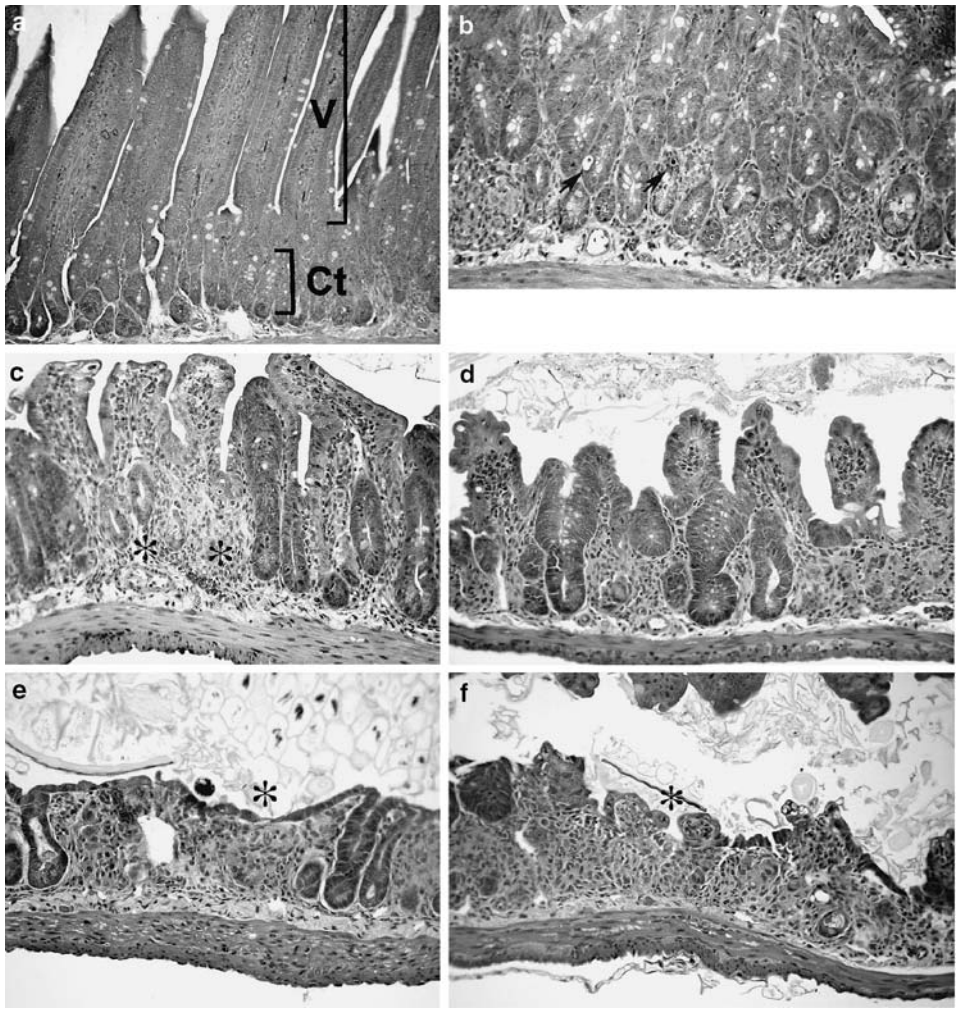

Fig. 2 Photomicrographs of mouse small intestine demonstrating morphological features for semi-quantitative grading of camptothecin-induced injury (as described in Materials and methods). **a** Normal control mouse intestine; note villus (*V*) to crypt (*Ct*) ratio. **b** Severity grade 1, minimal increase in the number of apoptotic cells (*arrowheads*) in the crypt. **c** Severity grade 2, some evidence of crypt injury and loss of crypts (*asterisk*). **d** Severity grade 3, moderate crypt injury and drop-out with muscosal atrophy (note V:C ratio). **e**, **f** Severity grade 4 demonstrating a marked shortening and/or loss of villi, a loss of crypts, and focal erosions (*asterisk*). H&E staining. Original magnification ×20

in male Sprague-Dawley rats ($n = 3$). BMS-422461 was dosed at 2.2 μmol/kg i.a. and p.o. in water. BMS-286309 was administered at 2.2 μmol/kg i.a. and 4.4 μmol/kg p.o. in cremophor:ethanol:water mixture (1:1:8). Serial blood samples were collected 48 h after dosing. Due to BMS-422461 instability in blood, samples (200 μl) were de-proteinized with two volumes of ice-cold acetonitrile immediately upon procurement. After centrifugation to remove precipitate, supernatants were immediately frozen on dry-ice and stored at −80°C.

Total concentrations (sum of lactone and carboxylic acid species) were analyzed by adding supernatant to an equal volume internal standard solution (750 ng/ml BMS–200785 and 1% formic acid in water). Standard and quality control samples were prepared in whole blood and processed in the same manner as study samples. The HPLC system consisted of two Shimadzu LC10AD pumps, a Perkin Elmer Series 200 autosampler and a Hewlett Packard Series 1100 column compartment. The column used was a Keystone BDS-C18, 2×20 mm^2, 3 μm particle size, held at 60°C. The mobile phase was A: 10 mM ammonium acetate in water, pH = 5.5 and B: acetonitrile, with 5% B initial changed to 90% B over 1 min, held for 1 min then re-equilibrated at 5% B for 0.5 min (total 2.5 min) at a flow rate of 0.3 ml/min. The system was interfaced to a Micromass Quattro LC tandem mass spectrometer with electrospray in a positive ionization mode. The following reactions were monitored: BMS-422461 m/z 656.1 → 438.8; BMS–286309 m/z 457.0 → 413.0; the 20-*β*-alanyl ester intermediate of BMS-286309 m/z 528.0 → 438.9; and BMS-200785 (internal standard) m/z 407.9 → 363.9. The area under the blood concentration versus time curve (AUC) data were analyzed by non-compartmental methods using the KINETICA software program.

Results

In vitro cytotoxicity

BMS-286309 was compared to camptothecin (BMS-193664) with regard to their in vitro cytotoxicity against a cell line panel consisting of mostly human tumors, but also human normal fibroblast cells, as well as mouse tumor and fibroblast cells and bovine normal endothelial cells (Fig. 3). The data are expressed as IC50 values and visually as a mean bar graph. BMS-286309 dem-

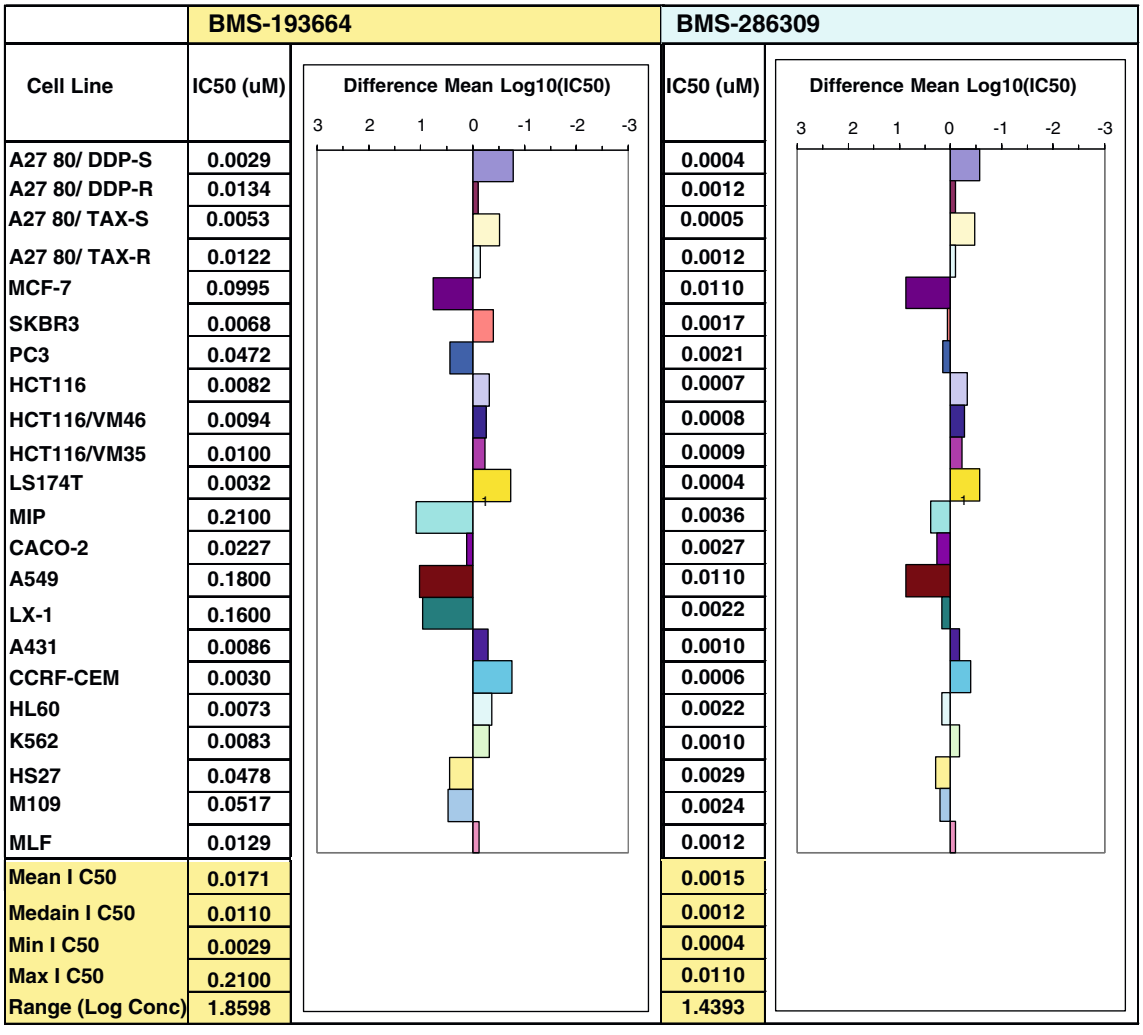

Fig. 3 Comparative cytotoxicity profiles of camptothecin (BMS-193664) and BMS–286309

onstrated potent in vitro cytotoxicity (mean IC50 value of 0.0012 μM) that was tenfold more potent than camptothecin and a robust cell line selectivity profile that was very similar to that for camptothecin. A similar selectivity pattern is commonly observed with compounds that possess the same mechanism of action, that is inhibition of topoisomerase I. A number of cell lines were particularly sensitive to BMS-286309 including A2780 ovarian carcinoma, HCT-116 and LS174T colon carcinomas, and CEM leukemia. BMS-286309 was active against two derivatives of the HCT-116 cell line, HCT-116/VM46, a MDR variant, which overexpresses *P*-glycoprotein, and HCT-116/VP35 that has low levels of topoisomerase II and is resistant to VP-16. Like camptothecin, BMS-286309 was not cross-resistant against either of these cell lines.

Induction of human topoisomerase I-mediated breaks in covalently closed phage DNA in vitro

BMS-286309 was found to be a potent inducer of topoisomerase I-mediated DNA breaks in vitro. Based on the average of two experiments, BMS-286309 had a potency (EC50 of 0.03 μM) similar to camptothecin (EC50 of 0.06 μM) and showed no signs of binding strongly to DNA in a manner that would inhibit the catalytic activity of the enzyme at high concentrations. The prodrug, BMS-422461, was not evaluated using this assay.

In vivo antitumor efficacy

BMS-422461 and BMS-286309 were evaluated in several s.c. distal site tumor models for their antitumor activity. They were compared to irinotecan, or occasionally to topotecan. A summary of the optimal antitumor effects for both BMS-286309 and BMS-422461 is shown in Table 1.

BMS-286309 test results

The parent camptothecin analog, BMS-286309, was initially evaluated by the i.v. route in mice bearing C3H mammary carcinoma. Treatment was given consecu-

Table 1 Summary of preclinical antitumor activities of camptothecin analogs BMS-286309 and BMS-422461 relative to irinotecan (or topotecan)

Tumor	Treatment			Optimal effect in LCK		
	Experiment no.	Schedule[a], route	Optimal dose (mg/kg/administration)	I.v.	P.o.	Irinotecan, i.v.
			BMS-286309			
16/C Mam	46	qdx5;4, i.v.	0.03[b]	0.9	–	1.1
A2780	99–11	q2dx5;10, i.v.	0.09[b]	3.9	–	2.5[d]
	99–14	q2dx5;10, i.v.	0.06[c]	1.8	–	1.0
HT-29	99–13	q2dx5;10, i.v.	0.13[b]	1.6	–	1.5
HCT-116	56	q2dx5;13, i.v.	0.06[b]	1.0	–	0.9
			BMS-422461			
16/C Mam	50	qdx5;4, i.v.	0.25[b]	0.6	–	0.8
	52	qdx5;4, i.v.	0.3[b]	0.6	–	0.2
A2780	138	q2dx5;12,i.v.	0.6[b]	2.3	–	2.2
	00–03	q2dx5;9, i.v.	0.6[b]	3.1	–	2.1 (LD25[e])
	00–03	q2dx5;9, p.o.	3.0	–	3.0	2.1 (LD25)
HCT-116	56	q2dx5;13, i.v.	0.6[b]	1.0	–	0.9
	57	q2dx5;14, p.o.	2.2	–	1.0	1.2
HT-29	00–02	q2dx5;10, i.v.	0.4[b]	1.4	–	1.4 (LD25)

[a]q2dx5;4, signifies treatment administered every 2 days (“q2d”), for a total of five treatments (“x5”) beginning on day 4 (the number after the semicolon) post-tumor implant. Interpretation of the other schedules shown on this table will follow this codification
[b]MTD likely reached
[c]MTD was slightly greater, 0.09 mg/kg/inj
[d]Topotecan used rather than irinotecan
[e]The dose used to achieve this effect was lethal to 25% of the treated mice

tively once daily for 5 days beginning on day 4 post-tumor implant (i.e., qdx5;4,i.v.). The consecutive daily treatment was chosen because of the rapid doubling time (2.0–2.8 days) of this tumor, and historical success using irinotecan with this schedule. At a dose of 0.03 mg/kg/inj, considered to possibly be a MTD, the analog produced 0.9 LCK, a borderline active result. In comparison, the optimal effect obtained using irinotecan, evaluated concomitantly, was 1.1 LCK. In general, without specifically describing its MTD in each experiment, the MTD (and optimal dose) for irinotecan ranged from 20 to 32 mg/kg/inj for C3H mammary experiments and between 36 and 48 mg/kg/inj for all the other experiments described herein.

Subsequent testing of BMS-286309 was done in several human tumor xenograft models using an intermittent schedule. The intermittent schedule was chosen because of the greater tumor volume doubling times (compared to the C3H mammary tumor) of these xenografts, and successful experience with such a schedule when evaluating irinotecan. In the first experiment involving the human ovarian carcinoma, A2780, topotecan was the reference compound utilized. When both compounds were administered i.v. on a q2dx5 schedule against established tumors (day 10 post-tumor implant), BMS-286309 at its MTD of 0.09 mg/kg/inj produced 3.9 LCK. This result was superior to the 2.5 LCK produced by the MTD of topotecan (9 mg/kg/inj) and at 1/100th the dose. These optimal effects of both camptothecins are shown in Fig. 4.

In a repeat experiment involving the A2780 tumor, optimal treatment with BMS–286309 yielded a therapeutic result that was comparable to irinotecan. BMS-286309 produced 1.8 LCK, at 0.06 mg/kg/inj on the same treatment schedule as before, compared to 1.0 LCK for irinotecan.

The next tumor model to be used for the evaluation of BMS-286309 was the human colon tumor, HT-29. At an MTD of 0.13 mg/kg/inj, q2dx5, i.v., the analog produced 1.6 LCK. This outcome was comparable to concomitantly tested irinotecan, which produced 1.5 LCK. The final antitumor test was conducted using another human colon carcinoma, HCT-116. The MTD of BMS-286309 in this experiment was only 0.06 mg/kg/

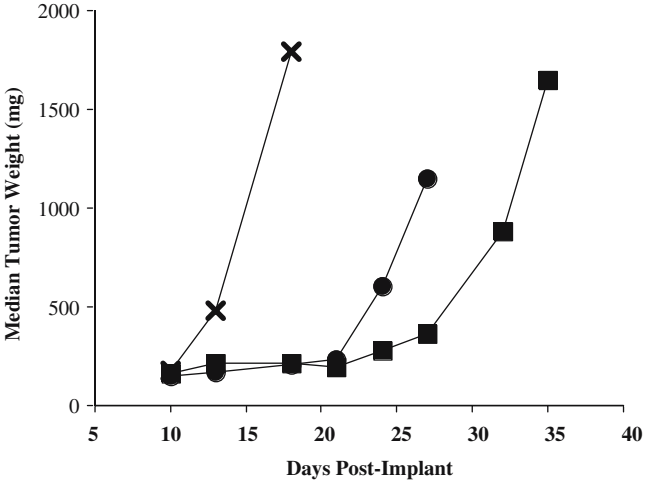

Fig. 4 Comparative antitumor activities of optimal therapies involving i.v. administration of topotecan (*filled circle*, 9 mg/kg/inj) and BMS-286309 (*filled square*, 0.09 mg/kg/inj) versus the human A2780 ovarian carcinoma (X untreated control). All treatments were q2dx5 beginning day 10 post-tumor implant

inj on a q2dx5, i.v. regimen. The maximum LCK produced by the analog was 1.0, which was nearly identical to the borderline active result, 0.9 LCK, caused by irinotecan tested in parallel.

BMS-422461 test results

The initial tumor model used for the evaluation of the prodrug, BMS-422461, was C3H murine mammary 16/C. In the first study, similar results were obtained for both the prodrug and irinotecan, maximum LCKs of 0.6 and 0.8, respectively. The MTD for BMS-422461 was 0.25 mg/kg/inj, qdx5, i.v. Neither camptothecin produced a firm active result. In a repeat experiment, the MTD of the prodrug was 0.3 mg/kg/inj, i.v., on the same schedule, and again it produced a maximum effect of 0.6 LCK. In comparison, irinotecan only produced an inactive 0.2 LCK. The doses of BMS-422461 tolerated in these studies were about tenfold higher than the MTD of the parent compound.

Several experiments were then conducted involving human tumor xenografts. Against the A2780 human ovarian tumor, an MTD for BMS-422461 was reached at 0.6 mg/kg/inj, given q2dx5, i.v., beginning on day 12 post-tumor implant (i.e., versus well-established s.c. tumors). At this dose level the prodrug produced 2.3 LCK. A nearly identical result, 2.2 LCK, was achieved with the MTD of concomitantly tested irinotecan. In the second A2780 experiment i.v. administration of the water-soluble analog yielded 3.1 LCK, again at a MTD of 0.6 mg/kg/inj, q2dx5. In comparison, irinotecan at a dose of 48 mg/kg/inj was slightly more toxic than usual, causing two of eight deaths, and even at this level caused but 2.1 LCK; lower doses were better tolerated but less effective. In this experiment, BMS-422461 was superior (≥1 LCK) to irinotecan when both were evaluated concomitantly at their MTDs (or slightly higher for irinotecan). In this same experiment, BMS-422461 was also evaluated orally. At the highest dose tested, 3 mg/kg/administration, the prodrug given p.o. produced 3.0 LCK. BMS-422461 achieved antitumor activity by the oral route of administration and produced a result comparable to what it had concomitantly produced when given parenterally. These optimum results for BMS-422461, p.o. and i.v., are shown in Fig. 5 relative to irinotecan.

Additional testing took place in the HCT-116 human colon carcinoma model. I.v. administration of BMS-422461 on a q2dx5 schedule produced a maximum LCK of 1.0 at a MTD of 0.6 mg/kg/inj. Irinotecan, tested in parallel, produced 0.9 LCK at its MTD. This was the only experiment in which both prodrug and parent analog were evaluated concomitantly, and each produced 1.0 LCK at their respective MTDs. The prodrug was tenfold less potent than the parent compound. In a second experiment involving HCT–116, BMS-422461 was evaluated orally. At the highest dose tested, 2.2 mg/kg/administration, p.o. BMS-422461 achieved 1.0 LCK. Irinotecan, in comparison, produced a similar 1.2 LCK.

The last antitumor experiment involved the HT-29 human colon carcinoma. Administration of BMS-422461 on a q2dx5, i.v. regimen, beginning on day 10 post-tumor implant, produced 1.4 LCK at an MTD of 0.4 mg/kg/inj. Concomitant testing with irinotecan yielded 1.4 LCK, but this comparison is made at an LD25 level; lower doses were better tolerated but were less effective.

In summary, the MTD of BMS-286309 was 0.03 mg/kg/inj when tested i.v. on a qdx5 schedule in C3H mice, and ranged from 0.06 to 0.13 mg/kg/inj when used i.v. on a q2dx5 schedule in nude mice. In contrast, the MTD of the prodrug, BMS-422461, given i.v. on a qdx5 schedule in C3H mice was 0.25–0.3, and 0.4–0.6 mg/kg/inj when administered i.v. to nude mice on a q2dx5 schedule. The prodrug was approximately fivefold to tenfold less potent given i.v. than the parent analog. When given p.o., the MTD of the prodrug was not definitely determined, but it was tolerated and produced good activity at doses of 2.2––3.0 mg/kg/administration, q2dx5 in nude mice.

GI toxicology

The drug-related histopathological characteristics of the GI tract observed in these studies were consistent with structural damage to the enterocyte cell population that most likely results in changes in absorption and/or other intestinal functions [3, 7]. In the comparison studies, the drug-induced lesions were similar in nature for BMS-286309, BMS-422461, and irinotecan, further supporting a common mode of action (anti-proliferative effect). For all compounds, there was an anterio-posterior gradient of injury that may just reflect the normal proximal–distal diminution in villous height. The severity of lesions was usually location-specific with the greatest injury in the ileum and cecum. Within each treatment group, inter-

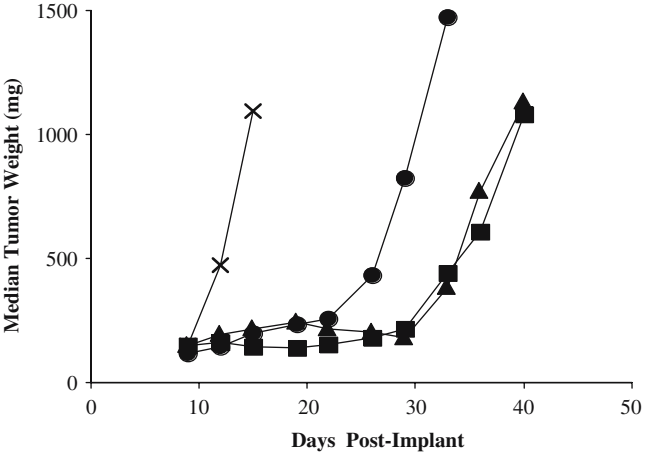

Fig. 5 Intravenous (i.v., *filled square*, 0.6 mg/kg/inj) and oral (p.o., *filled triangle*, 3 mg/kg/inj) administration of BMS-422461, relative to i.v. irinotecan (*filled circle*, 48 mg/kg/inj) versus human ovarian A2780 carcinoma (X untreated control). All treatments were q2dx5 beginning on day 9 post-tumor implant

Table 2 Comparative GI toxicity severity scores of irinotecan and camptothecin analogs in the C3H mouse GI toxicity assay

BMS no.	Average GI toxicity score (no. experiments)
Irinotecan	2.8 (3)
BMS- 422461	0.5 (2)
BMS- 286309	1.5 (4)

At their respective MTDs, the three camptothecins were evaluated for GI toxicity (scored for severity on a 0–4 scale) produced in mice using the methodology described in the Materials and methods section

animal variation in the degree of GI injury was often observed.

Based on semi-quantitative assessment, however, the degree of injury was less severe for both BMS–286309 and BMS-422461 as compared to the reference agent, irinotecan (Table 2). Irinotecan consistently yielded a mean GI toxicity severity score of ≥2.0 with an average severity score of 2.8 derived from three experiments. In comparison, the parent compound BMS-286309 produced a mean GI toxicity severity score of 1.5, with only one poor outcome out of four experiments conducted (a score of 3.3). The water-soluble prodrug, BMS-422461, was associated with an average GI toxicity severity score of 0.5 based on two experiments.

The MTDs for each compound derived from the GI toxicity experiments varied as follows: irinotecan (60–90 mg/kg/inj); BMS-286309 (0.06–0.12 mg/kg/inj); and BMS–422461 (0.25 mg/kg/inj).

Assessment of species differences in lactone-carboxylate equilibrium

The estimated percent of BMS-286309 existing as the lactone form at equilibrium in vitro was similar in mouse and human plasma (20.1 vs 23.7%, respectively) or in the presence of mouse or human albumin (31.8 vs 20.1%) (Table 3). For comparison, results reported by Burke and Mi [2, 13] on irinotecan, SN-38 and camptothecin are also shown in Table 3, which indicate that camptothecin under similar conditions exists as $<1\%$ lactone at equilibrium. These data suggest that albumin effects on in vivo lactone/carboxylate equilibrium for BMS-286309 should be similar in mouse and human.

Comparative release of parent from prodrug and intermediate in rodent versus human blood and liver S9 fractions

Metabolic conversion of prodrug BMS–422461 to the intermediate and parent BMS-286309 in liver S9 fractions and whole blood is shown in Figs. 6 and 7, respectively. Release of parent compound appears qualitatively similar for mouse, rat, and human blood and liver S9 fractions. Although the rate of conversion of prodrug BMS-422461 to the intermediate appears higher in rodents, the cumulative release of parent BMS-286309 is similar in human, mouse, and rat blood. Thus, the blood and liver enzymes mediating the reactions do not appear to display significant species differences in expression levels and/or overall catalytic competencies.

Metabolite profile of BMS-422461 in bile duct-cannulated rats

The 20-β- alanyl ester intermediate of BMS-286309, and BMS-286309, were the major derivatives detected in urine and 0–4-h bile samples following administration of BMS-422461. This intermediate was also identified in rat hepatocytes (data not shown). Parent drug was predominant in the 4–8-h bile sample. In total bile and urine, BMS-422461, the intermediate and BMS-286309 were recovered as 20, 17, and 53% of the administered

Table 3 Camptothecin lactone/carboxylic acid equilibrium in vitro

BMS data			Published data[a]		
Compound (concentration)	Matrix	Percent lactone at equilibrium	Compound (concentration)	Matrix	Percent lactone at equilibrium
Irinotecan (10 μM)	PBS	16.8	Irinotecan (1 μM)	PBS[b]	13.0
	HSA	27.3		HSA	24.0
	MSA	33.6		H plasma	19.0
BMS-286309 (20 μM)	PBS	27.6	SN-38 (1 μM)	PBS	15.3
	H plasma	23.7		HSA	36.0
	M plasma	20.1		H plasma	35.4
	HSA	20.9			
	MSA	31.8			
			Camptothecin (1 μM)	PBS	17.0
				HSA	<0.5
				H plasma	<0.2
				MSA	7.0
				M plasma	26.0

[a]References [2] and [13]
[b]*PBS* Phosphate buffered saline; *HSA* human serum albumin; *MSA* mouse serum albumin; *M* mouse

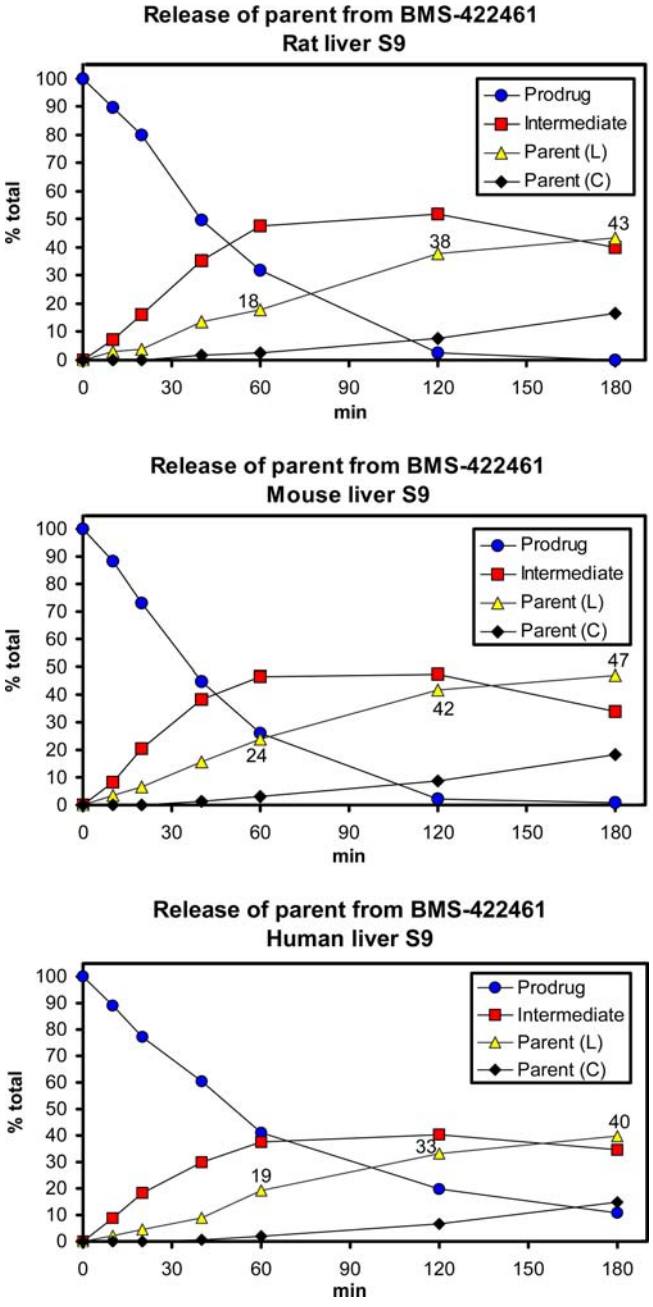

Fig. 6 Metabolic conversion of BMS-422461 in liver S9 fractions from various species (*L* lactone; *C* carboxylate)

dose (assuming similar UV extinction coefficients). No metabolites of BMS-286309 were detected. These results are summarized in Table 4. In all, 90% of the total dose administered as BMS–422461 was recovered in urine and bile over 8 h post-dose.

Pharmacokinetic evaluation in rats

Upon i.a. administration to the rat, BMS–422461 was rapidly cleaved to the 20-β-alanyl ester intermediate of BMS-286309 and BMS-286309. Plasma concentrations of BMS-422461 and the intermediate declined rapidly while those of BMS-286309 were sustained over several hours post-dose (Fig. 8). AUC for the parent compound was over fourfold greater than that of the prodrug (Table 5). The AUC of the intermediate was approximately half that of the prodrug. BMS-286309 was the only form detected following p.o. administration of the prodrug (Fig. 8). Oral administration of BMS–422461 yielded exposure to the parent compound at roughly 40% that obtained upon i.a. administration of the prodrug (based on 24 h AUCs).

BMS-286309 blood levels following its i.a. administration (Fig. 9) displayed rapid distribution followed by sustained concentrations similar to those observed following equimolar i.a. administration of the prodrug (Fig. 8). When administered p.o., BMS–286309 is approximately 22% bioavailable (Fig. 9 and Table 6) relative to its own i.a. administration. Following i.a. or p.o. administration of BMS-422461 or BMS–286309, the observed half-life of BMS-286309 was greater than 9 h. Apparent p.o. bioavailability of BMS-286309 following p.o. administration of BMS-422461 was also approximately 22% based upon AUC of the parent drug after p.o. administration of the prodrug and i.a. administration of the parent drug.

Based on the AUC of parent drug after i.a. administration of prodrug and parent, the fraction of BMS-422461 dose converted to BMS-286309 was estimated to be 70% in the rat. The remainder is likely excreted in bile or urine as prodrug or intermediate.

Discussion

The novel camptothecin analog, BMS-286309, and its water-soluble prodrug, BMS-422461 were evaluated extensively for pharmacologic, toxicologic, and pharmacokinetic developmental potential. BMS-286309 is a potent inducer of topoisomerase I-mediated DNA breaks in vitro and was comparable to camptothecin in this assay and tenfold more potent for in vitro cytotoxicity. Both analogs were found to be comparable to irinotecan with respect to preclinical antitumor activity based on parenteral administration to mice bearing established, distal site, tumors of both murine and human origin. BMS-422461 was also found to be active orally. Both compounds are >100-fold more potent than irinotecan in vivo.

Table 4 In vivo metabolic activation of BMS-422461 in BDC rats

	Percent recovery of BMS-422261 administered dose (0.5 mg/kg i.v.)		
	BMS-422461	Intermediate	BMS-286309
Bile 0–4 h	19	9	30
Bile 4–8 h	1	1	14
Urine 0–8 h	0	7	9

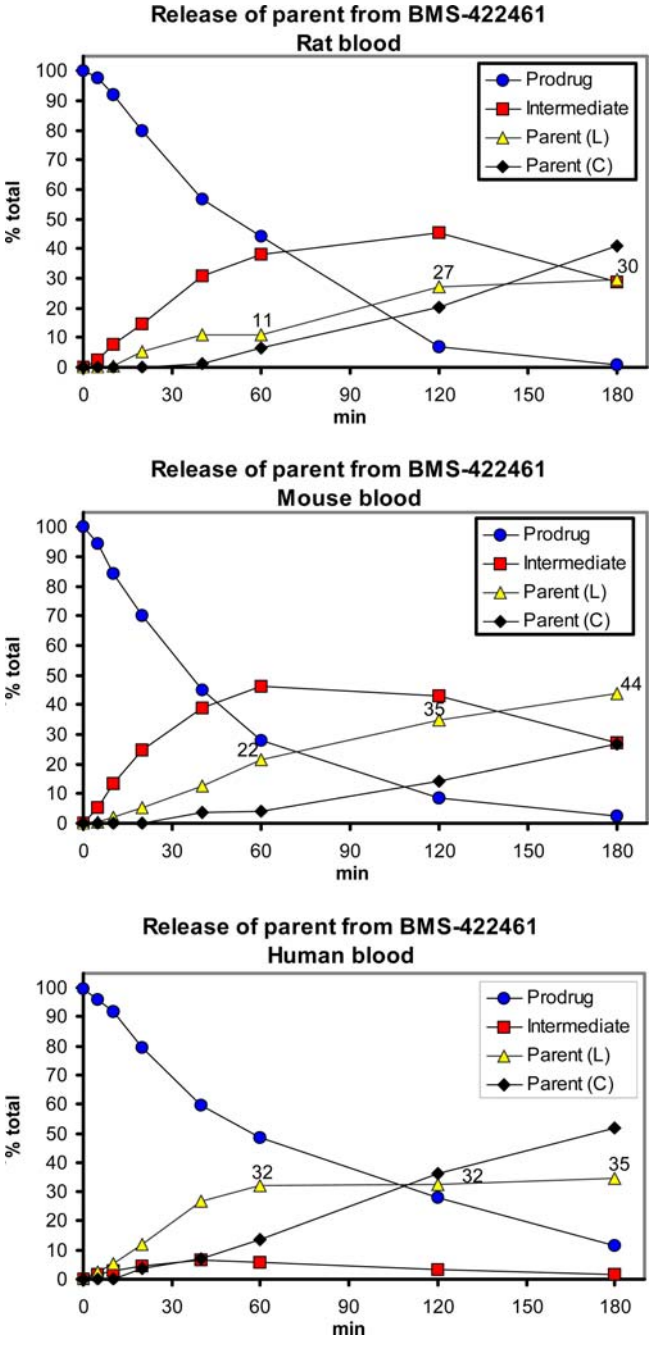

Fig. 7 Metabolic conversion of BMS-422461 in whole blood from various species (*L* lactone; *C* carboxylate)

An animal model was developed in order to assess the potential GI toxicity induced by BMS-286309 and BMS-422461 and to compare the severity of GI injury to that of irinotecan. It was the only model explored in which topotecan did not produce more GI toxicity than irinotecan at their respective MTDs (W. Rose and T. Monticello, unpublished data) and was therefore judged to best mimic the clinical situation with irinotecan. (For example, neither parenteral administration of the camptothecins to rats, nor i.v. administration of them to mice, provided the same profiling of irinotecan and topotecan as found in the model adopted.)

By using this model as a screen for potential clinical GI toxicity, a number of assumptions were made: (1) late onset irinotecan induced diarrhea observed in humans and is attributed to mucosal injury of the intestinal tract; (2) the GI mucosal injury is the result of irinotecan-induced apoptosis of enterocytes in the proliferative zone of the small and large intestines (i.e., crypts of small intestine and lower two-thirds of the large intestinal crypts); and, (3) accelerated apoptosis of enterocytes results in loss of mucosal integrity with subsequent malabsorption and maldigestion leading to diarrhea. These assumptions are not unlike those described by Ikuno et al. [9] in their study of irinotecan-induced changes in mouse ileum and cecum mucosal aberrations. Both BMS-286309 and BMS-422461 were found to be superior to irinotecan with respect to preclinical toxicological assessment of gastrointestinal injury to mice.

Structural modifications of the A, B, and E rings of the camptothecin core structure have yielded more stable camptothecins with restricted hydrolysis to the inactive carboxylate form in the bloodstream. In this manner, camptothecin opens more rapidly and completely in the presence of HSA than in the absence of the protein. Because a closed lactone ring has been shown to be a structural requirement both for effective drug interaction with the topoisomerase I target [8] and for antitumor activity [6], factors influencing the lactone-carboxylate equilibrium of camptothecin are regarded as important determinants of the function of the agent [16]. Therefore, the lactone to carboxylate ratios were compared for BMS-286309 with irinotecan. Burke and Mi [2, 13] have hypothesized that human but not mouse serum albumin shifts the lactone-carboxylate equilibrium to the latter inactive form for camptothecin and some of its analogs, contributing to diminished efficacy. Hence, this equilibrium was evaluated in PBS and in the presence of mouse and human serum albumin and plasma. Burke and Mi [2, 13] have shown that the presence of HSA significantly stabilizes the lactone form of irinotecan versus PBS. A similar shift was found in the percent lactone of irinotecan at equilibrium from 16.8% in PBS to 27.3% in the presence of HSA and 33.6% in the presence of MSA. For BMS-286309, the estimated percent lactone at equilibrium was comparable in the presence of HSA, MSA, and plasma. Importantly, the presence of human albumin did not dramatically shift the equilibrium to the inactive carboxylate form for BMS-286309, as has been reported for camptothecin.

BMS-422461 was developed as a water-soluble prodrug of BMS-286309. The aqueous solubility of the prodrug permitted i.v. administration in water for evaluation of efficacy and pharmacokinetics. However, it also became necessary to assess efficient conversion of the prodrug to the active BMS-286309 and to ensure lack of nonproductive metabolism. The conversion of the ester prodrug was assessed in liver S9 and whole

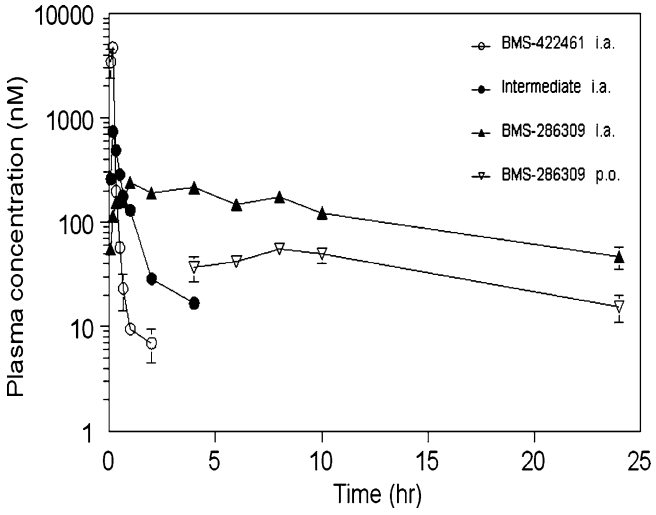

Fig. 8 Plasma concentration–time profiles after i.a. and p.o. administration of BMS–422461 to rats

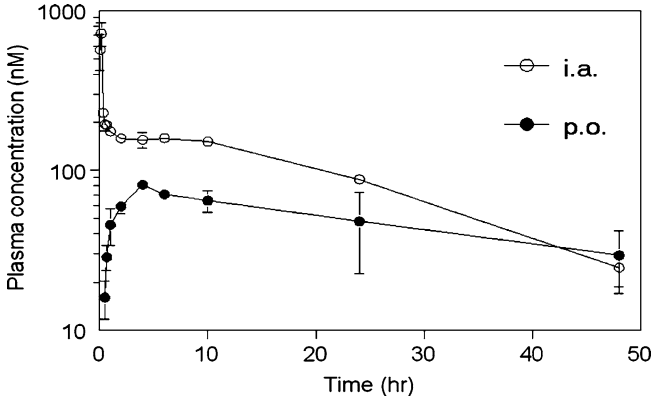

Fig. 9 Plasma concentration–time profiles after i.a. and p.o. administration of BMS-286309 to rats

blood from mouse, rat, and human. In all species and both matrices, similar conversion of the prodrug to a 20-β-alanyl ester intermediate of BMS-286309 was seen, followed by conversion to BMS-286309.

Pharmacokinetics of BMS-286309 and BMS-422461 was studied in the rat. The rat was chosen for these experiments rather than the mouse (the species used in efficacy and toxicity experiments) due to similar in vitro metabolic profiles in blood and liver S9 incubations, and for the added benefit of assessing excretory pathways *via* bile duct cannulation. The in vivo data after i.v. administration of BMS-422461 corroborated the in vitro data in whole blood and liver S9, in that the only drug-related species detected in urine and bile were the 20-β-alanyl ester intermediate of BMS-286309 and the parent compound. The prodrug BMS-422461 was detected only in the bile and accounted for 20% of the administered dose. The intermediate was detected in both urine and bile and accounted for 17% of the dose. The parent BMS-286309 was the major drug-related species recovered at 53% of the administered dose.

Pharmacokinetics of BMS-422461 was studied in the rat after i.a. and p.o. dosing to support the efficacy evaluation with the two routes of administration. BMS–422461 was rapidly cleaved to the intermediate and BMS-286309, forming sustained levels of the latter. The AUC for the parent compound was over fourfold greater than that of the prodrug. In contrast, it was recently reported that the 72 h plasma AUC of SN-38 following i.v. irinotecan administration to rats is approximately 4% that of the prodrug [21]. After p.o. administration of BMS-422461, the only circulating drug-related species was BMS-286309, illustrating the efficient in vivo conversion to the active moiety. The acceptable oral bioavailability of BMS–286309 upon administration of the prodrug is consistent with the oral efficacy data obtained against HCT-116 and A2780 human xenografts in mice. The apparent oral bioavailability of BMS-286309 upon administration of BMS-422461 was similar to that after p.o. administration of BMS-286309 itself, with the added benefit of enhanced water solubility for the prodrug.

The disposition of BMS-422461 thus appears to be relatively straightforward in the rat, with blood and liver esterase-mediated hydrolysis to the parent compound, followed by elimination in the urine and bile. This is in contrast to the complex pharmacokinetic profile described for irinotecan, which is dependent on a host of enzymes involved in metabolic transformation, as well as active transport proteins that regulate intestinal absorption and hepatobiliary secretion mechanisms [12 and references therein]. Among these enzymes, uridine diphospho-glucoronosyltransferases mediate glucuronidation of SN-38 to form the β-glucuronic acid conjugate (SN-38G), intestinal and endogenous β-glucuronidases

Table 5 Mean (SD) pharmacokinetic parameters in the rat after i.a. administration of BMS-422461 (2.2 μmol/kg, $n = 3$)

	BMS-422461	Intermediate	BMS-286309
C_{max} (nM)	4,673 (238)	735 (65)	241 (16)
T_{max} (h)		0.17	1
AUC (nM h)	760 (103)	439 (38)	3,484 (541)
$t_{1/2}$ (h)	0.16 (0.06)	0.66 (0.26)	9.4 (1.2)
Clearance (ml/min/kg)	49 (6)		
Vss (l/kg)	0.24 (0.06)		

Table 6 Mean (SD) pharmacokinetic parameters in the rat after i.a. (2.2 μmol/kg) and p.o. (4.4 μmol/kg) administration of BMS-286309

Parameter	I.a.	P.o.
C_{max} (nM)		81 (5)
T_{max} (h)		4
AUC (nM h)	4,508 (268)	1,945 (746)
$t_{1/2}$(h)	14.5 (2.0)	
Clearance (ml/min kg)	7.3 (0.6)	
Vss (l/kg)	9.6 (0.7)	
Oral bioavailability (%)		22

cause deconjugation of SN-38G, and cytochrome P450 enzymes convert irinotecan to inactive metabolites, 7-ethyl-10-(4-*N*-[5-aminopentanoic acid] −1-piperidino) carbonyloxycamptothecine and 7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecine. In addition, several drug-transporting proteins, such as canalicular multispecific organic anion transporter and *P*-glycoprotein can influence irinotecan elimination through hepatobiliary secretion and intestinal absorption [12]. Collectively, the favorable in vivo metabolic activation of BMS-422461, pharmacokinetic characteristics of BMS-286309, and comparable in vitro inter-species blood and liver metabolic profiles, suggest that the efficacy of BMS-422461 is derived from robust in vivo release of BMS–286309 in rodents, and that this characteristic is likely preserved in humans.

Relative to irinotecan, the comparable efficacy of BMS-422461 against a panel of tumors, its oral activity, reduced GI toxicity, enhanced potency, and favorable pharmacokinetics, offer a novel camptothecin analog with significant development potential.

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