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Report

Active transepithelial transport of irinotecan (CPT-11) and its metabolites by human intestinal Caco-2 cells

Wataru Yamamoto,[1,4] Jaap Verweij,[1] Peter de Bruijn,[1] Maja JA de Jonge,[1] Hiroshi Takano,[2] Masahiko Nishiyama,[2] Minoru Kurihara[3] and Alex Sparreboom[1]

[1]Department of Medical Oncology, Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital Rotterdam, 3008 AE Rotterdam, The Netherlands. [2]Department of Biochemistry and Biophysics, Research Institute for Radiation Biology and Medicine, Hiroshima University, 734-8553 Hiroshima, Japan. [3]Department of Internal Medicine, Toyosu Hospital, Showa University, 135-8557 Tokyo, Japan. [4]Present address: Department of Internal Medicine, Toyosu Hospital, Showa University, 135-8557 Tokyo, Japan.

Irinotecan (CPT-11) is a camptothecin analog with low (about 10–20%) and variable oral bioavailability in animal models. Here, Caco-2 cells were used to evaluate the transepithelial transport of CPT-11 and its metabolites. Caco-2 cells demonstrated significant expression of P-glycoprotein (P-gp), multidrug resistance-associated protein and canalicular multispecific organic anion transporter. Both the lactone and carboxylate forms of CPT-11 and SN-38 were actively transported across the cell monolayers, mainly by the apical-localized P-gp pump. Cellular permeability of CPT-11 at a concentration of 17 μM converted from active to passive-diffusional transport between the 2 and 6 h exposure time points. Antiproliferative effects of CPT-11 were related to permeability of the lactone form, whereas for SN-38 efficacy was dependent on lactone accumulation. Exposure of CPT-11 with cyclosporin A significantly enhanced its efficacy, whereas this was not observed with verapamil and R101933. In contrast, SN-38 efficacy decreased in the presence of P-gp inhibitors due to active transport toward the basolateral side, thereby reducing drug accumulation. Hence, multiple-active transport systems could be demonstrated to be responsible for not only accumulation profiles but also cytotoxic efficacy of CPT-11 and SN-38 in the intestinal Caco-2 cells. It is suggested that CPT-11 might act in a time-dependent manner and that SN-38-mediated cytotoxicity relates to (dose-dependent) lactone kinetics. The results detailed in this report could contribute toward the development of a clinically useful oral formulation of CPT-11 with improved absorption characteristics and suggest that cyclosporin A is a suitable agent for further research of this concept. [© 2001 Lippincott Williams & Wilkins.]

Key words: Caco-2 cells, intestinal transport, irinotecan (CPT-11), metabolism.

CPT-11, irinotecan (7-ethyl-10-[4-(1-piperidino)1-piperidino]carbonyl-oxycamptothecin); SN-38, 7-ethyl-10-hydroxycamptothecin; SN-38G, 7-ethyl-10-[3,4,5-trihydroxy-pyran-2-carboxylic acid]camptothecin (i.e. the β-glucuronic acid conjugate of SN-38); CYP3A4, cytochrome P-450 isoform 3A4; R101933, methyl-6, 11-dihydro-11-[1-[2-[4-(2-quinolinyl-methoxy)phenyl]ethyl]-4-piperidinylidene]-5H-imidazo-[2,1-β]-[3]benzazepine-3-carboxylate; HPLC, high-performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; P-gp, MDR1 P-glycoprotein (M_r, 170 000); MRP-1, multidrug resistance-associated protein; cMOAT, canalicular multispecific organic anion transporter (also referred to as MRP-2); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; TF, transport fraction.

Correspondence to A Sparreboom, Department of Medical Oncology, Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital Rotterdam, Groene Hilledijk 301, 3075 EA Rotterdam, The Netherlands.
Tel: (+31) 10 4391112; Fax: (+31) 10 4391053;
E-mail: sparreboom@onch.azr.nl

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Introduction

The water-soluble topoisomerase I poison CPT-11 is one of the most promising antitumor agents, displaying a broad spectrum of antitumor activity against various neoplasms and pleiotropically drug-resistant tumors both *in vitro* and *in vivo*.[1,2] CPT-11 exerts its antitumor activity following a metabolic conversion to SN-38 (Figure 1), mainly by hepatic microsomal carboxyl esterases, which is 100-fold more potent *in vitro* than the parent drug in inhibiting topoisomerase I activity, and inducing single-stranded DNA breaks that ultimately lead to DNA replication and transcription arrest and cell death.[3] SN-38 is known to undergo further metabolism to an inactive β-glucuronide derivative (SN-38G) mediated by a hepatic microsomal UDP glucuronosyl-transferase (isoforms 1A1 or 1A7).[4,5] We have shown recently that CPT-11, SN-38

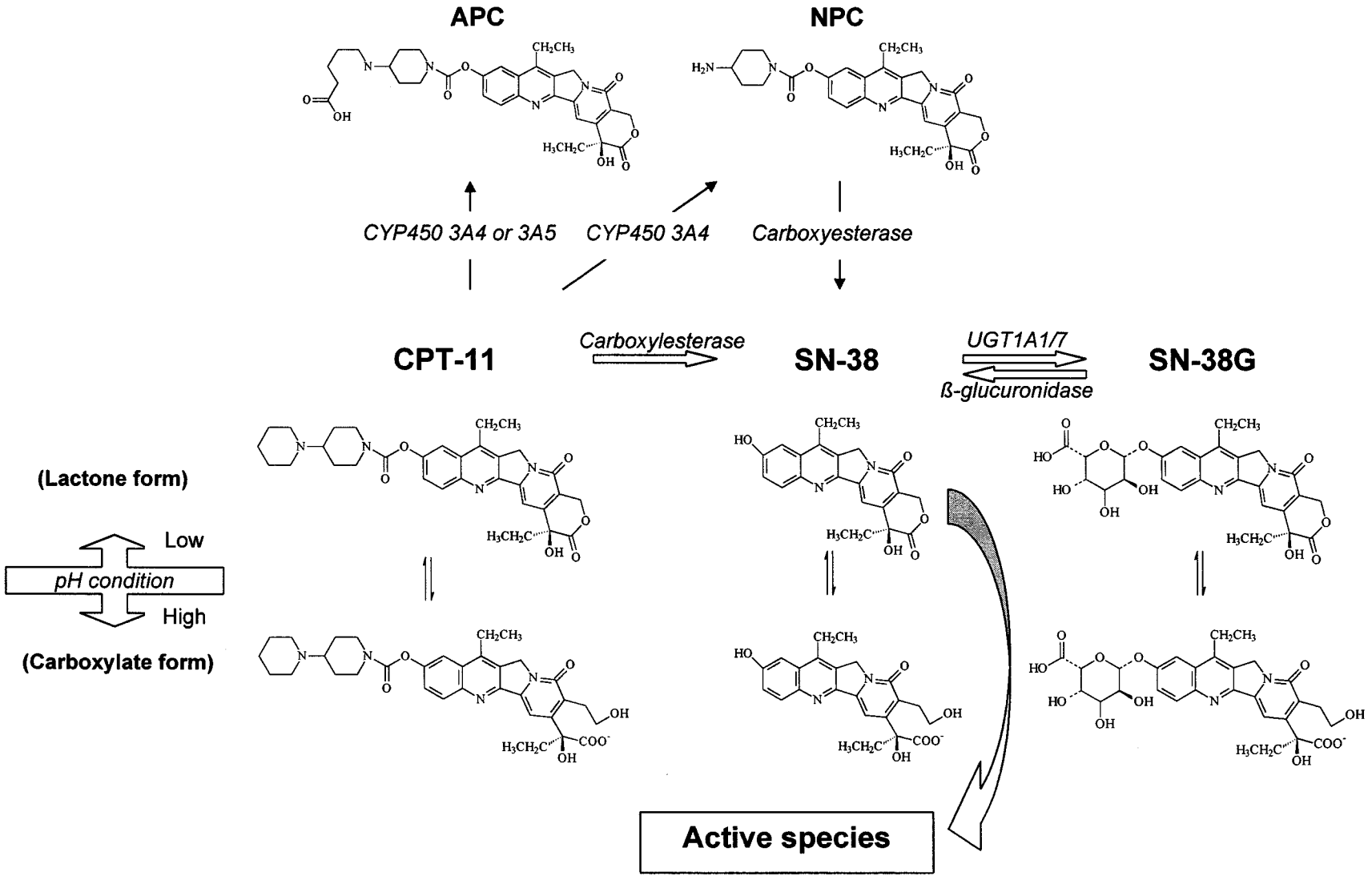

Figure 1. Metabolic pathways of CPT-11, showing hydrolysis to its ring-opened carboxylate form, carboxylesterase-mediated formation of the active metabolite SN-38, and its subsequent conversion to a glucuronide derivative (SN-38G) and deglucuronidation by (intestinal) β-glucuronidase. CPT-11 can also undergo CYP3A4- and CYP3A5-mediated oxidative metabolism to form APC (7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin) and NPC (7-ethyl-10-[4-(1-piperidino)-1-amino]carbonyloxy-camptothecin), of which the latter can be hydrolyzed to release SN-38.

and SN-38G are excreted into urine and feces, and that SN-38G in feces can be deconjugated to SN-38 by bacterial β-glucuronidase in the human intestine.[6] Preliminary evidence also indicated that, following biliary or intestinal secretion, SN-38 is reabsorbed by the intestinal cells, thus undergoing a re-circulation process that significantly reduces the effective clearance of the compound.[7]

As with all camptothecin analogs, both CPT-11 and SN-38 are liable to a reversible, pH-dependent hydrolysis at the α-hydroxy-δ-lactone moiety of the molecule, generating a pharmacologically inactive (ring-opened) carboxylate form (Figure 1), the formation of which is especially favored at physiologic pH or above.[8] For example, the variability in the disappearance half-life for the lactone forms of CPT-11 and SN-38 was as little as 29–32 min at pH 7.3 and 37°C.[9] Clearly, this process, as well as the susceptibility of CPT-11 to undergo CYP3A4-mediated biotransformation,[10] will have a serious impact on pharmacokinetic properties and pharmacodynamic actions, particularly with oral drug delivery, which is currently being studied both experimentally and clinically.[11,12] Indeed, recent work has shown that intestinal accumulation of the lactone forms of CPT-11 and SN-38 increased dose dependently, and was higher than that of the carboxylate forms at physiologic pH.[13] Furthermore, SN-38 accumulation into HT29 colon carcinoma cells was significantly correlated with its efficacy.[13]

Recently, various biochemical barriers have been implicated in the restriction of drug absorption that may be relevant for oral treatment with CPT-11. In particular, the drug-efflux pump P-gp, located on the brush-border membrane of intestinal enterocytes, and the drug-metabolizing enzyme CYP3A4 have been shown to limit the intestinal absorption of some drugs.[14,15] In the context of membrane permeability, it is noteworthy that CPT-11 carboxylate may be a substrate of P-gp and cMOAT, whereas SN-38 carboxylate can be transported by both MRP-1 and cMOAT.[16,17] Therefore, the overexpression of such pumps in intestinal cells, of which P-gp and cMOAT run countercurrent to the absorptive transport of substrate drugs,[18] can modulate the extent of oral CPT-11 or SN-38 (re)absorption. At present, no ATP-dependent cellular uptake has been observed for CPT-11 lactone, which is considered to be transported passively through hepatic and intestinal cells.[13,16] However, there is very little information on cellular permeability of the lactone forms of CPT-11 and SN-38, in particular with respect to relationships between pharmacokinetic profiles and activity. Furthermore, the limited aqueous solubility of SN-38 lactone and the lack of analytical methods with sufficient sensitivity[17] or the use of non-specific techniques based on radiolabeled drug[13] have precluded the use of experimental designs that mimic clinically relevant (physiological) conditions. To gain insight into mechanisms involved in the cellular permeability of CPT-11 and its metabolites, we studied transepithelial transport of the lactone and carboxylate forms of the drugs, and determined the role of the various membrane transporters using the colon cancer cell line Caco-2 as a model of the human intestinal epithelium.

Materials and methods

Chemicals and reagents

CPT-11 hydrochloride trihydrate (batch KO16) and SN-38 hydrochloride (batch LIE783) were kindly supplied by Aventis (Vitry-sur-Seine Cedex, France). Camptothecin (batch 93K05A), used as the internal standard for HPLC analysis, was obtained from Pharmacia-Upjohn (Albuquerque, NM). In the P-gp-inhibition assays, cyclosporin A was obtained from Sigma (St Louis, MO), verapamil from Bufa (Uitgeest, The Netherlands) and R101933 was supplied by the Janssen Research Foundation (Beerse, Belgium). All compounds were stored in DMSO at −80°C. All reagents for HPLC analysis, including acetonitrile, methanol, *n*-butyl chloride and DMSO were purchased from Rathburn (Walkerburn, UK). Hydrochloric acid, perchloric acid and ammonium acetate were obtained from Baker (Deventer, The Netherlands), sodium chloride from Merck (Amsterdam, The Netherlands), and tetrabuthylammonium sulfate from Serva (Heidelberg, Germany). The chemicals and reagents were of the highest purity available, and were used as received. All of the tissue-culture reagents were from Life Technologies (Breda, The Netherlands). Purified water was obtained by filtration and deionization with a Milli-Q-UF system (Millipore, Bedford, MA) and was used throughout.

Cell lines

The human intestinal epithelium adenocarcinoma Caco-2 cells were obtained from ATCC (Rockville, MD) and maintained according to the guidelines recommended by the agency. The human colon adenocarcinoma COLO201 and COLO320DM cells were from the Japanese Cancer Research Resources Bank (Tokyo, Japan), and the human doxorubicin-resistant leukemia K562/DOX cells (expressing *P-gp* and *MRP-1* mRNA) and the hepatic cancer Hep-G2 cells (expressing only *cMOAT* mRNA) were kindly provided by Dr Takashi Tsuruo (Tokyo University, Tokyo, Japan) and Dr Michihiko Kuwano (Kyushu

University, Fukuoka, Japan), respectively. Caco-2 cells and the other cells were passaged in Dulbecco's minimum essential medium and RPMI 1640 medium, respectively, containing 10% fetal bovine serum, 1 mM $NaHCO_3$, 2 mM L-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 at 37°C at a final pH value of 7.2 ± 0.05.

mRNA analysis

Total RNA isolation and Northern analysis was performed on all five cell lines as described.[19] In brief, 10 μg total RNA, isolated using an RNAeasy total RNA kit (Qiagen, Hilden, Germany) were separated electrophoretically on a 1.0% agarose gel containing formaldehyde and transferred onto a nylon membrane. The probes used for hybridization were a 0.7-kb *Pvu*II fragment of a human *MDR1* cDNA (*P-gp*) derived from K562 cells, a 0.9-kb *Eco*RI cDNA fragment of a human *MRP* gene (*MRP-1*), a 0.5-kb human *cMOAT* cDNA from K562 cells and a 0.85-kb fragment (containing the exons 4–7) of a human *GAPDH* gene. These probes were labeled with [α-^{32}P]dCTP using Ready-to-Go DNA Labeling Beads (Pharmacia, Piscataway, NJ). Radioactivity of the specific bands was quantified from a standard curve relative to expression levels of the *GAPDH* mRNA in the same cells using a BAS-2000 Bioimaging analyzer (Fuji Film, Tokyo, Japan).

Drug transport assays

Caco-2 cells were cultured in about 4 weeks until complete monolayers were obtained in Transwell clusters (Costar, Cambridge, MA) composed of a 12-well plate with 1-cm^2 polycarbonate membrane filters (0.4-μm pore size) in each well that contained a 0.5-ml upper compartment (the apical side) and a 1.5-ml lower compartment (the basolateral side). Transport assays were initiated with the addition of CPT-11 or SN-38 [both diluted from methanolic stock solutions in medium (pH 7.2) at final concentrations of 1.7 and 17 μM, and 2.5 and 25 μM, respectively], to either the apical or basolateral side. Experiments were conducted in triplicate at variable continuous exposure durations of 1, 2, 6 and 24 h in a controlled environment at a temperature 37°C. P-gp inhibition experiments were also performed in triplicate under identical conditions with a 24-h continuous exposure duration, by addition of cyclosporin A, verapamil or R101933 [all diluted in the medium (pH 7.2) at final concentrations of 0.1, 1.0 and 10 μM, 1.0 and 10 μM, and 0.01, 0.1 and 1.0 μM, respectively] at both the apical or basolateral side in the presence of CPT-11 or SN-38. The final concentration of methanol in the dosing medium was always below 1%. At the end of the indicated exposure time, the entire apical and basolateral solutions were collected, centrifuged for 5 min at 24 000 *g* (4°C) and immediately stored at −80°C until analysis by HPLC (see below). The mean TF, calculated as the amount of drug transported to the receiver compartment relative to the total amount added for each drug at the donor side, was used as a measure of drug permeability through the cells.

Measurement of cellular accumulation was performed as described for the related compound, 9-amino-20(*S*)-camptothecin,[20] with minor modifications. Briefly, Caco-2 cells were grown to 80% confluence or higher, plated in 12-well culture plates (Costar), and incubated in triplicate at 37°C with various concentrations of CPT-11 and SN-38 either alone or combined with a P-gp inhibitor for a total exposure duration of 24 h. The treated cells were washed twice rapidly with ice-cold phosphate-buffered saline, and stored as dry-pellets at −80°C until HPLC analysis after harvesting by scraping and centrifugation for 5 min at 24 000 *g* (4°C). Total drug accumulation was normalized by the sample dry weight and expressed as ng drug/mg dry weight of the cells.

Antiproliferative efficacies of CPT-11 and SN-38 when added combined with a P-gp inhibitor were measured by the MTT assay. Cells were seeded in quadruplicate at 5×10^4 cells/well in 96-well culture plates (Costar) and exposed to each drug diluted in the medium (pH 7.2) for 24 h at 37°C. Cell proliferation and growth inhibition in the presence of CPT-11 and SN-38 were evaluated as the percentage cell growth of treated cells versus untreated cells (T/C) and as the control rate minus T/C (i.e. 1−T/C), respectively.

HPLC analysis

Measurement of the lactone and the carboxylate (estimated as the difference of total drug, i.e. lactone plus carboxylate, and the lactone) forms of CPT-11 and SN-38 was performed by HPLC analysis as described,[21] with modifications. Briefly, for the pretreatment of the total form, a 250-μl aliquot diluted in the culture medium was acidified with 500 μl of a mixture of methanol:5% (v/v) aqueous perchloric acid (1:1, v/v). The sample was rocked on a multitube-vortex mixer for 10 min, followed by centrifugation at 24 000 *g* for 5 min (4°C) and an 80-μl aliquot of the clear supernatant from the extract was injected into the HPLC system. For the lactone form samples, a 1-ml aliquot of sample diluted with human plasma followed by addition of 2.5 ng camptothecin (used as internal

standard) and 0.8 g sodium chloride was extracted with 7.5 ml acetonitrile:*n*-butyl chloride (1:4, v/v). The sample was vortex-mixed for 2 min, followed by centrifugation at 3600 *g* for 7 min. The supernatant of the upper organic phase was transferred to a 10 ml glass tube containing 50 μl DMSO and evaporated under a stream of nitrogen at 65°C for 45 min. The residue was mixed with 200 μl methanol:0.15% (v/v) aqueous perchloric acid (1:1, v/v) and an 80-μl aliquot of the mixture was injected into the HPLC system. The system was composed of a constaMetric 3200 solvent delivery system, an autoMetric 4100 autosampling device (LDC Analytical, Riviera Beach, FL) and a FP-920 fluorescence detector (JASCO, Tokyo, Japan). Chromatographic separations were achieved using a Hypersil ODS column (100 × 4.6 mm inner diameter, 5-μm particle size; LC Service, Emmen, The Netherlands), protected by a LiChroCART guard column (4 × 4 mm inner diameter, 5 μm particle size; Merck, Darmstadt, Germany). The column temperature for analytical measurements of the total and lactone forms was maintained at 60 and 50°C, respectively, using a Column-Thermostat Jetstream Plus system (Alltech, Breda, The Netherlands). Injected samples were eluted isocratically with a mixture of methanol:0.1 M ammonium acetate containing 10 mM tetrabuthylammonium sulfate [35:65 (v/v) at pH 5.5 for the total form and 40:60 (v/v) at pH 4.5 for the lactone form, adjusted with hydrochloric acid]. The mobile phase was delivered at 1.0 ml/min, and the column effluent was monitored at 355 nm excitation and 515 nm emission wavelengths for both CPT-11 and SN-38. The detector signal was processed with the Fisons ChromCard data analysis system (Milan, Italy). The qualitative and quantitative determination of each compound was based on HPLC retention times and peak height measurements, respectively, in comparison with injected standards, over ranges of 2–200 ng/ml for the total form and 0.5–15 ng/ml for the lactone form of each drug. Calibration curves were computed using the ratio of the peak height for the total form and the normalized ratio of the internal standard (camptothecin) for the lactone form by weighted ($1x^2$) least-squares linear-regression analysis. The percentage deviation from calibrated values and the between-run and within-run variabilities at various concentration levels were always below 10% for both forms of each drug.

Statistical analysis

Differences in the TF for CPT-11 and its metabolites between the basolateral-to-apical and apical-to-basolateral direction were assessed by one-way analysis of variance. An unpaired Student's *t*-test was used to determine the difference between accumulation profiles of CPT-11 and SN-38 in the absence or presence of transport modulators. Relationships between the (logarithmic) cellular accumulation of CPT-11 and SN-38 and their respective growth inhibition rate in Caco-2 cells were analyzed by means of Pearson's correlation coefficient (*r*) and least-squares linear regression analysis. All statistical calculations were done using the Number Cruncher Statistical System 5.X series (J.L. Hintze, East Kaysville, UT, 1992). Statistical significance was considered to be reached when $p < 0.05$, with a two-tailed distribution. All data are presented as mean ± SD except where indicated otherwise.

Results

Expression of P-gp and MRP family transporters

When grown under conventional culture conditions, Caco-2 cells differentiate into polarized monolayers with characteristics of the human intestinal epithelium. Because P-gp and MRP family members such as MRP-1 and cMOAT are assumed to be involved in the hepatobiliary elimination of the caroboxylate forms of CPT-11 and SN-38 in rats and humans,[16] we hypothesized that membrane transporters expressed in the intestine might also mediate CPT-11 and SN-38 (ef)flux. Examination of the expression of these transporters by Northern-blot analysis with RNA from Caco-2 cells indicated that *P-gp* and *cMOAT* were highly expressed, whereas the expression of *MRP-1* was minimal (Figure 2), consistent with recent

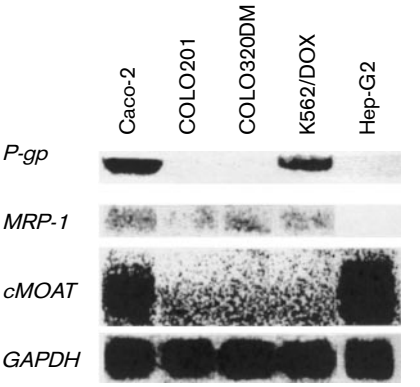

Figure 2. Northern-blot analysis to determine the expression level of the membrane transporters P-gp, MRP-1 and cMOAT in Caco-2, COLO201 and COLO320Dm cells. Expression levels were normalized by GAPDH mRNA. K562/DOX cells and Hep-G2 cells were used as the positive control of P-gp and MRP-1, and cMOAT, respectively.

findings.[18] Interestingly, after normalizing expression levels by *GAPDH* mRNA, *P-gp* levels in Caco-2 cells were still substantially higher than that in the P-gp-positive human doxorubicin-resistant leukemia K562/DOX cells. Normalized *MRP-1* and *cMOAT* mRNA levels in Caco-2 cells were also 1.2- and 1.3-fold and 2.1- and 2.7-fold higher, respectively, than observed in the other colon carcinoma cells tested, i.e. the COLO201 and COLO320DM cell lines. Hence, Caco-2 cells are likely to discharge many substrate drugs acting as a cellular poison and had already acquired chemotherapy resistance by expression of a combination of membrane transporters.

Metabolism of CPT-11 in Caco-2 cells

Pharmacokinetic studies in mice have shown increased SN-38 formation after oral as compared to i.v. CPT-11 administration, tentatively ascribed to activity of an intestinal epithelial carboxylesterase that metabolizes CPT-11.[11] Since carboxylesterase was previously detected immuno-histochemically and functionally in Caco-2 cells,[22] the possible conversion of CPT-11 into SN-38 was investigated. Analysis of the HPLC chromatograms of incubation mixtures from CPT-11 treated cells did not reveal SN-38 or any other compound with similar characteristics that might represent CPT-11 metabolites or decomposition products (Figure 3). In addition, no significant formation of the CYP3A4-mediated metabolites APC and NPC was detected following incubation with CPT-11, consistent with the insignificant expression of this isozyme in Caco-2 cells.[22] There was also no formation of SN-38 after exposure to SN-38G, in line with the undetectable levels of β-glucuronidase activity as measured by the colorimetric phenolphthalein glucuronic acid assay.[7]

Cellular permeability of CPT-11 and SN-38

Since during transport studies with CPT-11 and SN-38 no metabolites or degradation products could be found, the detected levels of the compounds were considered to be a direct measure of permeability. The transport of CPT-11 (lactone, Figure 4A; carboxylate, Figure 4B) and SN-38 (lactone, Figure 4C; carboxylate, Figure 4D) through Caco-2 cell monolayers increased in a time-dependent manner. However, the total TF (i.e. TF to the apical *plus* TF to the basolateral compartment) substantially decreased with higher concentrations for all drug forms. The highest TF of the CPT-11 and SN-38 lactone was achieved for both sides at the 2 and 6 h time points, respectively, when the lactones equilibrated cellular influx with efflux. At the lowest concentration of CPT-11 (1.7 μM), the TF of the lactone form in the basolateral-to-apical direction was 6.5- to 11-fold higher than in the reverse

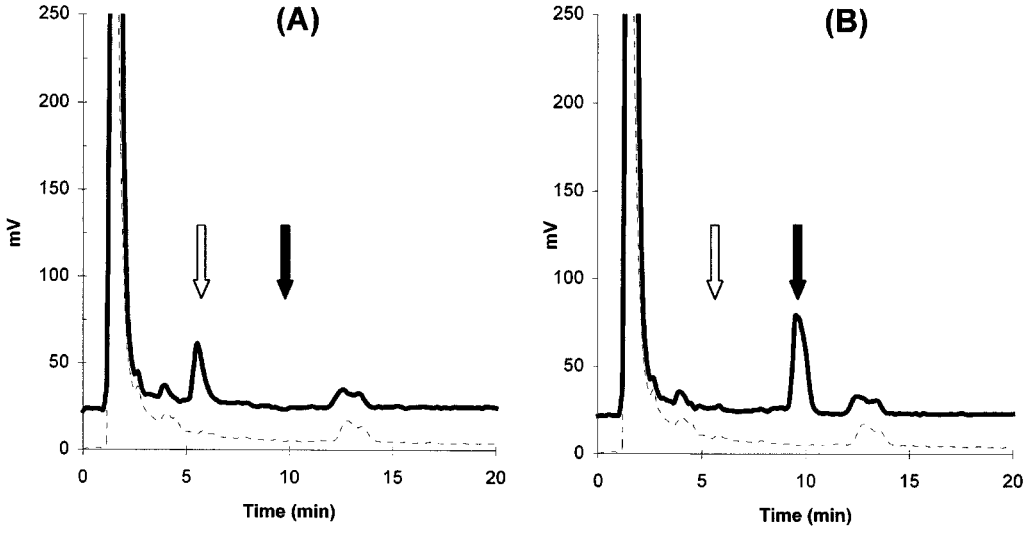

Figure 3. Reversed-phase HPLC tracing with fluorescence detection at excitation and emission wavelengths of 355 and 515 nm, respectively, of (A) a sample taken from the basolateral compartment after apical exposure of a Caco-2 cell monolayer to CPT-11 at a concentration of 1.70 μM and (B) a sample taken from the basolateral compartment after apical exposure of a Caco-2 cell monolayer to SN-38 at a concentration of 2.50 μM. Samples were processed for measurement of total drug forms (i.e. lactone plus carboxylate), and chromatographic peaks represent unchanged CPT-11 (white arrow, retention time, 5.5 min) and SN-38 (black arrow; retention time, 9.6 min). Concentrations of the two CYP3A4-mediated metabolites APC and NPC were below the lower limit of quantitation of the HPLC system (10 ng/ml).

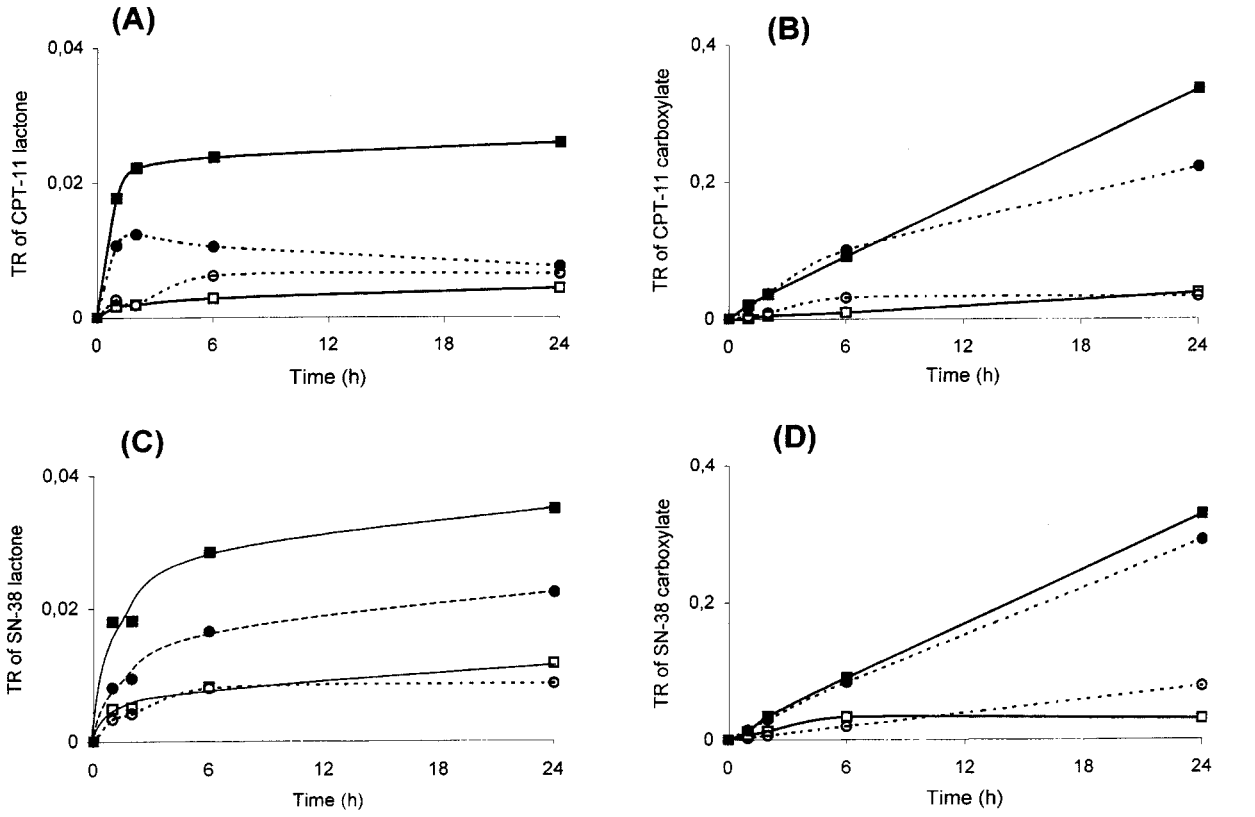

Figure 4. Transepithelial fluxes of CPT-11 and SN-38 measured in Caco-2 cells as a function of exposure time. The transport rate, defined as the amount of drug transported to the receiver compartment relative to the total amount added for each drug on the donor side, was used as a measure of drug permeability through the cells. Experiments were performed in three different experiments during 24-h exposure periods, and mean data are shown for CPT-11 lactone (A), CPT-11 carboxylate (B), SN-38 lactone (C) and SN-38 carboxylate (D). Fluxes were measured in the basolateral-to-apical (closed symbols) and apical-to-basolateral direction (open symbols) at either low concentrations (i.e. 1.7 μM for CPT-11 and 2.5 μM for SN-38; squares) or high concentrations (i.e. 17 μM for CPT-11 and 25 μM for SN-38; circles).

direction (p=0.0023; one-way analysis of variance) at the 24 h time point. However, the difference in TF of CPT-11 lactone at 17 μM between basolateral-to-apical and the reverse direction was less evident, particularly between the 2 and 24 h points. The CPT-11 carboxylate TF in the basolateral-to-apical direction was 7.2- to 9.2-fold higher (p=0.0010) and 3.2- to 6.7-fold higher (p=0.0051) at 1.7 and 17 μM, respectively. For SN-38, tested at concentrations of 2.5 and 25 μM, the lactone TF in the basolateral-to-apical direction was 2.9- to 3.6-fold (p=0.0567) and 2.1- to 2.7-fold (p=0.1350) higher than in the opposite direction, whereas for the carboxylate form, these increases were 1.7- to 11-fold (p=0.0007) and 3.8- to 4.7-fold (p=0.0252), respectively. This suggests that both lactone forms as well as the carboxylates could be transported through Caco-2 cells by an active mechanism, and that at the highest concentration tested for CPT-11, this mechanism converted from active to passive transport through Caco-2 cells.

Transport in the presence of P-gp inhibitors

A series of directional-transport experiments was performed to address the nature of the active efflux mechanisms. CPT-11 and SN-38 transport was determined after exposure to the apical or the basolateral side in the presence of cyclosporin A (as a P-gp and MRP family inhibitor), verapamil or R101933 (as selective P-gp inhibitors), during a 24-h incubation when transport of the lactone forms had achieved equilibrium. The difference in the permeability between both sides in combination with any modulator could not be detected (data not shown). The TF of CPT-11 lactone to the apical side at 1.7 μM, and the TFs of CPT-11 carboxylate to the apical side at 1.7 and 17 μM, respectively, were substantially reduced by 39, 62 and 32% in the presence of the modulator, although the TF of CPT-11 lactone to the apical side at 17 μM was significantly enhanced (Figure 5A). Interestingly,

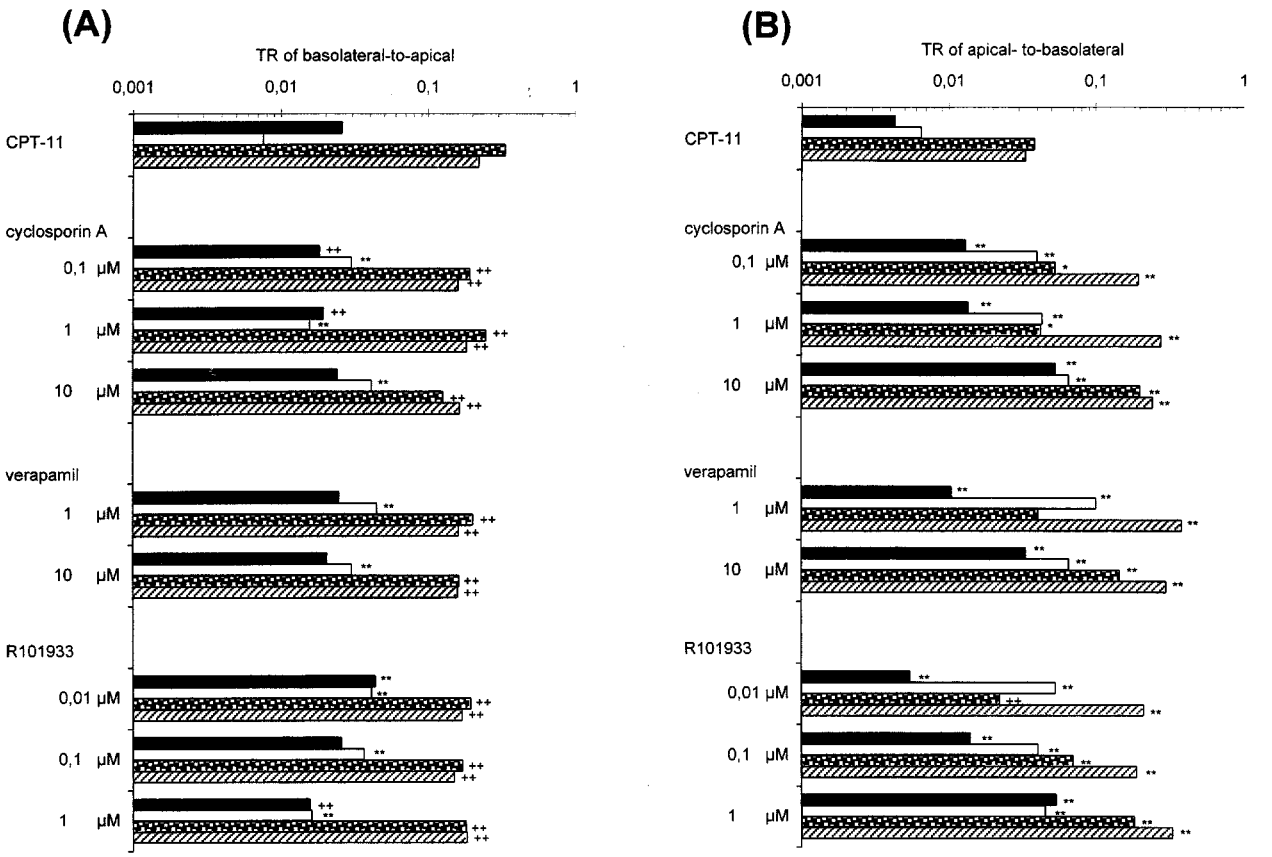

Figure 5. Transport characteristics of CPT-11 in the absence or presence of various modulating agents at the indicated concentration. Data are expressed as mean transport rates measured in triplicate of CPT-11 at 24 h in the basolateral-to-apical (A) or apical-to-basolateral direction (B). Black bar, transport rate of CPT-11 lactone at a tested concentration of 1.7 μM; white bar, transport rate of CPT-11 lactone at a tested concentration of 17 μM; checked bar, transport rate of CPT-11 carboxylate at a tested concentration of 1.7 μM; and hatched bar, transport rate of CPT-11 carboxylate at a tested concentration of 17 μM. */**Significantly higher; $^{+}$/$^{++}$significantly lower. */$^{+}p<0.05$; **/$^{++}p<0.01$ Student's *t*-test versus control.

the TFs of CPT-11 carboxylate to the apical side were affected to a lesser extent, with the strongest effect observed in the presence of R101933 at a concentration of 1 μM. Thus, the results indicate that countercurrent transport by P-gp increased the amount of CPT-11 (lactone and carboxylate) transported across the monolayer at low concentrations, with the transport mechanisms for the lactone form changing to an apparent P-gp-independent transport at higher levels. In contrast, the TFs of CPT-11 lactone and the carboxylate in the apical-to-basolateral direction were greatly increased by each modulator (1.3- to 15.6-fold and 1.1- to 12.6-fold, respectively), except with 0.01 μM R101933 (Figure 5B). These TFs, as well as the total side TF for CPT-11 lactone at both tested concentrations and CPT-11 carboxylate at 17 μM were found to increase with increased inhibition of P-gp-mediated transport. In addition, the TF of CPT-11 lactone in the apical-to-basolateral direction at 17 μM was significantly correlated with the TF in the opposite direction (r=0.75, p=0.0199) for the passive transport. The TFs of SN-38 lactone and carboxylate to the apical side at 2.5 and 25 μM were reduced 42 and 32%, and 55 and 19%, respectively, with stronger inhibition observed by escalated modulator dosing (Figure 6A). Similar to CPT-11, the TFs of SN-38 lactone and carboxylate in the basolateral direction were increased 1.1- to 5.4-fold and 1.4- and 8.7-fold, respectively (Figure 6B). Furthermore, inhibition of transport in the apical direction for SN-38 carboxylate at the lowest concentration influenced the TF of the lactone and carboxylate forms in the opposite direction (r=−0.82, p=0.0131 and r=−0.88, p=0.0037, respectively). Although the total side TF for the lactone form increased with stronger P-gp inhibition, as with CPT-11, the total side TF for the carboxylate form could be only weakly inhibited by modulation of P-gp-mediated transport. In contrast, the permeabil-

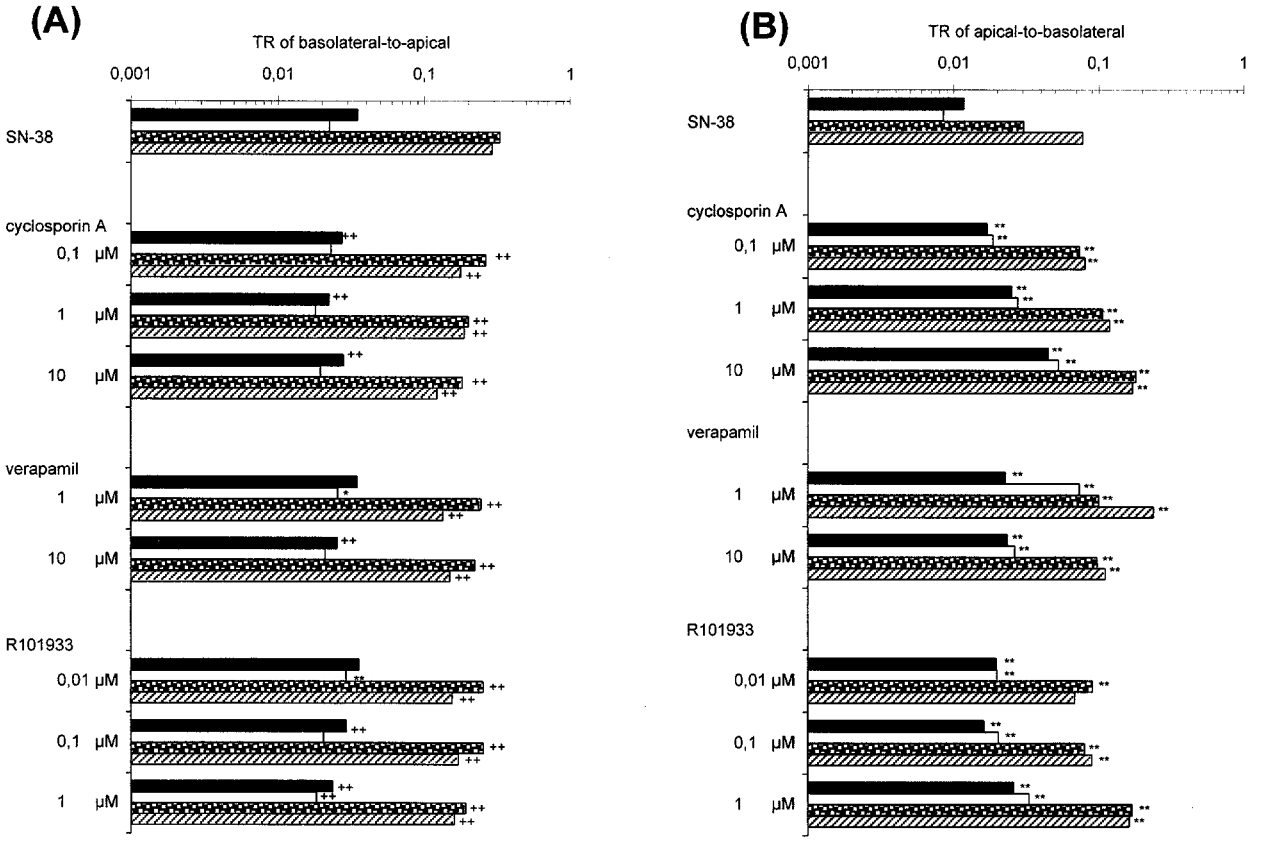

Figure 6. Transport characteristics of SN-38 in the absence or presence of various modulating agents at the indicated concentration. Data are expressed as mean transport rates measured in triplicate of SN-38 at 24 h in basolateral-to-apical (A) or apical-to-basolateral direction (B). Black bar, transport rate of SN-38 lactone at a tested concentration of 2.5 μM; white bar, transport rate of SN-38 lactone at a tested concentration of 25 μM; confetti-patterned bar, transport rate of SN-38 carboxylate at a tested concentration of 2.5 μM; and hatched bar, transport rate of SN-38 carboxylate at a tested concentration of 25 μM. */**Significantly higher; $^{+}$/$^{++}$significantly lower. */$^{+}p<0.05$; **/$^{++}p<0.01$ Student's *t*-test versus control.

ities of the lactone and carboxylate forms of both CPT-11 and SN-38 were indicated to be mainly dictated by P-gp activity in the basolateral-to-apical direction, although the permeabilities for CPT-11 and SN-38 could not be suppressed by combined exposure with a P-gp modulator due to accelerated permeability to the basolateral side, especially for the lactone forms.

Cellular uptake and efficacies

The amount of drug accumulated and the cell growth rates (T/C) for CPT-11 and SN-38 were differently influenced by modulation of P-gp activity in the Caco-2 cells (Tables 1 and 2). CPT-11 lactone accumulated in a dose-dependent manner and increased in the presence of the modulators, although its combined efficacy increased only with cyclosporin A, which is known to also inhibit the MRP family transporters. SN-38 used in combination with the modulators decreased not only its lactone accumulation, especially at the highest concentration of 25 μM, but also its combined efficacy. This is most likely because inhibition of the TF of SN-38 carboxylate in the basolateral-to-apical direction might affect the lactone accumulation by increasing the TF of the lactone form toward the basolateral side, particularly at 2.5 μM. Cellular accumulation of CPT-11 lactone was negatively correlated with its growth inhibition rate (1−T/C) at 1.7 μM ($r=-0.72$, $p=0.0434$), whereas this relationship was positive for SN-38 lactone ($r=0.72$, $p=0.0007$), as shown in Figure 7. Furthermore, accumulation of CPT-11 lactone (tested at 1.7 μM) was controlled by the total side TF for the lactone form ($r=0.78$, $p=0.018$), which did not correlate significantly with its combined efficacy. CPT-11 efficacy at 17 μM was not correlated to any measured factor in the Caco-2 cells. This suggests that the SN-38 efficacy is regulated by cellular accumulation of the lactone form, which can be influenced by differential accumulation of SN-38 carboxylate when the P-gp-mediated transport is

Table 1. Cellular accumulation and growth rate of CPT-11 in the absence and presence of various P-gp inhibitors[a]

CPT-11 (μM)	Inhibitor (μM)	Accumulated amount (ng/mg)		Cell growth rate T/C (%)
		Lactone form	Carboxylate form	
1.7	none	0.17±0.001	5.8±0.41	66±6.6
	cyclosporin A (0.1)	0.13±0.001	6.3±0.16[c]	77±3.6[e]
	cyclosporin A (1.0)	0.19±0.001[c]	4.9±0.11[d]	77±5.9[e]
	cyclosporin A (10)	0.20±0.002[c]	14±0.35[c]	71±6.4[e]
	verapamil (1.0)	0.27±0.002[c]	8.7±0.12[c]	87±7.2
	verapamil (10)	0.31±0.001[c]	11±0.11[c]	88±6.8
	R101933 (0.01)	0.25±0.001[c]	2.4±0.05[e]	88±6.6
	R101933 (0.1)	0.26±0.002[c]	10±0.25[c]	94±1.9[b]
	R101933 (1.0)	0.37±0.003[c]	13±0.15[c]	90±4.0
17	none	1.3±0.031	93±0.74	22±1.4
	cyclosporin A (0.1)	1.1±0.026[e]	106±2.6[c]	18±1.3[e]
	cyclosporin (1.0)	2.0±0.061	78±2.1[e]	19±1.1[e]
	cyclosporin A (10)	1.1±0.036	180±2.8[c]	20±1.5[d]
	verapamil (1.0)	2.9±0.087[b]	30±0.54[e]	20±1.1[d]
	verapamil (10)	3.9±0.15[c]	41±1.5[e]	21±1.5
	R101933 (0.01)	2.1±0.048	22±0.32[e]	15±0.56[e]
	R101933 (0.1)	3.3±0.11[b]	78±1.3[e]	22±2.0
	R101933 (1.0)	4.4±0.20[c]	68±1.3[e]	25±2.1[c]

[a]Data are expressed as mean±SD of accumulation data of three to four measurements. For details, see Materials and methods.
[b]Significantly higher at $p<0.05$.
[c]Significantly higher at $p<0.01$.
[d]Significantly lower at $p<0.05$.
[e]Significantly lower at $p<0.01$.

Table 2. Cellular accumulation and growth rate of SN-38 in the absence and presence of various P-gp inhibitors[a]

SN-38 (μM)	Inhibitor (μM)	Accumulated amount (ng/mg)		Cell growth rate T/C (%)
		Lactone form	Carboxylate form	
2.5	none	0.10±0.001	27±0.53	56±3.6
	cyclosporin A (0.1)	0.11±0.001	24±0.69[d]	63±2.9[c]
	cyclosporin A (1.0)	0.11±0.002[b]	35±1.1[c]	51±2.6[d]
	cyclosporin A (10)	0.070±0.001[e]	56±1.1[c]	50±3.4[d]
	verapamil (1.0)	0.053±0.001[e]	47±0.48[c]	56±4.0
	verapamil (10)	0.10±0.001	25±0.33	56±2.0
	R101933 (0.01)	0.021±0.002[e]	49±0.74[c]	66±3.9[c]
	R101933 (0.1)	0.26±0.001[c]	52±0.52[c]	53±3.4
	R101933 (1.0)	0.20±0.001[c]	54±0.70[c]	51±2.1[e]
25	none	6.5±0.066	1005±12	42±2.3
	cyclosporin A (0.1)	3.0±0.069[e]	1048±22	52±5.1[c]
	cyclosporin A (1.0)	4.7±0.087[e]	761±6.6[e]	46±4.3
	cyclosporin A (10)	2.8±0.035[e]	1329±3.7	45±3.1
	verapamil (1.0)	4.1±0.16[e]	1356±46	46±3.0[c]
	verapamil (10)	3.1±0.061[e]	1244±12	47±3.6[c]
	R101933 (0.01)	1.1±0.009[e]	1443±12[b]	56±2.6[c]
	R101933 (0.1)	3.5±0.22[e]	1107±3.2	53±4.4[b]
	R101933 (1.0)	2.5±0.033[e]	964±3.6	46±3.1[b]

[a]Data are expressed as mean±SD of accumulation data of three to four measurements. For details, see Materials and methods.
[b]Significantly higher at $p<0.05$.
[c]Significantly higher at $p<0.01$.
[d]Significantly lower at $p<0.05$.
[e]Significantly lower at $p<0.01$.

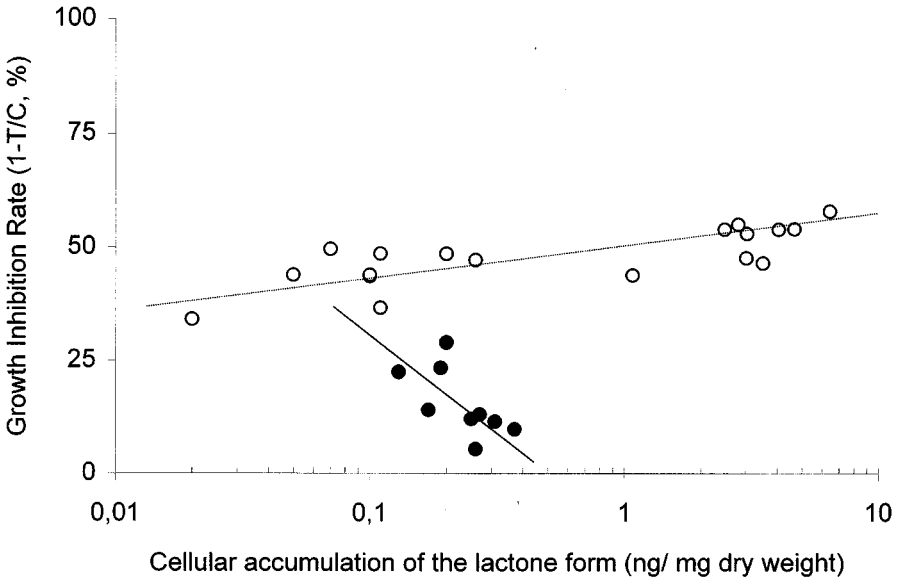

Figure 7. Relationship between the (logarithmic) cellular accumulation of CPT-11 (open symbols) or SN-38 (closed symbols) and the growth inhibition rate in Caco-2 cells. The percentage of the cell growth inhibition (1 − T/C) was calculated as the control value minus the cell growth rate. Relationships were fitted by least-squares log-linear regression analysis.

modulated. In contrast, for CPT-11 factors controlling changes in permeability were difficult to determine, although the low dose CPT-11 might be controlled by cellular permeability of the lactone form.

Discussion

Here, we have studied the transepithelial flux, cellular accumulation and antiproliferative effects of CPT-11 and SN-38 using Caco-2 cell lines, a well-established experimental model of human intestinal absorption. It has been proposed that the lactone forms of both CPT-11 and SN-38, in contrast to their respective carboxylate forms, are transported across cellular membranes by a passive diffusion mechanism when analyzed at extremely high concentration ranges up to 200 μM.[13] In the studies detailed here, however, an active transport regulating permeabilities of the lactone forms of CPT-11 and SN-38 was noted at lower concentrations, resembling the physiological condition, similar to that observed for the carboxylate forms with equilibrium between the lactone influx and efflux by cells after the 2-h treatment time point. This time period of exposure apparently represents a point when the lactone forms are equilibrated by the lactonolysis at pH 7.1–7.3 at these concentrations (around 2 μM).[9] This suggests that the lactone equilibrium for the CPT-11 and SN-38 permeabilities may be caused by achieving equilibrium of the lactone hydrolysis. In addition, transport of the lactone form of CPT-11 changed from an active to a passive transport mechanism at the highest tested concentration of 17 μM. Thus, the CPT-11 lactone permeability can be regulated by two different functions, especially under physiologically relevant conditions, i.e. active-membrane transport and passive diffusion.

To provide deeper insight into the active-membrane transport mechanisms involved in the permeability profiles of CPT-11 and SN-38, experiments were conducted in the presence of various compounds known to interfere with drug transporters. Northern-blot analysis with mRNA from the Caco-2 cells indicated that *P-gp* and *cMOAT*, normalized to expression levels of *GAPDH*, are highly expressed, whereas expression of *MRP-1* was minimal. This is consistent with previous data and with expression of these transporters under normal conditions in human intestinal tissues,[18] suggesting that the model system used here reflects the *in vivo* situation of normal enterocytes. Previously, it was shown that P-gp and cMOAT are expressed on the brush-border (apical) membrane of Caco-2 cells as well as *in vivo*,[18] whereas MRP-1 localizes to the basolateral side of polarized cells[23] (Figure 8). It has been known through the work of Sugiyama and coworkers that the carboxylate form of CPT-11 is a substrate of P-gp and cMOAT, whereas the carboxylate form of SN-38 can be transported by both MRP-1 and cMOAT.[16,17] Because of the expression of *P-gp*, *cMOAT* and *MRP-1* in Caco-2 cells it is

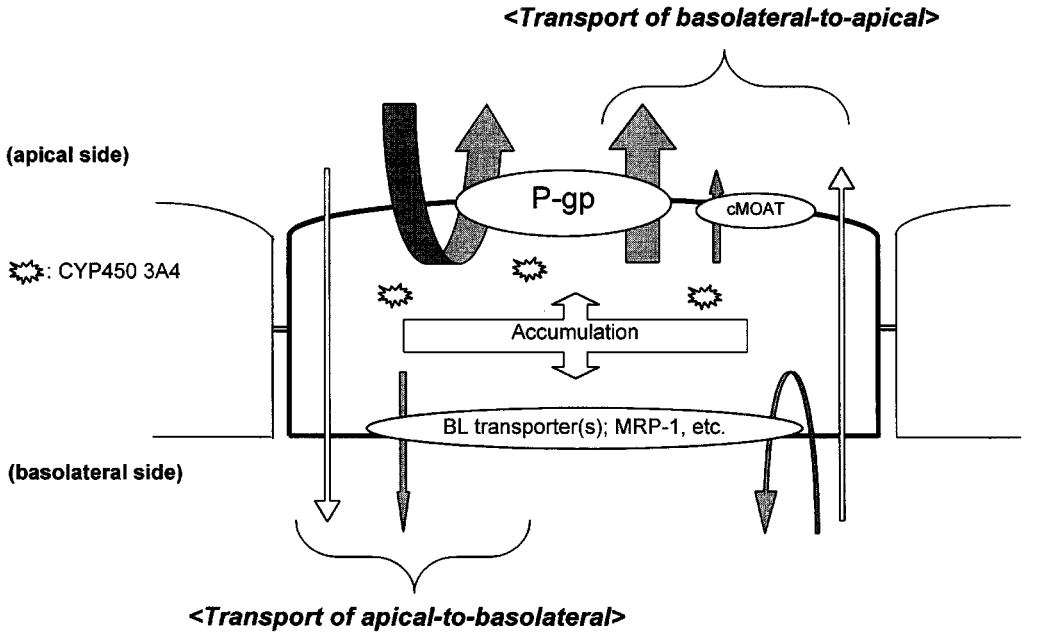

Figure 8. Model depicting the role of P-gp, MRP-1, cMOAT, CYP3A4 and passive membrane transport on drug (re)absorption profiles of CPT-11 and SN-38.

likely that the same transporters also mediate transepithelial fluxes of the carboxylate forms. However, we show now that, similar to CPT-11, the carboxylate form of SN-38 can be a substrate of not only MRP family transporters but also of P-gp. The involvement of P-gp in the extrusion of SN-38 is supported by the finding that efflux of SN-38 in Caco-2 cells is significantly inhibited by both cyclosporin A and specific inhibitors of P-gp, i.e. verapamil and R101933. Apparently, P-gp and a basolateral membrane transporter(s), presumably MRP-1, can control the apical membrane transport of the lactone forms of CPT-11 and SN-38. It is noteworthy, however, that Caco-2 cells are also known to express *MRP-3*,[18] a basolateral-localized membrane transporter that transports a variety of compounds, including anticancer drugs, and confers resistance to methotrexate and epipodophyllotoxins.[24] Although a role of MRP-3 expression in Caco-2 cells with respect to transport of CPT-11 and SN-38 cannot be ruled out, preliminary evidence suggests that camptothecin analogs (CPT-11, SN-38, and topotecan) are poor substrates of MRP-3.[24] Here, we found that the total permeabilities for both forms of CPT-11 and SN-38 could not be decreased by modulation of P-gp activity to the same extent as described for paclitaxel and vinblastine in the presence of verapamil.[25,26] As indicated, the apical transporter cMOAT, which plays an important role in the cellular extrusion of glucuronic acid and glutathione conjugated drugs,[16] can be also detected in Caco-2 cells by mRNA analysis. The function of cMOAT in the transport of CPT-11 lactone and both forms of SN-38 cannot be clearly established from this study. However, the possibility of its role in the transport in the basolateral-to-apical direction of CPT-11 carboxylate is indicated by the increased transport in the presence of the selective P-gp inhibitor, R101933 at a concentration of 1 μM. It has been proposed that cMOAT is also responsible for biliary secretion of the lactone and carboxylate forms of SN-38G.[16] Taking into consideration that SN-38G could not be transported through Caco-2 cell monolayers,[7] we speculate that cMOAT is predominantly responsible for this efficient active efflux mechanism. Furthermore, we found that the cellular accumulation of CPT-11 and SN-38 could not be augmented by combined exposure with specific P-gp inhibitors. Inhibition of the permeability to the apical side is thus guided to decrease the total permeability and the cellular accumulation of CPT-11 and SN-38. It is suggested that the decreased permeability in the apical direction by modulating P-gp activity may initially increase the cellular uptake, which in turn leads to enhanced transport toward the basolateral direction as a protective mechanism against CPT-11 and SN-38 accumulation. Indeed, whereas CPT-11-induced antiproliferative efficacy in the presence of cyclosporin A could surpass efficacy with single-agent treatment, SN-38 efficacy could not be increased in the presence of any

modulator. Hence, the Caco-2 cells may embody a variety of resistance mechanism(s), including the P-gp and MRP family membrane transporters, that have important protective functions by regulating drug uptake.

As indicated, the antiproliferative efficacies of CPT-11 and SN-38 are dependent on the total lactone permeability and the lactone accumulation, respectively. With respect to CPT-11, substantial antiproliferative efficacy could be provided by combined treatment with cyclosporin A, which can modulate the slow cellular permeability of CPT-11 by inhibition of both P-gp and the MRP family transporters. Although SN-38 efficacy could be affected by increasing lactone accumulation, e.g. by pH manipulation through a shift in the lactone–carboxylate equilibrium,[13] we did not observe increased SN-38-mediated antiproliferative effects in the presence of the modulators. This can be explained by assuming that modification of P-gp membrane transport (at the apical side) will eventually result in decreased accumulation of SN-38 lactone by a preferential outward-directed transport in the basolateral direction. Indeed, CPT-11 may display a time-dependent efficacy because the lactone permeability is increased with time up to 24 h and more antitumor effects by total membrane transport inhibition. Since for SN-38 efficacy is (dose-dependently) related to accumulation of the lactone form and given the fact that the carboxylate exhibits only minor topoisomerase I inhibitory activity,[3] increased efficacy could be achieved by maintaining low intracellular pH conditions, as in case of hyperglycemic acidosis.[19]

Clearly, cellular kinetics of the lactone forms of CPT-11 and SN-38 are not the sole processes influencing drug-induced antiproliferative properties. The cells may have already acquired other resistance mechanisms to CPT-11 and SN-38 induced toxicity by alteration of topoisomerase I expression or mutation, induced expression of the breast cancer resistance protein (BCRP),[27] expression of topoisomerase II, alterations in plasma transmembrane potentials and/or increased intracellular metabolic inactivation pathways mediated by glucuronosyltransferases.[28] In addition, the DNA repair enzyme, O^6-methylguanine-DNA methyltransferase (MGMT) has been shown to participate in the resistance of various topoisomerase I inhibitors, including CPT-11 and SN-38, against some glioblastoma cells, although its actual role here was not determined and remains to be elucidated.[29] Preliminary findings, though, indicate that Caco-2 cells also overexpress mRNA of *MGMT* (unpublished data). Currently, a detailed investigation into the actions of MGMT with respect to topoisomerase I poisons is a subject of intense interest.

One limitation to the present model system, particularly with respect to extrapolating our findings to the *in vivo* situation, is the fact that Caco-2 cells (under normal culture conditions) fail to express significant CYP3A4 activity. Indeed, previously we could not observe any formation of the major CYP3A4-mediated metabolites in the Caco-2 cells.[7] It has been proposed that P-gp can act in synergy with CYP3A4 in the intestine to restrict oral absorption by decreasing the rate of transport through repeated cycles of intracellular uptake and efflux, thereby increasing exposure of a drug to the enzyme before absorption in the systemic circulation.[30] Thus, inhibition of the countercurrent transport of CPT-11 and SN-38 by P-gp in patients treated with oral CPT-11 in combination with a modulator may also decrease the amount of APC and NPC formed intracellularly irrespective of competitive inhibition for CYP3A4 with, for example, cyclosporin A. Clinically, the combination of oral CPT-11 treatment with concomitant cyclosporin A would be particularly attractive in view of the fact that cyclosporin A interferes with hepatobiliary secretion of CPT-11 and its metabolites, thereby significantly reducing systemic clearance[31] and increasing CPT-11's therapeutic index by lowering the (dose-limiting) intestinal toxicity.[32] Taken together, these findings provide a rationale for attempts to improve the relatively low (around 10–20%)[33] and variable oral bioavailability of CPT-11 by concomitant administration of P-gp modulators.

References

1. Houghton PJ, Cheshire PJ, Hallman JC, Bissery MC, Mathieu-Boue A, Houghton JA. Therapeutic efficacy of the topoisomerase I inhibitor 7-ethyl-10-(4-[1-piperidino]-1-piperidino)carbonyloxy-camptothecin against human tumor xenografts: lack of cross-resistance in vivo in tumors with acquired resistance to the topoisomerase I inhibitor 9-dimethylaminomethyl-10-hydroxycamptothecin. *Cancer Res* 1993; **53**: 2823–9.
2. Shimada Y, Rothenberg M, Hilsenbeck SG, Burris III, HA, Degen D, Von Hoff DD. Activity of CPT-11 (irinotecan hydrochloride), a topoisomerase I inhibitor, against human tumor colony-forming units. *Anti-Cancer Drugs* 1994; **5**: 202–6.
3. Kawato Y, Aonuma M, Hirota Y, Kuga H, Sato K. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. *Cancer Res* 1991; **51**: 4187–91.
4. Iyer L, King CD, Whitington PF, *et al.* Genetic predisposition to the metabolism of irinotecan (CPT-11). Role or uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes. *J Clin Invest* 1998; **101**: 847–54.

5. Ciotti M, Basu N, Brangi M, Owens IS. Glucuronidation of 7-ethyl-10-hydroxycamptothecin (SN-38) by the human UDP-glucuronosyltransferases encoded at the UGT1 locus. *Biochem Biophys Res Commun* 1999; **260**: 199–202.
6. Sparreboom A, de Jonge MJ, de Bruijn P, *et al*. Irinotecan (CPT-11) metabolism and disposition in cancer patients. *Clin Cancer Res* 1998; **4**: 2747–54.
7. Kehrer DF, Yamamoto W, Verweij J, de Jonge MJ, de Bruijn P, Sparreboom A. Factors involved in prolongation of the terminal disposition phase of SN-38: clinical and experimental studies. *ClinCancer Res* 2000; **6**: 3451–8.
8. Akimoto K, Kawai A, Ohya K. Kinetic studies of the hydrolysis and lactonization of camptothecin and its derivatives, CPT-11 and SN-38, in aqueous solution. *Chem Pharm Bull* 2000; **42**: 2135–8.
9. Chourpa I, Millot JM, Sockalingum GD, Riou JF, Manfait M. Kinetics of lactone hydrolysis in antitumor drugs of camptothecin series as studied by fluorescence spectroscopy. *Biochim Biophys Acta* 1998; **1379**: 353–66.
10. Haaz MC, Rivory L, Riche C, Vernillet L, Roberg J. Metabolism of irinotecan (CPT-11) by human hepatic microsomes: participation of cytochrome P-450 3A and drug interactions. *Cancer Res* 1998; **58**: 468–72.
11. Zamboni WC, Houghton PJ, Thompson J, *et al*. Altered irinotetcan and SN-38 disposition after intravenous and oral administration of irinotecan in mice bearing human neuroblastoma xenografts. *Clin Cancer Res* 1998; **4**: 455–62.
12. Drengler RL, Kuhn JG, Schaaf LJ, *et al*. Phase I and pharmacokinetic trial of oral irinotecan administered daily for 5 days every 3 weeks in patients with solid tumors. *J Clin Oncol* 1999; **17**: 685–96.
13. Kobayashi K, Bouscarel B, Matsuzaki Y, Ceryak S, Kudoh S, Fromm H. pH-dependent uptake of irinotecan and its active metabolite, SN-38, by intestinal cells. *Int J Cancer* 1999; **83**: 491–6.
14. Sparreboom A, van Asperen J, Mayer U, *et al*. Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proc Natl Acad Sci USA* 1997; **94**: 2031–5.
15. van Asperen J, van Tellingen O, Beijnen JH. The pharmacological role of P-glycoprotein in the intestinal epithelium. *Pharmacol Res* 1998; **37**: 429–35.
16. Sugiyama Y, Kato Y, Chu X. Multiplicity of biliary excretion mechanisms for the camptothecin derivative irinotecan (CPT-11), its metabolite SN-38, and its glucuronide: role of canalicular multispecific organic anion transporter and P-glycoprotein. *Cancer Chemother Pharmacol* 1998; **42**(suppl): S44–9.
17. Chu XY, Suzuki H, Ueda K, Kato Y, Akiyama S, Sugiyama Y. Active efflux of CPT-11 and its metabolites in human KB-derived cell lines. *J Pharmacol Exp Ther* 2000; **288**: 735–41.
18. Hirohashi T, Suzuki H, Chu XY, Tamai I, Tsuji A, Sugiyama F. Function and expression of multidrug resistance-associated protein family in human colon adenocarcinoma cells (Caco-2). *J Pharmacol Exp Ther* 2000; **292**: 265–70.
19. Nishiyama M, Suzuki K, Kumazaki T, *et al*. Molecular targeting of mitomycin C chemotherapy. *Int J Cancer* 1997; **72**: 649–56.
20. Loos WJ, Verweij J, Gelderblom HJ, *et al*. Role of erythrocytes and serum proteins in the kinetic profile of total 9-amino-20(*S*)-camptothecin in humans. *Anti-Cancer Drugs* 1999; **10**: 705–10.
21. de Bruijn P, Verweij J, Loos WJ, Nooter K, Stoter G, Sparreboom A. Determination of irinotecan (CPT-11) and its active metabolite SN-38 in human plasma by reversed-phase high-performance liquid chromatography with fluorescence detection. *J Chromatogr B Biomed Sci Appl* 1997; **698**: 277–85.
22. Prueksaritanont T, Gorham LM, Hochman HJ, Tran LO, Vyas KP. Comparative studies of drug-metabolizing enzymes in dog, monkey, and human small intestines, and in Caco-2 cells. *Drug Metab Disp* 1996; **24**: 634–42.
23. Gutmann H, Fricker G, Torok M, Michael S, Beglinger C, Drewe J. Evidence for different ABC-transporters in Caco-2 cells modulating drug uptake. *Pharm Res* 1999; **16**: 402–407.
24. Kool M, van der Linden M, de Haas M, *et al*. MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci USA* 1999; **96**: 6914–9.
25. Hunter J, Jepson MA, Tsuruo T, Simmons NL, Hirst BH. Functional expression of P-glycoprotein in apical membranes of human intestinal Caco-2 cells. Kinetics of vinblastine secretion and interaction with modulators. *J Biol Chem* 1993; **268**: 14991–7.
26. Walle UK, Walle T. Taxol transport by human epithelial intestinal epithelial Caco-2 cells. *Drug Metab Disp* 1998; **26**: 343–6.
27. Maliepaard M, van Gastelen MA, de Jong LA, *et al*. Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. *Cancer Res* 1999; **59**: 4559–63.
28. Takahashi T, Fujiwara Y, Yamakido M, Katoh O, Watanabe H, Mackenzie PI. The role of glucuronidation in 7-ethyl-10-hydroxycamptothecin resistance *in vitro*. *Jpn J Cancer Res* 1997; **88**: 1211–7.
29. Okamura T, Kurisu K, Yamamoto W, Takano H, Nishiyama M. NADPH/quinone oxidoreductase is a priority target of glioblastoma chemotherapy. *Int J Oncol* 2000; **16**: 295–303.
30. Hochman JH, Chiba M, Nishime J, Yamazaki M, Lin JH. Influence of P-glycoprotein on the transport and metabolism of indinavir in Caco-2 cells expressing cytochrome P-450 3A4. *J Pharmacol Exp Ther* 2000; **292**: 310–8.
31. Gupta E, Safa AR, Wang X, Ratain MJ. Pharmacokinetic modulation of irinotecan and metabolites by cyclosporin A. *Cancer Res* 1996; **56**: 1309–14.
32. Gupta E, Lestingi TM, Mick R, Ramirez J, Vokes EE, Ratain MJ. Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea. *Cancer Res* 1994; **54**: 3723–5.
33. Stewart CF, Zamboni WC, Crom WR, Houghton PJ. Disposition of irinotecan and SN-38 following oral and intravenous irinotecan dosing in mice. *Cancer Chemother Pharmacol* 1997; **40**: 259–65.

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