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ABCG2 OVEREXPRESSION IN COLON CANCER CELLS RESISTANT TO SN38 AND IN IRINOTECAN-TREATED METASTASES

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Overcoming drug resistance has become an important issue in cancer chemotherapy. Among all known mechanisms that confer resistance, active efflux of chemotherapeutic agents by proteins from the ATP-binding cassette family has been extensively reported. The aim of the present study was to determine the involvement of ABCG2 in resistance to SN38 (the active metabolite of irinotecan) in colorectal cancer. By progressive exposure to increasing concentrations of SN38, we isolated 2 resistant clones from the human colon carcinoma cell line HCT116. These clones were 6- and 53-fold more resistant to SN38 than the HCT116-derived sensitive clone. Topoisomerase I expression was unchanged in our resistant variants. The highest resistance level correlated with an ABCG2 amplification. This overexpression was associated with a marked decrease in the intracellular accumulation of SN38. The inhibition of ABCG2 function by Ko143 demonstrated that enhanced drug efflux from resistant cells was mediated by the activity of ABCG2 protein and confirmed that ABCG2 is directly involved in acquired resistance to SN38. Furthermore, we show, for the first time in clinical samples, that the ABCG2 mRNA content in hepatic metastases is higher after an irinotecan-based chemotherapy than in irinotecan-naive metastases. In conclusion, this study supports the potential involvement of ABCG2 in the development of irinotecan resistance *in vivo*.

Key words: *colorectal cancer; ABCG2; SN38; drug resistance*

Chemotherapeutic drug resistance is a frequent cause of treatment failure in colorectal cancer patients. Understanding the cellular mechanisms that lead to this resistance should permit an improvement in the treatment of colorectal cancer. Irinotecan (CPT-11), a semisynthetic water-soluble derivative of camptothecin, is widely used for the treatment of metastatic colon cancer.[1]

CPT-11 is a prodrug converted by carboxylesterases into its active form, SN38.[2] Like other camptothecin derivatives, SN38 exerts its cytotoxic activity through the inhibition of topoisomerase I. Human topoisomerase I is a 100 kDa nuclear enzyme needed for replication and transcription and causing single strand breaks in DNA, thus permitting relaxation of supercoiled DNA.[3] SN38 interferes with topoisomerase I function by forming stable ternary complexes at the DNA breakage points and stopping the topoisomerase I-mediated religation.[4] Cellular resistance to camptothecin derivatives can result from a decrease in cellular drug accumulation, alterations in the structure or location of topoisomerase I, changes in the cellular response to the drug-DNA-enzyme ternary complex formation[5] or increased glucuronidation of SN38, resulting in an inactivation of the drug.[6]

Members of the ATP-binding cassette (ABC) transporters, notably MDR1 (ABCB1) and MRP1 (ABCC1), confer resistance to chemotherapeutic drugs by active drug efflux.[7,8] Recently, a new member of this family, the ABCG2 transporter also called BCRP[9] or ABCP[10] or MXR[11] has been discovered. This gene, localized in chromosome 4q22.1, encodes a 655 amino acid protein with an N-terminal ATP binding domain and a C-terminal region consisting of 6 transmembrane domains.[8] This ABC half-transporter requires dimerization to form a functional transporter and probably works as a homodimer.[12,13] The highest expression level of ABCG2 has been found in the placenta, and its normal physiological role is not yet known. Recently, a new protein named BMDP for brain multidrug resistance that shows high homology with ABCG2 has been isolated from pig brain capillaries,[14] and ABCG2 was demonstrated to be located at the human blood brain barrier,[15] suggesting a potential role for that ABCG2 transporter in the exclusion of xenobiotics from the brain. Another physiological role was suggested by Suzuki *et al*.,[16] who showed that ABCG2 is able to transport various sulfated conjugates through membrane vesicles and that it could be involved in the cellular extrusion of such compounds.

Overexpression of ABCG2 protein in various cancer cell lines confers high levels resistance to different anticancer drugs such as mitoxantrone, antracyclines and camptothecin derivatives.[9,17–21] Nevertheless, whereas the involvement of ABCG2 in resistance to chemotherapeutic drugs is clear *in vitro*, the clinical significance of such a mechanism has not yet been clearly demonstrated. Van den Heuvel *et al*.[22] reported a significant correlation between high levels of ABCG2 in acute myeloid leukemia (AML) and resistance to the treatment, while Van der Kolk *et al*.[23] found that ABCG2 was not consistently upregulated in relapsed/refractory AML. The implication of ABCG2 in clinical resistance was also studied in breast cancer but did not reveal any correlation between elevated ABCG2 and resistance to anthracycline treatment.[24]

The aim of our work is to determine the mechanisms involved in resistance to SN38 in colon cancer. To this end, we developed an *in vitro* model of resistance to SN38 by continuous and progressive exposure of human colon cancer cell line HCT116 to SN38. Investigation of efflux pumps showed that unlike PgP,

Abbreviations: ABCP, ATP-binding cassette transporter, placenta-specific; ABCG2, ATP-binding cassette, sub-family G, member 2; BCRP, breast cancer resistance protein; IC_{50}, drug concentration inhibiting 50% of cell growth; LRP, lung resistance protein; MDR1, multiple drug resistance 1; MRP1, multidrug resistance-associated protein 1; MXR, mitoxantrone resistance protein; Pgp, P170-glycoprotein; RF, resistance factor.

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MRP1 and LRP proteins, ABCG2 was overexpressed at the protein, transcriptional and genomic level in some of the SN38-resistant clones obtained during acquisition of resistance. Furthermore, ABCG2 overexpression was capable of conferring high levels of resistance and, as shown by inhibition of ABCG2 function, appeared to be partially responsible for resistance to SN38 in ABCG2-overexpressing resistant clones. Resistance in non-ABCG2 overexpressing clones does not involve decreased expression of topoisomerase I. We also studied ABCG2 expression in clinical samples from colon cancer patients and found that irinotecan-based chemotherapy led to an elevated ABCG2 mRNA level in human hepatic metastases. These results suggest that ABCG2 may be involved in irinotecan resistance, both *in vitro* and *in vivo*.

MATERIAL AND METHODS

Chemicals

SN38 was kindly provided by Aventis Pharma (Vitry-Sur-Seine, France). Irinotecan (CPT-11), topotecan, 5-fluorouracil, doxorubicin, mitoxantrone and oxaliplatin were kindly provided by Dr. F. Pinguet (CRLC Val d'Aurelle, Montpellier, France). Ko143 was kindly provided by Dr. Van Loevezijn (Institute of Molecular Chemistry, Amsterdam, the Netherlands).

Cell lines

HCT116 colon adenocarcinoma cell line was purchased from the ATCC (Manassas, VA). Cells were grown in complete medium, *i.e*., RPMI 1640 supplemented with 10% fetal calf serum and 2 mM L-glutamine at 37°C under a humidified atmosphere with 5% CO_2, and passaged by trypsinization. The parental cell line was first cloned to obtain a reference SN38 sensitive clone, referred to as HCT116-s. This sensitive clone was then subjected to a continuous exposure to SN38 with stepwise increased concentrations ranging from 1 nM to 15 nM over a period of approximately 8 months. The cell population growing in 10 nM SN38 was cloned to obtain the HCT116-SN6 clone, and the cloning of the population growing in 15 nM SN38 gave us the HCT116-SN50 clone. Drug-selected clones were maintained in the appropriate concentration of SN38. All the cell lines were cultured in drug-free medium at least 5 days prior to any experiment.

Drug sensitivity assay

Growth inhibition (IC_{50}) assays were performed using the sulforhodamine B assay as described by Skehan *et al*.[25]. Briefly, cells were seeded in 96-well plates (1,000 cells per well) in complete medium. After a 24 hr rest, drugs were applied in a dilution series, each concentration in triplicate wells. After 96 hours, cells were fixed by adding trichloroacetic acid solution to a final concentration of 10% and stained with a 0.4% sulforhodamine B solution in 1% acetic acid (Sigma Chemical Co., St. Louis, MO). Fixed sulforhodamine B was dissolved in 10 mM Tris-HCl solution and absorbance at 570 nm was read using an MRX plate reader (Dynex, Inc., Vienna, VA). IC_{50} was determined graphically from the cytotoxicity curves. Each experiment was performed at least 3 times.

Intracellular drug accumulation

Relative intracellular drug content was measured on a FacsVantage flow cytometer (Becton Dickinson, Franklin Lakes, NJ) with excitation at 345 nm and a 540 nm band-pass filter to detect emission for SN38, and on a FacsCalibur (Becton Dickinson) with excitation at 630 nm and emission at 670 nm for mitoxantrone. Subconfluent cells were harvested and incubated (500,000 cells) for 2 hr at 37°C under agitation in complete medium with or without 50 μM SN38 or 20 μM mitoxantrone. Accumulation was then stopped by cooling on ice and the cells were washed in ice-cold PBS before being submitted to FACS analysis. ABCG2 inhibition was obtained by adding 250 nM Ko143 to the incubation medium. To study accumulation under ATP-depleting conditions, glucose-free RPMI 1640 medium containing 10 mM sodium azide was used, and the cells were preincubated for 1 hr before accumulation. Assays were performed at least 3 times.

Efflux pumps immunoblotting

Whole cell lysates were prepared by homogenizing and sonicating cells at a density of 10^7/ml in sample buffer (0.7 M 2-mercaptoethanol, 2% SDS, 10% glycerol, 62.5 mM TrisHCl and bromophenol blue) followed by heating at 90°C for 5 min. Proteins from the extracts (10^5 cells per lane) were electrophoretically separated on 12% (ABCG2) or 8% (LRP, Pgp or MRP) SDS-PAGE. Proteins were electrotransferred onto a polyvynilidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). The nonspecific binding sites were blocked with 5% (wt/vol) nonfat milk in PBS-T (PBS with 0.1% (vol/vol) Tween 20) for 30 min. The membrane was then incubated for 2 hr with the appropriate monoclonal primary antibody: p170/p-Glycoprotein clone F4 (Neomarkers, Fremont, CA) at 0.5 μg/ml for Pgp ; LRP/MVP clone 1014 (Neomarkers) at 0.5 μg/ml for LRP; BXP-21[26] at 0.5 μg/ml for ABCG2 ; MRPm6 (Alexis Corp, San Diego, CA) at 10 μg/ml for MRP1. Membranes were then washed and incubated with the secondary antibody peroxidase-conjugated rabbit anti mouse IgG (Sigma Chemical Co.) at a 1:2,000 dilution for 1 hr. Chemiluminescence with ECL+ (Amersham Biosciences) was used for detection.

Top1 Immunoblotting

Whole cell extracts (100,000 cells per lane) were electrophoretically separated on 10% SDS-PAGE. Proteins were electrotransferred onto a polyvynilidene difluoride membrane (Amersham Biosciences). The nonspecific binding sites were blocked with 5% (wt/vol) nonfat milk in PBS-T [PBS with 0.1% (vol/vol) Tween 20] for 30 min. The membrane was then incubated for 2 hr with the topoisomerase I polyclonal antibody[27] at a dilution of 1:200. Membranes were then washed and incubated with a peroxidase-conjugated goat anti-rabbit antibody (Sigma Chemical Co.) at a 1:5,000 dilution for 1 hr. Chemiluminescence with ECL+ (Amersham Biosciences) was used for detection.

RNA extraction

Total RNA was isolated and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Exponentially growing cells were harvested, and 3 to 5 million cells were lysed. For human samples, 50 to 100 mg of tissue was lysed. Cells or tissue were disrupted using the Mixer Mill® MM300 (Qiagen). Remaining genomic DNA was removed by a DNAse treatment included in the kit. RNA integrity was checked by using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and quantified spectrophotometrically.

Reverse transcription

Reverse transcription was carried out on 1 μg total RNA diluted in a 10 μl mixture containing 0.5 μl pd(N)6 primers (0.2 μg/μl) (Amersham Biosciences), 2 μl 5× First Strand Buffer, 1 μl DTT (0.1 M), 0.5 μl dNTP mix (10 mM), 0.5 μl Superscript II Reverse Transcriptase (200 U/μl) (Gibco, Grand Island, NY) and water up to 10 μl. After incubation at 42°C for 50 min, Superscript II Reverse Transcriptase was inactivated for 15 min at 70°C, and cDNA was stored at −20°C or immediately used for real-time PCR.

Real-time PCR

Real-time PCR for ABCG2 mRNA was performed using the LightCycler thermal cycler system (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. A 1:100 dilution of cDNA prepared as described above (4 μl of each sample) was diluted to a volume of 20 μl with the PCR mix (LightCycler FastStart DNA Master SYBR Green I, Roche Diagnostics) containing a final concentration of 3 mM $MgCl_2$ and 500 nM of each primer. The primers used for ABCG2 were sense: 5′-CAGGAGGCCTTGGGATACTT-3′ and antisense: 5′-GCTATAGAGGCCTGGGGATT-3′. The GAPDH mRNA was used

as an internal standard, and primers were sense 5′-GAAGCAG-GCGTCGGAG-3′ and antisense: 5′-CATGTGGGCCATGAG-GTCCA-3′. For amplification, an initial denaturation at 95°C for 15 min, followed by 15 sec at 95°C, 5 sec at 60°C and 10 sec at 72°C for 40 cycles was used. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis from 65°C to 95°C with a step of 0.1°C/sec. The quantification data were analyzed with the LightCycler analysis software.

Nucleotide sequencing

The coding region of ABCG2 surrounding the codon 482 was sequenced using MWG Biotech sequencing service. A 400 bp region of ABCG2 surrounding codon 482 was amplified by PCR using the primers 5′-TGCCCTGTGGCTTCTTAAAC-3′ and 5′-TGCAAGCTCGAAGTCACTTC-3′. Purified PCR product was then sequenced using the same primers by the dideoxynucleotide method.

Patients

Normal colon, tumoral colon and hepatic metastases were obtained from 42 colorectal cancer patients at the CRLC Val d'Aurelle, Montpellier, France. A total of 57 samples were collected between 2000 and 2003 on patients having signed an informed consent document for a clinical trial approved by the French ethical committee CCPPRB (COD 03 and CPT GMA 301). Normal colon tissue, tumoral colon tissue and untreated hepatic metastases samples were obtained from colorectal cancer patients with synchronous hepatic metastases. All patients underwent surgery for primary tumor resection during which hepatic biopsies were taken. Treated metastases samples were obtained from patients diagnosed with unresectable hepatic metastases having received a 5-fluorouracil-, oxaliplatin- or raltitrexed-based treatment (nonirinotecan-treated metastases) or an irinotecan-based treatment (irinotecan-treated metastases) that ended less than 2 months before resection of metastases. All samples were frozen in liquid nitrogen within 20 min following surgery to ensure optimal RNA quality.

Statistical analysis

To compare ABCG2 expression between 2 different groups of patients, the nonparametric Mann-Whitney U-test was used. For the 3-group comparisons, the Kruskal-Wallis H-test was used followed by a Dunn's post test. Differences were considered significant when $p<0.05$.

RESULTS

Establishment of SN38-resistant cell lines

To obtain SN38-resistant variants, we exposed the colon adenocarcinoma cell line HCT116 to increasing concentrations of SN38. We first cloned the HCT116 cells and selected as reference one of the clones named HCT116-s (IC_{50} = 1.9 nM). This sensitive clone was submitted to selection by SN38. A low level resistant clone, HCT116-SN6 (IC_{50} = 11.2 nM), was obtained from the cell population growing in 10 nM SN38, and a high level resistant clone, HCT116-SN50 (IC_{50} = 98.4 nM) was obtained from the population growing in 15 nM SN38. Drug sensitivity of these 3 clones towards a panel of chemotherapeutic drugs is summarized in Table I. Compared to the sensitive clone, HCT116-SN6 and the further selected clone HCT116-SN50 were 6.1- and 53.2-fold resistant to SN38, respectively. None of the SN38-resistant clones showed any significant cross-resistance to doxorubicin, 5-fluorouracil or oxaliplatin. They appeared to be cross-resistant to the camptothecin derivatives CPT-11 and topotecan. Only HCT116-SN50 displayed a 4.0-fold cross-resistance to mitoxantrone.

To assess the stability of the acquired resistance, cells were grown in drug-free medium for 5 months and then SN38 and mitoxantrone resistance were determined. HCT116-SN6 resistance was stable, whereas HCT116-SN50 resistance towards SN38 and mitoxantrone was reduced by approximately 85% (RF = 7.7 and 0.5, respectively). The revertant cell line was named HCT116-SN50/rev (Table I).

Involvement of ABCG2 in high level resistance to SN38

Since some efflux proteins have been shown to transport camptothecin derivatives, Pgp, MRP1, LRP and ABCG2 expression was assessed by Western blotting (Fig. 1). The multidrug-resistant doxorubicin-selected MCF7-R breast cancer cell line was used as a positive control for Pgp and MRP1.[28] Compared to this cell line, none of our cell lines (neither SN38-sensitive nor the resistant ones) displayed any detectable amount of Pgp or MRP1. LRP was expressed at the same level in all the sensitive and resistant clones. ABCG2 was overexpressed in the HCT116-SN50 resistant clone. Thus, high level of resistance to SN38 was associated with overexpression of ABCG2. This overexpression was partially reversible as shown by the Western blotting on HCT116-SN50/rev cells (Fig. 1). The upregulation of ABCG2 was not induced by a short-term exposure to SN38 (data not shown).

To check the functionality of the overexpressed ABCG2 protein, we measured the intracellular content of SN38 and mitoxantrone by flow cytometry. After a 2 hr-long drug incubation, the HCT116-SN6 clone and the reverting cells HCT116-SN50/rev did not show any significant decrease in the intracellular accumulation of SN38 or mitoxantrone compared to HCT116-s. In contrast, the ABCG2-overexpressing resistant clone HCT116-SN50 demonstrated a marked decrease for both SN38 and mitoxantrone content, since the intracellular recorded fluorescence of both SN38 and mitoxantrone was about 65% lower than in HCT116-s (Table II). Furthermore, the SN38 intracellular accumulation was restored under ATP-depleting conditions (data not shown).

In order to confirm the involvement of ABCG2 protein in the resistance phenotype of HCT116-SN50, we performed ABCG2-inhibition experiments both in cytotoxicity and in intracellular drug accumulation assays. To this end, we used the fumitremorgin-type indolyl diketopiperazine Ko143, a specific and potent inhibitor of the ABCG2 transporter.[29] The addition of Ko143 restored SN38 accumulation up to 72% in HCT116-SN50 (Table II). ABCG2 inhibition also dramatically lowered the resistance to

TABLE I – DRUG SENSITIVITY OF THE HCT116 CELL PANEL

Anticancer drug	IC_{50}[1] ± S.D. (RF)[2]			
	HCT116-s	HCT116-SN6	HCT116-SN50	HCT116-SN50/rev
SN38[3]	1.9 ± 0.6	11.2 ± 2.5 (6.1)	98.4 ± 16.1 (53.2)	14.3 ± 1.6 (7.7)
Mitoxantrone[3]	2.8 ± 0.3	1.2 ± 0.3 (0.4)	11.1 ± 2.0 (4.0)	1.4 ± 0.1 (0.5)
Doxorubicin[3]	21.9 ± 3.2	21.3 ± 5.0 (1.0)	31.5 ± 3.3 (1.4)	ND
CPT-11[4]	0.08 ± 0.02	0.53 ± 0.10 (6.4)	1.38 ± 0.30 (16.7)	ND
5-FU[4]	3.6 ± 1.2	3.9 ± 1.1 (1.1)	4.3 ± 1.1 (1.2)	ND
Oxaliplatin[4]	0.27 ± 0.07	0.52 ± 0.23 (1.9)	0.24 ± 0.07 (0.9)	ND
Topotecan[3]	6.2 ± 0.2	118.3 ± 12.8 (19.1)	249.2 ± 61.6 (40.2)	ND

[1]IC_{50} values were determined using the sulforhodamine B assay. Data represent the mean ± SD values in at least 3 independent experiments.–[2]The resistance factor (RF) was determined by dividing the IC_{50} value of the resistant cell line by that of the sensitive clone HCT116-s and is indicated in brackets.–[3]IC_{50} values expressed in terms of nM.–[4]IC_{50} values expressed in terms of μM.

SN38 (from 53.2- to 8.9-fold) and to mitoxantrone (from 4.0- to 0.5-fold) in the ABCG2-overexpressing clone HCT116-SN50 and had no effect on HCT116-SN6 resistance (Table III). The resistance to SN38 and mitoxantrone was also slightly reduced by the use of Ko143 in HCT116-SN50/rev, confirming the remaining expression of ABCG2 found in Western blotting experiments.

Altogether, these results demonstrated the involvement of ABCG2 in the *in vitro* acquired resistance to SN38 of HCT116-SN50 cell line.

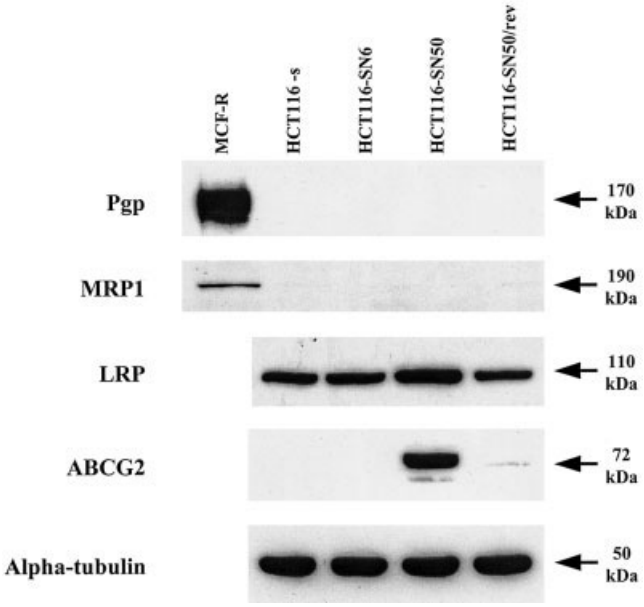

FIGURE 1 – Immunodetection of efflux pumps Pgp, MRP1, LRP and ABCG2 in SN38-sensitive and -resistant cells. Whole cell lysates (100,000 cells per lane) were electrophoresed; proteins were transferred to a PVDF membrane and detected as described in Material and Methods. The MCF7-R cell line was used as a positive control for Pgp and MRP1, and alpha-tubulin immunoblotting was performed as a loading control.

TABLE II – EFFECT OF ABCG2 INHIBITION BY KO143 ON THE INTRACELLULAR DRUG ACCUMULATION

Cell line	Relative intracellular drug accumulation (%)[1]			
	SN38		Mitoxantrone	
	−Ko143	+Ko143	−Ko143	+Ko143
HCT116-s	100	96 ± 5	100	103 ± 6
HCT116-SN6	90 ± 5	82 ± 1	108 ± 9	106 ± 2
HCT116-SN50	36 ± 8	72 ± 6	34 ± 10	101 ± 2
HCT116-SN50/rev	85 ± 12	98 ± 12	111 ± 3	113 ± 2

[1]Intracellular accumulation of SN38 or mitoxantrone was measured by flow cytometry. Drug content is expressed in percentage relative to the normal accumulation in the sensitive clone HCT116-s.

Transcriptional and genomic characterization of ABCG2

To further characterize the overexpression of ABCG2, we investigated ABCG2 mRNA expression. Semiquantitative RT-PCR studies showed that, relative to HCT116-s cells, ABCG2 mRNA expression was 0.9 ± 0.2 in HCT116-SN6 and 133 ± 9 in the HCT116-SN50 clone. FISH experiments were performed on cytogenetic preparations from the sensitive and the two SN38-resistant clones using the RP11-368G2 BAC clone containing the entire coding region of ABCG2. The experiment showed an amplification (4- to 6-fold) of this BAC in the HCT116-SN50 clone (data not shown), showing that ABCG2 mRNA and protein overexpression in HCT116-SN50 is possibly due to gene amplification.

It has been previously shown that ABCG2 overexpression in cancer cell lines results in a resistant phenotype that displays a high level of cross-resistance to doxorubicin and mitoxantrone.[9,11,30,31] In our study, the ABCG2-overexpressing HCT116-SN50 cell line showed cross-resistance to mitoxantrone but not to doxorubicin. The lack of cross-resistance to doxorubicin has been explained by a single mutation at codon 482 (doxorubicin is poorly transported by the wild-type variant of ABCG2 in which the amino acid at position 482 is an arginine but can be transported by the R482T mutant[32]). We checked for the status of this well-described mutation hotspot. The entire coding region of ABCG2 in the three HCT-116 clones was isolated by PCR amplification from mRNA and the nucleotide sequence of a 400 bp region surrounding codon 482 was determined. No mutation was found in this region, and all sequences were wild type at position 482 (data not shown).

Topoisomerase I expression

We investigated the expression level of the topoisomerase I protein, whose involvement in resistance to camptothecin derivatives has been extensively reported. Detection of the topoisomerase I protein in whole cell extracts by Western blotting (Fig. 2) revealed no significant changes in the expression level of this protein. This suggests that a decrease in the topoisomerase I expression level could not be the mechanism responsible for the resistance in HCT116-SN6 and for the irreversible resistance of HCT116-SN50/rev.

ABCG2 mRNA expression in clinical samples

To evaluate the potential relevance of ABCG2 in clinical resistance, we examined ABCG2 in 57 clinical samples including 8 samples of normal colon tissue, 10 tumoral colon tissues and 39 hepatic metastases. This latter group was subdivided into 3 groups: 23 hepatic metastases of patients who were untreated before the resection, 12 hepatic metastases of patients who recently received an irinotecan-based chemotherapy and 4 hepatic metastases of patients who recently received an irinotecan-free chemotherapy. The ABCG2 mRNA content of the samples was determined by semiquantitative RT-PCR and the data are presented in Figure 3 as a ratio to the mRNA level of the HCT116-s cell line. The median ABCG2 mRNA expression was 27.9 (10.6–62.8) in normal colon tissue, 0.3 (0.01–1.0) in tumoral colon tissue, 0.4 (0.05–1.8) in untreated metastases, 2.0 (0.18–9.7) in irinotecan-treated metastases and 0.3 (0.12–0.67) in metastases from patients who did not receive irinotecan. Thus, constitutive ABCG2 mRNA expression

TABLE III – EFFECT OF ABCG2 INHIBITION BY KO143 ON THE SENSITIVITY

Cell line	IC_{50} ± S.D. (RF)[1]			
	SN38		Mitoxantrone	
	−Ko143	+Ko143	−Ko143	+Ko143
HCT116-s	1.9 ± 0.6	2.1 ± 0.7	2.8 ± 0.3	4.1 ± 0.5
HCT116-SN6	11.2 ± 2.5 (6.1)	12.0 ± 6.1 (5.8)	1.2 ± 0.3 (0.4)	1.3 ± 1.0 (0.3)
HCT116-SN50	98.4 ± 16.1 (53.2)	18.5 ± 4.7 (8.9)	11.1 ± 2.0 (4.0)	2.2 ± 0.3 (0.5)
HCT116-SN50/rev	14.3 ± 1.6 (7.7)	7.5 ± 1.3 (3.6)	1.8 ± 1.1 (0.6)	0.9 ± 0.1 (0.2)

[1]IC_{50} and resistance factor (RF) were determined as described in Table I and Material and Methods. IC_{50} expressed in terms of nM. Inhibition experiments were performed by adding 100 nM Ko143 in the culture medium.

was significantly lower (about 100-fold) in tumoral tissue compared to the normal colon ($p=6.4\times10^{-4}$), while there was no difference between tumoral colon tissue and untreated hepatic metastases ($p=0.21$).

In addition to these results, we investigated the effect of drug exposure on metastases. Figure 3 shows that drug exposure is associated with significant differences in ABCG2 mRNA expression in hepatic metastases ($p=0.0050$). More precisely, irinotecan-based chemotherapy was associated with a 5-fold increase of the ABCG2 mRNA expression ($p<0.05$), whereas the expression observed in nonirinotecan-treated metastases did not seem to differ from untreated metastases ($p>0.05$). These results suggest that ABCG2 mRNA expression is specifically upregulated i*n vivo* by irinotecan treatment.

DISCUSSION

In this report, we established a human colorectal cancer cell line model made resistant to SN38, the active metabolite of CPT-11. The use of SN38 as the selecting agent permitted us to avoid differences in sensitivity of cells that may be due to altered metabolism of CPT-11, for instance by carboxylesterases.[2] As far as we know, to date only one model using SN38 as the selecting agent has been described[18,33] and was obtained by developing SN38 resistance on the PC-6 human lung carcinoma cell line. Whereas some cellular resistance models that show cross-resistance to SN38 have been established in the past few years,[19,21,34] it is for us of particular interest to study the mechanisms of acquired resistance in colon cancer cells to the active metabolite of irinotecan, a drug widely used in first- and second-line treatment of advanced colorectal cancer.

We selected clones with two levels of resistance: the first clone (HCT116-SN6) displaying about 6-fold resistance and the second one (HCT116-SN50) about 50-fold resistance. The level of resistance did not depend on the selecting concentration of SN38 but was rather correlated with an overexpression of the ABCG2 protein. Indeed, among all the clones we obtained, ABCG2 overexpression was found in both populations selected in 10 nM and 15 nM SN38 (data not shown). Low-resistance variants were obtained by cloning the cell population at an early stage, while a later cloning, at the same selecting concentration, led to the isolation of ABCG2-overexpressing cells as if ABCG2 conferred a selective advantage. Indeed, growth experiments demonstrated that the doubling time was significantly lower for ABCG2-overexpressing cells HCT116-SN50 than for non-ABCG2-overexpressing resistant cells HCT116-SN6 (data not shown).

ABCG2 overexpression was associated with a markedly decreased accumulation of SN38 and mitoxantrone, suggesting an enhanced drug efflux from resistant cells mediated by the activity of ABCG2 protein. The inhibition of ABCG2 function either by depleting ATP (not shown) or by exposure to Ko143 restored both drug accumulation and sensitivity of the resistant cells, indicating that ABCG2 is involved in the acquired resistance to SN38. These findings are consistent with those of Kawabata *et al.*,[18] who have also described the involvement of ABCG2 in resistance to SN38 in lung cancer sublines. Interestingly, as shown by SN38 sensitivity assays, the inhibition of ABCG2 by Ko143 failed to restore complete sensitivity of the cells and only lead to the low level of 5- to 6-fold resistance, suggesting that at least two mechanisms of resistance coexist and support the idea that resistance to chemotherapeutic drugs is multifactorial.

The topoisomerase I expression level was investigated in all the clones since the mechanism responsible for the resistance in HCT116-SN6 has not yet been identified and that altered expression of topoisomerase I is a well-known cause of resistance to camptothecin derivatives in cellular models.[35] The immunoblotting experiments showed that topoisomerase I expression was not associated with resistance. We can thus conclude that decreased expression of topoisomerase I protein is not the mechanism responsible for the resistance in HCT116-SN6. Nevertheless, topoisomerase I-mediated resistance to camptothecins is very common in cellular models[5] and can be due to genetic alterations in the topoisomerase I gene. We cannot exclude that mutations exist in the topoisomerase I gene of our HCT116-SN6 cells and that they could lead to a decreased topoisomerase I catalytic activity. Another explanation for the resistance in HCT116-SN6 would be that the metabolism of SN38 in tumor cells is modified: for instance,

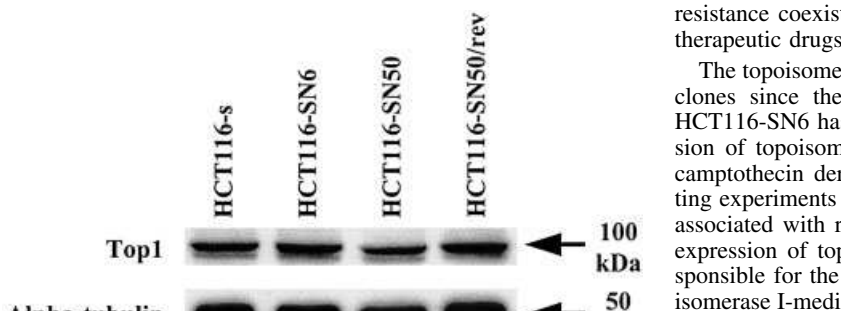

FIGURE 2 – Immunodetection of topoisomerase I in SN38-sensitive and -resistant cells. Whole cell extracts (100,000 cells per lane) were electrophoresed, transferred to a PVDF membrane and detected as described in Material and Methods.

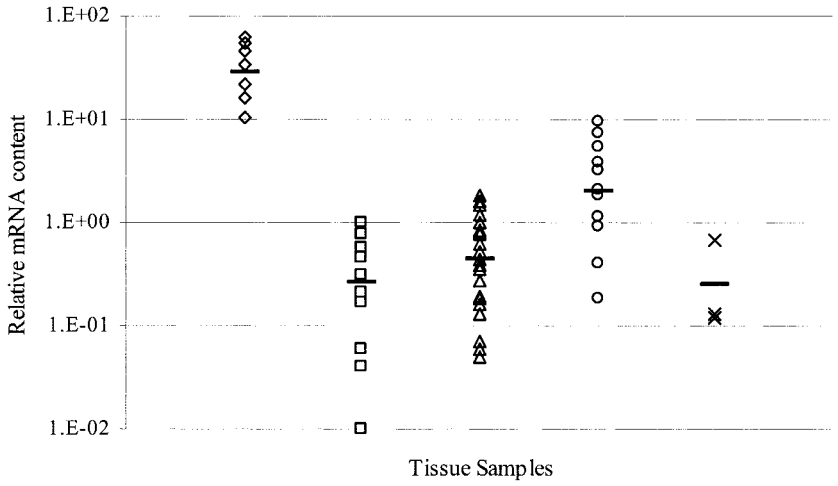

FIGURE 3 – ABCG2 mRNA content in clinical samples. The mRNA content in normal colon tissue (◇), tumoral colon tissue (□), untreated hepatic metastases (△), irinotecan-treated metastases (○) and nonirinotecan-treated metastases(×) samples was determined using the semi-quantitative real-time PCR (LightCycler). This ABCG2 mRNA value is expressed relative to the ABCG2 mRNA content in HCT116-s cells. Horizontal bars indicate the median value of each group.

SN38 could be inactivated by an increased UGT-mediated glucuronidation as previously shown.[6]

The ABCG2-overexpressing clone showed no cross-resistance to doxorubicin. Sequencing of our ABCG2 transcript revealed the presence of the wild-type arginine at position 482, which explains the lack of cross-resistance to doxorubicin. Concerning mitoxantrone, our results apparently showed a low cross-resistance to mitoxantrone (only 4-fold) compared to SN38 (53-fold) in HCT116-SN50 cells. If we assume that the unknown mechanism responsible for the resistance in HCT116-SN6 is conserved in HCT116-SN50, as suggested by our results, then the resistance factor due to the overexpression of ABCG2 is only 8.8-fold for SN38. Thus, the resistance level due to the overexpression of ABCG2 in HCT116-SN50 is in the same range for mitoxantrone and SN38, which is consistent with previously published studies, where information on the resistance to SN38 and mitoxantrone in ABCG2 overexpressing cells appears.[18,21,36]

The origin of ABCG2 overexpression is poorly understood. Gene amplification or chromosomal rearrangements that result in transcriptional activation have been observed in numerous *in vitro* models of MDR1- or MRP-overexpressing cells.[37,38] This kind of mechanism is thus thought to be responsible for efflux pump overexpression. The well-studied ABCG2-overexpressing cells MCF-7 AdVp3000 and MCF-7 MX showed an amplification peak at 4q21–q22, as demonstrated by comparative genomic hybridization (CGH) by Knutsen *et al*.,[39] whereas S1-M1-80 ABCG2-overexpressing cells contained only a simple reciprocal translocation as shown by FISH experiments. In FISH experiments HCT116-SN50 cells revealed a 4- to 6-fold increase in gene copy number. This gene amplification is not sufficient to explain the 133-fold increase in mRNA level. The cause of transcriptional activation seen in our model remains to be elucidated and could be due, for example, to mutations in the recently characterized ABCG2 promoter[40] or to modifications in ABCG2 transcription factors.

ABCG2 is expressed in various normal human tissues including placenta, liver canaliculi, small intestine, colon and endothelial cells. Its expression in tumors has been recently assessed by immunohistochemistry on 150 untreated solid tumors representing 21 different types of cancer and was seen in all tumor types including colon cancer.[41] Nevertheless, this study does not include samples from chemotherapy-treated patients. Another recent work aimed at detecting ABCG2 mRNA by semiquantitative real-time PCR on breast cancer samples from untreated, and previously treated patients[24] led to the conclusion that there was no difference in ABCG2 expression in anthracycline naive or pretreated samples, and no correlation was found between an elevated level of ABCG2 and resistance to the treatment. Our study was performed on 57 clinical samples composed of normal colon tissues, tumoral colon tissues and hepatic metastases. ABCG2 mRNA was detected using semiquantitative real-time PCR. We showed that ABCG2 was highly expressed in the normal colon and that this expression was dramatically lowered in tumoral cells, *i.e*., colon tumor cells as well as hepatic metastases. This finding is consistent with previous studies where ABCG2 was found to be expressed in normal colon,[9] and it suggests that the apical membrane of the colon may be a physiological localization for ABCG2, where it could play its potential role of protection against xenobiotics. The elevated ABCG2 mRNA level found in irinotecan-treated metastases provides evidence to support the hypothesis that ABCG2-expressing cells are selected during exposure to irinotecan *in vivo*. Despite the low number of samples in the group of nonirinotecan-treated metastases, we have a good indication that the elevation of the ABCG2 mRNA level is specific to irinotecan in colon cancer treatment. Moreover, oxaliplatin and 5-fluorouracil, the drugs commonly used in colon cancer chemotherapy, are not known substrates of ABCG2. Nevertheless, correlation between transcriptional overexpression of ABCG2 and resistance to irinotecan remains to be proven. Taking HCT116-s as reference, ABCG2 mRNA expression levels in irinotecan-treated metastases were less pronounced than in HCT116-SN50, but it is difficult to establish a relationship between mRNA expression level and protein function. Recently Kawabata *et al*.[42] assessed the question of functional analyses of ABCG2 in lung cancer samples. They chose to define a functional cut-off of ABCG2 mRNA expression causing resistance to topotecan *in vivo*, corresponding to the ABCG2 mRNA level measured in the NCI-H441 cell line displaying an IC_{50} above the maximum plasma concentration of topotecan in humans. On the basis of this hypothesis, the ABCG2 mRNA level measured in the irinotecan-treated metastases would not be great enough to explain the resistance to SN38. However, samples of hepatic metastases generally show heterogeneity, and ABCG2 overexpressing cells may only represent a low percentage of the sample, the cause of the low ABCG2 mRNA level measured. We hypothesize that a longer exposure to irinotecan could lead to a higher content of ABCG2 overexpressing cells in the tissue samples.

In conclusion, our study shows that exposure of the human colorectal cancer cell line HCT116 to SN38 upregulates the expression of ABCG2 protein. This overexpression confers high levels of resistance to SN38. We also propose irinotecan treatment selects for ABCG2-overexpressing cells *in vivo*. This parallel made between *in vivo* and *in vitro* studies confirms the relevance of cellular models and encourages us to further investigate the clinical and functional involvement of ABCG2 in resistance to camptothecin derivatives, notably to irinotecan and topotecan. If this involvement is confirmed, the next step would be to circumvent the ABCG2-mediated resistance, by using either ABCG2 inhibitors[29,43] or newly developed camptothecin derivatives that are not substrates of ABCG2.[20,21,34,43,44]

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