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ORIGINAL ARTICLE

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Determinants of the cytotoxicity of irinotecan in two human colorectal tumor cell lines

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Abstract *Purpose:* Irinotecan is a drug of the camptothecin family that has proven activity in advanced colon cancer, with about 20% responses in untreated as well as in 5-fluorouracil-resistant tumors. Irinotecan is considered as a prodrug which needs to be activated to SN-38 by carboxylesterases to become able to interact with its target, topoisomerase I. The work reported here intended to identify the determinants of the cytotoxicity of irinotecan in two human colorectal tumor cell lines, LoVo and HT-29, at the level of the target of the drug and at the level of the availability of the active metabolite to the target. *Results:* The cytotoxicity of irinotecan and SN-38 markedly differed in the two cell lines: irinotecan IC_{50} values were 15.8 μ*M* for LoVo cells and 5.17 μ*M* for HT-29 cells; SN-38 IC_{50} values were 8.25 n*M* for LoVo cells and 4.50 n*M* for HT-29 cells. Topoisomerase I expression (at the mRNA and the protein levels) and catalytic activity were similar in the two cell lines. Irinotecan induced similar amounts of cleavable complexes at its IC_{50} in both cell lines. SN-38 induced a concentration-dependent formation of cleavable complexes, which was not significantly different in the two cell lines. Expression of the carboxylesterase CES1 was higher in HT-29 than in LoVo cells. Expression of the carboxylesterase gene CES2 was comparable in the two cell lines and much higher than CES1 gene expression. Carboxylesterase activity was extremely low using *p*-nitrophenylacetate as a substrate (1.45 and 1.84 pmol/min per mg proteins) and could not even be detected using irinotecan as a substrate. Cell accumulation of irinotecan was markedly different, reaching consistently higher levels in HT-29 cells than in LoVo cells. *Conclusions:* Our results indicate that (1) the cytotoxicity of irinotecan was likely due to the drug itself and not to its metabolite SN-38, and (2) that irinotecan uptake was more predictive of its cytotoxicity than topoisomerase I availability and activity in these two cell lines.

Keywords Camptothecin · Irinotecan · Topoisomerase I · Drug activation · Colon cancer

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Introduction

In Western countries colorectal cancer represents 15% of all cancers and is the most frequently observed [2]. The majority of patients are metastatic at first presentation or later, and will require chemotherapy. Colon cancer chemotherapy has been based upon the use of 5-fluorouracil for 40 years. Recently, irinotecan (CPT-11), a derivative of camptothecin, has been shown to have efficacy against advanced colorectal cancers, both when used alone (20% responses in patients pretreated with 5-fluorouracil [6, 26]) or combined with 5-fluorouracil (near 50% responses [9]).

Irinotecan is a prodrug which needs to be transformed by carboxylesterases to its active metabolite SN-38 [19]. Camptothecin and its analogues are specific inhibitors of eukaryotic DNA topoisomerase I, a ubiquitous enzyme involved in DNA templating processes such as replication, transcription and repair [13]. These compounds can interfere with DNA-topoisomerase I cleavable complexes and stabilize them, leading to DNA damage by inducing single-strand breaks that are converted to double-strand breaks during S phase by collisions with replication forks [24].

Since the original work of Giovanella et al. [11], the level of topoisomerase I in human tumors has been considered as determining their sensitivity to camptothecin derivatives [16]. It has been shown that the amplification of the gene encoding topoisomerase I is able

to sensitize breast cancer cells to camptothecin [23]. Using another approach, Goldwasser et al. [12] have shown a relationship between the sensitivity of colon tumor cell lines and the amount of cleavable complexes formed in the presence of camptothecin. Studying colon cancer cell lines and xenografts, Jansen et al. [17] have also found topoisomerase I activity to be a determinant of irinotecan and SN-38 cytotoxicity. However, in lung cancer cells in culture, the same group have observed that the major determinant of irinotecan cytotoxicity is its conversion to SN-38 through carboxylesterase activity [29].

Concerning the involvement of carboxylesterases in irinotecan cytotoxicity, a recent study has shown that the transfection of a human liver carboxylesterase cDNA sensitizes by 17-fold the A549 human lung tumor cell line to irinotecan [21]. However, no evaluation of carboxylesterase activity has been systematically made in human tissues and tumors. It has not been established, therefore, whether the local generation of SN-38 by the tumor itself is of significance in determining irinotecan activity. Two distinct carboxylesterases have recently been characterized [15]. The first one, hCE1, isolated from macrophages, is relatively inefficient in activating irinotecan to SN-38 as compared to the second one, hCE2, isolated from the liver. The respective expression and activity of these enzymes have not yet been studied in tumors or tumor cell lines.

The importance of efflux pumps in determining drug resistance of tumor cells has been recognized for a long time. However, neither the *MDR*1 gene product, P-glycoprotein, nor the multidrug-resistance protein, MRP1, have been shown to be able to transport irinotecan or SN-38 out of tumor cells [18, 22]. Recently, it has been shown that irinotecan and SN-38, but not camptothecin itself, can be expelled by a new ABC pump, BCRP or ABCG2 [4, 30].

We studied the cellular determinants of irinotecan cytotoxicity in two colon tumor cell lines, LoVo and HT-29. We also studied the cellular determinants related to the availability of the active metabolite of irinotecan (irinotecan uptake and transformation into SN-38, carboxylesterase expression and activity, ABCG2 expression), and the cellular determinants related to the target of this drug (expression of topoisomerase I, catalytic activity, and amounts of cleavable complexes stabilized in the presence of irinotecan or SN38 in living cells). This in vitro study was undertaken as a prelude to an ex vivo study on tumor biopsies of colorectal cancers which is ongoing.

Materials and methods

Drugs and chemicals

Irinotecan and SN-38 were provided by Rhône-Poulenc Rorer (Antony, France). For growth inhibition and cell uptake studies, irinotecan was dissolved directly in culture medium from the stock solution at 20 mg/ml. SN-38 was first diluted in dimethylsulfoxide at 1 mg/ml, then in culture medium at appropriate concentrations.

Cell culture

The human colon tumor cell lines LoVo and HT-29 were obtained from the American Type Culture Collection (Rockville, Md.). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (10% for LoVo cells and 20% for HT-29 cells), an antibiotic cocktail (penicillin 1000 U/l, streptomycin 100 μg/ml) and 2 m*M* glutamine, and were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. The cell culture media and supplements originated from Seromed (Berlin, Germany).

Growth inhibition assays

Exponentially growing cells were seeded in 20 cm^2 Petri dishes with an optimal cell number for each cell line (20,000 for LoVo cells, 100,000 for HT-29 cells). They were treated 2 days later with increasing concentrations of irinotecan or SN-38 for one cell doubling time (24 h for LoVo cells, 40 h for HT-29 cells). After washing with 0.15 *M* NaCl, the cells were further grown for two doubling times in normal medium, detached from the support with trypsin-EDTA and counted in a hemocytometer (Coulter Counter ZX, Coultronics). The IC_{50} values were then estimated as the drug concentrations responsible for 50% growth inhibition as compared with cells incubated without drug.

Evaluation of cellular accumulation of irinotecan

Evaluation of the concentrations of irinotecan in cells after exposure to various extracellular concentrations (1, 5, 10, 25 μ*M*) was performed by HPLC as previously described [25]. The drug was added to the complete culture medium 16 h before incubation with cells in order to reach the lactone-carboxylate equilibrium. After a 4-h incubation, the cells layers were rinsed and the cells were recovered by scraping and pelleted. Cell extracts were obtained in methanol/acetonitrile (50/50 v/v) containing 1% HCl. Separation was carried out on a C-18 reversed-phase column (Nova-Pak, Radial Pak, Waters, Saint-Quentin-en-Yvelines, France) with a mobile phase consisting of a mixture of acetonitrile and 0.075 *M* ammonium acetate buffer, pH 6.0, containing 5 m*M* tetrabutyl ammonium phosphate (Pic-A, Waters). This mobile phase was delivered isocratically at a flow rate of 1.5 ml/min with a Spectra Systems P4000 XR pump (Thermo Quest, Les Ulis, France). Fluorometric detection was carried out with excitation and emission wavelengths set at 355 and 515 nm respectively, using the Spectra Systems FL 3000 (Thermo Quest) detector. Peaks were quantified by reference to a standard calibration curve obtained by spiking known amounts of drugs in untreated cell extracts, using PC1000 software (Thermo Quest).

Carboxylesterase activity

Carboxylesterase activity was evaluated in microsomal extracts of each cell line using two different substrates of the enzyme, irinotecan and *p*-nitrophenylacetate (PNPA), a generic substrate for carboxylesterases. Determination of carboxylesterase activity was performed according to a technique originally developed in our laboratory [14]. Human liver microsomes were used as a positive control of carboxylesterase activity. These microsomes were kindly provided by Dr C. Riché (University of Brest).

The cells (50×10^6) were mechanically detached from Petri dishes with 0.15 *M* NaCl and centrifuged at 250 *g* for 10 min. Further steps were performed on ice. Cell pellets were lysed by sonication three times for 10 s, and maintained on ice for 10 min. The cell suspensions were centrifuged two times at 7500 *g* for 30 min to eliminate nuclei and mitochondrial fractions, and the

supernatant was centrifuged at 100,000 *g* for 2 h to pellet the microsomal fraction. Microsomes were suspended in 40 m*M* Tris-HCl, pH 6.9, and kept at –80 °C until use. The amounts of protein were determined using the method of Bradford [3]. The purity of the microsomal fraction was checked by the relative enrichment in specific enzymatic activities: succinate dehydrogenase for mitochondria, sulfatase C for microsomes, phosphoglucomutase for cytosol. Standard enzymatic techniques were used for this control [1, 5, 7].

Microsomes (1 mg/ml) were incubated for 30 min with 5 μ*M* irinotecan lactone (diluted in 0.01 *M* citric acid) in 0.1 *M* Tris-HCl, pH 6.9, at 37 °C (final volume 40 μl). At the end of the reaction, 50 μl of a mixture of acetonitrile and methanol (1:1 v/v) were added to the tubes together with 10 μl 2.5 *N* HCl and 50 ng internal standard (camptothecin). After centrifugation at 10,000 *g* for 2 min to precipitate the proteins, the amount of SN-38 was determined by HPLC in 100 μl of the supernatant. A special technique allowing improved sensitivity for SN-38 quantification was used [10]. It differed from our standard method by replacing the mobile phase with a mixture of 0.1 *M* potassium buffer (pH 6.8) and acetonitrile (2:1 v/v), running isocratically at 1 ml/min, and excitation and emission wavelengths set at 228 and 540 nm, respectively. The limit of quantification was 0.005 μ*M* of SN-38 (limit of detection 0.2 ng).

For the determination of PNP formation, microsomes were first activated at 4 °C for 10 min with Triton X-100 (0.5% v/v). Incubations were performed with 100 μg microsomal proteins and 50 μ*M* PNPA in 1 ml 40 m*M* Tris-HCl, pH 6.9, at 37 °C. The kinetics of PNP formation were monitored spectrophotometrically at 405 nm for 4 min. A control without microsomal extract was used to subtract the spontaneous hydrolysis of PNPA. The results were analyzed and are expressed as the quantity of PNP formed per minute per milligram microsomal protein.

Western blotting of topoisomerase I

Exponentially growing cells (about 30×10^6 cells) were mechanically detached and centrifuged at 250 *g* for 5 min at 4 °C. All the following procedures were performed at 4 °C. Cell pellets were homogenized in a Potter homogenizer before centrifugation at 250 *g* for 5 min. The pellets were resuspended in 1 ml hypertonic solution (1 *M* NaCl) and centrifuged for 30 min at 18,000 *g* to eliminate DNA. Aliquots of the nuclear extracts of LoVo and HT-29 cells containing 100 μg proteins were loaded onto an 8% polyacrylamide gel and allowed to migrate at 40 V for 2.5 h at 4 °C in electrophoresis buffer. The proteins were then transferred to Immobilon P membrane (Millipore, Saint-Quentin-en-Yvelines, France) using an electroblotting system (Milliblot, Millipore) at 2.5 mA/cm^2 for 1 h.

The membranes were incubated at room temperature for 2 h with rabbit anti-human topoisomerase I antibody (TopoGen, Columbus, Ohio) diluted 1:2000, then for 1 h with horseradish peroxidase-labeled donkey anti-rabbit immunoglobulin as secondary antibody (Amersham-Pharmacia, Orsay, France) diluted 1:4000. Protein was detected using the peroxidase substrate Lumigen PS-3 acridan (ECL+, Amersham-Pharmacia), and visualized by autoradiography.

The chemoluminescence signals on the autoradiograms were analyzed using a video camera (Kodak DC120 Zoom Digital Camera, Rochester, N.Y.) coupled to a microcomputer, using the Kodak Digital Science 1D image analysis software. Signal intensities were recorded and expressed in arbitrary units.

Topoisomerase I catalytic activity

The catalytic activity of topoisomerase I was evaluated in 0.35 *M* nuclear extracts using its ability to relax supercoiled DNA. The nuclear extracts were prepared as described above. The substrate of the reaction was the pBSKS$^+$ phagemid (Clontech, Palo Alto, Calif.)

Serial dilutions of LoVo and HT-29 nuclear extracts were prepared so as to contain between 5 and 100 ng proteins, and incubated at 37 °C for 30 min with 1 μg pBSKS$^+$ in 20 μl reaction buffer. Samples containing 20 ng DNA were loaded onto a 1% agarose gel containing ethidium bromide (0.2 μl/ml) and submitted to electrophoresis at 80 V for 1 h. Electrophoresis allowed the separation of supercoiled DNA (substrate) and relaxed DNA (product of the reaction). A sample treated under the same conditions but without nuclear extract was used as a control.

Spot intensities were quantified by densitometry using the same camera and software as for Western blot analysis. The catalytic activity of topoisomerase I was evaluated as the amount in nanograms of DNA relaxed per nanogram protein in the nuclear extract.

Evaluation of cleavable complexes

DNA-topoisomerase I complexes were evaluated after 30-min incubations of the cells with irinotecan or SN-38 by a slot-blot method adapted from that of Subramanian et al. [27] using the Topo I Link Kit (TopoGen).

The cells ($1–10\times10^6$) were incubated for 30 min at 37 °C with different irinotecan or SN-38 concentrations, chosen as multiples of the IC_{50} values of the drugs. After removing the medium, the cells were lysed with 1 ml lysis buffer. Cell lysates were loaded at the top of a cesium chloride gradient, and centrifuged at 100,000 *g* for 16 h at 20 °C. Fractions of 200 μl were removed from the top of the gradient, and an aliquot of each fraction (10 μl) was diluted and loaded onto a 1% agarose gel containing ethidium bromide. In parallel, another aliquot of each fraction (50 μl) was diluted with an equal volume of 25 m*M* sodium phosphate buffer and loaded onto a nitrocellulose membrane (Schleicher and Schuell, Ecquevilly, France) using a slot-blot device.

Topoisomerase I was revealed in the slots with the immunoblotting technique used for the Western blots. A signal was seen in two different groups of slots, those not containing DNA (free topoisomerase I, top of the gradient) and those containing DNA (topoisomerase I-DNA complexes, bottom of the gradient). Signal intensities in the DNA-containing slots were normalized before comparing irinotecan or SN-38-treated and untreated cells. The results are expressed as the relative increase in topoisomerase I-DNA complexes, i.e. the amount of topoisomerase I-DNA complexes in treated cells as compared to that in untreated cells.

Evaluation of gene expression of topoisomerase I, carboxylesterases, and ABCG2

About 10×10^6 cells in exponential growth were recovered by scraping in 0.5 ml RNA extraction buffer and homogenized in a Dounce homogenizer. The homogenate was laid over a cesium chloride cushion and centrifuged at 150,000 *g* for 16 h at 20 °C. Pellets were then suspended in a buffer, extracted with 2.5 ml chloroform/butanol (4:1 v/v) and centrifuged for 10 min at 5000 *g*. The aqueous phase, containing RNAs, was submitted to a second extraction and purification cycle. RNAs were then pelleted with 3 *M* sodium acetate, pH 5.0, and absolute ethanol at a volume ratio of 0.1:2.

Reverse transcription was performed on 400 ng RNA in 20 μl 10 m*M* Tris-HCl buffer, pH 8.3, containing 50 m*M* KCl and 5.5 m*M* $MgCl_2$, with 0.5 m*M* dNTP mixture, 8 U RNase inhibitor, 2.5 μ*M* random hexamers and 25 U reverse transcriptase from Mule. The reaction was performed for 10 min at 25 °C followed by 30 min at 48 °C.

Real-time PCR was performed on 1- or 5-μl samples of cDNAs in 50 μl PCR buffer (TaqMan Universal Master Mix; PE Biosystems, Courtaboeuf, France) with 50, 300 or 900 n*M* oligonucleotide primers and 80 n*M* specific fluorogenic probe (all reagents from PE Biosystems). After a hot start (10 min at 95 °C), the denaturation steps were 15 s at 95 °C and the hybridization steps were 1 min at 60 °C in a thermocycler (GeneAmp 5700 Sequence

Detection System, PE Biosystems). A total of 40 cycles were performed.

The following primers and fluorogenic probes were used:

- For topoisomerase I (68 bp): *sense* 5′ tga cag ccc cgg atg aga 3′, *antisense* 5′ tgc aac agc tcg att ggc 3′, *fluorogenic probe* 5′ cat ccc agc aag atc ctt tct tat aac cgt 3′.
- For CES1 (66 bp): *sense* 5′ tgt ttt gtc tcc att ggc ca 3′, *antisense* 5′ gtg agg gcc acg cca ct 3′, *fluorogenic probe* 5′ acc tct tcc acc ggg cca ttt ctg 3′.
- For CES2 (65 bp): *sense* 5′ gtc cgc tgc gat ttg ca 3′, *antisense* 5′ ggt tcc atc cct cac acc ac 3′, *fluorogenic probe* 5′ ccc ctg agc ccc ctg aat ctt gg 3′.
- For ABCG2 (71 bp): *sense* 5′ tgc aac atg tac tgg cga aga T 3′, *antisense* 5′ tct tcc aca agc ccc agg 3′, *fluorogenic probe* 5′ ttg gta aag cag ggc atc gat ctc tca 3′.
- For GAPDH oligonucleotides and fluorogenic probe were purchased from PE Biosystems.

The fluorescence generated at each amplification cycle by liberation of the fluorochrome from the fluorogenic probe was detected by a computer-controlled cooled CCD camera (PE Biosystems). Values were then collected to determine the threshold cycle (Ct) of each reaction. GAPDH expression was used as a reference. The Ct of GAPDH was subtracted from the Ct of the gene of interest to determine a value called ΔCt. The lowest values of ΔCt correspond to the highest levels of expression of the gene of interest.

Results

Growth inhibition by irinotecan and SN-38

The IC_{50} values (mean ± SD) of irinotecan were 15.8 ± 5.1 and 5.17 ± 1.4 μ*M* for LoVo and HT-29, respectively. For SN-38, the values were 8.25 ± 1.1 and 4.50 ± 1.50 n*M* for LoVo and HT-29, respectively. This two- to threefold difference in sensitivity to both drugs prompted us to seek to identify the factors responsible.

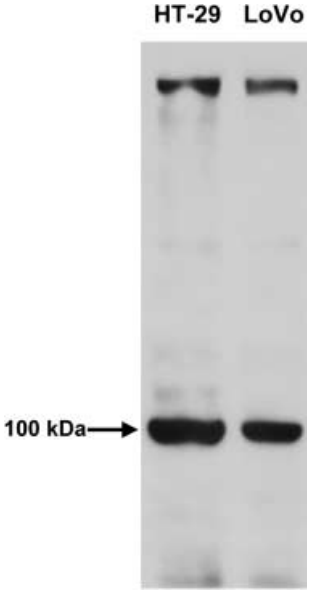

Fig. 1 Western blotting of topoisomerase I in nuclear extracts from LoVo and HT-29 cells

Evaluation of topoisomerase I expression by Western blotting and RT-PCR

Figure 1 presents the immunoblots of topoisomerase I present in the nuclear extracts from the two cell lines. Three independent nuclear extracts were analyzed. When compared by densitometry, the autoradiography signals provided by 50 μg of nuclear extracts proteins were similar, with a nonsignificant 20% higher intensity in HT-29 cells than in LoVo cells. When analyzed by real-time RT-PCR, the levels of topoisomerase I expression (average of three independent RNA preparations) were 30% higher in HT-29 cells than in LoVo cells (Table 1) but this difference did not reach significance.

Topoisomerase I catalytic activity

We performed the relaxation assays by incubating a constant amount of DNA substrate (1 μg) with increasing amounts of nuclear extracts (0 to 100 ng proteins) at 37 °C for 30 min. Three independent nuclear protein preparations were analyzed in duplicate (Fig. 2).

Table 1 Levels of gene expression of topoisomerase I, BCRP and carboxylesterases 1 and 2 as evaluated by real-time RT-PCR in cell RNA extracts. Three independent RNA extractions were performed and two PCRs were performed. The values presented are ΔCt (means ± SD) calculated as Ct(gene of interest)–Ct(GAPDH)

Gene	LoVo cells	HT-29 cells
Topoisomerase I	6.66 ± 0.71	4.48 ± 0.93
BCRP	11.0 ± 0.9	10.5 ± 1.5
CES1	16.8 ± 1.5	9.4 ± 0.8*
CES2	4.61 ± 0.79	5.53 ± 2.81

*$P < 0.01$

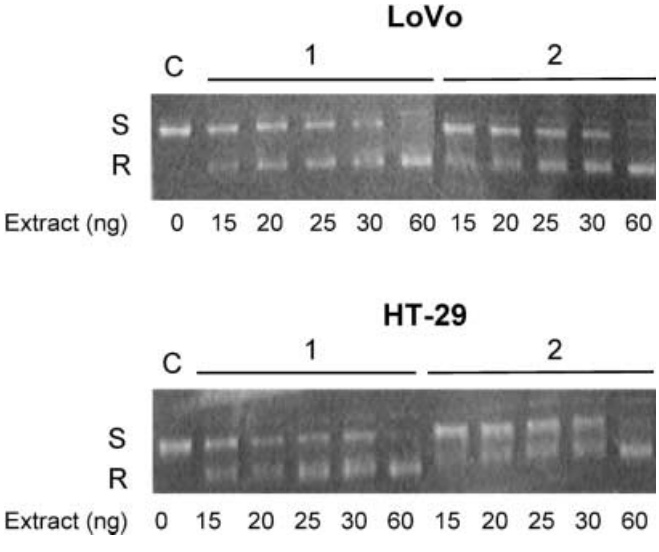

Fig. 2 Relaxation assays of pBSKS$^+$ DNA by LoVo and HT29 nuclear extracts (*C* control, *S* supercoiled DNA, *R* relaxed DNA). The amount of nuclear proteins was increased from 15 to 60 ng. For each cell line, two independent experiments are presented

Table 2 Formation of cleavable complexes in LoVo and HT-29 cells after 30-min incubations with irinotecan or SN-38. The concentrations are relative to the IC_{50} values of the drugs for each cell line. The values presented are the increases in topoisomerase I-DNA complexes relative to untreated cells, and are the means ± SD of three independent experiments (*ND* not determined)

Drug concentration	LoVo cells		HT-29 cells	
	Irinotecan	SN-38	Irinotecan	SN-38
$0.6\times IC_{50}$	ND	0.84 ± 0.22	ND	1.49 ± 0.44
$1\times IC_{50}$	1.32 ± 0.45	2.04 ± 1.41	1.53 ± 0.18	2.79 ± 1.67
$10\times IC_{50}$	ND	4.16 ± 1.04	ND	3.64 ± 1.18
$100\times IC_{50}$	ND	8.92 ± 2.00	ND	10.3 ± 1.7

Topoisomerase I catalytic activities were the same in the two cell lines: 12.7 ± 0.3 ng DNA relaxed per ng nuclear proteins in LoVo cells and 12.9 ± 0.2 in HT-29 cells (mean ± SD).

Evaluation of cleavable complexes

Table 2 presents the formation of cleavable complexes in LoVo and HT-29 cells. Irinotecan induced similar amounts of cleavable complexes at its IC_{50} value in both cell lines. SN-38 was studied over a wider range of concentrations, up to 100-fold the IC_{50} value, which was not possible for irinotecan because of the very high IC_{50} values (5–15 μ*M*). SN-38 induced a concentration-dependent formation of cleavable complexes, which was somewhat higher in the HT-29 cell line, but the difference observed did not reach significance.

Cellular accumulation of irinotecan

Cellular accumulation of irinotecan was linearly related to the dose in the LoVo and HT-29 cell lines up to 100 μ*M*. The accumulation of irinotecan was consistently twofold higher in HT-29 cells than in LoVo cells, but the kinetics of incorporation revealed a faster initial uptake in LoVo cells than in HT-29 cells (Fig. 3).

Carboxylesterase expression and activity

The expression of the CES1 gene was higher in HT-29 cells than in LoVo cells (Table 1), while the expression of the CES2 gene was quite similar in both cell lines and always much higher than the expression of the CES1 gene.

The enzymatic hydrolysis of PNPA was not significantly different in the two cell lines: 1.45 ± 0.31 and 1.84 ± 0.33 pmol/min per mg proteins in LoVo and HT-29 cells, respectively (mean ± SD). However, it was never possible to detect any formation of SN-38 with microsomal extracts of either cell line. Considering the limit of detection of SN-38 by the method used (0.2 ng), we can assume that the carboxylesterase activity transforming irinotecan was < 20 fmol/min per mg protein. In contrast, the activity in human liver microsomes was about 40 μmol/min per mg protein with PNPA and 4 pmol/min per mg protein with irinotecan as substrates.

ABCG2 expression

The expression of the ABCG2 protein was evaluated, after reverse transcription by real-time quantitative PCR using GAPDH as an internal standard of the reaction. No difference could be detected in the expression of this pump between the two cell lines (Table 1).

Discussion

LoVo cells were threefold less sensitive to irinotecan and twofold less sensitive to SN-38 than HT-29 cells. This difference can be seen as relatively minor but was quite significant and reproducible, and all the parameters studied were quantified with a reproducibility below this ratio. In the clinical setting, a two- to threefold

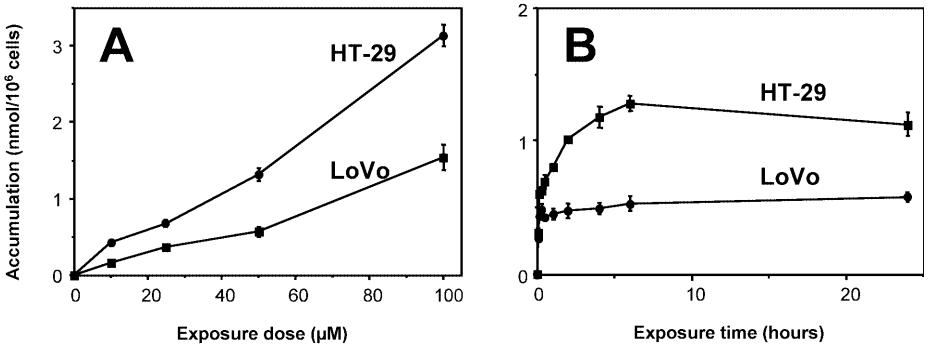

Fig. 3A, B Intracellular accumulation of irinotecan in LoVo and HT-29 cells as a function (**A**) of the external concentration of drug for an exposure time of 4 h, and (**B**) of the exposure time to the drug at a concentration of 50 μ*M*. Three independent incubations were performed and samples were analyzed twice. The *bars* represent the standard deviation of the mean values

difference in the dose administered is much larger than the difference between sensitivity and resistance to any anticancer drug, which justifies our detailed approach of the determinants of drug sensitivity. Irinotecan was about 1000-fold less cytotoxic than SN-38 in both cell lines, as already observed in vitro in cell cultures [19]. It was first remarkable that no SN-38 formation could be detected, either after incubation of the living cells with irinotecan or during the determination of carboxylesterase activity in microsomal extracts, using irinotecan as substrate. Since the limit of detection of our HPLC system was in that case especially enhanced to reach 0.2 ng SN-38, it can be concluded that the intracellular level of SN-38 formed from irinotecan during the evaluation of cytotoxicity was below the concentrations required for SN-38-induced growth inhibition [20]. Therefore, the cytotoxicity observed during irinotecan-induced growth inhibition can be attributed to irinotecan itself and not to its activated metabolic product, SN-38.

It has been shown in vitro that irinotecan can interact with topoisomerase I, leading to the same damage as SN-38, but with 1000-fold higher concentrations [8]. This means that contamination of an irinotecan preparation by SN-38 at a level as low as 0.1% could lead to the same result, which has sometimes led to the conclusion that irinotecan is totally devoid of activity. Indeed, it appears from our work that this is not true and that irinotecan can lead to cell growth inhibition in the absence of transformation to SN-38. Proof of this assertion would be given by measuring irinotecan cytotoxicity after switching off carboxylesterase activity. This is presently under study in our laboratory.

We observed that the expression of CES2 was similar in the two cell lines. This enzyme has been shown to be 50-fold more efficient in activating irinotecan than CES1 [15]. In addition, its expression in our cell lines was considerably higher than that of CES1, taking into account that the Ct scales are logarithmic. The higher expression of CES1 in HT-29 cells cannot, therefore, be considered responsible for the higher irinotecan activation, if any activation occurs in our cell lines.

No difference in topoisomerase I expression, both at the mRNA and the protein levels, could be detected between the two cell lines. As mentioned earlier, the level of topoisomerase I in a given tumor type has been related to sensitivity to topoisomerase I poisons [16, 23]. We can exclude in this study the contribution of target availability in the relative sensitivity of cell lines to irinotecan.

Cleavable complex formation was studied in the two cell lines after exposure to equitoxic concentrations of irinotecan and SN-38. This was explored in order to identify the responsibility of events occurring downstream of the drug-target interaction in mediating cytotoxicity. If the ultimate determinant of growth inhibition is the generation of cleavable complexes, then similar amounts of complexes should be obtained for similarly cytotoxic exposures. This was shown to be the case with SN-38, which induced nonsignificant differences in cleavable complex formation in the two cell lines over a wide range of concentrations. This was also the case for irinotecan after exposure at the IC_{50} value, but because of the very high IC_{50} values of irinotecan (5–15 μM), it was not possible to explore the effects of this drug at concentrations as high as could be done with SN-38. This shows that cleavable complex formation is the ultimate determinant of cytotoxicity, and that the two cell lines did not differ in their ability to undergo growth arrest upon drug-target interaction. The different p53 statuses of the two cell lines (wild-type in LoVo cells, mutated in HT-29 cells [28]) tends to indicate that this difference plays no role in the cytotoxicity of irinotecan or SN-38 in our cell lines.

The only difference we could identify between the cell lines which could account for the difference in sensitivity to irinotecan was the cellular accumulation of this drug during incubation. A twofold higher uptake of irinotecan was evident in HT-29 cells than in LoVo cells. This prompted us to study the expression of the only ABC protein that has been shown to transport drugs of the camptothecin family, the ABCG2 pump. However, a quantitative method (real-time PCR) failed to show any difference in ABCG2 expression between the two cell lines. This does not exclude the participation of other pumping systems in the regulation of intracellular accumulation of irinotecan and SN-38 in these tumor cell lines. This is presently under investigation in our laboratory. In addition, other unexplored factors, in combination with the difference in drug accumulation, may contribute to the difference observed in cytotoxicities.

Several factors studied are potential determinants of irinotecan activity and can also be studied in the clinical setting, using tumor biopsies or surgical samples. Topoisomerase I content and activity and carboxylesterase content and activity can easily be quantified in such specimens. However, cleavable complex formation or tumor drug uptake and accumulation cannot easily be determined, since this would require specific additional tumor samplings which might not be able to be carried out for ethical reasons.

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