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Sensitivity to CPT-11 of xenografted human colorectal cancers as a function of microsatellite instability and p53 status

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Summary Biological parameters influencing the response of human colorectal cancers (CRCs) to CPT-11, a topoisomerase 1 (top1) inhibitor, were investigated using a panel of nine CRCs xenografted into *nude* mice. CRC xenografts differed in their *p53* status (*wt* or *mut*) and in their microsatellite instability phenotype (MSI$^+$ when altered). Five CRC xenografts were established from clinical samples. All five had a functional p53, two were MSI$^+$ and three were MSI$^-$. Tumour-bearing *nude* mice were treated intraperitonealy (i.p.) with CPT-11. At 10 mg kg^{-1} of CPT-11, four injections at 4-day intervals, four of the five xenografts responded to CPT-11 (growth delay of up to 10 days); the non-responder tumour was MSI$^-$. At 40 mg kg^{-1} of CPT-11, six injections at 4-day intervals, the five CRCs displayed variable but marked responses with complete regressions. In order to assess the role of p53 status in CPT-11 response, four CRC lines were used. HT29 cell line was MSI$^-$/*Ala273-mutp53*, its subclone HT29A3 being transfected by *wtp53*. LoVo cell line was MSI$^+$/*wtp53*, its subclone X17LoVo dominantly expressed *Ala273-mutp53* after transfection. LoVo tumours (MSI$^+$/*mutp53*) were more sensitive than X17LoVo (MSI$^+$ /*mutp53*. HT 29 tumours (MSI$^-$*Imutp53*), were refractory to CPT-11 while HT29A3 tumours (MSI$^-$/*wtp53*) were sensitive, showing that *wtp53* improves the drug-response in these MSI$^-$ tumours. Levels of mRNA expression of *top1*, *fasR*, *TP53* and *mdr1* were semi-quantified by reverse transcription polymerase chain reaction. None of these parameters correlated with CPT-11 response. Taken together, these observations indicate that MSI and p53 alterations could be associated with different CPT-11 sensitivities; MSI phenotype moderately influences the CPT-11 sensitivity, MSI$^+$ being more sensitive than MSI$^-$ CRC freshly obtained from patients, *mutp53* status being associated with a poor response to CPT-11. © 2000 Cancer Research Campaign

Keywords: CPT-11; MSI; *p53*; *top1*; human colorectal cancer; *nude* mice

Human colorectal cancer is the second most frequent cancer in Western countries, and the third leading cause of death by cancer in both sexes (Wilke, 1996). Staging of tumour progression described by Dukes is the main basis for prognosis. For patients with advanced stage, adjuvant chemotherapy with 5-fluorouracil (5-FU) has been shown to decrease recurrence and improve overall survival (Moertel et al, 1990). Depending on 5-FU regimen, 20–30% of hepatic metastases are sensitive to 5-FU associated with leucovorin (de Gramont et al, 1997; Goldberg et al, 1997).

However, the progression of cancer is often associated with a chemoresistance to agents used as the first-line therapy and an effective second-line alternative is presently lacking. Thus, new anticancer drugs are being developed and biological determinants allowing one to predict response to therapy need to be established. CPT-11 (Campto®, Irinotecan) (Kunimoto et al, 1987), a semisynthetic water-soluble derivative of camptothecin (Wall et al, 1966), demonstrated potent anti-tumour activity against experimental colon cancer tumour models in vivo (Houghton et al, 1995) and also in phase I (Armand et al, 1995) and more recently, in phase II trials (Rougier et al, 1997). The anti-tumour activity is exerted by SN38, a metabolite of CPT-11 (Kaneda et al, 1990), which selectively inhibits DNA-topoisomerase1 (top1) (Tanizawa et al, 1994). Cytotoxicity is due to the formation of SN38-stabilized top1 DNA cleavable ternary complexes (Leteurtre et al, 1993), which impede the DNA-religation reaction (Kjeldsen et al, 1992), resulting in DNA single-strand breaks. Stabilized cleavable complexes are rapidly reversible upon lowering of the time of exposure to CPT-11 (Tanizawa et al, 1995). Elevated top1 protein levels increase the frequency of cleavable complexes (Pommier, 1996), thereby increasing DNA damage. As a consequence, DNA single-strand breaks tend to accumulate, and it is most likely that attempts to replicate and/or repair the damaged DNA enhances the formation of DNA double-strand breaks (Vamvakas et al, 1997).

Molecular characterization of genetic alterations in CRC led to the identification of two carcinogenesis pathways. In 85% of cases, CRC display chromosomal instability, allelic losses at 5q, 17p and 18q, p53 mutations are frequent, they are often left-sided and of poor prognosis. The second group (15%) is characterized by genetic instability, with widespread mutations in microsatellite sequences, a right-sided location and a better prognosis. These different pathways of carcinogenesis could account for a differential sensitivity of CRC to CPT-11. Indeed, the *TP53* gene encodes a multifunctional transcription factor that plays a critical role in cellular response after DNA damage, including G1 arrest followed by either DNA repair or apoptosis. Cells defective for p53 have a survival advantage by lacking the apoptotic programme induced by the toxic effects of anticancer drugs (Fan et al, 1994; Ceraline

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et al, 1998). Cells with functional *wtp53* are found to be dramatically more sensitive to many of the commonly used anticancer agents (Fujiwara et al, 1994; Lowe et al, 1993, 1994; McDonald and Brown 1998). In MSI$^+$ tumours, defects in mismatch repair genes could lead to the accumulation of unrepaired or misrepaired DNA during DNA replication, rendering them more sensitive for CPT-11 effect.

Other resistance mechanisms to CPT-11 could also be implicated. Overexpression of the multidrug resistance gene product, P-glycoprotein, encoded by the *mdr1* gene has been incriminated in tumour cell resistance to numerous compounds (Gottesman et al, 1996). Its role in the lack of sensitivity of tumour cells to CPT-11 has been described (Jansen et al, 1998). Two groups have recently shown that colorectal cancers of the colonic mucosa express the *fas* receptor (*fas*R), a cell membrane determinant which triggers the apoptotic cascade (Meterissian et al, 1997; Strater et al, 1997), and that camptothecin treatment activated the *fas*R in CRC cell lines (Shao et al, 1998). CPT-11, like several anticancer drugs (Micheau et al, 1997), might be able to render tumour cells highly susceptible to *fas*R-dependent apoptosis.

Our aim was to search for genetic alterations and biological parameters determining the response of CRCs to CPT-11. First, we evaluated the in vivo response of nine different xenografted CRCs, which differed in their MSI phenotype and their p53 status. Five of the nine xenografts were established from tumours in our laboratory. Four of the nine xenografts were derived from CRC cell lines: MSI$^+$/*wtp53* (LoVo) and its subclone (X17LoVo) transfected with a *mutp53* expression vector; and MSI$^-$/*mutp53* (HT29) and its subclone (HT29A3) transfected with a dominant *wtp53* expression vector. Second, expression of *top1*, the target of CPT-11, of *mdr1* and of *fas*R was investigated in these CRC xenografts.

MATERIALS AND METHODS

Human colon cancer tumours and cell lines

Three primary colorectal tumour specimens (TC1, TC7fal and TC71) and two liver metastases (TC118m and TC124m) were obtained from patients. After tumour resection, xenografts were established by subcutaneous (s.c.) implantation into the scapular area of *nude* mice. LoVo and HT29 cell lines were obtained from the American Type Cell Collection (Rockville, MD, USA). The LoVo cell line (MSI$^+$/*wtp53*) was previously transfected with an *Ala273-mutp53* geneticin resistance expression vector, as described (Pocard et al, 1996), producing the X17LoVo subclone which had stable integrated the plasmid. LoVo was arrested in the G1 phase of the cell cycle after 10-Gy irradiation, while no arrest was obtained after irradiation of X17LoVo cells with a mutated p53. The HT29 (MSI$^-$/*mutp53*) cell line was similarly transfected with a *wtp53* expression vector and a subclone HT29A3 was selected using geneticin as described above; HT29A3 had integrated the plasmid in chromosome 12 (12q11–q12), as evidenced by FISH. HT29A3 was arrested in the G1 phase after inhibition of pyrimidine synthesis, as published by Soulié et al (1999), while no arrest was obtained with HT29 parental cells, showing the dominant function of *wtp53* in the HT29A3 cell line. The four cell lines were established as transplantable tumours (s.c. injection of 2×10^6 cells). A summary of the clinical and biological information about CRCs and cell lines is given in Table 1.

Mononucleotide repeat microsatellite analysis

The MSI phenotype was determined by multiplex polymerase chain reaction (PCR) amplification of three microsatellite markers: BAT-26, as described (Hoang et al, 1997), BAT-25 and BAT-RII as described (Zhou et al (1998).

Detection of p53 mutations in CRCs

Characterization of the *TP53* gene was conducted according to the two-step procedure of (Hamelin et al, 1993).

Mice

Eight- to 10-week old, 25–30 g body weight, swiss *nu/nu* male or female mice, bred in the animal facilities of the Curie Institute, Paris, France were used in these assays. The animals were maintained under specified pathogen-free conditions. Their care and housing were in accordance with the institutional guidelines of the French Ethical Committee (Ministère de l'Agriculture et de la Forêt, Direction de la Santé et de la Protection Animale, Paris, France) and under the supervision of authorized investigators.

Growth inhibition studies

Mice were grafted with tumour fragments of approximately 15 mm^3 volume. Mice bearing growing tumours with a volume of 300–500 mm^3 were individually identified and randomly assigned to the control or treated group and the treatment started. The animals bearing tumours were sacrificed when their tumour volume reached 2000 mm^3, defined as ethical sacrifice. Volumes of individual tumours were calculated from the measurements of two perpendicular diameters using a calliper, performed every 2 days. Each tumour volume (V) was calculated according to the following formula: $V = a^2 \times b/2$, where a and b are the smallest and largest perpendicular tumour diameters. Relative tumour volumes were calculated form the formula: $RTV = (V_X/V_1)$, where V_X = the volume on day x and V_1 is the tumour volume at the initiation of therapy (day 1). Growth curves (Figure 1) were obtained for each individual tumour by plotting values of RTV against time (expressed as days after the start of treatment). The anti-tumour activity was evaluated according to four criteria: (i) the growth inhibition (GI), which was calculated according to the following formula: $\%\ GI = 100\text{-}(RTVt/RTVc) \times 100$ where RTVt is the mean RTV of the treated group and RTVc that of the control group, at the time of sacrifice of the first mouse in the group control when it attained a tumour volume of 2000 mm^3; (ii) the growth delay was calculated as the time necessary to increase of fivefold the tumour volume of individual mice. The cumulative percentage of individual tumours (mice) reaching this 5×-relative volume increase is plotted as a function of time on Figures 2 and 3. This corresponds to the survival time of mice. Statistical significance of the differences between the individual growth delays was tested by a log-rank test; (iii) a relative growth delay was calculated as the difference between the time in days required for control and treated tumours to reach a fivefold increase in volume (Houghton et al, 1995). A ratio between the growth delays of tumours in the treated group and that of the control group was also calculated and reported on Tables 2 and 3; and then (iv) the number of partial responses or complete tumour regressions (PR and CR); PR was

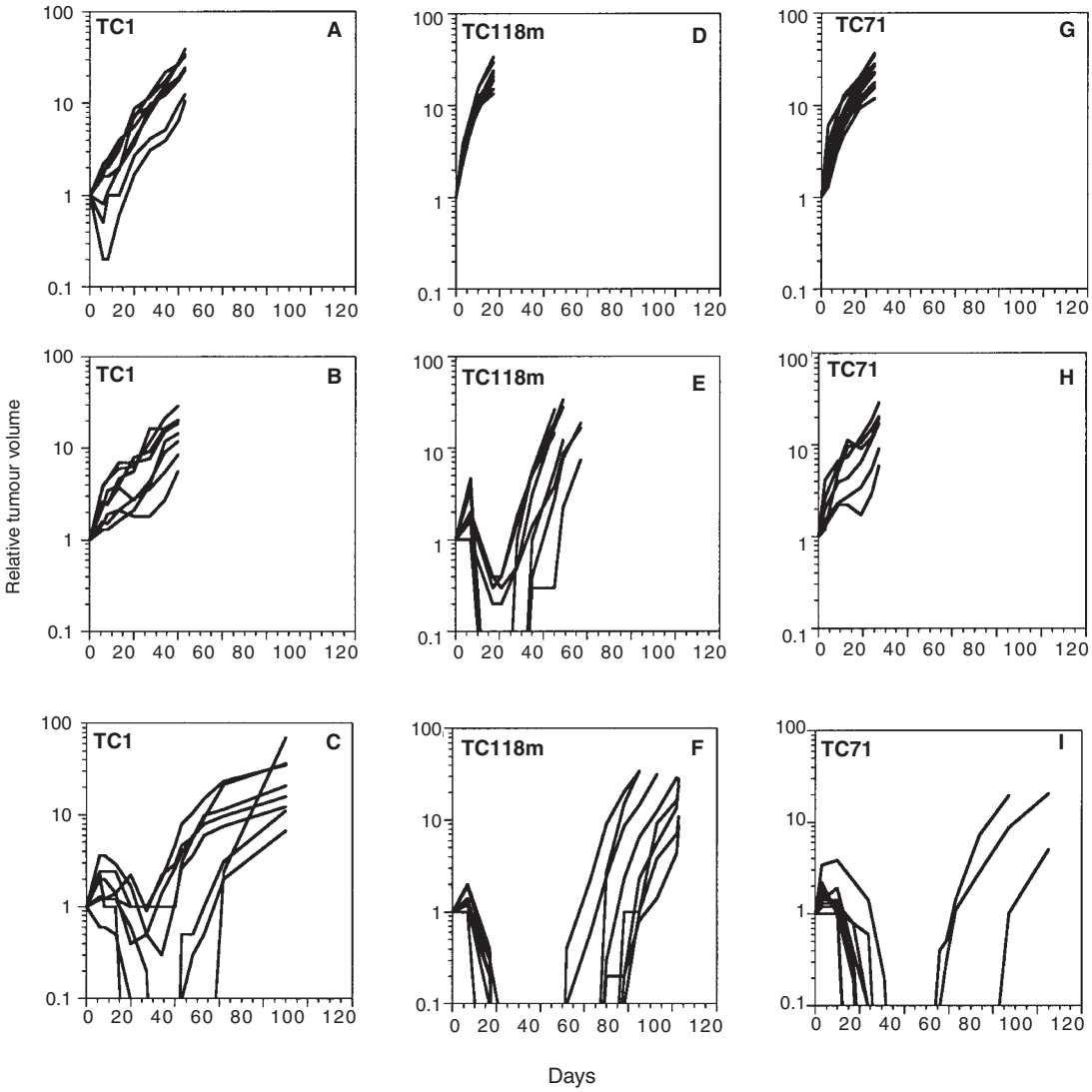

Figure 1 Anti-tumoural effects of CPT-11 on human colorectal cancers established from clinical samples and subcutaneously grafted into the scapular area of *nude* mice. Individual growth curves for TC1 (**A, B, C**), TC118m (**D, E, F**) and TC71 tumours (**G, H, I**). Tumour-bearing mice were treated with CPT-11 at low dose (**B, E, H**) (10 mg kg^{-1} at 4-day intervals × 4) or high dose (**C, F, I**) (40 mg kg^{-1} at 4-day intervals × 6)

defined as tumour growth inhibition greater than 50% and CR as tumours that had regressed and could no longer be palpated. Mice were considered to be cured if tumour-free after 180 days of observation.

Formulation and administration

CPT-11 (Campto®, Irinotecan) was generously supplied by Rhône Poulenc Rorer (Vitry sur Seine, France). Stock solution of CPT-11 was diluted in 0.9% sodium chloride solution and administered i.p. in a 0.2 ml volume to tumour-bearing mice using an intermittent schedule: two doses were tested, a high total dose of 240 mg kg^{-1} (6 injections of 40 mg kg^{-1} day^{-1} every 4 days) and a total low dose of 40 mg kg^{-1} (four injections of 10 mg kg^{-1} day^{-1} every 4 days). Mice in the control groups received 0.2 ml of the drug-formulating vehicle with the same schedule as given to the treated animals.

top-1, p53, mdr-1and fas-receptor PCR mRNA expression

Isolation of total RNA from frozen tumour samples weighing approximately 20 mg was done as follows: the frozen tissue was placed under liquid nitrogen and immediately pulverized with a mortar and pestle. The pulverized tissue was rapidly transferred to 1 ml-containing TRIZOL® Reagent (Gibco-BRL, Life Technologies). The resulting RNA pellet was resuspended in 40 µl sterile deionized water, and stored overnight at 4°C for complete dissolution. The concentration of the RNA was determined by spectrophotometry.

Synthesis of complementary DNAs from poly-adenosine (poly(A)) mRNA was done as follows: RNA at a final concentration of 2 µg in 20 µl of sterile water was incubated at 65°C for 10 min. A mix containing 4 µl of 5× RT buffer (Gibco-BRL, Life

Table 1 Clinical characteristics of the CRC tumours and cell lines used

Tumours	Origin	Stage[a]	HNPCC
TC1	Rectum	B	No
TC124m	Liver metastasis	D	No
TC118m	Liver metastasis	D	No
TC7fal	Right colon	C	No
TC71	Sigmoid	B	Yes[b]
LoVo and X17LoVo[c]	Lymph node metastasis	D	No
HT-29 and HT29A3[d]	Sigmoid	B	No

[a]According to Duke's classification. [b]With a familial story of HNPCC. [c]Subclone of LoVo cells transfected with *Ala273-mutp53* expression vector. [d]Subclone of HT29 cells transfected with *wtp53* expression vector.

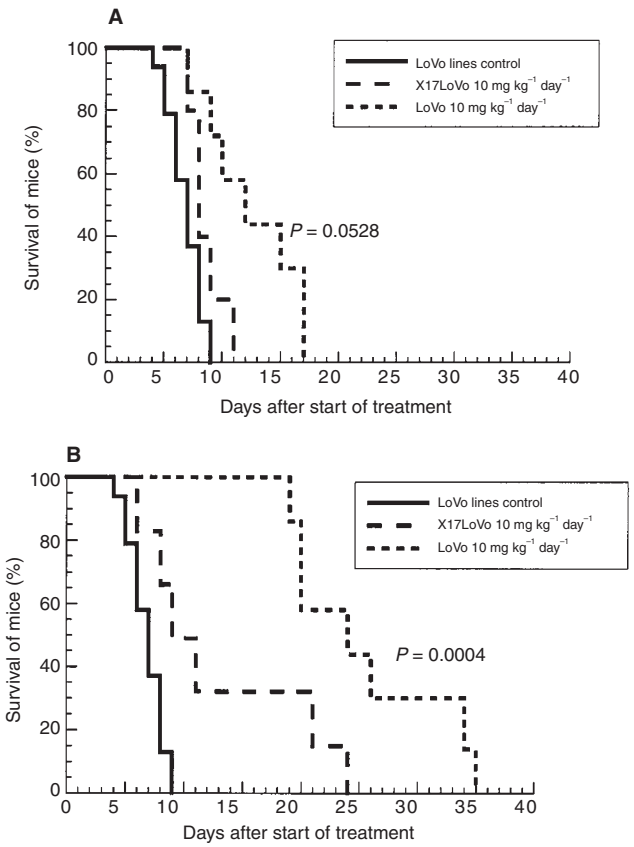

Figure 2 Effect of CPT-11 on the survival of mice bearing human colorectal cancers established from the cell lines LoVo and X17LoVo, a subclone of LoVo transfected with a dominant *mutp53*. Survival curves are plotted as a function of the survival time of mice, which were sacrificed as their tumour reached 2000 mm³. Mice were treated with CPT-11 at low dose (**A**) (10 mg kg^{-1} at 4-day intervals × 4) or high dose (**B**) (40 mg kg^{-1} at 4-day intervals × 6). *P*-values (log-rank test) corresponding to the differences between the growth curves of CPT-11 treated LoVo and X17LoVo tumours, are indicated

Technologies), 2 µl dithiothreitol (DTT) (100 mM), 1 µl of oligo-dT (200 ng µl^{-1}), 1 µl of a mix containing 12.5 mM of each dNTP (final concentration 2.5 mM) and 0.2 µl (200 U µl^{-1}) of RNAase H reverse transcriptase (Gibco-BRL, Life Technologies) was added to the RNA and incubated at 45°C for 60 min.

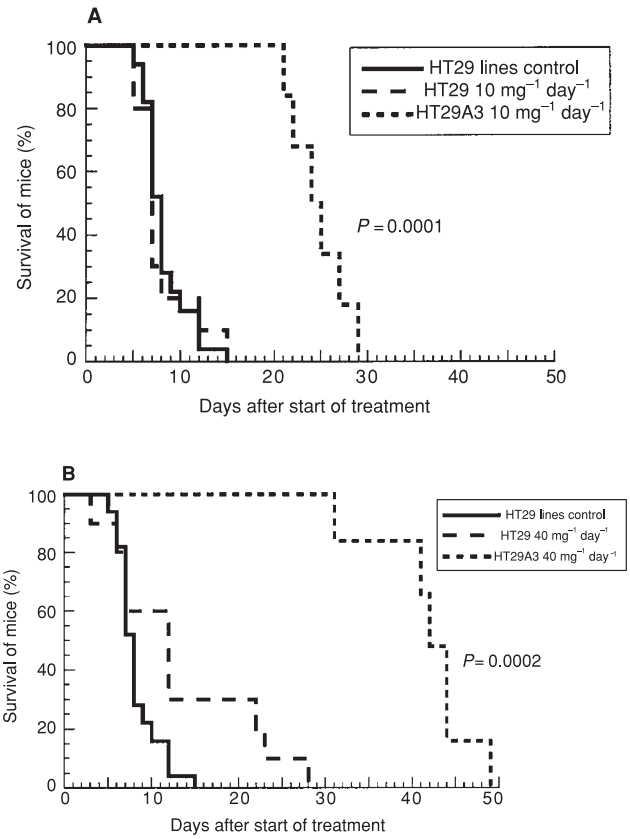

Figure 3 Effect of CPT-11 on the survival of mice bearing human colorectal cancers established from the cell lines HT29 and HT29A3, a subclone of HT29 transfected with a dominantly expressed *wtp53*. Survival curves are plotted as a function of the survival time of mice, which were sacrificed as their tumour reached 2000 mm³. Mice were treated with CPT-11 at low dose (**A**) (10 mg kg^{-1} day^{-1} at 4-day intervals × 4) or high dose (**B**) (40 mg kg^{-1} at 4-day intervals × 6). *P*-values (log-rank test) corresponding to the differences between the growth curves of CPT-11 treated HT29 and HT29A3 tumours, are indicated

PCR primers

The primers listed below were selected for their specificity and their selectivity towards human gene sequences (purchased from Oligo Express, Paris).

***top1*-1**: S 5′-AAA AGT CCA AGC ATA GCA ACA G-3′ (bases 345–365 of the topoisomerase-1 gene coding sequence)
***top1*-2**: AS 5′-AGG AAC AAA ATA GCC ATC ATC T-3′ (bases 574–595 of the topoisomerase-1 gene coding sequence)
***mdr1*-1**: S 5′-AGC GCG AGG TCG GGA TGG ATC TTG AAG GGG A-3′ (bases 411–441 of the *mdr*-1 gene coding sequence (Murphy et al, 1990))
***mdr1*-2**: AS 5′-TTG ACA TCA GAT CTT CTA AAT TTC CTG CAT TT-3′ (bases 664–695 of the *mdr*-1 gene coding sequence (Murphy et al, 1990))
***p53*-1**: S 5′-ACA CGC TTC CCT GGA TTG G-3′ (bases 168–186 of the *p53* gene coding sequence (el-Mahdani et al, 1997))
***p53*-2**: AS 5′-GGT CTT GGC CAG TTG GCA A-3′ (bases 616–634 of the *p53* gene coding sequence (el-Mahdani et al, 1997))
***fas* R-1**: S 5′-CAA GTG ACT GAC ATC AAC TCC-3′ (bases

Table 2 p53 status, MSI phenotype and responses to CPT-11 of CRC xenografts in *nude* mice established from clinical samples

Tumour xenografts	*p53*	MSI	CPT-11 total dosage mg kg^{-1}	Growth rate (days)[a] mean ±	Growth inhibition %[b] (days)	Growth delay in days (ratio)[c]	Nb mice per group	Nb PR[d]	Nb CR[e]	Nb cures[f]
TC1	*wt*	–	0	23 ± 8			8			
			40	22 ± 10	0	0	8	3	0	0
			240	54 ± 12**	>99 (30)	31 (2.3)	8	5	3	0
TC124m	*wt*	–	0	11 ± 3			6			
			40	21 ± 4**	>99 (20)	10 (1.9)	7	7	0	0
			240	31 ± 6**	>99 (20)	20 (2.8)	7	4	3	0
TC118m	*wt*	–	0	6 ± 1			8			
			40	41 ± 8***	>99 (17)	35 (6.8)	8	4	4	0
			240	86 ± 13***	100 (17)	80 (14)	8		8	0
TC7fal	*wt*	+	0	8 ± 3			8			
			40	27 ± 3***	>99 (17)	19 (3.4)	9	5	4	0
			240	44 ± 6***	>99 (17)	36 (5.5)	9	5	4	0
TC71	*wt/mut*	+	0	7 ± 2			10			
			40	15 ± 9*	>99 (24)	8 (2.1)	6	6	0	0
			240	86 ± 17***	100 (24)	79 (12.3)	10		10	7

[a]Delay in days to reach a fivefold increase of CRC xenograft volume from their initial size at the start of treatment (300–500 mm^3). [b]GI: treated/control growth inhibition, calculated at the day (in parentheses) of the ethical sacrifice of the first control mouse bearing a tumour with a volume of 2000 mm^3. [c]In parentheses, ratio between growth delays of the tumours in the treated group and that of the controls. [d]Up to 50% of growth inhibition, calculated at the ethical sacrifice of the first control mouse. [e]Complete regressions. [f]Tumour free mice were defined as bearing non palpable tumour after the end of the experiment (180 days). [g]Significantly different from control groups: *$0.01 < P < 0.05$; **$10^{-6} < P < 10^{-4}$; ***$P < 10^{-7}$.

Table 3 Responses to CPT-11 treatment of CRC cell lines varying for their p53 status and MSI phenotype established as xenografts in *nude* mice

Tumour xenografts	*p53*	MSI	CPT-11 total dosage mg kg^{-1}	Growth inhibition %[b] (days)	Nb mice per group	Nb PR[d]	Nb CR[e]	Nb cures[f]
LoVo	*wt*	+	0		7			
			40	60 (8)	7	6	0	0
			240	80 (8)	7	7		0
X17LoVo	*wt/mut*[h]	+	0		7			
			40	30 (7)	5	2	0	0
			240	50 (7)	6	4	0	0
HT29	*mut*	–	0		10			
			40	31 (22)	9	2	0	0
			240	66 (22)	9	7	0	0
HT29A3	*mut/wt*[h]	–	0		6			
			40	70 (20)	6	6		0
			240	>99 (20)	6	4	2	0

[a]Delay in days to reach a fivefold increase of CRC xenograft volume from their initial size at the start of treatment (300–500 mm^3). [b]GI: treated/control growth inhibition, calculated at the day (in parentheses) of the ethical sacrifice of the first control mouse bearing a tumour with a volume of 2000 mm^3. [c]In parentheses, ratio between growth delays of the tumours in the treated group and that of the controls. [d]Up to 50% of growth inhibition, calculated at the ethical sacrifice of the first control mouse. [e]Complete regressions. [f]Tumour free mice were defined as bearing non palpable tumour after the end of the experiment (180 days). [g]Significantly different from control groups: *$0.01 < P < 0.05$; **$10^{-6} < P < 10^{-4}$; ***$P < 10^{-7}$. [h]Dominant p53 in transfected lines.

270–290 of the *fas-apo*-1 receptor gene coding sequence (Laurence et al, 1996))

***fas*R-2**: AS 5′-CCT TGG TTT TCC TTT CTG TGC-3′ (bases 797–817 of the *fas-apo*-1 receptor gene coding sequence (Laurence et al, 1996))

***hprt*-1**: S 5′-CAA CAG GGG ACA TAA AAG TAA T-3′ (bases 414–435 of the *hprt*-gene coding sequence)

***hprt*-2**: AS 5′-AGT CCG TCA TAT TAG GTT TCT-3′ (bases 534–554 of the *hprt*-gene coding sequence).

PCR conditions

The final PCR reaction volume was 50 µl: 1 µl (1/20) of the cDNA solution was mixed with 5 µl of 10× *Taq* buffer (Appligene-Oncor, Illkirch, France), 1 µl dNTP (final concentration 2.5 mM each), 1 µl of each of the 5′ and 3′ primers (100 ng µl^{-1}), 40.5 µl water and finally 0.5 µl (2.5 U) *Taq* polymerase (Appligene-Oncor, Illkirch, France) was added. After preheating at 94°C (hot-start), the tubes were placed in a Perkin-Elmer 2400 thermocycler

(Yvelines, France) for 3 min at 94°C followed by a maximum of 35 cycles (1 min at 94°C, 1.5 min at 60°C and 2 min at 72°C), and a final elongation at 72°C for 7 min.

Quantitation of the PCR products

The linear range of amplification for each cDNA was established, according to a kinetic analysis (Köhler et al, 1995); 7 µl of the PCR products were consecutively pipetted at the end of each chosen cycle number within a preliminary chosen range of 10–15 cycles, the final elongation at 72°C for 7 min being performed immediately after, and stored at 4°C. Four microlitres of a gel loading solution (bromophenol blue and xylene cyanol) were added and the solution was electrophoresed on a 1.8% agarose gel stained with ethidium bromide (final concentration of 0.5 µg ml^{-1}). After running 50 min at 100V, the gel was washed in distilled water and placed on a UV illuminator (Imager, France) for a 6/30 s exposure, the image was then processed by software NIH image 1.60 version. These conditions were rigorously used in all experiments.

As the amplified PCR product is proportional to the initial amount of DNA only in the linear phase of the amplification reaction, we defined the linear range of expression for each gene. To calculate the relative expression of the genes of interest, a ratio was calculated between the fluorescent signal given by the BET-intercalated PCR product within the linear amplification range of the gene of interest and that given by that of the *hprt* gene.

RESULTS

Response to CPT-11 of human colorectal cancers xenografts varying for their MSI phenotype

Five CRC tumours obtained from clinical samples were used and established as xenografts. They originated from the left part of the colon or from the rectum. Their staging differed, stage B, B and C for TC1, TC71 and TC7fal respectively, and stage D for TC124m and TC118m. The TC71 tumour originated from a patient with a clinical history of hereditary non polyposis colon cancer (HNPCC) (Table 1). All had a functional p53, including the TC71 which was heterozygote with a mutation at the codon 176. The p53 status was determined by DGGE for TC118m and TC124m and has been previously described for TC1, TC7fal and TC71 (Hamelin et al, 1993; Cottu et al, 1996). These CRCs differed in their MSI phenotype, three being MSI^- (TC1, TC124m and TC118m) and 2 MSI^+ (TC7 and TC71). Microsatellite instability of tumours was measured by band shift of three probes (BAT 25, BAT 26 and BAT RII), as described by Olschwang et al (1997) (Table 4).

CPT-11 at a total dose of 240 mg kg^{-1} (high dosage) given as six i.p. injections of 40 mg kg^{-1} at 4-day intervals was well tolerated. CPT-11 was also given at a total dose of 40 mg kg^{-1} (low dosage) as four injections of 10 mg kg^{-1} at 4-day intervals. The responses of the five CRC tumours established from clinical samples to both CPT-11-treatment protocols are summarized in Table 2. Without treatment, the growth rates of CRC xenografts varied between 6 and 23 days for TC118m and TC1 respectively. Given at a high dose, CPT-11 caused significant growth delays of all CRCs: 31, 20, and 36 days for TC1, TC124m and TC7fal respectively, representing 2.3-, 2.8- and 5.5-fold decreases in growth rate respectively. Impressive growth delays were observed for TC118m and TC71, up to tenfold their control growth rate. Complete regressions produced by CPT-11 at high dosage were observable in all CRC tumour models, with the CR frequency varying from 3/8 to 10/10 for TC1 and TC71 respectively. Cures were observed only in the group of TC71 tumours, with a frequency of 70% (7/10): no palpable tumours at day 180 after start of the treatment and histological examination of the graft site revealed fibrosis tissue and the absence of remaining living tumour cells.

At low dosage, CPT-11 caused a growth delay of 35 days for TC118m, the effect was less marked for TC124m, TC7fal and TC71 and absent for TC1. Complete regressions were observable in TC118m and TC7fal, with the PR frequency varying from 3/8 to 6/6 for TC1 and TC71 respectively. All tumours regrew.

Individual growth curves of TC1, TC118m and TC71 in the control, low and high dose CPT-11 groups are shown in Figure 1. They allow the variability of drug responses in individual mice to be evaluated for the three CRC models. TC1 was the least aggressive of the three CRC, and mice survived 40 days in the control group. CPT-11 at low dose did not modify TC1 tumour progression. At high dose, all eight TC1 tumours were slowed down and three complete regressions were observed. No mice survived beyond day 90 after start of treatment. TC118m grew very rapidly in the control group and no mice survived beyond day 20. All TC118m-bearing mice responded to CPT-11 at low dose, with four complete regressions; at high dose, all had regressed completely at day 20. However, all regrew between days 50 and 80. Their growth rate was similar to that of untreated tumours. The growth rate of TC71 tumours was intermediate, all mice survived 30 days in the control group. The efficacy of CPT-11 at low doses was variable. With high dose CPT-11, complete regressions of all the tumours were induced and seven of ten TC71-bearing mice were cured. No acceleration of growth rate was detected in the relapses. Considering all the parameters of responsiveness, all these p53wt colorectal cancer xenografts respond to CPT-11, with variable sensivity; TC1(MSI^-) was the least sensitive CRC and TC71 (MSI^+) the most sensitive, as it was curable by high dose CPT-11.

Influence of p53 mutation on the response to CPT-11 of xenografted CRC established from MSI^+ cell lines

In order to evaluate the role of p53 mutation in CPT-11 response of CRC xenografts, a MSI^+ cell line (LoVo) was transfected with a mutated p53 vector. The LoVo cell line was derived from a lymph node metastasis of a CRC. LoVo cells were MSI^+ with a homozygous deletion of hMSH2 (Casares et al, 1995) and had a wild-type p53 (Hamelin et al, 1993). Microsatellite instability of tumours was measured by band shift of three probes (BAT 25, BAT 26 and BAT RII), as described by Olschwang et al (1997) (Table 5). LoVo cells were transfected with an *Ala273-mutp53* geneticin resistance expression vector, as described (Pocard et al, 1996), and one subclone, X17LoVo, was selected and established as a xenograft in *nude* mice.

LoVo and X17LoVo established as s.c. tumours were transplanted into mice for testing their sensitivity to CPT-11. The growth rates of LoVo and X17LoVo were very similar, of 6 and 7 days respectively (data not shown). Given at high dose, CPT-11 caused growth inhibitions of 80% and 50% observed for LoVo and X17LoVo respectively, but neither xenograft showed complete regression with high dose CPT-11. Given at low dose, CPT-11 caused growth inhibitions of 60% and 30% for LoVo and X17LoVo respectively, were observed. No complete regressions

Table 4 MSI phenotype and p53 status of the CRC tumours

	MSI phenotype			*p53* status
Tumours	**BAT-26**	**BAT-RII**	**BAT-25**	**mutated codon**
TC1	–	–	–	*wt*
TC124m	–	–	–	*wt*
TC118m	–	–	–	*wt*
TC7fal	Band shift	Band shift	Band shift	*wt*
TC71	Band shift	Band shift	Band shift	176/heterozygote

were achieved. Kinetics of the number of tumours (as a cumulative percentage of the total number of mice per group) reaching individually a fivefold increase of their initial volume at the start of the treatment, were reported on Figure 2, as a function of time. This parameter corresponds to the survival time of mice, which were sacrificed as their tumour reached 2000 mm^3. This parameter did not differ between the two control groups of non-treated tumours (LoVo and X17LoVo, $P = 0.07$) and data were pooled. Figure 2 visualizes the loss of sensitivity of X17LoVo tumours, at low dosage (Figure 2A) as well as at high dosage (Figure 2B). Differences between LoVo and X17LoVo were statistically significant, as calculated by the log-rank test ($P = 0.05$ and $P = 0.0004$), for low dose and high dose experiments respectively. Taking into account the different parameters of responsiveness to CPT-11, X17LoVo (*mutp53*) tumours were less sensitive than LoVo (*wtp53*) xenografts.

Influence of wtp53 on the response to CPT-11 of CRC xenografts established from MSI$^-$ cell lines

The HT29 cell line was derived from a sigmoid colon cancer of stage B1. HT29 cells were MSI$^-$ and had a mutated p53 in *Ala* 273 codon, as checked by us and published by Hamelin et al (1993) respectively. Microsatellite instability of tumours was measured as specified (Table 5). HT29 was transfected with a *wtp53* geneticin resistance expression vector, according to the methods described in Pocard et al (1996). A selected subclone, HT29A3, had integrated the plasmid in chromosome 12 (12q11–q12), as evidenced by FISH. After inhibition of DNA synthesis, HT29A3 cells were arrested in G1/S, while HT29 cells did not (data not shown). All cell lines were tumorigenic, with 100% tumour takes in our experimental conditions. The growth rates of HT29 and HT29A3 tumours did not significantly differ (of 8 and 10 days respectively $P = 0.25$). Given at high dose, CPT-11 caused growth inhibitions of 66 and 100% respectively. None of the HT29 xenografts regressed completely under treatment, whereas two of six HT29A3 achieved complete regression. Given at low dose, CPT-11 caused growth inhibitions of 31% and 70% for HT29 and HT29A3 tumours respectively. No complete regression was achieved. All HT29A3 responded at least partially to the treatment, whereas only two of the nine HT29 tumours were slowed down. Individual growth delays of HT29 tumours as calculated as the percentage of tumours (mice) reaching a fivefold increase of the initial tumour volume at the start of the treatment, were reported on Figure 3. Curves corresponding to the survival of non-treated tumours (HT29 and HT29A3) did not significantly differ ($P = 0.25$) and data were pooled. Figure 3 visualizes the prolongated survival of mice bearing the HT29A3 tumours as compared to that bearing the HT29 tumours, at low dosage (Figure 3A) as well as at high dosage (Figure 3B). These differences were statistically different, as calculated by the log-rank test ($P = 0.0001$ and $P = 0.0002$), for low dose and high dose treatments respectively. Taking into account the different parameters of responsiveness to CPT-11, HT29A3 tumours, *wtp53* transfected, were more sensitive than tumours established from HT29, its parental *mutp53* cell line.

top1, *fasR*, *mdr1* and *p53* gene expression in CRC xenografts

Relative expressions of the *top1*, *fasR*, *mdr1* and *p53* genes were determined with respect to the hypoxanthine phosphoribosyl transferase (*hprt*) gene, chosen as the internal standard. The *hprt* gene was expressed at a constant level among the different specimens (Figure 4). The relative expression of the genes of interest was calculated within the linear amplification range of the gene of interest and that given by that of the *hprt* gene (Figure 5). There was little variation of *top1* gene expression between the different tumours. A fourfold variation of *top1* expression among the different tumour samples was found (Table 3). Expression of *mdr1* showed a variation of greater than 80-fold in CRC xenografts, in the conditions described here. These large variations in *mdr1* gene expression were due to exceptionally high values in primary tumours and very low values in metastatic tumours. *p53* gene expression was very high in X17LoVo mutant tumours, 100-fold higher than its parental LoVo cell line. The level of expression of *p53* varied over a tenfold range among the other tumours. *fasR* expression showed a 20-fold variation. *top1*, *mdr1*, *p53* and *fasR* mRNA levels did not correlate with responsiveness of xenografts to CPT-11 (Table 6).

DISCUSSION

We have tested here the sensitivity to CPT-11 of a panel of nine human colorectal cancers (CRC) differing with respect to their genetic alterations concerning p53 function and/or DNA repair, hypothesizing that these genetic alterations influence their response to CPT-11. This might thereby orientate towards identification of groups of patients who could benefit from top1 inhibitors. CRC xenografts used here originated either from clinical CRC samples (without any intermediate cell culture) or from CRC cell lines, which have been experimentally modified for p53 status.

Among the xenografts established from clinical samples, two were MSI$^+$ and three were MSI$^-$, all were associated with a functional p53 and their responses to low or high dose CPT-11 were compared. At high doses, differences between the responses of the CRCs were seen: both MSI$^+$ tumours were highly sensitive, one (TC71) being curable in 70% of cases. The three MSI$^-$ CRCs

Table 5 MSI phenotype and p53 status of the cell lines used

	MSI phenotype			*p53* Status
Cell lines	BAT-26	BAT-RII	BAT-25	Mutated Codon
LoVo	–[a]	Band shift	Band shift	*wt*
X17LoVo	–[a]	Band shift	Band shift	273*mut* transfected
HT29	–	–	–	*Ala273*
HT29A3	–	–	–	*wtp53* transfected

[a]Homozygous deletion of hMSH2.

responded to high doses of CPT-11, but with only moderate responses for two of them (TC1 and TC124m). At low doses, one MSI⁻ tumour (TC1), did not respond at all; the four other CRC xenografts (MSI⁺ or MSI⁻) were responders, and one (TC118m) was highly sensitive to CPT-11 with four CR of eight mice, but none were cured by CPT-11. Growth rate slopes of recurrences were similar to that of the controls. The sensitivity of CPT-11 treated tumours (TC118m) to subsequent treatment with CPT-11 was not diminished (data not shown).

To test the respective role of MSI and *p53* in the response to CPT-11, MSI⁺ or MSI⁻ tumour cell lines were used. The HT29 cell line is MSI⁻/*mutp53* and resistant to CPT-11 at low dose, although at high dosage a mean growth inhibition of 66% could be obtained. HT29A3 is a tumorigenic subclone of HT29 cells transfected with *wtp53*. The transfected plasmid was located in chromosome 12 and dominant function of *wtp53* was attested to by arrest of the cell cycle when HT29A3 growth was inhibited by an inhibitor of pyrimidine synthesis. Transfection of *wtp53* in HT29 did not slow their growth in control mice and clearly increased their sensitivity to CPT-11, prolongating the survival of tumour-bearing mice, namely when treated at high dosage.

The LoVo cell line has a MSI⁺ phenotype (Malkhosyan et al, 1996) and a *wtp53*. Its subclone, X17LoVo, was selected for high expression of *mutp53* which behaved like a dominant mutated p53 conferring a gain of function, as described in other models (Gualberto et al, 1998). γ-irradiation did not induce cell cycle arrest in G1/S (Pocard et al, 1996). Expression of *mutp53* in X17LoVo led to a reduction of sensitivity as compared to LoVo as shown by reduced growth delay and decreased growth inhibition, and shortening their survival time.

Taken together, these results show that among the three MSI⁺ (TC7Fal, TC71 and LoVo) xenografts, all were sensitive at both dosages excepted LoVo which responded only at high dosage. Among the four MSI⁻-CRC xenografts (TC1, TC118m, TC124m and HT29), one (HT29) was completely refractory to both dosages, and another (TC1) to low dosage only; the two others (TC118m and TC124m) displayed a high sensitivity whatever the dosage used. In both types (MSI⁺ or MSI⁻) mutation of p53 was also associated with a lack of responsiveness, which can be overcome by re-expression of wtp53.

In recent years, it has been proposed that genotypic alterations acquired during tumour progression may provide additional prognostic information. In colorectal cancers, a correlation between survival, 17p allelic losses and/or p53 mutations has been established (Hamelin et al, 1994). Tumour prognosis is determined either by intrinsic aggressiveness and/or the potential sensitivity to chemotherapy. The p53 point mutations seem to be crucial in these two parameters, at least in colorectal cancers; indeed p53-deficient tumours are more invasive and less responsive. Goh et al showed a

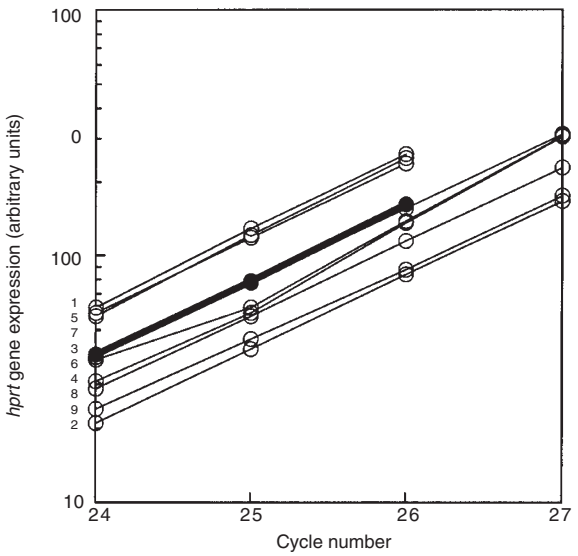

Figure 4 Comparison of the linear amplification range of *top1* and *hprt* gene mRNA expression in the CRC samples. *hprt* mRNA expression (internal standard) as a function of the number of amplification cycles (1, X17LoVo; 2, LoVo; 3, HT29; 4, HT29A3; 5, TC1; 6,TC7fal; 7, TC71; 8, TC118m; 9, TC124m)

shortened survival of *mutp53* CRC patients with post-operative therapies as compared to patients with CRCs with a functional p53 (Goh et al, 1995). This confirmed the study by Lowe showing that p53 plays a role in determining responsiveness to adjuvant chemotherapy (Lowe et al, 1994). Unresponsiveness of p53-deficient tumours has been ascribed to lack of apoptosis normally induced after G1/S-arrest following chemotherapy. Besides this clearly described programmed cell death, other alterations of cell cycle checkpoints in p53 deficient cells have been reported. Bypassing of the G2/M arrest in p53-deficient cells after drug treatment drives them either to survive or die, probably depending on the accumulation of DNA lesions (Kastan et al, 1991; Ceraline et al, 1998), and this is independent of the induction of apoptosis (Attalla et al, 1997).

Different combinations of genetic alterations lead to different pathways of CRC progression. In a series of 87 CRCs, Olschwang et al found 41 MSI-/*mutp53* (47%), nine MSI⁺/*wtp53* (10%), four MSI⁺/*mutp53* (4.5%) and 33 MSI⁻/*wtp53* (Olschwang et al, 1997). Fifteen per cent of CRCs were MSI⁺ and more often associated with a *wtp53*. Fifty per cent had a p53 mutation and were rarely associated with a MSI phenotype. This inverse correlation was also found in CRC cell lines (Cottu et al, 1996). Sporadic MSI⁺ CRCs and HNPCC have a better prognosis than cancers with MSI (Bubb et al, 1996; dos Santos et al, 1996; Vaurs and Bignon, 1997; Lukish et al, 1998) and reviewed by Arnheim and Shibata (1997). Their low progression potential is illustrated by the low frequency of MSI⁺ liver metastases (Ishimaru et al, 1995).

Advances in adjuvant chemotherapy of colorectal cancers must address a complex series of factors including not only the availability of new effective compounds but also which subgroups of patients may benefit from them. Inhibitors of topoisomerase 1 are a new category of putatively potent anti-tumour agents. CPT-11 is a water soluble analogue of camptothecin, which is metabolized to

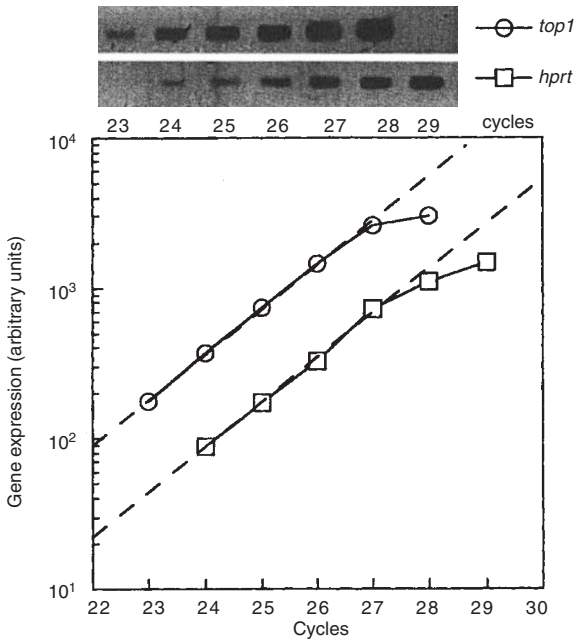

Figure 5 Comparative mRNA expression of *top1* and *hprt*, as a function of the amplification cycle number. Upper part, UV-irradiated BET signals on agarose gel with an increasing number of cycles. Lower part, linear amplification of *hprt* and *top1* gene in the same sample, as reported on a diagram where the number of amplification cycles is plotted on the *x* axis and the gene expression, as evaluated by arbitrary units (corresponding to the fluorescent signal given by intercalating-cDNA irradiated BET on agarose gel) on the *y* axis

an active compound, 7-ethyl-10-hydroxycamptothecin (SN38) β-glucuronide, by a carboxylesterase (Rivory et al, 1996). CPT-11 toxicity is a limiting factor (Armand et al, 1996). This contrasts with the tolerance in mice. Progresses in overcoming CPT-11 toxicity in humans should improve the clinical use of this compound (Takasuna et al, 1995, 1996). Nevertheless, even at the moderate doses tolerable by human hosts, top1 inhibitors are active agents in patients with metastatic colorectal cancers (Conti et al, 1996; Pazdur et al, 1997) and reviewed in Rougier and Bugat (1996); Rothenberg (1997).

CRC xenografts displayed a large range of sensitivity to CPT-11, from resistance to eradication. These differences were not due to different levels of top1, as evaluated by a semiquantitative method (Murphy et al, 1990), as already published (Goldwasser et al, 1995; van Ark-Otte et al, 1998). Jansen et al (1998) proposes a role for P-glycoprotein (encoded by *mdr1* gene) in lack of efficacy of numerous compounds, including CPT-11. Our study did not support such a role as no correlation was found between P-glycoprotein encoding gene expression (*MDR1*) and CPT-11 responses and, furthermore, a diversity of *mdr1* gene expression (a range of 80-fold) in our series of CRCs was shown.

The apoptotic process induced by drugs can involve the *fas* receptor pathway (Micheau et al, 1997) and it has been described that many CRCs express the fasR and initiate the death programme when activated (Meterissian et al, 1997; Tillman et al, 1998). Is CPT-11 activity mediated by activation of the fasR pathway in vivo, as shown in vitro in the HT29 cell line by Shao et al (1998)? In our series, HT29 tumours were the most CPT-11-resistant and they expressed a relatively low level of fasR, similar to that of HT29A3, its CPT-11-responsive counterpart, this excluding such correlation.

Table 6 Relative gene expression in CRC xenografts. Ratios ($\times 10^{-2}$) of each gene expression to that of *HPRT* gene

Samples	*top1*	*TP53*	*mdr1*	*fasR*
TC1	110	30	8	20
TC124m	105	ND	0.1	5
TC118m	290	300	0.2	70
TC7fal	140	120	4	100
TC71	160	50	0.05	40
LoVo	90	30	2	60
X17LoVo	120	2700	0.6	60
HT29	330	55	5	30
HT29A3	400	75	7	20

ND, Not detected at 35 cycles.

It was published that sensitivity to CPT-11 depends on the accumulation and stability of cleavable complexes, and this could be a parameter explaining the inter tumour differences. Cleavable complex levels were measured in vivo after camptothecin treatment in colon carcinoma cell lines by Goldwasser et al who showed a direct correlation between levels of cleavable complexes and cytotoxicity (Goldwasser et al, 1995). This has not been tested in vitro in our study as the methodology is in the process of being developed for in vivo samples but is not presently available.

In conclusion, our results indicate that CRCs are good candidates for CPT-11 chemotherapy whatever their MSI phenotype as long as they have a wild-type p53, whereas some, but probably not all mutations of p53, have the propensity to overcome CPT-11 sensitivity.

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