No topoisomerase I alteration in a neuroblastoma model with in vivo acquired resistance to irinotecan

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CPT-11 (irinotecan) is a DNA-topoisomerase I inhibitor with preclinical activity against neuroblastoma (NB) xenografts. The aim was to establish in vivo an NB xenograft resistant to CPT-11 in order to study the resistance mechanisms acquired in a therapeutic setting. IGR-NB8 is an immature NB xenograft with MYCN amplification and 1p deletion, which is sensitive to CPT-11. Athymic mice bearing advanced-stage subcutaneous tumours were treated with CPT-11 (27 mg kg⁻¹ day⁻¹ × 5) every 21 days (1 cycle) for a maximum of four cycles. After tumour regrowth, a new in vivo passage was performed and the CPT-11 treatment was repeated. After the third passage, a resistant xenograft was obtained (IGRNB8-R). The tumour growth delay (TGD) was reduced from 115 at passage 1 to 40 at passage 4 and no complete or partial regression was observed. After further exposure to the drug, up to 28 passages, the resistant xenograft was definitively established with a TGD from 17 at passage 28. Resistant tumours reverted to sensitive tumours after 15 passages without treatment. IGR-NB8-R remained sensitive to cyclophosphamide and cisplatin and cross-resistance was observed with the topoisomerase I inhibitor topotecan. No quantitative or qualitative topoisomerase I modifications were observed. The level of expression of multidrug resistance 1 (MDR1), MDR-associated protein 1 (MRP1) and breast cancer resistance protein, three members of the ATP-binding cassette transporter family was not modified over passages. Our results suggest a novel resistance mechanism, probably not involving the mechanisms usually observed in vitro.

Keywords: neuroblastoma; CPT-11; resistance; xenograft

Irinotecan (CPT-11), a semisynthetic water-soluble analogue of camptothecin, belongs to a new family of anticancer drugs, the DNA-topoisomerase I inhibitors. Topoisomerase I is an essential nuclear enzyme that relaxes DNA torsional tension during fundamental processes such as replication, transcription, recombination and repair, and represent a target for many anticancer drugs. Irinotecan settles on the DNA-topoisomerase I complex (cleavable complex), stabilises it and inhibits the religation of DNA. Cytotoxicity arises during the replication process when the advancing replication fork and cleavable complex collide, leading to irreversible DNA damage and to the initiation of a series of events that result in cell death (Chen and Liu, 1994; Pommier, 1996).

Neuroblastoma (NB) is one of the most common solid tumours in young children and is responsible for approximately 7.5% of all cancer among children younger than 15 years of age (Ries et al., 1999). It is a neural crest-derived embryonal cancer of the sympathetic nervous system. Biological parameters such as MYCN gene amplification (Brodeur et al., 1984), loss of heterozygosity of chromosome 1p (Fong et al., 1989), (Hayashi et al., 1989) diploidy (Look et al., 1991) and MDR1 gene overexpression (Bourhis et al., 1989) have been identified as strong predictors of a poor outcome.

CPT-11 has demonstrated antitumour activity against a wide spectrum of both adult and paediatric xenografts in preclinical studies (Komuro et al., 1994; Vassal et al., 1996; Thompson et al., 1997a). We have previously shown that i.v. treatment with CPT-11 resulted in extensive tumour regression and growth delay in three different NB xenograft models (Vassal et al., 1996). Similarly, Thompson et al. (1997b) showed that oral CPT-11 was active against a panel of six NB xenografts. Similar effects have been reported for the topoisomerase I inhibitor topotecan, which induced tumour regression and a significant tumour growth delay (TGD) in animals bearing NB xenografts (Vassal et al., 1997). CPT-11 is currently evaluated in children with NB using several doses and schedules, such as 600 mg m⁻² every 3 weeks, 50 mg m⁻² day⁻¹ × 5 every 3 week or 20 mg m⁻² day⁻¹ × 5 weekly for 2 weeks in a row every 3 weeks (Furman et al., 1999; Blaney et al., 2001; Vassal et al., 2003a).

The emergence of drug resistance in cancer is a major hurdle to successful chemotherapy. Drug resistance has been described in a
number of cell lines selected for their resistance to topoisomerase I inhibitors. These studies have shown that quantitative and qualitative changes in topoisomerase I can induce a resistance to topoisomerase I poisons (Li et al, 1996; Rubin et al, 1996). To date, resistance to CPT-11 has been studied mainly in vitro, a situation in which most of the variables are controlled. As this controlled situation cannot be equated with that likely to occur in a therapeutic setting, we established in vivo an NB xenograft model resistant to CPT-11, in order to study the mechanisms involved in acquired resistance in this context. It is believed that concrete results thus evidenced can be better translated into clinical applications.

MATERIAL AND METHODS

Drugs

CPT-11 was provided by Aventis Pharma SA (Vitry-sur-Seine, France). Cyclophosphamide was purchased from Asta-Medica (Mérignac, France), cisplatin from Bellon and etoposide from Novartis (Rueil-Malmaison, France). Drugs were dissolved in a 0.9% sodium chloride solution immediately before injection on each day of treatment.

Animals

Female specific pathogen-free Swiss athymic mice (6–8 weeks old) were bred in the Animal Experimentation Unit of the Institut Gustave-Roussy. Animals were housed in sterile isolators and fed with irradiated nutriments (UAR, Villemoisson/Orge, France) and filtered water. Experiments were carried out under the conditions established by the European Community directive no. 86/609/CEE and in accordance with the UKCCCR guidelines (Workman et al, 1998).

NB xenograft

IGR-NB8 xenograft model was derived from a newly diagnosed stage 3 abdominal NB in a 5-year-old boy, by direct subcutaneous transplantation of small tumour fragments into previously irradiated athymic mice (Vassal et al, 1996). The primary tumour of this patient was refractory to conventional chemotherapy that included platinum compounds, cyclophosphamide, doxorubicin, etoposide and vincristine. IGR-NB8 exhibited the classic microscopic appearance of an immature NB. In addition, this model elicited a high tumorigenicity (99%) and a mean tumour doubling time (DT) in vivo of 3.3 days. This xenograft displayed the biological features of poor-prognosis NB in children: MYCN amplification, near-diploid karyotype and chromosome 1p deletion. In addition, the karyotype showed pericentric inversion of chromosome 2 and additional material on the long arm of chromosome 6. The MDR1 gene was overexpressed. IGR-NB8 proved to be sensitive in vivo to CPT-11, topotecan, cyclophosphamide and cisplatin, but refractory to etoposide (VP16) (Vassal et al, 1996, 1997).

Tumour transplantation

For each experiment, 15–30 mm³ tumour fragments were xenotransplanted subcutaneously (unilaterally) into 50 athymic mice. On day 0 of the treatment, mice bearing a 100–300 mm³ subcutaneous tumour were randomly assigned to one treated and one control group of five to 10 mice each. Tumour perpendicular diameters were measured twice a week with a caliper, and tumour volume calculated according to the following equation: 

\[
V (\text{mm}^3) = (d^2 (\text{mm}) \times D (\text{mm}))/2,
\]

where \(d\) and \(D\) are the smallest and largest perpendicular tumour diameters, respectively. Each group of mice was treated according to the average weight of the group. Animal body weights were recorded twice weekly and mortality was checked daily. The experiments lasted until tumour volumes reached 1500–2000 mm³.

Treatment

CPT-11 was administered i.v. in a caudal vein at a dose of 27 mg kg⁻¹ day⁻¹ for 5 consecutive days. This dose was previously shown to induce 100% complete regressions (CR) and to be well tolerated (no treatment-related death and no body weight loss) (Vassal et al, 1996). This treatment was repeated every 21 days (one cycle) for a maximum of four consecutive cycles (one passage). During the establishment of in vivo resistance, the treatment was stopped either after the fourth cycle or when 50% of the tumours had reached a volume that was five-fold the initial volume. After tumour regrowth following discontinuation of treatment, tumour fragments were xenotransplanted subcutaneously into a new set of 50 athymic mice and the treatment was started according to the same methodology.

Four anticancer compounds were studied to evaluate cross-resistance phenotypes. Topotecan was administered i.p. daily × 5 at a dose of 3.2 mg kg⁻¹ day⁻¹. Etoposide was administered i.v. daily × 5 at a dose of 20 mg kg⁻¹ day⁻¹. Cyclophosphamide was administered as a single i.p. injection at a dose of 400 mg kg⁻¹. Cisplatin was administered i.v. on days 0 and 4 at a dose of 10 mg kg⁻¹ day⁻¹. Topotecan, cisplatin and etoposide were given at the highest nontoxic dose and cyclophosphamide was given at 90% of the historical LD10 dose, as previously evaluated in athymic mice (Vassal et al, 1996, 1997).

Evaluation of antitumour activity

The activity of each drug was evaluated according to three criteria: (1) the number of complete (CR) and partial (PR) tumour regressions; (2) the TGD; (3) the number of tumour-free survivors (TFS) (Bissery and Chabot, 1991). CR was defined as a tumour regression beyond the palpable limit (15 mm³) and PR as a tumour regression exceeding 50% of the initial tumour volume. At least two consecutive tumour measurements had to be observed in order to retain CR and PR. TGD was defined as the difference between the treated group and the control group, within the median time to reach a tumour volume that was five-fold the initial tumour volume (i.e. time to 5). For each tumour of the treated groups, the individual TGD was defined as the difference between the individual time to 5 and the median time to 5 of the control group. Since the duration of treatment was different from one passage to another, we also considered the TGD, corrected for the duration of treatment (TGDc), which was defined as the difference between TGD and the duration of treatment. TFS were defined as animals that were free of palpable tumour at the end of the experiment (at least 120 days).

Histological analysis

Xenograft tissue specimens were fixed in acetic acid–formalin–ethanol (Carlo-Erba, Milano, Italy) and embedded in paraffin. The paraffin-embedded sections were stained with haematoxylin–eosin–safranin for morphology.

MYCN amplification

MYCN copy number was measured using the TaqMan 5′ nuclease fluorogenic real-time quantitative PCR assay, as reported previously (Valent et al, 2001).
Comparative genomic hybridisation (CGH)

CGH was used to evaluate and characterise the genetic anomalies acquired during the establishment of the resistant phenotype. Genomic DNA from sensitive and resistant tumours at passages 1 and 24 was purified using the DNeasy tissue kit (Qiagen). Hybridisation of differentially labelled tumour and normal DNA to normal metaphase chromosomes was performed using previously published methods (Kallioniemi et al., 1992). Digital image analysis was used so that chromosomal regions with abnormal fluorescence ratios could be easily identified. The mean values of individual ratio profiles were calculated from at least 10 metaphases for each tumour specimen. CGH profile shifts were rated as gains and losses if they at least reached the 1.20 and 0.8 thresholds, respectively.

Protein expression analysis

Crude extracts were prepared from NB xenografts. Frozen tissues (50 mg) were grossly minced, suspended in lysis buffer (containing 0.15 NaCl, 1 mM KH₂PO₄, 5 mM MgCl₂, 1 mM EDTA pH 6.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM benzamidine, 1 μg ml⁻¹ aprotinin, 10 μg ml⁻¹ soybean trypsin inhibitor) and homogenised with a potter teflon-glass homogeniser. NaCl (0.55 mM) was added and incubated for 1 h on ice for total cell extraction. After centrifugation at 12 000 r.p.m. for 30 min, the supernatant was assayed for topoisomerase I activity. Protein concentration was determined by the BCA method (Pierce). Proteins (50 μg) were separated electrophoretically in 7.5% SDS-polyacrylamide gels and then transferred to a nitrocellulose membrane (Hybond P Amersham Life Science Les Ulis, France). Blots were incubated with human polyclonal topoisomerase I antibody (Topogen INC) diluted at 1:8300 followed by the anti-protein A horseradish-peroxidase-conjugated antibody (Amersham Pharmacia Biotech). Detection was performed using a chemiluminescence (ECL) enzyme immunoassay (Amersham Pharmacia Biotech).

Determination of topoisomerase I catalytic activity

Topoisomerase I catalytic activity of crude extracts was examined by a DNA relaxation assay using supercoiled pHOT1 plasmid DNA as the substrate (Topogen Inc., Columbus, OH, USA). For each sample, 10 extracts were serially diluted in buffer containing 10 mM Tris- HCI, 100 mM KCl, 1 mM PMSF, and 50 μg ml⁻¹ BSA, pH 7.5. Supercritical DNA (0.5 μg) was incubated with each diluted extract at 37 °C for 30 min in 10 μl Topoisomerase I assay buffer (Topogen Inc.). DNA topoisomers were separated by gel electrophoresis in 0.5% agarose. The fluorescence ratio of topoisomerase I activity was defined as the amount of DNA topoisomers that exhibited complete and partial regression, at first cycle.

Results

Acquisition of in vivo resistance to CPT-11

The influence of CPT-11 treatment on the growth of the IGR-NB8 xenografted tumour over 28 passages is shown in Figure 1. The tumour volume of all untreated controls increased rapidly. During the first three passages, the same total dose of CPT-11 (540 mg kg⁻¹) was administered over the same period of time (68 days) and tumour growth displayed the same pattern. Complete regression and PR were observed after the first cycle of treatment. Tumour volumes remained stable during the following three cycles. When treatment was discontinued, tumours remained stable for about 1 month and then started to grow again. Over the first three passages, IGR-NB8 tumour response to CPT-11 was significantly reduced with a TGD from 115 to 69, that is, a TGD corrected for the duration of treatment from 47 days at the first passage to 1 day at the third passage (Table 1). In contrast, neither complete or partial tumour regression nor any tumour stabilisation were observed beyond the third passage (Table 1 and Figure 1). At each passage, CPT-11 treatment was stopped as soon as at least 50% of tumours had reached a volume that was five-fold the initial

| Table 1 | Antitumour activity of i.v. CPT-I1 at dose of 27 mg kg⁻¹ day⁻¹ against IGR-NB8 xenografts |
|---------|---------------------------------------------|
| Passages | Number of cycles | Total dose (mg kg⁻¹) | Treatment duration (days) | DT (days) | CR* | PR* | TGD (days) | TGDc (days) | TFS |
| P1 | × 4 | 540 | 68 | 7.1 | 3/7 | 4/7 | 115 | 47 | 0 |
| P2 | × 4 | 540 | 68 | 8.5 | 3/14 | 8/14 | 97 | 29 | 1 |
| P3 | × 4 | 540 | 68 | 8.3 | 2/14 | 7/14 | 69 | 1 | 1 |
| P6 | × 3 | 405 | 47 | 3 | 0/13 | 0/13 | 54 | 7 | 0 |
| P9 | × 3 | 405 | 47 | 3.7 | 0/8 | 0/8 | 47 | 0 | 0 |
| P12 | × 2 | 270 | 26 | 4.6 | 0/7 | 0/7 | 29 | 3 | 0 |
| P15 | × 2 | 270 | 26 | 7 | 0/10 | 3/10 | 27 | 1 | 0 |
| P20 | × 2 | 270 | 26 | 2.4 | 0/8 | 0/8 | 28 | 2 | 0 |
| P28 | × 2 | 270 | 26 | 6.1 | 0/8 | 0/8 | 17 | 0 | 0 |

DT = doubling time; TGD = tumour growth delay; TGDc = tumour growth delay corrected for treatment duration; TFS = tumour-free survivors at 120 days. *CR, PR = complete and partial regression, at first cycle.
tumour volume. The number of treatment cycles was gradually reduced from 4 to 2, the total dose from 540 to 270 mg kg$^{-1}$ and the duration of treatment from 68 to 26 days from passages 3 to 28. Overall, the TGD was significantly reduced from 115 to 17 days after 28 in vivo passages and 68 cycles of treatment. Tumour DT gradually decreased during these 68 cycles of treatment.

We had established a NB xenograft that was resistant to CPT-11 (IGR-NB8-R).

Reverted resistance
In order to evaluate the stability of the resistance acquired, IGR-NB8-R tumours at passage 8 were further grown without treatment, while the prolonged treatment process was continued in parallel up to 28 passages. Sensitivity to CPT-11 was checked regularly (every 3 – 4 passages) by evaluating the TGD after one cycle (27 mg kg$^{-1}$ day$^{-1}$). As shown in Figure 2, the resistance acquired in vivo during the first eight passages was completely reverted after 15 growth passages of IGR-NB8-R without any further exposure to CPT-11. Thus, in vivo acquired resistance to CPT-11 was revertible.

Cross-resistance
In order to evaluate cross-resistance to other anticancer drugs in vivo, IGR-NB8-R xenografts (between passages 8 and 11) were grown in athymic mice. The sensitivity of the parental tumour (IGR-NB8) and the resistant tumour (IGR-NB8-R) to other anticancer drugs is shown in Figure 3. IGR-NB8-R and IGR-NB8 displayed similar tumour response to cyclophosphamide, an alkylating agent, (TGD, 24 days) and to cisplatin (TGD, 16 days). Conversely, IGR-NB8-R and IGR-NB8 failed to respond to etoposide, a DNA-topoisomerase II inhibitor. However, IGR-NB8-R was significantly less sensitive to the DNA-topoisomerase I inhibitor topotecan (TGD, 13 days) than the parental IGR-NB8 (TGD, 26 days at 2.7 mg kg$^{-1}$) (Vassal et al, 1997). Thus, IGR-NB8-R exhibited cross-resistance to topoisomerase I inhibitors, but not to DNA-damaging agents.

Characterisation of IGR-NB8-R
Fresh tumour tissues were collected from the parental xenograft and from the resistant xenograft at passages 17 – 20. The parental xenografts displayed the histological features of a poorly differentiated NB composed of undifferentiated neuroblastic cells (small uniform rounded cells) containing a high number of mitotic figures per high-power field and very little schwannian stroma (Figure 4A). During the first two passages, the stabilisation state was associated with tumour differentiation exhibiting features of a maturing ganglioneuroma (Santos et al, 2004) However, the IGR-NB8-R-resistant xenografts showed similar histological features to those observed in the parental xenografts (Figure 4A), namely those of a poorly differentiated NB (Figure 4B). These results indicate that there was no modification of histological features during the acquisition of resistance.
MYCN amplification

Neuroblastomas that overexpress MYCN due to amplification of the MYCN oncogene are aggressive tumours that become resistant to chemotherapy. High expression of MRP1 RNA has been reported to be associated with MYCN amplification and poor treatment outcomes (Norris et al., 1996). Analysis of the MYCN oncogene in IGR-NB8-R xenograft showed that in spite of very heterogeneous expression in a given passage, there was no major difference, between MYCN amplification at P1 (sensitive tumour), P9 (resistant tumour) and P8P16 (reverted tumour) exhibiting a median value of 26.5, 25, and 20 copies per haploid genome, respectively (Figure 5). However at passage 24 (resistant tumour), MYCN amplification was double that of the other passages studied. The modifica-

tions of topoisomerase I catalytic activity was not significant over these passages, while TGD decreased from 115 to 32 days. Moreover Western blot analysis of topoisomerase I showed no difference between the tumours studied (Figure 7). The human polyclonal anti-topoisomerase I antibody revealed two main bands located at 100 kDa, corresponding to a full-length enzyme and at 54 kDa, a form specific to human tissues (Santos et al., 2004) and perhaps the heavy chain of the immunoglobulin molecule (Bronstein et al., 1996). Thus, topoisomerase expression and activity was not implicated in the in vivo resistance acquisition.

Analysis of topoisomerase I

Topoisomerase I catalytic activity was quantified in four to five tumours at passages 1, 5, 11 and 13. (Figure 6). The average topoisomerase I activity was 7469 ± 2351, 7036 ± 4952, 7410 ± 1405 and 5334 ± 655 a.u. mg⁻¹ (mean ± s.d.), respectively. The modifications of topoisomerase I catalytic activity was not significant over these passages, while TGD decreased from 115 to 32 days. Moreover Western blot analysis of topoisomerase I showed no difference between the tumours studied (Figure 7). The human polyclonal anti-topoisomerase I antibody revealed two main bands located at 100 kDa, corresponding to a full-length enzyme and at 54 kDa, a form specific to human tissues (Santos et al., 2004) and perhaps the heavy chain of the immunoglobulin molecule (Bronstein et al., 1996). Thus, topoisomerase expression and activity was not implicated in the in vivo resistance acquisition.

ABC transporter superfamily analysis

MDR1 and MRP1 expression was quantified by RT–PCR before treatment (P1) and at passages 6 (P6) and 11 (P11). Three tumours were studied at each passage. MDR1-mRNA expression was detected in all tumour samples. As compared to GAPDH-mRNA expression, the MDR1-mRNA RER was 1.17 ± 0.46, 1.38 ± 0.43 and 1.23 ± 0.26 at P1, P6 and P11, respectively. These levels of relative expression were comparable with the positive control MCF7.
CPT-11 resistance were very positive in all tested samples (data not shown). Thus, et al P3, P7, P12, P18, P21 and P8P15). The positive control (T8 cells) undetectable in all the tumours analysed at various passages (P1, ern blotting confirmed these results. BCRP/MXR/ABCP sensitive, resistant and chemosensitivity-restored tumours. North- analysis of BCRP revealed relatively high expression in the positive significantly reduced from 115 to 25 days. Immunohistochemical did not increase from P1 to P11, while the TGD was being in IGR-NB8. However, expression of both MDR1 and MRP1 did not increase from P1 to P11, while the TGD was being significantly reduced from 115 to 25 days. Immunohistochemical analysis of BCRP revealed relatively high expression in the positive control (human placenta), and the absence of BCRP expression in sensitive, resistant and chemosensitivity-restored tumours. Nern blotting confirmed these results. BCRP/MXR/ABCP mRNA was undetectable in all the tumours analysed at various passages (P1, P3, P7, P12, P18, P21 and P8P15). The positive control (T8 cells) (Maliepaard et al, 1999) overexpressing BCRP mRNA and GAPDH were very positive in all tested samples (data not shown). Thus, CPT-11 resistance in vivo in IGR-NB8-R xenograft is not related to MDR1, MRP1 and BCRP expression.

**DISCUSSION**

Irinotecan has shown activity against colorectal, oesophageal, gastric, non-small-cell and small-cell lung cancer, leukaemia and lymphomas, as well as central nervous system malignant gliomas (Rothenberg, 2001). One of the major hurdles to clinical development of active agents is intrinsic or acquired chemoresistance. To date, the mechanisms that confer clinical resistance to camptothecin have not been characterised. In the field of oncology, knowledge of drug resistance mechanisms is based largely on *in vitro* studies. Several cell lines selected for resistance to irinotecan have been described and these studies have shed light on mechanisms such as topoisomerase I alteration or increased efflux of irinotecan from the cell (Xu and Villalona-Calero, 2002). However, *in vitro* analyses of resistance mechanisms remain limited. The majority of the variables are controlled and cellular interactions are not taken into account. Moreover, *in vivo* and *in vitro* phenotype of cells in culture do not always coincide. Thompson et al derived a series of alkylating agent-resistant variants (cis-diaminedichloroplatinum, cyclophosphamide, carboplatin and thiota) of the EMT-6 mouse mammary tumour through *in vivo* drug administration to syngeneic BALB/c tumour-bearing mice. The resistance observed was reversible after discontinuation of treatment, as demonstrated in our model. In spite of high levels of resistance *in vivo*, no significant resistance was observed when the cells from these tumours were exposed to the drugs *in vitro*, indicating that a very high level of resistance to anticancer drugs can develop through mechanisms that are exclusively *in vivo* (Teicher et al, 1996). Furthermore, Kobayashi et al (1993) have suggested that some forms of acquired drug resistance operate only at the multicellular level, as opposed to classic unicellular resistance mechanism.

Thus, our first objective was to establish a subcutaneous NB xenograft model that is resistant to CPT-11. After a series of 68 cycles of CPT-11 treatment and 28 passages *in vivo*, IGR-NB8 showed the characteristics of a CPT-11-resistant tumour. First, no CR or PR was observed after the third passage, whereas the treatment induced 100% of CR and PR at the first passage. Second, during passing, a very significant decrease (from 115 to 17 days) was observed in the TGD. Third, the number of consecutive cycles that were required for 50% of tumours to attain five-fold their initial volume was reduced from four to two cycles. The CPT-11-resistant IGR-NB8 NB model was designated IGR-NB8-R.

To our knowledge, only one model of *in vivo* acquired resistance to CPT-11 has been reported, which is also an *in vivo* NB (NB-1691) NB model established by Thompson et al. After 69 cycles of treatment/transplantation (5 mg kg−1 administration −1), authors observed a resistance to irinotecan and a partial resistance to topotecan. The mechanisms of resistance were not discussed in this paper (Thompson et al, 2002, p 541). Our *in vivo* model of acquired resistance evaluated in a therapeutic setting will allow us to confirm and clarify resistance mechanisms observed *in vitro* or to discover new resistance mechanisms by taking into account cellular interactions and thus rendering this approach more realistic from a clinical point of view.

First, IGR-NB8-R was characterised by the absence of cross-resistance to DNA-damaging agents and the presence of cross-resistance to another topoisomerase I inhibitor, topotecan. Both CPT-11 and topotecan are topoisomerase I inhibitors, which suggests a common resistance mechanism. Altering the topoisomerase I, the common target of these two drugs, can modify treatment efficacy. For example, some camptothecin-resistant cell lines have a mutation in topoisomerase I and this mutation may suggest a common resistance mechanism. Altering the topoisomerase I, the common target of these two drugs, can modify treatment efficacy. For example, some camptothecin-resistant cell lines have a mutation in topoisomerase I and this mutation may affect, the catalytic activity of the enzyme (Fujimori et al, 1995), or alter interactions with camptothecin or DNA cleavage (Li et al, 1996). In our xenograft model, however, qualitative or quantitative modifications of topoisomerase I cannot be responsible for the IGR-NB8-R resistance to CPT-11 and topotecan because no modification of topoisomerase I activity was demonstrated during the analysis with the DNA relaxation assay and the Western blot analysis showed no difference in topoisomerase I expression during the acquisition of resistance. Furthermore, the acquired
resistance of IGR-NB8-R was not permanent, since the tumour recovered its initial sensitivity after 15 passages without treatment. This suggests a resistance mechanism that is induced and maintained by CPT-11 and not a phenomenon such as mutation, which is not readily reversible.

We then evaluated the multidrug resistance phenomenon attributed to a change in drug efflux that could explain the cross-resistance of IGR-NB8-R to topotecan and CPT-11. Transmembrane proteins, belonging to the ABC superfamily, participate in energy-dependent drug efflux and confer multidrug resistance. Some transporters in this family, such as MRP and MDR1 that are involved in the active efflux of SN-38 and CPT-11, contribute to resistance to CPT-11 (Chu et al, 1999). Furthermore, in NB, it has been reported that high MRP RNA expression was associated with MYCN amplification and poor treatment outcomes (Norris et al, 1996). However, although the basal expression level of MDR1 and MRP1 was strong in sensitive tumours in our study, it did not increase during the acquisition of resistance. Maliepaard et al showed that BCRP, which also belongs to the ATP-binding cassette transporter family, appears to be a highly efficient transporter of topoisomerase I inhibitors in cell lines without overexpression of the multidrug resistance-associated pumps MDR1 and MRP1 (Maliepaard et al, 1999). Furthermore, van Hattum et al (2002) showed that DX-895, a derivative of camptothecin, is able to induce BCRP protein as a mechanism of resistance in the human ovarian cell line A2780. In our resistant model, which does not overexpress MDR1 or MRP1, we hypothesised that overexpression of BCRP could explain IGR-NB8-R resistance to irinotecan and topotecan without quantitative or qualitative modifications of topoisomerase I activity. This hypothesis prompted us to study BCRP expression; however, like MDR1 and MRP1, no overexpression of BCRP was demonstrated during the acquisition of resistance.

Among the various mechanisms of resistance to irinotecan characterised in vitro, we could eliminate those that were usually observed, such as intracellular drug accumulation or drug-target interaction. More recently, nuclear factor kappa B (NF-κB) activation was shown to play a role in sensitivity to this drug; treatment with camptothecin was reported to activate NF-κB (Wang et al, 1999; Cusack et al, 2000; Huang et al, 2000). Furthermore, the cytotoxic effect of SN-38 was strongly increased through the inactivation of NF-κB. The apoptotic response mediated by CPT-11 was dependent on NF-κB inhibition, establishing NF-κB as a principal mediator of inducible chemoresistance (Wang et al, 1999). We are currently exploring the potential role of NF-κB in our model of acquired resistance to CPT-11.

We have established a human NB xenograft resistant to CPT-11. This resistance mechanism seems to be novel. It does not imply any of the mechanisms of resistance usually observed in in vitro preclinical studies. Furthermore, it is a reversible mechanism that probably does not imply mutations. Our next objective is to investigate the basis of this mechanism of acquired drug resistance. Several genes are likely implicated; therefore, a genomic comparison of the transcriptome of sensitive and resistant tumours with macro- and microarray is in progress. This technology will allow us to rapidly identify differences in expression between genes. Clarifying the resistance mechanism in IGR-NB8-R will allow us to make a step forward in our understanding of acquired chemoresistance and to better target the clinical development of irinotecan.

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