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The novel lipophilic camptothecin analogue gimatecan is very active in vitro in human neuroblastoma: A comparative study with SN38 and topotecan

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Abstract

Neuroblastoma is one of the most common extracranial solid tumours in childhood with a poor prognosis in its advanced stage. Treatment failure is often associated to the occurrence of drug resistance. To date, treatment of paediatric neuroblastoma is still dismal, and therefore novel effective drugs are awaited. In recent years, an increasing interest has concentrated on camptothecin analogues. Topotecan and irinotecan, the only two clinically relevant camptothecin derivatives to date, have entered clinical trials in neuroblastoma but so far the results have been disappointing. Gimatecan (ST1481, LBQ707; 7-*t*-butoxyiminomethylcamptothecin), is a novel lipophilic camptothecin derivative that was selected from a series of lipophilic analogues rationally designed and synthesized in order to overcome some of the main drawbacks of conventional camptothecins, limiting their clinical efficacy. Gimatecan is endowed with potent antitumour activity, strong topoisomerase I inhibition, stable drug–target interactions and a better pharmacological profile. The present study deals with the comparative evaluation of cellular pharmacology features of gimatecan, topotecan and SN38 in neuroblastoma cell lines. We show that, despite the lowest intracellular accumulation, gimatecan was the most active among the camptothecin analogues studied. Our findings suggest that the high activity of gimatecan in neuroblastoma is related to the ability of this novel analogue to cause a very high number of DNA breaks as assessed by the Comet assay in both cellular or sub-cellular systems. We propose that DNA strand breaks efficiency as measured by the Comet assay might provide important information about the stability of the ternary complexes induced by camptothecin compounds.

Keywords: Gimatecan; Camptothecins; Topoisomerase I inhibitors; Neuroblastoma; Alkaline comet assay; DNA strand breaks

1. Introduction

Neuroblastoma is a paediatric tumour with a poor prognosis in its advanced stage. Although some progresses in therapy, neuroblastoma it is still responsible for about 15% of cancer-related deaths in children [1–4]. In recent years, different approaches to the treatment of advanced neuroblastoma have been attempted and new agents such as camptothecin analogues have shown some interesting results [5–8]. Camptothecins are semi-synthetic drugs derived from the alkaloid camptothecin that was first isolated from the Chinese tree Camptotheca acuminata. They represent an important class of anticancer drugs with a wide spectrum of activity in many solid tumours such as lymphoma, gastric cancer, small cell lung cancer, non small cell lung cancer, cervical cancer and colorectal cancer (see ref. [8] and references within). There is firm evidence that the molecular target of camptothecins is the nuclear enzyme topoisomerase I, that plays a key role in DNA replication,

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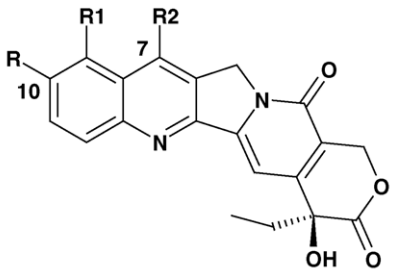
transcription and repair. Topoisomerase I acts by relaxing torsionally strained duplex DNA through the insertion of DNA single strand breaks. Camptothecins are able to form a labile cleavable complex drug–enzyme–DNA that inhibits the DNA relegation step. Because of the reversibility of these topoisomerase I–DNA complexes, toxicity occurs only when they are converted to irreversible DNA strand breaks [9]. The collision between the replication-fork and these complexes has been proposed to explain the camptothecin-driven S-phase specific cytotoxicity and the arrest in the G2-M phase of the cell cycle [10]. Furthermore, the blockage of the RNA polymerase elongation systems by topoisomerase I–DNA covalent complexes induces transcription arrest and triggers 26S proteasome-mediated degradation of both topoisomerase I and the large subunit of RNA polymerase II (RNA Pol II_0) [11–13]. This effect is considered a repair response to the cytotoxic action of camptothecins, as degradation of topoisomerase I results in the exposure of single strand breaks that can then be repaired through functional transcription-coupled repair (TCR) [13]. For their ability to transform topoisomerase I in a cytotoxin, camptothecins are often referred to as topoisomerase I poisons.

To date, two water-soluble derivatives of camptothecin, topotecan and irinotecan, represent the main clinically relevant drugs of this class for the treatment of many solid tumours. Topotecan is used as standard regimen treatment in ovarian and lung cancers [14,15], whereas irinotecan is used in first- and second-line treatments in advanced colorectal cancer [16,17]. Although topotecan has been rarely used in paediatric malignancies, there is a recent report of a phase II study in which the combination topotecan-cyclophosphamide administered i.v., showed significant activity in children with newly diagnosed neuroblastoma and this treatment was more efficacious than topotecan alone [18]. On the other hand, oral topotecan therapy showed antitumour activity only in a small percentage of patients with relapsed or refractory neuroblastoma where the drug was administered at a dose of 1 mg/(m^2 day) in two divided doses for 21 consecutive days. However, due the high toxicity reported, a dose adjustment was required in order to limit the side effects of the drug [7]. In fact, pre-clinical studies showed that, in several tumours, protracted schedules of daily administration of low-dose topotecan were more effective than more intense shorter schedules of administration [19]. Consistently, a recent phase I clinical trial using oral topotecan at a low dose (0.8 mg/(m^2 day)) in combination with oral cyclophosphamide for 10–17 days, showed reduced toxicity but only a partial response in one neuroblastoma patient [20]. So, the low-oral dose schedule of topotecan appears of use mainly as last-line therapy in pre-treated neuroblastoma patients. With irinotecan, phase I studies in paediatric tumours were conducted in Japan, USA and France [21–23] and Phase II studies are planned. Overall, irinotecan has shown some activity in neuroblastoma but a prolonged schedule and the i.v. administration route appeared necessary for better results [8].



Drug	R	R1	R2
Camptothecin	H	H	H
Gimatecan	H	H	$CH{=}NOC(CH_3)_3$
SN38	OH	H	CH_2-CH_3
Topotecan	OH	$CH_2N(CH_3)_2$	H

Scheme 1. Chemical structures of camptothecin analogues considered in this study.

The main drawbacks of camptothecin derivatives is the instability of the α-hydroxylactone ring (the active form of the drug) and the lability of the cleavable complex. In recent years, many efforts have been made in the medicinal chemistry field to overcome these limitations and to maintain antitumour potency. A series of modified lipophilic analogues was synthesized in order to stabilize drug-target interactions. Gimatecan (ST1481, LBQ707; 7-*t*-butoxyiminomethylcamptothecin; Scheme 1), is a novel analogue that was selected from this series as it is endowed with potent antitumour activity, strong topoisomerase I inhibition and a better pharmacological profile than other conventional camptothecins [24–26]. To date, there are already a few encouraging reports showing that gimatecan did not fail the initial expectations. Furthermore, there are evidences that unlike topotecan, gimatecan is able to overcome drug resistance mediated by the MDR phenotype [26] and BCRP [27]. The availability of the lipophilic derivative and orally active gimatecan showing both a better pharmacological profile and a lack of cross-resistance to topotecan and irinotecan has attracted our interest, and has prompted us to compare it to other conventional camptothecins in neuroblastoma. During our in vitro studies we selected topotecan and SN38, the active metabolite of irinotecan, as reference camptothecins.

2. Materials and methods

2.1. Drugs

Gimatecan and SN38 were kindly provided by Sigma Tau (Pomezia, Rome, Italy). The drugs were dissolved in

DMSO to make the stock solutions (10 mg/ml for gimatecan and 10 mM for SN38) that were stored in small aliquots at −20 °C protected from light. Topotecan (Hycamtin®, SmithKline Beecham S.p.A.) was dissolved in water to a final concentration of 10 mM. The day of the study, each drug was thawed and suspended in complete medium to the final dilutions required for treatment (DMSO in the final drug-suspension was less than 0.1%). The final concentrations of drugs were always lower than their solubility in complete medium.

2.2. Cell lines and drug sensitivity studies

A panel of five human neuroblastoma cell lines (SK-N-DZ, BE(2)M17, LAN-1, RNGA, SK-N-BE(2)c) were used for the cytotoxicity studies. RNGA and SK-N-BE(2)c cells were kindly provided by Prof. Susanna Scarpa (University "La Sapienza", Rome, Italy) and Dr. Raschellà (ENEA CR, Casaccia, Rome, Italy), respectively. All other neuroblastoma cell lines were from the ATCC. Cells were maintained as monolayers in RPMI-1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine under standard cell culture conditions (37 °C, 5% CO_2 in humidified incubator). Cell sensitivity to the drugs was assessed by either a growth inhibition assay (1 h treatment followed by 72 h) or a clonogenic survival assay (1 h treatment). In both assays, cells in the logarithmic growth were seeded 24 h before incubation with the drugs. After the required time, cells were washed once and left to grow in drug-free medium. The cytotoxicity was assessed as IC_{50}, that is the concentration of drug required to inhibit 50% of cellular growth/colony formation.

2.3. Cell uptake studies

After 1 and 6 h of treatment with 1 μM and with the IC_{50} concentrations of each drug, cells were collected (harvested, counted and the pellet frozen) for the gimatecan, SN38 and topotecan uptake analysis. The recovered pellets was resuspended in 50 μl of cold PBS, added with 10 μl of ST1966 (2.5 μg/ml) as internal standard and extracted by vortex mixing (1 min) with 240 μl of cold methanol: HCl 0.1 M (1:1). The drugs were quantified by using a recently developed HPLC method [28], slightly modified for the analysis of tissue and cells samples. The lower limit of quantification were 0.1 ng/10^6 cells for gimatecan and 0.5 ng/10^6 cells for topotecan and SN38.

2.4. Cell cycle studies

The SK-N-DZ cell line was selected for further studies (see Section 3). In the cell cycle analysis, about 1×10^6 cells were allowed to attach the day before, then were treated with cytotoxic concentrations of the drugs (IC_{30}, IC_{50} and IC_{70}) for 1 h. Then, the medium was removed and drug-free medium was added for increasing times (24, 48 and 72 h). Then adherent cells were harvested and pooled with the floating cells in the original medium, washed in PBS and stained with 2 ml of propidium iodide staining solution (20 μg/ml propidium iodide in 0.1% sodium citrate), 25 μl RNAse (1 mg/ml in water) and 25 μl Nonidet P40 (0.15% in water) for about 2 h in the dark at room temperature. Cell cycle analysis was performed with a FACScan flow cytometer equipped with an argon laser (Becton Dickinson, Mountain View, CA). Twenty thousand cells were acquired and analyzed for DNA content with ModFit Version 2 software (BD).

2.5. Assessment of DNA damage induction and repair

2.5.1. Cellular Comet

Exponentially growing cells were subcultured in 24-well plates at 2×10^4 cells/well and allowed to attach overnight. The following day, cells were treated with either equidose or equitoxic concentrations (IC_{50} multiple doses from the clonogenic assay) of gimatecan, SN38 and topotecan and left for the required time before samples processing. We observed that the highest damage was already detectable after 30 min following drug incubation, and therefore this time was used throughout the study. Drug-untreated and H_2O_2-treated (133 μM, 5 min on ice) samples were run as negative and positive controls for DNA damage, respectively. After drug treatment, cells were harvested, resuspended in ice-cold medium and then embedded in 1% low melting point agarose (Sigma) on agarose pre-coated microscope slides. Slides were carefully submersed in an alkaline lysing buffer (100 mM EDTA, 10 mM Tris–HCl, 1% Triton X100, 1% DMSO, 2.5 M NaCl; pH 10.5–11.5) for at least 1 h at room temperature in the dark. Following lysis and rinse, slides were transferred in the dark to an electrophoretic tank filled with alkali unwinding buffer (50 mM NaOH, 1 mM EDTA, pH 12.6–12.7) for 35 min and then electrophoresed at 21 V (0.7 V/cm) for 25 min. After electrophoresis completion, the slides were stained for 20 min in SYBR Gold (1:10000 solution in alkaline water, pH 8) (Bioproducts, Rockland, USA) For each sample, approximately 50 cells (25 per duplicate slide) were acquired using a Zeiss fluorescence microscope connected to a Ultrak CCD black and white camera. The statistical calculations of the %Tail DNA were carried out using the Kinetic Komet 5.0 software from Kinetic Imaging Ltd. (Liverpool, UK).

2.5.2. Sub-cellular Comet

The sub-cellular Comet assay was performed as described by Kasamatsu et al. [29]. Briefly, the untreated cells were processed as described for the Comet assay until the lysis step (1 h at 4 °C). Then the slides were placed twice in 0.4 M phosphate buffer (pH 7) for 5 min each time following treatment with the drugs at 1 μM dose at 37 °C in the same buffer for 30 min and 1 h. After drug treatment, the slides were washed twice in phosphate buffer 0.4 M for

5 min and left for 30 min at 4 °C in the high pH electrophoresis buffer used in the Comet study and then electrophoresed at 21 V (0.7 V/cm) for 25 min. After electrophoresis completion, the slides were processed as described in the Comet study.

2.6. ApoBrdU—Tunel assay

The induction of apoptosis following incubation of gimatecan, SN38 and topotecan on SN-K-DZ cells was evaluated with the Tunel (Tdt-mediated dUTP Nick-end labelling) assay according to the described protocol (APO-BRDU kit, Sigma). Briefly, about 0.6–0.8 × 10^6 exponentially growing cells were seeded the day before, then were treated with the compounds for 1 h at 1–10 μM doses or at the IC_{70} concentrations. After drug wash-out, cells were left for additional 72 h in drug-free medium and then harvested. Following two washes in 1 ml of PBS, cells were fixed in 1% paraformaldehyde in PBS and placed on ice for 15 min and then fixed with 5 ml of 70% (v/v) ethanol on ice for 30 min. Following washing in cold wash buffer (APO-BRDU kit, Sigma), cells were incubated for 1 h at 37 °C in the freshly prepared DNA labelling solution (APO-BRDU kit, Sigma) containing Br-dUTP and TdT enzyme. At the end of the incubation, cells were rinsed twice with rinsing buffer (APO-BRDU kit, Sigma), then were incubated for 30 min at room temperature in the dark with 0.1 ml of the antibody solution containing anti-BrdU-FITC monoclonal antibody. Following the incubation with the antibody, a solution of propidium iodide/RNase was added to the cell suspension for 30 min at room temperature in the dark. Ten thousand cells were then analyzed by a FACScan flow cytometer equipped with an Argon laser (Becton Dickinson, Mountain View, CA) where the analysis was performed with Cell Quest software (BD).

2.7. Active caspase-3

The assay was performed accordingly to the manufacturer protocol (BD Pharmigen). Essentially, SK-N-DZ cells were allowed to attach overnight before incubation with equidose concentrations of the drugs (0.01–0.1–1 μM). After 4 and 24 h, cells were harvested and washed twice with PBS then fixed with Cytofix/Cytoperm™ solution for 20 min on ice. Cells were then pelletted and washed twice with Perm/Wash™ solution before incubation with PE-conjugated anti-active caspase 3 antibody. After 30 min at room temperature in the dark, cells were washed with Perm/Wash™ and then analyzed by flow cytometer (FACScan, BD).

3. Results

3.1. Drug sensitivity studies

Drug sensitivities of gimatecan, topotecan and SN38 were compared in a panel of neuroblastoma cell lines. In the clonogenic survival assay, gimatecan showed a marked cytotoxic potency compared to topotecan (up to 40-fold more potent) whereas it was about 1.4–4 times more cytotoxic than SN38 (Table 1). However, these differences are even stronger if one considers the data from the cellular accumulation studies in the SK-N-DZ cells showing that at equipotent doses, gimatecan had the lowest cellular uptake (see next paragraph). In the SK-N-DZ cells, drugs sensitivity after 6 h of incubation was also explored with the clonogenic assay (Fig. 1B). Under these conditions, all analogues showed an enhanced cytotoxicity than after 1 h of drug-treatment suggesting the presence of a cumulative toxicity. As shown in Fig. 1B, gimatecan and SN38 have an IC_{50} (6 h) of about 6 and 12 nM, respectively, whereas for topotecan the dose had to be increased up to about 75 nM to get 50% of colony inhibition. Therefore, the differences in cytotoxicity between gimatecan/SN38 and topotecan were less marked after 6 h than after 1 h of incubation, although at the two time points the ratios in the cellular accumulations (expressed as ng/10^6 cells) between the drugs at their IC_{50} doses were similar (gimatecan:topotecan:SN38 = 1:1.4:4.8). Thus, longer incubation times (up to 6 h) appeared to enhance the potency of topotecan over the other two analogues, with no apparent correlation to drug accumulation. Also in the growth inhibition assay after 1 h incubation, gimatecan was still the most potent between the derivatives (Fig. 2). Moreover, similar drugs sensitivities were observed across the different neuroblastoma cell lines. Gimatecan and SN38 appeared consistently more potent in SK-N-DZ cells but less cytotoxic in RNGA and SK-N-BE(2)c cells. In our panel of cell lines, SK-N-DZ cells were one of the most sensitive to the action of gimatecan showing also a good difference in cytotoxicity between the compounds tested. Thus, we chose this cell line for a deeper evaluation of these camptothecin analogues.

Table 1
Drug sensitivity was assessed by clonogenic survival following 1 h incubation with the drugs and 6–12 days in drug-free medium, depending on colony formation rate

	IC_{50} (nM)				
	SK-N-DZ	BE(2)M17	LAN-1	RNGA	SK-N-BE(2)c
Gimatecan	20.85 ± 0.15	15.7 ± 4.8	14.12 ± 1.85	56.0 ± 0.2	40.1 ± 4.5
SN38	34.5 ± 4.0	22.0 ± 0.9	58.5 ± 8.3	112.5 ± 4.5	83.8 ± 2.7
Topotecan	620 ± 125	293 ± 85	597 ± 43	1003 ± 73	695 ± 55

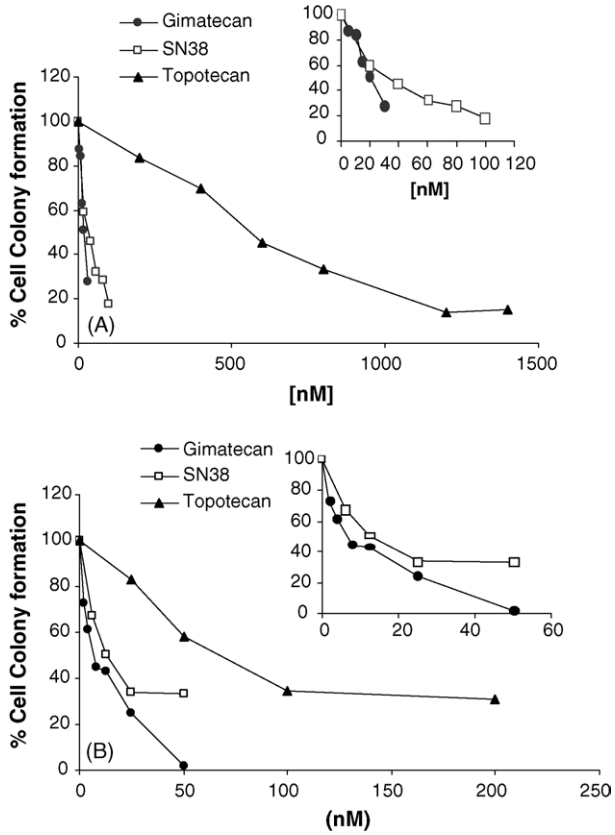


Fig. 1. Clonogenic survival after 1 h (Graph A) and 6 h (Graph B) of incubation with the drugs (gimatecan, SN38 and topotecan). The insets show more in detail the comparison between gimatecan and SN38.

3.2. Cell uptake studies in SK-N-DZ cells

In SK-N-DZ cells SN38 showed a superior cellular accumulation after 1 and 6 h at both IC_{50} (from the clonogenic assay) and 1 μM doses (Fig. 3). The superior accumulation of SN38 compared to gimatecan was statistically significant even after the treatment at the IC_{50} following both 1 and 6 h ($p < 0.01$, student's t-test). Following 1 h at 1 μM, SN38 detected in the cell pellet was about 13-fold and as high as 222-fold the amounts of gimatecan and topotecan, respectively. With time, we observed a reduction in the cellular uptake with all drugs at both doses, but at 1 μM SN38 showed a more persistent accumulation over the other analogues. However, it should be pointed out that 1 μM doses represent quite extreme experimental conditions as they correspond to doses up to 30- and 50-fold the IC_{50}s of SN38 and gimatecan, respectively. On the other hand, with topotecan, 1 μM concentration corresponds to just about two-fold its IC_{50} value. This likely explains why at the different doses used in our study, we did not observe any significant change in the cellular uptake of this analogue. Interestingly, raising the dose of topotecan up to 10 μM did not produce any significant increase in the intracellular accumulation of the drug (data not shown).

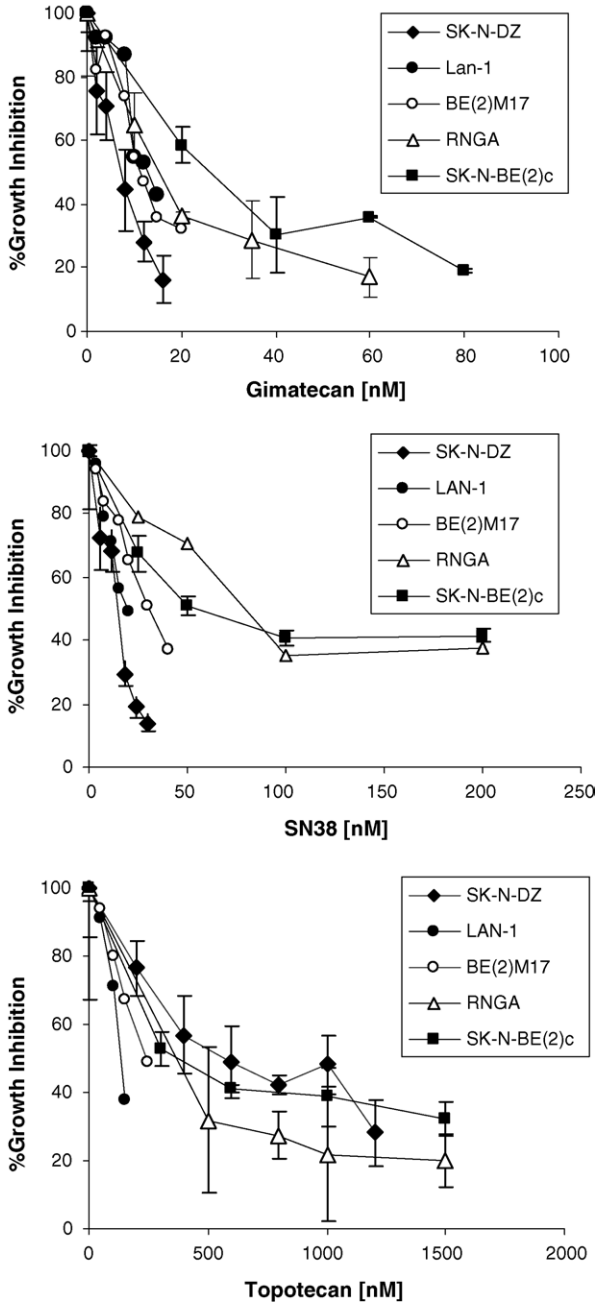


Fig. 2. Growth inhibition induced by the camptothecin derivatives (gimatecan, SN38 and topotecan) in the panel of neuroblastoma cell lines (short term assay, 1 h incubation following 72 h in drug-free medium).

3.3. Cell cycle analysis

Cell cycle perturbation was analyzed in SK-N-DZ cells at various times after 1 h treatment with equitoxic doses (IC_{30}, IC_{50} and IC_{70} from the clonogenic assay) of each compound. With all analogues we observed a dose-dependent block in the G2/M phase of the cell cycle after 24 h from drug wash-out. SN38 was the least efficient in producing this block (Fig. 4). With gimatecan the accumulation of cells in the G2/M phase was still observed at

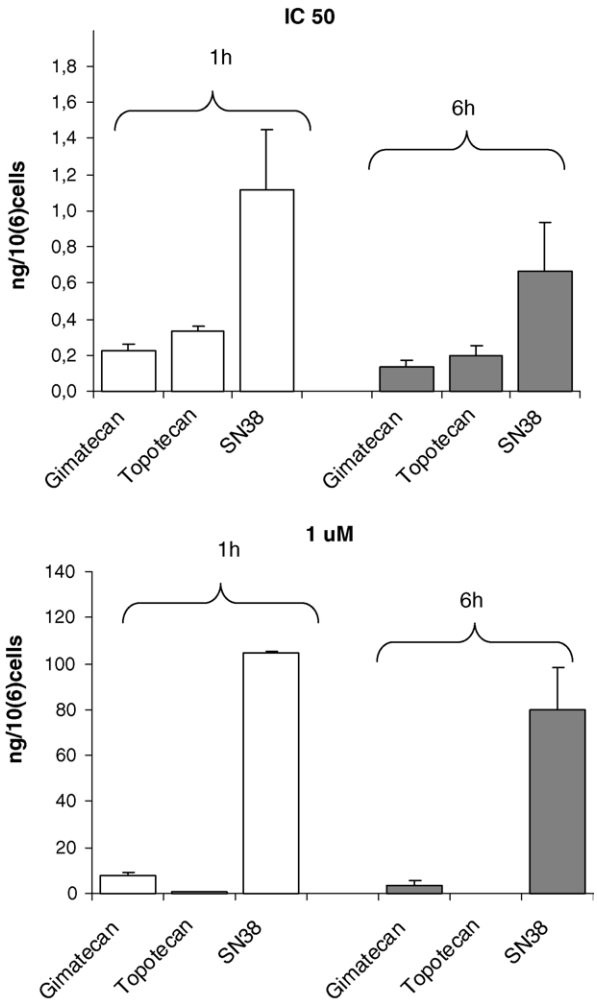


Fig. 3. Intracellular accumulation study. SK-N-DZ cells were treated with the drugs (gimatecan, SN38 and topotecan) at IC_{50} and 1 μM doses for 1 and 6 h. The IC_{50}s used in these experiments for 1 and 6 h were according to Fig. 1.The data are an average between at least three independent experiments. ±S.D. are indicated.

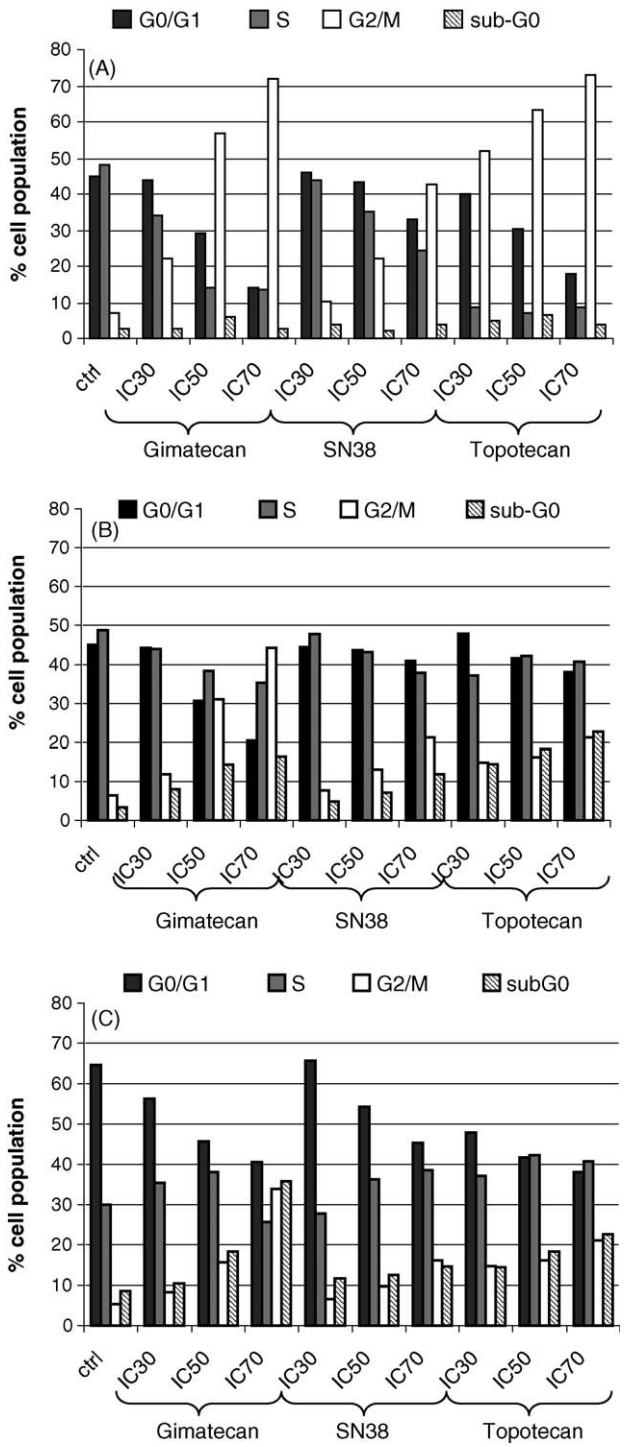


Fig. 4. Cell cycle analysis in SK-N-DZ cells at various times ((A) 24 h; (B) 48 h; (C) 72 h) after 1 h treatment with equitoxic concentrations of the drugs.

longer times (48 and 72 h). Furthermore, after 48 and 72 h all analogues produced a time-dependent growing hypoploid population (sub-G1 peak) that reached the highest value with gimatecan after 72 h from drug wash-out.

3.4. Cellular Comet studies

The alkaline Comet assay was used to monitor topoisomerase I-cleavable complexes in human SK-N-DZ neuroblastoma cell line. Additional experiments of DNA damage induction at equitoxic doses and of kinetic of repair of initial damage were also performed in the SK-N-BE(2)c cells. Tail% DNA was used to assess the damage. Extensive strand breaks on chromosomal DNA were detected following 2.5 h incubation with the drugs at equidose concentrations (0.01–1 μM) (Fig. 5A). With topotecan, the dose was increased up to 10 μM as the cells were less sensitive to this agent. With all compounds a dose-dependent DNA strand breaks production was observed. Gimatecan appeared the most efficient in inducing DNA damage also at the lowest doses. At 1 μM, the drug was about three-fold better than either SN38 or topotecan (Fig. 5A). In order to establish the relationship between the incubation time and the DNA damage detected, a kinetic of damage was explored at a fixed dose of gimatecan ($10 \times IC_{50}$) and at different incubation times

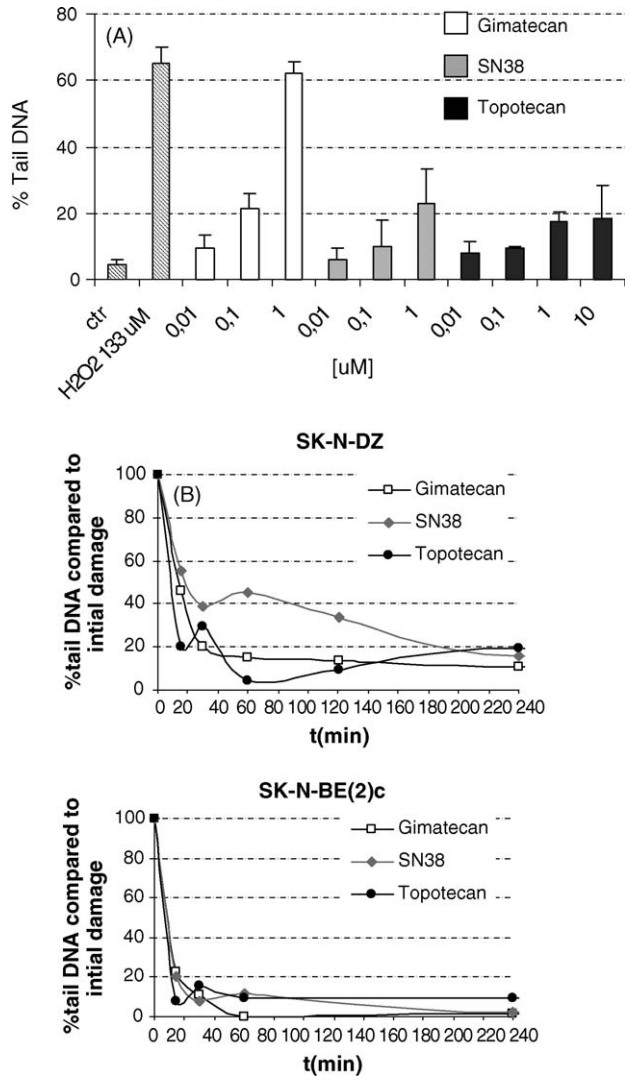


Fig. 5. Alkaline Comet assay. (A) DNA damage detected in SK-N-DZ cells treated with increasing concentrations of the drugs for 2.5 h (white, gimatecan; grey, SN38; black, topotecan). ±S.E. from two independent experiments are indicated. (B) Kinetic of DNA damage repair in SK-N-DZ and SK-N-BE(2)c cells treated with a fixed concentration of the drugs ($10 \times IC_{50}$) for 30 min and then left for increasing times in drug-free medium. The *Y*-axis shows the %Tail DNA of drug-treated samples compared to initial damage ($t = 0$). For each cell line a representative experiment is shown. ±S.E. below 5%.

ranging between 15 min up to 24 h. Interestingly the highest damage in both SK-N-DZ and SK-N-BE(2)c cells was observed after 30 min from drug addition (data not shown). We suggest that by this time an overlap of different effects might have taken place. Induction of topoisomerase I–cleavable complexes, reversal of the produced strand breaks and DNA repair mechanisms, might all contribute to the final DNA damage detected. In order to compare repair efficiency, we explored the kinetic of repair/reversal of the initial damage induced by a fixed concentration of the drugs ($10 \times IC_{50}$) and 30 min of incubation (time required to get the maximum of DNA damage) (Fig. 5B). In SK-N-DZ cells, we observed that with gimatecan and topotecan the rate of repair of the initial damage was quite fast, with about 60–70% of the initial damage being repaired within 15–20 min from drug washout. With SN38, the repair was slower, but for all compounds the DNA breaks were about 20% of the initial damage after about 4 h from drug removal. In SK-N-BE(2)c cells, repair was almost complete for all the analogues after the first 20 min following drug washout. The alkaline Comet assay was also used to assess whether there was a linear relationship between cytotoxic doses and topoisomerase I-mediated DNA cleavage. Fig. 6 shows that for all compounds in both SK-N-DZ and SK-N-BE(2)c cells, there was a dose-dependent production of chromosomal DNA strand breakage. The analogues' efficacy in inducing DNA damage at the same cytotoxic doses (multiples of IC_{50}) was the following: gimatecan $\gg$ topotecan $>$ SN38. This was an unexpected result as it shows a lack of correspondence between cytotoxicity and induction of topoisomerase I-cleavable complexes detectable as DNA strand breaks. The difference between SN38 and topotecan was less marked in the SK-N-BE(2)c cell line compared to SK-N-DZ cells.

3.5. Sub-cellular Comet studies

We have shown that the lipophilic agent gimatecan was more efficient than SN38 and topotecan at producing strand breakage on chromosomal DNA. Due to the differences in the drugs' cellular uptake between the camptothecins studied, the sub-cellular version of the Comet assay was exploited in order to detect DNA damage in a metabolism- and uptake-free model. Fig. 7 shows that gimatecan was still the stronger DNA damage inducer even in the sub-cellular assay. A similar behaviour was observed also in a rhabdomyosarcoma cell line (RD) where gimatecan appeared the most efficient DNA strand breaks inducer (data not shown), suggesting that this phenomenon might be common to other tumour cell lines.

3.6. ApoBrdU—Tunel assay

Data from the tunel assay showed that at the equitoxic doses (IC_{70}) gimatecan and SN38 produced similar percentages of tunel positive cells indicative of apoptosis (Fig. 8). Topotecan, which was between 20 and 30-fold less toxic than the other two analogues in the SK-N-DZ cell line, appeared slightly more pro-apoptotic at equipotent doses (IC_{70}). This was consistent with the data from the equidose studies where we had to use a dose only 10-fold the concentration of the other analogues in order to observe a similar amount of tunel positive cells.

3.7. Active caspase-3 studies

In order to ascertain whether the apoptotic process was caspase 3-dependent, we marked cells with an anti-active caspase 3 antibody. Fig. 9 shows that all camptothecin

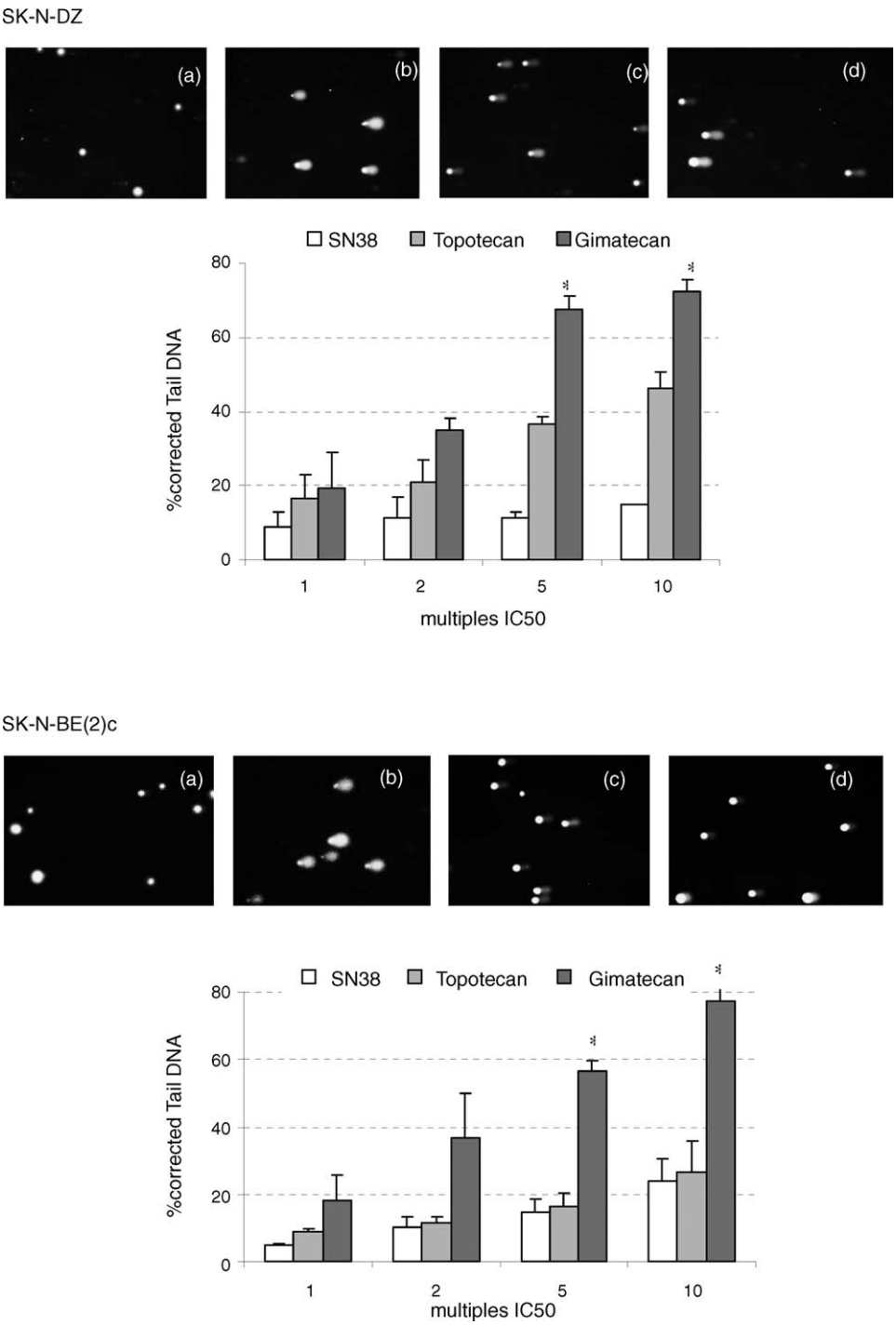


Fig. 6. Alkaline Comet assay in SK-N-DZ (top) and SK-N-BE(2)c cells (bottom) treated with gimatecan, SN38 and topotecan at equitoxic doses (multiples of IC_{50} from the clonogenic assay) for 30 min. Images: Comet pictures of untreated (a), gimatecan-treated (b), SN38-treated (c) and topotecan-treated cells (d) (30 min at 5xIC_{50}). Histogram: quantitative analysis (mean %Tail DNA) of the data from the study with equitoxic concentrations. The data of the drug-treated samples were corrected for the untreated control. $^{*}p < 0.05$ compared with SN38 (white bars) and topotecan (light grey bars). ±S.E. from two independent experiments.

analogues were able to induce a caspase 3 dependent apoptosis. Consistently with the tunel data, topotecan had to be used at a concentration only 10-fold higher (1 μM versus 0.1 μM) than that of the other analogues in order to induce about the same amount of active caspase-3 cells.

4. Discussion

In a panel of neuroblastoma cell lines gimatecan showed a superior antitumour potency over both SN38 and topotecan (Table 1; Fig. 2). In the SK-N-DZ cell line, longer incubation times (6 h) enhanced cell sensitivity to all drugs

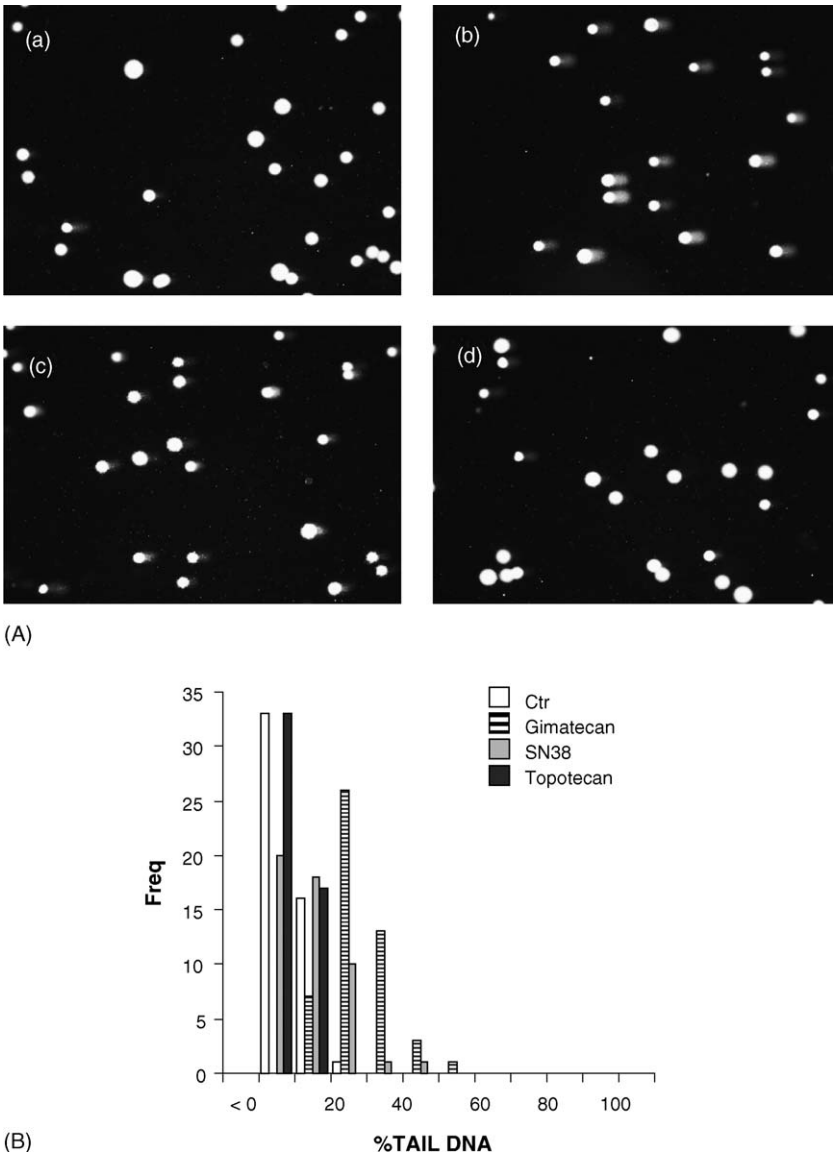


Fig. 7. Sub-cellular alkaline comet assay in SK-N-DZ cells. (A): Comet images of untreated (a), gimatecan-treated (b), SN38-treated (c) and topotecan-treated cells (d) (1 μM, 1 h). (B) Cells treated with the different agents at 1 μM for 1 h. 2D histograms showing the frequency of damaged cells vs. the amount of damage (measured as %Tail DNA) from a representative experiment.

but gimatecan was still the most potent (Fig. 1). The higher cytotoxicity of the novel derivative was consistent with cell cycle perturbations. At cytotoxic doses, a marked dose-dependent G2/M arrest was induced by all compounds in the SK-N-DZ cells after 24 h from drug removal (Fig. 4A). However, with gimatecan this block was still persistent after 48/72 h from drug wash-out (Fig. 4B and C). All compounds induced similar amounts of caspase-3 dependent apoptosis (Fig. 9), with the least cytotoxic agent topotecan appearing slightly more efficient at equitoxic doses (IC_{70}) and even when used at a 10-fold higher concentration compared to SN38 and gimatecan (see Section 3; Fig. 8). Taken all together, these initial findings suggest that the higher sensitivity of the neuroblastoma cell line SK-N-DZ to the novel lipophilic camptothecin analogue gimatecan is mediated by a more persistent G2/M block and that the cells die by apoptosis. On the other hand, the low sensitivity of our panel of neuroblastoma cell lines to topotecan might suggest the involvement of reduced drug accumulation as already reported in topotecan resistant cell lines [30]. Consistently, the data from the accumulation studies showed that at 1 μM concentrations, topotecan had the lowest intracellular content (Fig. 3). However, the most intriguing finding from this study was that at the IC_{50} doses, gimatecan showed the lowest intracellular amount following both 1 and 6 h incubations. These data are interesting as they indicate that, compared to the other two camptothecins, a much lower amount of intracellular gimatecan can produce a higher cytotoxic effect. Thus, gimatecan appears to be an appreciably more effective drug than either SN38 or topotecan in neuroblastoma.

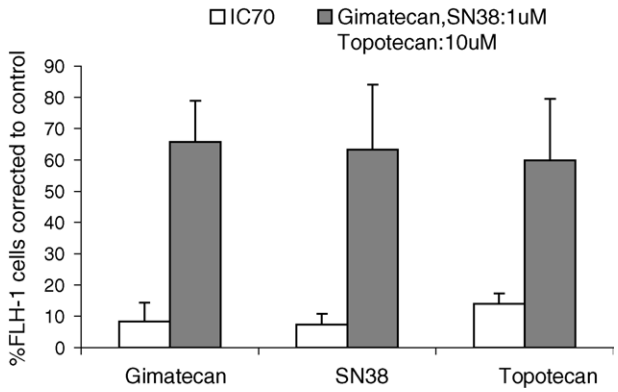


Fig. 8. ApoBrdU assay: induction of apoptosis in SK-N-DZ cells incubated for 1 h with the drugs (gimatecan, SN38 and topotecan) at 1–10 μM doses and left for additional 72 h in drug-free medium. The histogram shows the amount of tunel-positive cells. ±S.D. are from three independent experiments.

It could be argued that the low intracellular accumulation of gimatecan might decrease the amount of drug available for topoisomerase I inhibition. Thus, we evaluated the extent of drug-mediated DNA damage following incubation with the three analogues using the alkaline Comet assay. Intriguingly, we found that despite its low accumulation, gimatecan was an extraordinary strong inducer of DNA strand breaks compared to the other two conventional derivatives, either under equidose or equitoxic conditions (Figs. 5–6). The order of the efficiency as DNA strand breaks inducers after a short incubation time of 30 min at doses multiples of IC_{50} was the following: gimatecan ≫ topotecan > SN38. Interestingly, this sequence inversely correlated with the intracellular drugs contents at equipotent concentrations and after 1 h of incubation (Fig. 3). Furthermore, gimatecan appeared the most efficient DNA strand breaks inducer even in SK-N-BE(2)c cells (Fig. 6) suggesting that this is likely a common phenomenon to all neuroblastoma cell lines.

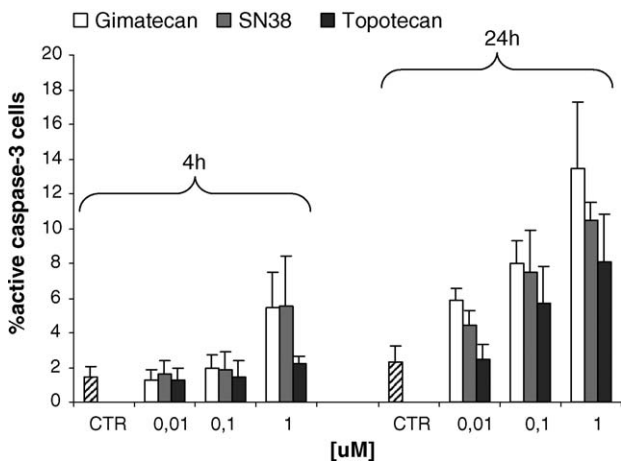


Fig. 9. Active caspase-3 experiments. SK-N-DZ cells were continuously treated with increasing doses (0.01–0.1–1 μM) of the drugs (gimatecan, SN38 and topotecan) for 4 and 24 h. ±S.D. are from three independent experiments.

So, these data suggest that despite the low amount of gimatecan available for topoisomerase I inhibition, the drug can efficiently target the enzyme–DNA complex producing the highest DNA damage. This is also consistent with the increased potency of this novel camptothecin analogue in the neuroblastoma cell lines. On the other hand, in the attempt to assess the role played by cell uptake on camptothecin-mediated cellular effects, it should be considered that the dramatic difference in intracellular accumulation between gimatecan and SN38 was produced under extreme conditions (up to 50 times IC_{50}). Thus, it cannot be ruled out that the sub-cellular localization more than the actual intracellular amount of the drug might play an important role to determine its final effects. In fact, there is recent evidence reporting on different sub-cellular sites of accumulation of topotecan and gimatecan. Topotecan was found mainly in mitochondria whereas gimatecan was mainly detected in lysosomes that are therefore suggested to represent a store of active drug [31].

The maximum DNA damage with the analogues was observed after just 30 min from drug addition (data not shown). This short incubation time was then used throughout the Comet study. Unexpectedly, the short time used did not appear to favour the rapidly accumulating analogue SN38 over the others. In order to better clarify this point, we also performed Comet studies using longer drug-incubation times to favour the slower accumulating agents topotecan and gimatecan. Interestingly, after 5 h of incubation at equidose concentrations, gimatecan appeared still the most efficient inducer of DNA strand breaks at the highest doses used (1 μM) (data not shown). We then wanted to evaluate the DNA strand breaks efficiency of these camptothecins analogues in a cell-uptake free model. The sub-cellular version of the Comet assay allowed us to measure the DNA damage produced at a single cell level on nude nuclei. We found that gimatecan was the strongest DNA strand breaks inducer even in the sub-cellular assay. Interestingly, similar data were obtained in another model, the rhabdomyosarcoma cell line RD (data not shown), suggesting that gimatecan might produce high DNA damage in different tumour types. Thus, taken all together, the Comet data appear to indicate that despite the superior cellular accumulation of SN38 and topotecan (Fig. 3) at equipotent doses, gimatecan is a more efficient DNA-damage inducer in neuroblastoma cells and nuclei. This is consistent with the superior potency of gimatecan in neuroblastoma cell lines. However, our cellular Comet data indicate a lack of linear correlation between the IC_{50} doses and the DNA damage induced by the analogues (Fig. 6). In fact, at the doses inducing the same cytotoxic effects (multiple of the IC_{50}), the three derivatives produced very different amounts of DNA damage. This finding was unexpected as it is well documented that the cytotoxic action of camptothecins occurs through the formation of the reversible cleavable complex and the induction of irreversible DNA damage [9]. In addition, Zuco et al.

[32] used the alkaline elution method to show that equitoxic concentrations of topotecan and gimatecan induced similar extent of DNA damage in human prostate carcinoma cell lines. A different group [33] found similar results with camptothecin and SN38 in breast and colon cancer cell lines. Therefore, our findings were not consistent with previous reports. However, it should be noted that we studied a different tumour model and that we used a more sensitive assay to detect DNA damage. Furthermore, our data could be explained with the hypothesis that after drug removal, differential repair rates might take place in each camptothecin-treated sample, ending in the different amounts of damage detected (gimatecan $\gg$ topotecan $>$ SN38). This hypothesis is supported by the fact that in our studies we likely favoured strand breaks reversal by washing out the drugs before harvesting and processing the cells through the assay [34]. In fact, unlike us, Brangi et al. added an additional step to the original procedure. Just prior to perform the elution process they added back to the samples the same concentration of camptothecin the cells where treated in. They stated that this additional step was required in order to take into account the rapid repair of DNA breakage following drug removal. However, our repair study of DNA strand breaks showed a similar repair rate and similar efficiency of strand breaks reversal in all analogues in both SK-N-DZ and SK-N-BE(2)c cells (Fig. 5B). In the SK-N-DZ cells, the repair process of the initial DNA breakage appeared indeed very rapid with about 60–70% of the damage induced by gimatecan and topotecan being reversed within 15–20 min from drug wash-out. With SN38, the repair was slower but for all compounds the DNA damage was about 20% of the initial strand breaks after about 4 h. In the SK-N-BE(2)c cells, the DNA damage was almost totally repaired after 20 min from drug wash-out for all analogues. Thus, we can conclude that on the basis of the complexity of the determinants involved in the neuroblastoma cell sensitivity to different camptothecin derivatives, the influence of intracellular drug content, topoisomerase I inhibition, DNA damage, cell cycle perturbations, mode of cell death could not be easily predicted.

As a whole, our data indicate that gimatecan is able to induce very high DNA damage that likely plays an important role in the enhanced sensitivity of neuroblastoma cells to the drug. Moreover, due to the rapid reversal of strand breaks occurring when camptothecins are removed from the cells, our procedure of washing out the drugs has likely allowed us to measure only the most ''persistent'' DNA damage still detectable despite rapid DNA strand breaks repair. Thus, we speculate that the gimatecan-mediated DNA strand breaks production is due to the formation of more persistent cleavable complexes compared to other camptothecin derivatives. This interpretation is consistent with the higher cytotoxicity of gimatecan, with the persistency of gimatecan-mediated block in the G2/M phase of the cell cycle up to 72 h from drug wash-out and with the data from the literature indicating the ability of the drug to induce more stable ternary complexes than topotecan and SN38 [24–27].

In conclusion, we showed that the lipophilic analogue gimatecan is interestingly more cytotoxic than SN38 and topotecan in a panel of neuroblastoma cell lines. Gimatecan superior cytotoxicity is likely expressed though a marked arrest in G2/M and induction of caspase 3-dependent apoptosis. We found that despite the slowest intracellular accumulation, gimatecan was still able to produce the highest DNA strand breaks. We suggest that DNA strand breaks efficiency as measured by the alkaline Comet assay is linearly correlated to the stability of the ternary complexes. We therefore propose that the alkaline Comet assay might represent an useful tool to evaluate the stability of camptothecin-mediated cleavable complexes in comparative studies.

In conclusion, the data presented in this manuscript support our initial interest towards this novel lipophilic camptothecin analogue. In addition, very preliminary data from ongoing in vivo studies in nude mice bearing neuroblastoma xenografts where gimatecan was administered orally, appear to confirm an interesting antitumour activity and a negligible toxicity of this novel compound. Therefore, we think that gimatecan warrants further investigation in neuroblastoma.

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References

[1] Matthay KK. Neuroblastoma: a clinical challenge and biologic puzzle. CA Cancer J Clin 1995;45:179–92.
[2] Israel MA, Thiele C.J. Tumor cell lines of the peripheral nervous system. In: Hay RJ, Park JG, Gazdar A, editors. Atlas of Human Tumour cell lines; 1994. pp. 42–78.
[3] Matthay KK, Villablanca JG, Seeger RC, Stram DO, Harris RE, Ramsay NK, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. N Engl J Med 1999;341:1165–73.
[4] Kawa K, Ohnuma N, Kaneko M, Yamamoto K, Etoh T, Mugishima H, et al. Long-term survivors of advanced neuroblastoma with MYCN amplification: a report of 19 patients surviving disease-free for more than 66 months. J Clin Oncol 1999;17:3216–20.
[5] Vassal G, Doz F, Frappaz D, Imadalou K, Sicard E, Santos A, et al. A phase I study of irinotecan as a 3-week schedule in children with refractory or recurrent solid tumours. J Clin Oncol 2003;21:3844–52.
[6] Langler A, Christaras A, Abshagen K, Krauth K, Hero B, Berthold F. Topotecan in the treatment of refractory neuroblastoma and other malignant tumours in childhood—a phase-II-study. Klin Padiatr 2002;214:153–6.

[7] Kramer K, Kushner BH, Cheung NK. Oral topotecan for refractory and relapsed neuroblastoma: a retrospective analysis. J Pediatr Hematol Oncol 2003;25:601–5.
[8] Shitara T, Shimada A, Tsuchida Y, Suzuki N, Toki F, Kuroiwa M. Successful clinical response to irinotecan in relapsed neuroblastoma. Med Pediatr Oncol 2003;40:126–8.
[9] Liu LF. DNA topoisomerase poisons as antitumor drugs. Annu Rev Biochem 1989;58:351–75.
[10] D'Arpa P, Beardmore C, Liu LF. Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons. Cancer Res 1990;50:6919–24.
[11] Bregman DB, Halaban R, van Gool AJ, Henning KA, Friedberg EC, Warren SL. UV-induced ubiquitination of RNA polymerase II: a novel modification deficient in Cockayne syndrome cells. Proc Natl Acad Sci USA 1996;93:11586–90.
[12] Ratner JN, Balasubramanian B, Corden J, Warren SL, Bregman DB. Ultraviolet radiation-induced ubiquitination and proteasomal degradation of the large subunit of RNA polymerase II Implications for transcription-coupled DNA repair. J Biol Chem 1998;273:5184–9.
[13] Desai SD, Zhang H, Rodriguez-Bauman A, Yang JM, Wu X, Gounder MK, et al. Transcription-dependent degradation of topoisomerase I-DNA covalent complexes. Mol Cell Biol 2003;23:2341–50.
[14] Ahmad T, Gore M. Review of the use of topotecan in ovarian carcinoma. Expert Opin Pharmacother 2004;5:2333–40.
[15] Cho LC, Choy H. Topoisomerase I inhibitors in the combined-modality therapy of lung cancer. Oncology (Huntingt) 2004;18:29–39.
[16] Cunningham D, Pyrhonen S, James RD, Punt CJ, Hickish TF, Heikkila R, et al. Randomised trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. Lancet 1998;352:1413–8.
[17] Douillard JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet 2000;355:1041–7.
[18] Kretschmar CS, Kletzel M, Murray K, Thorner P, Joshi V, Marcus R, et al. Response to paclitaxel, topotecan, and topotecan-cyclophosphamide in children with untreated disseminated neuroblastoma treated in an upfront phase II investigational window: a paediatric oncology group study. J Clin Oncol 2004;22(October (20)):4119–26.
[19] Houghton PJ, Cheshire PJ, Hallman 2nd JD, Lutz L, Friedman HS, Danks MK, Houghton JA. Efficacy of topoisomerase I inhibitors, topotecan and irinotecan, administered at low dose levels in protracted schedules to mice bearing xenografts of human tumors. Cancer Chemother Pharmacol 1995;36(5):393–403.
[20] Bowers DC, Aquino VM, Leavey PJ, Bash RO, Journeycake JM, Tomlinson G, et al. Phase I study of oral cyclophosphamide and oral topotecan for children with recurrent or refractory solid tumors. Pediatr Blood Cancer 2004;42(1):93–8.
[21] Furman WL, Stewart CF, Poquette CA, Pratt CB, Santana VM, Zamboni WC, et al. Direct translation of a protracted irinotecan schedule from a xenograft model to a phase I trial in children. J Clin Oncol 1999;17:1815–24.
[22] Vassal G, Doz F, Frappaz D, Imadalou K, Sicard E, Santos A, et al. A phase I study of irinotecan as a 3-week schedule in children with refractory or recurrent solid tumors. J Clin Oncol 2003;21:3844–52.
[23] Mugishima H, Matsunaga T, Yagi K, Asami K, Mimaya J, Suita S, et al. Phase I study of irinotecan in paediatric patients with malignant solid tumors. J Pediatr Hematol Oncol 2002;24:94–100.
[24] Dallavalle S, Delsoldato T, Ferrari A, Merlini L, Penco S, Carenini N, et al. Novel 7-substituted camptothecins with potent antitumor activity. J Med Chem 2000;43:3963–9.
[25] Dallavalle S, Ferrari A, Biasotti B, Merlini L, Penco S, Gallo G, et al. Novel 7-oxyiminomethyl derivatives of camptothecin with potent in vitro and in vivo antitumor activity. J Med Chem 2001;44:3264–74.
[26] De Cesare M, Pratesi G, Perego P, Carenini N, Tinelli S, Merlini L, et al. Potent antitumor activity and improved pharmacological profile of gimatecan, a novel 7-substituted camptothecin. Cancer Res 2001;61:7189–95.
[27] Perego P, De Cesare M, De Isabella P, Carenini N, Beggiolin G, Pezzoni G, et al. A novel 7-modified camptothecin analogue overcomes breast cancer resistance protein-associated resistance in a mitoxantrone-selected colon carcinoma cell line. Cancer Res 2001;61:6034–7.
[28] Pace S, Capocasa F, Tallarico C, Frapolli R, Zucchetti M, Longo A. Determination of new camptothecin derivative gimatecan and its metabolite (ST1698) in human plasma by high-performance liquid chromatography with fluorimetric detection. J Chromatogr B, submitted for publication.
[29] Kasamatsu T, Kohda K, Kawazoe Y. Comparison of chemically induced DNA breakage in cellular and subcellular systems using the comet assay. Mutat Res 1996;369:1–6.
[30] Ma J, Maliepaard M, Nooter K, Loos WJ, Kolker HJ, Verweij J, Stoter G, Schellens JH. Reduced cellular accumulation of topotecan: a novel mechanism of resistance in a human ovarian cancer cell line. Br J Cancer 1998;77:1645–52.
[31] Croce AC, Bottiroli G, Supino R, Favini E, Zuco V, Zunino F. Subcellular localization of the camptothecin analogues, topotecan and gimatecan. Biochem Pharmacol 2004;67:1035–45.
[32] Zuco V, Supino R, De Cesare M, Carenini N, Perego P, Gatti L, et al. Cellular bases of the antitumor activity of a 7-substituted camptothecin in hormone-refractory human prostate carcinoma models. Biochem Pharmacol 2003;65(8):1281–94.
[33] Brangi M, Litman T, Ciotti M, Nishiyama K, Kohlhagen G, Takimoto C, et al. Camptothecin resistance: role of the ATP-binding cassette (ABC), mitoxantrone-resistance half-transporter (MXR), and potential for glucuronidation in MXR-expressing cells. Cancer Res 1999;59:5938–46.
[34] Covey JM, Jaxel C, Kohn KW, Pommier Y. Protein-linked DNA strand breaks induced in mammalian cells by camptothecin, an inhibitor of topoisomerase I. Cancer Res 1989;49:5016–22.