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Original Paper

DNA-Topoisomerase I, a New Target for the Treatment of Neuroblastoma

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DNA-topoisomerase I is the nuclear target of new anticancer drugs, namely camptothecin and its derivatives. In order to establish the rational basis for their clinical development in paediatric oncology, the antitumour activity of irinotecan (CPT-11) and topotecan, two camptothecin water-soluble derivatives, was studied in nude mice bearing neuroblastoma xenografts. The panel was composed of 4 previously established subcutaneous xenograft lines (IGR-N835, IGR-N91, IGR-NB3, IGR-NB8) that exhibited the common biological markers of poor prognosis in children (*MYCN* amplification, 1p deletion, paradiploidy and/or *MDR1* overexpression). Irinotecan and topotecan were administered i.v. or i.p. over 5 consecutive days in animals bearing tumours. Irinotecan (40 mg/kg/day) induced 20–100% complete regressions with tumour growth delays ranging from 20 to 46 days. Two out of 10 IGR-N91 bearing animals were tumour free more than 120 days after treatment with the top dose (50 mg/kg/day). Topotecan (2.7 mg/kg/day) induced 0–67% complete regressions with tumour growth delays ranging from 23 to 50 days. One out of 8 IGR-NB3 bearing mice was tumour free at the end of the experiment. The antitumour activity of both drugs was clearly sustained at a lower dose level. Topoisomerase I activity was assayed in 15 neuroblastomas, 3 ganglioneuroblastomas and 2 normal adrenal glands, using a DNA relaxation assay. Topoisomerase I activity ranged from 69 to 1304 arbitrary units/mg of protein, and was significantly higher in immature neuroblastomas than in ganglioneuroblastomas and adrenal glands. In conclusion, irinotecan and topotecan are active against neuroblastoma xenografts. Their target is expressed in patients' tumour samples. Clinical development of topoisomerase I inhibitors in children with neuroblastoma is warranted. © **1997 Published by Elsevier Science Ltd.**

Key words: topoisomerase I, irinotecan, topotecan, neuroblastoma, xenografts
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INTRODUCTION

TOPOISOMERASE I is a nuclear enzyme capable of relaxing supercoiled DNA during replication, transcription and DNA repair. Topoisomerase I has been identified as the intracellular target of a new class of anticancer drugs, camptothecin (CPT) and its derivatives [1]. Irinotecan (CPT-11) and topotecan are two water-soluble derivatives in clinical development. They exhibit a wide spectrum of antitumour activity against murine tumours and human xenografts in preclinical studies [2, 3]. In clinical studies in adults, irinotecan has been shown to be active in colorectal cancer, lung cancer, squamous carcinoma of the uterine cervix and haematological malignancies [4]. Topotecan has been shown to be active in ovarian and lung cancer [2].

Irinotecan and topotecan may prove to be interesting new drugs for the treatment of cancer in children. In order to establish the rational basis for their clinical development in children with neuroblastoma, we studied the antitumour activity of irinotecan and topotecan against 4 neuroblastoma

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xenografts, along with the expression of their target, namely topoisomerase I, in tumour and normal tissue samples from patients.

MATERIALS AND METHODS

Animals

In vivo experiments were carried out in subcutaneous tumour-bearing female SPF-Swiss nude mice. Animals were bred in the Animal Experimentation Unit, at the Institut Gustave-Roussy (Villejuif, France), housed in sterile isolators and fed with irradiated nutrients and filtered water *ad libitum*.

Drugs

Irinotecan (CPT-11) was kindly provided by Bellon (Neuilly/Seine, France), and topotecan by SmithKline Beecham (King of Prussia, Pennsylvania, U.S.A.). Both drugs were dissolved in 0.9% sodium chloride solution, and administered i.v. or i.p. as a 0.2 ml volume of the appropriate solution. Animals of the control groups received 0.2 ml of 0.9% sodium chloride solution.

Xenografts

Four neuroblastoma xenografts have been previously established *in vivo* at the Institut Gustave-Roussy [5–7]. Their main biological characteristics are summarised in Table 1. These xenografts display most of the biological features known to be of poor prognosis in children. The xenografts are maintained *in vivo* by sequential subcutaneous transplantation in nude mice. Their *in vivo* take rate from an s.c. implant is greater than 90%.

Experimental design

The antitumour activity of irinotecan and topotecan was evaluated only against advanced-stage s.c. tumours. Both drugs were administered daily over 5 consecutive days at 1–3 dose levels per experiment. The experimental design has been previously described [5]. Briefly, mice bearing a 100–300 mm^3 tumour were randomly assigned to 1 control group and 1–3 treated groups (5–10 animals per group). In the topotecan experiments, animals bore bilateral tumours. Two tumour perpendicular diameters were measured three times weekly and each individual tumour volume was calculated according to the equation: $V\ (mm^3) = d^2\ (mm^2) \times D\ (mm)/2$, where d and D are the smallest and largest tumour diameters, respectively. Animal body weights were recorded three times weekly and the mortality was checked daily. The experiments lasted until tumour volumes reached 1500–2000 mm^3. The experiment was stopped after 120 days if there were tumour-free survivors.

The antitumour activity was evaluated according to the number of complete regressions (CR), i.e. tumour regression beyond the palpable limit at least at two consecutive tumour measurements; the tumour growth delay (TGD), defined as the difference between the treated group and the control group in the median time to reach a tumour volume that was five times greater than the initial tumour volume; and the number of tumour-free survivors (TFS), i.e. animals free of palpable tumour at the end of the experiment (at least 120 days).

Topoisomerase I activity in tumour and tissue samples

Topoisomerase I activity was measured in 20 freshly frozen tumour and tissue samples (15 immature neuroblastomas, 3 ganglioneuroblastomas, 2 normal adrenal glands) using a DNA-relaxation assay. Briefly, tissue specimens (30–150 mg) were grossly minced, suspended in a small volume of nucleus buffer containing protease inhibitors and then pulverised. Extraction of nuclear enzymes was performed by adding 0.55 M NaCl nucleus buffer (final concentration 0.39 M NaCl) for 60 min. After centrifugation, the supernatant was assayed for topoisomerase I activity by the ATP and Mg^{2+} independent relaxation of a supercoiled pHOT1 plasmid DNA (Topogen, Inc, Columbus, Ohio, U.S.A.). For each tumour, 10 serially-diluted extracts were performed in buffer containing 10 mM Tris-HCl, 100 mM KCl, 1 mM PMSF, and 50 μg/ml BSA, pH 7.5. Supercoiled DNA (0.5 μg) was incubated with each diluted extract at 37°C for 30 min in 10X TopI assay buffer (Topogen, Inc). DNA topoisomers were separated by gel electrophoresis in 1.25% agarose and stained with ethidium bromide. One arbitrary unit topoisomerase I activity was defined as the amount of topoisomerase I showing relaxation of 0.25 μg DNA under the above described conditions. Topoisomerase I activity was expressed in arbitrary units (a.u.) per mg of proteins. This assay was highly reproducible with a mean coefficient of variation of 8.5–11% when topoisomerase I activity was quantified in duplicate or triplicate. Statistical comparisons were carried out with non-parametric tests.

RESULTS

The five times daily i.v. administration of irinotecan has been previously shown to be the optimal short-term schedule

Table 1. *Characteristics of the four neuroblastoma xenografts*

Xenograft	IGR-N835	IGR-N91	IGR-NB3	IGR-NB8
Origin	Previously treated stage IV primary NB in a 2-year-old girl	Metastatic bone marrow in an 8-year-old boy	Stage III NB in a 4-year-old boy	Stage III NB in a 5-year-old boy
Histology	Immature NB	Immature NB	Immature NB	Immature NB
Ploidy	Paradiploid	Paradiploid	Paradiploid	Paradiploid
1p deletion	—	LOH	del(1)(p21)	del(1)(p35)
MYCN (copy/haploid genome)	25	60	14	5
MDR1 expression (a.u.)	—	5	10	22
In vivo doubling time (days)	3.5	6.5	10	3.3
Reference	[6]	[7]	[5]	[5]

NB, neuroblastoma; LOH, loss of heterozygosity.

Table 2. Antitumour activity of irinotecan against four neuroblastoma xenografts

Xenograft	Dose (mg/kg/day)	Schedule/route	Total dose (mg/kg)	BWL *n*	BWL (%)	Deaths	CR	TGD (days)	Tumour-free survivors
IGR-N835	27	daily × 5/i.v.	135	8	1.4	0	6	26	0/8
	40	daily × 5/i.v.	200	8	0.5	0	7	38	0/8
IGR-NB3	40	daily × 5/i.v.	200	8	11	0	3	42	0/8
IGR-NB8	27	daily × 5/i.v.	135	10	0	0	10	39	0/10
	40	daily × 5/i.v.	200	10	0.5	0	10	46	0/10
IGR-N91	27	daily × 5/i.v.	135	7	7	0	1	25	0/7
	40	daily × 5/i.v.	200	7	5	0	2	20	0/7
	50	daily × 5/i.v.	250	10	12	0	4	30	2/10

n, number of tumour-bearing nude mice; BWL, maximum body weight loss; CR, complete regression; TGD, tumour growth delay. Tumour-free survivors are observed more than 120 days after the start of treatment.

Table 3. Antitumour activity of topotecan against three neuroblastoma xenografts

Xenograft	Dose (mg/kg/day)	Schedule/route	Total dose (mg/kg)	BWL *n*	BWL (%)	Deaths	CR	TGD (days)	Tumour-free survivors
IGR-NB3	2.7	daily × 5/i.p.	13.5	8	12	0	3	49	1/8
	4	daily × 5/i.p.	20	8	20	4	2	50	0/8
IGR-NB8	2.7	daily × 5/i.p.	13.5	5	1	0	2	36	0/5
	4	daily × 5/i.p.	20	5	12.5	0	3	44	0/5
	6	daily × 5/i.p.	30	5	28	5	NA	NA	NA
IGR-NB8	1.8	daily × 5/i.v.	9	6	0	0	1	19	0/6
	2.7	daily × 5/i.v.	13.5	6	1.4	0	2	23	0/6
	4	daily × 5/i.v.	20	6	1.4	0	4	26	0/6
IGR-N91	1.8	daily × 5/i.p.	9	6	0	0	1	21	0/6
	2.7	daily × 5/i.p.	13.5	6	10	0	0	23	0/6
	4	daily × 5/i.p.	20	6	22	3	0	24	0/6

n, number of tumour-bearing nude mice; BWL, maximum body weight loss; CR, complete regression; TGD, tumour growth delay; NA, not available. Tumour-free survivors are observed more than 120 days after the start of treatment.

in our *in vivo* therapeutic experiments [6]. At the highest tested total dose (200 mg/kg), irinotecan was active against the four neuroblastoma xenografts, and induced 14–100% complete regressions with a tumour growth delay from 20 to 46 days (Table 2). This antitumor activity was clearly sustained at a lower dose level (135 mg/kg). At a total dose of 250 mg/kg, 2 out of 10 tumour free survivors were observed in IGR-N91 bearing mice.

Using a five times daily schedule, the optimal total dose of topotecan was 13.5 mg/kg. Topotecan induced 0–67% of complete regressions with a tumour growth delay from 23 to 50 days (Table 3). One tumour-free survivor was observed in IGR-NB3 bearing mice. Topotecan retained its antitumour activity at a lower dosage in 2 xenografts (IGR-N91, IGR-NB8).

Thus, irinotecan and topotecan were active *in vivo* against four subcutaneous neuroblastoma xenografts, three of them expressing the human MDR1 gene.

Topoisomerase I activity was measurable in all tumour and tissue samples, ranging from 69 to 1304 a.u./mg of protein.

Table 4. Catalytic activity of topoisomerase in tumour and normal tissue samples

Tissue	*n*	Mean	SD	Range
Adrenal gland	2	138	11	131–146
Ganglioneuroblastoma	3	171	101	69–272
Neuroblastoma	15	613	437	98–1304

Topoisomerase I activity was significantly lower in normal adrenal gland tissue (138 ± 11 a.u./mg) and ganglioneuroblastomas (171 ± 101 a.u./mg) than in immature neuroblastomas (613 ± 437 a.u./mg) ($P < 0.05$) (Table 4).

DISCUSSION

Among the new anticancer drugs in clinical development in adults, the DNA-topoisomerase I inhibitors are of particular interest for the treatment of children with cancer. These new drugs are semisynthetic derivatives of camptothecin, the leading compound in this new class [2]. DNA-topoisomerase I, their intranuclear target, has, hitherto, never been the target of any of the anticancer drugs currently used in chemotherapy protocols in paediatric oncology. Camptothecin activity is mainly directed against proliferating cells. Unlike adult cancers, most paediatric tumours are characterised by a rapid proliferation rate and thus may be sensitive to topoisomerase I inhibitors. Three camptothecin derivatives are under investigation in adult clinical trials: irinotecan, topotecan and 9-aminocamptothecin [2]. Irinotecan has been approved for the treatment of colon cancer. Recently, Tanizawa showed that SN38, the active metabolite of CPT-11, was the more potent compound in terms of *in vitro* cytotoxicity and the extent of DNA damage, compared with topotecan, camptothecin and 9-aminocamptothecin [8].

Human tumour xenografts are now well-established tools for the preclinical screening of anticancer drugs and an integral part of the current NCI and EORTC disease-oriented strategies for drug screening [9]. Xenografts are believed to

predict the histological type of human cancers likely to be sensitive or resistant to a new anticancer agent. This property lead to the design of so-called 'pre-clinical phase II studies' [10] of anticancer drugs which would serve to orient future clinical development targeting histology. Such disease-oriented preclinical development of new drugs is considered particularly applicable to paediatric oncology for two major reasons. Cancer is rare in children and survival rates are high. Consequently, the number of children suffering from cancer who are eligible for phase I and II studies is considerably lower than that of adults eligible for such studies. Preclinical phase II studies against specific paediatric tumour xenografts may help to select new drugs whose clinical development should be rapidly promoted in paediatric oncology.

Using a panel of four neuroblastoma xenografts in nude mice, we have shown that both irinotecan and topotecan displayed a significant antitumour activity when the drugs are given as a daily schedule over 5 consecutive days. These results are in good agreement with those of Komuro who showed that CPT-11, administered i.p., induced significant tumour growth inhibition in the TNB9 neuroblastoma xenograft model although no complete tumour regression was reported [11]. Irinotecan and topotecan were also found to be active against other paediatric cancer xenografts, including rhabdomyosarcomas [12, 13] and brain tumours [14, 15]. In addition, the use of protracted schedules may increase the therapeutic activity of topoisomerase I inhibitors [16].

Topoisomerase I levels have been shown to be elevated in human malignant tumours when compared with normal tissues or benign tumours. Topoisomerase I levels were found to be elevated in human colorectal tumours compared with normal colonic mucosa and seemed to parallel disease progression [17]. Topoisomerase I activity was greater in malignant ovarian tumours compared with benign and borderline tumours [18]. Husain confirmed that topoisomerase I protein levels, catalytic activity and mRNA content were significantly elevated in colon and prostate malignant tumours compared with matched normal counterparts [19]. In the present study, we showed that topoisomerase I catalytic activity is present in neuroblastomas and that its level is higher in immature neuroblastomas than in ganglioneuroblastoma and normal adrenal glands.

The sensitivity of malignant cells to topoisomerase I inhibitors has been in part related to the intranuclear amount of topoisomerase I as supported by the following observations: (1) baby hamster kidney cells overexpressing human topoisomerase I are hypersensitive to camptothecin [20]; (2) expression of human topoisomerase I in yeast cells lacking topoisomerase I restores the cell sensitivity to camptothecin [21]; campthotecin-resistant cell lines usually exhibit lower enzyme levels than the parental cell line [1, 22]; (4) for a large number of camptothecin derivatives, the level of drug-inducing cleavable complexes is directly proportional to drug cytotoxicity and antitumour activity [23]. If the presence of a functionally active topoisomerase I is necessary for the activity of topoisomerase I inhibitors, other biological factors, such as the percentage of proliferating cells, the regulation of cell progression throughout the cell cycle, the regulation of biological events involved in apoptosis may be important cellular determinants of sensitivity to topoisomerase I inhibitors [1].

Our preclinical data strongly suggest that further development of topoisomerase I inhibitors is warranted in paediatric oncology, especially in patients with neuroblastoma. To date, the preliminary report of an up-front phase II study of topotecan showed a 37% response rate in children with a stage IV neuroblastoma [24]. In addition, a paediatric phase I study of irinotecan is currently ongoing in France.

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