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ORIGINAL ARTICLE

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In vivo antitumor activity of S16020, a topoisomerase II inhibitor, and doxorubicin against human brain tumor xenografts

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Abstract New active drugs are needed for the treatment of primary brain tumors in both children and adults. S16020 is a cytotoxic olivacine derivative that inhibits topoisomerase II. The aim of the study was to determine its antitumor activity in athymic mice bearing subcutaneous medulloblastoma (IGRM33, 34, 57) and glioblastoma (IGRG88, 93, 121) xenografts treated at an advanced stage of tumor growth in comparison with that of doxorubicin. Animals were randomly assigned to receive i.v. S16020 or doxorubicin weekly for three consecutive weeks. The optimal dose was 80 mg/kg per week. S16020 demonstrated a significant antitumor activity in two out of three medulloblastoma xenografts. IGRM57 xenografts were highly sensitive with 100% tumor regressions and a tumor growth delay (TGD) of 102 days, while one of eight IGRM34 xenografts showed a partial regression with a TGD of 16 days. Doxorubicin was significantly more active than S16020 in these two models. IGRM33, a model established from a tumor in relapse after chemotherapy and radiotherapy, was refractory to both drugs. S16020 demonstrated a significant antitumor activity in the three glioblastoma xenografts evaluated. The wild-type p53 IGRG93 xenograft was highly sensitive with 100% tumor regressions and a TGD of 54 days. IGRG121 (wt p53) and IGRG88 (mutant p53) were moderately sensitive with TGDs of 33 and 23 days, respectively. Doxorubicin showed greater activity in two of these models. All six xenografts exhibited low expression of mdr1 as quantitated by RT-PCR, and no correlation was found with the activity of either drug. Conversely, a low activity of the two drugs was significantly associated with a high expression of MRP1 in medulloblastomas. Finally, no relationship was observed between drug sensitivity to either drug and expression of their target, topoisomerase IIα. In conclusion, S16020 and doxorubicin showed significant antitumor activity in brain tumor xenografts treated at an advanced stage of tumor growth. Their activity was related to MRP1 expression in medulloblastomas.

Keywords Medulloblastoma · Glioblastoma · Doxorubicin · Nitrosourea · MRP1

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Introduction

Primary malignant brain tumors remain an unresolved therapeutic problem and a major challenge for new drug development. In adults, malignant glial tumors, and mainly glioblastoma, are particularly refractory to most anticancer drugs and radiotherapy. Among anticancer drugs, chloroethylnitrosoureas, such as BCNU and fotemustine [13], and more recently temozolomide [35], display antitumor activity in human glioblastoma, but usually do not significantly prolong survival. New anticancer drugs and therapeutic strategies are urgently needed. In children, medulloblastomas, i.e. primitive neuroectodermal tumors (PNET) arising in the posterior fossa, are more chemosensitive and radiosensitive than malignant glial tumors. The more frequently used drugs are platinum compounds (cisplatinum or carboplatinum), etoposide, alkylating agents such as cyclophosphamide, and vincristine. Radiation therapy is delivered to the entire central nervous system. It induces profound long-term sequelae in young children, and new drugs are needed to improve both the cure rate and quality of life of children with brain tumor [9].

S16020 is a new pyridocarbazole derivative characterized by a basic *N*-dialkyl aminoalkyl-carboxamido group grafted onto an olivacine chromophore. S16020 is a cytotoxic compound that binds DNA by intercalation and inhibits topoisomerase II [16]. S16020 shows a wide spectrum of antitumor activity, both in vitro and in vivo, in a panel of murine and human experimental models [10, 17]. S16020 is particularly active in leukemias, lung and kidney cancers [15]. As compared with other anticancer compounds of the same class, S16020 has proved to be more active than ellipticine. Its activity is equivalent to, and even greater than, that of doxorubicin, another DNA intercalator and topoisomerase II inhibitor. In addition, S16020 shows potent antitumor activity in cell lines displaying a multidrug resistance (mdr) phenotype [17, 22].

The aim of the study was to evaluate the in vivo antitumor activity of S16020 against advanced stage human medulloblastoma and glioblastoma xenografts, in comparison with that of doxorubicin. These models were also characterized in terms of sensitivity to a class of anticancer drugs currently used for the treatment of brain tumors in humans, namely the nitrosoureas. In addition, this antitumor activity was characterized in relation to p53 gene status, the expression of two genes, namely mdr1 and MRP1, involved in the acquisition of a mdr phenotype, and the expression of topoisomerase II α, the nuclear target of S16020.

Material and methods

Drugs

S16020 and fotemustine were provided by IRI Servier (Courbevoie, France) and doxorubicin was purchased from Pharmacia (Saint-Quentin en Yvelines, France). Drugs were prepared in 5% dextrose immediately before administration to nude mice.

Animals

Female SPF-Swiss nude mice were bred in the Animal Experimentation Unit of the Institut Gustave-Roussy (Villejuif, France). Animals were housed in sterile isolators, and fed with irradiated nutriments (UAR, Villemoisson/Orge, France) and filtered water ad libitum. Experiments were approved by local committee and carried out under the conditions established by the European Community (directive no. 86/609/CEE) and in accordance with the UKCCCR guidelines [28].

Xenografts

The panel comprised three cerebellar PNET (IGRM33, IGRM34, IGRM57) and three glioblastoma xenografts (IGRG88, IGRG93, IGRG121). These xenografts were derived from primary tumors by subcutaneous transplantation of small fragments in previously irradiated athymic mice [29]. The human origin of these xenografts has been confirmed by the presence of abundant human LDH isoenzymes and by cytogenetic analysis. The characteristics of these six xenograft models are summarized in Table 1. IGRM34 was derived from a cerebellar PNET with a rhabdoid phenotype in an 8-month-old child. Since this xenograft was established, the hSNF5/INI1 gene mutation has been characterized as a molecular marker in rhabdoid tumors [32]. No hSNF5/INI1 gene mutation was found in IGRM34. All the xenografts were maintained in vivo by sequential passaging from a subcutaneous implant, with an engraftment success rate greater than 75%. The stability of the xenografts was checked at each passage by the analysis of their doubling time, and at each three to six passages by histological analysis.

Experimental design

Drug activity was evaluated against unilateral subcutaneous advanced stage tumors, as previously described [30]. For each experiment, 4×4-mm tumor fragments were xenotransplanted subcutaneously in 80 athymic mice aged 6 to 8 weeks. On day 0 of the treatment, mice bearing a 100–300-mm^3 subcutaneous tumor were pooled and randomly assigned to five or six treated groups of 8–10 animals, and one control group of 7–11 animals. Animals with tumor outside the desired volume range were excluded. In the earliest experiments, non-tumor-bearing animals were added in the treated groups to better evaluate toxicity in a larger number of animals. Two tumor perpendicular diameters were measured three times weekly with a caliper by the same investigator. Each tumor volume (in cubic millimeters) was calculated according to the following equation: $V = d^2 \times D/2$, where d and D are the smallest and largest perpendicular tumor diameters in millimeters, respectively. Each group of mice was treated according to the average weight of the group. Animal body weights were recorded three times a week and mortality was checked daily. Body weight loss (BWL) was reported as the maximum treatment-related weight loss. The experiments lasted until tumor volumes reached 1500–2000 mm^3, i.e. less than 10% of animal body weight. The experiment was stopped after 120 days when there were tumor-free survivors (TFS).

Treatment

S16020 and doxorubicin were given intravenously, weekly for three consecutive weeks. Four dose levels of S16020 (40, 60, 80 and 90 mg/kg per week) and five dose levels of doxorubicin (6, 8, 10, 15 and 20 mg/kg per week) were explored. Both drugs were always evaluated in the same experiments at several drug levels. Fotemustine, as a reference compound currently used in the treatment of malignant glial tumors in humans, was administered as a single intraperitoneal injection at doses of 34 and 50 mg/kg. The overall maximum tolerated dose (MTD) was defined as the dose inducing a maximum BWL less than 15% and/or no more than 10% treat-

Table 1 Characteristics of the human brain tumor xenografts

Xenograft	Histology	p53	Karyotype
IGRM33	Undifferentiated medulloblastoma derived from a relapsed tumor	Wild-type	45–47;1p−,1q−,5q+,t(10;11)(q11;q24), +multiple structural rearrangements
IGRM34	PNET with a rhabdoid phenotype	Wild-type	90–94,XXYY,der(1)t(1;17)(p31;q11)x2,del(2)(p24)x2, +2mars
IGRM57	Undifferentiated medulloblastoma	Wild-type	76–81,YY,der(1)t(1;?)(p11;?)x2; der(3)t(3;?)(p11;?), del9(p21)x2, der(9)t(9;13)(p11;q11),−10,−10, der(11)t(11;?)(p11;?), der(X)t(X;?)(q21;?)x2;+mar
IGRG88	Glioblastoma	Mutant	90–106,XXXX,+XX,+1,+7×2,−9, der9 t(0;22)(p21;q11)x3, −10×2,−11,−14×2, +16×2,+17, +18×2,+19×2,+21,−22
IGRG93	Glioblastoma	Wild-type	2n, dms
IGRG121	Glioblastoma	Wild-type	47–48,XY,1p−,+7,i(9q),−16, +2mars

ment-related death. Within each experiment, a dose level that induced a BWL greater than 15% or more than one treatment-related death was not considered in the statistical analysis.

Evaluation of antitumor activity

The activity of each drug tested was evaluated according to three criteria: (1) the number of complete and partial tumor regressions, (2) the tumor growth delay (TGD), (3) the number of TFS. Complete regression (CR) was defined as a tumor regression beyond the palpable limit (15 mm^3) and partial regression (PR) as a tumor regression greater than 50% of the initial tumor volume. CR and PR had to be observed for at least two consecutive tumor measurements in order to be retained. TGD was defined as the difference between the treated group and the control group in the median time to reach a tumor volume that was five times greater than the initial tumor volume [30]. TFS were defined as animals free of palpable tumor at the end of the experiment (at least 120 days).

Evaluation of p53 genotype

The functional assay of separated alleles in yeast (FASAY) evaluates the biological function of p53 to activate transcription in yeast. FASAY was conducted as described for the analysis of somatic p53 mutations in human tumors and cell lines [8]. P53 cDNA were obtained by RT-PCR. The primers used for p53 were located in exon 4 (P3) and exon 11 (P4), as follows:

- P3: (5′-ATT-TGA-TGC-TGT-CCC-CGG-ACG-ATA-TTG-AA(S)C-3′)
- P4: (5′-ACC-CTT-TTT-GGA-CTT-CAG-GTG-GCT-GGA-GT(S)G-3′)

where (S) represents a phosphorothioate link designed to avoid degradation by native DNA polymerase 3′-to-5′ exonuclease activity. Exponentially growing yeasts (strain yIG397) were co-transformed using the lithium acetate procedure with the crude PCR products, a linearized expression vector for gap repair, pSS16, and salmon sperm as DNA carrier. Yeast colonies carrying p53 sequences recombined in the pSS16 plasmid ($Leu2^+$) were selected on synthetic minimal medium lacking leucine and containing a minimal concentration of adenine. The yeast carries a reporter gene, ADE2, which allows it to grow in the absence of adenine when it is transactivated by a wild-type p53 protein. Yeast colonies expressing wt p53 are large and white, while those expressing mutant p53 are small and red because ADE2 gene expression is defective. If the proportion of red colonies is below 5% (i.e. below the threshold for mutation occurring during the procedure), the P53 protein is considered as wild type. The mutant p53 gene was then sequenced to identify the p53 mutation.

Expression of mdr1, MRP1 and topoisomerase IIα

During each experiment, three tumors (100 to 300 mm^3 in volume) were obtained from untreated animals, cut into small pieces, immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Five in vitro cell lines displaying different resistant phenotypes were used as controls. MCF7DXR is a doxorubicin-resistant human mammary adenocarcinoma cell line selected from MCF7 and overexpressing mdr1 [7]. The A549 cell line is a human lung carcinoma cell line and two variants, $A549^{T3}$ (resistant to paclitaxel [25]) and $A549^{V3}$ (resistant to etoposide [27]), have been established. These cell lines were maintained in paclitaxel- and etoposide-containing medium. Both cell lines overexpress the MRP1 gene. In addition, the $A549^{V3}$ cell line displays an underexpression of the topoisomerase IIα subunit. All these cell lines were cultured in RPMI-1640 medium containing fetal calf serum.

MRP1 and mdr1, and topoisomerase IIα mRNA expression were analyzed using RT-PCR. Total RNA were isolated using TRIzol Reagent (Life Technologies, Cergy Pontoise, France) then RNA (1 μg) was reverse-transcribed using Superscript II enzyme (200 U) (Life Technologies) and random hexanucleotide primers (Life Technologies). The expression of MDR1 and MRP1 mRNA was analyzed as previously reported [18].

The β_2-microglobulin gene was coamplified as reference gene using primers from Gussow et al. [11] with mdr1 (primers from Noonan et al. [20]), MRP1 (primers from Bordow et al. [4]) and topo2α using customized primers (forward primer 5′-TTTAAGGCCCAAGTCCAGTTAAACA-3′, reverse primer 5′-ATAAATTCCAGAAAACGATGTCG-3′). For topoisomerase IIα, the thermal cycling conditions were as follows: initial denaturation for 3 min at 94°C, annealing for 1 min at 58°C, synthesis for 1 min at 72°C, and each cycle consisted of 30 s at 94°C, 30 s at 58°C and 1 min at 72°C. A final synthesis step was run for 7 min at 72°C.

The expected PCR product sizes were 157, 140, 473 and 120 bp, respectively. Aliquots (5 µl) of the PCR products were analyzed using 2% agarose gel electrophoresis (ICN Pharmaceuticals, Orsay, France) containing ethidium bromide (50 ng/ml). Bands were visualized by UV transillumination and quantification was performed using a GelDoc1000 system (BioRad, Ivry sur Seine, France). Relative expression ratios (RER) were calculated by dividing the fluorescence intensity of the target gene band by that of the β_2-microglobulin control gene band.

Statistical analysis

For each experiment, the times for the tumors to attain a volume five times greater than the initial tumor volume in the treated and control groups were compared using a nonparametric Kruskal-Wallis test. When significance was raised, groups were further compared two-by-two using a Dunn's multiple comparison test. The aim of the study was first to evaluate the antitumor activity of S16020 alone, and the analysis compared all the groups treated by S16020 with the control group. The activity of doxorubicin was compared in the same way. The second aim was to compare the activity of S16020 to that of doxorubicin, and the statistical analysis was performed with all the experimental groups within each experiment.

In order to assess drug activity in relation to the expression of the different resistant genes, chemosensitivity of each xenograft was graded according to the grading shown in Table 2. The RER of each gene was compared within each tumor group by a nonparametric test.

Results

Toxicity

S16020 and doxorubicin exhibited different toxicity profiles in athymic mice. S16020 induced BWL and major cutaneous toxicity at the injection site without any other clinical features. The MTD of S16020 was 80 mg/kg per week with a maximum BWL of 8.7%, and 5 treatment-related deaths out of 57 animals. Doxorubicin induced a dose-dependent neurological toxicity, characterized by paraplegia occurring from day 25 up to day 112 after treatment. This toxicity was not predicted by early BWL and delayed treatment-related death occurred at a median of 72 days after treatment. The MTD of doxorubicin was 8 mg/kg per week with a maximum BWL of 2% and 4 treatment-related deaths out of 49 animals. The MTD of fotemustine was 34 mg/kg with a maximum BWL of 4.6% and 2 treatment-related deaths out of 23 animals.

Antitumor activity in medulloblastoma

S16020 demonstrated a significant dose-dependent activity in two out of three medulloblastoma xenografts (Table 3). IGRM57 was highly sensitive with 100% tumor regressions at 60 and 80 mg/kg, along with significant TGDs of 52 and 102 days, respectively (Fig. 1). IGRM57 was significantly more sensitive to doxorubicin (100% TFS at both 6 and 8 mg/kg dose levels). In addition, IGRM57 has previously been shown to be sensitive to fotemustine [31]. IGRM34 was moderately sensitive to S16020 with one PR out of eight animals at the MTD of 80 mg/kg, along with a significant TGD of 16 days. However, doxorubicin was significantly more active than S16020 in this model with seven tumor regressions out of eight animals at the MTD of 8 mg/kg along with a TGD greater than 82 days. IGRM34 has been shown previously to be sensitive to fotemustine and BCNU [31]. Finally, IGRM33, a medulloblastoma xenograft derived from a tumor relapse after chemotherapy and radiotherapy, was refractory to S16020, doxorubicin and fotemustine [31]. The experiments were duplicated for IGRM57 and IGRM34, and showed consistent results. Overall, S16020 proved to be less active than doxorubicin in two out of three medulloblastoma xenografts. The three medulloblastoma xenografts showed a wild-type p53 gene.

Antitumor activity in glioblastoma

S16020 demonstrated a significant antitumor activity in the three glioblastoma xenografts evaluated (Table 4). IGRG93, a p53-wt xenograft, was the most sensitive

Table 2 In vivo chemosensitivity grading.Antitumor activity was evaluated at the highest non-toxic dose (or maximum tolerated dose) defined as the dose inducing a maximum body weight loss less than 20% and/or no more that one treatment-related death, or the highest dose tested in the experiment

Grade	Tumor regressions	Tumor growth delay	Tumor-free survivors
0	Absent	Non-significant	None
1	Absent	Significant	None
2	< 50% of animals	Significant	None
3	> 50% of animals	Significant	None
4	> 50% of animals	Significant and more than four times the median time to five times the volume in the control group	None
5	> 50% of animals	Significant and more than four times the median time to five times the volume in the control group	< 50% of animals
6	> 50% of animals	Significant and more than four times the median time five times the volume in the control group	> 50% of animals

Table 3 Antitumor activity of S-16020 and doxorubicin in subcutaneous medulloblastoma xenografts

Xenograft		Treatment				Toxicity		Activity				
Name	Doubling time (days)	Drug	Dose (mg/kg)	Schedule (days)	Number of mice/tumors	Death	Maximum body weight loss (%)	Complete regression	Partial regression	Tumor growth delay (days)	*P* value[a]	Tumor-free survivors
IGRM33	8.8	S16020	40	0, 7,14	8/8	0	4	0	0	16	NS	0
		S16020	60	0, 7,14	8/8	1	5	0	0	21	NS	0
		S16020	80	0, 7,14	8/8	2	6.1	0	0	22	–	0
		Doxorubicin	6	0, 7,14	8/8	0	4.4	0	0	18	NS	0
		Doxorubicin	8	0, 7,14	8/8	2	5.7	0	0	21	–	0
IGRM34	7.5	S16020	40	0, 7,14	8/8	0	3.6	0	0	10	NS	0
		S16020	60	0, 7,14	8/8	0	7.7	0	0	16	< 0.05	0
		S16020	80	0, 7,14	8/8	1	9.6	0	1	16	< 0.01	0
		Doxorubicin	6	0, 7,14	8/8	0	0	0	0	26	< 0.05	0
		Doxorubicin	8	0, 7,14	8/8	0	6.4	4	3	> 82	< 0.001	0
IGRM57	4.8	S16020	60	0, 7,14	8/8	0	5.1	0	8	52	NS	0
		S16020	80	0, 7,14	8/8	1	7.7	1	6	102	< 0001	0
		S16020	90	0, 7,14	8/8	3	12.7	4	3	> 135	–	3
		Doxorubicin	6	0, 7,14	8/8	1	0.4	8	0	> 135	< 0.01	7
		Doxorubicin	8	0, 7,14	8/8	0	1.3	8	0	> 135	< 0.01	8

[a]Nonparametric test comparing S16020 and doxorubicin with the controls

model with 100% tumor regressions at the MTD along with a TGD of 54 days and one TFS out of seven animals (Fig. 1). The activity of doxorubicin was comparable to that of S16020 with 100% tumor regressions, a TGD of 47 days and one TFS at the MTD of 6 mg/kg. At the dose above the MTD, i.e. 8 mg/kg, two animals died and two survived tumor-free among the seven treated animals. IGRG93 was highly sensitive to fotemustine with 100% TFS at a dose of 34 mg/kg. The antitumor activities of S16020 and doxorubicin were equivalent in IGRG121, a p53-wt xenograft. Both drugs induced significant TGDs of 27 to 33 days. Interestingly, IGRG121 was refractory to fotemustine (no tumor regression with a TGD of 10 days). IGRG88, a p53-mutant xenograft (T → A transition at codon 113), was moderately sensitive to S16020 with a significant TGD of 23 days at 60 mg/kg. Doxorubicin proved to be significantly more active than S16020 with four PRs out of seven animals and a TGD of 51 days. Fotemustine induced 100% tumor regressions, with a TGD of 108 days and two TFS out of seven animals [31]. Overall, S16020 proved to be active in the three glioblastoma xenografts evaluated. Doxorubicin showed a greater activity than S16020 in two of these models. However, the activity profile of these two topoisomerase II inhibitors differed significantly from the activity profile of fotemustine.

Expression of mdr1 and MRP1 and sensitivity to S16020

The expression level of mdr1 was low in the six xenograft models studied (mean RER < 0.2), and was equivalent to the level of expression observed in the parental chemosensitive cell lines, namely MCF7 and A549 (Table 5). Conversely, the expression was high in the two resistant cell lines (RERs 3.5 in $MCF7^{DXR}$ and 0.79 in $A549^{T3}$).

The two A549-derived cell lines resistant to paclitaxel ($A549^{T3}$) and etoposide ($A549^{V3}$) showed RERs of MRP1 of 2.37 and 2.57, respectively, which were significantly higher than those of the parental cell lines, A549 (RER 1.53) and MCF7 (RER 1.77). None of the three glioblastoma models showed a RER greater than that of the parental cell lines. Conversely, the RER of MRP1 in the three medulloblastoma xenografts ranged from 1.56 to 3.55, and a high RER was significantly associated with resistance to S16020 (Fig. 2).

Expression of topoisomerase IIα and sensitivity to S16020

The RER of topoisomerase IIα ranged from 2.04 to 4.33 in all the control cell lines evaluated, but $A549^{V3}$, which is resistant to etoposide and exhibits a significantly decreased expression of topoisomerase IIα (RER 0.28,

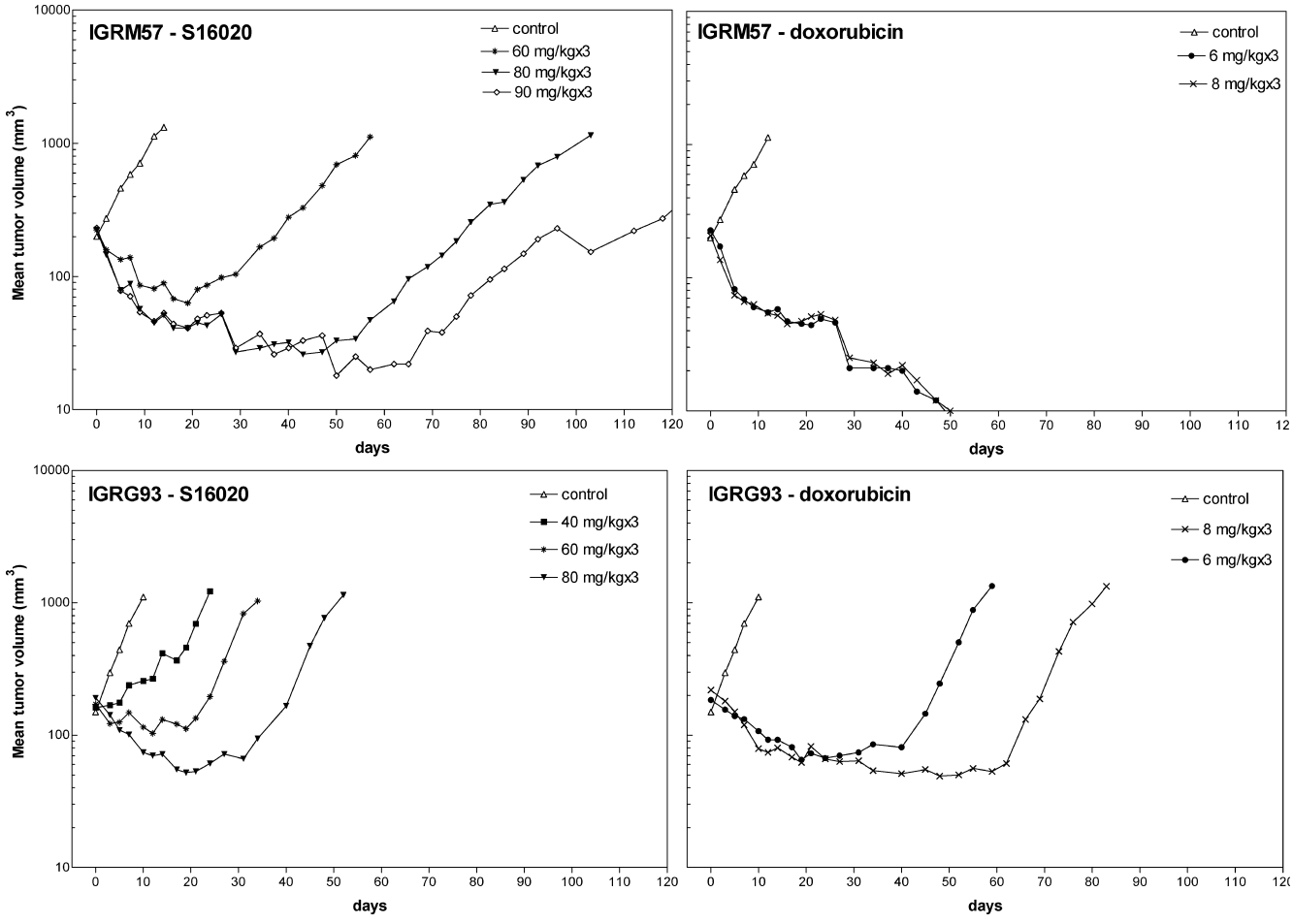

Fig. 1 Antitumor activity of S16020 and doxorubicin against the IGRM57 medulloblastoma xenograft and the IGRG93 glioblastoma xenograft. Both drugs were given intravenously weekly for 3 weeks. Animals received either the vehicle (△) or one of both drugs at the doses indicated. Each line represents the evolution of the mean tumor volume in each group

Table 5). The RER of topoisomerase IIα ranged form 2.25 to 7.77 in medulloblastoma xenografts, and from 1.37 to 1.52 in glioblastoma xenografts. In these tumor models, no relationship was observed between sensitivity to S16020 and doxorubicin, and expression of topoisomerase IIα.

Discussion

S16020 is a new topoisomerase II inhibitor. Its clinical development was started on the basis of a wide spectrum of antitumor activity in vitro and in vivo, and a favorable profile of cytotoxicity in cell lines expressing the mdr phenotype [10, 17, 22]. Here we showed that S16020 was also active in five of six human brain tumor xenografts growing subcutaneously in athymic nude mice and treated at an advanced stage of tumor progression. This activity was clearly dose-dependent in most of the models evaluated. We compared S16020 with doxorubicin, another topoisomerase II inhibitor and DNA intercalator, using the same schedule of administration. S16020 proved to be less active than doxorubicin in the medulloblastoma xenografts and in two of the three glioblastoma xenografts. The systemic exposure after a 90 mg/kg dose in B6D2F1 mice has been shown to be in the order of magnitude of the systemic exposure observed in humans during phase II studies at 100 mg/m^2. Finally, the profile of activity of S16020 and doxorubicin differed from that of fotemustine, a chloroethylnitrosourea used in the treatment of glioblastoma [13].

P53 plays a major role in the cellular response to several DNA-damaging processes, in particular in drug- or radiation-induced apoptosis [12]. P53 mutations are rare in medulloblastomas [6, 21] and the three xenografts used in our study showed a wild-type p53 gene using FASAY. Conversely, more than 50% of glioblastoma and malignant glial tumors display a p53 mutation in humans [33]. One of the three glioblastoma xenografts used in our study, i.e. IGRG88, showed a p53 mutation (T → A transition at codon 113). However, both S16020 and doxorubicin induced antitumor activity in IGRG88 to the same extent as in the other wild-type p53 IGRG121 xenograft. Thus, p53 mutation might not be a major mechanism of resistance to S16020

Table 4 Antitumor activity of S16020 and doxorubicin in subcutaneous glioblastoma xenografts

Xenograft		Treatment				Toxicity				Activity		
Name	Doubling time (days)	Drug	Dose (mg/kg)	Schedule (days)	Number of mice/tumors	Death	Maximum body weight loss (%)	Complete regression	Partial regression	Tumor growth delay (day)	*P* value[a]	Tumor-free survivors
IGRG88	5.4	S16020	60	0, 7, 14	9/7	1	0.4	0	0	23	<0.001	0
		S16020	80	0, 7, 14	9/7	0	3.3	0	0	14	<0.05	0
		Doxorubicin	6	0, 7, 14	9/7	0	0	0	4	51	<0.001	0
IGRG93	3.2	S16020	40	0, 7, 14	7/7	0	1	0	0	15	NS	0
		S16020	60	0, 7, 14	7/7	0	2.8	0	1	24	<0.01	0
		S16020	80	0, 7, 14	7/7	0	9.3	2	5	54	<0.001	1
		Doxorubicin	6	0, 7, 14	7/7	0	0	1	6	47	<0.0001	1
		Doxorubicin	8	0, 7, 14	7/7	2	0	5	2	73	–	2
IGRG121	5.5	S16020	60	0, 7, 14	8/6	0	7.5	0	0	27	<0.05	0
		S16020	80	0, 7, 14	8/6	1	14.5	0	1	33	<0.01	0
		Doxorubicin	6	0, 7, 14	8/6	0	1.7	0	0	23	<0.05	0
		Doxorubicin	8	0, 7, 14	8/6	0	2.6	0	0	31	<0.001	0

[a]Nonparametric test comparing S16020 and doxorubicin with the controls

and doxorubicin in glioblastoma. This needs further additional in vitro and in vivo data.

To better characterize the activity of S16020 and doxorubicin, we further evaluated the expression of two genes, namely mdr1 and MRP1, involved in the mdr phenotype, and commonly expressed in human brain tumors. In a series of 67 different brain tumors, mainly comprising malignant glial tumors, Mousseau et al. found by Northern blotting that mdr1 is occasionally overexpressed (2% of tumor samples) [19]. Using immunohistochemistry, Chou et al. observed expression of mdr1 gene in 16 out of 29 pediatric tumor samples [5]. Very low expression of mdr1 was found, using RT-PCR, in the six brain tumor xenografts we evaluated, as compared to the in vitro cancer cell lines used as control and sensitive to anthracyclines. No correlation was observed between the level of mdr1 expression, as evaluated by RT-PCR, and either drug, namely doxorubicin and S16020.

MRP1 is a 190-kDa ATP-binding membrane glycoprotein involved in the acquisition of the mdr phenotype. In vitro, the chemosensitivity of human glioma cell lines to doxorubicin and etoposide has been correlated with the level of MRP1 expression [1]. In addition, MRP1 is expressed in gliomas in humans, with up to 32% of positive cells per tumor [2]. In our series, the level of MRP1 expression was lower in glioblastoma xenografts than in medulloblastoma, as shown by RT-PCR. Interestingly, two of the three medulloblastoma xenografts showed a level of MRP1 expression equivalent to or even higher than that observed in the in vitro cells with a mdr phenotype, and were poorly sensitive or refractory to both doxorubicin and S16020 in vivo. Altogether, these results suggest that the cytotoxic effects of the two drugs were not related to the level of expression of mdr1 in the six brain tumor xenografts evaluated. However, it should be highlighted that the mdr1 expression levels were low. Thus a possible role for mdr1 overexpression in resistance to doxorubicin and S16020 in brain tumors is not ruled out. On the other hand, resistance to both drugs was associated with a high level of MRP1 expression in medulloblastoma xenografts.

S16020 has been shown to stimulate in vitro DNA topoisomerase II-mediated DNA cleavage and induction of protein-associated DNA breaks [16]. Topoisomerase II acts as a dimeric protein composed of two isoforms, namely α and β. Using a cancer cell line resistant to S16020, Le Mée et al. showed a correlation between cytotoxicity and DNA cleavage [16]. However, sensitivity to S16020 was not restored by transfection of either the α or the β isoform of the topoisomerase II gene, suggesting that cellular or nuclear cofactors are involved in drug resistance. Topoisomerase II α was expressed in the six xenografts evaluated at levels comparable to those in the cancer cell lines used as controls.

Anthracyclines are not used in the treatment of brain tumors in adults and children. Actually, there is no phase II study of doxorubicin as a single agent either in glioblastoma or in medulloblastoma. Indeed, these drugs

Table 5 Expression of mdr1, MRP1 and topoisomerase IIα in medulloblastoma and glioblastomaxenografts in relation to chemosensitivity grading

Xenografts	Sensitivity(grade)		Relative expression ratio (mean ± SD)			EpirubicinIC$_{50}$ (μ*M*) (mean ± SD)
	S16020	Doxorubicin	mdr1	MRP1	TopoIIα	
IGRM33	0	0	0.10 ± 0.07	3.55 ± 0.41	7.77 ± 0.83	
IGRM34	2	4	0.11 ± 0.03	2.50 ± 0.15	2.25 ± 0.11	
IGRM57	5	6	0.16 ± 0.06	1.56 ± 0.05	3.40 ± 0.50	
IGRG88	1	3	0.20 ± 0.03	0.41 ± 0.02	1.40 ± 0.10	
IGRG121	2	1	0.10 ± 0.02	1.39 ± 0.05	1.52 ± 0.27	
IGRG93	5	5	0.12 ± 0.01	0.96 ± 0.02	1.37 ± 0.10	
Cell lines						
MCF7			0.05 ± 0.01	1.77 ± 0.02	4.33 ± 0.58	1.9 ± 0.5
MCF7DXR			3.51 ± 0.11	–	–	787 ± 165
A549			0.16 ± 0.03	1.53 ± 0.03	2.37 ± 0.03	2.7 ± 0.5
A549^{T3}			0.79 ± 0.02	2.37 ± 0.03	2.04 ± 0.29	69 ± 13
A549^{V3}			0.05 ± 0.03	2.57 ± 0.02	0.28 ± 0.05	44 ± 12

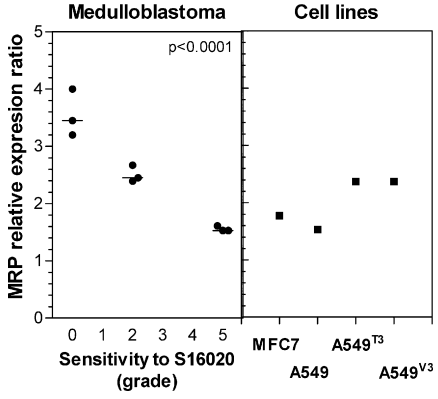

Fig. 2 Medulloblastoma sensitivity to S16020 in relation to MRP1 expression. Chemosensitivity was graded according to the scheme outlined in Table 2. In the *left graph*, each symbol represents one tumor (mean value of three quantifications). In the *right graph*, each symbol represents the mean of three quantifications

do not easily cross the blood-brain barrier, which is a major impediment to brain tumor chemotherapy [3]. Our preclinical data suggest that doxorubicin, and to a lesser extent S16020, may be active in both medulloblastoma and glioblastoma. However, because of their low brain distribution, they may not be active in humans. This is supported by the findings of a recent meta-analysis of all the studies in which the in vitro cytotoxic effects of anticancer drugs in glioma cells had been investigated [34]. The most active agents were actinomycin D, vincristine, mitoxantrone, vinblastine, doxorubicin. All these drugs are P-glycoprotein substrates and poorly distributed in normal brain. Conversely, in this in vitro analysis, nitrosoureas such as carmustine, nimustine and lomustine were less-active drugs.

In addition, Takamiya et al. have shown that resistance of intracranially transplanted human glioma xenografts to vincristine and doxorubicin is related to the overexpression of the murine mdr3 gene in brain microvessels, while the same tumor models are sensitive to these drugs when transplanted subcutaneously [26]. Enhanced brain distribution is likely to improve their activity in vivo. Siegal et al. have shown that vectorization of doxorubicin in long-circulating liposomes is able to increase the survival of rats bearing an intracranial tumor while free doxorubicin is not active [24]. In addition, when Stealth liposomal doxorubicin is administered to brain tumor patients, drug accumulation is 7 to 19 times higher in brain metastases and glioblastomas as compared to normal brain [14]. Thus, our findings suggest that doxorubicin and S16020 may be active in human brain tumors and that delivery systems such as liposomes, nanoparticles or tumor-specific drug immunoconjugates will have to be considered to improve drug distribution in human brain tumors [23, 24].

In conclusion, S16020 is a new olivacine that showed significant antitumor activity in five out of six human malignant brain tumor xenografts treated at an advanced stage of tumor progression. S16020 was less active than doxorubicin in medulloblastoma xenografts and in two of the three glioblastoma xenografts. In addition, their activity profile was different from that of the nitrosourea fotemustine. As far as clinical development of these topoisomerase II inhibitors in human brain tumors is concerned, vectorization using liposomes or nanoparticles could enhance brain distribution through the blood-brain barrier and make these drugs active in humans.

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