7β-Hydroxycholesterol Blocked at C-3-OH Inhibits Growth of Rat Glioblastoma *In Vivo*: Comparison between 7β-Hydroxycholesteryl-3β(ester)-oleate and 7β-Hydroxycholesteryl-3β-O(ether)-oleyl

CLOVIS RAKOTOARIVELO[1], MONIKA ADAMCZYK[1], MICHEL DESGEORGES[2], KEITH LANGLEY[1], JEAN-GEORGES LORENTZ[1], ANDRÉ MANN[3], DAMIEN RICARD[2], ELISABETH SCHERRER[1], ALAIN PRIVAT[1] and MARCEL MERSEL[1]

[1]*Institut des Neurosciences de Montpellier, Unité 583 de l'INSERM, 80 rue Augustin Fliche, 34091 Montpellier Cedex 05;*
[2]*Hôpital d'Instruction des Armées, Val-de-Grâce, 74 Boulevard de Port Royal, Cliniques de Neurologie et de Neurochirurgie, 75230 Paris Cedex 05;*
[3]*Laboratoire de pharmacochimie de la communication cellulaire, UMR 7081 du CNRS, Faculté de pharmacie, 74 route du Rhin, BP 24, 67401 Illkirch Cedex, France*

Abstract. *Background: It was previously demonstrated that the 7β-hydroxycholesteryl-3β(ester)-oleate (7β-ester) possesses antitumor properties against the experimental rat C6 glioblastoma. The effect of an analog of this molecule, 7β-hydroxycholesterol-3β-O(ether)-oleyl (7β-ether), was investigated. Materials and Methods: Liposomes containing no oxysterol (control), 7β-ether or 7β-ester were injected into tumors induced by C6 cells in rat brain cortex. At defined times, the animals were sacrificed, the tumors stained with cresyl violet and their volumes measured by densitometry. Oxysterol clearance was assessed by quantification from lipid extraction of treated tumors. Results: The clearance of the new compound was slower than that of the 7β-ester form. The 7β-ether and 7β-ester forms displayed similar antitumor activities against 3-day-old tumors. In contrast, the 7β-ether form was more active on well-developed glioblastoma: 75 nmol inhibited tumor growth by 70% compared to controls, while the 7β-ester had no effect under such conditions. The 7β-ether form had a cytostatic rather than a cytotoxic effect. In addition, the composition of the liposomes did not affect the antitumor activity. Conclusion: Only blockade of the C-3-OH group is required for the antitumor effect of this kind of oxysterol. It is suggested that the absence of "etherases" enhances the antitumor activity of this type of compound. Thus, an original therapeutic approach for glioblastoma treatment may be envisaged with such compounds.*

Correspondence to: Drs. Clovis Rakotoarivelo and Marcel Mersel, Institut des Neurosciences de Montpellier, Unité 583 de l'INSERM, 80 rue Augustin Fliche, 34091 Montpellier Cedex 05, France. Tel: +33 4 99 63 60 56, Fax: +33 4 99 63 60 20, e-mail: rakoto@montp.inserm.fr / mmersel@univ-montp1.fr

Key Words: C6 cells, stereotaxy, oxysterols, antitumor effect, glioblastoma.

Astrocytes are the most abundant cells present in the central nervous system. It is now established that their role is not restricted to structural and metabolic support for neurons, but also that they are sensors of neuronal activity and contribute to signal transmission. Two major pathologies derive from this cell type: an inflammatory process following brain injury, termed reactive gliosis, and a tumor process, termed astrocytoma or glioblastoma.

Glioblastomas are the most commonly diagnosed brain tumor in adults aged 45-74 yeast. In most European and North American countries, the incidence is approximately two to three new cases per 100,000 people per year (2,400 cases per year in France). Glioblastoma is essentially characterized by poorly-differentiated neoplastic astrocytes.

Based on the World Health Organization classification (WHO) which confers four grade degrees for astrocytoma, glioblastoma multiforme (GBM) is the most malignant (WHO, grade IV). Gliosarcoma and giant cell glioblastoma are variants of GBM. They typically contain more than one cell type. One therapeutic treatment may prevent division of some cell populations, but not that of the others. This characteristic makes glioblastomas very difficult to treat.

GBMs commonly overexpress the oncogenes for the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) and contain mutations and deletions of the tumor suppressor genes *PTEN* (phosphatase and tensin homolog) and TP53. Some

of these alterations lead to activation of the PI3K/Akt and Ras/MAPK pathways.

Some experiments of suicide gene therapy mediated by the thymidine kinase gene of the Herpes Simplex Type1 virus (HSV1) combined with ganciclovir have been performed on experimental gliomas and clinical protocols were established for the treatment of human glioblastoma 15 years ago (2-4). However, Hadaczek *et al.* (5) evoked two limitations to this strategy: expression of thymidine kinase in dividing tumor cells is rapidly decreased and there is a focal transduction of the brain tumor. Moreover, ganciclovir (a nucleotide analog) has adverse side-effects, such as neutropenia, thrombocytopenia, attention troubles, cutaneous rashes and also possesses teratogenic potential. Based on the fact that GBM expresses some antigens which differ from those of the normal brain tissue, other authors proposed stimulating a stronger immune response in patients with glioblastoma by using lymphokine-activated killer cells as cytotoxic T lymphocytes combined with interleukin-2 treatment (6, 7).

In spite of classic protocols combining surgical resection, radiotherapy and/or chemotherapy [Temodal agent/ radiotherapy (8) or surgery/external beam radiotherapy using GliaSite brachytherapy (9)] and new therapeutic approaches such as: (i) bi-/trichemotherapies [paclitaxel/ topotecan/ filgrastim; (10)] or (ii) dendritic cell vaccines (11) associated with Cilengitide (12), an angiogenesis inhibitor, the prognosis of malignant gliomas remains bleak, with a poor survival rate over a 2-year period, especially for glioblastoma (13). Currently, genetic, biological and pharmaceutical approaches are being undertaken for the detection of the tumor cells in their earliest stage of development.

Oxysterols are usually described as lethal for tumoral cells (14). This family of compounds consists of cholesterol bearing additional mono- or multi-oxygenated functions (alcohol, ketone, peroxide, epoxide). Among them, a variety of monohydroxylated forms constitute the major representatives of this family. One of them, 7β-hydroxy-cholesterol (7β-OHCH) has been thoroughly investigated, because of its marked cytotoxic effect toward hepatomas or lymphomas (15, 16). In addition, the lethal effect of 7β-OHCH on spontaneously transformed astrocyte cell lines (ACL), but not on the mother cells (primary cultures of neonatal rat astrocytes), has been previously demonstrated in our laboratory (17).

In recent years, a few groups have described oxysterols as antitumoral drugs *in vivo*. Christ *et al.* (18) intra-peritoneally administered two water-soluble phospho-diesters of 7β-OHCH to mice with subcutaneous P815 mastocytoma: between 20 and 40% of the treated mice recovered completely. 7β,22R-dihydroxycholesterol treatment of rats with mammary carcinomas, induced by an intravenous injection of dimethylbenzanthracene, reduced both the number and tumor size and increased animal survival (19).

7β-Hydroxycholesteryl-3β(ester)-oleate (7β-ester form) has been previously demonstrated to inhibit the growth of experimental rat C6 glioblastoma (1), a model developed by Kaye *et al.* (20) and San Galli *et al.* (21). An intra-tumor injection of 36 nmol of this compound, incorporated into liposomes, reduced the tumor size by 70% compared to control animals (1), while 7β-OHCH or oleate themselves did not affect tumor growth.

In the present study, the effect of another derivative of 7β-OHCH, the 7β-hydroxycholesteryl-3β-O(ether)-oleate (7β-ether form), was is investigated on the same tumor model. The aims of this study were: (i) to verify if the presence of the ester group on C3-OH is necessary for antitumor properties, and (ii) to test if this molecule is more effective than the ester form, since the lack of enzymatic hydrolysis of the ether link in the brain would predict a better effect. The galenic preparation and the *in vivo* model are also improved in view of clinical assays.

Materials and Methods

Drug synthesis. a) 7β-Hydroxycholesteryl-3β(ester)-oleate was prepared in the steps as described previously, with some modifications (1). In the first step, 4-dimethylaminopyridine was added to catalyze the esterification reaction. Then, the reaction mixture was initially heated at 80˚C for 4 h before being left at room temperature for 48 h. Diethyloxide (Et_2O) (30 ml) was used to stop the reaction. Before the reduction reaction, the organic phase was placed in an ice bath and washed successively with NaOH (40 ml of a 5% solution), HCl (40 ml of a 5% solution) and Na_2CO_3 (40 ml of a 5% solution). Sodium borohydride (18.5 mmol) dissolved in methanol (10 ml) was subsequently added dropwise to the organic phase containing 7-keto-cholesteryl-3β(ester)-oleate for 15 min at 0˚C and for 90 min at room temperature. The reaction was quenched by adding acetic acid. Et_2O was evaporated *in vacuo* and 7β-hydroxycholesteryl-3β(ester)-oleate was extracted with 21 ml of a mixture of 2,2,4-trimethylpentane (Aldrich, USA)/methanol/ bidistilled water (10/10/1; v/v/v). The organic phase was washed twice with saturated sodium chloride (50 ml). The product was purified as described previously (16, 22, 23).

b) 7β-Hydroxycholesteryl-3β-O(ether)-oleyl was also prepared in two steps. First, the etherification of 7-keto-cholesterol was performed according to Pouzar's procedure (24). 7-Keto-cholesterol (Sigma, USA) (1 mmol), oleyl bromide (Sigma) (3 mmol) and tetrabutylammonium hydrogen sulfate (0.48 mmol, Aldrich) were dissolved in 10 ml of benzene (Prolabo, France). 7-Keto-cholesteryl-3β-O(ether)-oleyl was purified by thin layer chromatography (TLC) using benzene/Et_2O (19/1, v/v) as eluant. The reduction protocol to convert 7-keto into 7β-hydroxy was the same as described above.

Preparation of treatment solution. Liposomes containing oxysterols (7β-hydroxycholesteryl-3(ester)-oleate or 7β-hydrochycholesteryl-3-O(ether)-oleyl) were prepared according to Holmberg *et al.* (25), with some modifications (1). Regarding the lipid composition, either monosialoganglioside (GM1, Fidia, Italy) or cholesteryl-3-sulfate (CHS, Sigma) was mixed with phosphatidylcholine (PC, Sigma). The liposome compositions were PC/oxysterol/GM1 at

molar ratios 10:5:1 or PC/oxysterol/CHS at 20:5:4. The molar lipids/detergent ratio was 4.

Lipids dissolved in solvents were initially mixed vigorously in a conical tube, then evaporated under a stream of nitrogen. The viscous residue thus formed was redissolved in sterile PBS (Gibco-BRL) containing 20 mM n-octyl-β-D-glucopyranoside (Sigma). Liposomes were obtained by dialyzing this mixture against PBS overnight at 4°C, using a microdialyzer and dialysis membranes with an exclusion limit of 12,000-14,000 (BRL, USA) to remove the detergent. Control liposomes, without oxysterols, were prepared in the same way. The liposomes were then concentrated under a stream of nitrogen to obtain the required oxysterol concentration.

C6 cell cultures. The C6 glioma cell lines, originally cloned from an *N*-nitrosomethylurea-induced glioma (26), were purchased from the American Type Culture Collection (Bethesda, MD, USA). The cells were seeded in 80-mm dishes (Falcon, PolyLabo, France) at 37°C under a humidified 5% CO_2-95% air atmosphere and kept in Dulbecco's modified Eagle's medium (DMEM) containing glutamex-1 (Gibco-BRL, France) supplemented with 10% decomplemented fetal calf serum (Dominique Dutscher, France) and a cocktail of 10 μg/ml of penicillin and 10 μg/ml of streptomycin (Sigma, France).

Generally, after 4 days in culture (proliferative phase of growth), the cells were washed twice with DMEM and were harvested mechanically in fresh culture medium, centrifuged at 450 x g and finally resuspended in a small volume of DMEM in order to obtain $2x10^5$ cells/5 μl. This cell suspension was kept in the incubator at 37°C until implantation in rat brain cortex.

Glioblastoma implantation. The C6 cell implantation procedure was essentially the same as described previously (1). New-born Wistar rats, 7-day-old and weighing 10-14 g, were used as tumor hosts. The animals were housed in a restricted temperature controlled vivarium, on a 12:12 light-dark cycle and the pups were left with their mother until transplantation. They were anesthetized with a cocktail composed of Ketalar (5%, Parke Davis, France) 20 μg/g and Rompun-Xylasine (2%, Bayer, Germany) 1.3 μg/g (in the proportion of 8/2, v/v). Each animal was securely placed in a stereotaxic David Kopf surgical frame and the brain was exposed by a short incision of the skin. A small burr hole was drilled 4.5 mm anterior to the lambda and 2.0 mm lateral to the median suture. The dura was pierced and a Hamilton syringe (PolyLabo) with a blunted tip was placed into the right frontal cortex 2.2 mm deep to the surface of the brain. The cell suspension containing $2x10^5$ C6 cells was inoculated slowly (30 sec) into the right frontal cortex. The needle was left in place for 2 min after injection and then slowly withdrawn. The skin was sutured. The pups were returned to the mother only after the anesthetic effect had worn off.

Tissue collection and histology. At indicated dates, the grafted rats were anesthetized with a lethal dose of sodium pentobarbital (Sanofi, France) and perfused through the heart, first with 0.9% NaCl and then with a fixative composed of 3.6% (w/v) paraformaldehyde (Sigma) and 2.5% (w/v) gluteraldehyde (Sigma) in 0.1 M phosphate buffer, pH 7.4. Sometimes 0.4% of Trypan Blue Solution (Sigma) was added to this solution. The brains were removed and immersed in the same fixative solution for 5-7 days, dehydrated in graded alcohol and embedded in histowax (Reichert-Jung, Germany). Ten-μm-thick sections were cut and stained with basic Cresyl Violet (Sigma).

Dose of treatment. The rats were anesthetized as described in the "Glioblastoma implantation" section and the liposomes were administered stereotactically. Liposomes containing 7.5, 60, 75 or 150 nmol of 7β-hydroxycholesteryl-3β-O(ether)-oleyl, 60 or 150 nmol of 7β-hydroxycholesteryl-3β(ester)-oleate were used; the injection volume was 5 μl.

Oxysterol extraction.

a) Liposomes. The lipid extraction of liposomes was carried out according to Folch *et al.* (27). Briefly, 400 μl of deionized water and 10 ml of chloroform (HPLC grade, Carlo Erba, Italy): methanol (HPLC grade, Carlo Erba), 2:1 v/v (C:M, 2:1) were added to 100 μl of liposome solution. After mixing, the single phase was allowed to settle for 30 min and then washed with 0.2 volume of NaCl (0.73%, w/v in water). After centrifugation, the upper phase was discarded and the organic phase evaporated to dryness under a stream of nitrogen.

b) Tumors. Tumors removed from non-fixed brains were homogenized (3000 t/min for 10 min, Bioblock Scientific, France) in H_2O at the ratio of 10-15 mg of fresh tissue/ml. Lipids were extracted according to Folch *et al.* (27) as described above, except that the "breaking" single phase was water only (0.2 volume). The extracts were kept at 37°C until organic/aqueous phase separation occurred (at least 2 hours). The organic phase was then evaporated under a stream of nitrogen.

Oxysterol quantification.

a) High performance thin layer chromatography (HPTLC). The lipid residue was dissolved in C:M, 2:1, and subjected to thin-layer chromatography (Precoated silica 60 HPTLC plates, Merck, Germany). Two-dimensional elution was carried out in chloroform/acetone (80/120, v/v) and benzene-ether (17/3, v/v). Lipids were visualized using the Macala reagent (28). HPTLC plates were heated at 135°C until purple (corresponding to cholesterol) and blue (7β-hydroxycholesterol and its derivatives) appeared. A quantitative analysis of spot intensity was performed by scanning the HPTLC plates (Densitometer, Molecular Analyst, Bio-Rad, USA) and comparing the scans with standards from 0.2 to 5.0 μg.

b) High pressure liquid chromatography (HPLC). Dry lipids were dissolved in 200-500 μl of acetonitrile (Merck, HPLC grade): isopropanol (Carlo Erba, HPLC grade): H_20 (44:54:2, v:v:v). The same mixture was used for HPLC in isocratic mode. Chromatography was carried out with a Waters Associates HPLC equipped with a reverse phase lichrosphere column (Interchim, USA; K10 OD2, 10 μm granulometry, 250 mm long and 2.2 mm internal diameter); the column was protected with a guard cartridge (Waters associates). The pressure was 500 PSI and the compounds were detected at 206 nm. The retention times for the 7β-ester and 7β-ether forms were 15.2 and 21.0 min, respectively; cholesterol, cholesteryl-esters, 7-keto-cholesterol and 7-hydroxy-cholesterol were used for HPLC calibration.

c) Control experiments. HPLC *versus* HPTLC analysis of oxysterol levels in liposomes and brain tumors gave the same values. Thus, the majority of oxysterol determinations were carried out with the HPTLC method. Analysis of the oxysterol content in liposomes was 90-100% of the initial oxysterol amounts added for liposome preparation.

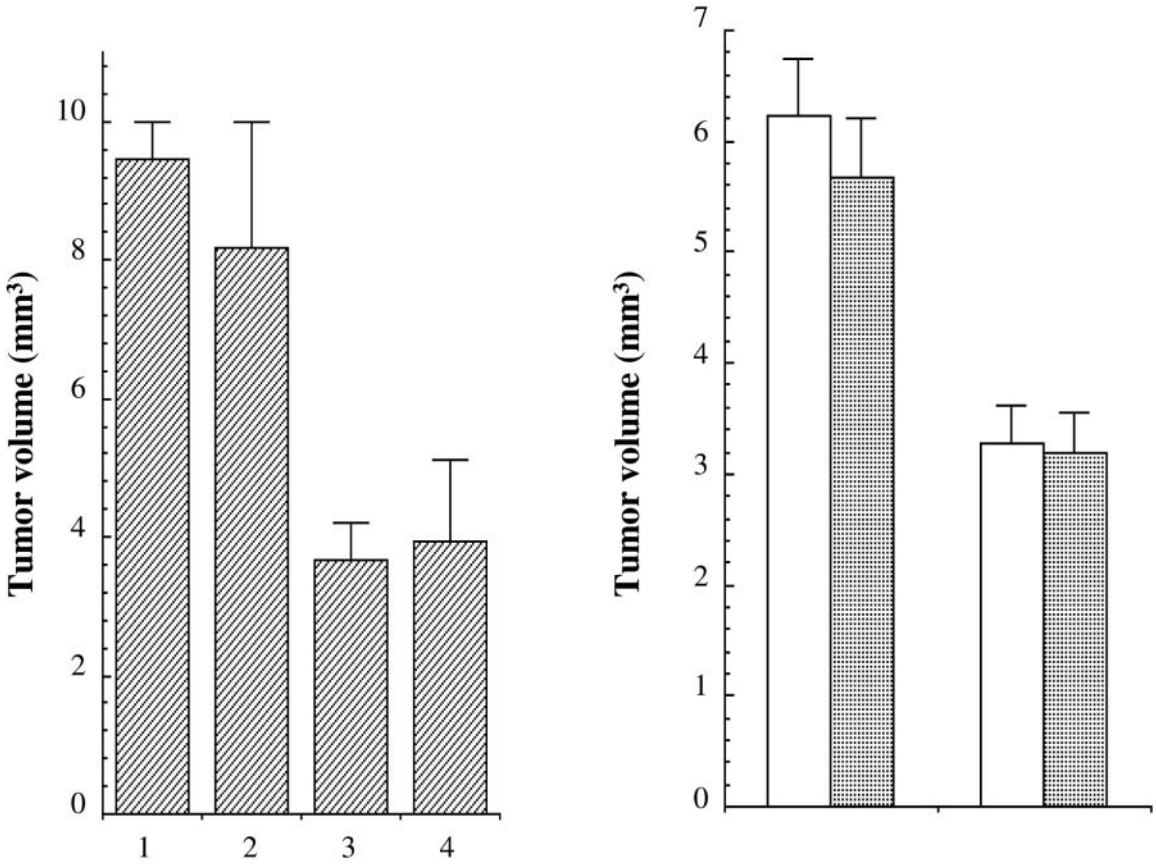

Figure 1. *Effect of 7β-hydroxycholesteryl-3β-O(ether)-oleyl (7β-ether) on C6 glioblastoma induced in Wistar rat brain cortex. (A) Dose/response evaluation. Treatments were performed on 3-day-old glioblastoma and tumor volumes calculated 10 days after the liposome injection. Liposomes composed of PC and GM1 contained: 0 nmol (control) (1); (2) 7.5 nmol; (3) 60 nmol; (4) 150 nmol of 7β-ether. (B) Comparison between the liposome composition on the 7β-ether activity (60 nmol) on 3-day-old glioblastoma; the effect was analyzed 9 days after the oxysterol injection. Open bars correspond to GM1/PC liposomes and solid bars to CHS/PC liposomes.*

Determination of tumor volume and statistics. The dimensions of the tumors on each slice were measured by microscopy and densitometry (Molecular Analyst, Bio-Rad). After approximating the shape of the tumor to an ellipsoid, the tumor volumes were estimated by the formula 4/6 x π x (largest width x largest height x number of slices x thickness of slices).

Data, expressed as means±SEM values, were compared using one way ANOVA; *post hoc* analysis was performed using Student-Newmann-Keuls multiple comparisons test (Instat, GraphPad, USA); $p<0.05$ was considered significant.

Results

Dose-response effect of 7β-hydroxycholesteryl-3β-O(ether)-oleyl on C6 glioblastoma growth. The inoculation of $2x10^5$ C6 cells into the frontal cortex of 7-day-old Wistar rats produced large tumors (mean volume 9.44±0.70 mm^3) 13 days later. Treatment solutions were injected 3 days after C6 implantation and the tumors were examined 10 days later. Three kinds of treatment were tested: PBS solution (control 1, data not shown), liposomes containing 0 (control 2) or 60 nmol 7β-ether. Treatment controls had no effect on the tumor size. In contrast, 60 nmol 7β-ether significantly reduced the tumor growth by 62% and, in 80% of cases, the tumor volume was decreased by at least 50% (Figure 1A).

To evaluate the dose-response relationship, two extreme doses were tested: 7.5 nmol (threshold) and 150 nmol (high dose). 7β-Ether at 7.5 nmol decreased the tumor size by at least 25% (8.18 mm^3±1.80, $p=0.54$), in 60% of cases. 7β-Ether at 150 nmol had the same effect as at 60 nmol ($p=0.83$) (Figure 1A).

The influence of the lipid carrier composition on treatment efficiency was also compared. For this, PC/GM1 *versus* PC/CHS containing 0 (control) or 60 nmol of 7β-ether were tested (Figure 1B). No significant differences were found: (i) between liposome control treatments (mean size volume at 6.23±0.51 and 5.68±0.52 mm^3, GM1- and CHS- based liposomes, respectively) or (ii) between oxysterol-based liposomes (3.27±0.34 mm^3 and 3.19±0.35

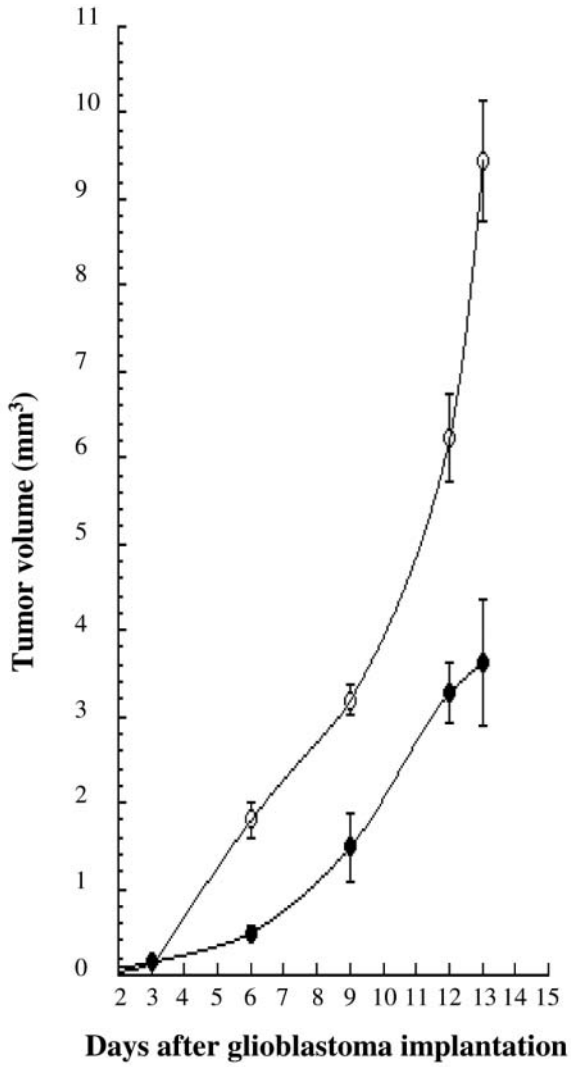

Figure 2. *Comparison between the effect of control liposomes and liposomes containing 7β-hydroxycholesteryl-3β-O(ether)-oleyl (7β-ether) on the development of C6 rat glioblastoma induced in Wistar rat brain cortex. The tumors were treated 3 days after C6 glioblastoma implantation with liposomes containing no oxysterol (control) or 60 nmol of 7β-ether. Open circles correspond to control tumors; dark circles to 7β-ether-treated tumors.*

mm^3, PC/oxysterol/GM1 and PC/oxysterol/CHS, respectively; $p=0.884$). In both cases, the scatter of tumor volumes was identical: 60% of oxysterol-treated tumors were 50% smaller than the controls.

Follow-up of tumor growth rate after treatment with 60 nmol of 7β-hydroxycholesteryl-3β-O(ether)-oleyl. The time-curve of tumor growth is shown in Figure 2. Tumors were treated with liposomes containing either 0 (control) or 60 nmol of 7β-ether 3 days after C6 cell implantation. The rats were sacrificed at 3, 6, 9, 10 or 12 days after oxysterol injection. Tumor growth patterns followed an exponential slope up to 13 days, as described previously by San Galli *et al.* (21). Up to 13 days after glioblastoma implantation, tumor growth was significantly inhibited by the 7β-ether form (Figure 3). Between the third and ninth days, the tumor mass for the controls increased by 0.94 mm^3/day, while that of oxysterol-treated tumors increased by 0.45 mm^3/day only. Calculation of the tumor size variation (=7β-ether - controls) gave: 1.3, 1.7, 2.96 and 5.8 mm^3 at 6, 9, 12 and 13 days, respectively. These data indicated that the 7β-ether form markedly inhibited growth (Figure 3) Serial sections were obtained on 13-day-old tumors.

Effect of 7β-hydroxycholesteryl-3β-O(ether)-oleyl versus 7β-hydroxycholesteryl-3β(ester)-oleate on undeveloped tumors. Nine days after treatment with 60 nmol of oxysterol-based liposomes, *i.e.,* 12 days after implantation, the rats were sacrificed and the tumors were measured. As compared with the controls (mean size 6.23±0.50 mm^3), the 7β-ester form reduced the tumor size by 75% and the 7β-ether form by 48% (Figure 4). Due to the large dispersion of tumor volume, the difference between these treatments was not statistically significant ($p=0.13$). However, the 7β-ester form lowered the tumor size by 50% in at least 85% of the experiments, whereas the 7β-ether treatment had a more homogenous effect, but in only 40% of the cases was the tumor size decreased by 50%.

Effect of 7β-hydroxycholesteryl-3β-O(ether)-oleyl versus 7β-hydroxycholesteryl-3β-O(ester)-oleate on well-developed tumor. The short-term effects of oxysterol treatment on well-developed glioblastoma were also investigated. Treatment was carried out 12 days after C6 cell implantation (tumor size 6.23±0.51) and the animals were sacrificed 3 days later, *i.e.,* 15 days after implantation. The results are represented in Figure 5. For control liposome treatment, the mean tumor size was 28.65±5.99 mm^3. At 150 nmol of oxysterol, tumor progression significantly stopped: the mean volumes were 8.66±2.59 ($p=0.0044$) and 11.10±5.01 ($p<0.05$) mm^3 for 7β-ester and 7β-ether, respectively. In 60% of the cases, the tumoral mass after treatment with each of these oxysterols was at least 50% smaller than when treated with control liposomes.

Subsequently, the double treatment, 150 nmol oxysterols, composed of a mixture of 75 nmol of the 7β-ester form and 75 nmol of the 7β-ether form, were tested. The tumor size was 8.52±3.97 mm^3 ($p<0.01$) *versus* 28.65±5.99 mm^3 (control liposomes). In parallel, single treatments at 75 nmol (7β-ester or 7β-ether) were carried out. The data in Figure 5 show clearly that the antitumoral effect is due to the ether (9.12±1.96 mm^3, $p=0.03$); 75 nmol of 7β-ester (Figure 5, bar 5) had no effect on the tumoral growth (29.60±3.19 mm^3, $p=0.9$) under these experimental conditions.

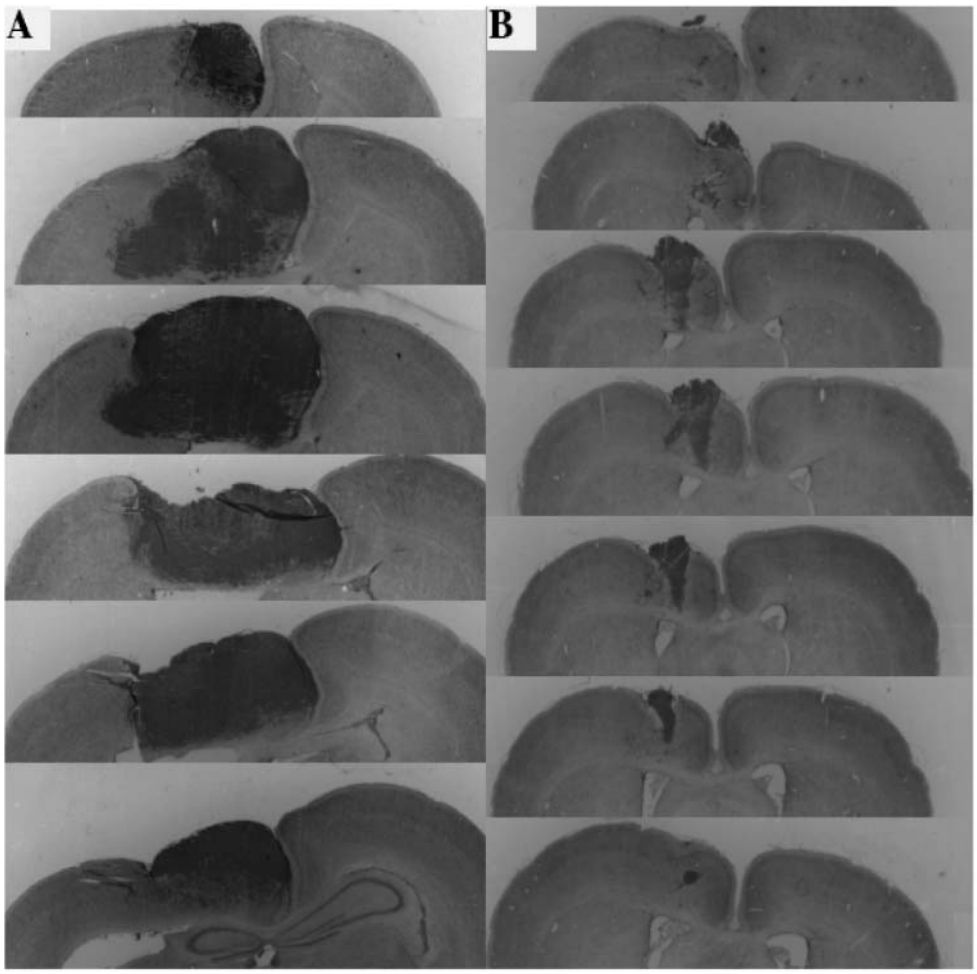

Figure 3. *Histochemistry of C6 glioblastoma induced in Wistar rat brain; 10-μm sections, embedded in paraffin, were stained with Cresyl Violet as described in Materials and Methods. Three days after tumor implantation, the rats were injected with liposomes containing no oxysterol (control) (A) or 60 nmol of 7β-hydroxycholesteryl-3β-O(ether)-oleyl (B). These serial sections correspond to a tumor at 13 days of development.*

Oxysterol clearance. In this set of experiments, the rats were double-treated (75 nmol of each oxysterol) 12 days after C6 cell implantation, as described above. At the defined time, oxysterol extraction was performed on the treated tumors. Despite optimizing oxysterol extraction techniques (data not shown), the oxysterol recovery was low: only 5.2% of the 7β-ether and 6.8% of the 7β-ester were recovered in tumors within 20 min of treatment. As indicated in Figure 6, the quantity of 7β-ether remained constant while the 7β-ester form decreased in the 3 days after treatment: only 25% of 7β-ester remained in the tumors.

Discussion

We previously demonstrated the antitumoral properties of 7β-hydroxycholesteryl-3β(ester)-oleate, synthesized in our laboratory, on C6 glioblastoma induced in rat brain (1).

We also compared the effects of 7β-ester and 7β-ether forms on spontaneously transformed astrocytes derived from primary rat astrocytes *in vitro* (29). Both compounds displayed an antiproliferative effect, but the decrease of the thymidine incorporation rate was more pronounced with 7β-ether. Moreover, we demonstrated, *in vitro,* that the level of the 7β-ether form remaining in the cells was markedly higher than that of the 7β-ester form. These results encouraged us to test the therapeutic potential of the 7β-ether form on C6 glioblastoma *in vivo*.

The procedures employed here for obtaining rat glioblastoma *in vivo* were modified compared to those previously used (1). For instance, the C6 cells were injected using DMEM instead of NaCl, in order to increase the tumor induction efficiency. However, the principal aims of this study were to compare two types of carriers by varying the lipid composition and to follow the tumor growth curve

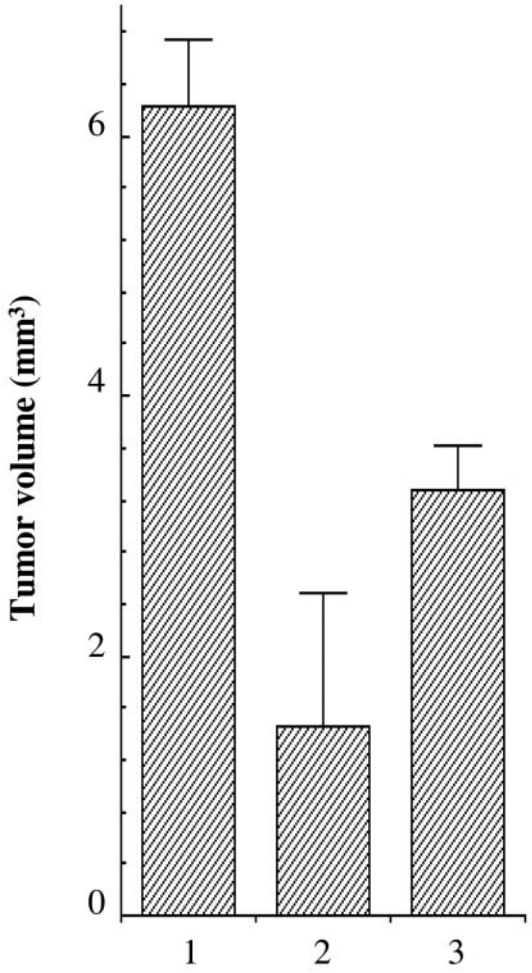

Figure 4. *Comparison between the effects of 7β-hydroxycholesteryl-3β-O(ether)-oleyl (7β-ether) and 7β-hydroxycholesteryl-3β(ester)-oleate (7β-ester) on 3-day-old glioblastoma. The animals were treated at 9 days before sacrifice; the liposomes contained no oxysterol (control) (1), 60 nmol of 7β-ester (2) or 7β-ether (3).*

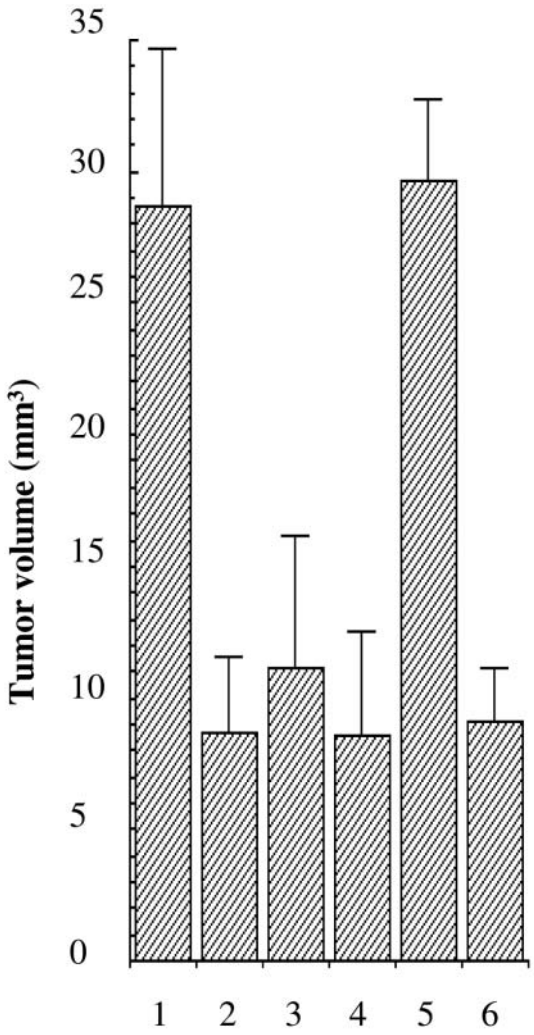

Figure 5. *The effects of 7β-hydroxycholesteryl-3β-O(ether)-oleyl (7β-ether) and 7β-hydroxycholesteryl-3β(ester)-oleate (7β-ester) on well-developed C6 glioblastoma. The tumors were injected 12 days after C6 cell implantation and the animals were sacrificed 3 days later: the liposomes contained no oxysterol (control) (1); 150 nmol of 7β-ester (2) or 7β-ether (3); 75 nmol of 7β-ester and 75 nmol of 7β-ether (4); 75 nmol of 7β-ester (5) or 7β-ether (6).*

for a long period in order to examine the antitumoral effect of the drugs on undeveloped and well-developed tumors.

The results indicated that a single local treatment with the 7β-ether form efficiently stopped tumor progression (Figure 1A). Our findings (Figure 2) indicated that this effect was more cytostatic than cytotoxic. We also demonstrated that the liposome composition did not change the oxysterol antitumoral efficiency (Figure 1B). We had previously used GM1 in order to enhance the hydrophilicity of liposomes, thereby further increasing drug uptake. The neuroactive properties of GM1 may also favor nervous tissue recovery, while retarding liposome clearance (1). However, GM1 was removed in view of clinical assays. Indeed, there are reports demonstrating, in a few cases, that injection of gangliosides (extracted from mammalian brain) may produce acute neuropathological illness such as the Guillain-Barré (30) or Kreutzfeld-Jacob syndromes. GM1 may also produce some immune responses (31). So, GM1 was replaced by CHS, a natural component of skin (32), used in cosmetic liposome preparations. Injection of liposome controls composed solely of PC/GM1 or PC/CHS does not affect tumor growth and substitution of GM1 by CHS does not modify the antitumor property of the 7β-ether form.

With regard to the 7β-ether form, dose/response studies showed that 7.5 nmol had little effect on tumor growth, which was not the case for the 7β-ester form (1). Surprisingly, a similar effect was observed for both 60 and

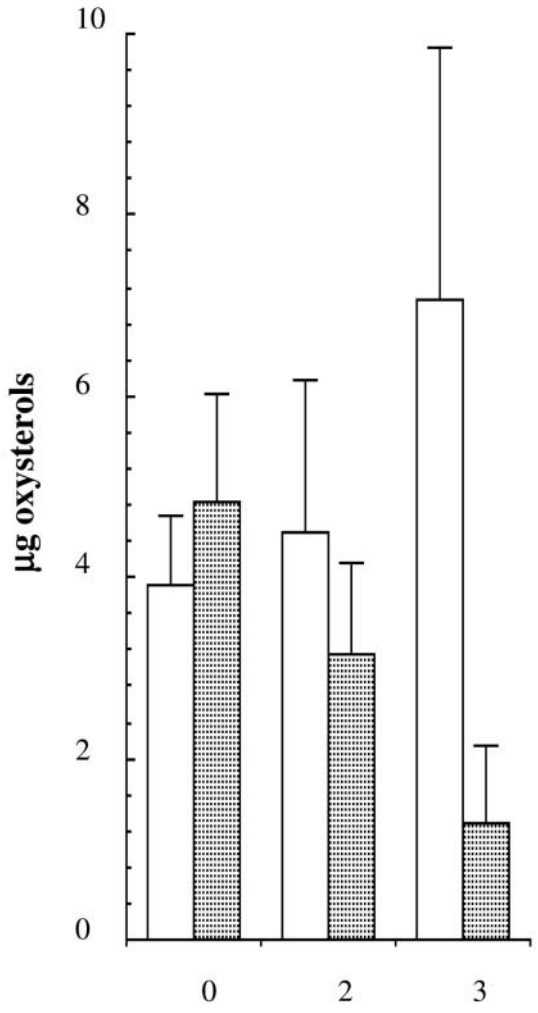

Figure 6. *Oxysterol clearance. Liposomes containing both 7β-hydroxycholesteryl-3β(ester)-oleate (7β-ester) and 7β-hydroxycholesteryl-3β-O(ether)-oleyl (7β-ether), 75 nmol each, were injected into the tumor site 12 days after C6 cell inoculation. The tumors were removed and the oxysterols were extracted as described in Materials and Methods. Open bars correspond to the treatment with 7β-ether and solid bars to 7β-ester.*

150 nmol of the 7β-ether form, suggesting that, under these experimental conditions, the antitumoral effect of the compound reaches a "plateau" between these two values. As for the 7β-ester form, a possible explanation is that the liposomes containing 60-150 nmol of the 7β-ether form were too concentrated and thus, too sticky to easily spread into the tumor site, since the injection volume was limited. The 7β-ether form is more hydrophobic than the ester and, thus, may be better incorporated into the liposomes. Hence, in unvascularized tumors, 7β-ester delivery may be better than that of the ether.

Comparing the mean tumor size after treatment of 3-day-old tumors with 60 nmol of the 7β-ester and 7β-ether forms demonstrated that the ether form was not more effective than the ester form; however, the statistics suggest that the 7β-ester form was more efficient.

Treatment of well-developed glioblastoma by both oxysterols resulted in the same efficiency at 150 nmol, but at 75 nmol, only the 7β-ether form showed an antitumor effect. In these experiments, the amount of ether in large tumors was higher than that of the 7β-ester form (Figure 6). Interestingly, 75 or 150 nmol of the 7β-ether form displayed the same antitumor effect on 12-day-old glioblastoma.

San Galli *et al.* (21) reported that, 3 days after C6 cell implantation, new blood vessels were observed in the tumor tissue. We suggest that vascularization in well-developed tumors (12 days) is well-established and this facilitates diffusion of the highly hydrophobic ether, which, in turn, is more active than the ester since "etherases" are not present in the tissue. Diffusion phenomenon may also explain the small quantity of oxysterols found in vascularized tumors only 20 min after double treatment (Figure 6).

In the normal adult brain, there is practically no synthesis of cholesterol, while in brain tumors this biosynthesis can be 15 times higher (33). Fumagalli *et al.* (34) observed an unusual accumulation of lanosterol and 24-dihydro-cholesterol in biopsies of glioblastoma, suggesting that a key enzyme in the biosynthetic pathway of cholesterol, the HMG-CoA reductase (EC 1. 1. 1. 3. 4), is activated. In the last decade, targeting glioblastoma cell lipid metabolism with lovastatine, a specific inhibitor of HMG-CoA reductase, has provided a novel approach to the treatment of malignant gliomas (35). Oxysterols are known to inhibit HMG-CoA reductase. Recent reports support another action mechanism of 7β-OHCH induced by apoptosis (36-38) in different *in vitro* tumor models. In addition to those mechanisms, alteration of plasma membrane fluidity, stability and function *in vitro* by 7β-OHCH should also be considered (39, 40). Our *in vitro* studies on spontaneously transformed astrocyte cell lines derived from primary cultures of neonatal rat astrocytes indicated that the lethal effect of 7β-OHCH was not correlated with: (i) changes of the biochemical composition or biophysical properties of the plasma membrane (41); or (ii) HMG-CoA inhibition (42). On the other hand, we demonstrated a clear-cut relationship between the cytotoxicity of 7β-OHCH and its esterification at C-3-OH by naturally-occurring fatty acids (43).

The cytotoxic effect of 7β-OHCH on C6 cells *in vitro* is correlated with the inhibition of mitogen-activated protein kinase (MAPK) activity, probably by inhibition of p21ras post-translational farnesylation. Oxysterol may indeed inhibit at the farnesyl level (44). To our knowledge, no data are available on the action mechanisms of oxysterols *in vivo*. *In vivo* and *ex vivo* experiments, using the ester or the ether as antitumor drugs, could address the question of whether the suggested action mechanism *in vitro* is also valid *in vivo*.

In conclusion, this is a first report to demonstrate the antitumor effect of 7β-hydroxycholesteryl-3β-O(ether)-oleyl. The main advantage of this compound is its resistance to enzymatic degradation, thus enabling it to persist at the site of the tumor. These findings demonstrate that the blockade of the C-3-OH group is sufficient for the antitumor effect of 7β-OHCH.

The 7β-ester form is currently authorized by l'AFSSAPS (Agence Française de Sécurité Sanitaire des Produits de Santé, Direction de l'évaluation des médicaments et des produits biologiques; Paris, France) to be used in clinical trials as an antiglioblastoma drug (Départements de Neurologie et de Neurochimie de l'hôpital d'Instruction des Armées; Val de Grâce, Paris, France). If the observations are encouraging, a request for the use of 7β-ether will be made. In addition, peripheral chemotherapy on glioblastoma with these oxysterols may be considered. Due to the low molecular weight and the hydrophobic properties of the molecules, the carriers are known to bypass the blood brain barrier.

Acknowledgements

This work was supported by the Association pour la Recherche contre le Cancer (Paris, France), Ligue contre le Cancer (Colmar, France) and INSTEL CHIMOS (MARSING group, Issy-les-Moulineaux, France).

Patent pending for 7β-hydroxycholesteryl-3β(ester)-oleate: CNRS and HRA PHARMA (Paris, France): n° WO 92/01803, 06.02.92 (Europe, Japan and USA).

References

1 Werthle M, Bochelen D, Adamczyk M, Kupferberg A, Poulet P, Lutz P, Privat A and Mersel M: Local administration of 7 beta-hydroxycholesteryl-3-oleate inhibits growth of experimental rat C6 glioblastoma. Cancer Res *54*: 998-1003, 1994.

2 Barba D, Hardin J, Sadelain M and Gage FH: Development of anti-tumor immunity following thymidine kinase-mediated killing of experimental brain tumors. Proc Natl Acad Sci USA *91*: 4348-4352, 1994.

3 Bensimon G, Philippon J, Valery CA and Klatzmann D: Clinical protocol. Gene therapy for glioblastoma in adult patients: safety and efficacy evaluation of an *in situ* injection of recombinant retroviruses producing cells carrying the thymidine kinase gene of the herpes simplex type 1 virus, to be followed with the administration of ganciclovir. Hum Gene Ther *7*: 109-126, 1996.

4 Chen SH, Shine HD, Goodman JC, Grossman RG and Woo SL: Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*. Proc Natl Acad Sci USA *91*: 3054-3057, 1994.

5 Hadaczek P, Mirek H, Berger MS and Bankiewicz K: Limited efficacy of gene transfer in herpes simplex virus-thymidine kinase/ganciclovir gene therapy for brain tumors. J Neurosurg *102*: 328-335, 2005.

6 Furnari FB, Huang HJ and Cavenee WK: Genetics and malignant progression of human brain tumors. Cancer Surveys *25*: 233-275,1995.

7 Iwadate Y, Yamaura A, Sato Y, Sakiyama S and Tagawa M: Induction of immunity in peripheral tissues combined with intracerebral transplantation of interleukin-2-producing cells eliminates established brain tumors. Cancer Res *61*: 8769-8774, 2001.

8 Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO; European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med *352*: 987-996, 2005.

9 Chan TA, Weingart JD, Parisi M, Hughes MA, Olivi A, Borzillary S, Alahakone D, Detorie NA, Wharam MD and Kleinberg L: Treatment of recurrent glioblastoma multiforme with GliaSite brachytherapy. Int J Radiat Oncol Biol Phys *62*: 1133-1139, 2005.

10 Pipas JM, Meyer LP, Rhodes CH, Cromwell LD, McDonnell CE, Kingman LS, Rigas JR and Fadul CE: A phase II trial of paclitaxel and topotecan with filgrastim in patients with recurrent or refractory glioblastoma multiforme or anaplastic astrocytoma. J Neurooncol *71*: 301-305, 2005.

11 Wheeler CJ and Black KL: Dendritic cell vaccines and obstacles to beneficial immunity in glioma patients. Curr Opin Mol Ther *7*: 35-47, 2005.

12 Smith JW: Cilengitide Merck. Curr Opin Investig Drugs: 741-745, 2003.

13 Davis FG, Freels S, Grutsch J, Barlas S and Brem S: Survival rates in patients with primary malignant brain tumors stratified by patient age and tumor histological type: an analysis based on surveillance, epidemiology, and end results (SEER) data, 1973-1991. J Neurosurg *88*: 1-10, 1998.

14 Parish EJ, Chitrakorn S, Luu B, Schmidt G and Ourisson G: Studies of the oxysterol inhibition of tumor cell growth. Steroids *53*: 579-596, 1989.

15 Moog C, Deloulme JC, Baudier J, Revel MO, Bischoff P, Hietter H and Luu B: Membrane-related oxysterol function: preliminary results on the modification of protein kinase C activity and substrate phosphorylation by 7 beta,25-dihydroxycholesterol. Biochimie *73*: 1321-1326, 1991.

16 Hietter H, Bischoff P, Beck JP, Ourisson G and Luu B: Comparative effects of 7 beta-hydroxycholesterol towards murine lymphomas, lymphoblasts and lymphocytes: selective cytotoxicity and blastogenesis inhibition. Cancer Biochem Biophys *9*: 75-83, 1986.

17 Kupferberg A, Teller G, Behr P, Leray C, Urban PF, Vincendon G and Mersel M: Effect of 7 beta-hydroxycholesterol on astrocyte primary cultures and derived spontaneously transformed cell lines: cytotoxicity and metabolism. Biochim Biophys Acta *1013*: 231-238, 1989.

18 Christ M, Ji Yh, Moog C, Pannecoucke X, Schmitt G, Bischoff P and Luu B: Antitumor activity of oxysterols. Effect of two water soluble monophosphoric acid diesters of 7β-hydroxycholesterol on mastocytoma P815 *in vivo*. Anticancer Res *11*: 359-364, 1991.

19 Iversen O. H, Kolberg A, Smith-Kielland I and Stabursvik A: Inhibitory effect of an oxygenated cholesterol on the induction and progression of DMBA-induced mammary carcinomas in the rat. Virchovs Arch (Cell Path) *51*: 313-320, 1986.

20 Kaye AH, Morstyn G, Gardner I and Pyke K: Development of a xenograft glioma model in mouse brain. Cancer Res *46*: 1367-1373, 1986.

21 San Galli F, Vrignaud P, Robert J, Coindre JM and Cohadon F: Assessment of the experimental model of transplanted C6 glioblastoma in Wistar rats. J Neuro-Oncol *7*: 299-304, 1989.

22 Behr P, Kupferberg A, Leray C, Schellenbaum L, Urban PF, Vincendon G and Mersel M: Effect of dibutyryl cyclic AMP and isoproterenol on 7β-hydroxycholesterol cytotoxicity and esterification in transformed cell lines derived from astrocyte primary cultures. FEBS Lett *313*: 151-154, 1992.

23 Bochelen D, Eclancher F, Kupferberg A, Privat A and Mersel M: 7β-Hydroxycholesterol and 7β-hydroxycholesteryl-3-esters reduce the extent of reactive gliosis caused by an electrolytic lesion in rat brain. Neuroscience *51*: 827-834, 1992.

24 Pouzar V, Schneiderova L, Drasar P, Strouf O and Havel M: Synthesis of 2-propynyl ethers of steroid alcohols. Collect Czech Chem Commun *54*: 1888-1902, 1989.

25 Holmberg E, Murayama K, Litzinger DC, Wright S, Davis M, Kabalka GW, Kennel S J and Huang L: Highly efficient immunoliposomes prepared with a method which is compatible with various lipid compositions. Bioch Biophys Res Commun *165*: 1272-1278, 1989.

26 Benda P, Lightbody J, Sato G, Levine L and Sweet W: Differentiated rat glial cell strain in tissue culture. Science *161*: 370-371, 1968.

27 Folch J, Lees M and Sloane-Stanley GH: A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem *226*: 497-509, 1957.

28 Macala LJ, Yu RK and Ando S: Analysis of brain lipids by high performance thin-layer chromatography and densitometry. J Lipid Res *24*: 1243-1250, 1983.

29 Rakotoarivelo C: Synthèse de dérivés du 7-hydroxycholestérol et étude de leurs effets *in vitro* et *in vivo* sur des cellules gliales transformées ou tumorales: mécanismes d'action et applications thérapeutiques. PhD manuscript in Cellular and Molecular Biology awarded by Université Louis Pasteur, Strasbourg, France, 1998.

30 Geisler FH, Dorsey FC and Colemann WP: GM1 gangliosides for spinal cord injury. N Engl J Med *326*: 494, 1992.

31 Ala TA, Perfetti PA and Frey WH II: Two cases of acute anti-GM1 antibody elevation in response to exogenous GM1 without neurological symptoms. J Neuroimmunol *53*: 109-113, 1994.

32 Epstein EH, Williams ML and Elias PM: The epidermal cholesterol cycle. J Am Acad Derm *10:* 866-868, 1984.

33 Paoletti P, Soloway AH, Whitman B and Messer JR: Lipid biosynthesis from labelled precursors in an experimental brain-tumor bearing mouse. Neurochirurgia (Stuttg.) *9*: 12-18, 1966.

34 Fumagalli R, Grossi E, Paoletti P and Paoletti R: Studies on lipids in brain tumors. Occurrence and significance of sterol precursors of cholesterol in human brain tumors. J Neurochem *11*: 561-565, 1964.

35 Prasanna P, Thibault A, Liu L and Samid D: Lipid metabolism as a target for brain cancer therapy: synergistic activity of lovastatine and sodium phenylacetate against human glioma cells. J Neurochem *66*: 710-716, 1996.

36 Bischoff PL, Holl V, Coelho D, Dufour P, Weltin D and Luu B: Apoptosis at the interface of immunosuppressive and anticancer activities: the examples of two classes of chemical inducers, oxysterols and alkylating agents. Curr Med Chem *7*: 693-713, 2000.

37 Roussi S, Winter A, Gosse F, Werner D, Zhang X, Marchioni E, Geoffroy P, Miesch M and Raul F: Different apoptotic mechanisms are involved in the antiproliferative effects of 7beta-hydroxysitosterol and 7beta-hydroxycholesterol in human colon cancer cells. Cell Death Differ *12*: 128-135, 2005.

38 Kang KA, Chae S, Lee KH, Park MT, Lee SJ, Lee YS and Hyun JW: Cytotoxic effect of 7beta-hydroxycholesterol on human NCI-H460 lung cancer cells. Biol Pharm Bull *28*: 1377-1380, 2005.

39 Verma SP, Philippot JR and Wallach DF: Chain length dependent modification of lipid organization by low levels of 25-hydroxycholesterol and 25-hydroxycholecalciferol. A laser Raman study. Biochemistry *22*: 4587-4591, 1983.

40 Smith LL and Johnson BH: Biological activities of oxysterols. Free Radic Biol Med *7*: 285-332, 1989.

41 Kupferberg A, Cremel G, Behr P, Van Dorsselaer A, Luu B and Mersel M: Differential sensitivity of astrocyte primary cultures and derived spontaneous transformed cell lines to 7β-hydroxycholesterol: effect on plasma membrane lipid composition and fluidity, and on cell surface protein expression. Mol Cell Biochem *101*: 11-22, 1991.

42 Behr P, Kupferberg A, Leray C, Urban PF and Mersel M: Effect of 7beta-hydroxycholesterol on astrocyte primary cultures and derived spontaneously transformed cell lines. Cytotoxicity and cholesterogenesis. FEBS Lett *280*: 202-206, 1991.

43 Kupferberg A, Behr P and Mersel M: Metabolism of 7 beta-hydroxycholesterol in astrocyte primary cultures and derived spontaneously transformed cell lines: correlation between the esterification on C-3 -OH by naturally occurring fatty acids and cytotoxicity. Biochim Biophys Acta *1046*: 106-109, 1990.

44 Adamczyk M, Scherrer E, Kupferberg A, Malviya AN and Mersel M: Inhibition of p42/p44 mitogen-activated protein kinases by oxysterols in rat astrocyte primary cultures and C6 glioma cell line. J Neurosci Res *53*: 38-50, 1998.

Received December 5, 2005
Revised February 23, 2006
Accepted March 1, 2006