A Lactosylated Steroid Contributes in Vivo Therapeutic Benefits in Experimental Models of Mouse Lymphoma and Human Glioblastoma

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Various mono- and disaccharides were grafted onto a steroid backbone. Whereas in vitro these glycosylated steroids had no cytotoxic effects on six different human cancer cell lines, several of the glycosylated steroids under study did significantly modify the levels of in vitro migration of the human U373 glioblastoma, the A549 non-small-cell-lung cancer (NSCLC), and the PC-3 prostate cancer cells, with more pronounced effects in the case of a monosubstituted β-L-fucopyranosyl-steroid (**19**), a monosubstituted β-D-isomaltosyl-steroid (**22**), and a monosubstituted β-D-lactosyl-steroid (**24**). These three compounds significantly increased the survival of conventional mice grafted subcutaneously with the P388 lymphoma, a lymphoma that metastasizes toward the liver. In vivo, the monosubstituted β-D-lactosyl-steroid (**24**) also increased the antitumor effectiveness of cisplatin, a cytotoxic pro-apoptotic drug, in the case of the P388 lymphoma model. This compound also increased the survival of immunodeficient mice into whose brains human U373 glioblastoma cells had been orthotopically grafted.

Introduction

One of four individuals dies from cancer in industrialized countries, and 90% of cancer patients die from their metastases. Once a metastatic cancer has been diagnosed in a patient, the probability that the patient will survive for more than a year after the initial diagnosis of his/her cancer is less than 50%. While glioblastomas do not metastasize outside the brain, they do remain associated with the most dismal prognosis for any type of human cancer because they diffusely invade the brain parenchyma during their development and, in so doing, avoid total surgical resection. Migrating cancer cells are protected against apoptosis.[1−4] Compounds that are able to reduce the migration levels of apoptotic-resistant cancer cells not only delay both the formation of metastases by cancer cells emerging from primary tumor sites and the invasion of the brain parenchyma by glioblastoma cells but also restore a certain level of sensitivity to apoptosis in these slowly migrating cells.[1−4] We have already shown that, in vitro, nontoxic 2-quinolone derivatives have an antimigratory effect on cancer cells and are a cause of additive in vivo benefits when combined with etoposide or adriamycin (two pro-apoptotic and cytotoxic compounds used to treat cancer patients) in the case of the MXT mouse mammary adenocarcinoma.[5] In the same way, we have shown in experimental glioblastomas that cimetidine (a H2 receptor antagonist[6]) increases the therapeutic effects of the pro-autophagic drug temozolomide by decreasing the levels of migration of malignant glial cells.[7]

In our quest to identify novel anticancer drugs with a potential antimigratory effect we decided to perform the bioguided fractionation of various marine sponges because these organisms are known to defend themselves against invaders by secreting cytotoxic and/or antimigratory compounds.[8,9] We observed that a number of compounds with steroid backbones from different sponges display both weak antimigratory and weak cytotoxic effects (unpublished data). We decided to start from a related and more accessible steroid skeleton (compound **1**, Scheme 1) in order to become independent of marine sponge sourcing. Derivatives of compound **1** that we have synthesized as detailed elsewhere[10] displayed some cytotoxic effects when added repeatedly to the human cancer cell culture media.[11,12] Antimigratory compounds that could be used to successfully combat migrating cancer cells have to be administered chronically to cancer patients. These compounds must therefore be noncytotoxic. We derived a series of novel steroids, of which compound **4** (see Scheme 1) was associated with a total absence of cytotoxicity and a certain level of antimigratory activity.[11,12] We then chose to synthesize novel glycosylated derivatives from compound **4** in order to endow this type of compound with real antimigratory, but noncytotoxic activity. The data obtained with the novel compounds that we describe in the present work show that we placed special emphasis on fucosylated and lactosylated analogues because (i) fucosylated moieties are known to be able to modulate tumor cell biology, especially in glioblastomas,[13,14] and (ii) β-lactoside moieties can bind to the galectins involved in the formation of metastases from epithelial cancers (carci-

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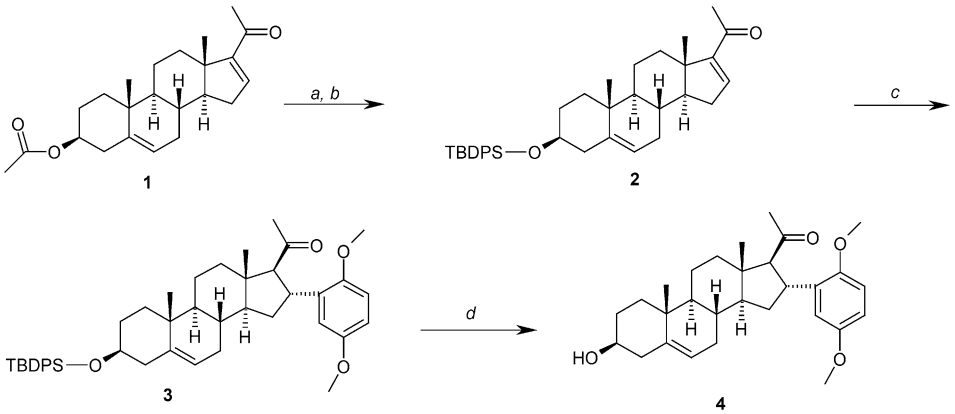
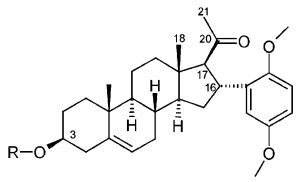
Scheme 1



(a) K_2CO_3, H_2O, MeOH; (b) *tert*-butyldiphenylsilylchloride, imidazole, DMF; (c) 1-bromo-2,5-dimethoxybenzene, Mg, 1,2-dibromoethane, CuI, Et_2O; (d) $FNtBu_4$, THF.

Table 1. (A) Compounds Synthesized from **4** and (B) Compounds Synthesized from **5**

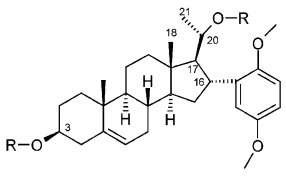
A



Compound	Glycosylation Method [a]	R
4	-	H
16	A	β-D-Glucopyranosyl
17	A	β-D-Mannopyranosyl
18	A	β-D-Galactopyranosyl
19	A	β-L-Fucopyranosyl
20	A	β-D-Cellobiosyl
21	A	β-D-Gentiobiosyl
22	A	β-D-Isomaltosyl
23	A	β-D-Maltosyl
24	A	β-D-Lactosyl
25	B	N-Acetyl-β-D-Glucosamine

[a] Method A used a silver catalyst and the method B a mercuric catalyst (see the SI section).

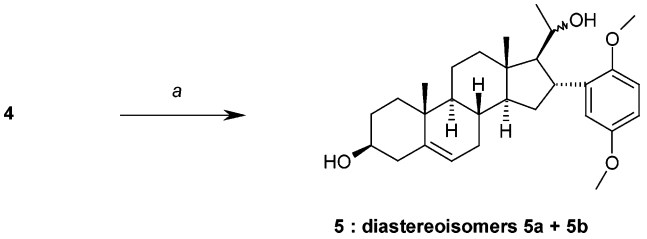
B



Compound	R
5 (5a+5b)	H
27	β-L-Fucopyranosyl
28	β-D-Cellobiosyl
29	β-D-Isomaltosyl
30	β-D-Lactosyl

nomas) and the development of malignancy in glioblastomas.[15,16] We in fact hypothesized that these glycosylated steroids could act as more or less specific antagonists for the cell adhesion receptors involved in cancer cell migration and the formation of metastases. We used the C-3 position of compound **4** to graft various oligosaccharide moieties (Table 1A). In addition, we used position C-20 to develop diglycosylated analogues from **5** (Scheme 2; Table 1B) in order to determine whether the grafting of two oligosaccharide moieties would enhance the antimigratory activity of monoglycosylated compounds.

Scheme 2



(a) $NaBH_4$, MeOH.

Chemistry

The steroid derivative **4** was prepared in four steps (Scheme 1). The intermediate **2** can be synthesized in four steps with an overall yield of 27%, as described previously.[17] We developed here a new method enabling us to obtain compound **2** in fewer steps with a higher overall yield (Scheme 1), as detailed in the Experimental Section.

The synthesized compounds under study are summarized in Tables 1A and 1B.

Pharmacology

In Vitro Cytotoxic Activity and in Vivo Tolerance. None of the glycosylated steroids under study had any significant cytotoxic effect on six different human cancer cell lines (including the Hs683 and U373 glioblastoma, the HCT-15 colon, the PC-3 prostate, the A549 NSCLC, and the MCF-7 breast cancer models). Indeed, the IC_{50} values of each of these compounds were higher than 10 μM for each of the six cancer cell lines under study. The IC_{50} value represents the concentration that reduces by 50% the overall growth level of a cell population after 3 days in the presence of the drug. In the same way, all these compounds were tolerated very well in vivo because they induced no toxic effects, at least in terms of both weight development and the survival of healthy mice for a minimum period of observation of 28 days after a single ip administration of 160 mg/kg.

Characterization of in Vitro Antimigratory Effects. Fourteen synthesized glycosylated steroids were submitted to in vitro cell migration analyses on three human cancer models in the shape of the A549 NSCLC, the PC-3 prostate cancer, and the U373 glioblastoma models, i.e., three of the six human cell lines on which the absence of cytotoxicity of these compounds had previously been demonstrated. Of the 14 compounds submitted to these cell migration analyses, only 7 had any significant ($p < 0.01$) antimigratory effect on at least one tumor cell line over

Table 2. Determination of the in Vitro Antimigratory Effects of Various Glycosylated Steroids on the Human A549 Non-Small-Cell-Lung, the PC-3 Prostate, and the U373 Glioblastoma Cancer Cell Lines[a]

	cancer cell lines						no. of cell lines on which a sustained (22 h) and significant ($p < 0.01$) antimigratory effect was obsd
	A549		PC-3		U373		
compd	12 h	22 h	12 h	22 h	12 h	22 h	
4	0 ± 5	6 ± 5	5 ± 9	10 ± 11	2 ± 10	7 ± 13	0
16	2 ± 7	9 ± 6	2 ± 8	6 ± 16	-6 ± 5	-8 ± 12	0
17	$20 \pm 6^{**}$	1 ± 8	2 ± 6	-1 ± 15	2 ± 5	-4 ± 7	0
18	2 ± 4	4 ± 4	7 ± 4	-8 ± 10	8 ± 6	1 ± 19	0
19	-8 ± 5	-8 ± 7	$-23 \pm 4^{***}$	$-15 \pm 6^{*}$	$-25 \pm 6^{**}$	$-38 \pm \mathbf{5}^{*}$**	1
20	$-30 \pm 5^{***}$	$-34 \pm \mathbf{5}^{*}$**	13 ± 10	-7 ± 9	8 ± 7	-9 ± 9	1
21	0 ± 7	-4 ± 5	$11 \pm 5^{*}$	-1 ± 9	0 ± 7	-1 ± 4	0
22	$-34 \pm 3^{***}$	$-40 \pm \mathbf{3}^{*}$**	-7 ± 9	-9 ± 6	$-18 \pm 4^{**}$	$-18 \pm \mathbf{5}^{*}$*	2
23	10 ± 6	5 ± 4	-5 ± 5	-5 ± 5	2 ± 6	-8 ± 9	0
24	8 ± 4	-5 ± 5	$-51 \pm 3^{***}$	$-34 \pm \mathbf{6}^{*}$**	$-29 \pm 4^{**}$	$-31 \pm \mathbf{5}^{*}$*	2
25	$-39 \pm 2^{***}$	$-39 \pm \mathbf{3}^{*}$**	11 ± 10	12 ± 7	$-17 \pm 4^{*}$	-3 ± 6	1
27	-6 ± 4	-4 ± 4	$-41 \pm 5^{***}$	$-45 \pm \mathbf{6}^{*}$**	-4 ± 6	-4 ± 4	1
28	4 ± 8	3 ± 9	9 ± 8	$-17 \pm 6^{*}$	-8 ± 4	4 ± 7	0
29	9 ± 5	0 ± 6	$8 \pm 3^{*}$	$10 \pm 4^{*}$	$-14 \pm 4^{*}$	-9 ± 7	0
30	$-26 \pm 5^{**}$	-12 ± 5	14 ± 8	$15 \pm 6^{*}$	$-12 \pm 5^{*}$	-13 ± 5	0

[a] The levels of migration were determined by means of computer-assisted videomicroscopy, with each of the compounds being tested for either 12 or 22 h in the presence (or absence for control) of a 100 nM concentration of each of the compounds under study. The control condition was arbitrarily normalized to "0%". The data are presented as mean values ± their standard errors. The statistical levels of significance are as follows: (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

at least 12 h of contact (Table 2). Of these 7 compounds, only 2 had a sustained (for at least 22 h) and significant ($p < 0.01$) antimigratory effect on at least 2 of the 3 tumor cell lines under study (Table 2). These two compounds were the monosubstituted β-D-isomaltosyl-steroid (**22**) and the monosubstituted β-D-lactosyl-steroid (**24**) (Table 2). The monosubstituted β-L-fucopyranosyl-steroid (**19**) gave rise to sustained antimigratory activity on the U373 glioblastoma model only (Table 2).

The bisubstituted β-D-isomaltosyl-steroid (**29**) and the bisubstituted β-D-lactosyl-steroid (**30**) displayed a lower level of antimigratory activity than their monosubstituted analogues (Table 2).

In Vivo Characterization of the Antitumor Effects of Compounds 19, 22, and 24 on a Subcutaneous Mouse Lymphoma Model Metastasizing to the Liver. When leukemic P388 cells of lymphoblastic origin[18] are grafted subcutaneously instead of intraperitoneally, they develop as anaplastic lymphomas.[19] The P388 lymphoma is very aggressive: its cells easily and rapidly cross the muscle wall of the peritoneal cavity and make enormous metastatic progress in the direction of the liver as early as one week post-tumor graft.[19]

As revealed by the in vitro cell migration analyses (Table 2), the two compounds under study associated with the greatest antimigratory activity were the monosubstituted β-D-isomaltosyl-steroid (**22**) and the monosubstituted β-D-lactosyl-steroid (**24**) (Table 1A). We therefore assayed these two compounds in vivo on the highly metastatic P388 lymphoma syngeneic model. Although the monosubstituted β-L-fucopyranosyl-steroid (**19**) displayed a lower level of antimigratory effects in vitro than the other two compounds (Table 2), we nevertheless decided to test this compound in the P388 lymphoma model as well because in vitro data cannot easily be extrapolated in vivo.[20] Figure 1A shows that while the monosubstituted β-D-isomaltosyl-steroid (**22**) and the monosubstituted β-L-fucopyranosyl-steroid (**19**) had no statistically significant ($p > 0.05$) effect on the growth of the subcutaneous P388 lymphoma, these two compounds were nevertheless the cause of significant ($p = 0.04$) increases in the survival of the P388 lymphoma-bearing mice. These antitumor effects could be considered as modest at first glance. It should nevertheless be emphasized that the antitumor effects associated with compounds **19** and **22** as observed here were more pronounced than the antitumor effects observed on the P388 lymphoma model with anticancer drugs widely used to treat cancer patients as, for example, adriamycin, vinblastin, vincristin, and paclitaxel.[19] The possibility remains that the increase in the survival periods brought about by the two glycosylated steroids relates to a decrease in the metastatic process of the subcutaneous P388 lymphoma cells toward the liver. We are now carrying out histological analyses to investigate this hypothesis. The P388 lymphoma is sensitive to platinum derivatives such as cisplatin,[19] a pro-apoptotic drug that is used to treat a broad panel of human malignancies.[21] The sensitivity of the P388 lymphoma to cisplatin is clearly evidenced in Figure 1B. As observed for the monosubstituted β-D-isomaltosyl-steroid (**22**) and the monosubstituted β-L-fucopyranosyl-steroid (**19**) (Figure 1A), the monosubstituted β-D-lactosyl-steroid (**24**) did not significantly ($p > 0.05$) decrease the growth of the subcutaneous P388 lymphoma (Figure 1B). However, as was also observed in the case of the monosubstituted β-D-isomaltosyl-steroid (**22**) and the monosubstituted β-L-fucopyranosyl-steroid (**19**) (Figure 1A), the monosubstituted β-D-lactosyl-steroid (**24**) also significantly increased the survival of the P388 lymphoma-bearing mice (Figure 1B). While the effects of cisplatin on the growth of the subcutaneous P388 lymphoma reached significant levels of statistical significance ($p < 0.05$ to $p < 0.01$ at the various days post-tumor graft), the increased survival rate of the P388 lymphoma-bearing mice brought about by cisplatin was no higher than the benefit brought about by the monosubstituted β-D-lactosyl-steroid (**24**) (Figure 1B). As for the monosubstituted β-D-isomaltosyl-steroid (**22**) and the monosubstituted β-L-fucopyranosyl-steroid (**19**), the possibility remains that the monosubstituted β-D-lactosyl-steroid (**24**) decreased the metastatic progress of the subcutaneous P388 lymphoma cells toward the liver, while cisplatin is known to have a number of direct cytotoxic effects on the growth of primary tumors.[19,21] When both cisplatin and the monosubstituted β-D-lactosyl-steroid (**24**) were used concomitantly to treat the mice bearing the P388 lymphoma, an additional therapeutical benefit was observed with respect to the growth of the primary subcutaneous P388 lymphoma and the survival level of the P388 lymphoma-bearing mice (Figure 1B). Such additional effects were not observed

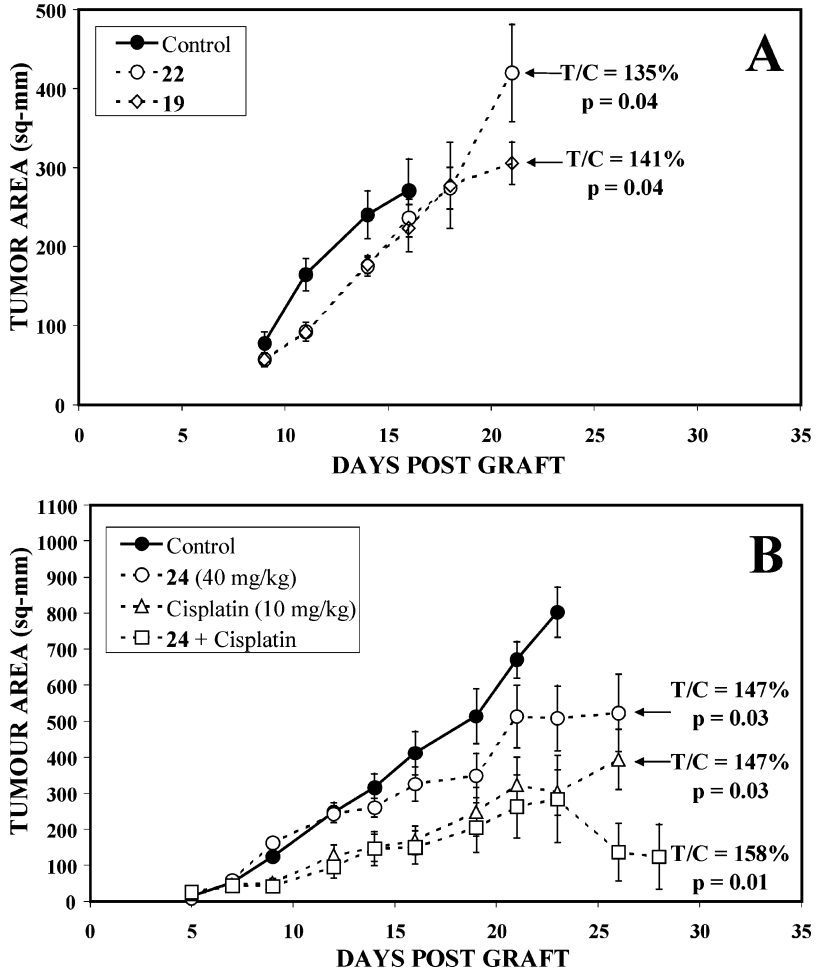


Figure 1. (A) The in vivo influence of a monosubstituted β-L-fucopyranosyl-steroid (**19**) and a monosubstituted β-D-isomaltosyl-steroid (**22**) on the growth (determined as the tumor area on the *Y* axis) of a subcutaneous P388 lymphoma metastasizing to the liver. The mice were grafted with the P388 lymphoma on day 0 (*X* axis) and were then treated (with intraperitoneal (ip) injections) or left untreated (control, with saline ip injections) with compounds **19** or **22** five times a week (from Monday to Friday) for three consecutive weeks, with the treatment starting at the 5th day post-tumor graft. The two compounds were administered at 40 mg/kg for each of the 15 ip injections. The T/C index represents the benefit over control contributed by the product in terms of the percentage increases in the median mouse survival period in the treated [T] group as compared to the median mouse survival period in the control [C] group. There were nine mice in each experimental group. (B) The in vivo influence on the P388 mouse lymphoma model of a monosubstituted β-D-lactosyl-steroid (**24**) in the presence or the absence of the pro-apoptotic cisplatin cytotoxic drug. The legend is identical to that for Figure 1A. Cisplatin was assayed ip at 10 mg/kg three times a week (Mondays, Wednesdays, and Fridays) for three consecutive weeks, with the treatment starting at the 5th day post-tumor graft.

when either the monosubstituted β-D-isomaltosyl-steroid (**22**) or the monosubstituted β-L-fucopyranosyl-steroid (**19**) was added to the cisplatin (data not shown).

In Vivo Characterization of the Antitumor Effects of Compound 24 on a Human Glioblastoma Xenograft Orthotopically Implanted into the Brains of Immunodeficient Mice. Previous data from our group have demonstrated the direct imvolvement of galectins in the progression of malignancy in human glioblastomas.[22−24] The fact that the monosubstituted β-D-lactosyl-steroid (**24**) could at least theoretically antagonize the biological functions of galectins in involvement in cancer cell migration therefore prompted us to investigate whether compound **24** could modify the survival of nude mice with orthotopic human U373 glioblastomas in their brains. The data in Figure 2 clearly show that compound **24** significantly increased the survival of these mice.

Discussion

Cancer cell migration lies behind tumor cell dispersal to points far distant from the primary tumor bulk and is therefore responsible for the formation of metastases in the case of cancers of the epithelial tissue (carcinomas), of the immune system (lymphomas), or of the soft tissue (sarcomas). Cancer cell migration also associates glioblastomas with very dismal prognoses because of their diffuse invasion of the brain parenchyma.[1,6,7] Migrating cancer cells are resistant to apoptosis because a number of anti-apoptotic signaling pathways are constitutively activated in these cells, which are therefore resistant to a large majority of currently used anticancer drugs because these drugs are pro-apoptotic agents.[1−4] New types of drugs are needed to combat the formation of metastasis from carcinomas or the dispersion of cancer cells into one or another organ such as glioblastoma cells into the brain.[1−7] Antimigratory drugs assume the role of potential antimetastatic and/or anti-invasive compounds because, in addition to reducing the locomotive abilities of migrating cancer cells, antimigratory compounds can also restore a certain level of sensitivity to pro-apoptotic drugs in the case of these restricted-migratory cells.[1−7]

Cell migration is a highly coordinated process involving cell adhesion, cell motility, and cell invasion. In the present study we focused our attention on cell adhesion, i.e., the interactions between cell surface receptors and extracellular matrix (ECM)

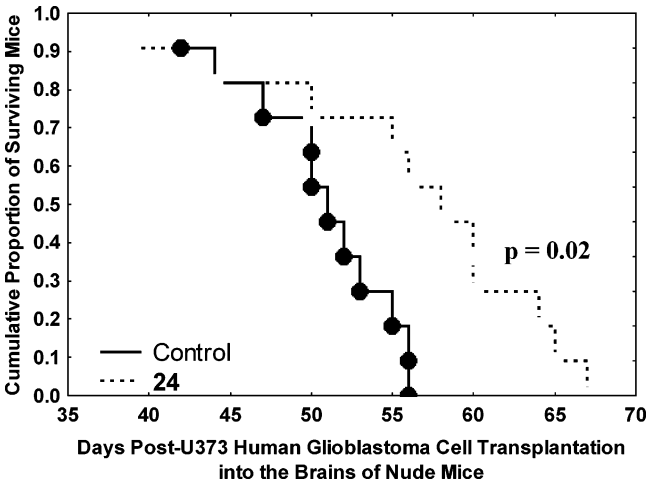
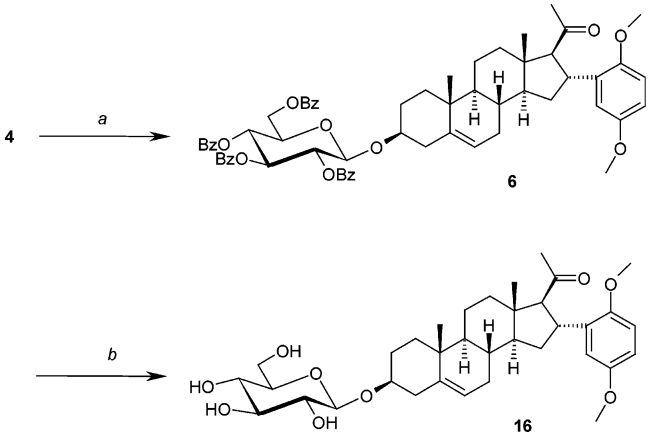


Figure 2. Illustration of the effects of compound **24** (a monosubstituted β-D-lactosyl-steroid) on the survival of immunodeficient mice with human U373 glioblastoma cells grafted orthotopically into their brains. Compound **24** (open dots) was administered iv at 40 mg/kg fifteen times (three times a week on Mondays, Wednesdays, and Fridays) for five consecutive weeks (with the treatment starting at the 14th day post-tumor graft), with the control mice (black dots) receiving 15 iv injections of saline.

components. While integrins employ protein−protein interactions with ECM components, a number of other proteins (including, for example, galectins, and selectins) use protein−carbohydrate interactions between themselves and ECM glycoproteins, with the core of carbohydrate ligands for galectins being represented by LacNAc moieties, i.e., Lewis antigens without fucosylation, and Lewis antigens alone in the case of selectins.[1,7] The present study shows that, of the 14 glycosylated steroids that we synthesized, the β-D-isomaltosyl-steroid and the β-D-lactosyl-steroid had the most marked effects on cancer cell migration in vitro. The endogenous cell surface receptors for the β-D-isomaltosyl-steroid have not been identified so far. In contrast, galectins can act as endogenous cell surface receptors for β-D-lactosyl-steroids. It has already been proved that galectins play a number of clearly defined roles in the development of malignancy and/or cell migration in gliomas[22−24] and lymphomas.[25−27] The present study clearly shows that the in vivo combination of a β-D-lactosyl-steroid (compound **24**) and the pro-apoptotic drug cisplatin[21] in an experimental metastasizing lymphoma gave rise to significant therapeutic benefits in addition to those deriving from cisplatin alone. Compound **24** also significantly increased the survival of immunodeficient mice bearing orthotopic xenografts of human U373 glioblastoma to their brains. The extent of the increase in survival brought about by compound **24** is of the same magnitude as temozolomide in the U373 orthotopic xenograft model.[28] Temozolomide is a pro-autophagic drug[29] currently associated with the a maximum level of efficiency in the treatment of glioblastoma patients.[30] We are synthesizing novel anti-galectin derivatives from **24**, and we plan to submit them to SAR studies. Indeed, the data from the present study reveal that a bisubstituted β-D-lactosyl-steroid (**30**) displayed a lower level of antimigratory activity than its monosubstituted analogue (**24**). Galectins are carbohydrate-binding proteins containing a C-terminal carbohydrate-recognition domain (CRD). Whereas the CRDs of all known galectins enjoy close structural homology,[31] there are subtle differences in binding affinities between specific galectins and lactosylated moieties.[32,33] The question of the specificity of galectins for saccharide ligands involves the investigation of the binding characteristics of this protein. Four subsites are described[34,35] in the carbohydrate-recognition domain of galectins. These subsites recognize monosaccharide units and are responsible for the affinity and specificity of galectins to ligands.[34,35] The defined orientations of the monosaccharide units are very important[34,35] for both the recognition and a good level of affinity of ligands to the CRD. Thus, the conformation of the ligands[36] can either enhance or eliminate the carbohydrate recognition process by the CRD of the galectins. In natural saccharides that bind to galectins, the galactose moiety is typically (1−4)-linked to glucose or *N*-acetylglucosamine or (1−3)-linked to *N*-acetylglucosamine or *N*-acetylgalactosamine. These natural saccharides cannot be designated as galectin inhibitors because they are difficult to synthesize, sensitive to hydrolysis, and too polar to be used as drugs. To circumvent these disadvantages Nilsson et al.[37−39] replaced the glucose moiety of the natural saccharides by less complex organic structures. Among the galactosyl oxime ethers[37] synthesized, an indole derivative was the best inhibitor with a good level of affinity. Other derivatives modified on anomeric carbon have since been synthesized[38,39] and have shown an affinity to galectins. In particular, the 1-phenyl-thio derivatives of galactose have a good level of affinity to galectin-7.[38] Galectin-7 is a potent modulator of lymphoma development[25−27] and also has a number of marked biological effects on colorectal cancers.[40] Nilsson et al.[39,41] also modified position 3 of the galactose moiety because the X-ray crystal structures[42] of the carbohydrate recognition domain of human galectin-3 with *N*-acetyllactosamine (LacNAc) showed extended binding sites close to the 3-OH function of the LacNAc. Several derivatives including 3-(1*H*-[1,2,3]-triazol-1-yl)-1-thio-galactoside[39] and 3,3′-bis-benzamido-thiodigalactosides[41] are inhibitors of galectin-3, which has a dramatic influence on both the development of metastases[15,43] and the resistance of carcinomas to apoptosis.[44]

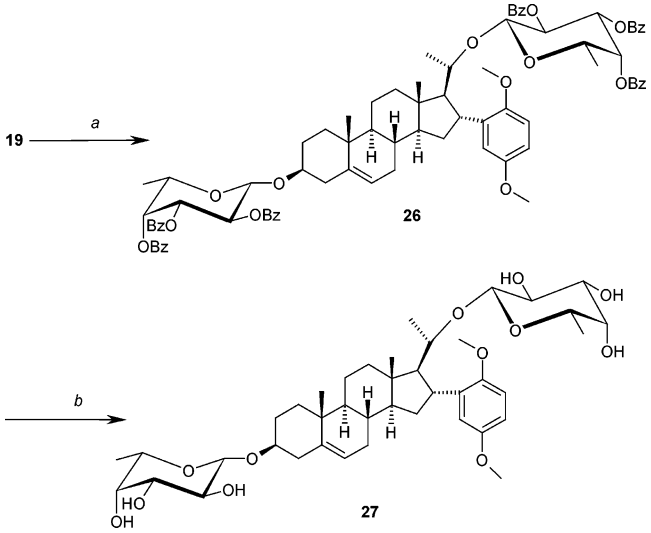
Interactions between fucose moieties present in glioma ECM and cell adhesion molecules present on the surface of glioma cells play a number of major roles in glioma cell migration.[13,14,45] Fucose-containing glycans with potential clinical applications are hypothesized as combating the development of malignant gliomas. Indeed, it has been known for a long time that, under normal circumstances, the astrocyte number remains constant in the mammalian central nervous system during adulthood and old age as a result of the balance of division promoters and division inhibitors.[46] Moreover, Nieto-Sampedro[47] has identified mitogen inhibitors as being immunologically related to blood group oligosaccharides (i.e. Lewis antigen-related structures) and the glycan epitopes of the epidermal growth factor receptor. On the basis of these data, Aguilera et al.[13] have synthesized fucosyl-LacNAc-related structures, a family of oligosaccharides with a common Lewis-X-type structure. These compounds display a significant level of antiproliferative activity against malignant glioblastoma cells.[14] Our recent study also revealed that cimetidine (a H2 receptor antagonist[6]) slightly, but nevertheless significantly, decreases the levels of expression of endogenous receptors for fucose moieties;[7] when combined with temozolomide this cimetidine-induced decrease in endogenous ligands for fucose gives rise to therapeutic benefits comparable to those brought about by temozolomide alone in the case of the U373 human glioblastoma model.[7] The present study shows that, in vitro, a β-D-fucopyranosyl-steroid (compound **19**) significantly modifies the levels of migration of human glioblastoma cells, but not of human NSCLC and prostate cancer cells. Whether compound **19** could endow temozolomide with additional therapeutic effects in vivo in the case of human glioblastoma xenografts remains to be seen.

Scheme 3



(a) 1-(α)-Bromoperbenzoylglucose, AgOTf, allylsilane, 4 Å molecular sieves, CH_2Cl_2/toluene; (b) NaOMe, MeOH.

Scheme 4



(a) 1-(α)-Bromoperbenzoylfucose, AgOTf, allylsilane, 4 Å molecular sieves, CH_2Cl_2/toluene; (b) NaOMe, MeOH.

Experimental Section

Chemistry. The deacetylation of the commercially available 16-dehydropregnenolone acetate **1** and its protection as *tert*-butyldiphenylsilylether gives compound **2** in two steps with a 90% overall yield (Scheme 1). The treatment of **2** with a large excess of the organomagnesium derivative of 1-bromo-2,5-dimethoxybenzene in the presence of copper iodide led to a 1,4-addition to enone **2** to give compound **3** (Scheme 1). The silyl group (TBDPS) was selectively removed by treatment with tetrabutylammonium fluoride in tetrahydrofuran to give compound **4** (Scheme 1). In the ^{1}H NMR spectrum (in $CDCl_3$) of isolated compound **4** the signal appearing at δ 2.88 (d, J_{17-16} = 9.6 Hz) was assigned to H-17 with the assistance of the ^{1}H−^{1}H COSY and HMBC spectra. The coupling constant of the signal revealed the transconfiguration of the substituents carried by carbons C-16 and C-17. The NOESY plot showed interactions between the H-18 and the H-21 protons, so indicating the β-orientation of the methyl ketone (carried by C-17). The interaction between the H-16 and the H-18 protons confirmed the α-orientation of the 2,5-dimethoxyphenyl substituent (carried by C-16). After the reduction of the ketone function of **4** in the presence of sodium borohydride in methanol, a mixture of diastereoisomers **5** (β-ol **5a** and α-ol **5b**; Scheme 2) was obtained (20β/20α ratio of 1/4 determined by ^{1}H NMR and HPLC analyses).

The glycosylation of the sterol derivatives was performed using the Koenigs−Knorr procedure.[48] The preferred conditions utilized glycosyl halides[49] as donors and the sterol under silver[50] (method A) or mercuric[51] (method B) catalysis in dry dichloromethane. When silver trifluoromethane sulfonate catalysis was used, allylsilane was added to the reaction mixture in order to trap the released triflic acid. Acceptable yields of protected glycosides were obtained with the β-configuration only. Starting from sterol **4**, this synthesis led first of all to the β-D-glucoside compound **6** (Scheme 3). Using this general procedure, we condensed several mono- or disaccharides to the hydroxyl function of **4** to obtain 10 additional glycosylated steroids **6**−**15** (see Supporting Information). Compounds **6**−**15** were not submitted to antimigratory analyses because their glycoside moieties are still protected. From diol **5** (a mixture of diastereoisomers **5a** and **5b**) we obtained the bis-β-L-fucoside derivative **26** (Scheme 4) along with three other protected bis-disaccharide derivatives that were not isolated any further. All the H-17, H-18, H-20, and H-21 signals in the ^{1}H NMR (in $CDCl_3$) of isolated compound **26** were assigned with the assistance of the ^{1}H−^{1}H COSY and HMBC spectra. The NOESY plot showed interactions between the H-18 protons and the H-20 and H-21 ones, with H-17 indicating the *S* stereochemistry at C-20. This NMR analysis showed that only the bis-fucosylated isomer of the α-ol **5a** derivative was isolated with a good yield. The other bis-fucosylated isomer of the β-ol **5b** derivative could not be isolated, probably because the amount was too small. This result was also confirmed in the case of the three other bis-glycosylated compounds (cellobiosyl, isomaltosyl, lactosyl) that we obtained. After the deprotection[51] of the acetates or benzoates of the glycosylated compounds with sodium methoxide in methanol we obtained 10 monoglycosylated (**16**−**25**; Scheme 3 and Table 1A) and four bis-glycosylated (**27**−**30**; Scheme 4 and Table 1B) final products.

The experimental procedures are detailed in the Supporting Information.

Pharmacology

Cell Lines and Culture Media. Six human cancer cell lines were used in the present work, namely, the Hs683 and U-373MG glioblastoma, the HCT-15 colon cancer, the PC-3 prostate cancer, the A549 non-small-cell-lung cancer (NSCLC), and the MCF-7 breast cancer cell lines. The manner in which we handled these cell lines in our laboratory is detailed in the Supporting Information.

Cytotoxicity Assay. Cytotoxicity analyses were performed by means of the colorimetric MTT assay, as previously detailed.[5,52,53] The experimental protocol is given in the Supporting Information.

Cell Migration Assay. The effects of various of the compounds synthesized during the course of the present research were analyzed at cell migration level on three different cell lines including the PC-3 prostate, the A549 NSCLC, and the U373 glioblastoma cancer cells (see above). The cancer cell migration levels were characterized quantitatively on a computer-assisted device, as detailed elsewhere.[5,7,54] This device enables the trajectories of culture-maintained living cells to be quantified. The greatest linear distance migrated by each cell was calculated from these trajectories. This distance is in fact the maximum relative distance from the point of origin, i.e. the MRDO quantitative variable.[5,7,54] The experiments were all performed over 24 h, and one image was recorded every 4 min. Since the analyses were carried out in triplicate, a minimum of 121 and a maximum of 184 cells were analyzed in each experimental condition. The influence of the various glycosylated steroids from the present study on human cancer cell migration was analyzed at 1, 10, and 100 nM.

In Vivo Testing. Maximum Tolerated Dose. The maximum tolerated dose (MTD) of a drug is defined as the maximum dose which can be administered acutely (i.e. in one ip, single dose) to healthy animals (i.e. not grafted with tumors). The survival periods and the weights of the animals are recorded

for up to 28 days postinjection. The detailed experimental protocols are given in the Supporting Information.

The P388 Subcutaneous Lymphoma Model. The P388 lymphoma model was developed in our laboratory, as detailed elsewhere.[19] Details of the treatments are provided in the captions to the figures. Additional information is also provided in the Supporting Information. The potential gain in survival obtained via a potential antitumor compound was evaluated by means of the T/C index. This index is the ratio between the median survival time of the treated animal group (T; $n = 9$) and that of the control group (C; $n = 9$).

The Orthotopic U373 Human Glioblastoma Xenograft. In vivo orthotopic xenografts of human U373 glioblastoma cells in nude mice were obtained as previously detailed.[7] The detailed protocol is given in the Supporting Information.

Statistical Analyses. Statistical comparisons between control and the treated groups were made by first carrying out the Kruskal-Wallis test (a nonparametric one-way analysis of variance), and, where this test revealed significant differences, we investigated whether any of the groups treated differed from control. For this purpose we applied the Dunn multiple comparison procedure (2-sided test) adapted to the special case of the comparisons of treatments and control, i.e. where only $(k - 1)$ comparisons were carried out among the k groups tested by the Kruskal-Wallis test (instead of the possible $k(k - 1)/2$ comparisons considered in the general procedure). The levels of statistical significance associated with the T/C-related survival indices were determined by using Gehan's generalized Wilcoxon test. All these statistical analyses were carried out using Statistica (Statsoft, Tulsa, OK).

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Supporting Information Available: The analyses covering all the compounds used in this work (melting points, elemental analyses, optical rotations, HPLC analyses, ^{1}H and ^{13}C NMR, mass spectra, and IR spectral data) and the pharmacology of the P388 subcutaneous model and the orthotopic U373 human glioblastoma model. This material is available free of charge via the Internet at http://pubs.acs.org.

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