VIP Tumor Targeting

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The First Generation of β-Galactosidase-Responsive Prodrugs Designed for the Selective Treatment of Solid Tumors in Prodrug Monotherapy**

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In memory of Gérard Déléris

The selective killing of tumor cells without affecting normal tissues is one of the main challenges of cancer chemotherapy. In recent years, the development of drug carriers designed to deliver potent cytotoxic compounds exclusively inside malignant cells has emerged as a valuable alternative to avoid dose-limiting adverse effects recorded with traditional anticancer agents.[1] Within this framework, the use of antibody–drug conjugates[2] targeting specific tumor-associated antigens is by far the best-explored approach. Many of these compounds are currently evaluated in humans, including Brentuximab Vedotin,[3] which reached the market in 2011 for the treatment of lymphomas. Another promising strategy relies on the use of nontoxic prodrugs that can be activated by an enzyme[4] previously targeted in cancerous tissues by the mean of a monoclonal antibody in the course of antibody-directed enzyme prodrug therapy (ADEPT).[5] In this case, the active compound is released extracellularly in the vicinity of cancer cells that are subsequently killed after drug uptake. Over the past decade, several galactoside prodrugs[6] have appeared as potential candidates to achieve selective chemotherapy of solid tumors in combination with antibody-β-galactosidase conjugates.[7] The best illustration of such an enzyme-responsive system is unambiguously the galactoside prodrugs of duocarmycin analogues developed by Tietze and co-workers[8] that meet all the main criterions required to be used in ADEPT.[9] However, the implementation of ADEPT procedures remains complex, costly, and with high risk of immune response linked to the administration of an antibody–enzyme conjugate. This is probably the reason why none of these promising galactoside prodrugs has been assessed for antitumor efficacy in animal models to date. Under such circumstances, the development of a simpler approach, allowing the selective activation of galactoside prodrugs by the endogenous β-galactosidase located inside malignant cells, is of great interest.

Herein, we present the first generation of galactoside prodrugs suitable for the treatment of solid tumors in prodrug monotherapy (PMT[10]), a strategy in which the use of an antibody-β-galactosidase is not needed. For this purpose, we designed the novel drug delivery system **1** composed of a galactoside trigger, a targeting ligand, and a potent cytotoxic compound articulated around a central self-immolative linker (Figure 1).[11]

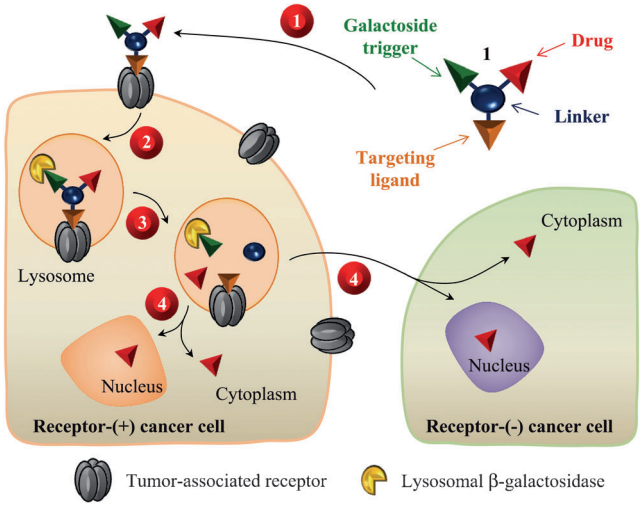

Figure 1. The principle of tumor targeting. Step 1: selective recognition of receptor-positive cancer cells; step 2: receptor-mediated endocytosis; step 3: β-galactosidase-catalyzed drug release; step 4: diffusion of the drug into the nucleus or the cytoplasm of both receptor-positive and receptor-negative cancer cells, leading to the death of each type of cell.

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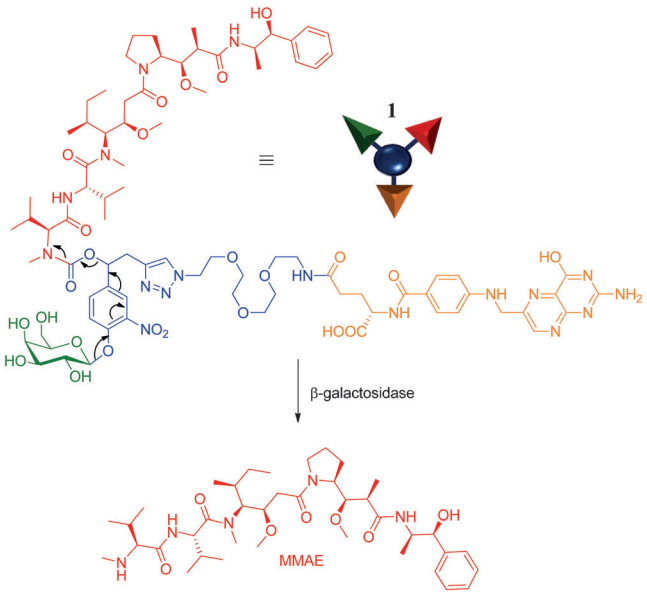
Such a targeting assembly is programmed to be selectively activated by the β-galactosidase present in the lysosomal compartment of cancer cells expressing a specific tumor-associated receptor.[1] Thus, as shown in Figure 1, recognition of the membrane receptor by the targeting ligand (step 1) will trigger the receptor-mediated endocytosis[12] of the whole device (step 2) that will be followed by the intracellular enzyme-catalyzed mechanism of drug release (step 3). As β-galactosidase is present in lysosomes of both healthy and malignant cells, this highly specific internalization process will allow the prodrug activation to occur exclusively inside receptor-positive tumor cells, thereby avoiding unselective drug release in non-malignant tissues. However, as the prodrug activation is catalytic, the β-galactosidase confined in the targeted cells will trigger the liberation of sufficient drug quantities to induce the death of both receptor-positive and surrounding receptor-negative tumor cells (step 4). It is worth mentioning that numerous tumor-associated receptors[1] have already been identified, and consequently this new generation of galactoside prodrugs could be adapted to target a wide variety of malignancies.

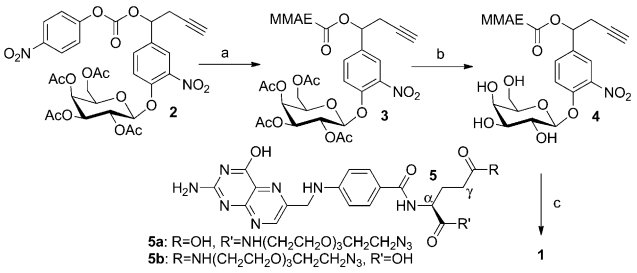
As proof of the concept, we developed the pilot prodrug **1** that can be activated selectively inside folate receptor-expressing tumor cells (Scheme 1). The folate receptor



Scheme 1. Structure of prodrug **1**, and the β-galactosidase-catalyzed MMAE release mechanism.

(FR)[13] is indeed overexpressed in several cancer types[14] while it is mainly undetectable in most normal tissues. Thus, the FR-expressing cells represent targets of choice to demonstrate the validity of this concept. With our design, the β-galactosidase-catalyzed cleavage of the glycosidic bond will release the potent antimitotic agent monomethyl auristatin E (MMAE)[15] in a stringently controlled fashion by the self-immolative mechanism depicted in Scheme 1.

The synthesis of prodrug **1** was carried out starting from the galactoside **2**, which is readily accessible as a mixture of two diastereoisomers through a multistep strategy that has been already described (Scheme 2).[16] Indeed, as we previously demonstrated with other enzyme-responsive systems,[17] glycosylated intermediates such as **2** are ideal platforms for



Scheme 2. Synthesis of the galactoside prodrug **1**. a) MMAE, diisopropylethylamine (DIPEA), hydroxybenzotriazole (HOBt), pyridine/DMF, RT, 36 h, 75%; b) LiOH, MeOH, 0°C, 20 min, 98%; c) **5**, $CuSO_4$, sodium ascorbate, DMSO, RT, 8 h, 85%.

the successive introduction of a cytotoxic compound on the activated carbonate at the benzylic position and a targeting entity on the terminal alkyne of the linker unit. Thus, coupling between **2** and MMAE undertaken by nucleophilic substitution gave the protected galactoside **3** in 75% yield. Full deprotection of the hydroxy groups furnished the clickable derivative **4** in nearly quantitative yield. Finally, introduction of the folate ligand was conducted in the presence of the azide **5**, using the well-known copper(I)-catalyzed azide–alkyne 1,3-cycloaddition to afford the prodrug **1**, which was then purified by preparative chromatography for biological evaluations (85%, as a mixture of four isomers).

We first investigated the ability of the prodrug **1** to target selectively FR-positive tumor cells by measuring its antiproliferative activity against both KB and HeLa cells, which overexpress the FR at various levels, as well as FR-negative A549 cells. As shown in Table 1, our enzyme-responsive

Table 1: IC_{50} values (nM) of MMAE and prodrug **1** on KB, HeLa, and A549 cell lines correlated with the FR level.[a]

Cell line	FR level	IC_{50} [nM] MMAE	**1**
KB	+++	0.240	0.240
HeLa	++	0.630	8.408
A549	–	0.872	195.230

[a] Values represent the mean ± SEM of seven experiments performed in triplicate. KB cells: human mouth epidermal carcinoma; HeLa cells: human cervix adenocarcinoma; A549 cells: human bronchial carcinoma. FR: folate receptor.

system dramatically affected the viability of KB and HeLa cells, with IC_{50} values of 0.240 and 8.408 nM, respectively. The cytotoxicities recorded in these experiments were consistent with the FR expression level that is higher in KB than in HeLa cells (see the Supporting Information). Interestingly, the antiproliferative activity of the galactoside prodrug **1** in KB cells is similar to that of MMAE ($IC_{50} = 0.240$ nM), making this compound the most potent folate–drug conju-

gate[18] developed to date. On the other hand, A549 cells that present only low level of FR were much less sensitive to the incubation of **1** ($IC_{50} = 195.230$ nM), whereas MMAE was highly toxic ($IC_{50} = 0.872$ nM). As expected, the hydrophilicity imparted by the galactoside trigger prevented passive cellular uptake and further intracellular activation of the prodrug in non-targeted cells. All together, these results indicated that the galactoside **1** can be selectively activated inside FR-positive tumor cells. Thus, as most normal tissues express low level of FR, such outcomes suggest that prodrug **1** should present only reduced toxicity toward safe tissues compared to untargeted MMAE in vivo.

To confirm that this selective toxicity was the consequence of the intracellular activation of prodrug **1** by lysosomal β-galactosidase, we examined the inhibition of tubulin polymerization, the mechanism by which MMAE exerts its antitumor activity (Figure 2). Thus, as shown by confocal

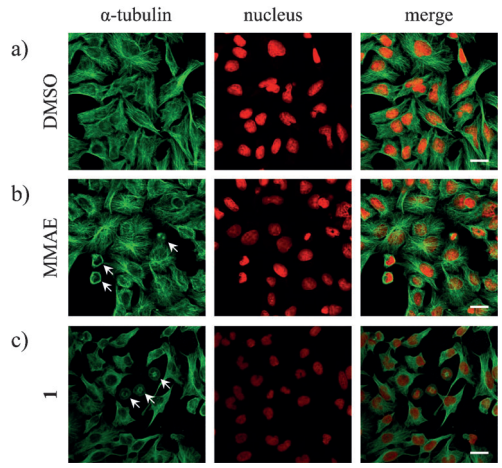


Figure 2. α-Tubulin immunodetection by confocal microscopy in HeLa cells treated for 24 h with a) DMSO, b) MMAE at 1 nM, and c) prodrug **1** at 1 nM. White arrows indicate cells blocked by MMAE or **1**.

microscopy imaging, incubation of the free MMAE with HeLa cells disturbed the microtubule network (Figure 2b), while this was not detected when cells were untreated (Figure 2a). Furthermore, as demonstrated by FACS analysis (see the Supporting Information), galactoside prodrug **1** produced a similar effect on cell division, demonstrating that its selective receptor-mediated endocytosis is followed by the β-galactosidase-catalyzed release of the antimitotic agent MMAE (Figure 2c). The role of lysosomal β-galactosidase in the prodrug activation process was also evidenced by comparing a galactoside conjugate of doxorubicin with its glucuronide analogue (see the Supporting Information).

As cancerous tissues are highly heterogeneous, the selective destruction of a particular population of malignant cells, such as those expressing a membrane receptor, is not sufficient to eradicate the wide diversity of tumor cells. However, an efficient intracellular enzymatic activation of prodrug **1** should release high quantities of MMAE that could then diffuse out of FR-positive cells to kill surrounding FR-negative cancer cells. To verify this hypothesis, we co-cultured KB FR-positive with A549 FR-negative cells using TransWell Boyden chambers (Figure 3).

Prodrug **1** was incubated with KB cells placed in the top chamber to trigger the release of MMAE, which can

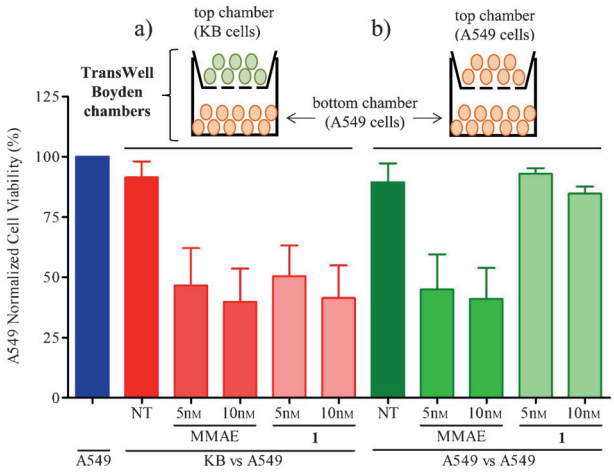


Figure 3. Viability of A549 cells in co-culture assay using TransWell Boyden chambers: a) in red, top chamber contains KB cells and bottom chamber contains A549 cells; b) in green, both top and bottom chambers contain A549; in blue, the viability of A549 cells alone was used as a control.

subsequently diffuse through the 0.4 μM filter in the bottom chamber containing A549 cells (Figure 3a). Under these conditions, the targeting system **1** induced a dramatic antiproliferative effect on A549 cells, which was comparable to that measured with MMAE at identical doses (5 and 10 nM). As a negative control, the same experiments were conducted with A549 cells in the top chamber (Figure 3b). In this case, while the antimitotic drug affected the viability of cells, prodrug **1** did not exhibit any significant toxicity. In accordance with the principle of tumor targeting illustrated in Figure 1, these results demonstrated for the first time that the activation of a galactoside prodrug such as **1** by lysosomal β-galactosidase located inside FR-expressing cells is an efficient catalytic process, enabling the release of suitable quantities of MMAE for the destruction of surrounding cancer cells, whatever their membrane characteristics.

The in vivo efficacy of the galactoside prodrug **1** in the course of PMT was assessed in nude mice bearing luciferase-transfected KB xenografts. The animals received several intravenous injections of 5 $mg\,kg^{-1}$ of the prodrug starting at day 5 after tumor implantation (for the full therapeutic procedure, see the Supporting information). Tumor progression was monitored by bioluminescence imaging three times per week and compared to that of mice treated with 0.1 $mg\,kg^{-1}$ of MMAE (Figure 4a).

As illustrated in Figure 4b, prodrug **1** induced a marked antitumor activity with almost total and durable disappearance of the luminescence from day 21, while treatment with MMAE led only to a moderate inhibition of tumor growth. With the aim of increasing the efficacy of the antimitotic agent in this animal model, MMAE was also evaluated at a higher dose of 0.5 $mg\,kg^{-1}$. However, in this case the first

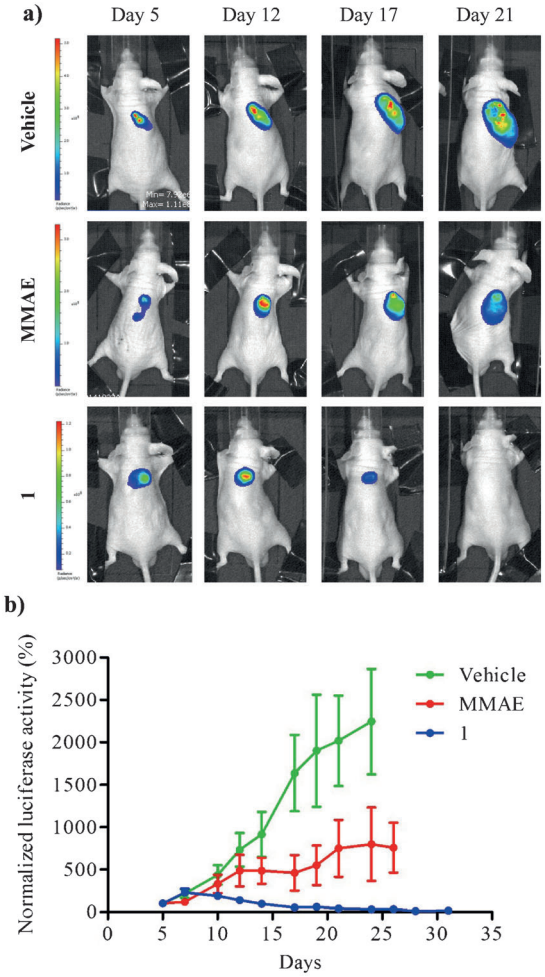


Figure 4. a) Representative bioluminescence imaging of luciferase-transfected KB xenografts at days 5, 12, 17, and 21 post-implantation when treated with vehicle (5% DMSO in PBS buffer), MMAE (intravenous administration at days 4, 7, 10, 14, and 17) or prodrug **1** (intravenous administration at days 5, 7, 10, 12, 14, 17, 19, 21, 23, and 26); b) Tumor growth inhibition over the time under therapy with prodrug **1** and MMAE.

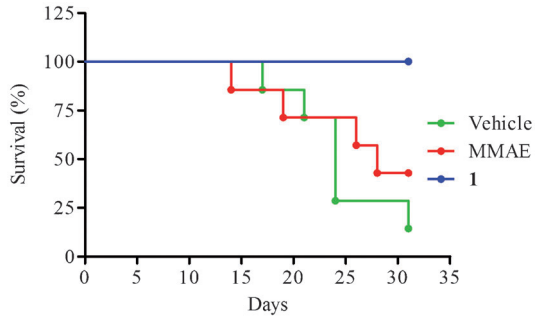


Figure 5. Survival curve representing the percent survival as a function of time in day.

administration of the free drug caused a body weight loss that did not permit to pursue the therapeutic procedure. In contrast, prodrug **1** was well tolerated without any sign of overt toxicity at the tested dose. Furthermore, all the mice in the group treated with **1** were still alive at the end of the study (7/7, day 31), whereas 4/7 mice of the MMAE group succumbed (Figure 5). Overall, these in vivo experiments demonstrated that the galactoside prodrug **1** is a promising candidate for selective treatment of solid tumors expressing the FR.

In summary, we developed the first β-galactosidase-responsive drug delivery system suitable for the treatment of solid tumors in PMT. Through the study of galactoside prodrug **1**, we demonstrated that such a targeting system can be selectively activated by lysosomal β-galactosidase located inside malignant cells expressing a specific tumor-associated receptor. This efficient enzymatic process triggers a potent cytotoxic effect, allowing the destruction of both receptor-positive and surrounding receptor-negative tumor cells. The prodrug **1** produces a remarkable antitumor effect when tested against FR-expressing KB xenograft without any detectable toxicity, showing the validity of this concept in vivo. Furthermore, as the synthetic strategy employed for the preparation of **1** allows the custom design of other β-galactosidase-responsive targeting assemblies, this approach could be easily adapted for the treatment of particular malignancies based on their tumor-associated membrane specificities. This new generation of low-molecular-weight galactoside prodrugs may offer a valuable alternative to the use of antibodies (in the form of either antibody-drug or antibody-enzyme conjugates) that exhibit poor tumor penetration. Thus, our finding may open a new door for selective chemotherapy of solid tumors.

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Communications

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T. Legigan, J. Clarhaut, I. Tranoy-Opalinski, A. Monvoisin, B. Renoux, M. Thomas, A. Le Pape, S. Lerondel, S. Papot* ____ ■■■■–■■■■

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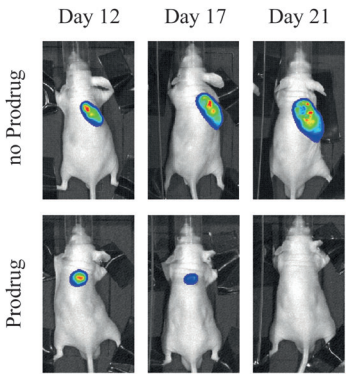

Massive attack: Galactoside prodrugs have been designed that can be selectively activated by lysosomal β-galactosidase located inside cancer cells expressing a specific tumor-associated receptor. This efficient enzymatic process triggers a potent cytotoxic effect, releasing the potent antimitotic agent MMAE and allowing the destruction of both receptor-positive and surrounding receptor-negative tumor cells.