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A β -glucuronidase-responsive albumin-binding prodrug programmed for the double release of monomethyl auristatin E†Brigitte Renoux,^a Laure Fangous,^a Camille Hötten,^a Elodie Péraudeau,^b
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We report on the synthesis, *in vitro* and *in vivo* biological evaluations of a dimeric β -glucuronidase-responsive albumin-binding prodrug designed for the double release of MMAE upon a single enzymatic activation step. This prodrug produced a significant antitumour activity in mice bearing subcutaneous LS174T colorectal adenocarcinoma xenografts without inducing side effects.

The selective delivery of anticancer drugs in tumour tissues is an emerging therapeutic strategy that limits the side effects usually associated with conventional chemotherapy. To date, this approach has been mainly explored with drug delivery systems designed to recognise a specific cell surface marker (*e.g.* antigens or receptors) and penetrate inside cancer cells through endocytosis to launch their cytotoxic activity.^{1,2} The potential of such targeted therapy has been confirmed in human with the approval of four antibody-drug conjugates (ADC) for applications in oncology.³ However, these internalising drug delivery systems can be employed only for the therapy of tumours expressing a high level of the targeted cancer cell membrane specificity. Additionally, their efficacy is limited by the high heterogeneity of malignant cells that present various concentrations of cancer cell surface hallmarks.⁴ Recently, we showed that β -glucuronidase-responsive albumin-binding prodrugs can be a valuable alternative to internalising drug delivery systems for the treatment of solid tumours.^{5,6} These prodrugs are composed of a glucuronide trigger,^{7,8} a potent anticancer drug and a self-immolative linker^{9,10} bearing a hydrophilic side chain with a maleimide functional group at the end (for an example see compound 1 Fig. 1). Once in the bloodstream, such molecular assemblies bind covalently to plasmatic albumin and accumulate passively in tumours where extracellular β -glucuronidase^{11–14} triggers the release of the active compound. In this approach, the drug is delivered in the tumour microenvironment and can

then enter the surrounding cancer cells regardless of their membrane specificities. By operating in this way, β -glucuronidase-responsive albumin-binding prodrugs mediated potent anticancer activities in several models *in vivo*, without inducing side effects.

Fig. 1 Structures of the β -glucuronidase-responsive albumin-binding prodrugs 1 and 2. Step (1) binding to plasmatic albumin through Michael addition; step (2) β -glucuronidase-catalysed hydrolysis of the glucuronide trigger; step (3) release of MMAE induced by the self-immolation of the linker followed, in the case of 2, by the chemical amplification process.

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With the aim to improve the efficiency of this targeting strategy, we now report on the dimeric β -glucuronidase-responsive albumin-binding prodrug of monomethyl auristatin E (MMAE) **2** (Fig. 1). In contrast to its monomeric analogue **1**, prodrug **2** includes a chemical amplifier^{15–17} allowing the release of two MMAE molecules after a single enzymatic activation step (for the full mechanism of drug release, see Fig. 2). While chemical amplifiers have been incorporated already in several drug delivery systems, our study represents the first *in vivo* evaluation of such molecular constructs.

The synthesis of prodrug **2** was carried out starting from carbonate **3**,⁶ previously described in the literature (Scheme 1). Aniline **4** was first introduced *via* nucleophilic substitution to give the diol **5** in 53% yield. The two primary alcohols were then activated in the presence of 4-nitrophenyl chloroformate affording the biscarbonate **6** (85%). The latter reacted with two equivalents of MMAE to produce the dimer

Scheme 1 Synthesis of **2**. Reagents and conditions: a) HOBt, DMF, 50 °C, 30 h, 53%; b) *para*-nitrophenyl chloroformate, pyridine, CH₂Cl₂, 0 °C to 20 °C, 3 h, 85%; c) MMAE, HOBt, pyridine, DMF, 20 °C, 24 h, 56%; d) O-(2-aminoethyl)-O'-(2-azidoethyl) nonaethylene glycol, Cu(MeCN)₄PF₆, CH₂Cl₂, 20 °C, 15 h, 47%; e) LiOH, H₂O/MeOH, 0 °C, 1 h 30; f) **9**, DMSO, 20 °C, 15 h, 33% over 2 steps.

Fig. 2 Enzyme-catalysed release mechanism of the two MMAE molecules from the β -glucuronidase-responsive albumin-binding prodrug **2**.

7 (56%). Copper(i)-catalyzed azide-alkyne 1,3-cycloaddition (CuAAC) undertaken with commercially available O-(2-aminoethyl)-O'-(2-azidoethyl) nonaethylene glycol furnished the amine **8** (47%). Deprotection of the glucuronide moiety using LiOH followed by the coupling of the primary amine with *N*-hydroxysuccinimide ester **9** yielded the dimeric prodrug **2** in 33% yield over two steps after purification by reverse phase column chromatography (purity >95%).

As the enzymatic cleavage of the glucuronide trigger is the initiating step in the process of drug release, our first aim was to ensure that the carbohydrate moiety was still accessible for β -glucuronidase when bound to albumin. For this purpose, prodrug **2** was incubated with human serum albumin (HSA) at 37 °C and the composition of the mixture was followed over time by HPLC-HRMS. After three hours under these conditions, **2** disappeared almost completely as the result of its binding to HSA (see the ESI†). In the absence of β -glucuronidase, the macromolecular **2**/HSA adduct appeared to be stable over time. On the other hand, when β -glucuronidase was added in the medium, the full release of MMAE was observed in five hours, therefore confirming that the glucuronide moiety was still a substrate for the activating enzyme despite the proximity of albumin (Fig. 3). However, it is worth mentioning that under the same conditions the liberation of MMAE proceeded faster from prodrug **1**, reaching completion in less than one hour.⁶ This slower kinetics of drug release may affect the therapeutic efficacy of the dimer

Fig. 3 Kinetics of MMAE release from **2** in the presence of β -glucuronidase (133 U mL^{-1}) at 37°C and pH 7.

2 *in vivo*. Indeed, it is well admitted that the active drug has to be released as quick as possible once the prodrug was activated to avoid the diffusion of an inactive intermediate outside the tumour site.⁹

We next evaluated the cytotoxicity of prodrug **2** in LS174T colon cancer cells under anchorage-independent conditions (Fig. 4). This model is indeed more predictive for MMAE activity under the 3D conditions of the tumour microenvironment.¹⁸ As shown in Fig. 3, a 5 nM dose of dimer **2** did not modify colony formation in the soft agar assay after 3 days of treatment. A similar result was obtained with the monomeric drug delivery system **1**, when incubated at the same dose. On the other hand, addition of β -glucuronidase reduced the

Fig. 4 Anchorage-independent colony formation of LS174T cells treated for 3 days with 5 nM of prodrugs **1** or **2** in the presence or in the absence of β -glucuronidase ($\approx 3700 \text{ U per well}$). Images are representative of 5 independent experiments. Scale bar: $250 \mu\text{m}$. Quantifications are presented as mean values $\pm$ s.e.m from 5 independent experiments.

LS174T colony size, demonstrating that the enzymatic activation of both prodrugs **1** and **2** occurred in the 3D cell culture medium. This effect was more pronounced with the dimer **2** due to the increased release of the active drug brought by the amplification process. Overall, these results revealed that **2** was a potential candidate for improving the therapeutic efficacy of β -glucuronidase-responsive albumin-binding prodrugs *in vivo*.

Thus, we next assessed the antitumour activity of the β -glucuronidase-responsive albumin-binding prodrug **2** in Balb/c athymic mice bearing subcutaneous LS174T colorectal adenocarcinoma xenografts (Fig. 5). Since LS174T tumours are known to exhibit a reduced level of extracellular β -glucuronidase,¹⁹ we anticipated that this model was appropriated for evaluating the potential of the dimer **2** versus the monomer **1** *in vivo*. Thus, compounds **1** and **2** were i.v. administered at the same molar dose which corresponds to 0.77 and 1.54 mg per kg per injection²⁰ of MMAE, respectively. The therapeutic efficacy of the free drug was also investigated at 0.50 mg per kg per injection, representing the maximal dose avoiding lethal toxicity.⁶

In this experiment, all the tested compounds produced significant antitumour activities as compared to the control group and similar efficacies (Fig. 5A). No detectable side effect or body weight loss has been observed in the animals (Fig. 5B). However, the dimeric prodrug **2** did not exhibit improved efficacy for reducing tumour growth compared to **1** in this model. Disappointingly, this result indicates that glucuronides **1** and **2** enabled the deposition of comparable MMAE concentrations in the tumour microenvironment.

Fig. 5 A) Tumour growth inhibition of subcutaneous LS174T xenografts in Balb/c athymic mice under therapy with MMAE (0.5 mg kg^{-1}), prodrugs **1** (2 mg kg^{-1}) and **2** (3 mg kg^{-1}) administered intravenously at days 17 and 24; B) mean body weights of each group of mice bearing LS174T xenografts.

Since **2** is not as efficient as expected *in vivo*, some hypotheses can be made to explain this outcome. First, the double release of MMAE did not proceed fast enough under the biological conditions prevailing in the tumour microenvironment. As pointed out by *in vitro* experiments, the complex mechanism of linker self-immolation delays the release of the drug after the enzymatic activation of the glucuronide trigger. Since the enzymatic step occurs in the extracellular space, one can postulate that inactive intermediates can diffuse outside the tumour rather than penetrate inside the surrounding cancer cells, thereby leading to reduced deposition of MMAE in malignant tissues. Second, the binding of **2** with plasmatic albumin in the bloodstream is not as effective as for its monomeric counterpart **1** and, consequently, reduces the passive accumulation of the prodrug at the tumour site. Indeed, while **1** and **2** bind albumin in a similar fashion *in vitro*, the coupling reaction in the circulation could be impacted by various parameters such as prodrug distribution, solubility and pharmacokinetics. However, as significant *in vivo* antitumour activity was obtained with compound **2** at a non-toxic dose for healthy tissues, new investigations deserve to be carried out with the aim to improve the efficacy of such drug delivery systems including a chemical amplifier.

In summary, we synthesised a dimeric β -glucuronidase-responsive albumin-binding prodrug programmed for the double release of MMAE in the tumour microenvironment. This prodrug is less toxic than the parent drug and readily activated in the presence of β -glucuronidase both *in vitro* and *in vivo*. While antitumour activity of the dimer **2** is similar to that of its monomeric analogue **1** in mice, our study represents the first *in vivo* evaluation of an enzyme-responsive drug delivery system bearing a chemical amplifier. Therefore, our results can be of interest for further development in this field, aiming to enhance the efficacy of targeted cancer chemotherapy.

Conflicts of interest

There are no conflicts to declare.

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