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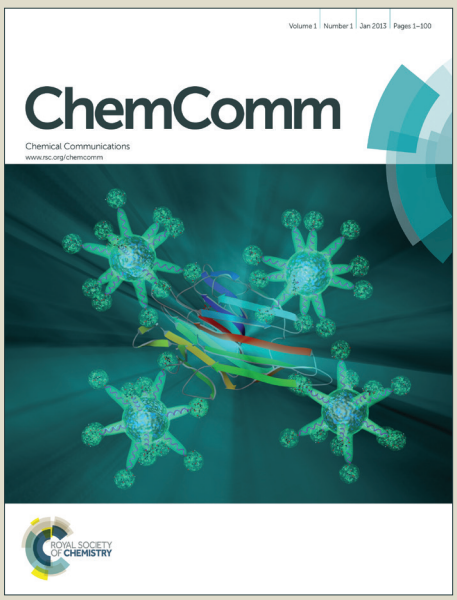

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A Dendritic β-Galactosidase-Responsive Folate-Monomethylauristatin E Conjugate

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We report the study of a new drug delivery system programmed for the selective internalisation and the subsequent enzyme-catalysed release of two monomethylauristatin E molecules inside FR-positive cancer cells. This targeting device is the most potent β-galactosidase-responsive folate-drug conjugate developed so far, killing cancer cells expressing a medium level of FR at low nanomolar concentrations.

The development of 'smart' drug carriers designed to deliver potent anticancer agents within malignant cells has recently emerged as a valuable alternative to enhance the selectivity of cancer chemotherapy. In this context, two antibody-drug conjugates targeting tumour-associated membrane antigens are already on the market (brentuximab vedotin[1] since 2011 and trastuzumab emtansine[2] since 2013) and more than forty are currently under evaluation in clinic.[3] Another promising strategy relies on the use of folate-drug conjugates that recognize cancer cells overexpressing the folate receptor (FR).[4] To date, the best illustration of this approach is the Vintafolide, a folate-desacetylvinblastine monohydrazine conjugate which is progressing through clinical trials.[5, 6] Recently, we studied enzyme-responsive folate-drug conjugates programmed for the selective internalisation and subsequent β-galactosidase-catalysed release of potent cytotoxic agents inside FR-positive cancer cells.[7-9] Amongst such targeting systems, a drug carrier designed for the delivery of the potent anti-neoplastic agent monomethylauristatin E (MMAE) induced a remarkable anticancer activity in mice.[8] In the course of these studies, we demonstrated that the efficiency of this strategy strongly depended on the FR expression level at the surface of cancer cells. A lower FR abundance indeed limits the amount of folate conjugate internalised through receptor-mediated endocytosis and therefore the concentration of active drug released enzymatically within the cells. Thus, with the aim to increase the potential of this targeting strategy, we now report on the study of the dendritic β-galactosidase-responsive folate-MMAE conjugate **1** (Fig. 1). By enabling the double release of MMAE through a single internalisation/enzymatic activation sequence, this novel drug delivery system is more efficient than its monomeric counterpart for the killing of cancer cells that express a medium level of FR.

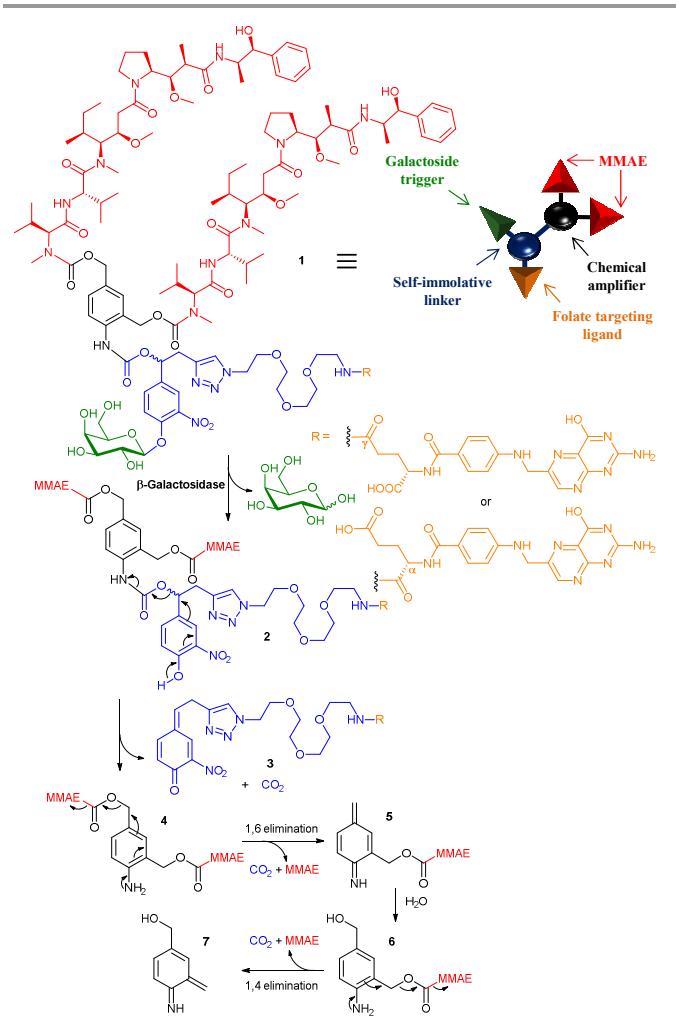
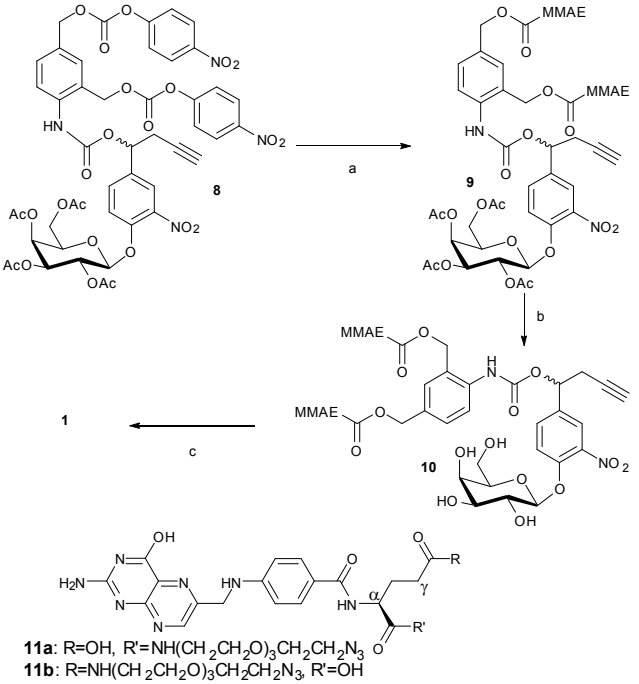


Fig. 1 Structure of the dendritic β-galactosidase-responsive folate-MMAE conjugate **1** and enzyme catalysed release mechanism of the two MMAE molecules.

Our functional molecular assembly **1** includes a galactoside trigger, a self-immolative linker,[10] a targeting ligand and two MMAE molecules articulated around a chemical amplifier (Fig. 1).[11-13] Once internalised inside FR-positive cancer cells, hydrolysis of the glycosidic bond by lysosomal β-galactosidase will generate the phenol **2** that will undergo a 1,6-elimination followed by a spontaneous decarboxylation to produce the quinone **3** concomitantly with the aniline intermediate **4**. This latter will then lead to the release of two MMAE units through successive 1,6- and 1,4-elimination processes as depicted in Fig. 1.

The synthesis of **1** was carried out starting from the biscarbonate **8** we described recently (Scheme 1).[13] Thus, MMAE was condensed on the two activated positions of **8** in the presence of HOBt to afford the expected dimer **9** in 64% yield. The galactoside moiety was then fully deprotected with LiOH in MeOH at 0°C. After 20 minutes under these conditions, the pH was adjusted to 7 and the resulting clickable derivative **10** was used in the next step without any purification. Finally, reaction between the terminal alkyne **10** and the azides **11** catalysed by copper (I) led to the amplified drug delivery system **1** (62% over two steps) that was further purified by preparative chromatography for biological evaluations (purity > 95%, as a mixture of 4 isomers, see the supporting information).



Scheme 1 Synthesis of **1**. *Reagents and conditions:* a) MMAE, HOBt, DMF/Pyr (4/1), RT, 24 h, 64%; b) LiOH, MeOH, 0°C, 20 min; c) **11**, $CuSO_4$, sodium ascorbate, DMSO/H_2O (9/1), RT, 20 h, 62% (2 steps).

We next investigated the release mechanism of MMAE from the targeting assembly **1** in the presence of β-galactosidase. For this purpose, galactoside **1** was incubated with the enzyme in phosphate buffer (pH 7.2, 0.02 M) at 37°C and the evolution of the mixture over time was followed by HPLC/HRMS (Fig. 2). The chromatograms showed the rapid disappearance (t = 35 min) of compound **1** and the emergence of four new peaks with M = 717.5040, 830.3096 and 896.5623 $g.mol^{-1}$, which correspond respectively to the free MMAE, the α- and γ-regioisomer of **3** and the aniline intermediate **6**. Thirty five minutes after the addition of β-galactosidase, traces of phenol **2** (M = 2514.3450 $g.mol^{-1}$) and dimer **4** (M = 1640.0456 $g.mol^{-1}$) were also detected in the mixture (see the supporting information). Taken together, these results confirm that the β-galactosidase-catalysed disassembly of **1** proceeds *via* the self-immolative mechanism depicted in Fig. 1. Since the release of MMAE was completed within two hours, it appeared that the whole process occurred relatively rapidly despite the complexity of the double drug release mechanism.

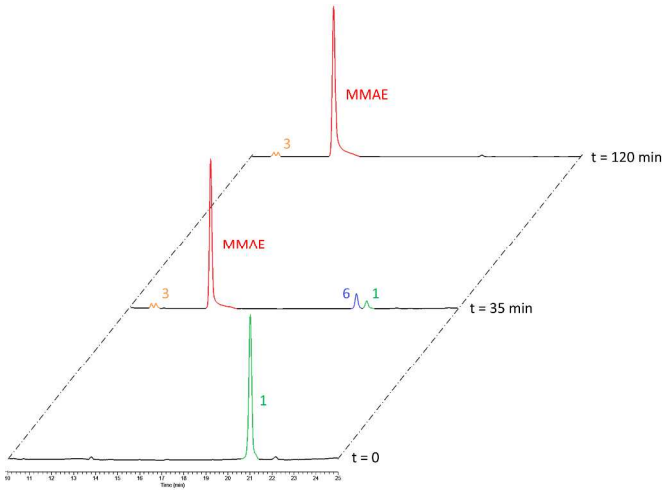


Fig. 2 Enzymatic hydrolysis of **1** with *E. coli* β-galactosidase in phosphate buffer (0.02 M, pH 7.2, 37°C) monitored by HPLC-HRMS at t = 0, t = 35 min and t = 120 min. Retention times: **1** (20.83 min), **3** (11.01 and 11.21 min), **6** (20.37 min), **MMAE** (13.76 min).

The antiproliferative activity of the dimeric β-galactosidase-responsive folate-MMAE conjugate **1** was then evaluated on HeLa, SKOV-3 and A2780 cancer cell lines and compared to that of the monomeric analogue **12**[8] (Fig. 3). These cancer cell lines were chosen in this study since they express far lower levels of FR than KB cells, usually used as a reference (see the supporting information).

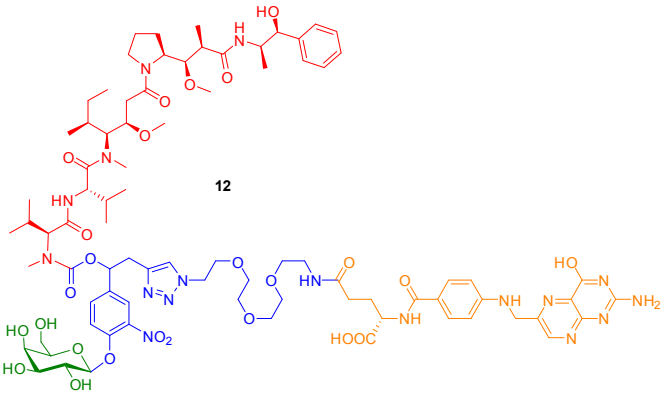


Fig. 3 Structure of the β-galactosidase-responsive folate-MMAE conjugate **12**.

As shown in Table 1, the dimer **1** dramatically affected the viability of cells, with IC_{50} values ranging from 9.62 to 64.51 nM. In comparison, the monomer **12** exhibited a 1.80 to 3.85-fold lower toxicity when incubated with the same cell lines. The superior cytotoxicity recorded with the targeting system **1** demonstrates the increased release of MMAE within tumour cells. The role of lysosomal β-galactosidase in the activation process of **1** was confirmed with a control experiment using a non-cleavable trigger (see the supporting information). However, these results also show that the [IC_{50} **12**]/[IC_{50} **1**] ratio depends on the tested cell line.

Surprisingly this ratio is approximately 2-fold higher for A2780 cells than for the other cancer cell types suggesting an enhanced amplification process. Thus, we decided to pursue our investigations by comparing the concentration of MMAE released from either dimer **1** or monomer **12** inside A2780 cells.

Table 1 IC_{50} values (nM)[a] of MMAE, **1** and **12** on HeLa, SKOV-3 and A2780 cell lines[b].

	IC_{50} (nM)			
Cell line	**MMAE**	**1**	**12**	**12/1**
HeLa	1.12±0.41	16.30±3.76	29.36±5.77	1.80
SKOV-3	0.64±0.08	9.62±1.12	20.16±1.45	2.10
A2780	6.67±3.54	64.51±14.13	248.23±87.09	3.85

[a] Values represent the mean ± SEM of five to six experiments performed in triplicate, [b] HeLa cells: human cervix adenocarcinoma, SKOV-3 and A2780 cells: human ovarian carcinoma.

For this purpose, cells were treated with 100 nM of targeting system **1** or **12** for 24 hours. Cells were then lysed and the free drug was dosed by HPLC/HRMS (Table 2). In these experiments, the galactoside dimer **1** led to the release of approximately four times more MMAE than its monomeric counterpart **12**, consistently with the observed amplification of cytotoxicity.

Table 2. Quantity of MMAE released from either **1** or **12** during treatment of A2780 tumour cells at 100 nM – Relative areas determined by HPLC/HRMS (AU).

	Relative area		
	1	**12**	**1/12**
Assay 1	56813491	13353647	4.25
Assay 2	58596545	14515391	4.04

This result indicated that a higher quantity of **1** was activated enzymatically in the culture medium. Indeed, if the same amount of drug delivery systems **1** and **12** had been hydrolysed by β-galactosidase, the quantity of MMAE released from the dimer should have been only doubled compared to the monomer. This effect could be due to extracellular activation of non-internalised folate-MMAE conjugates **1** by β-galactosidase liberated from dead cells. Since monomer **12** is less toxic for A2780 cells, the release of the activating enzyme should be less important upon treatment with this compound. This hypothesis is supported by the recent study of Antunes *et al.* who demonstrated that β-glucuronidase released from dead cancer cells can activate glucuronide prodrugs in the tumour microenvironment.[14]

Conclusion

In summary, we designed a new dendritic folate-drug conjugate including a chemical amplifier that enables the receptor-mediated endocytosis and the subsequent enzyme-catalysed release of two MMAE molecules inside cancer cells expressing the FR. This targeting system is the most potent β-galactosidase-responsive folate-drug conjugate reported so far, killing tumour cells with a medium level of FR at low nanomolar concentrations. As the FR is a target of clinical relevance, our study could be of great interest for the development of a new generation of enzyme-sensitive folate-drug conjugates that could widen the scope of FR-expressing tumours which could be treated by this therapeutic strategy.

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Notes and references

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† Electronic Supplementary Information (ESI) for experimental conditions and procedures, syntheses and compounds characterizations (^{1}H and ^{13}C NMR spectroscopic analyses and mass spectrometry data) as well as biological experiments see DOI: 10.1039/c000000x/

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