In situ targeted activation of an anticancer agent using ultrasound-triggered release of composite droplets

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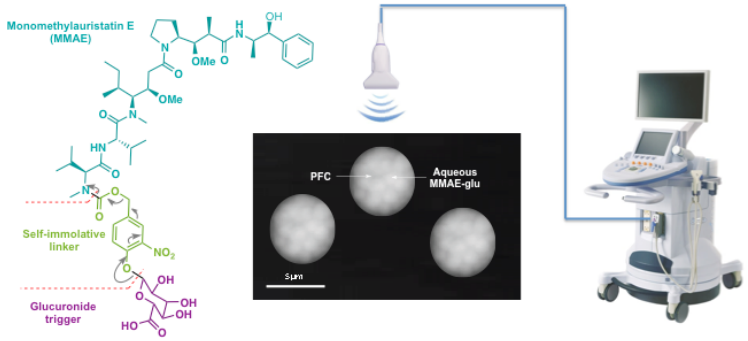

ABSTRACT: The efficiency of a drug is usually highly dependent on the way it is administered or delivered. As such, targeted-therapy, which requires conceiving drug-delivery vehicles that will change their state from a relatively stable structure with a very slow leak-rate to an unstable structure with a fast release, clearly improves the pharmacokinetics, the absorption, the distribution, the metabolism and the therapeutic index of a given drug. In this context, we have developed a particularly effective double stimuli-responsive drug-delivery method allowing an ultrasound-induced release of a monomethylauristatin E-glucuronide prodrug and its subsequent activation by a β-glucuronidase. This led to an increase of cytotoxicity of about 80% on cancer cells.

1. Introduction

The development of 'smart' carriers programmed for the localized delivery of highly cytotoxic drugs in response to extracorporeal physical stimuli offers promises for cancer chemotherapy [1-3]. Such systems are indeed designed to release most, if not all, the administered drug dose directly at the tumor site thereby avoiding any undesired toxicity in healthy tissues. To date, several stimuli-triggered release methods have been reported in the literature; these include ultrasound-activated structures [4,5], especially microbubbles [6-10] or thermosensitive liposomes [11-16]. Considering that ultrasound benefit from their high spatial (millimeter) and temporal (microsecond) resolution, their depth of penetration (up to 10 cm) and their ubiquity in hospitals worldwide [17], they represent a method of choice for achieving optimal spatial- and temporal-controlled release in the context of drug delivery. Within this framework, we previously demonstrated that ultrasound-sensitive perfluorocarbon (PFC) composite droplets injected systemically could transport large payload of fluorescein and deliver their content *in vivo* using a clinical ultrasound scanner with a millimetric resolution [18,19]. This strategy was also used by Fabiilli and co-workers to carry and release chlorambucil, a lipophilic chemotherapeutic [20], and thrombin, a serine protease used in the treatment of pseudoaneurysms [21]. More recently, Fabiilli *et al*. also reported the biodistribution of such composite droplets of perfluorocarbon (6 µm or less in diameter) in rats [22]. While this technology appeared very useful for internal tattooing of tissues, the slow leakage of the encapsulated compound observed from un-induced droplets appeared as a potential drawback to envision the adaptation of this concept to the delivery of highly toxic anticancer agents. Since the problem of unspecific release is inherent to all the carriers developed so far [23], we herein propose to evaluate a new generation of ultrasound-sensitive drug delivery systems based on the encapsulation, within ultrasound-sensitive perfluorocarbon composite droplets, of a none/less active drug precursor instead of the parent drug. Indeed, by specifically manufacturing the active agent within the ultrasound focus, not only would we be able to exclusively limit its activity within the zone delineated on the imaging scanner, but we would also limit any undesired side effects, which could occur downstream.

In order to evaluate the feasibility of this new drug delivery concept, we previously demonstrated that we were able to trigger a spontaneous reaction with a high spatial and temporal control [24]. A first proof of concept was achieved by promoting a Cu-free click reaction using a single ultrasound pulse specifically within the focus of a transducer (0.6 mm in width) and within a time frame of less than 3 ms. Since these acoustic

pulses were produced by a clinical ultrasonic scanner, the generation of a chemical reaction deep into the tissue could clearly be envisioned. We therefore decided to test this hypothesis by investigating the *in vitro* ultrasound-induced activation of a glucuronide anticancer prodrug on cancer cells. We report here the results of our endeavor.

2. Results and discussion

A glucuronide prodrug of monomethylauristatin E (MMAE-glu) [25-32] was chosen to perform this study [33,34]. Indeed, MMAE is a potent antimitotic agent currently employed in human for the treatment of lymphomas under the form of the brentuximab vedotin, an antibody-drug conjugate that reached the market in 2011 [35]. In contrast, the free MMAE cannot be used clinically because of its excessive toxicity towards non-malignant tissues. Under such circumstances, encapsulation of MMAE-glu instead of MMAE brings several advantages. Indeed, as the prodrug is much more hydrophilic than the parent drug, it is therefore a much better candidate for encapsulation in aqueous composite droplets. In addition, the glucuronide prodrug is 40-100 fold less toxic than the parent drug, which will largely reduce any non-selective toxicity caused by potential leakage of the composite droplets outside the area of interest and therefore limit potential damages of surrounding healthy tissues. Moreover, since the β-glucuronidase is over-expressed in the microenvironment of almost all solid tumors [26,36,37], ultrasound-triggered release of the prodrug in the vicinity of the tumor mass should be followed by the local enzyme-mediated release of the active drug *via* the self-immolative mechanism illustrated in Figure 1 [38,39].

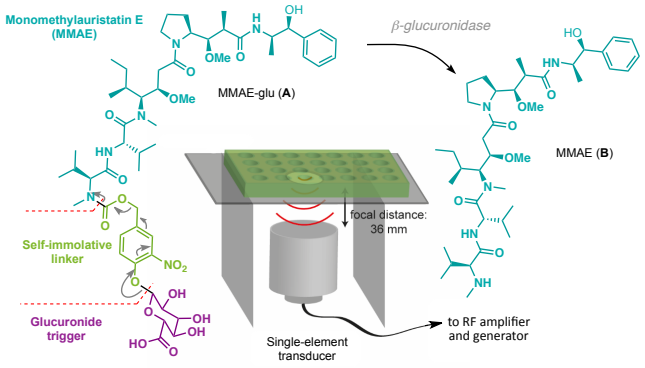

Fig. 1. Structure of MMAE glucuronide prodrug (MMAE-glu) and its self-immolative mechanism upon β-glucuronidase activation.

This strategy has proved its efficacy in numerous animal models over the years [29,37,40-45], as for example showed by Papot and co-workers in mice [30]. These prodrugs are rapidly eliminated by the kidneys, thus lowering their concentration in tumors. Nevertheless, the localized ultrasound-triggered delivery of an encapsulated prodrug should also improve its therapeutic index by both enhancing its concentration in the vicinity of the targeted tissues and minimizing its metabolization.

We first encapsulated solutions of MMAE and glucuronylated MMAE (at a concentration of 0.1 $mmol.L^{-1}$ in a RPMI1640 cell culture) in the inner phase of composite droplets of perfluorocarbon. These droplets were produced with a microfluidic system composed of parallel step emulsifiers (see experimental protocols for more details). A specific microfluidic device could yield a monodisperse population of droplets with sizes varying between 3 and 8 µm. In the context of this study, the droplets had a mean diameter of 3.3 µm with a standard deviation of 0.3 µm. Most importantly, these droplets are comparable in size and formulation to those previously exploited *in-vivo* by our group [18,19] and by the group of Fabiilli [20-22]. The *in vitro* study was carried out in 96-well plates specially designed to meet both the requirements of acoustic wave propagation and the biological needs (sterile medium, optical lecture of cell proliferation). Hence, the regular thick bottom of the plates was replaced by a thin Mylar-membrane. Those plates were immersed in a water bath thermo-stated at 37 °C, in which a single-element transducer was fixed at a distance from the plate corresponding to the focal distance of the transducer (36 mm). Four acoustic pulses were focused in each well (5 cycles ≈ 11 MPa PNP, 1.5 MHz, each pulse approximately one second apart).

Two different protocols were implemented (see experimental protocols for more details). In the first one, referred to as the "contact" protocol, KB cancer cells were grown in the wells for a 24 h period, before the droplets were added. Ultrasound pulses were then focused in the well in order to partially release the encapsulated compounds and the resulting suspension of unreleased droplets was left in contact with the cells and incubated at 37 °C for 72 h. The measurement of cell viability was then obtained through a XTT colorimetric assay. In the second protocol, referred to as the "transfer" protocol, a suspension of composite droplets was poured in an empty well. Part of the droplet's population was released using the same acoustic protocol. Only the droplets' supernatant, and therefore the ultrasound-released compounds, were transferred to the cancer cells, while the residual unreleased droplets were eliminated. After an incubation period of 72 h at 37 °C, cell proliferation was evaluated using the same XTT assay.

In order to rule out false positives, a number of control experiments were first carried out. It was indeed essential to assess the effect of the ultrasound pulses, the droplets' composition and the ultrasound-mediated vaporization protocol on the viability of KB cells. In other terms, we needed to ensure that the cytotoxic effect we would be measuring would be solely resulting from the local release of the prodrug and its specific activation. Interestingly, ultrasound exposure showed no significant alteration of the cell viability in all cases. Similarly, prolonged exposure ("contact" protocol, 72 h exposure) to a high concentration (0.5% v/v) of droplets containing only cell culture medium (CM) did not lead to significant change in mortality. Finally, the combination of droplets and ultrasound to induce local PFC vaporization appeared to be harmless as well (Figure 2).

Considering the results of the control experiments, we pursued our investigation further by studying the *in situ* release and activation of both monomethylauristatin E (MMAE) and its glucuronide analogue (MMAE-glu). To do so, we evaluated composite droplets containing MMAE and MMAE-glu, both prepared at the same initial inner concentration of 0.1 mM.

A suspension of droplets at 0.01% v/v was used in the course of this study. Regarding the *in situ* activation of MMAE-glu by the β-glucuronidase (β-glu), Legigan *et al*. [30] showed that complete conversion of the prodrug intro MMAE in the presence of β-glucuronidase was almost completely

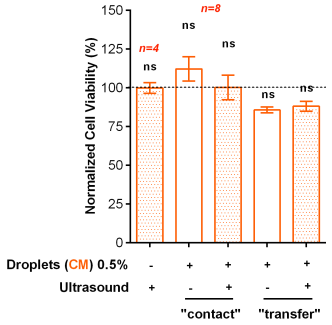

Fig. 2. Control experiments ("contact" protocol), using droplets containing pure cell culture medium (CM). When relevant, 4 ultrasound pulses were focused on the corresponding wells (5 cycles, 11 MPa PNP, 1.5 MHz). Cell viability was measured after a 72 h incubation period at 37 °C through a XTT colorimetric assay. All cell viability measurements were normalized by the viability obtained for non-treated cells. ns: no statistical difference. Experiment repeated n times.

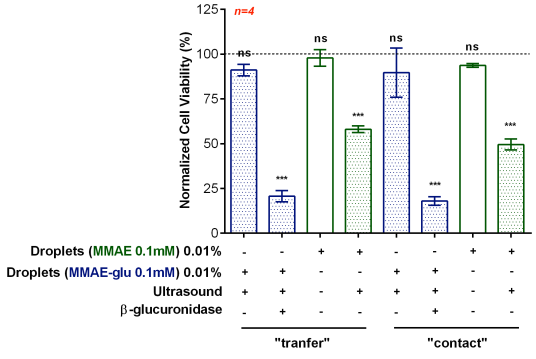

Fig. 3. In situ activation experiments ("transfer" and "contact" protocols), using droplets containing MMAE and MMAE-glu respectively. When relevant, β-glucuronidase was added (40u/well) and/or 4 ultrasound pulses were focused on the corresponding wells (5 cycles, 11 MPa PNP, 1.5 MHz). Cell viability was measured after a 72 h incubation period at 37 °C through a XTT colorimetric assay. All cell viability measurements were normalized by the viability obtained for non-treated cells. Statistical significance was determined by ANOVA test and Dunnet post-test. * p<0.05, ** p<0.01, *** p<0.001, ns: no statistical difference. Experiment repeated 4 times (n=4).

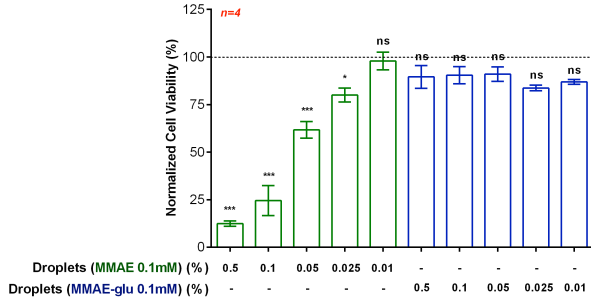

Fig. 4. Study of the droplets' stability and of the leakage phenomenon using the "transfer" protocol. Droplets containing either MMAE or MMAE-glu were used. Cell viability was measured after a 72 h incubation period at 37 °C through a XTT colorimetric assay without adding any β-glucuronidase in the wells. All cell viability measurements were normalized by the viability obtained for non-treated cells. Statistical significance was determined by ANOVA test and Dunnet post-test. * p<0.05, ** p<0.01, *** p<0.001, ns: no statistical difference. Experi-ment repeated 4 times (n=4).

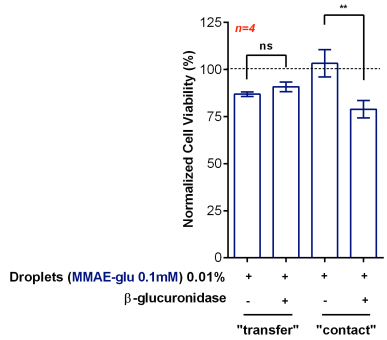

Fig. 5. Study of the droplets' stability and of the leakage phenomenon using the "transfer" protocol. Droplets containing either MMAE or MMAE-glu were used. Cell viability was measured after a 72 h incubation period at 37 °C through a XTT colorimetric assay without adding any β-glucuronidase in the wells. All cell viability measure-ments were normalized by the viability obtained for non-treated cells. Statistical significance was determined by ANOVA test and Dunnet post-test. * p<0.05, ** p<0.01, *** p<0.001, ns: no statistical differ-ence. Experiment repeated 4 times (n=4).

achieved within the first 10 minutes after contact. We were then able to compare the normalized cell viability of cells exposed to the ultrasound-released content of MMAE-glu droplets to the viability of cells exposed to both β-glucuronidase and ultrasound-released MMAE-glu. As a general trend, no statistically significant alteration of the cell viability was observed in the first case, while the exposure to both enzyme and released MMAE-glu led to a drop in viability of about 80% as a result of the *in situ* β-glucuronidase-catalyzed release of MMAE (Figure 3). A similar behavior was also observed in the case of a prolonged residence time of the droplets in the culture wells ("contact" protocol, 72 h exposure). Interestingly, however, the ultrasound-release of the encapsulated parent drug led to a smaller antiproliferative effect, which is most probably due to a slight difference in the respective MMAE and MMAE-glu droplets' formulation; the prodrug being more hydrophilic than the parent drug.

Concerned by the potential damage that could cause passive leakage of a highly toxic drug such as MMAE, we next evaluated the impact of the leakage of MMAE-glu containing droplets. To this end, KB cells were incubated for 72 h with increased concentrations of droplets containing either MMAE or MMAE-glu ("transfer" protocol) without adding any β-glucuronidase. As shown in Figure 4, increasing the concentration of MMAE-containing droplets dramatically affected the cell viability. In sharp contrast, MMAE-glu droplets exhibited no significant toxicity until the highest tested concentration of 0.5 % v/v thus showcasing the benefits of encapsulating the prodrug instead of the parent drug.

After demonstrating that MMAE-glu could be locally reactivated into MMAE upon ultrasound release and subsequent β-glucuronidase-mediated activation, and after confirming the limited impact of any residual leakage that could potentially stem from our prodrug-containing droplets, we brought additional evidence of the potential of our double-stimuli responsive strategy by showing the importance of the joint ultrasound and enzymatic activation. Remarkably, prolonged exposure of the cells to the MMAE-glu and MMAE-containing droplets ("contact" protocol, 72 h exposure) showed no statistically relevant drop in cell viability.

We also compared the viability of the cells treated with MMAE-glu-containing droplets in the presence and in the absence of β-glucuronidase without using any ultrasound. Interestingly, in the case of short-term leakage ("transfer" protocol), no significant change in cell viability was observed with both droplets in the presence or in the absence of enzyme in the culture medium (Figure 5). After a prolonged contact time of the MMAE-glu containing droplets with the cells ("contact" protocol, 72 h exposure), only a minute increase in cell mortality was detected, which showcased both the very good retention of the encapsulated compounds into the droplets and the absolute necessity of the β-glucuronidase to trigger the activation of the drug.

3. Conclusion

In conclusion, we managed to encapsulate an anti-cancer agent, monomethylauristatin E (MMAE), and its glucuronide prodrug, MMAE-glu, into composite droplets of perfluorohexane. A good retention of those compounds into the droplets was observed over three days. Moreover, cytotoxic activity of the parent drug MMAE was successfully restored by ultrasound-release of MMAE-glu droplets in the presence of β-glucuronidase with an 80% increase in cell mortality compared to the ultrasound release in the absence of the enzymatic partner. These *in vitro* results on KB cancer cells clearly validate our proof of concept. In the case of an enzymatic activation such as the one provided in this study, as the enzyme is specific of the extra-tumoral medium, the *in vivo* activation should only occurs on the tumor site. This *in situ* chemistry strategy could potentially be extended to other example of prodrug/enzyme couples as well as to other kind of chemically activatable systems [46].

4. Experimental protocols

4.1. Composite droplet preparation.

The composite droplets were fabricated in two steps. First, a primary nano-emulsion was obtained by using a sonic tip (Branson, 20 kHz, 70% of maximum amplitude, 8 cycles of 5 sec), with the fluids immersed in ice water. This nano-emulsion consists of an aqueous solution containing respectively MMAE (0.1 mM) and MMAE-glu (0.1 mM) in cell culture medium dispersed in perfluorohexane (FC-72, 3M) and stabilized by a fluorinated surfactant (PEG-di-Krytox, RAN Biotechnologies, 3% w/v in perfluorocarbon phase). The size of these nanoemulsions was evaluated on a Malvern Zetasizer Nano ZS. Typical diameter was found to be between 300 and 500 nm. The primary nano-emulsion was then encapsulated as micron-size droplets in a PBS solution (1X from 10X commercial solution), using a microfluidic device based on a parallelized system of step emulsifiers. The microfluidic system was made of polydimethylsiloxane. The droplets were stabilized by Pluronic-68® (polyoxyethylene-polyoxypropylene block copolymer, Sigma-Aldrich, St. Louis, MO, 3% w/v). The droplets were collected. Supernatant from the production step was removed and replaced by another PBS solution with various additives (Pluronic-68®, 3% w/v, Penicillin/Streptomycin 2X, Gentamycin 2X, Kanamycin 2X) to prevent any bacterial development. Droplets were then stored at 4 °C and used within 48 h.

4.2. Cell culture

KB (human oral squamous carcinoma) cells were grown in RPMI 1640 (Invitrogen) supplemented by 10% fetal bovine serum and 1% Penicillin/Streptomycin (Lonza) in a humidified incubator at 37 °C and 5% CO2. KB cells were purchased on ATCC (CCL-17) and used at early passages.

4.3. Ultrasound set-up

An ultrasound transducer (2.25 MHz, 38 mm focal length, f/d = 1, Imasonics, Voray-sur-l'Ognon, France) was fixed in a 3D-printed plastic setup, which allowed a 96-well plate immersed in a water bath thermostated at 37 °C to be moved through the acoustic focus (Figure 2a). The transducer was powered with a 40 dB RF amplifier (TOMCO, Stepney, Australia) driven by an arbitrary waveform generator (Tektronix, Beaverton, Oregon). Four acoustic pulses (5 cycles, 11 MPa PNP, 1.5 MHz) were triggered after aligning the target-well with the acoustic focus. The 96 well-plates were bottomless plates under which a Mylar membrane was glued under sterile conditions to provide an optically and acoustically transparent window.

4.4. Contact protocol

KB cells were seeded and grown for 24 h in poly-L-lysine-coated thin-bottom 96-well plates. After this first incubation period at 37 °C, 70 µL of a 0.01 % v/v suspension of droplets in cell culture medium was placed in each well, on the cells. When relevant, 4 ultrasound pulses were focused on the corresponding wells, followed when necessary by a solution of β-glucuronidase (E. coli, 40u/well). The plates were then incubated at 37 °C for 72 h, before XTT assay was performed to evaluate cell proliferation.

4.5. Transfer protocol

KB cells were seeded and grown for 24 h in regular 96-well plates. After this first incubation period at 37 °C, 90 µL of a 0.01% v/v suspension of droplets in cell culture medium was placed in thin-bottom 96-well plates. When relevant, 4 ultrasound pulses were focused on the corresponding wells (5 cycles, 11 MPa PNP, 1.5 MHz). The content of each well was centrifuged, and the non-released droplets were eliminated. Supernatant containing the ultrasound-released compounds (70 µL) was then transferred on the KB cells in the regular 96-well plates, followed when necessary by a solution of β-glucuronidase (E. coli, 40 u/well). The plates were then incubated at 37 °C for 72 h, before XTT assay was performed to evaluate cell proliferation.

4.5. Cell viability measurements

The cell viability kit II (XTT, Roche) was used to assess cell viability. This assay is based on the cleavage of XTT by metabolic active cells resulting in the production of an orange formazan dye quantified by spectrophotometry. Assays were carried out essentially as described by the manufacturer. Briefly, after the incubation specified in each protocol, 25 µL of the XTT labeling mixture were added per well. Cells were further incubated for additional 4 h at 37 °C before determination of the absorbance at 490 nm (Absorbance was read using a multimode microplate reader (Mithras LB940, Berthold Technologies). Experiments were performed n times ($4 < n < 8$) per condition. Statistical significance was determined by ANOVA test and Dunnet post-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns: no statistical difference.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at

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