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Histone deacetylase inhibitor-polymer conjugate nanoparticles for acid-responsive drug delivery

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ABSTRACT

We report the synthesis of acid–responsive polymeric nanoparticles (NPs) consisting of a polymer-histone deacetylase inhibitor conjugate. An innovative aspect of this drug delivery particle lies in the NP conjugation of a histone deacetylase (HDAC) inhibitor, CI-994 (Tacedinaline), introduced with a clickable acid-responsive prodrug during monomer synthesis, prior to polymerization. Another novelty lies in the selected norbornene (NB)-polyethylene oxide (PEO) macromonomer allowing standardization of the polymerization process by Ring-Opening Metathesis Polymerization (ROMP) and functionalization through azide-alkyne click chemistry. Herein we demonstrate that the synthesized polymer gave 300 nm core–shell spherical nanoparticles with low dispersity (0.04), high water dispersability thanks to the PEO shell and well controlled HDAC inhibitor prodrug loading. Bioluminescence Resonance Energy Transfer (BRET) assay in living cells and viability experiments demonstrated efficient cellular internalization without additional chemistry, drug release inside cells with restoration of the HDAC inhibition and induction of apoptosis. Such NPs should minimize drug release *in vivo* during blood circulation and trigger intracellular delivery after endocytosis, holding promises for improved efficacy of this class of epigenetic inhibitors. This standardized synthesis paves the way for multifunctional nanoparticles synthesis.

1. Introduction

Epigenetic modifications, which are responsible for heritable changes of genes activity that are independent of changes in DNA sequence, are hallmark of several pathologies. All these modifications are used to fine-tune gene expression through changes in chromatin structure. Deregulation of these subtle mechanisms, leading to the abnormal expression of key regulatory genes, is implicated in various diseases including cancers [1]. These modifications called epigenetic marks consist in reversible chemical modifications on DNA and post-translational modifications (PTMs) of histones, mediated by the opposite actions of several protein families defined as epigenetic marks writers, erasers and readers and leading to the "histone code" [2]. DNA methylation is regulated by DNA methyl transferases (DNMT) and for demethylation by cytosine oxidases (TET) as well as base excision repair mechanisms. Histones can be acetylated or methylated via histone acetyl transferases (HAT) and deacetylases (HDAC) whose opposite activities equilibrate histone acetylation, and histone or protein arginine methyl transferases (HMT and PRMT) with the histone

Abbreviations: ADCA, adenocarcinoma; BrD, bromodomain; BRET, bioluminescence resonance energy transfer; DDS, drug delivery system; DLS, dynamic light scattering; DMF, dimethyl formamide; FITC, fluorescein-isothiocyanate; HDAC, histone deacetylases; HDACi, histone deacetylase inhibitor; mBu, milliBRET units; MPM, malignant pleural mesothelioma; NB, norbornene; NPs, nanoparticles; PDI, polydispersity index; PEO, polyethylene oxide; PMDETA, pentamethyldiethylenetriamine; PTM, post-translational modifications; ROMP, ring-opening metathesis polymerization; SEC, size exclusion chromatography; THF, tetrahydrofuran; TLC, thin layer chromatography; TSG, tumor suppressor genes.

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demethylases counterparts (HDM) [3]. HDACs are one of the most studied epigenetic targets. Indeed, HDACs are overexpressed in numerous cancer cells, which leads to histone hypoacetylation involved in tumour suppressor genes (TSG) down regulation, like p21 [4]. Inhibitors of HDACs (HDACi) have been investigated in the past two decades as an alternative strategy to fight diseases resulting from the overexpression or modified activity of these epigenetic proteins. HDACi induce an increase of acetylated histones, which leads to chromatin relaxation along with an increase of gene transcription, notably TSG gene transcription [5]. In addition HDACi displayed interesting anti-tumor properties on a large number of different malignant cells [6]. These compounds are considered nowadays in clinic in single or combined therapies against various diseases like cancer [7] and in particular when current therapies failed [5,8]. However, HDACi, like many other chemotherapeutics, have weaknesses limiting their efficacy *in vivo*: clearance, fast metabolism and poor specific accumulation in tumour leading to side effects [8].

In the field of anticancer strategies using chemotherapeutics with limited bioavailability, several types of drug delivery systems (DDS) have been proposed to protect the molecules from fast clearance, to circumvent solubility limitations and/or to selectively deliver compounds in tumour in order to decrease systemic toxicity. Typical examples of clinically used DDS are Doxil®, a liposome formulation containing doxorubicin, or Abraxane®, an albumin-paclitaxel conjugate. Only few examples of DDS applications designed for epigenetics have been reported that are mainly, in reality, for epigenetically repositioned compounds [9]. Developing novel strategies to improve novel HDACi benefits could thus come from delivery systems adapted to such compounds. However, the strategy can fail due to the limited compound solubility in the solvent conditions when preparing DDS micelles. Loading could be improved by converting HDAC inhibitors into more soluble compounds by new design. This re-development stage could be avoided by a simpler approach where current inhibitors are converted to soluble prodrugs [10] used in turn to prepared DDS. Alternatively, the prodrug could be covalently linked to the DDS to avoid leaks, a well-known problem with liposomes.

The choice of the releasing and DDS connection strategies is thus particularly important. If glucuronidation or conversion to esters were often used to prepared prodrugs they are not convenient strategies for HDAC inhibitor. Glucuronidation is interesting because glucuronidases are overexpressed in the tumour environment; however glucuronidation of HDAC inhibitors is a major metabolic pathway. On the other hand esterases are ubiquitous enzymes in humans and selective release from ester prodrugs at the tumour site cannot be achieved this way. Finally, epigenetic targets are mostly nuclear and this implies that the DDS or the prodrugs must preferably enter the cells and then release the compounds close to the nucleus. In the past decades, DDS strategy based on tumour cell internalization via endocytosis [11] and release at acidic pHs has appeared to be a convenient answer to these challenges. This strategy is also particularly interesting to avoid release of the compounds at physiological pHs during blood circulation. Polymer conjugates were investigated with a particular emphasis for pH-mediated release [12,13] through endocytosis. pH-Mediated release is also a practical solution avoiding complex prodrug syntheses, like those developed for glucuronidase strategies.

We applied the DDS strategy associated with a pH mediated release system to CI-994 (Tacedinaline). This HDACi is a member of the benzamide-related group, demonstrating interesting anti-tumor effects on cancer cells in culture. CI-994 inhibits preferentially the nuclear HDAC class I (HDAC1-3) [14] directly participating in the stimulation of TSG expression in cancer cell lines. CI-994 has higher half live than other HDAC inhibitors, as shown by [^{3}H] radiolabelled derivative [15], which is in part due to moderate protein binding *in vivo* allowing a histone deacetylation effect in a wider time range. Despite these long-ranging effects, the results obtained in clinic were disappointing [8]. Indeed, the principal dose limiting toxicity was thrombocytopenia and no evident anti-tumor effect was observed [8,16]. This demonstrates that stability is not the major weakness of this class of drugs and that systemic toxicity and probably diffusion in tumour tissues also constitute major barriers for clinical efficacy. Thus, this HDACi seemed to be a good candidate for vectorization strategy using DDS. We previously synthesized the acid-responsive prodrug **3** (Scheme 1) for CI-994, with a tetraethylenoxide chain to improve water solubility [17]. This novel releasing system was designed for cleavage at mildly acidic pH corresponding to the pH found in endosomes/lysosomes generated during the endocytosis-mediated internalization. This type of prodrug has demonstrated convenient stability at physiological pH and effective release at low acidic pH. This CI-994 prodrug has also shown good restoration of the initial HDAC inhibition, correlated with cancer cell death. In this work, it was thus envisioned to attach this prodrug to DDS able to enter cancer cells and protecting the prodrug from external metabolism. Exploiting endocytosis for DDS internalization allows for both DDS and the prodrug to be respectively degraded and cleaved when exposed to the acidic endosome/lysosome pathways. Several methods are available to connect prodrugs to DDS, a currently popular one being the bioconjugation based on the click chemistry concept [18–20] involving the reaction between alkyne and azide groups. These rationales were the basis for initial introduction of an alkyne group on the PEO-end chain of the prodrug system **3** to allow future grafting to DDS by click chemistry, implying that the DDS should bear azide groups. Within the several polymeric nanoparticles systems available [21] living Ring-Opening Metathesis Polymerization (ROMP) has recently emerged as an alternative method to produce therapeutic DDS and offers opportunities for well-defined spherical core–shell polymeric nanoparticles. We demonstrated that such biopolymers can be obtained from norbornenyl-PEO macromonomers adapted to the click chemistry reaction and that the final nanoparticles can natively enter cancer cells by endocytosis without any additional chemistry [22,23].

We hypothesized that the generic azido-norbornenyl-PEO macromonomer **5** (Scheme 1) involved in our ROMP-DDS synthesis could be made functional with our alkyne-bearing CI-994 acid-responsive prodrug **3** [17]. In this contribution we report our investigations in the synthesis of such pH responsive CI-994-bearing DDS and its physical and chemical characterization. Delivery of the HDACi in cells and restoration of HDAC inhibition was evaluated. Anti-tumor properties of our DDS on malignant pleural mesothelioma (MPM) and lung adenocarcinoma (ADCA) cancer cells, two forms of aggressive thoracic cancers with poor chemotherapeutic options, was studied in comparison with free CI-994 and bare DDS.

2. Results and discussion

2.1. Syntheses

The key macromonomer **6** (Scheme 1) was synthesized by azide-alkyne click chemistry from macromonomer **5** [23] and derivative **3** [17] best suited for CI-994 release at endosomal/lysosomal pH 5. Good yields in Huisgen cycloaddition required 2 equivalents of CuBr and the ligand PMDETA and 1.5 equivalent of the alkyne **3**. The formation of the macromonomer **6** was confirmed by ^{1}H NMR (Fig. 1). Protons signals for the prodrug part were assigned according to the ^{1}H NMR of the starting prodrug **3**, as no major shifts were observed after cycloaddition. The two key difference were the disappearance of the alkyne proton signal