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Research paper

Delivery system for budesonide based on lipid-DNA

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ABSTRACT

Budesonide is a hydrophobic glucocorticoid with high anti-inflammatory activity for the treatment of asthma, inflammatory bowel disease and rheumatoid arthritis. A micellar drug-delivery system based on lipid-DNA may provide a strategy to maximize its drug efficacy and reduce adverse effects. In this work, we report the use of lipid-DNAA **(UU11mer)**, featuring two hydrophobic alkyl chains and forming micelles at a comparatively low critical micelle concentration, to render budesonide water-soluble with a high loading capacity (LC). The inhibition of interleukin-8 (IL-8) release shows that the new delivery system retains the inhibitory activity in cell-based assays. In conclusion, this research provides a novel approach to formulate and administer budesonide in a non-invasive manner, which dramatically improves its water-solubility while retaining its bioavailability.

1. Introduction

Asthma [1], inflammatory bowel diseases (IBDs, such as Crohn's disease [2] or ulcerative colitis [3]) and rheumatoid arthritis [4] are distinct disorders that are all characterized by chronic inflammation. The etiology of these diseases is not yet fully understood, but a complex interaction of environmental and genetic factors has been identified to contribute to the pathogenesis of these diseases [5,6]. As these diseases are incurable, the aim of current treatment is directed toward treatment of inflammation-induced symptoms [7–9]. Various anti-inflammatory regimens exist for the different disease states, yet, anti-inflammatory glucocorticoids are used in all. The anti-inflammatory activity of glucocorticoids is generally attributed to the repression of pro-inflammatory genes through signal transduction by the glucocorticoid receptors (GRs, NR3C1). The GRs are ligand-inducible transcription factors belonging to the nuclear receptor superfamily, which are expressed predominantly in the cytoplasm of virtually all cell types. Glucocorticoids exert their anti-inflammatory effects by binding to the GRs. The mechanisms modulating the repression of pro-inflammatory genes expression remain incompletely understood. Direct interaction of the GRs with inflammatory transcription factors to repress transcriptional activity of inflammatory genes, which subsequently results in decreased production of pro-inflammatory proteins, i.e. transrepression, represents the generally accepted mechanism of action for the most effective anti-inflammatory drugs presently used [10].

Budesonide is a glucocorticoid with high anti-inflammatory activity that is commonly used for the treatment of chronic inflammation. Depending on the disease that is treated, the delivery route for budesonide can be rectal [11], oral [12], intranasal [13], intravenous [14] or pulmonary [15]. Hence, various delivery strategies have been developed for each case [16–20]. Because of its hydrophobicity and poor water-solubility [21], which require organic solvents like DMSO for *in vitro* studies, budesonide presents low local bioavailability during treatment. As a result, higher doses have to be utilized, increasing the risk of systemic adverse effects [22,23]. Systemic bioavailability of orally administered budesonide is only 10–15% due to extensive first-pass metabolism [24], which limits the therapeutic potential of this efficacious glucocorticosteroid. In order to maximize drug efficacy and reduce the adverse effects, new delivery strategies are necessary.

Micelles have specific properties such as high efficiency, good reproducibility, simple preparation and stimuli-responsiveness [25–29], making them widely used nanocarriers of poorly water-soluble drugs.

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Amphiphilic molecules consist of a hydrophilic and a hydrophobic moiety and self-assemble into micelles in aqueous solution. Micelles have a hydrophilic external corona, and a hydrophobic interior in which hydrophobic drugs can be encapsulated through non-covalent interactions with minimal impact on the drug.

Despite various amphiphilic materials being used [30], it is still a challenge to construct a biocompatible and effective micellar drug-delivery systems. Previous studies have shown that lipid-DNA amphiphiles, consisting of a hydrophilic DNA moiety and hydrophobic alkyl tails, can undergo self-assembly into micelles, leading to potential nanocarriers of hydrophobic drugs [31,32]. Owing to their small size and the use of biocompatible DNA as a component, these nanocarriers provide several advantages including: (1) high drug loading capacity (LC) attributed to the interactions of hydrophobic interactions between the drugs and the hydrophobic interior; (2) improved biocompatibility by reducing the dose; (3) automated synthesis [33,34]; and (4) ease of modification by taking advantage of DNA hybridization to endow ligand-receptor-mediated drug targeting properties (such as folic acid to sites of inflammation [35]). For instance, de Vries et al. recently reported the first use of la amphiphiles in the field of ophthalmic drug delivery. These nanocarriers showed improved efficiency compared to the free drug and exhibited an excellent biosafety and biocompatibility with human tissue [36]. It demonstrates that these advantages can be of benefit to almost all drug-administration routes.

Based on the above considerations, the aim of this study is to investigate a lipid- DNA amphiphile, **UU11mer** [32], featuring two hydrophobic alkyl chains (Fig. 1d), that forms micelles at comparatively low critical micelle concentration (CMC) as solubilizers of budesonide (Fig. 1a) and to test the anti-inflammatory properties of the novel form of solubilized budesonide.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie N.V., Zwijndrecht, The Netherlands) or TCI Europe (TCI Europe N.V., Antwerp, Belgium) and were used without further purification, unless otherwise noted. In all experiments, MilliQ standard water (Millipore Inc., USA) with a typical resistivity of 18.2 MΩ/cm was used. Pristine oligonucleotide **11mer** (5′-TTTGGCGTCTT-3′) was purchased from Biomers.net (Ulm, Germany) at high-performance liquid chromatography (HPLC) purification grade.

2.2. Synthesis of lipid-DNA

Lipid-modified oligonucleotide **UU11mer** (5′-UUTGGCGTCTT-3′) with two modified uracils (U represents the modified uracils) was prepared by using solid-phase synthesis [32].

2.3. Preparation of budesonide loaded lipid-DNA micelles

Budesonide (107.63 μg, 0.25 μmol) (generous gift of H.W. Frijlink, University of Groningen) in ethanol (1 mg/mL) was loaded into a 2.0 mL vial. Ethanolwas removed by vacumn at 30°C for 3 h. In the mean time, an aqueous solution of **UU11mer** (50 μM) 1000 μL in 3 500 μL Eppendorf tubes was thermally cycled (90 °C, 30 min; −1 °C/2 min until room temperature) by using a polymerase chain reaction (PCR) thermocycler (Biorad, USA) before use. After that, **UU11mer** solution was added directly to budesonide, and the mixture was stirred (1000 r/min) for 12 h at room temperature. Then the mixture was centrifuged at the relative centrifugal force (RCF) of 8609 *g* for 15 min using an Eppendorf 5418 centrifuge (Eppendorf, Hamburg, Germany) and passed through a 0.22 μm pore-sized syringe filter (Millipore, Bedford, MA, USA).

2.4. Cryo-electron microscopy (Cryo-EM)

Cyro-EM as performed according to standard procedure. 3 μL of suspension was placed on a glow-discharged holy carbon coated grid (Quantifiol 3.5/1, Quantifiol GmbH, Jena, Germany) blotted and vitrified in a Vitrobot (FEI Company, Eindhoven, The Netherlands).

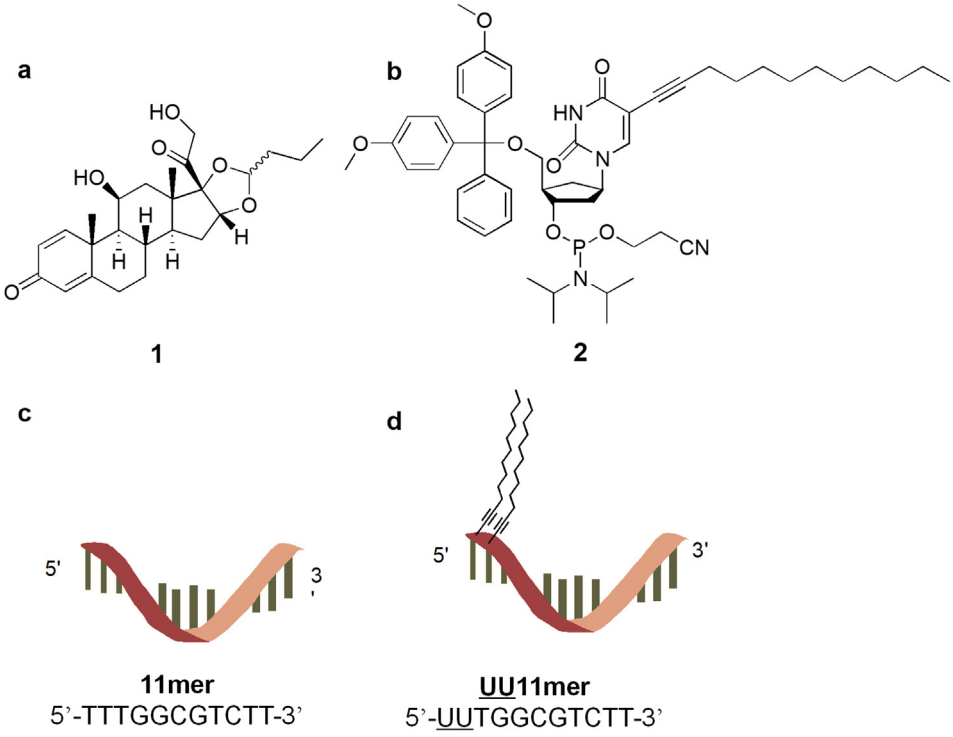

Fig. 1. Representation of (a) budesonide (1); (b) 5-(dodec-1-ynyl)uracil deoxyribophosphoramidite (2) used in solid-phase synthesis of **UU11mer**, this nucleotide building block is abbreviated as U in the corresponding sequence **UU11mer**; (c) pristine control **11mer**; (d) **UU11mer** used for the solubilization of **1**.

Table 1
RP-HPLC gradient.

Time (min)	%A	%B
0	95	5
3	95	5
30	5	95
35	5	95
40	95	5

Samples were observed in a Gatan 626 cryo-stage (Gatan, Pleasanton, CA) in a Philips CM120 (Philips, Eindhoven, The Netherlands) operating at 120 keV or in a FEI Tecnai T20 (FEI Company, Eindhoven, The Netherlands) operating at 200 keV. Images were recorded under low-dose conditions on a slow-scan CCD camera.

2.5. Determination of budesonide concentration and LC

The concentration of budesonide in the dispersions was determined by reversed-phase high-performance liquid chromatography (RP-HPLC). RP-HPLC conditions used: column, Xterra Prep MS C18 (Waters, Milford, MA, US), 10 μm, 7.8 × 150 mm; flow rate, 1.0 mL min^{-1}; wavelength 244 nm; eluent A, H_2O (0.1% trifluoroacetic acid (TFA, Iris Biotech GmbH, Marktredwitz, Germany)); eluent B, acetonitrile (0.1% TFA); injection volume, 20 μL; gradient, Table 1. The calibration curve of budesonide in ethanol was obtained by using the linear least square regression procedure of the peak area versus the concentration (Fig. S1). Each measurement was performed in triplicate; the average value was used for the calibration curve.

To determine the concentration of budesonide in the micellar dispersions, 500 μL of the supernatant was lyophilized, and cold ethanol 500 μL was added to extract budesonide. After being centrifuged at 8609 *g* for 15 min, 300 μL supernatant was removed to run the RP-HPLC measurement. Each measurement was performed in triplicate (Fig. S2). The budesonide concentration was obtained according to the calibration curve. Budesonide LC was calculated by the following equation:

$$\%LC = \frac{\text{Weight of budesonide loaded}}{\text{Weight of DNA}} \times 100\% \quad (1)$$

2.6. Cell culture

Human bronchial smooth-muscle cell lines, immortalized by stable expression of human telomerase reverse transcriptase (hTERT), were used for the Interleukin-8 (IL-8) determination experiments. hTERT airway smooth-muscle cells were generated from primary cultured human bronchial smooth muscle cells as described before [37].

2.7. IL-8 determination

Cells were plated in 24-well cluster plates (Costar, Corning Incorporated, NY, USA) and grown to confluence, using Dulbecco's modified eagle medium (DMEMsupplemented with 10% foetal bovine serum and antibiotics (50 U/mL streptomycin, 50 μg/mL penicillin and 1.5 μg/mL amphotericin B, (all from Gibco, Grand Island, NY, USA). Cultures were maintained in a humidified incubator at 37 °C, gassed with 5% CO_2.

Upon confluence, cells were washed two times with sterile phosphate-buffered saline (PBS) and serum-starved for 24 h in DMEM supplemented with antibiotics and ITS (5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenium (Gibco, Grand Island, NY, USA)). Cells were then washed with PBS and stimulated with interleukin-1β (IL-1β, 0.1 ng/mL) (Sigma-Aldrich Chemie N.V., Zwijndrecht, The Netherlands) in serum-free medium. **UU11mer** micelles loaded with budesonide (3 nM, 30 nM and 300 nM) were added 30 min before stimulation with IL-1β. Supernatants were collected 24 h after stimulation and stored at –80 °C until use.

IL-8 levels were determined using a specific sandwich enzyme-linked immunosorbent assay (ELISA) (Sanquin, Amsterdam, The Netherlands) according to the manufacturers' instructions.

3. Results and discussion

In the different protocols, we used a pristine DNA, **11mer** (Fig. 1c), that has the same nucleic acid sequence as **UU11mer** as a reference. **UU11mer**, which contains two modified uracil bases (Fig. 1b), where U represents the modified uracil base) was synthesized according to the published procedure [32]. The CMC was determined to be 29 μM for **UU11mer**. Therefore, we chose a concentration of 50 μM for the solubilization experiment because this concentration is greater than the CMC of **UU11mer**. We used Cryo-EM to visualize the empty and loaded **UU11mer** micelles and to corroborate their sizes and morphological aspects. Cryo-EM images (Fig. 2) show the formation of micelles with a narrow size distribution and regular shape both before and after budesonide loading. No obvious aggregation was visible and the diameter of **UU11mer** micelles increases slightly from 9.0 + 1.2 nm to 10.3 ± 1.5 nm after budesonide loading. Various methods have been reported for producing delivery systems of water-insoluble drugs [37–41]. In our research, budesonide was successfully incorporated into lipid-DNA micelles by simply mixing the solid budesonide with an

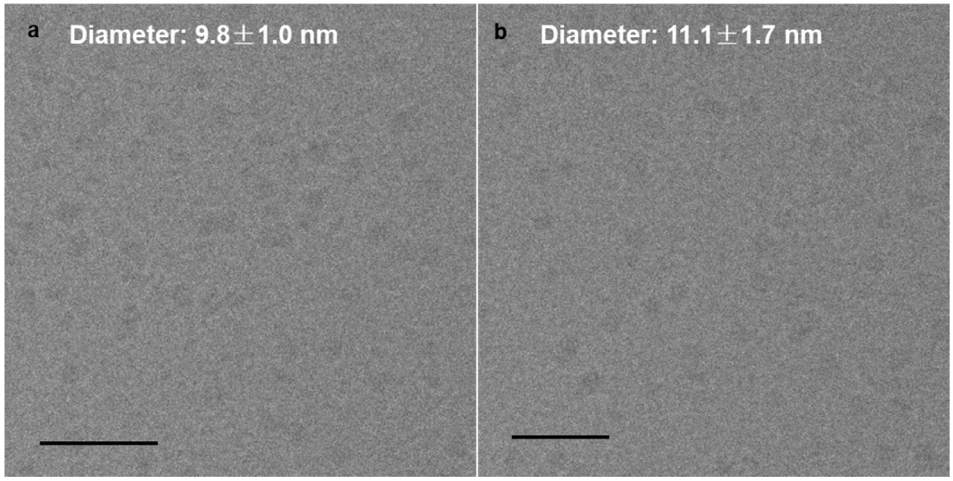

Fig. 2. Cryo-EM images of (a) **UU11mer** micelles; (b) **UU11mer** micelles loaded with budesonide. No stain was used and image acquisition was achieved at a 2 μm defocus. Scale bar = 50 nm.

Table 2
Concentrations and drug loading capacities (LCs) of budesonide-loaded samples.

Sample	Integral	Concentration (μM)	Average concentration (μM)	LC (%)	LC (%) reference correction
H_2O-1	635,340	45.1	44.9	–[a]	–[a]
H_2O-2	630,855	44.8			
H_2O-3	629,421	44.7			
11mer-1	671,172	47.6	47.4	12.3	–[a]
11mer-2	664,594	47.1			
11mer-3	664,594	47.4			
UU11mer-1	1,277,458	89.3	94.5	22.4	10.1
UU11mer-2	1,387,306	96.9			
UU11mer-3	1,391,454	97.2			

[a] Not applicable.

aqueous solution of the carrier and stirring at room temperature for 12 h. In this way, the budesonide was incorporated gradually into the micelles to reach the maximum LC and equilibrium. As a result, the new solution was stable without any precipitate being observed after one month at 4 °C.

We determined and calculated the loaded concentration of budesonide in H_2O, **11mer** micelles and **UU11mer** micelles by RP-HPLC according to the calibration curve (Fig. S1). We performed the solubilization of budesonide into H_2O or **11mer** following the same procedure as for **UU11mer** micelles. The solubility of budesonide is 44.9 μM in H_2O and 47.4 μM in **11mer** aqueous solution (50 μM), respectively (Table 2). The slight difference indicates that the usage of pristine oligonucleotide does not significantly improve the solubility of budesonide. However, the loaded concentration of budesonide into **UU11mer** micelles (94.5 μM) is much greater than that in **11mer**, which illustrates that the formation of micelles greatly improves the solubility of budesonide in aqueous solution. In conclusion, budesonide was loaded into **UU11mer** micelles at an improved concentration and a high LC (22.4%), leading to a new stable delivery system for budesonide.

Encouraged by this result, we studied the anti-inflammatory activity of the different delivery systems of budesonide by its effect on IL-1β-induced release of IL-8 from hTERT immortalized human airway smooth muscle (ASM) cells (Fig. 3). Their nanoscale size suggests the non-specific pinocytotic uptake of **UU11mer** micelles into the cells, which is followed by endosomal escape and the release of budesonide to complex with the GRs [42]. The diffusion of the budesonide-GR complex into the nucleus to target the pro-inflammatory genes can lead to the therapeutic effect through the repression of pro-inflammatory genes. IL-1β is a representative inflammatory stimulus. In this setting, we compared the effect of budesonide (3 nM, 30 nM and 300 nM) loaded **UU11mer** micelles to the effect of 30 nM budesonide in 0.3% dimethyl sulfoxide (DMSO) on basal and IL-1β-induced IL-8 release. Basal IL-8 release, without IL-1β stimulation, is not significantly affected by any of the budesonide solutions or vehicles used (Fig. 3a). Stimulation with IL-1β induces a strong increase in the release of IL-8 from ASM cells (basal: 0.12 ng/mL *vs.* IL-1β: 188 μg/mL, $p < 0.0001$). Whereas DMSO has no significant effect on this response, the empty **UU11mer** micelles induce a 30% inhibition of the IL-1β response ($p < 0.01$; Fig. 3b), demonstrating that empty **UU11mer** micelles by themselves have some inhibitory effect on IL-8 release. However, the reduction induced by the empty **UU11mer** micelles is not significantly different from the 22% reduction caused by DMSO. As expected, budesonide dissolved in DMSO (0.3% final concentration) at a concentration of 30 nM inhibits IL-1β induced IL-8 release by 78% ($p < 0.01$, Fig. 3b). Interestingly, budesonide solubilized using **UU11mer** micelles inhibits IL-1β induced IL-8 release in a concentration-dependent manner (Fig. 3b). At concentrations of 3 nM, 30 nM and 300 nM, the inhibition is 84.7% ($p < 0.01$), 90.1% ($p < 0.001$) and 92.2% ($p < 0.001$), respectively. In addition, the inhibition with 3 nM using the **UU11mer** micelles is stronger than previously described using DMSO [43]. These results demonstrate that **UU11mer** micelles are an effective way to solubilize budesonide while maintaining its anti-inflammatory properties.

4. Conclusions

In conclusion, we successfully incorporated the water-insoluble budesonide into biocompatible nanoparticles, lipid-DNA (**UU11mer**) micelles. The new delivery system features an improved concentration (up to 94.5 μM), a high drug LC and good stability. The study on the inhibition of IL-8 release showed that in this new delivery system, budesonide maintains its anti-inflammatory activity. This new way to solubilize budesonide offers opportunities to formulate and administer budesonide in novel ways and would potentially allow for the treatment of additional conditions that are currently limited by the poor solubility and very low bioavailability of budesonide.

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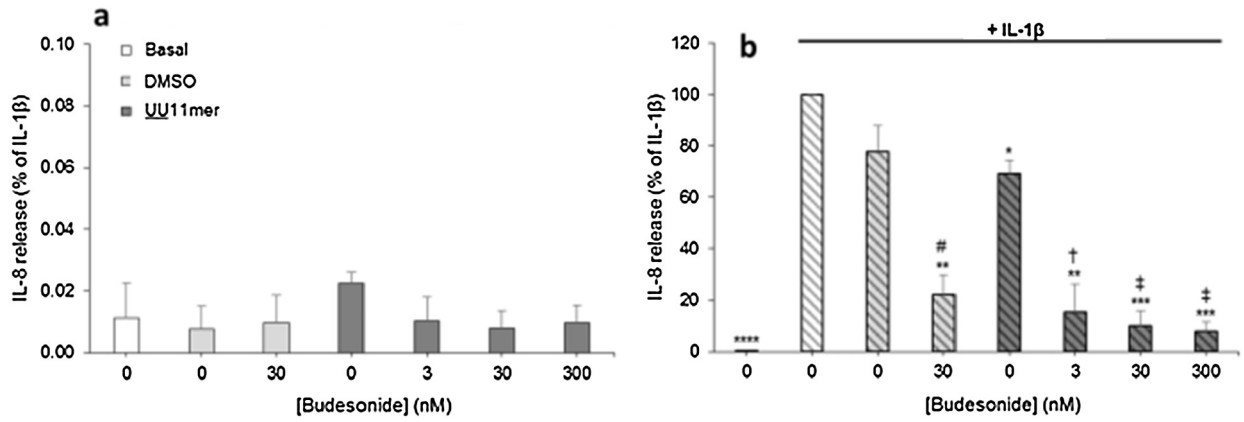

Fig. 3. IL-8 released by hTERT human airway smooth muscle (ASM) cells under basal (a) and IL-1β stimulated (b) conditions in the absence and presence of 0.3% dimethyl sulfoxide (DMSO) or **UU11mer** micelles alone or loaded with budesonide in indicated concentrations. Cells were pretreated with budesonide for 30 min and subsequently stimulated with 1 ng/ml IL-1β for 24 h. Supernatants were collected and IL-8 levels were analyzed by enzyme-linked immunosorbent assay (ELISA) and corrected for total protein content. Data represent means ± S.E.M. of 4 experiments performed $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}P < 0.001$ and $^{****}p < 0.0001$ compared to IL-1β-treated control; $^{\#}p < 0.05$ compared to DMSO; $^{\dagger}p < 0.01$ and $^{\ddagger}p < 0.001$ compared to **UU11mer**.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ejpb.2018.06.012.

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