Amphoteric liposomes are a platform for multi-organ delivery of oligonucleotides

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ABSTRACT

Oligonucleotides (ONs) such as antisense (ASO) or siRNA have created a need for new technologies that further enable the systemic and targeted delivery of these active principles. Here, we describe amphoteric, fully charge-reversible liposomes as a novel carrier concept that overcomes the limitations in biodistribution of cationic vectors systems while keeping functionality.

Keywords: oligonucleotides, delivery, liposomes, amphoteric.

1 INTRODUCTION

The majority of the current carriers for ONs are based on cationic liposomes or polymers; this is driven by the ease of complex formation between carrier and ON and the efficient delivery in vitro. However, these systems may suffer from limited organ penetration [1], induction of cytokine responses [2,3] or liver enzyme release [4].

Neutral or anionic liposomes would be safe, but suffer from low encapsulation of the payload. [5]

We here introduce amphoteric liposomes as a novel carrier principle that retains the favorable safety and biodistribution profile of anionic carriers while keeping the efficient cargo loading and cell transfecting properties of the cationic particles. Amphoteric liposomes still target the liver, but can also reach more distal sites such as tumors or inflamed tissues.

2 RESULTS

A series of amphoteric liposomes was prepared and best candidates were identified for each application. In all experiments, fluorescently labeled ONs were used to track the biodistribution and cellular uptake of the material and the efficacious delivery was analyzed with RT-PCR.

2.1 Delivery of siRNA to the liver

A Cy3-siRNA was encapsulated in amphoteric liposomes nov340 and nov582 and a single dose was injected intravenously in mice. With both types of liposomes, an intense staining of the liver parenchyme was observed.

While formulation nov582 was apparently taken up by all cell types, delivery using nov340 resulted in a more star-like pattern which resembles the cellular distribution of liver macrophages. Both carriers facilitated the knock-down of the target message after delivery of 2*4mg/kg of the respective siRNA, but no such effect was observed when carrier was given without such cargo ON. While nov340 resulted in about 60% target mRNA reduction, use of nov582 yielded about 85% target elimination. We attribute this more potent delivery of nov582 to its more homogeneous distribution in the liver and the improved exposure of hepatocytes.

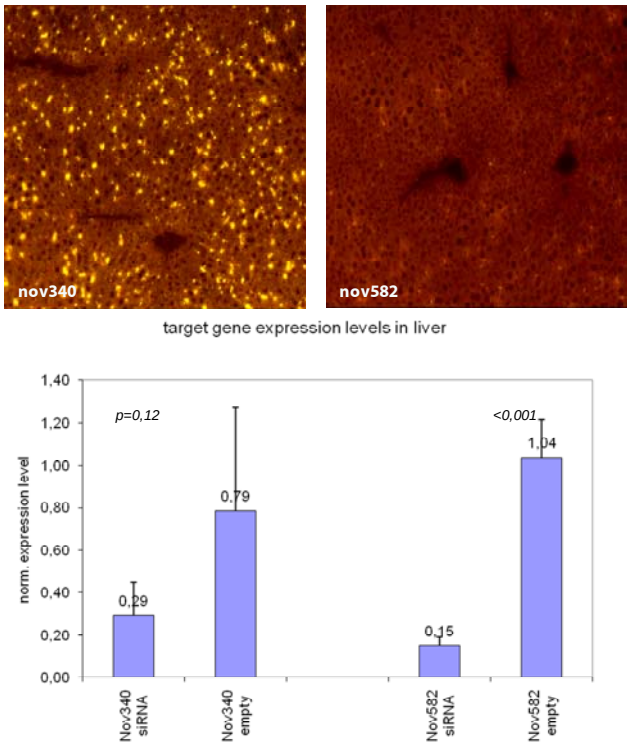

Figure 1: Delivery of siRNA to the liver. Animals received a single dose of Cy3 labeled siRNA in nov340 or nov582 and the cellular distribution and uptake was monitored by fluorescence microscopy. Mice treated with 2* 4mg/kg of the target siRNA were analyzed for inhibition of the target gene expression. Compared to the control group, the expression was reduced by 65 or 85%, respectively.

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2.2 Delivery of ASO or siRNA into tumors

ASO labeled with Cy5.5 was encapsulated in nov582 and used to analyze the exposure of tumor xenograft tissue after systemic dosing. Distribution to the tumor tissue was carrier-dependent since only background levels of the free Cy5.5 ASO could be detected. Amongst the carriers tested, nov582 achieved the highest uptake and mediated a homogenous staining throughout the tumor mass (figure 2). In a next experiment, nov582 was loaded with siRNA targeting the PLK1. The target gene is essential for cell division and inhibition of PLK1 was shown to inhibit tumor growth. [6] We observed a dose-dependent knockdown of the PLK1 protein after systemic dosing of the PLK1-specific siRNA, but not for an unrelated control; these effects started already after treatment with 2*1,25mg/kg and treatment of the animals with 2*5mg/kg resulted in a profound knockdown of the gene expression (figure 2, middle panel). We conclude that nov582 mediates the delivery of both ASO and siRNA into tumor xenografts and results in sequence-specific inhibition of gene expression.

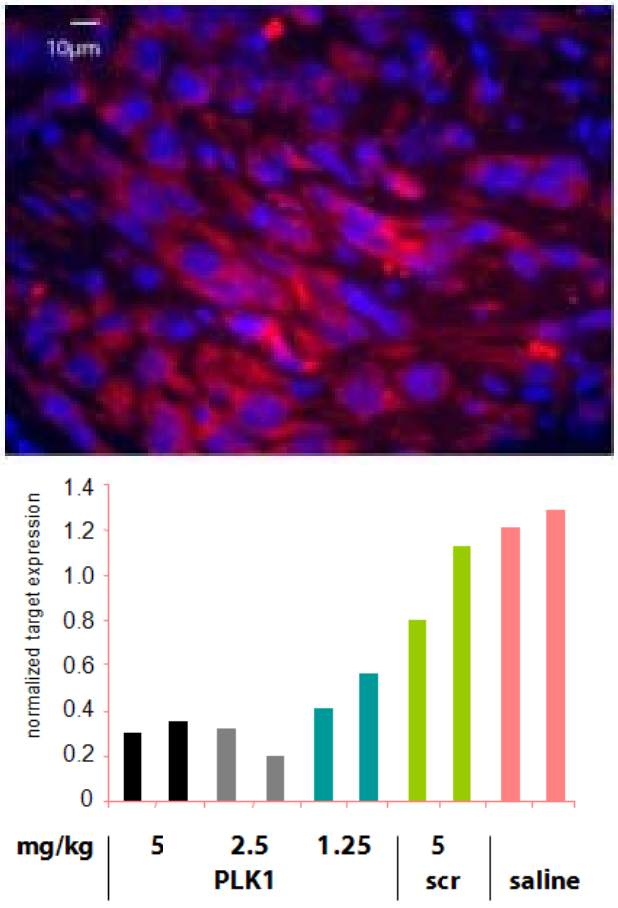

Figure 2: Delivery of ON into tumors. CD1 mice bearing Huh7 tumors received a single dose of Cy5.5 labeled ASO in nov582 and tumor section swere monitored for uptake. Cytosolic delivery was achieved throughout the tumor mass (left column, top). Delivery of a siRNA targeting PLK1, but not of an unrelated sequence results in inhibition of the target protein expression. Animals received two i.v. injections of siRNA in nov582 at 0 and 48h and were analyzed for PLK1 expression by western blot after 72h. Dosing is indicated in mg/kg per injection (left column, lower panel).

2.3 Delivery of CD40 ASO over the mucosal barrier

We here also report on the productive delivery of carrier:ASO complexes across the mucosal barrier Here, a single application of CD40 ASO in amphoteric liposomes prevented or greatly attenuated the severe inflammatory reaction following TNBS/ethanol enema, as judged by both macroscopic and microscopic evaluation. As revealed by immunohistochemistry, CD40 in the inflamed colon is mainly expressed by infiltrated monocytes and endothelial cells, which most likely represent the target of the ASO in this study. Expression of CD40 on these cells is markedly reduced after treatment with CD40 ASO in amphoteric liposomes, but not after treatment with an unrelated ON. (figure 3A-C)

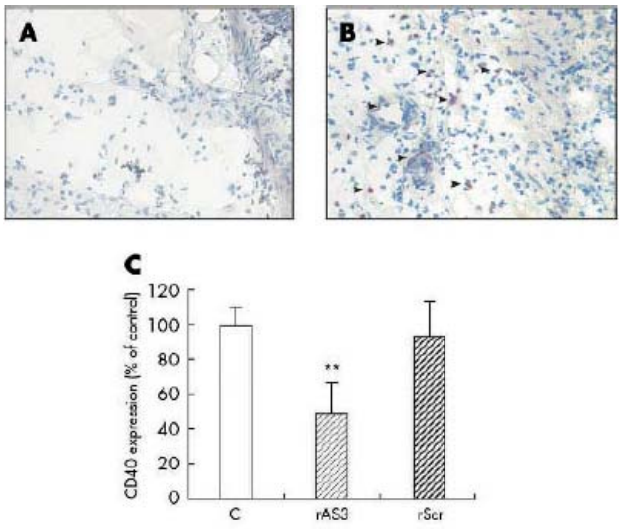

Figure 3: Blocking effect of rAS3(CD40 ASO) on CD40 expression in vivo. Animals were sacrificed on the second day after oligonucleotide administration and colitis induction. Colon sections were stained with the anti-CD40 antibody. In the unaffected ascending colon, only a few cells stained positive (A) while in the descending colon of 2,4,6-trinitrobenzene sulphonic acid (TNBS) treated controls an appreciable number of CD40 positive cells were detected (B, indicated by arrows), which were identified as monocytes and endothelial cells. Western blot analysis (C) was employed to quantify the decrease in CD40 protein in the descending colon of rAS3 treated compared with control or rScr treated rats. n = 3; **p,0.01 versus untreated control.

Moreover, it is well known that CD154/CD40 interactions between activated Th1 cells and APCs/endothelial cells in the affected mucosa greatly contribute to the secretion of proinflammatory cytokines, including IL-12, and TNF-α. At the same time, expression of adhesion molecules on endothelial cells such as VCAM-1 is enhanced. This may result from exposure to proinflammatory cytokines and/or CD154/CD40 inter-actions directly.

After treatment with CD40 ASO in amphoteric liposomes, a significant reduction in VCAM-1 expression was observed which in turn may have contributed to the decrease in granulocyte infiltration revealed by histochemistry. Although neither T cell infiltration nor CD154 expression in the inflamed descending colon was affected, expression of IL-12 p40, which is thought to be mandatory for induction of a Th1 immune response, was clearly blocked by CD40 ASO treatment. (figure 4)

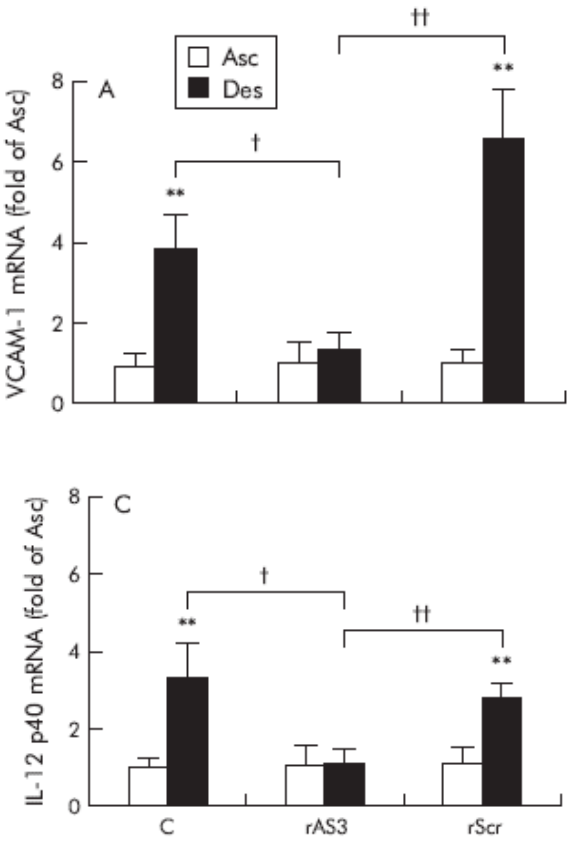

Figure 3: CD40 antisense oligonucleotide (rAS3) effects compared with control or rScr treatment, on VCAM-1 and IL-12p40 mRNA abundance in the colon of TNBS treated rats. Samples of both the ascending (Asc; that is, unaffected region) and descending (Des; that is, affected region) colon were collected seven days post colitis induction. mRNA abundance (relative to the mRNA level of the housekeeping gene EF 2) for each gene product is statistically summarized (n = 8, **p,0.01 versus Asc).

The aforementioned findings strongly suggest that CD40 ASO in amphoteric liposomes attenuates CD154/CD40 interactions in vivo, resulting in decreased mucosal inflammation in the subchronic IBD model employed. (figure 5). It should be noted that all these effects were observed at very low doses of about 24µg/kg of the CD40 ASO, supporting the power of delivery-enhanced ON techniques.

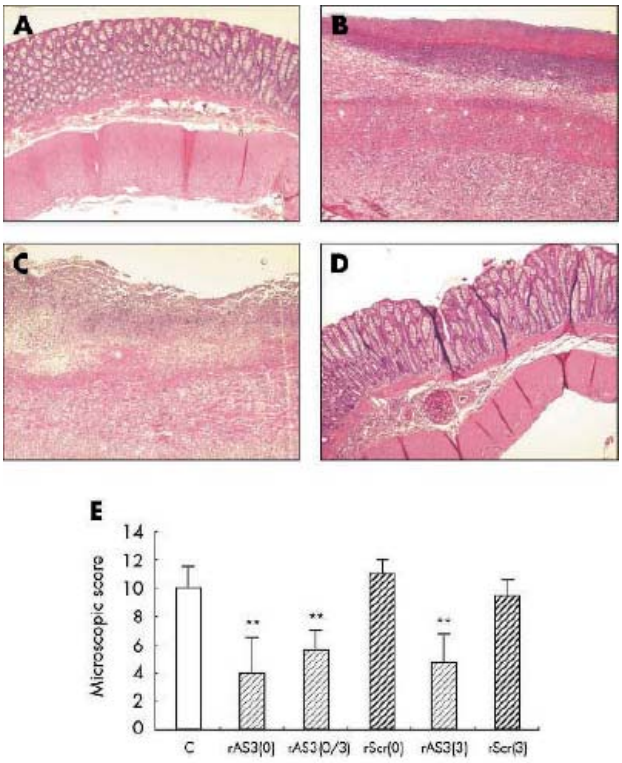

Figure 5: Histological analysis of CD40ASO effects in TNBS induced colitis. Normal structure of the bowel wall in the unaffected ascending colon (A) but major changes in the affected descending colon of control animals (B) were seen, revealed by necrosis and thickening of the bowel wall as well as a prominent leucocyte infiltrate. Treatment prior to colitis induction with CD40 ASO (rAS3) in amphoteric liposomes (D), but not rScr ASO in amphoteric liposomes (C), resulted in significant protection against colitis development. (E) Statistical summary comparing the histological analysis (n = 6–8, **p,0.01) for the different treatment groups (rAS3, CD40 ASO; rScr, scrambled control ASO; 0, treated at four hours prior to colitis induction on day 0; and 3, treated on the third day post colitis induction).

3 CONCLUSION

We here have demonstrated delivery of ON to multiple sites in animal models and were able to demonstrate target gene knockdowns with siRNA and ASO.

We relate the ability of amphoteric liposomes to target very specific organs or tissues to their inherent compatibility with the serum and tissue conditions. More specifically, the net negative surface charge of amphoteric carriers conceptually avoids the limitations of cationic carriers that either stick to endothelia or need PEGylation to reduce their surface charge.

Throughout the presented studies, different carriers have been identified to target different tissues or cell types. The organ specificity of amphoteric liposomes appears to be guided by particle size and lipid amount. The cellular specificity, however, appears to be more sensitive to the precise lipid composition of the carrier system and future studies will further exploit the flexibility of this approach to provide carriers with substantial targeting specificity. This in some cases might be achievable even without the use of external guiding ligands.

Regardless of their specific biodistribution properties, all of the carriers used herein share the amphoteric character and can functionally deliver ONs into the targeted cells. Additional data not presented here support a very favorable safety profile for this novel class of liposomes.

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