



Biodistribution of the GATA-3-specific DNAzyme hgd40 after inhalative exposure in mice, rats and dogs

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ABSTRACT

The DNAzyme hgd40 was shown to effectively reduce expression of the transcription factor GATA-3 RNA which plays an important role in the regulation of Th2-mediated immune mechanisms such as in allergic bronchial asthma. However, uptake, biodistribution and pharmacokinetics of hgd40 have not been investigated yet.

We examined local and systemic distribution of hgd40 in naive mice and mice suffering from experimental asthma. Furthermore, we evaluated the pharmacokinetics as a function of dose following single and repeated administration in rats and dogs.

Using intranasal administration of fluorescently labeled hgd40 we demonstrated that the DNAzyme was evenly distributed in inflamed asthmatic mouse lungs within minutes after single dose application. Systemic distribution was investigated in mice using radioactive labeled hgd40. After intratracheal application, highest amounts of hgd40 were detected in the lungs. High amounts were also detected in the bladder indicating urinary excretion as a major elimination pathway. In serum, low systemic hgd40 levels were detected already at 5 min post application (p.a.), subsequently decreasing over time to non-detectable levels at 2 h p.a. As revealed by Single Photon Emission Computed Tomography, trace amounts of hgd40 were detectable in lungs up to 7 days p.a. Also in the toxicologically relevant rats and dogs, hgd40 was detectable in blood only shortly after inhalative application. The plasma pharmacokinetic profile was dose and time dependent. Repeated administration did not lead to drug accumulation in plasma of dogs and rats. These pharmacokinetic of hgd40 provide guidance for clinical development, and support an infrequent and convenient dose administration regimen.

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Introduction

DNAzymes of the 10–23 family represent a class of small single-stranded oligodeoxynucleotides that are capable of catalytically cleaving single-stranded RNA (ssRNA) targets at purine–pyrimidine junctions. They consist of a sequence-conserved 15 base catalytic core that is flanked by binding arms typically 7–10 bases in length with sequence complementary to the target mRNA (Dass et al., 2002; Jason et al., 2004; Santoro and Joyce, 1997). Thus, DNAzymes combine cleaving specificity and catalytic activity of naturally occurring ribozymes with the stability of antisense deoxyoligonucleotides. This has attracted great interest to use the DNAzyme technology for

potential therapeutic applications as performed in several *in vitro* and *in vivo* studies (Darcan-Nicolaisen et al., 2009; Grimpe, 2011; Lee et al., 2007; Rivory et al., 2006; Verma et al., 2010). We have recently developed DNAzymes, among them hgd40, that specifically cleave the mRNA of the transcription factor GATA-3 and demonstrated their preventive and therapeutic efficacy in models of acute and chronic experimental asthma. In these models, intranasal administration of hgd40 prevented the development of hallmark features of experimental asthma including airway inflammation, mucus production and development of airway hyperresponsiveness to methacholine (Sel et al., 2008). Moreover, hgd40 did not trigger off-target effects such as macrophage activation or induction of proinflammatory cytokines like IL-12p40 or TNFα both *in vitro* and *in vivo* (Dicke et al., 2012).

Pharmacological properties of DNAzymes, as a novel class of compounds for therapeutic purposes, are still not fully investigated. Although pharmacokinetics of oligonucleotides in general have been characterized in a number of species (Geary et al., 1997, 2003) including man (Yu et al., 2007), little is known about dose-dependency of the

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pharmacokinetics of oligonucleotides as almost all published studies report results for a single dose level. In addition, there is only limited information on DNAzyme biodistribution in different organs especially after pulmonary application. Recently, a powerful tool offering the ability to integrate imaging with the investigation of novel therapeutics *in vivo* was developed. Single Photon Emission Computed Tomography (SPECT) and planar imaging offer the possibility of assessing real-time pharmacokinetics and biodistribution in living animals (Merkel et al., 2009a, 2009b, 2011). Following radiolabeling, a compound of interest may be visualized, measured and tracked to investigate its pharmacological properties in living animals. SPECT provides significant pharmacokinetic information on the labeled agent in tissues of interest (Saleem et al., 2011). Compound biodistribution may be investigated in all tissues using only trace amounts of the drug. There is no need for invasive biopsies and important clinically relevant information may be gained without studying large animal/patient groups.

In the present study we investigated the *in vivo* organ biodistribution of fluorescently and radioactively labeled GATA-3-specific DNAzyme hgd40 in healthy and asthma-induced mice. Furthermore, we evaluated the pharmacokinetics of hgd40 as a function of dose following single and repeated administration in rats and dogs. Altogether, the results represent a preclinical pharmacokinetic profile of hgd40 which provide guidance for the administration of hgd40 in human patients.

Materials and methods

DNAzyme hgd40 & fluorescently labeled hgd40. Unlabeled DNAzyme hgd40 (sequence 5'-GTGGATGGAGGCTAGTACAACGA GTCTTGGAG-3'-3'-T-5'), fluorescently labeled hgd40 coupled to rhodamine 6G at the 5'-end and amine-modified hgd40 with a C6-NH₂ linker at 5'-end were synthesized by BioSpring GmbH (Frankfurt am Main, Germany).

Radioactive labeling of hgd40. Radioactive labeling of amine-modified hgd40 was accomplished by a previously described method (Merkel et al., 2009a). Briefly, amino-modified hgd40 was coupled to the chelator p-SCN-Bn-DTPA [2-(4-Isothiocyanatobenzyl)]-diethylenetriaminepentaacetic acid (Macrocyclics, Dallas, USA) at pH 8.5 for 45 min. Spin columns from Plasmid Mini Kit (Qiagen, Hilden, Germany) were used together with buffers from the Absolutely miRNA Kit (Agilent Technologies, Santa Clara, USA) to purify p-SCN-Bn-DTPA coupled hgd40 from non-coupled hgd40. Radiolabeling of the p-SCN-Bn-DTPA coupled DNAzyme was achieved by incubation with ¹¹¹InCl₃ (Covidien Deutschland GmbH, Neustadt a.d. Donau, Germany), followed by size exclusion chromatography on PD-10 Sephadex G25 (GE Healthcare, Freiburg, Germany) column binding, washing, and elution. Fractions which showed both a high radioactive signal and the strongest UV absorption at 260 nm and do not contain any free p-SCN-Bn-DTPA or indium were used for animal studies.

Mice treatment protocols. Female BALB/c mice from 6 to 8 weeks old (Harlan Winkelmann, Hannover, Germany) were maintained under specific pathogen-free conditions. Ovalbumin-free diet and water were supplied *ad libitum*. All animal studies were performed under the approval of the local animal ethics committees (MR 43/2011).

For investigation of hgd40 uptake, mice with an OVA-induced allergic airway inflammation were used as previously described (Wegmann et al., 2005). Briefly, mice were intraperitoneally (i.p.) sensitized to ovalbumin (OVA) or PBS (control) at days 0, 7 and 14 and challenged with aerosolized OVA (1%) or PBS, respectively, on days 26, 27 and 28. Rhodamine 6G labeled hgd40 (200 µg) dissolved in PBS was once applied intranasally (i.n.) at 24 h after the last ovalbumin challenge. Mice were sacrificed at 1 h, 2 h, 6 h, 12 h and 24 h after DNAzyme application. Lungs were fixed *ex situ* with 4%

paraformaldehyde via the trachea, and stored in 4% paraformaldehyde. Lung tissues were embedded into paraffin, and 3 mm sections were counter-stained with DAPI (Sigma Aldrich, St. Gallen, Germany) according to manufacturer's recommendation. Subsequently, slides were evaluated using confocal laser scanning microscopy.

In vivo biodistribution and kinetics of hgd40 in mice. BALB/c control and OVA-treated mice were anesthetized with xylazine and ketamine. Radioactive labeled hgd40 (15 µg in 100 µl) was instilled into murine lungs intratracheally (i.t.), control animals were instilled with PBS only.

Biodistribution was analyzed by perfusion live imaging and static imaging using Single Photon Emission Computed Tomography (SPECT). Images were acquired with a Siemens e.cam gamma camera (Siemens AG, Erlangen, Germany) to which a custom built multiplexing multipinhole collimator was mounted. Three-dimensional SPECT and planar gamma camera images were recorded at different time points ranging from 5 min to 150 h after i.t. hgd40 administration. Planar images were analyzed for radioactivity in regions of interest (ROI) by Syngo software (Syngo, Siemens Medical Solutions, Erlangen, Germany).

From a set of mice blood samples were withdrawn retroorbitally at 1, 3, 5, 15, 30, 60, and 120 min after instillation. After 2 h, animals treated with ¹¹¹In-labeled hgd40 were sacrificed and biodistribution of radiolabeled material in dissected organs as well as in collected blood samples were measured using a Gamma Counter Packard 5005 (Packard Instruments, Meriden, CT). Radioactive decay of the isotope was factored into the calculations, and results are given as mean percentage of the applied dose.

Rats' treatment protocol. Wistar rats (10 male and 10 females per dose group), were analyzed in the course of toxicological studies (Fuhst et al., 2012). Animals were exposed to clean air (placebo control) or to 2 different aerosol concentrations of hgd40: 38.3 mg/m³ (corresponding to 303 µg/kg/day) or 113.2 mg/m³ (corresponding to 889 µg/kg/day) for 28 consecutive days (1 h per day). Serum samples were collected at different time points before and after the first exposure (day 0) and before and after exposure on day 27. From each dose group and gender six animals were used for toxicokinetic analysis and predose serum samples were taken from all these animals. Afterwards, blood was taken alternately from two subgroups (3 animals per dose and sex) to avoid too frequent interventions in individual animals. According to this procedure, serum samples were always taken from animals from 1 to 3 at the time points of 0.25 h, 1 h and 23 h, and samples at 0.5 h, 2 h and 4 h were taken from animals from 4 to 6 of each group. Samples were stored at –20 °C and later analyzed by the hgd40 hybridization ELISA method.

Dogs' treatment protocol. Beagle dogs (3 animals per sex and dose group including placebo) were analyzed in the course of toxicological studies (Fuhst et al., 2012). Animals received hgd40 by inhalative exposure for 30 min per day at doses of 40 mg/m³ (corresponding to 250 µg/kg/day) or 115 mg/m³ (corresponding to 750 µg/kg/day), respectively for 28 consecutive days. Plasma samples were collected on day 1 of exposure and on day 24 of the experiment at following time points: Predose, 0.10 h, 0.25 h, 0.5 h, 1 h, 2 h, 4 h, and 24 h after hgd40 exposure. Samples were stored at –20 °C and later analyzed by the hgd40 hybridization ELISA method.

Confocal laser scanning microscopy. A TCS SP5 2 microscope (Leica, Wetzlar, Germany) was used for laser scanning confocal microscopy. For excitation of rhodamine 6G fluorescence, an excitation wavelength of 514 nm was applied and fluorescence emission was detected using a 525 to 606 nm bandpass filter. Transmission images were obtained in the same scan. In a second scan of the multitrack image, DAPI was detected using a 405 nm laser and a 413 to 498 nm bandpass emission filter. All confocal images were acquired with the same settings with respect to laser intensity, filter block, and detector gain.

hgd40 hybridization ELISA. Mice, rats and dogs' serum or plasma samples were analyzed using a quantitative and sensitive hgd40 hybridization ELISA method which was developed based on a previously reported method for the detection of oligonucleotides (Brown-Augsburger et al., 2004). Briefly, 50 μ l of serum or plasma sample containing hgd40 was diluted with the same volume of serum dilution buffer (composition: 20 mM Tris HCl, 2.5% SDS, 0.5 mM MgCl₂, pH = 7.4). In the next step, the DNAzyme was hybridized to two complementary antisense oligonucleotide probes diluted in hybridization buffer (composition: 20 mM Tris HCl, 50 mM NaCl, 0.5 mM MgCl₂, pH = 7.4). One of these probes was labeled with biotin at the 3' end (5'-CTAGCCTCCATCCACAAA-3'-BioTeg-Q), the second probe was labeled with digoxigenin at the 5' end (Digoxigenin-5'-AAACTCCAAGACTCGTTG-3'), and both were synthesized by Eurofins MWG Operon (Ebersberg, Germany). 22 μ l of each serum/plasma sample were mixed with 198 μ l of the master mix (composition of master mix for single sample: 109.835 μ l of hybridization buffer, 88 μ l of serum dilution buffer, 0.055 μ l of 1 mg/ml biotin labeled oligonucleotide and 0.11 μ l of 0.5 mg/ml digoxigenin labeled oligonucleotide). Hybridization mixes were transferred to a 96-well PCR plate (BrandTech Scientific, Essex, USA) and incubated at 75 °C for 5 min in a heating block to form the hybridization complex. Next, 100 μ l of each hybridization mix were transferred to a 96-well Streptavidin-coated plate (Roche Diagnostics, Mannheim, Germany) and incubated for 2 h at 37 °C. For the detection of bound DNAzyme hybridization complex a horseradish peroxidase-conjugated, digoxigenin-specific antibody (100 μ l of 250 mU/ml Roche Diagnostics, Mannheim, Germany) was added and the plate was incubated for 1.5 h at 37 °C. After washing (composition of

washing buffer: 20 mM Tris-HCl, 50 mM NaCl, 0.1% Tween 20), 100 μ l of substrate solution (BM Blue POD Substrate 3,3',5,5'-tetramethylbenzidine, Roche Diagnostics, Mannheim, Germany) were added to each well. The plate was developed in the dark for a maximum of 30 min and finally the reaction was stopped by adding 50 μ l of 2 M sulphuric acid. The absorption was detected in a photometer (GENios; Tecan, Männedorf, Switzerland) at wavelength 450 nm. Prior to use, the assay was validated for precision, accuracy, selectivity, sensitivity, and stability of hgd40 quantitation. The detection limit of the assays was determined as mean plus 2-fold standard deviation of hgd40 negative samples and turned out to be 0.64 ng/ml for rat serum (based on 42 samples) and 0.35 ng/ml for dogs plasma (based on 154 samples), respectively.

Statistical analysis. Data were analyzed using GraphPad Prism version 4.01 (GraphPad Software San Diego, USA). Results are presented as means $\pm$ SEMs.

Results

Kinetics of DNAzyme hgd40 uptake in murine lungs after intranasal administration

In order to investigate the uptake of hgd40 into the lungs of mice, a single dose of fluorescently (rhodamine 6G)-labeled hgd40 was applied intranasally (i.n.). Strong fluorescence signals were distributed throughout the whole lung tissue observed throughout the whole lung of respectively treated mice at 1 h p.a. At this point in time the DNAzyme was detectable in the majority of cells in the

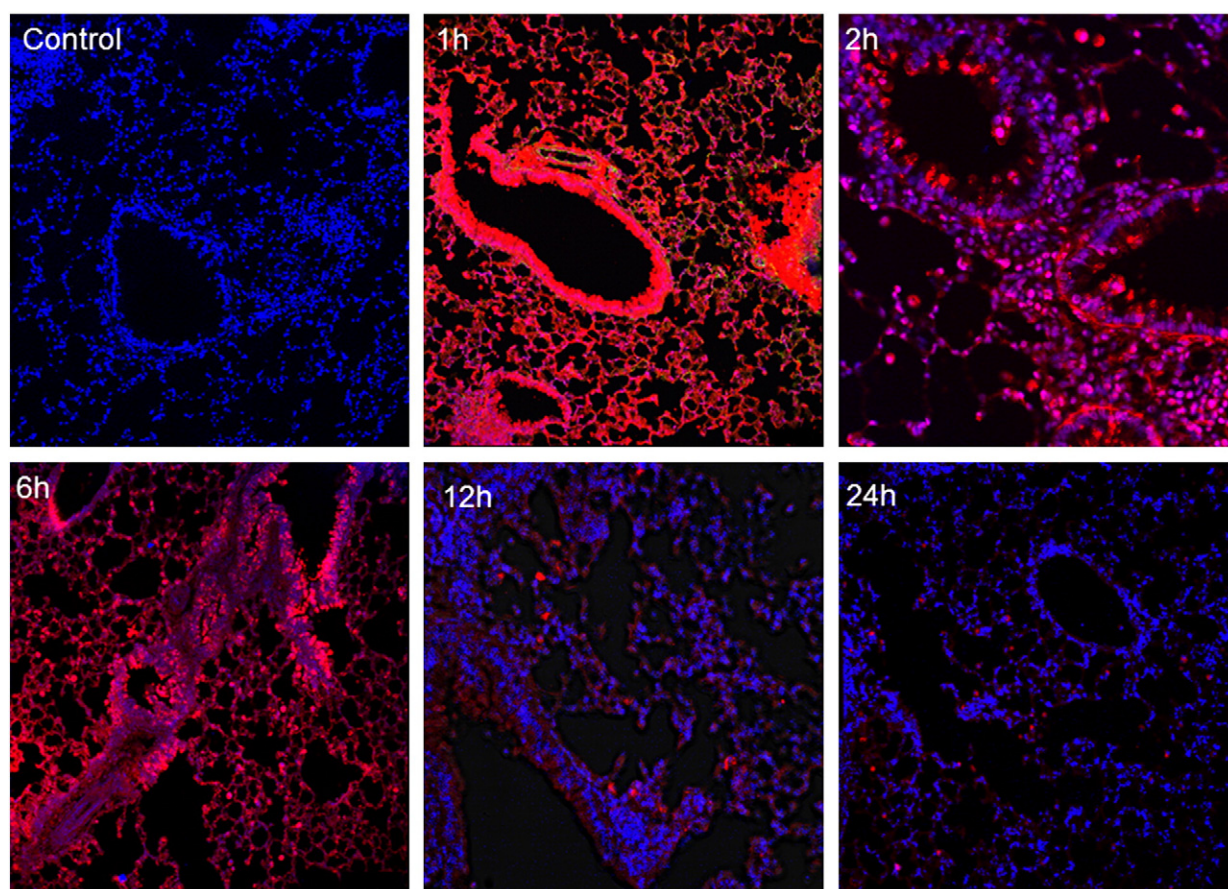


Fig. 1. Laser scanning confocal microscopic evaluation of rhodamine-labeled hgd40 (red color) uptake in mice lungs. Representative CLSM images were taken at different time points following i.n. hgd40 application. Nuclei were counterstained with DAPI (blue color).

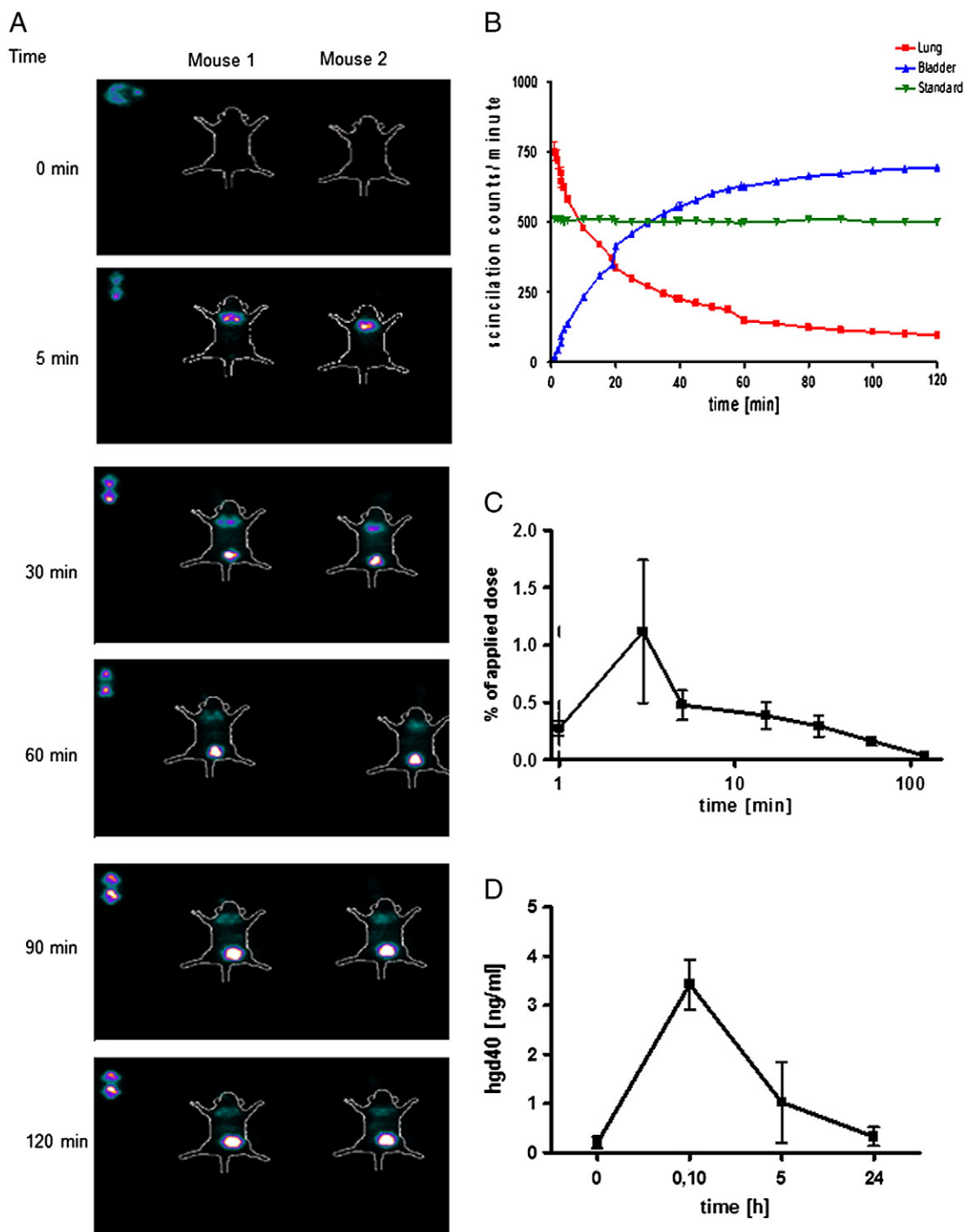


Fig. 2. Distribution kinetics of $^{111}\text{Indium}$ -labeled hgd40 in mice upon i.t. instillation. (A) SPECT imaging revealed rapid and sustained presence of hgd40 in the lungs followed by increasing signal intensity in the bladder. (B) Quantitation of $^{111}\text{Indium}$ -labeled hgd40 in lungs and bladder presented as function of time. Data are shown as mean $\pm$ SEM. Pharmacokinetics of $^{111}\text{Indium}$ -labeled hgd40 (C) and non-labeled hgd40 (D) in blood. As shown in both graphs, hgd40 was detectable in blood immediately after application with decreasing concentrations over time. Results are expressed as mean percent of applied dose $\pm$ SEM (C) or as mean concentration of hgd40 $\pm$ SEM (D) of three animals.

lungs. Over time, the intensity of the remaining fluorescence signal decreased, however, significant amounts of hgd40 were detectable up to 6 h p.a. and traces could still be observed at 24 h p.a. by this method (Fig. 1).

To further support these observations and to specifically quantify the kinetics of uptake and early distribution of the therapeutic DNAzyme, a real-time perfusion study with intratracheal application of $^{111}\text{Indium}$ -labeled hgd40 was performed. As shown in Fig. 2A,

rapid accumulation of the radioactive signal was observed in the lungs that appeared already at the first time point of analysis at 5 min p.a. Quantification of scintillation counts in the Region of Interest (ROI) as a function of time revealed that the signal in the lungs decreased steadily after initial accumulation (red line, Fig. 2B). These data obtained by both methods confirm that hgd40 was available in the lungs already within minutes after administration and seem to be rapidly taken up by the majority of lung cells.

Systemic and organ distribution of DNAzyme hgd40 following local application to the lung

In the blood, the highest amounts of radioactive labeled hgd40 (1.2% of applied dose) were detectable by gamma scintillation counting already at 5 min p.a., which decreased over time to non-detectable levels at 2 h p.a. (Fig. 2C). These results were confirmed by analysis of non-labeled DNAzyme serum levels following i.n. application using the hgd40 hybridization ELISA. Here, the highest serum concentration was detected at 6 min p.a. and a comparable time-dependent decrease was observed as with the radioactive method (Fig. 2D).

In addition, a fast and significant accumulation of hgd40 was also observed in the bladder, further pointing to a rapid systemic distribution and renal excretion of the compound. As demonstrated in Figs. 2A and B, radioactive labeled hgd40 was detectable in the bladder shortly after application and reached its plateau after about 60 min. As only minute amounts of radioactivity were detected in dissected emptied bladders (Fig. 3) at 2 h p.a. the accumulation occurred in the urine.

Furthermore, less than 4% of the total hgd40 dose was detected in the intestinum at 2 h p.a. and only some trace amounts were observed by SPECT imaging in livers and kidneys of healthy mice (Fig. 3). These data indicate that hgd40 is not accumulating in non-target organs and is preferentially removed from circulation via urinary excretion.

Pharmacokinetics of hgd40 in mice lungs after i.t. instillation

In order to determine the persistence of hgd40 in the lungs, long term analyses were performed in mice to which radioactive labeled hgd40 was administered. hgd40 was detectable up to 7 days after i.t. administration (Fig. 4A). ROI's (region of interest) analyses of images presented on Fig. 4A show that approximately 13% of the applied dose were present in the lungs at 2 h p.a. in mice with an OVA-induced allergic airway inflammation and this amount decreased significantly over time to 2% at day 7 p.a. Comparably, by scintillation analysis of dissected lungs, approximately 20% of the applied dose was detected at 2 h p.a. (Fig. 3).

The amount of hgd40 present in the lungs at 2 h p.a. in healthy mice was markedly lower in comparison to OVA-exposed mice (Fig. 4B) suggesting that disrupted epithelial barrier in asthmatic mice allows increased tissue absorption.

Pharmacokinetics of hgd40 after single and multiple inhalative applications in rats

Systemic bioavailability was assessed in rats and dogs following single and repeated exposure to inhaled hgd40 as part of toxicological studies (Fuhst et al., 2012). Levels of hgd40 were evaluated by hybridization ELISA at different time points following single and multiple administration of increasing hgd40 doses as described in detail in the materials and methods section.

As expected, analysis of samples at predose ($t = 0$) did not reveal any detectable hgd40 on day 0 (Fig. 5A). In the 303 $\mu\text{g/kg/day}$ dose group, hgd40 was detected in the serum at very low concentrations already at 0.25 h after end of exposure; levels peaked at 1 h with 2.58 ± 1.72 ng/ml following exposure and declined thereafter to levels below the detection limit of the analytical system after 23 h. Similarly, systemic levels of hgd40 were observed in the 889 $\mu\text{g/kg/day}$ dose group, however, higher concentrations were already observed at early time points and persisted for a longer time period. Also here, no detectable levels of hgd40 were found at 23 h after exposure (Fig. 5A).

In serum samples of rats obtained following repeated inhalation of the compound at a dose of 303 μg , low concentrations were detected that peaked again at 1 h after exposure as shown in Fig. 5B. Higher serum concentrations were found in the 889 $\mu\text{g/kg/day}$ dose group, the kinetics was similar to that after single exposure. After the last

hgd40 application at the 303 $\mu\text{g/kg/day}$ dose, generally only very low concentrations of hgd40 just above to the detection limit of the hybridization ELISA system were detected. The peak was again observed at 1 h after the last inhalation of the drug (Fig. 5B). About two-fold higher serum concentrations were found in the 889 $\mu\text{g/kg/day}$ dose group with comparable kinetics to the single dose exposure. The peak of hgd40 concentration in serum was observed at 0.5 h p.i. in this group (Fig. 5B).

Pharmacokinetics of hgd40 after single and multiple inhalative applications in dogs

Systemic hgd40 bioavailability was also assessed in dogs as second toxicologically relevant species. Plasma levels of hgd40 were evaluated by hybridization ELISA at different time points following single and multiple inhalations.

Within the 250 $\mu\text{g/kg/day}$ dose group hgd40 was detected only in few samples showing mean plasma levels close to or under the lower limit of detection at any time point of the observation period. However, higher amounts of hgd40 were detected in the higher dose group (750 $\mu\text{g/kg/day}$). Highest levels (0.72 ± 0.35 ng/ml) were observed directly after inhalation which rapidly declined afterwards. Already at 0.5 h after end of exposure hgd40 was no more detectable in plasma. As expected, analysis of plasma samples obtained before the first exposure to hgd40 (predose) revealed no detectable amounts of hgd40 (Fig. 6A).

Similar results were obtained at study on day 24. For all dose groups, predose plasma levels of hgd40 were below or in the range of the lower detection limit of the assay indicating that no hgd40 accumulation occurred due to inhalation on the preceding days. Within the 250 $\mu\text{g/kg/day}$ dose group, hgd40 was detected in the plasma of only one treated animal at the earliest time point. Within the high dose group, low but significant plasma levels of hgd40 (0.61 ± 0.50 ng/ml) were found again with the maximum directly after end of inhalation of the test compound. Also on this treatment day, plasma levels of hgd40 declined quite rapidly, 0.5 h after cessation of hgd40 inhalation the drug was no more detectable in plasma (Fig. 6B).

Discussion

Antisense oligonucleotides have attracted a great deal of attention as they can be applied for novel therapeutic product development.

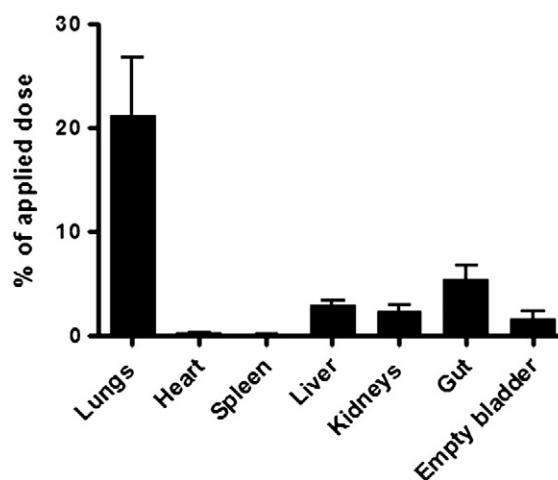


Fig. 3. Quantitative scintillation analysis of dissected organs upon i.t. instillation of $^{111}\text{Indium}$ -labeled hgd40. Organ analysis at 2 h after hgd40 application revealed highest signals in the lungs and minor amounts in liver, kidneys gut and emptied bladder. Results are expressed as mean percent of applied dose $\pm$ SEM of three animals per group.

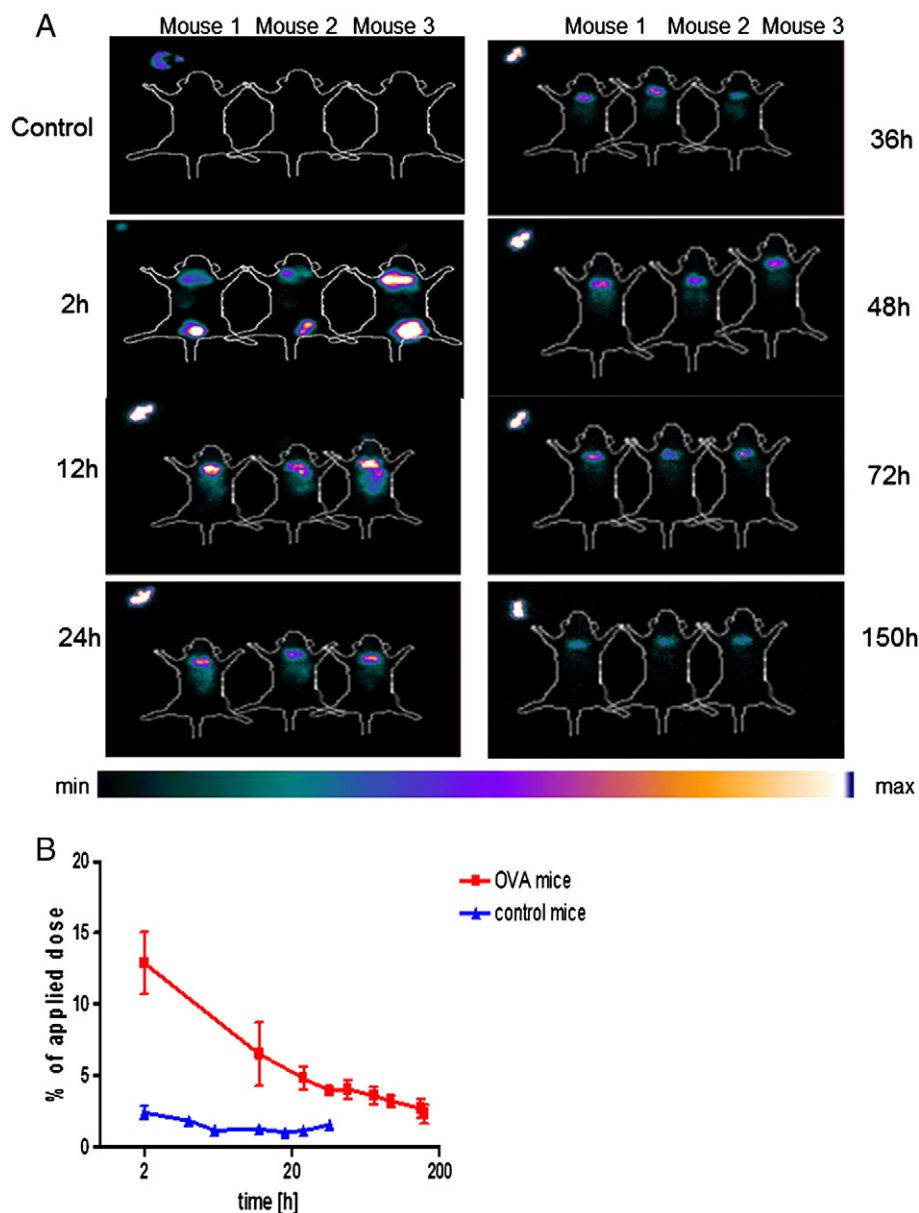


Fig. 4. Long-term kinetics of $^{111}\text{Indium}$ -labeled hgd40 biodistribution in mice after i.t. administration. (A) Highest signals were observed in the lungs at 2 h p.a. which sustained for up to 7 days p.a. Additionally, strong signals were also detected in the bladder pointing to excretion rather than accumulation of the compound. (B) ROI's analysis of radiolabeled hgd40 in healthy (blue line) vs. OVA-treated mice (red line) revealed higher uptake of hgd40 in mice with allergic airway inflammation. Data are presented as mean percent of applied dose $\pm$ SEM representative for three animals per group.

The highly efficient and specific gene silencing by DNazymes are generally applicable to all classes of molecular targets as a powerful new therapeutic approach. One of the main challenges in the development of antisense-based therapies however, is the efficient delivery of the active molecule to their target cells. Here we show that the GATA-3-specific DNzyme hgd40 can be efficiently delivered to the lung tissue without using additional delivery systems such as cationic lipid transfection reagents (Bitko et al., 2005) or nanocarriers such as surfactants or polyethyleneimine (PEI) and polyethylene glycol (PEG) which have been used for improved *in vivo* pulmonary delivery of siRNAs (Lee et al., 2010; Merkel et al., 2009a, 2009b). Administration of the 'naked' molecule avoids possible problems with accumulation of the delivery agent or putative side- or off-target effects caused by the delivery system itself.

We demonstrate that fluorescently labeled hgd40 is detectable in the lungs up to 24 h following inhalative application. This period is

sufficient to drive therapeutic effects in mice in a once daily application regimen as we have previously demonstrated (Sel et al., 2008). In addition, this study provides novel imaging data which describe for the first time the biodistribution of the hgd40 DNzyme in mice suffering from experimental asthma. Radiolabeling of the hgd40 molecule with $^{111}\text{Indium}$ allowed SPECT studies in mice during preclinical studies. Based on data obtained with radioactive labeled DNzyme additional real-time perfusion imaging studies were initially performed to record kinetics and biodistribution of the compound during the first 120 min after i.t. administration. This non-invasive procedure was performed by quantifying radioactivity as ROIs in living animals. We generally observed rapid accumulation of radioactivity in the lungs and in the bladder within the 1st hour p.a. At a later point in time, the amount of radioactive DNzyme, in the lungs, although lower, remained constant as shown in Figs. 2A and B. No significant accumulation of radioactive material in other

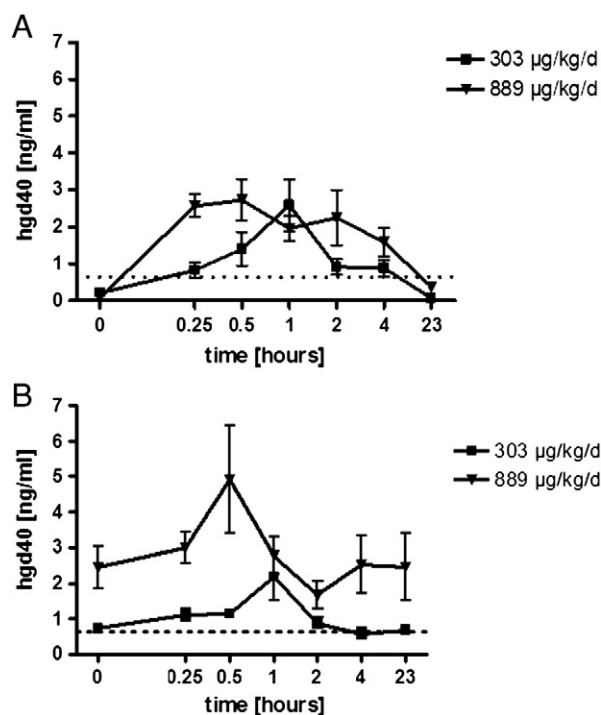


Fig. 5. Pharmacokinetic profile of hgd40 in rats after single (A) and multiple (B) inhalative exposure. Rats were exposed to two doses of the DNAzyme (303 µg/kg/day or 809 µg/kg/day) and hgd40 serum concentrations were analyzed at different time points after a single or the last of multiple exposures using a hybridization ELISA method. Highest serum concentrations of hgd40 were always detected within the first hour after inhalation. Data are presented as mean $\pm$ SEM of six animals per group (12 animals at time point 0).

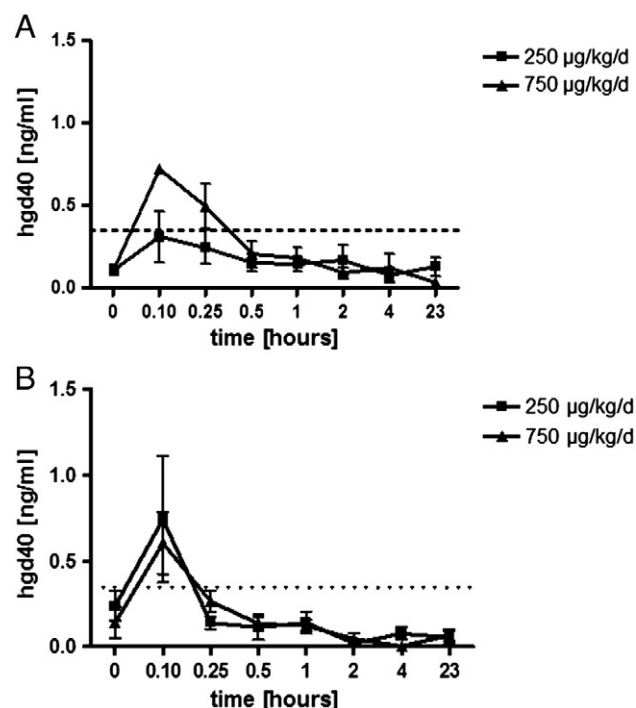


Fig. 6. Pharmacokinetic profile of hgd40 in dogs after single (A) and multiple (B) inhalative exposure. Dogs were exposed to two doses of the DNAzyme (250 µg/kg/day or 750 µg/kg/day) and hgd40 plasma concentrations were analyzed at different time points after a single or the last of multiple exposures using a hybridization ELISA method. Highest plasma concentrations of hgd40 were always detected at 0.1 h after end of inhalation. Data are presented as mean $\pm$ SEM of six animals per group.

organs was observed by this method. The high local bioavailability and low systemic distribution makes the direct administration route to the lungs an ideal approach for local asthma treatment. This is further supported by the notion that the small portion of extrapulmonary distributed hgd40 was previously shown to be associated with the complete absence of systemic toxicity (Fuhst et al., 2012).

Data obtained from non-invasive imaging studies, were further confirmed by direct assessment of ^{111}In labeled DNAzyme in blood samples and dissected organs. Again, highest amounts of DNAzyme were detected in the lungs, while other organs did not reveal a significant uptake of radioactivity as shown in Fig. 3. Further data from non-invasive imaging studies revealed that radioactive labeled hgd40 was detectable in the lungs in trace amounts up to 7 days after application. This observation is in agreement with data shown by Geary et al. (2003), where the tissue residence of a tumor necrosis factor-specific DNAzyme was generally longer than 1 week. Another interesting finding was that hgd40 uptake appeared to be higher in asthmatic OVA mice compared to healthy animals (Fig. 4B).

The disadvantage of the applied imaging techniques is that they do not allow differentiating between free label, degraded or intact labeled compound. Therefore, we verified the findings using three different method of hgd40 assessment: 6G rhodamine labeled, ^{111}In Indium labeled or non-labeled hgd40. Although the method of radioactive labeling of antisense oligonucleotides with ^{111}In Indium has been used in pharmacokinetics studies before (Merkel et al., 2009a, 2009b), the stability of oligonucleotide- ^{111}In Indium complexes has not been investigated so far under *in vivo* conditions. To address this issue we compared the pharmacokinetics of ^{111}In Indium labeled hgd40 with non-labeled hgd40 measured in the blood using a sensitive hgd40 hybridization ELISA. Overall, the pharmacokinetics data obtained with both methods are largely comparable. Thus, it appears

that there is no major stability or functional differences between labeled and unlabeled oligonucleotide molecules even though a change in cellular penetration properties due to the introduction of the fluorescence or radioactive labels cannot be ruled out. It would therefore be necessary to further investigate this aspect in future studies. Generally, potential differences between unlabeled and labeled molecules should be considered in the interpretation of results.

The serum/plasma profile following pulmonary administration of hgd40 differed from the typical biphasic profile (rapid distribution phase followed by a prolonged elimination phase) which has been described after intravenous administration of other oligonucleotides (Geary et al., 2003). After inhalative administration, hgd40 was detectable in the circulation already shortly after application. There was a clear time- and dose-dependent systemic bioavailability profile and hgd40 pharmacokinetics appeared to be quite similar in all tested species, *i.e.* mice, rats and dogs. Consistent with the relatively fast availability in and elimination from the plasma, there was no evidence for systemic compound accumulation even following multiple doses in dogs and rats.

Plasma distribution kinetics of hgd40 is rapid and, as such, is unlikely to influence dosing frequency strategies. Rather, frequency of dosing should be established based on the slow elimination from lung tissue with the goal to minimize accumulation and to ensure continued exposure for efficacy.

These favorable pharmacokinetic properties of hgd40 provide guidance for further clinical development and seem to support an infrequent and convenient dose administration regimen.

Conflict of interest

AT, JK, TD, and UH are employees of and HG and HR are shareholders of and consultants to sterna biologicals GmbH & Co. KG.

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