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Anti sense and sensibility : renal and skin effects of (antisense) oligonucleotides

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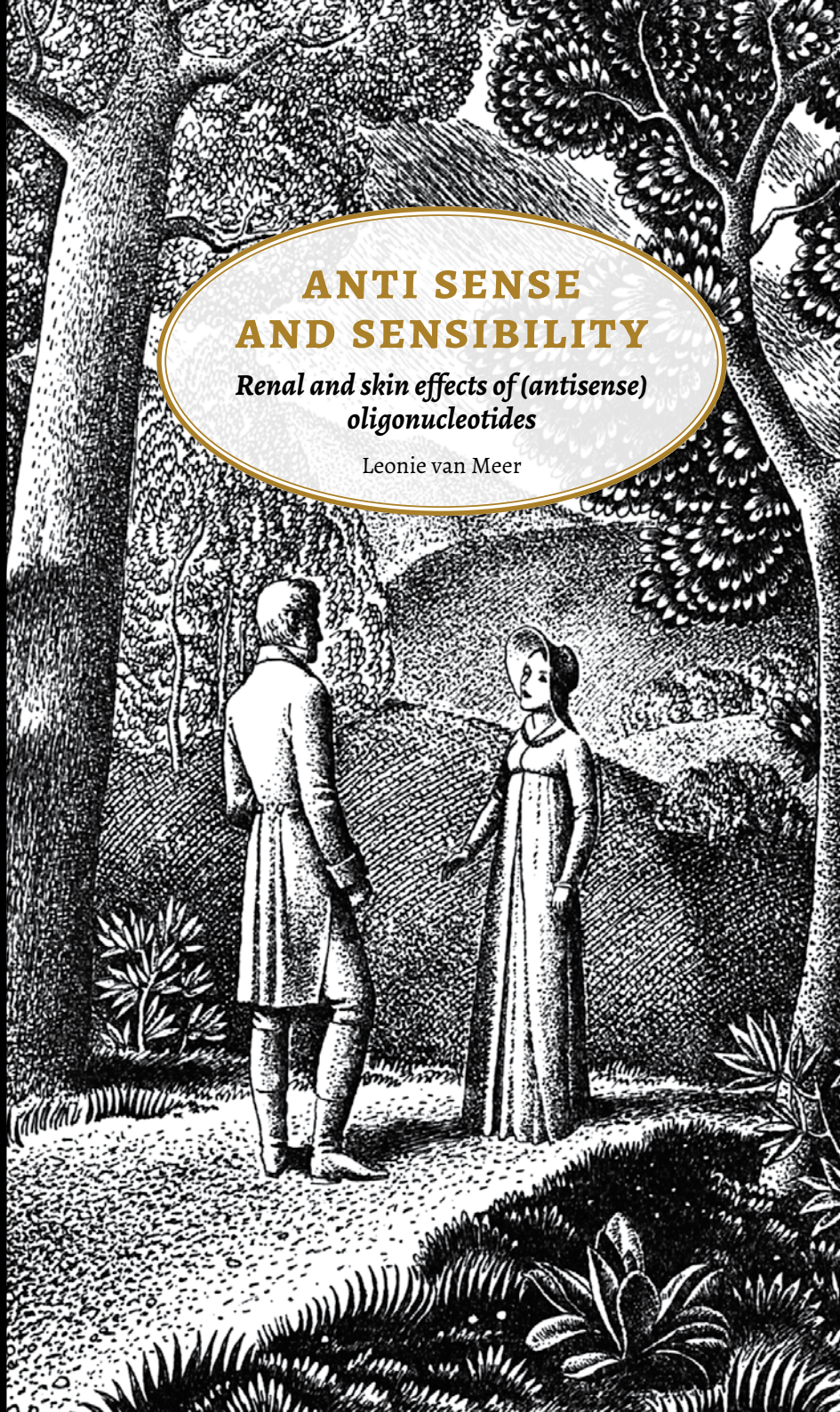

ANTI SENSE AND SENSIBILITY

Renal and skin effects of (antisense) oligonucleotides

Leonie van Meer

ANTI SENSE AND SENSIBILITY

Aan mijn grootouders,
die mij voor gingen en inspireerden

ANTI SENSE AND SENSIBILITY

Renal and skin effects of (antisense) oligonucleotides

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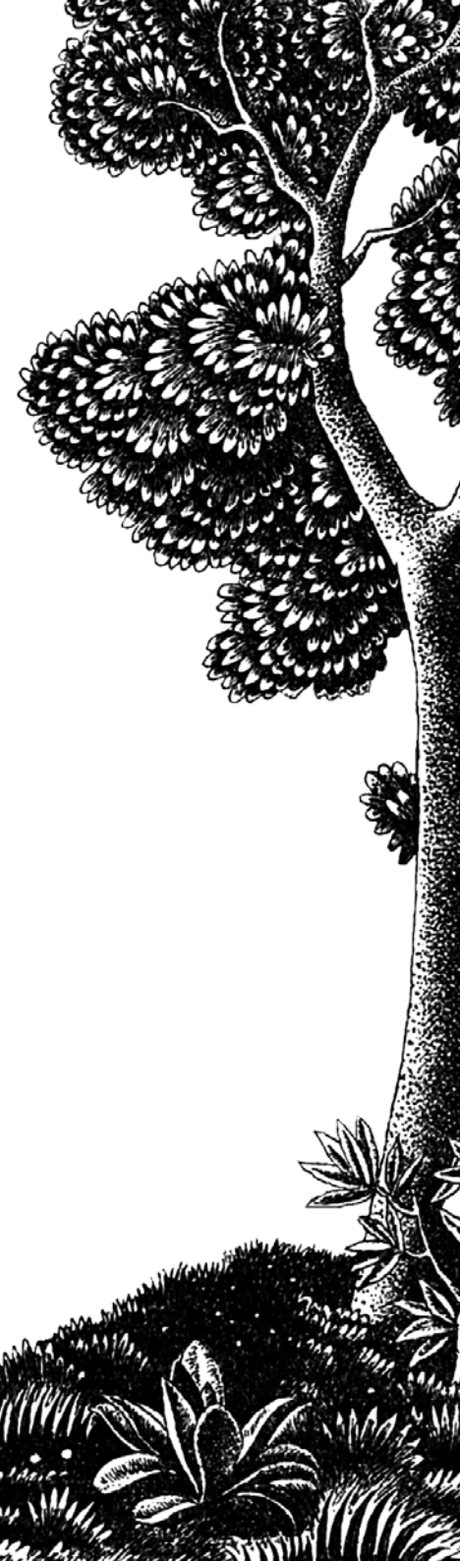
I

INTRODUCTION AND OUTLINE OF THIS THESIS



Introduction

This thesis describes the clinical investigation of a novel treatment strategy for type 2 diabetes mellitus (T2DM) using an antisense oligonucleotide (AON) to inhibit the SGLT2 receptor. The historical background of both treatment of T2DM and the development of oligonucleotide based therapeutics are covered in this introduction.

TREATMENT OF TYPE 2 DIABETES MELLITUS

About 387 million adults worldwide are living with type 2 diabetes mellitus (T2DM) [1]. The number of affected people is still growing at a fast pace, predicted to result in over 592 million people affected by 2035 [1]. The rise in the prevalence of T2DM in children and adolescents [2;3] contributes largely to this predicted trend. As the metabolic disease state of T2DM interferes with many physiological systems and organ functions, it is associated with a wide range of serious co-morbidities and increased mortality. Optimal treatment of T2DM with tight regulation of serum glucose will be of great importance the coming decades.

Treatment of T2DM began when insulin became available in 1923. Later, in 1955 the first sulfonylureas were developed (table 1). Both insulin and sulfonylureas are associated with burdensome side effects, such as weight gain and hypoglycemia. The next step was the development of metformin, which was introduced in Europe around 1975 and in the United States in 1995 [4]. Currently, metformin still is the preferred drug for initial therapy [5;6]. It is effective and safe and does not affect body weight, which is favorable compared to earlier treatments. Disadvantages include gastrointestinal side effects and its use is contraindicated in patients with severe renal insufficiency. Moreover, monotherapy with metformin is often not sufficient and within 3 years after start of the therapy, 50% of patients need additional drugs to achieve target HbA1c values [7].

Three treatments came available between 1995-1998; thiazolidinediones, α-glucosidase inhibitors and meglitinides. These treatments are less effective compared to metformin, and also have other disadvantages [8]. Thiazolidinediones are associated with weight gain and fluid retention. α-Glucosidase inhibitors and meglitinides need dosing three times daily and are expensive therapies. In 2005 incretin-mimetic compounds reached the market. These drugs have multiple antihyperglycemic actions and induce weight loss, which is promising. As monotherapy however, it is still less effective compared to metformin, insulin and sulfonylureas and long-term safety is not yet established [8].

Elaborating on the mechanisms of action of these drugs is beyond the scope of this thesis. The actions are summarized in table 1 and are more extensively described in the Teaching Resource Centre database [9].

The latest development in the treatment of T2DM are the small molecule SGLT2 inhibitors, which reached the market in 2013. SGLT2 co-transporters are responsible for reabsorption of up to 90% of the glucose filtered by the kidneys [10]. The pharmacological inhibition of SGLT2 co-transporters reduces hyperglycemia by decreasing renal glucose threshold and thereby increasing urinary glucose excretion. This mechanism of action, independent from the amount of circulating insulin or insulin sensitivity is very different from earlier developed therapies, making SGLT2 inhibition potentially useful for patients who are refractory to most other anti-diabetic therapies or as add-on therapy. SGLT2 inhibitors also induce weight loss and a reduction in triglycerides and systolic blood pressure [11;12]. Pharmacological inhibition of the SGLT2 receptor is an accepted concept which led to several registered and approved drugs (dapagliflozin [13] (Forxiga®), canagliflozin [14] (Invokana®) and empagliflozin [15] (Jardiance®)). As opposed to inhibition of the SGLT2 receptor function, antisense oligonucleotides enable inhibition of protein expression, which results in reduction of the abundance of the receptor. This concept led to the preclinical development of ISIS 388626, an antisense oligonucleotide designed to inhibit the SGLT2 receptor via targeting the SGLT2 mRNA. The clinical investigation of this drug candidate is described in this thesis.

OLIGONUCLEOTIDE BASED THERAPEUTICS

Oligonucleotides (ONs) are fragments of 12-24 nucleic acids in a target-specific sequence [16]. ONs have different mechanisms of action. They may be designed to either alter the reading frame by exon-skipping, work directly by inhibition of the targeted protein (antagonism), by binding to the receptor as an agonist or by inhibition of mRNA of the targeted protein using the antisense principle (figure 1). Antisense oligonucleotides (AONs) are complementary to a part of the mRNA of the target protein.

Watson-Crick hybridization of the AON with the mRNA, induces degradation of the mRNA and thereby prohibits translation of the mRNA into the target protein (figure 1).

Oligonucleotides found their origin by the discovery of the DNA structure by Watson and Crick [17] (table 2). It did take some time (25 years) before the first synthesis of short RNA fragments was accomplished [18]. And it lasted another 15 years before proof-of-concept of AONs was established by achieving successful inhibition of gene expression [19]. This led to the development of first generation AONs, of which fomivirsen (for intraocular treatment of CMV retinitis) was the first and only ever approved first generation AON [20]. These first generation compounds were AONs with a phosphorothioate backbone and were relatively stable in the bloodstream. However the duration of action of these compounds was limited and they induced various undesirable, non-specific in vivo side effects, such as immune stimulation and complement activation, which were mainly caused by their interactions with proteins. Around 1999 clinical investigation of 2nd generation AONs started. Many different modifications of the backbone sugar moieties were investigated [21]. The AONs with the most favorable modifications (2'-O-Methyl and 2'-O-Methoxyethyl) became the 2nd generation AONs. These demonstrated greater nuclease resistance and increased hybridization affinity due to more stable duplex formation with the target mRNA [22;23]. 2nd generation AONs are considered to be superior to first generation AONs, therefore the focus in drug development shifted accordingly. 2.5 or 3rd -generation oligonucleotides were developed from 2003 onwards to further improve the nuclease resistance and target affinity [24-26]. Locked nucleic acid (LNA), Peptide nucleic acid (PNA) and Morpholino phosphoroamidates (MF) are the three most commonly used 3rd generation AONs [27]. The clinical benefit of 3rd over 2nd generation AONs has not been established yet. The first and currently only approved 2nd generation compound, is mipomersen. Mipomersen was approved in 2013 by the FDA for the treatment of familial hypercholesterolemia [28].

Administration of all three generations of oligonucleotides, are associated with certain side effects in humans [29]. Intravenous (IV) administration may result in transient prolongation of aPTT [30-32]. This is thought to result from non-specific binding of the AONs with coagulation factors, phospholipids and calcium [33] and is considered to be non-clinically significant. aPTT is not affected when the oligonucleotide is injected subcutaneously probably due to lower plasma concentrations of the drug. Oligonucleotides have pro-inflammatory potential. In clinical studies with subcutaneous injection of the oligonucleotides, injection site responses are commonly observed [29]. IV infusion, as well as subcutaneous injection is associated with constitutional symptoms, such as fever and chills [34-36]. Oligonucleotides also rarely induce hypersensitivity reactions [29]. The pathophysiological and immunological pathways involved in these immune responses is unknown. Complement activation is common in monkeys and is rarely also present in humans [29]. Due to the known distribution pattern of oligonucleotides with accumulation in liver and kidney tissue, these organs are monitored carefully in clinical trials. Many oligonucleotides clearly have no effect on renal performance [29;37], however several other oligonucleotides do induce untoward kidney effects [38-40]. The reason for this discrepancy in unknown. Similarly, increases in liver transaminases have been observed after treatment with some, but not all, oligonucleotides [41-43]. In clinical studies with novel oligonucleotides, these side effects should be monitored closely.

ANTISENSE OLIGONUCLEOTIDE AS SGLT2 INHIBITOR

Inhibition of the SGLT2 transporter using small molecules has proven its efficacy by improving glycemic control in subjects with type 2 diabetes [9;10]. The maximal effect of these compounds is a modest inhibition of only 30-50% of renal glucose reabsorption, whereas complete inhibition of SGLT2 activity could theoretically induce 90% inhibition [10]. This limited effect is not well understood. This could be explained by compensatory increase of SGLT1 activity [44;45], however, another explanation could be that small molecules do not result in sufficient inhibition of the SGLT2 receptor. An alternative approach is to interfere with the synthesis of the receptor by antisense inhibition. ISIS 388626, a 2nd generation 2'-MOE-modified 12-mer oligonucleotide, is a very potent and selective inhibitor of SGLT2 and resulted in ≥ 80% reduction of renal SGLT2 mRNA expression in animal models. Therefore, it was anticipated to be more efficacious than small molecule SGLT2 inhibitors. Down regulation of the SGLT2 protein instead of SGLT2 inhibition with small molecules may mimic hereditary forms of glucosuria, with mutations in the SGLT2 gene [46;47]. Phenotypes resulting from these mutations are not associated

with hypoglycemia nor any other symptoms [46;47]. The specificity and selective renal distribution of ISIS 388626 resulted in potent anti-hyperglycemic effects in several preclinical models [48-50]. No AONs have been developed to date that have their target protein within the kidney. AONs generally have similar tissue distribution patterns with accumulation occurring in liver, kidney, lymph nodes and spleen, therefore the kidney has been suggested as potentially suitable target [51]. ISIS 388626 was designed to specifically distribute to the kidney, due to lower binding to plasma proteins compared to other 20-mer antisense oligonucleotides (88% to 60% bound over a concentration range of 4 to 400 µg/mL, respectively, in rodents, monkeys and dogs, data on file). The higher free fraction of ISIS 388626 in the circulation is subject to greater glomerular filtration and tubular reabsorption, leading to higher kidney concentration. This selective renal distribution was confirmed in animals using radiolabeled ISIS 388626; liver contained measurable levels of ISIS 388626, however these were approximately 5 to 100-fold lower than the levels in the kidneys in both mice and monkeys (data on file). ISIS 388626 did not produce any overt toxicities (specifically, no renal toxicities) in any of the species evaluated, even when SGLT2 mRNA levels were reduced by ≥ 80% [50]. No hypoglycemia was observed in euglycemic or hyperglycemic animals [48-50].

These data supported clinical development of ISIS 388626 for the treatment of T2DM. The objective of the phase 1 study was to provide an initial assessment of the effects of single and multiple subcutaneous doses of 50, 100, 200 and 400 mg ISIS 388626 in healthy subjects in order to select the effective doses for potential future trials in diabetic patients.

Outline of this thesis

ISIS 388626 is an antisense SGLT2 inhibitor, designed to treat Type 2 Diabetes Mellitus by inducing glucosuria. **CHAPTER 2** describes the results of the first-in-human trial with drug candidate ISIS 388626. This trial was halted early due to unexpected effects on renal function. Preclinical experiments revealed no effects on renal function, therefore the findings in this first in human trial were not well understood.

Additional and more extensive preclinical testing of ISIS 388626 with different dose regimens provided more insight into the renal effects. An adapted study design without loading dose was proposed to avoid these untoward effects. The study was restarted (with 50, 100 and 200 mg) and the findings are described in **CHAPTER 3**. Unexpectedly and despite changing the dose regimen, transient creatinine increases still occurred in the first cohort (50 mg). Therefore, renal clearance tests were performed in this study to evaluate the impact of 50 mg ISIS 388626 on GFR and renal plasma flow.

The cohort with the renal clearance tests confirmed the transient nature of the changes and revealed no indications for decreased GFR or renal plasma flow, therefore the trial continued with 100 and 200 mg ISIS 388626 treatment, as described in **CHAPTER 4**. These dose levels were expected to induce the pharmacodynamic effect. Renal effects were monitored closely. ISIS 388626 induced glucosuria, however the effect at the dose levels tested (up to 200 mg) was small. Higher doses of ISIS 388626 further increased the urinary levels of renal markers.

CHAPTER 5 explains why nephrotoxicity often is an important issue in early clinical drug trials. Limitations of the 'classic' renal markers such as serum creatinine and urea are addressed. An overview is provided on promising urinary kidney biomarkers. Finally, recommendations are made regarding the selection of suitable urinary biomarkers to monitor renal function and to detect changes early.

Apart from the unintended renal effects, ISIS 388626 treatment also caused skin reactions on the injection site. These injection site reactions (ISRs) are a commonly observed phenomenon after subcutaneous administered oligonucleotides, however little detailed information is available in the literature. **CHAPTER 6** is a review of all publicly available studies on skin reactions caused by oligonucleotide injection, supported by observations done in four clinical trials performed at the CHDR with four different oligonucleotides, including ISIS 388626. A comprehensive and detailed overview of the incidence, severity, clinical manifestations and pathophysiology of ISRs is provided.

Finally, **CHAPTER 7** combines the findings of the previous chapters, and addresses the risk-benefit assessment of oligonucleotide based therapies in general. Suggestions are done for further research in order to optimize the potential of oligonucleotide based therapies.

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Figure 1 The antisense oligonucleotide is transported across the plasma membrane (1). In the cytoplasm, the single-stranded oligonucleotide enters cell nucleus (2), where it bind to the target mRNA (3). The oligonucleotide/RNA duplex is a substrate for RNase H (4). RNase H cleaves the mRNA strand (5), creating dysfunctional mRNA that is not translated into protein. RNase H is also present in the cytosol, cleavage also occurs in that cellular compartment.

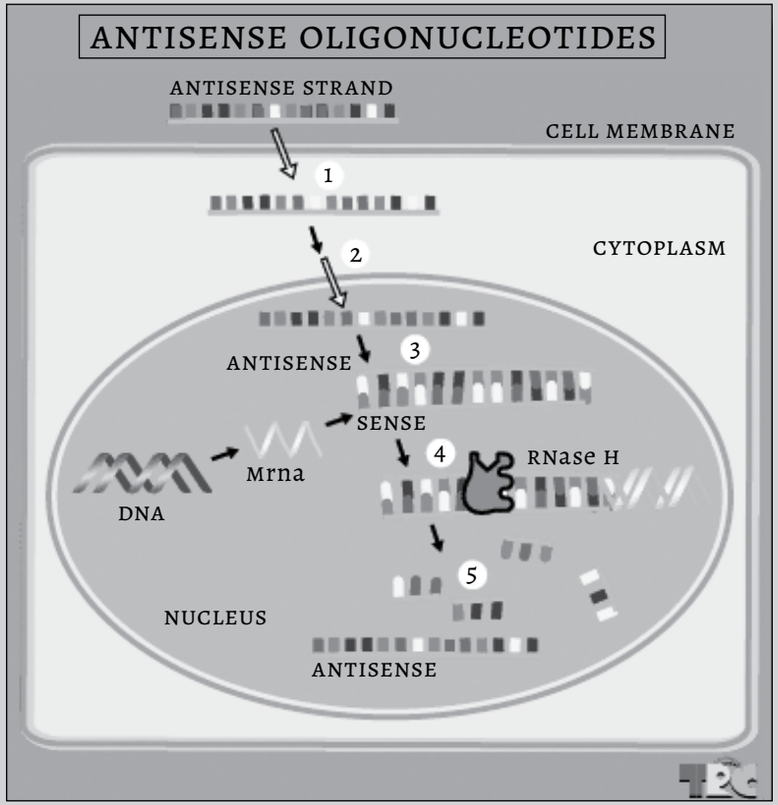

Table 1 Overview of historical development of drugs for the treatment of T2DM (↓ indicates inhibiting effect, ↑ indicates stimulatory effect)

Year	Drug	Actions
1923	Insulin	Hepatic glucose production ↓ Glycogen storage liver muscles ↑ Glucagon ↓
1955	Sulfonylureas	Production insulin β cells ↑
1975	Biguanides available Europe	Hepatic glucose production ↓
1983	2nd generation Sulfonylureas	Production insulin β cells ↑
1995	Biguanides available in the US	Hepatic glucose production ↓
1996	α-Glucosidase inhibitors	Digestion carbohydrates ↓
1997	Thiazolidinediones	Insulin sensitivity liver ↑
1998	Meglitinides	Production insulin β cells ↑
2005	GLP1 analogues	Production insulin β cells ↑ Insulin sensitivity ↑ Glucagon ↓ Gastic emptying, appetite, food intake ↓
2006	DPP4 inhibitors	GLP1 ↑ Production insulin β cells ↑ Insulin sensitivity ↑ Glucagon ↓ Gastic emptying, appetite, food intake ↓
2013	Small molecule SGLT2 inhibitors	Urinary glucose excretion ↑

Table 2 Overview of historical development of oligonucleotide drugs

Year	Drug	Actions
1923	Insulin	Hepatic glucose production ↓ Glycogen storage liver muscles ↑ Glucagon ↓
1955	Sulfonylureas	Production insulin β cells ↑
1975	Biguanides available Europe	Hepatic glucose production ↓
1983	2nd generation Sulfonylureas	Production insulin β cells ↑
1995	Biguanides available in the US	Hepatic glucose production ↓
1996	α-Glucosidase inhibitors	Digestion carbohydrates ↓
1997	Thiazolidinediones	Insulin sensitivity liver ↑
1998	Meglitinides	Production insulin β cells ↑
2005	GLP1 analogues	Production insulin β cells ↑ Insulin sensitivity ↑ Glucagon ↓ Gastic emptying, appetite, food intake ↓
2006	DPP4 inhibitors	GLP1 ↑ Production insulin β cells ↑ Insulin sensitivity ↑ Glucagon ↓ Gastic emptying, appetite, food intake ↓
2013	Small molecule SGLT2 inhibitors	Urinary glucose excretion ↑

Table 3 Overview of generation oligonucleotides and chemical modifications

Generation	Chemical modifications
First generation	Phosphorothiate backbone
Second generation	2-O-Methyl (2-OME) 2′-O-Methoxyethyl (2′-MOE)
Third generation (2.5 or 3rd)	Locked nucleic acid (LNA) Peptide nucleic acid (PNA) Morpholino phosphoroamidates (MF)

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II

FIRST-IN-MAN STUDIES WITH NOVEL SGLT2 INHIBITOR: ANTISENSE COMPOUND IS ASSOCIATED WITH UNEXPECTED RENAL EFFECTS

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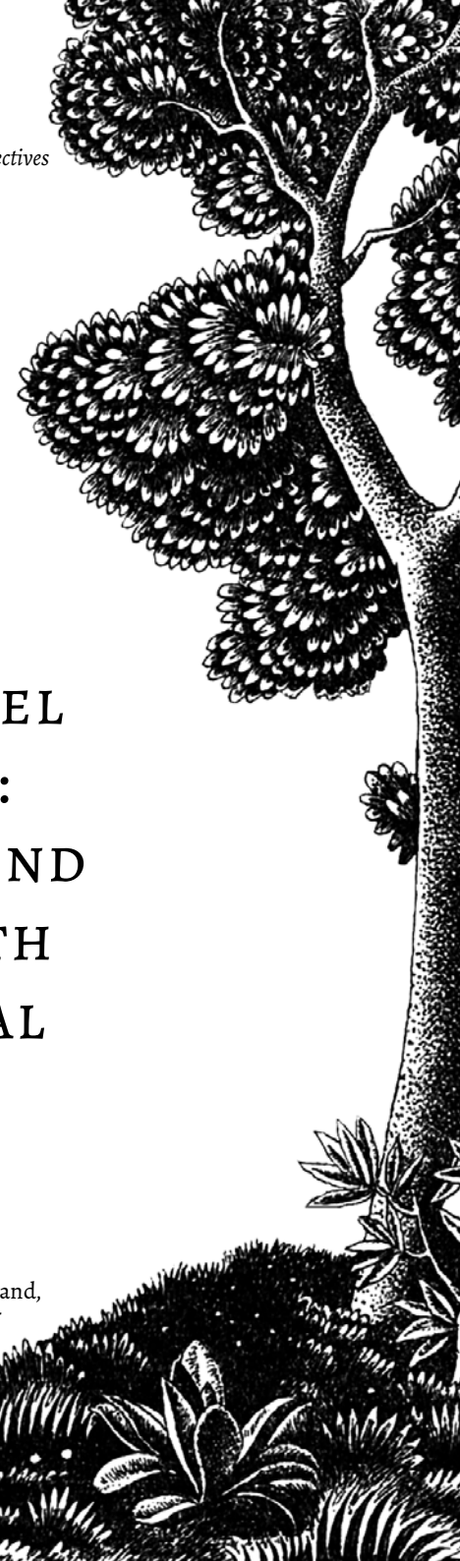

Abstract

The antisense compound ISIS 388626 selectively inhibits renal glucose reabsorption by inhibiting the sodium-glucose cotransporter-2 (SGLT2) mRNA expression. It is developed as an insulin-independent treatment approach for type 2 diabetes mellitus (T2DM). The safety, tolerability, pharmacokinetics, and pharmacodynamics after subcutaneous administration of the drug were planned to be evaluated in healthy volunteers in a single-ascending-dose study (50-400 mg) and a multiple-ascending-dose study (6 weeks; weekly doses of 50-400 mg with loading dose regimen of three doses during the first week). The study was halted early because increases in serum creatinine occurred in the subjects participating in the 100 mg multiple dose cohort. The pronounced changes in serum creatinine were accompanied by increased urinary excretion of Beta-2-Microglobulin and KIM1. The possible mechanisms for these findings remain elusive and are in contrast to preclinical findings as comparable treatment with ISIS 388626 of animals did not reveal similar changes. Although exposure was limited there was an indication that glucosuria increased upon active treatment. Before the concept of antisense-mediated blocking of SGLT2 with ISIS 388626 can be explored further, more preclinical data are needed to justify further investigations.

Introduction

The renal SGLT2 transporter accounts for 90% of the reabsorption of glomerular filtrated glucose [1]. In type 2 diabetes mellitus (T2DM) insulin resistance and the subsequent chronic hyperglycemia increases the amount of filtrated glucose. T2DM is associated with increased SGLT2 transporter expression and activity, thereby contributing to the hyperglycemic state [2]. These findings render the SGLT2 transporter a promising target for patients with diabetes mellitus. Indeed, inhibition of SGLT2 using small molecules has proven its efficacy by improving glycemic control in subjects with T2DM [3;4]. In addition this treatment improves other cardiovascular risk measures such as blood pressure, weight and levels of triglycerides [4]. This has led to the registration of several drugs (dapagliflozin [5] (Forxiga®), canagliflozin [6] (Invokana®) and empagliflozin [7] (Jardiance®). It was recently shown that adding empagliflozin to standard care favors cardiovascular outcome [8]. The use of clinically relevant doses of these compounds result in a modest 30-50% inhibition of renal glucose reabsorption [9;10]. An alternative approach is reduction of the synthesis of the transporter by antisense interference which theoretically could reduce the transporter to 80-90%. Targeting the kidney with antisense oligonucleotides should be possible as these compounds generally distribute to the kidney [11]. One of the antisense compounds developed to selectively knock-down the SGLT2 receptor is ISIS 388626, a second generation 2'-methoxyethyl (MOE)-modified 12-mer phosphorothioate oligonucleotide. This oligonucleotide is structurally complementary to a portion of the coding region of SGLT2 mRNA in multiple species including, mouse, rat, rabbit, monkey, dog and human. This compound was developed to inhibit the synthesis of the renal SGLT2 receptor by utilizing its short length (12-mer *vs.* the typical 20-mer), that enables fractional glomerular clearance and thus selective targeting of the proximal tubular epithelium [12].

Weekly subcutaneous (SC) injection of ISIS 388626 showed to be an effective and safe treatment in preclinical studies ranging from 6 weeks to 6 months in duration with a dose range of 0-30 mg/kg/week [12;13]. Following SC injection in animals, peak plasma concentrations and AUC increased in a dose-dependently and were similar for single and repeated dosing for up to 13 weeks in mice and monkeys. Peak plasma concentrations occurred within 0.25 to 1.5 hours after SC injection in mice, rats and monkeys, then decreased in an apparent multi-exponential fashion with time. ISIS 388626 was initially predominantly cleared by distribution to the kidneys with a plasma half-life ($t_{1/2\alpha}$) ranging between 1.6 and 2.9 hours. The initial rapid distribution phase was followed by a slow elimination phase with an elimination half-life between 5-8 days. In dogs, diabetic rodents, monkeys, ≥ 80% reduction of renal SGLT2 mRNA expression was observed without affecting SGLT1 expression [13]. In normoglycemic animals the reduction in SGLT2 mRNA expression that occurred at doses of 1-3 mg/kg weekly for 13 weeks translated into effective glucosuria (at 3 mg/kg a 60-fold increase in mice and 7-fold increase in monkeys in urine glucose creatinine ratio) [12]. Interestingly, ISIS 388626 also slowed progression of ocular cataract formation, glomerular damage and pancreatic islet cell deterioration in diabetic rodents [14]. Signs of toxicity of ISIS 388626 were not observed in any of the animal models. In a 6-month study in diabetic rats, no accumulation of

ISIS 388626 was observed in cardiac, liver or intestinal tissues, demonstrating the specificity and selective renal distribution of ISIS 388626 [14]. In 6 to 13 weeks treatment studies in mice and monkeys the only histological changes consisted of dose- dependent accumulation of basophilic granules in tubular epithelial cells, which is a result of H&E staining of the oligonucleotide itself in the cytoplasm and is an expected effect [15;16]. Also no indications for long term changes of general kidney function were noted [12].

Based on this pre-clinical information, it was considered safe to evaluate the compound in humans. Here we describe the results of the first in human trial with ISIS 388626, performed to assess its effects after single ascending doses (SAD) and multiple ascending doses (MAD).

Methods

SUBJECTS

Inclusion and exclusion criteria were similar for both parts of the trial. Adult, male or (post-menopausal or surgically sterile) female subjects (18-65 yrs) with BMI < 30 kg/m^2 were eligible if fasting plasma glucose and HbA1c was below the upper limit of normal and it was agreed to maintain steady hydration throughout study participation. Excluded were pregnant or nursing women, and subjects with significant abnormalities regarding medical history, physical examination findings, 12-lead electrocardiogram findings, and clinical laboratory evaluations (including positive protein in urine dipstick analysis and calculated eGFR below 60 ml/min [17]. Studies were conducted in accordance with good clinical practice guidelines and were approved by the national ethics committee as well as the competent authority.

CHOICE OF DOSING

It was anticipated to explore 50, 100, 200 and 400 mg of ISIS 388626. The doses were based on a MABEL approach, taking into account a No Adverse Effect Level estimated to be 10 mg/kg/week (including a loading dose regimen) in monkeys. In preclinical studies across multiple species, the pharmacologically active dose range of ISIS 388626 was 1-3 mg/kg/week. At this exposure, a significant reduction in SGLT2 mRNA occurred (74 to 97% in mice and approximately 30 to 90% in monkeys over the dose range 1-30 mg/kg/week), accompanied by a 25-200 fold increase in urinary glucose excretion [12-14]. Based on this, estimation of the equivalent human effective dose falls in the range of 1-3 mg/kg/week. Experience with other 2'-MOE-modified antisense oligonucleotides, safely administered (intravenously and subcutaneously) in multiple clinical studies at doses up to weekly 750 mg (which translates into 10.7 mg/kg/week assuming an average weight of 70 kg), with treatment durations exceeding one year [18], further supports the safety of this dose range. In the MAD part of the study it was planned to administer these doses weekly for 6 weeks after a loading dose regimen of 3 doses in the first week to achieve effective steady-state tissue concentrations.

STUDY DESIGN

The first study was a double-blind, randomized, placebo-controlled single ascending dose (SAD) study. Sixteen subjects were randomly assigned in a 3:1 ratio to receive either a single dose of 50, 100, 200 or 400 mg ISIS 388626 or placebo, administered as SC injection in the abdominal region. The SAD study part was followed by a double-blind, randomized, placebo-controlled multiple ascending dose (MAD) study administered as subcutaneous injection in the four abdominal quadrants (i.e. upper left, upper right, lower right, lower left), or upper lateral arms and thighs. Subjects were to receive eight doses of study drug (or placebo) over a six-week period, three doses in study week 1 followed by once weekly dosing for five weeks. At the higher dose levels in this study part (>50 mg), the intended pharmacodynamic effects of ISIS 388626 (inhibition of renal urinary glucose reabsorption and lowering of plasma glucose concentrations) were to be estimated by evaluation of glucose handling after an oral glucose tolerance test (OGTT), performed before the first administration of ISIS 388626/placebo and at Week 6. In both study parts, dose escalation was only permitted when the preceding dose regimen did not raise any safety concerns.

SAFETY MEASUREMENTS

The safety assessments were similar for both study parts and consisted of recording adverse events and measurement of vital signs,

electrocardiograms, physical examinations, and clinical laboratory tests (including clinical chemistry, hematology, coagulation, complement tests and urinalysis (including excretion of B2M)) throughout the study period. Adverse events were captured in MEDDRA terms.

RENAL MARKERS

The biomarkers Beta-2-Microglobulin (B2M), Kidney Injury Molecule (KIM1), Alpha-Glutathione S-Transferase (AGST) and N-Acetyl-β-(D)-Glucosaminidase (NAG) were chosen based on their performance on detecting injury to the proximal tubule where SGLT2 is located [19]. Analysis of renal damage markers AGST and NAG was performed using quantative enzyme immunoassays (NEPHKITO immunoassay for AGST, Argutus Medical Dublin, Ireland, and Diazyme 70010 Rev. F (Poway, USA) for NAG). KIM1 was measured using a microsphere-based immunoassay.

PHARMACOKINETIC ANALYSIS

For the quantification of ISIS 388626, plasma samples were collected frequently after administration of the single dose for 48 hrs after first and sixth dose in the multiple dose part. In the MAD part samples were also taken before dosing in week 2 and 4 and during 5 weekly follow-up visits). In addition, PK urine collections were done after first and sixth dose (up to 24 and 48-hours post-dose). Plasma samples were analyzed using a validated hybridization enzyme-linked immunosorbent assay and urine samples were analyzed by a validated Capillary Gel Electrophoresis method. Both assays were performed at PDD laboratories (Richmond, USA).

The plasma pharmacokinetics of ISIS 388626 were evaluated using non-compartmental analyses. The analyses were performed to determine the maximum observed plasma concentration (Cmax), the time to maximum plasma concentration (Tmax), the area under the plasma concentration-time curve from dosing to 48 hours after dosing (AUC0-48h) using WINNONLIN (version 5.3, Pharsight Corporation, USA).

DATA ANALYSES AND STATISTICAL METHODS

Safety and tolerability evaluation was based on descriptive statistics. The sample sizes (4 subjects per cohort for the SAD part, 8 subjects per cohort for the lowest dose range in the MAD part) were selected to allow descriptive analysis of safety and pharmacodynamics of ISIS 388626, and were not supported by any statistical rationale. The sample size of 12 subjects for the cohorts treated at dose levels exceeding 50 mg ISIS 388626 was based on observed changes in plasma glucose (AUC0-120min) upon an OGTT challenge in healthy volunteers, with an estimated standard deviation 85 mmol*min/L. At a sample size of 6 subjects per treatment group, at least 80% power would be achieved to detect a 170 mmol*min/L difference in plasma glucose AUC0-120min between treatment groups at an alpha level of 0.05. Based on this power calculation, a sample size of 12 was selected to ensure sufficient power.

The pharmacodynamic evaluation was based on descriptive summary statistics only for the MAD 50 mg cohort, as previous experience with second generation oligonucleotides suggests that the minimal pharmacologically effective dose exceeds 100 mg/week [20].

Pharmacodynamic effects of ISIS 388626 at dose levels above 50 mg were statistically evaluated using a two-sided T-test, with placebo subjects from different cohorts pooled as a group. Serum glucose, 24h urinary glucose excretion (UGE) and fractional glucose excretion (defined as (UGE/ filtered glucose load (GFR*fasted plasma glucose) *100) at the end of treatment (week 6) were compared between ISIS 388626 treatment groups and placebo group. For the cohorts with doses of 100, 200, and 400 mg, it was planned to analyze change from baseline of the pharmacodynamic endpoints to Week 6 assessments among treatment groups using ANOVA.

Results

SUBJECTS

The study was performed at the Centre for Human Drug Research in the Netherlands. Sixteen subjects were enrolled and completed the SAD study. 23 subjects enrolled the MAD study, of whom all subjects assigned to the 50 mg cohort completed the study (8) and the 15 subjects assigned to the 100 mg cohort terminated early due to a premature halt of the study. The 6 actively treated subjects of the 50 mg cohort received all 8 doses. In the 100 mg cohort, the first 4 doses were received by all 12 subjects. Thereafter 9 subjects received the 5^{th} dose and 4 subjects received the

6th dose and no subjects in the 100 mg cohort received the 7th or 8th dose. Subject demographics are presented in table 1.

SAFETY OUTCOMES

Adverse events (AEs) were reported in 26 (87%) subjects who received single and multiple doses of ISIS 388626, and in 6 (67%) subjects who received placebo. All AEs reported were classified as mild in intensity and were transient. The most common AE was fatigue which was reported by eighteen of thirty subjects (60%) treated with single or multiple SC doses of ISIS 388626. Incidence of this AE did not increase as dose increased. Fatigue was also reported in the placebo group in two of nine subjects (22%). Other than fatigue, the only other AEs that were reported more than once were headache (13%), nasopharyngitis (33%) and dizziness (10%). These adverse events are unlikely to be drug-related as these AEs occurred in placebo and both dose groups with a similar incidence (table 2). No hypoglycemia occurred at any dose level. In the MAD part of the study, injection site reactions (ISRs) were observed in the treated groups. When expressed as Local Cutaneous Reactions at the Injection Site (LCRIS; defined as erythema, swelling, itching, pruritus, pain or tenderness with an onset on the day of injection which did not resolve on the day of the injection or the day after the injection), the above mentioned skin reactions occurred in 2 out of 6 subjects at doses of 50 mg and in 6 out of 12 subjects at doses of 100 mg ISIS 388626. All ISRs were mild in severity. Most ISRs resolved completely and spontaneously during the study period with a duration ranging from 14 days-2 months. In the two female subjects, the ISRs did not completely resolve before the last follow up visit. The ISRs were not progressive, not accompanied by local lymphadenopathy, and no study discontinuations occurred due to ISRs.

In the SAD study, some isolated values outside the normal range values were observed in individual biochemistry or hematology parameters, but these were not considered clinically significant. This specifically applied to markers of hepatic and renal function (liver enzymes, serum creatinine, blood urea nitrogen (BUN), estimated glomerular filtration rate (eGFR) and urinalysis parameters) which are organs known for antisense oligonucleotide accumulation. In the MAD study, several hematology parameters were outside the normal range at isolated time points, but considered not to be clinically significant. Parameters of coagulation, complement and cytokines did not change significantly at any time point after dosing. Also, no clinically relevant changes in vital signs, ECG-derived parameters or body temperature were observed. However, marked changes were observed in serum creatinine levels for several subjects of both cohorts of the MAD study. In the 50 mg cohort a minimal increase in average serum creatinine of 0.13 ± 0.09 mg/dl (13% increase) was observed during the first three weeks after treatment initiation (figure 1), with increases over baseline in individuals ranging from 0.11 to 0.23 mg/dL. Since the individual changes in serum creatinine values were not considered clinically significant, study drug administration in this cohort was continued. Indeed, serum creatinine values returned to baseline levels after the third treatment week, despite continued dosing (figure 1). Subjects did not have any other clinically significant finding until follow-up, including no change in serum electrolytes or proteinuria. Variability in serum creatinine over time was large, as demonstrated by the placebo-treated group, with changes over baseline in serum creatinine levels ranging from -0.11 to 0.21 mg/dL. Nonetheless, the changes in serum creatinine observed in the ISIS 388626 treated subjects were more pronounced compared to the placebo group. In the 100mg cohort, during the first three weeks of study drug treatment an increase in average serum creatinine of 0.14 ± 0.12 mg/dl (16% increase) was observed compared to baseline values, ranging on an individual level from 0 to 0.47 mg/dl (figure 1). As the inter-individual variability in serum creatinine upon ISIS 388626 treatment was high, supplemental individual narratives are provided below, focusing on two subjects with the most pronounced increase in serum creatinine level.

The changes in serum creatinine were often accompanied by a small rise in BUN without clinically meaningful changes in serum electrolytes, albumin, aldosterone, or plasma renin activity. Besides creatinine and BUN, no other chemistry parameter, including parameters of liver biochemistry changed after dosing. Urine flow and urinalysis parameters did not change significantly in the subjects with increased creatinine, except for one subject, whose urinary protein excretion was 1.48 g/24 hr after the fourth dose (week 2) (See narratives below).

Based on the observed creatinine increases in the then running 100 mg cohort, further dosing of all subjects was discontinued, for safety reasons. All 15 subjects of the 100 mg cohort (12 on active treatment and 3 on placebo) entered the follow-up period. Therefore, it was impossible to

assess if continued dosing in the 100 mg dose group would have resulted in a resolution of the renal effect as observed in the 50 mg cohort.

To provide mechanistic insight into the observed changes in serum creatinine, analysis of potential renal damage markers KIM1, NAG, alpha-GST (aGST) and beta-2-microglobulin (B2M) was performed in biobanked urine samples (figure 2). After 4 doses, a dose-dependent increase in B2M excretion was observed in nearly all ISIS 388626-treated subjects. In the 50 mg cohort an average of 843 ± 1027.5 ug/24 hrs was observed, versus 69.8 ± 27.6 ug/24 hrs in the placebo group. For the 100 mg cohort, the increase was even more pronounced with an average of 2200 ± 2956.2 ug/24 hrs (versus 69.8 ± 27.6 ug/24 hrs in the placebo group). Also KIM1 and aGST excretion increased upon ISIS 388626 treatment in the majority of treated subjects, although there were no clear dose relationships observed for these renal markers. No significant changes were observed in urinary NAG excretion upon ISIS 388626 treatment. B2M, KIM1 and aGST excretion returned to baseline levels during follow-up (data not shown). No significant changes in absolute creatinine clearance were observed, however inter and intra subject variability was large (data not shown).

Individual subject narratives

A 63-year old female received 4 doses of ISIS 388626 at a dose level of 100 mg (Day 1, 3, 5 and 8). A 75% increase in serum creatinine level was observed in a blood sample collected prior to administration of the fourth dose (1.1 mg/dL versus 0.61 mg/dL at baseline). There were no additional clinically remarkable findings, but monitoring of the subject was intensified. Three days after administration of the fourth dose, total urine protein excretion was 1.5g/24 h, and further treatment was discontinued. During the intensive follow-up period serum creatinine levels and urine protein excretion decreased and returned to baseline levels within 6 weeks after the last drug administration. A week after the last drug administration, glucosuria (approximately 1200 mg/24h) was observed. Post-hoc analysis showed that creatinine changes were accompanied by transient increases of potential renal damage markers (B2M, NAG, and KIM1), that also returned to baseline during the follow-up period. Apart from the transient serum creatinine and urine protein elevations, other adverse events for this subject (obstipation, fatigue, common cold, and ISRs at the four injection sites) were of mild intensity and resolved without intervention. The subject's ISIS 388626 AUC 0-24hr levels after the first dose and trough levels on day 3, 5, and 8 were comparable to the other subjects in the 100 mg treatment group.

In a 39 year old male who received 5 doses of ISIS 388626 at a dose level of 100 mg (day 1, 3, 5, 8, and 15), a 54 % increase in serum creatinine level was observed prior to administration of the fifth dose (1.39 mg/dL versus 0.90 mg/dL at baseline). ISIS 388626 treatment was discontinued and an intensive follow-up period was started in which serum creatinine values returned to baseline levels. Renal damage markers (proteinuria, B2M and aGST) transiently increased, peaking after the fourth dose and returning to baseline levels during the follow-up period. No change in urinary glucose excretion was observed in this subject. Apart from the serum creatinine elevations, the other adverse events (one ISR and migraine-like symptoms after the first dose) relieved with paracetamol and ibuprofen treatment) were of mild intensity. Also for this subject, ISIS 388626 AUC 0-24hr after the first dose and trough levels on day 3, 5, and 8 were comparable to the other subjects in the 100 mg treatment group.

PHARMACOKINETICS

Following single SC injection, ISIS 388626 was rapidly absorbed as demonstrated by reaching maximum plasma concentrations (Cmax) between 1.2 and 1.5 hours (figure 3, table 3). Plasma concentrations of ISIS 388626 decreased rapidly after reaching Cmax, and distributed to tissues. The total amount of ISIS 388626 in urine increased more than dose-proportionally (figure 4).

After single administration of ISIS 388626, AUC increased in a dose-proportional manner, whereas the increase in Cmax was less than dose-proportional (table 3). Repeated pharmacokinetics in the multiple dose study part could only be assessed for the 50 mg dose, as other dose levels were prematurely terminated or not executed. The PK analysis showed that no accumulation of ISIS 388626 occurred with repeated dosing, as demonstrated by Cmax and AUC (table 4). Plasma pharmacokinetic parameters were comparable between the first dose (day 1) and last dose (day 36). The differences in plasma AUC and Cmax between subjects in the single dose cohort and multiple dose cohorts are likely explained by inter-subject variability.

PHARMACODYNAMICS

Due to the early halt of this part of study, the statistical analysis was constrained and a formal efficacy analysis could not be performed. Descriptive statistics are provided, describing effects of ISIS 388626 on UGE, FI and serum glucose following the OGTT in the MAD 50 mg cohort, and the MAD 100 mg cohort that was halted prematurely. Repeated administration of 50 mg ISIS 388626 did not alter urinary glucose excretion or fractional glucose excretion after 6 weeks of treatment, compared to baseline levels or compared to placebo treatment (figure 5 and 6). At follow-up, six weeks after the last administration of ISIS 388626, a possible trend towards an increased urinary glucose excretion and increased fractional glucose excretion was observed in the treatment group compared to placebo. However, this observation could not be confirmed by the results of MAD 100 mg cohort, most likely because of the premature study termination.

Discussion

The objectives of these first-in-human study was to assess the safety, pharmacokinetic and pharmacodynamic effects of single and multiple SC doses of ISIS 388626. It was shown that the pharmacokinetics of single doses of the compound were characterized by a rapid absorption after SC administration, almost dose-linear increases in Cmax and AUC and urinary excretion as expected for 20-mer antisense oligonucleotides of this chemical class [20-22], and without safety signal suggesting untoward effects.

Although the multiple dose part of the study was prematurely halted because unexpected findings regarding creatinine increases, this part of the study showed that the concept of SGLT2 inhibition by antisense ONs may be promising. The limited data that could be collected showed that antisense-mediated SGLT2 inhibition may result in glucosuria without hypoglycemia. This is in accordance with findings in small molecule SGLT2 inhibitors [23] and genetic disruption/absence of SGLT2 gene [24].

The unexpected renal findings demonstrated by the substantial increase in serum creatinine and increase in urinary excretion of B2M, AGST and KIM1 obviously warrant further evaluation.

Although the increase in serum creatinine levels could be explained by increased production, this is unlikely also because CPK levels in blood and urinary creatinine excretion did not differ significantly between groups (data not shown). This suggests that reduced kidney clearance, based on decreased filtration or decreased tubular secretion of creatinine are likely explanations for our observations. Creatinine clearance is likely to be affected, however this was not detectable, possibly due to a large inter and intra subject variability of creatinine clearance.

The findings of a dose-dependent increased urinary excretion of B2M further supports the view that tubular dysfunction explains our findings. This type of tubular dysfunction has been described also for cimetidine and is thought to reflect tubular adaptation to altered function, whereby impaired tubular creatinine secretion results in decreased re-absorption of proteins such as albumin and B2M. This is strengthened by the observation that B2M is filtrated and reabsorbed by proximal tubular cells via similar pathways as luminal uptake of antisense oligonucleotides [16;25]. It may be hypothesized that ONs impair megalin and cubulin mediated uptake. However, it cannot be ruled out that our findings are explained by tubular damage rather than functional adaptation, as increases of urinary KIM1 and AGST, the latter seemingly related to cumulative dose, were also observed. KIM1 is a transmembrane protein expressed by tubular epithelial cells in response to injury and acts by increasing the capacity to clear cell debris by phagocytosis. Similarly, the increase in urinary excretion of AGST, an intracellular lysosomal enzyme not filtrated by glomerulus, may reflect damage of the organelles of tubular cells. It remains unknown if this is related to the accumulation of oligonucleotide within phagolysosomes of proximal tubule cells, which is a common microscopic finding after oligonucleotide administration in animals [15;16]. For the majority of ONs this commonly is benign, although there seem to be a few exceptions. Animal studies with other phosphorothioate ONs have shown an association with proximal tubular degeneration and necrosis in the kidneys [26-28]. This renal toxicity commonly occurs only at doses that are at least 10- to 100-fold higher than administered in clinical trials, therefore the relevance of these findings for humans is unknown. Whether accumulation of ONs in tubular cells is innocuous when the target is also in the tubuli should be explored further. Thus, tubular dysfunction appears to be a reasonable explanation for our findings. We consider it unlikely that extensive tubular degeneration and necrosis occurred in our study as the

changes in renal function were rapidly reversible. It is more likely that a milder form of tubular dysfunction occurred in which the tubuli, which had taken up the ON, showed functional impairment in secreting creatinine and a decreased capacity to reabsorb B2M. A third explanation of the observed signals could be temporary disruption of membrane function. Loss of the SLGT2 transporter could impact expression or function of other transporters in the proximal tubules, needed to maintain the complex balance of apical and basolateral transport. The changes observed were relatively mild and reversible of nature, nonetheless could well reflect sub lethal cellular injury taking into account the KIM1 increase.

Due to the early halt adequate assessment of the pharmacodynamic properties of ISIS 388626 was not possible. It appears that there was a trend towards higher urinary glucose excretion during follow-up after six weeks of treatment with 50 mg as compared to placebo, but the variability between subjects precludes strong conclusions. The doses and duration of treatment with ISIS 388626 should be increased in future clinical studies in order to achieve exposure comparable with pharmacological effective doses in animals [12]. Obviously, this can only be justified with a better understanding and possibly avoidance of the unexpected adverse renal effects. In this respect it should be noted that similar effects after comparable treatment regimens with ISIS 388626 in rodents and monkeys did not lead to these similar changes. This may be explained by the fact that sampling time points in these studies were taken after 6 and 13 weeks of dosing [13]. The observed creatinine increases in our study occurred earlier in time and reduced with continued dosing (in the 50 mg cohort). If in animals the renal changes also occurred early, these apparently abated.

More extensive preclinical investigation, focused on early changes and comparing different dose regimens such as abandoning a loading regimen and/or less frequent dosing, could be considered as future experiments. Further, it might be of interest to include in such experiments other drugs that increase serum creatinine, such as cimetidine. Comparing ISIS 388626 to small molecule SGLT2 inhibitors could be used as benchmark and confirm or refute the hypothesis that the renal effects are related to SGLT2 inhibition. Further development of antisense-mediated knockdown of SGLT2 can only be justified when possible mechanisms causing the increase in serum creatinine and urinary excretion of B2M, AGST and KIM1 are understood and manageable.

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Figure 1 Average change from baseline of serum creatinine over time in the multiple ascending dose study with sd error bars. Timing of dosing is indicated with D's. The 6 subjects of the 50 mg cohort received all 8 doses. In the 100 mg cohort, the first 4 doses were received by all 12 subjects. Thereafter 9 subjects received the 5th dose and 4 subjects received the 6th dose and no subjects in the 100 mg cohort received the 7th or 8th dose, due to the early halt of the study. In the 50 mg cohort a minimal increase in average serum creatinine of 0.13 ± 0.09 mg/dl (13% increase) was observed during the first three weeks after treatment initiation. The values returned to baseline levels after the third treatment week, despite continued dosing. In the 100 mg cohort an increase in average serum creatinine of 0.14 ± 0.12 mg/dl (16% increase) was observed during the first three weeks.

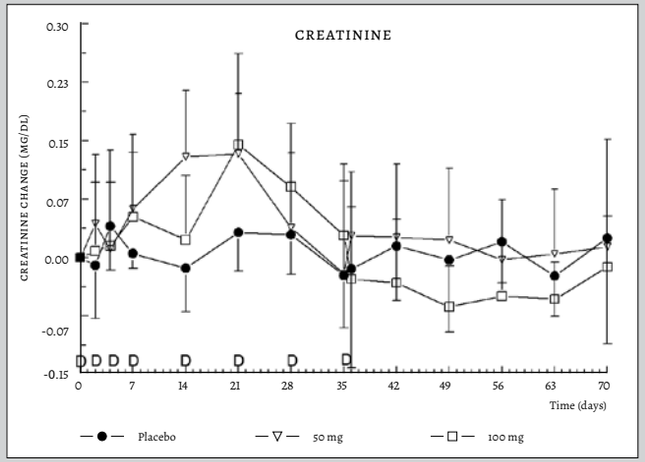

Figure 2 Box-Whisker plot of urinary renal damage markers Pre-Dose and Post-Dose (after 4 doses). The bottom and top of the box are the 25th and 75th percentile, respectively and the band inside is the median. The ends of the whiskers represent the minimum and maximum of all the data. Dots are individual results, grey being pre-dose measurements, black being values measured after 4 doses of study drug. After 4 doses, a dose-dependent increase in B2M excretion was observed in nearly all ISIS 388626-treated subjects. An average of 843 ± 1027.5 ug/24 hrs was observed for the 50 mg cohort and 2200 ± 2956.2 ug/24 hrs for the 100 mg cohort, versus 69.8 ± 27.6 ug/24 hrs in the placebo group. Also KIM1 and AGST excretion increased upon ISIS 388626 treatment in the majority of treated subjects, although there were no clear dose relationships observed for these renal markers. No clear changes occurred in urinary NAG.

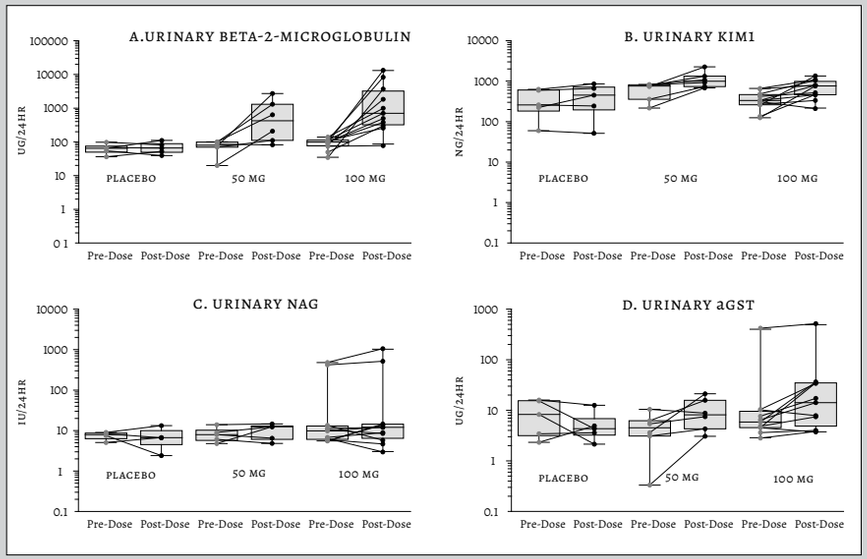

Figure 3 Profile of plasma pharmacokinetics during the first 48 hours after a single dose of study drug. Average concentrations with SD error bars. Following single SC injection, ISIS 388626 was rapidly absorbed as demonstrated by reaching maximum plasma concentrations (Cmax) between 1.2 and 1.5 hours.

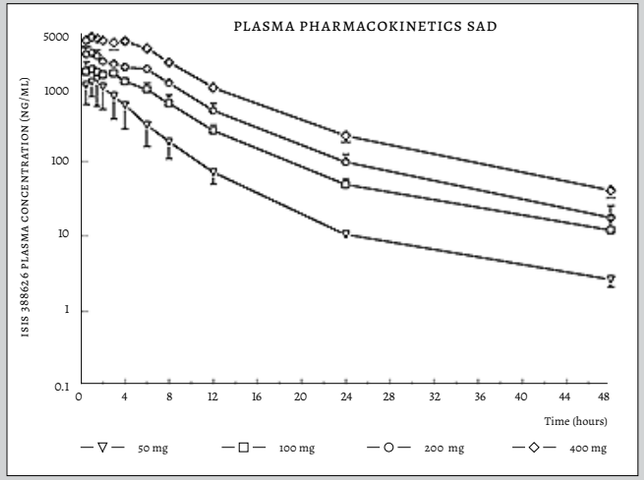

Figure 4 Average ISIS 388626 urinary excretion (mg), with SD error bars after one dose of study drug during the first 24 hours and during the 24-48 hour interval. N=3 subjects per dose level. The total amount of ISIS 388626 in urine increased more than dose-proportionally.

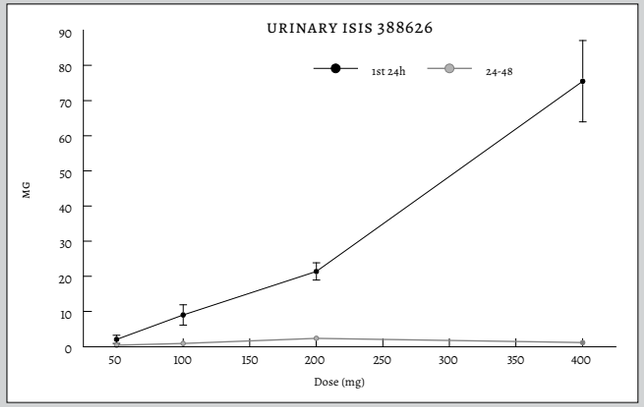

Figure 5 Average urinary glucose excretion time profile graph, with SD error bars. W=week. EOT= End Of Trial. OGTT= Oral Glucose Tolerance Test. Repeated administration of 50 mg ISIS 388626 did not alter urinary glucose excretion after 6 weeks of treatment. At follow-up, six weeks after the last administration of ISIS 388626, a possible trend towards an increased urinary glucose excretion was observed.

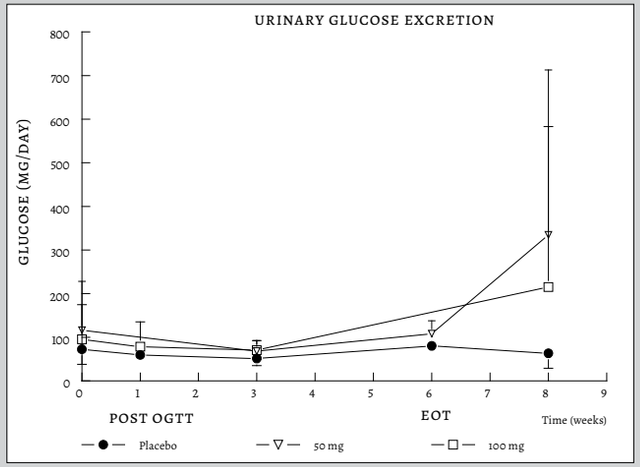

Figure 6 Average inhibition of glucose reabsorption time profile graph, with SD error bars. W=week. EOT= End Of Trial. OGTT= Oral Glucose Tolerance Test. Repeated administration of 50 mg ISIS 388626 did not alter inhibition of glucose reabsorption after 6 weeks of treatment. At follow-up, six weeks after the last administration of ISIS 388626, a possible trend towards an increased inhibition of reabsorption was observed.

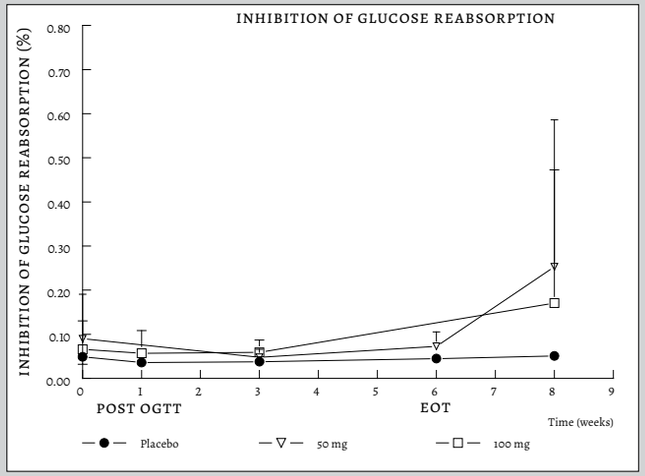

Table 1 Summary of subject demographics. SAD: Single Ascending Dose. MAD: Multiple Ascending Dose

	Number	Age (yrs ± Sd)	BMI (Kg/m2 ± Sd)	% Male
SAD 50 mg	3	31.3±13.7	23.6±1.7	100
SAD 100 mg	3	22.0±5.3	23.4±2.1	100
SAD 200 mg	3	24.7±5.0	23.5±1.5	100
SAD 400 mg	3	26.7±8.0	24.3±1.5	100
SAD PLACEBO	4	21.0±0.8	21.3±0.8	100
MAD 50 mg	6	50.7±16.4	24.6±3.0	83.3
MAD 100 mg	12	40.1±14.4	23.9±2.8	83.3
MAD PLACEBO	5	40.8±16.4	25.4±3.3	100

Table 2 Frequency overview of adverse events reported more than once (%). SAD: Single Ascending Dose. MAD: Multiple Ascending Dose

	SAD 50-400 mg (N=12)	SAD PLACEBO (N=4)	MAD 50 mg (N=6)	MAD 100 mg (N=12)	MAD PLACEBO (N=5)
Fatigue	50	25	66.7	66.7	20
Headache	0	0	33.3	16.7	40
Nasopharyngitis	8.3	0	0	33.3	60
Dizziness	0	0	0	25	40
ISRs	0	0	33.3	50	0

Table 3 **Plasma pharmacokinetics Single Ascending Dose study. N=number**

Dose	N	AUC 0-48hr (ug*hr/mL)	Cmax (ug/mL)	Tmax (hr)
50 mg	3	5.82 ± 2.18	1.31 ± 0.59	1.17 ± 0.58
100 mg	3	13.0 ± 0.78	1.72 ± 0.35	1.17 ± 0.76
200 mg	3	22.2 ± 0.88	2.88 ± 0.52	0.83 ± 0.29
400 mg	3	42.8 ± 2.47	4.58 ± 0.19	1.5 ± 1.32

Table 4 **Plasma pharmacokinetics Multiple Ascending Dose study. N=number**

Dose	Study Day	N	AUC 0-48hr (ug*hr/mL)	Cmax (ug/mL)	Tmax (hr)
50 mg	1	6	9.58 ± 1.39	1.65 ± 0.37	1.25 ± 0.27
	36	6	9.78 ± 1.53	1.67 ± 0.26	1.33 ± 0.61
100 mg	1	12	16.1 ± 3.09	2.23 ± 0.36	1.21 ± 1.01

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III

NOVEL ANTISENSE SGLT2 INHIBITOR CAUSES SERUM CREATININE INCREASES WITHOUT AFFECTING RENAL BLOOD FLOW OR GLOMERULAR FILTRATION

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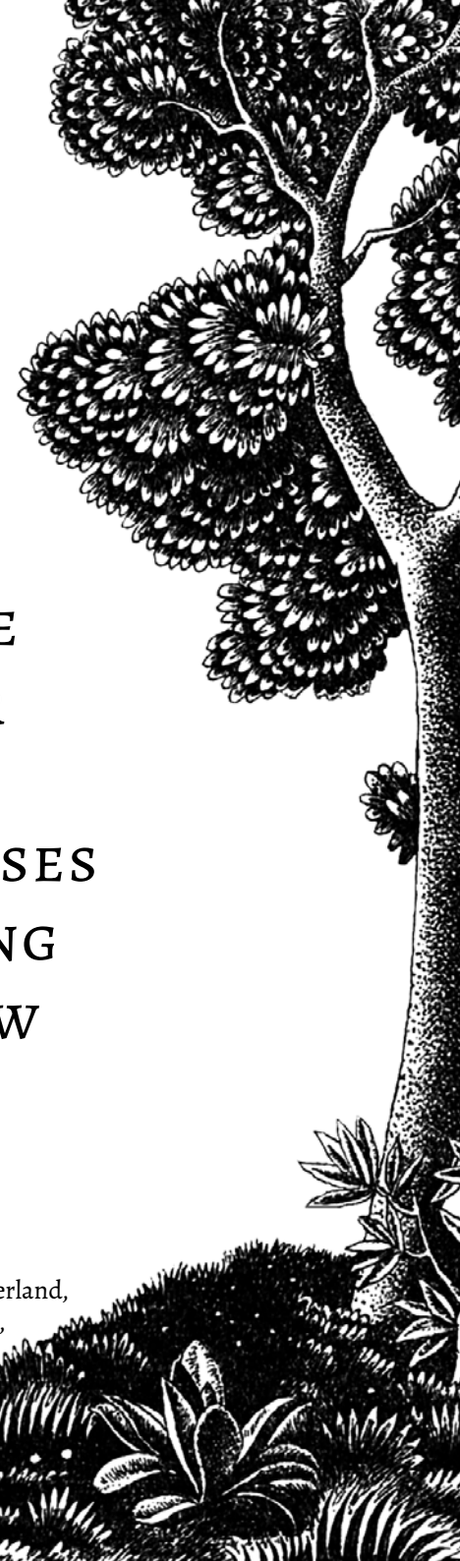

Abstract

ISIS 388626 is an antisense oligonucleotide designed to inhibit the renal SGLT2 receptor to treat Type 2 Diabetes Mellitus by inducing glucosuria. The first-in-human trial with this drug candidate was halted early due to unexpected effects on renal function. Additional and more extensive preclinical testing of ISIS 388626 with different dose regimens provided more insight into the renal effects. An adapted study design without loading dose was proposed to avoid these untoward effects and the study was restarted with a multiple ascending dose design with weekly 50, 100 and 200 mg for 13 weeks. Despite changing the dose regimen, treatment with 50 mg ISIS 388626 induced serum creatinine increases and increases in urinary renal markers. These changes prohibited dose escalation to the 100 mg dose. Instead, a new cohort of volunteers was treated at the 50 mg dose level to explore if the observed transient increases in renal damage markers coincided with functional renal changes. This was assessed with renal clearance tests to evaluate the impact of ISIS 388626 on GFR and renal plasma flow. Weekly ISIS 388626 treatment at a dose level of 50 mg for 13 weeks increased average serum creatinine (with 0.15 mg/dl) and renal damage markers. The changes were relatively mild and fully reversible upon cessation of dosing. The renal clearance test revealed no indications for impairment of glomerular filtration or renal perfusion. No increase in renal glucose excretion was observed at the 50 mg dose level, as was expected based on preclinical data. To elicit pharmacological activity exposure is to be increased. Exploration of higher ISIS 388626 dose levels in healthy volunteers is possible as long as a careful approach is applied with close monitoring of renal function and damage markers.

Introduction

The strategy of SGLT2 Inhibition in the treatment of Diabetes Mellitus Type 2 has proven its efficacy and has led to the registration and approval of several small molecule SGLT2 inhibitors [1-3]. An alternative to SGLT2 inhibition by small molecules, which result in a moderate 30-50 % inhibition only, could be antisense-mediated SGLT2 knock-down. ISIS 388626 is such an antisense oligonucleotide which in animal models causes ≥ 80% reduction of renal SGLT2 mRNA expression at doses of 1-3 mg/kg/week (rodents) to 30 mg/kg/week (monkeys), resulting in effective glucosuria [4;5].

As ISIS 388626 appeared to be effective and safe in animal studies ranging from 6 weeks to 6 months in duration the compound was tested in humans. These first clinical studies with 50-100 mg SC weekly after a loading dose regimen of three doses during the first week showed, contrary to expectations, possibly untoward renal effects (chapter 2 of this thesis, *L. van Meer et al.*). In summary, 3-4 week treatment with ISIS 388626 resulted in dose-dependent, transient, fully reversible, and variable (range; 0 - 73 % at 100mg ISIS 388626) increases in serum creatinine that was accompanied by increased urinary excretion of renal markers such as beta-2-microglobulin (B2M) and Kidney Injury Molecule 1 (KIM1). This was unexpected as in animals assessments at 6 and 13 weeks had not shown such results. The pertaining clinical study was halted early and further pre-clinical data were collected. First, bio-banked samples collected earlier than 6 weeks in previously performed pre-clinical experiments were analyzed. This showed that also in animals, relatively early during weekly treatment with ISIS 388626 after using loading doses resulted in transient increases in serum creatinine and urinary excretion of B2M and protein. The changes were reversible even upon continuation of dosing (unpublished data, on file). Thus, the apparent discrepancy of renal effects of ISIS 388626 treatment in rodent and monkeys and humans could be explained by the timing of the assessments [4-6]. In a further dedicated experiment in monkeys it was explored if the renal effects by ISIS 388626 could be explained by the loading dose. Animals were dosed for 13 weeks with either 30 mg/kg every other day or a single dose in the first week, followed by weekly dosing for another 12 weeks. This experiment showed that changes in renal markers occurred only with the loading dose regimen. Importantly, this study also showed that abandoning the loading dose ISIS 388626 still resulted in glucosuria, while changes in serum creatinine did not occur.

Based on these findings, the clinical study was restarted with the aim to investigate the effects of 13 weekly SC doses of 50, 100 and 200 mg ISIS 388626. However, omitting the loading dose did not prevent increases in renal markers, as described here. Dose escalation after 50mg was stopped and further explorations were done to investigate whether the transient increases in renal markers in humans could be explained by functional changes in renal blood flow and/or glomerular filtration rate.

Materials and Methods

SUBJECTS

Adult subjects (18-65 yrs), male or female (post-menopausal or surgically sterile) with a BMI < 30 kg/m2 and a fasting plasma glucose and a normal HbA1c could participate in this study. Subjects with significant abnormalities in medical history, physical examination, 12-lead electrocardiogram, and clinical laboratory evaluations (including positive protein in urine dipstick analysis and calculated eGFR below 60 ml/min by MDRD equation[7] were excluded The study was conducted in accordance with good clinical practice guidelines, after approval by the national ethics committee.

STUDY DESIGN

This was a double-blind, randomized, placebo-controlled multiple ascending dose study of 12 weeks duration and 5 weeks follow-up, with weekly administration of ISIS 388626 to establish the safety profile and pharmacodynamics of the compound, performed at the Centre for Human Drug Research in the Netherlands. Per cohort, 16 randomly assigned subjects received multiple doses of either ISIS 388626 or placebo (in a 3:1 ratio), administered as subcutaneous injection. An oral glucose tolerance test (OGTT) was performed before the first administration of ISIS 388626 (or matching placebo) and at week 9 and 13. The OGTT consisted of ingestion of a 75 mg glucose solution, given after an overnight fast. Subsequently blood was drawn regularly during 4 hours for determination of glucose, insulin and C-peptide concentrations. It was anticipated to investigate the effects of 50, 100 and 200 mg ISIS 388626, but due to unexpected findings in the 50 mg cohort, execution of the 100 and 200 mg cohort was cancelled. To explore the nature of the observed safety signals in more detail, renal clearance tests (using PAH and sinistrin infusions) were performed regularly in an additional cohort treated with the same dose regimen (weekly administration of 50 mg ISIS 388626 or placebo).

DOSE RATIONALE

It was anticipated to explore 50, 100 and 200 mg of ISIS 388626. The doses were based on a MABEL approach, taking into account a No Adverse Effect Level estimated to be 10 mg/kg/week (including a loading dose regimen) in monkeys. In preclinical studies across multiple species, the pharmacologically active dose range of ISIS 388626 was 1-3 mg/kg/week. At this exposure, a significant reduction in SGLT2 mRNA occurred (74 to 97% in mice and approximately 30 to 90% in monkeys over the dose range 1-30 mg/kg/week), accompanied by a 25-200 fold increase in urinary glucose excretion [5;6;8]. Based on this, estimation of the equivalent human effective dose falls in the range of 1-3 mg/kg/week. Experience with other 2'-MOE-modified antisense oligonucleotides, safely administered (intravenously and subcutaneously) in multiple clinical studies at doses up to weekly 750 mg (which translates into 10.7 mg/kg/week assuming an average weight of 70 kg), with treatment durations exceeding one year [9], further supports the safety of this dose range.

The dose regimen was chosen because the loading dose (3 doses in the first week) resulted in creatinine increases in prior human studies (chapter 2 of this thesis, *L. van Meer et al.*) and dedicated experiments in monkeys showed that changes in renal markers occurred only with the loading dose regimen. The treatment duration of 12 weeks (13 doses) was selected, which was expected to be safe and resulting in sufficient steady state tissue concentrations, based on animal studies.

CLINICAL MEASUREMENTS

Safety assessments, performed throughout the study period, included vital signs, electrocardiograms, physical examinations, and clinical laboratory tests (including clinical chemistry, hematology, coagulation, cytokines, complement tests and urinalysis (including B2M and protein)) as well as registration of adverse events. Adverse events were defined as any new medical occurrence or worsening of a pre-existing condition after administration of the study drug or placebo. Predefined stopping rules regarding renal parameters were defined as changes in serum creatinine change from baseline of more than 0.3 mg/dL or more than 40% on two consecutive weeks, or proteinuria of more than 0.5 g/24hr occurring on two consecutive weeks.

RENAL MARKERS

The biomarkers B2M, aGST and NAG were chosen based on their performance on detecting injury to the proximal tubule where SGLT2 is located [10]. Analysis of renal damage markers aGST and NAG was performed batch-wise upon study completion by quantative enzyme immunoassays

(Argutus Medical NEPHKITO immunoassay for aGST, and Diazyme 70010 Rev. F, colorimetric end point assay for NAG).

RENAL PERFUSION AND GLOMERULAR FILTRATION

Renal clearance tests to assess RPF and GFR were performed using established and validated techniques [11-13]. Sinistrin infusion allows calculation of glomerular filtration rate (GFR) as it is not secreted or reabsorbed in any appreciable amount by the kidney. PAH infusion allows calculation of renal plasma flow (RPF) as it is completely secreted and not reabsorbed by the tubules. The intravenous infusion of sinistrin and PAH started 90 minutes after administration of ISIS 388626 or placebo. Infusion rates were calculated with the aim to obtain a steady state concentration that was comparable between subjects and within the measurable range. PAH and sinistrin doses, corrected for lean body mass, serum creatinine and age were administered via a continuous infusion of 120 minutes (infusion rates ranging from 400 to 750 mg/hr and from 380 to 740 mg/hr for sinistrin and PAH, respectively), preceded by a 10-minute priming dose that was corrected for body surface area (ranging from 825 to 1200 mg and from 840 to 1200 mg for sinistrin and PAH respectively). Plasma samples for PAH, sinistrin and hematocrit measurement and urine samples for PAH and sinistrin measurement were collected at 30 minute intervals. During the infusion period, hydration was maintained by subjects drinking amounts of water matching urinary output, with a maximum of 4 L, to ensure sufficient urine production. Serum and urinary sinistrin levels were analyzed according to the method described by Looye ([14]) PAH levels were measured according to the method described by Waugh et al. [15].

No formal power calculation was performed, however group size (12 treated subjects) was considered to be sufficient as expected effect size of change in GFR (in case present) was around 17% and as previously shown, differences of 10% GFR can be detected with a group size of 9 healthy volunteers [12].

PHARMACOKINETICS

ISIS 388626 plasma levels were measured in using a validated hybridization enzyme-linked immunosorbent assay (PDD laboratories, Richmond, USA) frequently for a 24 hour profile after the first and 13th ISIS 388626 dose, and predose on weeks 3, 8, 10 during treatment and on 5 weekly follow-up visits. In addition, ISIS 388626 urine levels were measured using a validated Capillary Gel Electrophoresis method (PPD Laboratories, Richmond, USA), in 24 hour collections after the first and 13th dose (up to 24 and 48 hours post-dose).

DATA ANALYSIS AND STATISTICAL METHODS

Safety and tolerability evaluation was based on descriptive statistics. ISIS 388626 plasma concentrations were subjected to non-compartmental pharmacokinetic evaluation in order to determine the maximum observed plasma concentration (Cmax), the time to maximum plasma concentration (Tmax), the area under the plasma concentration-time curve from dosing to 24 hours after dosing (AUC0-24h) using WINNONLIN (version 5.3, Pharsight Corporation, USA).

Results

SUBJECTS

Sixteen subjects were enrolled and thirteen subjects completed the first study part (50mg/placebo). One subject withdrew consent for personal reasons after receiving 12 doses, and in two subjects dosing was stopped due to safety findings; in one subject after 5 doses due to increases in serum creatinine, in the other subject after 7 doses due to increased liver biochemistry parameters (see safety results for details). Another sixteen subjects were enrolled in the second study part study which included renal clearance tests (RCT); demographics are presented in table 1. Subjects were randomized to 13 weekly SC injections of 50 mg ISIS 388626 (n=12) or placebo (n=4).

SAFETY

All reported adverse events (AEs) were of mild intensity and transient (table 2). The most common AEs were headache, gastrointestinal complaints (diarrhea, nausea or abdominal discomfort) and mild upper respiratory complaints (nasopharyngitis and flu-like symptoms). Since the incidence of AEs was comparable between active treatment

and placebo, it is considered unlikely that these AEs are related to ISIS 388626 administration. Injection site reactions (ISRs) were observed in 2 out of 12 ISIS 388626-treated subjects (17%) in the first study part. No ISRs were observed in the second study part (table 2). In one subject hyperpigmentation was reported. The ISRS were not progressive and not accompanied by local lymphadenopathy. ISRs were considered to be related to administration of study drug and all resolved completely and spontaneously during the study period.

ISIS 388626 treatment did not result in any clinically relevant changes in vital signs, ECG-derived parameters, body temperature, hematology, coagulation, complement or cytokines. Also chemistry parameters, including hepatic enzymes and glucose levels, were generally unchanged with the exception of elevated serum creatinine levels in multiple subjects. It was also observed that in one participant after 7 doses of ISIS 388626 transiently increased liver biochemistry parameters occurred (maximal change from baseline AST 3.6-fold ULN, ALT 4-fold ULN, confirmed by repeated measurement). This laboratory finding was suspect for a viral infection as it coincided with mild joint pain and tonsillitis. Study drug administration was discontinued and hepatic chemistry normalized within 2 weeks. Before treatment start, serum creatinine levels were comparable for the ISIS 388626 group and placebo group (table 1). ISIS 388626 treatment resulted in a rapid and sustained increase in serum creatinine concentrations, peaking at the end of dosing (figure 1A) with an average increase over baseline of 0.17 ± 0.08 mg/dL (+20 %). The observed increase in serum creatinine was variable between subjects, ranging from 0.10-0.33 mg/dL at week 13, but the increase was observed in all ISIS 388626-treated subjects and in none of the placebo-treated subjects. Study drug administration was discontinued for one subject in whom one of the predefined stopping criteria was met; 41% increase in serum creatinine after five ISIS 388626 doses. Upon cessation of ISIS 388626 administration, serum creatinine levels returned to baseline in all subjects within 5 weeks. The observed changes in serum creatinine were not accompanied by rises in BUN or any clinically meaningful changes in serum electrolytes, albumin, aldosterone, plasma renin activity (data not shown).

Urine flow and urinalysis parameters did not change significantly in the subjects with increased serum creatinine levels. ISIS 388626 treatment did not result in changes in renal damage markers NAG and aGST (data not shown). Creatinine increases did coincide with increase in urinary B2M (figure 1B) with a maximal average change from baseline of 1250 ± 1361 µg/24hr (15-fold increase) at week 12. Although the inter-individual variability in urinary B2M was substantial, in 9 out of 12 ISIS 388626-treated subjects an increase was observed, returning to baseline levels within 5 weeks after treatment cessation. Average excretion of urinary protein was larger in the ISIS 388626 -treated group, but variability was substantial in both treatment groups (figure 1C).

RENAL PERFUSION AND GLOMERULAR FILTRATION

To explore the nature of the observed renal findings in more detail, an additional cohort was treated with the same dose regimen e.g. weekly administration of 50 mg ISIS 388626 or placebo. Renal clearance tests (with PAH and sinistrin) were performed to assess kidney function. Comparable ISIS 388626-induced effects were observed as in the first study part, with transient increases in serum creatinine and urinary B2M and mildly elevated urinary protein levels (figure 2A,B,C). aGST was slightly elevated in the ISIS 388626-treated group, but variability was substantial (data not shown). RPF and GFR were in the expected range, as was the filtration fraction (GFR/RPF*100) of approximately 20%. Neither RPF nor GFR changed during the entire 13-weeks ISIS 388626 treatment period (figure 3).

PHARMACOKINETICS

After the first dose average maximal plasma concentrations of 1240±274.4 ng/mL were reached at 1.2±0.32 hours, and the AUC 0-24hr was 7627±1202 ng*hr/mL. Following the 13th dose comparable values were found (Tmax 1.4±0.56 hours, Cmax 1275±470.6 ng/ml, AUC0-24hr 8626±1037 ng*hr/mL), suggesting little or no accumulation of the study drug. Mean urinary excretion after the first dose was low (1.9±0.72%) during the first 24 hours after dosing and also during the subsequent 24 hours (1.6±0.60%).

PHARMACODYNAMICS

To explore the pharmacological activity of ISIS 388626, oral glucose tolerance tests were performed before the first administration of ISIS 388626 or placebo and at week 9 and 13. The observed time-concentration curves for serum glucose, insulin and c-peptide after OGTT were not different

between the treatment groups (data not shown). During the first four hours after glucose intake of the OGTT urinary glucose excretion was increased in the ISIS 388626 treated groups (figure 4A). At week 13 the observed difference in change from baseline values was 1.41 g/4hr compared to 0.04 g/4hr in the placebo group.

The average 24 hr urinary glucose excretion and the fractional glucose excretion over time during the treatment period did not differ between the treatment groups (Figure 4B).

Discussion

This clinical study was performed to investigate the effects of multiple ISIS 388626 doses, administered weekly for a period of 13 weeks, without using a loading dose regimen. It was anticipated to explore dose levels of 50, 100 and 200 mg. Changes in renal parameters at the 50 mg dose precluded dose escalation to the 100 mg dose. Instead, a new cohort of volunteers was treated with the same dose to explore if the observed transient increases in renal markers coincided with functional renal changes.

In the first 50 mg cohort commonly observed adverse events such as headache, mild upper respiratory complaints and mild gastrointestinal complaints occurred in the placebo group at a similar incidence, and were not considered to be ISIS 388626-related. In several ISIS 388626-treated subjects injection site reactions were observed. The observed ISRs were of mild severity and regressed spontaneously. The frequency and severity of the observed skin reactions appears to be lower than described for other phosphorothioate oligonucleotides at similar doses [16;17]. Furthermore, in one ISIS 388626-treated subject transiently increased liver biochemistry parameters were observed, resulting in discontinuation of study drug. Although no explanation was found, it is unclear if the event was related to ISIS 388626 treatment.

Repeated administration of 50 mg ISIS 388626 resulted in gradually increasing serum creatinine concentrations that were maximal (+20%) after the final dose, concomitant with an increase in urinary B2M. B2M is generally considered to be a marker of glomerular injury, although certain types of tubular dysfunction also result in increases [18-20]. The urinary excretion of NAG and AGST did not change upon ISIS 388626 treatment. Generally, phosphorothioate antisense compounds are known to induce renal changes including degeneration and regeneration effects in proximal tubules, but these are generally observed at least 10- to 100-fold higher dose levels (exceeding 50 mg/kg) [21-23]. Moreover, the observed creatinine increases were unexpected, since dedicated animal experiments had previously demonstrated that ISIS 388626-induced creatinine changes were dependent on the application of a loading dose regimen, which was avoided in this clinical study.

To explore whether the observed transient increases in renal markers coincided with functional changes, a new cohort of volunteers exposed to the same ISIS 388626 dose level and regimen, and PAH and sinistrine clearance was assessed to estimate RPF and GFR. The effects on serum creatinine and urinary markers were reproduced in this second cohort, but we found no indications that the changes can be explained by ISIS 388626-induced changes in GFR and RPF. This may suggest that the observed increase in serum creatinine and other renal markers are more likely related to changes in tubular function.

No effect of ISIS 388626 treatment on glucose handling was observed which was not surprising given the relatively limited exposure, and the fact the study was not designed to demonstrate pharmacodynamic effects. In general, pharmacologically effective dose levels for second generation oligonucleotides exceed 100 mg/week [16;24;25]. Small molecules targeting SGLT2 induced glucose excretion in the range of 10 to 80 grams per 24 hrs, even in healthy subjects [26]. The glucose excretion observed in our study ranged from 0.03 to 9.84 grams, which indicates that the maximal ISIS 388626 dose level tested may be far below dose levels anticipated to have intended pharmacological activity.

In summary, weekly ISIS 388626 treatment at a dose level of 50 mg for 13 weeks increased serum creatinine and renal damage markers in a relatively mild, fully reversible manner and was not associated with impairment of glomerular filtration or renal blood flow. This dose level and regimen did not significantly alter renal glucose handling. Exploration of higher ISIS 388626 dose levels in healthy volunteers is possible as long as a careful approach is applied with close monitoring of renal function.

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Figure 1 First study part with 50 mg. A. Average serum creatinine (mg/dl), change from baseline values with SD error bars. B. Average urinary B2M excretion (ug), change from baseline values with SD error bars. C. Average urinary protein excretion (g), absolute values with SD error bars.

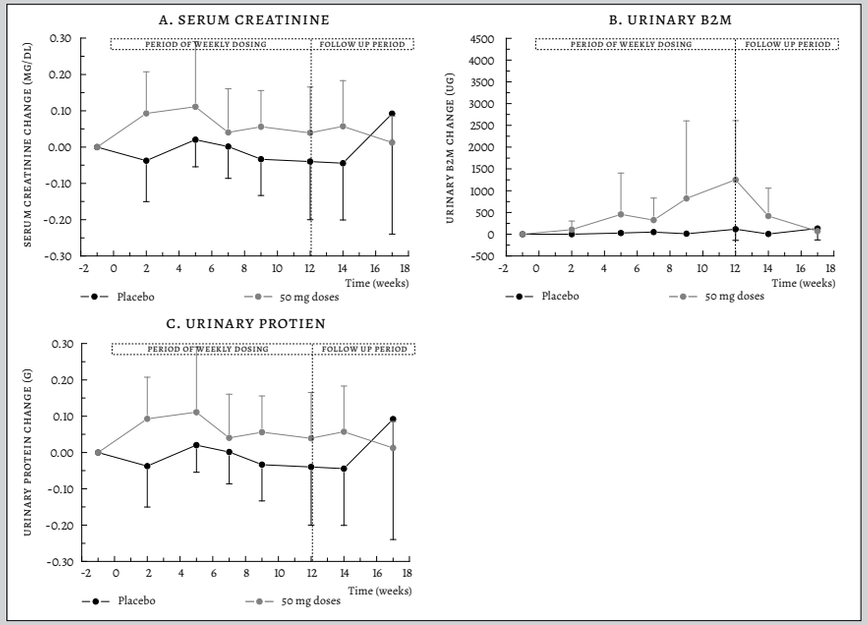

Figure 2 Repeat study with 50 mg. A. Average serum creatinine (mg/dl), change from baseline values with SD error bars. B. Average urinary B2M excretion (ug), change from baseline values with SD error bars. C. Average urinary protein excretion (g), absolute values with SD error bars.

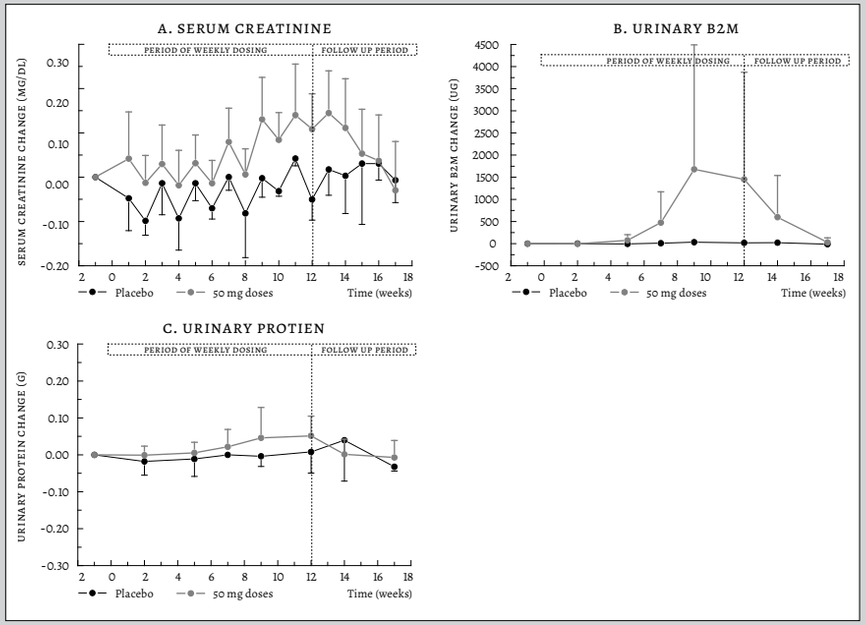

Figure 3 A. GFR calculated by sinistrin clearance B. RPF calculated by PAH clearance

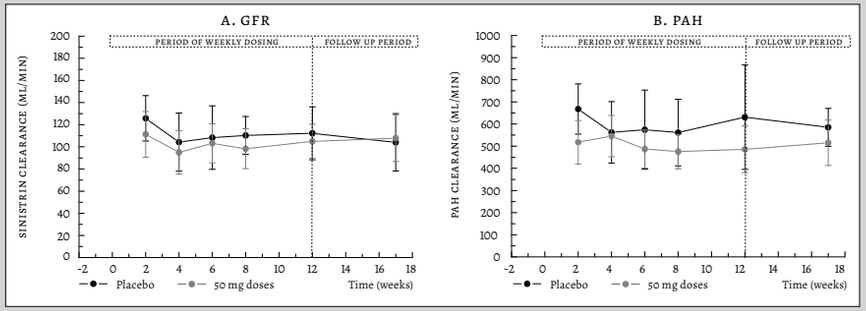

Figure 4 **A. urinary glucose excretion during the first 4 hour interval of the OGTT (4hr). The gray bar indicates the treatment phase. B. Urinary 24 hr glucose excretion. The grey bar indicates the treatment phase. The black bar indicates the 5 weeks of follow-up**

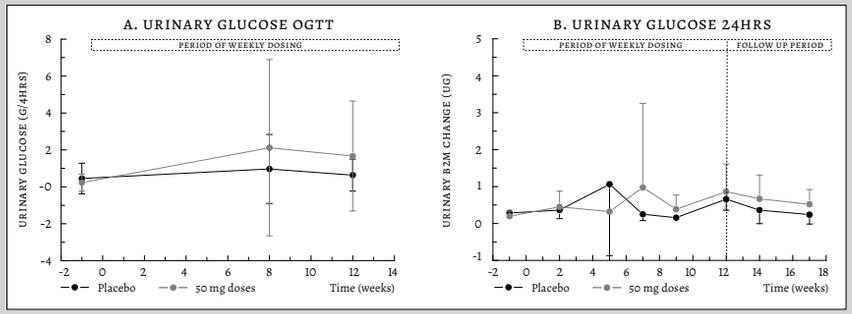

Table 1 **Summary of subject baseline characteristics.**

Treatment	N	Age (yrs, Std)	BMI (Kg/m2, Std)	% Male	Early termination of subjects*	Serum Creatinine (mg/dl, Std)	HbA1c (%, Std)
50 mg	12	33.9 +/- 14.23	23.5 +/- 3.13	100	2	0.88 +/- 0.098	5.2 +/- 0.21
Placebo	4	40.7 +/- 16.78	22.7 +/- 4.10	75	1	0.91 +/- 0.149	5.4 +/- 0.29
50 mg with RCT	12	35.2 +/- 14.68	23.1 +/- 2.62	92	None	0.92 +/- 0.120	5.0 +/- 0.27
Placebo with RCT	4	31.3 +/- 8.06	24.1 +/- 2.32	100	None	0.87 +/- 0.104	+/- 0.29

** In one subject dosing was stopped due to increases in serum creatinine after five doses and another subject was stopped due to increased liver biochemistry parameters after seven doses (see safety results) and one subject stopped after 12 doses due to personal reasons.*

Table 2 **Frequency overview of adverse events reported in more than one subject (%).**

	50 mg (n=12)	PLACEBO (n=4)	50 mg with RCT (n=12)	Placebo with RCT (n=4)
Headache	33% (n=4)	50% (n=2)	50% (n=6)	25% (n=1)
Mild upper respiratory complaints	58% (n=7)	75% (n=3)	75% (n=9)	75% (n=3)
Mild gastrointestinal complaints	58% (n=7)	75% (n=3)	33% (n=4)	25% (n=1)
ISRS	17% (n=2)	0% (n=0)	0% (n=0)	0% (n=0)

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IV

RENAL EFFECTS OF SGLT2 INHIBITION WITH A NOVEL ANTISENSE OLIGONUCLEOTIDE

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Abstract

The aim of this study was to study the effects of the SGLT2 inhibitor ISIS 388626. ISIS 388626 is an antisense SGLT2 inhibitor, designed to treat type 2 diabetes mellitus. ISIS 388626 was demonstrated to be safe and effective in preclinical trails, reducing renal SGLT2 mRNA expression in rodent and monkeys, translating into effective glucosuria. A randomized, placebo-controlled, dose-escalation Phase 1 study was designed to evaluate the effects of ISIS 388626 in healthy volunteers. Twenty-nine subjects were enrolled sequentially into 1 of 2 dose cohorts at a 3:1 (active/placebo) ratio. Subjects received 13 weekly doses of 100 or 200 mg over 12 weeks. The primary pharmacodynamic endpoint was change in urinary glucose excretion. In addition, biomarkers of kidney toxicity were assessed throughout the dosing period to explore the safety profile. ISIS 388626 increased 24 hour urinary glucose excretion dose-dependently with 508.9 ± 781.45 mg/day in the 100 mg and 1299.8 ± 1833.4 mg/day in the 200 mg cohort, versus 88.7 ± 259.29 mg/day in the placebo group. ISIS 388626 also induced a reversible increase in serum creatinine, with the largest effect after 8 doses of 200 mg ISIS 388626 (0.38 ± 0.089 mg/dL; 44% increase over baseline). Three subjects were discontinued due to creatinine increases. The creatinine increases were accompanied by a rise in the levels of urinary renal damage markers (B2M, total protein, KIM1, AGST, NAG). Other treatment related AEs included mild ISRs, occuring in 8-19% of the subjects. In conclusion, ISIS 388626 treatment induced glucosuria at a dose level of 200 mg/week. This intended pharmacological effect was small, amounting approximately 1% of total amount of filtered glucose. Changes in serum and urinary markers were indicative of transient renal dysfunction, most likely of tubular origin. Whether the glycosuria is caused by specific SGLT2 inhibition or general tubular dysfunction or a combination remains uncertain.

Introduction

The antisense oligonucleotide ISIS 388626 inhibits the synthesis of the renal SGLT2 receptor, which accounts for 90% of the reabsorption of glomerular filtrated glucose [1;2]. SGLT2 inhibition affects renal glucose reabsorption, resulting in glucosuria and lowered serum glucose levels. The strategy of SGLT2 inhibition for the treatment of type 2 diabetes mellitus is efficacious and has led to the registration and approval of a number of small molecule SGLT2 inhibitors [3-5]. ISIS 388626 treatment was effective and safe in animals, in studies ranging from 6 weeks to 6 months in duration [1;2]. Reductions of 80% and more in renal SGLT2 mRNA expression were observed at doses of 1-3 mg/kg/week (rodents) to 30 mg/kg/week (monkeys), resulting in effective glucosuria [1;2]. The initial animal models did not show signs of ISIS 388626-induced toxicity [2]. In spite of the favorable preclinical data, the first clinical ISIS 388626 studies halted early due to unexpected increases in serum creatinine *(manuscript First-in-man studies with novel SGLT2 inhibitor: antisense compound is associated with unexpected renal effects, L. van Meer et al.)*. Discovery of these effects of the oligonucleotide led to analysis of banked serum samples collected at an earlier timepoint in the monkey studies, which revealed transient rises in serum creatinine and proteinuria. These early effects were not observed in the initial (more infrequent) sample analysis. A subsequent preclinical study that focused on the renal effects of ISIS 388626 demonstrated that no rises in serum creatinine and proteinuria occurred at a dose level of 30 mg/kg/week ISIS 388626 when abandoning the earlier applied loading dose regimen (unpublished data, on file). Based on these results, clinical studies were re-initiated, exploring the effects of weekly subcutaneous doses of 50, 100 and 200 mg ISIS 388626 for a period of 12 weeks (13 doses), avoiding the loading dose. Nonetheless, weekly ISIS 388626 treatment at a dose level of 50 mg induced increases in serum creatinine and renal damage markers. Further dose escalation was halted and instead a dedicated clinical experiment was performed to explore whether ISIS 388626 (50 mg, weekly during 12 weeks) affected renal blood flow and/or glomerular filtration. This appeared not to be the case (*manuscript Novel antisense SGLT2 inhibitor causes serum creatinine increases without affecting renal blood flow or glomerular filtration, L. van Meer et al.)*. It was judged to be safe and rational to perform additional clinical experiments to study higher doses of ISIS 388626 that may exert the intended pharmacodynamic effects. In this study, healthy volunteers received treatment with 100 and 200 mg ISIS 388626 weekly for a period of 12 weeks. The study design included oral glucose tolerance tests (OGTT) to estimate the intended pharmacodynamic effect (the induction of glucosuria) and close monitoring of renal function and injury, applying strict predefined stopping criteria. This paper reports on the findings from

these clinical experiments. For reference, the results of the earlier cohort of healthy volunteers exposed to 50 mg ISIS 388626 (chapter 3 of this thesis, *L. van Meer et al.)* have been included.

Materials and Methods

SUBJECTS

Adult subjects (18-65 yrs), male or female (post-menopausal or surgically sterile) with a BMI < 30 kg/m² and a fasting plasma glucose and HbA1c below the upper limit of normal could participate in this study. Significant abnormalities in medical history, physical examination, 12-lead electrocardiogram, and clinical laboratory evaluations (including positive protein in urine dipstick analysis and calculated eGFR below 60 ml/min by MDRD equation [6] led to exclusion. The study was conducted in accordance with good clinical practice guidelines, after approval by the national ethics committee.

STUDY DESIGN

This was a double-blind, randomized, placebo-controlled multiple ascending dose study of 12 weeks duration and 5 weeks follow-up, with weekly administration of ISIS 388626 to establish the safety profile and pharmacodynamics of the compound, performed at the Centre for Human Drug Research in the Netherlands. Per cohort, 16 randomly assigned subjects received multiple doses of either ISIS 388626 or placebo (in a 3:1 ratio), administered as subcutaneous injection. An oral glucose tolerance test (OGTT) was performed before the first administration of ISIS 388626 (or matching placebo) and at after the 9th and 13th dose. The OGTT consisted of ingestion of a 75 mg glucose solution, given after an overnight fast. Subsequently blood was drawn regularly during 4 hours for determination of glucose, insulin and c-peptide concentrations.

SAMPLE SIZE

The selection of a total of 12 subjects per treatment group was based on previous data obtained after conducting an OGTT in normal subjects. It is estimated that the standard deviation of change in plasma glucose AUC0-120min during the OGTT is approximately 85 mmol*min/L. With 6 subjects in the pooled placebo group and 6 subjects in the ISIS 388626 treated group, this would result in at least 80% power to detect a 170 mmol*min/L difference in plasma glucose AUC0-120min at an alpha level of 0.05. To ensure sufficient power of the efficacy analysis also in case of more than expected variation, additional subjects were included.

DOSE RATIONALE

It was anticipated to explore 50, 100 and 200 mg of ISIS 388626. The doses were based on a MABEL approach, taking into account a No Adverse Effect Level estimated to be 10 mg/kg/week (including a loading dose regimen) in monkeys. In preclinical studies across multiple species, the pharmacologically active dose range of ISIS 388626 was 1-3 mg/kg/week. At this exposure, a significant reduction in SGLT2 mRNA occurred (74 to 97% in mice and approximately 30 to 90% in monkeys over the dose range 1-30 mg/kg/week), accompanied by a 25-200 fold increase in urinary glucose excretion [1;7;8]. Based on this, estimation of the equivalent human effective dose falls in the range of 1-3 mg/kg/week. Experience with other 2'-MOE-modified antisense oligonucleotides, safely administered (intravenously and subcutaneously) in multiple clinical studies at doses up to weekly 750 mg (which translates into 10.7 mg/kg/week assuming an average weight of 70 kg), with treatment durations exceeding one year [9], further supports the safety of this dose range.

The dose regimen was chosen because the loading dose (3 doses in the first week) resulted in creatinine increases in prior human studies *(manuscript First-in-man studies with novel SGLT2 inhibitor: antisense compound is associated with unexpected renal effects, L. van Meer et al.)* and dedicated experiments in monkeys showed that changes in renal markers occurred only with the loading dose regimen. The treatment duration of 12 weeks (13 doses) was selected, which was expected to be safe and resulting in sufficient steady state tissue concentrations, based on animal studies.

CLINICAL MEASUREMENTS

Safety assessments, performed throughout the study period, included vital signs, electrocardiograms, physical examinations, and clinical

laboratory tests (including clinical chemistry, hematology, coagulation, cytokines, complement tests and urinalysis) as well as registration of adverse events. Adverse events were defined as any new medical occurrence or worsening of a pre-existing condition after administration of the study drug or placebo. Predefined stopping rules regarding renal parameters were defined as changes in serum creatinine change from baseline of more than 0.3 mg/dL or more than 40% on two consecutive weeks, or proteinuria of more than 0.5 g/24hr occurring on two consecutive weeks.

RENAL DAMAGE MARKERS

The biomarkers KIM1, Cystatin C, EGF, NGAL/LCN2, Osteopontin, Uromodulin, aGST and NAG were chosen based on their performance on detecting injury to the proximal tubule where SGLT2 is located [10]. Analysis of renal damage markers aGST and NAG was performed batch-wise upon study completion by quantative enzyme immunoassays (Argutus Medical NEPHKITO immunoassay for aGST, and Diazyme 70010 Rev. F, colorimetric end point assay for NAG) and KIM1, Cystatin C, EGF, NGAL/LCN2, Osteopontin, Uromodulin by enzyme-linked immunosorbent assay (R&D Systems ELISA).

PHARMACOKINETICS

ISIS 388626 plasma levels were measured in using a validated hybridization enzyme-linked immunosorbent assay (PDD laboratories, Richmond, USA) frequently for a 24 hour profile after the first and 13th ISIS 388626 dose, and predose on weeks 3, 8, 10 during treatment and on 5 weekly follow-up visits. In addition, ISIS 388626 urine levels were measured using a validated Capillary Gel Electrophoresis method (PDD laboratories, Richmond, USA), in 24 hour collections after the first and 13th dose (up to 24 and 48 hours post-dose).

DATA ANALYSIS AND STATISTICAL METHODS

Safety and tolerability evaluation was based on descriptive statistics. ISIS 388626 plasma concentrations were subjected to non-compartmental pharmacokinetic evaluation in order to determine the maximum observed plasma concentration (Cmax), the time to maximum plasma concentration (Tmax), the area under the plasma concentration-time curve from dosing to 24 hours after dosing (AUC0-24h) using WinNonLin (version 5.3, Pharsight Corporation, USA).

Pharmacodynamic evaluation was based on descriptive statistics as well as statistical analysis using ANCOVA with baseline as a covariate. Endpoints were urinary glucose excretion (UGE) and fractional glucose excretion (defined as (UGE / filtered glucose load (GFR*fasted plasma glucose) *100), and plasma glucose, insulin and c-peptide concentrations.

Results

SUBJECTS

Twenty one subjects participated in the 100mg/placebo cohort, of which fifteen subjects completed the study. Another sixteen subjects participated in the 200mg/placebo cohort, of which fourteen subjects completed the study. Subject demographics are presented in table 1.

PHARMACODYNAMICS

ISIS 388626 dose-dependently increased 24 hour urinary glucose excretion (figure 1A, to 575.6 ± 789.5 and 1413.1 ± 1804.6 mg/day glucose excretion at end of treatment in the 100 and 200 mg cohort, versus 172.3 ± 387.0 mg/day in the placebo group). During the five week follow-up period, values returned to baseline, although not completely for the 200mg cohort. Average values of fractional glucose excretion, calculated from 24-hour urine glucose excretion and serum glucose, showed a very similar pattern (data not shown).

The OGTT resulted in an expected strong increase in serum glucose, insulin and c-peptide levels, followed by a rapid decline towards normal values (data not shown). ISIS 388626 treatment did not affect the OGTT-induced increase in serum glucose. ISIS 388626 treatment resulted in a dose-dependent enhancement of insulin and c-peptide release (Figure 1B and C). Urinary glucose excretion during the first four hours of the OGTT was increased in ISIS 388626 treated groups compared to placebo (figure 1D, 347.3 ± 411.4 and 745.6 ± 1122.4 mg/4hr for the 100 and 200 mg cohort, versus 133.9 ± 327.5 mg/4hr in the placebo groups). Increases were statistically different from placebo.

PHARMACOKINETICS

ISIS 388626 rapidly entered the circulation upon SC injection, with maximum plasma concentrations (Cmax) occurring within the first 2 hours after dosing and rapidly declining concentrations thereafter (figure 2A, B, C and table 2). Cmax and area under the plasma concentration time curve (AUC0-24hr; total exposure) showed no accumulation of ISIS 388626 upon repeated administration at dose levels 100 mg. Some accumulation might be present at 200 mg as Cmax and AUC increased slightly from the 1st to the 13th dose (figure 2C, table 2).

The total amount of ISIS 388626 in urine (0-24 hours) ranged from 3.7 to 18.4 mg over the dose range tested and increased in a dose-proportional manner (figure 2D). Urinary excretion was approximately 2-fold higher after the 13th dose compared to the first dose.

SAFETY

Adverse events (AEs) occurred in all subjects who received multiple doses of ISIS 388626, and in 85% of subjects who received placebo (table 3). All AEs reported were classified as of mild intensity and transient. The most common AE was nasopharingitis. Other AEs commonly reported in all groups were headache, fatigue, a range of gastrointestinal complaints (such as diarrhea, nausea or abdominal discomfort) and musculoskeletal complaints (such as myalgia and back pain). These AEs occurred in the active treatment groups and placebo groups with a similar incidence, thus considered unlikely to be ISIS 388626-related. Injection site reactions (ISRs) occurred at frequencies of 19% in the 100mg cohort and 8% in the 200mg cohort. ISRs consisted of mild erythema at the site of the SC injection without itch. In three subjects hyperpigmentation was reported after the initial erythema had resolved. In two subjects re-appearance of erythema occurred after initial resolution. The ISRs were not progressive, not accompanied by local lymphadenopathy, and no study discontinuations occurred due to ISRs. All ISRs resolved completely and spontaneously, ranging from within 12 hours to 50 days.

ISIS 388626 treatment did not result in any clinically relevant changes in vital signs (blood pressure, ECG-derived parameters, body temperature) or parameters of hematology and coagulation. No increases in circulating cytokines (IFNα, IL6, MCP-1 and MIP1α) were observed at any dose level. Analysis of complement factor C5a and Bb revealed no changes, except for an increase in factor Bb in the 200mg dose group, maximally at 24 hours after the 13th dose (from 0.74 ± 0.336 mg/mL at baseline to 0.93 ± 0.394 mg/mL at 24hrs after dose of week 13).

Treatment with 100 and 200mg ISIS 388626 did not result in clinically significant changes in chemistry parameters, such as hepatic parameters and glucose levels. However, ISIS 388626 dose-dependent changes in serum creatinine occurred (figure 3A). Baseline serum creatinine levels were comparable between all treatment groups. The largest average increase of 0.38 ± 0.089 mg/dL (44% increase) was reached after 8 doses of ISIS 388626 (200 mg). The effect of ISIS 388626 on serum creatinine was highly variable between subjects ranging individually from no increase at all to a 0.49 mg/dL increase over baseline levels. Despite continued dosing, creatinine levels started to decline after ten doses, returning to baseline during the follow up period. However, at the 200 mg dose average creatinine levels remained above baseline levels at week 17. Three subjects met the protocol-specified stopping rule for renal function tests and were discontinued. The elevation in serum creatinine did not coincide with obvious changes in urea levels. At the highest dose level tested ISIS 388626 induced a short transient increase in aldosterone levels, although the inter- and intra-subject variability was significant (figure 3B).

ISIS 388626 treatment did not result in significant changes in urine flow and urinalysis parameters (data not shown), but did increase the levels of urinary renal damage markers (B2M, total protein, KIM1, aGST, NAG). ISIS 388626 treatment dose-dependently increased urinary B2M (figure 3C). In both the 100 and 200 mg group, B2M levels increased gradually with repeated ISIS 388626 administration, to decline after treatment stop during the five week follow-up period. Urinary protein also showed a dose-dependent increase, although the inter- and intra-subject variability was large (figure 3D). ISIS 388626 treatment, at all dose levels tested, resulted in an increase in urinary KIM1, assessed after the 8th administration (figure 3E). Also urinary levels of aGST and NAG increased upon ISIS 388626 treatment (figure 3F,G).

Discussion

The antisense oligonucleotide ISIS 388626, targeting the renal SGLT2 receptor, did not induce pharmacological effects (i.e. induction of glucosuria) when applying dose levels up to 50 mg, with weekly dosing for 12 weeks. This was not unexpected since in preclinical studies across multiple species, the pharmacologically active doses were doses exceeding 1 mg/kg/week, which translates into 70 mg/kg/week assuming an average weight of 70 kg [1;7;8]. Repeated ISIS 388626 administration with 50 mg did induce transient and mild rises in serum creatinine. To explore whether the observed transient increases in renal markers coincided with functional changes, a new cohort of volunteers exposed to the same ISIS 388626 dose level and regimen, and PAH and sinistrine clearance was assessed to estimate RPF and GFR. This study revealed no indications that the changes can be explained by ISIS 388626-induced changes in GFR and RPF (chapter 3 of this thesis, *L. van Meer et al.)*. This enabled dose escalation to elicit possible pharmacological effects.

ISIS 388626 dose-dependently increased urinary glucose excretion. After 13 weekly doses urinary glucose increased, which ceased after therapy cessation. On average, the maximal level of urinary glucose excretion was 1.4 gram per day, observed in the 200 mg ISIS 388626 treatment group after 13 weekly doses, compared to 0.2 gram in the placebo group (7-fold increase). The observations suggest an ISIS 388626-induced increase in glucosuria, but the effect is rather small, since the average amount of glucose that is filtered daily is approximately 144 grams (800 mmol/day [11]). Furthermore, compared to treatment with small molecule SGLT2 inhibitors the observed level of urinary glucose excretion is minimal, as these compounds induce urinary glucose excretion in the range of 50-80 grams per day [12]. In spite of the relatively small effect observed, the findings do suggest that ISIS 388626 doses of 200 mg and beyond exert intended pharmacodynamic activity. Animal data demonstrated that a certain threshold level of SGLT2 MRNA exists maintaining tubular glucose reabsorption and limiting glucosuria, and that once this threshold is exceeded, glucose reabsorption strongly declines resulting in sudden increases in glucosuria: an ISIS 388626-mediated SGLT2 MRNA reduction of nearly 70% did not induce a significant increase in glucosuria, whereas an SGLT2 MRNA reduction of 85% resulted in a 100-fold increase in glucosuria (data on file; monkey treated with 2 mg/kg/week versus 24 mg/kg/week ISIS 388626). This is supported by the observation that a phenotype of hereditary SGLT2 mutations with 50-60% loss of SGLT2 does not display significant glucosuria [13]. Compensation by an increase in SGLT1 mediated transport is likely to play a role in the maintenance of renal glucose reabsorption, as was demonstrated in animals after genetic and pharmacological SGLT2 inhibition [14].

In line with the mild glucosuria observed in 24 hour urine collections, urinary glucose excretion after the OGTT also increased in the ISIS 388626 treated groups. Furthermore, ISIS 388626-treatment resulted in elevated levels of circulating insulin and c-peptide during the first hours after the glucose load. These increases seem counter-intuitive, as increased urinary glucose loss is expected to result in lower insulin levels needed to compensate for the glucose load. Probably changes in other mechanisms involved in glucose homeostasis occur simultaneously. Treatment with SGLT2 inhibitors dapagliflozin and empagliflozin resulted in similar paradoxical findings of altered glucose homeostasis and increased c-peptide/insulin levels, probably compensating an increased endogenous glucose production [15-18].

Although the observed glucosuria after ISIS 388626 treatment may result from antisense mediated inhibition of SGLT2 in humans, it may also relate to ISIS 388626-induced tubular dysfunction. At the highest dose level, ISIS 388626 treatment resulted in increases in serum creatinine levels of 40-50% over baseline, with concomitant induction of other markers indicative of renal damage or dysfunction. The correlation between induction of renal damage markers and glucosuria was explored (table 4), which demonstrated a correlation between the renal markers serum creatinine, urinary B2M and urinary protein (with coefficients of 0.46, 0.47 and 0.66, table 4), but no correlation with urinary glucose excretion (with coefficients of 0.086, 0.43 and 0.23 table 4). The absence of a correlation between renal side effects and intended pharmacodynamic effect suggests that the observed mild glucosuria probably results from antisense mediated inhibition of SGLT2, and not from tubular dysfunction. Moreover, it makes it unlikely that a general membrane dysfunction occurs as a result of knock-down of the SGLT2 receptor. This is further supported by the absence of signs of membrane dysfunction with other oligonucleotides directed to transmembrane receptors [19;20]. ISIS 388626 is, however, the first antisense oligonucleotide targeted to a renal receptor, and it remains uncertain if knock down interferes with

membrane function. On the other hand, subjects with homozygous SGLT2 mutations are largely asymptomatic and have no signs of renal tubular dysfunction, hypovolemia or electrolyte imbalance [21].

We hypothesize that the changes in serum creatinine and urinary renal markers induced by ISIS 388626 treatment most likely reflect transient tubular dysfunction. Although urinary B2M and protein may also increase in case of glomerular injury due to increased filtration [22], the reversible nature of the changes in our study suggest interference with tubular reabsorption. This is supported by the observation that elevations in KIM1, AGST and NAG occurred, all markers known to increase in response to different tubulo-toxic agents in animal models [22-26]. Accumulation of antisense oligonucleotides occurs in proximal tubular cells as basophilic granules [27;28] and this was indeed also seen in animal studies with ISIS 388626 [1]. In primates, tubular accumulation of oligonucleotides is usually not associated with renal toxicity and tubular functional changes, unless extremely high doses are used [1;27;28]. In accordance, these compounds are not associated with adverse renal effects in humans during subsequent clinical investigations [19;29]. However, it is of note that the 12-mer chemistry of ISIS 388626 enables more selective distribution to the kidney compared to other 18- to 20-mer second generation oligonucleotides [8], resulting from lower plasma protein binding, higher free fraction and increased renal filtration. It is uncertain if this increased selectivity contributes to the observed adverse renal effects, but it should be taken in account that this is a theoretical possibility. No comparable other 12-mer oligonucleotides have been investigated in humans to date. Despite the observed increases in renal markers, no functional loss occurred at weekly doses of 50 mg demonstrated in the dedicated cohort with renal clearance tests (chapter 3 of this thesis, *L. van Meer et al.)* supporting the hypothesis that the observed changes reflect adaptation and possibly regeneration of tubular cells. Also no trend was detected in regression analysis exploring the relation between values of GFR/RPF and renal damage markers (data not shown). Examples of other drugs affecting tubular creatinine secretion without decreasing glomerular filtration rate include cimetidine and pyrimethamine [30]. Finally, the observation that the effect of ISIS 366828 on serum creatinine diminishes after ten doses, despite continued dosing, supports the likelihood that antisense treatment is associated with a process of tubular adaptation.

Treatment with small molecule SGLT2 inhibitors also increases serum creatinine [31;32]. However, these effects were smaller and considered to be secondary to volume depletion due to osmotic diuresis. These mild effects on serum creatinine coincided with a much larger pharmacological effect, therefore this does not provide sufficient explanation for our findings. Renal side effects are commonly observed for clinically tested oligonucleotide compounds, such as PRO051 (developed for Duchenne Muscular Dystrophy, and associated with proteinuria [33]), LY2181308, (developed for treatment of melanoma and caused a case of reversible kidney damage [34]) and SPC5001 (developed for familial hypercholesterolemia, associated with increased renal markers and a case of acute tubular necrosis [35]). Although these observations suggest an unintended class effect of oligonucleotides, several other antisense compounds with chemical characteristics comparable to ISIS 388626 were free of renal side effects in humans, such as mipomersen [36], ISIS 325568 [19], ISIS 2302 and ISIS 104838 [9]. Recently, it has been shown in monkeys that chronic administration of a compound similar in class, drisapersen, results in C3 glomerulopathy most probably due to an immune-based mechanism [37]. This appears to be consistent with the observation that antisense oligonucleotides may activate the alternative pathway of the complement system via transient inhibition of factor H [38].

Conclusion

Taken together, our data suggest that ISIS 388626 exerts its intended pharmacological effect in humans: treatment for a period of 12 weeks at a dose level of weekly 200 mg resulted in a small but significant elevation in urinary glucose. Concomitantly, changes in serum and urinary markers were indicative of transient renal dysfunction, most likely of tubular origin. Theoretically, the ISIS 388626-induced tubular dysfunction may have contributed to the glucosuria, but as the increases in renal markers do not correlate with the glucosuria, the latter is more likely an effect of oligonucleotide-induced SGLT2 inhibition. The pharmacodynamic effect of ISIS 388626 in type 2 diabetes mellitus patients, with elevated SGLT2 expression and a larger renal glucose load, may outweigh the effect observed in healthy volunteers. However, the efficacy is probably insufficient with the dose levels currently tested, considering the glucosuria

observed with existing small molecule SGLT2 inhibitors. Moreover, the mechanisms underlying the transient renal dysfunction warrant more detailed exploration. In the first place, because the aimed patient population, being subjects with diabetes type 2 are known to be particularly prone to renal injury and chronic kidney disease may develop as a result. And secondly, as we know renal effects are also observed for other oligonucleotides, this might be prohibitive of using this promising drug class for chronic conditions that are not directly life threatening.

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Figure 1 Dose-dependent pharmacodynamic effects of ISIS 3886262. Increased urinary glucose excretion measured in 24-hour urine collections (A). Increased OGTT insulin (B) an c-peptide. The change after the 13th dose in weighted AUC 0-4hour (C) Response, calculated from the change in weighted AUC 0-4hour. Increased glucose excretion during the 4 hour-OGTT, calculated as absolute change from baseline (D). All values expressed as average with SD error bars. P values (tested to placebo). Calculated as absolute change from baseline after the 13th dose. * = < 0.05, * = < 0.001**

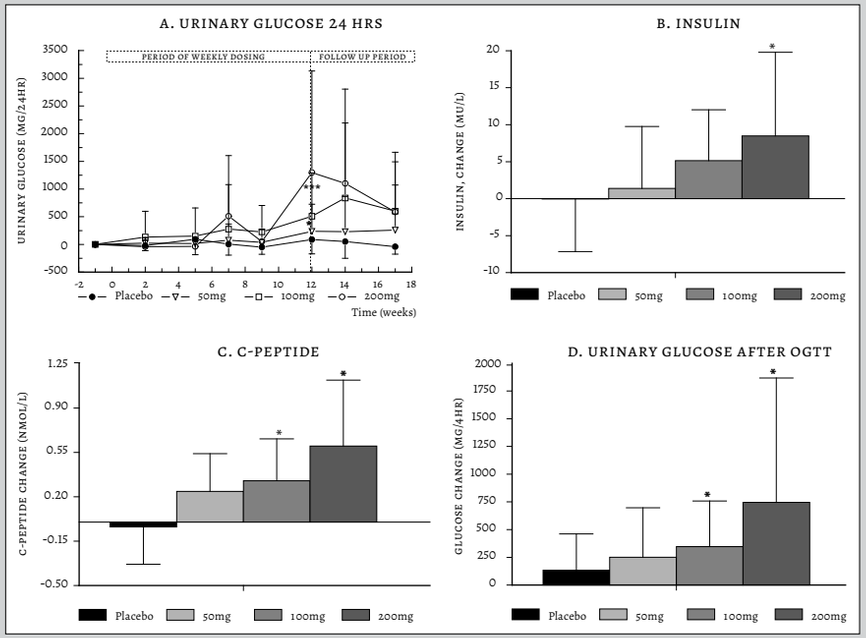


Figure 2 Pharmacokinetic properties of ISIS 388626. The grey line represents the values measured after the 1st dose, the black line after the 13th dose. Average values with SD error bars. Rapid absorption upon injection (Cmax within 2 hours) and rapidly declining concentrations thereafter at all dose levels (A, B and C). No accumulation at dose levels of 50mg and 100mg, possible accumulation for 200mg, with an increased AUC after 13th dose. 24hr excretion of ISIS 388626 in urine increased dose-proportionally, with an increased excretion after the 13th dose compared to the 1st dose.

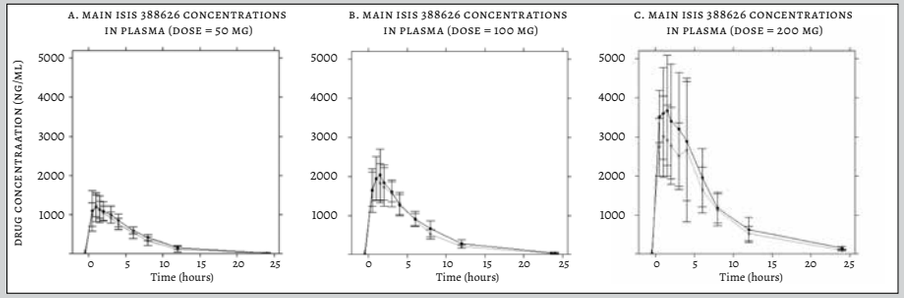
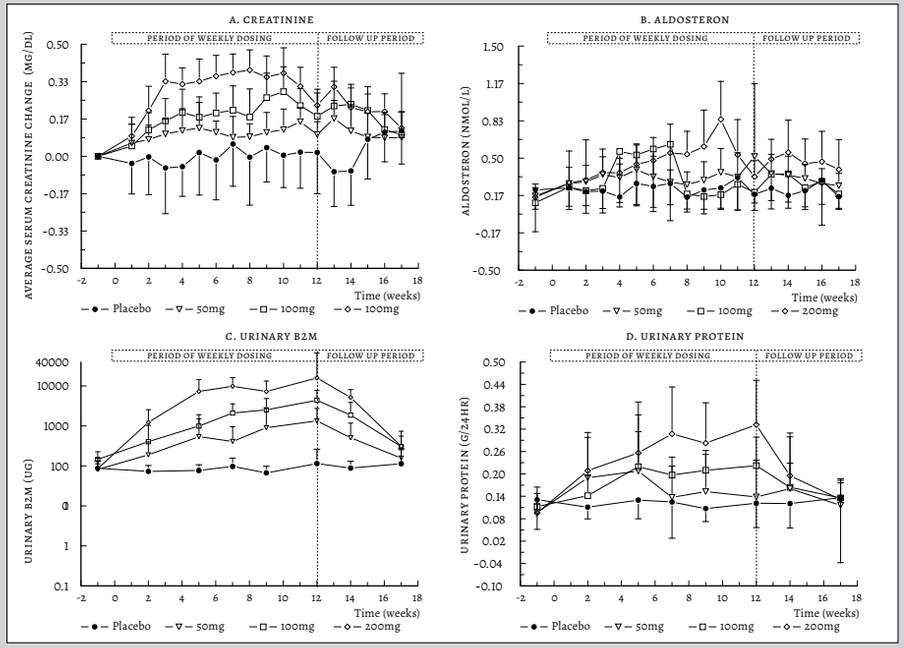


Figure 3 Dose-dependent effects of ISIS 388626 on renal markers and recovery during the follow up period Average values with SD error bars. Logarithmic Y-axis used for B2M. Serum creatinine increases at all dose levels over time, expressed as change from baseline (A). Increases absolute serum aldosteron for dose levels of 100 and 200mg (B). Increases in urinary B2M (C) and urinary protein (D) over time at all dose levels. Increases in urinary KIM1, expressed as change after the 8th dose compared to baseline (E). Increases in urinary AGST (F) and urinary NAG (G) over time. Increases visible at a dose level of 200mg.



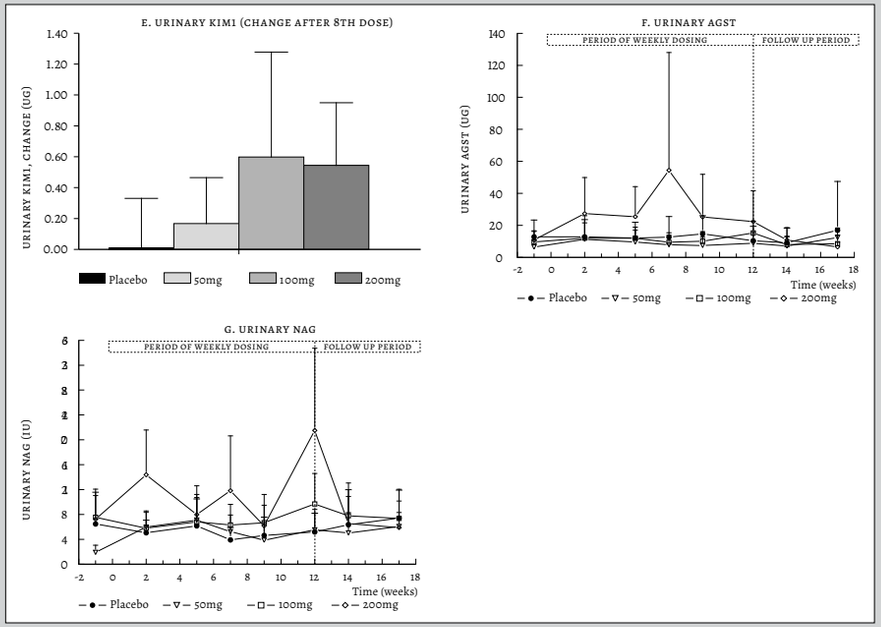


Table 1 Summary of subject demographics

	N	Age (yrs, Std)	BMI (Kg/m2, Std)	Number female	Early termination of subjects
50 mg ISIS 388626	12	33.9 ± 14.23	23.5 ± 3.13	0	2*
100 mg ISIS 388626	16	37.5 ± *16.18*	23.7 ± 3.49	0	5**
200 mg ISIS 388626	12	27.2 ± 12.27	23.7 ± 2.82	1	2***
Pooled Placebo	13	36.1 ± 16.18	23.8 ± 2.87	1	1****

in one subject dosing was stopped after 5 doses due to increases in serum creatinine and one subject was stopped after 7 doses due to increased liver biochemistry parameters. ** Stopped due to personal reasons (after 1, 2, 4, 5 and 12 doses). Therefore subjects were replaced. ***Stopped (after 7 and 10 doses) due to increases in renal parameters. ** Stopped after 1 dose due to personal reasons. Therefore subject was replaced.*

Table 2 **Plasma pharmacokinetics after first and 13th dose**

	50 mg		100 mg		200 mg	
After dose no	1	13	1	13	1	13
N	12	10	16	12	12	9
AUC 0-24hr (ug*hr/mL)	7627 ± 1202	8626 ± 1037	12650 ± 2085	13970 ± 2041	24250 ± 8796	28320 ± 9875
Cmax (ug/mL)	1240 ± 274.4	1275 ± 470.6	2048 ± 487.5	2185 ± 558.2	3333 ± 1602	4155 ± 1266
Tmax (hr)	1.17 ± 0.324	1.40 ± 0.566	1.38 ± 0.97	1.50 ± 0.88	1.63 ± 1.11	1.56 ± 1.37

Table 3 **Frequency overview of adverse events reported in more than one subject (%)**

	50mg (n=12)	100mg (n=16)	200mg (n=12)	Placebo (n=13)
Nasopharyngitis	42% (n=5)	38% (n=6)	67% (n=8)	46% (n=6)
Headache	33% (n=4)	56% (n=9)	33% (n=4)	38% (n=5)
Mild gastrointestinal complaints	58% (n=7)	31% (n=5)	42% (n=5)	38% (n=5)
Mild musculoskeletal complaints	17% (n=2)	25% (n=4)	33% (n=4)	38% (n=5)
Fatigue	33% (n=4)	6% (n=1)	8% (n=1)	23% (n=3)
ISRS	42% (n=5)	19% (n=3)	8% (n=1)	0% (n=0)

Table 4 **Correlation coefficients of the exploratory analysis of correlations between serum creatinine (s.creat), urinary B2M (u.B2M), urinary protein (u.prot) and urinary glucose (u.gluc), calculated with change from baseline values (CFB)**

	CFB s.creat	CFB u.B2M	CFB u.prot
CFB s.creat	X	0.46**	0.47**
CFB u.B2M	0.46**	X	0.66**
CFB u.prot	0.47**	0.66**	X
CFB u.gluc	0.086	0.43	0.23

*** indicates a positive correlation that is considered significant.*

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V

URINARY KIDNEY BIOMARKERS FOR EARLY DETECTION OF NEPHROTOXICITY IN CLINICAL DRUG DEVELOPMENT

Leonie van Meer, Matthijs Moerland, Adam Cohen, Jacobus Burggraaf

Abstract

Early detection of drug-induced kidney injury is vital in drug development. Generally accepted biomarkers such as creatinine and BUN lack sensitivity and early injury responses are missed. Many new biomarkers to detect nephrotoxicity for pre-clinical utilization have been described and their use is adopted in regulatory guidelines. However, guidance on appropriate biomarkers for clinical trials is minimal. We provide an overview of potentially useful kidney biomarkers that can be used in clinical trials. This includes guidance to select biomarkers suitable to capture specific characteristics of the (expected) kidney injury. We conclude that measurement of urinary Kidney Injury Marker-1 (KIM1) serves many purposes and is often an appropriate choice. Cystatine C captures effects on glomerular filtration rate, but this marker should preferably be combined with more specific markers to localize the origin of the observed effect. Untoward effects on tubules can be captured relatively well with several markers. Direct detection of glomerular injury is currently impossible since specific biomarkers are lacking. Indirect assessment of toxic effects on glomeruli is possible by using carefully selected panels of other injury markers. We conclude that it is possible to obtain appropriate information on nephrotoxicity in clinical drug development by using carefully selected panels of injury markers and suggest that identification and validation of specific glomerular biomarkers could be of great value.

Introduction

As illustrated by the case, described on the right hand side, early detection of drug-induced nephrotoxicity and prevention of clinical manifestations such as tubular necrosis are vital in early drug development in humans. The problem of drug-related renal toxicity is important as renal drug toxicities in animal studies account for more than 30 percent of the attrition of compounds from drug development [2]. Despite this pre-selection, prevalence of (acute) kidney injury due to drug toxicity in clinical practice is as high as 18 - 27% of all episodes of acute kidney injury [3]. In addition, efficacious interventions to reverse kidney damage are non-existing and clinicians can only apply supportive therapies while awaiting recovery of renal function. Animal models have shown that intervention

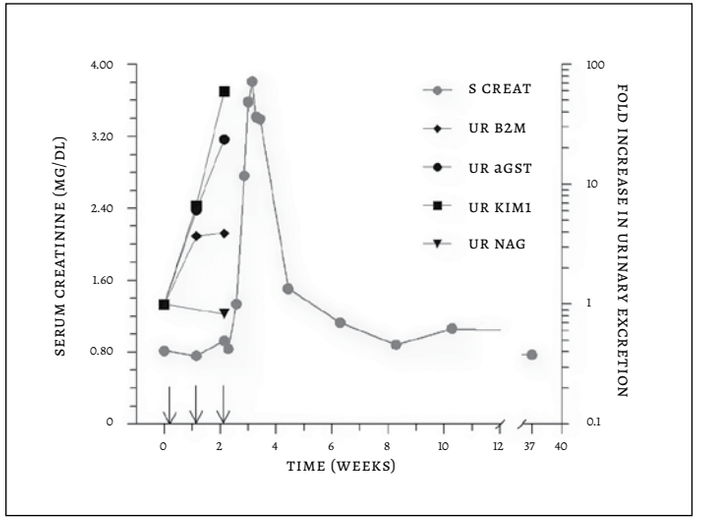

Figure 1 Time course of serum creatinine and urinary kidney damage markers. Arrows denote drug administration on study days 1, 8 and 15.

CASE

Administration of a low dose locked nucleic acid antisense oligonucleotide caused toxic acute tubular necrosis in a 56 year old otherwise healthy female volunteer. She received 3 weekly subcutaneous doses of experimental drug SPC5001, a PCSK9 inhibiting antisense oligonucleotide (developed to lower LDL-cholesterol). Five days after the last dose serum creatinine reached 2.67 mg/dL (a 238% change from baseline). Urine microscopy revealed red blood cells, white blood cells, and granular casts. Renal biopsy showed multifocal tubular necrosis. Kidney biomarkers were measured retrospectively and were already increased after the first dose, clearly preceding changes in serum creatinine, urea, dipstick, or urine microscopy (see figure 1). Serum creatinine peaked 1 week after the last antisense dose and was 3.81 mg/dL at that time. Upon supportive treatment, serum creatinine decreased gradually. Baseline levels were observed from 44 days after last antisense administration onwards. She recovered completely without any persisting abnormalities. Which biomarkers for kidney injury would have prevented this?

Van Poelgeest et al. Acute tubular necrosis during PCSK9 antisense oligonucleotide therapy.[1]

directly after early changes have been noted is preferable as the window of opportunity to apply treatment appears to be limited to a few hours [4]. Unfortunately, signals of early injury responses and the underlying mechanism of damage are often missed by traditional methods for monitoring renal function, such as serum creatinine, serum BUN, estimated GFR and actual GFR. Serum creatinine and BUN are easy to obtain and the assays are part of standard care measurements rendering the results readily available. However, as markers for kidney injury, both have several shortcomings. Creatinine concentration in serum depends on multiple other factors than decline in renal function that differ intra- and inter-individually, such as age, gender, muscle mass, protein intake, and certain drugs [4;5]. Also, BUN concentrations are influenced variable factors, such as diet, dehydration, liver function and tissue breakdown [6]. This lack of specificity complicates the interpretation of these markers. The problems can be partially overcome by calculating Glomerular Filtration Rate (GFR). Based upon data of large groups of patients different equations have been derived to estimate the actual (eGFR) using the serum creatinine value [7]. The eGFR is commonly used in clinical practice as an overall index for kidney function [8]. The Cockcroft-Gault formula, described in 1976 is based on a cohort of 249 patients and takes into account the factors age, weight and gender [9]. The eGFR Modification of Diet in Renal Disease (MDRD) was derived from a study with renal disease patients and includes age, race, gender, serum BUN and serum albumin as input parameters [10]. In addition, the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) was derived from two study cohorts, one consisting of patients with known renal disease (mGFR < 90 mL/min), and a second cohort made up of kidney transplant donors all of whom had an mGFR > 90 mL/min [11]. The CDK-EPI formula includes the factors gender, age and race. Thus all formulas correct for certain variables and offer a tool to estimate GFR and monitor kidney function in chronic kidney disease patients. However, they still lack accuracy [12;13] and miss early and relatively small changes in GFR [14]. This is particularly true when monitoring healthy subjects with normal renal function and normal baseline serum creatinine values. In this situation very small changes in plasma creatinine, which are in the noise of the assay, may still reflect considerable loss of renal function, as illustrated in figure 2. Another disadvantage of serum creatinine (and eGFRs derived from this marker) and BUN is that a considerable time delay exist between the onset of kidney injury and the moment that the signal is observed. For creatinine this is for example illustrated in figure 1. BUN levels correlate closely with histopathological changes in the kidney [15], however, the signal is delayed; by the time the damage is revealed by this marker 70-80% of renal epithelial mass is already lost [16].

It has been advocated to not estimate but measure GFR for a more reliable assessment of kidney function. This can be achieved by measuring clearance after intravenous administration of exogenous markers that are solely removed by glomerular filtration. This approach also avoids GFR estimates influenced by extra-glomerular clearance such as tubular secretion. Different markers are used among which inulin [17] and radio isotopic agents such as 99mTc-diethylenetriaminepentaacetic acid (DPTA), 169Yb-DTPA, 125I-iothalamate and 51Cr-ethylenediaminetetraacetic acid (51Cr-EDTA). These markers have a high accuracy and can detect subtle changes in GFR [17;18]. These methods are time consuming and therefore the results may not be readily available [19;20]. More importantly, however, GFR is only one measure of kidney function while many different mechanism of drug induced kidney injury exist, such as reduction in renal perfusion, direct tubular toxicity, intratubular obstruction, allergic interstitial nephritis and hemolytic-uremic syndrome [21]. All these mechanisms will lead directly or indirectly to reduction in the GFR, for instance by tubuloglomerular feedback after tubular toxicity [22]. However, these changes in GFR may be detected too late for adaptations in dose or schedule of dosing, and do not provide information on the mechanism of toxicity. In this review we focus on measures that may provide earlier and more specific warning signals of renal damage than a decreased GFR.

In preclinical research detection of early signs of kidney injury is done by histological examination, which is considered to be the gold standard. Histopathology provides accurate anatomical information on the kidney injury as well as its severity and leads to a diagnosis. However, performing a kidney biopsy in humans is rarely an option and is an inappropriate tool to be used in drug development trials.

These difficulties regarding early detection of kidney injury may substantially influence the path of drug development. For instance, development of compounds for which renal toxicity is observed during preclinical experiments may be ceased in order to avoid safety issues during the clinical development. However, it is known that only 40-60% of animal findings are predictive of toxicities in humans [23;24]. This results

in the undesirable situation in which potentially efficacious compounds are abandoned for the wrong reason. Moreover, as these compounds do not reach the clinical phase, no insights are gained regarding the (potentially avoidable) nephrotoxic mechanisms of injury. The development of immortalized human proximal tubule cell lines expressing functional influx and efflux transporters [25], which can be challenged with toxins [26] appears to be a promising approach that can be used preclinically. However, it is not yet confirmed that these cell lines and the subsequent incubations sufficiently mimic the in vivo situation. Therefore translating findings from these cell-lines directly to the clinical situation seems premature. Adequate use of renal biomarkers could thus be of great value during early drug development.

For preclinical assessment, many new potential biomarkers of toxicity have been identified and their possible benefit has been evaluated by comparing their performance to the traditional markers. This extensive research has resulted in an EMEA/FDA guideline (published in 2009), that identified acceptable biomarkers that can be used to detect drug-induced nephrotoxicity in preclinical research [27]. This panel includes the urinary excreted biomarkers Kidney Injury Molecule-1 (KIM1), albumin, total protein, Beta-2-microglobulin (B2M), clusterin, Trefoil Factor 3 (TFF3), and Cystatin C (CysC). These markers can be used to capture acute drug-induced nephrotoxicity of tubular or glomerular (with associated tubular involvement) origin [27]. These markers were shown to provide additional and complementary information to BUN and serum creatinine and correlate with histopathological alterations. However, it was recognized that for the clinical setting these markers were insufficiently qualified to justify their general use. It was suggested to further explore their potential as clinical biomarkers for acute drug-induced kidney injury and recovery/reversibility. The guideline also advised to consider the biomarkers in clinical trials on a case-by-case basis to gather further data on their usefulness to monitor drug-induced renal toxicity in man.

With this review, we aim to offer guidance to select the biomarkers that suit the specific characteristics of the (expected) kidney injury. We provide an overview of promising biomarkers for nephrotoxicity, focusing on their possible use and limitations in clinical trials during early drug development. Certain properties of biomarkers of kidney injury are considered to be critical. First, the origin of the biomarker and the mechanism and/or site of injury should be clarified as much as possible.

Furthermore the biomarker should be sensitive to early injury in order to outperform the traditional biomarkers and enable early intervention. High specificity and correlation with established outcome measures such as histopathology are also vital to avoid confusion on the value of the observed signal. As the majority of data on kidney injury biomarkers originates from preclinical research, the translational step to humans is of great importance. This could be achieved if supportive clinical evidence on biomarker profiles reflecting kidney injury in humans is available. Finally, reliable assays must be readily available.

The biomarkers were selected using the previously mentioned EMEA/FDA guidelines on preclinical markers, and on the condition that the pertaining assay is validated for human use and commercially available. The selection was expanded with the biomarkers for which promising data have been reported. These markers are Neutrophil Gelatinase-associated Lipocalin (NGAL), alpha Glutathione S-transferase (aGST), N-acetyl-beta-glucosaminidase (NAG) and Interleukin-18 (IL18). Established markers such as serum creatinine, serum BUN, urinary albumin and protein were not considered in detail, although these are used as reference. Based on the literature, several considerations and recommendations regarding selection of kidney biomarkers in clinical drug development are given.

Summary on selected markers

Cystatin C (CysC) is a small molecule cysteine proteinase inhibitor synthesized by all nucleated cells and filtered freely by the glomerulus. After filtration it is not secreted nor reabsorbed by the tubules, but catabolized completely and thus reflects true GFR when measured in blood. Preclinically, CysC appears to be the most sensitive marker for early proximal tubular damage in animals, although a consistent dose response relation is lacking [28]. CysC is suitable to assess kidney function in general, regardless of specific lesion site, as this marker is devoid of extra-glomerular clearance, variability in production and limited sensitivity that apply for BUN and serum creatinine [29]. It has been suggested that CysC measured in blood could be a suitable translational biomarker as it avoids laborious urine collection in animals [29]. Possible disadvantages of CysC are its dependency on factors other than decline of renal function alone, such as age, gender, weight and height, smoking, and

high serum C-reactive protein levels [5]. In the clinic, CysC has been shown to be a sensitive marker of early renal dysfunction following ischemic injury [30].

Neutrophil Gelatinase-associated Lipocalin (NGAL) is an acute-phase protein secreted as a response to acute injury of proximal and distal tubular epithelial cells. It is freely filtered by the glomerulus after which rapid clearance occurs via receptor binding and endocytosis[4]. NGAL has been reported to be the most sensitive marker for proximal tubular damage in the preclinical setting [28]. After gentamicin exposure in rats a clear signal is detected as early as 24 hours after exposure. However, specificity to the location of injury must be questioned, since in a rodent glomerular damage model increased NGAL levels were also observed [31]. Clinical research has demonstrated that urinary NGAL is increased in several forms of chronic kidney injury [32;33] and in patients with urinary tract infections [34].

Interleukin-18 (IL18) is a proinflammatory cytokine produced by leukocytes and renal parenchymal cells such as tubular epithelial cells, podocytes and mesangial cells. It plays an important role in the exacerbation of acute tubular necrosis [35] and the inflammatory pathways involved are partly clarified [36]. The IL18 receptor (IL-18R) is expressed on these cells in cisplatin-induced acute kidney injury and urinary IL18 excretion has proven to be an early diagnostic marker for acute kidney injury in humans, particularly in critically ill patients [37]. However, at present it is unclear if IL18 reflects location-specific injury and therefore its potential for preclinical and clinical use in drug development is unclear. An obvious disadvantage is that increased IL18 levels can also be observed upon many forms of inflammation not limited to the kidney.

N-acetyl-beta-glucosaminidase (NAG) is a lysosomal enzyme which is contained abundantly in the renal tubular epithelia and involved in the degradation of mucopolysaccharides and glycoproteins. Its size precludes glomerular filtration and elevated urinary concentrations are considered to reflect tubular dysfunction. In preclinical research the sensitivity of NAG is higher compared to serum creatinine and comparable to BUN [38]. The NAG response profile is dependent on the toxin causing proximal tubule injury [39]. For example, gentamicin triggers an early response that last for 8 hours after dosing, whereas chromium triggers a response after 8 hours and mercury does not trigger a significant NAG increase at all [39]. Nevertheless, clinical evidence supports the usefulness of NAG as an early marker of mild tubular injury [40] and demonstrates that it has predictive properties regarding the development of acute tubular necrosis [41].

Alpha Glutathione S-transferase (aGST) is a detoxification enzyme that is produced in numerous tissues. Urinary aGST levels are very low under physiological conditions, but substantial amounts are excreted in case of various manifestations of tubular injury, including cisplatin- [42] and gentamicin-induced [43] toxicity and in acute tubular necrosis after mercuric chloride and potassium dichromate exposure [44]. aGST appears to be an adequate preclinical early toxicity biomarker to detect onset of epithelial necrosis, but is less suitable to monitor reversibility [29]. In drug-induced injury of proximal tubular cells with cisplatin and gentamicin, aGST correlated more closely to histopathological confirmed injury compared to NAG, BUN and serum creatinine [45]. In a preclinical model of tubular injury limited to the pars recta of proximal tubule cells, it was demonstrated that aGST-excretion reflects injury of low grade toxicity, outperforming numerous other markers, and with equal sensitivity as KIM1 [46]. However, injury to the collecting duct was associated with a decreased aGST, which is not well understood yet. As a consequence it has been suggested that qualification of this biomarker has to await further results [45]. Although limited information is available, clinical evidence suggests that aGST is informative for tubular dysfunction or injury [47;48].

Kidney Injury Molecule-1 (KIM1) is a transmembrane protein expressed by proximal tubular epithelial cells. KIM1 functions as a phosphatidylserine receptor and has phagocytic capacity [28]. Expression is markedly upregulated in response to injury [49]. Urinary KIM1 concentration provides a more sensitive predictor of histopathological confirmed injury in 11 well-established rat models of acute kidney injury when compared to BUN, serum creatinine or urinary NAG, even in cases of low grade toxicity [38]. One study reported that urinary KIM1 levels also correlate with different grades of kidney tubular histopathologies. This was supported by the finding of dose-dependent upregulation of the KIM1 gene in segment-specific toxicity models [50]. Whereas aGST appears to be a good early toxicity biomarker for epithelial necrosis, KIM1 and clusterin levels persist during regeneration and appear to reflect the triggering and continuation of the repair process [29;51]. KIM1 responses seem to depend on toxin, for example Sasaki et al. [52] report that after cisplatin exposure KIM1 increases (together with clusterin and aGST) after 3 days

(confirmed by Vinken et al.[15]), whereas in a model for papillary necrosis using 2-bromoethylamin hydrobromide KIM1 levels (together with clusterin, albumin and osteopontin) are convincingly increased as early as day 1 after exposure. Interestingly, measurement of urinary KIM1 also enables detection of subchronic and chronic injury and correlated closely with histopathology [28]. This study also showed that CysC and NGAL are the most sensitive markers for early kidney damage of proximal tubular damage, but subchronic or chronic injury was best reflected by KIM1 levels. In keeping with the notion that urinary KIM1 may be an early marker for chronic nephrotoxicity in animals, increased levels of urinary KIM1 levels were reported in an experiment using cadmium [53]. Clinical evidence that the results in animals translate to humans is currently limited. KIM1 did indeed show a significant signal following acute kidney injury, although sampling for KIM1 in this study was rather late, precluding assessment of its suitability as early marker [54].

Beta-2-microglobulin (B2M) is produced by all cells expressing major histocompatibility complex (MHC) class I antigen. Under normal conditions the main source are activated lymphocytes from which shedding from cell surface of the MHC occurs through proteolysis. Synthesis is stimulated in various conditions characterized by proliferation of lymphoid cells that occurs in various disease states, such as neoplasms, (auto-) immune disorders or infections [55-57]. B2M is filtered freely across the glomerulus and complete reabsorption occurs by proximal tubular cells [28]. Impaired tubular uptake results in increased B2M urinary excretion. Glomerular protein loss may also increase urinary B2M excretion as B2M shares a common rate-limited tubular reabsorption pathway with other proteins. In the preclinical setting B2M has a better diagnostic performance than BUN and serum creatinine to detect glomerular injury (together with urinary total protein and CysC) [58]. As tubular dysfunction without glomerular impairment also increases B2M [59;60] specificity regarding the location of damage using B2M alone is questionable. Interestingly, B2M might be excreted via other pathways as compared to other markers, as can be concluded from the findings on a model for papillary necrosis. This massive injury gave rise to impressive increases in KIM1, clusterin, albumin and osteopontin, but levels of B2M remained close to normal [52]. In the clinical setting, B2M has proven to be a marker for disease severity in autosomal polycystic kidney disease [61] and for renal damage by fumaric acid esters [62].

Clusterin is a glycosated protein associated with apoptosis and clearance of cellular debris and can be found in several tissues. Within kidney cells, clusterin has been suggested to possess anti-apoptotic properties and facilitates cell protection, lipid recycling and cell attachment, and aggregation [63]. Clusterin cannot be filtered by glomeruli due to its size and therefore urinary levels are specific for kidney injury. Clusterin performed better to detect proximal tubular injury than CysC, B2M and total protein [58] and evidence suggests it can be used as an early marker with a profile similar to KIM1 [15;52]. The clusterin response correlates well with tubular injury regardless of the location, particularly when regeneration is present [45]. Elevated clusterin levels persist during regeneration and appear to reflect the triggering and continuation of the repair process [29]. Clinical data on urinary clusterin in relation to kidney injury is not extensively available, however, it has been shown that clusterin expression is increased in renal injury and cystic diseases [64].

Trefoil factor 3 (TFF3) is a small peptide hormone secreted by mucus-producing and other epithelial cells [65]. In the kidney it is produced/secreted by cells of the collecting ducts [66]. TFF3 is involved in many functions including restoration of intestinal epithelium [67], but its physiological function within the kidney is still elusive. Because in ageing rats decreasing amount of kidney TFF3 are found, it has been suggested that TFF3 may have a general protective function [68]. Significantly decreased levels of TFF3 have been observed in different rat models of proximal tubular injury. Combining TFF3 with urinary albumin increases sensitivity to early injury compared to traditional markers [69]. However, studies comparing TFF3 to other novel biomarkers are lacking. In humans, it has been reported that certain populations (African descent, diabetes and antihypertensive medication use) have higher baseline urinary TFF3 levels, and that increase in urinary levels might indicate ongoing repair of chronic damage in the kidney [70].

Comparison & Considerations

Concerns regarding nephrotoxicity are often encountered during the development of novel drugs. Particularly when the compound is about to be tested in humans for the first time, all indications of possible kidney injury are weighed. This may regard compounds belonging to drug

classes that are notoriously associated with renal injury, drugs specifically targeted to the kidney, but also drugs that are considered 'suspicious' because of their mechanism of action or pharmacokinetic properties. Preclinical suggestions for nephrotoxicity can be present. In case of clearly dose-related adverse renal effects at the high end of the tested dose range, it is usually possible to estimate a safe range for dosing in humans provided that adequate monitoring is possible. However, it is more difficult if preclinical data point towards an incidentally occurring event. This might lead to a more variable risk for susceptible human subjects, which is difficult to catch. Whatever the findings in animals, close monitoring of the kidney in clinical trials to detect untoward effects as soon as possible is necessary in all cases.

Every drug has its specific features and other factors such as dosing schedule, cumulative dose, patient- and/or disease characteristics may play a role. Thus, each drug requires the selection of an appropriate biomarker or most likely a panel of biomarkers to provide comprehensive information. As subject safety is a primary goal in first into human trials, sensitivity for the event is crucial. A strategy for selecting biomarkers in humans could be to first focus on the localization of the site of injury and the mechanism by which the compound causes injury. This may be achieved by using histopathological information on the compound and comparing this to histopathological and biomarker profiles of known nephrotoxic agents that target similar sites (table 1).

The selection of a panel of biomarkers should obviously be based on the possibilities and limitations of individual markers and the aim should be to compose a panel of which the combination of biomarkers provides complimentary information (table 2).

We first point out that currently a specific marker for glomerular injury is unavailable and thus glomerular damage is frequently made by excluding other causes. Identification of a biomarker specific for glomerular injury would be of great value. CysC can be used to monitor general kidney function as it reflects GFR. Further, CycC and NGAL are the most sensitive markers for early kidney damage of proximal tubular damage [28]. However, it should be realized that the organ-specificity of CysC is poor and other biomarkers are recommended to differentiate between tubular and glomerular damage. The dependency of CysC levels on other factors than renal function is important in diagnostic work-ups, but not so much in clinical drug research as these circumstances can be taken into account by choice of population and by focusing on the time course and relative changes from baseline. If this is unfeasible or undesired, CysC appears to be a less suitable biomarker.

Although NGAL seems more tubular injury-specific compared to CysC, this has been challenged by the finding that NGAL also increases in a model of glomerular injury [31]. This could reflect generalized (glomerular and tubular) injury or that the biomarker is not injury-site specific. NGAL is not recommended as a monitoring tool, as it appears to possess equal sensitivity as serum BUN. aGST and KIM1 exhibit similar sensitivity to detect tubular damage to the pars recta of the proximal tubule [46]. Nevertheless, KIM1 is preferred as there is more evidence regarding its performance compared to other biomarkers [38]. Moreover, the close correlation of KIM1 to histopathology [38;50] and the finding that it can be used to also monitor reversibility [29] suggest KIM1 to be the biomarker of choice. Furthermore, KIM1 can be used to detect subchronic or chronic injury [28;71].The limited information available on TFF3 suggests that this biomarker does not have a clear advantage over other biomarkers for proximal tubular injury. We suggest to not use this marker unless more information is available on its profile and its performance compared to other biomarkers in animals and humans.

B2M is generally considered to be a glomerular marker, but increased urinary excretion could also reflect impaired reabsorption by proximal tubular cells. It appears that B2M as single measure does not have great value [72]. However, when included in a panel of biomarkers it allows distinguishing between glomerular and tubular dysfunction. The choice to incorporate clusterin depends largely on the expected toxicity. Clusterin is a sensitive marker and its urinary excretion increases as a response to tubular injury irrespectively of the site. This feature of non-specificity can be of value, when tubular injury to distal tubules is suspected or localization is unclear. It is advised to use clusterin only in a panel of biomarkers. The information on IL18 as suitable marker is too limited to justify its use in early clinical drug development. However, the finding that IL18 can be used to detect acute kidney injury very early in hospitalized patients [37] and its role in the pathophysiology of kidney injury [36] suggests that IL18 is potentially a useful marker but this should be explored further.

It is important to take into account that biomarkers might increase for other reasons than kidney injury (alone), which potentially results in

an inaccurate conclusion. For instance, increased levels of acute-phase proteins such as NGAL, but also B2M and IL18 may be observed upon any inflammatory condition regardless of the site of inflammation. Also the population that is studied is important as illustrated by the finding that patients with an impaired glucose tolerance show a higher urinary excretion of NAG [73]. These problems may be minimized by assessment of (changes in) renal function while taking all available information into account. In practice, it is advised to careful consider the population that is studied, to have good information on the biomarkers at baseline, and to always measure chemistry and hematology at similar time points as the panel of renal biomarkers. This comprehensive approach prevents erroneous attribution of findings to the kidney.

Recommendation

We conclude that measurement of urinary KIM1 suits many purposes and is therefore often an appropriate choice. It allows for early detection of proximal tubule injury, differentiation between glomerular and tubular damage and assessment of reversibility and regeneration provided this occurs. KIM1 can also be used to detect sub-chronic and chronic kidney injury. NGAL is useful if tubular involvement is suspected, but no clear notion of tubular localization is present. Clusterin may add value in case of suspected injury in the distal tubule. If general kidney function and thus a measure of clearance is required, it is useful to include CysC, preferably combined with more specific markers to localize the origin of the observed effect. IL18 is considered less suitable for early phase trials, but might be helpful in phase II/III trials to be able to detect ATN in a very early stage. It may be useful to include B2M and aGST in the panel of biomarkers as this enables to relate findings on new compounds to existing knowledge of known nephrotoxic agents.

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Figure 2 Theoretical relationship between serum creatinine and glomerular filtration rate (GFR) calculated with creatinine clearance for a subject with normal muscle mass (12 mmol creat/24 hours). In the range of creatinine for healthy subjects large declines in GFR are associated with relatively small increases in serum creatinine. For example, if serum creatinine increases from 60 to 90 mmol/l (black arrow), which is a 50% increase (a cut off value for safety often used in clinical trials), GFR decreases with 50 ml/min (grey arrow), which reflects a considerable functional loss of over 35%. When the GFR decreases below 60 mL/min, further decrements are associated with larger increases in serum creatinine, which makes the marker more sensitive for change.

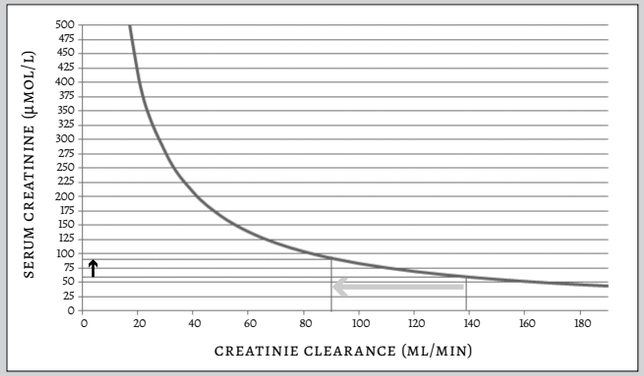
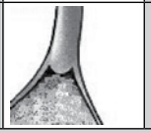


Table 1 Established nephrotoxic agents and accompanying biomarker signal

		Injury Models		Kidney Biomakers		
				Sensitive		Specific
Glomerulus		Puromycin aminonucleoside (PAN) Doxorubicin		B2M, CysC		
Proximal Convoluted Tubule s1		Gentamicin	Cisplatin, Carbapenem a		TFF3, CysC, NGAL, (IL-18)	NAG
Proximal Convoluted Tubule s2						
Proximal Straight Tubule s3		Vancomycin Hexachloro-1:3-butadiene (HCBD)		KIM-1, aGST		
Distal Convoluted TUBule		AmphotericIN B		clusterin, ngal	agst	
Collecting Duct						
Renal Papilla		2-bromoethylamin hydrobro-mide (BEA)		KIM-1, clusterin*		

** no biomarker identified to date covering this area*

Table 2 Aims of research and appropriate biomarkers

Aim:	Suitable marker
Monitoring general kidney function (GFR)	CysC
Differentiate between glomerular and tubular damage	KIM1, clusterin , sGST
Monitor toxicity with suspected glomerular localization	B2M
Monitor toxicity with suspected non-glomerular localization	CysC, NGAL,
Detect early kidney injury with suspected proximal tubular localization	aGST, KIM1
Monitor reversibility/regeneration with suspected proximal tubular localization	KIM1
Monitor toxicity with suspected distal tubular localization	Clusterin
Monitor reversibility/regeneration with suspected distal tubular localization	Clusterin
Elucidate pathophysiological mechanism with known proximal tubular injury site	NAG
High risk of ATN (Phase II/III trials)	IL-18

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VI

INJECTION SITE REACTIONS AFTER SUBCUTANEOUS OLIGONUCLEOTIDE THERAPY

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Abstract

Oligonucleotides (ONs) are short fragments of nucleic acids, currently being investigated as therapeutic agents. When administered subcutaneously (SC) ONs cause a specific local reaction originating around the injection site, such as erythema, itching, discomfort and pain, including more severe manifestations such as ulceration or necrosis. These injection site reactions (ISRs) are common, but rather poorly described in the literature. With this review, we aim to provide an overview on the extent of the problem of ISRs, based on reported incidence. A structured literature search was performed to identify reported incidence and clinical features of ISRs which yielded 70 manuscripts that contained information regarding ISRs. The data from literature was combined with data on file available at our institution. All SC administered ONs described in literature lead to the occurrence of ISRs. The percentage of trial subjects that developed ISRs differed per ON and ranged from 22 to 100%. The majority of ONs caused ISRs in more than 70% of the trial subjects. Severity of the observed reactions varied between different ONs. Occurrence rate as well as severity of ISRs increases with higher doses. For chemistry and target of the compounds no clear association regarding ISR incidence or severity was identified. All ONs developed to date are associated with ISRs. Overcoming the problem of ISRs might add greatly to the potential success of SC administered ONs. Knowledge of these skin reactions and their specific immunostimulatory properties should be increased in order to obtain ONs that are more suitable for long-term use and clinical applicable in a broader patient population.

Introduction

Oligonucleotides are fragments of 12-24 nucleic acids in a target-specific sequence [1]. These compounds are designed to either inhibit mRNA of the targeted protein using the antisense principle, altering the reading frame by exon-skipping, directly inhibit the targeted protein (antagonist) or bind as an agonist on the receptor (figure 1). The latter ONs are under investigation for their immunostimulatory properties, such as C-phosphate-Guanine ONs (CpGs) [2]. ONs are an attractive class of compounds since the drug synthesis and production has become fully automated, rapid and inexpensive, whereby every desired nucleic acid sequence can be generated. Over the years, different ON subclasses with distinct molecular structures have been generated. Initial ONs, dating from the early 1990s, were unmodified deoxyoligonucleotides. Around the year 2000, a phosphorothioate backbone was added to many ONs. This led to major improvement as phosphorothioate ONs are highly soluble in water, have increased nuclease stability and show excellent biologic activity [1].

Despite the numerous ON drug candidates identified and studied over the last 20 years, up to the highest clinical trial phases, only two ONs achieved marketing approval by the FDA, mipomersen (in 2013) and fomivirsen (in 1998, for the treatment of CMV retinitis). This discrepancy may be explained by frequently untoward effects induced by ONs, including nephrotoxicity, hepatotoxicity, thrombocytopenia and inflammatory responses [3-6]. Subcutaneous (SC) administration of ONs also results in the occurrence of injection site reactions (ISRs), specific local skin reactions originating around the injection site manifesting itself as erythema, induration, itching, discomfort and pain, or more severely as ulceration or necrosis. ON-induced ISRs are considered to be common, nonetheless detailed information in the literature regarding these skin effects is limited.

For example mipomersen (Kynamro®; previously ISIS 301012) the only FDA-approved ON currently available is known to cause ISRs. Mipomersen targets the mRNA for apolipoprotein B to treat homozygous familial hypercholesterolemia. Mipomersen carries a boxed warning for the serious risk of liver toxicity, which is considered to be an off-target effect [7]. Although it is known that in phase 3 trials, 5% of all treated subjects discontinued mipomersen treatment due to ISRs [8], detailed public information on ISR severity, incidence and causal mechanism is scarce. The full prescribing information of mipomersen states that the local reactions typically consist of erythema, pain, tenderness, pruritus and/or local swelling. However, no notification is made that reactions may be more severe, and/or lead to irreversible skin changes. The occurrence of ISRs is unlikely to be a specific feature of mipomersen, but a class effect of oligonucleotides.

With this review we aim to provide a comprehensive and detailed overview of the incidence, severity, clinical manifestations and pathophysiology of ON-induced ISRs after SC administration.

Materials and Methods

A structured literature search was performed to identify reported incidence and clinical features of ISRs in clinical trials up to and including February 2015. The following databases were used: PubMed, MEDLINE, Embase, Embase meeting abstracts, Web of Science, Web of Science meeting abstracts, COCHRANE, CENTRAL, CINAHL, Academic Search Premier (free text), ScienceDirect (free text), Wiley, SAGE (free text), HighWire (free text), LWW (free text). Search terms were injections site reaction or related terms and oligonucleotides or related terms. 514 hits were found, only original trials reporting on phosphorothioate ONs were included. By screening of the manuscript title 255 papers were excluded (animal/preclinical data, oligonucleotide used as adjuvant, no oligonucleotide therapy, compounds with different chemistry or no subcutaneous administration), by abstract screening another 189 results were excluded (review articles e.g. on mipomersen and CpG-structures, and above mentioned reasons for exclusion). Of the 70 results the complete papers (when available) were studied and information regarding ISRs was extracted and reported here. A cross-check was performed by a search for ONs using the Integrity Database [9] (last accessed 18 February 2015). This yielded information on 7 additional ONs clinically tested, not (yet) reported on in the public domain. Further, publicly available documents from manufacturers of ONs and regulatory agencies were screened for relevant information on ISRs.

These data were combined with safety data on file available at the Centre for Human Drug Research in Leiden, the Netherlands, where a total of 204 subjects participated in trials with 4 different ONs. These studies were conducted in accordance with good clinical practice guidelines, after approval by the Central Committee on Research Involving Human Subjects (CCMO) of the Netherlands. Two of these compounds were made anonymous by naming them ON_CHDR to protect intellectual property.

Results

INCIDENCE

The literature search yielded no review papers on ISRs. Twenty-four (24) different SC administered ONs were identified in the papers found by the literature search and the information from the Integrity Database [9]. For 19 compounds reporting was available, and for all these compounds ISRs occurred. For the other 5 compounds identified, no reporting was (yet) available (PRO044, PRO045, PRO053, IsisGCCRRx and IsisTTRRx). The data found in the search was supplemented with CHDR data on file regarding four other ONs for which also ISRs were invariably noted. An overview of incidence of ISRs associated with these ONs is provided in figure 2. The percentage of trial subjects that develops ISRs differs per ON and ranged from 22 to 100%. The majority of ONs causes ISRs in more than 70% of the trial subjects and for two ONs it was reported that all treated individuals developed ISRs. For almost all ONs the incidence of ISRs is clearly dose-dependent. This is illustrated by the incidence for mipomersen [10], ON_CHDR2, IMO-8400 and ISIS325566 (figure 3). For these four ONs higher doses are associated with higher incidence of ISRs. Although the shape of the curve differs, the trend towards higher incidence with higher dose is clear and a plateau at 100% seems to occur from a certain dose level onwards. The only ON that did not show direct dose-dependency was ISIS14803 with a 100% occurrence rate at all dose levels tested, which may reflect that the doses studied were too high to detect dose-dependency.

ISRs following SC injection of ONs are characterized by a symmetrical erythemous skin lesion around the injection site, with a diameter ranging from 4 - 15 cm. The erythema may be accompanied by discomfort, pain, itch, induration and/or ulceration (figure 4), but is usually not accompanied by lymphadenopathy. The erythema generally appears 24-96 hours after the injection. It often reaches a maximal intensity around 48 hours after injection. These data appear to be corroborated by publicly available sources reporting that the most common injection site reactions (incidence between brackets) for mipomersen consisted of erythema (59%), pain (56%), hematoma (32%), pruritus (29%), swelling (18%) and discoloration (17%) [7]. Information on severity and duration of ISRs is not readily available, but it appears that the resolution of a skin lesion differs greatly among individuals. A total of 204 subjects were actively treated with one of 4 different ONs at our centre. ISRs were reported in 122 (60%) of the subjects and complete resolution occurred in this group in over 80% of the cases. The duration to resolution ranged from 14 to 90 days. Approximately 20% of the participants developed a (semi)permanent discoloration of the skin. This manifested itself as persistent mild erythema or hypo/hyperpigmentation of the skin, which was usually smaller

in diameter than the original erythemous lesion (figure 5). Interestingly, the pooled phase III trials with mipomersen report that 7.7% (20/261) of individuals experienced reactions at a previous injection site when subsequent injections were administered at a different site; a so-called injection site recall reaction.

The severity of ISRs is generally described in the literature as 'mild to moderate'. However, in most papers the definitions of the concepts 'mild' and 'moderate' are not specified and usually no information is provided on how many of each were reported. A notable exception was the reporting on the severity of the ISRs occurring for PF3512676 for which a grading system was used (table 2) [11]. The majority of patients were reported to have an ISR of Grade 2 or lower (mild to moderate), up to 10% of patients reported an ISR of Grade 3 or greater which was defined as severe and requiring dose modification. In the combined phase III 6-month trials 5% of all mipomersen-treated individuals discontinued due to ISRs [8]. Other patient drop-outs as a result of ISRs were reported for PF 3512679 and ISIS2302 [12]. The severity profile may differ between compounds and between dose levels, however this is difficult to assess as no grading system is consistently used throughout different studies.

HISTOLOGY

Little is known of the histopathology of ISRs. The largest series currently available is from a dedicated ISR study performed with mipomersen. In this study 32 individuals had post-treatment skin biopsies of injection sites. Histological analyses of these injection sites showed that 9 of the 32 individuals had findings consistent with leukocytoclastic vasculitis e.g. infiltrating neutrophils, prominent nuclear dust, lymphocytes, and eosinophils with local macrophage infiltration [7]. The histology of a biopsy of an erythematous ISR observed in our center showed a non-specific spongiotic appearance with few eosinophils (figure 6A,B,C). The inflammatory influx was mainly perivascular and to small extent also present in pre-existing collagen, and the basal layer of the epidermis. The subcutaneous fat tissue demonstrated necrosis and infiltration with eosinophils (figure 6D).

Discussion

Since detailed information on oligonucleotide-induced ISRs is currently lacking, we conducted a systematic review of all data available in the public domain, and supported the findings with relevant data collected in clinical studies with subcutaneously administered ONs performed at the CHDR. All SC administered ONs described in literature and studied at the CHDR resulted in the induction of ISRs. ON-induced ISRs appear as symmetrical erythematous skin lesions, often accompanied by discomfort, pain, itch, induration and/or ulceration, variable in size, and with variable resolution times between compounds and individuals. This local immune response is in line with the general pro-inflammatory potential of ONs in humans, since flu-like symptoms and elevated CRP are common after SC administration of ONs [7]. Also systemic adverse reactions directly following IV infusion (fever, nausea, malaise) are commonly observed in clinical trials with ONs [13;14]. Based on the data available, it is concluded that ON dose level is an important determinant for the induction of ISRs as occurrence rate and severity increases with higher dose levels. ONs differed mutually with respect to the incidence and severity of the induced ISRs, although no clear association with a specific ON subclass was observed. Although many efforts have been made to design ONs lacking immune-stimulating effects, none of the established chemical modifications did result in the desired effect. The 5-methyl-cytosine substitution is claimed to reduce immune stimulation [7;15]. Locked nucleic acid (LNA) modifications are intended to enhance the antisense binding affinity to the mRNA target and increase its biological half-life, reducing the probability of off-target effects [16]. However, the observed incidence and severity of the skin reactions induced by these newer compounds does not differ from older ON subclasses (table 1). It is remarkable that the ONs with the highest ISR rates, ISIS 14803 and mipomersen, both include a 5-methyl-cytosine substitution. No other potential correlation between ON subclass or length and ISR incidence or severity was observed (table 1). These findings demonstrate that it is at least uncertain that further chemical modification of ONs may result in the desired lack of immune-stimulating activity. Nevertheless, it appears that chemical modifications are pursued. Examples of this approach are to introduce a so-called steric bulk at 5'-position of the sugar-phosphate backbone [17], to conjugate the oligonucleotide with peptides, proteins, carbohydrates,

aptamers and small molecules [18], or introduce receptor-binding molecules such as folate, anisamide or N-acetyl galactosamine (GalNac) or dynamic polyconjugates to the oligonucleotide [19]. An alternative strategy may be to alter the delivery of ONs in a manner which may bypass local immune responses. Altered delivery can be obtained using chelation [20] or the use of nanoparticles or liposomes [21]. Moreover, avoiding ISR s could be achieved by oral administration of ONs, a concept that is currently investigated [22]. Whether these strategies are safe and efficacious in humans remains to be determined.

Our review demonstrates that ISRs after SC administration of ONs are a serious problem. Obviously, severe and long-lasting local inflammatory responses upon SC use of ONs are debilitating for potential future patient populations. In addition, relatively mild discomfort such as itch and cosmetic aspects like erythema and altered pigmentation may jeopardize adherence to therapy, particularly upon chronic use. This is illustrated by the relatively high percentage of participants in phase 3 mipomersen trials discontinuing therapy early due to the occurrence of ISRs [8]. Ultimately, the potential value of SC ON therapy is dependent on the severity of a particular disease and availability of alternative therapies. For example, ISR-inducing SC ON therapy may be acceptable for patients suffering from an otherwise untreatable malignancy or lethal muscular dystrophy, whereas hypercholesterolemic patients would not readily consider the use of such a therapy.

For the future clinical application of SC ONs, detailed understanding of the mechanisms causing the skin reactions is essential. It is unlikely that the occurrence of ISRs can be explained by the ON target or the target distribution. For example, ISIS 14803 and miravirsen both target RNA replication of the Hepatitis C Virus (HCV), however the compounds have the highest and second lowest ISR rates (table 1). It does not seem that the dermal localization of the ON target predisposes to a higher ISR occurrence rate: the target of the ON with the highest incidence is expressed hepatically (ISIS 14803), whereas CPG10101 that has a target in the skin shows a remarkably low incidence in ISRs. This is interesting given the fact CpG ONs are intended to act immunostimulatory. Unlike assumed earlier however, it was shown that TLR9 activation is not confined to the compounds containing unmethylated CpG, but depends on backbone structure [23;24]. In conclusion, the induction of ISRs by SC administration of ONs may be a class effect inherent to the physical-chemical nature of the compounds, which can potentially be circumvented by further chemical modification, although published evidence is currently lacking. Further, we argue that rational development of tailored ON subclasses could benefit from in-depth knowledge on the relationship between chemical modifications and the molecular pathways involved in the immune responses causing the ISRs.

MOLECULAR TARGETS

Which molecular targets may be implied in the observed ON-induced immune responses? The skin functions as a mechanical barrier, and also as a first line immune defense, comprising innate and adaptive immune mechanisms [25]. The skin contains a mixture of immune cells: keratinocytes releasing cytokines and chemokines in response to injury, resident dendritic cells, macrophages, innate, NK- and helper T lymphocytes, and mast cells in the dermis [26]. ON-mediated activation of the immune system is likely to start in the dermis. Unfortunately, relatively limited information is available on the specific pathophysiology of ISRs. In general, drug-induced skin reactions are non-specific hypersensitivity-like responses, characterized by dermal edema with perivascular and interstitial acute and chronic inflammation with involvement of neutrophils, eosinophils and lymphocytes [27-29]. This is compatible with the findings from biopsies performed upon occurrence of ISRs after SC administration of mipomersen [7]. Similar histological responses were observed in a clinical study with a ribozyme construct, chemically resembling an ON [30]. Specific features of ON-induced ISRs include the involvement of immunological memory, as demonstrated by the injection site recall reactions related to previous exposure to the ON [7]. Also, hyper- and hypopigmentation is incidentally observed after administration of mipomersen. They should be considered non-specific post-inflammatory clinical sequellae, also occurring in skin diseases including acne vulgaris, atopic dermatitis, skin infections and allergic reactions [31]. The response is known to result from the activation of melanocytes with overproduction of melanin or an irregular dispersion of pigment, but the exact mechanism underlying post-inflammatory hyperpigmentation is unknown [32]. Some scattered histological information is available on immune responses in ON-induced ISRs, but this information does not tell which specific molecular pathways are driving the initial immune

response. The innate immune system, being the first line of defense, comprises different subsystems for the protection of the body against pathogens. The most likely initial drivers of the ON-induced immune response are innate cytosolic sensors and Toll-like receptors (TLRs) and the complement system, two innate immune pathways that may act synergistically [33]. The TLR system comprises different pattern recognition receptors sensing a variety of pathogens ranging from bacteria to fungi, protozoa, and viruses [34]. More particularly, the cell-membrane bound TLR2 and TLR4 can be activated by viruses and viral components, as are the endosomal TLR3, TLR7/TLR8 and TLR9, which sense double-strand RNA, single-strand RNA, and CpG DNA, respectively. Since ONs are short nucleic acid sequences, which may mimic viral sequences, they could theoretically trigger innate cytosolic sensors such as retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA-5), TLR3, TLR7/8, resulting in MyD88 (TYPE I IFN) and NF-KAPPA-B and the expression of pro-inflammatory cytokines by dendritic cells and macrophages [35-39]. Complement activation is known to play a key role in dermatological inflammatory conditions [40]. Leukocytoclastic vasculitis has been demonstrated in ISR biopsies of mipomersen-treated human subjects [7], which implies the potential involvement of complement since in this type of vasculitis complement complexes and perivascular complement deposits are commonly observed [41]. Leukocytoclastic vasculitis (also known as hypersensitivity vasculitis / angiitis) is commonly confined to the skin and is caused by vascular damage due to nuclear debris from infiltrating neutrophils. The most common cause is secondary to medications. The common clinical observations fit the findings for ON-associated ISRs as the majority of the lesions are acute and show resolution in weeks to months, but 10% turns into a chronic condition characterized by persistent lesions or intermittent recurrence. These chronic lesions eventually develop into morphea (also known as localized scleroderma or circumscribed scleroderma), a condition consisting of patches of hardened skin with no internal organ involvement. Interestingly, the treatment for leukocytoclastic vasculitis is to stop the causative agent and to avoid steroids. The latter would explain why attempts to reduce IISR using steroids have failed. However, based on the data available in the public domain it remains difficult to draw firm conclusions on potential TLR and complement activation in the skin upon SC ON treatment. The occurrence of ISRs appears to be species-dependent, which is not surprising given the large differences in the immune system response between species [42]. For example, monkeys are more sensitive to oligonucleotide-induced complement activation than humans [43;44]. However, dedicated animal studies may shed light on the mechanisms underlying ISRs: in mice and non-human primates immunostimulatory effects of ONs were observed [2;45-47], which demonstrates the potential relevance of these animal models for mechanistic studies. Subcutaneous administration of phosphorothioate ONs to rodents resulted in local swelling and induration at the injection site, with mononuclear cell infiltrates [46;48], lymphoid hyperplasia and multiorgan lymphohistiocytic cell infiltrates [43;49;50]. Administration of CpG-containing ONs to mice resulted in TLR9 activation [35]. Furthermore, experiments in non-human primates have shown that phosphorothioate ONs may activate the alternative complement pathway [51;52], possibly by interaction with Factor H [52;53]. It is uncertain how these preclinical data translate to humans, but when combined with tailored preclinical studies applying human cell cultures or mouse models with a humanized immune system, potentially involved mechanisms in ON-induced ISR development in man may be identified. The involvement of specific TLR pathways and complement in ON-induced ISRs could be systematically explored in a clinical trial with mipomersen, a commercially available oligonucleotide that is considered to be safe, but does induce ISRs. Thorough investigation of biopsies from skin lesions with dedicated immunohistochemical staining may shed light on the exact immunological mechanisms involved. In addition, we would advocate standardized quantitative and qualitative assessments of skin reactions in all future clinical studies with SC administration of ONs including immunohistochemistry and electronmicroscopy. This would provide more insight into the course of the development of the lesions, and allow a more structured comparison between different compounds.

In summary, all ONs tested in clinical studies have been reported to induce ISRs, reflecting a drug class effect. Detailed information on ISRs in the experimental setting is currently lacking. It is recommended to perform a uniform and standardized assessment of the skin reactions for all future studies with ONs, to gain more insight and to allow comparison between different compounds. This assessment should include a standardized way of reporting the clinical features, scoring severity and reporting duration (table 3). Also performing standardized medical photography and

biopsies from affected skin could add greatly to the current knowledge. More recent ON subclasses with specific chemical modifications aiming to avoid immunological skin responses have at present not been successful to completely prevent occurrence of ISRs. The pathophysiology underlying the ISRs and the causative immune pathways remains speculative. The initial immunological activation is likely to be driven by specific TLRs and complement. However, the exact involvement of these pathways has not been studied in detail, or alternatively not reported upon in the public domain. We therefore advocate a systematic approach to elucidate the immune-stimulatory effects of oligonucleotides, by performance of dedicated clinical and preclinical studies. In-depth knowledge on the exact mechanisms underlying these skin reactions will be of importance for the future of all ONs, not only the ones administered subcutaneously. In parallel, strategies to diminish or limit the skin response induced by ONs should be considered. It appears that neither systemic or locally applied corticosteroid treatment prevent development of ISRs [54], but other treatments that have been explored for leukocytoclastic vasculitis may be considered [55]. Further, local ON exposure should be limited by restricting the dose to the minimal level exerting the desired clinical effect, and possibly by spreading an effective total dose over multiple administrations, an approach demonstrated to be effective for mipomersen [56].

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Figure 1 The different modes of actions for therapeutic oligonucleotides. A. activation or inhibition of the target protein to induce or inhibit immune activation B. exon skipping to induce alternative splicing. C. inhibition of mRNA with antisense ON, inhibits production of the protein.

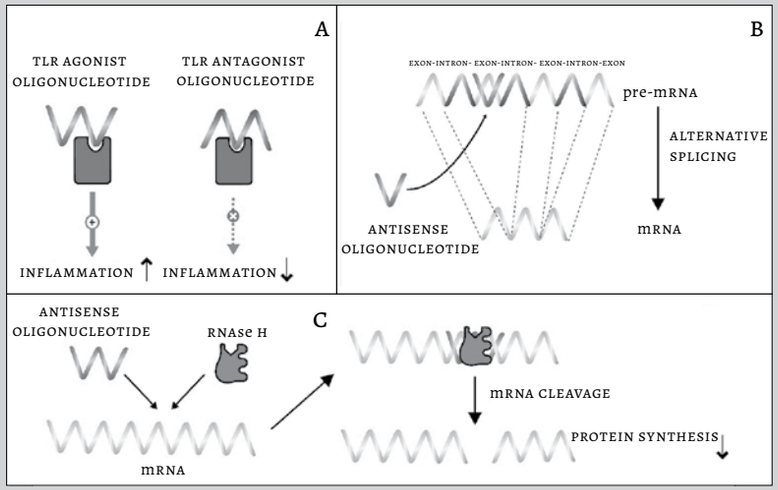


Figure 2 All 21 SC administered ONs resulted in ISRs. Incidence ranged from 22 to 100%. For 4 ONs no incidence numbers were reported, namely ISISAPO(A)RX, ISIS113715, ATL-03 and ISISGCGR-RX. For ONs that were studies at different dose levels and/or multiple trials, an average ISR occurrence was calculated.

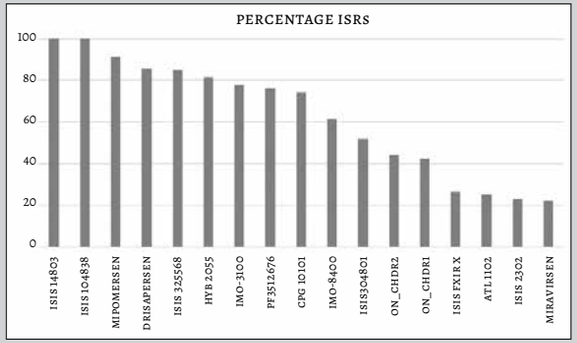
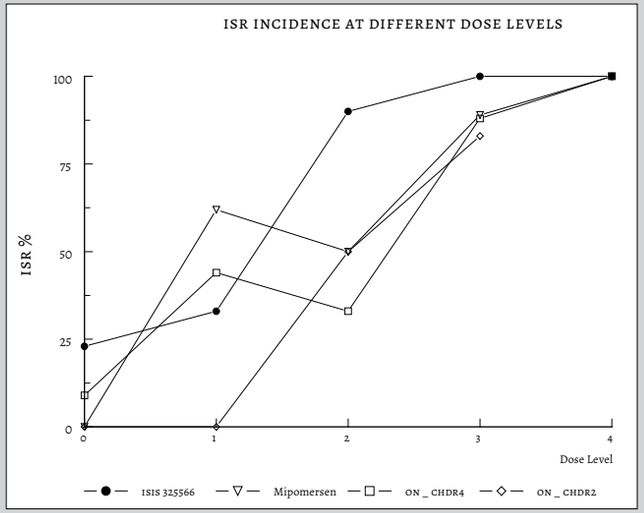


Figure 3 Dose-dependent occurrence of ISRs after administration of four different ONs. Higher dose levels result in increased incidence of ISRs up to 100% at the highest dose level. The dose levels tested for ISIS32566 and Mipomersen [10] were placebo (0), 50, 100, 200 and 400 mg. For IMO-8400 dose levels were: placebo (0), 0.075, 0.15, 0.3 and 0.6 mg/kg, and for ON_CHDR2 dose levels were placebo (0), 0.5, 1.5 and 5 mg/kg.



For figure 4, 5,6: see inside cover.

Table 1 Listing of clinically tested oligonucleotides. PHON = Phosphorothioate Oligonucleotide, LNA = locked nucleid acid structure, 2MOE = 2'-O-MOE structure, 5MCS = 5-methyl-cytosine substitution, CPG = Cytosine triphosphate deoxynucleotide-Guanine triphosphate deoxynucleotide

Name	Structure	Length	MOA	Indication	N*	Ref.	ISR %
ISIS 14803	PHON 5MCS	20 units	Inhibits HCV RNA synthesis	Chronic HCV Infection	1	(19)	100
ISIS 104838	PHON, 2MOE	20 units	Inhibits TNFα	Rheumatoid arthritis, Crohn's disease and psoriasis	1	(56)	100
Mipomersen	PHON, 2MOE 5MCS	20 units	apoB synthesis inhibitor	Hyper-cholesterolemia	19	(13; 55; 57-73)	91.2
Drisapersen	PHON, 2MOE	20 units	Induces Exon 51 skipping in DMD	Duchenne's Muscular Dystrophy	6	(54; 74-78)	85.5
ISIS325568	PHON, 2MOE	20 units	Inhibits GCCR	Diabetes Mellitus Type 2	1	(79)	85
HYB 2055	NR**	NR	Activates TLR 9	Cancer	1	(80; 81)	81.3
IMO-3100	PHON	18 units	Inhibits TLR 7,9 activation	Psoriasis	1	(82)	65
PF 3512676	PHON, CPG	24 units	Activates TLR 9	As adjuvant of vaccination/ chemotherapy	5	(83-87)	76.3
CPG 10101	PHON, CPG	22 units	Activates TLR 9	Hepatitis C (HCV) Infection	3	(88-90)	74.5
ON_CHDR2	PHON, LNA	14 units	undisclosed	undisclosed	-	-	72.2
IMO-8400	PHON, 2MOE	18 units	Inhibits TLR 7, 8, 9 activation	Psoriasis	1	(91)	61.5
ISIS304801	PHON, 2MOE	20 units	Inhibits Apolipo-protein C-III	Dyslipidemia	1	(92; 93)	52
ON_CHDR1	PHON, 2MOE	12 units	undisclosed	undisclosed	-	-	42.7
ISISFIXRX (Isis416858)	PHON, 2MOE	20 units	Reduces human factor XI	Prevention of thrombosis	2	(94; 95)	33.3
ATL1102	PHON, 2MOE	20 units	Inhibits CD49d	Relapsing-remitting multiple sclerosis	1	(96)	25
ISIS 2302	PHON	20 units	Inhibits ICAM-1 expression	Crohn's Disease	1	(16)	23.3
Miravirsen	PHON, LNA	15 units	Inhibits miR-122	HCV Infection	2	(97; 98)	22.2
ISISApo(a) RX	NR	NR	Inhibits apolipo-protein (a) protein	Coronary Artery Disease	1	(99)	NR
ISIS113715	PHON, 2MOE	20 units	Inhibits PTP-1B protein	Diabetes Mellitus Type 2	2	(100; 101)	NR
ATL-03	PHON, 2MOE	20 units	Inhibits GHR Expression	Acromegaly	1	(12; 102)	NR
ISISGCCR-RX	Not reported	NR	Inhibits GCCR	Diabetes Mellitus Type 2	1	(103)	NR

** # Number of Studies found in literature ** Not Reported*

Table 2 An example of an ISR grading system to score the severity used for PF3512676 [11]

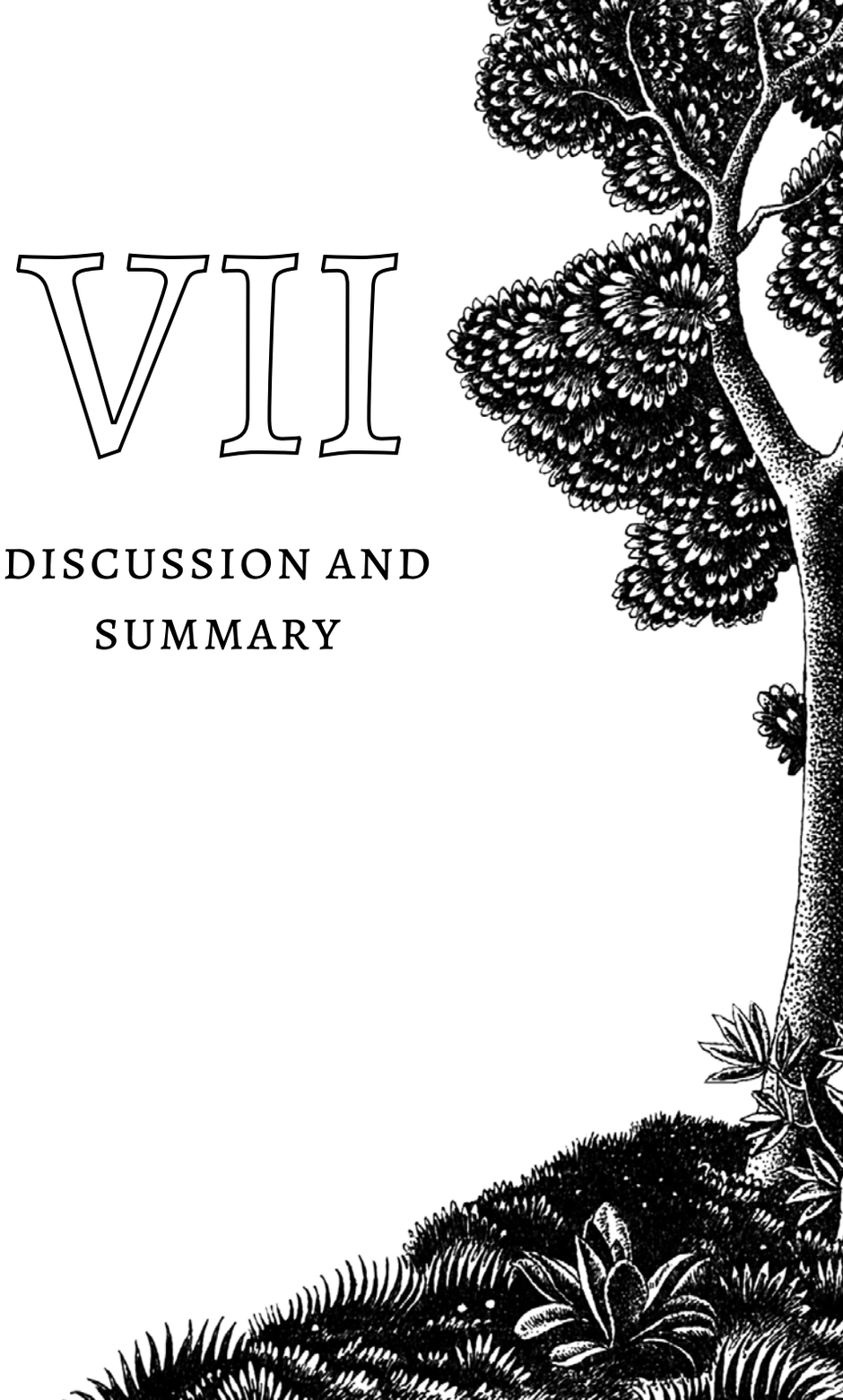
Grade 1	mild (does not interfere with daily life)
Grade 2	moderate (interferes with daily life but no dose modification)
Grade 3	severe (requires dose modification)
Grade 4	disabling (requires drug discontinuation)

Table 3 Suggested uniform standardized ISR scoring system. ADL = 'Activities of Daily Living' and are defined as bathing, dressing and undressing, feeding self, using the toilet, taking medications, preparing meals, shopping for groceries or clothes, using the telephone etc.

	0 = **No**	1 = **Mild**	2 = **Moderate**	3 = **Severe and undesirable**
Injection site reaction	None	Erythema OR Tenderness OR Itching	As 1 and Pain OR Swelling OR Signs of inflammation	Ulceration or necrosis
Maximal diameter ISR	NA	Max 5 cm	Max 10 cm	Max 15 cm OR any diameter and systemic reaction OR flare up previous IS
Duration of symptoms	≤1 day	2-14 days	2-6 weeks, reversible	Permanent
Sequelae	None	Minimal and tolerated by patient	Hardly tolerated OR wish for treatment by patient	Permanent despite treatment OR no treatment options
Likely impact on next dose	None	Injection site can be used in rotation AND no dose adaptation	Injection site should be avoided in rotation OR change dose regimen	Injection site cannot be used anymore OR discontinuation
ADL limitations	None	Minimal	Functional	Self-care limitations

VII

DISCUSSION AND SUMMARY



Introduction

This thesis describes the clinical testing of the antisense oligonucleotide ISIS 388626. Oligonucleotides (ONs) are fragments of 12-24 nucleic acids in a target-specific sequence [1]. The sequences of antisense ONs are complementary to part of the mRNA of the targeted protein. The ON binds to the mRNA, prohibits mRNA translation and thereby inhibiting the formation of the targeted protein. The concept of antisense ONs is very promising as it allows highly specific inhibition of any target for which the mRNA sequence is established. ISIS 388626 is complementary to a region of the mRNA of the SGLT2 protein. SGLT2 is a transporter that enables glucose reabsorption in the kidney and inhibition of this transporter is a validated strategy in the treatment of type 2 diabetes [2-4].

PRECLINICAL DATA

In preclinical studies, ranging from 6 weeks to 6 months in duration, weekly subcutaneous (sc) injection of ISIS 388626 showed to be an effective and safe treatment [5;6]. In normoglycemic animals the reduction in SGLT2 mRNA expression of ≥ 80% that occurred at doses of 1-3 mg/kg weekly for 13 weeks translated into effective glucosuria (at 3 mg/kg a 60-fold increase in mice and 7-fold increase in monkeys in urine glucose creatinine ratio) [5]. In none of the animal models, signs of toxicity of ISIS 388626 were observed. ISIS 388626 demonstrated a specific and selective renal distribution [7] and no indications for long term changes of general kidney function were noted in studies up to 6 months in duration [5].

TRANSLATION TO THE CLINIC

The effects of treatment with ISIS 388626 were explored clinical studies in the dose range of 50-200mg. The chosen doses were based on a minimal anticipated biological effect level (MABEL) approach. In preclinical studies across multiple species, the pharmacologically active dose range of ISIS 388626 was 1-30 mg/kg/week. At this exposure, a significant reduction in SGLT2 mRNA occurred (74 to 97% in mice and approximately 30 to 90% in monkeys over the dose range 1-30 mg/kg/week), accompanied by a 25-200 fold increase in urinary glucose excretion [5-7]. Based on this, estimation of the equivalent human effective dose falls in the range of 1-3 mg/kg/week. The No Adverse Effect Level (NOAEL) was estimated to be 10 mg/kg/week in monkeys. The safety of the 1-3 dose range was further supported by previous experience with 2'-MOE-modified antisense oligonucleotides [8-10]. Multiple clinical studies showed that these compounds could be safely administered (intravenously and subcutaneously) at dose levels up to weekly 750 mg (which translates into 10.7 mg/kg/week assuming an average weight of 70 kg), with treatment durations exceeding one year [8-11]. Pharmacokinetic data from preclinical studies showed that a loading dose regimen of 3 doses in the first week ensured rapid achievement of steady state tissue concentrations, therefore in the MAD part of the study this regimen was also applied, followed by a maintenance schedule of weekly dosing for 5 weeks. The results from the preclinical experiments with ISIS 388626 are summarized in table 1.

TRIAL DESIGN

The initial study design consisted of a double-blind, randomized, placebo-controlled study, starting with a single ascending dose (SAD) study, in which sixteen subjects were assigned a single dose in a 3:1 ratio (50, 100, 200 or 400 mg ISIS 388626 or placebo), followed by a multiple ascending dose (MAD) study, in which subjects were planned to be enrolled in a 12:3 ratio (50, 100, 200 or 400 mg ISIS 388626 or placebo). In the MAD study, the dose levels exceeding 50 mg, the intended pharmacodynamic effects of ISIS 388626 (inhibition of renal urinary glucose reabsorption and lowering of plasma glucose concentrations) were to be estimated by evaluation of glucose handling after an oral glucose tolerance test (OGTT), performed before the first administration of ISIS 388626/placebo and at Week 6. Safety profile was to be continually monitored by weekly evaluation of blinded clinical laboratory assessments and adverse events throughout the cohort. Before escalating the dose the safety profile of the preceding cohort had to be evaluated and considered acceptable.

FIRST CLINICAL STUDY: UNEXPECTED FINDINGS

The first study started with a single ascending dose study, which was completed without safety problems. However, the following 6 week multiple ascending dose part was halted early because increases in serum creatinine occurred in the subjects participating in the 100 mg multiple

dose cohort. Evaluation of the pharmacodynamic effect was limited due to the small exposure in this study, but there was an indication that glucosuria increased upon active treatment. The pronounced changes in serum creatinine were accompanied by increased urinary excretion of beta-2-Microglobulin (B2M) and KIM1. These findings were unexpected as experiments in animals treated with ISIS 388626 with safety assessments at 6 and 13 weeks had not shown such results. As the possible mechanisms for the changes in serum creatinine were unknown, it was felt more preclinical data were needed to justify further clinical investigations.

Therefore bio-banked samples collected earlier than 6 weeks of the previously performed pre-clinical experiments were analyzed. This showed that in animals treated with a loading dose similar transient increases in serum creatinine and urinary excretion of B2M and protein had occurred. Thus, the apparent discrepancy of renal effects of ISIS 388626 treatment between rodent and monkeys on the one hand and humans on the other hand could be explained by the suboptimal timing of the initial assessments [5;7;12]. This prompted a further dedicated experiment in monkeys in which it was explored whether the renal effects by ISIS 388626 were related to the loading dose. Animals were dosed for 13 weeks with either 30 mg/kg every other day or a single dose in the first week, followed by weekly dosing for another 12 weeks. This experiment showed that changes in renal markers in monkeys occurred only with the loading dose regimen. Importantly, in this study it was also shown that abandoning the loading dose did not impair the primary pharmacodynamic effect, as glucosuria still occurred, although onset was delayed.

SECOND CLINICAL STUDY: NO IMPAIRED RENAL FUNCTION

Based on the clinical findings and the new animal data, clinical studies were restarted with the aim to investigate the effects of SC doses of 50, 100 and 200 mg ISIS 388626, administered without a loading dose as 13 weekly injections. However, omitting the loading dose did not prevent increases in renal markers in humans. Treatment with 50 mg ISIS 388626 induced serum creatinine increases and increases in urinary renal markers. These changes prohibited dose escalation to the 100 mg dose and further explorations were done to investigate whether the transient increases in renal markers in humans could be explained by functional changes in renal blood flow and/or glomerular filtration rate. A new cohort of volunteers was treated at the 50 mg dose level to explore if the observed transient increases in renal damage markers coincided with functional renal changes. This was assessed with renal clearance tests to evaluate the impact of ISIS 388626 on GFR and renal plasma flow. Weekly ISIS 388626 treatment at a dose level of 50 mg for 13 weeks increased average serum creatinine (with 0.15 mg/dl) and renal damage markers. The changes were relatively mild and fully reversible upon cessation of dosing. The renal clearance test revealed no indications for impairment of glomerular filtration or renal perfusion. No increase in renal glucose excretion was observed at the 50 mg dose level, as was expected based on preclinical data. To induce the intended pharmacological activity, a higher level of drug exposure was required. Exploration of higher ISIS 388626 dose levels in healthy volunteers was considered acceptable using a careful approach with close monitoring of renal function and damage markers.

THIRD CLINICAL STUDY: INTENDED PHARMACOLOGICAL ACTIVITY OR TUBULAR DYSFUNCTION?

In the continuation of the clinical evaluation of ISIS 388626, repeated doses of 100 and 200 mg were applied. ISIS 388626 induced glucosuria in these studies, with a dose dependent increase in average 24 hour urinary glucose excretion (average increase of 508.9 and 1299.8 mg/day, in the 100 and 200 mg groups respectively, comparing baseline values with end of treatment). As the average amount of glucose that is filtered and reabsorbed amounts to approximately 144 grams per day (800 mmol/day [13]) and the average inhibition of glucose reabsorption that we observed amounted to only 0.8 % for the 200 mg dose regimen, it appears that the intended effect is very small indeed. The observed level of urinary glucose excretion is significantly higher upon treatment with small molecule compounds that target SGLT2, which have been reported to induce urinary glucose excretion in the range of 60-70 g/day [14-17]. This robust glucosuria results in an effective glucose-lowering therapy and has led to the registration and approval of a number of small molecule SGLT2 inhibitors [2-4]. In patients with type 2 diabetes addition of an SGLT2 inhibitor to standard therapy resulted in lower risk for cardiovascular events and death [18].

Kidney biomarkers further increased at the 100 and 200 mg dose levels. Maximal average changes in serum creatinine of 0.28 ± 0.11 and 0.38 ± 0.09 mg/dl occurred in for 100 and 200 mg respectively (versus 0.15 ± 0.06

mg/dl after 50 mg). Urinary renal markers of tubular damage, such as B2M, protein and KIM1, increased in parallel in a significant and dose-dependent manner. The only other ISIS 388626 related adverse events were mild injection site reaction (ISRs), occurring in 8-19% of the subjects. Interestingly, an effect on renal function is also reported for small molecule SGLT2 inhibitors [19], suggesting a target-related effect. However, this effect consists solely of a small decline in eGFR and is considered to be secondary to the mild reduction in intravascular volume as a result of the natriuresis that accompanies the glucosuria, although it cannot be excluded that this effect is also caused by diminished tubular secretion of creatinine. However these effects in small molecule SGLT2 inhibitors were mild, not accompanied by increases in other renal markers and coincided with a strong pharmacodynamic effect, therefore this observation shows little similarity to the ISIS 388626-induced effect. Nonetheless, it cannot be excluded that natriuresis adds to the creatinine increase observed with ISIS 388626. Similarity to the effects of small molecule SGLT2 inhibitors on glucose homeostasis is further supported by the significant increases in insulin and C-peptide levels (after OGTT) observed in ISIS 388626 treated subjects, suggesting increased endogenous glucose production, as also observed for dapagliflozin and empagliflozin [20-23].

The low-grade glucosuria, that is considered a pharmacological effect, could also be (partly) caused by tubular dysfunction. The adverse renal effects were unexpected. For some oligonucleotide compounds effects on the kidney have been described [24-26] and in one case an oligonucleotide has caused acute tubular necrosis in a healthy volunteer, however, several others have no renal effects [11;27;28]. The available data to date are too limited to draw conclusions on the relationship between renal adverse effects and compound specific factors, such as size or chemistry. We suggest that comprehensive screening for renal effects using tubular damage markers and functional tests is performed whenever oligonucleotides are administered systemically as animal experiments clearly cannot produce certainty about the absence of these effects in humans.

As the glucosuria observed upon ISIS treatment was minimal, and was accompanied by adaptations of or injury to renal cells due to unclarified mechanisms, the therapeutic window of ISIS 388626 is narrow. It is possible that subjects with type 2 diabetes mellitus (with higher SGLT2 expression and greater renal filtered glucose load) could benefit more from ISIS 388626 compared to the healthy volunteers investigated in these studies. However, the mechanisms underlying the transient renal dysfunction warrant more detailed exploration before ISIS 388626 could be considered for further clinical development.

MONITORING RENAL EFFECTS

The clinical testing of ISIS 388626 resulted in unexpected effects on markers for kidney injury. In preclinical studies, including studies in multiple animal species, no signal was initially detected. This illustrates that monitoring renal function in first in human studies is of great importance, particularly because only 40-60% of animal findings are predictive of toxicities in humans [29]. Generally accepted and routine biomarkers such as serum creatinine and BUN are often used. However they lack sensitivity and early injury responses may be missed [29]. In the relatively long term dosing regimen tested for ISIS 388626 (13 weeks), a clear response of serum creatinine was detected and led to adaptation of the study design and also allowed decisions about dose-escalation. Other potentially useful kidney biomarkers that can be used in clinical trials include KIM1, B2M, aGST and NAG. These urinary biomarkers may outperform serum creatinine, as illustrated by a case of acute tubular necrosis after treatment with an antisense oligonucleotide [26]; KIM1 increases obviously precede serum creatinine increases. However, to catch these early changes frequent urine sampling is required as well as rapid analysis of the samples. The biomarkers KIM1, B2M, aGST and NAG were assessed during the clinical investigations with ISIS 388626 and KIM1 and B2M correlated closely with serum creatinine responses. The markers were not included to create opportunity for early intervention, but were analyzed in a batch at the end of the cohort and were included to gain maximal insight in the nature of the observed effect on serum creatinine.

A serious limitation of novel renal biomarkers is the lack of their validation for clinical use. Due to limited or contradictive information on their clinical performance, choosing the most appropriate biomarker is challenging. Also, when data is obtained, interpretation is complicated by uncertainty on normal ranges and normal variability. It would be helpful if more data on clinical performance and large validation studies on novel biomarkers would be publicly available, to be able to confirm superiority over the more classical choices of serum creatinine and BUN. Also measurement of the markers should become more readily available and lower in cost. The ideal renal biomarker, does not seem to exist or is not yet identified. Nonetheless, some have promising qualities. An

example is KIM1, which suits different purposes and thus often seems to be a right choice. And the field of renal biomarkers is evolving and even newer biomarkers such as TIMP2 and microRNAs appear to outperform the biomarkers currently used [30;31]. A marker specific for glomerular injury is yet to be identified, as this is currently lacking. As glomerular involvement often leads to irreversible damage early identification of this type of kidney injury would be of added value.

INJECTION SITE REACTIONS AFTER OLIGONUCLEOTIDE THERAPY

As mentioned, the subcutaneous (SC) injection of ISIS 388626 induced Injection site reactions (ISRs) in a subset of subjects. At this moment, no oligonucleotides (ONs) are available that are completely devoid of ISRs. Although all ONs that are administered SC result in ISRs, incidence and severity of the observed reactions vary between different ONs. From the relevant literature as well as experience within CHDR, it can be concluded that dose level plays an important role. Higher doses probably result in higher local exposure and more intense reactions. Low-grade ISRs usually consists of erythema and discomfort, but may also progress into more severe manifestations such as induration, ulceration and necrosis. ISRs appear to be common, but are poorly described in literature. The pathophysiology of ISRs remains unclear at his point. Immunological activation is likely to involve recognition of the compounds by innate immune receptors such as TLRs and possibly complement activation. Overcoming the problem of ISRs might add greatly to the potential success of SC administered ONs. Knowledge on these skin reactions in specific and immunostimulatory properties of ONs in general should be increased. Thorough investigation of biopsies from skin lesions after ON therapy with dedicated immunohistochemical staining may shed light on the exact immunological mechanisms involved. Also, to increase knowledge on ISRs, the study design of clinical trials with SC ONs, should include systematic reporting of skin reactions, including photography, scoring of the lesions and proper follow-up.

FUTURE PERSPECTIVES

The favorable preclinical profile of ISIS 388626 combined with the upcoming target of SGLT2 inhibition to treat diabetes, rendered ISIS 388626 a promising drug candidate. Small molecule SGLT2 inhibitors are promising agents that are already implemented in the treatment of diabetes. The latter are known to have a limitation of inhibition of less than 50% of the filtered glucose load. This limitation is hypothesized to result from a compensatory increased SGLT1 activity, when SGLT2 is completely blocked. It remains unknown if the maximal pharmacodynamic effect of antisense inhibition of SGLT2 would exceed the maximal effect of SGLT2 inhibition using small molecule compounds. However, the unexpected and incompletely understood effects on the kidney of ISIS 388626, makes further augmentation of the dose unacceptable from a safety perspective. Therefore clinical development of the compound was stopped before a robust pharmacodynamic effect was achieved.

In general antisense oligonucleotides are an attractive class of compounds. The compounds are 'custom-made', highly selective to the specific target, creating inhibition on a whole different level by interfering with the synthesis of the target protein. However, the promise of many drug candidates tested clinically has not rendered many successes. Currently the only approved oligonucleotide is mipomersen used to treat homozygous familial hypercholesterolemia. There are several important challenges to overcome in order to move towards broader implementation of oligonucleotide treatment in clinical practice. Some of these challenges are illustrated in this thesis. Accumulation of the oligonucleotides seems to play an important role in both the renal and cutaneous effects of oligonucleotides. Alternative delivery systems are being investigated. Avoiding ISRs for example could be achieved by oral administration of ONs a concept that is currently being investigated [32]. Renal accumulation is known to be dependent on chemistry [5;33;34], therefore this should be taken into account in future studies with ONs. Strategies to avoid accumulation of ONs also include modification of the distribution properties using oligonucleotide conjugates, such as peptides, proteins, carbohydrates, aptamers and small molecules [35]. The thin line between accumulation of oligonucleotide needed for the effect and the accumulation that induces toxicity should be further explored. Preclinical and early clinical studies should focus on increasing knowledge on how to avoid unintended effects. This has the potential to lead to ONs with a better profile, more suitable for chronic use and applicable for broader patient populations.

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Table 1 Summary of the results of preclinical experiments with ISIS 388626

Study NR	Description	Model	Route	Regimen	dose (mg/kg)	PD effect MNRA reduction(%)	PD effect Urine glucose increase	Other PD	Cmax (ug/mL)	Hypo-glyce-mia	ISR	Specific Toxicity
APK01	Pharmacokinetics, Distribu-tion,Metabolism Excretion and MassBalance of Radio-activity Study inRats	Rats	SC	Single Dose	3 and 10				3	NO	NO	
	4-Week Study in Mice	CD-1 Mice	SC	Weekly	30					NO	NO	
AS02	13-Week Toxicity Study of Mice	CD-1 Mice	SC	week 1: 3 wk, week 2-13: 1 wk	0 1 3 10 30	no effect 75% 88% 87% 94%	no effect 14x 30x 63x 125x		 1.52 7.14	NO	NO	10 and 30 mg/kg: kidney and spleen weight increased 30 mg/kg: 1.3-1.6 fold increase of ALT and AST
	12-Week study in Rats	Sprague-Dawley Rats	SC		0.8 3.2	80%	>50x			NO	NO	
	16-Week Study in Mouse-Model of Diabetes	db/db Mice	SC	Weekly	2	88%		36% blood glucose reduction 43% HbA1c reduction		NO	NO	
	26-Week Study in Rat-Model ofDiabetes	Zucker Diabetic Fatty Rats	SC		1.6	85%		50% blood glucose reduction 40% HbA1c reduction		NO	NO	
AS03	13-Week Toxicity Study inMonkeys	Cynomolgus-Monkey	SC	week 1: 3 wk, week 2-13: 1 wk	0 1 3 10 30	no effect 42% 65% 70% 75%	no effect no effect 6x 16x 90x		 0.6 5.66 20.6 44.6	NO	NO	10 and 30 mg/kg: at day 14 creat increase of 15% and 40%, respectively. No pathology findings.
	6-Week Pharmacodynamic-Evaluation Monkeys	Cynomolgus-Monkey	SC	week 1: 3/wk, week 2-6: 1 wk	2 24	66% 85%	no effect >100x			NO	NO	
AS06	8-Week Repeated Dose Pharmacokinetic and Pharmaco-dynamic Evaluation in Dogs	Beagle Dogs	SC oral oral SC SC	Daily Daily Daily week 1: 3/wk, week 2-8: 1/wk week 1: 3/wk, week 2-8: 1/wk	0.5 25 100 0 30				0.42 27.6	NO	NO	
	6.5-Week Pharmaco-dynamicEvaluation in Dogs	Beagle Dogs	SC	Weekly	1 10	85% 95%	7.5x >250x			NO	NO	
AS09	8-Week Study to Assess RenalFunction in Monkeys	Cynomolgus-Monkey	SC	wk 1: 3/wk, wk 2-13: 1/wk Weekly	0 10 30							30 mg/kg with loading dose: 1. 44% increase creat, and B2M increase 15 fold 2. Pathology findings: tubular nephropathy at week 2 in 3/4 animals 30 mg/kg without loading dose: no significant findings

VIII

NEDERLANDSE SAMENVATTING

In dit proefschrift worden de eerste experimenten in mensen beschreven met een nieuw kandidaat-geneesmiddel, ISIS 388626. Deze experimenten hebben tot doel om te onderzoeken of ISIS 388626 veilig aan mensen kan worden gegeven en de beoogde werking heeft. ISIS 388626 is een antisense oligonucleotide. De werking van traditionele geneesmiddelen berust veelal op remming of stimulatie van bepaalde eiwitten, waardoor de functie ervan wordt beïnvloed. Het werkingsmechanisme van antisense oligonucleotiden is anders, aangezien het de aanmaak van het gehele eiwit tracht te voorkomen. Antisense oligonucleotiden zijn opgebouwd uit een reeks van 12 tot 24 nucleïne zuren, die precies past aan een deel van het MRNA van een eiwit, bijvoorbeeld een enzym, transporteiwit of receptor [1]. Door te binden aan het MRNA, kan de translatie niet plaatsvinden en wordt het eiwit minder geproduceerd. Het concept is veelbelovend omdat het specifieke remming mogelijk maakt van alle eiwitten waarvan de bijbehorende MRNA opbouw bekend is. ISIS 388626 bindt aan het MRNA van het SGLT2 eiwit. SGLT2 is een transporteiwit dat zorgt voor reabsorptie van glucose uit de voorurine in de nier. Remming van SGLT2 leidt tot toegenomen glucose uitscheiding in de urine en is een manier om type 2 diabetes te behandelen [2-4].

In de dieronderzoeken die werden verricht met ISIS 388626 bleek dit een effectieve en veilige strategie [5;6]. In deze proeven werd aangetoond dat bij doseringen van 1-3 mg/kg het SGLT2 MRNA effectief werd geremd, hetgeen leidde tot een aanzienlijke toename van de renale uitscheiding van glucose [5]. Er werden geen tekenen van toxiciteit of achteruitgang van nierfunctie waargenomen [5;7]. Op basis hiervan werd de equivalente dosering voor mensen die veilig en effectief zou zijn geschat te liggen tussen 1 en 3 mg/kg/week [5-7]. Dit vertaalt zich naar doseringen tussen de 50 en 200 mg/week (0.7-2.8 mg/kg uitgaande van 70kg gewicht). Dit is in lijn is met doseringen van vergelijkbare antisense oligonucleotiden die het gewenste effect lieten zien en veilig waren bij mensen [8-10]. Het oorspronkelijke ontwerp voor de klinische studie bestond uit een dubbelblind, placebo-gecontroleerd onderzoek waarbij eerst enkelvoudige doses in opklimmende sterkte (*single ascending doses;* SAD) aan de proefpersonen zouden worden toegediend om de veiligheid van het middel te onderzoeken. Dit werd gevolgd door onderzoek met een meervoudige wekelijkse dosering in opklimmende sterkte, met in de eerste week een oplaaddosis van 3 doseringen (*multiple ascending dose;* MAD). In deze MAD-studie werd naast veiligheid ook gekeken naar het beoogde effect, door de glucose excretie in de urine te testen en ook de metabole reactie op een grote glucose inname ('orale glucose tolerantie test'). De enkelvoudige doseringen in de SAD studie leidden niet tot veiligheidsproblemen. Echter, in de MAD studie werden stijgingen in serum creatinine opgemerkt bij 50 en 100 mg doseringen, die kunnen wijzen op een nadelig effect op de nier. Om deze reden werd het onderzoek voortijdig gestopt. Ook andere markers voor nierschade waren toegenomen bij deze proefpersonen. Farmacodynamisch effect (toename van glucose in de urine, glucosurie) werd nog niet gezien bij deze doseringen. Deze effecten, die suggestief waren voor nierschade waren in dieren niet opgemerkt. Daarom werd besloten nader onderzoek in dieren te verrichten. Deze onderzoeken toonden aan dat ook in dieren dergelijke effecten ontstonden, die voorbijgaand waren en daardoor in eerdere proeven waren gemist [5;7;11]. Ook bleek in extra onderzoek bij apen dat deze effecten alleen optraden als de oplaaddosis werd toegepast. In de groep proefdieren die alleen wekelijkse doseringen kregen zonder oplaaddosis, werd geen creatinine stijging gezien, terwijl er wel voldoende glucosurie ontstond. De klinische proeven werden herstart om doseringen van 50, 100 en 200 mg ISIS 388626 gedurende 13 weken zonder oplaaddosis te onderzoeken. Niettemin ontstond in de groep met 50 mg dosering een gemiddelde stijging in serum creatinine en urine markers. Deze stijging verdween na het stoppen met doseren. Er werd een extra onderzoek verricht met een wekelijkse dosis van 50 mg gedurende 13 weken om eventuele functionele nierschade bij de mens nader te onderzoeken. De nierfunctie werd bepaald door middel van een meting van de glomerulaire filtratie snelheid en de renale doorbloeding. Dit onderzoek toonde geen functionele achteruitgang van de nier, terwijl er wel creatinine stijgingen optraden. In de groepen behandeld met 50 mg trad de beoogde glucosurie nog niet op. Gezien de milde en volledig omkeerbare effecten en het ontbreken van verlies van nierfunctie, werden vervolgens de doseringen 100 en 200 mg getest, waarbij wel glucosurie ontstond. Echter, het effect was klein; bij een dosering van 200 mg werd maximaal 1.3 g/dag glucose in de urine uitgescheiden, terwijl met andere SGLT2 remmers tot 60-70 g/dag wordt bereikt [12-15]. De biomarkers voor nierschade liepen verder op in de groepen deelnemers die doseringen van 100 of 200 mg kregen toegediend. Als enige andere belangrijke bijwerking werd in 8-19% van alle deelnemers roodheid van de injectieplekken gezien (Injection Site Reactions, ISRS). Het effect van ISIS 388626 op de nier biomarkers

is onbegrepen. Andere SGLT2 remmers geven ook een serum creatinine stijging, maar deze kan plausibel verklaard worden door de natriurese die ontstaat als gevolg van de uitgesproken glucosurie [16] en heeft waarschijnlijk dus een andere origine. De milde glucosurie die ISIS 388626 teweeg bracht kan worden geduid als gewenst farmacodynamisch effect, maar zou ook kunnen passen bij milde tubulaire dysfunctie. Sommige eerder onderzochte oligonucleotiden zijn ook geassocieerd met stijging van nier biomarkers [17-19]. Weer andere vergelijkbare oligonucleotiden geven dergelijke effecten niet [20-22]. De oorzaak van dit verschil is nog onduidelijk. In dat licht is het raadzaam om bij klinische experimenten met oligonucleotiden altijd nier biomarkers en eventueel functionele testen van de nier in te bouwen. Zeker gezien het feit dat effecten op de nier in dieren maar weinig voorspellend zijn voor de effecten in de mens.

De glucosurie die werd bereikt was minimaal en het effect op de nier aanzienlijk en onbegrepen. Daarmee is het therapeutisch venster van ISIS 388626 smal. Mogelijk heeft de beoogde doelgroep meer baat bij deze therapie, omdat patiënten met type 2 diabetes mellitus een hogere SGLT2 expressie hebben. Echter, meer onderzoek naar de effecten op de nier is nodig voordat verdere klinische ontwikkeling van ISIS 388626 verantwoord zou zijn.

De effecten van ISIS 388626 op de nier waren onverwacht, aangezien initieel geen enkel signaal werd gezien gedurende de uitgebreide preklinische studies in verschillende diersoorten. Dit illustreert het belang van het bepalen en volgen van effecten op de nier in zogenaamd *vroege fase* klinisch geneesmiddelonderzoek. Vaak worden bepalingen serum creatinine en ureum ingezet, maar deze markers zijn weinig gevoelig en vroege schade kan worden gemist [23]. Daarom verdient het aanbeveling ook markers te overwegen waarvan in dieronderzoek is aangetoond dat ze potentieel informatiever zijn, zoals KIM1, B2M, aGST en NAG. Van deze markers is beschreven dat ze eerder reageren dan serum creatinine en daarnaast geven deze biomarkers inzicht in de mogelijke aard en plaats van de nierschade. Een belangrijke beperking van nier biomarkers is het ontbreken van validatie voor het klinisch gebruik, aangezien vooral dierstudies beschikbaar zijn in de huidige literatuur. Gedurende de klinische studies met ISIS 388626 correleerde vooral KIM1 en B2M met de creatinine respons, hetgeen het vermoeden van de tubulaire origine van het effect leek te bevestigen. Er zou echter meer informatie beschikbaar moeten zijn over de klinische bruikbaarheid van de markers. Ook zou het meten van de markers laagdrempeliger en efficiënter moeten worden. Een andere beperking is het ontbreken van een geschikte biomarker die specifiek is voor schade aan de glomeruli van de nier.

De onderhuidse injectie van ISIS 388626 leidde in een aantal proefpersonen tot ISRS. Alle oligonucleotiden die subcutaan worden toegediend geven dergelijke reacties, de mate en ernst van de reacties verschilt per oligonucleotide. Vanuit de literatuur en ervaring binnen CHDR kan worden geconcludeerd dat hoogte van dosis een belangrijke rol speelt. Hogere doses resulteren in hogere lokale blootstelling in de huid en ernstiger reacties. Milde reacties bestaan meestal uit erytheem en ongemak. Er kunnen ook ernstiger reacties optreden die zich manifesteren als induratie, ulceratie en necrose. ISRS veroorzaakt door oligonucleotiden zijn zeer beperkt beschreven in de literatuur en de pathofysiologie is onbekend. Vermoedelijk speelt immunologische activatie via de aangeboren immuunreceptoren zoals *Toll-Like Receptoren* (TLRS) een rol en mogelijk ook complement activatie. Als er oligonucleotiden ontwikkeld zouden kunnen worden die geen ISRS geven, zou dit het potentiele succes van oligonucleotiden aanzienlijk kunnen vergroten. Hiertoe is meer kennis nodig over deze specifieke huidreacties en immuno-stimulatoire eigenschappen van oligonucleotiden in het algemeen. Weefselonderzoek van de laesies met immuun-kleuringen zou mogelijk inzicht bieden. Ook zouden alle klinische studies systematisch en bij voorkeur op een uniforme manier het optreden van ISRS moeten rapporteren om zo de gedeelde kennis te vergroten.

Het gunstige profiel van ISIS 388626 in dierproeven, gecombineerd met de recente succesvolle ontwikkeling van andere SGLT2 remmers, maakte ISIS 388626 een veelbelovend kandidaat-geneesmiddel. De SGLT2 remmers die momenteel op de markt zijn hebben de beperking dat er niet meer dan 50% remming van terug reabsorptie van gefiltreerd glucose wordt bereikt. Mogelijk door een compensatoire toename van SGLT1 activiteit. Het blijft onduidelijk of een maximaal farmacodynamisch effect van antisense remming van SGLT2 dit plafond zou ontstijgen. Echter, de onverwachte en onbegrepen effecten op de nier van ISIS 388626, maakt het klinisch testen van hogere doseringen onacceptabel vanuit een veiligheidsperspectief. Om deze reden is de klinische ontwikkeling stopgezet.

In het algemeen zijn antisense oligonucleotiden een aantrekkelijke klasse geneesmiddelen. Ze zijn 'op maat gemaakt', zeer specifiek voor het doeleiwit, en bewerkstelligen remming op een geheel ander niveau dan

klassieke geneesmiddelen door te interfereren met synthese van eiwit. Maar de belofte die uitging van vele kandidaat-geneesmiddelen van deze klasse die klinisch werden getest heeft nog niet tot veel successen geleid. Momenteel is mipomersen het enige geregistreerde oligonucleotide. Dit wordt gebruikt voor de behandeling van patiënten met een homozygote familiaire hypercholesterolemie. Er is een aantal belangrijke uitdagingen te overwinnen om bredere implementatie van oligonucleotide therapie in de klinische praktijk te bereiken. Enkele uitdagingen zijn geïllustreerd in dit proefschrift. Accumulatie van oligonucleotiden lijkt een rol te spelen bij zowel de nadelige effecten op nier als bij de huideffecten. Alternatieve toedieningsroutes worden onderzocht, waarbij bijvoorbeeld ISRs zouden kunnen worden voorkomen door orale toediening [24]. De mate van accumulatie in de nier hangt af van de chemische structuur [5;25;26]. Dit zou meegewogen moeten worden in toekomstige studies met oligonucleotiden. Strategieën om accumulatie te voorkomen zijn bijvoorbeeld het veranderen van de distributie eigenschappen door het gebruik van oligonucleotide conjugaten, zoals peptiden, proteïnes, koolhydraten en aptameren [27]. De nauwe grens tussen accumulatie die nodig is om effect te bewerkstelligen en de accumulatie die toxiciteit geeft zou verder moeten worden bestudeerd. Preklinische en vroege klinische studies zouden zich moeten richten op het vermijden van ongewenste effecten. Dit zou de weg kunnen banen voor oligonucleotiden met een beter profiel, geschikt voor chronisch gebruik en toepasbaar voor brede patiënt groepen.

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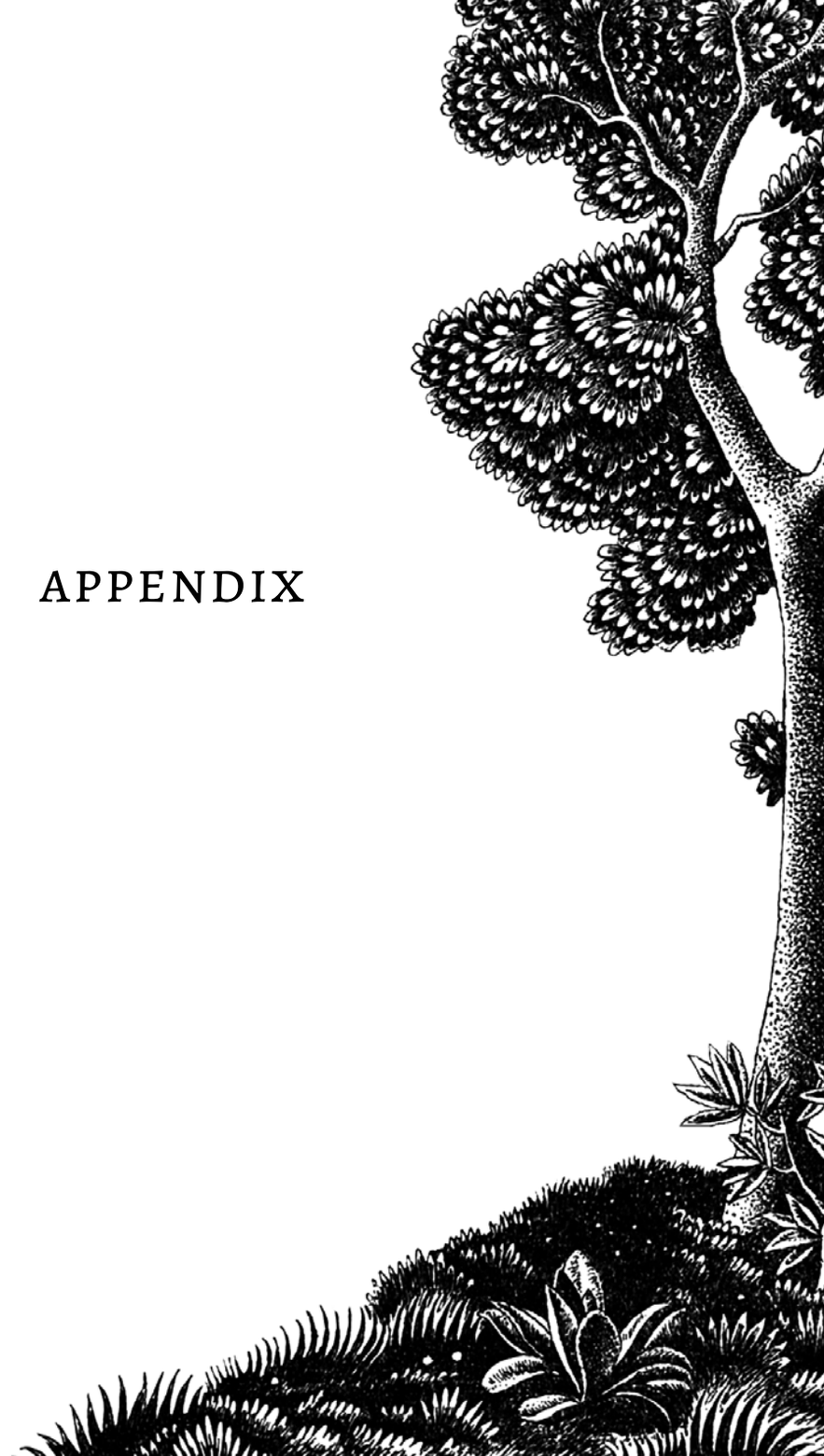
APPENDIX



CURRICULUM VITAE

Leonie werd geboren te Haarlem in 1984. Na het behalen van haar Gymnasium diploma aan het Kennemer Lyceum in Overveen, startte ze met haar studie Geneeskunde in Leiden. Tijdens haar derde studie jaar stortte ze zich op de organisatie van het Veerstichting Symposium 2005 en was ze werkzaam bij Eurotransplant. Na het afronden van de examens, vertrok ze voor een jaar naar Parijs om Frans te leven en daarbij basaal wetenschappelijk onderzoek te doen op een nefrologisch/fysiologisch laboratorium van Université Paris-Descartes. Bij terugkomst startte ze met haar co-schappen, eindigend met een semi-arts stage op de interne geneeskunde. Aangetrokken door het onderzoek, begon ze eind 2010 als promovendus bij het Centre for Human Drug Research (CHDR) te Leiden. In 2014 werd ze aangenomen voor de opleiding tot internist in het LUMC en is in dat kader werkzaam in het HMC Bronovo te Den Haag sinds mei 2015. Leonie heeft de aantekening klinische farmacologie. Ze woont in Den Haag met haar man Bertolt de Vos van Steenwijk en haar drie kinderen, Floor, Justus en Bénine.

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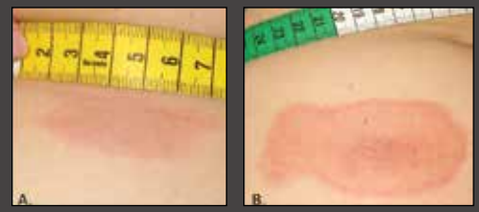
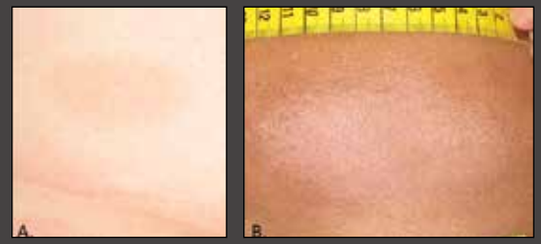
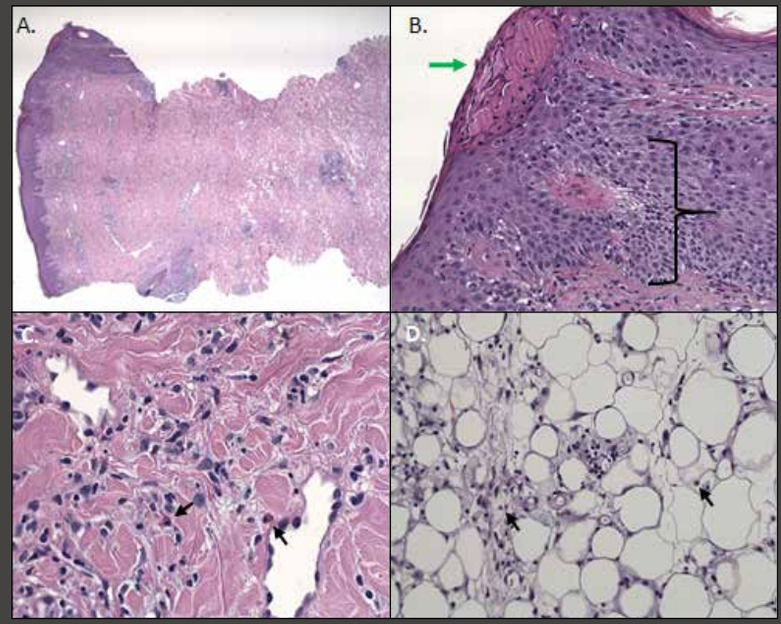
NOTES

CHAPTER 6 – Figure 4 Examples of ISRs, ranging from mild erythema of several cm's (A) to pronounced erythema of > 10cm with central ulceration (B)



CHAPTER 6 – Figure 5 Examples of persisting discoloration of the skin. Mild hyperpigmentation (A) and hypopigmentation (B)



CHAPTER 6 – Figure 6 The histology of a biopsy of an erythematous ISR. A. Overview of biopsy. B. Spongiosis with exocytosis of lymphocytes and parakeratosis with serumcrustae. C. infiltration with eosinophilic granulocytes. D. Necrosis of the subcutaneous fat tissue and infiltration with eosinophilic granulocytes.