available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Cross-species comparison of in vivo PK/PD relationships for second-generation antisense oligonucleotides targeting apolipoprotein B-100

Rosie Z. Yu *, *Kristina M. Lemonidis, Mark J. Graham, John E. Matson, Rosanne M. Crooke, Diane L. Tribble, Mark K. Wedel, Arthur A. Levin, Richard S. Geary*

Primary Laboratory of Origin, Isis Pharmaceuticals, Inc., 1896 Rutherford Road, Carlsbad, CA 92008, United States

ARTICLE INFO

Article history:
Received 24 July 2008
Accepted 6 November 2008

Keywords:
Pharmacokinetics
Pharmacodynamics
ApoB
Lipid lowering
Cross-species
Antisense

ABSTRACT

The in vivo pharmacokinetics/pharmacodynamics of 2′-O-(2-methoxyethyl) (2′-MOE) modified antisense oligonucleotides (ASOs), targeting apolipoprotein B-100 (apoB-100), were characterized in multiple species. The species-specific apoB antisense inhibitors demonstrated target apoB mRNA reduction in a drug concentration and time-dependent fashion in mice, monkeys, and humans. Consistent with the concentration-dependent decreases in liver apoB mRNA, reductions in serum apoB, and LDL-C, and total cholesterol were concurrently observed in animal models and humans. Additionally, the long duration of effect after cessation of dosing correlated well with the elimination half-life of 2′-MOE modified apoB ASOs studied in mice ($t_{1/2} \cong 20$ days) and humans ($t_{1/2} \cong 30$ days) following parental administrations. The plasma concentrations of ISIS 301012, observed in the terminal elimination phase of both mice and monkeys were in equilibrium with liver. The partition ratios between liver and plasma were similar, approximately 6000:1, across species, and thus provide a surrogate for tissue exposure in humans. Using an inhibitory E_{max} model, the ASO liver EC_{50s} were 101 ± 32, 119 ± 15, and 300 ± 191 μg/g of ASO in high-fat-fed (HF) mice, transgenic mice containing the human apoB transgene, and monkeys, respectively. The estimated liver EC_{50} in man, extrapolated from trough plasma exposure, was 81 ± 122 μg/g. Therefore, extraordinary consistency of the exposure–response relationship for the apoB antisense inhibitor was observed across species, including human. The cross-species PK/PD relationships provide confidence in the use of pharmacology animal models to predict human dosing for second-generation ASOs targeting the liver.

1. Introduction

Applying pharmacokinetic and pharmacodynamic analyses to guide and expedite drug development is well recognized and has received increasing interest in recent years [1]. Although these principles are well accepted and widely used for low molecular weight drugs and proteins, there have been a growing number of reports describing pharmacokinetic and

* *Corresponding author*. Tel.: +1 760 603 2549; fax: +1 760 603 3862.
E-mail address: Ryu@isisph.com (R.Z. Yu).

Abbreviations: ASO, antisense oligonucleotide; ApoB-100, apolipoprotein B-100; VLDL-C, very low density lipoprotein cholesterol; IDL-C, intermediate density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HF-fed, high-fat diet fed; IACUC, Institutional Animal Care and Use Committee; s.c., subcutaneous; i.p., intraperitoneal; ELISA, enzyme-linked immunosorbent assay; LLOQ, the lower limit of quantitation; CGE, capillary gel electrophoresis; SPE, solid phase extraction; MOE, 2′-O-(2-methoxy) ethyl.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.11.005

pharmacodynamic relationships with antisense therapeutic agents [2–5].

The pharmacokinetics of 2′-methoxyethyl (2′-MOE) chimeric phosphorothioate antisense oligonucleotides (ASOs), or second-generation ASOs, have been described in a number of species including man [6–10]. The structure of the second-generation ASOs is characterized by five 2′-MOE modifications on the ribose sugar at both 5′- and 3′-termini, flanking a 10-nucleotide oligodeoxynucleotide gap (5-10-5 2′MOE chimeras) with phosphorothioate backbone. Although there are slight sequence dependent differences, the pharmacokinetics of the second-generation ASOs are remarkably similar and are characterized by predictable distribution and prolonged tissue elimination (14–30 days) as compared to first-generation oligonucleotides.

Although, ASOs have been shown to be able to work by multiple mechanisms once bound to the target RNA [11], all of the 2′MOE chimeric ASOs in development have been shown to work by forming RNA–ASO duplexes that serve as a substrate for RNase H_1 [12,13]. RNase H_1 cleaves the RNA to which the ASO is bound, resulting in loss of the target RNA and eventually the protein, both of which have been measured as a direct means of evaluating pharmacodynamic effects.

Consequently, the ASO prevents the translation of the encoded protein product in a highly sequence-specific manner. Because of the unique mechanism of action of antisense therapeutics, investigations of the pharmacological effects of antisense oligonucleotide in vivo have focused primarily on target mRNA reduction and the subsequent reduction in protein translation and the downstream effects resulting from target protein reduction, which are dependent on the target studied. Moreover, establishment of the correlation between plasma equilibrium concentrations with the concentrations at the target sites is pertinent, enabling plasma concentrations to be used as a surrogate in clinical studies to establish the pharmacodynamics and pharmacokinetics relationships. Translation of preclinical to clinical PK/PD relationships require predictive pharmacokinetics and reliable PD biomarkers that can be assessed in real time. In this report, we present data from multiple species for potent apolipoprotein B-100 (apoB-100) 2′-MOE chimeric antisense compounds that provides both of these prerequisites; predictable cross-species pharmacokinetics [9] and reliable biomarkers, apoB itself and its related LDL-cholesterol particles measured in serum in real time.

ApoB-100 is the protein component of atherogenic lipids and triglycerides, including LDL-cholesterol. ApoB-100 is synthesized and packaged into lipoprotein particles principally in the liver of all species [14–16]. Circulating LDL-C, which typically constitutes 60–70% of serum cholesterol, is widely recognized as a major risk factor for coronary heart disease (CHD) and has been implicated in the inflammation associated with the pathogenesis of atherosclerosis. Similarly, apoB-100 is now recognized as a risk factor for atherosclerosis. This led to the development of an antisense inhibitor of the molecular target, apolipoprotein B (apoB), for use in lowering apoB-100 and subsequent lowering LDL-C.

Since the sequence of mRNAs for apoB-100 differs depending on the species and 2′-MOE ASOs are highly specific, we have used species-specific apoB-100 ASOs to demonstrate potent dose-dependent reduction of apoB-100 mRNA and protein in the liver of all species tested and concomitant reductions in plasma apoB and apoB-100 containing lipoproteins [17,18]. Species-specific apoB antisense inhibitors had been evaluated in multiple animals species, including mice, hamsters, rabbits and monkeys (lean and HF-fed) and it had been demonstrated that administration of the apoB-100 antisense inhibitor produced significant pharmacological effects, i.e., significant reductions in mRNA and liver protein with concomitant reductions in serum apoB, LDL-C, and total cholesterol [18]. All the apoB antisense inhibitors evaluated are 20-mer phosphorothioate oligonucleotide with 2′-O-(2-methoxy) ethyl (MOE) modification on the 5 nucleotides on both 3′ and 5′ termini. MOE modifications provide enhanced resistance to nucleases, a longer target organ half-life, and reduced toxicity [19,20]. In addition, these modifications increase the affinity of an antisense oligonucleotide for complementary target mRNA, resulting in enhanced potency and specificity [20–22].

ISIS 301012 (Mipomersen), the human specific apoB-100 antisense inhibitor, is currently in Phase 3 clinical development in familial hypercholesteremia. ISIS 301012 has been shown to produce consistent and predictable dose-dependent and exposure-dependent reduction in serum atherogenic lipids and lipoproteins in human subjects [23–26]. These dose- and exposure–response correlations have been demonstrated in all clinical subject populations studied to date, including healthy volunteers, subjects with mild hypercholesterolemia, polygenic hypercholesterolemia subjects on stable statin therapy, and homozygous familial hypercholesterolemia subjects on stable statin therapy.

In this paper, we present for the first time remarkable cross-species correlates of PK/PD associations at the molecular level for apoB-100 antisense oligonucleotides. Herein we will summarize the pharmacokinetics and pharmacodynamics of apoB antisense inhibitors in fat fed mice, transgenic human apoB-100 mice, monkeys and humans using species-specific apoB antisense inhibitors. The comparisons in pharmacodynamics across species provide guidance in selection of predictive animal models for the development of future antisense oligonucleotides in this chemical class.

2. Methods and materials

2.1. Materials and reagents

2.1.1. Oligonucleotides

ISIS 147764, ISIS 326358 and ISIS 301012 are 20-nucleotide second-generation antisense oligonucleotides targeting apoB mRNA in mice, monkeys and humans, respectively. All the compounds have MOE modifications at positions 1–5 and 15–20 (Table 1). ISIS 13866, a 2′MOE -modified oligonucleotide at positions 15–21 (underlined) with a sequence of 5′-GCG TTT GCT CTT CTT^{M}CTTG^{M}CG TTT TTT-3′, was used as the internal standard for quantitation of ASO in tissues. In addition, all the cytosines of the compounds were modified to contain a 5-methyl group (5-methyl cytosine, ^{M}C). The sequence of the binding site in the monkey differs by only two nucleotides in the region that binds the 3′-MOE wing. Thus, ISIS 301012 is

Table 1 – Testing compounds targeting apoB mRNA across species.

Isis compound number	Species	Sequence (5′ → ′3)
ISIS 147764	Mouse	GT^{M}C^{M}C^{M}CTGAAGATGT^{M}CAATG^{M}C
ISIS 326358	Monkey	G^{M}C^{M}CT^{M}CAGT^{M}CTG^{M}CTTTA^{M}CA^{M}C^{M}C
ISIS 301012	Human	G^{M}C^{M}CT^{M}CAGT^{M}CTG^{M}CTT^{M}CG^{M}CA^{M}C^{M}C

Underline denotes 2′-O-methoxyethyl-modification. The cytosines in the compounds were modified to contain a 5-methyl group (5-methyl cytosine, ^{M}C).

pharmacologically active in the monkey, albeit much lower potency than the monkey-specific ASO, ISIS 326358.

All the studied ASOs were synthesized using an automated DNA synthesizer Model 380B (Applied Biosystems, Inc., Foster City, CA) and purified as previously described [22]. The purity of the compounds used in this study was >90%.

2.1.2. *Chemicals and reagents*

Tween 20 and phenol:chloroform:isoamyl alcohol 25:24:1 was obtained from SIGMA Chemical Co. (St. Louis, MO). Reacti-Bind™ NeutrAvidin coated polystyrene 96-well plates and SuperBlock TBS Blocking Buffer were purchased from Pierce (Rockford, IL). Tris–HCl and Na_2HPO_4 was obtained from J.T. Baker, (Phillipsburg, NJ), NaCl from Fisher Scientific (Fair Lawn, NJ), anti-digoxigenin-AP (conjugated with alkaline phosphatase, Fab fragments) was obtained from Roche Diagnostics Corporation (Indianapolis, IN). The alkaline phosphatase fluorescent substrate AttoPhos® and its reconstitution solution were purchased from Promega Life Science (Madison, WI).

2.2. *Animals and treatments*

All animal studies were conducted utilizing protocols and methods approved by the Institutional Animal Care and Use Committee (IACUC) and carried out in accordance with the Guide for the Care and Use of Laboratory Animals adopted and promulgated by the U.S. National Institutes of Health.

2.2.1. *Mice*

2.2.1.1. HF-fed C57BL/6 mice. Four to 6-week-old male C57BL/6 mice (Charles River Laboratories, Inc., Wilmington, MA) fed a HF diet were administered ISIS 147764 5, 25, or 50 mg/kg or control ASO at 50 mg/kg intraperitoneally (i.p.), twice weekly for 6 weeks. Animals were sacrificed at 48 h and 1, 2, 4, and 6 weeks after the last dose. Oligonucleotide concentrations in the liver, hepatic apoB mRNA and protein levels, serum apoB-100, total-C, and LDL-C levels were measured at each time point.

2.2.1.2. Human apoB expressing transgenic mice. Six-week old female human apoB expressing transgenic mice (C57BL/6 hemizygous for full length human gene-Taconic, Hudson, NY) were administered ISIS 301012 at 2.5, 5, 10 and 25 mg/kg or control ASO at 25 mg/kg i.p., twice weekly for 6 weeks. Animals were sacrificed 48 h after receiving the last dose and samples collected were measured for hepatic oligonucleotide concentrations and human apoB mRNA expression.

2.2.2. *Monkeys*

2.2.2.1. Lean cynomolgus monkeys. ISIS 301012 was administered to male and female cynomolgus monkeys (*Macaca fascicularis*; Sierra Biomedical Animal Colony, Sparks, NV) at 4 mg/kg by 1-h i.v. infusion every other day for 4 doses (1 week). On Days 7, blood was collected for quantitation of ISIS 301012 in plasma by peripheral venipuncture into EDTA containing vacutainers just prior to dosing and at 1, 2, 4, 8, 24 and 48 h following i.v. infusion. Following the 4th dose of 4 mg/kg ISIS 301012, monkeys were euthanized and additional plasma samples and liver samples were taken 3, 4, 8, 16, 32 and 48 days after the fourth dose to assess the terminal elimination half-life in plasma and liver (1/sex/time point).

2.2.2.2. High-fat-fed (HF-fed) cynomolgus monkeys. The cynomolgus monkeys (*M. fascicularis*; Charles River Laboratories Animal Colony, Sparks, NV) were fed an HF diet (~43.9% fat, 21.3% protein, and 35.2% carbohydrate), monkeys for 3 weeks prior to treatment and continued throughout the study period. Monkey-specific apoB antisense inhibitor, ISIS 326358 was administered subcutaneously (s.c.) at 5, 10 or 35 mg/kg/week, to monkeys for 5 weeks. The oligonucleotide sequences for ISIS 326358 and ISIS 301012 (human apoB inhibitor) differ by only two bases. Doses were given on alternate days for the first 3 doses (loading) and twice weekly thereafter (maintenance). Oligonucleotide concentrations in the liver, liver apoB mRNA/protein levels, serum apoB-100, total-C, and LDL-C levels were measured at various time points.

2.2.3. *Humans*

Healthy human volunteers received ISIS 301012 as a 2-h i.v. infusion and s.c. injections at doses that ranged from 50 to 400 mg [9,26] in a Phase I clinical study. Briefly, 29 healthy volunteer subjects in this study received either 50 mg (n = 8), 100 mg (n = 8), 200 mg (n = 9), or 400 mg (n = 4) per dose day; seven subjects received placebo. The multiple-dose (MD) period consisted of three i.v. infusions (on Days 1, 3 and 5) over 2 h every other day during the first week, followed by once weekly s.c. injections for 3 weeks (total of 6 doses over 22 days on Days 8, 15 and 22). Intensive pharmacokinetic blood sampling occurred for 24 h following the first i.v. dose (at 0, 0.5, 1, 2, 2.25, 2.5, 3, 4, 6, 8, and 24 h), and again following the last s.c. dose (at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h). Additionally, trough blood samples for determination of elimination half-life were collected at 3, 17, 33, 47, 61, 75 and 89 days after the last s.c. dose. Serum lipoprotein and cholesterol profiling was performed after fasting at pre-dose on Days 1, 8, 15, and 22 and during all the follow-up time points. Selected pharmacokinetic and pharmacodynamic data from the Phase I study are included in this report to compare with preclinical data.

2.3. Methods

2.3.1. Sample extraction and analysis of oligonucleotide concentrations in liver

Liver samples from mice and monkeys were analyzed using a quantitative capillary gel electrophoresis method which is a variation on the method reported previously [27]. The assay was validated for precision, accuracy, selectivity, sensitivity and stability of ISIS 301012 in liver tissues. Quantitation of the other species-specific apoB antisense inhibitors in liver were qualified but not fully validated. These analyses were conducted at Southwest Bio-Labs (Las Cruces, NM) and Isis Pharmaceuticals, Inc. (Carlsbad, CA). Briefly, liver samples were weighed, homogenized in a Bio Savant (Bio 101, Inc., Vista, CA) and then the material was extracted as described [27] with the exception that a phenyl-bonded SPE column (Supelco Inc., Bellefonte, PA) was used. An internal standard (ISIS 13866, a 27-mer 2′-O-methoxyethyl modified phosphorothioate oligonucleotide) was added prior to extraction. Extracted samples were analyzed by CGE using a Beckman P/ACE Model 5010 capillary electrophoresis instrument (Beckman Instruments, Irvine, CA) with UV detection at 260 nm. The limit of quantitation for this assay has been estimated to be 1.52 μg/g oligonucleotide in liver.

2.3.2. Analysis of oligonucleotide concentrations in plasma

Plasma samples in mice, monkeys and humans were analyzed using a quantitative, sensitive hybridization ELISA method which is a variation on the method reported previously [28]. The assay was validated for precision, accuracy, selectivity, sensitivity and stability of ISIS 301012 in plasma. Quantitation of the other species-specific apoB antisense inhibitors in plasma were qualified but not fully validated. Plasma sample analyses were conducted at PPD Development (Richmond, VA) and Isis Pharmaceuticals, Inc. (Carlsbad, CA). The assay conducted with synthesized putative shortened oligonucleotide metabolite standards showed no measurable cross-reactivity confirming the assays specificity for the parent oligonucleotide. The lower limit of quantitation (LLOQ) was determined to be 1.52 ng/mL.

2.3.3. RNA isolation and RT-PCR analysis

Total RNAs were extracted from liver samples using the RNeasy kit (Qiagen, Santa Clarita, CA). RT-PCR analysis was performed using a Prism 7700 Sequence Detector (Applied Biosystems, Inc., Foster City, CA). The species-specific primer probe sets for apoB quantitation were used and values were normalized to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and/or Ribogreen levels.

2.3.4. Lipid and lipoprotein analysis

Serum lipoprotein cholesterol in animal studies was analyzed using a Beckman System Gold 126 HPLC system, with 126 photodiode array detector (Beckman Instruments; Fullerton, CA) on a Superose 6 HR 10/30 column (Pfizer; Chicago, IL). VLDL, LDL, and HDL fractions were measured at a wavelength of 505 nm and validated with a cholesterol calibration kit (Sigma). For each experiment, a three-point standard curve was performed in triplicate to determine the absolute concentration of each lipoprotein fraction [18]. For clinical samples, apoB levels were determined by an immunoturbimetric method (MDS PharmaServices, Belfast). Total cholesterol, LDL-C, HDL-C and triglycerides were measured using standard enzyme based colorimetric assays (MDS PharmaServices, Belfast; and LipoScience, Raleigh, NC) [26].

2.3.5. Pharmacokinetic analysis

Both compartmental and non-compartmental analysis methods were used for pharmacokinetic characterization of the plasma concentration data (WinNonlin 4.0 or 5.0, Pharsight Corporation, Mountain View, CA). First-order elimination rate constants for apoB antisense inhibitors in plasma or liver were calculated using non-compartmental nonlinear regression of the decay curves for plasma or liver. Half-life was calculated by dividing 0.693 by the first-order elimination rate. In animals, the elimination of apoB antisense inhibitors from liver was measured directly, and the liver concentration–time data were analyzed using a non-compartmental model.

2.3.6. Pharmacodynamic analysis

The relation between apoB mRNA, serum apoB reductions and liver concentrations or plasma trough concentrations of ASOs was characterized using a pharmacodynamic model. ApoB mRNA levels following apoB antisense inhibitor treatment were normalized with the ApoB mRNA levels from control animals (treated with saline):

$$E = \frac{\mathrm{mRNA_{treatment}}}{\mathrm{mRNA_{control}}} \times 100 \quad (1)$$

Similarly, serum apoB levels following ASO treatment were normalized with the baseline serum apoB levels of the same animal or subject.

The relationship between inhibitory activity of apoB mRNA or serum apoB (E) and liver concentrations or plasma trough concentrations of ASOs was best described by adopting an inhibitory sigmoidal E_{max} model:

$$E = E_0 - \frac{E_{max} C^n}{EC_{50}^n + C^n} \quad (2)$$

where E_0 is the baseline level; E_{max} is the maximum reduction of apoB mRNA; EC_{50} is the concentration of ISIS 301012 required for half-maximal reduction of apoB mRNA; C is liver concentrations or plasma trough concentrations of ASOs; and n is the sigmoidicity factor.

3. Results

3.1. Pharmacokinetics

The clearance of apoB ASOs from tissues was slow in all species studied. The elimination half-life for the mouse-specific apoB antisense inhibitor, ISIS 147764 in mouse liver was 20 days, while the elimination half-lives for ISIS 301012 were 24 and 34 days in mice and monkeys, respectively (Table 2). Elimination half-life was not determined for ISIS 326358 because ISIS 326358 is a monkey-specific apoB inhibitor and is not going to be developed for use in humans. The

Table 2 – Estimated elimination half-life (in days) of apoB ASOs in mice, monkeys and man.

Species	Compound	Dose	Elimination half-life (days)		Liver/plasma trough concentration ratio
			Plasma	Liver	
Mouse	ISIS 147764	25 mg/kg	NA	20	NM
Mouse	ISIS 301012	2.5–25 mg/kg	NM	24[a]	5861 ± 2342
Monkey	ISIS 301012	4 mg/kg	31.3 ± 0.3[b]	34[b]	5825 ± 2882
Human	ISIS 301012	200 mg (~2.7 mg/kg)	31 ± 11[c]	NA	NM

NA = not applicable; NM = not measured.
[a] Measured in CD-1 mice (internal data).
[b] Animals received 30 mg/kg/week doses of ISIS 301012 for 1 year. For determination of plasma elimination half-life, plasma samples were collected at various time points up to 182 days after last dose (n = 3).
[c] Data presented are mean ± standard deviation (n = 8).

monkey study with ISIS 326358 was to demonstrate pharmacology and evaluate target knock down-related toxicities. The plasma concentration–time profile for ISIS 301012 was multiphasic with a rapid distribution phase and at least one additional much slower elimination phase (Fig. 1). The plasma concentrations of ISIS 301012 observed in the terminal elimination phase in monkeys represent ISIS 301012 that is in equilibrium with liver (Fig. 1) as well as other tissues and thus, equilibrium plasma concentrations provide a measure of tissue elimination rate. Because such a high fraction of the administered dose is retained by the liver and kidney, the majority of the oligonucleotide in circulation at equilibrium should be proportional to the concentrations in those tissues. Indeed, the elimination half-life of ISIS 301012 studied in monkey liver of approximately 30 days was very similar to the elimination half-life observed in monkey plasma, as well as in human plasma (Table 2). Moreover, a similar partition ratio of ISIS 301012 between liver and plasma at equilibrium was observed across species, and was 5861 and 5825 in mice and monkeys, respectively (Table 2). Therefore, it is reasonable to assume this liver to plasma partition ratio at equilibrium in humans would be similar, thus providing a surrogate for liver exposure in the clinic. Taken together, these results suggest

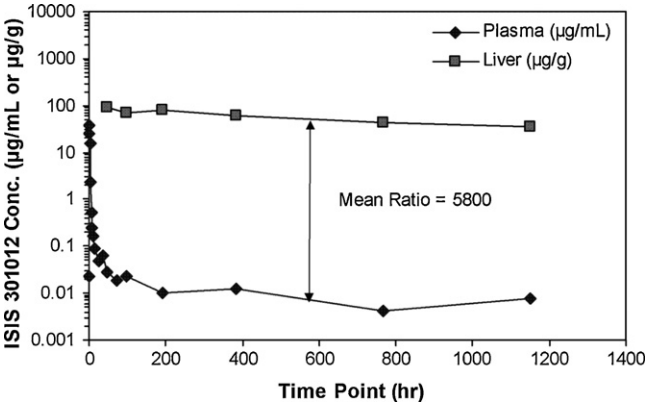

Fig. 1 – Post-distributional phase plasma and liver concentrations of ISIS 301012 in monkeys. Each tissue data point represents average concentrations in two animals. Note that both plasma and tissue concentrations decay similarly over time following cessation of intravenous administration.

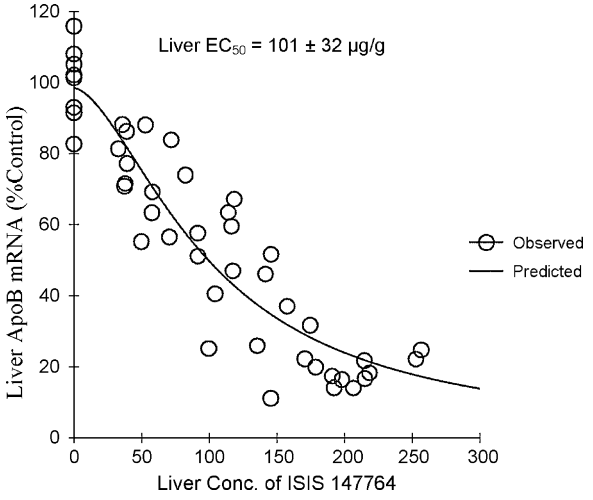

Fig. 2 – Best fit inhibitory effect E_{max} model of ApoB mRNA levels in liver as a function of liver concentrations of ISIS 147764 in HF-fed mice. Each data point represents a single animal (n = 48). Solid lines represent predicted apoB mRNA levels using nonlinear regression.

that the pharmacokinetic behavior of ISIS 301012 in animals is predictive of that in man.

3.2. *Pharmacodynamics of ApoB ASO inhibitors in various species*

ApoB antisense inhibitors act as antagonists to apoB-100 mRNA expression. To elicit their effect on mRNA, they must by definition gain access to the target cell, in this case the hepatocyte. ApoB antisense inhibitors bind to apoB mRNA via Watson–Crick hybridization to form RNA–DNA duplex, subsequently, RNases H cleave apoB RNA strand of the duplex [12,13]. Therefore, the relationships between liver apoB mRNA levels to ASO concentrations in the liver can be described by a typical sigmoidal inhibitory effect E_{max} model after administration of apoB-100 ASOs in animals. The obtained EC_{50} in high-fat-fed mice administered ISIS 147764 was 101 ± 32 µg of ISIS 147764 per gram of liver (Fig. 2). Furthermore, the duration of effect was correlated with the tissue elimination half-life from liver ($t_{1/2}$ = 20 days) in this model (Fig. 3). Approximately 8 weeks after cessation of treatment, apoB mRNA levels had returned to their

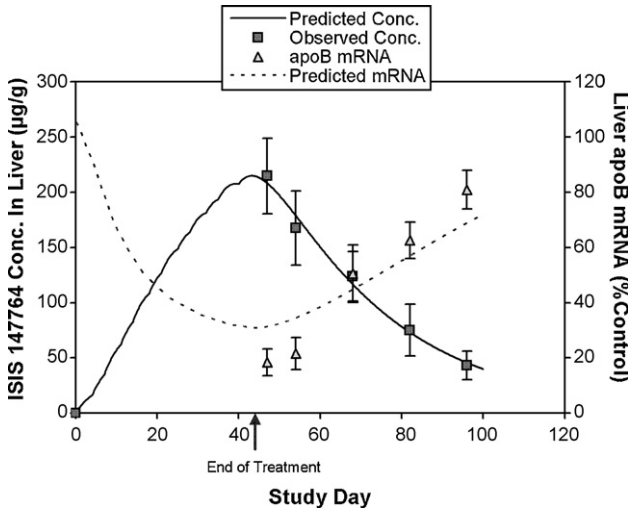

Fig. 3 – Pharmacokinetic/pharmacodynamic relationship of ISIS 147764 in mouse liver following 6 weeks of treatment with mouse specific ApoB antisense oligonucleotide ISIS 147764 (error bars representing standard deviation, *n* = 3). Solid lines represent predicted concentrations in liver using nonlinear regression.

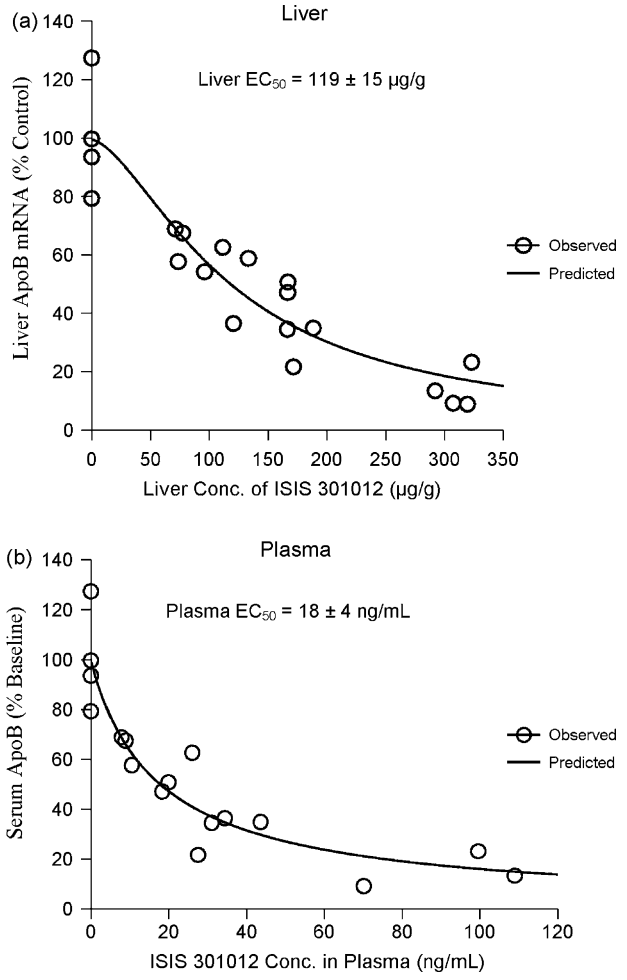

Fig. 4 – Best fit inhibitory effect E_{max} model of ApoB mRNA levels in liver as a function of liver concentrations of ISIS 301012 (a) or plasma trough concentrations of ISIS 301012 (b) in human ApoB transgenic mice. Each data point represents a single animal (*n* = 20). Solid lines represent predicted apoB mRNA levels using nonlinear regression.

pre-dose levels in mice. Similarly, in the human apoB transgenic mouse model, the relationship between human apoB hepatic mRNA expression and concentrations of ISIS 301012 in the mouse liver and in trough plasma (C_{min}) was observed with estimated EC_{50} of 119 ± 15 μg/g in liver and 18 ± 4 ng/mL in plasma (Fig. 4, Table 3). The ratio of the EC_{50} in liver to plasma was consistent with the partition ratio of ISIS 301012 between liver and plasma at equilibrium (Tables 2 and 3). The apoB mRNA reductions in human transgenic mice treated with human specific ISIS 301012 were specific for human apoB mRNA, as no change in murine apoB mRNA was observed in this model (Fig. 5). Additionally, in these animals, the reduction in hepatic apoB mRNA also produced corresponding reductions in liver apoB protein, serum apoB, LDL-C, and total cholesterol (data not shown). In all experiments, control ASOs had no effect on apoB mRNA in the liver, indicating that inhibition of apoB expression was both target- and sequence-dependent.

The liver EC_{50} determined in high-fat-fed monkeys was 300 ± 191 μg/g of species-specific apoB ASO (ISIS 326358) per gram of liver (Table 3, Fig. 6a). Although the estimate of EC_{50} was two-fold higher when compared to the EC_{50} measured in mice, it is not possible to assign a real difference because of the large standard error associated with the estimates. Treatment with ISIS 326358, the monkey-specific ASO, produced a dose- and liver concentration-dependent decrease in liver apoB mRNA, with the corresponding downstream effects, such as serum apoB (Fig. 6a and b) and LDL-C following 5 weeks of treatment at doses ranging from 5 to 33 mg/kg/week. Indeed, by the end of

Table 3 – In vivo pharmacodynamics of ApoB antisense inhibitors across species.

Species	Isis compound number	Liver EC_{50} (μg/g) (estimate ± SE)	Plasma EC_{50} (ng/mL) (estimate ± SE)
Mouse	ISIS 147764	101 ± 32	NM
Human ApoB transgenic mouse	ISIS 301012	119 ± 15	18 ± 4
Monkey	ISIS 326358	300 ± 191	52 ± 33[b]
Human[a]	ISIS 301012	81 ± 122[b]	14 ± 21

SE = standard error of the estimate; NM = not measured.
[a] Phase 1 healthy volunteer study.
[b] A liver/plasma partition ratio of 5800 was used.

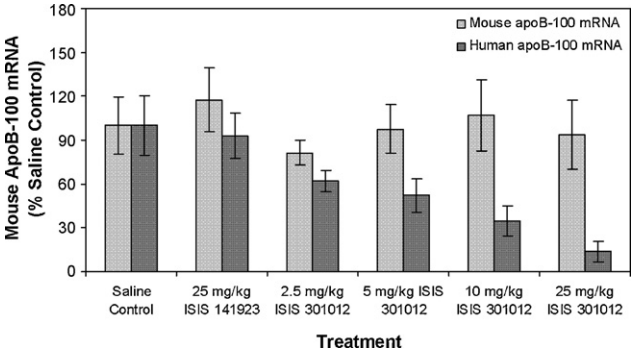

Fig. 5 – Lack of inhibitory effects of control ASO (ISIS 141923) and ISIS 301012 treatment on mouse ApoB-100 mRNA in human apoB transgenic mice following 6 weeks of treatment administered twice a week (error bars representing standard deviation, $n = 4$).

treatment, the mean reduction in serum apoB and LDL-C ranged from 39% to 60% and 46% to 71%, respectively.

The exposure–response relationships were ultimately studied in human volunteer subjects administered ISIS

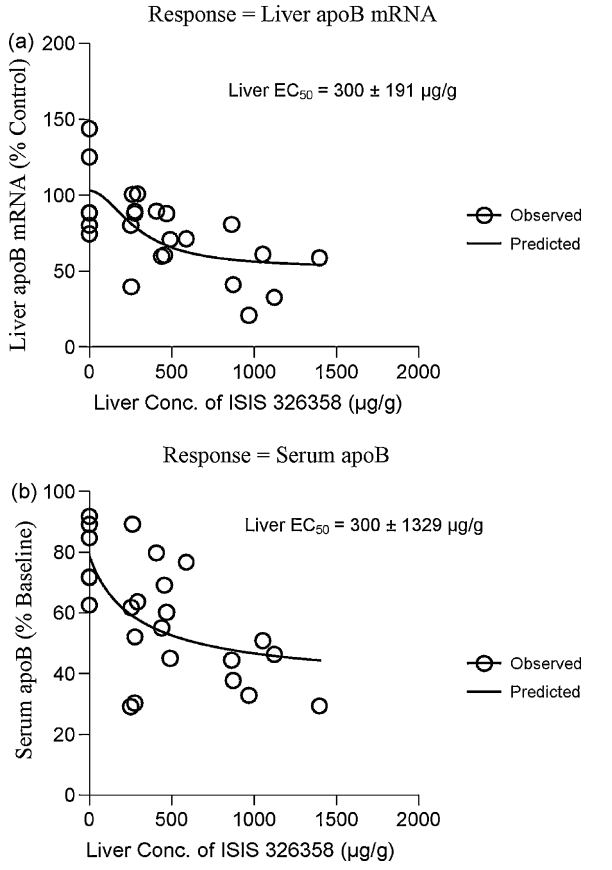

Fig. 6 – Best fit inhibitory effect E_{max} model of ApoB mRNA levels in liver as a function of liver concentrations of ISIS 326358 (a) and serum ApoB levels as a function of liver concentrations of ISIS 326358 in HF-fed monkeys (b). Each data point represents a single animal ($n = 24$). Solid lines represent predicted apoB mRNA levels using nonlinear regression.

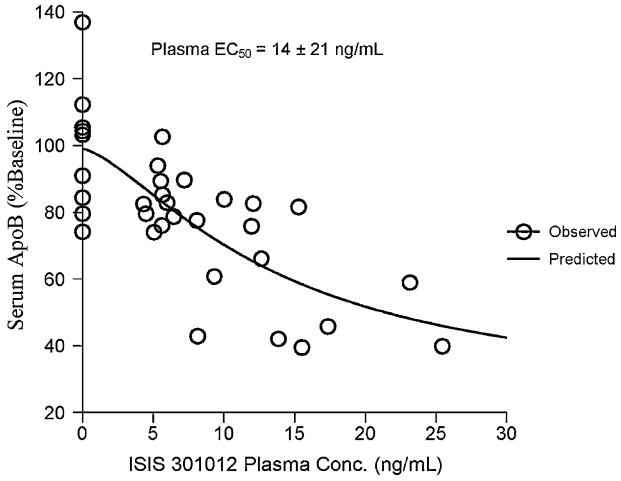

Fig. 7 – Best fit inhibitory effect E_{max} model of serum ApoB (%baseline) as a function of plasma trough concentrations of ISIS 301012 measured at the end of treatment (on MD25) in healthy human volunteers. Each data point represents an individual volunteer subject ($n = 33$). Solid lines represent predicted apoB mRNA levels using nonlinear regression.

301012 in a Phase 1 study in healthy volunteers with mildly elevated LDL-C. ISIS 301012 demonstrated dose-dependent exposure/responses as measured by reductions in both serum apoB and LDL-C [26]. Because ISIS 301012 concentrations in human liver cannot be directly measured easily, the correlation between plasma and liver drug concentrations measured in preclinical models was utilized to estimate the levels of ISIS 301012 in the livers of human subjects (Table 3).

The disposition relationship between ASO plasma trough levels and liver levels were the same as observed in preclinical animal models which led us to determine the relationship between serum apoB levels and plasma trough levels in man. The relationship between serum apoB levels and plasma trough concentrations of ISIS 301012 were effectively described with a sigmoidal inhibitory effect E_{max} model using the data collected on PD14 (17 days after last treatment). The estimated trough plasma concentration that produced 50% of maximum effect (EC_{50}) and predicted liver concentrations, were 14 ng/mL (Table 3, Fig. 7). This plasma level corresponds to an estimated liver exposure of approximately 81 µg/g.

The duration of effects on apoB and related lipids in plasma following cessation of treatment was highly correlated with the slow elimination of ISIS 301012 (Fig. 8). For example, significant reduction ($p < 0.02$) of serum apoB levels from baseline for the 200-mg treatment cohort was achieved for up to 75 days after last dose, consistent with the slow elimination of ISIS 301012 (elimination $t_{1/2}$ of approximately 31 days) (Table 2). Therefore, the pharmacologic effects were prolonged, consistent with the long tissue half-life of the drug.

4. Discussion

Because the direct pharmacological response of antisense therapeutics is target mRNA reduction, establishment of the

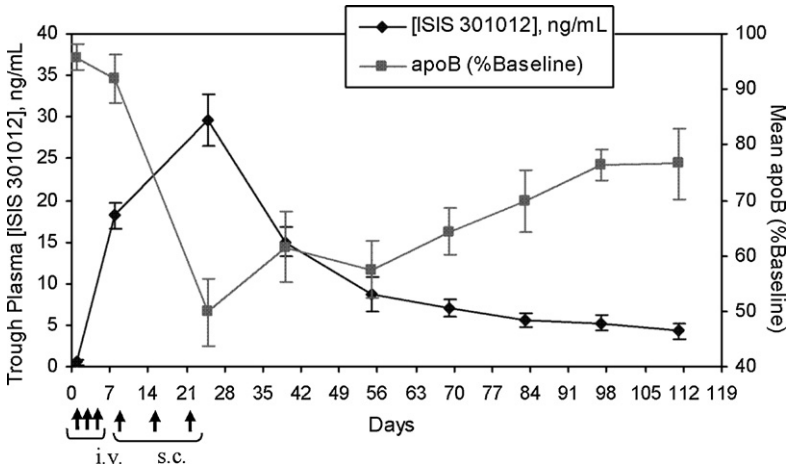

Fig. 8 – Prolonged exposure–response relationships between ISIS 301012 trough plasma concentrations and serum ApoB levels (%baseline) with time in human healthy volunteers during and following six (6) doses of ISIS 301012 at 200 mg per injection. Trough plasma concentrations of ISIS 301012 were measured at ≥72 h from last dose. Data presented are mean ± standard error. Arrows represent dosing times. Last dose was administered on Day 22.

correlation of target organ concentration and target mRNA reduction is most widely used in studying the pharmacokinetic/pharmacodynamic relationships of antisense oligonucleotides in animal models. The pharmacological effect of antisense inhibitors occurs in cells when the antisense inhibitor binds to its cognate mRNA and the RNA strand of the heteroduplex is degraded by RNase H [13]. RNase H_1 cleavage is very rapid, occurring within minutes [13]. Therefore, a direct response pharmacodynamic model was used to describe the liver concentration (exposure) relationship with liver apoB mRNA expression (response) in all species.

We have previously reported that the pharmacokinetics of ISIS 301012 in monkeys is predictive for man on the basis of body weight. Thus a dose of 2 mg/kg ISIS 301012 in the monkey results in similar plasma concentrations at the same dosage (2 mg/kg) in man [9]. It is this remarkable similarity in pharmacokinetics across species that allows for the prediction of exposure from animals models and further, based on these studies across species, allows prediction of response in humans from the preclinical models. Additionally, the lack of impact of mode of parenteral administration on the ultimate tissue pharmacokinetics independent of species has been established for multiple antisense oligonucleotide molecules in this chemical class [8,9,29,30] as well as for ISIS 301012 [9]. The predictive pharmacokinetic behavior of these molecules, independent of there sequence, has been shown in these published accounts to be primarily a consequence of their common biopharmaceutical and physical chemical properties.

Similarities in the pharmacodynamics and potency of apoB antisense inhibitors between species were observed for the apoB-100 ASOs, including mice (both wild type and human transgenic), monkeys, and humans. The observed similarities in pharmacodynamics across species related to the previously observed similarities in pharmacokinetics for ASOs across species [10,31–33]. Peak plasma concentrations of the antisense oligonucleotides like ISIS 301012 do not correlate with drug effect as the effect is post-distribution and intracellular, not in the plasma compartment. This is unlike small molecules and protein therapeutics where plasma concentrations (C_{max} or AUC) directly correlate the drug response [34–37] due to either a rapid equilibrium with intracellular compartments or direct effects occurring in the circulation, Antisense oligonucleotides rapidly and extensively transfer from plasma to tissues following parenteral administrations. However, distribution to the ultimate site of action within the cells of interest takes hours with maximum activity seen 24–48 h after administration [38]. Since the site of activity for the antisense oligonucleotides is intracellular, ASO drug effects are closely related with target tissue concentrations only after distribution from circulation [2–5,33]. Thus, the plasma concentrations of ASO observed in post-distribution phase (plasma trough ASO concentration) represent the ASO that is in equilibrium with target tissue. Because tissue concentrations in human are not usually accessible, establishing the relationship of plasma trough concentration of ASOs with target tissue concentrations (liver) is required to provide a surrogate of target tissue drug exposure. As shown in mice and monkeys, plasma trough concentrations directly correlated with target organ (liver) concentrations with similar partition ratios. This similarity across species provides confidence that similar relationships may exist for humans. It is this relationship that was used to estimate human liver concentrations based on measured plasma trough levels in human clinical trials. Comparison of the measured trough plasma EC_{50} values in humans ultimately confirmed that data generated in animal models are predictive of the potency of the human apoB antisense inhibitor (ISIS 301012). Because the mechanism of action and ADME characteristics are similar for other ASOs in the same chemical class as ISIS 301012, these data further provide confidence in the use of preclinical models for prediction of clinical effect for other ASOs.

The strength of the human volunteer data has been extended to hypercholesteremic subjects in multiple Phase 2 studies. Consistent with the Phase I healthy volunteer study, similar exposure–response relationships in patients with hypercholesterolemia, either used alone or when co-adminis-

tered with statins, had been demonstrated in several Phase II studies [23–25].

ISIS 301012 has a long tissue half-life in animals and humans. The elimination half-lives observed in monkeys were highly predictive of the half-lives measured in humans (greater than 30 days). Consistent with the long elimination half-life of ISIS 301012, it had a long duration of action in lipid lowering effect. Based on the long half-life coupled with the duration of action of ISIS 301012, current dosing regimens being evaluated in clinical development involve once weekly s.c. injection. Furthermore, the excellent relationship of trough plasma levels to apoB-100 and other downstream effects allows for the design of more complex PK/PD models. As clinical experience with this compound grows, development of predictive population models that further incorporate demographic or physiologic covariates should be possible. The use of these models in clinical trial planning and design has been widely adapted in novel drug development strategies and shown to greatly expedite drug development process [39].

In conclusion, these data demonstrated that apoB antisense inhibitors have consistently exhibited exposure-dependent reduction on liver apoB mRNA, as well as the downstream effects such as serum apoB, LDL-C and total cholesterol in multiple preclinical models and human. The exposure–response relationships in HF-fed mice using the mouse-specific apoB antisense inhibitor, ISIS 147764, in human apoB transgenic mice using human-specific apoB antisense inhibitor, ISIS 301012, and monkeys using monkey-specific apoB antisense inhibitor, ISIS 326358, were predictive of the effects of ISIS 301012 in man. The long duration of action of apoB antisense inhibitors in animals and humans is consistent with the prolonged tissue half-lives. These favorable PK/PD properties for apoB antisense inhibitors provide guidance for clinical development and appear to support infrequent dose administration.

Acknowledgements

The authors wish to thank Drs. Stanley Crooke and Jeff Jonas for scientific discussion and critical review of the manuscript. Finally, this manuscript would not be possible without the administrative support provided by Robert Saunders, for which we are grateful.

Authors are employees of ISIS Pharmaceuticals and own shares in ISIS Pharmaceuticals.

REFERENCES

[1] Galluppi GR, Rogge MC, Roskos LK, Lesko LJ, Green MD, Feigal Jr DW, et al. Integration of pharmacokinetic and pharmacodynamic studies in the discovery, development, and review of protein therapeutic agents: a conference report. Clin Pharmacol Ther 2001;69:387–99.

[2] Geary RS, Yu RZ, Siwkowski A, Levin AA. Pharmacokinetic/pharmacodynamic properties of phosphorothioate 2′-O-(2-methoxyethyl)-modified antisense oligonucleotides in animals and man. In: Crooke ST, editor. Antisense drug technology: principles, strategies and applications. Boca Raton, FL: Taylor & Francis Group; 2007. p. 305–26.

[3] Callies S, Andre V, Vick A-M, Graff J, Patel B, Brail L, et al. Modelling pharmacokinetic and pharmacodynamic properties of second generation antisense-oligonucleotides (ASOs). Copenhagen: PAGE (Population Approach Group in Europe); 2007.

[4] Yu RZ, Gibiansky L, Gibiansky E, Geary RS. Population pharmacokinetics and pharmacodynamics of ISIS 2302 (Role of Population Analysis in Drug Development). ASCPT Annual Meeting. Orlando, Florida; 2001.

[5] Yu RZ, Matson J, Geary RS. Terminal elimination rates for antisense oligonucleotides in plasma correlate with tissue clearance rates in mice and monkeys. Annual Meeting of American Association of Pharmaceutical Scientists. Denver, Colorado; 2001.

[6] Geary RS, Mathison B, Ushiro-Watanabe T, Savides MC, Henry SP, Levin AA. Second generation antisense oligonucleotide pharmacokinetics and mass balance following intravenous administration in rats. San Francisco, CA: Society of Toxicology, Oxford University Press; 2001. p. 343.

[7] Geary RS, Ushiro-Watanabe T, Truong L, Freier SM, Lesnik EA, Sioufi NB, et al. Pharmacokinetic properties of 2′-O-(2-methoxyethyl)-modified oligonucleotide analogs in rats. J Pharmacol Exp Ther 2001;296:890–7.

[8] Geary RS, Yu RZ, Watanabe T, Henry SP, Hardee GE, Chappell A, et al. Pharmacokinetics of a tumor necrosis factor-alpha phosphorothioate 2′-O-(2-methoxyethyl) modified antisense oligonucleotide: comparison across species. Drug Metab Dispos 2003;31:1419–28.

[9] Yu RZ, Kim T-W, Hong A, Watanabe TA, Gaus HJ, Geary RS. Cross-species pharmacokinetic comparison from mouse to man of a second generation antisense oligonucleotide ISIS 301012. Targeting human ApoB-100. Drug Metab Dispos 2007;35:460–8.

[10] Levin AA, Yu RZ, Geary RS. Basic principles of the pharmacokinetics of antisense oligonucleotide drugs. In: Crooke ST, editor. Antisense drug technology: principles, strategies and applications. Boca Raton, FL: Taylor & Francis Group; 2007. p. 183–215.

[11] Crooke ST, Vickers T, Lima W, Wu H. Mechanisms of antisense drug action, an introduction. In: Crooke ST, editor. Antisense drug technology: principles, strategies and applications. Boca Raton, FL: Taylor & Francis Group; 2007. p. 5–46.

[12] Wu H, Lima W, Crooke ST. Molecular cloning and expression of cDNA for human RNase H. Antisense Nucleic Acid Drug Dev 1998;8:53–61.

[13] Wu H, MacLeod AR, Lima WF, Crooke ST. Identification and partial purification of human double strand RNase activity: a novel terminating mechanism for oligoribonucleotide antisense drugs. J Biol Chem 1998;273:2532–42.

[14] Davidson NO, Shelness GS. Apolipoprotein B: mRNA editing, lipoprotein assembly, and presecretory degradation. Annu Rev Nutr 2000;20:169–93.

[15] Marsh JB, Welty FK, Lichtenstein AH, Lamon-Fava S, Schaefer EJ. Apolipoprotein B metabolism in humans: studies with stable isotope-labeled amino acid precursors. Atherosclerosis 2002;162:227–44.

[16] Das HK, Leff T, Breslow JL. Cell type-specific expression of the human apoB gene is controlled by two cis-acting regulatory regions. J Biol Chem 1988;263:11452–8.

[17] Crooke R. Second-generation antisense drug for cardiovascular disease demonstrates significant and durable reductions in cholesterol. In: The 9th Drug Discovery Technology World Congress; 2004.

[18] Crooke RM, Graham MJ, Lemonidis KM, Whipple CP, Koo S, Perera RJ. An apolipoprotein B antisense oligonucleotide

lowers LDL cholesterol in hyperlipidemic mice without causing hepatic steatosis. J Lipid Res 2005;46:872–84.
[19] Henry SP, Stecker K, Brooks D, Monteith D, Conklin B, Bennett CF. Chemically modified oligonucleotides exhibit decreased immune stimulation in mice. J Pharmacol Exp Ther 2000;292:468–79.
[20] McKay RA, Miraglia LJ, Cummins LL, Owens SR, Sasmor H, Dean NM. Characterization of a potent and specific class of antisense oligonucleotide inhibitor of human protein kinase C-α expression. J Biol Chem 1999;274:1715–22.
[21] Altmann K-H, Fabbro D, Dean NM, Geiger T, Monia BP, Muuler M, et al. Second-generation antisense oligonucleotides: structure–activity relationships and the design of improved signal transduction inhibitors. Biochem Soc Trans 1996;24:630–7.
[22] Baker BF, Lot SS, Condon TP, Cheng-Flournoy S, Lesnik EA, Sasmor HM, et al. 2'-O-(2-Methoxy)ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. J Biol Chem 1997;272:11994–2000.
[23] Stein E. High low-density lipoprotein cholesterol on three drugs. ACC06: 56th Scientific Session of the American College of Cardiology ACC Symposium: Common challenges in preventive cardiology; 2007. p. 603–8.
[24] Stein E, Wedel M, Bradley J. Statin-like dose-dependent reductions in LDL cholesterol and apolipoprotein B with ISIS, 301012, an antisense inhibitor of apolipoprotein B, in subjects with polygenic hypercholesterolemia. J Am Coll Cardiol 2007;49:1206–78.
[25] Kastelein J, Akdim F. Trip M. ISIS, 301012, an antisense inhibitor of apolipoprotein B, produces significant additional reduction of low-density lipoprotein cholesterol and apolipoprotein B in hypercholesterolemic subjects on statins not meeting target. J Am Coll Cardiol 2007;49:820–7.
[26] Kastelein JJ, Wedel MK, Baker BF, Su J, Bradley JD, Yu RZ, et al. Potent reduction of apolipoprotein B and low-density lipoprotein cholesterol by short-term administration of an antisense inhibitor of apolipoprotein B. Circulation 2006;114:1729–35.
[27] Leeds JM, Graham MJ, Troung L, Cummins LL. Quantitation of phosphorothioate oligonucleotides in human plasma. Anal Biochem 1996;235:36–43.
[28] Yu RZ, Baker B, Chappel A, Geary RS, Chueng E, Levin AA. Development of an ultrasensitive noncompetitive hybridization-ligation enzyme-linked immunosorbent assay for the determination of phosphorothioate oligodeoxynucleotide in plasma. Anal Biochem 2002;304:19–25.
[29] Geary RS, Leeds JM, Henry SP, Monteith DK, Levin AA. Antisense oligonucleotide inhibitors for the treatment of cancer. 1. Pharmacokinetic properties of phosphorothioate oligodeoxynucleotides. Anticancer Drug Des 1997;12:383–93.
[30] Zhang RW, Iyer RP, Yu D, Tan WT, Zhang XS, Lu ZH, et al. Pharmacokinetics and tissue disposition of a chimeric oligodeoxynucleoside phosphorothioate in rats after intravenous administration. J Pharmacol Exp Ther 1996;278:971–9.
[31] Geary RS, Yu RZ, Leeds JM, Ushiro-Watanabe T, Henry SP, Levin AA, et al. Pharmacokinetic properties in animals. In: Crooke ST, editor. Antisense drug technology: principles, strategies, and applications. New York: Marcel Dekker Inc.; 2001. p. 119–54.
[32] Geary RS, Yu RZ, Levin AA. Pharmacokinetics of phosphorothioate antisense oligodeoxynucleotides. Curr Opin Investig Drugs 2001;2:562–73.
[33] Yu RZ, Geary RS, Levin AA. Pharmacokinetics and pharmacodynamics of antisense oligonucleotides. In: Meyers RA, editor. Encyclopedia of molecular cell biology and molecular medicine. Weinheim, Germany: Wiley-VCH; 2007.
[34] Boothe DM, Boeckh A, Simpson RB, Dubose K. Comparison of pharmacodynamic and pharmacokinetic indices of efficacy for 5 fluoroquinolones toward pathogens of dogs and cats. J Vet Intern Med 2006;20:1297–306.
[35] Svetyi LI, Alekhin SN. Pharmacokinetic and pharmacodynamic effects of isradipine in patients with arterial hypertension. Eksp Klin Farmakol 2002;65:35–8.
[36] Zhou H, Choi L, Lau H, Bruntsch U, Vries EE, Eckhardt G, et al. Population pharmacokinetics/toxicodynamics (PK/TD) relationship of SAM486A in phase I studies in patients with advanced cancers. J Clin Pharmacol 2000;40:275–83.
[37] Ebert U, Grossmann M, Oertel R, Gramatte T, Kirch W. Pharmacokinetic–pharmacodynamic modeling of the electroencephalogram effects of scopolamine in healthy volunteers. J Clin Pharmcol 2001;41:51–60.
[38] Yu RZ, Zhang H, Geary RS, Graham M, Masarjian L, Lemonidis K, et al. Pharmacokinetics and pharmacodynamics of an antisense phosphorothioate oligonucleotide targeting Fas mRNA in mice. J Pharmacol Exp Ther 2001;296:388–95.
[39] Barrett JS, Gupta M, Mondick JT. Model-based drug development applied to oncology. Expert Opin Drug Discov 2007;2:185–209.