Biochemical Pharmacology 78 (2009) 284–291

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Effect of dose and plasma concentration on liver uptake and pharmacologic activity of a 2′-methoxyethyl modified chimeric antisense oligonucleotide targeting PTEN

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ARTICLE INFO

Article history:
Received 10 February 2009
Accepted 15 April 2009

Keywords:
Pharmacokinetics
Antisense oligonucleotide
mRNA
Pharmacodynamics
Liver

ABSTRACT

The role of dose and plasma concentration on liver tissue uptake and resulting antisense pharmacology using a chemically modified antisense oligonucleotide (ASO) targeting PTEN was assessed in mice. A single bolus s.c. dose of 60 mg/kg in mice showed a time-dependent reduction in liver PTEN mRNA that was maximal at 48–72 h and returned to near control levels by 20 days after administration. These pharmacodynamics are in good agreement with liver concentrations of ASO and are consistent with slow elimination ($t_{1/2}$ = 8 days) of the PTEN ASO from Balb/C mouse liver. As expected, highest ASO concentrations in liver resulted from the s.c. slow infusion at all doses tested. Unexpectedly, the liver EC_{50} for the 24-h s.c. slow infusion was approximately twofold higher than the two bolus routes of administration. Based on plasma concentration analysis it appears that 1–2 μg/mL ASO plasma concentration is a threshold that, if exceeded, results in robust antisense effects and below which there is reduced or complete loss of antisense pharmacology in liver even though bulk uptake in the organ is improved. Co-administration of a nonsense ASO competed for liver uptake, but unexpectedly increased pharmacodynamic response for the active oligonucleotide (ISIS 116847) supporting inhibition of a nonproductive bulk uptake pathway while simultaneously improving productive uptake (pharmacodynamics). This competition effect was similar whether the nonsense oligonucleotide was co-administered with ASO or administered up to 24 h prior to active ASO injection.

1. Introduction

Continued advancements towards identification of chemical modifications that improve upon the pharmacokinetic and pharmacodynamic properties of single-strand antisense oligonucleotides (ASOs) have been achieved over the years [1–4]. Amongst the vast majority of these modified phosphorothioate (PS) oligonucleotides, the 2′-O-methoxyethyl (2′-MOE) modified PS oligodeoxynucleotides have consistently demonstrated greater biological stability, and greater in vitro and in vivo potency. The improved potency is due in part to higher binding affinity of the 2′-MOE modification to its target mRNA while maintaining RNase H activity by using a chimeric design strategy [4]. This modification also simultaneously decreased general non-hybridization toxicities [5,6]. Several so-called second generation 2′-MOE modified oligonucleotides have entered clinical development, including ISIS 104838 for the treatment of rheumatoid arthritis [7,8], ISIS 113715 for the treatment of diabetes [9], OGX-011 for the treatment of prostate cancer [10], LY2181308 for the treatment of solid tumors [11], ATL-1102 in multiple sclerosis [12], and mipomersen (ISIS 301012) for hypercholesteremia [13].

While second generation 2′-MOE modified ASOs are relatively early in their development, the number of published accounts describing their pharmacokinetics is growing [7,14–16]. The in vivo disposition of second generation 2′-MOE partially modified phosphorothioate ASOs as a class of compounds are primarily driven by the phosphorothioate chemistry in the backbone of the ASO [17]. Thus, similar to first generation phosphorothioate oligodeoxynucleotides, the pharmacokinetic properties of these ASOs are remarkably similar across various sequences [18]. 2′-MOE chimeric ASOs all exhibit rapid transfer (distribution $t_{1/2}$ of 1–2 h) from blood to tissues with urinary excretion playing a minor role. The organs with the highest natural distribution of ASOs in this chemical class are kidney and liver. Spleen, lymph nodes and bone marrow also exhibit relatively high concentrations of ASO following parenteral administration. Little to no ASO can be found

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doi:10.1016/j.bcp.2009.04.013

in skeletal muscle or brain. All of these chimeric ASOs circulate in plasma loosely bound (>85% in all species) to plasma proteins.

ASOs must cross a lipid bilayer to access the compartment(s) necessary to bind target mRNA and ultimately elicit a pharmacological response. ASOs access intracellular compartments by multiple mechanisms that are time and temperature dependent [19]. Absorptive and fluid phase endocytosis has been identified as a mechanism for uptake into cells in vitro [20]. However, in vitro cell uptake has traditionally proven to be a poor predictor of organ or cellular uptake in vivo [21]. Scavenger receptors have been implicated in vivo as a mechanism for uptake of ASOs [22], however knock-out mouse models continue to exhibit excellent cell uptake and sequence specific ASO pharmacology [23]. The difficulty in predicting in vivo uptake with in vitro data may be, at least in part, attributed to cell surface proteins that are expressed in vivo, but down-regulated in an in vitro setting. This has been the case for specific nucleic acid transporters that appear to function in multiple tissues [24] in vivo or in situ, but are not expressed in vitro.

Antisense oligonucleotides (ASOs) in this chemical class are highly protein bound. Thus, it is likely that they traffic into and through cells via a protein to protein shuttling mechanism driven by binding affinity gradients [17,25]. Since binding events are likely required for transport, uptake into cells should be a saturable event. In fact, nonlinear and saturable uptake has been reported in numerous in vivo pharmacokinetic reports [15,26–28]. Because protein binding begins in plasma there are at least three compartments of interest for distribution of ASOs from plasma to the intracellular target, mRNA. A diagram that illustrates the three compartments of potential protein binding interaction is shown in Fig. 1. It is clear from the literature reviewed that there are likely multiple uptake mechanisms for single-strand ASOs.

Liver, a primary target site for ASO accumulation and cell uptake, is a complex organ with multiple cell types represented. Previous suborgan distribution of oligonucleotides in liver has been described with the lowest concentrations per cell being reflected in hepatocytes, while highest concentrations are seen in Kupffer and sinusoidal endothelial cells [29–31]. Kinetic observations further illustrate that the cells in liver that take up the most ASO (Kupffer and sinusoidal endothelial cells) are also the cells that take up ASO first with the hepatocyte uptake being secondary to the higher affinity cell types. Thus, ASO distribution to the liver is further complicated by competing differential cell uptake in multiple suborgan compartments.

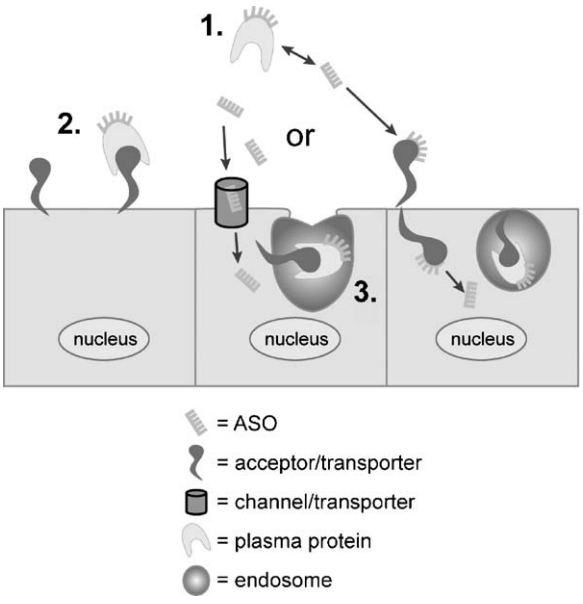


Fig. 1. Cartoon illustrating the multiple ASO:protein interactions that may play a role in distribution. (1) Plasma proteins, (2) cell surface protein(s), and (3) intracellular compartment(s) and protein(s).

In this report we describe for first time the role of plasma concentration on hepatocellular uptake. A series of in vivo experiments was designed to elucidate the uptake properties of a 2′-MOE modified ASO, ISIS 116847, targeting putative protein tyrosine phosphatase (PTEN) mRNA, a ubiquitously expressed protein apparent in most, if not all cell types, in the body [32]. The antisense sequence attributed to ISIS 116847 targets a region of the PTEN mRNA conserved from mouse to man. Competition for hepatic uptake is also demonstrated for the first time using nonsense oligonucleotides in the same chemical class. The effect of dose, route, rate, and resultant ASO plasma and liver concentration on ASO pharmacodynamics in the whole liver are characterized and reported.

2. Methods

2.1. ASO synthesis and chemistry

2′-O-methoxyethyl modified antisense phosphorothioate oligonucleotides (2′-MOE ASOs) were synthesized at Isis Pharmaceuticals, Inc. (Carlsbad, CA) as described previously [33]. ASOs used were 20 nucleotides in length and contained 2′-MOE modifications at the terminal residues at both the 5′ and 3′ ends of the molecule with at least nine contiguous deoxynucleotide residues in the center. The sequences for the ASOs were as follows: ISIS 116847, 5′-**CTGCT** agcctctgga **TTTGA**-3′ (active anti-PTEN sequence) and ISIS 13920, 5′-**TCC** gtcatcgct **CCTCAGGG**-3′ (nonsense sequence), where the bolded/italized nucleotides are 2′-MOE modified and the "middle", lower case nucleotides are—deoxy. All of the cytosines in both sequences were methylated at the C-5 position.

2.2. In vivo mouse dosing

Balb/C male mice weighing between 20 and 25 g were purchased from Charles River Laboratories (Wilmington, MA) and housed 5 per cage in shoe box cages. Mice were maintained on feed and water ad libitum. 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.

Intravenous (i.v.) injection (tail vein) and subcutaneous (s.c.) bolus injection (sub-scapular) were compared. Subcutaneous (s.c.) infusions were accomplished using implanted Alzet minipumps (Durect Corp., Cupertino, CA) that provided either 24, 72 or 168 h (1 week) continuous infusion. Dose volumes were less than or equal to 10 mL/kg (approximately 200–250 μL per mouse).

All dosing studies involved a single dose injection or infusion of ISIS 116847 ASO in saline solution at a dose of 0 (saline control), 15, 30, 60 or 100 mg/kg. ISIS 13920 doses of 15, 30, 60, 90 and 120 mg/kg were co-infused with a dose of 30 mg/kg ISIS 116847 ASO to characterize the effects of direct competition using an inactive, nonsense oligonucleotide (ISIS 13920). ISIS 116847 was also administered as a bolus s.c. injection 0 (immediately after) and 24, 48 and 72 h after a 24-h infusion of ISIS 13920 oligonucleotide in saline solution to test the kinetics of competition following completion of ISIS 13920 distribution.

Plasma was collected and frozen at −70 °C or less for determination of ASO concentration at previously determined maximal concentration time points (immediately after i.v. injec-

tion, 0.5 h after s.c. injection, and approximately 6 h for all 24 h infusions). Urine was collected on ice for measuring oligonucleotide excretion over the 24-h period beginning with the start of dosing. Urine was stored at −70 °C or less until analyzed. Liver and kidneys were collected at sacrifice approximately 72 h after dosing was completed. A portion of each liver was homogenized in guanidine isothiocyanate buffer for mRNA extraction and RT-PCR analysis of PTEN mRNA. The remaining liver and one kidney were flash frozen and stored at −70 °C or less until assayed for oligonucleotide concentration.

2.3. Quantitative reverse transcription-PCR

Tissues were homogenized in guanidine isothyocyanate buffer (4 M guanidine isothiocyanate, 25 mM EDTA, 1 M β-mercaptoethanol, 50 mM Tris–HCl, pH 6; Invitrogen, Carlsbad, CA) immediately following sacrifice. RNA was extracted using RNeasy columns (Qiagen, Valencia, CA) according to manufacturer's protocol. RNA was eluted from the columns with water. RNA samples were analyzed by fluorescence-based quantitative RT-PCR using an Applied Biosystems 7700 sequence detector. Levels of target RNAs as well as those of cyclophilin A, a housekeeping gene, were determined. Target RNA levels were normalized to cyclophilin levels for each RNA sample. Primers used for determination of PTEN RNA level are as follows: FP 5′ ATGACAATCATGTTGCAGCAATTC 3′, RP 5′ CGATGCAATAAATATGCACAAATCA 3′, and PR 5′ 6FAM-CTGTAAAGCTGGAAAGGGACGGACTGGT-TAMRA 3′ (Integrated DNA Technologies, Coralville, IA). Primers used for determination of cyclophilin A RNA level are as follows: FP 5′ TCGCCGCTTGCTGCA 3′, RP 5′ ATCGGCCGTGATGTCGA 3′, and PR 5′ 6FAM-CCATGGTCAACCCCACCGTGTTC-TAMRA 3′ (Integrated DNA Technologies, Coralville, IA).

2.4. Bioanalytical methods

Plasma, urine and tissue ASO concentrations were measured using capillary gel electrophoresis (CGE) [34,35] with UV detection at 260 nm. ASO from plasma and urine were extracted by solid phase extraction methods (SPE) followed by desalting by dialysis prior to electrokinetic introduction of the extracts on column. Tissues were weighed, minced and spiked with a known amount of internal standard (ISIS 13866, a 27mer 2′-MOE modified oligonucleotide, synthesized at Isis Pharmaceuticals, Inc., Carlsbad, CA) prior to homogenization. Tissue homogenates were initially extracted using phenol:chloroform:isoamyl alcohol (Sigma–Aldrich Co., St. Louis, MO). Additional sample cleanup varied dependent on the matrix, but generally involved solid phase extraction (SPE) followed by dialysis prior to electrokinetic introduction of analytes from the extract on a Beckman PACE 5000 capillary electrophoresis (CE) instrument (Beckman Instruments).

For competition studies, a sequence specific hybridization ELISA method was used as previously described (modified from Yu et al., 2002 [48]) to determine concentrations of ISIS 116847 and ISIS 13920 separately. Cross-reactivity of the respective hybridization-based ELISA methods were found to be <0.0001%. Both CGE and hybridization ELISA methods have been shown to be specific for full-length parent ASO (20mer) [34,36].

2.5. Calculations and statistics

Descriptive statistics were used to summarize plasma, urine and tissue PTEN ASO concentrations and PTEN mRNA levels relative to control in liver following ASO treatment. All statistical comparisons utilized either pair-wise Student's *t*-tests or ANOVA with the appropriate control for each experiment.

3. Results

3.1. Single dose PK/PD

PTEN mRNA levels decreased significantly (approximately 50%) by 24 h after a single s.c. dose of 60 mg/kg (approximately 1.2 mg per animal) ISIS 116847 (Fig. 2). Maximal decrease in target PTEN mRNA (approximately 70%) was observed at 48 and 72 h. The

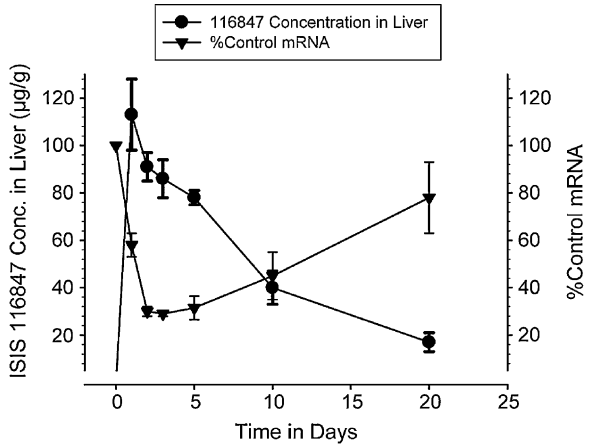
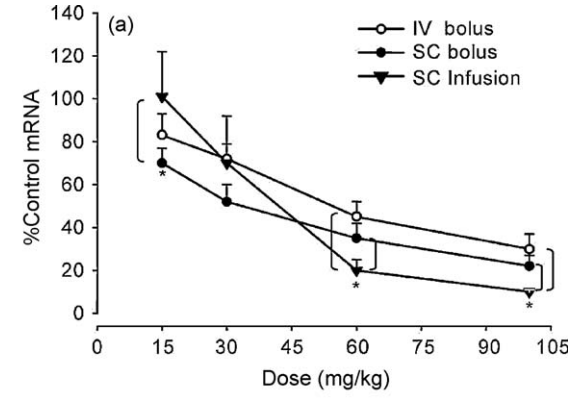
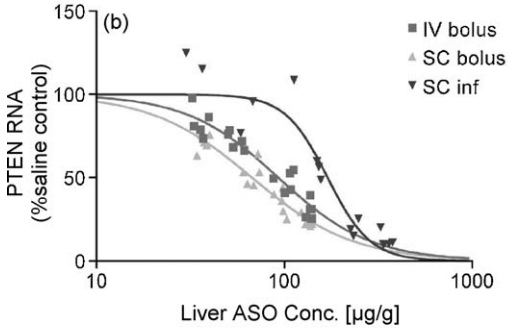


Fig. 2. Liver ISIS 116847 concentrations and PTEN mRNA response as a function of time following a single subcutaneous injection dose of 60 mg/kg (approximately 1.2 mg/mouse). Data represent the average of five animals at each time point. Error bars are standard deviation.





	IV bolus	SC bolus	SC inf
IC_{50}	93	67	170
95% CI	85 to 102	62 to 74	148 to 195

Fig. 3. (a) Dose- and (b) concentration-dependent PTEN mRNA reductions at 72 h after administration. Asterisks indicate statistically significant difference ($p < 0.05$) in effect from comparator treatment at each dose denoted by the bracket. Each point in (a) represents the average of four to five animals. The error bars are standard deviation. The individual animal data is plotted in (b).

duration of effect was quite long with maximal effect still apparent for at least 5 days (120 h) after injection (Fig. 2). PTEN mRNA levels were still reduced by approximately 50% 10 days after injection. The effect was ultimately reversible as drug cleared from the liver, and by 20 days levels had nearly returned to control. For subsequent experiments a single time point was sampled at 72 h to ensure maximal effect of treatment.

While maximal effect was observed 48–72 h after injection, maximum concentrations of ISIS 116847 in liver were observed by 24 h (Fig. 2). After 24 h oligonucleotide concentrations decreased slowly over the next several weeks with a half-life of approximately 8 days. The decay of drug levels correlated well with the duration of activity following single dose administration in mice (Fig. 2).

3.2. Dose and route response

Dose-dependent PTEN mRNA reductions were observed by all routes of administration (Fig. 3). Slow s.c. infusion of ISIS 116847 which produced the lowest maximum plasma concentrations and resulted in the highest liver concentrations (Fig. 4) and lowest urine excretion (Fig. 5), consistent with saturable uptake kinetics in tissues of distribution. However, and counter to expectations, concentration–effect curves comparing the three regimens (i.v. bolus vs. s.c. bolus vs. s.c. infusion) indicate approximately a twofold loss of activity at the lower s.c. infusion doses (Fig. 3). The change in the concentration–effect curves for the slow infusion regimen results in a higher EC_{50} for the s.c. slow infusion regimen. The observed loss of liver pharmacologic effect at the lower s.c. infusion doses (15 and 30 mg/kg) occurred at very low plasma concentrations (<1 μg/mL; Fig. 4)).

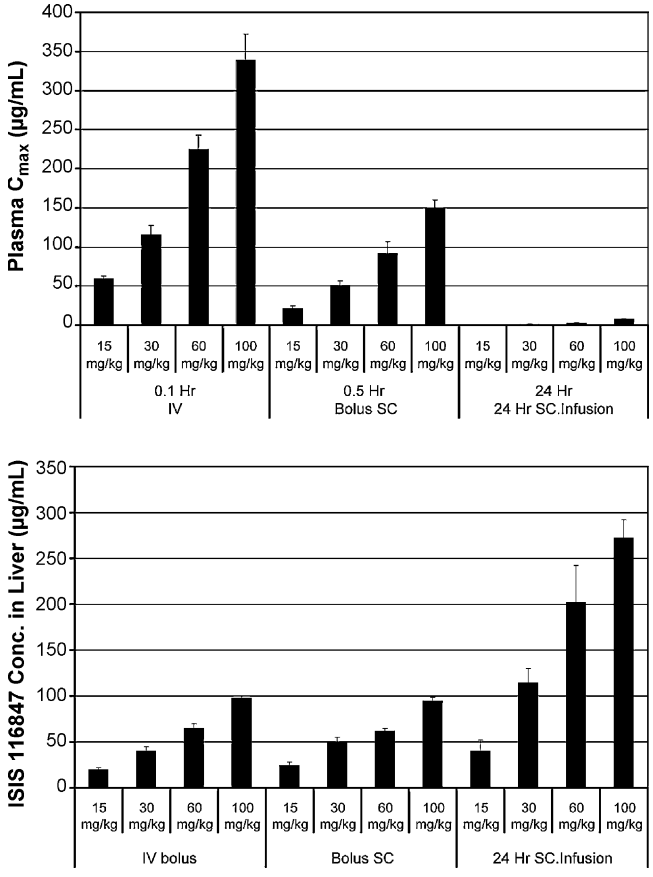


Fig. 4. Plasma ASO C_{max} (observed approximately 2 min after i.v. bolus administration, 30 min after s.c. bolus administration and 6 h after start of continuous infusion) and liver ASO concentrations measured 72 h after single dose escalation study. Each bar represents the average from four animals. The error bars are standard deviation.

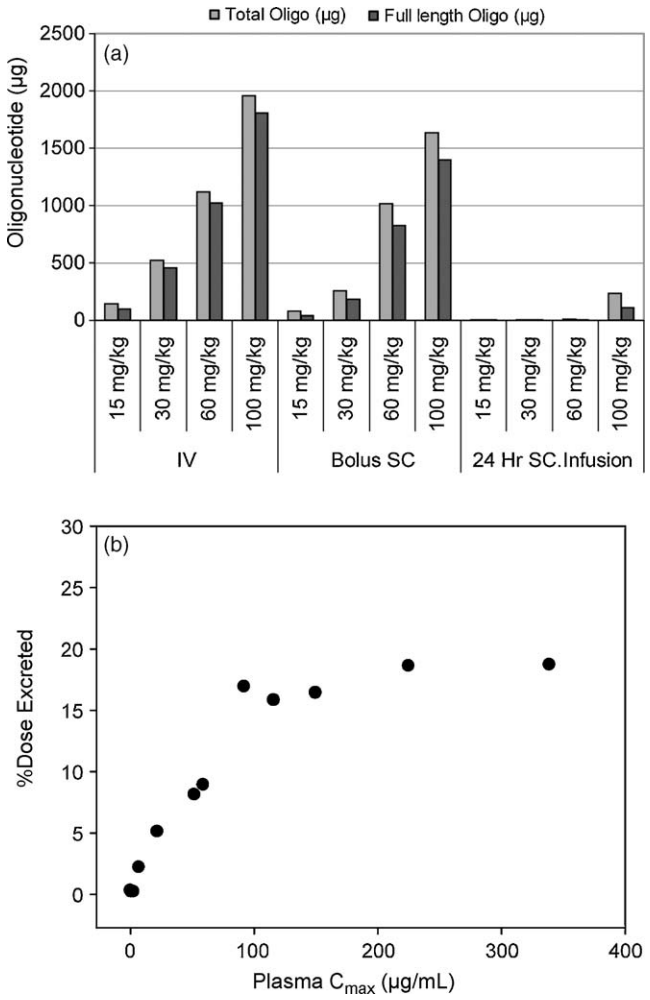


Fig. 5. Urinary excretion of ISIS 116847: (a) 24 h cumulative amount of ASO excreted in urine as total or parent oligonucleotide as a function of dose and regimen (represents total amount excreted from 5 mice per metabolism cage) and (b) percent of administered dose excreted in 24 h as a function of plasma C_{max}. Each filled circle represents the average of 5 mice.

To assess the effect of plasma concentration at equivalent doses on active liver uptake of ISIS 116847, a second experiment compared a highly active dose of 60 mg/kg (70–90% reductions independent of regimen) administered as a slow infusion over 24, 72 and 168 h to allow delivery of a similar dose but with progressively lower plasma concentrations (Fig. 6 and Table 1). In the previous experiment the 60 mg/kg dose administered over 24 h yielded a plasma concentration >1 μg/mL. Administering the same dose over 72 and 168 h resulted in plasma concentrations much less than 1 μg/mL but yielded similar total ASO concentra-

Table 1
Plasma and tissue concentrations as a function of infusion rate at a constant total dose of 60 mg/kg in Balb/C mice ($n = 5$). Data is presented as mean (standard deviation).

Infusion length (h)	Plasma conc.[a] (μg/mL)	Liver conc.[b] (μg/g)
24	1.7 (0.6)	155 (6)
72	0.27 (0.02)	101 (23)
168	0.13 (0.02	116 (6)

[a] Samples taken during continuous infusion (6 h).
[b] Samples collected at sacrifice, 72 h after each infusion ended.

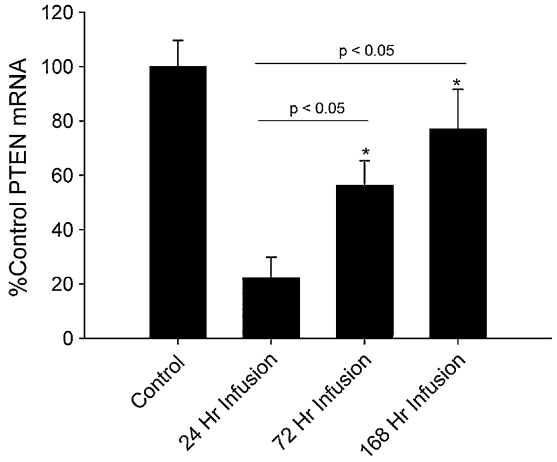


Fig. 6. Relative PTEN mRNA levels in liver following administration of 60 mg/kg ISIS 116847 as a function of infusion rate. A significant loss in activity was observed at slower infusion rates. Each bar represents the average of five animals. The error bars are standard deviation.

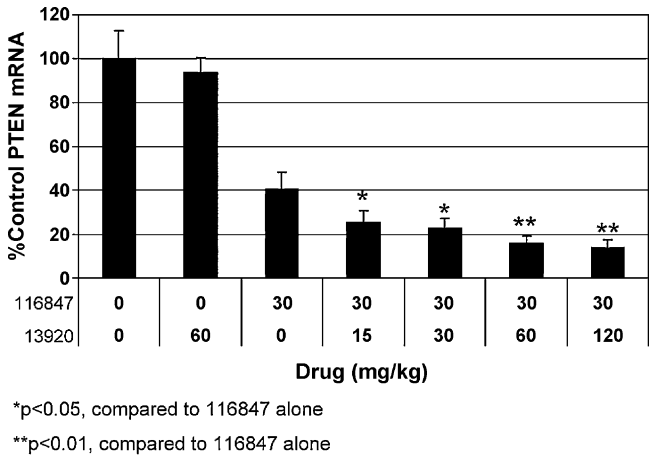


Fig. 7. Significantly increased effect on PTEN liver mRNA observed when ISIS 116847 was co-administered at increasing dose of a non-active oligonucleotide, ISIS 13920. Each bar represents the average from four animals. The error bars are standard deviation.

tions in liver (Table 1). While liver concentrations remained essentially constant independent of the infusion duration/rate, a significant loss in pharmacologic activity was observed as plasma steady-state concentration decreased (Table 1), shown by less of an effect on PTEN mRNA knockdown (Fig. 6), confirming a plasma concentration relationship with target reduction.

3.3. Competition studies

Competition experiments were conducted to test if another oligonucleotide could compete for uptake of active ISIS 116847 in the liver. The "nonsense" oligonucleotide (ISIS 13920) had no effect on the PTEN mRNA or protein target when dosed alone, as expected (Fig. 7). While plasma steady-state concentrations and overall clearance were not altered (Table 2), the amount of ISIS 116847 taken up in liver decreased as simultaneous administration of the nonsense oligonucleotide, co-administered with ISIS 116847, was increased (Table 2) indicating an ability to compete for bulk drug uptake. However, the whole liver mRNA knockdown resulting from the PTEN ASO significantly improved (Fig. 7), suggesting that the "nonsense" oligonucleotide competes for uptake into an apparent negative or nonproductive sink ultimately resulting in improved pharmacology by perhaps improving uptake by a more productive pathway.

To test the kinetics of competition (extra- or intracellular), simultaneous and delayed administration of ISIS 116847 was tested together with a 24 h infusion of ISIS 13920. Significantly improved mRNA knockdown was achieved up to 24 h after administration of the "nonsense" oligonucleotide (Fig. 8a), a time at which little to no measurable ISIS 13920 remained in plasma. ASO concentrations in liver was either somewhat lower or equivalent while pharmacology was improved (Fig. 8b).

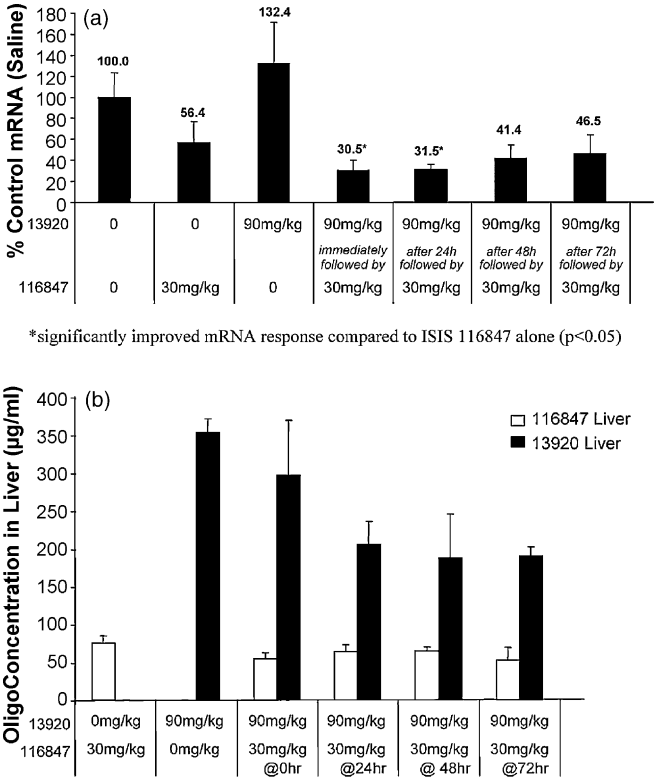


Fig. 8. Kinetics of co-administration effects with bolus active ASO (ISIS 116847) administered immediately or at 24, 48 or 72 h after competing nonsense oligonucleotide infusion was stopped (ISIS 13920). (a) Liver PTEN mRNA levels at 72 h and (b) liver drug concentrations at 72 h. Each bar represents the average of four animals and the error bar is standard deviation.

Table 2
Plasma (during infusion) and liver tissue concentration of ISIS 116847 and ISIS 13920 with increasing ISIS 13920 co-administered doses.

ISIS 116847 dose (mg/kg)	ISIS 13920 dose (mg/kg)	116847 plasma conc. (μg/mL)	13920 plasma conc. (μg/mL)	116847 liver conc. (μg/g)	13920 liver conc. (μg/g)
30	0	1.7 (0.2)	0 (0)	138 (14)	0 (0)
30	15	1.4 (0.3)	0.7 (0.2)	99 (17)	44 (8)
30	30	2.1 (0.4)	2.4 (1.0)	98 (13)	85 (22)
30	60	2.0 (0.4)	3.8 (1.2)	74 (15)	175 (23)
30	120	2.3 (0.2)	9.5 (1.5)	35 (14)	252 (111)

Data is presented as average (S.D.); $n = 4$.

4. Discussion

Onset and duration of action at the mRNA level following single dose administration of the PTEN targeted ASO, ISIS 116847, was similar to that observed previously for a FAS antisense oligonucleotide [31,37]. In these papers, the authors described a 2′-MOE modified chimeric ASO targeting FAS in mice that showed a single s.c. injection of approximately 1 mg (~50 mg/kg) produced profound and durable reduction in FAS mRNA and resulting translation of protein [31,38] in liver. Onset of action was relatively rapid (within 24 h of administration) and maximal effect was observed by 48–72 h. Duration of action for the FAS ASO was closely correlated with the hepatocyte ASO half-life consistent with the expression of FAS solely in the hepatocyte compartment. The similarity in onset of action suggests a sequence and target independence to cell uptake and ultimate antisense initiated binding and degradation of the cognate mRNA target. The duration of action appeared to exceed 10 days consistent with elimination kinetics of the ASO which exhibited a half-life in liver of approximately 8 days. By 20 days after a single dose the mRNA levels had returned to near pre-treatment levels confirming reversible and concentration dependent antisense effects on the target mRNA.

ISIS 116847, like other 20mer ASOs in this chemical class, is approximately 7 kDa in size and the phosphorothioate backbone confers multiple negative charges at physiological pH. Oligonucleotides are not expected to passively diffuse across lipid bilayer membranes but rather are thought to enter cells by a series of protein binding interactions that ultimately result in binding to the target mRNA [25]. Following parenteral administration reversible protein binding interactions begin in the blood plasma where phosphorothioate oligonucleotides bind to primarily albumin and α_2-macroglobulin with the bound fraction exceeding 85% [39,40] in mouse plasma and greater than 90% in monkey and man. Blood plasma then bathes multiple organs and cells and the oligonucleotides are observed to be associated with cell surface proteins and extracellular matrix by 1 h after parenteral administration [41,42]. While the specifics of the pathways or transporters involved in the process are not yet elucidated, it is clear that oligonucleotide appears in the cytoplasm of many cells within a very short time after administration including Kupffer cells and sinusoidal endothelial cells [29,30] in the liver, and renal proximal epithelial cells in the kidney [43]. In this study, reproducible antisense-mediated reduction of target PTEN mRNA is observed within hours of administration further validating the intracellular disposition of ISIS 116847 in vivo. Thus, this rapid cellular uptake in vivo appears to be a class effect and has been observed for this class of oligonucleotides independent of sequence.

The ultimate intracellular disposition of oligonucleotides is believed to be a process of protein binding interactions leading from high capacity low affinity proteins in plasma to higher affinity binding in organs of disposition. Thus, one would predict uptake kinetics would be saturable and nonlinear. We have demonstrated that uptake of ISIS 116847 in whole liver of mice was favored at lower plasma concentrations consistent with saturable, nonlinear uptake. Reduced urinary excretion plays at least some role in the improvement of whole organ uptake at lower doses and plasma concentrations. All of the pharmacokinetic observations observed were expected based on the known properties of antisense oligonucleotides. Unexpectedly, however, an increase in liver drug concentration due to slower infusion did not confer improved pharmacodynamics, perhaps suggesting that the low plasma concentration-favored an uptake pathway that is, at least in part, a nonproductive pathway. The plasma concentration threshold above which improved pharmacodynamics were observed consistent with the amount of oligonucleotide distributed to the liver appeared to be approximately 1–2 μg/mL in plasma. The extent of binding to plasma proteins does not saturate at these low concentrations [40], so it is unlikely that the plasma binding compartment represents the nonproductive sink. We cannot, however, rule out a high affinity, low-capacity plasma protein that is saturated at lower concentrations may play a role as a negative sink. Nevertheless, these data appear to implicate saturable binding to a nonproductive uptake process within the target organ or cell(s). The competition kinetic results (seen up to 24 h after administration of the competing nonsense oligonucleotide) further suggest that competition is at the level of the cell itself, rather than in plasma. In other words the continued protection from uptake by the nonproductive pathway may be a consequence of congestion of the saturable pathway. Continued congestion over time is consistent with the long tissue residence half-life of this class of compound, including the nonsense oligonucleotide. We cannot rule out the alternative possibility of a trans-stimulatory effect on the productive uptake transporter. However, the co-administration effects appear contrary to this mechanism.

It is tempting to speculate that certain binding sites on the cell surface may be saturated at or above threshold plasma concentrations that are presented to the tissue continuously via slow infusion. In this manner, surface proteins involved in endosomal incorporation of soluble large molecules (such as scavenger receptors or other proteins involved in an endocytotic mechanism) may be easily saturated and likely represent, at least in part, the nonproductive uptake pathway. When co-administered with a nonsense oligonucleotide that would be expected to compete for the uptake process, the uptake of the active ASO was decreased. Again, unexpected improved antisense activity of the active antisense oligonucleotide, ISIS 116847, was observed. These data taken together suggest that there may be a second cellular uptake pathway that is less saturable (perhaps of lower affinity) that can be favored by competing for the nonproductive pathway in liver. While it is possible that both productive and nonproductive pathways exist within the same cell, we must also consider suborgan complexities [30,31] of the liver in which the saturable, nonproductive pathway, may predominate in high uptake cells (Kupffer and sinusoidal endothelial cells). Saturation of these higher affinity cell types may promote subsequent distribution to hepatocytes. Most likely, multiple pathways exist in all cells while the nonproductive endocytotic pathways may dominate in cells with endocytotic functionality.

Application of the pharmacokinetic/pharmacodynamic model described by Yu et al. [31] fails to predict the outcome of a series of studies we have conducted with ISIS 116847. The Yu et al. model predicts that greater uptake in the organ would result in greater ASO pharmacological effect. In these studies, exactly the opposite was seen once plasma concentrations presented to the organ fell below 1 μg/mL. Other PK/PD modeling papers have suggested models that take into consideration target-mediated disposition and dynamics [44,45] may better predict PD outcomes. These papers take into account high affinity binding targets that are generally assumed to be the primary pharmacological target of the biopharmaceutical agent, such as antibody with antigen interactions or a small molecule binding a receptor target. In the case of an ASO, the primary pharmacological binding site for the ASO is with its cognate intracellular targeted mRNA. Target-mediated disposition would predict that the disposition of the ASO would follow the location of its target. This is clearly not the case for ASOs given distribution is similar across multiple sequences independent of target or location of target [17,18]. However, a secondary protein binding target for ASOs, unassociated with direct pharmacological binding to its target mRNA, may drive the disposition of these compounds. For example, a high affinity, low-capacity binding to a nonproductive uptake pathway may be required in tandem with a

lower affinity high capacity productive pathway. The high affinity path would drive disposition at low dose or with slower infusion, but result in poor distribution to the active intracellular site of mRNA. Additional work elucidating models that are predictive for ASOs will likely need to take into consideration the fundamental findings we have observed here and may be directed by determination of the multiple pathways elucidated in this work.

When considering active uptake pathways, recent work by Shi et al. in the CNS [24] combined with the identification of a nucleotide transporter in kidney by Hanss [46,47] is suggestive of a common productive transporter in multiple tissues or cells selective for single-strand oligonucleotides. One might speculate that this transporter or ones like it may be involved in productive uptake of single-strand ASOs in liver. Our data suggest that a nonproductive pathway resulting in poor intracellular trafficking of the ASO dominates when the ASO is dosed slowly as a slow infusion and that co-administration with a nonsense ASO can block the nonproductive pathway while simultaneously improving selection to the productive uptake pathway in liver. Elucidation of the mechanism for the productive pathway for antisense oligonucleotide uptake represents a significant opportunity to further advance the design of ASOs. If elucidated at the molecular level, we posit that it would then be possible to target such a pathway, by chemistry or formulation, to further improve the potency of these selective and important pharmacological compounds.

5. Conclusion

A combination of dose, dose-rate and resulting plasma ASO concentration data together with liver target mRNA effects and ASO liver uptake, have revealed at least two uptake pathways: the first pathway that is preferred at low plasma concentrations, is thus saturable, but results in sequestration of nonproductive ASO in the cells; the second pathway that is accessed upon saturation of the first pathway and optimized by competition with the higher affinity but nonproductive first pathway. Blocking the nonproductive high affinity and saturable pathway or by optimizing binding to the currently lower affinity but more productive uptake pathway present promising opportunities for improved ASO pharmacokinetics. Successful application will require further research to elucidate mechanistic and molecular components of the uptake pathway(s).

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