Review

Antisense oligonucleotides on neurobehavior, respiratory, and cardiovascular function, and hERG channel current studies

Tae-Won Kim a,⁎,1, Ki-Suk Kim b,1, Joung-Wook Seo b, Shin-Young Park b, Scott P. Henry a

a Isis Pharmaceuticals, Inc., 2855 Gazelle Ct., Carlsbad, CA 92010, USA
b Korea Institute of Toxicology, Korea Research Institute of Chemical Technology, Yuseong, Daejeon 305-600, Republic of Korea

Abstract

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Introduction: Safety Pharmacology studies were conducted in mouse, rat, and non-human primate to determine in vivo effects of antisense oligonucleotides (ASOs) on the central nervous system, respiratory system, and cardiovascular system. Effects on the hERG potassium channel current was evaluated in vitro. Methods: ASOs contained terminal 2′-O-methoxyethyl nucleotides, central deoxy nucleotides, and a phosphorothioate backbone. Neurobehavior was evaluated by Functional Observatory Battery in rodents. Respiratory function was directly measured in rodents by plethysmograph; respiratory rate and blood gases were measured in monkey. Basic cardiovascular endpoints were measured in rat; cardiovascular evaluation in monkey involved implanted telemetry units. In single and repeat dose studies ASOs were administered by subcutaneous injection at up to 300 mg/kg, 250 mg/kg, and 40 mg/kg in mouse, rat, or monkey, respectively. Assays were performed in HEK293 or CHO-K1 cells, stably transfected with hERG cDNA, at ASO concentrations of up to 300 μM. Results: No apparent effects were noted for respiratory or CNS function. Continuous monitoring of the cardiovascular system in monkey demonstrated no ASO-related changes in blood pressures, heart rate, or ECG and associated parameters (i.e., QRS duration). Specific assessment of the hERG potassium channel indicated no potential for actions on ventricular repolarization or modest effects only at excessive concentrations. Discussion: The absence of direct actions on neurobehavior and respiratory function associated with the administration of ASOs in safety pharmacology core battery studies is consistent with published toxicology studies. The combination of in vitro hERG studies and in vivo studies in rat and monkey are consistent with no direct actions by ASOs on cardiac cell function or electrical conduction at relevant concentrations and dose levels. Taken as a whole, dedicated studies focused on the safety pharmacology of specific organ systems do not appear to add significant data for interpretation of potential adverse effects. The need for dedicated studies for future ASOs in the same class is questionable, as a more encompassing data set can be collected in repeat dose and longer-term toxicology studies.

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Abbreviations: ASO, Antisense Oligonucleotide; CNS, Central Nervous System; ECG, Electrocardiography; FOB, Functional Observatory Battery; GLP, Good Laboratory Practice; hERG, Human Ether-a-go-go related gene; ICH, International Conference on Harmonisation; IC50, The half maximal inhibitory concentration; LSmean, Least squares mean; 2′MOE, 2′-O-methoxyethyl; PCO2, Partial pressure of CO2; PO2, Partial pressure of O2; S.E.M, Mean Standard Error; SO2%, Percent hemoglobin saturation.

⁎ Corresponding author. Tel.: +1 760 603 2313; fax: +1 760 603 2502.
E-mail address: tkim@isisph.com (T.-W. Kim).
1 Equal contributors.

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1. Introduction

Antisense oligonucleotides (ASOs) have demonstrated therapeutic benefit for the treatment of various cancers, and more recently this dynamic drug platform has been expanded to include chronic and non-life-threatening conditions such as diabetes and cardiovascular disease. A significant advantage of this class of drugs is the direct targeting of a specific mRNA through Watson–Crick base pairing, and subsequent inhibition of disease-causing proteins. Therapeutic intervention is the ultimate goal for the technology, and as such, treatment involves a diversity of schedules, doses, and routes. This requires a thorough understanding of the molecular mechanisms, pharmacologic activity, pharmacokinetic properties, toxicology and clinical efficacy.

Pharmacology has been reported in animal models of disease (Hassing et al., 2012; Lentz et al., 2013; Wheeler et al., 2012). Evidence of antisense activity in patients has been demonstrated for several targets in early clinical trials (Miner, Wedel, Xia, & Baker, 2006; Monteleone et al., 2012; Stein et al., 2012), and in definitive trials for hypercholesterolemia (Gelsinger, Steinhagen-Thiessen, & Kassner, 2012; McGowan et al., 2012; Ricotta & Frishman, 2012). General toxicity has been reviewed (Farman & Kornbrust, 2003; Henry et al., 2012; Jason, Koropatnick, & Berg, 2004; Levin, Henry, Monteith, & Templin, 2001); similarly, reviews on the therapeutic potential of ASOs have been published (Bennett & Swayze, 2010; Malik & Roy, 2011).

Safety pharmacology is one area that has not been as well outlined for ASOs. The conduct and objectives of safety pharmacology assessment are described in ICH M3 (R2), ICH S7A, and ICH S7B. In general, these guidance documents are focused on small molecules. Safety assessment of large molecules, i.e., biotechnology-derived pharmaceuti- cals, is outlined in ICH S6 (R1). Guidance for pharmaceuticals intended for cancer indications, as described in ICH S9, may also be considered. The manufacturing of ASOs is a chemical process, as is that for small molecules; whereas the high degree of single-target specificity and the potential for exaggerated pharmacology are more closely aligned with large molecules, such as monoclonal antibodies (mAb).

Although ASOs are not specifically described in guidance documents, the evaluation for potential undesirable functional effects of ASOs on major physiological systems involve the same concepts and considerations. Pharmacologic activity is a primary consideration in species selection; evaluation and selection of an ASO for an endogenous target almost exclusively includes activity in non-human primates. Similar to that for biologics, the optimal candidate ASO for humans may or may not have activity in rodents. Study design parameters, e.g., dose ranges, routes of administration, and numbers of animals, are also often more similar to biologics, as compared to those for small molecules.

In regard to safety pharmacology, the core battery of the central nervous system, the cardiovascular system, and the respiratory system are of highest interest. Additional systems such as renal/urinary, autonomic nervous, gastrointestinal and others may be appropriate under certain conditions. While the focus of these studies remains consistent across drug classes, there are specific points for consideration in the evaluation of an ASO. The non-human primate has been a key toxicology species for the development of ASOs, whereas safety pharmacology studies have not been common in monkey, in particular neurobehavioral-based assessments. Therefore, the evaluation of an ASO may require both rodent and non-rat studies, and scientifically sound judgments on which species, studies, and assays best fulfilling the objectives of the regulatory guidelines for addressing safety pharmacology within a development program.

The compilation of data described here is focused on the results of six distinct ASOs with known targets and a scrambled ASO with no known biologic target. Each was evaluated for effect in the core battery systems of in vivo neurobehavior and respiratory and cardiovascular function. The hERG assay is the most common in vitro means of assessing the potential for repolarization abnormalities that can lead to cardiovascular events, and although no inhibition was anticipated, this analysis was included to provide a more robust analysis for safety pharmacology.

It should be recognized that the ASOs described herein representing biologic targets were intended for diverse clinical indications, and as such the development programs for the ASOs each had a specific focus. The objectives of each program are beyond the scope of this manuscript, but these do have an influence on the designs of the studies and the data sets for consideration. Finally, studies were conducted within different facilities and the specific details, e.g., endpoints measured in a FOB and data analysis, were based on the facilities’ standard operating procedure[s], with prior review by the development team for assurance of addressing any concerns and a robust scientific assessment.

As noted above, ASOs are not specifically called out in ICH guidance documents, and therefore, the level of evaluation and approach for safety pharmacology is often on a case-by-case basis. The regulatory review for ASOs also appears to involve case-by-case considerations with scientific rationale allowing for differences among programs. In light of this, for certain ASOs discussed herein specific portions of safety pharmacology were addressed within toxicology studies and the studies described here cover only those studies dedicated to safety pharmacology. Furthermore, it is not the authors’ intent to make a strict head-to-head
comparison among ASOs. Compilation of data is essential for the evaluation of an ASO as a pharmaceutical class and for determining the context of specific sequences. A similar data set has not yet been published for ASOs, and this and future publications are expected to increase the knowledge base for these compounds.

2. Methods

2.1. Animal care and use

All procedures involving animals were approved and performed in accordance with guidelines of the Institutional Animal Care and Use Committee (IACUC) of the facility in which each study was conducted.

2.2. ASOs, doses, and time points used in the evaluation

The sequence, modification pattern, targeted gene, and cross-species activity for each of the ASOs is provided in Table 1. Each ASO can be described as having 2′′-O-methoxymethyl (2′′MOE) nucleotides in the first five positions in both the 5′ and 3′ termini, deoxy nucleotides in the central region, and a phosphorothioate backbone throughout the sequence. ASOs were synthesized by Isis Pharmaceuticals, Inc. (Carlsbad, CA, USA) and provided for each study as stock or as individual concentrations.

The dose ranges evaluated represent doses from toxicology studies in mice (up to 300 mg/kg) rats (up to 250 mg/kg), or monkey (up to 40 mg/kg), and the highest doses are multiples, -10 to 30 X for rodent and -3 to 10 X for monkey, above potential clinical doses. Specific observations for clinical observations, serum chemistry and hematology, and histopathology have been described (Farman & Kornbrust, 2003; Henry et al., 2012; Jason et al., 2004; Levin et al., 2001), and these doses are similar to those for which the hepatic, renal, immune stimulation effects and complement activation (monkey only) occur with ASOs. Likewise, the concentrations for the hERG assay are similar to (25 to 50 μM) or greater than (> 100 μM) peak concentrations achieved in toxicology studies, and greatly above the < 5 μM concentrations found with common clinical doses.

The pharmacokinetics of an ASO are best described by rapid clearance from the systemic circulation followed by uptake into tissues. Yu et al. (2008) outlined the cross-species pharmacokinetics of an ASO in mouse, rat, monkey, and human. Peak concentration (Cmax) occurs immediately upon intravenous injection or at or near the end of an infusion; following subcutaneous injection Cmax is approximately 30 min post-dose. The elimination half-life (t1/2) in the systemic circulation is ~1 h or less across species. Elimination from the systemic circulation occurs with a combination of nuclease-based metabolism, renal elimination, and uptake and deposition into tissues, primarily liver, kidney, and other organs of the reticuloendothelial system. It is during the period of systemic circulation of an ASO that is considered to be the most likely for adverse effects upon the CNS and respiratory and cardiovascular systems. Therefore, data collection and monitoring were focused on early time periods following dosing.

2.3. Evaluation on neurobehavior in mice

ISIS 420476 was dissolved in phosphate buffered saline (PBS) at 5, 10, or 25 mg/mL to achieve dose levels of 50, 100, or 250 mg/kg, respectively, and administered as a single subcutaneous dose. Controls were administered PBS at the same dose volume of 10 mL/kg. Eight Crl:CD1(ICR) male mice (Charles River UK Limited), 6 to 7 weeks of age and body weight range 27.8 to 37.4 g at study initiation, were assigned to each group. Neurobehavioral observations were recorded pre-dose (0 min) and at 30, 90, 150 and 300 min post-dose. The specific endpoints for evaluation were: death, restlessness, apathy, writhing, fighting, stereo typed behavior (described type), tremor, twitches, convulsions, exophthalmos, respiration, alertness, startle response, loss of righting reflex, abnormal body carriage (described type), abnormal gait (described type), straub tail, piloerecton, pupil diameter, touch response, fearfulness, pinna reflex, corneal reflex, catalepsy, passivity, aggressiveness, body tone, grip strength, cutaneous blood flow, cyanosis, ptosis, lacrimation, salivation, pain response, paralysis, grooming, diarrhea, vocalization, and increased urination.

ISIS 487660 was given subcutaneously at 50, 100, or 300 mg/kg, as a single dose, using a dose volume of 10 mL/kg and concentrations of 4, 10, or 30 mg/mL in PBS. Controls received only PBS. Ten males and 10 females were assigned to each of the dose or control groups. Observation for neurobehavioral changes were recorded pre-dose and at 60, 120, and 360 min and 24 h post-dose. Animals were evaluated for locomotor activity, tail elevation, tremors, convulsion, abdominal tone, catalepsy, traction, righting reflex, pinna reflex, piloerecton, skin coloration, respiration rate (observed), palppebra size (eyelid), exophthalmos, lacrimation, salivation, diarrhea, startle reflex, and death.

Data collected in the mouse was analyzed for statistical significance using StarTox version 3.2.1 (Graham Laboratories, New Braunfels, TX, USA). Analysis of variance (ANOVA) was used to test for group differences. When the results were statistically significant (p < 0.05) inter-group comparisons were made using Student’s t-tests, with the error mean square from one-way ANOVA. Body temperature and spontaneous locomotor activity results were compared to controls using ANOVA followed by Williams’ test.

2.4. Evaluation on neurobehavior in rats

2.4.1. Single dose

ISIS 353512 was dissolved in PBS at 20, 40, or 100 mg/mL to achieve dose levels of 50, 100, or 250 mg/kg, respectively. Controls were administered PBS at the same dose volume, 2.5 mL/kg, and dose route, subcutaneous injection. Ten male and 10 female Sprague–Dawley rats (Orient Bio Co., Ltd., Kyunggi-do, Korea) were assigned to each group. At the time of dosing, rats were approximately 5 weeks of age with body weights ranging from 130 to 159 g and 123 to 145 g for males and females, respectively. Neurobehavior of the animals were observed and recorded pre-dose and at 30, 90, 180, and 360 min and 24 h post-dose.

2.4.2. Repeat dose

ASOs were dissolved in PBS at a concentration of 50 mg/mL. ISIS 116847 or ISIS 379804 was administered at 50, 100 or 150 mg/kg (1, 2, and 3 mL/kg, respectively). ISIS 345198 or ISIS 141923 was administered at 150 mg/kg (3 mL/kg). Controls were administered PBS (3 mL/kg) only. Each group was composed of four male (132–147 g) and four female (113–132 g) Sprague–Dawley rats (Orient Bio Co., Ltd., Kyunggi-do, Korea) approximately 6 weeks of age at study initiation. Doses were administered by subcutaneous injection on Days 1, 4 and 8. On Day 8, observations were recorded pre-dose and at 45, 90, 180, and 360 min post-dose.

| Table 1 | Sequence, chemical structure, target, and cross-species activity of the ASOs. |
|-------------|-----------------|-----------------|-----------------|
| ASO ID no.  | Sequencea       | Target          | Cross-species activity |
| 116847      | CTCCCTACCTCTGGAATTCGA | PTEN            | Human, monkey, rat, mouse |
| 141923      | CTTTCTCATGAGGACTCC | Scramble ASO    | No perfect matches in any species |
| 345198      | GCTAACGTGCTCTGCTG | GCCR            | Human, monkey, rat, mouse |
| 353512      | TCCATTTGAGGACCTGGT | CRP             | Human, monkey |
| 379804      | CCTCTCTGATTCCTCCAC          | ApoB       | Mouse, rat |
| 420476      | TTCATCTGCTCTGACTATG | GCCR            | Human, monkey |
| 487660      | CACGCTCAACCCCTTTA      | α1-Antitrypsin  | Human, monkey |
2.4.3. Observations and statistical analysis

Neurobehavior was evaluated by observation of signs during visual inspection or elicited by specified handling of the animals. Animals were observed for the presence of locomotor activity, tail elevation, tremors, convolution, abdominal tone, catalepsy, righting reflex, grip strength (traction), pinna reflex, piloerection, skin color, respiration rate, palpebra size (eyelid), exophthalmos, lacrimation, salivation, diarrhoea, startle reflex, and death. Body temperature was also measured at each time point.

Statistical analysis was performed using the GraphPad Instat program (GraphPad Software, Inc., La Jolla CA, USA). Variance of numerical data was checked by the Bartlett test; if the variance was homogeneous, the data was subjected to ANOVA; otherwise, they were analyzed by the Kruskal–Wallis H test. Dunnett’s multiple comparison tests were conducted and data were considered to be significant at \( p < 0.05 \).

2.5. Evaluation of respiration in conscious mice

Eight Crl:CD1(ICR) male mice/group (Charles River UK Limited), 6 to 7 weeks of age and 29.6 to 38.0 g at study initiation, were given a single subcutaneous dose of ISIS 420476 at 50, 100 or 250 mg/kg (6, 12 and 30 mg/mL in PBS, respectively; 8.33 mL/kg). Baclofen (Sigma-Aldrich Co., St. Louis MO, USA) was dosed at 20 mg/kg as a study positive control.

Prior to dose administration, respiration rate, tidal volume, and minute volume were measured for a 60 minute period in unrestrained animals in a whole body plethysmograph (Buxco Research Systems, Wilmington NC, USA). Pre-dose data was taken as the mean value of the last 30 min of data collection. Post-dose time points were 30, 60, 90, 120, 150, 180, 210 and 240 min and 24 h. Data were the mean of ten 2 minute intervals around each time point, with the exception of the 240 minute and 24 hour time points which were the mean of the last 20 min of data collection for each.

Analysis of covariance (ANCOVA) was carried out on the data for each post-dose time point with pre-dose data included as a covariate in the analyses. Following the ANCOVA, control (PBS) was compared to the ASO-treated groups using Williams’ test. Comparisons were made between the PBS group and the positive control (baclofen) group using Student’s t-test based on the error mean square from the ANCOVA. A significance level of \( p < 0.05 \) was used for all evaluations.

2.6. Evaluation of respiration in conscious rats

2.6.1. Single dose

Four groups of eight male Sprague–Dawley rats/group (Orient Bio Co., Ltd., Kyunggi-do, Korea) were used for evaluation of respiration rate, tidal volume, and minute volume. At study initiation rats were approximately 6 weeks of age and ranged in weight from 112 to 128 g. ISIS 353512 was evaluated following a single subcutaneous dose of 0 (control), 50, 100, or 250 mg/kg (2.5 mL/kg; 20, 40, and 100 mg/mL in PBS, respectively).

2.6.2. Repeat dose

On Days 1, 4 and 8, ISIS 116847 or ISIS 379804 was administered subcutaneously at an ASO dose of 50, 100 or 150 mg/kg (1, 2, and 3 mL/kg, respectively; 50 mg/mL in PBS); ISIS 345198 or ISIS 141923 was administered at 150 mg/kg (3 mL/kg; 50 mg/mL in PBS). Controls were administered PBS (3 mL/kg) only. Male Sprague–Dawley rats (Orient Bio Co., Ltd., Kyunggi-do, Korea) were used for the study and were approximately 6 weeks of age and body weights ranged from 171 to 184 g at study initiation. Eight animals were assigned to each of the ASO-treated or control groups.

2.6.3. Measurement and statistical analysis

Before initiation of dosing on Day 1 (ISIS 353512) or Day 8 (other ASOs), baseline data for respiration rate, tidal volume and minute volume was obtained for a 10 minute period in unrestrained animals in a whole body plethysmograph (Buxco Research Systems, Wilmington, NC USA). Pulmonary function was then measured for a 10 minute period at 45, 90, 180, and 360 min post-dose. Statistical analysis was performed using the GraphPad Instat program (GraphPad Software, Inc., La Jolla CA, USA). Variance of numerical data was checked by the Bartlett test; if the variance was homogeneous, the data was subjected to ANOVA; otherwise, they were analyzed by the Kruskal–Wallis H test. Dunnett’s multiple comparison tests were conducted and data were considered to be significant at \( p < 0.05 \).

2.7. Evaluation of the cardiovascular system in rats

Each group was composed of five male rats (Orient Bio Co., Ltd., Kyunggi-do, Korea). Controls were dosed subcutaneously with PBS (3 mL/kg) only. On Days 1, 4, and 8, groups assigned to receive ISIS 116847 or ISIS 379804 were dosed subcutaneously with 50, 100 or 150 mg/kg (1, 2, and 3 mL/kg, respectively); ISIS 345198 and ISIS 141923 were administered at 150 mg/kg (3 mL/kg). On Day 7, all animals were anesthetized with 50 mg/kg pentobarbital sodium and a polyethylene cannula was surgically inserted into the femoral artery.

Prior to dosing on Day 8, baseline data was collected in freely moving conscious animals via the arterial catheter connected to a blood pressure (BP) transducer (BIPAC System Inc., Goleta CA, USA), and mean BP and heart rate were recorded for a 10 minute period using a BP recording system (BIPAC System Inc., Goleta CA, USA). Post-dose data were collected at 45, 90, 180, and 360 min. Statistical analysis was performed using the GraphPad Instat program (GraphPad Software, Inc., La Jolla, CA, USA). Variance of numerical data was checked by the Bartlett test; if the variance was homogeneous, the data was subjected to ANOVA; otherwise, they were analyzed by the Kruskal–Wallis H test. Dunnett’s multiple comparison tests were conducted and data were considered to be significant at \( p < 0.05 \).

2.8. Evaluation on cardiovascular system in unrestrained non-human primates

An ascending dose study was used for the evaluation of ISIS 487660. There was a minimum washout period of 3 days between the dosing of the control (PBS; 0 mg/kg) and low (12 mg/kg) dose, and a 1 week washout between the dosing of the low and high dose (40 mg/kg) groups. All doses were given by subcutaneous injection.

Four male cynomolgus monkeys of Cambodian origin (SNBL USA, Alice TX, USA) were surgically implanted with telemetry transducers (Data Sciences International, St. Paul MN, USA) for continuous collection of hemodynamic, body temperature, and ECG data; and with arterial vascular access ports (Access Technologies, Skokie IL 60076, USA) for collection of arterial blood. Collection of cardiovascular data began at least 2 h before each dose, and ended after the last time point. Data was specifically analyzed at — 60 min (prior to dose) and 60, 120, 180, 240, 300, 360, and 720 min and 24 h post-dose. Measurement of systolic, diastolic, and mean arterial pressure, heart rate, respiratory rate, body temperature, and lead II ECG parameters (PR interval, QRS duration, RR interval, and QT interval) were acquired from each animal at each time point. Following established procedures for the laboratory, the lead II QT interval was corrected for variations in heart rate using Fridericia QTc interval (QTcF) and an individual animal correction factor (QTcI). Representative ECG tracings were evaluated for disturbances in rhythm and waveform morphology TcI prior to dosing, at the 240 minute post-dose time point, and at the end of the evaluation period. Blood samples were collected at 240 min post-dose for ISIS 487660 concentration analysis. Arterial blood samples were taken prior to dose and at 360 min post-dose for PO2, PCO2, pH, and SO2% assessments.

Statistical analysis was performed using GraphPad Prism Version 4.03 (GraphPad Software, Inc., La Jolla CA, USA). Comparisons were made between the control and ASO-treated groups using Friedman
Test and Dunn's post-hoc test statistical procedures, except arterial blood gases which were compared using one-way ANOVA. Differences between the control and ASO treatment groups were considered to be statistically significant at \( p < 0.05 \).

Eight female cynomolgus monkeys of Chinese origin (approximately 3–5 years of age, weight range 2.7–3.3 kg; Covance Research Products, Inc.) were surgically implanted with telemetry units (Data Sciences International, St. Paul MN, USA) for evaluation of ISIS 420476. All eight animals received the vehicle control (subcutaneous injection of PBS) on Day 1; on Day 4, four animals each received a subcutaneous dose of 12 or 40 mg/kg (0.4 mL/kg dose volume). Systolic, diastolic, and mean arterial blood pressures, heart rate (derived from blood pressure and ECG), and ECG were monitored continuously from at least 2 h prior to dosing until at least 24 h post-dose. ECG parameters included RR interval, PR interval, QRS duration, QT interval, and corrected QT (QTc) interval. For this study and data collection methods the QTc interval was calculated as described by Spence, Soper, Hoe, and Coleman (1998) and modified by Miyazaki and Tagawa (2002). Representative ECG tracings were printed at approximately 1 h prior to dosing, at approximately 3.5 h post-dose, and within the last hour of the data recording sessions, for visual evaluation.

A Repeated Measures Analysis of Covariance (rANCOVA) was performed for each endpoint, with post-treatment group mean results (Day 4) compared to the group mean results collected on Day 1 (PBS treatment) from the matching animals. Akaike's Information Criterion (AIC) was utilized for model selection. This approach takes into account statistical goodness of fit and the number of parameters that have to be estimated to achieve a particular degree of fit. The AIC penalizes for the addition of parameters, and thus selects a model that fits well but has a minimum number of parameters. Lower values indicate the preferred model, and results from the smallest AIC were used. The first order Kenward–Roger (KR) degrees of freedom approximation was utilized. When the ‘treatment by time’ interaction was significant (\( p < 0.05 \)), each treatment group Least Squares mean (LSmean) was compared to the control group LSmean using a 2-sided Student’s t-test; if this was not significant (\( p > 0.05 \)), the treatment main effect was evaluated. If the treatment main effect was significant a 2-sided Student’s t-test was used to compare treated to control, using the overall segment LSmeans; if not significant no further analysis was conducted. For respiratory rate no additional analysis was performed following the rANCOVA.

### 2.9. Evaluation on hERG channel current

#### 2.9.1. Cell culture and expression of hERG in HEK293 cells

HEK293 cells were stably transfected with hERG cDNA, the lineage, procedures, and historical data for which was established within the facility which testing occurred. Stable transfectants were selected by coexpression with the G418 resistance gene incorporated into the expression plasmid. Cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (D-MEM/F-12) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. Selection pressure was maintained with 500 μg/mL G418 in the culture medium. All reagents were obtained from Sigma-Aldrich (St. Louis MO, USA).

#### 2.9.2. Electrophysiological recordings in HEK293 cells

Cells were transferred to the recording chamber and superfused with HEPES-buffered physiological saline (HB-PS: 137 mM NaCl; 4.0 mM KCl; 1.8 mM CaCl\(_2\); 1 mM MgCl\(_2\); 10 mM HEPES; 10 mM glucose; pH adjusted to 7.4 with NaOH). The Micropipette Solution for whole cell patch clamp recordings was composed of 130 mM potassium aspartate; 5 mM MgCl\(_2\); 5 mM EGTA; 4 mM ATP; 10 mM HEPES; pH adjusted to 7.2 with KOH. All reagents were obtained from Sigma-Aldrich (St. Louis MO, USA).

Assays were performed at 33 to 35 °C. A patch-clamp amplifier was used for whole cell recordings. Cells were held at −80 mV prior to study start. Onset and steady state inhibition of hERG potassium current were measured using a pulse pattern with fixed amplitudes (conditioning prepulse +20 mV for 1 s; repolarizing test ramp to −80 mV (−0.5 V/s) repeated at 5 s intervals). Each recording ended with a final application of a supramaximal concentration of E-4031 (0.5 μM supplemented with 0.3% DMSO) to assess the contribution of endogenous currents. The remaining uninhibited current was subtracted to determine the potency for hERG inhibition. ISIS 487660 was tested at 108 and 324 μM. For ISIS 420476, the concentration range was 25, 50, 100 and 150 μM. Steady state was defined by the limiting constant rate of change with time (linear time dependence). The steady state before and after ASO application was used to calculate the percentage of current inhibited at each concentration.

Data acquisition and analyses were performed using the suite of pCLAMP programs (Ver. 8.2; Molecular Devices, LLC, Sunnyvale CA, USA). Percent inhibition at each ASO concentration was compared to control using one-way ANOVA followed by Dunnett’s multiple comparison test (JMP Version 5.0.1, SAS Institute, Cary NC, USA). Significant inhibition was defined at \( p < 0.05 \).

#### 2.9.3. Cell culture and expression of hERG in CHO-K1 cells

ISIS 116847, ISIS 141923, ISIS 345198, ISIS 353512, and ISIS 379804 were tested in CHO-K1 cells. Cells were maintained in 90% Minimum Essential Medium supplemented with 9% fetal bovine serum, 1% penicillin–streptomycin, and 50 μg/mL Hygromycin B. Reagents were obtained from Invitrogen Corporation (Carlsbad CA, USA). The hERG cDNA, in a pcDNA3.1/zeo(−) expression vector, was cotransfected with a surface marker protein, green fluorescence protein (GFP), to allow assessment of the transfection efficiency and identification of cells for electrophysiological study. Plasmids were transiently expressed in cells using Lipofectamin2000 (Invitrogen Corporation, Carlsbad CA, USA).

#### 2.9.4. Electrophysiological recordings in CHO-K1 Cells

All testing was conducted in whole-cell patch clamp experiments performed in the voltage–clamp mode. With the exception of the evaluation of ISIS 345198, cells were superfused with a control external solution containing 143 mM NaCl; 5.4 mM KCl; 5.0 mM HEPES; 0.5 mM MgCl\(_2\); 16.6 mM Glucose; and 1.8 mM CaCl\(_2\) (pH 7.4; titrated with NaOH). While within the acceptance range, the salt content for the lot of material used for evaluation of ISIS 345198 was considered to be elevated and the superfusing solution was modified to 137 mM NaCl; 4.0 mM KCl; 10.0 mM HEPES; 1.0 mM MgCl\(_2\); 10.0 mM Glucose; and 1.8 mM CaCl\(_2\) (pH 7.4 was titrated with NaOH). For all the ASOs, the micropipettes were filled with an intracellular solution containing 130 mM KCl; 1 mM MgCl\(_2\); 5 mM EGTA; 5 mM MgATP; and 5 mM HEPES (pH 7.2; titrated with KOH). Reagents were obtained from Sigma-Aldrich Co. (St. Louis MO, USA), with the exception of KOH (Junsei Chemical Co. Ltd., Tokyo, Japan).

All experiments were conducted at 37 °C. Current signals were amplified and digitized by an Axopatch 200B amplifier (Axon Instruments, Inc., Union City CA, USA) and analyzed using the pCLAMP software package (version 8.0, Molecular Devices, LLC, Sunnyvale CA, USA) and SigmaPlot2000 (SPSS Inc., Chicago IL, USA). Cells were clamped at a holding potential of −80 mV. After establishing the whole-cell configuration, stimulation protocols were applied successively and the induced currents recorded. Cells were depolarized for 2 to 20 mV followed by a 3 second repolarization back to −40 mV. After the control stimuli were applied, the extracellular solution was changed to a solution containing ASO. Additional stimuli were then applied and the peaks of the inward tail-currents analyzed.

For ISIS 116847 and ISIS 379804, concentrations were 15, 75, and 150 μM; ISIS 345198 concentrations were 15, 50, 75, and 150 μM; ISIS 141923 was tested at 150 μM, and ISIS 353512 concentrations were
3. Results

For the in vivo studies, a summary of the study type, species used for evaluation, the specific ASO, and the dose range evaluated is given in Table 2. A similar summary of the concentration range for each ASO in the hERG assay is provided in Table 3.

3.1. Effects of ASOs on neurobehavior

Subcutaneous administration of ISIS 487660 at up to 300 mg/kg was not associated with changes in neurobehavior or body temperature in male or female mice. Similarly, male mice receiving up to 250 mg/kg ISIS 420476 produced no meaningful changes in behavior, locomotor activity, or body temperature. There were no primary neurobehavioral effects noted in male or female rats administered a single dose of up to 250 mg/kg ISIS 353512, or in rats administered repeat doses (3 doses total) of ISIS 116847, ISIS 379804, ISIS 345198 or ISIS 141923 at up to 150 mg/kg/dose.

Transient changes in body temperature, and an increased incidence of diarrhea, were noted in association with ASO administration. A statistically significant, but relatively minimal (0.7 °C), decrease in body temperature was found at 30 min post-dose for female rats, but not male rats, with a single 250 mg/kg dose of ISIS 353512 (Table 4). Also shown in Table 4, potential treatment-related observation of an increased incidence of diarrhea was noted in males at 30 to 180 min and in females at 30 and 90 min post-dose. With repeated administration of ISIS 116874, ISIS 379804, ISIS 345198, or ISIS 141923, mild (<1 °C) and occasionally statistically significant differences of increased or decreased mean body temperature were noted post-dose on Day 8 (Table 5); however, statistically significant differences in mean body temperature were also found pre-dose for ISIS 116847, ISIS 345198, and ISIS 141923. In monkey, a mild (<1 °C) and occasionally statistically significant decreased body temperature was noted for the 12 and 40 mg/kg ISIS 420476 groups proximate to 2 to 3 h post-dose (Table 6).

3.2. Effects of ASOs on respiration

No treatment-related changes in respiratory rate, tidal volume or minute volume were found in mice administered up to 250 mg/kg ISIS 420746. Following a single dose of ISIS 353512 at up to 250 mg/kg in the rat, no changes in respiratory rate, tidal volume, or minute volume were noted. Repeat dosing of ISIS 116847, ISIS 379804, ISIS 345198, or ISIS 141923 at up to 150 mg/kg/dose was also found to be without meaningful change in respiratory function in rat. There were no changes in respiratory rate (collected via telemetry) or arterial blood gases (PO2, PCO2, pH, and SO2%) associated with the administration of ISIS 487660 in monkeys.

3.3. Effects of ASOs on cardiovascular system

In the rat, a statistically significant decrease in heart rate was noted with 150 mg/kg ISIS 345198, ISIS 141923, and ISIS 116847 at 45 min post-dose, and with 100 and 150 mg/kg ISIS 379804 and 150 mg/kg ISIS 345198 at 90 min post-dose (Table 7). The relatively modest and transient changes in heart rate were not associated with changes in mean blood pressure. No differences in heart rate were noted at 180 or 360 min post-dose, and no changes in heart rate or blood pressure were noted at 50 mg/kg for any of the four ASOs or time points evaluated. Of note, the 45 and 90 minute time frame is the same in which

Table 2
Summary of the in vivo studies for CNS, respiratory, and cardiovascular systems with various ASOs.

<table>
<thead>
<tr>
<th>Study type</th>
<th>Species (strain)</th>
<th>ASO</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS</td>
<td>Mouse</td>
<td>420476</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(CD-1)</td>
<td>353512</td>
<td></td>
</tr>
<tr>
<td>CNS (single dose)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNS (repeat dose)</td>
<td>(SD)</td>
<td>116874</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>379804</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>345198</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>141923</td>
<td></td>
</tr>
<tr>
<td>Respiration</td>
<td>Mouse</td>
<td>420476</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(CD-1)</td>
<td>353512</td>
<td></td>
</tr>
<tr>
<td>Respiration (single dose)</td>
<td></td>
<td>116847</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(SD)</td>
<td>379804</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>345198</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>141923</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Rat</td>
<td>116847</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(SD)</td>
<td>379804</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>345198</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>141923</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Monkey</td>
<td>487660</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(cynomolgus)</td>
<td>420476</td>
<td></td>
</tr>
</tbody>
</table>

SD = Sprague–Dawley. All doses were administered by subcutaneous injection. For repeat dosing, rats were dosed on Days 1, 4 and 8.
decreased body temperature was noted among these ASOs. Therefore, considering that there is no clear evidence of a direct ASO-dependent effect on cardiac function, the biological significance of these observations for heart rate is in question.

Continuous monitoring in telemeterized monkeys allowed for greater dynamics in the evaluation of ASOs for cardiovascular effects. Blood collected at 4 h post-subcutaneous injection confirmed dose dependent systemic exposure of ISIS 487660 with group mean levels of 34.5 μg/mL and 84.5 μg/mL for the 12 and 40 mg/kg doses, respectively. No significant differences between these groups were found for the HR corrected QT intervals (QTcF and QTcI) at any other time points.

Table 5: Effects of ISIS 116847, ISIS 379804, ISIS 345198 and ISIS 141923 on body temperature in rats pre-dose and following subcutaneous administration on Day 8.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Pre-dose</th>
<th>45 min</th>
<th>90 min</th>
<th>180 min</th>
<th>360 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature (°C)</strong> males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>0</td>
<td>38.8 ± 0.1</td>
<td>38.2 ± 0.1</td>
<td>38.2 ± 0.1</td>
<td>38.1 ± 0.2</td>
<td>38.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>38.5 ± 0.2</td>
<td>38.3 ± 0.1</td>
<td>38.3 ± 0.1</td>
<td>38.2 ± 0.2</td>
<td>38.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>38.6 ± 0.3</td>
<td>38.2 ± 0.4</td>
<td>38.2 ± 0.4</td>
<td>38.2 ± 0.6</td>
<td>38.1 ± 0.2</td>
</tr>
<tr>
<td>ISIS 379804</td>
<td>50</td>
<td>38.3 ± 0.1*</td>
<td>37.7 ± 0.1*</td>
<td>37.8 ± 0.5</td>
<td>37.3 ± 0.0</td>
<td>37.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>38.4 ± 0.2</td>
<td>38.3 ± 0.3</td>
<td>38.1 ± 0.3</td>
<td>37.4 ± 0.2</td>
<td>37.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>38.4 ± 0.2*</td>
<td>38.0 ± 0.1</td>
<td>37.6 ± 0.1</td>
<td>37.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>ISIS 345198</td>
<td>150</td>
<td>37.9 ± 0.4**</td>
<td>37.9 ± 0.3</td>
<td>38.0 ± 0.2</td>
<td>37.1 ± 0.4</td>
<td>37.1 ± 0.2*</td>
</tr>
<tr>
<td>ISIS 141923</td>
<td>150</td>
<td>38.1 ± 0.4*</td>
<td>38.2 ± 0.4</td>
<td>38.1 ± 0.5</td>
<td>37.3 ± 0.4</td>
<td>37.1 ± 0.3</td>
</tr>
<tr>
<td><strong>Temperature (°C)</strong> females</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>0</td>
<td>39.0 ± 0.5</td>
<td>39.1 ± 0.5</td>
<td>38.9 ± 0.6</td>
<td>38.0 ± 0.3</td>
<td>37.4 ± 0.1</td>
</tr>
<tr>
<td>ISIS 116847</td>
<td>50</td>
<td>38.9 ± 0.2</td>
<td>38.9 ± 0.3</td>
<td>38.7 ± 0.6</td>
<td>37.9 ± 0.2</td>
<td>37.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>39.0 ± 0.3</td>
<td>38.7 ± 0.2</td>
<td>38.6 ± 0.2</td>
<td>38.5 ± 0.3</td>
<td>37.8 ± 0.4</td>
</tr>
<tr>
<td>ISIS 379804</td>
<td>50</td>
<td>39.1 ± 0.1</td>
<td>38.4 ± 0.2*</td>
<td>38.7 ± 0.2</td>
<td>37.3 ± 0.2</td>
<td>37.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>39.1 ± 0.3</td>
<td>38.8 ± 0.3</td>
<td>38.7 ± 0.2</td>
<td>37.6 ± 0.3*</td>
<td>37.6 ± 0.2</td>
</tr>
<tr>
<td>ISIS 345198</td>
<td>150</td>
<td>39.2 ± 0.2</td>
<td>39.0 ± 0.2</td>
<td>39.1 ± 0.1</td>
<td>37.8 ± 0.7*</td>
<td>37.5 ± 0.3</td>
</tr>
<tr>
<td>ISIS 141923</td>
<td>150</td>
<td>39.0 ± 0.5</td>
<td>39.1 ± 0.5</td>
<td>38.9 ± 0.6</td>
<td>38.0 ± 0.3</td>
<td>37.4 ± 0.1</td>
</tr>
</tbody>
</table>

Rats were dosed on Days 1, 4 and 8 of the study. Each value represents the mean ± S.D.; n = 4/group; statistically significant difference from control group (phosphate buffered saline (PBS)): *p < 0.05 and **p < 0.01.
Table 6

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>0.5 h</th>
<th>1 h</th>
<th>1.5 h</th>
<th>2 h</th>
<th>2.5 h</th>
<th>3 h</th>
<th>3.5 h</th>
<th>4 h</th>
<th>4.5 h</th>
<th>5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>38.90 ± 0.24</td>
<td>38.75 ± 0.12</td>
<td>38.68 ± 0.16</td>
<td>38.98 ± 0.11</td>
<td>38.98 ± 0.13</td>
<td>38.78 ± 0.14</td>
<td>38.55 ± 0.14</td>
<td>38.60 ± 0.13</td>
<td>38.45 ± 0.14</td>
<td>38.73 ± 0.13</td>
</tr>
<tr>
<td>12</td>
<td>38.80 ± 0.24</td>
<td>38.42 ± 0.12</td>
<td>38.80 ± 0.16</td>
<td>38.45 ± 0.11*</td>
<td>38.37 ± 0.13*</td>
<td>38.60 ± 0.14</td>
<td>38.57 ± 0.13</td>
<td>38.62 ± 0.14</td>
<td>38.70 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>38.77 ± 0.22</td>
<td>38.40 ± 0.14</td>
<td>38.40 ± 0.13</td>
<td>38.75 ± 0.10</td>
<td>38.62 ± 0.08</td>
<td>38.45 ± 0.10</td>
<td>38.47 ± 0.08</td>
<td>38.30 ± 0.11</td>
<td>38.30 ± 0.11</td>
<td>38.55 ± 0.12</td>
</tr>
<tr>
<td>40</td>
<td>38.51 ± 0.22</td>
<td>38.10 ± 0.14</td>
<td>38.38 ± 0.13</td>
<td>38.10 ± 0.10*</td>
<td>38.00 ± 0.08*</td>
<td>38.18 ± 0.10</td>
<td>38.25 ± 0.08</td>
<td>38.30 ± 0.11</td>
<td>38.38 ± 0.11</td>
<td>38.50 ± 0.12</td>
</tr>
</tbody>
</table>

Values represent the LSmean ± LSM s.e.; n = 4; statistically significant difference from control: *p < 0.05. A pre-dose value was not collected, comparison for ISIS 420476-treated monkeys is made to the corresponding value for the control group at each time point.

3.4. Effects of ASOs on hERG current in HEK293 and CHO-K1 cells

Representative tracings from the hERG assay in HEK293 cells for ISIS 420476 at the 150 μM concentration and E-4031 at 0.5 μM are displayed in Fig. 3. The concentration-response for ISIS 420476 (expressed as mean ± s.e.m.) demonstrated a hERG current reduction of 5.2 ± 1.4%, 6.1 ± 1.3%, 8.9 ± 1.6%, and 8.6 ± 1.8% at 25, 50, 100 and 150 μM, respectively; the values for the 100 and 150 μM concentrations were statistically significantly different from control (1.2 ± 0.7%).

A clear reduction in hERG current was found for the positive control, 60 nM terfenadine, with values ranging from 77.9% to 81.4%. No apparent reduction in the hERG current was found for ISIS 487660 in HEK293 cells with values of 0.2 ± 0.8% at 108 μM and 0.9 ± 0.4% at 324 μM, as compared to 1.0 ± 0.6% for the negative control and approximately 84% reduction for the positive control (60 nM terfenadine). An IC50 value could not be calculated for ISIS 420476 due to the modest changes across the concentration range, and data was too limited for calculations for ISIS 487660; however, the inhibitory effect of hERG current was estimated to be greater than 150 μM and 324 μM, respectively.

In CHO-K1 cells, reduction in the hERG-current (mean ± s.e.m.) with ISIS 353512 was found to be 0.8 ± 0.6%, 0.2 ± 1.0%, 4.1 ± 1.6% and 6.6 ± 1.4% at concentrations of 10, 50, 150, and 300 μM, respectively, as compared to 3.5 ± 1.6% for the superfusing solution only, or an 88 ± 1.4% reduction with 100 nM E-4031. Values for ISIS 116847 (−2.0 ± 0.6%, −0.4 ± 1.9% and 2.1 ± 1.1%) or ISIS 379804 (−1.9 ± 1.0%, −0.7 ± 0.6% and 3.4 ± 0.7%) at 15, 75 or 150 μM, respectively, and ISIS 141923 (2.9 ± 1.6%) at 150 μM were not statistically or biologically significant for reduction in hERG potassium current. As described, the superfusing solution for ISIS 345198 contained a lower concentration of NaCl and KCl, nevertheless, ISIS 345198 was associated with a statistically significantly reduced hERG current at the higher concentrations of 75 μM (22.5 ± 3.4%) and 150 μM (30.2 ± 0.8%), a modest decrease (9.5 ± 2.4%) at 50 μM, and no apparent reduction (1.9 ± 2.1%) at 15 μM. With the latter four ASOs, the hERG current for the superfusing solution group was found to be 0.8 ± 0.6%, and for E-4031 (100 nM) the reduction in hERG current was 94.1 ± 0.1% for each of the ASOs evaluated in CHO-K1 cells, the IC50 was predicted to be >150 μM.

Table 7

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>0 min</th>
<th>45 min</th>
<th>90 min</th>
<th>180 min</th>
<th>360 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0</td>
<td>91.8 ± 7.91</td>
<td>94.3 ± 10.36</td>
<td>94.1 ± 7.91</td>
<td>93.8 ± 8.36</td>
<td>94.4 ± 7.93</td>
</tr>
<tr>
<td>ISIS 116847</td>
<td>50</td>
<td>93.1 ± 9.29</td>
<td>98.6 ± 5.48</td>
<td>98.3 ± 6.43</td>
<td>96.3 ± 6.35</td>
<td>95.1 ± 4.18</td>
</tr>
<tr>
<td>ISIS 379804</td>
<td>50</td>
<td>97.4 ± 2.20</td>
<td>97.9 ± 3.90</td>
<td>97.8 ± 3.97</td>
<td>99.4 ± 5.01</td>
<td>99.8 ± 4.68</td>
</tr>
<tr>
<td>ISIS 141923</td>
<td>150</td>
<td>89.2 ± 11.76</td>
<td>88.9 ± 9.41</td>
<td>88.3 ± 10.99</td>
<td>88.8 ± 11.31</td>
<td>90.0 ± 9.12</td>
</tr>
</tbody>
</table>

Values represent the mean ± S.D. n = 6; PBS, ISIS 116847 150 mg/kg, ISIS 379804 50 mg/kg and ISIS 141923 150 mg/kg, n = 5 others. Statistically significant difference from control group: *p < 0.05 and **p < 0.01.
4. Discussion

The respiratory system was specifically evaluated for six ASOs, one in mouse and five in the rat. Consistent with ICH S7A, respiratory rate and a second measurement, tidal volume, were directly measured by plethysmograph. With up to 250 mg/kg in mouse or rat, there were no effects that were considered to be of toxicological concern or biologically meaningful. Furthermore, respiratory end points were included for assessment of ISIS 487660 in telemeterized monkeys at up to 40 mg/kg, with no effect on respiratory rate or blood gases, including hemoglobin oxygen saturation (SO₂%), a core battery endpoint for the respiratory system. These results are consistent with published reports on the toxicology of similar ASOs, e.g., phosphorothioate (PS) backbone DNA sequences and PS mixed DNA/2′MOE sequences (Henry et al., 2002, 2012; Levin et al., 2001; Zanardi et al., 2012), in which no indications of adverse respiratory effects have been reported.

Neurobehavior was directly assessed through Functional Observation Battery (FOB) tests of two ASOs in mouse and five in rat. While the specific endpoints varied among the studies, each study involved multiple endpoints spanning observation of signs (e.g., locomotor activity) to physical manipulation (e.g., grip strength) for potential CNS effects. No adverse effects were noted among the seven ASOs evaluated for this core physiological system.

Body temperature is listed within the evaluation parameters for assessment of the CNS, as per ICH S7A. As such, this endpoint was included in the evaluation in the mouse (one ASO) and rat (five ASOs). Body temperature was also measured within the cardiovascular studies in monkey (two ASOs). There were no apparent changes in body temperature in mouse. In rat, body temperatures were increased or decreased with no clear pattern post-dose. ISIS 487660 had no apparent impact on body temperature in monkey, whereas ISIS 420476 was noted with decreased mean body temperature. Considering that the magnitude of change was minimal, never exceeding a 1 °C difference between treated and controls for rat or monkey, the change in body temperature was considered to be not adverse.

The proximity to dosing and trend for higher dose levels suggest an underlying reason for fluctuation in body temperature. Similarly, transient observations of diarrhea for ISIS 353512 in rat was most prominent at the highest dose. The absence of alteration in the multiple FOB endpoints suggests that the variations in body temperature and diarrhea are not mediated by a direct action within the CNS. Furthermore, these observations occurred with sequences to diverse targets, varied

| Table 8 | Effects of ISIS 487660 or ISIS 420476 on QRS duration (milliseconds) following subcutaneous dosing in cynomolgus monkey. |
| Dose (mg/kg) | 0 | 12 | 40 | 0 | 12 | 40 | 0 | 12 | 40 | 0 | 12 | 40 |
| 60 min | 60 min | 60 min | 120 min | 180 min | 240 min | 300 min | 360 min | 720 min | 24 h | 60 min | 60 min | 120 min | 180 min | 240 min | 300 min | 360 min | 720 min | 24 h |
| 0 | 39.5 ± 1.3 | 39.0 ± 1.3 | 39.0 ± 1.8 | 38.9 ± 1.6 | 39.3 ± 1.7 | 39.0 ± 1.7 | 38.5 ± 1.3 | 39.2 ± 1.4 | 39.1 ± 1.6 |
| 12 | 37.6 ± 1.8* | 37.9 ± 1.8 | 38.5 ± 1.6 | 37.3 ± 2.3 | 37.4 ± 2.1* | 37.6 ± 1.8 | 38.0 ± 1.9 | 39.5 ± 2.3 | 37.8 ± 2.0 |
| 40 | 38.7 ± 1.6 | 38.6 ± 1.9 | 39.1 ± 2.2 | 39.2 ± 2.1 | 39.0 ± 2.1 | 38.4 ± 2.0 | 38.4 ± 1.9 | 39.5 ± 2.2 | 38.0 ± 2.2 |

Values represent the mean ± s.e.m.; n = 4; statistically significant difference from control group: *p < 0.05.

Fig. 1. Left panel: Mean arterial pressure (MAP) in cynomolgus monkey pre-dose (−60 min) and to 360 min post-dose following subcutaneous administration of ISIS 487660. Right panel: Heart rate in cynomolgus monkey during the same time interval. Values are the group mean ± s.e.m. (n = 4).
species homology, and with a sequence of no known homology (ISIS 141923), indicating no target-related (on- or off-target) association. A more likely reason for changes in body temperature is secondary to a non-specific inflammatory response. Immune stimulation in rodents has been consistently reported to occur with ASOs (Farman & Kornbrust, 2003; Henry, Templin, Gillett, Rojko, & Levin, 1999; Monteith & Levin, 1999), but secondary actions, such as changes in body temperature, may demonstrate temporal variation and were detected in these studies as a result of the frequent repeated measurements post-dose.

Fig. 2. Mean arterial pressure (MAP) and heart rate in cynomolgus monkey following subcutaneous administration of 12 mg/kg (upper left and lower left panels, respectively) or 40 mg/kg (upper right and lower right panels, respectively) ISIS 420476 on Day 4, as compared to matching control data from Day 1. Values are expressed as the Least Square Mean; n = 4/group.

Fig. 3. Representative trace for the effects 150 µM ISIS 420476 and 0.5 µM E-4031 on hERG potassium current in HEK293 cells. Left panel: upper panel [Current (pA)] shows hERG potassium currents obtained in a single cell as evoked by the voltage protocol shown in the lower panel [Voltage (mV)]. Right panel: peak current amplitude during application, horizontal bars indicate the control period, and ASO and E-4031 exposure periods.
The transient decrease in heart rate among the four ASOs was of uncertain biological significance as there was no change in blood pressure. Only ISIS 345198 at 150 mg/kg had two consecutive data points of statistical significance. ISIS 345198 has not been evaluated for impacts on cardiovascular function in non-human primates and may warrant additional follow-up studies to determine if there is a biological significance to these observations.

Continuous monitoring of cardiovascular parameters in telemeterized monkeys administered ISIS 420476 or ISIS 487660 demonstrated no effects on the mechanical function, e.g., vascular pressure and rate of contractions, in the heart. Examination of the ECG tracings and calculation of key intervals (e.g., QRS) further supports an absence of any ASO-dependent actions on electrical conductance in the heart. The occasional value of statistical significance found among the various time points and parameters within or across studies did not demonstrate a trend or consistency. Moreover, when examined with consideration of the variability in each measured parameter found in the control group across time points or across the control and treated groups at pre-dose, the values found to be statistically significant were within the normal expected range further confirming no biological significance and no direct actions on cardiovascular function. Cardiac-based adverse effects reported with ASOs in monkey have been directly associated with activation of the complement system, most prominently the alternative pathway (Henry et al., 1997, 1999, 2002).

Consistent with the absence of any change in quantitative ECG measurements in treated monkeys, there was no biologically meaningful change in hERG function noted among the seven ASOs. This was not unexpected as the most common means of adverse effects on hERG is direct actions within the channel pore (Stanfield, Sutcliffe, & Mitcheson, 2006; Thomas, Karle, & Kielh, 2006), and such as the relatively large molecular size and hydrophobicity of an ASO suggests a low potential for inhibition.

No inhibition of hERG channel current was noted for five of the ASOs. Although the results for ISIS 345198 indicate a modest impact of 20% to 30% inhibition, this occurred at concentrations of 75 μM or higher. The change in hERG channel current with ISIS 420476 was less than 10% with concentrations of >100 μM. While the observations for the latter two ASOs were of interest, the magnitude of the response must be taken into consideration. Katchman, Koerner, Tosaka, Woosley, and Ebert (2006) proposed four categories for hERG inhibition: high-potency blockers (IC50 ≤ 0.1 μM), moderate-potency blockers (0.1 μM < IC50 < 1.0 μM), low-potency blockers (IC50 > 1.0 μM), and ineffective blockers (IC50 > 300 μM). The positive controls, E-4031 and terfenadine, found in the assays fell within the high-potency category. Each of the ASOs, including ISIS 345198, had an IC50 of >150 μM or >300 μM, placing them in the low-potency to ineffective category.

The hERG assay is one for which properties of ASOs and the test system should be considered. Temperature, salt content, and osmolality have been implicated as confounding factors (Kirsch et al., 2004; Yabuuchi, Beckmann, Wettwer, Hegele-Hartung, & Heubach, 2007). Temperature was well controlled in these studies, and would not be expected to be impacted by an ASO. As relatively large molecules (MW of 5000 to 8000 Da) that are highly charged, ASOs can impart oncolytic pressure. Furthermore, they are known to contain sodium and other monovalent cationic salts as counter ions to the 20 negatively charged PS backbone residues. These secondary factors would demonstrate an increased potential for influencing the results with increased concentration, i.e., an apparent dose–response, and thus may play an additive role in the observations of modest changes in hERG channel current at high concentrations.

Furthermore, a cell-based system does not reflect the influence that protein binding may have on ASO-cell interactions. ASOs have been shown to be highly protein bound in plasma (Geary et al., 2001; Watanabe, Geary, & Levin, 2006), with free ASO <10% of the total plasma concentration. Yu et al. (2007) reported a Cmax of approximately 21 μg/mL following a 200 mg intravenous dose of an ASO in humans.

Based on this information and MW of 7000 Da for a standard ASO, this roughly equates to a 3 μM² total concentration, and 0.3 μM free concentration. Thus, the highest concentrations evaluated in the hERG assay exceeded a reasonable total plasma concentration by up to 100× and a free plasma concentration by 1000×. This is well above the 30× margin that has been proposed by Redfern et al. (2003) for compounds that may interfere with hERG potassium channels. Subcutaneous dosing results in much lower plasma concentrations of an ASO, and thus lower potential for interactions with cell surface proteins.

The ASOs can also be examined for general characteristics. In regards to the hERG assay, all seven of the ASOs were evaluated with at least one concentration in the range of 100 μM to 150 μM. This concentration greatly exceeds plasma levels of ASO in studies in monkey as noted for ISIS 487660 with levels of ~5 μM (~35 μg/mL) or ~12 μM (~85 μg/mL), the ~5 μM, and that reported for a 4 mg/kg intravenous infusion of an ASO in monkey or ~3 μM concentration described above for a representative human dose (Yu et al., 2007).

Of interest, mAb have been identified as a class of drugs for which hERG testing is likely to be of limited or no direct benefit (Vargas et al., 2008). This was based on low potential to interact with the extracellular or intracellular domains of the hERG channel, target-specific binding properties, and large molecular size (~140,000 Da). ASOs share the high specificity for binding and structural attributes (high molecular size and nucleic acid composition) that appear to limit the potential for direct hERG inhibition, and at least warrant consideration of a similar approach to that summarized by Vargas et al. (2008). As the published knowledge base for PS DNA/MOE ASOs increases this point should be reviewed for a consensus among the various parties involved in Research and Development of nucleic acid-based pharmaceuticals. The Oligonucleotide Safety Working Group (OSWG) has been established that includes a consortium of individuals working in drug development companies and university settings, and this is a likely source of a consensus review on safety pharmacology testing for ASOs.

In vivo studies are, of course, essential for evaluation of potential cardiac vascular effects, and for effects on other key systems. ASOs were evaluated in the mouse or rat with at least one dose level in the range of 100 to 150 mg/kg, a range that represents a ≥30-fold multiple above clinical doses (McGowan et al., 2012; Ricotta & Frishman, 2012; Stein et al., 2012; Yu et al., 2007). No effects were noted for respiration, and no primary effects were noted for CNS. The 40 mg/kg dose in monkey is 4- to 10-fold above common clinical doses, and inclusion of the results for respiration and the cardiovascular systems further indicates no unwanted actions on these core systems. The absence of direct adverse effects on CNS, and the respiratory and cardiovascular systems is consistent with published data (Farman & Kornbrust, 2003; Henry et al., 1997, 1999, 2002, 2012; Levin et al., 2001; Monteith & Levin, 1999; Zanardi et al., 2012) covering the toxicology for a number of ASOs within the class of PS DNA/MOE ASOs.

While the objectives of safety pharmacology studies encompass key information required for a thorough analysis, much of the data needed to meet regulatory guidance can be obtained within other studies, e.g., the inclusion of cardiac function and cardiac parameters (e.g. QT interval), in GLP toxicology studies. This point is addressed for safety pharmacology in ICH S9, in which it states that safety pharmacology could be included in general toxicology studies when the conduct of stand-alone studies is not called for. In consideration of PS DNA/MOE ASOs, dedicated studies focused on specific organ systems do not appear to add significant data for the interpretation of potential adverse effects. Therefore, the need for dedicated studies for future ASOs in the same class is questionable, rather more encompassing data for cardiac and respiratory function and neurobehavior can be collected in repeated dose and longer-term toxicology studies.

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2 0.021 mg/mL ‡ (1 mmol/7000 mg) ÷ (1000 mL/1 L) ÷ (1000 μmol/mmol) = 3 μM
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References


Pharmacodynamic and subchronic toxicity in mice and monkeys of ISIS 388026, a second-generation antisense oligonucleotide that targets human sodium glucose cotransporter 2. Journal of Pharmacology and Experimental Therapeutics, 343(2), 489–496.