Toxicology Reports 8 (2021) 839–845

Contents lists available at ScienceDirect

Toxicology Reports

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

Safety pharmacology of self-assembled-micelle inhibitory RNA-targeting amphiregulin (SAMiRNA-AREG), a novel siRNA nanoparticle platform

Tae Rim Kim [a,1], Hyeon-Young Kim [b,1], In-Hyeon Kim [b], Ki Cheon Kim [b], Youngho Ko [a], Jun Hong Park [a], Sungil Yun [a], In-Chul Lee [c], Sung-Hwan Kim [b,*], Han-Oh Park [a,*]

[a] siRNAgen Therapeutics and Bioneer Corporation, Daejeon, 34302, Republic of Korea
[b] Jeonbuk Branch Institute, Korea Institute of Toxicology, Jeongeup, 56212, Republic of Korea
[c] Functional Biomaterial Research Center, Korea Research Institute of Bioscience and Biotechnology, Jeongeup, 56212, Republic of Korea

ARTICLE INFO

Edited by Dr. A.M. Tsatsaka

Keywords:
Self-assembled-micelle inhibitory RNA nanoparticle
Amphiregulin
Safety pharmacology
Core battery

ABSTRACT

The present safety pharmacology core battery studies (neurobehavior, respiratory, cardiovascular system, and human ether a-go-go (hERG) channel current) investigated the potential harmful effects of self-assembled-micelle inhibitory RNA-targeting amphiregulin (SAMiRNA-AREG). The SAMiRNA-AREG was administered by single intravenous injection at up to 300 mg/kg and 100 mg/kg in mice and monkeys, respectively. The hERG assay was performed in Chinese hamster ovary (CHO) cells at SAMiRNA-AREG concentrations of up to 200 μg/mL. In the evaluation on neurobehavior, a transient decrease in body temperature was found at 0.5 h (30 min) post-dose at both sexes in mice, with a single 300 mg/kg dose of SAMiRNA-AREG. However, these effects had returned to normal at 1 h post-dose. In the evaluation on hERG channel current, there were statistically significant differences in the inhibition of peak hERG potassium channel current between the 20, 100, and 200 μg/mL SAMiRNA-AREG treatment groups and the vehicle control group. However, these effects were less potent than that of E-4031, a positive control article. For the respiratory and cardiovascular systems, no treatment-related changes were observed in mice or monkeys. Thus, under these experimental conditions, these studies suggest that SAMiRNA-AREG showed no adverse effects on the neurobehavior, respiratory, and cardiovascular function.

1. Introduction

Fibrotic diseases, which include idiopathic pulmonary fibrosis (IPF), nephrogenic systemic fibrosis, and systemic sclerosis, are characterized by enhanced extracellular matrix components and excessive accumulation of collagen in inflamed or damaged tissues [1–5]. The IPF is a relentlessly progressive and inevitably fatal lung disease with high mortality and morbidity, primarily occurring in older people. The annual prevalence and incidence of IPF in the United States is about 100,000 people, and over 30,000 new cases are found each year [6–8]. However, there is are no effective therapeutics for IPF patients yet.

RNA-based gene silencing using RNA interference (RNAi) demonstrates powerful potential as an innovative therapeutic strategy for eliminating expression of pathogenic gene. It was reported that RNAi mediated by small interfering RNA (siRNA) can inhibit the specific target genes of several fibrotic diseases [9–15]. A previous study reported that silencing of amphiregulin (AREG) significantly inhibit collagen deposition in animal model [16]. Recently, the modified siRNA, self-assembled-micelle inhibitory RNA-targeting AREG (SAMiRNA-AREG), demonstrated therapeutic effects for the suppression of the bleomycin-induced lung injury in the pulmonary fibrosis model [17].

In general, the safety pharmacology core battery evaluations (central nervous, respiratory, and cardiovascular systems) are indispensable part of the therapeutic development in rodent and non-rodent animals. These are an essential process in evaluating the pivotal risk before human usage. Despite the great therapeutic potentials for pulmonary fibrosis of SAMiRNA-AREG, there have been no reports on the evaluation of safety pharmacology.

This studies aims to confirm the undesirable pharmacodynamics effects of SAMiRNA-AREG for the central nervous, respiratory, and cardiovascular systems in mice and cynomolgus monkeys. The safety pharmacology studies were performed according to the guidelines

* Corresponding authors.
E-mail addresses: sunghwan.kim@kitox.re.kr (S.-H. Kim), hpark@bioneer.com (H.-O. Park).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.toxrep.2021.03.022
Received 18 December 2020; Received in revised form 26 March 2021; Accepted 27 March 2021
Available online 31 March 2021

provided by the Ministry of Food and Drug Safety (MFDS), Organization for Economic Cooperation and Development (OECD), and the International Conference on Harmonization (ICH) M3 (R2), ICH S7A, and ICH S7B under modern Good Laboratory Practice (GLP) regulations.

2. Materials and methods

2.1. Animal care and use

Crl:CD1 (ICR) mice (5–6 weeks of age at initiation injection drug use) were purchased from Orient Bio Inc. (Seongnam, Republic of Korea). Cynomolgus monkeys (4–6 weeks of age at initiation injection drug use) were obtained from Worldwide Primates Inc. (Miami, FL, USA) and Covance research products Inc. (Denver, PA, USA).

ICR mice were housed 2–4 mice in each polycarbonate cage (180 W × 240 L × 143 H mm) under standardized conditions (relative humidity 50 ± 20 %, temperature 23 ± 3 °C, 10–20 air changes/hr, 12 h light/dark cycle). Cynomolgus monkeys were housed individually in stainless cages under standardized conditions (relative humidity 50 ± 20 %, temperature 24 ± 5 °C, 10 or greater air changes/hr, 12 h light/dark cycle). The animals were allowed standard sterile diets and sterilized tap water *ad libitum*. All experimental procedure involving animals were performed and approved in accordance with guidelines of the test facility in which each study was conducted.

2.2. Reagents

The SAMiRNA-AREG was provided by Bioneer Inc. (Daejeon, Republic of Korea). The chemicals and solvents used throughout the experiments were of analytical grade. According to a previous study, the sizes of SAMiRNA-AREG nanoparticles are uniformed and similar to each other (100 ± 20 nm and 0.2 ± 0.01 nm for average size and PDI, respectively). Also, the stability of SAMiRNA nanoparticles was excellent because no significant degradation was noted in size distribution or polydispersion index in solution over 12 months [17]. Fetal bovine serume (FBS), dulbecco's modified eagle's medium nutrient mixture F-12 (DMEM/F-12), penicillin/streptomycin, Hygromycin B, and 0.05 % trypsin-EDTA were purchased from Invitrogen Co. (Carlsbad, USA). Dimethyl sulfoxide (DMSO), normal tyrode solution, and intracellular solution were obtained from Sigma Aldrich Co. (MO, USA). Dulbecco's phosphate buffered saline (DPBS) was purchased from WelGENE Inc. (Seoul, Republic of Korea). The Chinese hamster ovary (CHO) cell line was purchased from the bSysGmBH (Witterswill, Switzerland).

2.3. Evaluation on neurobehavior in mice

The evaluations on neurobehavior were performed in accordance with the ICH S7A safety pharmacology guideline [18] according to previously described methods [19]. SAMiRNA-AREG was a suspended in DPBS and intravenously injected to four groups of ICR mice. Eight males and eight females per group were administrated with doses of 0, 100, 200, and 300 mg/kg of SAMiRNA-AREG by single intravenous injection. In this study, highest dose was selected as 300 mg/kg (a 60-fold of efficacy dose), and low and middle doses were selected as 100 and 200 mg/kg, respectively, using a common ratio of 1.5 or 2. The vehicle control group received DPBS only at the same dose-volume of 10 mL/kg. Mice were evaluated for death, startle reflex, locomotion, respiratory rate, tail elevation, eyelid, piloeretion, exophthalmos, righting reflex, skin coloration, pinna reflex, abdominal tone, salivation, lacrimation, tremor, convulsion, diarrhea, catalepsy, and traction. Measurement of rectal body temperature was carried out simultaneously with neurobehavioral evaluations. Changes in neurobehavior and body temperature were monitored at before dosing (0) and 0.5 (30 min), 1, 3, 6, and 24 h after dosing following the test article administration.

2.4. Evaluation of respiration in conscious mice

The evaluation of respiration was conducted in accordance with the ICH S7A safety pharmacology guideline [18]. SAMiRNA-AREG was suspended in DPBS and intravenously injected to four groups of ICR mice. Eight male mice per group were single intravenously injected with doses of 0, 100, 200, and 300 mg/kg of SAMiRNA-AREG. In this study, highest dose was selected as 300 mg/kg (a 60-fold of efficacy dose), and low and middle doses were selected as 100 and 200 mg/kg, respectively, using a common ratio of 1.5 or 2. The vehicle control group received DPBS only at the same dose-volume of 10 mL/kg. Prior to intravenous injection of the test article, male mice were stabilized at least 30 min in the whole-body plethysmography chamber (BUXCO, CT, USA), and then minute volume, tidal volume, and respiration rate were measured for 10 min per each time point.

2.5. Evaluation on human ether a-go-go (hERG) channel current in CHO Cells

The evaluation on hERG channel current was performed in CHO cells in accordance with ICH S7B safety pharmacology guideline [20]. CHO cells were maintained in reconstituted 90 % DMEM/F-12 supplemented with 9% FBS, 0.9 % penicillin/streptomycin, and 0.1 % 50 μg/mL Hygromycin B. The CHO cells were cryopreserved as a 0.9 mL cell suspension containing 0.1 mL of DMSO. At least 7 days prior to use, a vial of frozen CHO cells was thawed and cultured and the absence of mycoplasma contamination was verified. The CHO cells were incubated at 37 °C in a 5% CO_2 atmosphere as a monolayer in 75 cm^2 culture flasks and then subcultured twice weekly using 0.05 % trypsin-EDTA. Mycoplasma testing was performed again on the date of cell seeding.

All testing was performed using whole-cell patch-clamp experiments conducted in the voltage-clamp mode [21]. Cells were trypsinized with 0.05 % trypsin-EDTA, placed in the bath chamber, and then allowed to settle for approximately 20 min. Cells were perfused with normal tyrode solution (5.4 mM KCl, 5.0 mM HEPES, 143 mM NaCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 16.6 mM glucose, adjusted to pH 7.4 NaOH). The intracellular solution for filling micropipettes contained 130 mM KCl, 5 mM MgATP, 1 mM $MgCl_2$, 5 mM EGTA, and 5 mM HEPES, adjusted to pH 7.2 KOH. The electrode tip was attached to the cell membrane using a micromanipulator. Voltage-clamp mode was selected on the Axopatch 200B (Axon Instrument, CA, USA) and negative pressure was applied to form a giga-seal to rupture the cell membrane and achieve whole-cell status. After whole-cell mode, the membrane potential of the cell was held at −80 mV, and the cells were hyperpolarized for 100 ms to −90 mV then depolarized the membrane for 2 s to +20 mV followed by a 3 s repolarization to −40 mV for hERG potassium channel current evaluation. The pulse interval used was approximately 20 s, and the temperature of the normal tyrode solution in the application chamber was monitored and maintained at approximately 37 °C using a TC344B controller (Warner Instruments, CT, USA). Data was acquired using pipette electrodes fabricated from borosilicate-glass capillaries (Harvard Apparatus Ltd., Edenbridge, UK) using a PP-830 puller (Narishige Scientific Instrument Lab., Tokyo, Japan). SAMiRNA-AREG concentrations were 0, 2, 20, 100, and 200 μg/mL. Current amplitudes were measured before and after application of SAMiRNA-AREG. Relative current value was calculated as current amplitude in the presence of SAMiRNA-AREG / initial current amplitude. Compensated suppression rate was calculated as (suppression rate in the test article, positive control, or vehicle control groups – mean suppression rate in the vehicle control group) / (100 – mean suppression rate in the vehicle control group). The positive control E-4031 was used at a concentration of 100 nM.

2.6. Evaluation on cardiovascular system in unrestrained monkeys

The evaluation on the cardiovascular system was conducted in accordance with the ICH safety pharmacology guidelines [18,20,22]

following previously described methods [23–25]. Four groups of male monkeys were anesthetized using intramuscular administration of ketamine. Surgical placement of the transmitter and postoperative care were conducted in accordance with testing facility SOP, using IACUC and veterinary-approved procedures. Four male monkeys per group were intravenously injected once with doses of 25, 50, and 100 mg/kg of SAMiRNA-AREG (2, 2, and 3.33 mL/kg/day, respectively). The animals were unrestrained and monitored for selected physiological parameters *via* telemetry. The vehicle control group received DPBS only at the same dose-volume of 3.33 mL/kg. For assessment of the cardiovascular parameters including systolic pressure, diastolic pressure, arterial pressure, QT interval, heart rate, RR interval, PR interval, and QRS duration, analysis time points were set before and at 10-min intervals for the first 1 h and in approximately 60-min intervals until at least 24 h after administration. The QT interval was normalized for the heart rate by conversion to the corrected QT (QTc) interval using a procedure based on the previously described methods [26,27].

2.7. Statistical analysis

For general safety pharmacology, statistical analysis was performed using the Pristima system (version 7.3., Xybion Medical System Co., NJ, USA). Data were collected for variance homogeneity using the Bartlett's test during the study. If the variance was homogenous, the data was subjected to analysis of variance (ANOVA). Heterogeneous data were analyzed using the Kruskal–Wallis test and the significance of intergroup differences between the vehicle control and treated groups was assessed using post hoc Dunn's Rank Sum test. The data were expressed as mean ± standard deviation (SD). For cardiovascular system evaluation, statistical analysis was performed using the SAS/STAT system (SAS) software (version 14.1., SAS Institute Inc., Cary, NC, USA). A repeated measures analysis of covariance (rANCOVA) was performed for each endpoint. Akaike's Information Criterion (AIC) was utilized for model selection. The first-order Kenward–Roger (KR) degrees of freedom approximation was utilized. For hERG assay, current amplitudes were measured before and after application of the respective test article. Effects were calculated from 1 to 4 experiments per concentration of test article and data were presented as mean ± standard error of mean (SEM). The results of the comparisons are indicated for $p < 0.01$.

3. Results

3.1. Effects of SAMiRNA-AREG on neurobehavior in mice

During the test period, a single mouse found dead was observed in the male 200 mg/kg group. As the results of necropsy examination, there were no physical abnormalities and there were no abnormalities in abdominal and thoracic cavities. However, rectum perforation was observed when organs examined, which was considered a spontaneous finding. Thus, the other test data for this animal were excluded in the results of the present study. There was no treatment-related neurobehavior in any test article treated groups at any time points (data not shown). As shown in Table 1, transient changes in body temperature were noted in association with SAMiRNA-AREG administration. Statistically significant decreases in body temperature were observed at 0.5 h (30 min) post-dose in the 300 mg/kg group at both sexed compared with the vehicle control group (male: 0.9 °C, female: 1.4 °C, mean value). However, the effects were returned to normal at 1 h post-dose. At other time points, there were no changes in body temperature compared with the vehicle control group in both sexes.

3.2. Effects of SAMiRNA-AREG on respiration in conscious mice

There was no significant difference in respiratory rate, tidal volume, and minute volume in any test article treated groups (Tables 2–4).

3.3. Effects of SAMiRNA-AREG on hERG current in CHO cells

As shown in Table 5, there was no inhibition of hERG with 2 μg/mL SAMiRNA-AREG, but higher concentrations of SAMiRNA-AREG significantly suppressed hERG current in a concentration-dependent manner (9.2 ± 0.9, 15.8 ± 1.2, and 24.9 ± 2.9 % suppression at 20, 100, 200 μg/mL, respectively). In comparison, hERG potassium channel current of E-4031 was suppressed by 81.5 % at 100 nM. Based upon these data, the IC_{50} for SAMiRNA-AREG is > 200 μg/mL.

3.4. Effects of SAMiRNA-AREG on cardiovascular system in unrestrained monkeys

There were no effects of SAMiRNA-AREG on blood pressure (Fig. 1), heart rate, QT interval, QTc interval, PR interval, RR interval, and QRS duration (Fig. 2) in male monkeys as compared with the vehicle control group.

4. Discussion

The newly developed SAMiRNA-AREG provides *in vivo* effective and safe delivery of siRNA targeting AREG with suppression of collagen deposition and restoration of lung function in pulmonary fibrosis [17,28]. However, despite its therapeutic potential for pulmonary fibrosis, the adverse effects of SAMiRNA-AREG have not been fully characterized. Thus, the present studies evaluated the potential harmful effects of SAMiRNA-AREG on the central nervous, respiratory, and cardiovascular systems in accordance with the current test guidelines, as well as the

Table 1
Body temperature following SAMiRNA-AREG intravenous injection in mice.

Dose (mg/kg)	Time (hr)					
	0	0.5	1	3	6	24
Male						
0	39.2 ± 0.4[a]	39.0 ± 0.2	38.9 ± 0.2	38.3 ± 0.2	38.0 ± 0.1	39.1 ± 0.2
100	39.2 ± 0.3	39.1 ± 0.3	38.8 ± 0.3	38.3 ± 0.1	38.1 ± 0.2	39.1 ± 0.3
200[b]	39.1 ± 0.2	39.0 ± 0.1	38.8 ± 0.1	38.3 ± 0.2	38.1 ± 0.2	39.1 ± 0.1
300	39.2 ± 0.3	38.1 ± 0.4**	38.7 ± 0.2	38.3 ± 0.2	38.2 ± 0.3	39.0 ± 0.3
Female						
0	39.0 ± 0.2	38.9 ± 0.3	38.8 ± 0.2	38.2 ± 0.2	38.1 ± 0.1	39.0 ± 0.2
100	39.1 ± 0.2	39.0 ± 0.3	38.9 ± 0.3	38.3 ± 0.2	38.3 ± 0.3	39.1 ± 0.2
200	39.0 ± 0.1	38.9 ± 0.1	38.8 ± 0.2	38.3 ± 0.2	38.2 ± 0.2	39.0 ± 0.2
300	39.0 ± 0.1	37.5 ± 0.4**	38.8 ± 0.2	38.3 ± 0.2	38.3 ± 0.3	39.0 ± 0.1

$n = 8$.
[a] Values are presented as mean ± SD (℃).
[b] One male mouse was excluded, since it considered spontaneous dead ($n = 7$).
** $p < 0.01$, compared with the vehicle control group.

Table 2
Respiratory rate following SAMiRNA-AREG intravenous injection in male mice.

Time (hr)	SAMiRNA-AREG (mg/kg)			
	0	100	200	300
0	276.51 ± 129.40[a]	283.48 ± 146.14	198.49 ± 89.85	243.63 ± 105.88
0.5	236.69 ± 123.27	246.60 ± 75.68	236.38 ± 72.23	257.47 ± 97.91
1	197.92 ± 76.87	209.69 ± 45.34	191.88 ± 70.76	170.32 ± 30.97
3	203.52 ± 64.26	178.82 ± 51.72	192.08 ± 38.10	221.44 ± 94.80
6	211.11 ± 94.66	228.42 ± 117.39	196.28 ± 45.31	232.26 ± 92.01
24	375.33 ± 121.42	288.07 ± 160.42	232.59 ± 83.39	276.68 ± 83.87

$n = 8$.
[a] Values are presented as mean ± SD (beats/min).

Table 3
Tidal volume following SAMiRNA-AREG intravenous injection in male mice.

Time (hr)	SAMiRNA-AREG (mg/kg)			
	0	100	200	300
0	0.25 ± 0.03[a]	0.24 ± 0.03	0.24 ± 0.04	0.23 ± 0.03
0.5	0.24 ± 0.03	0.22 ± 0.05	0.23 ± 0.04	0.22 ± 0.03
1	0.23 ± 0.02	0.24 ± 0.04	0.24 ± 0.03	0.24 ± 0.03
3	0.22 ± 0.02	0.23 ± 0.05	0.22 ± 0.03	0.23 ± 0.03
6	0.22 ± 0.03	0.22 ± 0.03	0.21 ± 0.02	0.22 ± 0.03
24	0.25 ± 0.02	0.25 ± 0.06	0.26 ± 0.03	0.25 ± 0.03

$n = 8$.
[a] Values are presented as mean ± SD (mL).

Table 4
Minute volume following SAMiRNA-AREG intravenous injection in male mice.

Time (hr)	SAMiRNA-AREG (mg/kg)			
	0	100	200	300
0	63.38 ± 24.71[a]	63.20 ± 30.11	43.72 ± 12.72	52.29 ± 19.67
0.5	53.31 ± 27.25	46.70 ± 4.72	46.15 ± 8.56	50.23 ± 13.13
1	42.60 ± 12.08	45.45 ± 10.11	40.70 ± 8.83	38.05 ± 5.91
3	42.38 ± 12.21	38.19 ± 6.96	36.36 ± 5.00	45.66 ± 18.42
6	43.82 ± 16.28	47.83 ± 26.28	37.71 ± 8.46	48.04 ± 21.82
24	87.42 ± 28.47	62.28 ± 29.39	52.87 ± 12.86	65.36 ± 27.94

$n = 8$.
[a] Values are presented as mean ± SD (mL).

Table 5
Inhibitory effects of SAMiRNA-AREG on hERG currents.

Test compound	Concentration	Relative value (%)	Compensated suppression rate (%)
Vehicle	0 μg/mL	0.99 ± 0.01[a]	–
SAMiRNA-AREG	2 μg/mL	0.97 ± 0.00	2.0 ± 0.2
	20 μg/mL	0.90 ± 0.01**	9.2 ± 0.9
	100 μg/mL	0.83 ± 0.01**	15.8 ± 1.2
	200 μg/mL	0.74 ± 0.03**	24.9 ± 2.9
E-4031	100 nM	0.18 ± 0.03**	81.5 ± 2.9

[a] Values are presented as mean ± SEM (%).
** $p <$ 0.01, compared with the vehicle control group.

GLP regulations. The results of our studies clearly showed that SAMiRNA-AREG did not cause any adverse effects under the experimental conditions.

The modified Irwin test is a common tool to evaluate the neurobehavior effects of drug and chemical on mice and is recommended by the ICH S7A guideline, to help protect clinical trials patients and participants from potential adverse effects [18,19,29–31]. In the present study, there were no adverse effects at any doses of SAMiRNA-AREG up to 300 mg/kg for this center nerve system in mice. In body temperature, the significant decrease at 0.5 h (30 min) post-dose in the 300 mg/kg group compared with the vehicle control group was considered as

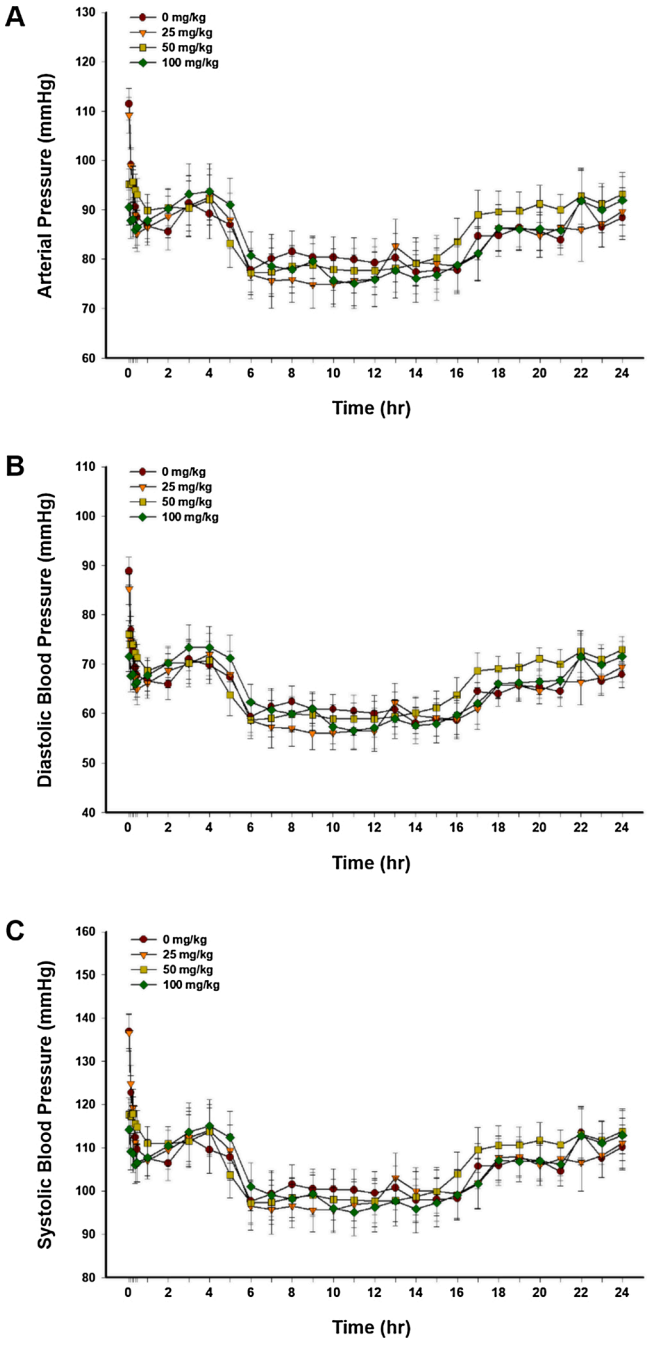

Fig. 1. Effects of intravenous injection of a vehicle or SAMiRNA-AREG on arterial pressure (A), diastolic blood pressure (B), and systolic blood pressure (C). Values are presented as mean ± SD.

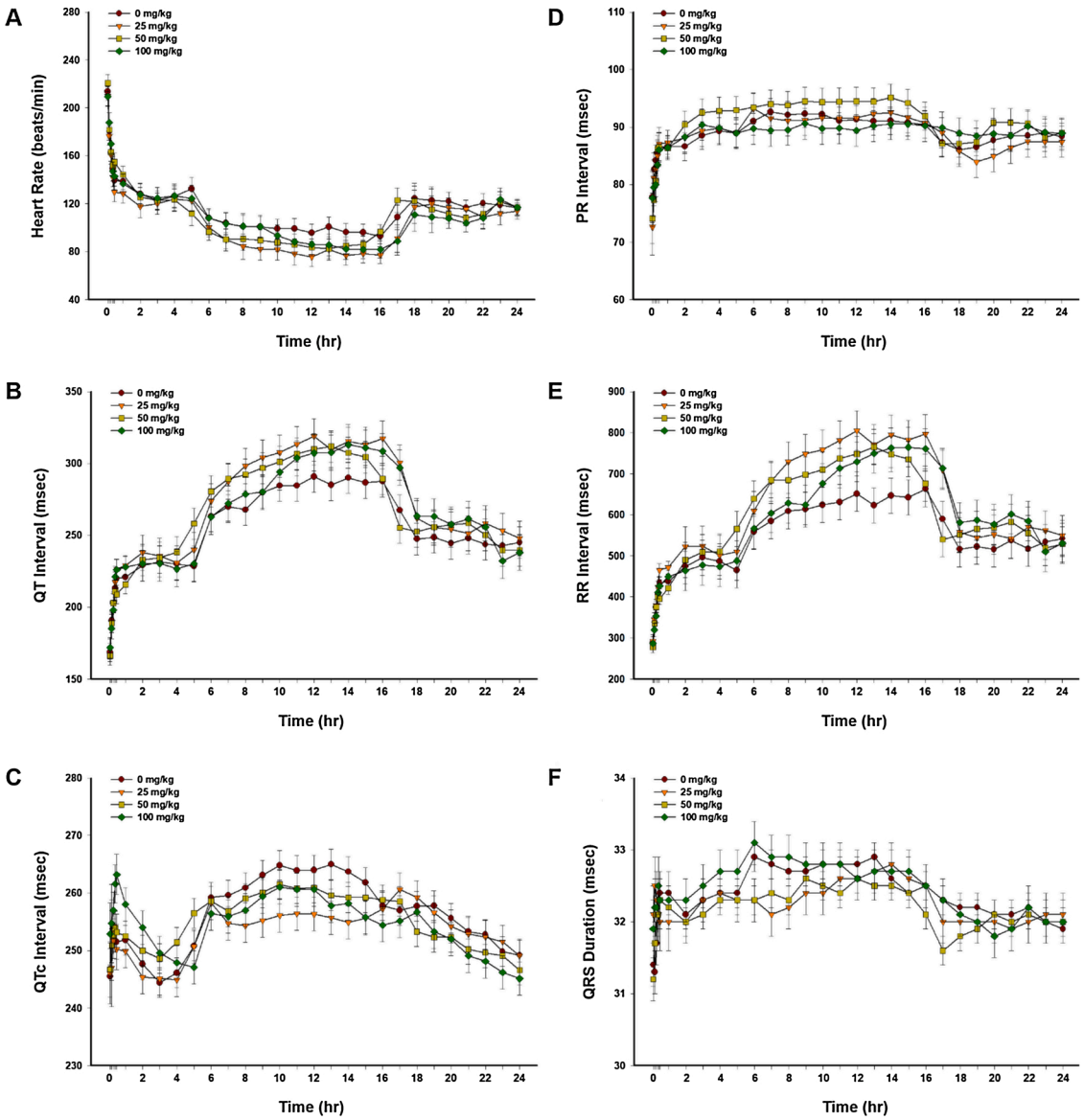

Fig. 2. Effects of intravenous injection of a vehicle or SAMiRNA-AREG on heart rate (A), QT interval (B), QTc interval (C), PR interval (D), RR interval (E), and QRS duration (F). Values are presented as mean ± SD.

treatment-related, because these changes are observed in the highest dose group and both sexes. However, it was of no toxicological significance because the changes were minimal and recovered by 1 h after dosing. Moreover, there were no significant changes in the 300 mg/kg group in both sexes compared with the vehicle control group at any other time point.

Respiratory assessment is a part of the safety pharmacology core battery study performed to evaluate transient respiratory dysfunction secondary to drug exposure. In our study of respiratory system, there was no change in tidal volume, respiratory rate, and minute volume in any test article treated groups. The highest dose of 300 mg/kg in mice is 60-fold the efficacy dose, and these findings were indicates no unwanted actions on the respiratory system. Furthermore, the absence of direct adverse effects on respiration is consistent with published data [32,33].

The hERG assay is a useful method for the detection of chemical-induced adverse actions within the channel pore [34,35]. It can be used for the risk evaluation of cardiac proarrhythmic. In our evaluation on hERG channel current, there were statistically significant differences in the inhibition of peak hERG potassium channel current between the 20, 100, and 200 μg/mL SAMiRNA-AREG treatment groups and the vehicle control group. The hERG potassium channel current of E-4031 was suppressed by 81.5 % at 100 nM. Although the result for SAMiRNA-AREG occurred at a concentration of 20 μg/mL or higher, this indicates a modest impact of 9.2–25% inhibition. In preliminary study, the maximum observed peak serum concentration (C_{max}) of approximately 8.4 μg/mL following intravenous dose of 5 mg/kg SAMiRNA-AREG in cynomolgus monkeys (data not shown). Based on this information, these studies suggested that SAMiRNA-AREG showed low risk on the hERG channel inhibition, but it was of no toxicological significance because the highest concentration evaluated in the hERG assay exceeded C_{max} by up to 20×. Moreover, a previous study reported that no effects on *in vitro* cardiovascular system of other similar oligonucleotide-based investigational therapeutic agents were observed [32].

The use of cardiovascular safety assessments in the conscious telemetered animals was first described by Kinter et al. [36]. The introduction of the telemetry system facilitated the conduct of *in vivo* study in unanesthetized and unrestrained animals, enabling assessment of the standard cardiovascular system. A previous study reported that the QT prolongation is generally known as a potential surrogate marker for

ventricular arrhythmias [37]. A lot of drugs have been withdrawn from the clinical market due to their potential to cause sudden cardiac death and fatal ventricular arrhythmias induced by QT interval prolongation [38,39]. In our study showed no significant differences in blood pressure, QT interval, heart rate, QTc interval, PR interval, RR interval, and QRS duration after treatment with SAMiRNA-AREG at doses up to 100 mg/kg in the monkeys. These cardiovascular telemetry results in our study were consistent with published report on the toxicology of other antisense oligonucleotide therapeutics, which show no indications of adverse effects in cardiovascular parameters [40].

Taken together, these results suggest that SAMiRNA-AREG has no side effects on the nervous, respiratory, and cardiovascular systems with regard to potential risk for ventricular arrhythmias under the present experimental conditions. These findings support continued development of SAMiRNA-AREG and provide important information for clinical trials in humans.

Author statement

All authors participated in the design expriments, interpretation of the studies, analysis of the results and review of the manuscript. Tae Rim Kim and Hyeon-Young Kim: contributed to writing the paper, Youngho Ko, Jun Hong Park, and Sungil Yun: contributed to design experiments, In-Hyeon Kim and Ki Cheon Kim: contribution analyzed the results, In-Chul Lee: responsible for data discussion; Sung-Hwan Kim and Han-Oh Park: involved with the manuscript correction.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This research was supported by a grant funded by Ministry of Science and ICT (Grant No KK-2004, Republic of Korea) and Korea Drug Development Fund (KDDF) funded by Ministry of Science, ICT, and Future Planning (MSIP); the Ministry of Trade, Industry, and Energy (MOTIE); the Ministry of Health and Welfare (MOHW) (Grant No KDDF-201706-03, Republic of Korea). It was also supported by Bioneer Corporation and siRNAgen Therapeutics Research Fund.

References

[1] D. Strehlow, J.H. Korn, Biology of the scleroderma fibroblast, Curr. Opin. Rheumatol. 10 (1998) 572–578.
[2] D.J. Abraham, J. Varga, Scleroderma: from cell and molecular mechanisms to disease models, Trends Immunol. 26 (2005) 587–595.
[3] R. Bataller, D.A. Brenner, Liver fibrosis, J. Clin. Invest. 115 (2005) 209–218.
[4] J. Varga, D. Abraham, Systemic sclerosis: a prototypic multisystem fibrotic disorder, J. Clin. Invest. 117 (2007) 557–567.
[5] T.A. Wynn, Integrating mechanisms of pulmonary fibrosis, J. Exp. Med. 208 (2011) 1339–1350.
[6] D.B. Coultas, R.E. Zumwalt, W.C. Black, R.E. Sobonya, The epidemiology of interstitial lung diseases, Am. J. Respir. Crit. Care Med. 150 (1994) 967–972.
[7] G. Raghu, D. Weycker, J. Edelsberg, W.Z. Bradford, G. Oster, Incidence and prevalence of idiopathic pulmonary fibrosis, Am. J. Respir. Crit. Care Med. 174 (2006) 810–816.
[8] A.L. Olson, J.J. Swigris, D.C. Lezotte, J.M. Norris, C.G. Wilson, K.K. Brown, Mortality from pulmonary fibrosis increased in the United States from 1992 to 2003, Am. J. Respir. Crit. Care Med. 176 (2007) 277–284.
[9] G. Li, Q. Xie, Y. Shi, D. Li, M. Zhang, S. Jiang, H. Zhou, H. Lu, Y. Jin, Inhibition of connective tissue growth factor by siRNA prevents liver fibrosis in rats, J. Gene Med. 8 (2006) 889–900.
[10] J. George, M. Tsutsumi, siRNA-mediated knockdown of connective tissue growth factor prevents N-nitrosodimethylamine-induced hepatic fibrosis in rats, Gene Ther. 14 (2007) 790–803.
[11] Y. Takabatake, Y. Isaka, M. Mizui, H. Kawachi, S. Takahara, E. Imai, Chemically modified siRNA prolonged RNA interference in renal disease, Biochem. Biophys. Res. Commun. 363 (2007) 432–437.
[12] G.H. Luo, Y.P. Lu, J. Song, L. Yang, Y.J. Shi, Y.P. Li, Inhibition of connective tissue growth factor by small interfering RNA prevents renal fibrosis in rats undergoing chronic allograft nephropathy, Transplant. Proc. 40 (2008) 2365–2369.
[13] D.R. Brigstock, Strategies for blocking the fibrogenic actions of connective tissue growth factor (CCN2): from pharmacological inhibition in vitro to targeted siRNA therapy in vivo, J. Cell Commun. Signal. 3 (2009) 5–18.
[14] B.A. Molitoris, P.C. Dagher, R.M. Sandoval, S.B. Campos, H. Ashush, E. Fridman, A. Brafman, A. Faerman, S.J. Atkinson, J.D. Thompson, H. Kalinski, R. Skaliter, S. Erlich, E. Feinstein, siRNA targeted to p53 attenuates ischemic and cisplatin-induced acute kidney injury, J. Am. Soc. Nephrol. 20 (2009) 1754–1764.
[15] H. Shimizu, Y. Hori, S. Kaname, K. Yamada, N. Nishiyama, S. Matsumoto, K. Miyata, M. Oba, A. Yamada, K. Kataoka, T. Fujita, siRNA-based therapy ameliorates glomerulonephritis, J. Am. Soc. Nephrol. 21 (2010) 622–633.
[16] Y. Zhou, J.Y. Lee, C.M. Lee, W.K. Cho, M.J. Kang, J.L. Koff, P.O. Yoon, J. Chae, H. O. Park, J.A. Elias, C.G. Lee, Amphiregulin, an epidermal growth factor receptor ligand, plays an essential role in the pathogenesis of transforming growth factor-β-induced pulmonary fibrosis, J. Biol. Chem. 287 (2012) 41991–42000.
[17] P.O. Yoon, J.W. Park, C.M. Lee, S.H. Kim, H.N. Kim, Y. Ko, S.J. Bae, S. Yun, J. H. Park, T. Kwon, W.S. Kim, J. Lee, Q. Lu, H.R. Kang, W.K. Cho, J.A. Elias, J. S. Yang, H.O. Park, K. Lee, C.G. Lee, Self-assembled micelle interfering RNA for effective and safe targeting of dysregulated genes in pulmonary fibrosis, J. Biol. Chem. 291 (2016) 6433–6446.
[18] ICH S7A, Safety Pharmacology Studies for Human Pharmaceuticals, Available from:, 2001 http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S7A/Step4/S7A_Guideline.pdf.
[19] S. Irwin, Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse, Psychopharmacologia 13 (1968) 222–257.
[20] ICH S7B, The Non-clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals, Available from:, 2005 http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S7B/Step4/S7B_Guideline.pdf.
[21] O.P. Hamill, A. Marty, E. Neher, B. Sakmann, F.J. Sigworth, Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches, Pflugers Arch. 391 (1981) 85–100.
[22] ICH M3 (R2), Note for Guidance on Non-clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals, Available from:, 2009 http://www.ich.org/LOB/media/MEDIA5544.pdf.
[23] K. Ando, T. Hombo, A. Kanno, H. Ikeda, M. Imaizumi, N. Shimizu, K. Sakamoto, S. I. Kitani, Y. Yamamoto, S. Hizume, K. Nakai, T. Kitayama, K. Yamamoto, QTPRODACT: In vivo QT assay with a conscious monkey for assessment of thepotential for drug-induced QT interval prolongation, J. Pharmacol. Sci. 99 (2005) 487–500.
[24] S. Authier, J.F. Tanguay, D. Gauvin, R.D. Fruscia, E. Troncy, A cardiovascularmonitoring system used in conscious cynomolgus monkeys for regulatory safetypharmacology. Part 2: pharmacological validation, J. Pharmacol. Toxicol. Methods 56 (2007) 122–130.
[25] E. Moscardo, G. McPhie, N. Fasdelli, R. Dorigatti, K. Meecham, An integratedcardiovascular and neurobehavioural functional assessment in the consciousteleметered cynomolgus monkey, J. Pharmacol. Toxicol. Methods 62 (2010) 95–106.
[26] S. Spence, K. Soper, C.M. Hoe, J. Coleman, The heart rate-corrected QT interval of conscious beagle dogs: a formula based on analysis of covariance, Toxicol. Sci. 45 (1998) 247–258.
[27] H. Miyazaki, M. Tagawa, Rate-correction technique for QT interval in long-term telemetry ECG recording in beagle dogs, Exp. Anim. 51 (2002) 465–475.
[28] S. Falsini, S. Ristori, L. Ciani, E. Di Cola, C.T. Supuran, A. Arcangeli, M. In, Time resolved SAXS to study the complexation of siRNA with cationic micelles of divalent surfactants, Soft Matter 10 (2014) 2226–2233.
[29] A.J. Hunter, J. Hatcher, D. Virley, P. Nelson, E. Irving, S.J. Hadingham, A. A. Parsons, Functional assessments in mice and rats after focal stroke, Neuropharmacology 39 (2000) 806–816.
[30] S. Roux, E. Sablé, R.D. Porsolt, Primary observation (Irwin) test in rodents for assessing acute toxicity of a test agent and its effects on behavior and physiological function, Curr. Protoc. Pharmacol. (Suppl. 27) (2004), 10.10. 1-10.10. 23.
[31] W.S. Redfern, I.D. Wakefield, Toxicological Testing Handbook Principles, Applications and Data Interpretation, 2006.
[32] T.W. Kim, K.S. Kim, J.W. Seo, S.Y. Park, S.P. Henry, Antisense oligonucleotides on neurobehavior, respiratory, and cardiovascular function, and hERG channel current studies, J. Pharmacol. Tocicol. Methods 69 (2014) 49–60.
[33] F. Li, W. Wang, H. Xiao, The evaluation of anti-breast cancer activity and safety pharmacology of the ethanol extract of Aralia elata Seem. leaves, Drug Chem. Toxicol. 26 (2019) 1–10.
[34] P.J. Stansfeld, M.J. Sutcliffe, J.S. Mitcheson, Molecular mechanisms for drug interactions with hERG that cause long QT syndrome, Expert Opin. Drug Metab. Toxicol. 2 (2006) 81–94.
[35] D. Thomas, C.A. Karle, J. Kiehn, The cardiac hERG/IKr potassium channel as pharmacological target: structure, function, regulation, and clinical applications, Curr. Pharm. Des. 12 (2006) 2271–2283.
[36] L.B. Kinter, D.J. Murphy, W.A. Mann, T.B. Leonard, D.G. Morgan, Major organ systems toxicology: an integrative approach to pharmacodynamic safety assessment studies in animals, in: I.G. Sipes, C.A. McQueen, A.J. Gandolfi (Eds.), Comprehensive Toxicology., Elsevier Science, New York, 1997, pp. 155–168.
[37] W.S. Redfern, L. Carlsson, A.S. Davis, W.G. Lynch, I. MacKenzie, S. Palethorpe, P. K. Siegl, I. Strang, A.T. Sullivan, R. Wallis, A.J. Camm, T.G. Hammond, Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development, Cardiovasc. Res. 58 (2003) 32–45.

[38] B. Darpö, Spectrum of drugs prolonging QT interval and the incidence of torsades de pointes, Eur. Heart J. Suppl. 3 (2001) K70–K80.
[39] S.M. Al-Khatib, N. Allen LaPionte, J.M. Kramer, R.M. Califf, What clinicians should know about the QT interval, JAMA 289 (2003) 2120–2127.
[40] P. Sazani, D.L. Weller, S.B. Shrewsbury, Safety pharmacology and genotoxicity evaluation of AVI-4658, Int. J. Toxicol. 29 (2010) 143–156.