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Non-clinical development of Ozanezumab: a humanised antibody targeting the amino terminus of Neurite Outgrowth Inhibitor A (Nogo-A).

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ABSTRACT

Ozanezumab (GSK1223249) is a humanised, Fc-disabled, monoclonal antibody (mAb) which targets the amino terminus of Neurite Outgrowth Inhibitor A (Nogo-A) which is currently being developed for the treatment of amyotrophic lateral sclerosis (ALS). Here we report on the biochemical and structural characterisation of Ozanezumab together with an assessment of pharmacology and non-clinical safety. A minimal binding epitope was characterised and emerging biology and pre-clinical pharmacology provide confidence that targeting the amino terminus of the neurite outgrowth inhibitor A (Nogo-A) through passive immunization may offer a promising approach to treat various neurodegenerative diseases, including ALS. A comprehensive non-clinical assessment of safety has been completed based on a package of in vitro and in vivo studies in rodents, non-human primates and female rabbits (reproductive toxicology only). There was no evidence for toxicological, cardiovascular, respiratory, neurobehavioural, immunogenic, reproductive or delayed toxicity effects in any species following repeat dose treatment with Ozanezumab (biweekly up to doses of 500 mg/kg iv for 52 weeks in the non-human primate). Based on these studies there are no non-clinical safety findings that would preclude the development of Ozanezumab in patients.
INTRODUCTION

Ozanezumab (GSK1223249) is a humanized IgG1-type monoclonal antibody (mAb) that specifically binds to the transmembrane protein Nogo-A, which is a myelin-associated neurite outgrowth inhibitor, and thereby neutralizes and/or antagonizes its biological function. Anti-Nogo-A antibodies have been reported to enhance neurite outgrowth in vitro and functional recovery in vivo of neurons in a number of different animal models of human neurodegenerative diseases.\(^1\)\(^-\)\(^4\) It is hypothesised that by neutralizing Nogo-A-mediated inhibition of neuroregeneration, ozanezumab may be a useful therapy for neurological diseases characterized by CNS axonal injury. Ozanezumab is currently being developed for the treatment of amyotrophic lateral sclerosis (ALS).\(^5\)

Nogo-A

Nogo-A is a high molecular weight transmembrane protein that was initially identified as a potent myelin-associated inhibitor of axonal growth and regeneration expressed mostly by oligodendrocytes.\(^6\)\(^,\)\(^7\) It stimulates a receptor complex consisting of the Nogo66 receptor NgR1, the adaptor molecule Lingo-1, and effector components p75/Troy, which leads to the intracellular activation of the small GTPase RhoA that mediates actin depolymerisation and the collapse or retraction of neurites.\(^4\)\(^,\)\(^8\)\(^,\)\(^9\) Nogo-A is the longest of several splice forms of the Nogo/reticulon (RTN)-4 gene\(^10\) and protein expression is enriched in the brain and spinal cord of rats\(^11\) and humans\(^12\); for review see Schwab.\(^9\)

Neuronal Nogo-A plays a role in central nervous system development and plasticity (see reviews\(^13\)\(^,\)\(^14\)) and significant time- and region-specific changes in Nogo-A expression are observed during the development of the human brain.\(^15\) Although the exact role of Nogo-A protein in development is not well understood, there is evidence to suggest that Nogo-A is associated with the regulation of neuronal synapse morphology and architecture,\(^16\)\(^,\)\(^17\) possibly via cytoskeletal re-organization,\(^18\) with emerging roles for Nogo-A in the stabilisation of neuronal networks, either developmentally\(^16\) or during the processes of memory consolidation in the mature nervous system.\(^19\) Indeed, Nogo-A may represent an important regulator of neuronal plasticity associated with learning\(^20\) and Nogo-A down regulation is associated with altered synaptic plasticity during aging and may play a role in age-related cognitive decline.\(^21\)\(^,\)\(^22\) There are also reports linking Nogo-A and NgR with schizophrenia (reviewed by Willi and Schwab\(^23\)). Other research suggests a role for Nogo-A in the neuronal response to hypoxia and oxidative stress\(^24\) and as a negative regulator of CNS angiogenesis.\(^25\) These findings demonstrate the complexity of the emerging biological functions of Nogo-A protein in the CNS.
Nogo-A and ALS

Amyotrophic Lateral Sclerosis (ALS), also known as motor neurone disease, is a degenerative disorder characterised by gradual degeneration and progressive loss of the upper and lower motor neurons throughout the central nervous system, and is associated with severe neurologic morbidity and death.

Whilst Nogo-A is not appreciably expressed in healthy skeletal muscle, there is increasing evidence that increased expression of Nogo-A in skeletal muscle may be involved in the pathophysiology of ALS. Nogo-A is present in skeletal muscle biopsies from ALS patients, but not in those from healthy controls or patients with sensorimotor peripheral neuropathy or myopathy. The expression of Nogo-A in skeletal muscle appears to correlate with the severity of ALS although expression is not considered to be specific for the disease. Nevertheless, in patients with spinal lower motor neuron syndrome (LMNS) the presence of Nogo-A in skeletal muscle biopsy samples correctly identified those patients who further progressed to ALS. Furthermore, in a mouse model of ALS, exogenous expression of Nogo-A in skeletal muscle promotes denervation and instability of the neuromuscular junction. These data are highly suggestive of a role of Nogo-A in the pathophysiology of ALS.

Recent research has provided insights into the pathogenesis of amyotrophic lateral sclerosis (ALS), with some observations shifting previous thinking about the disease. One idea is that interference with normal proteasomal or autophagic protein degradation in ALS, due to causal mutations in proteins such as superoxide dismutase 1 (SOD1), valosin-containing protein (VCP), ubiquilin 2 (UBQLN2), charged multivesicular body protein 2b (CHMP2B), etc, results in proteinopathy (reviewed by Robberecht and Philips). In turn, these primary pathogenic changes can lead to progressive cellular failure characterized by protein clumping, aggregate formation, endoplasmic reticulum (ER) stress and Golgi and mitochondrial failure in the motor neurones and neuromuscular endplates. The discovery of superoxide dismutase1 (SOD1) mutations in 20% of familial ALS patients led to the generation of the first animal models of ALS. SOD1 mice (particularly SOD1-G93A) are the most widely studied animal models of ALS and have been used by GlaxoSmithKline (GSK) to study the effect of Nogo-A inhibition on disease onset and time of death in vivo. In this model, the murine parent anti-Nogo-A antibody, GSK577548, significantly delayed the time to onset of symptoms, prolonged survival and improved muscle strength. Thus, based on the human disease and emergent biological data there is a compelling rational for the development of anti-Nogo-A antibodies, such as ozanezumab, for the treatment of ALS.
Ozanezumab
Ozanezumab (GSK1223249) is a humanised, Fc-disabled, monoclonal antibody (mAb) which targets the amino terminus of Neurite Outgrowth Inhibitor A (Nogo-A). Here we report on the biochemical and structural characterisation of Ozanezumab together with an assessment of pharmacology and non-clinical safety. The rat, rabbit and cynomolgus monkey were selected as the most relevant species for toxicological assessment of Ozanezumab because of the conservation of the Nogo-A sequence in mammals and because the toxicology of most biopharmaceuticals is typically related to their target and generally associated with exaggerated pharmacology at supra therapeutic doses.

MATERIALS AND METHODS

Ozanezumab (GSK1223249)
Ozanezumab (CAS 1310680-64-8) is a humanized IgG1-kappa type antibody consisting of two heavy chains and two light chains linked by a single disulphide bond. The Fc portion of the antibody includes two amino acid substitutions at positions 235 and 237 (EU numbering according to Kabat et al) which disable the ability of monoclonal antibodies to recruit immune effector cells and complement. Ozanezumab was prepared according to current Good Manufacturing Practice (cGMP) for use in the GLP non-clinical safety studies. In addition, some studies used GSK577548, the murine parental antibody to Ozanezumab (GSK1223249), which binds Nogo-A protein.

Animal Care, Use and Regulatory Compliance
Toxicology studies were conducted in experimentally naive, purpose-bred, young adult to adult male and female Sprague-Dawley rats and cynomolgus monkeys and female Dutch Belted rabbits.

All animal studies used written study protocols and facility standard operating procedures and were ethically reviewed and performed in accordance with the GSK Policy on the Care, Welfare and Treatment of Animals and conform to local laws and regulations. Generally, rats were group housed in cages whilst rabbits were individually housed. Monkeys were individually or pair housed. All animals were maintained with a 12 hour light-dark cycle with temperature ranges of 19-26°C and relative humidity between 30-74%. Cages were generally provided with environmental enrichment e.g. objects for manipulation for monkeys. Animals were allowed free access to water and were fed a diet of rodent (rat or rabbit) or monkey chow, free of animal protein, as appropriate. Additional fruits were offered, usually twice daily, to monkeys. For all non-clinical safety studies the drug was...
formulated as a solution in 50 mM sodium acetate, pH 5.5 containing 0.02% Polysorbate 80™ (which also served as vehicle). Stability and concentration analysis were performed to confirm doses.

Toxicology studies were conducted in accordance with regulatory recommendations for the nonclinical safety evaluation of monoclonal antibodies and products derived from recombinant DNA technology (i.e. International Conference on Harmonisation (ICH), European Medicines Agency (EMEA) and Food and Drug Administration (FDA) Guidelines).

Pharmacology

Primary and Secondary Pharmacology

Epitope characterisation
To map the human Nogo-A epitope a set of overlapping peptides (each 12 amino acids in length) corresponding to amino acids 586-785 of Nogo-A were assessed for Ozanezumab binding. Each peptide was biotinylated via a C-terminal linker and peptides were captured onto strepavidin-coated surfaces. Antibody binding to the immobilised overlapping peptides was measured using a Biacore 3000 Surface Plasmon Resonance optical biosensor (GE Healthcare Life Sciences).

Complement C1q binding
ELISA plates were coated overnight with 1 μg/mL Ozanezumab (6.89 nM) or 1 μg/mL control human IgG antibodies (IgG1 and IgG4), and human C1q was added at various concentrations (0.5 to 20 μg/mL). Binding of human C1q to Ozanezumab was detected with goat anti-human C1q peroxidase conjugate and measured using a Biacore 3000 Surface Plasmon Resonance optical biosensor (GE Healthcare Life Sciences).

Pharmacodynamics
The ability of Ozanezumab to neutralise myelin-mediated inhibition of neurite outgrowth (NO) by glutathione s-transferase-Nogo-A protein (corresponding to amino acids 586-785) was evaluated in vitro in rat post-natal cerebellar granule neurons (CGNs), which produce robust NO when cultured on poly-D-lysine or glutathione S-transferase (GST) protein. Poly-D-lysine 96 well plates were coated with recombinant human GST-Nogo-A or GST protein (control). GSK1223249 [0.7 to 100 μg/mL (4.67 to 667 nM)] or the murine parent GSK577548 [0.7 to 100 μg/mL (4.67 to 667 nM)] or buffer (control) was added to the wells coated with GST-Nogo-A and incubated for 1 hour. Freshly prepared rat CGNs were added to the wells (4x10^4 cells/well) and incubated for 24 hours. Subsequently, cells
were stained with Hoechst for nuclear staining and with rabbit anti-beta tubulin antibody for neurite staining. Average neurite length was measured using the Arrayscan machine (Cellomics). The in vivo activity of GSK577548, the murine parental antibody to Ozanezumab (GSK1223249), was also evaluated in the SOD1 mouse model of ALS. These studies are reported elsewhere.\textsuperscript{36}

\section*{Safety Pharmacology}

The potential for cardiovascular and respiratory systems toxicity were assessed in conscious male monkeys (aged 4-7 years). Cardiovascular evaluations were obtained in restrained monkeys fitted with surgically implanted telemetric devices (TL11M3-D70-PCTP, Data Sciences International, St. Paul, MN) whilst respiratory evaluations were measured by means of a clear plastic helmet adapted to serve as a volume displacement plethysmograph. Monkey pairs were administered single bolus IV injections of vehicle control (n=8) or Ozanezumab (30 or 300mg/kg, n=4 per group) on one of two consecutive days in a crossover study design with 21 days between dosing. Cardiovascular and respiratory function parameters were acquired for up to 7 days after dosing and analyzed using the Life Science Suite™ PO-NE-MAH Physiology Platform P3 software system (Version 4.2 with Open ART™, Version 2.3, DSI, Valley View, OH).

Arterial blood pressures, heart rate, electrocardiographic intervals, ventilatory function (tidal volume, respiratory rate or minute volume), airway resistance and body temperatures were recorded. Blood pressure, electrocardiographic (ECG) and respiratory waveforms, were monitored continuously from \textasciitilde 15 minutes prior to dosing (to establish a baseline) to approximately 3 hours postdose (i.e. est. $C_{\text{max}}$ after completion of dosing) and for approximately 15 to 30 minutes on days 1 and 7 after dosing to evaluate reversibility and/or the potential for delayed effects. ECG waveform tracings (\textasciitilde 5-minute duration) were recorded prior to dosing and at various time intervals. Exposure was estimated from other studies conducted in monkeys at the same dose.

In addition, ECG’s were monitored on Day 15 (following the second of 2 doses) or on Days 67 and 72 (prior to and following the last of 6 doses) in the 4 and 12 week repeat dose toxicity studies in monkeys, respectively. Neurological safety evaluations were conducted as part of the 12 and 52 week repeated dose toxicology study (see below for details).

\section*{Toxicology}

\textit{In vitro assessments}
Ozanezumab cross-reactivity with a range of normal rat, monkey, rabbit (CNS only) and human tissue samples was assessed by immunohistochemistry (IHC) using standard methodology. The isotype human IgG1 antibody served as the negative control. In addition, the binding of Ozanezumab to rat, monkey and human peripheral blood leucocytes, erythrocytes or platelets was determined by flow cytometry using standard methods. Binding of biotinylated Ozanezumab to beads coated with Nogo-A peptide (GSK Biopharm Biology, UK) was used as a positive control while assay specificity was determined by the absence of binding to uncoated beads.

**In vivo assessments**

The toxicity and toxicokinetics (TK) of Ozanezumab in young, healthy SD rats and monkeys was evaluated following repeat dose intravenous administration for 4, 12 and 52 weeks (monkey only) as shown in Table 1. The highest doses selected were based on guidance in ICH S6-R1\(^{40}\) or multiples of the anticipated clinical dose, as appropriate. Formulation analysis confirmed all doses were within the specified nominal concentration (100 ± 10%).

Male and female rats (n=10/sex/group) were administered IV (bolus) doses of Ozanezumab once every 2 weeks at 0 (vehicle) or 1000 mg/kg (i.e. maximum feasible dose based on the formulation of 200 mg/mL) for 4 weeks (Days 1 and 15) or at 0, 30, 100 or 300 mg/kg (Days 1, 15, 29, 43, 57 and 71) for 12 weeks. In the 12 week study, additional rats (n=6/sex) were included at 0 and 300 mg/kg to investigate immunogenicity and the potential for delayed toxicity, following an 8 week “off-dose” period after the final dose.

Male and female cynomolgus monkeys (n=3 or 4/sex/group) were administered IV (bolus) doses of Ozanezumab once every 2 weeks at 0 (vehicle) or 1000 mg/kg (limit dose) for 4 weeks (Days 1 and 15); or at 0, 30, 100 or 300 mg/kg for 12 weeks (Days 1, 15, 30, 43, 57 and 71); or at 0, 20, 100 or 500 mg/kg once every 2 weeks for 51 weeks (26 doses in total). A higher top dose was selected in the 52 week monkey study compared with the 12 week study in order to further increase exposure. In the 12 and 52 week studies, additional monkeys (n=2/sex) were included in the vehicle and high dose groups (i.e. 300 or 500 mg/kg) to investigate immunogenicity and the potential for delayed toxicity following 8 and 16 week “off-dose” periods, respectively, after the final dose.

Evaluations in both species included clinical observations, food consumption, body weight, electrocardiography, ophthalmology, clinical pathology, anatomic pathology, organ weights, and microscopic pathology (described in the Appendix – see online). In addition, blood samples were
collected and assayed for serum Ozanezumab concentrations and anti-GSK1223249 antibody formation. An assessment of neurobehavioral function (Irwin test) was performed as part of the repeat dose toxicology studies in monkeys. The potential for any delayed neurobehavioural effects was also investigated during the final week of the “off-drug” recovery periods in these studies.

Reproductive and developmental toxicity studies.
Female rats (n=25/group) were administered IV doses of Ozanezumab at 0 (vehicle), 30, 100 or 500 mg/kg once weekly for a total of 5 weeks (i.e. a pre-mating phase and post coitum (pc) during gestation). Mated females and their litters were euthanized on Day 21 pc to evaluate potential effects on female fertility and early embryonic development to implantation (see Appendix online for further details).

The effects of Ozanezumab on pregnancy and embryofetal development were also investigated in female rabbits following IV administration once weekly at 0 (vehicle), 30, 100 or 500 mg/kg to mated female Dutch Belted rabbits (n=22/group) on Days 7 and 14 pc, resulting in sustained systemic exposure over the major period of organogenesis. The rabbits and their litters were killed on Day 29 pc and assessed by standard methodology (see Appendix online for further details). Blood samples (predose and prior to necropsy) were collected at various intervals to determine the presence of anti-GSK1223249 binding antibodies and for TK analysis.

Pharmacokinetics & Toxicokinetics

i. Single dose pharmacokinetics

The pharmacokinetics of Ozanezumab were assessed in single dose studies in male rats and monkeys following IV (1 hour infusion) and SC dosing at 1 mg/kg for rats (n=3/route) or 1.5 mg/kg for monkeys (n=4/route), respectively. Serial blood samples were collected after dosing (up to 14 days for rat or 70 days for monkey) and plasma samples were analyzed for Ozanezumab concentrations using a validated chemiluminescent immunoassay (CLIA). Plasma samples (100uL) were diluted 20-fold with assay buffer. The lower limit of quantification (LLQ) was 1 ug/mL and the higher limit of quantification (HLQ) was 25 ug/mL. Non-compartmental pharmacokinetic analysis was performed using WinNonlin™, Enterprise edition. Systemic exposures were determined by calculating the area under the plasma concentration time curve (AUC) using the linear-logarithmic trapezoidal rule. Maximum plasma concentrations (Cmax) were obtained by inspection of the
observed data. In addition, total plasma clearance (CL); volume of distribution at steady-state (VSS) and apparent terminal half-life ($t_{1/2}$) were determined.

ii. Repeat dose toxicokinetics

Additional rats (n=3 to 6/sex/group) were included in each repeat dose toxicity study for toxicokinetic evaluations. In both rats and monkeys, serial blood samples were collected on Days 1 and 15 in the 4 week studies and on Days 1, 43 and 71 in the 12 week studies; and at selected time points in the 52 week study (monkey only). In addition, blood samples were taken during the “off-dose” periods at selected time points after the final dose. In rabbits, serial blood samples (~ 0.5 mL) were obtained from an ear vessel at nominal times of 5 mins and 3, 24, 48, 72, and 168 hours after dosing (i.e. prior to dosing on Day 14 pc). In addition, single blood samples were obtained on Day 21 pc. Plasma Ozanezumab concentrations were determined in blood samples for all three species using a similar CLIA method as above.

Data analyses. In the repeat-dose toxicity studies, numerical data from dosed animals were compared with those from the control group. Group means and standard error of the mean (SEM) were calculated for all quantitative data.

RESULTS

Antibody characterisation

Ozanezumab was found to only bind to the peptides with amino acids sequences TSPVLPDIVMEAPLN and VLPDIVMEAPLNSAVP respectively, from which a minimal binding epitope of VLPDIVMEAPLN was derived. Ozanezumab displayed significantly reduced C1q binding activity compared to a wildtype IgG1 sequence, indicating that the substitutions present in CH2 domain reduce the likelihood of triggering ADCC and CDC.

Pharmacodynamics

Ozanezumab decreased GST-Nogo-A protein mediated inhibition of neurite outgrowth (NO) in cultured primary CGNs in a dose-dependent manner as indicated by a corresponding increase in neurite length (Figure 1). A control IgG had no effect on NO.

Ozanezumab Pharmacokinetics & Toxicokinetics
**Single dose studies:** Data are summarised in Table 2.

In the rat, $t_{1/2}$ was approximately 193 hours and plasma CL was low (0.47 mL/hr/kg) and $V_{ss}$ of 0.1 L/kg following IV dosing, indicating distribution was confined to the systemic circulation. Following SC dosing, bioavailability was estimated to be 68% with $C_{max}$ at 96 hours and only 30% of the observed IV dose.

In the monkey $t_{1/2}$ ranged from 50 to 147 hours following IV dosing and CL was again low (0.95 mL/hr/kg). $V_{ss}$ was also very low (0.08 L/kg), suggesting Ozanezumab was again mainly confined to the systemic circulation. Following SC dosing bioavailability was 100% and $C_{max}$ was observed at 30 hours. The $C_{max}$ was only 50% of the observed IV dose.

**Repeat dose studies:** Data are summarised in Table 3.

In both rats and monkeys the systemic exposure ($AUC_{0-\tau}$ and $C_{max}$) was similar in males and females with $T_{max}$ observed immediately after dosing. $AUC_{0-\tau}$ and $C_{max}$ values increased dose proportionally and Ozanezumab was cleared from the plasma very slowly with a $t_{1/2}$ of approximately 100-180 hours (rat) and 249 to 388 hours (monkey). The PK profile in monkeys appeared biphasic, with most of the dose (~80%) cleared from the systemic circulation within 7 days, and the remainder eliminated with an apparent half-life of 14 days. Despite this, no marked (>2-fold) changes in $AUC_{0-\tau}$ and $C_{max}$ were observed with repeat dosing in either species. Anti-GSK1223249 antibodies were observed in one monkey given 100 mg/kg/biweekly in the 52 week study. As plasma exposure values remained within the range seen in the other monkeys, both prior to and after anti-drug antibodies were detected the impact on study interpretation was considered to be negligible.

A similar TK profile was also observed in pregnant Dutch Belted rabbits, albeit with a relatively shorter $t_{1/2}$ (< 72 hours). Systemic exposures ($AUC_{0-168h}$ and $C_{max}$) were again dose-proportional and similar between Days 7 and 14 pc. All drug-treated rabbits had measurable plasma concentrations of Ozanezumab on Day 21 pc (168 hours after the last dose was given on Day 14 pc). Anti-GSK1223249 antibodies were detected in a small number of rabbits and these animals generally showed more rapid plasma clearance of Ozanezumab.

**Non-clinical safety assessment**

**Safety Pharmacology**

**Evaluation of cardiovascular and respiratory systems in vivo**
Ozanezumab (300 mg/kg) produced a mild decrease in group mean heart rate (up to 17% compared to vehicle) in the telemetered monkey study. The effect was observed from approximately 0.5 to 3 hours after dosing on day 1 and persisted to day 7. In the lower dose group (30 mg/kg), the effect on group mean heart rate was milder (up to 12% compared to vehicle) and transient i.e. from ~1 to 3 hours after dosing on day 1 only [see Figure 1, Supplementary data]. The effects were not considered adverse because the decreases in the absolute heart rate values in the Ozanezumab treatment groups remained within the range of the absolute heart rate values for the vehicle control group. A transient (1 to 3 hours after dosing) and mild increase in body temperature (up to 0.55°C) was also recorded in the high dose group. No other effects on respiratory or cardiovascular function were observed in this study.

In repeat dose toxicity studies there were no test article-related effects on cardiovascular function in either species (rat or monkey) in any study.

**Evaluation of neurological systems in vivo**

Neurobehavioral function and body temperature was unaffected by Ozanezumab in either of the 12 or 52 week monkey studies during treatment or after the 8 or 16-week ‘off–dose’ periods.

**Toxicological Assessment**

**In vitro**

There was good concordance between the rat, monkey and human in terms of the range of tissues in which specific Ozanezumab immunocytochemical staining was observed. The following tissues and associated histological structures were noteworthy in all three species; central and peripheral nervous tissue including glial cells in the brain and spinal cord, retinal cells in the eyes and ganglion cells in the myenteric plexus (gastrointestinal tract). Muscle tissues including smooth muscle in blood vessels, muscularis mucosae, and muscle layers in many tissues, myofibres in striated muscle and myoepithelial cells in many tissues. Occasional staining was also seen in stromal cells in many tissues.

In monkey and rat, Ozanezumab specific staining was also observed in Purkinje cells and other neurons (brain-cerebellum, brain-cortex and spinal cord), fibroblasts or Schwann cells (peripheral nerves), stromal cells in the pituitary (pars nervosa and pars distalis) and cardiomyocytes (heart). In contrast, such staining was not observed in humans. Some inconsistencies in staining were observed in a few tissues (e.g. blood smear, kidneys, ovaries, pancreas, pituitary and epithelial layers) in one
or the other animal species and were most likely due to high concentrations of biotinylated GSK1223249 required for staining. There was no evidence of specific surface binding of Ozanezumab to leucocytes, red blood cells or platelets from rat, monkey or human. In a separate study, Ozanezumab-specific staining was observed in central nervous tissue (cerebellum and hippocampus) of rabbit.

In vivo

Rat 4 and 12 repeated-dose toxicity studies

In the rat, Ozanezumab was well tolerated and there was no evidence of toxicity or irritancy in either the 4 or 12 week studies. One male rat in the 12 week study (30 mg/kg low dose group) was found dead on Day 83, but in the absence of test-article related changes or otherwise significant macroscopic or microscopic differences this death was not considered to be attributable to drug treatment. During Week 1 of the 4 week study there was reduced body weight gain and food consumption in males treated with Ozanezumab (1000 mg/kg), but overall weight gain was similar to controls over the duration of the study. No differences were seen in females. In the 12 week study, there was no effect on body weight or food consumption in either gender at doses of up to 300 mg/kg Ozanezumab.

Increases in serum gamma globulin 2 concentrations were seen in males and females given 1000 mg/kg Ozanezumab in the 4 week study, and females also showed increases in serum globulin concentration and decreases in the ratio of albumin to globulin. All these changes were considered to reflect the circulating drug, consistent with the administration of such high doses of Ozanezumab. There was a slight increase in the incidence of mild mammary gland lobular hyperplasia in 4 females (compared to 1 of 10 in controls). Otherwise, there were no significant clinical observations or toxicities observed based on the various assessments made in either study. There was no evidence of any toxicity on the male and female reproductive organs (including stage dependent evaluation of spermatogenesis in the male) nor was there evidence for immunogenicity or delayed toxicity. However, compared with concurrent controls, there was a slight decrease in mean activated partial thromboplastin time in females in the 12 week study. A similar change was not noted in males. Slight increases (<13%) in mean heart weight (male and female) and mean liver weight (female only) were also noted. In the absence of other pathology (clinical or histological), these minor changes were considered to be of no toxicological concern.

Monkey 4, 12 and 52 week repeated-dose toxicity studies
Ozanezumab was well tolerated in all the repeat dose (4, 12 and 52 week) studies in monkey. There were no deaths or unscheduled terminations and there was no evidence for toxicity, irritancy or any adverse treatment-related findings for any parameter evaluated. There was no evidence of any toxicity on the male and female reproductive organs (including stage dependent evaluation of spermatogenesis in the males). In the 12 week study, there was no evidence for immunogenicity or delayed toxicity at the highest dose (300 mg/kg) at the end of the 8 week “off-dose” period. In the 52 week study there was no evidence for delayed toxicity at the highest dose (500 mg/kg) at the end of the 16 week “off-dose” period. Development of anti-GSK1223249 antibodies was observed, however, in one monkey in the 100 mg/kg treatment group. This was not considered to have impacted study interpretation because plasma exposure values (AUC) in that monkey remained within the range seen in the other monkeys, both prior to and after anti-drug antibodies were detected.

Reproductive toxicity studies
Reproductive toxicology studies were performed in female rats and rabbits. Ozanezumab was well tolerated in both species and there were no adverse maternal effects or changes in pregnancy or foetal parameters at any dose.

DISCUSSION
Assessment of non-clinical primary pharmacology
Ozanezumab binds to a novel epitope corresponding to amino acids VLPDIVMEAPLN within the Nogo-A amino terminus. Binding of Ozanezumab causes potent inhibition of neurite outgrowth (NO) in vitro and these data are consistent with the notion that neutralisation of myelin-mediated inhibition of NO by Ozanezumab is specific for Nogo-A protein (amino acids 586-785). Efficacy has also been observed with a murine homologue of Ozanezumab in SOD1 mice\textsuperscript{36} which is a model of ALS in humans. Drug treatment resulted in prolonging disease free and overall survival in these animals and a concomitant increase in muscle force and motor unit survival.

Assessment of non-clinical safety
The safety profile of Ozanezumab was evaluated in a number of in vitro and in vivo studies. The rat and monkey were selected for toxicological evaluation because of similar binding affinities of Ozanezumab for rat, monkey and human Nogo-A, consistent with the evolutionary conservation of the target. The rabbit was selected for reprotoxicology assessment.
The in vitro tissue cross-reactivity studies showed good overall concordance between rat, monkey and human in terms of the range of tissues in which Ozanezumab specific staining was observed. Importantly, all tissues/histological structures in which specific staining was observed in humans were also positive in the rat, rabbit (CNS) and monkey, confirming the relevance of these species for toxicological evaluation. No in vitro safety pharmacology studies were conducted with GSK1223249. Assays to assess the potential for delayed ventricular repolarisation (e.g., hERG assay) are considered inappropriate for proteins, such as monoclonal antibodies. In addition, it is unlikely that large proteins, such as Ozanezumab, would block the hERG pore or any other cardiac channel, which is the main mechanism by which hERG is inhibited.41

Ozanezumab had no significant effects on cardiovascular or respiratory function in male telemetered monkeys. There was no evidence for adverse ECG or temperature changes in repeat dose toxicity studies in monkeys.

Ozanezumab was well tolerated in repeat dose toxicity studies in rats and monkeys and there was no evidence for toxicity or irritancy in any animal in any study based on the results from a wide range of clinical, anatomical, physiological and histo-pathological investigations. In female rats given 1000 mg/kg/fortnight ozanezumab for 4 weeks there was a slight increase in the incidence of mild mammary gland lobular hyperplasia in 4/10 animals compared to controls (1/10 animals). In a second repeat dose study, there was no increase in the incidence of mammary gland lobular hyperplasia in female rats given Ozanezumab at doses of up to 300 mg/kg/biweekly for 12 weeks (0/30 animals). Complete Nogo-A inhibition would have been anticipated at all of the doses used in both studies and therefore these data do not support a direct pharmacological link. As such, and given the minimal severity of the observation together with its isolation from any other associated effect on the endocrine and reproductive tract in Ozanezumab-treated female rats, this finding is considered to be non adverse. This conclusion is further supported by the lack of any effects in the female fertility studies in the rat (estrous cycle evaluations are very sensitive to endocrine disturbances). In addition, there were no effects on male rat mammary glands and other endocrine sensitive organs (uterus, prostate) which indicates that there is no endocrine basis for this observation.

Therefore, the no observed adverse effect level (NOAEL) in the monkey was 1000 mg/kg (i.e., the limit dose in non-rodent species) administered once every 2 weeks for a period of 4 weeks, or 500 mg/kg administered once every 2 weeks for 52 weeks. In the 52 week monkey study, the NOAEL
(500 mg/kg) represents mean AUC_{0-t} values of 890 and 947 mg.h/mL in Weeks 1 and 51, respectively. These data provided good safety margins of exposure (> 10 x fold for both C_{max} and AUC) based on the clinical doses of Ozanezumab (i.e. up to 15mg/kg IV every 2 weeks) used in the phase I and II studies in ALS patients (Meininger et al; ClinicalTrials.gov identifiers NCT00875446 and NCT01753076).

Monoclonal antibodies generally do not interact directly with DNA or chromosomes; therefore, genetic toxicology studies were not performed with Ozanezumab. Similarly, carcinogenicity studies have not been performed with Ozanezumab in accordance with ICH S6(R1). No proliferative, hyperplastic or pre-neoplastic changes were observed in the 52 week repeat dose study in monkeys. Furthermore, targeted disruption (gene knockout) of Nogo-A has shown no evidence for increased susceptibility to tumourigenesis in 3 independent mouse models. Nogo-A is not associated with human tumours other than those of neurological origin, where strong expression is observed only in human oligodendrogliaomas (71% tumours) and a subset (25%) of glioblastoma multiformae. To date, there is no evidence that Nogo-A gene mutation is associated with tumour susceptibility whereas the role of Nogo-A in human oligodendrogliaomas, if any, remains unknown. Ozanezumab is a highly specific monoclonal antibody and given the nature of the Nogo-A protein target there is no published evidence to suggest that the induction or progression of a tumour would be affected by Nogo-A blockade. Therefore, Ozanezumab is considered to have low or negligible carcinogenic risk.

**Assessment of impact on neuronal plasticity**

Nogo-A is an influential molecular modulator of synaptic plasticity and a regulator for learning of skilled movements in the motor cortex. Although the learning of cortically controlled precision movements can be improved in the rat upon anti-Nogo-A antibody treatment there remains a theoretical concern that inhibition of Nogo-A might facilitate unwanted and unnecessary (and even maladaptive) neuronal connections. Indeed, in the chicken embryo, in ovo injection of anti-Nogo-A antibodies has been associated with aberrant innervations of the hindlimb and Nogo-A-deficient transgenic rats show deficits in higher cognitive functions. In Nogo-A knockout mice specific behavioural abnormalities resembling schizophrenia-related endophenotypes have also been reported recently, but not in adult wild-type mice following acute antibody-mediated Nogo-A blockade (reviewed by Willie and Schwab). Interestingly, the infusion of anti-Nogo-A antibodies in adult rats increased the expression of growth and synapse related proteins in the hippocampus, a brain region which might be particularly sensitive to Nogo-A depletion due to the high expression level of Nogo-A. Despite these changes, Nogo-A blockade was not associated with any pronounced
cognitive-behavioural changes indicative of hippocampal functional deficiency across several critical tests. These results indicate that anti-Nogo-A antibody therapy appears safe in the adult CNS in rat over 4 weeks of continuous administration.

Nevertheless, it remains important to evaluate whether Nogo-A blockade by GSK1223249 represents a potential hazard to neuronal plasticity during development. In mammalian reproductive toxicity studies conducted to date, Ozanezumab treatment had no effect on female fertility or early embryonic development in rats and there were no effects on pregnancy or embryofoetal development in rabbits. Tissue cross reactivity studies confirmed that Ozanezumab binds Nogo-A in both the rat and in the rabbit and the no observed adverse effect level (NOAEL) in both species was 500 mg/kg/week. Since antibodies of the IgG1 class are known to be transferred across the placenta in the second half of gestation further peri-postnatal studies are planned for the future to assess the effects of Ozanezumab treatment on the developing foetus in utero.

Conclusions

Emerging biology and pre-clinical pharmacology provides confidence that targeting the amino terminus of the neurite outgrowth inhibitor A (Nogo-A) through passive immunization may offer a promising approach to treat various neurodegenerative diseases. Ozanezumab (GSK1223249) is a humanised, Fc-disabled, monoclonal antibody (mAb) that targets the amino terminus of Nogo-A. Non-clinical biochemical and pharmacological characterisation of the monoclonal antibody supported the clinical development of Ozanezumab in patients with ALS. A comprehensive non-clinical assessment of safety has been completed based on a package of in vitro and in vivo studies in rodents, non-human primates and female rabbits (reproductive toxicology only). There was no evidence for toxicological, cardiovascular (ECG including QTc), respiratory, neurobehavioural, immunogenic, reproductive or delayed toxicity effects in any species following treatment with Ozanezumab. As such, the non-clinical safety profile is supportive of a positive benefit–risk assessment for the development of Ozanezumab in patients with ALS.

SUPPLEMENTARY DATA
Supplementary data are available online at http://

Conflict of interest statement
The authors declare that there are no conflicts of interest.
ACKNOWLEDGMENTS

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REFERENCES


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Fig. 1 Anti-Nogo antibodies neutralise neurite outgrowth inhibitory activity of Nogo. Rat primary cerebellar granular neurons were cultured on GST-Nogo(5+6) coated plates in the presence of anti-Nogo antibody (GSK1223249) or control antibody for 24 hours. Cells were fixed with formaldehyde and dual stained with Hoechst and FITC labelled anti-β tubulin antibodies. The neurons were imaged, and neurite outgrowth per cell was measured by using Cellomics ArrayScan imaging platform. Data was normalised using neurite outgrowth on GST-Nogo as a control.
<table>
<thead>
<tr>
<th>Study type</th>
<th>Species</th>
<th>Route</th>
<th>Regimen</th>
<th>Dose (mg/kg)</th>
<th>Euthanized (weeks)</th>
<th>Terminal</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>General toxicology</td>
<td>Monkey 3 M/F</td>
<td>iv</td>
<td>Q2W</td>
<td>0, 1000</td>
<td>4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>General toxicology</td>
<td>Monkey 3-4 M/F</td>
<td>iv</td>
<td>Q2W</td>
<td>0, 30, 100, 300</td>
<td>12</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>General toxicology</td>
<td>Monkey 4 M/F</td>
<td>iv</td>
<td>Q2W</td>
<td>0, 20, 100, 500</td>
<td>52</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>General toxicology</td>
<td>Rat 10 M/F</td>
<td>iv</td>
<td>Q2W</td>
<td>0, 1000</td>
<td>4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>General toxicology</td>
<td>Rat 10 M/F</td>
<td>iv</td>
<td>Q2W</td>
<td>0, 30, 100, 300</td>
<td>12</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Reproductive toxicology</td>
<td>Rat 25 F</td>
<td>iv</td>
<td>Q1W</td>
<td>0, 30, 100, 500</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Reproductive toxicology</td>
<td>Rabbit 26 F</td>
<td>iv</td>
<td>Q1W</td>
<td>0, 30, 100, 500</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cardiovascular/Respiratory safety pharmacology&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Monkey</td>
<td>iv</td>
<td>Single dose</td>
<td>0, 30, 300</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>PK&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Monkey</td>
<td>sc, iv</td>
<td>Single dose</td>
<td>1.5</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Note. Q2D, every other week; animals were randomly assigned to treatment groups based on stratified body weight. All animals were humanely euthanized after the periods described.

<sup>a</sup> Animals were retreated after a wash-out period as described in the Methods section.

<sup>b</sup> Each monkey received two doses, separated by a 2-week wash-out period.

<sup>c</sup> Rats were dosed once weekly during the pre-mating treatment phase (Days 1, 8 and 15) and gestation phase [Days 6 and 13 post coitum (pc)] for a total of 5 doses.
Table 2. Mean Pharmacokinetic Parameters of GSK1223249 Following Single Intravenous (Bolus) or Subcutaneous (SC) Administration of GSK1223249 to Male Rats (1 mg/kg Nominal) or Cynomolgus Monkeys (1.5 mg/kg Nominal)

<table>
<thead>
<tr>
<th>Species</th>
<th>Route</th>
<th>Dose</th>
<th>$C_{\text{max}}$</th>
<th>$\text{AUC}_{0-t}$</th>
<th>$\text{CL}_p$</th>
<th>$V_{ss}$</th>
<th>MRT</th>
<th>$T_{\text{max}}^a$</th>
<th>$t_\frac{1}{2}$</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>IV</td>
<td>1</td>
<td>25.0</td>
<td>2083</td>
<td>0.47</td>
<td>0.10</td>
<td>121</td>
<td>ND</td>
<td>193</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SC</td>
<td>1</td>
<td>6.93</td>
<td>1552</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
<td>ND</td>
<td>ND</td>
<td>68</td>
</tr>
<tr>
<td>Monkey</td>
<td>IV</td>
<td>1.5</td>
<td>23.8</td>
<td>2000</td>
<td>0.95</td>
<td>0.08</td>
<td>87.9</td>
<td>7.0</td>
<td>92.2</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>SC</td>
<td>1.5</td>
<td>11.3</td>
<td>1990</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
<td>30</td>
<td>155</td>
<td>118</td>
</tr>
</tbody>
</table>

Key:
- AUC$_{0-t}$ where $t = 336$ and 1680 hours for rat and monkey, respectively.
- Values are mean (n=3 for rats; n=4 for monkeys except as otherwise noted).
- F = Bioavailability.  IV = Intravenous.   MRT = Mean residence time.  NA = Not applicable.  ND = Not determined.
- SC = Subcutaneous.
- a. Expressed as median.
- b. n=3 monkeys.
Table 3. Mean Plasma Toxicokinetic Parameters for GSK1223249 Following Once Every 2 Week IV (Bolus) Administration in Male and Female Rats and Monkeys and in Female Rabbits

<table>
<thead>
<tr>
<th>Species/Study (No. of Doses)</th>
<th>Dose (mg/kg)</th>
<th>Sex</th>
<th>C_{max} (mg/mL)</th>
<th>AUC_{0-336h} (mg.h/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Days 1 to 15</td>
<td>Days 15 to 29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat/4 week (2 doses)^a</td>
<td>1000 (NOAEL)</td>
<td>M</td>
<td>30.6 [28.6-33.8]</td>
<td>32.9 [29.8-35.5]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>28.4</td>
<td>32.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30.1 [24.8-33.8]</td>
<td>30.4 [27.3-32.6]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25.5 [22.8-30.6]</td>
<td>26.3 [22.8-32.3]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1570 [1320-1710]</td>
<td>1530 [1430-1930]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey/4 week (2 doses)^a</td>
<td>1000 (NOAEL)</td>
<td>M</td>
<td>30.6 [28.6-33.8]</td>
<td>30.4 [27.3-32.6]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25.5 [22.8-30.6]</td>
<td>26.3 [22.8-32.3]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1220 [1150-1320]</td>
<td>1200 [1080-1390]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species/Study (No. of Doses)</td>
<td></td>
<td></td>
<td>C_{max} (mg/mL)</td>
<td>AUC_{0-168h} (mg.h/mL)^b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 7pc</td>
<td>Day 14pc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit EFD (2 doses)^c</td>
<td>30</td>
<td>F</td>
<td>1.03</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.61</td>
<td>287</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>F</td>
<td>4.74</td>
<td>287</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species/Study (No. of Doses)</td>
<td></td>
<td></td>
<td>C_{max} (mg/mL)</td>
<td>AUC_{0-336h} (mg.h/mL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Days 1</td>
<td>Days 43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Days 71</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat/12 week (6 doses)^h</td>
<td>30^e</td>
<td>M</td>
<td>0.932</td>
<td>1.050</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.885</td>
<td>1.260</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td></td>
<td>1.260</td>
</tr>
<tr>
<td></td>
<td>100^e</td>
<td>M</td>
<td>3.53</td>
<td>3.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.71</td>
<td>4.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td></td>
<td>4.26</td>
</tr>
<tr>
<td></td>
<td>300^f (NOAEL)</td>
<td>M</td>
<td>8.30 [7.97-8.86]</td>
<td>11.40 [10.6-12.6]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7.27 [7.10-7.55]</td>
<td>11.10 [10.9-11.3]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td></td>
<td>11.10 [10.2-13.1]</td>
</tr>
<tr>
<td></td>
<td>100^f</td>
<td>M</td>
<td>2.21</td>
<td>2.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.70</td>
<td>2.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td></td>
<td>2.88</td>
</tr>
<tr>
<td></td>
<td>300^f (NOAEL)</td>
<td>M</td>
<td>8.45 [7.81-8.96]</td>
<td>9.01 [6.43-10.2]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7.29 [5.89-8.97]</td>
<td>9.61 [7.75-10.9]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td></td>
<td>9.61 [7.75-10.9]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10.1 [8.76-11.4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.55 [8.19-10.2]</td>
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<td></td>
<td>377</td>
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<td>289</td>
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<td>201-394</td>
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Table 3 (Continued)  Mean Plasma Toxicokinetic Parameters for GSK1223249 Following Once Every 2 Week IV (Bolus) Administration in Male and Female Rats and Monkeys and in Female Rabbits

<table>
<thead>
<tr>
<th>Species (No. of Doses)</th>
<th>Dose (mg/kg)</th>
<th>Sex</th>
<th>(C_{\text{max}}) (mg/mL)</th>
<th>AUC(_{0-336\text{h}}) (mg.h/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Week 1</td>
<td>Week 13</td>
</tr>
<tr>
<td>Monkey/52 week (26 doses)a</td>
<td>20g</td>
<td>M</td>
<td>0.571</td>
<td>0.528</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>0.550</td>
<td>0.588</td>
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<td>100h</td>
<td>M</td>
<td>3.11</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>3.30</td>
<td>3.67</td>
</tr>
</tbody>
</table>

**Key:** Bolded doses are no observed adverse effect levels (NOAELs) with mean [range] except for composite samples.

a = Doses were administered once every 2 weeks for 4, 12 or 52 weeks (2, 6 or 26 doses in total).
b = AUC\(_{0-t}\) was calculated using \(t = 168\) hours.
c = Doses were administered once weekly on Days 7 and 14 post coitum.
d = To enable comparison with Day 1 and 43 AUC\(_{0-t}\) data, the AUC\(_{0-t}\) for Day 71 was calculated using \(t = 336\) hours.
e = n=3/sex.
f = n=6/sex.
g = n=4/sex.
h = n=8/sex.
EFD = Embryofoetal development. IV = Intravenous. pc = Post coitum.