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Nonclinical Safety Evaluation of a Transforming Growth Factor β Receptor I Kinase Inhibitor in Fischer 344 Rats and Beagle Dogs

Anja J Stauber[1*], Kelly M Credille1, Lewis L Truex[1], William J Ehlhardt[1] and Jamie K Young[2]

[1]*Lilly Research Laboratories, Toxicology and Pathology, Eli Lilly and Company, Indianapolis, IN, 46285, USA*

[2]*Covance Laboratories Inc., Greenfield, Indiana, 46140, USA*

Corresponding author:Anja J Stauber, Ph.D, D.A.B.T, Lilly Corporate CenterEli Lilly and Company, Indianapolis, Indiana 46285, USA,Tel: 317-433-9486; E-mail: anja_stauber@lilly.com

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Abstract

Objective: The transforming growth factor β (TGF-β) pathway regulates diverse cellular functions and plays a prominent role in diseases such as cancer, autoimmune disorders and cardiovascular disease. LY2157299 monohydrate (LY2157299) is a potent and selective inhibitor of TGF-β receptor I kinase that is under clinical evaluation for the treatment of advanced cancer.

Methods: This paper characterizes the toxicity profile of LY2157299 in Fischer 344 rats and beagle dogs for up to six months of daily oral dosing. LY2157299 is well tolerated in the rat and dog for up to one month of daily dosing at doses of 150 and 20 mg/kg, respectively.

Results: In the rat, LY2157299 is well tolerated after three months of 2 weeks on/2 weeks off intermittent dosing schedule at 50 mg/kg. Chronic (≥3 months) oral administration results in multiple target organ toxicities involving the cardiovascular, gastrointestinal, immune, bone/cartilage, reproductive, and renal systems.

Conclusion: Defining the appropriate dose and schedule led to a better understanding of how to define safety margins and thus enable the clinical investigation of LY2157299 in cancer patients.

Keywords: TGF-β Inhibitor; Kinase; Drug-associated cardiovascular toxicity; Rats; Dogs

Introduction

Transforming growth factor β (TGF-β) signaling leads to pleiotropic activation, including regulation of cell growth, migration, and differentiation [1-3]. TGF-β ligands are differentially expressed across a variety of tissues including hematopoietic, cartilage, bone, cardiac, and neuronal tissues in both endothelial and epithelial cells [4]. The ligands TGF-β1, TGF-β2, and TGF-β3 signal through binding to the TGF-β type I and type II Ser/Thr kinase receptors. Upon ligand binding-mediated activation, phosphorylation of the type II receptor activates the Type I receptor resulting in phosphorylation and activation of downstream Smad proteins, which are the intracellular effectors of TGF-β signaling [5].

Because of its pleiotropic role, genetically altered animals can provide useful insights on the relevance of TGF-β signaling during development and normal physiology. Genetic manipulation of the TGF-β signaling pathway in mice demonstrates its crucial role in embryonic development in controlling vasculogenesis, angiogenesis, and inflammation. The phenotypes of TGF-β1, β2, or β3 knockout models all result in high embryonic lethality or mortality shortly after birth. TGF-β1 and TGF-β2 knockouts have vascular, aortic arch, and cardiac septal defects [6,7]. TGF-β1 knockout mice die shortly after birth due to cardiopulmonary complications with severe tissue inflammation [6]. Ablation of TGF-β receptors I and II results in embryonic lethality with vascular and angiogenesis defects [8,9]. The Smad proteins are the downstream signal transducers of TGF-β signaling, and genetic knockdown of key regulators such as Smad 2 and 3 are similarly embryonic lethal or affected mice experience a reduced lifespan with abnormalities consisting of mucosal inflammation, skeletal defects, and colon cancer [10]. Based upon the key role that TGFβ signaling plays in embryonic development and survival as well as its central role in certain cancers and other disease states, inhibition of this important signaling pathway may be anticipated to have adverse consequences on animals.

Blocking TGF-β signaling can be achieved via different mechanisms, including neutralizing antibodies and small molecule inhibitors that interrupt the phosphorylation of the TGF-β receptor I kinase (RIK) [11]. Within the last 5 years, a small number of publications have disclosed the effects of TGF-β inhibition in nonclinical toxicology species. Inhibition of TGF-β signaling results in a physeal dysplasia that is observed in rats administered small molecule inhibitors of TGF-β RIK [12,13]. Rats administered repeated doses of small molecule TGF-β RIK inhibitors develop a valvulopathy that is characterized by inflammation, hemorrhage and stromal hyperplasia [12-14]. Valvulopathy in rats is likely a result of direct inhibition of the TGF-β signaling pathway, rather than an "off-target" effect, because multiple structurally diverse small molecule inhibitors have produced similar effects. In contrast, large molecule inhibitors of this pathway appear to have a somewhat different toxicity profile. Monkeys administered GC1008, a human IgG4 pan-neutralizing TGF-β antibody for six months developed a dose- and time-dependent increase in epithelial hyperplasia of the gingiva and bladder [15].

These nonclinical findings from pharmaceutical companies have led to termination of several small molecule drug development programs targeting the TGF-β signaling pathway based on an apparent lack of acceptable safety margins for clinical testing [16].

Here, we disclose the nonclinical toxicology profile of LY2157299, a potent and selective oral TGF-β RIK inhibitor in Fisher 244 rats and Beagle dogs. This inhibitor is being developed for the treatment of patients with advanced cancer. Administration of LY2157299 was associated with several toxicological findings that involved multiple organ systems including cardiovascular, gastrointestinal, immune, bone/cartilage, reproductive, and renal. Toxicities were dose and duration dependent, some evident within days at high doses, whereas others were only evident in studies of 6 months' duration. Despite these serious adverse nonclinical toxicities, careful evaluation of various dosing regimens provided sufficient data to ensure the safety of patients in clinical trials.

Materials and Methods

Test compound

LY2157299, (4-[2-(6-methyl-2-pyridyl)-5,6-dihydro-4H-pyrrolo[1,2-b]pyrazol-3-yl]quinoline-6-carboxamide hydrate), a specific and potent inhibitor of TGF-β receptor 1 kinase, was synthesized by Lilly Research Laboratories, a division of Eli Lilly and Company. Dosing suspensions were prepared and stored within established stability parameters in 1% (w/v) carboxymethylcellulose, 0.25% (v/v) Polysorbate 80, and 0.05% (v/v) Dow Corning® Antifoam 1510-US in purified water. LY2157299 monohydrate was prepared in 500mM phosphate buffer (pH 1.8 to 2.2) for administration in dogs.

Animals and husbandry

Eight oral nonclinical toxicology studies are described in this manuscript: a 2-week pilot study; 1-month, two 3-month studies, and a 6-month study in rats; two 1-month studies and a 6-month study in dogs. With the exception of the 2-week rat pilot study, all studies were conducted to conform to the United States Food and Drug Administration (CFR 21 – Part 58) and Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice standards in place at the time of study initiation.

Male and female Fischer 344 rats (obtained from Taconic, Germantown, NY) were used for nonclinical toxicity studies up to 6 months in duration. All animals were between 6-11 weeks old at the start of treatment with initial body weights ranging from 100 to 300 grams. Clinically acceptable male and female rats were randomly assigned to dose groups to ensure equivalent distribution of body weights. Rats were housed individually in stainless steel cages and offered Certified Rodent Diet and were provided water ad libitum. Animals were fasted overnight prior to euthanasia.

Beagle dogs obtained from Marshall Farms (North Rose, NY) for the three dog studies were approximately 7-10 months of age at the start of treatment. Clinically acceptable male and female dogs were randomly assigned to dose groups to ensure equivalent distribution of body weights. Dogs were individually housed in stainless steel cages with suspended mesh floors and offered Certified Canine Diet and were provided water ad libitum. Animals were fasted overnight prior to euthanasia.

In all studies, animal care and use were done in accordance with federal and local laws, policies, regulations, and standards in effect at the time of their conduct, e.g., Animal Welfare Act and Regulations. Laboratories conducting these studies were accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and all study protocols were approved by each laboratory's Institutional Animal Care and Use Committee.

Experimental procedures

Nonclinical toxicology studies were conducted in Fisher 344 rats and Beagle dogs. Table 1 describes the study designs for all studies conducted with LY2157299 reported in this paper. Rats were administered control vehicle or compound orally via gavage, while dogs were administered control vehicle or LY2157299-filled capsules. Plasma for measuring systemic exposure were taken on the first day of dosing and on the last day of dosing in all studies in addition to periodic intervals in studies lasting 3 months or longer. Plasma was analyzed for LY2157299 concentration using the same validated liquid chromatography/mass spectrometry method used for analysis in human plasma [17], except the method was validated over the range of 0.5-500 ng/mL in rat and dog plasma.

Species	Duration (weeks)	Recovery (weeks)	Dosing Regimen	N (sex/group)	Doses (mg/kg)
Rat	2	None	Daily	5	0, 50, 300, 1200
	4	4	Daily	10	0, 15, 50, 150
	12	None	Daily	10	0, 50, 150, 250
	12	None	2 weeks on 2 weeks off	10	0, 50, 150, 250
	8	6	Daily	10 (control) 35 (treated)	0, 250
	26	None	Daily	15	0, 50, 150, 250
Dog	4	4	Daily	3	0, 50, 250, 500
	4	None	Daily	3	0, 2, 8, 20
	26	None	Daily	4	0, 8, 20, 60

Table 1: Nonclinical Studies Conducted with LY2157299.

Animals were observed at least once daily for mortality and clinical signs; body weights and food consumption were analyzed weekly. Electrocardiograms (ECGs) were collected at pretest in order to screen for preexisting ECG abnormalities and to provide baseline ECG data for later comparisons. During the live phase, ECGs were collected before dosing and after dosing during the first week of treatment and at 1 month and near the end of the reversibility phase (for 1-month study containing reversibility). For the 6-month study, ECGs were also collected at 3 months and 6 months.

Blood samples for complete blood counts, coagulation, and serum biochemical assays were collected from rats and dogs at the end of the

treatment and/or reversibility phases. In the dog, these analyses were evaluated prior to initiation of treatment, around the start of the study and at other time points during the study. Cardiac troponin I was also evaluated in some studies.

Urine samples were collected by cystocentesis at necropsy. Urine was collected from rats overnight using metabolism cages at the end of treatment in the 6-month study and from a subset of rats in the 1-month study. Samples were analyzed for appearance/color, microscopic examination of sediment, bilirubin, blood, glucose, ketones, pH, protein, specific gravity, urobilinogen, and volume.

Rats were anesthetized with isoflurane or carbon dioxide inhalation, euthanized by exsanguination and necropsied. Dogs were administered a barbiturate overdose, exsanguinated and necropsied. Necropsies were conducted in all rats and dogs and any observations were recorded. Tissues collected at necropsy for microscopic evaluation included adrenals, aorta, bone marrow smear, bone with marrow, brain, brown fat (interscapular), cecum, cerebellum with pons or medulla oblongata, cerebrum, epididymides, esophagus, eyes with optic nerves, femur with knee joint, gastrointestinal tract (cecum, colon, duodenum, esophagus, ileum, jejunum, stomach), gross lesions, heart, kidneys, liver, lungs (including bronchi), lymph nodes (mandibular and mesenteric), mammary gland, ovaries with oviducts, pancreas, sciatic nerve, pituitary, prostate, salivary glands, seminal vesicles, skeletal muscle (rectus femoris), skin, spinal cord (cervical), spleen, sternum, testes, thymus, thyroids with parathyroid, tongue, trachea, urinary bladder, uterus with cervix, and vagina. Tissues were preserved in 10% buffered neutral formalin, trimmed, processed routinely, and embedded in paraffin. Paraffin blocks were microtomed and sections stained with hematoxylin and eosin (H&E). The trimming procedure for hearts consisted of longitudinally bisecting these along a plane perpendicular to the plane of the pulmonary artery to expose the right atrioventricular, left atrioventricular, and aortic valves. Each hemisection was embedded in paraffin with the cut surface down. In addition to H&E stained sections, some sections of heart immunostained for alpha-smooth muscle actin (α-SMA) using standard immunohistochemical methods. The tissue sections were examined by light microscopy by board-certified members of the American College of Veterinary Pathologists (ACVP). All studies were peer reviewed by ACVP-certified veterinary pathologists [18].

Results

LY2157299 was dosed to Fischer 344 rats and Beagle dogs for up to 6 months (Table 1). Daily oral administration was the dose schedule in the rat and the dog except for a study in the rat in which an intermittent dose schedule of 2 weeks on/2 weeks off was investigated. LY2157299 was well tolerated in the rat as a single dose up to 2000 mg/kg (data not shown). In repeat dose studies in the rat, LY2157299 caused mortality at progressively lower doses as the duration of treatment increased. Mortality was observed in rats administered 1200 mg/kg of LY2157299 in a 14-day study. In the 3- and 6-month studies, mortality occurred in animals receiving daily doses of 150 mg/kg (6-month study) and 250 mg/kg (3- and 6-month study) beginning on days 83 and 54, respectively. At a dose of 1200 mg/kg in rats, a gross change of sternal deviation consistent with acquired pectusexcavatum occurred, rarely with concurrent rib deformities, as early as nine days after initiation of dosing.In the longer term (>3 months) rat studies, most of the deaths that occurred prior to the schedule termination were attributed to compound-related inflammation and rupture of the aorta and/or distributing arteries at or near the base of the heart resulting in hemothorax and/or hemoabdomen.

LY2157299 was evaluated in beagle dogs up to 500 mg/kg. All animals tolerated LY2157299 for the duration of the study with the exception of a single animal administered 500 mg/kg which was euthanized shortly following the end of the treatment period in a 1-month study due to deteriorating condition. This animal was found to have severe necrotizing cholecystitis.

LY2157299 is well absorbed following oral dosing and exposures appear to be dose linear in the rat and dog. In rats, exposures in females are higher than in males and are consistent with the toxicity data in which female rats are more affected than males. No exposure differences are observed between sexes in the beagle dog.

Cardiovascular toxicology

In both rats and dogs, the heart and great vessels were identified as the major target organs for toxicity. Cardiovascular findings in F344 rats treated with LY2157299 included degenerative and inflammatory valvular lesions (valvulopathy), myocardial degeneration and necrosis, aortitis with rupture, vasculitis/perivasculitis, and increased heart weights. The cardiovascular lesions were dose- and duration-dependent in rats and dogs, respectively (Figures 1 and 2). These cardiovascular lesions were not accompanied by chronic pulmonary or hepatic congestion that would have been suggestive of congestive heart failure, even after 6 months of daily dosing.

Valvulopathy

The earliest microscopic changes included heart valve lesions and myocardial degeneration at the insertion point of the valves which occurred in less than 2 weeks in the rat at a 1200-mg/kg dose and was also observed in the 3-month and 6-month daily dosing studies at ≥50 mg/kg. No cardiac lesions were observed following 1 month of daily dosing study in the rat at doses of 15 mg/kg, 50 mg/kg, and 150 mg/kg. In the dog, valve lesions were observed following one month of daily dosing at ≥50 mg/kg or at doses as low as 8 mg/kg following 6 months of daily dosing.

In both species, the valvulopathy was characterized by valve thickening, hemorrhage, hemosiderin-laden macrophages, inflammation, stromal and endothelial hyperplasia and increased myxomatous matrix (Figure 3). The inflammation was typically a mixture of lymphocytes and macrophages, but occasionally included neutrophils. The stromal hyperplasia and valve thickening were similar to spontaneous mitral valvulopathy [19], but occurred at a high incidence in the rat and were usually associated with other lesions such as hemorrhage or inflammation. Both atrioventricular and semilunar valves had lesions, and often multiple valves were affected in a given animal. Increased smooth muscle actin (SMA) immunolabeling of the valve consistently occurred when light microscopic lesions were present and indicated valvular interstitial cell activation with transdifferentiation into myofibroblast-like cells. However, because increases in SMA expression were always concurrent with morphologic changes, this finding was not useful as a premonitory marker for the morphologic changes (data not shown).

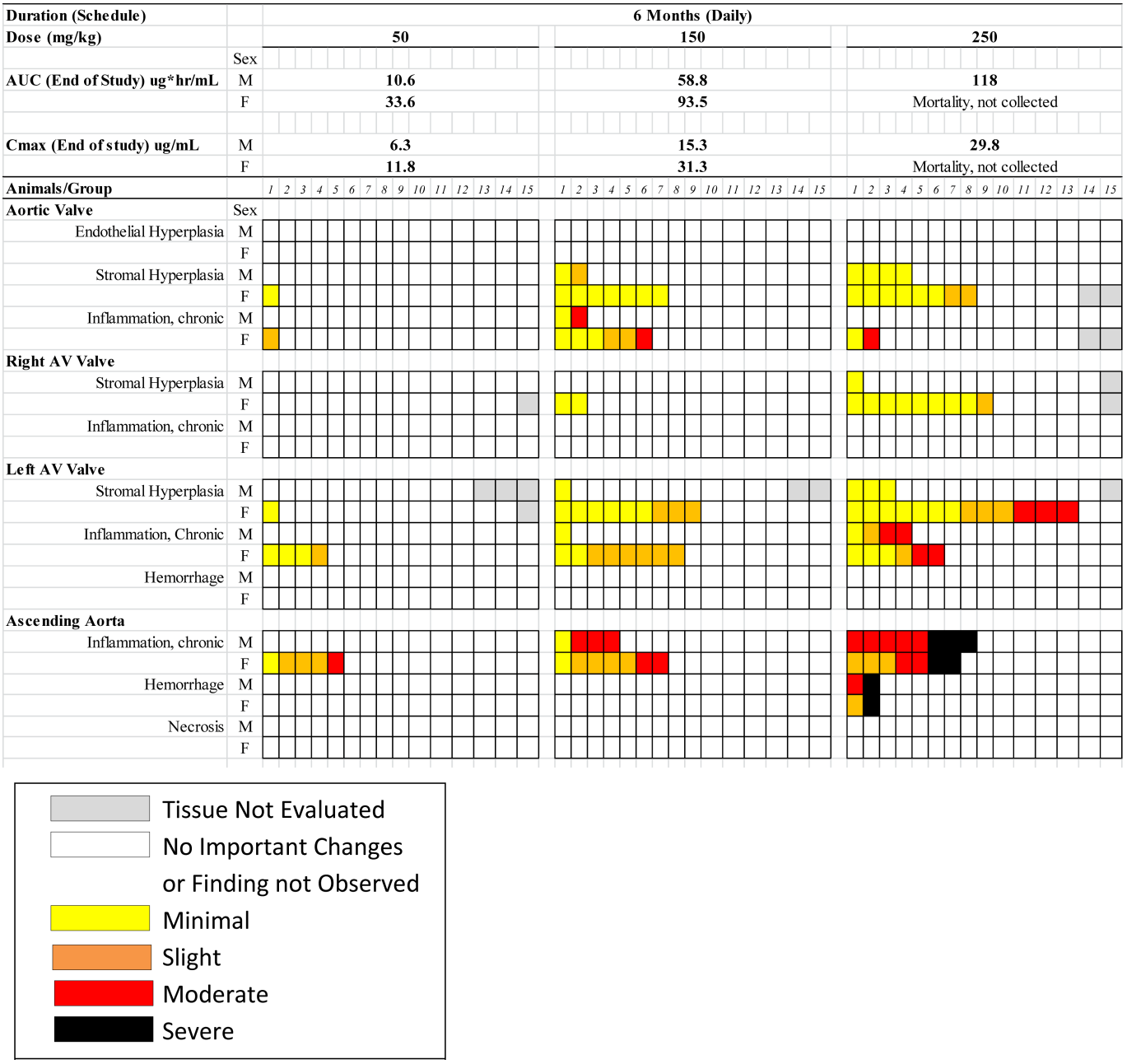

Figure 1: Incidence and severity of cardiac lesions in rats following daily administration of LY2157299 for six months. Exposure was determined at the end of the study in a group of satellite animals receiving compound for six months.

The incidence, severity, and chronicity of valvulopathy were similar in the 3- and 6-month daily dosing studies in rats at all dose levels. In the dog at the end of the 6-month dosing period, most animals in the 20 mg/kg and 60 mg/kg dose groups had at least one valve affected, whereas the 8-mg/kg dose group had fewer affected animals. The valvular lesions were still present in the rat and dog following a treatment-free recovery period. A partial recovery in the rat was observed when rats were dosed with 250 mg/kg LY2157299 daily for 2 months, a dose known to produce virtually 100% incidence of valvular lesions. Rats allowed a 6-week recovery period still had lesions present; however, there was a decrease in severity and incidence, indicating partial reversibility. A chronic end stage lesion characterized by scarring and valvular insufficiency was not recognized microscopically, although valvular function was not assessed in vivo. This could suggest that the onset of the valvular injury occurred at different time points throughout the dosing period.

Vascular lesions

Vascular lesions occurred when dogs and rats were administered LY2157299 at doses of ≥8 and ≥50 mg/kg in the dog and rat,

respectively, for prolonged time periods (≥3 months). In the rat, the earliest lesions were typically periarteritis and intramural hemorrhage that progressed to transmural inflammation, necrotizing vasculitis, and vascular rupture (Figure 4). Vascular lesions were most prominent in the thoracic cavity and involved the aortic arch, coronary arteries, and other distributing arteries originating from the aorta in the thoracic and cranial abdominal cavities. Lesions were often pronounced at the base of the aorta and where distributing arteries branched from the proximal aorta, a site of high pressure and increased turbulence. In a subset of rats, severe degenerative and inflammatory changes were associated with vascular rupture resulting in fatal hemothorax or hemoabdomen. The intramural hemorrhage was compatible with aortic dissection, although vascular dilation and aneurysms were not apparent. There were no lesions in the standard section of thoracic descending aorta.

Changes in the dog aorta did not occur in the 1-month studies but were noted in the 6-month study. While the valvular lesions in the dog were similar to those described in the rat, the aortic vascular change differed. The changes in the base of the ascending aorta of dogs were characterized by focal to multifocal degeneration and disorganization of the mural elastic lamellae, increased prominence of mucopolysaccharide-rich ground substance, but without accompanying inflammation or intramural hemorrhage (Figure 5). In the most affected animals, irregular separation between the elastic layers occurred. There were no findings in the standard section of thoracic descending aorta. Although the microscopic changes likely compromised aortic structural integrity, there were no changes diagnostic of aneurysm.

Duration (Schedule)		**1 Month (Daily)**			**1 Month (Daily)**						**6 Months (Daily)**											
Dose (mg/kg)		**2**			**8**			**20**			**50**			**250**			**500**			**8**				**20**				**60**			
	Sex																														
AUC (End of Study) ug*hr/mL	M	2.7			10.2			23.3			23.4			55.4			160			10.6				44.9				155			
	F	1.9			9.5			24.5			12.2			70.1			307			11.7				36.1				148			
Cmax (End of study) ug/mL	M	1.2			4.7			8.2			5.3			10.4			21.8			3.84				12.8				37.8			
	F	1.2			3.6			10.9			3.9			21.2			49.6			4.5				14.3				37.1			
Animals/Group		*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*4*	*1*	*2*	*3*	*4*	*1*	*2*	*3*	*4*
Valve, not specified																															
Stromal Hyperplasia	M																														
	F																														
Inflammation	M																														
	F																														
Hemorrhage	M																														
	F																*														
Aortic Valve																															
Hemorrhage	M																														
	F																														
Left AV Valve																															
Inflammation	M																														
	F																														
Hemorrhage	M																														
	F																														
Ascending Aorta																															
Connective Tissue Degeneration	M																														
	F																														

* n=4

Not Evaluated

No Important Changes or Finding not Observed

Minimal

Slight

Moderate

Severe

Figure 2: Incidence and severity of cardiac lesions in dogs following daily administration of LY2157299 for one and six months. Exposure was determined at the end of the study.

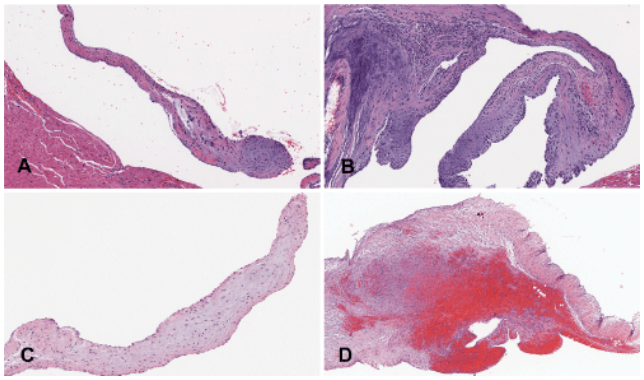

Figure 3: Hematoxylin and eosin stain of atrioventricular heart valves (A) Control rat demonstrating some mild spontaneous myxomatous change at the distal end of the valve. (B) Rat dosed for 3 months with LY2157299. A chronic valvulopathy characterized by expansion of the valve leaflet with increased myxomatous matrix, inflammatory cells, and hemorrhage. (C) Control dog. (D) Valve from a dog dosed for 1 month with LY2157299. An acute valvulopathy characterized by marked expansion due to hemorrhage, edema, and inflammatory cells.

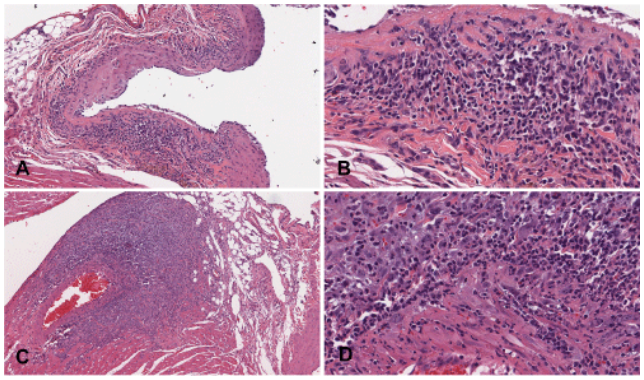

Figure 4: (A and B) Low- and high-magnification images (hematoxylin and eosin stain) of the ascending aorta including the origin of the coronary artery from a rat dosed for 14 weeks with LY2157299. The origin of the coronary artery has disruption of the tunica media with transmural infiltration by mixed inflammatory cells. (C and D) Low- and high-magnification images from a rat dosed for 6 months with LY2157299. The coronary artery has a pronounced perivascular infiltration of mixed inflammatory cells with only limited involvement of the tunica media.

Additional cardiac findings

Myocardial degeneration and necrosis occurred in rats that had the most pronounced valvular and/or aortic changes, and were interpreted as secondary to the valvular and coronary artery changes rather than a primary test article effect.

Multifocal myocardial degeneration and necrosis typically involved the base of the heart, at the base of the valves, and the ventricles, and was generally minimal in severity. Microscopic cardiovascular changes were rarely associated with increases in cardiac troponin I concentrations, most likely due to timing of the serum collection and the relatively mild myocardial degenerative changes.

LY2157299 induces slight-to-moderate decreases in blood pressure and increases in heart rate in dogs. These changes were not considered adverse. No biologically relevant change in QT/QTcf, test-article related arrhythmia, or altered waveforms were identified.

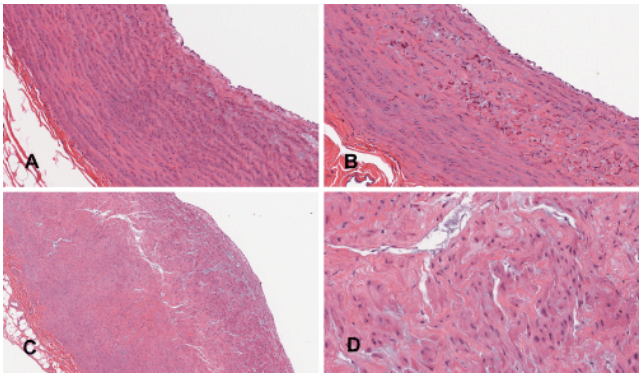

Figure 5: Ascending aorta from dogs dosed for 6 months with LY2157299 (hematoxylin and eosin). (A) Control dog. (B) Focally extensive disruption of the internal elastic laminae within the tunica media. (C and D) Marked disruption of the tunica media resulting in disorganization with loss of the laminar arrangement, increased prominence of mucopolysaccharide-rich ground substance, and cleft formation.

Characterizing a Non Observed Effect Level for Cardiac Lesions

In order to support the clinical development of LY2157299 with the cardiovascular changes described above, an additional nonclinical toxicology study was conducted in rats to determine if a no observed effect level (NOEL) for cardiovascular changes could be identified using an alternative dosing schedule consisting of 2 weeks on treatment followed by 2 weeks off treatment.

The duration of this study was 3 months and compared daily dosing to the alternative dosing schedule. This duration supports the clinical time interval relevant to patients who are enrolled and then reassessed for disease status 2 months after receiving study drug. Based on the previous 6-month study in rats that identified when early mortality occurred due to cardiac events, a 3-month treatment duration was considered sufficient to induce a high incidence of cardiac lesions with a daily dosing schedule. The doses used for this study were 50 mg/kg, 150 mg/kg, and 250 mg/kg. As expected, mortality due to vascular rupture was observed in rats administered 250 mg/kg daily. In contrast, the rats on the 2-weeks-on/2-weeks-off dosing schedule at all doses survived until the scheduled termination of the study.

Rats administered 150 mg/kg and 250 mg/kg on a 2-weeks-on/2-weeks-off dosing schedule had cardiovascular lesions similar to those in the daily dosing groups, however, the incidence and often severity were lower in rats administered LY2157299 on the intermittent dosing schedule (Figure 6).

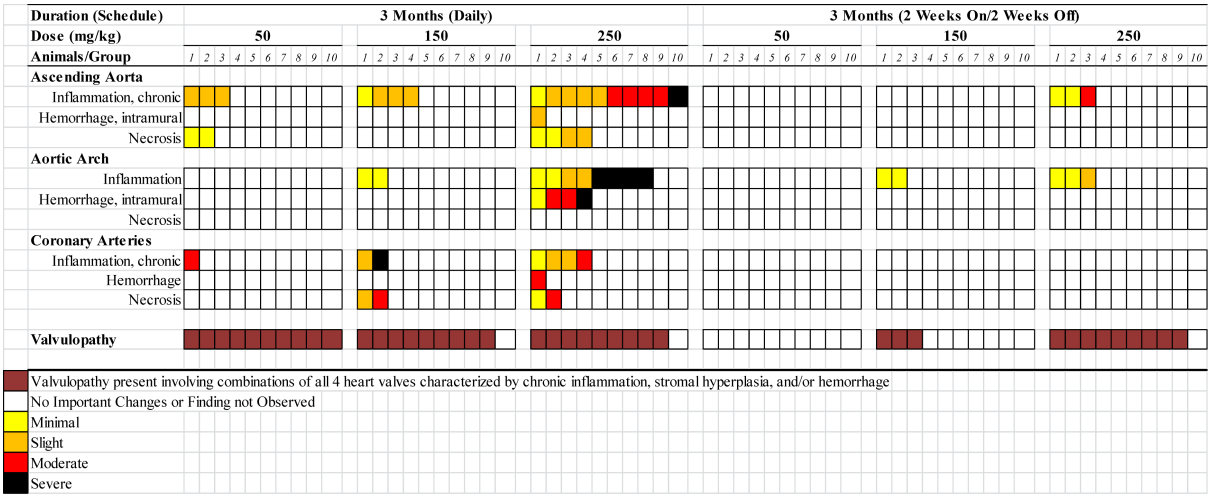

Figure 6: Comparison of incidence and severity of the cardiac lesions in female Fischer 344 rats given LY2157299 in two different dosing schedules for three months.

Immune system

Numerous changes occurred in multiple organs of the rat and dog that were consistent with altered immune responses, both proinflammatory and also suggestive of immune dysfunction. These patterns occurred most often in organs such as the gastrointestinal tract and skin that are exposed to environmental antigenic stimulation and host commensal bacteria.

Increased mixed inflammatory cell infiltrates within the lamina propria of the large intestines, and less commonly the small intestines and stomach, occurred in studies of at least 1 month in duration. Additionally, increased inflammatory cell infiltrates occurred in the kidneys, lung, gallbladder, and/or prostate gland depending on the species. Rats in the 6-month toxicity study also had multiple subcutaneous abscesses, and acute inflammation involving the preputial and clitoral glands, suggesting immune dysfunction and/or alterations in the innate immune responses.

Gastrointestinal system

Gastrointestinal changes frequently involved the large intestine, but at higher doses also included the small intestines, stomach, and gallbladder. Findings in the large intestine were most apparent in the 6-month studies and included mucosal inflammation in dogs and rats, and mucosal epithelial hyperplasia and neoplasia in rats. In the cecum of the rat, a continuum was identified with numerous rats showing increased mononuclear inflammatory cell infiltrates within the lamina propria of the mucosa, and fewer animals displaying enterocyte hyperplasia, and adenoma formation. In the colon, fewer rats had increased mucosal inflammation, possibly because of higher background infiltrates; however, some rats had epithelial hyperplasia and adenocarcinoma (Figure 7). Some of the adenocarcinomas were quite large and were the cause of adverse clinical signs, early euthanasia and/or death. Similar inflammatory and hyperplastic changes also involved the rectum.

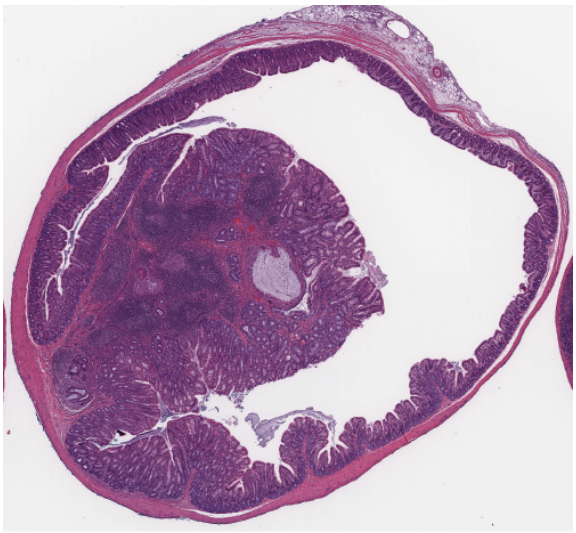

Figure 7: Adenocarcinoma of the colon from a rat that was euthanized after 23 weeks of daily dosing with LY2157299 (hematoxylin and eosin stain).

Dogs in the 1-month toxicity study at high doses (≥50 mg/kg) had acute intestinal changes with more pronounced lesions in the small intestine than the large intestine. These microscopic findings included inflammation and hemorrhage, and increased numbers of mucosal cysts. These dogs also had inflammation of the gallbladder that in one dog included necrotizing cholecystitis with rupture that resulted in bile peritonitis.At the highest dose, some dogs had hepatic inflammation and hepatocellular degeneration, changes that may have been secondary to the intestinal changes.Intestinal lesions in dogs in the 6-month toxicity study were uncommon and limited to mild inflammatory cell infiltrates within the large intestine that ranged from acute to chronic, and sometimes included increased numbers of

eosinophils and/or plasma cells. These dogs also had chronic inflammation of the gastric mucosa with glandular atrophy and vasculitis that was most prominent in the cardia.Increased amounts of mucus within the gallbladder occurred in some dogs.

Skeletal system

Osseous changes included physeal and subphyseal changes in the femur, tibia, and sternum of rats that occurred after just 14 days of dosing and were similar to those previously described with TGF-β inhibition [12,13]. The zones of maturation and hypertrophy were expanded in the physes of the long bones and endplates of the sternabrae, and the primary and secondary spongiosa were denser with increased connectivity (Figure 8). These changes were orderly and appeared to result in relatively normal endochondral ossification. In animals that had a recovery phase, these osseous changes were characterized by a band of metaphyseal hyperostosis (increased trabecular density) that was separated from the physis by a zone of normal endochondral ossification, presumably the result of an earlier alteration in endochondral ossification that had reversed. Microscopic osseous changes were often more pronounced in rats that had the pectus excavatum defect. Similar physeal changes occurred in the sternebrae of the dog after 6 months of dosing. In addition to the physeal lesions, rats dosed for 6 months had additional changes of multifocal cartilaginous degeneration in the joints of the sternebrae and stifle.

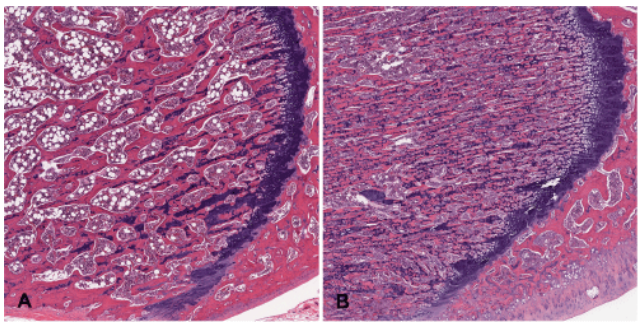

Figure 8: Proximal femur including the physis (hematoxylin and eosin stain). (A) Control rat. (B) Rat dosed for 6 months with LY2157299 had expansion of the physeal zones of maturation and hypertrophy, increased density and interconnectivity of the subphyseal primary and secondary spongiosa, and an orderly increase in the trabecular bone density.

Discussion

TGF-β plays a key role in several disease states, including cancer, cardiovascular disease, and fibrosis, which has made the TGF-β signaling pathway a logical and attractive target of pharmaceutical development. However, TGF-β signaling has posed a challenge with respect to understanding safe therapeutic doses that do not confer toxicities associated with prolonged inhibition of this critical pathway [16]. We characterized the toxicity profile of LY2157299 in nonclinical species for up to 6 months of daily dosing. LY2157299 was well tolerated in the rat and the dog for one month of daily dosing and in the rats for 3 months of intermittent dosing. Long-term daily dosing of ≥3 months caused alterations in the gastrointestinal, immune, musculoskeletal, renal and cardiovascular systems of animals, which is anticipated based on the integral role for TGF-β signaling for maintaining homeostasis.

Other pharmaceutical companies have published similar valvular toxicities with inhibitors from their development efforts, indicating that the lesions described are likely an on target effect [12,13]. Many explanations have been considered to understand mechanism of this phenomenon; no definitive explanation has been provided at this time [12]. TGF-β is a required mediator for valve structural and functional homeostasis [20]. In response to injury, valvularmyofibroblasts are activated by TGF-β and results in increases in α-SMA expression [21]. This paradoxical inhibition of TGF-β signaling with LY2157299 - treated animals resulting in increased SMA in their heart valves is not understood and awaits further clarification.

We have characterized cardiac effects in nonclinical species that extend beyond the previously reported valvulopathy and, in contrast to the valvulopathy, constituted a pathologic lesion defined as dose limiting within the context of a given study. In both species, the aorta and arteries of the heart are major target organs for toxicity. The vascular lesions in the rat are characterized by inflammation and hemorrhage with associated disruption of the mural organization, sometimes resulting in vessel rupture and hemorrhage into the thoracic cavity. In contrast to the rat, the dog showed changes in the base of the ascending aorta, characterized by degeneration and disorganization of the mural elastic lamellae and often increased prominence of mucopolysaccharide-rich ground substance without accompanying inflammation. The reason for this apparent species difference is unknown.

Our findings support that TGF-β plays a pleiotropic role in cell growth and on the extracellular matrix. It is important in both production of matrix proteins and the degradation of the matrix [22]. Two well-studied genetic diseases Marfan syndrome (MFS) and Loeys-Dietz syndrome (LDS) affect the skeletal, ocular, and cardiovascular systems [23]. Clinically, these syndromes present with overlapping symptoms including aortic aneurysms, dissections and mitral valve prolapse [24]. MFS is caused by mutations in the gene encoding fibrillin-1 that interacts with latent TGF-β binding protein to upregulate TGF-β bioavailability and activity [25]. LDS is an autosomal dominant aortic aneurysm syndrome caused by mutations in the promoter of TGF-β R1 and R2 with increased TGF-β activity [23,26]. In LDS, the microscopic aortic lesion is characterized by fragmentation of elastic fibers, loss of elastin content, and accumulation of amorphous matrix components in the aortic media, changes very similar to those observed in the 6-month dog study with LY2157299 [23]. In cardiac tissue from affected patients and mouse models of MFS, TGF-β signaling is increased, potentially due toaltered receptor processing or alternative pathways responsible for upregulation of TGF-β [27]. Mutations in the TGF-β signaling pathway that should lead to decreased TGF-β signaling resulting in an upregulation of this pathway underscores that in vivo, precise regulation of multiple members is required to maintain homeostasis and that canonical and non-canonical signaling pathways play an important role [28,29].

The findings described in the aorta and cardiac arteries in the toxicology studies in the rat and the dog, based on similarity with genetic syndromes, appear to be more consistent with an upregulation rather than inhibition of the TGF-β pathway. The contributions of the non-canonical TGF-β signaling are not yet understood. Activation of the ERK pathway contributes to progression of aneurysms in *Fbn1*mice. RDEA119, a selective MEK inhibitor, reduced aortic root

growth independent of increased *Smad2* activation, suggesting that non-canonical pathway modulation may contribute to progression to aneursyms in *Fbn1*mice [28]. As with MFS and LDS, which are genetic mutations resulting in a clinical phenotype, additional genetic differences may also play a role in susceptibility to cardiac injury observed in nonclinical studies with inhibitors of TGF-β signaling. Behmoaraset al. evaluated seven genetically different strains in rats for their differences in composition of the extracellular matrix and noted a differentiation among strains with respect to matrix composition and occurrence of internal elastic lamina rupture [30].

The changes to the heart following long-term treatment of LY2157299 at toxicologically relevant doses are of largest concern with respect to clinical safety, inhibition of TGF-β signaling resulted in changes to several other organ systems in both the rat and dog. LY2157299 resulted in changes to the bone similar to previous reports [12,13]. Reversibility of bone changes was assessed following the 1-month study in rats revealing a band of increased trabecular density that was separated from the normal physis by a zone of normal endochondral ossification. Following 6 months of treatment in the rat, degeneration in the joints of the sternebrum and stifle was reported. Dominant negative mutant TGF-β receptor II mice are reported to have bifurcated xiphoid process and sternum as well as a progressive osteoarthritis-like disease [31]. Dogs used in the 6-month study were between 6-8 months of age and are expected to still have active longitudinal growth in their bones; therefore, alterations in the endochondral cartilage similar to what was described in the rat may be anticipated [32].

TGF-β also plays a critical role in epithelial biology, acting as a tumor suppressor or promoter [33]. Alteration in TGF-β signaling is common in many cancers [34]. Disruption of TGF-β signaling in either epithelial or stromal cells increases inflammatory responses that promote tumor initiation, progression, and metastasis [35]. *TGFβ1-/-Rag2 -/-* mice develop inflammation-associated adenomas and carcinomas through an inability to maintain epithelial tissue organization [36]. Mice genetically deficient in TGF-β R2 signaling have increased susceptibility to cancer [37]. Similarly, inactivation of downstream proteins *Smad3*and *Smad4* results in carcinomas of the intestine [38,10]. The observations of hyperplasia, adenoma, and carcinoma of the intestine in rats treated with LY2157299 for 6 months are consistent with the well-characterized biology.

The use of intermittent dosing is common practice in oncology: clinicians will reduce the dose or permit a drug holiday to allow the patient to recover from toxicities prior to receiving another dose. Studies have shown that anticancer agents were more tolerable and efficacious when administered in intermittent dosing schedules [39,40]. In contrast to a dosing schedule based on clinical findings, clinical dosing with LY2157299 proactively applied an indirect model to support a clinical dosing regimen that would support a desired pharmacologic effect and avoid unwanted toxicity. Buenoet al. have been able to relate plasma concentrations to pSmad inhibition [41]. Tumor growth inhibition was then linked to pSmad inhibition, providing a tool to titrate the dose required for efficacy. Furthermore, the data support the concept of intermittent dosing in maintaining a similar tumor response. The ability to maintain plasma concentrations required for efficacy lower than those observed to cause adverse toxicities in nonclinical species is important, especially when considering drugs targeting signaling pathways such as TGF-β that have been demonstrated to be critical in maintaining normal function. We demonstrate continuous administration of LY2157299 at high doses or durations ≥3 months in nonclinical species results in adverse toxicities to numerous organ systems including cardiovascular, gastrointestinal, immune, bone/cartilage, reproductive, and renal. The cardiovascular system appears to be most sensitive to abrogation of TGF-β signaling with the small molecule inhibitor LY2157299. Our data have defined a NOEL for cardiovascular lesions of 150 mg/kg and 20 mg/kg, in rat and dog, respectively, after one month of daily dosing and 50 mg/kg for 3 months on a 2-week-on/2-week-off dosing schedule in the rat. An intermittent dosing paradigm demonstrated that the severity and incidence of cardiac lesions could be lessened. The data from the 3-month intermittent dosing study in rats along with the absence of cardiovascular lesions in the 1-month rat and dog study established a nonclinical data package that supports a clinical dosing schedule of 2 weeks on/2 weeks off LY2157299. Successful development of such inhibitors will need to have a thorough characterization of nonclinical toxicity and an understanding of schedule and plasma concentrations needed to demonstrate anti-tumor effect and determine if a sufficient therapeutic window exists to test the clinical efficacy of molecules involved in inhibiting key pathways.

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