

Clinical development of galunisertib (LY2157299 monohydrate), a small molecule inhibitor of transforming growth factor-beta signaling pathway

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Abstract: Transforming growth factor-beta (TGF- β) signaling regulates a wide range of biological processes. TGF- β plays an important role in tumorigenesis and contributes to the hallmarks of cancer, including tumor proliferation, invasion and metastasis, inflammation, angiogenesis, and escape of immune surveillance. There are several pharmacological approaches to block TGF- β signaling, such as monoclonal antibodies, vaccines, antisense oligonucleotides, and small molecule inhibitors. Galunisertib (LY2157299 monohydrate) is an oral small molecule inhibitor of the TGF- β receptor I kinase that specifically downregulates the phosphorylation of SMAD2, abrogating activation of the canonical pathway. Furthermore, galunisertib has antitumor activity in tumor-bearing animal models such as breast, colon, lung cancers, and hepatocellular carcinoma. Continuous long-term exposure to galunisertib caused cardiac toxicities in animals requiring adoption of a pharmacokinetic/pharmacodynamic-based dosing strategy to allow further development. The use of such a pharmacokinetic/pharmacodynamic model defined a therapeutic window with an appropriate safety profile that enabled the clinical investigation of galunisertib. These efforts resulted in an intermittent dosing regimen (14 days on/14 days off, on a 28-day cycle) of galunisertib for all ongoing trials. Galunisertib is being investigated either as monotherapy or in combination with standard antitumor regimens (including nivolumab) in patients with cancer with high unmet medical needs such as glioblastoma, pancreatic cancer, and hepatocellular carcinoma. The present review summarizes the past and current experiences with different pharmacological treatments that enabled galunisertib to be investigated in patients.

Keywords: TGF- β , TGF- β RI kinase inhibitor, ALK5, galunisertib, LY2157299, cancer, clinical trials

Introduction

Since the discovery of the cytokine transforming growth factor-beta 1 (TGF- β 1),¹ a diverse family of ligands, corresponding receptors, and signal transduction proteins known as the TGF- β superfamily were identified (Table 1).² Although the members of the TGF- β superfamily share genetic and protein structures, some of these members have different physiological functions and play distinct roles in diseases, including cancer (Figure 1). For example, the activin receptor-like kinase 1 (ALK1) receptor and the ALK5 receptor, also known as TGF- β receptor type I (TGF- β RI), can both promote angiogenesis but have different intracellular activation pathways with different effects on angiogenesis (for details, see the section TGF- β /ALK5 signaling pathway and its role in cancer). Here, we will focus on the role of the TGF- β /ALK5-mediated pathway and describe the experiences of developing galunisertib.



Table 1 TGF- β receptors, their respective ligands, and canonical intracellular signaling proteins currently targeted by clinical investigation

TGF- β receptor	TGF- β receptor ligands	Canonical intracellular signaling activated by TGF- β receptor
TGF-β type I receptor (TGF-β superfamily receptors: seven type I receptors [ALK1–ALK7])		
Activin receptor-like kinase I (ACVRL1, ALK1)	BMP9 BMP10 TGF- β 1 TGF- β 2 TGF- β 3	SMAD1 SMAD5 SMAD8
Alternative names: Serine/threonine-protein kinase receptor R3 (SKR3) TGF- β superfamily receptor type I (TSR-I) Activin receptor-like kinase I (ALK1)		
TGF-β receptor type-I (TGF-βRI)	TGF- β 1 TGF- β 2 TGF- β 3 GDF11 Myostatin (GDF8)	SMAD2 SMAD3
Alternative names: Activin A receptor type II-like protein kinase of 53 kD Activin receptor-like kinase 5 (ALK-5, ALK5) Serine/threonine-protein kinase receptor R4 (SKR4)		
TGF-β type II receptor (TGF-β superfamily receptors: four type II receptors [TGF-βRII, BMPRII, ActRIIA, and ActRIIB])		
TGF-β receptor type II (TGF-βRII)	TGF- β 1 TGF- β 2 TGF- β 3 GDF15	SMAD2 SMAD3

Notes: The TGF- β superfamily includes the following ligands: TGF- β 1, TGF- β 2, and TGF- β 3; BMPs (BMP1–10); GDFs (GDF1–10); activins/inhibins (activin 1–5); nodal; leftys (lefty 1 and lefty2); and anti-muellerian hormone.

Abbreviations: TGF- β , transforming growth factor-beta; BMP, bone morphogenetic protein; GDF, growth and differentiation factor.

TGF- β /ALK5 signaling pathway and its role in cancer

In the early 1980s, TGF- β was biochemically isolated from tumor cells and was named after its ability to transform normal rat kidney fibroblasts.¹ This approximately 13 kDa polypeptide was later designated as TGF- β 1.³ TGF- β ligands include TGF- β 1, TGF- β 2, and TGF- β 3 (Table 1), all of which regulate diverse biological functions.^{4,5} All three ligands can

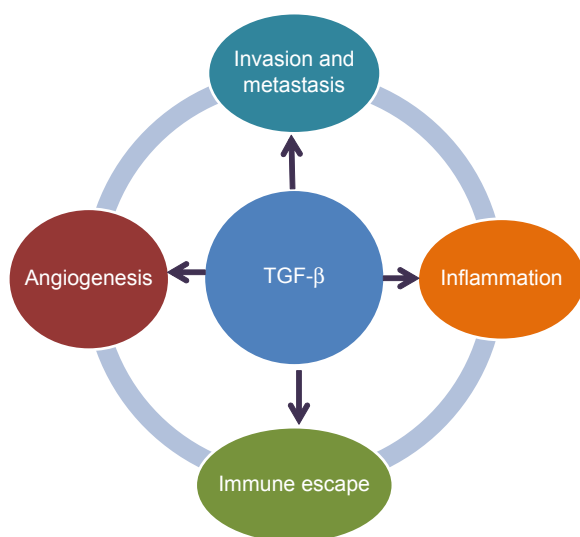


Figure 1 TGF- β signaling and hallmarks of cancer.

Note: TGF- β signaling plays an important role in inducing angiogenesis, inflammation, invasion/metastasis, and immune escape.

Abbreviation: TGF- β , transforming growth factor-beta.

independently engage the specific receptor TGF- β RI/ALK5, which then undergoes dimerization with TGF- β receptor type II (TGF- β RII).⁶ Recent studies have shown that two TGF- β ligand molecules bind as homodimers to two TGF- β RI and two TGF- β RII molecules (ie, a homodimer bound to a heterotetramer), creating a heterodimer complex consisting of six distinct molecules.⁷ For simplicity reasons, figures often do not show the duplicity of the heterodimer (Figure 2). This heterodimer complex phosphorylates the intracellular proteins SMAD2 and SMAD3, activating a signaling cascade to induce several nuclear transduction proteins. The induction of these proteins leads to cellular proliferation, differentiation, motility, survival, and apoptosis in tumor cells.⁸ This TGF- β /ALK5 signaling pathway is commonly referred to as the canonical signaling pathway (Figure 1). In addition to this canonical pathway, a number of accessory receptors have been identified, which can separately interact with the TGF-RI and TGF-RII. One example is the TGF- β RIII, to which the glycoprotein endoglin (CD105) can bind with high affinity.⁹

Given the complexity of the TGF- β signaling pathway, its physiological role is diverse and appears to be dependent on the disease setting and cellular context. In cancer, TGF- β can affect tumor growth directly (referred to as intrinsic effect of TGF- β signaling) or indirectly (referred to as extrinsic effect) by promoting tumor growth, inducing epithelial-mesenchymal transition (EMT), blocking antitumor immune responses, increasing tumor-associated fibrosis and

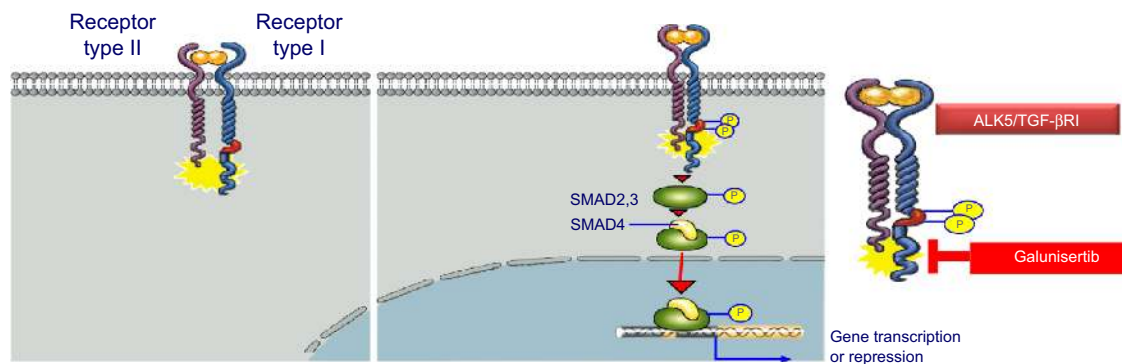


Figure 2 TGF- β RI (ALK5) and TGF- β RII canonical activation.

Note: Galunisertib blocks the kinase of the ALK5 pathway reducing the pSMAD2 activation in cells.

Abbreviations: ALK5, activin receptor-like kinase 5; pSMAD2, phosphorylation of SMAD2; TGF- β , transforming growth factor-beta.

enhancing angiogenesis (Figure 1).¹⁰ Although TGF- β 1 was originally found to transform normal cells,¹ subsequent studies revealed that it also behaved as a tumor suppressor in epithelial cells. The factors determining whether TGF- β signaling has a tumor promoter or suppressor function are a matter of intense research.⁸ Currently, it is postulated that the tumor suppressor function of TGF- β signaling is lost in early stages of cancer similar to recessive loss-of-function mutations in other tumor suppressors.⁵ Such a loss of function has been associated with drug resistance in tumors and reported in colon and lung cancer cell lines.^{11–14} The switch to becoming a tumor promoter with resulting drug resistance often occurs in the presence of EMT.¹⁵ TGF- β signaling plays a prominent role in EMT by influencing key transcription factors, including Snail, zinc-finger E-box-binding, and basic helix–loop–helix transcription factors. Besides these genetic and physiological changes, TGF- β signaling plays a critical role in the regulation of immune cell function in normal and tumor-associated lymphocytes, in particular, the activation of T-regulatory cells.^{16,17}

In addition to its role in regulating immune cells, TGF- β signaling is a strong inducer of fibrosis.¹⁸ It is an activator of cancer-associated fibroblasts (CAFs)¹⁹ that can be a major source of collagen type I and other fibrogenic factors. CAFs and their associated production of fibrogenic factors may further foster a microenvironment that decreases immune responses and increases drug resistance and tumor angiogenesis.²⁰

The role of TGF- β signaling in angiogenesis is well recognized during ontogeny and in tumor growth.²¹ For example, murine embryos deficient in TGF- β RI exhibit severe defects in vascular development; TGF- β signaling acts as a key regulator of development of both vascular endothelial and smooth muscle cells.²¹

In summary, TGF- β signaling plays a key role in pathways that are considered hallmarks of cancer²² by aiding cancer

cells in their evasion of immunosurveillance, increasing inflammation (in part by modulating cytokine responses, tumor migration, and angiogenesis) (Figure 1).^{5,23,24} Thus, targeting this TGF- β -mediated signal transduction in tumors may provide a novel approach to controlling tumor growth by blocking a central activation node with its connected downstream signaling pathways.²⁵

Pharmacological approaches: blocking the TGF- β signaling pathway

Specific inhibitors are key for the pharmacological development of ALK5 inhibitors:

Given the structural and genetic similarities of the different TGF- β signaling pathways, inhibitors must be highly specific to block the intended activation pathway. For example, ALK5 and ALK1 pathways both increase tumor angiogenesis (Table 1).²⁶ ALK1 inhibitors block the interaction of bone morphogenetic protein (BMP)-9 and -10 with ALK1, which interrupts the subsequent phosphorylation of SMAD1 (pSMAD1)/pSMAD5/pSMAD8 (Table 1). PF-03446962 is an ALK1 neutralizing antibody that does not bind other ALKs. Currently, PF-03446962 is being evaluated in Phase II trials in patients with solid tumors to determine its ability to block angiogenesis (Table 2). Another molecule directed against ALK1 is dalantercept/ACE-041, a chimeric protein consisting of the ALK1 ligand binding extracellular domain and an Fc portion (ALK1-Fc). This ligand trap is a soluble receptor that can bind circulating ligands and prevents their engagement with cell surface receptors. Dalantercept efficiently blocks BMP-9 and BMP-10-induced SMAD1 phosphorylation and SMAD1-dependent transcription. Dalantercept is currently in Phase II trials as monotherapy and in combination with vascular endothelial growth factor

Table 2 TGF- β inhibitors in clinical development

Target/mode of action	Generic name	Clinical development phase	Indications in clinical trials	Company	References
TGF-β receptor serine/threonine kinase inhibitors					
TGF- β RI	Galunisertib	II/III	Pancreatic carcinoma, glioblastoma, hepatocellular carcinoma, myelodysplastic syndromes (MDS)	Eli Lilly and Company	NCT01746004 NCT01965808 NCT01582269 NCT01722825 NCT02008318 NCT01220271 NCT01246986 NCT01373164 NCT01682187 NCT02160106
ALK5	LY2157299				
TGF- β RI	TEW-7197	I	Melanoma, breast cancer, hepatocellular carcinoma, prostate cancer	MedPacto Inc. (South Korea)	
ALK5					
Antibodies and protein therapeutics against TGF-β and TGF-β receptors ALK1, ALK5, ALK6					
TGF- β RII	LY3022859	I	Advanced solid tumors	Eli Lilly and Company	NCT01646203
	IMC-TR1				
TGF- β -I	LY2382770	II	Diabetic kidney disease, diabetic nephropathy, diabetic glomerulosclerosis	Eli Lilly and Company	NCT01113801
Pan TGF- β	Fresolimumab (GC-1008)	II	Systemic sclerosis, metastatic breast cancer, focal segmental glomerulosclerosis, glioma, renal cell carcinoma, melanoma, malignant pleural mesothelioma, myelofibrosis	Genzyme, a Sanofi Company	NCT01284322 NCT01401062 NCT01665391 NCT01472731 NCT00356460 NCT00923169 NCT01112293
ALK-I	PF-03446962	II	Hepatocellular carcinoma, urothelial cancer, malignant pleural mesothelioma	Pfizer	NCT01911273 NCT01620970 NCT01486368 NCT01458392 NCT01727336 NCT01720173
BMP9/BMP10 ALK-I interaction (soluble form of ALK-I)	Dalantercept ACE-041	II	Squamous cell carcinoma of the head and neck, renal cell carcinoma, ovarian epithelial, fallopian tube, or primary peritoneal cavity cancer	Acceleron Pharma Inc.	http://www.acceleronpharma.com/products/dalantercept/ NCT01736683 NCT01571635 NCT01146574 NCT01999582 NCT01464164 NCT01712308 NCT01562405
Soluble fusion protein of receptor type IIA (ActRIIA) linked to the Fc protein of human IgG1	Sotatercept ACE-011	II	Anemia in rare blood diseases, including β -thalassemia, MDS, and diamond blackfan; chronic kidney disease; multiple myeloma	Acceleron Pharma Inc. and Celgene Corporation	http://www.acceleronpharma.com/products/sotatercept/

Modified type II activin receptor fusion protein that acts as a ligand trap for members in the TGF- β superfamily	Luspatercept ACE-536	II	β -thalassemia; anemia in patients with MDS	Acceleron Pharma Inc. and Celgene Corporation	NCT01749540 NCT01749514 http://www.acceleronpharma.com/products/luspatercept/
TGF- β -I blocking peptide	P144	II	Skin fibrosis in systemic sclerosis	Digna Biotech S.L.	NCT00781053 NCT00574613 NCT00656825
Direct and indirect inhibitors of TGF-β synthesis and secretion					
TGF- β mRNA antisense	Trabedersen (AP-12009), intravenous (convection enhanced delivery formulation terminated)	II	Pancreatic neoplasms, melanoma, colorectal neoplasms	Isarna Therapeutics GmbH	NCT00844064 NCT00431561 NCT00761280 http://www.isarna-therapeutics.com/fileadmin/user_upload/documents/press-releases/2013/20130226_EN_Press_Release_CorporateStrategy_2013.pdf
TGF- β synthesis	Belagenpumatucef-L Cancer vaccine (TGF- β antisense) Lucanix	III	Nonsmall-cell lung cancer	NovaRx Corporation	NCT00676507
TGF- β synthesis	Pirfenidone Etury F-647 Esbriet® Pirespa®	III	Idiopathic pulmonary fibrosis, pulmonary fibrosis, focal glomerulosclerosis, diabetic nephropathy, neurofibromatosis type I and progressive plexiform neurofibromas, systemic sclerosis-related interstitial lung disease, hypertrophic cardiomyopathy, radiation-induced fibrosis, uterine leiomyoma (fibroids)	InterMune Inc.	NCT00063583 NCT00076102 NCT00287716 NCT01933334 NCT01366209 NCT00011076 NCT00754780 NCT00020631 NCT00053937 NCT02009293 NCT00332033
"bi-shRNA(furin)/GMCSF DNA/autologous tumor cell" vaccine (FANG)	FANG™ vaccine	II	Melanoma, Ewing's sarcoma, ovarian cancer, colorectal carcinoma	Gradalis Inc.	NCT01453361 NCT01061840 NCT01505166 NCT01309230 NCT01867086 NCT01551745 NCT01505153

Abbreviations: ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; TGF- β , transforming growth factor- β ; NCT, national clinical trial.

inhibitors.²⁷ In contrast to the ALK1 inhibitors, the inhibition of the ALK5 pathway blocks activation of different intracellular proteins (eg, SMAD2/3) and alters the vascular and smooth muscle cell compartment.²¹ This may improve delivery of chemotherapy²⁸ although it may have a reduced inhibitory effect on pro-angiogenic factors, such as vascular endothelial growth factor and basic fibroblast growth factor. In contrast to ALK1 inhibitors, ALK5 inhibitors increase angiogenesis in cell cultures of normal endothelial cells.²⁹

Monoclonal antibodies: Arguably, monoclonal antibodies are considered highly specific and can provide the best approach to develop selective inhibitors to the TGF- β signaling pathway. Fresolimumab (formerly GC1008) is one of the first pan-TGF- β ligand monoclonal antibodies to be investigated in cancer patients.³⁰ Fresolimumab is being developed for patients with renal fibrosis and until recently, for patients with metastatic cancer.^{30–33} In a Phase I trial, fresolimumab was given to 29 patients with malignant melanoma and renal cell carcinoma administered in doses up to 15 mg/kg given first every 28 days then subsequently by biweekly dosing to patients with stable disease. Reversible cutaneous keratoacanthomas/squamous cell carcinoma (four patients) and hyperkeratosis were reported as major drug-related adverse events. One patient with malignant melanoma achieved a partial response lasting 44.4 weeks, and six melanoma patients had stable disease. Of the seven patients with partial response or stable disease, six patients had received ≤ 3 mg/kg of fresolimumab. Given that patients with higher doses (up to 15 mg/kg) had no responses or stable disease, the authors postulated that clinical activity was associated with lower doses.³⁰ In a second Phase II study, fresolimumab was given to 13 patients with malignant pleural mesothelioma at 3 mg/kg given every 21 days, the anticipated most active dose. None of the patients had radiographic responses, and three patients had stable disease at 3 months. However, five patients developed new antibodies against tumor lysates isolated from malignant pleural mesothelioma, suggesting a possible immune response. After the 13 patients were treated, the manufacturer terminated further development of fresolimumab for oncology indications.³³ Eli Lilly and Company also developed a pan-TGF- β ligand inhibitor but did not pursue its development due to severe toxicity in animals (data on file, Eli Lilly and Company, Indianapolis, IN, USA). In addition, a TGF- β 1-specific monoclonal antibody (T β M1 or LY2382770) was evaluated in a Phase I study in patients with cancer. LY2382770 showed no radiographic responses once escalated to the predefined dose of 240 mg (flat dose) given every 28 days, which was thought to be efficacious

based on a pharmacokinetic/pharmacodynamic (PK/PD) model for LY2382770.³⁴ There are also monoclonal antibodies that were designed to block the TGF- β receptors, such as the monoclonal antibody TR1.³⁵ TR1 blocks the TGF- β R2; this monoclonal antibody is currently being evaluated in a first-in-human dose (FHD) study. Given the toxicity observed with small molecule inhibitors (SMIs) blocking ALK5 (see section SMIs of the TGF- β R1/ALK5 and the early development of galunisertib), specific inhibitors of TGF- β R2 using monoclonal antibodies were thought to have more manageable toxicity or perhaps even a reduced toxicity profile.

Antisense oligonucleotides (ASOs): Another approach to selectively blocking the TGF- β signaling is the use of ASOs. For example, the first-generation ASO trabedersen (formerly AP12009) was developed to block the TGF- β 2 production in glioma cells and was advanced to clinical investigation.³⁶ Although the safety profile was manageable, the antitumor activity of trabedersen seemed to be limited to a subset of patients, mainly those with World Health Organization grade III glioma. Similar to fresolimumab, lower doses appeared to be associated with better radiographic responses. However, the clinical development of trabedersen with its convection-enhanced delivery of compound in patients with glioblastoma was terminated. The convection-enhanced delivery was necessary for trabedersen because first-generation ASOs generally achieve their optimal pharmacological activity if they are continuously applied and administered directly to the tumor. Because of this added complexity for the administration of an ASO, newer ASOs with improved chemistry are being generated for future investigation in a range of indications, including cancer.³⁷

Vaccines strengthening the patient's microenvironment:

Another approach to blocking the TGF- β signaling pathway is based on vaccines. For example, belagenpumatucel-L is a cancer vaccine (NovaRX Corporation, San Diego, CA, USA) that inhibits the synthesis of TGF- β 2. Genetically modified whole tumor cells stimulate the patient's own immune system to attack the tumor. The vaccine contains four allogenic and irradiated nonsmall-cell lung cancer (NSCLC) cell lines expressing a TGF- β 2 synthetic antisense gene. The recently reported Phase III "STOP" trial in patients with stage III/IV NSCLC did not meet its predefined primary endpoint of overall survival (OS). However, an increase in OS was observed in a subgroup of patients who had started treatment with belagenpumatucel-L within 12 weeks of completing frontline chemotherapy. Also, patients with nonadenocarcinoma NSCLC (= squamous cell carcinoma and large cell

lung cancer) had an OS of 19.9 months when treated with belagenpumatucel-L compared with an OS of 12.3 months for patients treated with placebo (hazard ratio 0.55, $P=0.036$). The authors of the study concluded that belagenpumatucel-L should be further evaluated in lung cancer.³⁸

SMIs of the TGF- β RI/ALK5 and the early development of galunisertib (LY2157299 monohydrate)

Among the TGF- β inhibitors, SMIs represent a large and diverse group of chemical entities that are designed to block the activation of the signaling cascade downstream of the TGF- β receptor type I kinase (TGF- β RI or ALK5) or type II (TGF- β RII) by inhibiting the serine/threonine kinase. There is a growing list of SMIs blocking the TGF- β RI,³⁹⁻⁴² including recent SMIs such as Ki26894⁴³ and TEW-7197.^{44,45}

At Eli Lilly and Company, several SMIs were identified in the past years (Table 3). A large library of SMIs was screened in vitro using a TGF- β -dependent cell-based assay. Selected compounds were further evaluated for their ability to inhibit autophosphorylation of the isolated human TGF- β RI kinase domain. The TGF- β RI kinase domain was expressed as a constitutively active construct (T204D mutation) by Sf9 insect cells.^{46,47} For example, the diheteroaryl-substituted pyrazole 1 (LY364947) was identified as a potent inhibitor ($IC_{50}=51$ nM) (Table 3). Compounds were further evaluated by measuring their inhibitory effect in a TGF- β -dependent luciferase assay produced in mink lung cells (p3TP Lux) and their growth inhibition in mouse fibroblasts (NIH3T3).⁴⁸ Compounds such as LY580276,⁴⁹ LY364947,⁵⁰ and LY2109761⁵¹ share with LY2157299 monohydrate the selectivity profile and inhibition of the ALK5.⁵²⁻⁵⁴ Overall, the kinase selectivity profile of galunisertib met the desired characteristics (Table 4).

Compared with other SMIs, galunisertib (LY2157299) monohydrate had reduced cardiovascular toxicity in animals and appeared to be less potent in inhibiting pSMAD2 levels in vitro (Table 3).^{49,55-57} Galunisertib (pronounced: gal-ue' ni-ser-tib) is now the United States Adopted Name and International Nonproprietary Name designation for LY2157299 monohydrate. The first part of the name refers to the Greek-Roman physician Aelius Galenus (born: 129 AD; died: c.200/c.219 AD), also known as Galen of Pergamon.⁵⁸ Apart from galunisertib that started its clinical development in 2006, only TEW-7197 is known to be in clinical investigation. In mid-2014, a Phase I study of TEW-7197 was initiated in patients with breast cancer, melanoma, hepatocellular carcinoma (HCC), and glioblastoma.⁵⁹

Table 3 Clinical and surrogate compounds developed by Eli Lilly and Company (data on file)

	Ki (μ M)	TGF- β RI kinase IC_{50} μ M Mean values \pm SEM for a minimum of three determinations (n)	p3TP lux, IC_{50} μ M Mean values \pm SEM for a minimum of three determinations (n)	NIH3T3, IC_{50} μ M Mean values \pm SEM for a minimum of three determinations (n)	p38MAPK, IC_{50} μ M Single-point determination	Half-life mean (hours)		Toxicity other than known toxicities for ALK5 SMIs in animals
						Rat	Dog	
Galunisertib (LY2157299)	0.086	0.051 \pm 0.005 (258)	0.047 \pm 0.016 (55)	0.089 \pm 0.010 (68)	0.53	0.3	2.26	-
LY2109761	0.038	0.069 \pm 0.031 (2)	0.18 \pm 0.093 (5)	0.21 \pm 0.170 (4)	1.8	Not done	1-2	Not done
LY364947	0.028	0.051 \pm 0.005 (258)	0.047 \pm 0.016 (55)	0.089 \pm 0.010 (68)	0.74	Not done	Not done	Not done
LY580276	0.037	0.175 \pm 0.088 (4)	0.096 \pm 0.016 (3)	0.339 \pm 0.349 (4)	> 20	Not done	Not done	Not done

Abbreviations: ALK, activin receptor-like kinase; IC_{50} , half maximal inhibitory concentration; MAPK, mitogen-activated protein kinases; SMIs, small molecule inhibitors; SEM, standard error of the mean; TGF- β , transforming growth factor- β .

Table 4 Kinase selectivity profile of galunisertib (LY2157299) (data on file, Eli Lilly and Company)

Kinase	IC ₅₀ (μM)
TGF-βRI	0.17
TGF-βRII	0.21
ALK4	0.08
MINK	0.19
RIPK2	0.22
CSNK1A1	0.26
MAP4K4	0.28
GAK	0.31
CSNK1E1	0.4
BMPRI1B	0.47
BRAF	0.50
TNIK	0.51
RSK4	0.72
Abl1	0.86
ZAK	0.86
NLK	0.91

Abbreviations: IC₅₀, half maximal inhibitory concentration; TGF-β, transforming growth factor-beta; TGF-βRI, TGF-β receptor type I; TGF-βRII, TGF-β receptor type II; ALK, activin receptor-like kinase; MINK, misshapen/NIK-related kinase; RIPK2, receptor-interacting serine-threonine kinase; CSNK1A1, casein kinase alpha 1; MAP4K4, mitogen-activated protein kinase kinase kinase kinase 4; CSNK1E1, casein kinase epsilon 1; BMPRI1B, bone morphogenetic protein receptor type 1B; TNIK, TRAF2 And NCK Interacting Kinase; RSK4, ribosomal S6 kinase 4; Abl1, Abelson interactor 1; ZAK, Sterile alpha motif and leucine zipper containing kinase AZK; NLK, Nemo-Like Kinase.

Despite the long list of ALK5 SMI inhibitors, few ALK5 inhibitors have been moved to clinical investigation, perhaps because of the observed severe cardiac toxicities in animals.^{41,60} Galunisertib overcame this barrier by addressing

the following: (a) running preclinical toxicology studies with administration schedules that identified a sufficient margin of safety, (b) developing a predictive PK/PD model using animal pharmacology information, and (c) developing assays of PD markers in patients with cancer to confirm the therapeutic window as predicted by the PK/PD model (Figure 3).

Toxicology

In animal studies, several ALK5 inhibitors showed an increased incidence of hemorrhagic, degenerative, and inflammatory lesions in heart valves. Galunisertib was selected for clinical investigation based on its profile in animal toxicology studies in rats and dogs.^{49,50,57,61,62} The lesions appeared either at very high doses (1 month of continuous dosing) or during continuous dosing for 6 months. Intermittent dosing provided a sufficient margin of safety to advance galunisertib into clinical development.⁵⁷ In general, rats appeared to be more sensitive to galunisertib toxicity than dogs. Because of the difference in heart rate and also intravascular blood pressure, there is a possibility that the cardiac lesions were related to shear stress-associated intravascular remodeling. Under such conditions, the TGF-β signaling pathway is activated in endothelial and smooth muscle cells.^{63,64} Based upon these findings, it is possible that the lesions observed in the valves resulted from an inhibition of a physiologic TGF-β-dependent mechanism, which is normally needed to repair

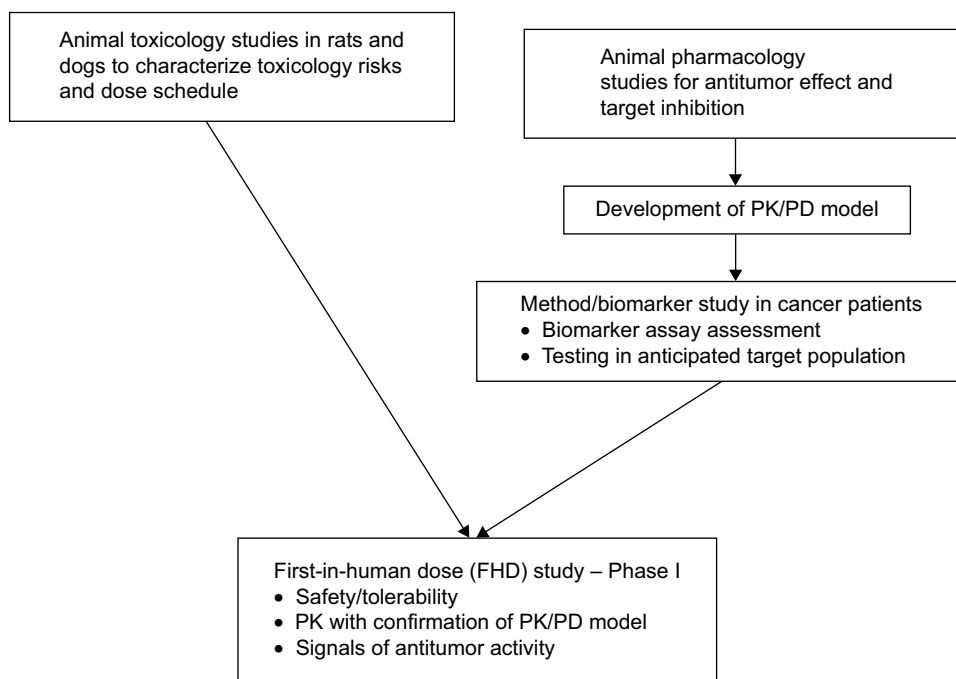


Figure 3 Preparing for the first-in-human dose (FHD) study.

Note: Standard Good-Laboratory Practice (GLP) animal toxicology studies were performed as well as a pharmacokinetic/pharmacodynamic (PK/PD) model established prior to the FHD study.

the injury caused by shear/stress at the outflow of the heart. When valves and ascending aorta were examined, they showed activation of the TGF- β pathway after administration of a TGF- β inhibitor as measured by pSMAD2 staining. Patients with Loeys–Dietz syndrome have a similar paradox where a loss of functional mutation in the TGF- β signaling gene is present; however, in the tissue, an activation of the TGF- β pathway is observed.^{65,66} One hypothesis for this paradox is that alternative pathways are activated.⁶⁷ A second hypothesis proposes a changed receptor/ligand processing as observed in studies with cells.^{68,69} If the second hypothesis based on a possible pathway adaptation is correct, then the intermittent dosing of TGF- β inhibitors provides a safe dosing regimen.

Galunisertib affects bone development and alters inflammatory responses in the skin or gut of rats and dogs. It is well known that blocking TGF- β signaling can cause chronic inflammation in skin and gut, which in turn can lead to pre-cancerous conditions.^{70,71} Intermittent or continuous regimens with low doses may reduce such a risk of developing chronic inflammation and thus allow long-term administration of a TGF- β inhibitor.

Pharmacology in cellular assays and animals

In addition to the animal toxicology studies, evidence of target inhibition and preclinical antitumor efficacy was needed for developing a predictive PK/PD model.^{72,73} The initial antitumor activity of galunisertib was characterized in three different cancer models: two breast cancer models, MX1 and 4T1, and a NSCLC model using Calu6. Dosing (75 mg/kg twice daily by oral gavage) was initiated 4–6 days postinoculation and continued for 20 days in the xenograft models (MX1 and Calu6) for the entire length of the survival study with the 4T1 model.⁷⁴ A statistically significant tumor growth delay of 10.1 days was observed in the MX1 model, and the 4T1 tumor model exhibited a 4.5-day survival advantage. In the Calu6 xenograft, galunisertib significantly delayed tumor growth.

Despite these encouraging observations with some cell lines in standard xenografts (eg, Calu6 and U87MG), it appears that only select tumor cell lines respond to galunisertib treatment in standard, immune-deficient xenografts. In patient-derived xenografts (PDXs), the antitumor response to galunisertib treatment is also limited.⁷⁵ Furthermore, in some immune-deficient PDX models, tumor growth appeared to be enhanced, which was previously raised as a potential risk for administering TGF- β inhibitors to patients.^{76,77} However, it is possible that such growth is only observed

in immune-compromised animals; in immune-competent murine models, no such tumor growth augmentation has been observed. Tumor-bearing, immune-competent mice treated with the monoclonal antibody against TGF- β R2, TR1, had antitumor effects that were at least partly dependent on the presence of T-cell subsets.³⁵ Finally, mice receiving long-term exposure (over 1 year of administration) of a soluble TGF- β antagonist had no adverse events.⁷⁸ All these observations imply that immune-competent animal models may be more predictive to evaluate the activity of TGF- β inhibitors. It appears that an active immune response is essential to assess the effect of TGF- β signaling inhibition in animal models; thus, models using immune-compromised animals may have limited use in screening for TGF- β inhibitors.

Because of the difficulties of using animal models as a screening method for identifying novel agents, alternative *in vitro* models were considered. Tumor clonogenic assays with PDX were evaluated, but it became evident that many PDX had a disrupted canonical pathway.⁷⁵ Hence, this assay was found not to be adequate to screen large libraries of novel SMI TGF- β inhibitors. Recently, experiments with a 3D *in vitro* model were used to assess the effect of galunisertib.⁷⁹ In such 3D culture systems, galunisertib had activity whereas it had none in standard monolayer assays. An advantage of the 3D culture model was the possibility to evaluate some aspects of the immune response. Whether such 3D *in vitro* assays may also offer an alternative to *in vivo* models is not yet determined.

Drug resistance mechanism and implications on drug combination

Based on the biology of TGF- β signaling, the associated mechanisms that affect drug resistance are likely to be dependent on the tumor microenvironment. One such example are the CAFs: these produce large amounts of the TGF- β ligands and are associated with fibrosis and with resistance to 5-fluorouracil (5-FU).²⁰ Recently, drug resistance related to TGF- β signaling was reported to be associated with loss of the *MED12* gene in tumors.⁸⁰ *MED12* loss induces not only an EMT-like phenotype that results in chemotherapy resistance to 5-FU but also resistance to the epidermal growth factor receptor tyrosine kinase inhibitor (EGFR TKI) gefitinib. Treatment with galunisertib in *MED12*-deficient cells restored the sensitivity to both chemotherapy and EGFR TKI. In addition to drug resistance to 5-FU and EGFR TKIs, there were reports connecting TGF- β signaling to paclitaxel resistance in triple-negative breast cancer.⁸¹ In all these observations, it appears that EMT or EMT-like phenotype of the

tumor cells plays a critical role to drug resistance associated with TGF- β signaling.

PK/PD model – predicting a therapeutic window in patients with an acceptable safety profile

The development of preclinical PK/PD models have been invaluable in guiding early clinical trial design.^{82,83} A similar model was built using preclinical data on pSMAD2 inhibition, antitumor activity of galunisertib in Calu6 xenografts, and the observed PK in mice, rats, and dogs.^{72,73} The half-life of galunisertib in animals was less than 3 hours (Table 3). An observed moderate variation in PK was, in part, attributable to the formulation of galunisertib.⁸⁴ Allometric PK scaling of galunisertib allowed a reliable prediction of both the exposure in humans within the expected range to produce antitumor activity. The drug effect continued even after the systemic disappearance of the drug: the PD effect of reducing pSMAD2 was still detectable in tumor tissue and peripheral blood mononuclear cells (PBMCs) up to 7 days after stopping galunisertib and when galunisertib was no longer detected in the plasma. This delayed PD effect was also seen when treated with the monoclonal antibody against TGF- β R2, TR1, suggesting that this phenomenon is not limited to SMIs (data on file, Eli Lilly and Company).

The simultaneous inhibition of pSMAD2 inhibition in tumor and surrogate tissue (ie, PBMCs) led to the development of a PD detection assay using peripheral blood. This assay was developed to monitor and confirm the PK/PD relationship during the FHD study. To avoid toxicity and maintain antitumor activity, the galunisertib exposure had to be limited to a pSMAD2 inhibition of approximately 30% over 24 hours, combined with a maximum inhibition of 50%. This was achieved by a twice-daily (BID) dose schedule that produced a modulatory exposure.⁸⁵

Dosing considerations for galunisertib

Based on the PK/PD modeling and the toxicity observation, we decided to use a BID dosing schedule and a 14-day on/14-day off schedule. In preclinical models and later in the Phase I study, we had observed that pSMAD2 inhibition was extended up to 7 days after galunisertib was stopped. Given that continuous dosing may increase the risk for chronic toxicity, the 14-day treatment with an anticipated prolonged pSMAD2 inhibition of 7 days was the most acceptable regimen for long-term treatment. To avoid high single-day exposures, a morning and evening dosing schedule was

instituted. All these interventions were designed to avoid a steady-state or continuous on-target inhibition.

Early biomarker development

The biomarker work early in development focused on two main objectives: a) biomarkers for patient selection and b) pharmacodynamic response markers. For patient selection, three groups were considered: those whose tumors produced high amounts of TGF- β 1, (eg, in renal cell carcinoma,⁸⁶ prostate cancer,⁸⁷ and breast cancer²⁵); those in whom TGF- β inhibition had shown clinical responses with other TGF- β inhibitors (such as glioma³⁶), and those with skeletal metastasis. In such conditions, TGF- β is being mobilized from the bone matrix, and increased TGF- β 1 can serve as a marker of tumor progression.⁸⁸

A pSMAD2 assay to measure the reduction of pSMAD2 in PBMCs during the FHD trial was established.⁸⁹ This pSMAD2 enzyme-linked immunosorbent assay (ELISA) used a polyclonal antisera and was tested on serum from patients with skeletal metastasis (a nondrug interventional trial). The intra-patient variability was determined to be less than 30%.⁹⁰ This variability was considered acceptable for the FHD trial.

A plasma TGF- β 1 ELISA was developed and subsequently used for PD assessments. This assay provided reliable information on plasma TGF- β 1 levels when citrate-theophylline-adenosine-dipyridamole tubes were used for collection.⁹¹ The assay detects the activated TGF- β 1 form in a standard ELISA format (R&D Systems, DB100B). The original hypothesis assumed that TGF- β 1 levels will be reduced if galunisertib interrupts a possible autocrine or paracrine growth signal.

Other plasma proteins such as parathyroid hormone-related protein, von Willebrand factor, and interleukin-10 correlated with TGF- β 1 levels.⁹⁰ Given this connection, the measurements of such indirect plasma markers were thought to help with understanding of the downstream effect of the TGF- β signaling inhibition with galunisertib. Thus, they were considered as alternative PD markers for future clinical trials.

Gene expression profiling of PBMCs was initially pursued as an additional option to assess PD changes,^{92,93} but previously observed changes in PBMCs in ex vivo experiments were not able to be reproduced in subsequent clinical trials. In earlier ex vivo studies, T-cells were activated with CD3/CD28 costimulation to obtain reliable gene expression profiles.⁹² By contrast, PBMCs obtained from patients with skeletal metastasis were only stimulated with exogenous TGF- β 1.⁹³ The stimulation with exogenous TGF- β 1 led to a rapid and subtle change in gene expression, which may

have been too transient to be detected in patients treated with galunisertib.

Several attempts were also made to develop patient selection strategies based on gene expression profiles from tumor-bearing animal or PDX models.^{75,94,95} These profiles were compared with publicly available gene expression data sets, but it was not possible to define a clear profile. In part, this was due to the minimal antitumor activity with galunisertib in immune-deficient animal models or monolayer cell line experiments.

FHD study and early clinical development of galunisertib in cancer patients, including patients with glioblastoma

Given the potential for toxicity and general safety concerns, throughout the course of the FHD study the predictive PK/PD model was continuously updated, and results were shared with the scientific community (a summary of all clinical studies with galunisertib is presented in Table 5). The decision to select galunisertib as a clinical candidate was presented in 2005,⁷² and the FHD study was disclosed at a conference in 2006.⁵⁶ Over the subsequent years, the progress of the FHD study and the patient safety information were routinely presented.⁹⁶⁻⁹⁹ Additionally, preclinical studies of galunisertib were regularly updated.^{72,74,94}

One of the most important objectives of the FHD study was to assess the safety of galunisertib when administered to reach the predicted therapeutic window. Based on the PK/PD model, the therapeutic window was anticipated to be achieved when compound was dosed between 160 and 360 mg.⁷³ Within the initial cohorts of the FHD study, galunisertib was given daily continuously based on the standard 1-month animal toxicology studies. Although not generally required for oncology Phase I trials, 6-month animal toxicology studies were begun to assess the risk of more prolonged exposures. This study started concurrently with enrollment of the patients into the FHD study. Unexpectedly, new toxicities emerged after the evaluation of 6-month animal toxicology studies. As a result of these new findings, the FHD study was placed on clinical hold and additional animal toxicology studies began using an intermittent dosing schedule. The intermittent dosing schedule in the most sensitive species (rats) provided an acceptable margin of safety. These data supported lifting the clinical hold; the FHD study resumed but was restricted to patients with glioblastoma, in part because of the previously reported activity of trabedersen.³⁶

During the FHD study, all patients underwent comprehensive cardiac monitoring. These studies consisted of baseline and every-other-month echocardiography/Doppler imaging, baseline and monthly serial plasma assessment for Troponin I, brain natriuretic peptide, and additional cardiac measurements.¹⁰⁰ No drug-related cardiovascular toxicity was observed during the entire course of the FHD study. The use of a central echocardiography/Doppler assessment along with cardiac serial serum marker evaluations proved valuable and was supplemented by the help of dedicated cardiologists on call at each site. In this study, one postmortem examination was performed on a patient who died from progressive malignant glioma: although the aorta was found to be abnormal, the ante-mortem echocardiography/Doppler was normal. There was an absence of pathological cardiac serum markers, and there were no histopathologic changes in the heart valves. The case was also peer reviewed by an external vascular pathologist who found the changes to be consistent with routine degenerative findings in age-matched patients.¹⁰¹

During the course of the FHD study, radiographic responses were observed; some subjects obtained complete responses that proved durable with patients on study drug more than 2 years. Some of these individuals with glioblastoma included patients with relapsed disease (World Health Organization grade II and III).^{102,103} Responses tended to occur after a minimum of two cycles of galunisertib. Also, in two patients with radiographic responses, a site-specific imaging study found that tumor blood flow was changed, similar to that seen with antiangiogenic agents.¹⁰⁴ However, compared with other antiangiogenic drugs, the blood flow changes occurred at a later time.

Tissue was available from 8 of 20 patients with secondary/low-grade glioma. Of these eight patients, five had an IDH1/2 mutated tumor; within this cohort, four patients (80%) had either radiographic response or stable disease \geq six cycles. This suggests that the TGF- β pathway plays an important role in this subgroup of glioma patients.¹⁰³ This observation is intriguing because of the similarities described with trabedersen.³⁶ Furthermore, *in vitro*, the IDH1 R131H variant has been associated with induction of mesenchymal gene expression phenotype.¹⁰⁵ In addition to the patients with an IDH1 mutation and response, the other patients who appeared to benefit had no clear genetic pattern of a mutation. The recently defined mesenchymal subtype of glioma may be driven by TGF- β signaling given the well-recognized association between TGF- β and the EMT.¹⁰⁶ As such, the mesenchymal phenotype or IDH1 variant may enrich for patients likely to respond to galunisertib.

Table 5 Clinical trials with galunisertib (LY2157299)

Trial	Phase	Disease	Number of patients	Treatment	Primary endpoint	NCT#
Phase II/III study of monotherapy LY2157299 monohydrate in very low-, low-, and intermediate-risk patients with myelodysplastic syndromes	II/III	Very low-, low-, and intermediate-risk myelodysplastic syndromes	140	LY2157299 plus BSC vs placebo plus BSC	<ul style="list-style-type: none"> Percentage of participants with hematological improvement (HI) based on International Working Group (IWG) 2006 criteria Percentage of participants who are transfusion free or have hemoglobin (Hb) increase ≥ 1.5 g/dL maintained for 8 weeks Relationship of change in response biomarker to clinical benefit 	NCT02008318
Phase II study of LY2157299 in patients with hepatocellular carcinoma	II	Hepatocellular carcinoma	190	LY2157299 vs LY2157299 plus sorafenib	<ul style="list-style-type: none"> Relationship of change in response biomarker to clinical benefit Time to progression Overall survival 	NCT01246986
A Phase II study of LY2157299 monohydrate monotherapy or LY2157299 monohydrate plus lomustine therapy compared with lomustine monotherapy in patients with recurrent glioblastoma	II	Recurrent glioblastoma	180	LY2157299 plus lomustine vs LY2157299 vs lomustine plus placebo	Overall survival	NCT01582269
Phase Ib/IIa study combining LY2157299 with standard temozolomide-based radiochemotherapy in patients with newly diagnosed malignant glioma	Ib/IIa	Newly diagnosed glioblastoma	62	LY2157299 plus radiation plus temozolomide	<ul style="list-style-type: none"> Phase I: recommended dose for Phase II portion Phase II: relationship of change in response biomarker to clinical benefit 	NCT01220271
A Phase Ib/II study with gemcitabine and LY2157299 for patients with metastatic cancer (Phase Ib) and advanced or metastatic unresectable pancreatic cancer (Phase II)	Ib/IIa	Metastatic cancer and advanced or metastatic unresectable pancreatic cancer	168	LY2157299 + gemcitabine	<ul style="list-style-type: none"> Phase Ib: recommended Phase II dose Phase 2: overall survival 	NCT01373164
Disposition of [¹⁴ C]-LY2157299 monohydrate following oral administration in healthy subjects	I	Healthy volunteers	8	[¹⁴ C]-LY2157299	Urinary and fecal excretion of LY2157299 radioactivity	NCT01746004
Effect of food on the pharmacokinetics of LY2157299 monohydrate in healthy subjects	I	Healthy volunteers	16	LY2157299	Pharmacokinetics	NCT01965808
Phase I dose-escalation study of LY2157299 monotherapy in Japanese patients with solid tumors	I	Advanced metastatic cancer	12	LY2157299 monohydrate	Safety and side effects of LY2157299 in Japanese patients	NCT01722825

Abbreviations: BSC, best supportive care; NCT, national clinical trial; vs, versus.

In PBMCs, pSMAD2 was reduced in most patients treated with galunisertib.⁹⁷ A post-treatment biopsy in one patient showed a reduction in Id1 and CD44 gene expression, both of which are upregulated by TGF- β signaling.¹⁰⁷ These observations support that galunisertib achieved its pharmacologic effect by its presumed central mode of action (by blocking the TGF- β signaling). The data from PBMCs further indicated that peripheral blood cells could be used to determine the PD effect of TGF- β inhibitors; however, we did not identify whether this effect is mainly related to the effect on neutrophils, macrophages or lymphocyte subsets.

Using continuous assessment of PK and PD, the preclinical prediction of PK and PD was confirmed in humans, and the therapeutic window was deemed to be safe for further development.⁸⁵ For the Phase II studies, a 150-mg BID dose (300 mg/day; given intermittently 14 days on/14 days off on a 28-day schedule) was selected, with few Grade 3 or 4 toxicities observed to date.¹⁰³

Glioblastoma (glioma)

Nonclinical observations

The TGF- β signaling pathway and, especially, the pSMAD2 expression are associated with poor prognosis in glioblastoma.^{108–110} Thus, it was hypothesized that tumors overexpressing the TGF- β signaling pathway will respond to a TGF- β SMI. Also, data suggested that patients with low-grade glioma have higher plasma TGF- β 1 levels than patients with high-grade gliomas. If TGF- β 1 levels are decreased after treatment to the primary (such as surgical tumor removal), patients may benefit even if the surgery is not definitive.¹¹¹ The source of the TGF- β signaling in glioma has not been identified and may not originate from the tumor cell itself. For example, TGF- β signaling is found in cancer-initiating stem cells, which are a small subset within the tumor tissue. Galunisertib may inhibit these cancer-initiating stem cells and thus arrests TGF- β -dependent tumor cell growth and migration.^{107,112,113}

Although we observed no responses to galunisertib in most PDX and tumor cell lines, the glioma models such as U87MG did show direct treatment responses to galunisertib. Hence, the combination of galunisertib with concurrent temozolomide (TMZ) and radiation was evaluated in this model: an additive antitumor effect was observed.^{114,115} Also, the combination of galunisertib and lomustine showed additive antitumor activity in this same xenograft model. Interestingly, lomustine by itself reduced pSMAD2 in glioblastoma cell lines.¹¹⁶ This was an unexpected finding, further suggesting that a combination of lomustine and galunisertib may have additive pharmacologic effect.

Clinical experiences

Based on the totality of the data,¹⁰² galunisertib received orphan drug designation by the European Medical Agency (EMA) (May) (EMA/COMP/175329/2013) and the Food and Drug Administration (March) in 2013.¹¹⁷ Two Phase II studies in glioblastoma patients were concurrently initiated: 1) a blinded, randomized three-arm study comparing galunisertib to lomustine and the combination of galunisertib and lomustine in patients receiving second-line systemic treatment after first relapse¹¹⁸ and 2) a randomized Phase I/II study in patients with newly diagnosed glioblastoma receiving standard first-line therapy of chemoradiation with TMZ (Table 5).¹¹⁹ Although both studies are still ongoing, interim data have been presented. At American Society of Clinical Oncology 2013, safety data of the second-line study were presented, and no added toxicity across the entire study was reported.¹²⁰ The first-line study investigates clinical benefit in relationship to biomarker changes such as total lymphocyte, CD3⁺, CD4⁺, CD4⁺CD25⁺, CD127⁺, Foxp3⁺, and CD8⁺ T-cells counts.¹²¹ At interim, galunisertib administration appeared to conserve or even increase CD8⁺ counts after standard chemoradiation had been completed, and patients were receiving adjuvant TMZ and galunisertib. This finding was not observed in patients receiving only adjuvant TMZ.

Hepatocellular carcinoma

Nonclinical observations

TGF- β expression is associated with both early and late progression of HCC.^{122–126} HCC cells can secrete TGF- β 1 in an autocrine manner; blocking this feedback loop may have a beneficial impact on controlling tumor progression.¹²³ In contrast to other HCC treatments, in vitro galunisertib had no cytotoxicity at the putative therapeutic concentrations; however, galunisertib was inhibitory in cell invasion and migration assays.^{125,127–133} This effect is seen at a 1–10 nanomolar concentration in several HCC cell lines.

Similar to the impact of sorafenib, galunisertib reduces alpha-fetoprotein (AFP) and Ki67 expression in patient-derived HCC liver slices.¹³⁴ Recently, the combination of sorafenib and galunisertib has demonstrated an additive effect in immune-competent C57BL6/ASV-B mice,¹³⁵ a murine model that spontaneously develops HCC as a result of SV40 antigen overexpression in the liver. Whether the additive effect of sorafenib and galunisertib is also related to the ability of sorafenib to inhibit the TGF- β pathway at high concentrations is unclear.¹³⁶ Galunisertib is also active in HCC cells that are resistant to multikinase inhibitors such as sorafenib and sunitinib.^{137,138} This implies that galunisertib

may have activity in conditions where other kinase inhibitors are inactive. *In vitro* studies further suggest that galunisertib has inhibitory activity on kinases downstream of SMAD2 when treatment is extended beyond 24 hours.¹³² Although the relevance of this finding on the antitumor activity of galunisertib remains to be further evaluated, these long-term cultures with galunisertib had no cytotoxic effect on cells, despite the changes of multiple kinases. This observation further supports that galunisertib exerts its antitumor effect not by cell killing but by altering the phenotype of the cells and by reducing their EMT phenotype. In summary, galunisertib has shown an antitumor effect in HCC that appears mainly based on the ability to inhibit migration and invasion rather than direct tumor cell killing.

Clinical studies

In 2013, galunisertib received orphan drug designation in HCC by the EMA (March) (EMA/COMP/95768/2013) and the Food and Drug Administration (April).¹¹⁷ Using the preclinical information, a two-arm dose comparison Phase II study¹³⁹ was designed to compare the lower (160 mg/day) dose with the higher (300 mg/day) dose. The objective was to determine whether the lower dose was superior to the higher dose given that other TGF- β inhibitors may have been more effective at lower doses.^{30,36} All patients in this dose-comparison study had to have elevated AFP ($\geq 1.5\times$ upper limit of normal) and reduction in AFP was initially used to select the dose for future clinical development. All patients had to have failed prior sorafenib or be ineligible to receive sorafenib (Table 5). Preliminary results of this trial showed that there were no differences between the doses in an intent-to-treat analysis, although patients receiving 300 mg/day (including patients with poor prognosis) had slightly different demographic characteristics, such as Child Pugh B7 and prior liver transplantation. Given this lack of difference between the doses, all new patients were enrolled on 300 mg/day.^{140,141} Consistent with the *in vitro* observations, some patients had a reduction in plasma AFP, E-cadherin, and TGF- β 1 levels. Patients with reduction in any or all of the three markers had a longer time-to-tumor progression and OS. Patients with $\geq 20\%$ reduction from baseline in AFP had a median OS of approximately 21 months. This was observed in about 25% of all patients who had elevated AFP at baseline. Furthermore, 50% of the patients had elevated TGF- β 1 levels (above 3,400 pg/mL). In 66% of these patients, a TGF- β 1 reduction of $\geq 20\%$ was observed, and median OS was about 12 months. Given the poor prognosis generally associated with elevated AFP, the survival information suggested that

galunisertib was a potentially active agent in a difficult-to-treat population with poor prognosis.

Pancreatic cancer

Nonclinical observations

TGF- β signaling pathway is active in metastatic/advanced pancreatic cancer.¹⁴² In a xenograft model, LY2109761 was reported to have antitumor activity in combination with gemcitabine.¹⁴³ This observation was confirmed using galunisertib (data on file, Eli Lilly and Company). One possible explanation for this additive antitumor effect is the improved delivery of chemotherapy via vessel normalization.²⁸ It is also possible that TGF- β signaling blockade reduces the metastasis to the liver, which may independently improve survival.¹⁴⁴ Galunisertib was able to block the renewal of cancer stem cells isolated from pancreatic cancers and was additive in the combination with a Hedgehog inhibitor.¹⁴⁵ In addition, blocking the TGF- β signaling in the stroma alone in immune-competent or immune-deficient orthotopic pancreatic models was shown to reduce metastasis, reduce pancreas weight, and inhibit protumoral fibroblast activity.¹⁴⁶ Thus, TGF- β inhibition appears to have antitumor activity by improving chemotherapy delivery, blocking metastasis, and inhibiting renewal of cancer-initiating stem cells.

Clinical studies

A blinded, randomized Phase I/II study was initiated to determine whether the activity observed in animals is also observed in patients with advanced or metastatic pancreatic cancer (Table 5). The combination (Phase I) had the expected manageable toxicity of gemcitabine,¹⁴⁷ but the final efficacy data are not yet available.

Myelodysplastic syndromes

Nonclinical observation

Myelodysplastic syndrome (MDS) is characterized by bone marrow cytological dysplasia and ineffective hematopoiesis, in which TGF- β (through induction of inflammatory and myelosuppressive cytokines) may play a role.¹⁴⁸ The natural inhibitor of the TGF- β signaling pathway, SMAD7, is suppressed in MDS progenitor cells due to increased miR21 expression, which in turn upregulates the TGF- β pathway. This leads to increased expression of myelosuppressive cytokines and decreased burst forming unit-erythroid (BFU-E) and colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM). These same cytokines are immunosuppressive and likely contribute to the fatigue seen in MDS. Blockade of the TGF- β pathway

by galunisertib in vitro and in vivo murine models resulted in increased erythropoiesis and myeloid cell lineage CFUs, restoring normal hematopoiesis.¹⁴⁹ Animals overexpressing TGF- β were treated with galunisertib, and hemoglobin levels improved in these mice. Human CD34⁺ cells from patients with MDS were also cultured with and without galunisertib; cells cultured with galunisertib demonstrated restoration of normal BFU-E and CFU-GEMM colony formation.

Clinical studies

The preclinical observation together with its favorable toxicity profile (<5% of drug-related Grade 3/4 toxicities) of galunisertib justified the start of a Phase II trial in patients with very low, low, and intermediate risk MDS to evaluate the activity of galunisertib (Table 5).^{150,103} The study is currently enrolling patients.

Leukemia

LY2109761 was evaluated in models of leukemia with the hypothesis that it may block leukemic growth from the microenvironment.¹⁵¹ However, the antileukemic effect was limited, and no additional experiments were conducted. Currently, there are no studies evaluating the interdependency between the leukemic cells and the microenvironment in the bone marrow. However, the importance of the stroma as a source of TGF- β signaling has recently been highlighted. Blocking the TGF- β signaling after chemotherapy accelerated the hematopoietic reconstitution and delayed the return of cycling hematopoietic stem cells to quiescence. This was not observed during homeostasis, suggesting that TGF- β signaling is context dependent.¹⁵²

Lung cancer

The TGF- β inhibition with LY2109761 reduced metastatic spread of NSCLC cell lines.¹⁵³ The TGF- β inhibitor LY364947 was also able to overcome the EGFR resistance in cell lines that had no T790M mutation.¹⁵⁴ Furthermore, LY2109761 showed antimigratory effects on a murine adenocarcinoma NSCLC cell line.¹⁵⁵ Given these early observations, TGF- β signaling may play an important role in drug resistance in NSCLC and hence not be limited to certain lung cancer subtypes, as defined either by EGFR mutations or by histology (such as adenocarcinoma or squamous cell carcinoma).

Colorectal cancer

Using gene expression profiling, a mesenchymal subgroup among patients with colorectal cancer was recently identified.¹⁵⁶ This subgroup had poor prognosis and did not

benefit from adjuvant chemotherapy (5-FU). Additionally, MED12 loss can result in activation of TGF- β signaling and a mesenchymal phenotype that is also associated with resistance to fluoropyrimidine-based therapy.⁸⁰ In cell-line experiments, the addition of galunisertib restored chemosensitivity.⁸⁰ Galunisertib was also used to alter the gene expression of cells from the microenvironment, such as cancer-adjacent fibroblasts.¹⁵⁷ From these experiments, a different gene expression profile for colorectal cancer was developed. Overall, it appears that the mesenchymal phenotype is the key characteristic for resistance to standard treatment in colorectal cancer. Whether the aggressiveness of the colorectal cancer is driven by the mesenchymal phenotype of the tumor cells or the microenvironment remains unclear at this time.

Breast cancer

In breast cancer, TGF- β signaling appears to act as an auto-crine growth factor.^{158–160} Thus, TGF- β inhibition may have multiple effects, including the reduction of bone metastasis.⁸⁸ For example, LY2109761 can minimize osteolytic bone metastasis.¹⁶¹ Because of its inhibition on metastatic spread, TGF- β inhibitors were active across different breast cancer subtypes.^{162,163} Basal-like breast cancer responds to TGF- β inhibition that prevents metastatic spread in animal models.¹⁶⁴ In addition, models with human epidermal growth factor receptor 2 (HER2)/neu expression respond to TGF- β inhibition.^{165–167} Furthermore, paclitaxel combined with galunisertib has additive antitumor activity in ER/PR/HER2 nonexpressing “triple-negative” cell lines.¹⁶⁸ Because triple-negative cell lines also have characteristics of cells undergoing EMT, TGF- β inhibition may be effective in limiting EMT in such cells. Hence, it is possible that the main target of TGF- β inhibitors is the alteration of the tumor cell phenotype rather than direct tumor cytotoxicity. Although these observations support the use of a TGF- β inhibitor in breast cancer, long-term administration of LY2109761 has also been associated with the outgrowth of chemoresistant breast cancer tumors.¹⁶⁹ Whether this is an artifact of the model used in this particular study or reflects a possible long-term adverse effect related to continuous dosing remains controversial.

Prostate cancer

In prostate cancer, TGF- β expression in tissue or plasma has been associated with poor survival.^{87,170–172} LY2109761 has shown antitumor activity in animals by inhibiting bone metastasis in both MDAPCa2b and PC-3.¹⁷³ At the same time, the bone density was increased in severe combined immunodeficiency mice harboring either tumor cell lines.

Melanoma

There are several mechanisms by which TGF- β may promote tumor growth in melanoma, including immune modulation/inhibition, remodeling, and drug resistance. For example, TGF- β can cause resistance to proto-oncogene B-Raf (BRAF) inhibition, and melanoma cell lines with a BRAF mutation also often have a mesenchymal phenotype.^{174,175} While pSMAD2 was inhibited in PDX of melanoma, no growth inhibitory effect was observed in the same PDX using a standard tumor colony assay.⁷⁵ However, no antimigratory or anti-invasive effect of galunisertib was evaluated in these PDX. Recently, an antitumor effect of the combination galunisertib and ipilimumab was reported in a murine melanoma model, while each inhibitor had no antitumor effect in this immune-competent mouse model.¹⁷⁶

Radiation and fibrosis

Radiation therapy is associated with both tumor cell killing and normal tissue fibrosis within the radiation field and with associated increases in TGF- β 1 in the plasma and tissues.^{177,178} Also, increases in TGF- β signaling are not only associated with fibrosis or tumor cell killing but also with tumor cell activation inducing tumors to metastasize after radiation.^{179–181} LY2109761 administration before, during, and after radiation treatment inhibited lung fibrosis in irradiated animals.¹⁷⁹ This provides a rationale to investigate the role of galunisertib in radiation-associated fibrosis or other fibrotic diseases.

Conclusion

The preclinical and clinical research on galunisertib, including the treatment of over 300 patients, has taught us some important lessons. First, SMIs of TGF- β can safely be developed for clinical testing, provided there is an adequate understanding of the PK/PD relationship. Most of the toxicities in animal models that were of concern prior to the start of clinical development of galunisertib have not been observed in humans. Second, the biology of the TGF- β inhibition is largely dependent on the microenvironment, perhaps more than originally anticipated. Thus, a focus on direct tumor cell cytotoxicity may be misleading and provide inconclusive observations that will not be helpful to advance clinical development of future TGF- β inhibitors. Consequently, more novel preclinical testing assays are required than those traditionally used in oncology research. Third, it appears that the activity of TGF- β inhibition is dependent on a subtle modulation of the EMT, stem cell function, and immune function. Hence, it will be important to investigate new biomarkers that are related to EMT, stem cell function, and immune responses.

For example, it appears that mesenchymal phenotypes contain TGF- β -associated gene expression profiles regardless of the histologic tumor. Whether this applies to all tumors is not clear at this time but it may help with patient selection in future studies. For clinical development, radiographic tumor responses will likely occur only in those patients who have the opportunity to receive longer treatment periods. Thus, patient selection tools, defining who will most likely benefit from TGF- β inhibition, remain one of the most challenging questions to date. Alternatively, combining TGF- β inhibitors with other agents will pose other challenges.

Disclosure

All authors are employees or were employees of Eli Lilly and Company. The authors report no other conflicts of interest in this work.

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