



REVIEW ARTICLE

The clinical trial landscape in amyotrophic lateral sclerosis—Past, present, and future

Heike J. Wobst¹ | Korrie L. Mack^{2,3} | Dean G. Brown⁴ |
Nicholas J. Brandon¹ | James Shorter²

¹Neuroscience, BioPharmaceuticals R&D, AstraZeneca, Boston, Massachusetts

²Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

³Merck & Co, Inc, Kenilworth, New Jersey

⁴Hit Discovery, Discovery Sciences, BioPharmaceuticals R&D, AstraZeneca, Boston, Massachusetts

Correspondence

Heike J. Wobst, Jnana Therapeutics, Northern Avenue, Boston, MA 02210.

Email: heike.j.wobst@gmail.com

James Shorter, Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104.

Email: jshorter@pennmedicine.upenn.edu

Present address

Heike J. Wobst, Dean G. Brown and Nicholas J. Brandon, Jnana Therapeutics, Northern Avenue, Boston, MA 02210.

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease marked by progressive loss of muscle function. It is the most common adult-onset form of motor neuron disease, affecting about 16 000 people in the United States alone. The average survival is about 3 years. Only two interventional drugs, the antiglutamatergic small-molecule riluzole and the more recent antioxidant edaravone, have been approved for the treatment of ALS to date. Therapeutic strategies under investigation in clinical trials cover a range of different modalities and targets, and more than 70 different drugs have been tested in the clinic to date. Here, we summarize and classify interventional therapeutic strategies based on their molecular targets and phenotypic effects. We also discuss possible reasons for the failure of clinical trials in ALS and highlight emerging preclinical strategies that could provide a breakthrough in the battle against this relentless disease.

KEYWORDS

ALS preclinical strategies, amyotrophic lateral sclerosis, clinical trials, edaravone, riluzole

1 | INTRODUCTION

It was 1938 when the Yankees' first baseman Lou Gehrig, arguably one of the greatest baseball players of all time, showed signs of a dip in form. Or rather, it was more than that. In the words of the sports journalist James Kahn: "I think there is something wrong with him. Physically wrong, I mean. I don't know what it is, but I am satisfied that it goes far beyond his ball-playing. I have seen ballplayers 'go' overnight, as Gehrig seems to have done. But they were simply washed up as ballplayers. It's something deeper than that in this case, though. I have watched him very closely and this is what I have seen: I have seen him time a ball perfectly, swing on it as hard as he can, meet it squarely—and drive a soft, looping fly over the infield. In other words, for some reason that I do not know, his old power isn't there... He is meeting the ball, time after time, and it isn't going anywhere."¹ Shortly after, he was diagnosed with amyotrophic lateral sclerosis (ALS).

ALS is a disorder characterized by the death of motor neurons in the motor cortex and spinal cord as well as spinal interneurons. The loss of motor neurons, which control voluntary movement, in turn, causes progressive muscle weakness. Over time, patients lose the ability to walk, speak, and swallow.

ALS is the most common adult-onset form of motor neuron disease, with a prevalence of around five to six in 100 000 people in the United States and Europe.^{2,3} Since the first disease-associated gene, superoxide dismutase 1 (SOD1), was identified in 1993, more than 30 genes have been genetically linked to familial (5%-10% of cases) and sporadic forms (ie, without ascertainable family history; 90%-95% of cases) of the disease.^{4,5} By far the most common mutation identified to date is a G₄C₂ hexanucleotide repeat expansion in the gene C9ORF72, which is linked to 30% to 50% of familial and 5% to 7% of sporadic cases.⁶ Histopathologically, ALS is characterized by inclusions of mislocalized, aggregated proteins. In around 97% of ALS cases—excepting those caused by mutations in the genes SOD1 and FUS—the principal constituent of these inclusions is a RNA-binding protein (RBP) with a prion-like domain (PrLD), TDP-43.^{7–9}

Most ALS patients die within 3 to 5 years after diagnosis,⁴ usually as a result of respiratory failure. Sadly, Lou Gehrig was no exception: in 1941, he succumbed to the disease aged 37. Today, almost 80 years later, ALS remains almost as intractable as it was back then. Scores of clinical trials have only yielded two interventional treatments: Riluzole, approved in 1995; and edaravone, approved by the FDA in 2017 (Table 1). In this review, we summarize the state of play of interventional drugs currently or formerly in the clinic for the treatment of ALS. We also highlight exciting novel therapeutic avenues built on our rapidly expanding understanding of the pathogenesis of this devastating disease.

2 | APPROVED INTERVENTIONAL DRUGS

2.1 | Riluzole and other antiexcitatory therapeutic strategies

In the late-1980s and early-1990s, studies first implicated impaired glutamate homeostasis in ALS.^{48,102,103} Today, excitotoxicity—excessive, deleterious glutamate signaling—is believed to underlie or contribute to motor neuron degeneration in ALS.¹⁰⁴ Consistent with this hypothesis, transcranial magnetic stimulation experiments have revealed a cortical hyperexcitability phenotype in ALS patients.^{105,106} Consequently, many therapeutic efforts have focused on stemming excessive excitatory signaling (Figure 1). To date, however, riluzole remains the only antiexcitatory therapy that has proven effective. It was approved by the FDA in 1995 based on positive survival data in two clinical trials (chemical structures of riluzole and other antiexcitatory small molecules, see Figure 2).^{34,35} A more recent meta-analysis combining results of three trials concluded that riluzole, at a dose of 100 mg per day, improves the probability of surviving 1 year by about 10% and extends median survival by about 3 months.³⁶ Limited bioavailability in the central nervous system due to efflux via the P-glycoprotein transporter may limit the efficacy of riluzole.¹⁰⁷ A recent study by Abrahao et al¹⁰⁸ demonstrated that the blood-brain barrier (BBB) can be

TABLE 1 Overview of disease-modifying therapies in clinical trials

Modality	Drug name	Mechanism of action	Phase/outcome	Primary endpoint	References
Anti-inflammatory/immune-modulatory					
Antibody	Tocilizumab	Antibody targeting IL-6R	Ph 2	Safety, tolerability	10,11
Polypeptide	ACTH/Acthar gel	ACTH—stimulates release of glucocorticoids	Ph 2 halted	ALSFRS-R	12,13
Polypeptide	IL-2	Promotes differentiation of regulatory T cells	Ph 2	Time to death (18 mo)	14
SM	Ibuprofen	PDE inhibitor, suppresses proinflammatory microglial activation	Ph 2	Safety, tolerability	15
SM	Pentoxifylline	PDE inhibitor	Ph 3 failure	Time to death (18 mo)	16
SM	Masitinib	CSF-1R/c-kit inhibitor, suppresses microglial activation and proliferation	Ph 2/3	ALSFRS-R	17–20
SM	NP001	Stabilized form of sodium chloride, downregulates NF- κ B expression and IL-1 β production	Ph 2 failure	ALSFRS-R	21,22
SM	Thalidomide	Suppresses production of TNF- α	Ph 2 failure	Copimary ALSFRS-R + PFT	23
Peptide mimetic	Glatiramer acetate	MBP decoy, modulates T cell reactivity	Ph 2 failure	ALSFRS-R	24
SM	CC100	Caffeic acid phenethyl ester, suppresses NF- κ B and MAPK signaling	Ph 1	Safety, tolerability	25
SM	Fingolimod	S1PR1 agonist, reduces circulating lymphocytes	Ph 2	ALSFRS-R + SVC	26
SM	Minocycline	Suppresses proinflammatory microglial activation	Ph 3 failure	ALSFRS-R	27
SM	Pioglitazone	PPAR- γ agonist, suppresses NF- κ B signaling	Ph 2 failure	Overall survival	28
SM	DNL747	RIPK1 inhibitor, suppresses proinflammatory microglial cytokine production	Ph 1	Safety, tolerability	29
SM	Fasudil	ROCK inhibitor, suppresses proinflammatory microglial cytokine production	Ph 2A	Safety, tolerability	30
SM	Ono-2506	Arundic acid, modulates astrocyte activity	Ph 2 failure	SVC	31
Protein	Anakinra	IL-1 receptor antagonist	Ph 1	Safety, tolerability	32
Nanobubbles	RNS60	Suppresses microglial NF- κ B	Ph 2	ALSFRS-R	33

(Continues)

TABLE 1 (Continued)

Modality	Drug name	Mechanism of action	Phase/outcome	Primary endpoint	References	
Antiepileptory	SM	Riluzole	Reduces glutamatergic neurotransmission, Na ⁺ channel blocker	Approved	Tracheostomy-free survival	34–36
	SM	Retigabine	K ⁺ channel agonist	Ph 2	SICI	37,38
	SM	Memantine	NMDA receptor antagonist	Ph 2 failure	ALSFRS-R	39
	SM	Talampanel	AMPA receptor antagonist	Ph 2 failure	Isometric arm strength	40
	SM	Perampanel	AMPA receptor antagonist	Ph 2	ALSFRS-R	41,42
	SM	Mexiletine	Na ⁺ channel blocker	Ph 2 failure	ALSFRS-R	43,44
	SM	Ceftriaxone	Increases astrocytic EAAT2	Ph 3 failure	Coprimary survival + ALSFRS-R	45
	SM	Celecoxib	COX2 inhibitor, suppresses astrocytic glutamate release	Ph 2 failure	MVIC	46
	SM	Lamotrigine	Na ⁺ channel blocker	Ph 2 failure	Norris scale	47
	SM	Nimodipine	Ca ⁺⁺ channel blocker	Ph 2 failure	FVC	48
	SM	Gabapentin	Blocks glutamate synthesis, Ca ⁺⁺ channel blocker	Ph 3 failure	Arm muscle strength	49
	Antioxidants	SM	Edaravone	Free radical scavenger	Approved	ALSFRS-R
SM		Epi-589	Modulates levels of glutathione	Ph 2	Safety	N/A
SM		N-Acetylcysteine	Radical oxygen scavenger (glutathione precursor)	Ph 2 failure	Survival*	51
Vitamin		Vitamin E	Radical oxygen scavenger	Ph 2 failure	Norris limb score, survival*	52,53
SM		Coenzyme Q10	Radical oxygen scavenger, cofactor in mitochondrial electron transport chain	Ph 2 failure	ALSFRS-R	54
SM		Selegiline	MAO-B inhibitor; upregulates SOD, catalase activity	Ph 2 failure	AALRS	55
SM		Rasagiline	MAO-B inhibitor	Ph 2 failure	Time to death, ALSFRS-R	56,57
SOD1-targeting		SM	Cu(II)-ATSM	Ph 1/2	Safety, tolerability	58
		ASO	BIIB067	Ph 1	Safety, tolerability	59,60
		SM	Pyrimethamine	Ph 1/2	Change in CSF SOD1	61
Troponin binders		SM	Levosimendan	Ph 3	Supine SVC	58,62
		SM	Tirasemtiv	Ph 3 failure	SVC	43,63
	SM	CK-2127107	Ph 2	SVC	64	

TABLE 1 (Continued)

Modality	Drug name	Mechanism of action	Phase/outcome	Primary endpoint	References
(Neuro)trophic factors					
Polypeptide	IGF-1	Recombinant human IGF-1	Ph 3 mixed/failure	AALSRS, MMT score	65-67
SM	Xaliproden	5-HT _{1A} agonist, nonpeptide neurotrophic factor enhancer	Ph 3 failure	Survival,* time to VC <50%	68
Polypeptide	EPO	Recombinant human erythropoietin	Ph 3 failure	Survival*	69
Polypeptide	BDNF	Recombinant human BDNF	Ph 3 failure	FVC, 9-mo survival	70
Peptide	GM604	Peptide fragment of motoneurotrophic factor	Ph 2A	Safety, tolerability, biomarkers	71
Polypeptide	CNTF	Recombinant human CNTF	Ph 2/3 failure	Isometric muscle strength, MVIC + FCV	72,73
Plasmid	SB509	ZFP transcription factor for expression of VEGF	Ph 2	ALSFRS-R	74
Plasmid	VM202	Expression of HGF	Ph 1/2	Safety, tolerability	75
Plasmid	sNN0029	Expression of VEGF165	Ph 1/2	Safety, tolerability	76
Polypeptide	KP-100	Recombinant human HGF	Ph 1	Safety, tolerability	77
Modulators of mitochondrial function					
SM	Dexpropimexole	Inhibits mitochondrial permeability transition pore	Ph 3 failure	CAFS	78
SM	Olexosime	Inhibits mitochondrial permeability transition pore	Ph 2/3 failure	18-mo survival	79
SM	Creatine	Increases mitochondrial ATP production	Ph 2 failure	Survival,* MVIC	80-83
SM	Acetyl-L-carnitine	Transports long-chain fatty acids across mitochondrial membrane	Ph 2	Loss of self-sufficiency	84
Autophagy inducers					
SM	Lithium	Inhibits inositol monophosphatase	Ph 3 failure	18-mo survival	85,86
SM	Rapamycin	mTOR inhibitor	Ph 2	Treg number	87
SM	Tamoxifen	Increases beclin-1	Ph 2	Arm strength	88
Other therapeutic approaches					
Antibody	Ozanezumab	Targets NOGO-A	Ph 2 failure	Joint rank score ALSFRS-R + 48-wk survival	89
SM	GDC-0134	DLK inhibitor, suppresses JNK-dependent stress response	Ph 1	Safety, tolerability	90
N/A	YAM80	N/A	Ph 2	Safety, ALSFRS-R	91

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TABLE 1 (Continued)

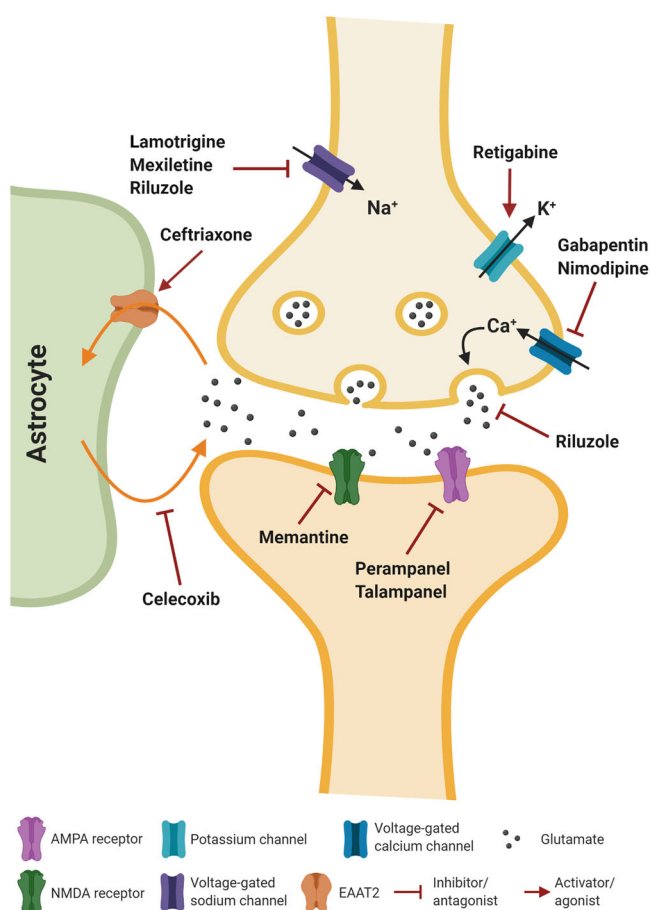
Modality	Drug name	Mechanism of action	Phase/outcome	Primary endpoint	References
Vitamin	Methylcobalamin	Active vitamin B12, decreases levels of homocysteine	Ph 2/3 failure	Survival* + ALSFRS-R	92
SM	Pimozide	T-type calcium channel blocker, strengthens NMJs	Ph 2	ALSFRS-R	93
SM	Omigapil/TCH346	Antiapoptotic, binds GAPDH	Ph 2/3 failure	ALSFRS-R	94
SM	Deferiprone	Iron metal chelator	Ph 2, 3	CAFS	95
SM	AMX0035	Upregulation of heat shock proteins	Ph 2	Safety, tolerability, ALSFRS-R	96,97
SM	Arimoclomol	Induces heat shock protein expression—targeting of misfolded proteins	Ph 3	ALSFRS-R, CAFS, survival*	98
SM	EH301	Nicotinamide riboside, boosts levels of NAD+	Ph 2	ALSFRS-R	99
ASO	BIIB078	ASO targeting C9ORF72	Ph 1	Safety, tolerability	100
SM	MD1003	High dose biotin, supports remyelination	Ph 2	Safety, tolerability, hematology panel	101

Note: The two drugs in bold indicate that they are the approved drugs.

Abbreviations: AALSRS, Appel amyotrophic lateral sclerosis rating scale; ACTH, adrenocorticotrophic hormone; ALSFRS-R, revised amyotrophic lateral sclerosis functional rating scale; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ASO, antisense oligonucleotide; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; CAFS, combined assessment of function and survival; CNTF, ciliary neurotrophic factor; CSF, cerebrospinal fluid; CSF-1R, colony stimulating factor 1 receptor; DLK, dual leucine zipper kinase; EAAT2, excitatory aminoacid transporter 2; EPO, erythropoietin; FVC, forced vital capacity; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1; IL-6R, interleukin-6 receptor; JNK, c-Jun N-terminal kinase; MAALS, mean Appel ALS score; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MMT, manual muscle testing; MAO-B, monoamine oxidase-B; mTOR, mammalian target of rapamycin; MVIC, maximum voluntary isometric contraction; NF- κ B, nuclear factor κ B; NMJ, neuromuscular junction; PDE, phosphodiesterase; PFT, pulmonary functional testing; PPAR- γ , peroxisome proliferator-activated receptor γ ; RIPK1, receptor-interacting serine/threonine-protein kinase 1; S1PR1, sphingosine-1-phosphate receptor 1; SICI, short-interval intracortical inhibition; SVC, supine vital capacity; TNF- α , tumor necrosis factor α ; VC, vital capacity; VEGF, vascular endothelial growth factor; ZFP, zinc finger protein.

*Survival defined as time to death, long-term ventilation, or tracheostomy.

FIGURE 1 Antiexcitatory clinical treatment strategies for ALS. Lamotrigine, mexiletine, and riluzole inhibit voltage-gated sodium channels. Riluzole furthermore inhibits the release of glutamate into the synaptic cleft through sodium channel-independent mechanisms. Retigabine is an activator of voltage-gated potassium channels. Gabapentin and nimodipine inhibit voltage-gated calcium channels. Memantine is an NMDA receptor antagonist approved for the symptomatic treatment of Alzheimer's disease. Perampanel and telampanel are AMPA receptor inhibitors. Ceftriaxone promotes the uptake of glutamate by astrocytes while the COX2 inhibitor celecoxib inhibits the release of glutamate from astrocytes. ALS, amyotrophic lateral sclerosis; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EAAT2, excitatory aminoacid transporter 2; NMDA, *N*-methyl-D-aspartate [Color figure can be viewed at wileyonlinelibrary.com]



safely and reversibly opened using transcranial magnetic resonance-guided focused ultrasound (MRgFUS).¹⁰⁸ Thus, in the future, MRgFUS may be coupled with therapeutics with limited BBB permeability such as riluzole to improve therapeutic efficacy.

The exact mechanism of action (MOA) of riluzole is not entirely understood, as a multitude of studies, many of them carried out *in vitro* and at nonphysiological concentrations, have identified various antiexcitatory features of this drug (for a detailed review of riluzole's MOA, see Reference¹⁰⁹). At concentrations approximating those reached in the human brain, riluzole inhibits fast and persistent sodium currents, inhibits presynaptic glutamate release and may also increase glutamate reuptake.

Despite intense efforts to identify other therapeutic interventions targeting excitatory signaling, riluzole remains the only approved drug in this class. Other therapies that modulate concentrations of glutamate at synapses were not effective. For example, removing excess glutamate from the synaptic cleft by increasing expression of the glutamate transporter EAAT2/SLC1A2 with ceftriaxone did not improve functional decline or survival in a phase 3 trial.⁴⁵ Another trial examining the effects of nonsteroidal anti-inflammatory celecoxib also disappointed.⁴⁶ Celecoxib is an inhibitor of cyclooxygenase-2, an enzyme in the synthesis pathway of prostaglandin E2, which stimulates the release of glutamate from astrocytes.¹¹⁰

Other potential therapeutic approaches that directly target glutamate receptors also fared poorly (Figure 1). Memantine, an *N*-methyl-D-aspartate receptor antagonist used to improve cognitive function in Alzheimer's disease, failed to slow the rate of functional decline in the ALS functional rating scale revised (ALSF_{RS}-R),³⁹ a compound score comprising

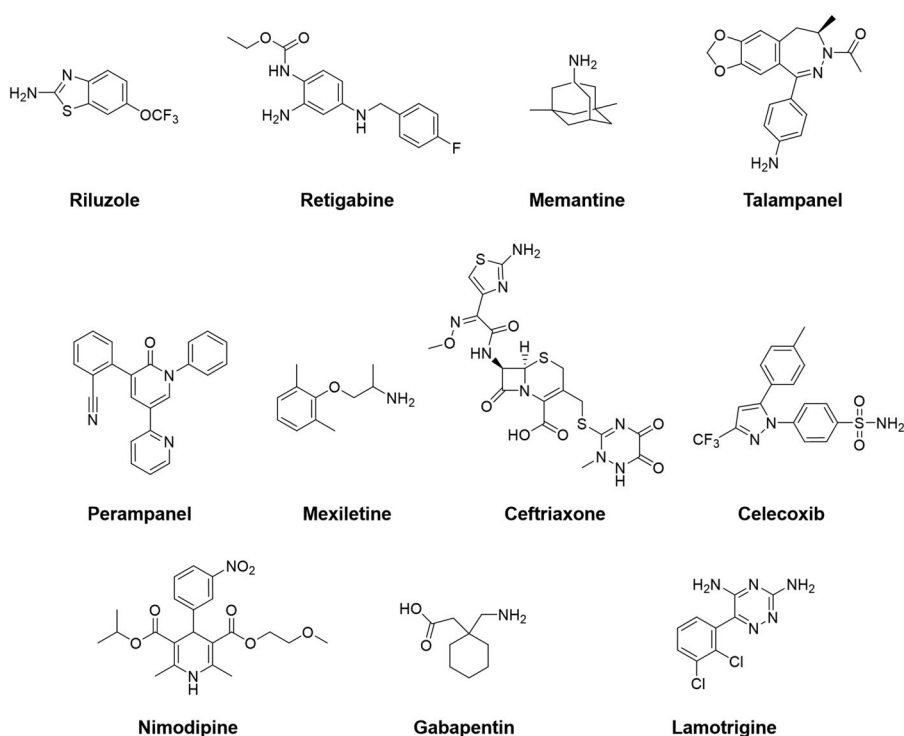


FIGURE 2 Antiexcitatory small molecules in clinical trials for amyotrophic lateral sclerosis

12 functional categories including speech, handwriting, swallowing, salivation, walking, stair climbing, and respiration.¹¹¹ An α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, talampanel, showed a potential effect on muscle strength and function in a small phase 2 study, but failed a subsequent larger, unpublished trial.⁴⁰ Perampanel, another AMPA receptor antagonist with a longer half-life than talampanel, demonstrated preservation of motor neurons and motor function in an ALS mouse model,⁴¹ and is under investigation in a phase 2 study.⁴² Compounds that modulate voltage-gated ion channels have also undergone clinical testing. For example, the potassium channel opener retigabine, an approved antiepileptic drug recently withdrawn from the market, is being tested in the clinic based on data showing improved neuronal excitability and survival of ALS patient-derived induced pluripotent stem cell (iPSC)-derived motor neurons harboring the SOD1 A4V mutation.³⁷ The sodium channel blockers lamotrigine and mexiletine failed to slow down the rate of functional decline but may prove beneficial as a symptomatic treatment for muscle cramps.^{43,44} The γ -aminobutyric acid analogue gabapentin is thought to suppress excitotoxicity by inhibiting branched-chain aminotransferase, an enzyme in the glutamate synthesis pathway, as well as by inhibiting L-type calcium channels.¹¹² However, gabapentin failed to slow the decline of arm muscle strength in a phase 3 trial.⁴⁹ Another L-type calcium channel blocker, nimodipine, did not progress to a larger study after a trial of 87 patients suggested that it was ineffective in slowing down the decline in forced vital capacity (FVC), a measure of respiratory function.⁴⁸

2.2 | Edaravone and other antioxidant therapeutic strategies

The only other current ALS therapy, edaravone, was approved based on a significant effect on the rate of decline in the ALSFRS-R in a subset of Japanese ALS patients.⁵⁰ Furthermore, a recently published retrospective study of a small number of patients suggested that edaravone prolongs tracheostomy-free survival in human patients.¹¹³

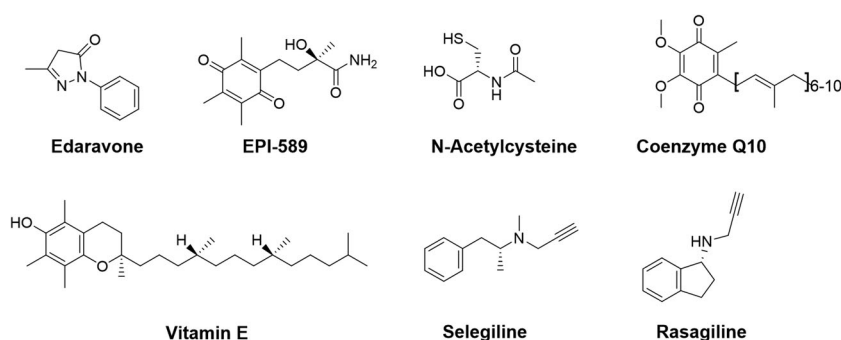


FIGURE 3 Antioxidants small molecules in clinical trials for amyotrophic lateral sclerosis

However, the small number of subjects and imbalances in the number of patients with the more aggressive bulbar-onset form of the disease between the edaravone and control groups could have skewed the survival outcome numbers. A 5-year prospective observational study (SUNRISE) investigating the effects of edaravone on survival is ongoing.¹¹⁴

The precise MOA of edaravone is unknown. It is a radical oxygen scavenger shown to protect both neurons and glia from oxidative stress.¹¹⁵ Oxygen radicals are thought to contribute to the degeneration of motor neurons in ALS, and markers of oxidative stress are upregulated in patient tissue.¹¹⁶ Edaravone may reduce oxidative stress in ALS and other models of disease.^{117–119} In cells exposed to oxidative stress, edaravone increases the expression of peroxiredoxin 2, which reduces hydrogen peroxide.¹²⁰ Furthermore, edaravone increases expression of nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor that regulates the expression of antioxidant proteins.¹²¹ Overexpression of NRF2 in astrocytes improved survival in the SOD1-G93A mouse model of ALS.¹²²

However, with the exception of edaravone, antioxidant therapeutic strategies have thus far failed (chemical structures of edaravone and other antioxidants, see Figure 3). The radical oxygen scavengers *N*-acetylcysteine, vitamin E, and coenzyme Q10 did not show significant beneficial effects in their respective clinical studies.^{51–54} One reason for the failure of these compounds might be poor or no BBB permeability. Monoamine oxidase-B (MAO-B) inhibitors have also been studied as potential interventional treatments for ALS. MAO-B inhibitors such as selegiline and rasagiline are approved for the management of Parkinson's disease and work by suppressing the breakdown of dopamine. A byproduct of this enzymatic reaction is hydrogen peroxide. Thus, inhibition of MAO-B in ALS could decrease the oxidative stress burden on motor neurons. Furthermore, *in vivo* studies demonstrated that selegiline displayed antioxidant properties in the striatum of aged rats at high concentrations by increasing the activity of SOD and catalase.^{123,124} Selegiline failed to decelerate functional decline measured using the Appel ALS rating scale,¹²⁵ but was administered in doses far below those used to elicit its antioxidative effects.⁵⁵ More recently, the second-generation MAO-B inhibitor rasagiline also showed no beneficial effects on survival and disease progression in two studies.^{56,57} In a different strategy to combat oxidative stress in ALS, Epi-589 purportedly increases levels of the antioxidant glutathione and is now being trialed in a phase 2 study. However, to date, there have been no publications detailing its MOA or its effects in animal models of ALS.¹²⁶

3 | CLINICAL CANDIDATES

3.1 | Anti-inflammatory/immune-modulatory drugs

Motor neuron death and skeletal muscle denervation are the defining, though not the only, pathological features of ALS. The factors that drive this selective degeneration in ALS patients are still poorly understood and may vary

between cases. Many lines of evidence suggest that cell autonomous as well as noncell autonomous mechanisms can drive the degeneration of motor neurons. In recent years, neuroinflammation has emerged as a process closely linked to neurodegeneration. Aberrant activation of astrocytes^{127,128} and microglia,^{129,130} as well as infiltration of CD4⁺ and CD8⁺ T lymphocytes,¹³¹ have been demonstrated in ALS patient tissue. Furthermore, coculture systems using astrocytes, microglia, or T cells of mouse primary or human iPSC origin have shown that these cells exert direct cytotoxic effects on motor neurons.^{127,128,132–134} These cytotoxic effects might at least in part be mediated through proinflammatory cytokines or other factors released as a result of inflammatory signaling.

Various classes of anti-inflammatory drugs are aimed at suppressing the production of proinflammatory cytokines and chemokines as well as the proliferation and polarization of glial cells, through mechanisms that are not always completely understood (Figures 4 and 5). Several of these drugs have failed to prove efficacious in ALS clinical trials. Two approved drugs with anti-inflammatory properties, the peroxisome proliferator-activated receptor γ agonist pioglitazone, used for the treatment of type 2 diabetes, and the antibiotic minocycline, protected neurons from degeneration and improved survival in SOD1 mouse models of ALS.^{135,136} However, both failed in clinical trials, with minocycline proving harmful to patients as evidenced by a faster rate of decline in the ALSFRS-R compared with placebo-treated patients.^{27,28} NP001, a pH-adjusted intravenous formulation of sodium chlorite, which has been shown to decrease expression of nuclear factor κ B (NF- κ B), did not slow down functional decline in a phase 2 study.^{21,22} Other drugs that target glial responses through inhibition of rho kinase (ROCK; fasudil), NF- κ B, and mitogen-activated protein kinase signaling (CC100) or phosphodiesterases (PDEs; ibudilast) are being trialed in ALS patients.^{15,25,30} Ibudilast recently garnered positive feedback from the FDA to commence a phase 3 study. Previously, treatment with pentoxifylline, another nonselective PDE inhibitor, led to a worse outcome in 18-month survival in a phase 3 trial.¹⁶ Thalidomide, which reduces messenger RNA (mRNA) levels of the inflammatory cytokine tumor necrosis factor α , was not effective in a small, open-label study compared with historical data, and may have had adverse effects on disease outcome.²³ Ono-2506 was developed as a modulator of astrocyte activity and was shown to suppress the expression of S110b and GFAP. A phase 2 study failed to show a therapeutic benefit in pulmonary function.³¹ The most unconventional anti-inflammatory therapeutic agent undergoing clinical testing in a phase 2 trial for ALS is perhaps RNS60. It consists of charge-stabilized, oxygen-filled nanobubbles in a saline solution. RNS60 has been shown to exert its anti-inflammatory effects by suppressing activation of the NF- κ B pathway.^{33,137}

The receptor-interacting serine/threonine-protein kinase 1 (RIPK1) kinase inhibitor DNL747 was recently shown to be safe in a phase 1 study.²⁹ It is thought to work by suppressing RIPK1-dependent production of proinflammatory cytokines in microglia. It has also been shown to prevent RIPK1-dependent death of oligodendrocytes in two ALS mouse models.^{138,139} Masitinib, a CSF1R/c-kit inhibitor that reduces proliferation of inflammatory microglia in the SOD1-G93A rat model of ALS,¹⁴⁰ met its primary endpoint, a change in the ALSFRS-R score in a phase 2/3 study.^{17,18} However, the European Medicine Agency's Committee for Medicinal Products for Human Use subsequently recommended refusal of marketing authorization due to concerns about the reliability of the results and its safety.^{19,20}

Other anti-inflammatory treatment strategies in ALS focus on T lymphocytes (Figure 4). Fingolimod, an approved drug for the treatment of relapsing-remitting multiple sclerosis (MS), binds to and causes the internalization of the sphingosine-1-phosphate receptor 1. As a result, T cells become trapped in lymph nodes, reducing the number of circulating cells.^{26,141} Another approved MS drug, glatiramer acetate, which is thought to exert its effects by modulating the proliferation, migration, and balance of T helper cells¹⁴² failed to slow the rate of decline in the ALSFRS-R score in a phase 2 trial.²⁴ More recently, the MIROCALS study was launched to investigate the efficaciousness of interleukin 2 (IL-2), which promotes the differentiation of regulatory T cells.¹⁴

The aforementioned drugs seek to modulate proinflammatory activation and cytokine release by glia, T cells, or macrophages. By contrast, other interventional therapeutic strategies are aimed at suppressing the downstream effects of proinflammatory cytokines. Tocilizumab is an antibody targeting the interleukin-6 receptor (IL-6R) and is approved for the treatment of several forms of arthritis. A phase 2 trial was recently completed, but no results have been published as of the date of this publication.^{10,11} Another approved rheumatoid arthritis drug, the IL-1 receptor antagonist anakinra, was deemed safe in a single-arm pilot study in ALS patients.³²

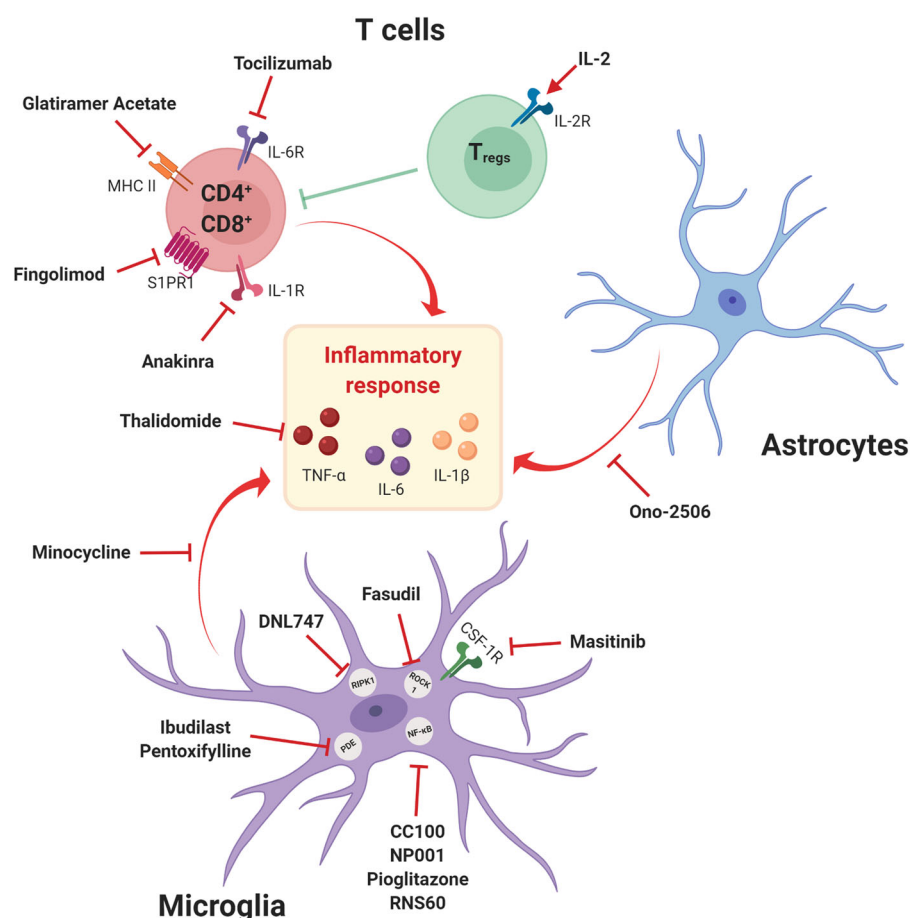


FIGURE 4 Anti-inflammatory clinical treatment strategies for ALS. Astrocytes, microglia, and T cells promote inflammation in ALS, causing immune cell infiltration and the production of inflammatory cytokines. Anti-inflammatory strategies suppress proinflammatory signaling. Ono-2506 suppresses astroglial activation. Various small molecules are under investigation that suppress microglial activation including masitinib (inhibitor of CSF-1R), fasudil (ROCK1 inhibitor), DNL747 (RIPK1 inhibitor), and the PDE inhibitors pentoxifylline and ibudilast. Several therapeutics are aimed at suppressing NF-κB signaling through various and sometimes unknown mechanisms including CC100, NP001, pioglitazone (an approved drug for the treatment of diabetes), and RNS60. Minocycline suppresses microglial cytokine production through unknown mechanisms. Thalidomide decreases levels of TNF-α. Other therapies are aimed at suppressing T cell response including antibodies that target IL-1R (anakinra) and IL-6R (tocilizumab) and thus suppress signaling through these receptors. Glatiramer acetate, approved for the treatment of multiple sclerosis, is a mixture of peptides resembling myelin basic protein and modulates the reactivity of T cells. IL-2 peptide activates regulatory T cells that modulate T cell response. ALS, amyotrophic lateral sclerosis; IL, interleukin; IL-1R, interleukin receptor 1; MHC II, major histocompatibility complex class II; NF-κB, nuclear factor κB; RIPK1, receptor-interacting serine/threonine-protein kinase 1; PDE, phosphodiesterase; TNF-α, tumor necrosis factor α [Color figure can be viewed at wileyonlinelibrary.com]

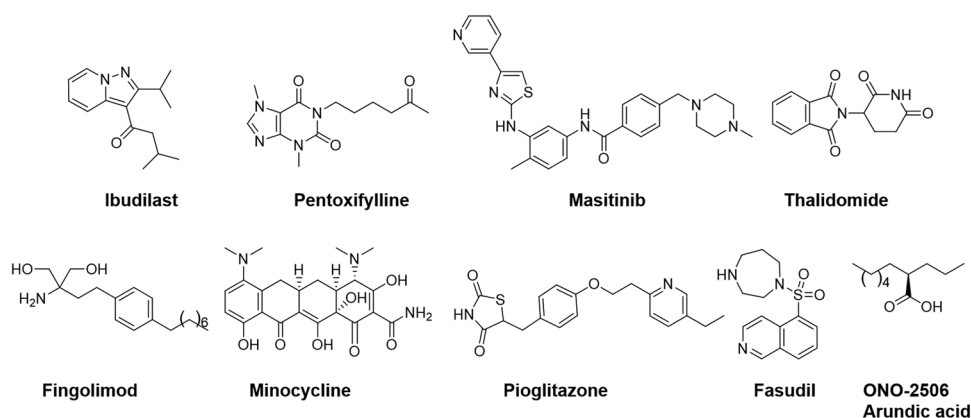


FIGURE 5 Anti-inflammatory/immune-modulatory small molecules in clinical trials for amyotrophic lateral sclerosis

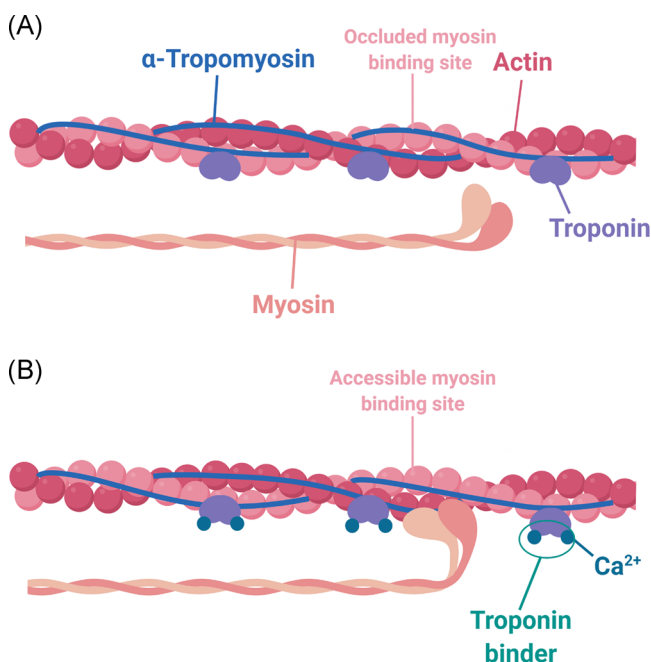
3.2 | Troponin binders

Dysfunction and degeneration of spinal cord motor neurons underlie the progressive muscle atrophy in ALS, as muscle fibers do not receive sufficient electrical input. Thus, increasing muscle contraction in response to a given input signal could slow down the deterioration of muscle strength in ALS. With this hypothesis in mind, the first-generation fast skeletal muscle activator tirasemtiv (CK-2017357) was developed. Tirasemtiv binds to fast skeletal muscle troponin and increases its affinity for calcium (Figures 6 and 7A).¹⁴³ While a phase 2 study failed to show an effect of tirasemtiv on the rate of functional decline in the ALSFRS-R, muscle strength and slow vital capacity (SVC) declined more slowly.¹⁴⁴ However, these results could not be confirmed in a subsequent phase 3 study, possibly due to a high rate of dropouts in the high dose cohort as a result of poor tolerability.⁶³ A second-generation fast skeletal muscle troponin activator, CK-2127107 (Figure 7A), is thought to be more potent and better tolerated⁶⁴ and is under investigation in a phase 2 trial. Oral levosimendan, a troponin binder and calcium sensitizer in fast and slow skeletal muscle fibers, recently completed a phase 2 trial. The primary outcome—SVC when sitting—did not show significant differentiation compared with the placebo. The secondary efficacy variable, supine SVC, improved in the levosimendan groups while it deteriorated in the placebo group.⁶² Despite missing its endpoints, a phase 3 trial was launched in 2018 to investigate positive trends in supine SVC observed in the phase 2 study.⁵⁸

3.3 | SOD1-targeting therapeutic approaches

SOD1 is a cytoplasmic copper/zinc-binding enzyme that converts superoxide radicals (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2). Mutations in SOD1 were the first identified genetic cause of ALS, accounting for 5% to 10% of familial cases.¹⁴⁵ Thus, therapeutic strategies aimed at mutant SOD1 present a targeted therapeutic approach tailored to a well-defined patient population (chemical structures of SOD1-targeting small molecules, see Figure 7B). Several ALS-associated mutations confer poor binding affinity for copper and zinc ions to SOD1,¹⁴⁶ which may affect SOD1 stability, aggregation behavior, and binding to other proteins. Thus, delivery of zinc or copper to mutant SOD1 may be beneficial in SOD1-ALS. Indeed, treatment of mutant SOD1 mouse models with Cu(II)-ATSM has been shown to improve locomotor function and survival and preserve motor neurons, despite increased levels of total SOD1 in the spinal cord.^{147,148} A phase 1 dose escalation and pharmacokinetic study began in 2016, and is open to both familial ALS patients with SOD1 mutations as well as sporadic patients.¹⁴⁹

FIGURE 6 The actin-myosin cross-bridge enables muscle contraction. A, Relaxed muscle. The myosin-binding site on actin is partially occluded by tropomyosin, preventing myosin from forming an actin-myosin cross-bridge. B, Muscle contraction. Ca^{2+} binds to troponin, causing a conformational shift that causes tropomyosin to unblock the myosin-binding site on actin. Myosin binds to the accessible binding site, forming an actin-myosin cross-bridge. Small-molecule troponin binders increase sensitivity to Ca^{2+} , thus increasing the force of muscle contraction [Color figure can be viewed at wileyonlinelibrary.com]



Several lines of evidence suggest that mutant SOD1 exerts its detrimental effects through a gain-of-function rather than a loss-of-function mechanism. While mutant SOD1-expressing mice develop ALS phenotypes, SOD1 knockout mice do not.¹⁵⁰ Furthermore, while some ALS-associated SOD1 mutations cause a decrease in dismutase activity, others have little effect or even increase activity.¹⁵¹ Perhaps most tellingly, knocking out murine SOD1 in an ALS mouse model expressing the enzymatically inactive G85R mutant has no effect on survival.¹⁵² Thus, rather than altering the metal-binding status of SOD1, other interventional strategies are aimed at reducing total SOD1 protein levels. Pyrimethamine, a drug used to treat toxoplasmosis and human immunodeficiency virus-associated pneumonia, was shown to reduce SOD1 levels in cerebrospinal fluid (CSF) of ALS patients with SOD1 mutations by ~13.5%.⁶¹ Antisense oligonucleotides (ASOs) targeting SOD1 mRNA were beneficial in the SOD1-G93A rat model of ALS.⁵⁹ A first study in man demonstrated that intrathecal delivery of a SOD1 ASO was safe but did not decrease SOD1 protein levels in the CSF at the low doses used.⁶⁰ A phase 1 trial of the second-generation ASO BIIB067 (IONIS-SOD1Rx) commenced in 2016. While detailed results of the study were not publicly available at the time of the submission of this article, an interim analysis showed that treatment with BIIB067 over a 3-month period led to lower SOD1 levels in the CSF and a trend toward a slower rate of decline in the ALSFRS-R.¹⁵³ In addition to ASOs, SOD1 mRNA can also be targeted by RNA interference approaches. A single administration of an adeno-associated virus gene therapy vector (VY-SOD101) has been shown to reduce levels of SOD1 mRNA in spinal cord motor neurons by greater than 70% in non-human primates.¹⁵⁴ However, this therapeutic strategy has not yet entered the clinic.

3.4 | Autophagy enhancers

Autophagy is the process by which long-lived, misfolded and aggregated proteins, as well as organelles, and pathogens are cleared from cells under homeostatic and pathological conditions.^{155,156} Several lines of evidence indicate that impaired autophagy is fundamentally implicated in the pathogenesis of ALS. As in other age-related neurodegenerative diseases, a

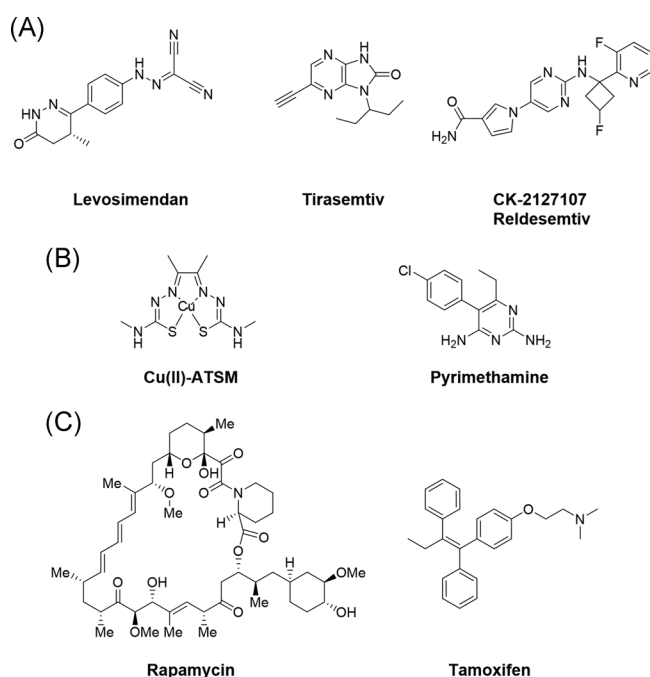


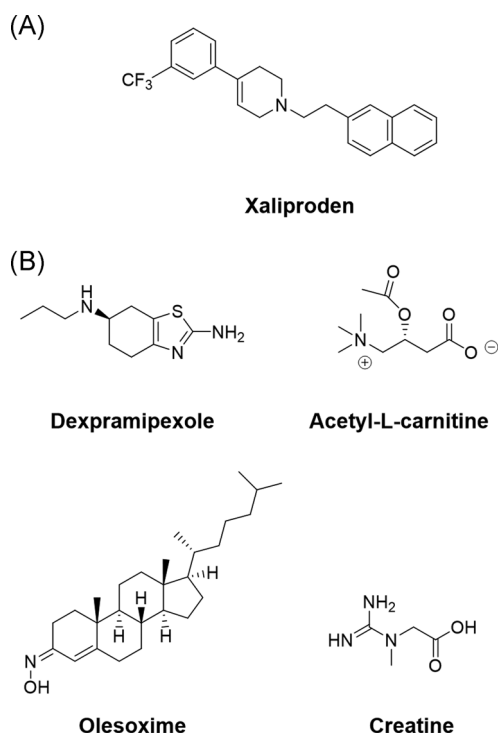
FIGURE 7 Small molecules in clinical trials for amyotrophic lateral sclerosis. A, Troponin binders. B, SOD1-targeting small molecules. C, autophagy enhancers. SOD1, superoxide dismutase 1

major histopathological hallmark of ALS is the accumulation of misfolded proteins in insoluble aggregates, suggesting that autophagic clearance of these misfolded proteins is insufficient. In 97% of ALS cases, the major protein found in these inclusions of aggregated protein is a RBP with a PrLD, TDP-43.⁹ Second, mutations in a remarkable number of autophagy genes cause ALS, including *C9ORF72*, *UBQLN2*, *TBK1*, *VCP*, and *OPTN*.⁴ Last, *post mortem* tissues of human patients and ALS mouse models display markers of impaired autophagy.^{157–160} Thus, therapeutic strategies aimed at enhancing autophagy could be a viable therapeutic avenue for the treatment of ALS (chemical structures of small-molecule autophagy enhancers, see Figure 7C). Lithium is an inhibitor of inositol monophosphatase, leading to decreased levels of inositol 1,4,5-trisphosphate, a negative regulator of mammalian target of rapamycin (mTOR)-independent autophagy. However, lithium did not improve survival in phase 2 and 3 studies.^{85,86} A phase 2 trial investigating the effects of rapamycin is ongoing.⁸⁷ Rapamycin is an inhibitor of the mammalian target of rapamycin complex 1, a complex containing the kinase mTOR that serves as a master regulator of cell growth, survival, metabolism, autophagy, and protein synthesis.¹⁶¹ mTOR inhibits autophagy by phosphorylating Unc-51 like autophagy activating kinase 1, which regulates the initiation of autophagy. However, mTOR inhibitors do not robustly stimulate autophagy in neurons,^{162,163} and thus in the brain are only likely to stimulate autophagy in neighboring glia. It is unclear whether stimulating glial autophagy alone will be neuroprotective in ALS. Novel compounds that stimulate neuronal autophagy of TDP-43 are beginning to emerge.¹⁶⁴ A clinical study examining tamoxifen, which increases levels of beclin-1, a central protein in autophagosome formation and maturation, was completed with no results yet published.^{87,88}

3.5 | Neurotrophic factors

Growth factors have long been studied as a therapeutic avenue for the treatment of neurodegenerative diseases including ALS. Neurotrophic factors promote prosurvival signaling, axon outgrowth, synapse formation, and metabolic function, and confer protection against toxic or oxidative insults and injury.^{165,166} However, clinical studies thus far

FIGURE 8 Small molecules in clinical trials for amyotrophic lateral sclerosis. A, Xaliproden, a small-molecule neurotrophic factor. B, Modulators of mitochondrial function



have not yielded any viable therapies. Subcutaneously administered brain-derived neurotrophic factor, ciliary neurotrophic factor (CNTF), and insulin-like growth factor 1 were not shown to be beneficial in large-scale studies.^{65–67,70,72,73} This could be due to low brain exposure resulting from insufficient dosing, short protein half-life, or lack of BBB penetration.¹⁶⁵ Furthermore, intravenous injection of the hematopoietic growth factor erythropoietin did not improve survival in a phase 3 study.⁶⁹ Intravenously administered GM604, a peptide sequence identical to an active site of motoneurotrophic factor, was recently found safe,⁷¹ as was intrathecal injection of human recombinant hepatocyte growth factor.⁷⁷ Xaliproden is a 5-HT_{1A} agonist and the first discovered nonpeptide neurotrophic factor enhancer (Figure 8A). It prolongs neuronal survival in vitro and in vivo and enhances the secretion of neurotrophic factors. In two phase 3 trials to assess xaliproden as a monotherapy and in combination with riluzole, primary outcome measures (survival and vital capacity <50%) did not justify clinical approval.⁶⁸

Given issues of short half-lives or poor BBB penetration, producing neurotrophic factors at the site of motor neuron degeneration may result in better outcomes. This could be achieved by intramuscular or intracerebroventricular administration of plasmids encoding neurotrophic factors or transcription factors that regulate the expression of neurotrophic factors, and trials investigating this approach have been initiated.^{74–76}

3.6 | Modulators of mitochondrial function

Mitochondrial dysfunction is a central feature of ALS and manifests in a variety of ways. Disturbances in energy metabolism, impairment of mitochondrial fission and transport and abnormal mitochondrial morphology have been described in various cell and animal models (for a thorough review describing the role of mitochondria in ALS, see Reference¹⁶⁷). Thus, therapeutic treatment strategies have been aimed at rescuing mitochondrial function. Dexpramipexole and olesoxime are inhibitors of the mitochondrial permeability transition pore, a nonselective mitochondrial inner membrane channel that

opens in response to insults such as excitotoxicity, excess oxidative stress, or decreased adenosine triphosphate (ATP) levels (Figure 8B). Opening of the transition pore causes the breakdown of the electrochemical proton gradient, increases the production of reactive oxygen species and disrupts ATP production.¹⁶⁸ Both drugs failed to improve survival in their respective trials.^{78,79} Creatine supplementation to boost the intracellular pool of phosphocreatine, which acts as an energy buffer for regeneration of ATP, was also not found to be efficacious in several clinical studies (Figure 8B).^{80–83} A small study investigating the effects of acetyl-L-carnitine, which improves transportation of fatty acids across mitochondrial membranes via the carnitine shuttle,⁸⁴ found a significant effect on the proportion of patients becoming non-self-sufficient—an unusual primary endpoint, but thus far no larger follow-up study has been reported.

4 | STEM CELL APPROACHES

Another way of improving trophic support locally in affected tissues is stem cell transplantation. Stem cells can differentiate into support cells such as astrocytes, oligodendrocytes, or microglia, which may benefit degenerating motor neurons by producing growth factors and anti-inflammatory cytokines, providing nutrients and buffering excessive glutamate. A variety of stem cell therapies have entered early clinical phases (for a comprehensive overview of stem cell trials in ALS see Reference¹⁶⁹). Mesenchymal stem cells (MSCs) can be harvested from bone marrow or adipose tissue.¹⁷⁰ As they are an autologous source of stem cells, transplant recipients do not require treatment with immunosuppressants. The most common delivery routes are intrathecal or intramuscular. Most current trials are small and primarily aimed at evaluating safety. However, a phase 3 study of bone-marrow derived MSCs induced to secrete neurotrophic growth factor was initiated and will assess efficacy using the ALSFRS-R.^{171,172}

Neural stem cells (NSCs) are pluripotent stem cells capable of differentiating into neural and glial cell types. Early theories posited that NSC transplantation could replace degenerating motor neurons in ALS. However, these neurons would need to integrate into and form functional circuits while projecting axons over great distances.¹⁷³ It is thus more likely that NSC transplantation therapies could confer therapeutic benefit by differentiating into glial cells and interneurons that provide trophic and structural support and suppress excitotoxicity. Results of phase 1/2 studies have demonstrated that intraspinal transplantation of human spinal cord-derived NSCs is safe and well-tolerated, and that grafts can survive up to 2.5 years.^{174–177} Comparison of a phase 1/2 study to historical datasets suggested a significant clinical benefit in functional decline measured using the ALSFRS-R.¹⁷⁸ Due to the invasive nature of stem cell implantation, early phase trials should be carried out without a sham surgery control group. Rather, outcome measures are compared with historical controls or to data gathered from trial participants before the procedure. Where the data suggest a significant benefit of the stem cell procedure, a controlled follow-up trial with a sham surgery control group is then warranted to substantiate the findings.

4.1 | Why have drugs failed in ALS clinical trials?

In over 20 years of clinical trials only two drugs—riluzole and edaravone—have been approved for the treatment of ALS. Many more failed to efficaciously stall the disease, or worse had deleterious effects. There are many possible reasons for these failures. First, the drug target itself may not be appropriate to treat the disease. It is difficult to either prove or disprove this argument, and the intention of this review is not to critique the choice of target of any one clinical trial. However, the question of appropriate target selection leads to another problem: the choice of animal models for in vivo experiments. The most frequently used mammalian models of ALS are mutant SOD1 transgenic animals. However, given that only ~10% of familial and 1% of sporadic patients carry SOD1 mutations,⁶ efficacy in mutant SOD1 transgenic mice may be inadequate to predict efficacy in a heterogeneous human patient population.¹⁷⁹ Furthermore, inadequate study designs may have confounded results from animal studies. In 2008, Scott et al¹⁸⁰ demonstrated that when various drugs that had failed in the clinic after showing efficacy in

SOD1-G93A mouse studies, such as minocycline, creatine, and ceftriaxone, were retested in an optimized testing paradigm controlled for sex, transgene copy number, litter, and exclusion criteria, none provided a significant survival benefit. In addition, treating animals before onset of symptoms is a common strategy for mouse efficacy studies, but does not reflect the human situation.

Flawed clinical trial designs may have also contributed to candidate failures. ALS is a heterogeneous disease, both phenotypically and genetically. Since the discovery of SOD1 as the first gene linked to familial forms of ALS in 1993, mutations in over 30 genes have been found to be causative of both familial and sporadic forms of the disease.⁶ Hexanucleotide repeat expansions in C9ORF72, which account for up to 30% to 50% and 5% to 7% of sporadic and familial ALS, were only described in 2011.^{181,182} Different genetic contributions may indicate that mechanisms leading to the disease state are distinct, despite apparent similarities in presentation of symptoms. While older trials may not have distinguished different patient populations based on genetic background, it will likely become a key inclusion/exclusion criterion in the future. In trials to evaluate the efficacy of ASOs targeting disease-associated genes such as SOD1 and C9ORF72, genetic determination is already a prerequisite for trial enrolment.^{59,60,100} Indeed, SOD1 ASOs are likely to be ineffective in non-SOD1 ALS cases. Additionally, eligibility criteria based on phenotype may also become more stringent, such as disease stage, initial site of onset or individual progression rate, defined during a pretrial observational period. For example, following an unsuccessful phase 3 trial for the now approved edaravone, enrollment eligibility criteria were adjusted based on a posthoc subgroup analysis. In addition to the previous criteria, which among others included a decrease in the ALSFRS-R score of 1 to 4 points during a 12-week observation period, a second phase 3 trial also specified a disease duration of less than or equal to 2 years, scores of greater than or equal to 2 on all 12 items of the ALSFRS-R, FVC of at least 80% and definite or probable ALS according to the El Escorial and revised Airlie House criteria.⁵⁰

Last, unsuccessful clinical trials could be due to insufficient exposure to the therapeutic agent at the site of action. For example, insufficient brain exposure may have hampered trials investigating antioxidants such as coenzyme Q10.¹⁸³ The troponin binder tirasemtiv was poorly tolerated at higher doses, causing patients to drop out of the study.⁶³ The growth factor CNTF has a very poor half-life of under 3 minutes when administered intravenously.¹⁸⁴ Finding appropriate biomarkers to confirm target engagement and rule out lack of exposure should be a vital aspect of early clinical development.

5 | EMERGING PRECLINICAL STRATEGIES

In addition to the wide array of treatment strategies currently under clinical investigation for the treatment of ALS, exciting preclinical research has uncovered a variety of possible disease mechanisms that could be targeted with novel therapeutic interventions. While a thorough discussion of these novel preclinical strategies for ALS is outside the scope of this review, we want to highlight a few approaches aimed at transforming the therapeutic landscape of this intractable disease.

5.1 | Emerging ASO approaches

ASOs are a powerful technology enabling long-lasting (several months) modulation of gene expression by binding to RNA targets with complementary sequence. ASOs exert their therapeutic effects either by targeting RNAs for degradation, correcting splicing defects or sequestering miRNA.¹⁸⁵ They are particularly attractive for the treatment of diseases known or thought to arise from the dysfunction of a single gene product. Since their inception, a number of ASOs targeting neurological disorders including ALS, Huntington's disease and familial amyloid polyneuropathy have entered the clinic.^{59,60,186,187} One of the biggest success stories thus far is nusinersen (Spinraza), an ASO approved for the treatment of spinal muscular atrophy (SMA), a disease characterized by lower motor neuron degeneration resulting in progressive

muscle atrophy. The most common form of SMA is caused by mutations in the gene survival of motor neuron 1 (SMN1), leading to loss of SMN1 function.¹⁸⁸ Humans possess a SMN1 paralog, SMN2. SMN2 differs from SMN1 by only a few nucleotides including a cysteine-to-threonine mutation that causes skipping of exon 7, resulting in the expression of an unstable protein.¹⁸⁹ Nusinersen targets the splice site containing this mutation, thus enhancing the inclusion of exon 7, which leads to the expression of more stable, full-length SMN protein.¹⁹⁰ In the interim analysis of its phase 3 study, nusinersen demonstrated significant effects on motor function and survival, prompting early termination of the trial and subsequent approval of the drug.¹⁹¹

To date, no ASO therapy has been approved for the treatment of ALS, but phase 1 trials investigating ASOs that reduce levels of mutant SOD1⁶⁰ and C9ORF72 with the hexanucleotide repeat expansion mutation¹⁰⁰ were recently initiated. Furthermore, in May 2019 the US House of Representatives passed *Jaci's Bill*. The bill's passage and subsequent approval by the FDA allows treatment of Jaci Hermstad, a patient suffering from early-onset ALS, with a personalized ASO targeting the FUS mutation P525L she carries.¹⁹² This effort, spearheaded by Neil Shneider, marks only the second instance of a personalized ASO approach made public in the United States.¹⁹³

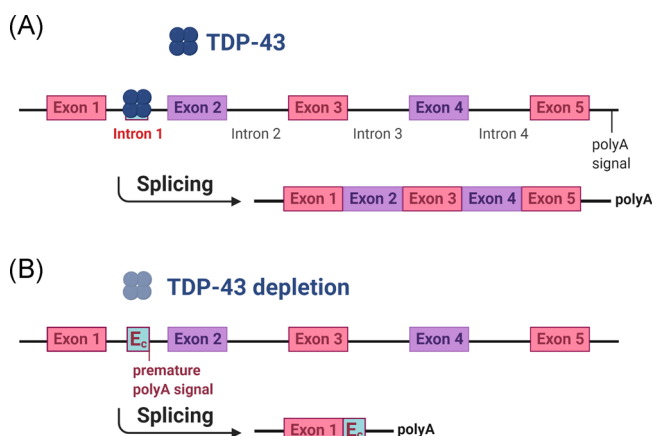
In addition to ASOs aimed at lowering the expression of ALS-causative genes or even specific mutations in genes such as described above, preclinical efforts are also focused on ASOs targeting mRNAs that encode disease-modifying proteins downstream in the disease cascade. One ASO has been developed to reduce expression of ataxin-2, a protein involved in the assembly of stress granules (SGs). Expansions in the polyglutamine (polyQ) stretch of ataxin-2 beyond 34 to 36 repeats cause the neurodegenerative disease spinocerebellar ataxia type 2^{194,195} while intermediate-length expansions (22-33 repeats) are associated with an increased risk of developing ALS.^{196,197} The role of ataxin-2 in mediating toxicity of TDP-43 was first discovered in yeast, when it was shown that upregulation of the yeast ataxin-2 homolog Pbp1 enhanced, while knocking out Pbp1 suppressed, TDP-43 toxicity. Furthermore, heterozygous deletion of the ataxin-2 homolog Atx2 was shown to rescue eye degeneration and extend lifespan in *Drosophila* expressing TDP-43.¹⁹⁶ In a subsequent study, reduction in ataxin-2 by genetic knockout or a single administration of an ASO resulted in improved survival and motor function in a TDP-43 transgenic mouse.¹⁹⁸ As the vast majority of ALS patients present with TDP-43 proteinopathy, an ataxin-2 ASO approach could thus target the broadest possible patient base.

Another potential target for a therapeutic ASO approach is stathmin-2 (STMN2). RNAseq studies to identify transcripts regulated by TDP-43 found that levels of STMN2, which is required for normal outgrowth of motor neurons, are greatly downregulated in TDP-43-depleted cells. Furthermore, STMN2 was found decreased in human motor neurons and spinal cord sections of ALS patients.^{199,200} Reduced binding of TDP-43 to a site within intron 1 uncovers a cryptic exon encoding a polyadenylation (polyA) site in STMN2, and inclusion of this polyA sequence leads to the translation of a truncated, nonfunctional protein (Figure 9). Rescue of STMN2 levels by post-transcriptional stabilization or expression of a construct without the cryptic polyA site rescued the axonal outgrowth defect caused by loss of TDP-43.^{199,200} Thus, an ASO designed to prevent the inclusion of the cryptic exon to restore expression of STMN2 is an interesting therapeutic strategy. Crucially, as the cryptic polyA site within intron 1 is absent in mouse STMN2,²⁰⁰ mouse neuronal cells or in vivo models would not reveal a decrease in STMN2 expression upon TDP-43 depletion. This key difference between mouse and human demonstrates the importance of incorporating human cell systems such as iPSC-derived motor neurons or motor neurons generated by direct conversion.

5.2 | Targeting deleterious phase separation

Liquid-liquid phase separation (LLPS) is the process by which macromolecules condense into structures with dynamic fluid-like properties within a surrounding liquid phase. LLPS underlies the formation of membrane-less organelles, self-organizing complexes without delineating lipid bilayer that form functional compartments within cells.^{201,202} Membrane-less organelles exist both in the nucleus (eg, nucleoli, Cajal bodies) and cytoplasm (eg, SGs, P bodies).²⁰²

FIGURE 9 TDP-43-dependent loss of stathmin-2. A, In healthy cells, nuclear TDP-43 binds to a cryptic splicing site in intron 1 of stathmin-2 pre-mRNA. Intron 1 (and following introns) are spliced and the mature mRNA contains all exonic sequences. B, Upon TDP-43 depletion, the cryptic exon (E_c), which harbors a premature polyA site, is included in the mRNA transcript, resulting in truncation of the transcript and loss of expression of stathmin-2 protein. mRNA, messenger RNA [Color figure can be viewed at wileyonlinelibrary.com]



SGs are transient structures that form in response to activation of stress pathways and sequester RBPs and untranslated RNA. RBPs implicated in ALS such as TDP-43, FUS, hnRNP A1 are recruited to SGs^{203–206} and can undergo LLPS *in vitro*.^{205,207–211} While these LLPS modalities are beneficial, LLPS can also have deleterious consequences in disease.

Critical research over the past several years has suggested that liquid assemblies of RBPs can solidify over time and transition to an insoluble state *in vitro* and in cells.^{9,210,212–216} Furthermore, SGs, as well as SG-independent cytoplasmic droplets, can have detrimental effects linked to disease. For example, G3BP1 and TIAR-2 form liquid-like granules in neurons that suppress axon regeneration.^{217,218} Cytoplasmic TDP-43 droplets that form independently of SGs deplete nuclear TDP-43, sequester components of the nucleocytoplasmic transport machinery and cause cell death²¹⁴ while repetitive, persistent assembly of SGs causes a cell death phenotype.²¹³ Thus, therapeutic strategies that drive disassembly or disaggregation or interfere with the genesis of cytoplasmic liquid-like droplets may be a viable option for the treatment of ALS.

In a recently published study, Fang et al.²¹⁹ screened for small molecules that modulate the number, size, and composition of SGs or have the ability to disassemble preformed SGs. The screen identified a number of planar compounds that reduced the accumulation of TDP-43 in SGs as well as the puromycin-induced formation of cytoplasmic TDP-43 puncta that persist after SGs are resolved. In another study, using a light-inducible model of TDP-43 LLPS, Mann et al. demonstrated that binding of RNA suppresses TDP-43 LLPS and inclusion formation. Excitingly, treatment of neuronal cells with a bait RNA oligonucleotide with high affinity for TDP-43 inhibited aberrant phase transition of TDP-43 and rescued neuronal survival, suggesting that RNA oligonucleotides that antagonize aberrant TDP-43 phase separation may be a possible therapeutic strategy.²¹⁵ These RNA oligonucleotides are intriguing therapeutic candidates as they might be readily delivered to patients like an ASO. A challenge for therapeutics targeting phase separation, however, will be to ensure that beneficial phases are preserved and deleterious phases are eliminated. Overcoming this challenge will require a detailed structural and mechanistic understanding of what differentiates beneficial phases from deleterious phases.

5.3 | Poly(ADP-ribose) polymerase inhibition

A number of studies have provided evidence that poly(ADP-ribose) polymerases (PARPs) may be a viable therapeutic target for ALS and other neurodegenerative diseases.^{211,220–223} PARPs catalyze a type of posttranslational modification called ribosylation, the addition of monomers or polymers of ADP-ribose to proteins. The role of poly(ADP-ribosylation) (PARYlation) has been extensively studied in the context of DNA damage response

signaling, where auto-PARYlation of PARP-1 triggers recruitment of DNA damage repair factors.²²⁴ PARP-1 inhibitors prevent efficient DNA damage repair and are used to treat BRCA-mutant forms of breast and ovarian cancer.²²⁵ More recently, it was shown that TDP-43 is a PAR-binding protein and that PAR promotes TDP-43 LLPS.^{211,221} Furthermore, inhibition of PARP-1/2 reduced SG formation, TDP-43 recruitment to SGs and formation of cytoplasmic TDP-43 foci, and rescued TDP-43-induced cell death of NSC-34 and spinal cord motor neurons.^{221,223} In TDP-43 *Drosophila* models of ALS, knockout of PARP-1 or tankyrase, another member of the PARP family, rescued retinal degeneration and extended survival.^{211,221} Thus, inhibiting members of the PARP family could reduce aberrant TDP-43 phase transition and prevent TDP-43-associated cytotoxicity. Since PARP inhibitors are already safely in the clinic for various cancers it would seem that a clinical trial with PARP inhibitors for ALS is warranted.

5.4 | Stimulating protein disaggregation

The accumulation of misfolded proteins is the defining hallmark of neurodegenerative proteinopathies including ALS. Aggregation of misfolded proteins can cause toxicity either by loss-of-function or gain-of-function mechanisms. Thus, reversing protein aggregation could be a viable therapeutic strategy. Hsp104 is a AAA⁺ (ATPases associated with diverse cellular activities) ATPase with disaggregate activity found in *Saccharomyces cerevisiae* where it modulates the physiological aggregation of yeast prion proteins.^{226–230} However, wild-type Hsp104 only displays moderate disaggregate activity against human proteins that undergo aberrant misfolding associated with neurodegenerative diseases.²²⁶ By introducing specific single missense mutations, potentiated Hsp104 variants have been engineered that efficiently suppress the toxicity and aggregation of TDP-43 and FUS as well as α -synuclein, the major protein component of Lewy body inclusions associated with Parkinson's disease, in yeast (Figure 10).^{231–236} Curiously, while Hsp104 is conserved between bacteria and many eukaryotes, no homolog exists in the animal kingdom,²³⁷ so Hsp104 may be a possible exogenous therapeutic candidate.^{238,239} In support of this possibility, expression of enhanced Hsp104 variants reverses ALS-linked FUS aggregation in mammalian cells.²⁴⁰ Further studies will be required to

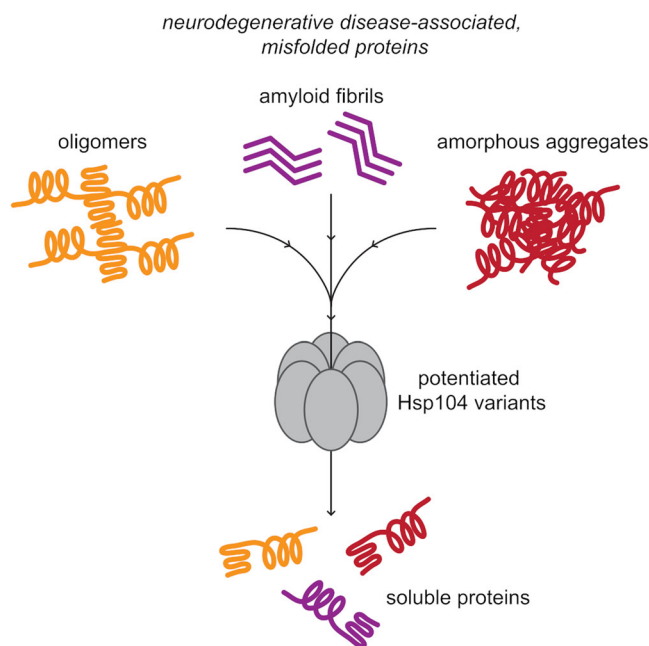


FIGURE 10 Hsp104 is a protein disaggregase. Hsp104 extracts misfolded proteins from soluble oligomers, amyloid fibrils, and amorphous aggregates and returns them to a natively folded state in an ATP-dependent manner. Humans (or any other members of the animal kingdom) have no Hsp104 homolog. Thus, a therapeutic disaggregase strategy would depend on delivery of Hsp104 or DNA encoding Hsp104 exogenously. Potentiated Hsp104 variants have been engineered with enhanced activity against different misfolded proteins. ATP, adenosine triphosphate [Color figure can be viewed at wileyonlinelibrary.com]

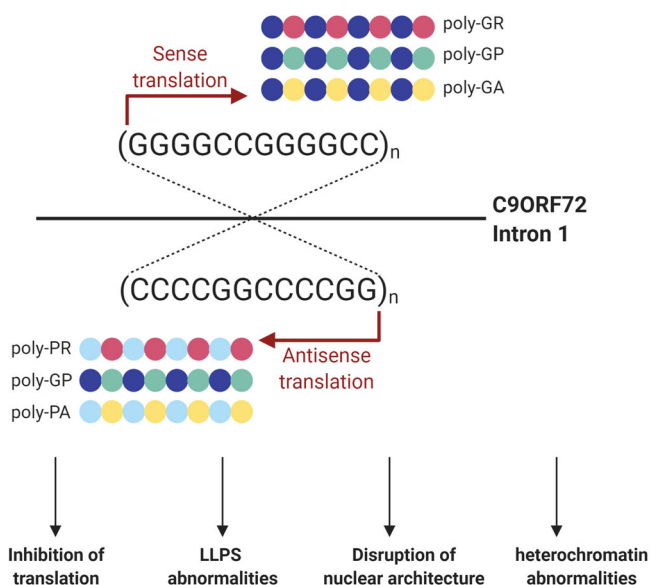
investigate (a) whether potentiated Hsp104 variants can effectively disassemble aggregates of TDP-43 in degenerating neurons, (b) whether expression of potentiated Hsp104 variants is effective in mammalian ALS models *in vivo*, and (c) whether expression of potentiated Hsp104 variants interferes with normal cellular folding processes in mammalian systems.

In addition to potentiated Hsp104 variants, nuclear import receptors with chaperone activity have emerged as another class of proteins capable of driving protein disaggregation.²⁴¹ Indeed, nuclear-import receptors with chaperone function can prevent and even reverse aberrant phase transition and fibrillization of a number of ALS-associated RBPs harboring a nuclear-localization signal. Transportin 1 (TNPO), also known as karyopherin- β 2, prevents fibrillization as well as disassembles preformed fibrils, liquid droplets, and hydrogels of FUS, and reduces the accumulation of FUS in SGs.^{241–245} Furthermore, a combination of importin- α and karyopherin- β 1 prevents fibrillization and promotes disassembly of TDP-43.^{241,245} Thus, strategies that boost the disaggregase function of mammalian nuclear-import receptors could provide a viable therapeutic avenue.

5.5 | Inhibition of repeat-associated non-ATG translation

G₄C₂ hexanucleotide repeat expansions in C9ORF72 are the most common genetic mutation associated with ALS, accounting for 30% to 50% of familial and 5% to 7% of sporadic cases.^{246,247} Three potential mechanisms could explain how C9ORF72 repeat expansions cause ALS: loss-of function of C9ORF72 protein, gain-of-function toxicity caused by C9ORF72 repeat RNA or gain-of-function toxicity by expression of dipeptide repeat proteins (DPRs). DPRs are translated from RNA with nucleotide repeat expansions, a process termed repeat-associated non-ATG (RAN) translation.²⁴⁸ Bidirectional RAN translation of the repeats yields five DPRs (poly-GA, poly-GP, poly-PR, poly-GR, and poly-PA; Figure 11), which are detected in pathological inclusions in patients' brains.^{249,250} Poly-GA, poly-PR, and poly-GR are toxic *in vitro* and in animal models.^{251–256} Poly-PR interacts with ribosomal proteins and inhibits translation, disrupts nucleolar function by affecting LLPS of nucleolar proteins, causes heterochromatin abnormalities and disrupts nuclear architecture.^{256–259} Thus, therapeutic strategies aimed at lowering the production of DPRs may be beneficial to stop or slow down the progression of ALS caused by mutations in C9ORF72.

FIGURE 11 RAN translation of dipeptide repeats in C9ORF72-ALS. G₄C₂ hexanucleotide repeat expansions in intron 1 of the C9ORF72 gene are translated in the absence of a start codon (RAN translation). Translation from the sense and antisense strand yields five dipeptide repeats (poly-GR, poly-GP, poly-GA, poly-PR, poly-PA). Dipeptide repeats have detrimental effects in cells, causing heterochromatin abnormalities, translational stalling, disturbed LLPS and cytotoxicity. Preclinical therapeutic strategies are aimed at suppressing RAN translation. LLPS, liquid-liquid phase separation; RAN, repeat-associated non-ATG [Color figure can be viewed at wileyonlinelibrary.com]



In one recent study, Yamada et al²⁶⁰ showed that RPS25, a eukaryotic-specific, nonessential component of the 40S ribosomal subunit, is required for RAN translation. Knockout of RPS25 in immortalized cells led to a reduction in poly-GP, poly-GR, and poly-GA levels without affecting RNA foci formation or total C9ORF72 mRNA levels. Furthermore, an ASO targeting RPS25 reduced the number of nuclear poly-GR and poly-PR foci in iPSC-derived motor neurons from C9ORF72 patients.²⁶⁰ An ASO approach targeting RPS25 or other modulators of RAN translation may thus represent a therapeutic strategy.

Another recent study suggested that DDX3X, an RNA helicase, remodels G_4C_2 repeats to inhibit RAN translation.²⁶¹ DDX3X directly binds to $(G_4C_2)_n$ RNAs but not the antisense $(C_4G_2)_n$ RNAs.²⁶¹ Indeed, elevating DDX3X expression decreased DPR levels, rescued nucleocytoplasmic transport defects, and enhanced survival of patient iPSC-differentiated neurons.²⁶¹ Thus, agents that increase DDX3X expression or activity could be another therapeutic strategy.²⁶¹ Finally, small-molecule screens to identify RAN translation inhibitors may also provide novel compounds and targets.²⁶²

5.6 | Selective inhibitors of nuclear export

A major pathological hallmark in the majority of ALS cases is the mislocalization of TDP-43 from the nucleus to the cytoplasm. Deletion of the TDP-43 nuclear localization signal causes a loss of nuclear function, cytoplasmic accumulation, and aggregation of TDP-43 and is deleterious both in vitro and in vivo.^{263,264} Furthermore, nucleocytoplasmic transport deficits have been described in a variety of cellular ALS models.^{265–267} Preventing the egress of TDP-43 from the nucleus might thus be a potential therapeutic strategy. TDP-43 harbors a leucine-rich nuclear export signal (NES) predicted to be recognized by the nuclear export factor exportin-1 (XPO1), which can be inhibited by selective inhibitors of nuclear export (SINE) compounds. In 2019, the FDA approved the first-in-class XPO1 inhibitor KPT-330 (selinexor) as a fifth line treatment for multiple myeloma.²⁶⁸ The inhibition of XPO1 causes the accumulation of tumor suppressor proteins in cancer cells, which eventually kills the cells. Inhibition of XPO1 was shown to reduce TDP-43-induced cell death in primary neuron cultures, ameliorate larval locomotor deficits in a TDP-43 *Drosophila* model, and improve grip strength in a rat TDP-43 model.^{267,269} However, recent studies have demonstrated that the NES of TDP-43 is not functional and that TDP-43 egress is the result of passive diffusion rather than active transport.^{270,271} Furthermore, at concentrations shown to effectively rescue TDP-43-induced cell death in neuronal cultures, no increase in nuclear TDP-43 signal was observed.²⁶⁹ Thus, the mechanisms of action of XPO1 SINEs that protect against TDP-43-induced cell death and locomotor deficits may be independent of the localization of TDP-43. In addition to its effects in TDP-43 cell and in vivo models of ALS, inhibition of XPO1 also rescued an eye degeneration phenotype in a *Drosophila* model expressing the C9ORF72 G_4C_2 hexanucleotide repeat expansion and improved survival in mutant SOD1-expressing flies.^{272,273} While the mechanism through which XPO SINEs protect cells against ALS gene-induced cell death remains to be fully elucidated, a recent study suggested that XPO1 inhibition stimulates autophagy.²⁷²

6 | CONCLUSIONS

Despite extensive research, dedication, and a multitude of clinical trials, ALS remains a devastating, debilitating, and ultimately fatal disease. Approved treatments only provide limited benefits. In this review, we sought to give an overview of the major types of interventional treatments that have been or are being investigated in the clinic. There are many possible explanations for the high failure rates of ALS clinical trials, such as choice of the wrong target, flawed clinical trial design, and patient selection or insufficient exposure to the drug. It will be crucial to address these reasons for failure for new drugs entering the clinic, such as by selecting patients based on genetic status, which is already a key criterion in trials investigating ASOs targeting SOD1 and C9ORF72. In addition, emerging preclinical strategies with novel mechanisms of intervention provide hope for more effective treatment options in the future.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ORCID

Heike J. Wobst  <http://orcid.org/0000-0002-9041-3241>

Nicholas J. Brandon  <http://orcid.org/0000-0002-3917-0096>

James Shorter  <http://orcid.org/0000-0001-5269-8533>

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AUTHOR BIOGRAPHIES

Heike J. Wobst obtained a BSc in molecular biotechnology from Heidelberg University in 2007, an MSc in molecular medicine from Charité Universitätsmedizin Berlin in 2009 and a DPhil in Physiology, Anatomy and Genetics from the University of Oxford in 2014. After a postdoc in the AstraZeneca-Tufts Lab for Basic and Translational Neuroscience, she joined AstraZeneca as a Principal Scientist in Neuroscience. She is currently a

Senior Scientist at Jnana Therapeutics. Her scientific interests are focused on molecular mechanisms of neurodegeneration and therapeutic intervention strategies targeting these mechanisms.

Korrie L. Mack graduated from Syracuse University in 2013 with a BS in biochemistry. She earned her PhD in biochemistry and molecular biophysics from the University of Pennsylvania, where she was an NSF Graduate Research Fellow in the lab of James Shorter. Her dissertation work focused on engineering a protein disaggregase to selectively counter toxic, misfolded proteins in neurodegenerative disease. Korrie is currently a neuroscience postdoctoral fellow at Merck.

Dean G. Brown is Vice President and Head of Chemistry at Jnana Therapeutics. He was previously Director of Chemistry at AstraZeneca within the Hit Discovery Department, Discovery Science, R&D BioPharmaceuticals Unit. He obtained a BS in chemistry at Abilene Christian University (Abilene, TX) and a PhD at the University of Minnesota (Minneapolis) in organic chemistry. He has over 22 years of industrial drug discovery experience. Dean has been responsible for building many new scientific programs in both neuroscience and infection, several which have resulted in successful transition to clinical trials. Dean is listed as an author or coauthor on more than 50 publications and patent applications in medicinal chemistry and drug design, including granted patents on clinical candidates.

Nicholas J. Brandon graduated from Pembroke College, Cambridge in Natural Sciences (Genetics Part II) in 1995. After a PhD and postdoctoral studies at UCL, Nick started his industry career at Merck and after time at Wyeth and Pfizer he led Discovery Neuroscience at AstraZeneca before joining Jnana Therapeutics as SVP and Head of Biology in 2019. His research interests span drug discovery across neuropsychiatric and neurodegenerative disorders.

James Shorter graduated with distinction in biology from Keble College, University of Oxford in 1995. He received his PhD from University College London in 2000. After postdoctoral studies at Yale and MIT, James established his own group at the University of Pennsylvania. In 2018, James became Professor of biochemistry and biophysics. The Shorter lab studies how to counter deleterious protein misfolding and aberrant phase transitions in neurodegenerative disease.

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