Translational Therapeutic Str ategies in Amyotrophic Lateral Sclerosis

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Abstract: Amyotrophic lateral sclerosis (ALS) is a clinically severe and fatal neurodegenerative disease characterized by a loss of both upper and lower motor neurons, resulting in progressive muscle loss and paralysis. While the exact cause of neuronal death in ALS remains unknown, it is proposed that multiple molecular defects trigger motor neuron cell death. These pathophysiological mechanisms include oxidative stress, mitochondrial impairment, protein aggregation, glutamate cytotoxicity, transcription dysfunction, inflammation, and apoptotic cell death. An understanding of how these potential therapeutic targets interrelate will provide direction both in the development of a pharmacotherapy and in the design of clinical trials in ALS. Important issues related to therapeutic development are the principals that should be followed in designing and conducting experiments using genetic animal models and what body of evidence is desirable to fully inform clinical decision making. In the context of ALS, we review some of the salient issues related to the use of genetic models in providing a guide to assessing studies in translating therapeutic strategies to patients with ALS and discuss therapeutic targets and pharmacological approaches to slowing disease progression. As in other neurodegenerative diseases, the most effective neuroprotection may result from combined treatment strategies.

Key Words: Amyotrophic lateral sclerosis, therapy, neuroprotection, motor neuron, transgenic mice, clinical trials.

1. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a clinically severe, fatal neurodegenerative disorder characterized by a loss of upper and lower motor neurons, resulting in progressive muscle wasting and paralysis [1]. The incidence of ALS is 1-2/100,000/year and may be rising. Death occurs within 2-5 years of diagnosis. It has recently been reported that there is a significant increased risk of developing ALS in Gulf War veterans and within the military service outside of the Gulf War [2-4]. The vast majority of ALS cases occur sporadically, but about 5-10% of ALS cases are familial. The genetic linkage of several mutations in the gene for Cu/Zn superoxide dismutase (SOD1) with some cases of familial ALS [5] provided the first indication of a potential causal factor in the disease process. The similarity in the course and pathological features of familial (FALS) and sporadic ALS (SALS) has led a number of investigators to search for genetic mutations associated with FALS as a strategy for elucidating disease pathogenesis and defining novel treatments in both sporadic and inherited forms of the disease. Current medical care focuses on symptom management. Supportive care ameliorates symptoms and makes ALS more manageable for patients and their families, but does not significantly affect the primary disease process. Riluzole, the only FDA-approved ALS therapy, is associated with a 2-3 month prolongation of survival [6, 7]. To date, no other drug therapies slow or abrogate the disease process in ALS.

Missense mutations in the enzyme copper/zinc superoxide dismutase (SOD1) cause about 25% of FALS cases [5], whose clinical and pathological features are indistinguishable from those in sporadic ALS. This has prompted the view that all forms of the disease may be better understood and ultimately treated by studying pathogenesis and therapy in rodent models of ALS transgenic mice and rats expressing mutant forms of SOD1 [8, 9]. Transgenic mice that express high levels of FALS mutant SOD genes develop clinical and pathological features similar to those seen in the human disease, including hindlimb weakness and loss of motor neurons [10]. Beneficial therapeutic trials in transgenic ALS mice have generated trials of treatments in humans with ALS [12-16].

2. METHODOLOGICAL CONSIDERATIONS FOR MOUSE THERAPEUTIC TRIALS

Genetic animal models of inherited neurological diseases provide an opportunity to test potential treatments and explore their promise for translation to humans experiencing these diseases. An important advance in clinical trials in ALS patients has been the introduction of genetic mouse models of ALS [17, 18]. Transgenic mice expressing the G93A or G37R human SOD1 mutations with elevated levels of SOD1 activity or mice expressing G85R mutant SOD1 with protein levels and activity levels essentially equal to endogenous levels develop progressive hind limb weakness, muscle wasting, and neuropathological sequelae similar to that observed in both SALS and FALS patients [17-19]. In addition, mutations in SOD1 from two different ALS-associated mutations (G93A and H46R) develop a striking ALS phenotype of motor neuron degeneration and paralysis in rats [20, 21].

Therapeutic trials conducted in mouse models of ALS have identified a growing number of potential therapies that are candidates for clinical trials [11]. There is increasing

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concern about the feasibility and desirability of taking each and every compound that may work in mice and testing them in patients. There is a need to begin to prioritize leads emerging from transgenic mouse studies, however, it is difficult to compare results between compounds and laboratories and there are also many additional factors that can affect translation to humans. The translation of findings in cell models is a tenuous scientific bridge to cross given the complexity of the intact neuronal system in mammalian models, and *in vitro* analyses are further removed from the human disease process that requires years to evolve. Admittedly, there are issues in translating murine findings to ALS patients, but these are significantly less than in other model systems. Transgenic mice and rats expressing mutant forms of SOD1 have been useful in studying pathophysiological pathways and in developing candidate ALS therapies [9, 11].

Phenotype homogeneity is essential in testing potential therapies in rodent models. Minimizing measurement variability in mouse models increases the power to detect differences between the test groups, with the potential of improving their utility in therapeutic trials. Whereas in human trials there is enormous variability; in mouse trials it is possible to minimize the variability and thereby increase the power to detect smaller differences. It is important to understand the genetic, physical, and environmental sources of variability to take advantage of this.

In human clinical research, inclusion and exclusion criteria are critical to the success of the trial and should be predetermined. This is true of mouse trials as well. Monitoring of the transgene copy number to ensure that drift has not occurred, weight criteria to exclude runts (mice below 7 gm at weaning), mice with injuries, and mice unable to perform some of the planned evaluations, such as those including motor performance, are important issues to assess prior to assignment into experimental cohorts. In addition, treatment groups should be genetically comparable. The mice should be distributed into experimental groups to prevent overrepresentation of sibs in any group. The environment in which these mice are housed and treated also needs to be uniform. Environmental enrichment slows disease progression. The practice of using environmental enrichment can be considered as a therapeutic treatment that may confound mouse trials. Enrichment results in greater heterogeneity of the clinical and neuropathological phenotype, lowering the power to detect differences. Another important issue is the onset and duration of treatment in mouse trials. Initiation of treatment at weaning, as has been most common, is analogous to treating presymptomatic humans. Initiation of treatment once motor or other phenotypic symptoms are evident would be more analogous to human trials for ALS.

Potential outcome measures used in mouse therapeutic trials bear close attention because they can differ greatly in relevance, with some outcomes much more informative than others. Achieving neuroprotection, which at its most basic level is the preservation of neuronal processes, somata, and function, is of utmost import. Measures that assess these directly are spinal cord weight, gross atrophy, cellular atrophy, neuronal counts, gliosis, and volumetric imaging. These should be considered as the primary outcome measures. While improving behavioral symptoms may correspond to neuroprotection, they can be modified without affecting neurodegeneration, and, as such, assessments of symptoms should be considered as secondary outcome measures. This information is important when considering how informative the results of a mouse therapeutic trial are for translation to humans. In the absence of ameliorated neuropathological evidence, a treatment cannot be considered to be neuroprotective in mice. Therefore, the quality of neurohistopathology is of utmost importance in mouse therapeutic trials. In addition, the expression of molecules of interest are only meaningful if neuropathological sequelae are measured to provide a context for interpreting them. Quantitative methods using stereological procedures are essential, as observation alone can only detect large differences and semi-quantitative methods are prone to many types of errors.

Mouse survival and other surrogate outcomes are especially useful secondary outcome measures. While correlating well with neuropathology, they provide a relevant measure of the magnitude of benefit that enables ready comparison with other therapies and provides context to the preclinical trial. A dose ranging versus survival study is recommended, using at least three doses demonstrating the presence or absence of efficacy. While body weight shows early and significant improvements in some mouse therapeutic trials, little effect has also been observed in other trials despite dramatic improvement in survival [16]. We measure body weight weekly, on the same day and time, usually from the time of weaning.

While a number of motor performance tasks have been used in mice to assess the clinical phenotype, rotarod, grip strength measurement, and gait analysis may provide the greatest correlation with improved survival and neuroprotective outcomes. The rotarod test, in which mice are required to walk along an elevated rotating rod, is generally accepted as the most sensitive and encompassing and is widely used. The length of time that the mice remain on the rotating rod is used as the measure of competency on this task. Mice are tested until they are unable to perform.

Methodological variances in testing paradigms can also result in differing expression of outcome responses that may produce differences in data interpretation, with the potential to obscure therapeutic efficacy. In addition, longitudinal biomarkers of disease that measure pathological phenotype can validate therapeutic efficacy. Biomarkers are urgently needed for diagnosis, disease progression, and for potential disease-modifying therapies that are being developed and evaluated in clinical trials. The development of early biomarkers is of great importance, as these may improve the power and cost-effectiveness of drug trials. While many different approaches have been undertaken to identify biomarkers, profiling objective biomarker measurements of ALS has proven difficult at the present time**.** There have, however, been recent inroads into this issue [23].

While neuroprotective therapies that target specific neurotoxic molecular mechanisms in the ALS mice have the potential to delay the onset and slow the progression of disease in ALS, the predictive value of the murine models for human ALS is unclear. Successful preclinical trials demonstrating improved phenotype in ALS transgenic mice have

yet to be validated in ALS patients. Indeed, there are drug therapies that are efficacious in ALS mouse models that do not show efficacy in ALS patients [24]. This may be the consequence of insufficient fully-powered clinical trials in humans to permit a comparison of therapeutic efficacy between mouse and man. Alternatively, optimal therapeutic dosing may be underestimated. Human Equivalent Dose extrapolation measurements derived from body surface area criteria in animals may not accurately predict the maximum-recommended safe dose in neurological disorders. This is evident in human trials where human equivalent dosing of bioenergetic agents comparable to that given to mice, while considered safe and tolerable, have not demonstrated significant efficacy in ALS patients [25]. Higher doses of bioenergetic agents, such as coenzyme Q_{10} and creatine, may result in slowing disease progression in neurodegenerative disorders. It has been reported that higher doses are well tolerated in Parkinson's disease and that they slow the rate of deterioration in the UPDRS score [77]. In addition, doses up to 3,000 mg per day appear safe and well tolerated in ALS patient [78].

There is strong evidence to suggest that the phenotypes of ALS transgenic rodent models correlate with human neurological disease and that they may validate known CNS drug targets in a therapeutically relevant manner. The strengths of the ALS mouse models are in their utility to provide parallel pathophysiological targets that are present in ALS patients, in their potential as sensitive predictors for therapeutic intervention, and their promise in the development of novel drug agents. While drug trials in mice confirm therapeutic direction, the challenge is in determining what dose might be of value in patients since the pharmacokinetics of mice and man is dissimilar.

Moreover, a more definitive understanding of the molecular events leading to motor neuron death in ALS remains to be elucidated. Many pathological pathways have been incriminated in ALS: perturbed mitochondrial function and oxidative injury, formation of various aggregates of ubiquitinated proteins, glutamatergic excitotoxicity and impaired axonal transport, altered transcriptional function, and activation of programmed cell death cascades (Fig. (**1**)) [8, 26]. The progressive motor neuronal degeneration might be associated with any one of these specific pathways or concurrent impairments of multiple pathways.

3. CURRENT THERAPEUTIC TARGETS FOR ALS

3.1. Oxidative Stress and Mitochondrial Dysfunction

Oxidative stress is closely linked to oxidative phosphorylation dysfunction and further implicated in the pathogenesis of FALS. Metabolic processes involving the mitochondrial electron transport chain are known to contribute to the formation of harmful reactive oxygen species (ROS). For example, an unavoidable by-product of respiration is the generation of the superoxide anion, a potentially toxic radical that has been implicated in cellular damage such as DNA oxidation, protein oxidation, and lipid peroxidation. In the cytoplasm, the superoxide anion is neutralized by SOD1, an anti-oxidant enzyme that catalyzes the conversion of two

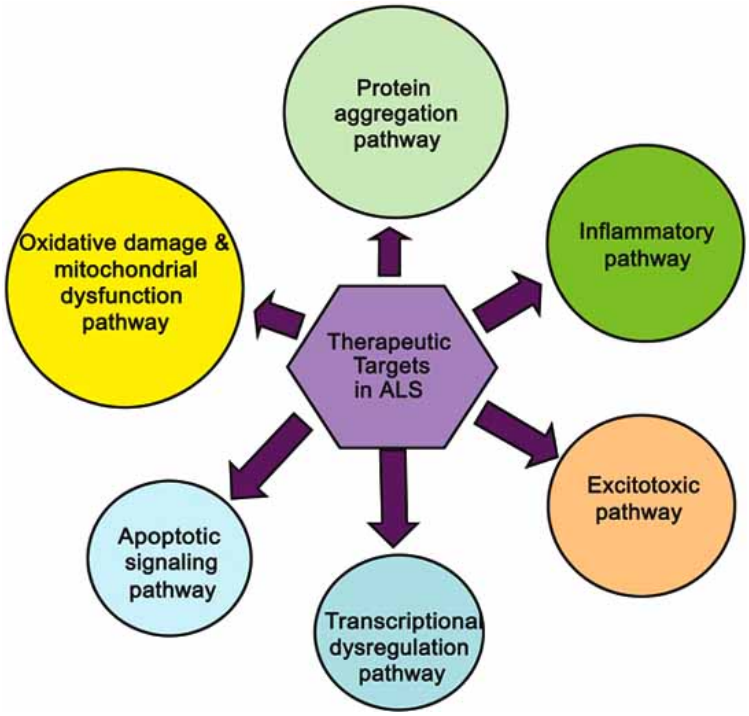

Fig. (1). Proximal and distal therapeutic targets for amyotrophic lateral sclerosis (ALS). There is strong evidence that the mutations of SOD1 in both familial ALS (FALS) patients and animal models cause toxic effects in motor neurons, leading to a cascade of pathogenic mechanisms associated with oxidative stress and mitochondrial dysfunction, with subsequent bioenergetic defects. Additionally, protein aggregation, excitotoxicity, transcriptional dysfunction, and pro-apoptotic signals play roles in the untimely death of motor neurons in ALS. The size of each sphere suggests proximal and distal events, with oxidative stress and mitochondrial dysfunction being most proximal.

superoxide radicals to hydrogen peroxide and molecular oxygen. The reactive hydrogen peroxide is then further broken down by the enzyme catalase to yield two relatively benign products. When a cell loses the ability to effectively neutralize ROS, homeostasis is disturbed and a state of oxidative stress ensues (Fig. (**2**)). In ALS, motor neurons are particularly vulnerable to oxidative stress, a phenomena attributed to a low level of antioxidant enzymes, a high content of easily oxidized substrates (e.g. membrane polyunsaturated lipids), and an inherently high flux of reactive oxygen species generated during energy metabolism. Therefore, based on the well characterized and essential function of SOD1 in limiting free radical accumulation, research has examined the association between SOD1 mutations and the generation of pathological oxidative damage, which results in subsequent motor neuron degeneration. Oxidative damage to spinal cord proteins has been shown to occur in both human SALS and FALS. Protein carbonyl and nuclear DNA 8-hydroxy-2 '-deoxyguanosine (OH8dG) levels have been reported to be increased in SALS motor cortex, but not FALS patients [27]. Malondialdehyde levels showed no significant changes, however, immunohistochemical studies showed increased neuronal staining for hemeoxygenase-1, malondialdehyde modified protein, and OH8dG in both SALS and FALS spinal cord. In addition, previous studies have shown that transgenic mice expressing mSOD1 develop a progressive accumulation of 8-hydroxy-2-deoxyguanosine, a marker of oxidative DNA damage, and have elevated levels of mitochondrial oxidative damage. A proteomics approach has recently showed that SOD1, translationally controlled tumor protein, ubiquitin carboxyl-terminal hydrolase-L1 , and alphaB-crystallin are highly carbonylated in the spinal cord of G93A ALS mice [28]. Other oxidative modifications, such as nitrosylation of proteins, could also be important pathogenic mechanisms of ALS. Either excessive or deficient levels of protein S-nitrosylation may contribute to onset of ALS. Recently, deficient S-nitrosylation has been found in the mitochondria of cells expressing SOD1 mutants [29]. In this paradigm, S-nitrosothiol donor compounds rescue cells from mutant SOD1-induced cell death and suggest that this protective mechanism may provide a novel therapeutic strategy in ALS.

3.2. Protein Aggregation

Protein aggregates and ubiquitinated inclusions are associated with motor neuron degeneration and are recognized as

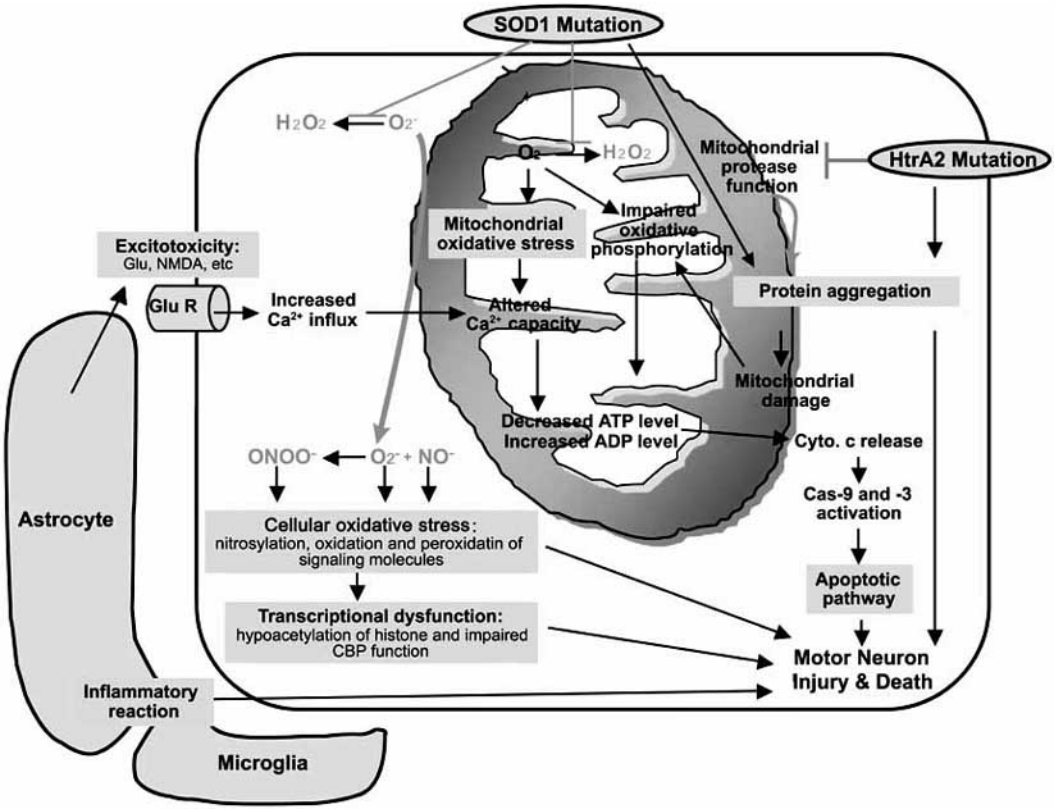

Fig. (2). Cellular and molecular mechanisms of action of therapeutic compounds in ALS. The mitochondrial cofactor coenzyme Q_{10} and creatine restore putative bioenergetic defects due to mitochondrial dysfunction and inhibit cellular oxidative stress. Creatine significantly increases intracellular concentrations of creatine and ATP. Sodium phenylbutyrate inhibits histone deacetylase (HDAC) activity and modulates gene transcription to compensate for the transcriptional dysregulation in ALS. Scriptaid disrupts *in vitro* aggresome formation with mutant SOD1-GFP, while sodium butyrate reduces ubiquinated protein aggregates in the G93A mice. Riluzole inhibits glutamate release, post-synaptic glutamate receptor activation and reduces excitotoxicity. Pioglitazone (PPAR gamma inhibitor), nimesulide (COX-2 inhibitor), thalidomide, and lenalidomide modulate neuroinflammatory pathways and prevent motor neuron damage. Minocycline inhibits caspase-1 and –3 activity and mRNA upregulation and, as a result, has the potential to correct the apoptotic pathway in ALS.

important pathological features of ALS (Fig. (**2**)) [30, 31]. Several hypotheses have been proposed to explain the mechanism of protein aggregate formation in motor neurons. First, mSOD1 proteins are prone to either aggregate with itself or other proteins due to their molecular instability in familial ALS and mSOD1 ALS mice [28, 32]. Second, bioenergetic failures, due to oxidative stress and mitochondrial dysfunction, result in the impairment of the ubiquitin-proteasome system, which in turn contributes to cellular protein aggregation. Accordingly, many signaling molecules and structural proteins have been detected within the inclusions found in spinal cord motor neurons from ALS patients. Patients with ALS have a higher concentration of both intra- and extra-nuronal tau aggregates [32]. In addition, heavy subunits of neurofilaments (NFs), major structural elements of neuronal cytoskeleton, are accumulated in neuronal perikarya and dilated axons (spheroids) [33]. It is noteworthy that the elimination of assembled NFs from axons or misaccumulated NFs in motor neuron cell bodies markedly slows disease in G37R SOD1 transgenic mice [34]. This finding suggests that removing NFs reduces axonal crosslinking, and restores axonal transport and motor neuron functionality. Other studies show that a protein kinase is colocalized with phosphorylated NFs and with ubiquitinated inclusions in degenerating motor neurons of ALS mice [35]. Thus, intracellular kinase aggregates might also contribute to the pathogenesis of motor neurons.

3.3. Excitotoxicity

Glutamate-mediated toxicity has long been associated with neuronal death, particularly in ALS. Several converging lines of inquiry have established glutamate regulation defects in ALS [36, 37, 40]. These investigations have given currency to the notion that excessive synaptic glutamate is an initiator or propagator of motor neuron loss in ALS (Fig. (**2**)) [38]. Glutamate receptors have been subdivided into two types: N-methyl-D-aspartate (NMDA) receptors and alpha-amino-3-hydroxy-5 methyl-4 isoxazole proprionic acid (AMPA/Kainate receptors). These are multi-subunit receptors that gate calcium to varying degrees depending on the subunit composition. Both types of receptors are present on motor neurons, but *in vivo* and *in vitro* data suggests that motor neurons are more vulnerable to AMPA/Kainate toxicity. However, *in vitro* data also shows that AMPA receptor activation in organotypic cultures can be partially blocked by NOS inhibitors. Furthermore, Kimura and colleagues have shown that NMDA and non-NMDA agonists induce motor neuron death *in vitro* when motor neuron cultures are exposed to a low level of glutamate plus a glutamate transport inhibitor. Of note, motor neuron death in this paradigm can be abrogated by NOS inhibitors. In addition, a key component that prevents continued neuronal firing is the removal of glutamate by gluamate transporters, of which the excitatory amino acid transporter 2 (EAAT2 glial glutamate transporter) removes the majority of synaptic glutamate [39]. There is evidence showing a significant loss of the astroglia EAAT2 protein and that this loss results in neuronal death [40].

3.4. Inflammatory Pathway

The neuroinflammatory pathway is believed to be responsible for disease onset and progression in ALS [26, 41]. The elevation of inflammatory genes is highly induced in the presymptomatic stage of G93A ALS mice, the FALS animal model [42]. However, either in the motor neurons or ventral horns of sporadic ALS patients, there was no significant upregulation of inflammatory cytokine genes [43]. Chronic inflammation with an increased level of oxidative stress and cytokine production may contribute to motor neuron damage. A marked increase of tumor necrosis factor alpha with increased CSF level of interleukin (IL)-6 has been reported in ALS patients [44]. It has been proposed that the imbalance of multiple cytokines leads to severe inflammatory reactions within the degenerating spinal cord. The ALS spinal cords also showed infiltration of macrophages and mast cells that indicate the activation of innate immune response is involved in neuropathological abnormalities of ALS [41]. In addition, activation of microglia was found in patients with ALS (Fig. (**2**)). The presence of CD40 ligand –positive T lymphocytes in the perivascular region also suggests that an adaptive immune response triggers the inflammatory process in ALS by T cells [41].

3.5. Transcriptional Dysregulation

Recent evidence suggests that transcriptional dysregulation may play a role in the pathogenesis of ALS [16, 45-50]. Microarray analysis shows distinct changes in the molecular signature of gene expression in both ALS animal models and patients [45, 46, 50]. For example, upregulation in TAFII30, one of the TATA-binding protein-associated factors required for transcription of a subset of genes, may alter the activity of cellular transcription and contribute to neuronal toxicity in ALS [46].

Transcription is regulated by complex interactions between many proteins, among them transcription factors and histones that ultimately affect the actions of RNA polymerase II on individual genes. Many of these interactions, in turn, are regulated by covalent modifications, such as acetylation, methylation, and phosphorylation. Recruitment of histone deacetylases to DNA alters the nucleosome structure locally and inhibits transcription. Histone deacetylase [HDAC] inhibitors increase acetylation of histones, thereby promoting transcriptional activation. Of the five classes of HDAC inhibitors, the butyrates are the most developed for clinical use in humans [51, 52]. Since the butyrates penetrate the blood-brain barrier, they are candidates for HDAC inhibition therapy relative to disorders of the brain and spinal cord.

3.6. Apoptosis

While mechanisms precipitating neuronal death in ALS are not fully defined, it is now clear that apoptotic cell death is a common cellular event in both animal models and patients with ALS (Fig. (**2**)) [26, 53, 54]. The potential importance of apoptotic death pathways in this disease is suggested by the observations that increased expression and activity of caspases, and administration of tetrapeptide caspase inhibitors delay disease onset and prolong survival in ALS mice [55-57]. Nitric oxide (NO) has been implicated in the induction of apoptotic cell death in ALS. Ventral root avulsion in the rat results in the induction of nitric oxide synthase and motor neuron loss 6 weeks after treatment [58]. Inhibitors of NOS activity prevent motor neuron loss after

ventral root avulsion [58, 59]. Finally, trophic factor deprivation of motor neurons leads to *de novo* synthesis of nNOS and subsequent apoptotic death; and death can be suppressed by inhibitors of nNOS. In this context, as well as others, the neurodestructive effects of NO are mediated by its diffusion limited interaction with superoxide to form the potent oxidant, peroxynitrite [60-64]. While high levels of NO interacting with superoxide to form peroxynitrite can be neurodestructive [61, 64], low levels of NO interacting with guanylate cyclase to form cGMP can be neuroprotective [65]. NO donors or NO produced by constitutive nNOS mediates the survival-promoting effects of BDNF in primary motor neurons**.** The mechanism of protection by NO appears to be mediated through activation of guanylate cyclase, leading to increased cGMP levels and prevention of activation of the apoptosis related protease, caspase 3 [66]. NO has also been demonstrated to directly nitrosylate caspases and thereby prevent their activation [67]. Further elucidation of cellular mechanisms of NO regulation that prevent the formation of toxic levels of NO, but permit protective NO concentrations in the appropriate subcellular compartments and cell types is of import.

4. PHARMACOLOGICAL APPROACHES TO ALS

Despite the fact that many different therapeutic strategies have been applied in the clinic to slow or prevent disease progression, no cure or effective therapy is currently available for ALS. In this section, we will discuss proposed mechanisms of action by different types of therapeutic agents that have been applied to both ALS mice and patients.

4.1. Bioenergetic Compounds Ameliorating Oxidative Stress and Mitochondrial Dysfunction

Recent evidence suggests that bioenergetc defects may be a proximal effect in the G93A transgenic SOD1 mouse model of FALS [68]. Mitochondria are particularly vulnerable to oxidative stress, and mitochondrial swelling and vacuolization are among the earliest pathologic features found in two strains of transgenic ALS mice with SOD1 mutations [69-70]. Mice with the G93A human SOD1 mutation have altered electron transport enzymes, and expression of the mutant enzyme *in vitro* results in a loss of mitochondrial membrane potential and elevated cytosolic calcium concentration. Mitochondrial dysfunction may lead to ATP depletion, which could contribute to cell death. If so, then buffering intracellular energy levels should exert neuroprotective effects. Creatine kinase and its substrates creatine and phosphocreatine constitute an intricate cellular energy buffering and transport system connecting sites of energy production with sites of energy consumption. Creatine administration stabilizes mitochondrial creatine kinase and inhibits opening of the mitochondrial transition pore.

Creatine (methylguanidino-acetic acid) is a guanidino compound synthesized endogenously in the body from arginine, methionine, and glycine, largely in the liver, as well as in the kidneys, pancreas, testes [71] and the brain [72] (Fig. (**3**)). It is also supplied exogenously through consumption of meat and fish [73]. Oral administration of creatine produced a dose-dependent improvement in motor performance and extended survival in G93A transgenic mice, while it protected the loss of both motor neurons and substantia nigra neurons at 120 days of age [69]. Creatine administration also protected G93A transgenic mice from increases in biochemical indices of oxidative damage. It has recently been reported that the neuroprotective effects of creatine may be additively enhanced with minocycline and COX-2 inhibitors in ALS mice [74, 75].

Coenzyme Q_{10} (CoQ_{10}) is a lipid-soluble benzoquinone derivative structurally similar to vitamin K. It is composed of a benzoquinone ring and an isoprenyl side chain, which contains 10 isoprenyl groups (Fig. (**3**)). It resides in the inner mitochondrial membrane and is essential for Complex I and II electron transfer activities during oxidative phosphorylation. CoQ_{10} shuttles electrons from complexes I [nicotinamide adenine dinucleotide] and II (succinate dehydrogenase) to complex III (ubiquinone-cytochrome c reductase) and plays a vital role in ATP production. CoQ_{10} also has membrane-stabilizing properties and acts as an antioxidant in both mitochondrial and lipid membranes. In the inner mitochondrial membrane, it directly scavenges free radicals. CoQ_{10} has also been shown to interact with mitochondrial uncoupling proteins mediating uncoupling through superoxide production, thereby reducing free radical generation. Administration of CoQ_{10} significantly increases brain mitochondrial concentrations of CoQ_{10} in mature and older animals. Low dose coenzyme Q_{10} prolonged median survival of G93A ALS transgenic mice [76]. Although experimental evidence shows that CoQ_{10} is efficacious in animal models of neurodegeneration and has potential as a therapeutic in patients, it remains unclear whether optimal CoQ_{10} dosing has been determined. It may well be that higher doses of CoQ_{10} are necessary to significantly slow the disease process in ALS patients. As evidence, a double-blind, randomized, controlled study trial in Parkinson's disease patients, using CoQ_{10} at 1200 mg/d, slowed the rate of deterioration in the UPDRS score, with higher tolerated doses [77]. Of great interest, doses up to 3,000mg per day appear safe and well tolerated in ALS patients [78]. Compounds that buffer neuronal energy demands, such as creatine and coenzyme Q_{10}, are highly attractive candidates for targeting this important disease mechanism. There are some advantages to creatine that include lower cost, more straightforward bioavailability, and biomarkers that can be used *in vivo*. Given creatine's apparent safety and tolerability, it may be especially well suited for long-term use in ALS. For the same reasons, creatine is also well suited for use in combination with neuroprotective agents targeting other pathologic mechanisms of disease [74, 75]. A high dose creatine trial in ALS is being planned.

4.2. HDAC Inhibitors Targeting Nuclear Transcriptional Dysfunction

Histones are important nuclear proteins that bind DNA in the formation of nucleosomes. They help to regulate transcription. Chromatin remodeling and transcription regulation is controlled in part by histone acetylation, which is regulated by the opposing activities of histone acetyltransferases and histone deacetylases (HDAC). Altered nucleosome dynamics may play a role in the pathogenesis of ALS. Recruitment of HDACs to DNA alters the nucleosome structure locally and inhibits transcription, presumably because acetylation neutralizes the positive charge on lysines in the his-

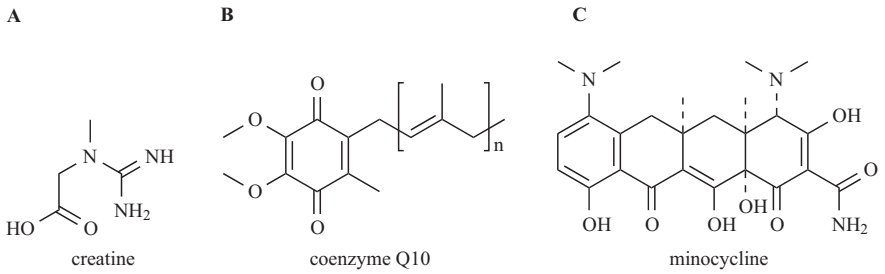

Fig. (3). Structure of chemotherapeutic compounds for ALS. A, Creatine (*N*-aminosarcosine; methylguanidino-acetic acid). B, Coenzyme Q_{10} (ubiquinone; 2,3-dimethoxy-5-methylbenzoquinone). C. Minocycline (7-dimethylamino-6-demethyl-6-deoxytetacycline).

tone tails and alters intra- and/or inter-nucleosomal structure. HDAC inhibitors can promote either transcription activation or suppression by relaxing DNA conformations. Transcription repression *via* the recruitment of HDACs occurs with many transcription factors. Because HDAC inhibitors induce growth arrest in cell culture models, they are considered potential anticancer agents. It has been reported that HDAC inhibition is neuroprotective in mouse models of spinal muscular atrophy and Huntington's Disease (HD) [79, 80]. Of relevance is the finding that sodium butyrate improves gene transcription and the clinical symptoms in these mice. We have showed that sodium phenylbutyrate significantly improved the clinical and neuropathological characteristics in G93A transgenic mice, restored histone acetylation to near normal levels, and induced NF-κB p50 and bcl-2 [16]. Cell culture studies from the G93A mice and *in-vitro* experiments confirmed and expanded these findings. The mechanism and efficacy of phenylbutyrate was confirmed after onset of symptoms. The effect of phenylbutyrate, an HDAC inhibitor, is more than two-fold greater than that of riluzole in the transgenic SOD1 mice. Increased expression of NF-κB p50 and bcl-2 is neuroprotective in these experiments, presumably because these factors reduce levels of key components of the intrinsic apoptotic pathway. NF-κB may directly alter the transcriptional events associated with apoptosis and prevent programmed cell death [81]. Alternatively, NF-κB or improved acetylation may increase transcription of bcl-2 that, in turn, prevents the subsequent release of cytochrome c, resulting in a post-transcriptional event that prevents apoptosis (Fig. (**2**)) [82, 83].

In addition, HDAC inhibitors may also target protein aggregation. Neuronal protein aggregates containing SOD1, ubiquitin, and proteosome are observed in human ALS spinal cords and in mouse models [17, 84]. Although it is unclear if these aggregations are cytotoxic or protective to cells, agents that decrease aggregation have been hypothesized to be neuroprotective. While the pathogenic significance of protein aggregation remains unclear, as with other neurodegenerative disorders in which protein aggregates are a hallmark of disease, additional levels of experimental analyses are required to provide a salient context to the human condition. It is difficult, however, to reason that such factors such as the mass effect of aggregate burden, the potential for sequestration of critical transcription factors and neuronal proteins that are essential for neuronal survival and their subsequent reduced activity, and altered cellular organelle function, do not have a deleterious effect upon neuronal function and survival. Scriptaid is a known HDAC inhibitor that was identified in a screen for small molecules that disrupt *in vitro* aggresome formation in cultured COS cells transfected with mutant SOD1-GFP [85]. While sodium butyrate reduces ubiquinated protein aggregates in the G93A mice [16], animal studies using scriptaid confirming the *in vitro* data of aggregate inhibition are of import, in addition to determining which genes are upregulated. Safety, optimum dose, and pharmacokinetic human data remain to be determined for this drug. A safety and tolerability trial using sodium phenylbutyrate in ALS patients is underway.

4.3. Compounds Targeting Excitotoxicity

Riluzole acts in a number of physiologic pathways, including both inhibition of glutamate release and post-synaptic glutamate receptor activation, inactivation of voltage-sensitive sodium channels, neurotrophic and anti-apoptotic effects, and reduces mitochondrial swelling (Fig. (**2**)). It is the only FDA-approved ALS therapy and is associated with a 2-3 month prolongation of survival [6, 17, 86, 87]. To date, no other drug therapies slow or abrogate the disease process in ALS. The drug is well tolerated, though liver function monitoring is necessary especially during the first year of therapy.

Rasagiline, an antiapoptotic compound with neuroprotective potential, has shown neuroprotective effects alone and in combination with the putative glutamate release blocker riluzole in the G93A ALS mice [88]. The drug had a significant dose-dependent therapeutic effect on both preclinical and clinical motor function and survival of the animals. Of note, the combination of rasagiline with riluzole is safe and extends survival by about 20% in a dose-dependent manner. Therefore, the combination of rasagiline and riluzole is a promising clinical combination for the improvement of current neuroprotective treatment strategies of ALS.

4.4. PPAR Gamma and COX-2 Inhibitors Directed at Neuroinflammation

The presence of activated microglia and astrocytes at the presymptomatic stage of ALS mice suggests that a neuroinflammatory reaction may contribute to the onset of disease. The modulation of neuroinflammatory cells and pathways may be neuroprotective (Fig. (**2**)). Oral treatment using pioglitazone, a peroxisome proliferators-activated receptor gamma agonist, in G93A ALS mice demonstrated complete protection of motor neurons in the spinal cord and significantly extended survival [89]. Additionally, activated micro-

glia were reduced at the site of neurodegeneration in pioglitazone-treated mice.

Nimesulide is a specific COX-2 inhibitor with anti-inflammatory properties [90]. Drachman and Rothstein have found that COX-2 related neuroinflammation may be important in the pathogenesis of ALS [91]. COX-2 is increased in spinal cords of transgenic SOD1 mice and in the postmortem spinal cords of ALS patients [92]. Nimesulide administration decreased PG-E2 levels in the spinal cord of mSOD1 [G93A] mice and preserved motor performance [93]. However, the COX-2 inhibitor celecoxib failed to show benefit in a recent clinical trial in ALS patients [22]. Conversely, it has been hypothesized that COX-2 inhibitors may be harmful in patients with ALS because they can block the natural upregulation loop of VEGF during hypoxemia, which occurs with rapid and progressive restrictive respiratory failure in ALS [44]. The reciprocal interactions between COX-2/prostaglandin E-2 and VEGF may act as a homeostatic reaction to inflammatory signals in ALS. However, further studies are required to assess the details of action mechanism of these drugs.

Beal and colleagues have examined the neuroprotective effect of thalidomide and its analog lenalidomide, pharmacological agents that inhibit the expression of TNF-alpha and other cytokines by destabilizing their mRNA [94]. Thalidomide is a non-barbiturate sedative that was introduced in the 1950s and withdrawn from the world market on discovery of its teratogenic effects. Treatment with either thalidomide or lenalidomide attenuated weight loss, enhanced motor performance, reduced motor neuronal death, and significantly extended the survival of G93A ALS mice [94]. TNF-alpha and FasL immunoreactivity, as well as their mRNA, in the lumbar spinal cord were reduced in treated G93A mice. It seems possible that thalidomide and lenalidomide may be promising therapeutic agents for ALS, however, with some caution.

4.5. Minocycline Inhibition of the Apoptotic Cell Death Pathway

The scientific rationale for using minocycline is in its anti-apoptotic effects. Minocycline is a second-generation tetracycline anti-apoptotic compound capable of inhibiting caspase-1 and –3 activities and expression levels as well as inducible nitric oxide synthase [95] (Figs. (**2** and **3**)). Although minocycline does not directly inhibit these enzymes, the effects may result from interference with upstream mechanisms. Minocycline inhibits the release of cytochrome c, an apoptogenic factor from mitochondria and also inhibits reactive microgliosis, both of which are activated in the spinal cord of ALS mice, and have been implicated in disease pathogenesis [96]. It effectively crosses the blood brain barrier and is neuroprotective in experimental models of cerebral ischemia, brain trauma, HD, amyotrophic lateral sclerosis, and Parkinson's disease. Four SOD1 transgenic mouse studies show enhanced median survival ranging between 6.4% and 16% [95-98]. Interestingly, the combination of minocycline and creatine resulted in additive neuroprotection and may well be a novel polytherapeutic strategy for the treatment of ALS [97]. In a recent study by Pontieri, the safety of combined treatment with minocycline and riluzole in ALS was investigated [98]. Twenty ALS patients were randomized into two groups and administered either riluzole (50 mg b.i.d.) or riluzole and minocycline [100 mg i.d.] for 6 months. Combined treatment with minocycline and riluzole resulted in no significant adverse events. This pilot study provides evidence that minocycline and riluzole can be taken safely together. Further clinical trials are needed to assess the efficacy of such treatment in ALS patients.

4.6. Other Therapeutics for ALS

4.6.1. Gene Therapy Using Neurotrophic Factors

Vascular endothelial growth factor (VEGF) is a cytokine that has protective function *via* angiogenic, neurotrophic, gliotrophic and anti-apoptotic activities. VEGF has been shown to inhibit neurodegeneration in ALS mice and may have therapeutic potential in patients with ALS [99, 100]. Intracerebroventricular delivery of recombinant VEGF in the G93A rat model of ALS delays onset of paralysis by 17 days, improves motor performance, and prolongs survival by 22 days [99]. The neuronal expression of a transgene expressing the VEGF receptor prolongs the survival of G93A ALS mice. In addition, VEGFR2 staining is reduced in the neuropil of ALS patients associated with a reduction of synaptophysin. A greater proportion of AHCs in ALS patients show low expression of VEGF and VEGFR2 compared with controls. A similar expression pattern of VEGF and VEGFR2 in ALS suggests autocrine/paracrine effects on spinal motor neurons. The reduction in VEGF and VEGFR2 expression seen in ALS patients supports the hypothesis that, as in mouse models of the disease, reduced VEGF signaling may play a role in the pathogenesis of ALS. Although a number of human neurotrophic molecules have been administered to ALS patients with slowing of functional progression, a recent trial using BDNF in 10 ALS patients concluded that there was no improvement in autonomic function tests and serum norepinephrine levels [101]. VEGF gene therapy, however, may be used in clinical trials in the near future.

4.6.2. Vaccine Therapy

Schwartz and colleagues have reported that vaccination with Copaxone (glatiramer acetate, Cop-1) protects motor neurons against acute and chronic degenerative conditions [102]. Cop-1 vaccination extended the life span in G93A ALS mice by 24%, compared to untreated matched controls. Furthermore, Cop-1 vaccination markedly improved motor function. These findings suggest that Cop-1 vaccination boosts the local immune response necessary in modulating destructive self-compounds associated with motor neuron death. Although autoimmunity as a mechanism of pathogenesis has been controversial in ALS, recent studies have refocused research efforts in this area. Cop-1 administration is an approved therapy for multiple sclerosis.

4.6.3. Stem Cell Therapy

Stem cell therapy has been tested to treat incurable neurodegenerative diseases including ALS [103]. Autologous mesenchymal bone marrow stem cells have been safely transplanted into the spinal cord and well tolerated by ALS patients [104]. Moreover, human umbilical cord blood cells provide therapeutic potential in noninvasive cell-based treatment of ALS by providing cell replacement and protec-

tion of motor neurons [105]. Although stem cell transplantation has been effective in ALS patients, the underlying mechanisms are yet to be determined. Several mechanisms such as cell fusion, neurotrophic factor release, endogenous stem cell proliferation, and transdifferentiation may explain positive therapeutic results, in addition to replacement of lost cells [103, 106]. Encouragingly, a recent clinical study has shown that intraspinal cord implantation of autologous mesenchymal stem cells (MSCs) in ALS patients shows a significant slowing of the linear decline of the forced vital capacity 36 months after MSCs transplantation. This study demonstrates that direct injection of autologous expanded MSCs into the spinal cord of ALS patients is safe, with no significant acute or late toxicity, and well tolerated [107]. The biological issues need to be clarified in order to maximize the potential for effective therapies. It may be necessary, however, to combine stem cell therapy with other treatments and agents to improve efficacy.

CONCLUSION

Specific neuroprotective strategies targeted at identified molecular mechanisms have the potential to dramatically delay the onset and slow the progression of ALS. As such, the compounds described above may emerge as relatively safe therapeutics for the treatment of ALS. Although the drug agents and their analogs described in this review are available for human use and represent immediate candidates as neuroprotective agents for clinical trials in ALS, an important implication of the multiple levels of molecular pathology and treatment is that it will most likely be necessary to combine neuroprotective therapies to maximize neuroprotection in order to reach the greatest efficacy.

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