Investigation of the novel small molecule HSP90 inhibitor, NXD30001, in a mouse model of amyotrophic lateral sclerosis

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Abstract

Heat shock proteins (HSPs) are attractive therapeutic targets for neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), that are characterized by aberrant formation of protein aggregates. Small molecule HSP90 inhibitors are effective inducers of *HSP* gene expression in eukaryotic cells, including motor neurons, the primary cell type affected in ALS. Our lab has evaluated a novel small molecule HSP90 inhibitor, NXD30001, for *in vitro* efficacy using a primary culture model of fALS1 due to mutations in the *SOD1* gene. Treatment with NXD30001 induced the expression of HSP40 and the inducible form of HSP70 (iHSP70) through activation of HSF1 and protected against several measures of mutant SOD1 (mSOD1) toxicity in primary motor neurons, including: protein aggregation and accumulation, mitochondrial fragmentation and motor neuron death. This study assessed NXD30001's ability to induce *HSP* gene expression in the $SOD1^{G93A}$ transgenic mouse model of ALS. Symptomatic $SOD1^{G93A}$ transgenic mice and nontransgenic wild-type mice were dosed intraperitoneally (*i.p.*) with NXD30001 and their tissues were analyzed for drug penetrance and expression of HSPs (HSP90, iHSP70, HSP60, HSP40 and HSP25). NXD30001 distributed to brain, spinal cord and peripheral tissues in $SOD1^{G93A}$ transgenic and nontransgenic mice after acute and subacute *i.p.* injections. The profile of NXD30001-mediated induction of *Hsp* gene expression differed *in vivo* from *in vitro*. NXD30001 induced expression of iHSP70 in skeletal muscle, cardiac muscle and, to a lesser extent, in kidney, but not in liver, spinal cord or brain with either single or repeated administration. NXD30001 is thus a useful experimental tool for culture work, but these data highlight the complex nature of *Hsp* gene regulation *in vivo* and the need for early evaluation of the efficacy of novel HSP inducers in target tissues.

Résumé

De par leur rôle dans le repliement des protéines, les protéines chaperonnes (HSPs) représentent des cibles thérapeutiques intéressantes pour le traitement des maladies neurodégénératives, comme la sclérose latérale amyotrophique (ALS), caractérisés par la formation des agrégats protéiques. Les molécules inhibitrices de HSP90 sont des inducteurs de l'expression des gènes codant pour les HSPs notamment dans les neurones moteurs affectés dans l'ALS. Nous avons déterminé l'efficacité *in vitro* d'un nouvel inhibiteur de HSP90, le NXD30001, sur l'induction de l'expression de HSP40 et de HSP70 et son effet sur des mesures phénotypiques (agrégation protéique, fragmentation mitochondrial et mort neuronale) du à l'expression de SOD1 mutants. Cette étude se propose de déterminer les propriétés *in vivo* du NXD30001 en utilisant un modèle murin d'ALS. Les souris transgéniques $SOD1^{G93A}$ ont été traitées avec le NXD30001 délivré par injection intra-péritonéales (*i.p.*) afin de déterminer l'expression des gènes des protéines chaperonnes (HSP90, iHSP70, HSP60, HSP40 and HSP25) et la distribution tissulaire du NXD30001. La pénétrance tissulaire du NXD30001 dans le cerveau, la moelle épinière et les tissus périphériques est effective à la suite d'injection *i.p.* En revanche le profil d'expression des gènes HSPs est diffèrent de celui observe *in vitro*. NXD30001 induit l'expression de HSP70 dans le muscle squelettique, le muscle cardiaque et les reins mais est inefficace en regard de l'induction de HSP70 dans le foie, la moelle épinière ou le cerveau en dépit de différents protocoles d'injection. En conclusion, le NXD30001 est un outil intéressant pour la culture cellulaire mais notre étude met en évidence la régulation complexe des gènes des HSPs *in vivo* et le besoin d'une évaluation de leur efficacité dans les tissus ciblés.

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List of Abbreviations

°C	degrees Celsius
µg	microgram
µm	micrometer
µM	micromolar
17-AAG	17-allylamino-17-desmethoxygeldanamycin
17-DMAG	17-(2-dimethylaminoethyl)-amino-17-desmethoxygeldanamycin
ALS	amyotrophic lateral sclerosis
ALS-PDC	amyotrophic lateral sclerosis with associated Parkinsonism-dementia complex
ANG	angiogenin
AR	androgen receptor
ASK1	apoptosis signal-regulating kinase 1
ATP	adenosine triphosphate
BAG-1	Bcl-2-associated-athanogene
BBB	blood brain barrier
C9ORF72	open reading frame 72 on chromosome 9
CAF	cytosine β-D-arabinofuranoside
CHIP	carboxyl-terminus of the HSC70-interacting protein
CHMP2B	charged multivesicular body protein 2B
DCTN1	dynactin 1
DMSO	dimethyl sulfoxide

DRG	dorsal root ganglia
EDTA	ethylenediaminetetraacetic acid
EMEM	Eagle's minimum essential medium
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
fALS	familial ALS
fALS1	familial ALS associated with mutations in the *SOD1* gene
FTD	frontotemporal dementia
FUS/TLS	fused in sarcoma/translated in liposarcoma
G	gravity
g	gram
GLT1	glial glutamate transporter 1
hr	hour
HRP	horseradish peroxidase
HSC70	heat shock cognate protein 70 kDa
HSE	heat shock element
HSF	heat shock factor
HSF1	heat shock factor 1
HSR1	heat shock RNA 1
hSOD1	human superoxide dismutase 1
HSP	heat shock protein
HSP90	heat shock protein 90 kDa

HSR	heat shock resposne
i.p.	intraperitoneal
i.v.	intravenous
iHSP70	inducible heat shock protein 70 kDa
kDa	kilodalton
kg	kilogram
L	liter
LCMS	liquid chromatography - mass spectrometry
M	molar
MEM	minimum essential medium
mg	milligram
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
mSOD1	mutant superoxide dismutase
NFH	neurofilament heavy chain subunit
ng	nanogram
NGF	nerve growth factor
nM	nanomolar
PBS	phosphate-buffered saline
PDC	Parkinson dementia complex

PK	pharmacokinetic
PON	paraxonase
rhHSP70	recombinant human HSP70
sALS	sporadic ALS
SBMA	spinal-bulbar muscular atrophy
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
sec	seconds
sHSP	small heat shock proteins
SMN	survival of motor neuron
SNP	single nucleotide polymorphism
SOD1	superoxide dismutase 1
$SOD1^{G93A}$	glycine-to-alanine substitution at codon 93 of the *SOD1* gene
TBS	tris-buffered saline
TDP43	TAR DNA-binding protein 43
UPR	unfolded protein response
UPS	ubiquitin-proteasome system
V	volt
VAPB	VAMP-associated protein B
VCP	valosin-containing protein
VEGF	vascular endothelial growth factor
wtSOD1	wild-type superoxide dismutase 1

µL	microliter

Introduction

The increasing burden of neurodegenerative disease on our increasingly-aging population has emerged as a serious healthcare issue. Diseases such as Alzheimer's disease, Parkinson's disease and ALS typically strike patients late in life, causing a variety of devastating symptoms that progressively destroy quality of life. Despite significant strides in understanding the clinical progression of these diseases, their pathogeneses remain poorly understood and few therapies are currently available to patients. Although these diseases affect different areas of the central nervous system, several commonalities have been identified in their pathogeneses at the molecular level. For example, proteotoxicity, or the cell's inability to effectively regulate protein homeostasis, has been observed in nearly all neurodegenerative diseases and has emerged as a target for the development of small molecule therapeutics.

ALS is characterized by the preferential degeneration and death of motor neurons, which innervate skeletal muscle and control voluntary muscle movement. Their progressive degeneration causes a gradual loss of voluntary muscle movement and, eventually, paralysis. Some patients also develop cognitive and behavioural abnormalities associated with degeneration of neurons in the frontal and temporal lobes of the brain (1). End-stage ALS is characterized by respiratory failure and necessitates external breathing support. ALS is a universally fatal disease with no effective treatment; the only FDA-approved pharmacotherapeutic, Riluzole, extends lifespan by 3-6 months in only some ALS patients. As such, most patients progress from diagnosis to end-stage within 2-5 years. Roughly 90% of ALS cases occur sporadically, that is, without any family history of disease, with the remaining 10% of cases demonstrating Mendelian familial inheritance patterns. The most commonly-studied

form of familial ALS is caused by mutations in the gene encoding the superoxide dismutase 1 (SOD1) enzyme. Until recently, mutations in SOD1 were the most common identified cause of fALS, accounting for 20% of all fALS cases. Recently, however, hexanucleotide expansions in the open reading frame 72 on chromosome 9 (C9ORF72) were identified and are now the most common known genetic cause of ALS (2, 3). The genetic climate of ALS is therefore rapidly evolving, with new ALS-associated genes providing insights into pathogenic mechanisms.

Despite the multifactorial nature of ALS pathogenesis, the accumulation of misfolded proteins could represent an upstream process that is amenable to therapeutic intervention. Indeed, the upregulation of heat shock proteins (HSPs), a family of proteins with chaperoning activity, has extended neuronal viability in primary motor neurons and transgenic mice overexpressing mutant SOD1 (4-7). Translation of this strategy to the clinic has, however, been challenged by the lack of safe and stable inducers of HSP expression that can penetrate the blood-brain-barrier (BBB). Small molecule inducers of HSP expression with more favourable pharmacokinetic (PK) and safety profiles are therefore in development. This study examined NXD30001, a novel small molecule HSP inducer from NexGenix Pharmaceuticals that disributes to the CNS (8), for its ability to induce HSPs in the tissues of the $SOD1^{G93A}$ transgenic mouse model of ALS.

Chapter 1: Literature Review

1.1 Amyotrophic Lateral Sclerosis

1.1.1 What is ALS?

Described by the French neurologist Jean-Martin Charcot in 1869 (9), amyotrophic lateral sclerosis (ALS, Lou Gehrig's Disease) is an adult-onset, fatal neurodegenerative disease of the human motor system. ALS is characterized by the preferential degeneration of upper and lower motor neurons resulting in progressive muscular weakness, paralysis and eventual death due to respiratory failure typically within 1-5 years of diagnosis (10). ALS cases are classified as familial (fALS) or sporadic (sALS) based on the presence or absence of a family history of disease, respectively. Despite substantial progress elucidating the mechanisms and origins of its pathogenesis, ALS remains a poorly understood disease for which there is no cure or effective treatment. The only approved pharmacotherapeutic agent for ALS is Riluzole, which extends lifespan by approximately 3 months in a subset of ALS patients (11). A clear need thus exists for clinically-applicable, disease-modifying therapies.

1.1.2 Clinical Aspects of ALS

Diagnosis is established through the revised El Escorial criteria of the World Federation of Neurology which, in short, identify the progressive and specific loss of upper and lower motor neurons in the absence of electrophysiological, pathological or clinical signs of non-ALS disease (12). Neurologists diagnose patients as having suspected, possible, probable or definite ALS (12). With a disease prevalence of 4-6 per 100,000 and an incidence of 1-2 per 100,000, approximately 3,000 Canadians currently live with ALS (13).

Symptom onset is heterogeneous and may be bulbar, impairing swallowing and speech; cervical, impairing arm and shoulder movements; or lumbar, causing mobility problems. The onset of symptoms reflects the location of the motor neurons most affected in each patient, with bulbar-onset patients having the worst clinical prognosis (10). Motor neuron degeneration increases with disease progression, resulting in increased muscle weakness and paralysis. Consequently, swallowing becomes challenging, resulting in reduced caloric intake producing nutrition problems and an increased choking risk. Communication and movement also progressively deteriorate due to speaking, writing and walking difficulties, and eventually become impossible without assistance. End-stage ALS occurs when the motor neurons innervating the diaphragm and intercostal muscles degenerate, necessitating permanent external breathing support.

Some ALS patients also show cognitive symptoms (1). Cognitive impairments were measured in roughly half of a sALS patient cohort, with 6% showing severe deficits (1, 14). Of the patients assessed in this study, 15% were classified as having frontotemporal dementia (FTD) in addition to ALS. ALS-FTD patients show a range of social, emotional and sexual dysfunctions, and the severity of these symptoms is correlated with the amount of frontal and temporal lobe degeneration (15). This overlap of distinct neurodegenerative diseases suggests the existence of a spectrum of neurodegenerative disease encompassing ALS, FTD and ALS-FTD. Indeed, mutations in the genes encoding charged multivesicular body protein 2B (CHMP2B), dynactin 1 (DCTN1), fused in sarcoma/translated in liposarcoma (FUS/TLS), TAR DNA binding protein 43 kDa (TDP43) and open reading frame 72 on chromosome 9 (C9ORF72) have been

identified in ALS and FTD (2, 3, 16-18); how these mutations lead to multiple neurodegenerative diseases remains unclear.

1.1.2 Sporadic ALS

Approximately 90% of ALS diagnoses are classified as sporadic. While the causes of sALS are unknown, several genetic and environmental factors have been suggested as risk factors. In fact, with identification of genes in fALS, some individuals classified as sporadic are found to have a mutation.

An environmental contribution to sALS was first recognized in 1945 following a physician's report of an unusually high incidence of motor neuron disease with associated Parkinsonism and Dementia Complex (PDC) in the indigenous population of Guam (19). This endemic was later associated with the consumption of flour derived from cycad seeds, which contain high amounts of toxic plant sterol glucosides (20). Indeed, mice fed cycad flour developed ALS-PDC-like symptoms with associated degeneration in several regions of the brain and spinal cord (21, 22). Outside of Guam, the search for environmental contributors to ALS has yielded disappointing results, possibly due to the retrospective design of the studies, their inherent biases, and the small number of cases studied (low power of the studies). Nevertheless, several risk factors have been suggested, including: solvent exposure, cigarette smoking, prior poliovirus infection, physical activity, trauma, alcohol consumption, residence in rural regions, farm work and active military service during the Gulf War (23-25).

Much of the knowledge regarding what, if any, genetic factors contribute to sALS have come from twin studies. A study examining mono- and dizygotic twins where one sibling had

ALS found that 4 of 26 monozygotic twins were concordant-affected, compared to 0 of 51 dizygotic twins, translating to an estimated heritability value of 0.38 to 0.85 (26). A more recent study of larger sample size produced similar results, reporting an estimated heritability value of 0.38 to 0.78 for concordant development of ALS disease in monozygotic twins (27). Mutations or single nucleotide polymorphisms (SNP) in several genes have been associated with a possibly increased risk of ALS. These genes include those encoding vascular endothelial growth factor (VEGF) (28), angiogenin (ANG) (29, 30), paraoxonases (PON1, PON2, PON3) (31-33), ataxin 2 (34) and the neurofilament heavy subunit (NFH) (35, 36). Low expression of the survival of motor neuron proteins (SMN1, SMN2) or a haploinsuffciency of SMN1 are also associated with an increased risk of developing sALS (37, 38).

1.1.3 Familial ALS

An estimated 10% of ALS patients have a family history of ALS. Mutations in several genes have proven to be causative of fALS, including those encoding Cu^{2+}/Zn^{2+}-superoxide dismutase (SOD1) (39), TDP43 (40), FUS/TLS (41, 42), VAMP-associated protein B (VAPB) (43), Valosin-Containing Protein (VCP) (44), ubiquilin 2 (45), senetaxin (46), optineurin (47), dynactin (16), C9ORF72 (2, 3) and profilin-1 (48). Mutations in these genes cause a variety of ALS subtypes characterized by different ages of onset, penetrance, inheritance patterns and modes of toxicity (gain-of-function, loss-of-function, unknown). Some ALS subtypes are characterized by mutations in genes encoding proteins implicated in common pathways such as RNA processing (TDP43, FUS, senataxin) and cellular trafficking (VAPB, optineurin, dynactin), providing insight into possible disease-causing mechanisms. Familial and sporadic forms of ALS

are clinically indistinguishable, aside from observations of earlier symptom onset and a longer course of disease progression in some fALS patients (49). The pathogeneses of familial and sporadic forms also share common molecular abnormalities in affected cell types, including protein misfolding and aggregation, impaired calcium (Ca^{2+}) homeostasis, elevated oxidative stress, abnormal mitochondrial morphology and mitochondrial dysfunction (10).

1.1.4 Familial ALS linked to SOD1 mutations

An estimated 20% of fALS cases are linked to mutations in the gene encoding SOD1, with the glycine-to-alanine substitution at codon 93 ($SOD1^{G93A}$) being the most extensively studied mutation. Over 150 ALS-causing mutations have been identified in the *SOD1* gene, with the majority being missense mutations occurring in various regions of the protein (50).

Human SOD1 (hSOD1) is a ubiquitously expressed, predominantly cytosolic enzyme encoded by a gene of five exons and four introns on chromosome 21 (51). SOD1 homodimerizes to catalyze the conversion of the superoxide radical, a toxic by-product of oxidative phosphorylation (52), to hydrogen peroxide. FALS1 is not caused by a loss of SOD1 function, as the majority of known ALS-associated SOD1 mutants maintain enzymatic activity (53), SOD1 knock-out mice do not develop ALS-like disease (54, 55), and overexpression of wild-type hSOD1 (wtSOD1) in transgenic mice expressing mSOD1 protein did not prevent or ameliorate disease progression; in fact, co-expression of wtSOD1 has been linked to faster disease progression in $SOD1^{G93A}$ transgenic mice (56, 57). ALS-linked mutant SOD1 protein is therefore pathogenic through a gain of toxic function mechanism that is independent of changes in dismutase activity (58).

While ALS is a motor neuron disease, its pathogenesis likely results from the manifestation of many disease pathways in multiple cell types. This non-cell autonomous theory is supported by the absence of motor phenotypes in mice with neuron-specific or astrocyte-specific expression of mSOD1 (59, 60). Additionally, expression of mSOD1 in non-neuronal cells exacerbates disease in motor neurons in mice, illustrating that toxic mechanisms in several cell types likely converge to cause fALS1 (61, 62).

SOD1-immunoreactive inclusions are found in the motor neurons of fALS and sALS patients (63, 64), transgenic mice and rats expressing mutant SOD1 (65, 66), and in fALS1 culture models using primary, dissociated spinal cord from embryonic mice (67). Although the pathogenic contribution of these inclusions is debated, the accumulation of misfolded mutant SOD1 protein is believed to contribute to ALS pathology. In addition to protein accumulation, mutant SOD1 toxicity also involves increased oxidative stress, endoplasmic reticulum (E.R.) stress, glial cell activation, disruption of cytoskeletal networks, glutamate-mediated excitotoxicity, impaired proteasomal activity, mitochondrial network fragmentation and mitochondrial dysfunction (58). Interestingly, SOD1-immunoreactive protein inclusions have been identified in the motor neurons of sALS patients, suggesting that the accumulation of SOD1 could be a common pathogenic contributor regardless of ALS subtype (63, 64, 68, 69).

1.1.5 Protein misfolding and aggregation in fALS1

Substantial evidence points to a common propensity of mSOD1 to misfold and aggregate as its primary toxic gain of function (70). Indeed, the majority of ALS-causing mutations in SOD1 have been shown to destabilize the protein's native fold *in silico* (71) and *in*

vitro (72-74), and the severity of this destabilization is correlated with decreased survival time in fALS1 patients (75, 76). It thus appears that mSOD1-induced proteotoxicity is a central contributor to fALS1 and that misfolded, non-mutant SOD1 could contribute to the pathogenesis of other ALS subtypes.

Several groups are investigating the molecular determinants of SOD1 misfolding and aggregation. In its mature, homodimeric state, SOD1 is extremely stable and retains dismutase activity in the presence of 10M urea *in vitro* (77). This stability is largely mediated by the associated Zn^{2+} ion, which tethers the relatively unstructured electrostatic and zinc-binding loops, protecting the eight-stranded Greek key β-barrel enzymatic core (70, 72, 78, 79). *In vitro* assays suggest that dissociation of the SOD1 homodimer is a necessary initiating step in SOD1 aggregation (80, 81). Monomeric SOD1 is more prone to dissociation from the stabilizing Zn^{2+} ion, leading to exposure of β-barrel edge strands (78, 79) and freer movement of the electrostatic loop (82). Metal-free, monomeric SOD1 has increased surface hydrophobicity, and this increase in surface hydrophobicity appears to precede its aggregation (83, 84). A general model of ALS-associated SOD1 aggregation has thus emerged in which dissociation of the SOD1 homodimer and subsequent Zn^{2+} loss promote structural changes favoring assembly of the protein into non-native oligomers (70).

Metal-free, monomeric SOD1 has been observed in both insoluble, detergent-resistant aggregates (also called inclusion bodies when microscopically visible) and soluble oligomers in cell culture, transgenic mice overexpressing mSOD1 and fALS1 patients (63, 64, 70, 85-87). Which of these species contributes most to motor neuron death remains a controversial subject in the field. The large, insoluble aggregates appear coincidentally with symptom onset in brain

stem and spinal cord motor neurons and accumulate with disease progression in mice overexpressing mSOD1 (88, 89). Furthermore, in cell lines overexpressing mSOD1, 90% of cells that formed inclusion bodies died within 48 hours of their formation, whereas 70% of cells without visible inclusion body formation survived this time period (90). However, immunohistochemical analyses using antibodies immunoreactive to misfolded SOD1 show that soluble SOD1 oligomers are present from birth and are selectively enriched in the motor neurons of transgenic mice overexpressing mutant SOD1 (91-93). Small, misfolded SOD1 oligomers are thus hypothesized to be the contributing toxic species, accumulating throughout life and affecting multiple cellular pathways (70). Under this paradigm, the consolidation of misfolded SOD1 into inclusion bodies is considered to be neuroprotective, as has been observed with the aggregation of Aβ into plaques in Alzheimer's disease (94) and the aggregation of huntingtin into inclusion bodies in Huntington's disease (95). Nevertheless, the relative toxicities of the large-scale insoluble SOD1 aggregates or small soluble SOD1 oligomers remain unknown and, as such, no clear model exists to explain SOD1's proteotoxicity. It appears that proteotoxicity plays a central role in ALS pathogenesis, with adverse effects on multiple cellular pathways.

1.2 Protein Quality Control Mechanisms

1.2.1 Protein quality control in fALS1

Many of the pathological mechanisms shown to contribute to ALS culminate in the appearance of proteinaceous aggregates, suggesting that overloading or malfunction of protein quality control machinery is a common feature across ALS disease subtypes (70). Indeed, several protein quality control mechanisms are impaired in models of ALS and in the tissues of ALS patients.

The ubiquitin-proteasome system (UPS) plays a major role in cellular protein homeostasis, degrading many normal and damaged proteins (96, 97). The UPS serves two functions: protein recruitment and degradation. Protein recruitment is accomplished through the coordinated interplay of three enzymes, the first of which activates the cellular protein ubiquitin in an ATP-dependent reaction allowing for its transfer to the second enzyme, a ubiquitin carrier. Activated ubiquitin is then transferred from the ubiquitin carrier to the target protein via the third enzyme, a ubiquitin protein ligase (98). The ubiquitin tag acts as a signal for the target protein's transport to the proteasome. The proteasomal machinery comprises two 19S cap structures and one 20S core, forming a 26S complex. The 19S caps serve primarily regulatory roles, while the 20S supplies proteolytic activity (98). The proteasome is largely responsible for mSOD1 degradation (99), and mSOD1 colocalizes with proteasomal subunits and ubiquitin ligases (99-101). Ubiquitin- and ubiquitin-ligase positive inclusion bodies have been identified in the motor neurons of both transgenic mice overexpressing mutant SOD1 protein (65, 102, 103) and postmortem spinal cord tissues from sALS patients (104-107), indicating activity of the UPS and, possibly, the sequestration of its machinery due to increased

substrate load (70). Furthermore, proteasomal activity was found to be impaired in the brainstem and spinal cord of transgenic mice overexpressing mSOD1 both before and after onset of symptoms (108, 109), and this impairment was detectable in extracts from the lumbar spinal cord, the region most sensitive to ALS pathology, long before other regions (109). Presymptomatic reductions of structural β3 subunit and β5 catalytic subunit protein levels were also observed in these mice, as were postsymptomatic, motor neuron-specific decreases in 20S protein levels as measured through immunohistochemical labeling (110). A decrease in proteasomal catalytic activity was also observed in spinal cord homogenates from postmortem sALS patients, and this decrease was concomitant with a reduction in β5 subunit protein levels (111). Evidence thus supports that proteasomal activity is reduced in ALS-affected tissues.

Protein quality control through ER-associated degradation (ERAD) is also impaired in ALS. During nascent protein synthesis and maturation in the endoplasmic reticulum (ER), mis-folded proteins are removed from the ER lumen through the ERAD pathway. Overloading or inhibition of ERAD leads causes an accumulation of mis-folded proteins in the luminal space, triggering the Unfolded Protein Response (UPR) (112). ERAD is inhibited directly by mSOD1 through its binding of derlin-1 (113), a transmembrane protein responsible for the transport of mis-folded proteins from the ER lumen to the cytoplasm for proteasomal degradation, and BiP (114), a chaperone protein localized to the ER lumen. SOD1-mediated inhibition of ERAD creates ER stress which activates apoptosis signal-regulating kinase 1 (ASK1), a pro-apoptotic protein kinase (113). Interestingly, mutations in VAPB, a protein involved in the UPR, are linked to some ALS cases (43). ER stress is thus a likely contributor to ALS pathogenesis.

In addition to these proteasomal and ERAD deficits, protein homeostasis in motor neurons is challenged in ALS by a higher threshold of activation for stress-induced protein quality control mechanisms (115), known as the Heat Shock Response (HSR), compared to other cell types in the spinal cord, which may contribute to a preferential vulnerability of motor neurons to proteotoxic insult.

1.2.2 The heat shock response

The HSR is an evolutionarily-conserved orchestrated activation of protective responses by the cell upon exposure to stress (116). Originally described as the biochemical response of cells to elevated temperatures (117), the HSR has since been observed to respond to a variety of cellular stresses including, amongst others, oxidative stress, altered pH, heavy metals and detergents (118). A common consequence of cellular stressors is the presence of denatured or misfolded proteins, often resulting in protein aggregation. The cell responds by arresting the synthesis of non-essential proteins via the sequestration of mRNAs into protein structures known as stress granules, or by directing them to P bodies for degradation (116). This inhibition of general protein synthesis serves to reduce the intracellular protein load, reducing the likelihood of further protein aggregation and accumulation.

The cell also responds to stress by selectively activating Heat Shock Factors (HSFs). HSFs are transcription factors that mediate the stress response through interactions with conserved DNA binding regions called Heat Shock Elements (HSE). The nuclear translocation of HSFs and their subsequent interactions with HSEs produce an upregulation of Heat Shock Protein (HSP) expression, a group of primarily chaperone-type proteins which act to alleviate the aggregation

of mis-folded proteins (116, 119). In mammals, Heat Shock Factor-1 (HSF1) is the main HSF responsible for stress-induced transcriptional activation. Under resting conditions, HSF1 is cytoplasmic, monomeric and is bound with Heat Shock Protein 90 (HSP90) and the co-chaperone p23. Cellular stress stimulates the dissociation of HSF1 from the HSP90 complex. HSF1 then trimerizes and becomes phosphorylated, activating the protein's DNA-binding ability (120), and accumulates in the nucleus where it binds to HSEs in the promoters of target genes, leading to *HSP* gene expression (116, 121).

In addition to dissociation of the HSP90 complex, the activation of HSF1's pro-transcriptional functionality involves heat shock RNA-1 (HSR1). HSR1 is a large, noncoding RNA thermosensor that is required for HSF1 activation in human cell lines, functioning in a ribonucleoprotein complex containing translation elongation factor eIF1A that promotes trimerization of HSF1 and expression of *HSP* genes at temperatures greater than 37°C (122). Although the precise mechanism of HSR1's contribution to HSF1 activation is unknown, most known RNA thermosensors are complex nucleic acid structures found in the 5' UTR of mRNAs that inhibit translation of the mRNA (123). High temperature induces melting of the secondary structure, allowing for ribosomal access to the mRNA (123). These temperature-labile 5' structures thus provide "thermosensing" functionality (123).

HSPs are highly conserved, ubiquitously expressed proteins belonging to a multi-gene family. HSPs are grouped into sub-families based on their molecular weight and range in size from 8 to 150 kilodaltons (kDa). The five major mammalian HSP sub-families are HSP90, HSP70, HSP60, HSP40 and the small heat shock proteins (sHSP) (124). HSPs aid in the folding of nascent polypeptides, or with the refolding of mis-folded proteins, and thus serve to prevent protein

aggregation. HSPs also function in a variety of other cellular roles, including the transport and degradation of proteins, signal transduction, cellular growth and differentiation, and the prevention of apoptosis (125, 126). Isoforms of HSPs from the same sub-family can localize to different subcellular compartments, and their expression can be constitutive or stress-dependent. Within the HSP70 sub-family, for example, heat shock cognate protein 70 (HSC70) is constitutively expressed, whereas inducible HSP70 (iHSP70) is expressed at very low levels or only in response to stress, depending on cell type (127).

HSPs bind to mis-folded or unfolded proteins at exposed areas of high hydrophobicity. HSPs may then attempt to re-fold the polypeptide in an ATP-dependent process that requires the function of co-chaperone proteins. A common example of this HSP/co-chaperone interplay is that of HSP70-family proteins, which bind unfolded or mis-folded proteins through their C-terminal domain, and their HSP40-family co-chaperone proteins, which activate HSP70's ATPase activity (128). Should HSP-mediated refolding of a mis-folded polypeptide fail, HSPs will shuttle their substrate to proteasomal or autophagic degradation pathways. For example, HSP70 can associate with Bcl-2-associated athanogene (BAG-1) and the arboxyl terminus of HSC70-interacting protein (CHIP) (129) co-chaperones, which coordinate transfer of the HSP70-HSP40 complex's protein substrate to proteasomal machinery by stimulating the unloading of the protein substrate from the complex (130) and its ubiquitination (131), respectively.

1.2.3 The heat shock response in ALS

The threshold for stress-induced induction of HSP70 varies amongst neuronal subpopulations (132). Motor neurons appear to be particularly resistant, as no upregulation of

HSP70 was observed in primary dissociated motor neurons after heat shock, exposure to excitotoxic glutamate or mSOD1-induced proteotoxicity (115). Similarly, immunohistochemical analysis of post-mortem spinal cord slices using antibodies immunoreactive to HSP70 revealed no upregulation of HSP70 in the motor neurons or astroglia of fALS1 and sALS patients (115) or transgenic mice overexpressing mSOD1 (115, 133). It is likely this resistance stems from an impaired activation of HSF1, as primary motor neurons expressing a constitutively active form of HSF1 significantly upregulated expression of HSP70 as assessed by immunofluorescent labeling of iHSP70 (115).

In addition to this reduced ability of motor neurons to respond to stress, mSOD1 is believed to bind and sequester HSPs. Indeed, HSP27, HSP40 and HSP70 have been shown to colocalize with SOD1-immunopositive aggregates in the spinal cord motor neurons of transgenic rats overexpressing mSOD1 (90, 134, 135). However, the formation of inclusion bodies likely reflects the failure of chaperones in motor neurons to handle proteotoxic insult, as evidenced by an observed decrease in chaperoning activity in the lumbar spinal cord of transgenic mice overexpressing mSOD1 (4). FALS1-affected motor neurons are thus challenged both in their ability to handle housekeeping proteostasis due to insufficient proteasomal and chaperoning activity and in their ability to respond to stress due to their high threshold for HSF1 activation. Improving the chaperoning capabilities of motor neurons has therefore emerged as a target for therapeutic intervention.

1.2.4 The heat shock response as a therapeutic target for fALS1

To investigate the effects of increased *HSP* expression on ALS pathogenesis, several studies have used transgenic mice overexpressing *HSP* genes. In transgenic mice overexpressing both HSP70 and mSOD1, no increases in time to symptom onset or survival were observed compared to transgenic mice overexpressing mSOD1 alone (136). Similarly, transgenic mice overexpressing both HSP27 and mSOD1 showed no improvements in time to symptom onset or survival (137, 138), although an early protection of the neuromuscular junction was observed in one study (138). These results suggest that upregulation of a single HSP is insufficient when attempting to improve overall protein quality control (116). This is corroborated by *in vitro* data demonstrating that overexpression of both HSP40 and HSP70 in a primary culture model of fALS1 conferred substantially increased neuroprotection compared to the overexpression of HSP70 alone (4, 5). Strangely, presymptomatic intraperitoneal (*i.p.*) injection of recombinant human HSP70 (rhHSP70) significantly delayed the onset of symptoms, improved motor function and increased lifespan in the $SOD1^{G93A}$ transgenic mouse model of fALS1 (139). No increase in the number of surviving motor neurons was observed at 120 days of age in rhHSP70-treated $SOD1^{G93A}$ mice, and no rhHSP70 protein was detected in cortex or spinal cord tissues of the same mice (139). The authors therefore attributed the beneficial effects of *i.p.* rhHSP70 to the protection of muscle fibers and peripheral motor nerves (139, 140). Overall, genetic manipulation of single *HSP* gene expression does not appear to offer substantial benefit in models of ALS. Pharmacological agents that upregulate expression of HSPs therefore represent a more attractive therapeutic strategy, and several have been assessed in models of ALS.

The only FDA-approved pharmacotherapeutic for ALS, Riluzole, has been shown to increase lifespan in some ALS patients (11). Riluzole's mechanism of action is poorly understood

and is thought to involve inhibition of glutamate release and, consequently, protection of motor neurons from excitotoxicity. Interestingly, Riluzole's neuroprotection may also involve the upregulation of HSPs. For instance, Riluzole increases the protein levels of cytosolic HSF1 monomers and nuclear HSF1 dimers and trimers in stressed HeLa cells, producing an amplified HSR (141). Riluzole was also shown to increase the activity of the glial glutamate transporter 1 (GLT1) promoter, which harbors a HSE, possibly through activation of HSF1 (142). A conclusive link between Riluzole and the HSR has yet to be established, but this initial link between HSF1 activation and increased survival warrants further evaluation of HSR-modulating compounds.

1.3 HSP90 Inhibition as a Therapeutic Strategy

1.3.1 HSP90

HSP90 is a ubiquitously expressed, multifunctional family of chaperones that are among the most abundantly expressed proteins in mammalian cells. Five human HSP90 isoforms have been identified which differ in substrate specificity, subunit structure and subcellular localization (143). HSP90 functions in a multicomponent complex of chaperones that includes HSP70, HSP40, Cdc37 and p23 to regulate several cellular processes including cell cycle, signal transduction, maturation of client proteins, cellular proliferation and apoptosis (144-146). Unsurprisingly, HSP90's client proteins include a variety of cell signaling molecules,

transcription factors and hormone receptors, many of which are implicated in oncogenesis (146).

The structure of HSP90 is organized into three functional domains: the C-terminal domain, which is essential for HSP90's dimerization; the middle domain, which contains binding sites for HSP90 client proteins; and the N-terminal domain, which contains an ATP-binding site and harbours ATPase activity (147). The conformation of HSP90 is closely linked to its ATP-binding ability. Briefly, when the ATP-binding sites in the HSP90 dimer's N-terminal domains are occupied by ATP, the N-terminal domains tightly associate creating a "closed" HSP90 complex. In this closed conformation, HSP90 client proteins are sequestered within the HSP90 complex. ATPase-catalyzed hydrolysis of the bound ATP molecules drives movement of the HSP90 complex which causes the N-terminal domains of each subunit to dissociate, creating an "open" state that promotes release of client proteins from the HSP90 complex (147). The ATPase activity of HSP90 is modulated by the cohort of co-chaperones associated with the complex. For example, Cdc37 and p23 inhibit ATPase activity, promoting an open-state HSP90 complex, while Aha1 and Cpr6 stimulate ATPase activity, promoting a closed-state complex (148, 149). Regulation of HSP90 activity is thus a complex, dynamic process involving the recruitment of many co-chaperone proteins that either drive or inhibit ATPase activity. The ATP-binding site has thus emerged as the main target for the development of therapeutics targeting HSP90 function.

1.3.2 HSP90 inhibitors

Small molecule HSP90 inhibitors have been assessed in models of both cancer and neurodegeneration. These molecules target the ATP binding site in the N-terminal region of HSP90, blocking ATP binding. This locks the HSP90 in the "open" conformation as the N-terminal regions of each monomer cannot associate in the absence of ATP. Blocking the ATP binding site thus promotes the release of HSP90 client proteins, which include many pro-survival transcription factors and HSF1. HSF1 is then free to enter the nucleus and associate with HSEs (see section 1.2.1). Two classes of HSP90 inhibitors exist; those based on the ansamycin backbone, and those based on the radicicol backbone. Geldanamycin, an ansamycin-based compound, was the first HSP90 inhibitor identified (150). Geldanamycin effectively induces the HSR *in vitro*, but has limited *in vivo* utility due to its narrow therapeutic window and its inability to cross the BBB (151). Derivatives of geldanamycin, such as 17-allylamino-17-desmethoxygeldanamycin (17-AAG) and 17-(2-dimethylaminoethyl)-amino-17-desmethoxygeldanamycin (17-DMAG) demonstrate improved activity *in vitro* and BBB-penetrance *in vivo*, but cause hepatotoxicity (152). Radicicol does not cause hepatotoxicity, but is highly unstable *in vivo* and therefore not appropriate for therapeutic purposes (153). Furthermore, both radicicol and 17-AAG are cytotoxic to primary motor neurons at concentrations necessary for HSR induction (5). HSP90 inhibitors are thus clear inducers of the HSR, but require further development before their translation to the clinic is possible.

1.3.3 HSP90 inhibitors in protein folding diseases

The HSR induced by HSP90 inhibition has been assessed for therapeutic efficacy in models of several neurodegenerative diseases where protein misfolding contributes

pathologically. In a primary culture model of fALS1, geldanamycin induced the expression of HSP70 and HSP40, prevented the formation of mutant SOD1 inclusions and prolonged motor neuron viability (5). Furthermore, an *in vitro* screen of small molecules for efficacy in preventing α-synuclein aggregation and toxicity identified 17-AAG and other geldanamycin derivatives (154). 17-AAG was also effective *in vivo*, where it reduced the toxicity of polyglutamine-containing repeats in a mouse model of spinal-bulbar muscular atrophy (SBMA) as reflected by an increase in motor performance, body weight and survival in transgenic mice overexpressing polyglutamine-expanded androgen receptor (AR) (155). This improvement in the SBMA mouse phenotype likely involved an HSR-independent protective mechanism, as 17-AAG-mediated induction of HSP40 and HSP70 was not as marked as the reduction in AR protein level (155). Indeed, AR is stabilized by HSP90, and opening of the HSP90 complex releases AR to be degraded by the proteasome (156). HSP90 inhibition can therefore provide cytoprotection through two pathways: the upregulation of HSPs through release of HSF1 and the liberation and degradation of toxic HSP90 client proteins. It is worth noting, however, that disruption of the HSP90 complex alters several cellular signaling pathways. Indeed, geldanamycin treatment increased the abundance of several protein kinases, including p38 MAP kinase, in a cancer cell line (157). Given that activation of p38 MAP kinase is a pathogenic contributor to mSOD1 toxicity (158), it is important to consider the possible potentiation of mSOD1-related disease pathways when assessing HSP90 inhibitors in fALS1 models.

1.3.4 A novel HSP90 inhibitor, NXD30001

The development of more tolerable HSP90 inhibitors stemmed from their investigation as possible therapeutics for cancer. In collaboration with Dr. Nicolas Winssinger of Université Louis Pasteur, NexGenix Pharmaceuticals engineered a series of radicicol-based HSP90 inhibitors. The lead compound of this series, NXD30001, exhibits *in vivo* stability, BBB permeability, high binding affinity to HSP90, accumulation in nervous tissues and an improved safety profile (8, 159, 160). In the BT474 human breast cancer cell line, NXD30001 dose-dependently decreased the protein levels of HSP90 client proteins with an efficacy comparable to 17-AAG and radicicol (8). NXD30001 also induced the expression of iHSP70 and HSP40 in the motor neurons of dissociated murine spinal cord-dorsal root ganglia (DRG) cultures in an HSF1-dependent manner (8). Furthermore, NXD30001 delayed the accumulation of mSOD1, prevented the formation of SOD1 inclusion bodies, maintained mitochondrial morphology and prolonged motor neuron viability in a primary culture model of fALS1 (8). Thus, we proceeded to evaluate NXD30001's ability to induce the expression of HSPs *in vivo* using the $SOD1^{G93A}$ transgenic murine model of fALS1.

Chapter 2: Hypothesis and Rationale

2.1 Hypothesis

The novel small molecule HSP90 inhibitor NXD30001 will induce *Hsp* gene expression in the tissues of transgenic mice overexpressing mutant SOD1 ($SOD1^{G93A}$) and their non-transgenic littermates.

2.2 Rationale

Heat shock proteins have proven to be neuroprotective in several models of neurodegenerative disease, including ALS. Our lab has demonstrated that HSP90 inhibitor-mediated induction of *HSP* expression and/or liberation of HSP90 client proteins protects against several endpoints of toxicity in primary motor neurons overexpressing mSOD1 (5, 8). Commercially available HSP90 inhibitors do not have a favourable profile for *in vivo* administration due to their narrow therapeutic windows, their inability to cross the BBB and/or their low stability *in vivo*. NXD30001, a novel small molecule HSP90 inhibitor licensed by NexGenix Pharmaceuticals for development, exhibits *in vivo* stability, high binding affinity to HSP90, BBB-penetrance and an improved safety profile relative to the parent compound. NXD30001 readily induced the degradation of HSP90 client proteins in the BT474 human cancer cell line, and induced the expression of iHSP70 and HSP40 in a primary spinal cord culture (8). NXD30001 thus demonstrated a favorable profile for *in vivo* use. The aim of this study was to investigate the ability of NXD30001 to induce the expression of HSPs *in vivo* using the $SOD1^{G93A}$ transgenic model of fALS1.

Previous studies have examined the effect of HSP modulation in SOD1^{G93A} transgenic mice through overexpression of HSPs, injection of recombinant HSP70 or administration of small molecule HSP co-inducers. The overexpression of HSP70 in SOD1^{G93A} transgenic mice failed to confer neuroprotection, as no improvements in time to symptom onset or survival were observed (136). Overexpression of HSP27 also failed to extend time to symptom onset or survival in SOD1^{G93A} transgenic mice (137, 138). Thus, systemic overexpression of a single HSP does not appear to confer substantial benefit in genetic models of ALS. Interestingly, *i.p.* injection of rhHSP70 extended the lifespan of SOD1^{G93A} transgenic mice, a result attributed to protection of peripheral nerves and muscle fibers (139, 140). Administration of arimoclomol, a small molecule HSP co-inducer that potentiates but does not constitutively induce *HSP* gene expression, has also extended lifespan in SOD1^{G93A} transgenic mice when administered orally at early (6) or late (7) stages of disease. Increasing HSP protein levels through either injection of active HSP70 or pharmacological modulation of *HSP* gene expression can therefore confer neuroprotection in genetic models of ALS.

Motor neurons have a high threshold for stress-induced induction of HSPs; indeed, motor neurons in SOD1^{G93A} transgenic mice do not express iHSP70 (115). This high threshold has been linked to a failure of motor neurons to activate the HSF1 transcription factor (115). Targeting HSF1 activation pharmacologically could therefore provide neuroprotection by upregulating *HSP* expression in motor neurons. NXD30001's ability to constitutively induce multiple HSPs *in vitro* through activation of HSF1 thus supported its investigation using the SOD1^{G93A} transgenic model of fALS1. The first step was to conduct a preliminary dose-finding study to assess penetration of NXD30001 into nervous tissue and induction of HSPs prior to

evaluating efficacy against the motor neuron disease phenotype in the $SOD1^{G93A}$ transgenic mouse model.

2.3 Specific Aims:

1. To assess the distribution of NXD30001 compound in the tissues (lumbar spinal cord, brain, skeletal muscle, liver) of nontransgenic and $SOD1^{G93A}$ transgenic mice

2. To assess NXD30001-mediated HSF1 activation and *HSP* gene expression in the tissues (lumbar spinal cord, brain, skeletal muscle, liver) of $SOD1^{G93A}$ transgenic mice

3. To assess the effect of NXD30001 on SOD1 protein levels in the tissues of $SOD1^{G93A}$ transgenic mice (lumbar spinal cord, skeletal muscle)

4. To assess the dose-response and time course of NXD30001-mediated *HSP* gene expression in the tissues (lumbar spinal cord, brain, skeletal muscle, cardiac muscle, liver, kidney) of nontransgenic and $SOD1^{G93A}$ transgenic mice

Chapter 3: Materials and Methods

3.1 SOD1^{G93A} transgenic mice

3.1.1 Generation and maintenance of transgenic mouse line

C57BL6-TgN(SOD1^{G93A})1Gur, transgenic for human SOD1 with the ALS-associated mutation, G93A and nontransgenic littermates were used in this study. Initial breeders were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Lines were maintained in the animal facility at the Montreal Neurological Institute and mice hemizygous for the transgene were obtained by breeding hemizygous males with non-transgenic C57BL6 females. All experiments were approved by the McGill University Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care. Mice were genotyped for human SOD1 using the JAX protocol, as previously described (134). Litters including at least three transgenic and three non-transgenic littermates were used at symptomatic stage (approximately P120). SOD1^{G93A} transgenic mice were designated "symptomatic" by positive hind- limb extensor reflex. Mice were killed by deep anesthesia with intraperitoneal injection of a mixture of 180 µL ketamine and 20 µL xylazine.

3.1.2 Dosing of mice with NXD30001

Mice were injected *i.p.* with 50, 100, or 150 mg/kg NXD30001 in 10% dimethyl sulfoxide (DMSO), 5% Tween20, 15% Cremaphor EL and 70% saline, whether as a single dose or repeated 3X weekly for two weeks. Solid NXD30001 compound was stored at -20°C and solutions for injection were prepared fresh at room temperature approximately 30 min before administration.

3.1.3 Measuring tissue levels of NXD30001 compound

Early symptomatic mice (D115-D139) treated with 50 mg/kg *i.p.* NXD30001 three times weekly for two weeks were euthanized by deep anesthesia via *i.p.* injection of ketamine/xylazine 1 hr or 6 hr after the last injection. Blood was obtained by cardiac puncture (ethylenediaminetetraacetic acid (EDTA) anticoagulant); subsequently, mice were perfused with normal saline to flush blood from the tissues. Brain, spinal cord, liver, kidney, heart and skeletal muscle (quadriceps) were harvested and samples were divided in two for separate analysis of drug level and expression of HSPs. Plasma was collected following centrifugation of blood samples at 2000 x G. Plasma and tissues were placed immediately on ice and then frozen and stored at -80°C.

Plasma and tissue levels of NXD30001 were analyzed under subcontract to Cerep (Redmond, WA, USA). Each tissue sample was homogenized in 0.75 mL cold phosphate-buffered saline (PBS), pH 7.4 for 10 sec on ice using Power Gen 125. The homogenized tissue was then stored at -20^{o}C until further processing. NXD30001 was extracted from plasma and tissue homogenates using acetonitrile precipitation. Calibration standards (1-5000 ng/mL) were generated by spiking 2.5 or 10 µL of each 20X standard solution of the test compound into 47.5 or 190 µL of drug-free plasma or tissue homogenate, respectively. The spiked sampled were processed together with the unknown samples. Briefly, 200 µL of acetonitrile was added to each 50 µL plasma sample, and 400 µL of acetonitrile was added to each 200 µL sample of tissue homogenate. Samples were mixed for 5 min on the plate shaker and then centrifuged at 6000 x G for 15 min at 4^{o}C. NXD30001-containing supernatant was transferred into a new tube and was centrifuged at 3900 x G for 15 minutes. 20 µL of the supernatant was subjected to

liquid chromatography – mass spectrometry (LCMS) analysis. A Gemini NX C18 column (2 x 50 mm, 5 µm) was used (Phenomenex, Torrance, CA, USA). The mobile phase A was 12 mM ammonium formate/6 mM formic acid in water/methanol and the mobile phase B was 6mM ammonium formate/3 mM formic acid in water/methanol (1/9, v/v). The flow rate was 0.5 mL/min and the gradient was 60% B for 0.5 min, 60-100% B in 1.5 min, and 100% B for 0.9 min. A TSQ Quantum mass spectrometer was used for tandem mass spectrometry analysis.

3.2 Analysis of HSP expression in murine tissues

3.2.1 Tissue preparation

Ice-cold excised tissue samples were homogenized in 2% SDS sample buffer (20 mM Tris, 2 µM EDTA) containing protease inhibitor cocktail (Roche, Mississauga, ON, Canada) using a 2 mL Wheaton hand homogenizer. Tissue homogenates were sonicated for 5 sec at 50% cycle using a Vitro Cell Sonicator and centrifuged at 15,000 x G for 15 min. Supernatants were collected and sample protein concentrations were determined using the Bradford protein assay. 25 µg of protein from each sample was prepared with Laemmli loading buffer containing 5% β-mercaptoethanol and boiled for 5 min.

3.2.2 SDS-PAGE and Western Blotting

Protein samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide resolving gel, 4% acrylamide stacking gel) at 100 V. Gels were run until the loading dye front reached approximately 1 cm from the bottom of the resolving gel. Separated proteins were transferred to a nitrocellulose membrane at 100 V at 4°C

for 1 hr followed by 30 V at 4°C overnight. Transfer efficiency was assessed using Ponceau protein staining of the nitrocellulose membrane. Following transfer, the nitrocellulose membranes were blocked for 30 min at room temperature via gentle rocking in 5% skim milk in tris-buffered saline (TBS) at room temperature. The blocked membranes were probed with primary antibody overnight at 4°C with gentle rocking, and with horseradish peroxidase (HRP)-conjugated secondary antibody for 45 min at room temperature with gentle rocking. The membranes underwent three 15 min washes in 0.3% Tween-TBS after each probing. HRP activity was stimulated by HyGlo Chemiluminescent HRP detection reagent (Denville Scientific, Metuchen, NJ, USA). Immuneoreactive protein bands were visualized using the INTAS imaging system (INTAS Science Imaging Instruments, Göttingen, DE).

3.2.3 Statistics

The optical densities of immunoreactive protein bands were normalized to loading controls and statistics were performed using a one-way, parametric ANOVA for independent samples. Statistical significance was defined as $p < 0.05$.

3.3: Positive controls and antibodies

3.3.1 Heat shock of dissociated spinal cord-DRG cultures

Two positive iHSP70 controls were used: Recombinant human iHSP70 (see below) and extracts of heat shocked murine embryonic spinal cord-DRG cultures. The latter are routinely used in our laboratory and, although the motor neurons do not upregulate *HSP* gene expression following heat shock, other cells do (115). Cultures were prepared as previously described

(161). Briefly, spinal cords with attached DRGs were dissected from embryonic day 13 CD1 mouse embryos (Charles River Laboratories, Wilmington, MA), dissociated using trypsin and trituration, plated on glass coverslips coated with poly-D-lysine (Sigma-Aldrich, Oakville, ON) and Matrigel (Invitrogen Life Technologies, Burlington, ON) at cell densities of 350,000 cells/coverslip for 12 mm coverslips and 950,000 cells/coverslip for 18 mm coverslips. Culture medium was minimum essential medium (HyClone Minimum Essential Medium, Thermo Scientific, Nepean, ON), 1% N3 stock solution (1.74 µM insulin, 200 µg/mL transferrin, 10 µg/mL BSA, 198 µM putrescine, 150 nM selenium, 30 nM triiodothyronine, 25 nM hydrocortisone, 40 nM progesterone), 3% horse serum (Invitrogen Life Technologies), 2.5 ng/mL nerve growth factor (NGF) (UBI, Lake Placid, NY, USA), 5 g/L dextrose and 1.5 g/L sodium bicarbonate. At confluency (approximately 5 days after plating), cultures were treated with 1.4 µg/mL cytosine-β-D-furanoside (CAF) to arrest mitosis of non-neuronal cells. Cultures were allowed to mature over three to four weeks.

For heat shock, spinal cord-DRG cultures on coverslips were transferred to individual culture dishes containing Eagle's Minimum Essential Medium (EMEM) without bicarbonate, pH 7.4 (HyClone EMEM, Thermo Scientific, Nepean, ON) at 37°C. Coverslips were placed in a 43°C water bath for 30 min, with each dish wrapped in parafilm to prevent leakage and contamination. Coverslips were then transferred back into regular culture medium and incubated at 37°C for 6 hr to allow cellular recovery. Total cell lysates were collected by scraping the coverslips in 2% SDS sample buffer (20 mM Tris, 2 µM EDTA) using a 25 cm disposable cell scraper (Fisher Scientific, Ottawa, ON, CA). Cell lysates were sonicated for 5 sec at 50% cycle using a Vitro Cell Sonicator and centrifuged at 15,000 x G for 15 min. Supernatants

were collected and sample protein concentrations were determined using the Bradford protein assay. 25 µg of protein from each sample was prepared with Laemmli loading buffer containing 5% β-mercaptoethanol and boiled for 5 min. Samples were subjected to SDS-PAGE and Western blotting as described above.

3.3.2 Recombinant human HSP70

Recombinant human HSP70 (HSP72) protein was purchased from Stressgen (Victoria, BC, USA). 0.1 µg of protein was prepared in 2% SDS sample buffer (20 mM Tris, 2 µM EDTA) and Laemmli buffer containing 5% β-mercaptoethanol prior to SDS-PAGE.

3.3.3 Antibodies

Antibodies used for the detection of HSPs on Western blots were: monoclonal mouse anti-iHSP70 (Enzo Life Sciences, Framingdale, NY, USA. SPA-810; 1:1000), rabbit anti-HSP40 (Enzo Life Sciences. SPA-400; 1:1000), goat anti-HSP25/27 (Santa Cruz Biotechnology, Santa Cruz, CA, USA. SC-1049; 1:500), rabbit anti-HSP60 (Santa Cruz SC-13966; 1:500), and rabbit anti-HSP90 (Santa Cruz SC-7947; 1:1000). For analysis of HSF1 activation, monoclonal rat anti-HSF1 was used (Enzo Life Sciences SPA-950, clone # 10H8; 1:1000). Mouse anti-β-actin (MP Biochemicals, Solon, OH, USA; 1:10000) was used for loading control.

Chapter 4: Results

4.1 To assess the distribution of NXD30001 compound in the tissues (lumbar spinal cord, brain, skeletal muscle, liver) of nontransgenic and SOD1^{G93A} transgenic mice

4.1.1 Tissue penetrance of NXD30001 after an acute dose in wild-type mice

Currently-available small molecule HSP90 inhibitors are either unstable *in vivo* or have difficulty crossing the BBB. Investigating the tissue penetrance and stability of NXD30001 was therefore necessary prior to any functional analysis. Studies by NexGenix Pharmaceuticals had established *i.p.* administration as the best route of administration for NXD30001, oral bioavailability being very low likely due to the low aqueous solubility of the compound (Table 1). The distribution of NXD30001 in CNS tissue after a single, 100 mg/kg *i.p.* dose in nontransgenic mice is presented in Table 2. The concentration of NXD30001 in brain exceeded 40 nM, a concentration that induced *HSP* expression in a culture model of fALS1 (8), and persisted for at least 12 hr after dosing.

To compare NXD30001's BBB permeability to another HSP90 inhibitor, a parallel group of mice was dosed *i.p.* with 70 mg/kg of 17-AAG. 17-AAG has been reported to reach µM concentrations in brain tissues following single *i.v.* doses of 40 or 60 mg/kg in mice (162). Unlike NXD30001, 17-AAG was barely detectable in brain tissue 6 hr after dosing, and was undetectable by 12 hr after dosing. NXD30001 thus accumulates in the brain tissues of mice following a single *i.p.* dose at substantially greater levels compared to 17-AAG.

4.1.2 Tissue distribution of NXD30001 after subacute dosing in symptomatic SOD1^{G93A} transgenic mice

Having established accumulation of NXD30001 in brain of wild type mice, the next step was to determine a dosage regimen and the tissue distribution of NXD30001 in the $SOD1^{G93A}$ model of fALS1, an established preclinical ALS model for evaluating therapeutics. Measuring NXD30001's CNS penetrance was necessary in this model due to the altered BBB function in $SOD1^{G93A}$ transgenic mice (163). A subacute dosing regimen was pursued to provide preliminary information for the development of a chronic, therapeutic-oriented dosing strategy in disease-relevant *in vivo* models. Previous studies by NexGenix established a tolerable dosing schedule in nontransgenic, wild-type mice of 50 mg/kg NXD30001 delivered *i.p.* three times per week without clinical toxicity. This dosing regimen was followed for two weeks in symptomatic $SOD1^{G93A}$ transgenic mice, as measured by abnormal extension reflex beginning around P120. Early symptomatic mice were chosen for this analysis as they parallel the typical stage of ALS disease when pharmacological intervention is initiated in patients. NXD30001 drug levels were measured in plasma, brain, lumbar spinal cord, skeletal muscle and liver tissues of these mice collected 1 hr or 6 hr after the final dose (table 3). NXD30001 was cleared rapidly from plasma, with drug levels falling to near-undetectable levels 6 hr after the final dose. NXD30001 rapidly accumulated in tissues. In brain and lumbar spinal cord tissues, the concentration of NXD30001 exceeded 40 nM at both time points, with maximum concentrations ranging from 459 to 1689 nM at 1 hr in brain, and from 536 to 1709 nM at 1 hr in spinal cord. Much higher concentrations were observed at both time points in skeletal muscle and liver tissues, with maximum concentrations occurring at 1 hr in both tissues. It is also worth noting that no phenotypic differences were observed between NXD30001-treated mice and their untreated littermates at any dose or time point.

4.2 To assess NXD30001-mediated HSF1 activation and *HSP* gene expression in the tissues (lumbar spinal cord, brain, skeletal muscle, liver) of SOD1^{G93A} transgenic mice

4.2.1 Tissue profile of NXD30001-mediated induction of iHSP70 expression in SOD1^{G93A} transgenic mice dosed for 2 weeks

Having demonstrated distribution of NXD30001 to nervous tissue, tissues from the SOD1^{G93A} transgenic mice described in section 4.1.2 (50 mg/kg, NXD30001, 2 weeks, 3x weekly dosing regimen) were analyzed for expression of iHSP70 to provide a preliminary measure of NXD30001's efficacy in inducing *HSP* gene expression *in vivo*. iHSP70 is a convenient biomarker of NXD30001's ability to induce *HSP* gene expression as the protein is barely- or un-detectable *in vivo* by Western blot under normal conditions. Total tissue extracts of brain, lumbar spinal cord, liver and skeletal muscle were subjected to Western analysis using antibodies against iHSP70 and β-actin as loading control. Recombinant human iHSP70 and heat-shocked dissociated murine spinal cord-DRG cultures were loaded as positive controls. NXD30001 induced the expression of iHSP70 in the 1 hr and 6 hr skeletal muscle samples, but iHSP70 expression was not induced by NXD30001 at any time point in liver, brain or lumbar spinal cord tissues (Fig. 1). NXD30001-mediated induction of iHSP70 expression reached statistical significance only after 6 hr ($p < 0.05$, one-way ANOVA, Fig. 2). Very low levels of iHSP70 were detected in the vehicle-treated and NXD30001-treated 6 hr lumbar spinal cord samples.

4.2.2 NXD30001 induced activation of HSF1 transcription factor in skeletal muscle, but not lumbar spinal cord tissue of SOD1^{G93A} transgenic mice

Previous *in vitro* work demonstrated that NXD30001's ability to induce expression of HSPs in motor neurons was dependent on activation of the HSF1 transcription factor (8).

NXD30001's ability to induce activation of HSF1 *in vivo* could therefore reflect its ability, or lack thereof, to induce expression of *HSP* genes in tissues. HSF1 activation is visualized through Western immunoblot via "gel-shift" of the 82 kDa protein band; that is, the appearance of a higher molecular weight smear that corresponds to a hyperphosphorylated form of the transcription factor. Whole tissue extracts of skeletal muscle and lumbar spinal cord from the same mice analyzed in sections 4.1.2 and 4.2.1 were subjected to Western analysis using an antibody immunoreactive to HSF1. Gel-shift of HSF1 was observed in NXD30001-treated skeletal muscle after 1 hr, but not after 6 hr and never in vehicle-treated mice (Fig. 2). Gel-shift of HSF1 was not, however, observed in lumbar spinal cord samples from NXD30001- or vehicle-treated mice at any time point (Fig. 3). Activation of HSF1 was therefore concordant with NXD30001-induced induction of iHSP70 expression.

4.2.3 NXD30001 did not induce expression of HSP90, HSP60 or HSP40 in skeletal muscle or lumbar spinal cord of SOD1^{G93A} transgenic mice

NXD30001 induced the expression of both iHSP70 and HSP40, but not other HSPs, *in vitro* (8). To assess whether NXD30001 produced a similar pattern of *HSP* gene expression *in vivo*, the blots prepared in section 4.2.2 were re-probed using antibodies immunoreactive to other HSPs (HSP90, HSP60, HSP40, HSP25). In skeletal muscle, no increases in HSP90, HSP60, HSP40 or HSP25 were observed with NXD30001 treatment (Fig. 2). In lumbar spinal cord, no increases in HSP90, HSP60 or HSP40 were observed, but an increase in HSP25 protein levels was concordant with administration of NXD30001 without reaching statistical significance at $p < 0.05$ ($p = 0.06$, one-way ANOVA, Fig. 3). The profile of NXD30001-mediated induction of *HSP* gene expression therefore differs *in vivo* from *in vitro*.

4.3 To assess the effect of NXD30001 on SOD1 protein levels in the tissues (lumbar spinal cord, skeletal muscle) of symptomatic SOD1^{G93A} transgenic mice

4.3.1 NXD30001 did not reduce SOD1 protein levels in skeletal muscle or lumbar spinal cord tissues of SOD1^{G93A} transgenic mice

The accumulation and aggregation of mSOD1 protein is posited to be a central contributor to pathogeneses in fALS1 patients and SOD1^{G93A} transgenic mice (70). Reducing the levels of mSOD1 protein is therefore a target for therapeutic intervention and could represent a biochemical marker of therapeutic efficacy. Indeed, NXD30001 reduced mSOD1 aggregate load in primary motor neurons overexpressing mSOD1 through upregulation of *HSP* gene expression and/or liberation and degradation of mSOD1 bound to the HSP90 chaperone complex, and this reduction was associated with increased neuronal viability (8). To determine if treatment with NXD30001 had a similar effect *in vivo*, the blots prepared in section 4.2.2 were re-probed using an antibody immunoreactive against SOD1 protein. In skeletal muscle and lumbar spinal cord tissues, no decrease in SOD1 protein level was observed following administration with NXD30001; in fact, a statistically significant increase in SOD1 protein level was observed in the 6 hr lumbar spinal cord samples following treatment with NXD30001 ($p < 0.05$, one-way ANOVA, Fig. 3). NXD30001-mediated induction of iHSP70 expression in skeletal muscle and/or NXD30001-mediated dissociation of the HSP90 chaperone complex in skeletal muscle and lumbar spinal cord therefore do not reduce SOD1 protein load in either tissue, as detectable by Western immunoblot. Note that a high molecular weight band immunoreactive with SOD1 antibody appeared in vehicle- and NXD30001-treated spinal cord samples after 1 hr; these bands were included for quantitation.

4.4 To assess the dose-response and time course of NXD30001-mediated *HSP* gene expression in the tissues (lumbar spinal cord, brain, skeletal muscle, cardiac muscle, liver, kidney) of nontransgenic and SOD1^{G93A} transgenic mice

4.4.1 Acute dosing with NXD30001 induced expression of iHSP70 in skeletal muscle, cardiac muscle and kidney, but not liver or CNS tissues

The dose-response and time course of NXD30001-induced *HSP* gene expression was further investigated in wild-type, nontransgenic C57BL6 and symptomatic SOD1^{G93A} transgenic mice. Cardiac muscle, quadriceps (skeletal) muscle, kidney, liver, brain and lumbar spinal cord tissues were excised from mice dosed with 50, 100 or 150 mg/kg NXD30001 after 4, 16, 36, or 72 hr. Whole tissue extracts were subjected to Western analysis using antibodies against iHSP70, HSP25 and β-actin as loading control. In the skeletal and cardiac muscles of mice dosed with 100 mg/kg NXD30001, iHSP70 protein levels appeared to be highest after 16 hr, with minimal iHSP70 protein observed in the 4 hr samples (Fig. 4A). 100 mg/kg NXD30001 also induced iHSP70 expression in the skeletal and cardiac muscle tissues of SOD1^{G93A} transgenic mice after 16 hr (Fig. 4B). iHSP70 protein levels persisted in the cardiac and skeletal muscle tissues of nontransgenic C57BL6 mice for up to 72 hr postinjection after 50 mg/kg and 100 mg/kg doses (Fig. 5). Weak induction of iHSP70 was also observed in the kidney of NXD30001-treated mice after 16 hr, but protein levels were undetectable by 36 hr (Fig. 6). No iHSP70 expression was observed in liver at any dose or time point (Fig. 6).

No induction of iHSP70 was observed in brain or lumbar spinal cord tissues at any dose or time point (Fig. 7), and no induction of HSP25 was observed in brain or lumbar spinal cord tissues from mice dosed with 100 mg/kg NXD30001 after 4 or 16 hr (Fig. 8). A higher dose of

NXD30001 was therefore administered to determine if the lack of *HSP* expression was due to a higher threshold of NXD30001-mediated HSP induction in nervous tissues. Nontransgenic C57BL6 mice were dosed *i.p.* with 150 mg/kg NXD30001, and brain and lumbar spinal cord tissues were harvested after 16 hr. No increase in iHSP70 expression was seen in NXD30001-treated mice in either tissue (Fig. 9). 150 mg/kg is the upper limit of drug solubility and tolerability, making higher doses impossible.

Table 1. Bioavailability of NXD30001 by different routes of administration

NXD30001 in mouse	C_{max} ng/mL	T_{max} hr	$AUC_{(0-t)}$ ng/mL*hr	$AUC_{(0-\infty)}$ ng/mL*hr	$t_{1/2}z$ hr	CL L/hr/kg	Vz L/kg	F(%)
i.v. (4 mg/kg)	1023.3	0.08	405.4	413.9	0.89	9.66	12.34	
i.p. (100 mg/kg)	1006	1	6258.0	6260.2	2.13	NA	NA	60.5
p.o. (100 mg/kg)	63.1	8	213.5	353.8	1.51	NA	NA	3.42

Bioavailability of NXD30001 in plasma was assessed in preliminary single-dose pharmacokinetic analyses in nontransgenic mice. The doses of NXD30001 for both oral and *i.p.* administrations were 100 mg/kg, and 4 mg/kg *i.v.* Concentrations of NXD30001 were measured using LC/MS.

Definitions: C_{max}: Maximum concentration; T_{max}: Time of the maximum concentration; $AUC_{(0-t)}$: Area under the curve from the time of dosing to the time of the last observation; $AUC_{(0-\infty)}$: Area under the curve from the time of dosing to infinity; $t_{1/2}z$: Terminal half-life; CL: Clearance; Vz: Volume of distribution at terminal phase; F(%): Bioavailability.

Table 2. CNS penetration of NXD30001 compared to 17-AAG

NXD30001 concentration (ng/g)			17-AAG concentration (ng/g)
Time (hrs)	**100 mg/kg, *i.p.***	**150 mg/kg, *i.p.***	**75 mg/kg, *i.p.***
0	27.1	28.8	0.0
6	675.5	1439.5	2.6
12	823.0	3424.0	0.0
24	271.0	899.5	0.0
48	10.7	115.5	0.0
AUC (0-48 hr.) ng x hr/g	**16554**	**57135**	

CNS penetrance of NXD30001 and 17-AAG was assessed by measuring drug levels in brain tissues of perfused nontransgenic mice via LC/MS. Mice were dosed *i.p.* with 100 mg/kg and 150 mg/kg NXD30001 or 75 mg/kg 17-AAG and tissues were extracted up to 48 hr. later. To convert ng/g to µM, multiply value by 2.

Table 3. Accumulation of NXD30001 in murine tissues, including CNS

Animal	Plasma (ng/ml)	Brain (ng/g)	Spinal cord (ng/g)	Liver (ng/g)	Muscle (ng/g)
NXD300001 tissue levels: 1-h postadministration					
Mouse 36/6	455	810	815	9930	1812
Mouse 36/8	246	254	290	5451	12208
Mouse 36/9	189	219	256	4791	2182
NXD30001 tissue levels: 6-h postadministration					
Mouse 36/1	18	68	501	3921	330
Mouse 36/2	12	247	339	410	632
Mouse 36/4	14	105	124	2347	253

Early symptomatic SOD1^{G93A} transgenic mice were dosed *i.p.* with 50 mg/kg NXD30001 on a three times per week schedule for 2 weeks and were euthanized 1 hr. or 6 hr. after the last dose. Excised tissues were snap-frozen and stored at -80°C. Tissue levels of NXD30001 were measured by LC/MS (performed on contract by Cerep (Redmond, WA, USA), administered by NexGenix Pharmaceuticals).

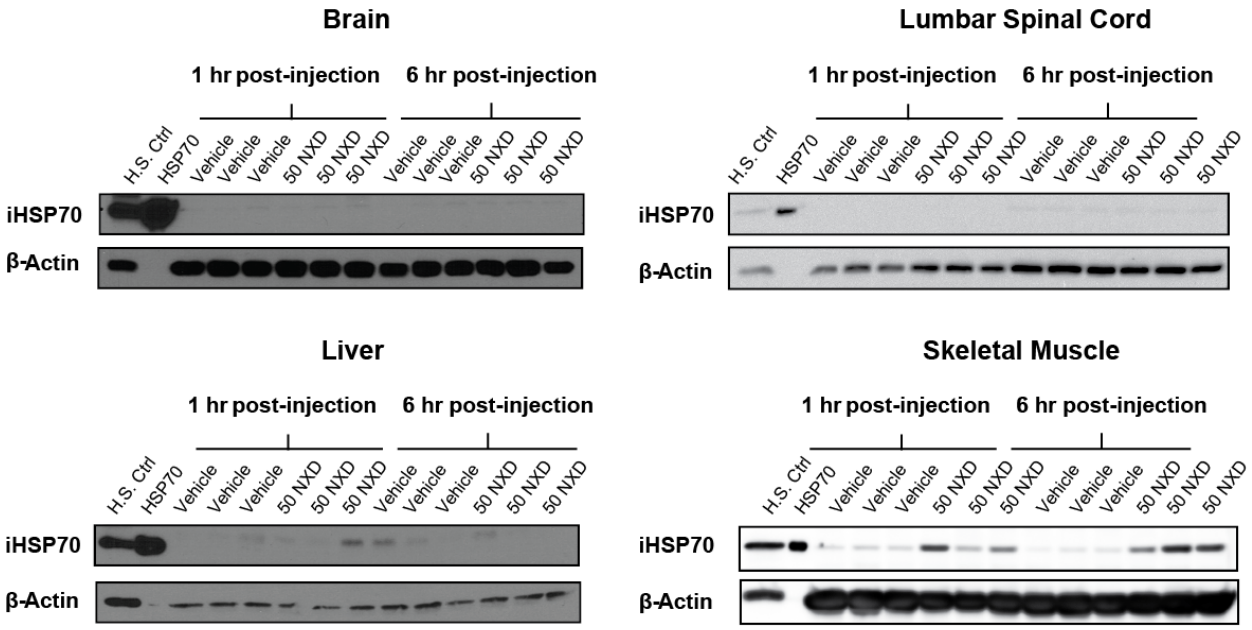

Figure 1. NXD30001 induced expression of iHSP70 expression in skeletal muscle of SOD1^{G93A} transgenic mice, but not in CNS or liver. Early symptomatic SOD1^{G93A} were dosed *i.p.* with 50 mg/kg NXD30001 on a three times per week schedule for two weeks (tissue levels of NXD30001 were analyzed in the same mice; see Table 3). Tissues were harvested 1 or 6 hr. after the final dose of NXD30001. Whole tissue extracts were subjected to Western analysis using antibodies immunoreactive to iHSP70 and β-actin as loading control. Extracts of heat-shocked spinal cord-DRG cultures (H.S. Ctrl) and recombinant iHSP70 (HSP70) were run as positive controls.

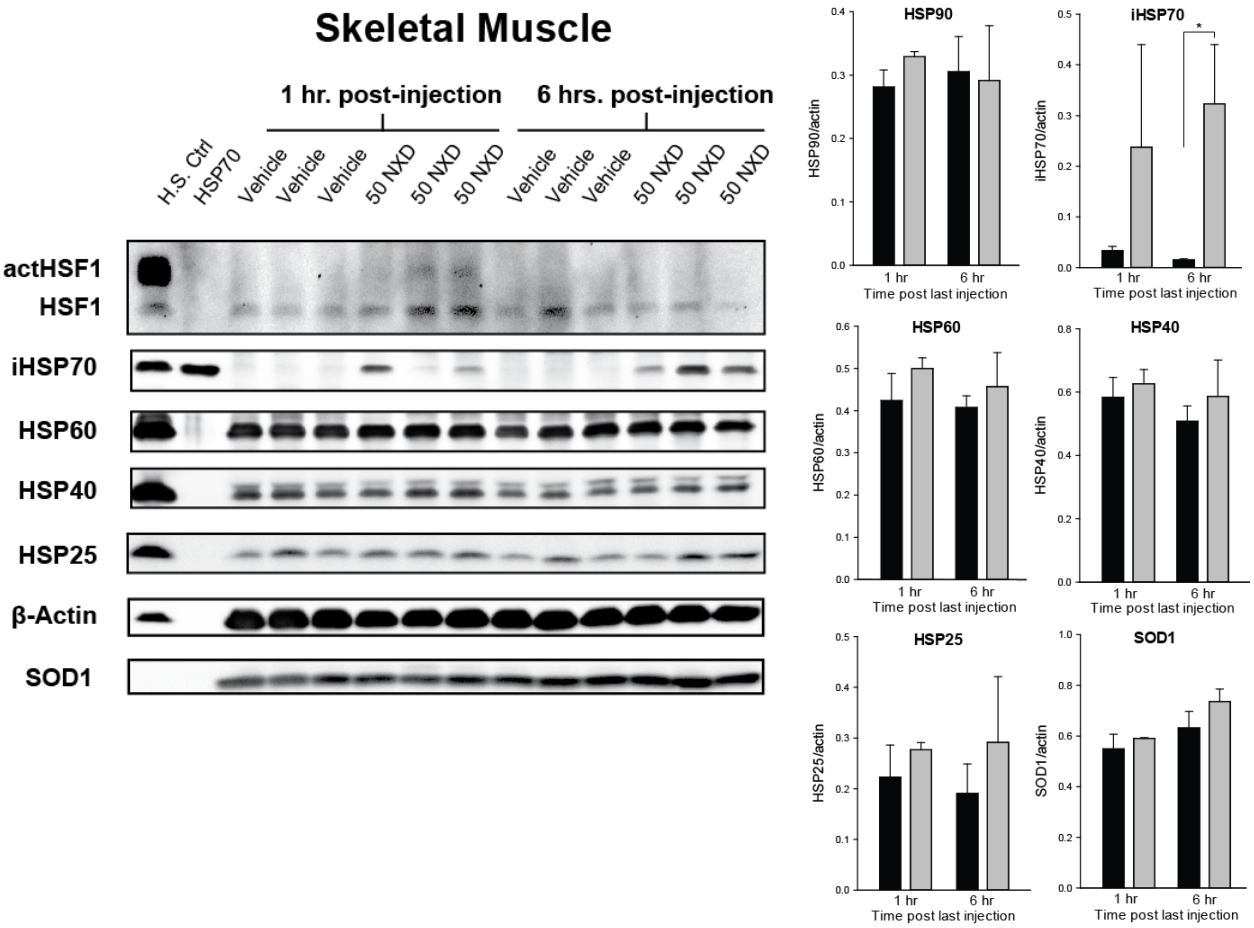

Figure 2. Treatment with NXD30001 caused transient phosphorylation/activation of HSF1 (*actHSF1*) in skeletal muscle of early symptomatic SOD1^{G93A} transgenic mice. Mice were administered vehicle or 50 mg/kg NXD30001 three times per week for two weeks and tissues were harvested 1 or 6 hr. after injection of the last dose (same mice as in Table 3 and Fig. 1). Whole tissue extracts were subjected to Western analysis using antibodies reactive to HSF1, HSPs (iHSP70, HSP60, HSP40, HSP25), SOD1 and β-actin as loading control. Extracts of heat-shocked spinal cord-DRG cultures (H.S. Ctrl) and recombinant iHSP70 (HSP70) were run as positive controls.

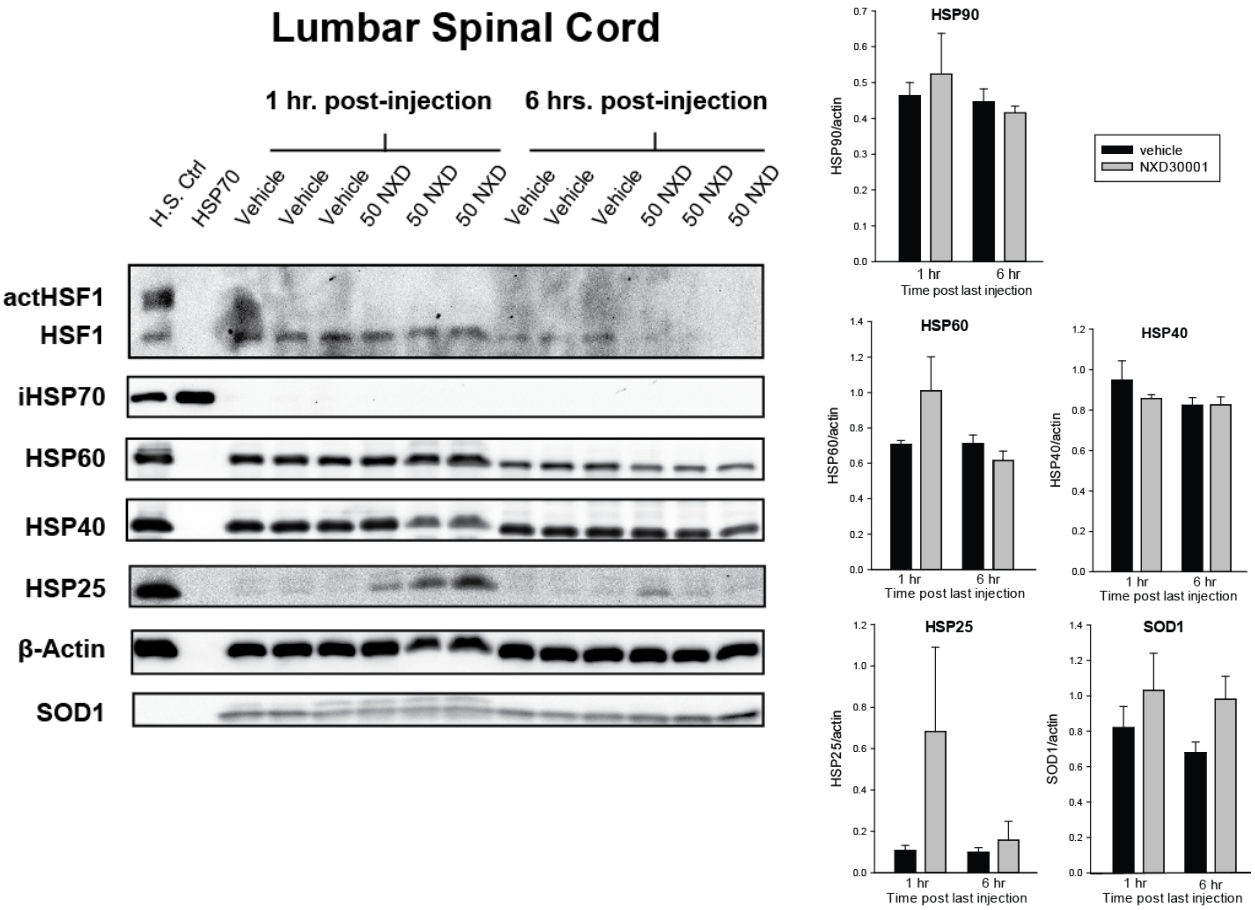

Figure 3. Treatment with NXD30001 did not induce iHSP70 expression or activation of HSF1 (*actHSF1*) in lumbar spinal cord of early symptomatic SOD1^{G93A} transgenic mice. Mice were administered vehicle or 50 mg/kg NXD30001 three times per week for two weeks and tissues were harvested 1 or 6 hr. after injection of the last dose (same mice as in Table 3 and Fig. 1). Whole tissue extracts were subjected to Western analysis using antibodies reactive to HSF1, HSPs (iHSP70, HSP60, HSP40, HSP25), SOD1 and β-actin as loading control. Extracts of heat-shocked spinal cord-DRG cultures (H.S. Ctrl) and recombinant iHSP70 (HSP70) were run as positive controls.

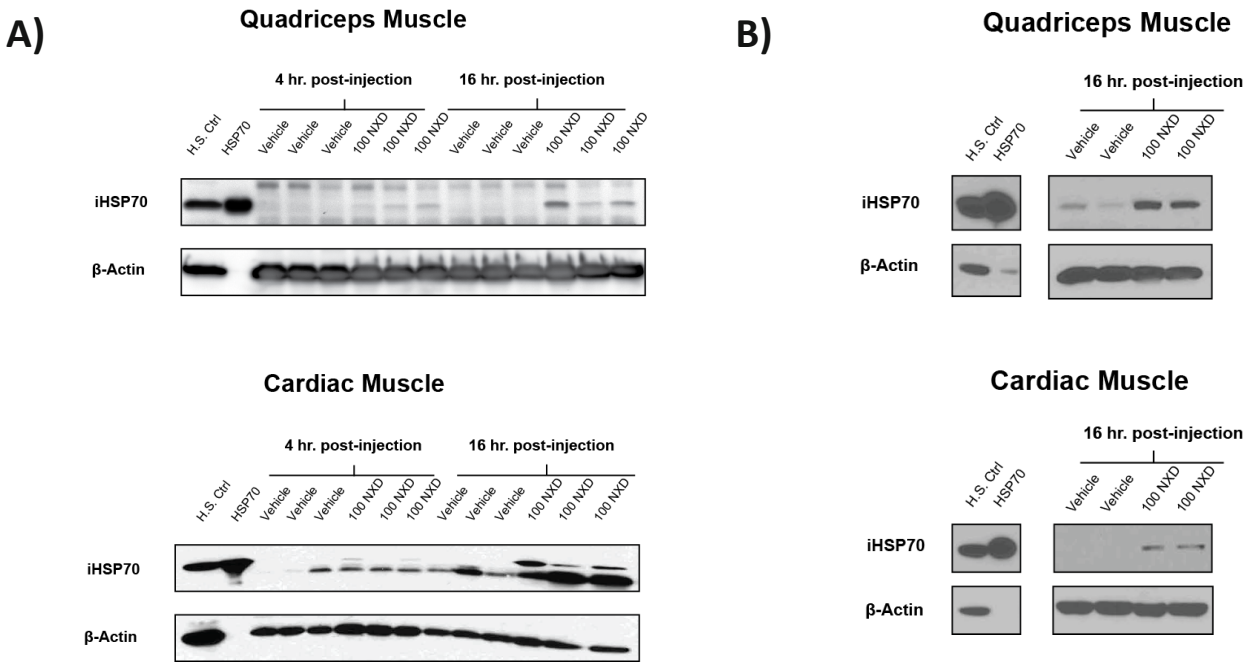

Figure 4. A) Single dose *i.p.* administration of NXD30001 induced iHSP70 expression in quadriceps and cardiac muscle tissues after 16 hr., with minimal iHSP70 protein detected after 4 hr. Nontransgenic C57BL6 mice were dosed *i.p.* with a single dose of 100 mg/kg NXD30001 and tissues were extracted 4 or 16 hr. later. Whole tissue extracts were subjected to Western analysis using antibodies immunoreactive to iHSP70 and β-actin as loading control. Extracts of heat-shocked spinal cord-DRG cultures (H.S. Ctrl) and recombinant iHSP70 (HSP70) were run as positive controls. **B)** Single dose *i.p.* administration of NXD30001 induced iHSP70 expression in quadriceps and cardiac muscle tissues after 16 hr. in $SOD1^{G93A}$ transgenic mice. Early symptomatic $SOD1^{G93A}$ transgenic mice were dosed *i.p.* with a single dose of 100 mg/kg NXD30001 and tissues were extracted 16 hr. later. Whole tissue extracts were subjected to Western analysis using antibodies immunoreactive to iHSP70 and β-actin as loading control. Extracts of heat-shocked spinal cord-DRG cultures (H.S. Ctrl) and recombinant iHSP70 (HSP70) were run as positive controls.

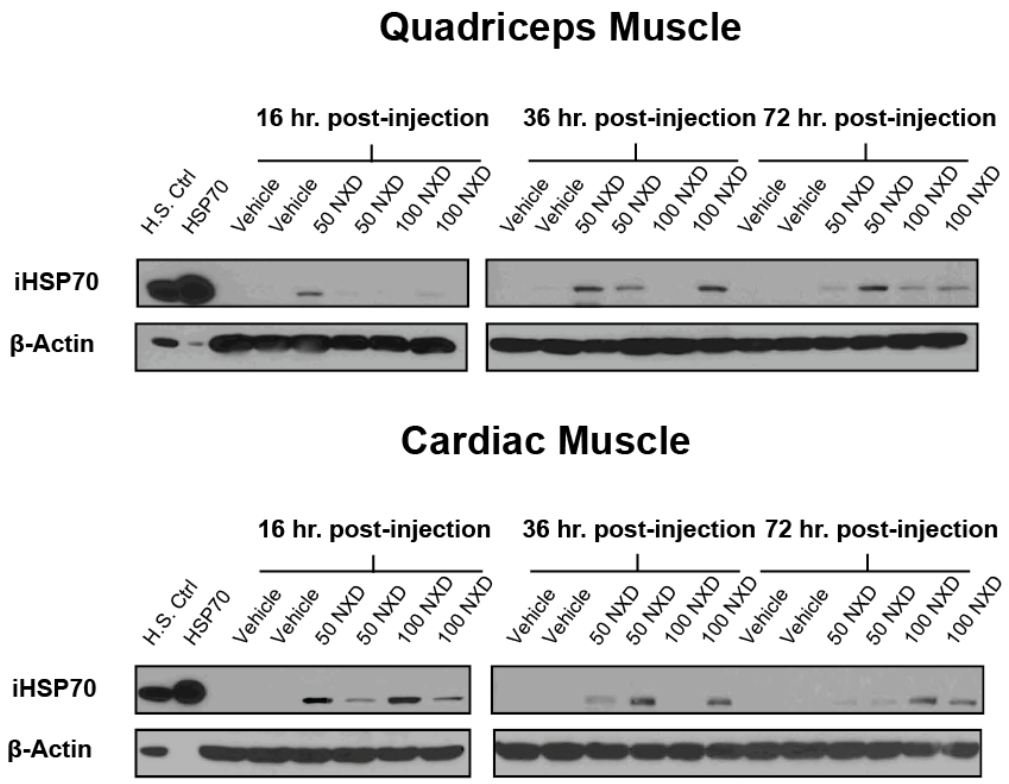

Figure 5. NXD30001-induced iHSP70 protein levels persisted up to 72 hr. in skeletal and cardiac muscle tissues after a single *i.p.* dose. Nontransgenic C57BL6 mice were dosed *i.p.* with a single dose of 50 mg/kg or 100 mg/kg NXD30001 and tissues were extracted 16, 36 or 72 hr. later. Whole tissue extracts were subjected to Western analysis using antibodies immunoreactive to iHSP70 and β-actin as loading control. Extracts of heat-shocked spinal cord-DRG cultures (H.S. Ctrl) and recombinant iHSP70 (HSP70) were run as positive controls.

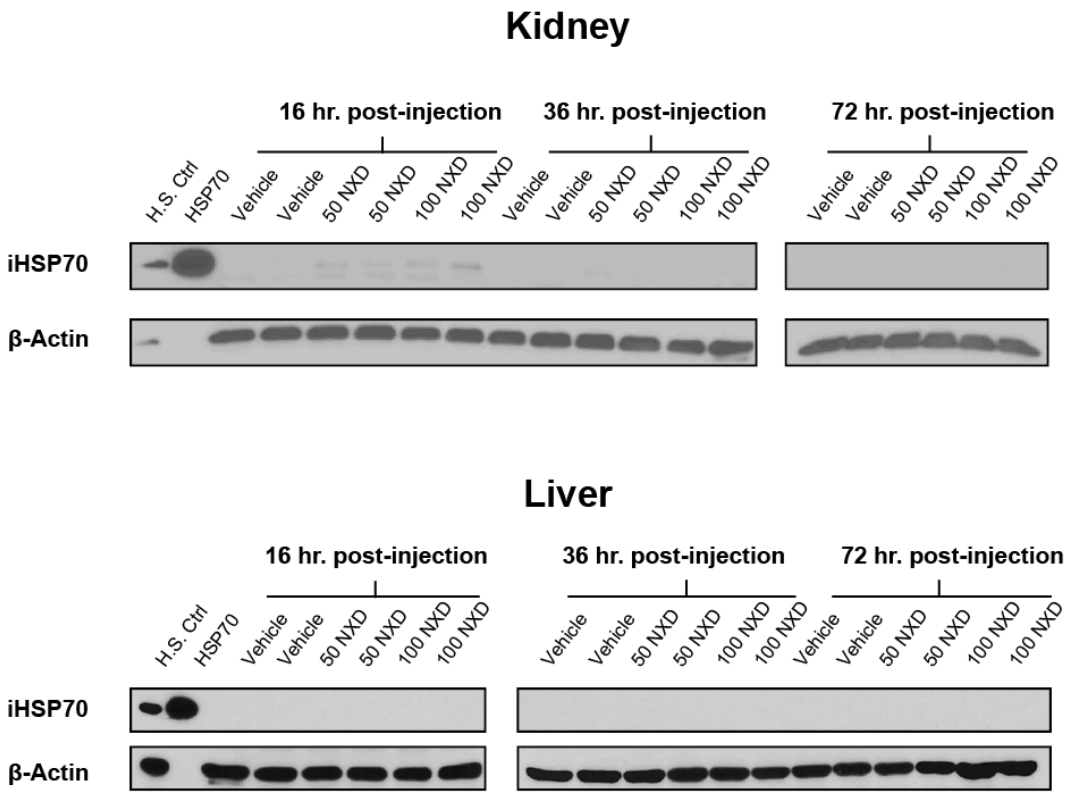

Figure 6. NXD30001-induced transient iHSP70 expression in kidney, but not liver after a single *i.p.* dose. Nontransgenic C57BL6 mice were dosed *i.p.* with a single dose of 50 mg/kg or 100 mg/kg NXD30001 and tissues were extracted 16, 36 or 72 hr. later. Whole tissue extracts were subjected to Western analysis using antibodies immunoreactive to iHSP70 and β-actin as loading control. Extracts of heat-shocked spinal cord-DRG cultures (H.S. Ctrl) and recombinant iHSP70 (HSP70) were run as positive controls.

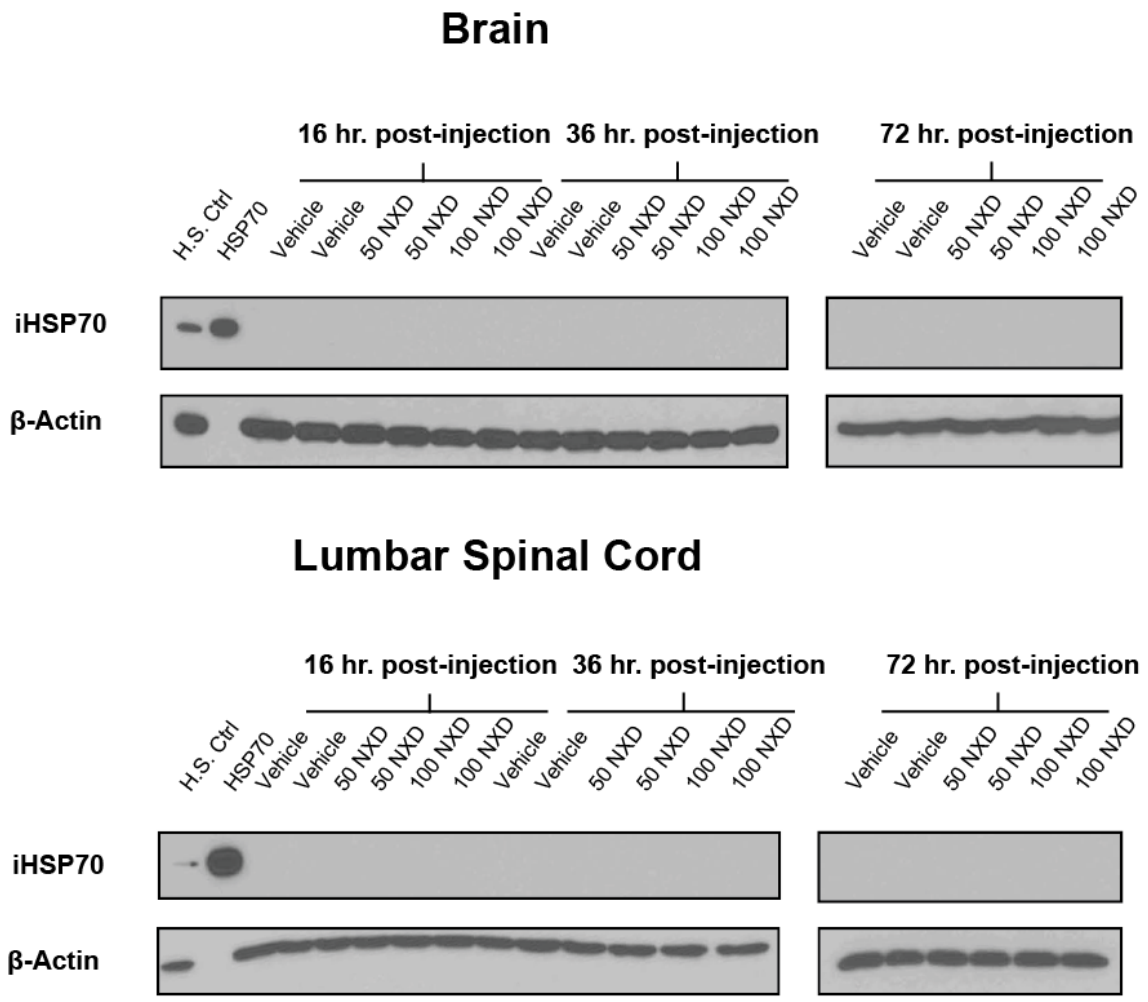

Figure 7. NXD30001 did not induce iHSP70 expression in CNS tissues (brain, lumbar spinal cord) at any dose or time point. Nontransgenic C57BL6 mice were dosed *i.p.* with a single dose of 50 mg/kg or 100 mg/kg NXD30001 and tissues were extracted 16, 36 or 72 hr. later. Whole tissue extracts were subjected to Western analysis using antibodies immunoreactive to iHSP70 and β-actin as loading control. Extracts of heat-shocked spinal cord-DRG cultures (H.S. Ctrl) and recombinant iHSP70 (HSP70) were run as positive controls.

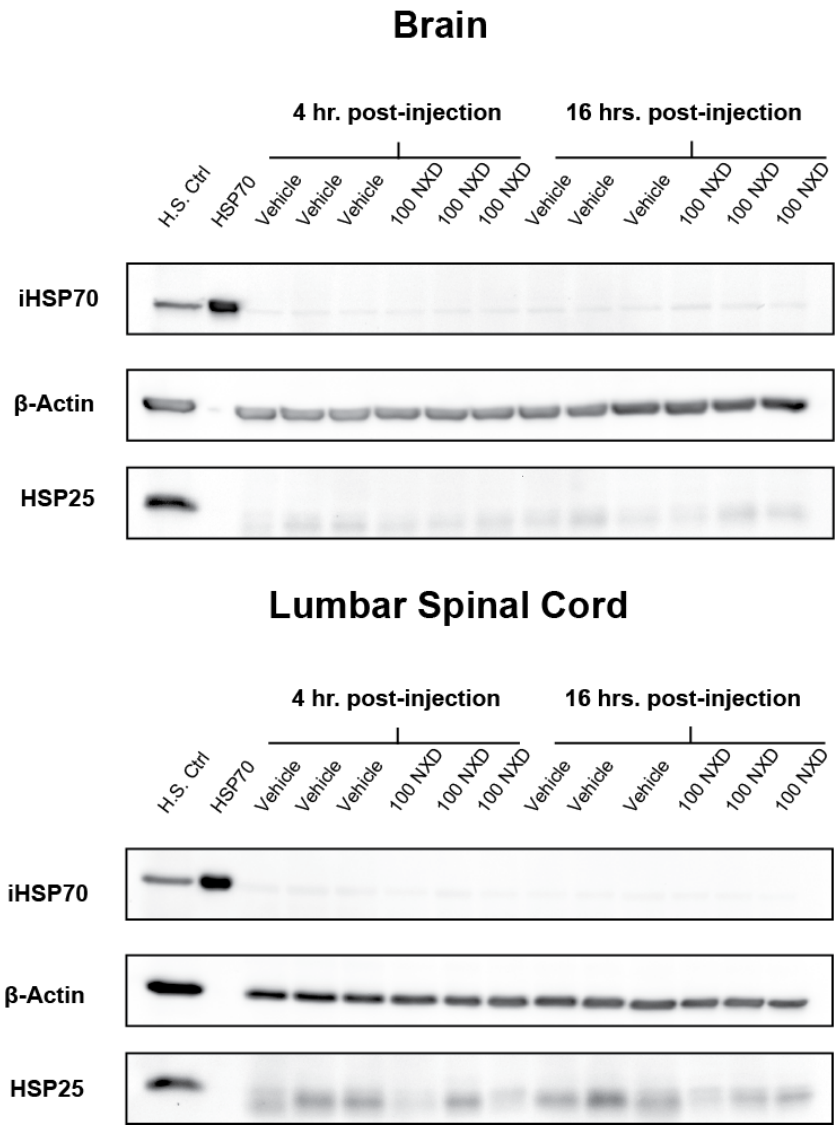

Figure 8. Single dose *i.p.* administration of NXD30001 did not induce iHSP70 or HSP25 expression in CNS tissues (brain, lumbar spinal cord). Nontransgenic C57BL6 mice were dosed *i.p.* with a single dose of 100 mg/kg NXD30001 and tissues were extracted 4 or 16 hr. later. Whole tissue extracts were subjected to Western analysis using antibodies immunoreactive to iHSP70, HSP25 and β-actin as loading control. Extracts of heat-shocked spinal cord-DRG cultures (H.S. Ctrl) and recombinant iHSP70 (HSP70) were run as positive controls.

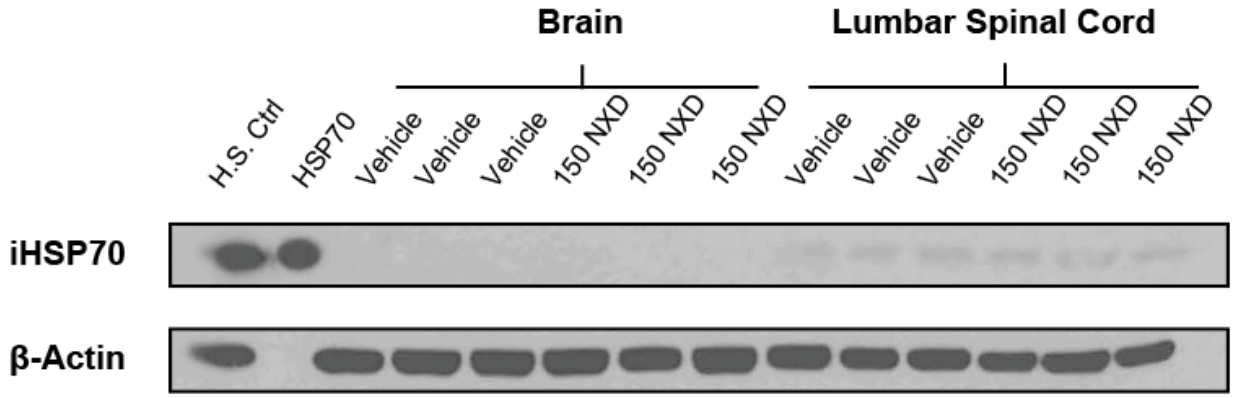

Figure 9. NXD30001 did not induce iHSP70 expression in CNS tissues (brain, lumbar spinal cord) at its maximum tolerable *i.p.* dose. Nontransgenic C57BL6 mice were dosed *i.p.* with a single dose of 150 mg/kg NXD30001 and tissues were extracted 16 hr. later. Whole tissue extracts were subjected to Western analysis using antibodies immunoreactive to iHSP70 and β-actin as loading control. Extracts of heat-shocked spinal cord-DRG cultures (H.S. Ctrl) and recombinant iHSP70 (HSP70) were run as positive controls.

Chapter 5: Discussion, Conclusions and Future Directions

5.1 Discussion:

The study reported in this thesis investigated the ability of NXD30001, a novel small molecule HSP90 inhibitor, to induce the expression of HSPs in various tissues of wild type mice and in $SOD1^{G93A}$ transgenic mice, a model of fALS1 due to SOD1 mutation. The experiments were conducted in preparation for an efficacy study of the compound in $SOD1^{G93A}$ transgenic mice.

Mutant SOD1 toxicity is complex and involves multiple cellular pathways in several cell types. ALS-causing mutations increase the propensity of the SOD1 protein to mis-fold and aggregate, promoting inappropriate interactions of the protein with cellular constituents and the accumulation of mutant SOD1 oligomers in motor neurons. Mutant SOD1 is hypothesized to challenge cellular mechanisms of protein homeostasis. Indeed, protein chaperoning activity and proteasomal activity, two measures of cellular ability to maintain protein homeostasis, are preferentially compromised in the spinal cords of $SOD1^{G93A}$ transgenic mice (4, 108-110). Proteasomal activity is also compromised in postmortem spinal cord tissues from sALS patients (111). Increased demands on protein chaperoning and degradation pathways therefore contribute to ALS pathogenesis due to multiple causes and are interesting targets for therapeutic intervention.

HSP90 inhibitors are a class of small molecules initially evaluated as cancer therapeutics due to their anti-tumorigenic ability. Their mechanism of action involves the dissociation of the HSP90 chaperone complex, resulting in the release and degradation of several proliferative and pro-survival signaling molecules. Dissociation of the HSP90 complex also releases the HSF1 transcription factor. Trimerized, phosphorylated HSF1 then accumulates in the nucleus, where

it induces the expression of *HSP* genes through association with HSEs in the promoter regions. This pharmacological induction of chaperoning activity has emerged as a strategy for treatment of neurodegenerative disorders involving proteotoxicity, such as ALS. Therapeutic use of these compounds has, however, been limited by their lack of CNS penetrance and narrow therapeutic window. NexGenix Pharmaceuticals has developed a line of HSP90 inhibitor compounds based on the radicicol backbone, the lead compound from which, NXD30001, demonstrating distribution to CNS tissues and an improved safety profile relative to the parent compound. NXD30001 was therefore investigated for therapeutic utility in models of fALS1.

NXD30001 was first evaluated *in vitro* using a primary spinal cord culture model of fALS1 derived from mouse embryos (67). NXD30001's profile of *HSP* induction was similar to that observed with geldanamycin, another small molecule HSP90 inhibitor, with both compounds inducing the expression of iHSP70 and HSP40, but not other HSPs (5, 8). Both NXD30001 and geldanamycin also improved several measures of mSOD1-associated toxicity in vulnerable cell types. Indeed, both molecules prevented the formation of mutant SOD1 inclusions, prevented motor neuron death and maintained mitochondrial morphology in primary motor neurons overexpressing $SOD1^{G93A}$ protein (5, 8). The efficacy of NXD30001 *in vitro* coupled with its improved safety profile *in vivo* compared to geldanamycin prompted an investigation of its efficacy in an *in vivo* model of fALS1, the $SOD1^{G93A}$ transgenic mouse.

Initial pharmacokinetic analysis showed that NXD30001 distributed to the CNS and peripheral tissues of both $SOD1^{G93A}$ transgenic mice and their wild-type littermates after single and multiple *i.p.* dosings. A different profile of NXD30001-induced *HSP* expression was, however, observed *in vivo* compared to *in vitro*. Despite accumulation of the compound in CNS

tissues, no induction of HSPs was detected in brain or spinal cord tissues of NXD30001-treated animals, with the possible exception of a transient induction of HSP25 expression with repeated dosing. No iHSP70 expression was detected in nervous tissues despite the administration of maximum tolerable doses (maximum due to the irritant properties of the vehicle solution and the solubility of the drug) and peak CNS tissue concentrations of NXD30001 surpassing the minimum effective *in vitro* concentration.

Despite lack of efficacy in inducing HSPs in the CNS, NXD300001 routinely induced the expression of iHSP70 in cardiac and skeletal muscle. This disparity was not due specifically to differences in biodistribution of the drug since no HSP induction was detected in liver, despite much higher accumulation of the compound in liver compared to muscle tissues. This points to potential differences in sensitivity of the mechanisms underlying induction of HSPs.

Induction of iHSP70 by NXD30001 in muscle appeared to result from activation of the transcription factor HSF1. HSF1 phosphorylation, a marker of the transcription factor's activation, was concordant with iHSP70 expression in muscle tissue. Additionally, NXD30001 did not induce HSF1 activation or *HSP* gene expression in lumbar spinal cord tissues. This was consistent with *in vitro* data showing a dependence of NXD30001-mediated induction of *HSP* expression on HSF1 activation (8). In that study, primary motor neurons overexpressing a dominant negative form of HSF1 that prevented endogenous HSF1 activation were unable to upregulate expression of iHSP70 following treatment with NXD30001 (8). The threshold for NXD30001-mediated induction of HSPs via activation of HSF1 thus appears to vary with tissue or cell type *in vivo*. To gain DNA-binding ability, HSF1 proceeds through a series of steps involving liberation from the HSP90 complex, trimerization, posttranslational modification and

localization to the nucleus. The specific post-translational modifications required for HSF1 activation are not clear. In addition, it is unknown whether HSF1 activation pathways are conserved through all mammalian tissues, and little is known of HSR regulation in multicellular organisms. Indeed, the extent of differential HSR regulation across different tissues is largely unexplored. A recent genome-wide RNAi screen for regulators of the HSR in *Caenorhabditis elegans* revealed tissue-specific negative regulators of the HSR, although positive HSR regulators functioned ubiquitously in all tissues (164). HSR regulation thus appears to reflect the functional requirements of each tissue, at least in primitive multicellular organisms (164). NXD30001's muscle-specific efficacy could, then, be reflective of tissue-specific regulation of the HSR. Skeletal and cardiac muscle tissues are subject to high thermal and contractile stress during function, and the induction of HSPs following exercise is well documented in both tissues in rodents (165, 166). Muscle tissues could therefore be "primed" for HSF1-mediated induction of *HSP* gene expression due to their functional requirements in organisms. Tissue-specific HSR regulation could also explain the transient increase in HSP25 observed in the lumbar spinal cord tissues of NXD30001-treated $SOD1^{G93A}$ mice. The HSP25 promoter contains binding sites for other transcription factors, including SP1 elements, and induction of HSP25 could therefore proceed through a pathway independent of HSF1 (167). NXD30001's tissue specificity could also be attributed to cell-type specificities in *HSP* gene accessibility. Indeed, another HSP90 inhibitor, HSP990, induced iHSP70 expression in CNS tissues of mice, but its efficacy desensitized over time due to chromatin changes that limited accessibility of HSF1 to HSEs (168).

HSP90 inhibitors improve protein homeostasis through two mechanisms: the release of HSP90 client proteins which include HSPs capable of chaperoning misfolded proteins, and the release of HSF1 which induces the expression of multiple *HSP* genes via HSEs. In motor neurons overexpressing $SOD1^{G93A}$, NXD30001 delayed the accumulation of mSOD1 protein, but did not affect SOD1 protein levels in motor neurons overexpressing wtSOD1 (8). No reduction of SOD1 protein levels occurred after 2 weeks of *i.p.* NXD30001 dosing in $SOD1^{G93A}$ transgenic mice. In fact, total SOD1 protein levels were higher in the lumbar spinal cords of NXD30001-treated mice sacrificed 6 hr after the final dose relative to vehicle-treated animals, possibly due to NXD30001-mediated disaggregation of SDS-insoluble SOD1 protein aggregates. This suggests that NXD30001 could influence mSOD1 protein levels independently of HSF1 activation and *HSP* gene expression *in vivo*. A higher molecular weight band immunoreactive with SOD1 antibody was also observed in the lumbar spinal cord of both NXD30001- and vehicle-treated mice sacrificed 1 hr. after the final *i.p.* dose, suggesting a toxic effect of the solvents in the vehicle. This higher molecular weight band could represent a posttranslationally modified or crosslinked form of mSOD1 protein. Although the appearance of higher molecular weight SOD1-immunoreactive bands is known to occur in the late stage of disease, cross-linked mutant SOD1 is not usually present at the relatively early symptomatic stage of the mice used in this study; it remains unknown whether the solutions administered accelerated this process. Given the statistically significant increase in total SOD1 protein levels in the lumbar spinal cords of NXD30001-treated mice, investigating the effect of multiple NXD30001 treatments on SOD1 aggregate load in the tissues of older symptomatic mice (when SOD1 aggregates typically appear with immunoblotting) could prove interesting.

5.2 Conclusions and Future Directions:

In conclusion, this study intended as a prelude for a full efficacy study in the fALS1 mouse actually precluded continuing on with this investigation. The project did highlight the complexities of pharmacological induction of *HSP* gene expression *in vivo*. Targeting the HSR for therapeutic application necessitates the use of an effective clinical biomarker of drug efficacy and early analysis of *in vivo* drug efficacy. Care must also be taken when interpreting *in vitro* results for the design of *in vivo* investigations given the apparent tissue-specific differences in the threshold for pharmacological induction of *HSP* expression. Given NXD30001's lack of ability to induce *HSP* expression and activate HSF1 in CNS tissues, further study of its therapeutic application in neurodegenerative disease is futile. Its ability to induce iHSP70 in skeletal muscle tissue points to a possible use in muscle proteinopathies, such as inclusion body myositis and oculopharyngeal muscular dystrophy, although modifications of the structure to improve solubility and oral availability would be recommended. Indeed, the small molecule HSP co-inducer BPG-15 preserves muscle function and slows disease progression in a mouse model of muscular dystrophy (169). The NXD30000 series of compounds is therefore a useful pharmacological inducer of *HSP* gene expression *in vitro* with possible *in vivo* applications in skeletal and cardiac muscle tissues.

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