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Brain permeable small molecule inhibitors of Hsp90 prevent alpha-synuclein oligomer formation and rescue alpha-synuclein-induced toxicity

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a) **Running Head:** Hsp90 inhibitors reduce toxic alpha-synuclein oligomers

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d) **List of Abbreviations**

αsyn = alpha-synuclein

PD = Parkinson's Disease

AD = Alzheimer's Disease

ALS = Amyotrophic Lateral Sclerosis

PolyQ = polyglutamine

LB = Lewy Body

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DLB = Dementia with Lewy Bodies

S1 = αsyn-hGLuc(1)

S2 = αsyn-hGLuc(2)

Hsp = Heat Shock Protein

HSF-1 = Heat Shock Factor 1

GA = Geldanamycin

17-AAG = 17-(Allylamino)-17-demethoxygeldanamycin

17-DMAG = 17-dimethylaminoethylamino-17-demethoxy-geldanamycin

e) **Section Assignment**: 1. Cellular and Molecular

2. Neuropharmacology

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Abstract

Aggregation of alpha-synuclein (αsyn) is a hallmark of sporadic and familial Parkinson's disease (PD) as well as dementia with Lewy Bodies (DLB). Lewy Bodies contain αsyn as well as several heat shock proteins (Hsp), a family of molecular chaperones upregulated by the cell under stress. We have previously shown that direct expression of Hsp70 as well as pharmacological upregulation of Hsp70 by geldanamycin (GA), an Hsp90 inhibitor, are protective against αsyn-induced toxicity and prevent aggregation in culture. Here we used a novel protein complementation assay to screen a series of small molecule Hsp90 inhibitors for their ability to prevent αsyn oligomerization and rescue toxicity. Using this assay, we found that several compounds prevented αsyn oligomerization as measured by decreased luciferase activity, led to a reduction in high molecular weight oligomeric αsyn, and were protective against αsyn cytotoxicity. A lead compound, SNX-0723, was determined to have an EC_{50} for inhibition of αsyn oligomerization of approximately 48 nM and was able to rescue αsyn-induced toxicity. *In vivo* assessment of SNX-0723 showed significant brain concentrations along with induction of brain Hsp70. With a low EC_{50}, brain permeability, and oral availability, these novel inhibitors represent an exciting new therapeutic strategy for PD.

Introduction

Cytoplasmic depositions of alpha-synuclein (αsyn) aggregates are a major component of Lewy bodies (LBs) (Spillantini et al., 1997), the characteristic inclusions observed in both sporadic and familial cases of Parkinson's disease (PD) and related disorders. Point mutations in the αsyn gene (Polymeropoulos et al., 1997; Krüger et al., 1998; Zarranz et al., 2004), as well as gene duplication and multiplication of the αsyn gene locus (Singleton et al., 2003), can lead to αsyn deposits and toxicity, although the mechanism of this process is unclear. However, recent evidence implicates prefibrillar, oligomeric species of αsyn as a vital toxic intermediate in the process of neurodegeneration (Conway et al., 2000; Uversky et al., 2001; Volles and Lansbury, 2003; Dedmon et al., 2005; El-Agnaf et al., 2006; Danzer et al., 2007; Outeiro et al., 2008). If oligomeric species of αsyn are toxic, an important therapeutic strategy would be to reduce rates of oligomerization or oligomer levels, thereby potentially rescuing αsyn-induced cell death and preventing disease progression.

In general, protein misfolding and aggregation can be prevented by the machinery of the molecular chaperone system. Hsp70 in particular is upregulated as part of a stress response to protein misfolding and aggregation and protects against misfolded αsyn-induced toxicity and neurodegeneration by refolding pathogenic species of αsyn and/or directing the misfolded species toward proteasomal or lysosomal degradation (Klucken et al., 2004; McLean et al., 2004; Auluck et al., 2005; Dedmon et al., 2005; Shin et al., 2005; Tetzlaff et al., 2008) We and others have previously shown that Hsp70 interacts with overexpressed αsyn in *in vitro* H4 neuroglioma cells (Klucken et al., 2004; Shin et al., 2005), in *Drosophila* (Auluck et al., 2005) and in Masliah line D mouse models (Klucken et al., 2004) in a neuroprotective manner by decreasing higher molecular weight αsyn species as well as rescuing αsyn-induced toxicity.

Hsp90 is a molecular chaperone involved in the folding, stabilization, and binding of many client proteins, and is believed to be critical for maintaining the integrity of many signaling cascade pathways in response to cellular stress and perturbations of the pathways by aberrant expression and/or mutation (Schulte and Neckers, 1998; Xiao et al., 1999). Inhibition of Hsp90 chaperone activity results in activation of Heat Shock Factor-1 (HSF-1) and subsequent activation of protective stress-induced HSPs such as Hsp70 (Dickey et al., 2005; Fujikake et al., 2008).

Geldanamycin (GA), a naturally occurring Hsp90 inhibitor, has been found to upregulate Hsp70 and is cytoprotective in many assays of misfolded protein-related toxicity (McLean et al., 2004; Fujikake et al., 2008). GA itself cannot cross the blood brain barrier and has considerable toxicity in cancer trials (Waza et al., 2006; Fujikake et al., 2008). 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG) are much less toxic derivatives of GA that are blood brain barrier permeable (Waza et al., 2006; Fujikake et al., 2008), but they have been difficult to formulate, have limited oral availability, or cause varying degrees of hepatotoxicity in clinical cancer trials, presumably due to the reactivity of the chemical core (Chiosis and Tao, 2006; Cysyk et al., 2006; Okawa et al., 2009).

SNX-2112 represents a class of novel, orally available, non chemically reactive and potent Hsp90 inhibitors that exhibit excellent anti-tumor activity in vitro and in vivo (Chandarlapaty et al., 2008; Okawa et al., 2009). In this study, we screened a group of synthetic, orally active, small molecule Hsp90 inhibitor compounds in this drug class in an *in vitro* model of αsyn oligomerization and toxicity as well as for brain penetration. These compounds are chemically dissimilar to GA and derivatives. We show that novel Hsp90 inhibitors can rescue αsyn-induced toxicity and decrease oligomerization *in vitro*

in a dose-dependent manner at a lower dose than 17-AAG. *In vivo* pharmacokinetic (PK) and pharmacodynamic (PD) studies also indicate that members of this class of Hsp90 inhibitors have good brain absorption and excellent oral bioavailability, thus making them good candidates for further evaluation. Together, these data provide important preclinical information that validates inhibition of Hsp90 as a strong therapeutic strategy in Parkinson's disease and other neurodegenerative disorders linked to protein misfolding.

Methods

Plasmids:

Syn-Luc1 (S1) and Syn-Luc2 (S2) were generated as described previously (Outeiro et al 2008) by subcloning αsyn into the Not1/ClaI sites of humanized Gaussia Luciferase constructs provided by Dr. Stephen Michnick of University of Montreal (Remy and Michnick, 2006). The Hsp70 and wild-type αsyn (pSI-WTsyn) plasmids used in this study have been described previously (Klucken et al 2004). Full-length gaussia luciferase cDNA was kindly provided by Dr. Bakhos Tannous of the Massachusetts General Hospital (Tannous et al., 2005).

Cell Culture and Transfection:

Human H4 neuroglioma cells (HTB-148;ATCC) were maintained in OPTI-MEM growth media with 10% Fetal Bovine Serum (both from Invitrogen, Carlsbad, CA) and incubated at 37°C in 5%CO_2 conditions. H4 cells were plated to 80-90% confluency 16-24H prior to transfection. They were transfected using Superfect (Qiagen, Chatsworth, CA) according to manufacturer protocol. Equimolar plasmid ratios for all constructs were used.

Toxicity Assay:

Toxicity was measured 24 hours after transfection utilizing the Toxilight™ cytotoxicity assay kit (Lonza, Rockland, ME) according to the manufacturer's instructions.

Gaussia Luciferase Protein Complementation Assay:

H4 neuroglioma cells were cotransfected with S1 and S2 in 96 well plates as described above. At 24 hours post transfection, existing cell media was replaced with

serum-free, phenol red-free OPTI-MEM (Invitrogen, Carlsbad, CA). The cell permeable substrate, native Coelenterazine (Prolume Ltd, Pinetop, AZ) was resuspended in methanol to 1 mg/mL and dispensed per well by an automated plate reader, the Wallac 1420 Victor2 (Perkin Elmer, Waltham, MA) to a final concentration of 20 μM. The signal generated from substrate-enzyme interaction was integrated over 2 seconds before measurement at 480 nm.

Enzyme Linked Immuosorbant Assay (ELISA) for αsyn:

α-Synuclein concentration was quantified using ELISA according to the manufacturer's instructions (Invitrogen, Camarillo, CA). Briefly, a monoclonal antibody specific for αsyn was coated onto the wells and αsyn binds simultaneously to the immobilized monoclonal (capture) antibody and to the solution phase rabbit polyclonal (detection) antibody specific for αsyn. After washing, a horseradish peroxidase-labeled anti-rabbit IgG (anti-rabbit IgG-HRP) is added which binds to the detection antibody to complete the four-member sandwich. After incubation and washing, substrate solution is added and absorbance is read at 450 nm. The absorbance is directly proportional to the concentration of αsyn present in the original specimen. αSyn concentration was determined by plotting sample absorbances against standards using Graph Pad Prism fitting software (four parameter algorithm).

Native and denatured PAGE:

H4 cells were plated in 60mm or 100mm tissue culture dishes and transfected 18-24 hours later using transfection procedures described above. 24 hours post-transfection, samples were harvested in 4°C lysis buffer. All samples were lysed with detergent-free

lysis buffer containing protease inhibitors (50 mM Tris/HCl pH 7.4, 175 mM NaCl, 5 mM EDTA pH 8.0).

For denatured SDS-PAGE, samples were sheared with a 27-gauge 1 mL syringe 3-4 times and centrifuged for 2 minutes. The supernatant was then boiled in 2x tris glycine sample buffer (Invitrogen, Carlsbad, CA) containing beta-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) for 5 minutes at 100° C, and then run in SDS-containing buffer on 10 well 10-20% tris glycine gels (Invitrogen, Carlsbad, CA).

For native blots, samples were harvested 24 hours post transfection in the detergent free lysis buffer detailed above. The supernatant was then mixed with 2x SDS-free native tris glycine sample buffer and run on 4-12% tris glycine gels. For both denatured and native blots, protein concentrations were determined prior to electrophoresis using the BCA protein assay and 30μg of each sample was loaded in per lane.

After electrophoresis, gels were transferred to PVDF membrane (Perkin Elmer, Waltham, MA) and blocked in 5% milk in tris-buffered saline containing tween-20 (TBS-T) for 1 hour at room temperature or overnight at 4°C. Membranes were immunoblotted with primary antibodies (Mouse anti-alpha synuclein, 1:1000, BD Biosciences, San Jose, CA; rabbit anti-gaussia luciferase, 1:1000, Prolume Ltd, Pinetop, AZ; rabbit anti-Hsp70, 1:10,000, StressGen/Assay Designs, Ann Arbor, MI) for 2 hours at room temperature or overnight at 4°C. After three TBS-T washes, membranes were incubated in horseradish peroxidase-conjugated secondary antibodies (1:2000). Immunoblots were analyzed using the ECL chemiluminescent detection system (Amhersham/GE Healthcare, Buckinghamshire, UK).

Pharmacological Treatments in vitro:

Pre-treatment: H4 cells were plated into 96 well plates and treated 6-8 hours later with 500nM 17-AAG or 10nM-10μM synthetic Hsp90 inhibitors, followed by transfection of S1 and S2 16-24 hours later.

Co-treatment: H4 cells were plated into 96 well plates, transfected with S1 and S2 16-24 hours later, then immediately treated with 500nM 17-AAG or 10nM-10μM synthetic Hsp90 inhibitors

pcDNA 3.1+ vector was used as a control for Hsp70 plasmid transfections, and DMSO was used as a control for drug treatments.

Pharmacokinetics and pharmacodynamics:

The in-life part of the animal studies was conducted at Washington Biotech (Columbia, MD). For each timepoint, 3 animals were sacrificed and samples collected. For pharmacokinetic studies, SNX-0723 was administered by oral gavage at 10 mg/kg to female Sprague-Dawley rats weighing 160–190g. Samples were collected at 0, 3, 6, 12, and 24 hours. Each animal was immediately necropsied. Brain and lung tissues were collected and flash-frozen. All specimens were stored at -80°C. Extracts from 40-100 mg brain and lung tissue were prepared with a glass dounce in 100% acetonitrile containing an internal standard. The extracts were centrifuged and a 10 μL aliquot of the supernatant was injected directly into the LC-MS/MS system [4000 QTRAP (Applied Biosystems, Foster City, CA) with Shimadzu HPLC (Columbia, MD). Tissue concentrations were calculated from the sample response ratios and tissue masses. Pharmacokinetic parameters were determined using the WinNonlin software package (Pharsight Corporation, St. Louis, MO).

For pharmacodynamic studies, SNX-0723 was administered by oral gavage at 10 mg/kg to each of 3 female Sprague-Dawley rats weighing 160–190g. Samples were

collected at 0, 1, 3, 6, 12, and 24 hours. Lysates were prepared with modification to the MSD multi-array total Hsp70 assay kit protocol. Brain tissue was homogenized in 1:6 wt:vol of complete lysis buffer using a glass dounce on ice. Protein concentration was determined using the BCA protein assay (Pierce, Rockland, IL). Hsp70 levels were measured according to kit protocol using the SI6000 SECTOR imaging instrument (Meso Scale Discovery, Gaithersburg, MD).

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Results

To examine the effect of novel compounds on αsyn oligomerization and toxicity we have developed a bioluminescent protein-fragment complementation assay (bPCA). In this strategy, αsyn is fused to non-bioluminescent amino-terminal or carboxy-terminal fragments of *Gaussia princeps* luciferase (hGluc) (Remy and Michnick, 2006) that can reconstitute when brought together by αsyn-αsyn interactions (Outeiro et al., 2008), thus providing a readout of αsyn oligomerization (Figure 1A & B). Using this assay, cotransfection of Syn-Luc1 (S1) and Syn-Luc2 (S2) results in reconstituted luciferase activity more than 5-fold above background (Figure 1C), indicative of αsyn dimer and oligomer formation. Furthermore, like untagged αsyn (Klucken et al., 2004) and αsyn fluorescent protein-fragment complementation pairs (Outeiro et al., 2008), cotransfection of S1 and S2 results in significant αsyn-induced cytotoxicity (Figure 1D). Importantly, although we have previously shown that cotransfection of αsyn and synphilin-1 results in the formation of intracellular inclusions (McLean et al., 2002; Shin et al., 2005), the oligomeric forms described herein remain soluble and do not lead to macroscopic aggregate formation (Outeiro et al., 2008; Tetzlaff et al., 2008).

We have previously demonstrated that overexpression of Hsp70 or pharmacological upregulation of Hsp70 with GA can prevent αsyn aggregation into cell inclusions (McLean et al., 2004). We now examined the effect of Hsp70 on αsyn oligomerization in our αsyn PCA assay by overexpressing Hsp70 or treating with the Hsp90 inhibitor 17-AAG. H4 cells were either pre-treated (16-18 hours before transfection) or co-treated (at time of transfection) with 17-AAG, or cotransfected with Hsp70. Both pre- and co-treatment with 17-AAG prevented αsyn oligomerization (85% ± 8.3% and 59% ± 9.5% respectively) (Figure 2A & B), as did cotransfection with Hsp70 (50% ± 13.7%). Although pre-treatment with 17-AAG resulted in a significant decrease in monomeric

protein levels (Fig. 2C), which could also account for the decrease in luciferase activity (Fig 2A), co-treatment with 17-AAG did not significantly reduce protein levels (Fig. 2D) even though it significantly decreased luciferase activity (Fig 2B). These data suggest that multiple mechanisms including degradation and refolding may be at play. To quantify the amount of αsyn in cells following 17-AAG treatment and Hsp70 coexpression we used an αsyn specific ELISA and found that there was no significant decrease in αsyn levels following *co-treatment* of 17-AAG and coexpression of Hsp70 (Fig. 2E). Furthermore, in accord with our previous studies (McLean et al., 2004), pre-treatment of 17-AAG was found to be effective in rescuing αsyn-induced toxicity (Figure 2F).

Because 17-AAG and GA have limited CNS bioavailability and as ansamycins have the potential for problems with chemical reactivity and oral bioavailability, we next screened a novel series of highly selective synthetic Hsp90 inhibitors for their effect on αsyn oligomerization and toxicity. These compounds were discovered *de novo* from a designed library for targeting purine-binding proteins which, like Hsp90, have binding sites for purine co-factors such as ATP. They represent a novel and specific chemical platform for Hsp90 inhibition, as they are unrelated to all known Hsp90 inhibitors including 17-AAG, radicicol, and the purine-based analogs (Rodina et al., 2007). This novel series of small molecule inhibitors of Hsp90 are highly selective and do not bind to other purine-binding proteins (Barta et al., 2008; Chandarlapaty et al., 2008; Rice et al., 2008; Okawa et al., 2009) (Figure 3a).

We tested a panel of ten Hsp90 inhibitors in the αsyn PCA assay that were selected for chemical properties predicted to favor brain penetration. H4 neuroglioma cells were plated in 96 well plate format, pre-treated with 500 nM of each inhibitor, and transiently transfected with S1 and S2. Twenty four hours after transfection, cells were assayed for

luciferase activity in an automated plate reader to determine the effect of the inhibitors on αsyn oligomerization as measured by reconstituted luciferase activity. Interestingly, a wide range of effectiveness in preventing αsyn oligomerization was observed (Figure 3A), with several compounds preventing αsyn oligomerization by more than 75% (from 68% ± 20.52% to 82.64% ± 2.53%). Four of the most effective compounds, SNX-8891, SNX-3113, SNX-3723, and SNX-0723, were selected for further co-treatment screening whereby H4 cells were transfected with S1 and S2 and then treated with 500nM of the inhibitors (Figure 3B). All four of the inhibitors (Fig. 3C-F) also prevented αsyn oligomerization in this treatment paradigm. As a control for all compound treatments, we determined that full length gaussia luciferase activity was not affected by Hsp90 inhibitor treatment (not shown).

Hsp90 inhibition is thought to be therapeutically effective by specifically degrading misfolded or mutated client proteins that contribute to disease pathology. To determine if this class of Hsp90 inhibitors were targeting αsyn for degradation, we treated S1/S2 transfected cells (pre- and co-treatment paradigms) with 17-AAG, SNX-8891, 3113, 3723, and 0723 and examined αsyn protein levels via both native and denatured Western blot 24 hours post transfection. Interestingly, both high molecular weight (HMW) and monomeric forms of S1 and S2 were almost completely degraded by four out of the five Hsp90 inhibitors tested following pre-treatment (Figure 4A), while levels of the housekeeping gene GAPDH (Figure 4A) were intact. By contrast, co-treatment was more selective with some decrease in HMW αsyn species evident, especially with SNX-0723 (Figure 4B). Interestingly, SNX-3113 appeared to shift the αsyn species from HMW to monomers suggesting that refolding mechanisms may be at play. As expected, pre- and co-treatment with Hsp90 inhibitors also resulted in concomitant increase of Hsp70 levels by 6-8 fold and 2-3 fold respectively (Figure 4A & B).

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To determine if the effect of the Hsp90 inhibitors occurred in a dose dependent manner, we selected a lead compound, SNX-0723, which prevented αsyn oligomerization by 82.6%, and performed a dose response experiment. H4 cells were pre-treated with 10nM-1μM SNX-0723 and assayed for luciferase activity 24H post transfection. These data yielded a dose response curve that places the EC_{50} for inhibition of αsyn oligomerization by SNX-0723 at approximately 48.2 nM (Figure 5A).

To determine if SNX-0723 treatment was simultaneously able to rescue αsyn -induced toxicity, we examined cytotoxicity levels following αsyn overexpression and SNX-0723 treatment. In agreement with SNX-0723's ability to prevent oligomerization, it was also effective in preventing αsyn-induced toxicity by up to 40% (Figure 5B) at doses of 100 – 500mM. Importantly, SNX-0723 was also effective in preventing αsyn-induced toxicity from overexpression of wild-type, untagged αsyn (Figure 5C).

The selectivity of SNX-0723 was profiled on over 120 different receptors, enzymes and transporters in the CEREP BioPrint panel and 36 different kinases using the Invitrogen Kinase Selectivity panel. SNX-0723 showed nM potency at inhibiting Hsp90 (IC_{50}=14 nM), inducing Hsp70 (IC_{50}=31 nM) and decreasing expression of several known Hsp90 client proteins: Human epidermal growth factor receptor 2 (Her2) (IC_{50}=9.4nM), ribosomal protein s6 (pS6) (IC_{50}=13nM), and protein kinase R-like endoplasmic reticulum kinase (pERK) (IC_{50}=5.5nM). At 10μM, no inhibition above 30% was observed for any of the kinases profiled. In addition broad receptor profiling revealed μM potency at the $GABA_A$ receptor (IC_{50}=23μM), the melatonin 2 receptor (IC_{50}=2.4μM), the CYP2C19 enzyme (IC_{50}=53μM) and weak binding activity was also observed for phosphodiesterase 11 (PDE11) (47% at 10μM).

Pharmacokinetic (PK) and pharmacodynamic (PD) assessment of Hsp90 inhibitors: Many currently available Hsp90 inhibitors, including 17-AAG, will not be useful as therapies for neurodegenerative disorders such as Parkinson's disease because of low solubility, and limited CNS and oral availability (Chiosis and Tao, 2006). Regardless, several studies now support Hsp90 as an important target for PD therapeutics. *In vivo* pharmacokinetic studies were performed to determine if SNX-0723 has the potential to be active and brain permeable. Rats orally dosed with 10 mg/kg of SNX-0723 were found to reach maximal brain concentrations 6 hours after oral dosing with almost complete clearance by 24 hours (Figure 6A). To validate that the PK properties for SNX-0723 were relevant, a pharmacodynamic evaluation was performed assessing Hsp70 induction in the brain. These studies reveal that SNX-0723 causes a 5-fold induction of Hsp70 in rat brain following a single oral dose of 10 mg/kg (Figure 6B).These PK and PD data support the potency and brain permeability of this class of Hsp90 inhibitors, making them viable compounds for *in vivo* validation or further derivation.

Discussion

Many neurodegenerative diseases such as Parkinson's disease (PD), dementia with Lewy bodies (DLB), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and polyglutamine (polyQ) diseases are thought to be caused by misfolding and subsequent accumulation of toxic proteins (Waza et al., 2006; Fujikake et al., 2008). Direct upregulation of HSF-1 and Hsp70, as well as pharmacological induction by Hsp90 inhibitors in cell culture and animal models, are protective against toxicity induced by pathogenic proteins, ameliorate abnormal transgenic phenotypes in fly and murine models, and suppress protein aggregate formation in several models of neurodegeneration (Dickey et al., 2005; Fujikake et al., 2008).

Hsp90 inhibitors such as 17-AAG and radicicol have been shown to have neuroprotective effects similar to direct HSF-1 and Hsp70 upregulation (Auluck et al., 2005; Waza et al., 2006), but also have limited oral bioavailability and blood brain barrier permeability (Chiosis and Tao, 2006; Taldone et al., 2008). 17-AAG is currently in phase II trials as an anti-tumor compound (Heath et al., 2005; Ramanathan et al., 2005) and is neuroprotective in pre-clinical models of Huntington's disease and spinocerebellar ataxias (Waza et al., 2006; Fujikake et al., 2008). Furthermore, Hsp90-CHIP complexes can target and selectively degrade phosphorylated tau protein implicated in AD (Petrucelli et al., 2004), and Hsp90 inhibitors are also neuroprotective against toxicity caused by Aβ aggregation (Ansar et al., 2007) as well as existing and induced oxidative toxicity (Xiao et al., 1999; Ouyang et al., 2005).

PD is characterized by dopaminergic cell loss in the substantia nigra and the presence of Lewy Bodies comprised of aggregated αsyn and heat shock proteins amongst other proteins. We and others have shown that Hsp90 inhibition may play an important role in PD synucleinopathies, where increasing evidence implicates a pre-

fibrillar αsyn species as the toxic moiety, by protecting against αsyn-induced toxicity (McLean et al., 2004; Auluck et al., 2005). In this study we used a bioluminescent protein-fragment complementation assay to study prefibrillar, oligomeric αsyn species. In accord with our previously described fluorescent PCA (Outeiro et al., 2008), no macroscopic aggregates of αsyn are observed under these conditions; rather, prefibrillar, intermediate αsyn species are present. The manipulations measured by the PCA assay are therefore directed towards a range of oligomeric, pre-aggregate αsyn species.

Like geldanamycin, both 17-AAG and the novel Hsp90 inhibitors decreased αsyn oligomerization and toxicity. At lower doses SNX-0723 was more effective at rescuing αsyn-induced toxicity than 17-AAG (i.e. 100 nM compared to 500 nM), suggesting greater therapeutic potential. However, the data presented herein demonstrate that Hsp70 overexpression, 17-AAG treatment and the novel Hsp90 inhibitors had a greater effect on αsyn oligomerization than on toxicity rescue. There are several possible explanations for the observed differential effects observed including the fact that other species of αsyn, or other interacting proteins, that are not directly measured by the PCA assay nor affected by the inhibitors, may contribute to αsyn-induced toxicity. This suggests that multiple facets contribute to neurodegeneration in PD and raises the possibility that oligomeric αsyn may not represent the only toxic moiety. Indeed, although our own data support oligomeric species as a toxic species, they do not do so conclusively.

We have previously established that pharmacological upregulation of Hsp70 with GA pre-treatment protects against αsyn-induced toxicity and leads to degradation of higher molecular weight αsyn (McLean et al., 2004). In line with previous data, we demonstrate here that a novel series of brain permeable Hsp90 inhibitors can prevent αsyn

oligomerization in a dose-dependent manner and concomitantly rescue αsyn-induced toxicity by potentially refolding and/or degrading αsyn. In accord with our previously published studies, significant rescue of αsyn-induced toxicity is only observed using a pre-treatment paradigm, even though decreased oligomerization occurs with a co-treatment paradigm. The reason for this phenomenon is unclear, however, it is possible that specific species of αsyn may have to be ameliorated before significant rescue is achieved and that pre-treatment achieves this more effectively than co-treatment.

Current therapies for PD relieve symptoms by restoring levels of dopamine in the brain, but with several side effects including decreased impulse control, hallucinations, increased risk of cardiovascular side effects, and drug-induced dyskinesia after prolonged treatment (Jankovic and Stacy, 2007; Moller et al., 2008). SNX-0723 exhibits promising PK properties, including robust brain concentration and induction of Hsp70 in rat brains. It is orally available and reaches maximal brain concentrations at 6 hours post oral dose, suggesting it could be taken as a therapeutic oral regimen. Although it is uncertain what the side-effects of prolonged Hsp90 inhibition will be, the data here support further investigation *in vivo* to determine if Hsp90 inhibition can rescue αsyn-induced cell death in animal models.

These novel compounds represent a potential alternative to existing therapeutics, which treat the symptoms of the disease rather than the underlying cause. They specifically induce Hsp70 *in vitro* as well as *in vivo*, possess a chemically distinct scaffold with good target specificity, and are orally available. They rescue αsyn- induced toxicity while preventing oligomerization, suggesting a consistent therapeutic effect that is capable of targeting the accumulation and resulting cytotoxicity associated with αsyn in PD. Taken together, these data suggest that these novel compounds may represent an exciting new neuroprotective therapeutic strategy for PD.

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FOOTNOTES

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Legends for figures

Figure 1: αSyn bioluminescent Protein Complementation Assay (bPCA). **A & B**) Schematic representation of the αsyn bPCA constructs. Nonbioluminescent halves of humanized gaussia luciferase is fused to αsyn monomers. **C**) Cotransfection of S1 and S2 results in reconstituted luciferase signal at 5X greater than background. n=3, ***p<0.001 **D**) Like αsyn, overexpression of S1 and S2 results in cytotoxicity. n=3, * p<0.05

Figure 2: Hsp70 and 17-AAG reduce αsyn oligomerization and rescue toxicity. A) Pre-treatment with 500 nM 17-AAG or overexpression of Hsp70 reduces αsyn oligomerization as measured by the αsyn bPCA. n=3, ***p<0.001; **B**) Co-treatment with 500 nM 17-AAG or overexpression of Hsp70 reduces αsyn oligomerization as measured by the αsyn bPCA. n=3, ***p<0.001; **C**) Pre-treatment with 500 nM 17-AAG or overexpression of Hsp70 reduces αsyn protein levels while upregulating Hsp70 levels; **D**) Co-treatment with 500 nM 17-AAG or overexpression of Hsp70 does not affect αsyn protein levels while upregulating Hsp70 levels; **E**) Intracellular αsyn protein levels are not reduced following co-treatment with 500nM 17-AAG or cotransfection of Hsp70 as measured by αsyn ELISA. **F**) Pre-treatment with 17-AAG and coexpression of Hsp70 rescues αsyn-induced toxicity. n=3, * p<0.05. C) & D) are representative western blots from n=3.

Figure 3: Novel Hsp90 inhibitors prevent αsyn oligomerization and rescue toxicity. **A**) Pretreatment prevents αsyn oligomerization as measured by decreased luciferase

complementation. Of the compounds tested, six significantly reduced αsyn oligomerization. n=3, ** p<0.01, *** p<0.001 **B**) Cotreatment with the 4 most effective compounds also prevents αsyn oligomerization as measured by decreased luciferase complementation. n=3, *** p<0.001. **C-F**) Structure of 4 most effective Hsp90 inhibitors used in the study.

Figure 4: **Novel Hsp90 inhibitors reduce high molecular weight oligomeric αsyn species.** Western blots of S1 and S2 transfected H4 cells treated with various Hsp90 inhibitors. **A**) Composite native and denatured PAGE of *pre-treatment* with Hsp90 inhibitors. Blots were probed for αsyn (native & denatured), Hsp70 (denatured only), and GAPDH (denatured only). A reduction in higher molecular weight (HMW) and monomeric αsyn species with a concomitant 6-8 fold upregulation in Hsp70 following pre-treatment with Hsp90 inhibitors was observed. **B**) Composite native and denatured PAGE of *co-treatment* with Hsp90 inhibitors. Blots were probed for αsyn (native & denatured), Hsp70 (denatured only), and GAPDH (denatured only). Co-treatment resulted in some reduction in HMW species (SNX-8891, SNX-3113, SNX-0723) as well as an apparent redistribution of αsyn species from HMW to monomeric (SNX-3113) with a concomitant 2-3 fold upregulation in Hsp70 following Hsp90 inhibition. Representative blots from n=3 of each condition.

Figure 5: SNX-0723 prevents αsyn oligomerization and rescues αsyn-induced toxicity in a dose-dependent manner. A) H4 cells were treated with 10nM-10μM doses of SNX-0723 and assayed for luciferase activity 24h post transfection. SNX-0723 prevents αsyn oligomerization in a dose dependent manner with an EC_{50} of 48.2 nM. n=4 **B**) SNX-0723 rescues αsyn-induced toxicity in a dose-dependent manner. n=3, **

p<0.01, ***p<0.001. **C**) SNX-0723 also rescues toxicity induced by overexpression of wild-type, untagged αsyn. n=4, * p=0.05

Figure 6: SNX-0723 is absorbed by the brain and causes Hsp70 induction in vivo. **A**) SNX-0723 is brain permeable. Rats orally dosed with 10mg/kg SNX-0723 reached maximal brain at 6 hours post-dose with almost complete clearance by 24 hours. **B**) SNX-0723 causes a 5-fold induction of Hsp70 in the rat brain following a single oral dose of 10 mg/kg.

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Figure 1

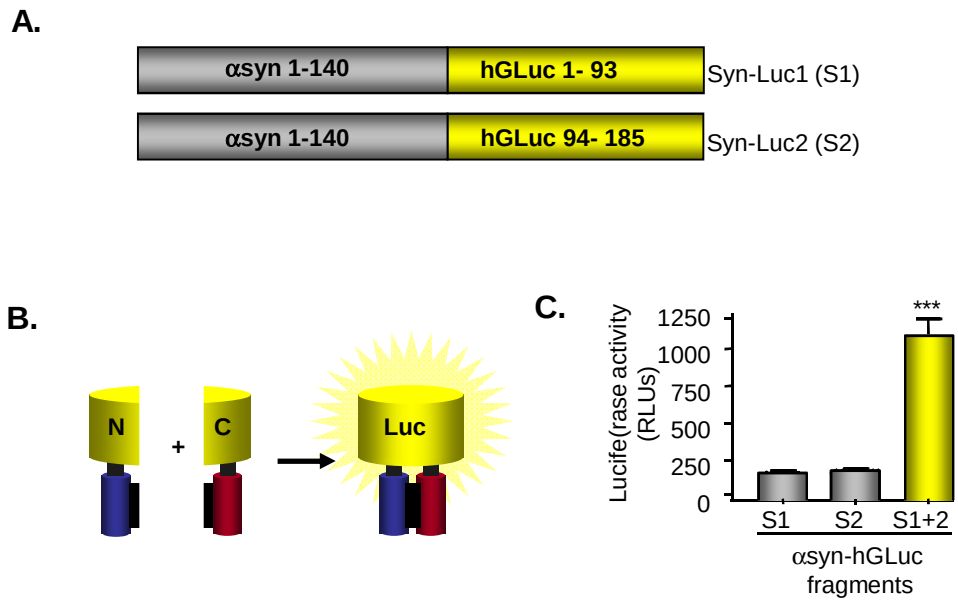

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Figure 2

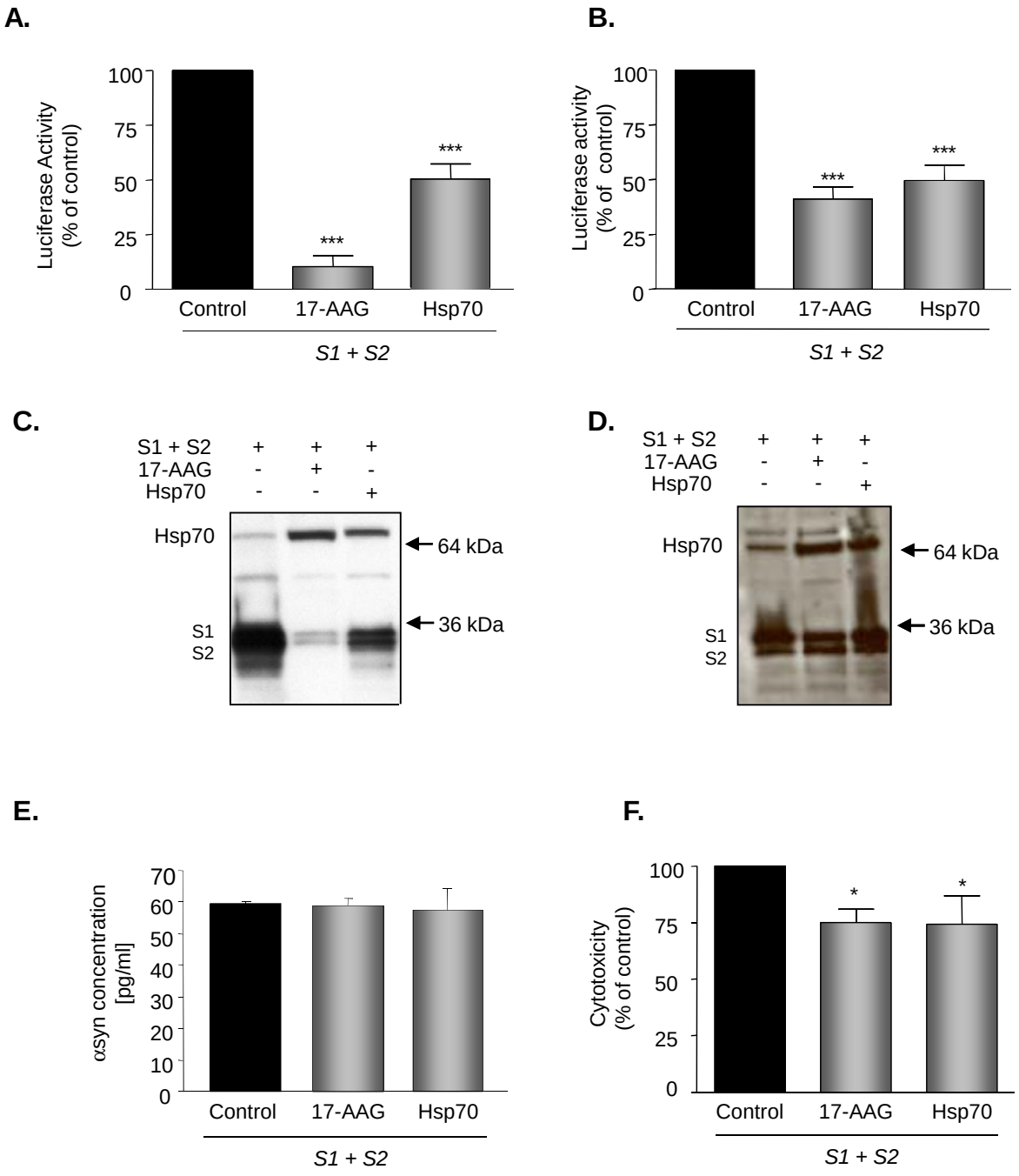

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Figure 3

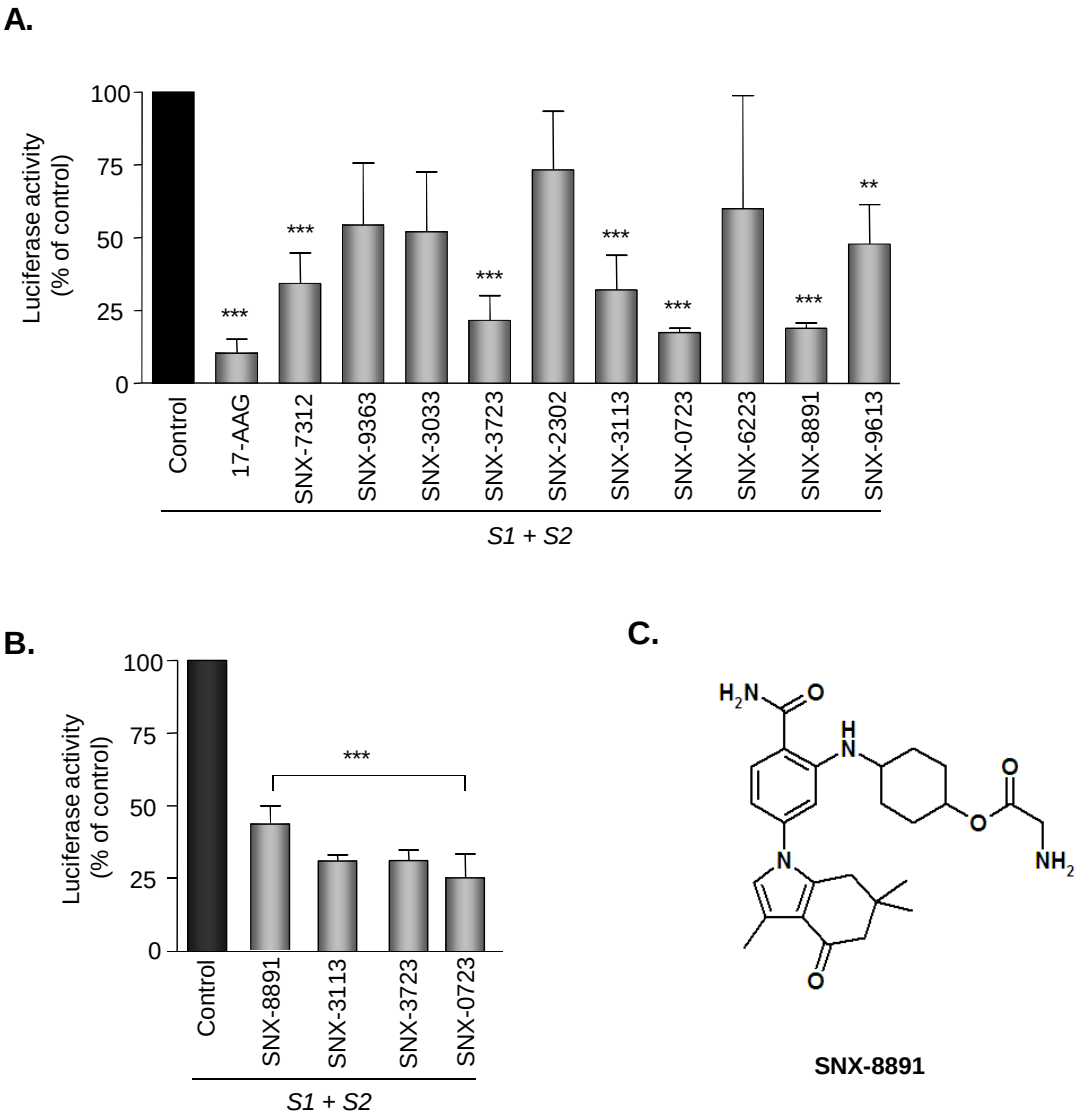

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Figure 4

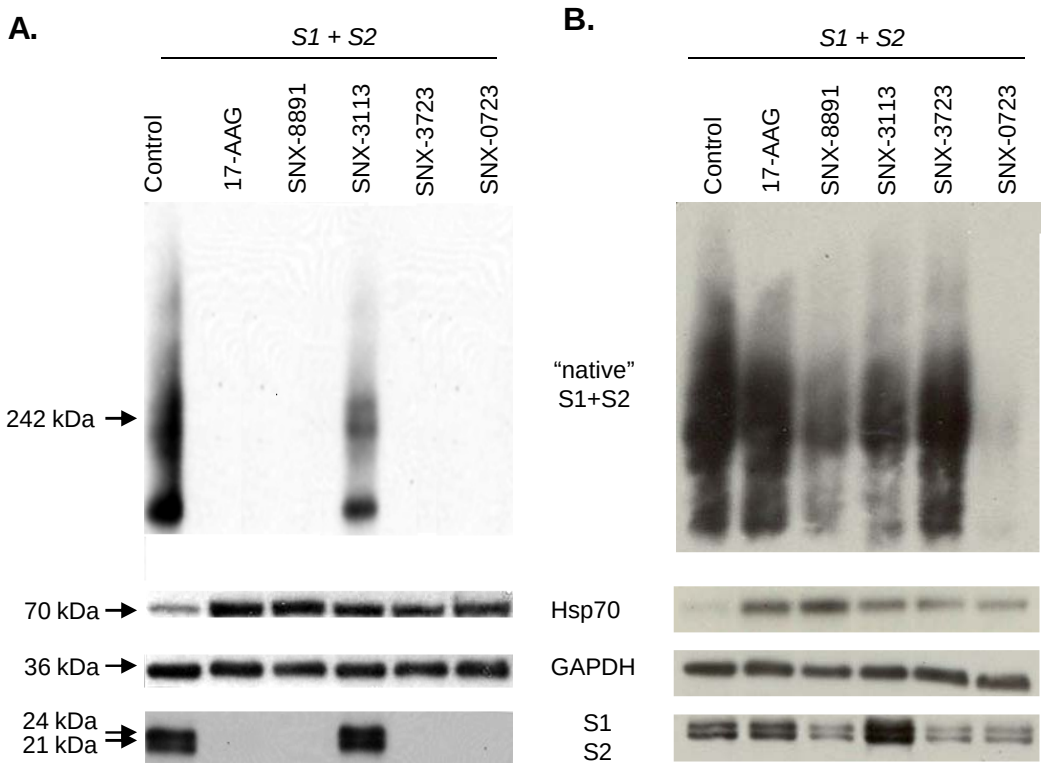

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Figure 5

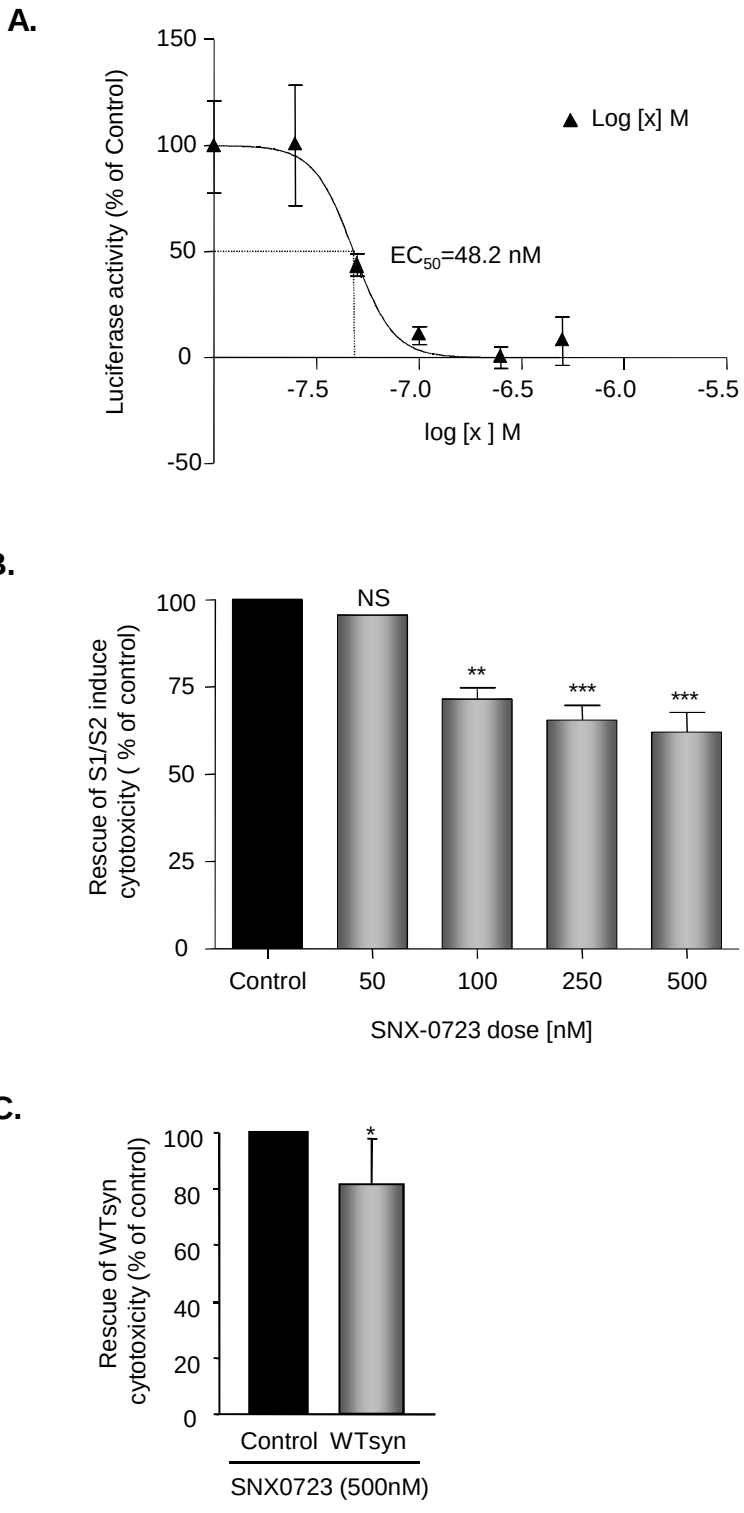

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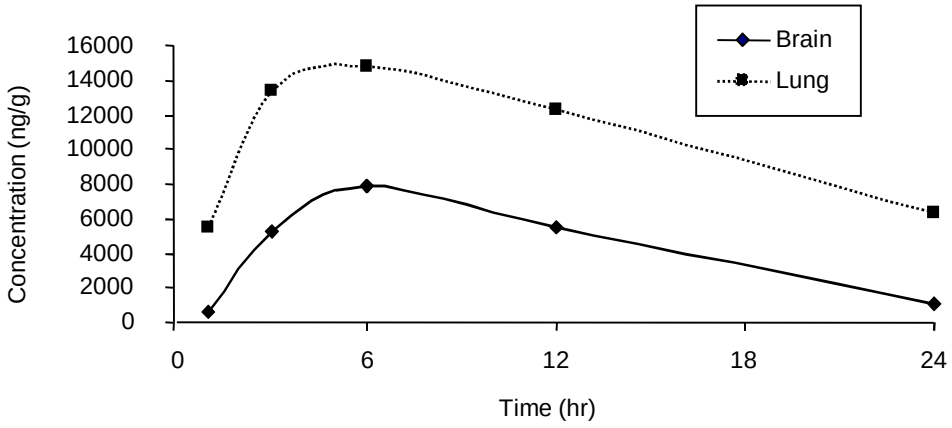
Figure 6

A.



B.