CHAPTER 6

NEUROPROTECTIVE FEATURES OF HSP90 INHIBITORS EXHIBITING ANTI-INFLAMMATORY ACTIONS: IMPLICATIONS FOR MULTIPLE SCLEROSIS

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Abstract: Multiple sclerosis is a T-cell mediated, autoimmune disease of the central nervous system, affecting over 400,000 people in the US. While the exact causes of MS remain to be elucidated, several anti-inflammatory treatments can reduce disease severity in MS as well as in its animal model experimental autoimmune encephalomyelitis (EAE). It has been known for many years that induction of a heat shock response (HSR), either by thermal or pharmacological means, can suppress inflammatory responses, and indeed induction of a HSR can reduce the incidence and severity of EAE. More recently, it was reported that drugs which bind to the nucleotide binding pocket of HS protein 90 (Hsp90) induce a HSR, which led to testing of several naturally occurring and synthetic Hsp90 inhibitors in EAE. In this article, we will review possible mechanisms by which Hsp90 inhibitors could provide benefit in EAE, and potentially in MS

Keywords: Hsp90; ansamycin; EAE; nitric oxide; T-cells; cytokines

Abbreviations: 17-AAG, 17-allylamino-17-demethoxygeldanamycin; CPD, client protein degradation; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; EC, experimental compound; HSR, heat shock response; HSF, heat shock factor; IFN, interferon; IL, interleukin; IκB, inhibitor of kB; IKK, IκB kinase; LPS, lipopolysaccharide; MHC, major histocompatability class; MOG, myelin oligodendrocytes protein; NOS2, nitric oxide synthase type 2; NFκB, nuclear factor kB

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INTRODUCTION

MS is an autoimmune T-cell mediated disease, influenced by genetic background, geographic location, and having a possible viral involvement. There are over 400,000 people diagnosed with MS in the US, and an estimated 2.5 million worldwide. Symptoms may be mild, such as numbness in the limbs, or severe, involving paralysis or loss of vision. MS is classified into four main subtypes, Relapsing-Remitting (RRMS, characterized by partial or total recovery after attacks), secondary progressive MS (SPMS, a relapsing-remitting course which becomes steadily progressive), primary progressive MS (PPMS, characterized by a progressive course from onset), and progressive relapsing MS (PRMS, a progressive course from the outset, also characterized by acute attacks). Current treatments for MS are limited, and include use of the anti-inflammatory cytokine interferon – β (IFNβ); the synthetic repeating polypeptide glatiramer acetate, the immune suppressant mitoxantrone, and recently the monoclonal antibody natalizumab which blocks binding of leukocytes to endothelial cells. However, these interventions either show limited efficacy, can only be used for short periods, need to be injected, or have significant associated risks, thus warranting development of newer and safer drugs.

ANTI-INFLAMMATORY EFFECTS OF THE HSR

It has been known for many years that the Heat Shock Response (HSR) can protect cells and tissues from a variety of noxious stimuli, including thermal, chemical, and physical stress, possibly by facilitating re-naturation of denatured proteins or by chaperoning nascent proteins to specific subcellular locations. Observations that HSPs are induced during stroke, and that ischemic damage is reduced in transgenic mice constitutively expressing Hsp70 demonstrated that the HSR is neuroprotective (Sharp et al. 1999; Giffard and Yenari 2004). However, the HSR can also reduce cell and tissue damage by inhibiting pro-inflammatory gene expression (Jaattela et al. 1992; Hotchkiss et al. 1993; Marber et al. 1995; Simon et al. 1995). In addition, induction of a HSR can prevent expression of the inducible form of nitric oxide synthase (NOS2) whose production of NO contributes to neurological damage in various diseases and conditions (Boje 2004). We showed that thermal induction of the HSR reduced NOS2 in cultured astrocytes (Feinstein et al. 1996), a finding replicated in numerous cell types including hepatocytes (de Vera et al. 1996), islet cells (Scarim et al. 1998), smooth muscle cells (Joly et al. 1997) as well as in whole animals (Hauser et al. 1996).

There are several mechanisms by which the HSR could inhibit inflammatory gene expression. A common feature of inflammatory responses is activation of transcription factor NFκB, and the pathways leading to NFκB activation and de-activation are well known (Traenckner et al. 1995; Simeonidis et al. 1999). In brief, activation of NFκB involves degradation of an inhibitory IκB protein (IκBα being most common in glial cells) which normally maintains NFκB in the

cytoplasm. Inflammatory stimuli leading to activation of IκB kinases (IKKs) allows for phosphorylation of IκBs, ubiquination, degradation by the 26S proteosome, and release of NFκB. The rapid re-expression of IκBα restricts prolonged NFκB activation. The HSR prevents NFκB activation in diverse cell types and by various stimuli (Wong et al. 1997; Scarim et al. 1998). We described that the cytokine induced loss of IκBα in C6 cells was blocked by the HSR, suggesting inhibition of degradation or increase of synthesis (Feinstein et al. 1996, 1997). At the same time, Wong et al. showed that HS blocked cytokine-dependent IκBα loss, and later that HS or arsenite prevented IκBα loss due to incubation with TNFα (Wong et al. 1997). Both groups demonstrated that expression of inhibitory IκB proteins (IκBα and IκBβ) is increased by the HSR, most likely due to the presence of a HS element in their promoter regions (Thomas et al. 1998). More recent studies demonstrate that the HSR inhibits IKK activity (Curry et al. 1999; Kohn et al. 2002; Pittet et al. 2005), thus providing another means to prevent IκB phosphorylation and degradation. HSR effects may also be in part due to other activities, such as repression of gene expression by binding of the HSF-1 transcription factor to upstream regions of inflammatory transcription factor cytokine promoters (Cahill et al. 1996). Together, these studies demonstrate that the HSR blocks NFκB activation via increasing the levels of inhibitory IκB proteins, and thus helps to explain the broad spectrum of inflammatory responses it can suppress.

EFFECTS OF THE HSR IN AN ANIMAL MODEL OF MS

The demyelinating autoimmune disease experimental autoimmune encephalomyelitis (EAE) is an often-used animal model to study possible causes and therapeutic interventions for MS. Active immunization with myelin oligodendrocyte glycoprotein (MOG) or a 21 residue peptide (MOG 35–55) in C57BL6 mice yields a chronic monophasic disease that lasts for up to several months. The central steps leading to disease progression are well known and in general are very similar to those responsible for symptoms in MS, namely priming and proliferation of T-cells in lymph nodes and spleen, their migration into brain, release of pro-inflammatory Th1 type cytokines (including IFNγ and TNFα) in brain, induction of chemokines and of cell adhesion molecules on brain microvasculature, and production of reactive oxygen (RO) and nitrogen (RN) species from glial cells. Infiltration of an additional T-cells and macrophages augments expression of pathological cytokines, ROS, and RNS, and leads to demyelination and axonal damage. Numerous studies have shown that several treatments which reduce the symptoms of EAE also reduce activation of transcription factor NFκB, consistent with the idea that NFκB plays a critical role in EAE (Du et al. 2001; Heneka et al. 2001; Dasgupta et al. 2004). Studies from our lab and others have described various means to prevent NFκB activation, including treatment with anti-inflammatory drugs such as NSAIDs, tetracycline derivatives, steroids (Li et al. 2005; Stanislaus et al. 2005;), endogenous neurotransmitters such as noradrenaline (Feinstein et al. 2002), natural products such as green tea extracts (Aktas et al. 2004), and agonists of the peroxisome proliferator-activated receptors

(PPARs) (Feinstein 2003; Kielian and Drew 2003). Interestingly, most, if not all of these treatments can also induce a HSR.

We therefore tested if induction of a HSR would provide benefit in the EAE model, and showed that a brief period of hyperthermia (body temperature raised to 42°C for 20 minutes), given 2 days after the MOG immunization injection (and therefore before the appearance of clinical symptoms) prevented disease onset in the majority of mice, and the few animals which became ill showed mild, transient symptoms (Heneka et al. 2001). The HSR reduced T-cell infiltration into brain, cortical chemokine and NOS2 expression, and brain NFκB activation. Subsequently, we confirmed that hyperthermia decreased inflammation within the brain (Heneka et al. 2000), since inflammatory responses (NFκB activation; NOS2 expression) to intracortical injection of the robust inflammatory agent lipopolysaccharide (LPS) were blunted and IκB (as well as Hsp70) expression was increased following treatment. These findings raised the intriguing possibility that inducing a HSR could be of therapeutic benefit in MS; however since hyperthermia is not well tolerated in MS patients, an alternative paradigm was needed.

HSP90 AND ANSAMYCINS

The HSR can be induced by a wide array of metabolic insults including exposure to elevated temperatures, heavy metals, ionophores, amino acid analogs, and metabolic poisons. These stressors adversely affect protein conformation, and the intracellular accumulation of abnormally folded proteins initiates a HSR by activating heat shock factor (HSF-1). HSF-1 is present in unstressed cells as an inactive monomer, which rapidly trimerizes in response to metabolic stress (Morimoto 2002). Trimerization enables HSF-1 to bind to a consensus DNA sequence, the heat shock element (HSE), located within the promoter element of genes encoding stress proteins, and thereby induces rapid HSP transcription. However, a HSR can also be elicited by treating cells or tissue with drugs that bind to Hsp90, which is expressed at high levels in the cytoplasm under normal conditions, and is further induced after stress (Burrows et al. 2004; Kamal et al. 2004). Hsp90 normally forms a complex with HSF-1 (Zou et al. 1998) and upon inhibition of Hsp90, HSF-1 dissociates allowing induction of the HSR.

Mammalian cells contain four distinct members of the Hsp90 family, the major forms being cytosolic Hsp90a and Hsp90b which share 76% homology. Hsp90b is generally constitutively expressed, whereas Hsp90a is inducible. Hsp90 proteins contain a low affinity ATP/ADP binding pocket in the N-terminal domain, which exhibits low ATPase activity. When ATP is bound, Hsp90 forms a multi-protein complex which binds to and stabilizes various Hsp90 client proteins. When ADP is bound (or ATPase inhibited), Hsp90 adopts a conformation that destabilizes the complex, inducing dissociation of client proteins and targeting some of them for degradation by the 26S proteasome. In addition to the N-terminal ATP binding site, the presence of a second nucleotide site and an independent substrate binding site has been also demonstrated (Scheibel et al. 1999). This region mediates formation of

Hsp90 dimers, the presumed active conformation of the chaperone and regulates the ATPase activity of the N-terminal region. Moreover, drugs that bind the C-terminal ATP binding region have been found to interfere with the Hsp90 chaperone activity (Marcu and Neckers 2003).

An increasing number of proteins have been identified as Hsp90 client proteins (Burrows et al. 2004; Zhao and Wang 2004; Millson et al. 2005); including steroid hormone receptors (SHRs), protein kinases, and transcription factors. In some cases, such as that for the steroid hormone receptors, the released proteins are not degraded, but instead can dimerize and activate gene transcription (Bagatell et al. 2001). Amongst the Hsp90 client proteins some have a clear role in regulating inflammatory gene expression, including IKK IκB kinase (IKK) (Pittet et al. 2005), PPARs (Sumanasekera et al. 2003), and NOS2 (Yoshida and Xia 2003). Thus, Hsp90 inhibitors can exert their effects by inducing a HSR or by inducing client protein degradation (CPD).

HSP90 PROTEINS AND BRAIN

There is relatively little known about the role of Hsp90 in the brain, and even less regarding Hsp90 regulation of brain functions. Hsp90 is expressed ubiquitously throughout brain, with preferential localization to neurons (Quraishi et al. 1996; Jeon et al. 2004). Hsp90 has been shown to play a role in neurite extension (Ishimoto et al. 1998), neural survival (Lee et al. 2001; Jeon et al. 2004), cell migration (Sidera et al. 2004), and neurotransmitter release (Gerges et al. 2004). Hsp90 is relatively less abundant in glial cells (Itoh et al. 1993; Uryu et al. 2006) but increased can be readily increased after induction of an excitotoxic lesion in mouse brains (Jeon et al. 2004), and may contribute to mechanisms of glial cell protection and adaptation in response to damage. Several studies demonstrated that Hsp90 forms a complex with the two constitutive forms of NOS (NOS1 and NOS3), and regulates their stability and activity (Osawa et al. 2003). More recently, a similar interaction of Hsp90 with NOS2 was reported (Yoshida and Xia 2003), demonstrating that Hsp90 normally increases NOS2 activity, and that Hsp90 inhibitors reduce it. Thus, Hsp90 inhibitors may modulate brain inflammatory damage by directly reducing NOS2 activity.

HSP90 AND T-CELL FUNCTIONS

Little is known regarding Hsp90 interactions with T-cell signaling in either EAE and MS. However, Hsp90 client proteins may play an important role in T-cell activation and co-stimulation. Current work on T-cell activation demonstrates that two distinct signals are needed for activation; ligand presentation by an antigen presenting cell (APC) cell to the T-cell receptor (TCR; CD3); and activation of a co-stimulatory molecule on T-cells. Together, the 2 signals induce T-cell proliferation,

differentiation, and cytokine production. In the absence of co-stimulation, T-cells die or become refractory to antigen stimulation (Chitnis and Khoury 2003; Allen et al. 2005). There are several identified costimulator pairs, however some of the more well-characterized interactions include binding of the ligand B7-1/2 to its receptor CD28; and binding of CD154 (a member of the TNFα family) to its receptor CD40. These interactions lead to activation of anti-apoptotic proteins, activation of inflammatory transcription factors such as NFκB, increased cell survival, and cytokine production. The signaling pathways involved are not entirely worked out but activation of the PI3Kinase: AKT/PKB system has been implicated in some cases (Ward 1999). CD28 through PI3K augments NFκB activation and BclX expression (Wu et al. 2005), and increases IL2 expression (Okamoto et al. 2003; Sanchez-Lockhart et al. 2004). A primary target of PI3K is the prosurvival kinase AKT/PKB, and several studies show that AKT influences T-cell function and survival (Jones et al. 2002).

Likewise, certain costimulatory interactions reduce T-cell activation, including binding of B7 to CTLA-4 (a receptor that is closely related to CD28), and these are thought to terminate T-cell function. CTLA-4 ligation signaling is less well characterized than that of CD28, but may involve different downstream targets of PI3K. Modulation of costimulatory interactions either pharmacologically or genetically has profound effects on EAE disease (Sporici and Perrin 2001; Chitnis and Khoury 2003; Howard and Miller, 2004); thus costimulatory molecules are now recognized as therapeutic targets for disease intervention. In view of the fact that AKT is a well characterized Hsp90 client protein, and considering that lymphocytes use similar signal transduction pathways as tumor cells do, Hsp90 inhibitors could be potential immunosuppressant drugs. Indeed several reports (Schnaider et al. 1998, 2000; Yorgin et al. 2000) have shown that the Hsp90 inhibitor geldanamycin inhibits CD28 mediated T-cell activation and that degradation of the non-receptor tyrosine kinase $p56^{lck}$ seems to be one of the molecular mechanisms involved.

The above studies suggest that Hsp90 inhibitors could modulate T-cell function interfering with binding of Hsp90 to client proteins involved in T-cell signaling pathways (AKT, NFκB). However several reports have demonstrated a more specific role for Hsp90 in mediating immune functions. Hsp90 participates in the transport, trimming, and presenting of antigenic peptides to the MHC class I molecules to evoke T-cell immune responses (Binder et al. 2001; Chen and Androlewicz 2001). Recently a role for Hsp90 in the ability of antigen presenting human dendritic cell (DC) phenotype and function has been reported (Bae et al. 2007). The authors showed that Hsp90 inhibition significantly decreased cell surface expression of costimulatory (CD40, CD80, CD86), maturation (CD83), and MHC (HLA-A, B, C and HLA-DP, DQ, DR) markers in both immature DC and mature DCs, and decreased the ability of mature DC to present antigen to T-cells. Thus, Hsp90 may modulate not only T-cell signaling but the ability of antigen presenting cells to activate the T-cells themselves.

HSP90 INHIBITORS AND EAE

The discovery of naturally occurring Hsp90 inhibitors followed characterization of the bacterial product geldanamycin, which had been identified as a tyrosine kinase inhibitor with anti-tumorigenic properties (Whitesell et al. 1994), yet exerted effects at concentrations that did not inhibit oncogenic kinase activities. Geldanamycin was shown to form a complex with Hsp90, disrupting the Hsp90:Src Kinase complex needed for cellular transformation (Bagatell et al. 2001). As a consequence, geldanamycin and its derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) are in clinical trials for treatment of breast cancer (Neckers 2002; Ramanathan et al. 2005).

In view of the fact that ansamycins were known to induce a HSR, we tested the effects geldanamycin in MOG induced EAE (Murphy et al. 2002). Both geldanamycin, as well as its less toxic derivative 17-AAG induced a HSR in primary rat astrocytes as well as rat C6 glioma cells, and both dose-dependently reduced NOS2 expression and activity (with IC_{50} values in the low nanoMolar range). The ansamycins increased expression of the inhibitory IκBα protein, suggesting their effects were mediated, iun part by suppression of NFκB activation. In the MOG EAE model, treatment with geldanamycin (a single injection of 300 ng i.p., given 3 days after the MOG booster) reduced disease onset by over 50% Although results with geldanamycin were promising, its known side effects (hepatotoxicity (Supko et al. 1995)) limit its potential.

In subsequent study we more carefully evaluated the ability of 17-AAG to influence the course of EAE (Dello Russo et al. 2006). In primary glial cultures, 17-AAG dose-dependently reduced LPS-dependent expression and activity of NOS2, reduced IL-1β expression and release, increased inhibitory IκBα protein levels, and induced HSP70 expression. 17-AAG prevented disease onset when given to MOG immunized mice at an early time during disease progression, and importantly could reduce clinical symptoms when given during ongoing disease. The effects of 17-AAG were due in part to actions on T-cells, since T-cells derived from 17-AAG treated-mice showed a reduced response to immunogen restimulation, and 17-AAG reduced the ability of T-cells to produce IL-2 (necessary for T-cell proliferation). Treatment with 17-AAG also provided neuroprotection, as determined by silver staining for axonal damage. Taken together, treatment with 17-AAG appeared to exert numerous protective effects in the MOG EAE model, including direct anti-inflammatory effects on brain glial cells, inhibition of T-cell activation, and possibility direct neuroprotective effects, suggesting that similar treatments could be of value in treating MS patients.

HSR AND NEUROPROTECTION

There is existing evidence that the HSR can provide neuroprotection against a variety of stimuli and in a variety of neurological conditions, with most studies describing protection in stroke (Sharp et al. 1999; Yenari 2002; Giffard

and Yenari 2004). Evidence that Hsp90 inhibitors can provide neuroprotection come from studies that show geldanamycin inhibits glutamate-induced toxicity in mouse hippocampal cells (Xiao et al. 1999) and blocks caspase activation in stressed neurons (Lee et al. 2001). Upregulation of Hsp90 (and Hsp70) reduced motoneuron degeneration following peripheral nerve axotomy in neonatal rats (Kalmar et al. 2002) and Hsp90 promoted neurite outgrowth of dissociated neurons (Ishimoto et al. 1998). As described above, neuronal pathology, including axonal transection, occurs in MS and EAE, and may be due to a combination of TNFα cytotoxicity, perforin-mediated damage, free radicals, matrix metalloproteinases, or NO production (Coleman and Perry 2002; Bjartmar et al. 2003), and more recently axonal damage in EAE was reported to be due in part to excitotoxic effects of glutamate since AMPA/kainate receptor antagonists reduce EAE disease (Werner et al. 2000; Gilgun-Sherki et al. 2003). Induction of a HSR may therefore provide neuroprotection in EAE by reducing glutamate induced excitotoxicity (Rordorf et al. 1991; Snider and Choi 1996). Recently, neural damage in EAE was shown to involve activation of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), since blocking antibodies to TRAIL reduced clinical signs of EAE (Song et al. 2000) and since TRAIL deficient T-cells were less able to induce symptoms (Aktas et al. 2005). Since the HSR can block TRAIL-induced apoptosis in other cell types (Ozoren and El Deiry 2002), this may be another means by which Hsp90 inhibitors could provide neuroprotection in EAE and MS.

CHARACTERIZATION OF HIGH AFFINITY HSP90 INHIBITORS

In hopes to obtain Hsp90 inhibitors with improved bioavailability and reduced cytotoxic effects, several geldanamycin derivatized analogs have been synthesized (Le Brazidec et al. 2004). In addition, structural studies have shown that ansamycins including geldanamycin bind to the amino-terminal nucleotide pocket of Hsp90 and destabilize interactions with Hsp90 client proteins, and several panels of high affinity fully synthetic drugs have been designed based on the crystal structure of the Hsp90:17-AAG complex (Le Brazidec et al. 2004; Biamonte et al. 2006; Chiosis and Tao 2006; Chiosis et al. 2006; Kasibhatla et al. 2007). These newer Hsp90 inhibitors act at lower (nanoMolar) concentrations, have fewer known side effects, and have improved solubility properties compared to first generation ansamycins and thus may be of greater therapeutic value.

In collaboration with the Conforma Therapeutics Corporation (San Diego, CA), we carried out an initial screening of a panel of novel geldanamycin derivatives, hopes to identify drugs which could reduce inflammatory activation of glial cells, suppress T-cell activation, and potentially allow us to distinguish the relative importance of inducing a HSR versus client protein degradation (e.g. loss of AKT) in protective effects in EAE. We screened experimental compounds (ECs) for their effects on IFNγ production from rat splenic T-cells and on LPS-stimulated nitrite production from primary rat astrocytes (Figure 1). Several compounds (EC78, 119, 86, and 82) were observed to be more potent than 17-AAG (EC72) to reduce

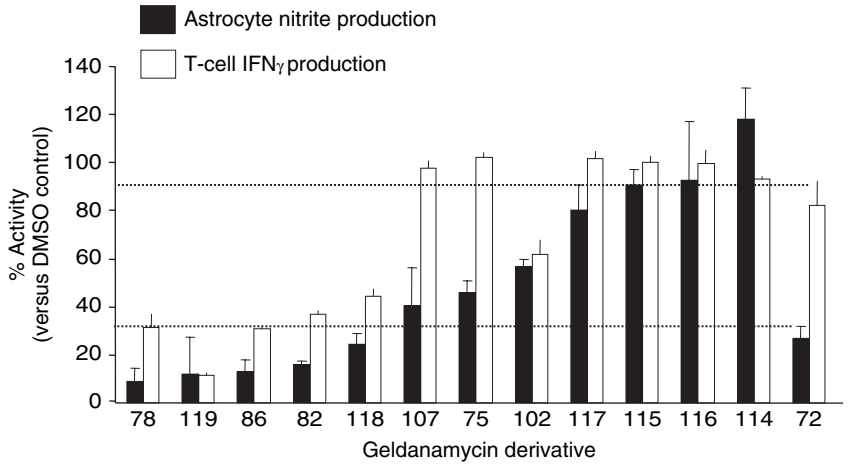

Figure 1. Anti-inflammatory effects of novel geldanamycin derivatives. Splenic rat T-cells were stimulated with Concanavalin A in the presence of 100 nM of the indicated geldanamycin derivative (the experimental identification numbers are given); IFNγ levels were measured by specific ELISA after 48 hr. Primary rat astrocytes were stimulated with a combination of IFNγ plus LPS to induce NOS2 expression in the presence of 100 nM drug, and nitrite levels were measured after 24 hr. The data is mean ± se of $n=3$ samples in each group and shown as the % response compared to vehicle (DMSO) controls

nitrite and IFNγ production. Moreover at the dose tested (100 nM), two drugs (EC107, 75) showed little effect on IFNγ production but >50% inhibition of nitrite production. Further studies with these selected drugs (those with highest potency and with differential effects on glial cells versus T-cells) are ongoing; as are studies using purine-derivatives which can selectively induce a HSR versus inducing client protein degradation.

CONCLUSIONS

Although the induction of a HSR and of HSPs is well known to provide neuroprotection, much less attention has been placed on the fact that induction of a HSR can have direct anti-inflammatory actions. Studies from our group and others demonstrate that inducing a HSR (either chemically or by use of Hsp90 inhibitors) can reduce a variety of pro-inflammatory molecules (including the NOS2 enzyme), and can suppress T-cell activation. The more recent findings that the Hsp90 inhibitors may be working via induction of a HSR as well as by inducing degradation of client proteins known to be involved in T-cell signaling or glial cell activation suggest that these drugs could have important effects on the progression of autoimmune diseases such as MS. In view of the fact that 17-AAG is already in phase II/III clinical trials, initial studies in MS seem feasible.

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