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Targeted heat shock protein 72 (HSP72) for pulmonary cytoprotection

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Abstract

Heat shock protein 72 (HSP72) is perhaps the most important member of the HSP70 family of proteins, given that it is induced in a wide variety of tissues and cells to combat stress, particularly oxidative stress. Here we review independent observations of the critical role this protein plays as a pulmonary cytoprotectant and discuss the merits of developing HSP72 as a therapeutic for rapid delivery to cells and tissues after a traumatic event. We also discuss the fusion of HSP72 to a cell-penetrating single-chain Fv (scFv) antibody fragment derived from mAb 3E10, referred to as Fv-HSP70. This fusion construct has been validated *in vivo* in a cerebral infarction model and is currently in testing as a clinical therapeutic to treat ischemic events and as a fieldable medical countermeasure to treat inhalation of toxicants caused by terrorist actions or industrial accidents.

Keywords

HSP70; HSP72; oxidative stress; apoptosis inhibitor; medical countermeasure; reperfusion therapy

Introduction

The HSP70 family consists of 11 genes encoding a highly conserved group of proteins involved in critical aspects of cellular function, including protein folding processes and apoptosis inhibition.¹ Whereas redundancy exists amongst the HSP70 family members, many have evolved unique characteristics. HSP72 and HSP73 have nearly identical molecular weights, yet *HSP72* is an intron-less gene that is induced upon receiving a stress signal; in comparison, *HSP73* has introns and is constitutively expressed.² Some HSP70 members have specific subcellular locations: BiP, also known as glucose regulated protein 78 (GRP78), is responsible for protein folding in the endoplasmic reticulum;³ in contrast, GRP75 localizes in the mitochondria.^{4,5}

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Conflicts of interest

The authors declare they are employees of Rubicon Biotechnology, which is developing the Fv-HSP70 technology. Two of them (M.H.P. and R.A.R.) are also owners of Rubicon. All of the articles concerning Fv-HSP70 discussed in this review were published before the authors' and Rubicon's involvement with development of the Fv-HSP70 technology, except for Ref. 59. The pulmonary data discussed in the review was obtained either at Rubicon or in the laboratory of our collaborators under NIH Grant R21ES024028.

This review focuses on perhaps the most interesting member of this family, the inducible isoform commonly referred to as HSP70i or HSP72 in the scientific literature.² The first gene for HSP72 was isolated in 1985,⁶ and further characterization determined that HSP72 is transcribed by two genes (*HSPA1A* and *HSPA1B*) 11 kb apart.^{7,8} Although a harmonizing nomenclature has been proposed for the HSP70 family, the use of original names is still quite prevalent (for a useful table correlating each HSP70 gene to the various historic names used for each protein see Table 1 in Ref. 2). Many of the publications referenced herein use the term HSP70 generically, although careful examination of the papers reveals that they are specifically working with HSP72. In this report, the term HSP70 will be used when referring to the entire protein family and HSP72 will be used when referring to the specific member of this family that has great potential as a therapeutic agent.

What does HSP72 do in the Cell?

Like all HSP70s, HSP72 helps prevent protein misfolding within the cellular milieu by binding and refolding those proteins improperly assembled or in the process of denaturing owing to cell stress.¹ HSP72 also inhibits at least three different pathways leading to apoptosis: (1) the Apaf-1 apoptosome,⁹ (2) AIF,¹⁰ and (3) NF- κ B¹¹ (Fig. 1). Because HSP72 is induced in response to a variety of stresses (e.g., heat shock, oxidative stress), it has been considered as a therapeutic agent.¹² HSP72 is not only triggered by reactive oxygen species (ROS); it also helps prevent further generation of oxidative stress. Recent *in vivo* work in rats using a kainic acid seizure model to elucidate HSP72's mechanism of action inhibiting neuronal death has uncovered an interaction with the NF- κ B transcription factor. HSP72 blocks NF- κ B from upregulating nitric oxide synthase (NOS) II (iNOS) and thus triggering a cell death cascade that includes the generation of ROS and peroxynitrite and the activation of caspases (Fig. 2).¹¹ The broad applicability of this protein as a general cytoprotectant is demonstrated by its identification as a key regulator of apoptosis in diverse areas of medical research.

HSP72 as a pulmonary cytoprotectant

Whereas oxygen is critical to human survival, prolonged exposure to elevated (i.e., > 21%) oxygen concentrations can lead to inflammation, edema, and vascular leakage in the lungs.¹³ Damage occurs to the lung endothelial cells, leading to barrier disruption between the lung surface and the vasculature.¹⁴ In studies investigating the connection between hyperoxia and lung barrier disruptions, researchers demonstrated increased HSP72 protein expression in pulmonary artery endothelial cells exposed to 95% oxygen¹⁵ and specific inhibition of the ATP-dependent, caspase-directed apoptosis pathway and the ATP-independent AIF pathway¹⁵ by HSP72. Disruption of HSP72 activity, either with a general heat shock protein inhibitor (KNK437) or specific disruption using siRNA, increased endothelial cell apoptosis as measured by TUNEL assay, caspase-3 activity, and AIF translocation from the cytoplasm into the nucleus. Interestingly, the researchers also found that induction of HSP72 activity during hyperoxia only occurred from one of the two nearly identical genes encoding the protein. The mechanism responsible for the selective *HSPA1B* induction is not clear at this time.

In addition to hyperoxia, HSP72 induction occurs in acute lung injuries. In 2003, the Environmental Protection Agency (EPA) identified 123 chemical plants in the nation where a terrorist attack or accident could potentially expose more than 1 million people to a cloud of toxic gas.¹⁶ This includes high-priority threat agents, such as phosgene, chlorine gas, and sulfur mustard. Preservation of cellular function in the lungs and airways is crucial for survival following exposure to hazardous chemical fumes, either in the workplace or on the battlefield. Providing victims with supplemental oxygen appears to be the only treatment resulting in improved survival, improved arterial oxygenation, and reduced edema in the lungs.^{17,18} However, a major cause of cell death is from oxidative stress, so endogenous HSP72 induction is not sufficiently effective to counter this process. But it is not only industrial accidents or terrorist events that result in acute lung injury: millions of Americans injure their lungs voluntarily either through the excessive use of alcohol¹⁹ or the smoking of tobacco²⁰ or marijuana.²¹ The oxidative stress and damage caused by the induction of ROS in the lungs due to these activities can lead to severe health conditions, such as chronic obstructive pulmonary disease (COPD)²² and cancer.²³

Current strategies to prevent oxidative stress include small molecule antioxidant (e.g. *N*-acetylcysteine) treatment directly to the lungs.²⁴ These reagents lack specificity and may suffer from a double-edged problem. Without targeting specific cells, product potency can be diminished unless excess material is delivered to the lungs. Conversely, excessive material delivered to pulmonary tissues can result in dangerous side effects. So, finding an optimal therapeutic window can often be challenging. An alternative approach is hypobaric hypoxia preconditioning (HHP) of the lungs for up to 2 weeks to induce HSP72.²⁵ HHP has been shown to combat lung damage caused by a simulated high-altitude exposure of 6000 m for 24 h. Unfortunately, such a pretreatment strategy would be impractical to implement in a mass casualty toxicant exposure.

The induction process for HSP72 requires 12 h, although it remains at a level above baseline for up to 72 h after the initial insult to the lungs.²⁶ This long induction period may have evolved in order to deliver a chronic cytoprotectant to the cells following an acute injury in which secondary effects, such as apoptosis, can occur hours later. In the case of hyperoxic exposure of the lungs, HSP72 induction reaches its maximum 24 h after exposure.¹⁵ Hyperoxia causes a biphasic barrier disruption of the endothelial monolayer, with the first phase occurring 1–3 h after exposure and the second phase, largely attributable to apoptosis, occurring at 24 h.¹⁵ Therefore, the timing of HSP72 induction may be well suited to counter the effects of apoptosis in chronic disease conditions; however, this strategy is not well suited for acute conditions that require immediate attention. And, as our next example illustrates, the induction process can be impaired by other factors.

Thinking of sepsis as a pulmonary disease may be unorthodox; however, acute respiratory distress is an end-organ dysfunction in patients suffering from this malady.²⁷ HSP72 induction in septic lungs is not a new observation, having been first reported in 1994.²⁸ Only ~30% of *Hsp72*^{-/-} knockout mice survived up to day 3 in one sepsis induction model; in comparison, ~65% of *Hsp72*^{+/+} wild-type mice survived in the same model.²⁷ Surprisingly, HSP72 induction may be linked to blood glutamine levels: deficiency in blood glutamine levels at the time of admission to an intensive care unit is a strong indicator of mortality.²⁹

One team of investigators studying this phenomenon reported that administration of glutamine can help signal HSP72 induction, in turn combatting NF- κ B activation, inflammation, and general lung injury caused by sepsis.²⁷ Administration of glutamine to *Hsp72*^{-/-} knockout mice failed to reduce any of these symptoms. Unfortunately, although septic conditions cause Hsp72 induction, it is often insufficient and can simultaneously suppress HSP72 expression,³⁰ possibly because glutamine transport into lung cells via plasma membrane vesicles is impaired.³¹

Targeted HSP72 delivery into cells

Rapid intracellular delivery of exogenous HSP72 can protect cells from acute lung injuries detailed above. A simple solution already exists that avoids the lag time inherent in HSP72 induction. Fusion of HSP72 to the scFv fragment of a cell-penetrating antibody, mAb 3E10,³² delivers this cytoprotectant to damaged tissues. This fusion molecule is referred to in the literature as Fv-HSP70 and was found safe when the whole IgG was used in phase I clinical trials for the treatment of lupus.³³ The 3E10 monoclonal antibody targets extracellular DNA and nucleosides.^{34,59} These targets are abundant, stable, and quite accessible during tissue damage, where there is cell necrosis.^{35,36} The 3E10 antibody's unique cell-penetration pathway has been described.^{34,37,59} It binds an antigen that includes the 1'-thymine base and the 2'-deoxyribose sugar moiety of DNA, a determination made by studying inhibition of 3E10 binding to DNA using several competitors.⁶⁰ Double- and single-stranded DNA, in particular strings of poly-deoxythymidine, interfere with 3E10 binding in competitive-inhibition assays. Neither RNA nor oligonucleotides of deoxyadenosine, deoxycytidine, or deoxyguanosine appear to do so.⁶⁰ Binding extracellular DNA allows 3E10 penetration of cellular membranes through a specific nucleoside salvage channel found in most cells, known as the equilibrative nucleoside transporter 2 (ENT2) channel^{34,37,38} (Fig. 1, green channel). Penetration occurs through the plasma membrane³⁸ surrounding the cell and through the nuclear membrane as well.⁶¹ In fact, the intact 3E10 antibody (~ 150 kD) has been shown to penetrate both actively dividing COS-7 cells and non-dividing primary rat cortical neurons *in vitro*.³² Attachment of the antioxidant enzyme catalase to intact 3E10 shepherds the entire antibody–enzyme conjugate across the plasma membrane, where the exogenously supplied catalase protects hydrogen peroxide (H₂O₂)–exposed cells by breaking H₂O₂ down into water and rescuing them from apoptosis caused by oxidative stress.³⁹ Selectivity of this agent *in vivo* is based on the simple concept that tissues undergoing significant cell injury, such as vascular ischemia or pulmonary intoxication, possess a high concentration of extracellular DNA. Salvaging of the DNA by surrounding cells, through the ENT2 channel, provides 3E10 the opportunity to enter those cells still hanging on to life (for an *in vitro* illustration of this phenomenon, see Fig. 1 in Ref. 59).

The Fv–HSP70 treatment concept has been validated *in vivo* in a cerebral infarction model.⁴⁰ A common mechanism of action for apoptosis in neural cells undergoing stress, both in seizure and stroke models, has been established in several studies.^{11,41–44} Like seizures, reperfusion of an occluded cerebral artery leads to oxidative stress, calcium dysfunction, and cell death.⁴⁵ Rats treated with Fv–HSP70 after reperfusion, 2 and 3 h after the onset of ischemia, had significantly improved sensorimotor function at 24 h poststroke

compared to rats treated with a saline control. Furthermore, histological staining of brain tissue using triphenyl tetrazolium chloride (TTC) revealed a 68% reduction of neural cell death (the infarct volume).⁴⁰ Such a result further indicates the blood–brain barrier is sufficiently disrupted under conditions of stress that penetration of the 100-kD Fv–HSP70 is not hindered. However, histology only tells part of the story. Whether Fv–HSP70 provides any neurological benefit to the rats was also investigated using a vibrissae-induced forelimb-placement test to assess sensorimotor function. This test was specifically chosen because both the vibrissae sensory cortex and the forelimb motor cortex lie within the vascular bed of the occluded cerebral artery. Briefly, each rat was held parallel to the edge of a bench and one set of whiskers was brushed against its edge. Movement of the front left forelimb, ipsilateral to the whisker stimulation, was monitored as a response and recorded as successful if the paw was placed on the bench surface with or without pad contact. An inverse correlation between the infarct volume in control and Fv–HSP70–treated rats with the successful placement of impaired forelimbs on the benchtop ($n = 10$ rats/group) was established. Placement was unsuccessful for nearly all trials in the saline treated rats—only 5% successfully placing the impaired left forelimb on the benchtop—whereas $> 30\%$ ($P = 0.038$) of Fv–HSP70–treated rats successfully responded.⁴⁰ Placement scores for the right forelimb were near perfect for both groups.

With the successful demonstration of Fv–HSP70 efficacy in a rat stroke model, we have surmised that administration of this fusion molecule to animals experiencing oxidative stress and apoptosis in the pulmonary tissues may provide similar benefits. Preliminary *in vitro* studies using either human primary alveolar cells or an alveolar carcinoma line (A549) confirmed our suspicions. Cells were exposed to H_2O_2 , a potent ROS generator,⁴⁶ and then treated with Fv–Hsp70 30 min later. H_2O_2 exposure results in increased apoptosis and cell death over the course of 24 h compared to control cells, yet treatment with Fv–HSP70 inhibited cell death (manuscript in preparation). Armed with these results, we chose to demonstrate the rapidity of exogenous HSP72 supplementation using an *in vivo* phosgene intoxication model in (rats). Toxic inhalation results in oxidative stress and tissue necrosis in the lungs; in the case of phosgene, it is lethal.¹⁷ Early indications suggest that the Fv–HSP70 approach is a viable treatment for pulmonary exposure to toxic vapors. In a study with rats exposed to an LD_{50} concentration of phosgene vapor, 60% of animals treated with Fv–HSP70 intravenously 30 min after the exposure survived at 24 h, compared to only 20% of subjects treated with a saline control. Furthermore, those rats treated with Fv–HSP70 and surviving the phosgene challenge exhibited less pulmonary edema and lacked the red mottled appearance indicative of hemorrhaging (manuscript in preparation). Although the intravenous approach helped establish proof of principle, other drug administration modalities are currently being evaluated in the event that Fv–HSP70 must be deployed in a mass casualty scenario.

Targeted Hsp72 delivery versus gene induction

The targeting of biologic cytoprotectants, such as Fv–HSP70, has advantages over small-molecule strategies designed to induce HSP72 synthesis. For a small molecule approach, the need to evaluate the unintended induction of other genes is critical if the drug is going to be used in the clinic. Furthermore, induction of HSP70s is attenuated with aging.^{47,48} If that is

not enough, heat shock protein induction in response to oxidative stress is complicated by the recent finding that, ironically, some ROS can impair HSP72 induction⁴⁹ through RNA interference with microRNAs.⁵⁰

Bimoclomol was considered a promising inducer of HSP72 synthesis and was evaluated in the clinic for diabetic neuropathy. It failed to show significant advantages over a placebo in phase II clinical trials. Bimoclomol only induces HSP72 synthesis in the presence of cell stress⁵¹ and requires a significant lag time to take effect,⁵² limiting its usefulness as a therapeutic agent. Another HSP72 inducer, geranylgeranyl acetone, requires lag times varying from 8 to 24 h in *in vivo* models, depending on the organ being targeted.⁵³ On the basis of studies conducted in cardiac ischemia models, induction of some *HSP70* mRNAs is reported to occur between 2 and 4 hours after reperfusion,⁵⁴ but specific *HSP72* mRNA induction above baseline has not been detected until 3 h after reperfusion.⁵⁵ Unfortunately, HSP72 induction or gene therapy approaches for boosting HSP72 production⁵⁶ are unrealistic when a physician must deal with the immediate trauma occurring to heart and brain tissue in the wake of a heart attack or stroke. Such options that require induction of HSP70 waste valuable time as left ventricular function begins to fail in the heart or as the stroke transitions from an acute phase into the subacute phase with complications of edema and hemorrhagic transformation. Fv–HSP70 is designed to be administered to a patient for immediate delivery of the HSP72 cytoprotectant into the stressed cells.

Fv–HSP70: a unique approach for rapid HSP72 delivery to stressed cells

Compared to HSP72 transport using the 3E10 antibody fragment, other cell-penetrating peptides (CPPs) studied *in vivo* for the treatment of human disease have revealed a highly inflammatory nature, as demonstrated by the TAT protein.⁵⁷ The TAT peptide, derived from the HIV virus, has been shown to upregulate expression of inflammatory signaling molecules and facilitate monocyte invasion into the brain,⁵⁷ as well as to markedly increase cellular oxidative stress,⁵⁸ two physiological phenomena that should not be exacerbated in neural tissue that have incurred some form of trauma. CPPs enter cells through an energy-driven process known as endocytosis, whereas 3E10 enters cells in a diffusion-driven process through the ENT2 channel. Originally classified as a transporter, which would require energy to transport molecules, ENT2 actually is a channel through which molecules can pass based on equilibration. That means energy-deficient, dying cells can still take up 3E10 when endocytosis and transporters cease to function. As an aside, although glutamine is not considered a CPP, as mentioned previously, its effectiveness in inducing HSP72 in dying lung cells is also compromised due to impaired transport via plasma membrane vesicles.³¹ Since CPPs are capable of penetrating any healthy cells using endocytosis, their use as targeting agents is problematic. 3E10 only penetrates cells expressing the ENT2 channel.³⁴ However, the presence of the ENT2 channel is not sufficient. The 3E10 must bind nucleosides from DNA in order to enter the cells;⁵⁹ hence, its specificity is for tissues undergoing significant damage with abundant and easily accessible extracellular DNA.

Several versions of the Fv–HSP70 fusion construct are currently undergoing evaluation as cytoprotectants in pulmonary, cardiovascular, and neural disease models. Given the universal applicability of binding extracellular DNA for entry into viable cells in damaged tissue, Fv–

HSP70 should, theoretically, prevent further cell damage regardless of the event triggering cell stress and apoptosis, be it genetic or environmental. Therefore, rapid intracellular delivery of HSP72 should find broad applicability, both as a clinical therapeutic to treat ischemic events and as a medical countermeasure in the field or hospital to treat toxicant exposures caused by terrorist actions or industrial accidents.

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Figure 1.

HSP72/Fv–HSP70 and its mechanisms of action. Endogenous HSP72 induction within the cell or delivery of exogenous HSP72, via the ENT2 channel in the form of the Fv–HSP70 fusion molecule, results in the same cellular effects. (A) HSP72 can help with the proper folding of new proteins during protein synthesis. (B) During cell stress, HSP72 binds to critical proteins that are misfolding and protects them from aggregation and cellular destruction. (C) Once the crisis has passed, these critical proteins are released by HSP72 in their correct structural forms. (D) HSP72 also helps directly inhibit apoptosis pathways. During apoptosis, mitochondria release cytochrome c (yellow spheres), triggering the construction of a molecular machine known as an apoptosome. Apoptosomes cleave procaspase-9 proteins (purple spheres) into their active caspase-9 form, which go about triggering a cascade of cellular destruction, including other caspases, leading to the death of the cell. (E) HSP72 binds the apoptosome, preventing conversion of pro-caspase-9 into its active caspase-9 form.⁹ (F) During apoptosis, mitochondria may also release apoptosis inducing factor (AIF). Sufficient AIF in the cell cytoplasm can also trigger apoptosis. Unlike the apoptosome, this apoptotic process triggered by AIF does not require ATP, therefore it occurs even under low-energy conditions. (G) HSP72 binds AIF, inhibiting this ATP- and caspase-independent apoptotic pathway.¹⁰ (H) Recent studies have now identified another cell death pathway (NF-κB) that HSP72 interacts with in neural cells.¹¹

Figure 2.

HSP72 inhibits the NF-κB apoptosis pathway. (A) Transcription factor NF-κB is activated and triggers a cell death cascade upon phosphorylation of IκB by its kinase, IκK, under conditions of neural cell stress. (B) Immunoprecipitation data indicates that HSP70 blocks IκK's access to IκB, preventing translocation of NF-κB to the nucleus.⁴³ HSP72 knock-down experiments using antisense RNA increased IκB phosphorylation and increased NF-κB activation in CA3 hippocampal neurons and glial cells, leading to increased CA3 cell death after experimental status epilepticus.¹¹