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Modification of Hsp90 by 4-Hydroxynonenal in a Rat Model of Chronic Alcoholic Liver Disease

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Abbreviations: ALT, alanine aminotransferase; ALD, alcoholic liver disease; 4-HNE, 4-hydroxy-2-nonenal; Hsp70, heat shock protein 70; Hsp90, heat shock protein 90; NEM, N-ethylmaleimide; 4-ONE, 4-oxo-2-nonenal; RRL, rabbit reticulocyte lysate

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ABSTRACT

Lipid peroxidation during oxidative stress leads to increased concentrations of thiol-reactive α, β-unsaturated aldehyde including 4-hydroxy-2-nonenal (4-HNE) and 4-oxo-2-nonenal (4-ONE). These aldehydes have a documented ability to disrupt protein function following adduct formation with specific residues. Therefore, to identify 4-HNE-modified proteins in a model of ethanol-induced oxidative stress, a proteomic approach was applied to liver fractions prepared from rats fed a combination high-fat/ethanol diet. The results revealed that essential heat shock protein 90 (Hsp90) was consistently adducted by 4-HNE in the alcohol-treated animals. In vitro chaperoning experiments using firefly luciferase as a client protein were then performed to assess the functional effect of 4-HNE modification on purified recombinant human Hsp90, modified with concentrations of this aldehyde ranging from 23 to 450 μM. Modification of Hsp90 with 4ONE also led to significant inhibition of the chaperone. Because 4-HNE and 4-ONE react selectively with Cys, a thiol-specific mechanism of inhibition was suggested by these data. Therefore, thiol sensitivity was confirmed following treatment of Hsp90 with the specific thiol modifier N-ethylmaleimide (NEM), which resulted in over 99% inactivation of the chaperone by concentrations as low as 6 μM (1:1 molar ratio). Finally, tryptic digest of 4-HNE-modified Hsp90 followed by LC-MS/MS peptide analysis identified Cys 572 as a site for 4-HNE modification. The results presented here thus establish that Hsp90 is consistently modified by 4-HNE in a rat model of alcohol-induced oxidative stress, and that the chaperoning activity of this protein is subject to dysregulation through thiol modification.

INTRODUCTION

The Hsp90 family of heat shock proteins accounts for nearly 2% of total protein in most unstressed cells (Lai et al., 1984), and is involved in essential physiological processes including protein trafficking and signal transduction (Bell and Poland, 2000), protein degradation (Goasduff and Cederbaum, 2000) and regulation and stabilization of a wide range of client proteins (Wegele et al., 2004). In humans, Hsp90 exists in either the β or the more prominent α isoform, which share approximately 85% homology (Hickey et al., 1989). Each isoform consists of an ATPase domain (N-terminal), middle domain, and dimerization domain (C-terminal) (Wegele et al., 2004). Although Hsp90 typically exists as a homodimer, experiments in defined systems have documented a chaperoning activity by self-oligomerization of Hsp90 and client protein binding in a thermally denaturing environment, thus maintaining the substrate protein in a folding-competent form (Yonehara et al., 1996). Refolding of the substrate is accomplished upon the addition of Hsp70 along with other essential chaperones and co-chaperones such as Hsp60, Cpn10, and Hsp organizing protein (Johnson et al., 1998).

In yeast expressing non-functional Hsp90, it was shown that absence of this chaperone was lethal (Borkovich et al., 1989). This observation has led to the emergence of Hsp90 as a chemotherapeutic target for the treatment of multiple carcinomas including breast cancer (Beliakoff and Whitesell, 2004), prostate cancer (Solit et al., 2003), and acute myelogenous leukemia (George et al., 2004). However, while Hsp90 may be a lucrative target for the treatment of cancer, inhibition of Hsp90 in non-neoplastic disorders, such as alcoholic liver disease (ALD), would likely impact cell survival, further complicating the disease.

Oxidative stress, such as that documented to occur during ethanol metabolism, is typically accompanied by the production of electrophilic aldehydes, which are formed following the peroxidation of lipid membranes (Niemela, 1999). Of these compounds, 4-HNE (Fig. 1A) has been widely studied, and the ability of this aldehyde to disrupt cellular processes through 1, 4 Michael addition with Cys, His, and Lys residues, as well as Schiff base formation with primary amines has been reported (Esterbauer et al., 1991). In addition to 4-HNE, the aldehyde 4-ONE is emerging as an especially reactive electrophile (Doorn and Petersen, 2002), likely due to the ketone group (Fig. 1B) which allows nucleophilic attack at both the second and third carbons, as opposed to 4-HNE which can undergo nucleophilic attack only at the third carbon.

Lipid aldehydes such as 4-HNE are recognized elements of oxidative tissue injury (Esterbauer et al., 1989), with a well-established ability to disrupt protein function (Uchida and Stadtman, 1993; Luckey et al., 1999; Alderton et al., 2003). Because of their pathogenic potential, these aldehydes were recently the focus of a proteomic study (Carbone et al., 2004) in which several crucial proteins were found to be modified by 4-HNE in a rat model of early-stage alcoholic liver disease (ALD). The current study represents an extension of the earlier report, and documents consistent modification of Hsp90 by 4-HNE in the animals receiving an ethanol-containing diet. Because Hsp90 has an established role in maintaining cell homeostasis (Wegele et al., 2004), disruption of this molecular chaperone would impact multiple cell housekeeping pathways, and would likely be detrimental to cell survival. Further experimentation was therefore conducted to test the hypothesis that lipid aldehyde modification of Hsp90 results in decreased chaperoning efficiency. Specifically, in a system using purified recombinant Hsp90 with

heat denatured firefly luciferase as a client protein, inhibition of Hsp90-mediated chaperoning by the Cys-reactive aldehydes 4-HNE and 4-ONE was established. Given the importance of Hsp90 in cellular homeostasis, the results presented here suggest that modification of this protein by lipid aldehydes produced by oxidative stress associated with ALD, may contribute to progression of the disease. Together, this and the previous report (Carbone et al., 2004) provide further evidence that modification of intracellular proteins by lipid peroxidation products, such as 4-HNE and 4-ONE, may contribute to disease progression.

Materials and Methods

Reagents. Unless stated otherwise, all reagents were purchased from Sigma-Aldrich Chemical Co. (Saint Louis, MO). Untreated RRL was purchased through Promega Corporation (Madison, WI). 4-HNE and 4-ONE were synthesized according to procedures described previously, and purity and concentration were confirmed by TLC, UV/Vis spectrophotometry, and LC-MS (Doorn and Petersen, 2002; Mitchell and Petersen, 1991). Recombinant firefly luciferase and a luciferase assay system were purchased through Promega Corp. (Madison, WI). Human recombinant Hsp90 was purchased from StressGen Biotechnologies (Victoria BC, Canada).

Animals. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Colorado, and were performed in accordance with published National Institutes of Health guidelines. Male Harlan Sprague-Dawley rats were fed a modified Lieber-Decarli documented previously to initiate liver injury consistent with the early stages of ALD (Carbone et al., 2004). Upon

completion of the feeding protocol, plasma alanine aminotransferase (ALT) determination, tissue harvesting, sectioning, and staining, and subcellular fractionation were performed as described in a previous report (Carbone et al. 2004; Little and Petersen, 1983). Assessment of liver histology was performed by fixing whole liver tissue in 10%(v/v) sodium phosphate buffered formalin, pH 7.4. Fat, accumulation and necrosis were estimate in each Hemotoxylin and Eosin-stained section by evaluation of four randomly chosen fields. Fat was assessed by estimating the percentage of cells in each field containing micro- or macrovesicular fat with higher scores indicating a higher percentage of cells containing fat: 0 (absent); 1+ (1-25%); 2+ (25-50%); 3+ (50-75%); 4+ (100%). Necrosis was scored as: 0 (absent); 0.5+ (rare); 1+ (scattered necrotic cells); 2+ (small foci of >10 necrotic cells). Total pathology scores were determined by summing the scores for steatosis and necrosis. The extent of oxidative injury was also evaluated through immunohistochemical detection of aldehyde-protein adducts in tissue sections from the harvested livers (Sampey et al., 2003), using antibodies shown previously to specifically detect 4-HNE sulfhydryl adducts (Hartley et. al., 1999).

Two-Dimensional Electrophoresis and In-Gel Digest. Two-dimensional electrophoretic separation of proteins from rat liver mitochondrial fractions, immunodetection of 4-HNE adducts, and spot harvesting were performed as described previously (Carbone et al., 2004; Shevchenko et al., 1996).

In Vitro Hsp90 Modification by 4-HNE, 4-ONE, or NEM, and Tryptic Digest. Because the concentration of purified, recombinant Hsp90 varied between lots, pretreatment of the chaperone was performed as a function of molar ratio to preserve, as well as possible, the consistency of the extent to which the protein was modified. Using

this approach, human recombinant Hsp90 was incubated in the presence of aldehyde ranging from 0 to approximately 500 μM (5:1, 10:1, or 100:1 aldehyde/protein molar ratio) in 50 mM sodium phosphate (pH 7.4) overnight (approximately 16 hours) at 37°C. Treatment with concentrations of NEM ranging from 6 to 60 μM, corresponding to molar ratios ranging from 1:1 to 10:1, were performed similarly. Free aldehyde or NEM was removed prior to the addition of the client protein (luciferase) using Pierce Protein Desalting Columns (Rockford, IL), ensuring no carry-over of the thiol modifiers to the protection and refolding reactions. This step was taken to prevent inhibition of luciferase or other components of the refolding system by the thiol modifiers. For tryptic digest, following the aldehyde pretreatment (500 μM 4-HNE) Hsp90 was heat denatured at 100°C for 5 minutes in the presence of 2 mM β-mercaptoethanol and cooled on ice. The chaperone was then digested overnight at 37°C in 10% (v/v) ACN, 50 mM ammonium bicarbonate, and 0.3 μg trypsin.

Mass Spectral Analysis. Peptides (8 μl) from each in-gel digest, or purified Hsp90 which had previously been treated with 4-HNE and subject to trypsin digest, were analyzed by liquid chromatography, tandem mass spectrometry (LC-MS/MS) as described previously (Carbone et al., 2004). Following in-gel digest, peptides within the mass range of 500 to 1500 Da were subject to MS/MS analysis, and MS/MS ion search was performed on deconvoluted spectra using MASCOT for protein identification (Perkins et al., 1999). Peptides from tryptic digest of Hsp90 modified by 4-HNE *in vitro* were identified based on a mass shift of the parent peptide equal to that of 4-HNE (156 D). Identity of the aldehyde-modified peptide and location of the adduct were determined

via MS/MS analysis. Fragment ions were calculated using the MS-Product feature of Protein Prospector version 4.0.5.

Western Blotting. Immunodetection of 4-HNE adducts following electrophoresis and transfer to PVDF membrane was performed according to procedures published previously (Carbone, et al., 2004). Immunodetection of Hsp90 was performed using antibodies purchased from Stressgen Biosciences, and protein was visualized using a GE Biosciences STORM 860 imaging system along with the software package ImageQuant version 5.2.

Luciferase Refolding. Recombinant firefly luciferase was diluted to a concentration of 100 nM in 25 mM Tricine, pH 7.8, 8 mM magnesium sulfate, 10% (v/v) glycerol, and 10 mg/ml bovine serum albumin (BSA), and was stored in aliquots at −80°C (Raynes and Guerriero, 1998). Hsp90-mediated chaperoning was measured using an adaptation of procedures published elsewhere (Minami et al., 2001). Briefly, Luciferase (20 nM) was denatured in the presence of control (i.e. untreated) or 4-HNE treated Hsp90 by heating the enzyme to 50°C for 5 minutes. Luciferase refolding was performed by adding 10 µl of the Luciferase/Hsp90 mixture to a solution consisting of 50% RRL, 30 mM HEPES (pH 7.0), 50 mM potassium chloride, 2 mM dithiothrietol, 5 mM magnesium chloride, and 1 mM ATP in a final volume of 65 µl. Refolding was allowed to proceed for up to 40 min, at which time 5 µl aliquots were removed from the refolding buffer and added to 100 µl luciferase assay reagent (Promega) in borosilicate glass tubes. Luminescence was immediately read using a Los Alamos Diagnostics 535 luminometer.

Statistical Methods. Statistical analysis was performed using the software package GraphPad Prizm version 3.02 (GraphPad Software, San Diego, CA). Plasma ALT, body-

to-weight ratio, and body weight were compared using a two-tailed t-test. Representative figures demonstrating luciferase refolding were sub-sampled no less than three times, and compared by one-way ANOVA with Bonferroni post-test. These representative figures demonstrate a consistent trend observed in no less than three independent experiments. In all cases, mean differences between a treated and the control (untreated) group is indicated (*, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$).

Results

The extent of oxidative injury induced by the ethanol-containing diet was assessed through the appearance of adduct formation between hepatic proteins and the lipid peroxidation product 4-HNE. Figure 2, panels A-D are representative of the immunohistochemical detection of 4-HNE protein modification in liver tissue sections harvested from control (Panels A, C) and ethanol-fed rats (Panels B, D). The immunopositive staining in Figure 2 B, D clearly demonstrates extensive protein modification by this aldehyde in the ethanol-fed animals as compared to controls. Additionally, protein modification by 4-HNE within the hepatocytes appears to be pan-lobule, as immunoreactive staining is observed in zones 1-3 between the hepatic triad (T), and the central vein (CV). Localization of 4-HNE adduction was observed under higher magnification (40x), in which lipid accumulation was detected in proximity to the antibody staining. Intense immunoreactivity was especially clear in the areas surrounding several larger lipid droplets, and is indicated by the arrows in Figure 2D. The severe antibody staining observed in liver tissue sections harvested from rats fed the ethanol-containing diet thus demonstrate a marked increase in lipid peroxidation.

Indicators of liver injury evaluated in this study include elevated levels of plasma ALT, increased liver-to-body weight ratio, and decreased body weight. These parameters were measured in animals from both ethanol-fed and control groups, and the data are presented in Table 1. As compared to isocaloric controls, the body weights of ethanol-treated animals were approximately 10% less at the end of the study. The data also reveal a significant elevation in liver-to-body weight ratio (1.3-fold), and plasma ALT (4.0-fold). Microscopically, liver injury in animals receiving the ethanol diet was confirmed through hemotoxylin and eosin staining of tissue sections taken from harvested livers (Figure 2 E, F). Specifically, the results shown in these panels indicate the presence of micro, and to a lesser extent macrosteatosis in the livers of animals receiving the ethanol diet, while significant necrosis and the appearance of inflammatory infiltrate were notably absent. These data are summarized in Table 2, demonstrating significant pathology in the livers of animals which had received the ethanol-containing diet. Together, these findings of micro- and macrosteatosis are consistent with the hepatocellular changes taking place during the early stages of alcohol-induced liver injury (Diehl, 2001; Tsukamoto and Lu, 2001). The data presented by Tables 1 and 2 thus demonstrate elevated tissue damage in the livers isolated from rats receiving the ethanol-containing diet accompanied by an elevation in protein modification by the lipid peroxidation product 4-HNE (Figure 2), demonstrating increased lipid peroxidation.

It is well established that aldehyde modification can dysregulate protein function (Esterbauer et al., 1991). A proteome-wide scan was thus used to locate and identify proteins modified by 4-HNE. Two-dimensional electrophoresis of proteins isolated from rat livers followed by immunoblot against 4-HNE-modified proteins has revealed an

array of consistently modified targets. The aldehyde-modified proteins thus located were identified using in-gel tryptic digest and LC-MS/MS ion search. Figure 3 A-B presents typical two-dimensional electrophoresis and immunodetection of 4-HNE protein adducts in control (Figure 3A) or ethanol-fed animals (Figure 3B), confirming the presence of more severe aldehydic protein modification in animals fed the ethanol-containing diet. Additionally, the location of the chaperone Hsp90 is indicated in Figure 3 B (arrow), however, immunoreactivity of this protein is noticeably absent in protein harvested from animals receiving the control diet. Although several co-migrating proteins were also identified along with Hsp90 (Figure 3B; Table 3), these proteins were not assigned a MASCOT score deemed significant, nor was their location consistent with the actual mass and isoelectric point of the spot (Table 3). Both mass and isoelectric point are, however, consistent with that which would be expected of Hsp90. Together, Figure 3 B and Table 3 suggest that Hsp90 is indeed the prominent protein in the spot harvested from the gel.

Finally, a previous report by Carbone et al. (2004) demonstrated a slight induction of Hsp72 in the same animals used for the present study. Therefore, to ensure that the elevated immunoreactivity observed in Figure 3 B is not simply the result of Hsp90 induction, hepatocellular concentrations of this chaperone were compared via western blot between animals receiving the control diet (Figure 3 C, Lanes 2-5), and their respective ethanol-fed pairs (Figure 3 C, Lanes 6-9), demonstrating no detectable induction of the protein. A recombinant control was loaded in Lane 10, which served as a positional control. Therefore, Figure 3 indicates that the enhanced immunoreactivity by Hsp90 likely represents aldehydic modification of the protein by 4-HNE, which is

consistent with the increased immundetection in tissue sections observed in Figure 2 B-D.

It has been documented previously (Carbone et al., 2004) that modification of the inducible heat shock protein Hsp72 by thiol-reactive aldehydes such as 4-HNE and 4-ONE results in inactivation of this chaperone. Because Hsp90 plays an important role in many cell processes, a similar approach was taken to assess the effect of 4-HNE on Hsp90-mediated chaperoning of thermally-denatured luciferase. Specifically, previous reports have demonstrated the ability of Hsp90 to maintain recombinant firefly luciferase in a folding-competent form throughout co-incubation under thermally denaturing conditions (Minami et al., 2001). When cooled and placed in a system such as the RRL used here, which contains Hsp/Hsc70 and the other required co-chaperones, such as the, the Hsp90-protected luciferase is refolded more efficiently than unprotected luciferase. Following pretreatment of Hsp90 with concentrations of 4-HNE ranging from 23 to 450 μM (5 to 100-fold molar excess aldehyde), a significant and concentration-dependent inhibition of Hsp90-mediated chaperoning was observed, as presented in Figure 4. Finally, 4-HNE has a documented ability to inactivate Hsp72 (Carbone et al., 2004); therefore, free aldehyde was removed prior to co-incubation of 4HNE-adducted Hsp90 with luciferase, thus ensuring that any inhibition of luciferase refolding was due to modification of Hsp90, and not the effect of 4HNE on either luciferase or Hsp70-mediated refolding.

4-ONE is a recently discovered lipid peroxidation product, with documented thiol reactivity exceeding that of 4-HNE. The effects of 4-HNE and 4-ONE on Hsp90 chaperoning were therefore measured. The data presented in Figure 4 also summarize a

side-by-side comparison of inhibition following pretreatment of Hsp90 with 45μM (10× molar excess aldehyde) 4-HNE or 4-ONE, resulting in a similar extent of protein inactivation. Interestingly, the slight difference in the extent of inhibition by 4-ONE was deemed to be significantly different ($p < 0.05$) from that following pretreatment of the chaperone with an identical (45 μM or 10× molar excess) concentration of 4-HNE, and is likely the result of increased thiol reactivity by 4-ONE (Doorn and Petersen, 2002).

The increased extent of Hsp90 inactivation by 4-ONE thus suggests inhibition of this chaperone as a function of Cys modification. This notion was subsequently confirmed through pretreatment of Hsp90 with the Cys modifier NEM (Figure 5). Specifically, the data in Figure 5 demonstrate that treatment of Hsp90 with concentrations of NEM as low as 6 μM, representing a 1:1 molar ratio between the thiol modifier and the chaperone, almost completely abolished the activity of the Hsp. Higher concentrations were similarly effective at inhibiting Hsp90 chaperoning activity. Again, as with experiments performed with 4-HNE or 4-ONE, excess NEM was removed prior to co-incubation with the client protein (luciferase), ensuring that the observed effect was not due to NEM interference with luciferase or the RRL.

The nearly complete inactivation of Hsp90 chaperoning activity by a 1:1 molar ratio between NEM and the Hsp suggests that consistent modification of a specific Cys residue will lead to inactivation of this chaperone. Indeed, a previous report (Nardai et al., 2000) has suggested a functional role for Cys residues based on the slight reductase activity demonstrated by Hsp90. Therefore, tryptic digest of purified recombinant Hsp90 previously treated with 4-HNE, followed by LC-MS/MS peptide analysis were performed, confirming modification of a peptide corresponding to amino acids 568-573

(FENLCK; 753.4 Da). A shift in *m/z* of the parent peptide equal to the mass of 4-HNE (156 Da) is demonstrated by Figure 6A, and the adduct confirmed through MS/MS of *m/z* 909.4 (Figure 6B), in which neutral loss of the adduct reveals the mass of the parent peptide, or *m/z* 753.4. Finally, the identity of peptide 568-573 (FENLCK) and the site of the 4-HNE adduct were confirmed through the appearance of the following fragment ions: y_4 (477.3), b_4-NH_3 (487.2), b_4 (504.3), a_5-NH_3 (562.2), b_5-NH_3 (590.2), and b_5 (607.3). Although sequence coverage of Hsp90 following tryptic digest was incomplete, and typically resulted in only 40%, modification of Cys 572 was consistently observed. Interestingly, a previous report had predicted that Cys 572 was among the least reactive of the seven Cys residues possessed by Hsp90 (Nardai et al., 2000). However, the possibility exists that other Cys residues were modified by 4-HNE as well, and could not be detected due to loss of the adduct during protein digest and LC-MS/MS procedures, or simply due to the fact that the appropriate peptide could not be identified. Also, despite repeated attempts, due to incomplete digest of Hsp90 the modification of Cys 572 in protein isolated from the ethanol-fed animals could not be confirmed, as the limited number of peptides isolated following in-gel digest of the protein did not included this residue. The spectra in Figure 6, along with the data presented in Figure 4-6, thus suggest a role for this amino acid in the chaperoning functions of client proteins.

Discussion

Oxidative injury is a component of many diseases, including ALD, atherosclerosis, diabetes, and ischemic injuries such as stroke and myocardial infarction. A portion of this injury is thought to be mediated by an array of electrophilic aldehyde

species, which are spontaneously formed following the peroxidation of lipid membranes (Esterbauer et al., 1989). As a logical extension of a previously published proteomic study (Carbone et al., 2004), the present report demonstrates that an essential molecular chaperone, Hsp90, was consistently modified by the lipid peroxidation product 4-HNE in a rat model of alcohol-induced oxidative liver injury. Because aldehydes such as 4-HNE have a well-established ability to modify proteins and consequently disrupt protein function (Uchida and Stadtman, 1993; Luckey et al., 1999; Alderton et al., 2003), and because Hsp90 is critical to cell survival (Borkovich et al., 1989), it is conceivable that dysregulation of this Hsp could initiate or augment progression of an existing disorder such as ALD. Given the prevalence of alcoholism in the United States (Li, 2004), as well as the dismal outcome associated with later (cirrhotic) stages of the ALD (Stinson et al., 2001), characterizing the effect of the lipid peroxidation product 4-HNE on Hsp90 was the immediate focus of present report, with the hope of gaining further insight into the molecular mechanisms behind the ALD.

In the studies described here, Hsp90-assisted firefly luciferase refolding was significantly inhibited in an RRL system following modification of Hsp90 with 45 μM 4-ONE (5:1 molar ratio), as well as concentrations of 4-HNE ranging from 23 to 450 μM (5:1 to 100:1 aldehyde/protein ratio; Fig. 4). It should be noted that these concentrations were chosen strictly due to the molar ratio between the aldehyde and the chaperone, and do not necessarily reflect physiological or pathological concentrations of 4-HNE or 4-ONE. In fact, intracellular concentrations of 4-HNE have been estimated to reach cytosolic concentrations of up to 10 μM (Poli and Schaur, 2000). Unfortunately, due to the fact that 4-ONE has only recently been recognized as a potentially pathogenic

compound, similar estimates regarding intracellular concentrations are lacking. However, although the concentrations used in the experiments presented here exceed estimates for intracellular 4-HNE concentrations, evidence is provided by this report that Hsp90 is indeed modified by 4-HNE under conditions of oxidative stress, and that this modification very likely leads to inactivation of the protein.

A previous report that measured the ability of 4-ONE to modify various amino acids demonstrated higher reactivity of this aldehyde towards Cys than that reported for 4-HNE (Doorn and Petersen, 2002). Additionally, a recent report characterizing the effects of both 4-HNE and 4-ONE on the inducible chaperone Hsp72 demonstrated a thiol-specific mechanism of inactivation (Carbone et al., 2004). The data presented in Figure 4 support the notion that inactivation of Hsp90 is proceeding through a Cys-based mechanism, due to the fact that treatment of the chaperone with 4-ONE resulted in significantly greater inhibition ($p < 0.05$) than that observed by an identical concentration of 4-HNE. Therefore, to test the hypothesis that inactivation of Hsp90 by lipid peroxidation products such as 4-HNE and 4-ONE is a function of Cys modification, the chaperone was treated with the specific thiol modifier NEM. Confirmation of this notion is presented in Figure 5, demonstrating nearly complete (i.e. > 99%) inactivation of Hsp90 following treatment of the protein with concentrations of NEM as low as 6 μM. Because this concentration represented a 1:1 molar ratio between the Cys modifier and Hsp90 in the pretreatment, the data also suggest that modification of a single Cys residue results in inactivation of the chaperone.

Reasons behind the extreme potency of NEM when compared with either 4-HNE or 4-ONE mediated Hsp90 inactivation remain unknown. However, a plausible

explanation is that these two lipid peroxidation products are reactive with amino acids other than Cys. Specifically, both aldehydes, which have the highest reactivity for Cys, are capable of modifying His and Lys, and in the case of 4-ONE, Arg as well (Doorn and Petersen, 2002; Oe et al., 2003). It is therefore possible that these other amino acids may also serve as targets for aldehyde modification. Attempts to test this possibility were unfortunately hindered by the relative resistance of Hsp90 to tryptic digest, which is required for LC-MS/MS peptide analysis and adduct characterization. Despite sequence coverage typically in the range of 40%, a stable Cys modification by 4-HNE was observed, lending credibility to the claim of thiol modification as the mechanism behind Hsp90 inactivation.

If indeed modification of Cys residues is responsible for inactivation of Hsp90 by the lipid peroxidation products 4-HNE and 4-ONE, as is strongly suggested through differential inhibition by 4-HNE and 4-ONE, inhibition by NEM, and mass spectral identification of Cys modification by 4-HNE, then a mechanism distinct from that observed with the classic Hsp90 inhibitor geldanamycin is likely. This rationale stems from the fact that geldanamycin interacts with Hsp90 in the ATPase (N-terminal) domain (Whitesell et al., 1997). However, because Hsp90 lacks Cys residues in this domain (Yamakazi et al., 1989), modification of the ATPase region by thiol-reactive aldehydes such as 4-HNE is unlikely. The formation of 4-HNE adducts are therefore probably occurring in a region other than the ATPase domain of the heat shock protein, leading to a mechanism of inhibition which is probably distinct from that of geldanamycin.

Although the exact mechanism of Hsp90 inhibition by 4-HNE and 4-ONE remains unknown at this time, the data presented here demonstrate susceptibility of this

chaperone to inactivation by lipid peroxidation products through a pathway distinct from classic Hsp90 inhibitors. Furthermore, the consistent appearance of 4-HNE-immunoreactive Hsp90 in the livers of rats chronically ingesting alcohol underscores the importance of characterizing the effect of lipid aldehyde modification on this protein in diseases associated with persistent oxidative stress. Potentially, inactivation of Hsp90 by 4-HNE and other Cys-reactive aldehydes may be a result of impaired substrate or chaperone binding, or ATP hydrolysis. As such, elucidation of these possible pathways is the focus of ongoing research.

Lipid peroxidation occurring under conditions of oxidative stress yields a series of reactive aldehyde species, some of which have received considerable attention regarding the disruption of protein function through modification at critical residues (Esterbauer et al., 1989). However, despite the prevalence and apparent pathogenic potential of these aldehydes, a definite link between lipid peroxidation products and disease progression has remained elusive. Both the study presented here, as well as the previous report characterizing the effects of lipid peroxidation products on Hsp72 function represent an extension of a proteome-wide scan for aldehyde-modified proteins in a rat model of early stage alcoholic liver injury (Carbone et al., 2004). This proteomic study was conducted under the premise of providing further evidence for a link between lipid aldehydes and disease progression. Specifically, it is the general goal of this work to provide evidence that crucial cellular proteins are targets for modification and inactivation by these aldehydes during disease conditions, thus providing a foundation for more complex experiments examining an *in vivo* effect.

As stated earlier, the present study represents a logical extension of a previously reported proteome-wide scan (Carbone et al., 2004) to identify substrates for 4-HNE modification in the livers of rats receiving a combination high-fat/ethanol diet. This diet has been shown here and elsewhere to induce early stage ALD (Diehl, 2001; Tsukamoto and Lu, 2001). Among the proteins modified by this aldehyde is the essential heat shock protein Hsp90, which is involved in crucial cell processes including protein chaperoning, protein degradation, and protein trafficking. The progression of ALD beyond the steatotic (fatty liver) stage includes the appearance of insoluble cytokeratin aggregates, or Mallory bodies, thus suggesting impairment of protein chaperoning or degradation in this disease. Because Hsp90 is involved in these processes, and has been shown to be modified and inactivated by the lipid peroxidation product 4-HNE, the contribution to disease progression through impairment of Hsp90 by lipid peroxidation products is a distinct possibility.

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Footnotes

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Legends for Figures

Fig. 1. Chemical structures for 4-HNE (A) and 4-ONE (B). While both aldehydes may undergo 1, 4 Michael addition at the third carbon, the ketone possessed by 4-ONE also allows this unsaturated aldehyde undergo addition at the second carbon.

Fig. 2. Oxidative stress was measured through immunohistochemical detection of 4-HNE (Panels A-D) protein adducts. Pan-lobule protein modification by this aldehyde, observed in all three zones between the central vein (CV) and hepatic triad (T), was markedly increased in animals fed the ethanol (B; 10×) versus the control diet (A; 10×). Higher magnification (40×) revealed localization of 4-HNE staining to the lipid accumulation in the ethanol fed animals (D; 40×), and is indicated by the arrows surrounding several areas of several larger lipid droplets. Hemotoxylin and eosin staining of liver sections from control (E; 10× magnification) and ethanol-fed (F; 10× magnification) revealed micro, and to a lesser extent macrosteatosis in the livers of ethanol-fed animals, while inflammatory infiltrate was notably absent from both groups.

Fig. 3. Two-dimensional electrophoresis of proteins isolated from the livers of rats receiving the high-fat/ethanol diet, with immunoblot using antibodies against 4-HNE. More severe protein modification is demonstrated following ethanol feeding (B), indicating extensive lipid peroxidation in the livers of these animals. Additionally, the presence of immunoreactive Hsp90 is observed in protein harvested from the ethanol fed animals (B), but is noticeably absent in control animals (A), demonstrating modification

of this protein by 4-HNE. In addition to the MASCOT identification of Hsp90, the mass and isoelectric point of the spot are consistent with Hsp90, supporting the claim that Hsp90 is the predominant protein in this spot. Total hepatocellular Hsp90 content was compared (C) between control animals (Lanes 2-5) and their respective ethanol-fed pairs (Lanes 5-10), demonstrating no detectable induction of Hsp90, further suggesting that the observed increase in immunoreactivity presented in panel B is the effect of more extensive Hsp90 modification, rather than protein induction.

Fig. 4. Inhibition of Hsp90-mediated luciferase chaperoning was observed following pretreatment of the Hsp with concentrations of 4-HNE ranging from 23 to 450 μM, corresponding to molar ratios of 5 to 100-fold molar excess aaldehyde. Sensitivity of Hsp90 to inactivation by 4-HNE is demonstrated by significant inhibition of chaperoning following treatment with aldehyde at a 5:1 aldehyde/protein ratio. The effects of 45 μM 4-ONE (10:1 molar ratio; dashed line) were also tested, resulting in significant inhibition of Hsp90. Statistical significance is indicated where a treated group differs from the untreated control.

Fig. 5. Over 99% inhibition of Hsp90-mediated luciferase protection was observed following treatment of the chaperone with concentrations of the specific thiol modifier NEM as low as 6 μM (1:1 molar ratio), demonstrating sensitivity to thiol modification. Significance is indicated where a treated group differs from the untreated control.

Fig. 6. Mass spectral identification of 4-HNE modification of the peptide FENLCK (residues 568-573), located through a shift in *m/z* of the parent peptide equal to the mass of 4-HNE (156 Da; Panel A). Peptide sequence and the presence of the adduct were confirmed through fragment analysis following MS/MS of *m/z* 909.4,in which neutral loss of the adduct has resulted in regeneration of the parent peptide (Panel B).

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Table 1. Effect of chronic ethanol feeding on body weight, liver-to-body weight ratio, and blood alanine aminotransferase.

Treatment	Body Weight (g)	Liver/Body	ALT (U/L)
Control	430.1 ± 5.311	2.744 ± 0.1049	29.37 ± 3.8
Ethanol	383.9 ± 4.008 *	3.581 ± 0.1645 *	116.05 ± 16.11 *

All values are expressed as mean ± SEM (n=6)

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Table 2. Histopathology Scoring.

	Steatosis			
Treatment	Micro	Macro	Necrosis	Total
Control	1.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	2.1 ± 0.2
Ethanol	4.5 ± 0.6 *	2.9 ± 0.3 *	0.6 ± 0.2	7.8 ± 0.5 *

All values are expressed as mean ± SEM (n=6)

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Table 3. Profiles of proteins co-migrating with Hsp90.

Protein	Score[a]	Peptides Identified[b]	Significant[c]	Mass (kDa)	IEP (pH)
Rat Hsp90	75	12	+	83.3	5.1
Rat IL-5 Precursor	38	5	-	15.2	7.7
Rat MHCII	28	3	-	29	4.7
Rat Keratin	28	3	-	29.1	4.8

a Mowse score determined from peptide mass fingerprinting using MASCOT, as described in Materials and Methods.

b Number of peptides matched.

c Scores greater than 54 are significant ($p < 0.05$; Perkins et al., 1999)

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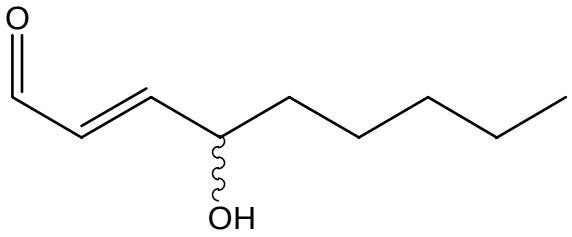
Figure 1

A.



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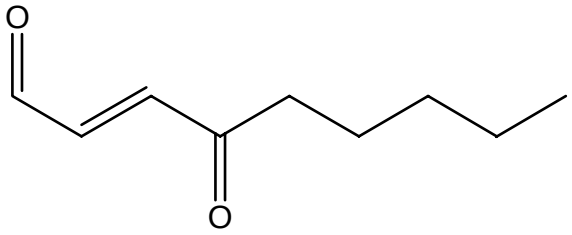

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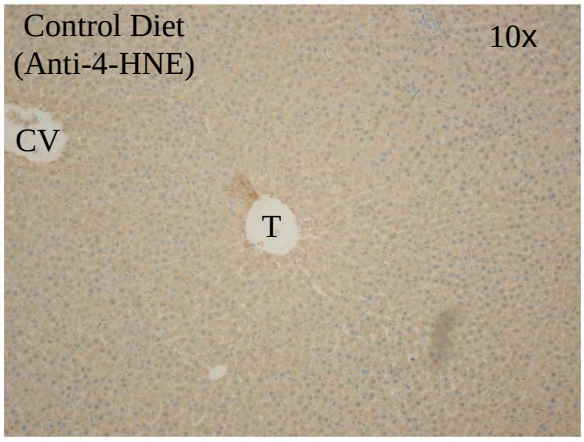
Figure 2



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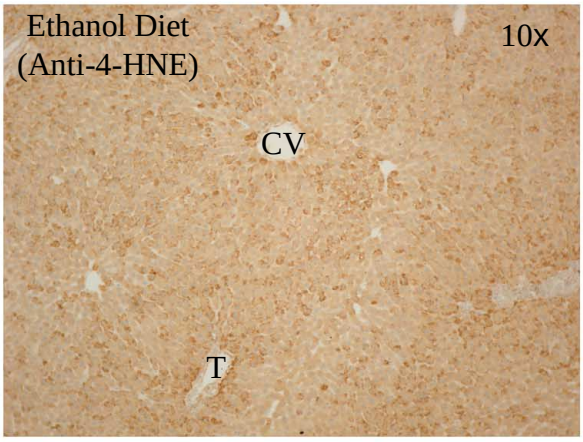

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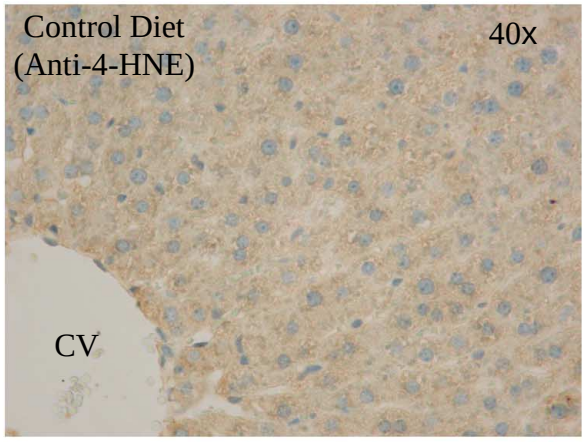

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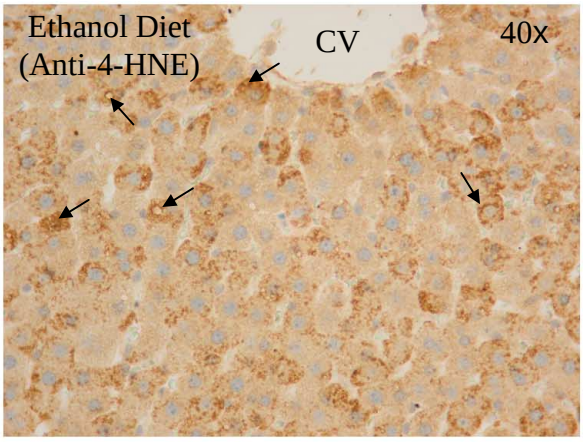

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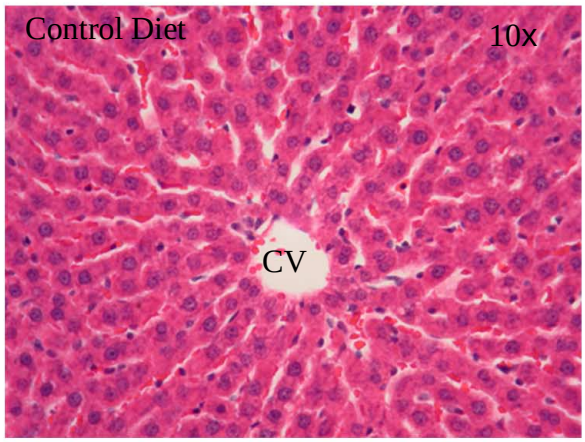

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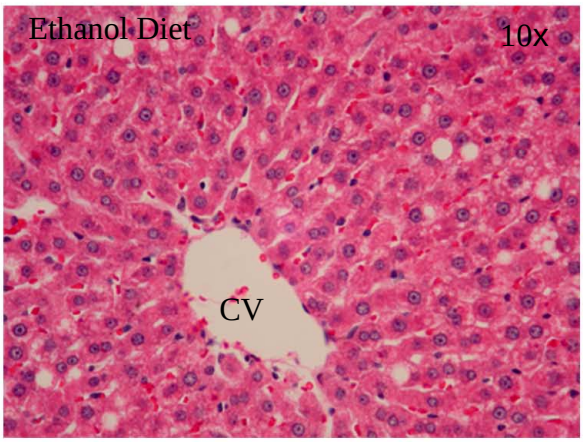

A.

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Figure 3

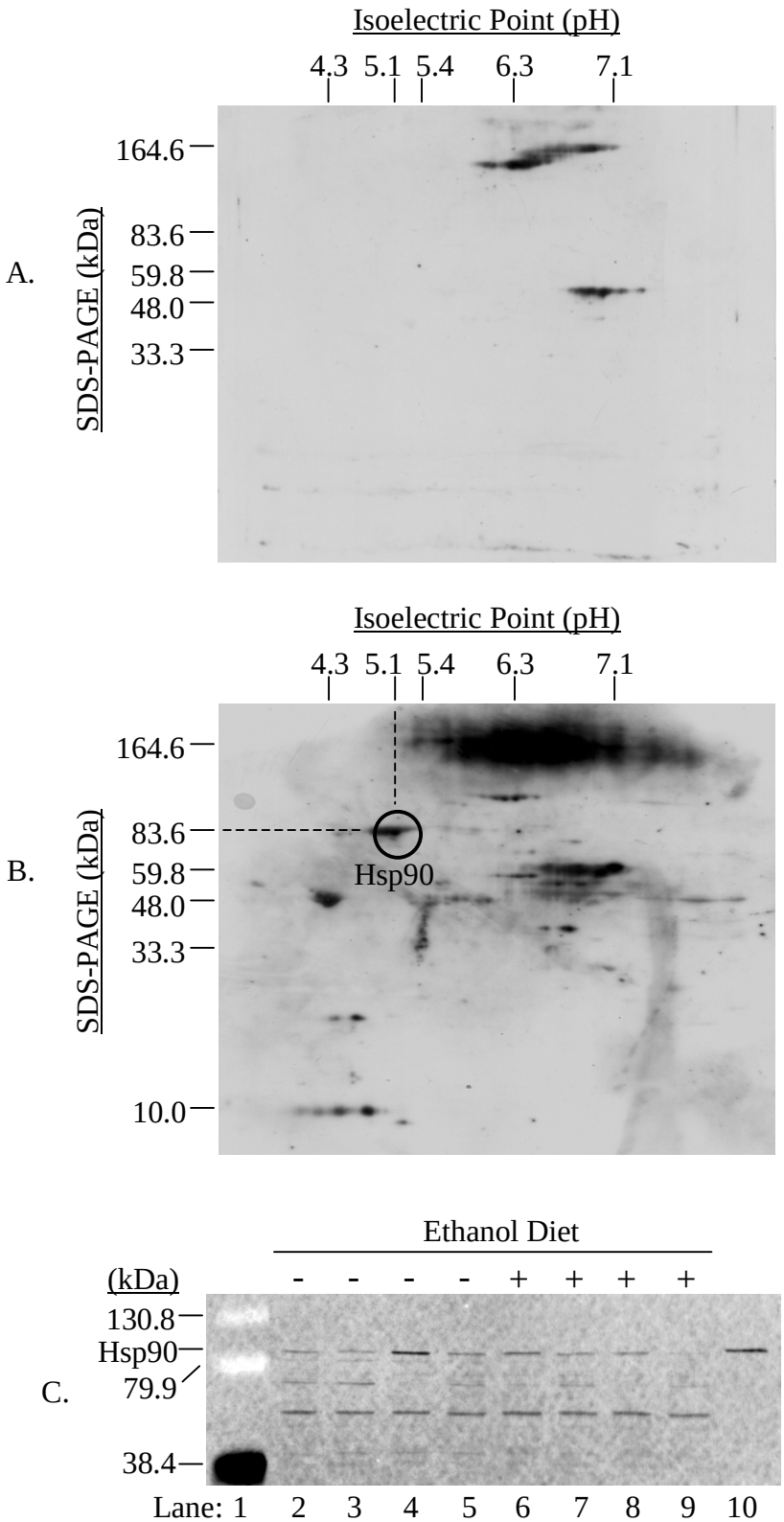

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Figure 4

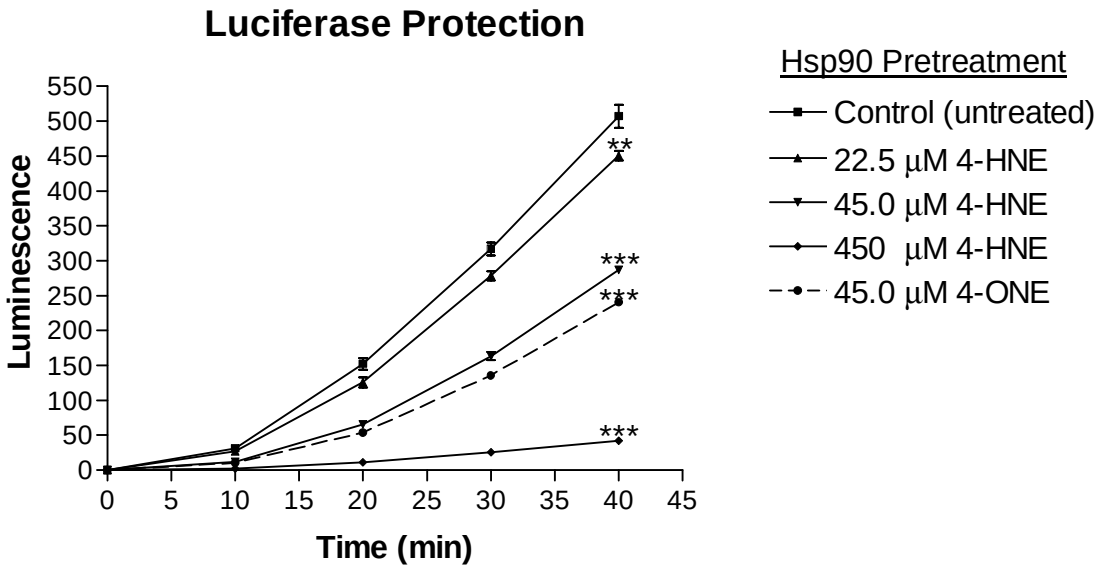

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Figure 5

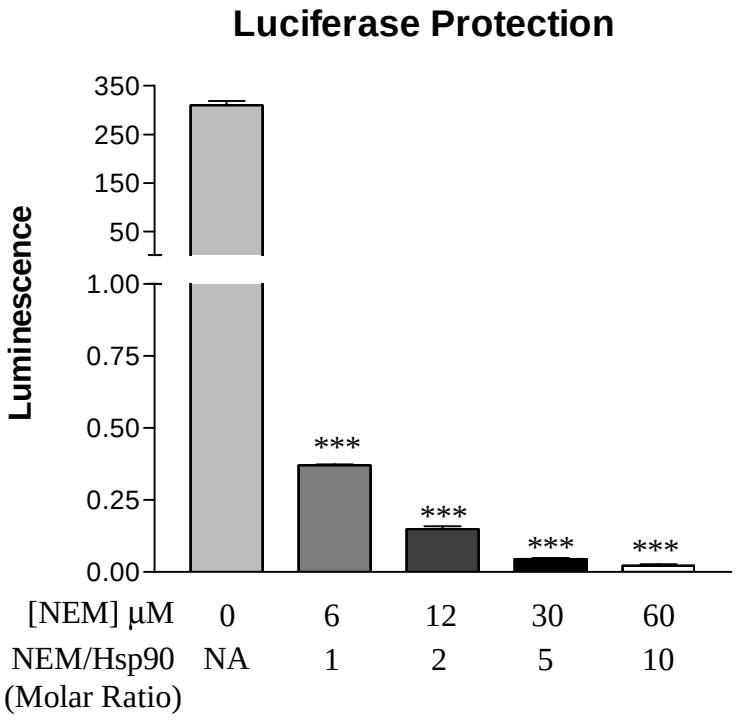

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Figure 6