

Strong Protein Adduct-Trapping Accompanies Abolition of Acrolein-Mediated Hepatotoxicity by Hydralazine in Mice*

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Abstract:

Acrolein is a highly reactive α,β -unsaturated aldehyde that readily alkylates nucleophilic centers in cell macromolecules. Typically, such reactions proceed via Michael addition chemistry, forming adducts that retain an electrophilic carbonyl group. Since these species participate in secondary deleterious reactions, we hypothesize that inactivation of carbonyl adducts may attenuate acrolein toxicity. Indeed, we recently established that the nucleophilic antihypertensive drug hydralazine readily “traps” acrolein adducts in cell proteins and strongly suppresses acrolein-mediated toxicity in isolated hepatocytes. This work sought to determine whether hydralazine prevents the *in vivo* hepatotoxicity of the acrolein precursor allyl alcohol in whole mice, and whether adduct-trapping accompanies any such hepatoprotection. Mice received allyl alcohol alone or in conjunction with several doses of hydralazine. Four hours later, mice were sacrificed to allow determination of liver enzymes in plasma as markers of hepatic injury, while livers were assessed for glutathione and hydralazine-stabilized protein adducts. Hydralazine afforded strong, dose-dependent protection against the increases in plasma marker enzymes but not the hepatic glutathione depletion produced by allyl alcohol. Western blotting revealed intense, dose-dependent adduct-trapping by hydralazine in numerous liver proteins over a broad 26 to 200 kDa mass range. In keeping with these findings, immunohistochemical analysis of liver slices indicated diffuse, extranuclear adduct-trapping by hydralazine that was uniformly distributed across the liver lobule, with partial localization in parenchymal cell membranes. These findings concur with our hypothesis that hydralazine readily inactivates reactive carbonyl-retaining protein adducts formed by acrolein, thereby preventing secondary reactions that trigger cellular death.

Allyl alcohol causes severe periportal necrosis in rodent liver (Piazza, 1915). Its pronounced toxicity is mediated by acrolein, a reactive α,β -unsaturated aldehyde formed via direct oxidation by alcohol dehydrogenase (Rikans, 1987). Consistent with acrolein's key role in the toxic process, alcohol dehydrogenase inhibitors abolish allyl alcohol toxicity in isolated hepatocytes (Ohno et al., 1985).

A dramatic loss of hepatic glutathione (GSH) occurs in the early stages of allyl alcohol intoxication, reflecting the pronounced readiness with which acrolein alkylates the thiol group possessed by GSH (Miccadei et al., 1988). Acrolein also readily modifies cysteine, lysine and histidine residues in proteins, typically via Michael additions involving attack by nucleophilic amino acids on the unsaturated β -carbon (Esterbauer et al., 1991; Uchida et al., 1998). Since these reactions generate carbonyl-retaining adducts, pronounced protein carbonylation accompanies allyl alcohol toxicity (Burcham and Fontaine, 2001). The chemistry underlying carbonylation at lysine is unexpectedly complex, since initial adduction is followed by Michael addition of a second acrolein molecule to form a *bis*-adducted species (Fig. 1) (Uchida et al., 1998). This *bis*-adduct then undergoes cyclization and dehydration to a novel 6-membered heterocyclic carbonyl-retaining species, N^ε-(3-formyl-3,4-dehydropiperidino)lysine (Fig. 1).

At present, the role of such chemistry in the pathogenesis of allyl alcohol hepatotoxicity is ill defined. We hypothesize that since protein modification generates reactive carbonyl-retaining species, these adducts propagate cell injury by participating in deleterious secondary reactions. For example, the strong cross-linking potency of acrolein indicates carbonyl-retaining adducts readily react with neighboring nucleophiles in the same or adjacent macromolecules (Kuykendall and Bogdanffy, 1992; He et al., 1995; Kozekov et al., 2001; Kurtz and Lloyd, 2003). Since capacities for the cellular repair of cross-linked macromolecules are limited, such reaction

products are likely key contributors to the pathogenesis of acrolein toxicity. Consequently, we hypothesize that since reactive carbonyl-retaining adducts are precursors to cross-linked macromolecules, they are logical targets for chemico-pharmacological intervention using nucleophilic, adduct-trapping drugs (Shapiro, 1998).

During a search for nitrogen-containing nucleophiles that could block acrolein toxicity, we identified the vasodilatory antihypertensive hydralazine as a powerful inhibitor of allyl alcohol toxicity in mouse hepatocytes (Burcham et al., 2003a). This property appeared to reflect hydralazine's ability to efficiently scavenge free acrolein in buffered solutions (Burcham et al., 2003a). Such actions are consistent with the chemical properties of hydralazine, which as a strong nucleophile readily forms hydrazones with several biogenic keto compounds (Reece, 1981). Indeed, we recently isolated two novel hydrazones formed during reactions between acrolein and hydralazine.¹ Figure 1 (panel A) shows the structure of (1*E*)-acrylaldehyde 1-[1-phthalazinyl]-hydrazone, the main isolable product from reactions between free acrolein and hydralazine, as well as that of a minor *Z*-isomer. However, our most recent work suggests that rather than simply scavenging free, unadducted acrolein, hydralazine reacts extensively with acrolein-derived protein adducts (Burcham et al., 2004). Using electrospray ionization-mass spectrometry to identify products formed during reactions of an acrolein-adducted model peptide with hydralazine, we detected hydrazones derived from each of the 3 carbonyl-retaining adducts generated during modification of lysine (Fig. 1, Panel B). Employing rabbit antiserum raised against such hydralazine-stabilized acrolein adducts, we detected intense adduct-trapping in proteins from allyl alcohol-pretreated hepatocytes exposed to low micromolar concentrations of hydralazine (Burcham et al., 2004).

The aim of the present study was to explore the *in vivo* relevance of these findings, firstly establishing whether hydralazine could protect mice against the liver injury and hepatic glutathione depletion caused by an acutely toxic dose of allyl alcohol. Also, antibody-based methods were used to determine whether adduct-trapping accompanied any hepatoprotection afforded by hydralazine against allyl alcohol hepatotoxicity in Swiss mice.

Experimental Procedures:

Animals. Male Swiss mice (aged 4-6 weeks), obtained from Laboratory Animal Services at the University of Adelaide, were used in all experiments. Animals were housed at 21 °C on a 12 hour light/dark cycle with ad libitum access to food and water. All procedures were approved by the Animal Ethics Committee of the University of Adelaide (M-83-01) and were consistent with criteria in the “*Guide for the Care and Use of Laboratory Animals*” published by the NIH.

Animal treatments. To diminish inter-animal variability in hepatic responsiveness to allyl alcohol, food was withheld for 15 hours prior to commencing experiments (Jaeschke and Wendel, 1985). On the day of experimentation, mice received allyl alcohol (60-100 mg/kg [approx. 1100-1800 µmol/kg]) either alone or in conjunction with hydralazine (100-300 µmol/kg) via a single intraperitoneal injection (the dosing volume was 10 mL/kg). Control mice received either vehicle only (phosphate buffered saline [PBS], 50 mM, pH 7.4) or 300 µmol/kg hydralazine. In a related experiment, the time dependence of hydralazine-induced hepatoprotection was explored, with 200 µmol/kg hydralazine administered to mice either 0, 20 or 30 minutes after a single 90 mg/kg dose of allyl alcohol. Four hours after hydralazine administration, mice were anaesthetized with pentobarbitone (6 mg/animal, i.p.) and blood was collected via open cardiac puncture. Plasma was prepared and stored at -20 °C until use. After perfusion with 25 % sucrose, the right medial lobe was removed for use in immunohistochemical studies or Western blotting procedures. The remaining tissue portions were homogenized in 9 volumes of cold 3 % perchloric acid and then centrifuged at 7,000 x g for 5 minutes. The resulting supernatant was used for GSH determination as outlined below.

Biochemical Analyses. Plasma activities of sorbitol dehydrogenase (SDH) and glutamate pyruvate transaminase (GPT) were measured to assess the severity of liver injury (Asada and Galambos, 1963; Bergmeyer and Bernt, 1974). For SDH determination, 0.1 mL plasma was diluted with 0.5 mL Tris-HCl buffer (0.1 M, pH 7.5) containing 0.4 mM NADH. After a 10 min incubation at room temperature to allow removal of interfering metabolites, reactions were started by adding 0.1 mL fructose solution (4.0 M prepared in the abovementioned Tris-HCl buffer). NADH oxidation was then followed for 3 minutes at 340 nm using a Metertek SP-830 spectrophotometer (Analytical Equipment Co., Adelaide, South Australia). SDH activity was then expressed as Units/L, where 1 Unit is the activity producing 1 mol of NAD⁺ per minute at 25 °C. For the estimation of GPT activity, a 2-step reaction was used where pyruvate, the product of GPT-catalyzed alanine deamination, was reduced to lactate in a NADH-dependent reaction catalysed by lactate dehydrogenase (Bergmeyer and Bernt, 1974). Briefly, 0.1 mL plasma was added to a 0.6 mL reaction mixture that comprised 1.0 M alanine and 10 Units/mL lactate dehydrogenase (Sigma, Type II, rabbit muscle) prepared in potassium phosphate buffer (0.1 M, pH 7.4). A 10 µL volume of stock NADH solution (13 mM, prepared in 120 mM sodium bicarbonate) was then added to each sample. After mixing, the samples were allowed to stand at room temperature for 3 minutes, after which reactions were started by adding 20 µL of α-ketoglutarate solution (0.66 M). NADH oxidation was then followed for 3 minutes at 340 nm using the Metertek SP-830 spectrophotometer. To assess any possible interference by hydralazine with the enzyme assays, 0.3 mM hydralazine was added to cuvetts containing aliquots of serum from allyl alcohol-treated mice. This treatment had no effect on either SDH or GPT activity (data not shown). GSH estimation was via the procedure of Hissin and Hilf (1976), which measures a fluorescent isoindole formed upon derivitization of GSH by *o*-

phthalaldehyde. Briefly, a standard curve was prepared over the range of 100 to 1000 ng GSH using 3 % perchloric acid. Samples and standards were then neutralized by adding 0.16 mL of 2.5 M NaOH for each mL of perchloric acid extract. Next, 50 μ L volumes of samples or standards were added to individual wells of 96-well microplates which contained 0.2 mL Tris-HCl buffer (0.2 M, pH 8.0, containing 1 mM EDTA). After the addition of 10 μ L *o*-phthalaldehyde (1 mg/mL stock in methanol), the reactions were allowed to proceed for 20 min in the dark. The fluorescence of each sample was then determined via a *PolarStar Galaxy* microplate reader (BMG Labtechnologies, Durham, NC) using respective excitation and emission wavelengths of 340 and 420 nm. The hepatic GSH content was then expressed as μ g/mg liver.

Western Blotting. Fresh liver tissues were homogenized for 60 seconds in 9 volumes (w/v) of chilled buffer comprising 250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.2 mg/mL phenylmethylsulfonyl fluoride and 1 mM benzamidine. Following centrifugation of the homogenate for 10 minutes at 1500 x g at 4 °C, an aliquot of supernatant was assayed for protein content using the bicinchoninic acid assay (Bollag et al., 1996). Aliquots of supernatant were then diluted with 4 volumes of buffer comprising 1 % SDS, 25 % glycerol and 1 % (octylphenoxy)polyethoxyethanol (Igepal CA-630). Following further dilution (1:3) with SDS/PAGE Loading buffer and heating at 90 °C for 5 min, aliquots containing 125 μ g protein were resolved overnight on a 12.5 % polyacrylamide gel. After transfer to nitrocellulose, membranes were blocked for 30 min using 5 % nonfat milk powder in PBS and then treated with 1/1000 dilutions of rabbit antiserum that had been raised against hydralazine/acrolein-modified KLH (Burcham et al., 2004). After extensive washing and treatment with horseradish peroxidase-coupled goat anti-rabbit IgG (1/10,000, Immunopure, Pierce, Rockford, IL),

membranes were treated with Pierce *SuperSignal West Pico* Chemiluminescence Reagent for 5 minutes. The resulting membranes were exposed to Kodak *BioMax Light* film (Eastman Kodak Company, Rochester, NY) and the film was developed as per recommended procedures.

Immunohistochemical Methods. Following drug treatments as described above, mice were anaesthetized and their livers were perfused with 25 % sucrose. The right medial lobe was removed and frozen in liquid nitrogen before storage at -20°C . Liver sections ($5\ \mu\text{m}$) were prepared using a cryostat maintained at -20°C and following drying they were fixed in methocarn solution (methanol: chloroform: acetic acid, 6:3:1) for 20 minutes. Following brief rehydration in ethanol, slices were blocked in 10 % skim milk/PBS for 1 hour. After treatment overnight at 4°C with primary antibody (rabbit antiserum raised against hydralazine/acrolein-modified KLH diluted 1/750 in 10 % nonfat milk/PBS), the sections were washed in PBS before they were treated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Pierce, 1/400 in 10 % nonfat milk/PBS) at room temperature for 1 hour. The slices were washed in 0.05 % Tween 20 in PBS, mounted in 50 % glycerol/PBS and then viewed using an Olympus BX50WI fluorescence microscope (Olympus Optical Company, Japan). In a related experiment, liver tissue was analyzed following recovery from mice 4 hours after they received a $300\ \mu\text{mol/kg}$ (i.p.) dose of (1*E*)-acrylaldehyde 1-[1-phthalazinyl]-hydrazone, the main product formed during trapping reactions between free acrolein and hydralazine. (1*E*)-acrylaldehyde 1-[1-phthalazinyl]-hydrazone was synthesized from acrolein and hydralazine and its purity confirmed via NMR and mass spectrometric analysis.¹

Statistical Analysis. Serum levels of SDH and GPT in treated mice were compared to values from vehicle-treated control mice using Kruskal Wallis analysis with a Dunns test. Liver GSH levels were compared to control values via 1 way ANOVA followed by Dunnett's post hoc test.

Results

Hydralazine Affords Dose-Dependent Protection Against Allyl Alcohol Hepatotoxicity in

Mice. To facilitate later experiments, we conducted a pilot study to identify a dose of allyl alcohol that consistently elevated plasma SDH and GPT activities within a 4 hour period in the mouse strain used in this study. The 4 hour duration was based on results from other laboratories as well as our own (*data not shown*) indicating that maximal elevation of liver enzymes in plasma occurs 2 to 3 hours after allyl alcohol administration in mice (Jaeschke et al., 1987). For our pilot study, plasma SDH and GPT activities were measured 4 hours after mice received 0, 60, 80, 90 or 100 mg/kg allyl alcohol (*data not shown*, N = 6 - 13). Since 90 mg/kg allyl alcohol (*i.p.*) elevated plasma SDH and GPT activities 30- and 24-fold over controls at this time, and had decreased hepatic GSH stores by 65 %, this dose was judged suitable for use in subsequent experiments. Also, no fatalities accompanied the 90 mg/kg dose, compared to 30% mortality in mice receiving 100 mg/kg allyl alcohol (*data not shown*).

In recent studies, hydralazine afforded strong, concentration-dependent cytoprotection against allyl alcohol toxicity in isolated mouse hepatocytes (Burcham et al., 2003a, 2004). Fig. 2 likewise indicates that hydralazine afforded clear, dose-dependent protection against allyl alcohol-induced changes in plasma enzymes in whole mice, with 300 $\mu\text{mol/kg}$ hydralazine almost totally abolishing the changes in both SDH (Panel A) and GPT (Panel B) activities ($p < 0.01$). Using the dose-response data shown in Fig. 2, hydralazine doses affording half-maximal protection against liver injury were estimated as 160 and 80 $\mu\text{mol/kg}$ for SDH and GPT, respectively.

Hepatoprotective Doses of Hydralazine Do Not Prevent Hepatic GSH Depletion. Other

investigators have established that GSH depletion is of fundamental importance in allyl alcohol

toxicity, with irreversible liver injury typically occurring after hepatic GSH is diminished below a critical threshold (Jaeschke and Wendel, 1985). Moreover, allyl alcohol hepatotoxicity is abrogated by interventions that either increase hepatic GSH or upregulate glutathione-S-transferase expression (Jaeschke et al., 1987; Haenen et al., 1988). Notwithstanding these considerations, hepatoprotective doses of hydralazine had no effect upon the hepatic GSH depletion caused by allyl alcohol (Figure 2C). Hence the hepatic GSH content in mice that received the fully hepatoprotective dose of 300 $\mu\text{mol/kg}$ hydralazine was unchanged from that in allyl alcohol-only treated mice ($p > 0.05$).

Delayed Administration of Hydralazine Abolishes the Hepatoprotection Against Allyl

Alcohol Hepatotoxicity. In recent work, hydralazine protected against acrolein-mediated toxicity when added to cells that had been pre-exposed to allyl alcohol for 25 min, yet were washed free of toxin prior to irreversible membrane rupture (Burcham et al., 2004). This was consistent with the possibility that hydralazine targets “early” steps in the modification of cell macromolecules by acrolein, a likelihood that emerged during experiments in which we found that the epitope for an antibody raised against acrolein-adducted KLH displayed transient instability to nucleophilic buffer constituents (Burcham et al., 2003b). Using this antibody, we found that acrolein adducts were highly susceptible to hydralazine when the drug was added 15 minutes after commencing protein modification by acrolein, but that they became progressively resistant to the drug at later time points (e.g. by 60 min they completely resisted attack by hydralazine) (Burcham et al., 2004). To determine whether the protective effects of hydralazine *in vivo* might involve comparable interference with “early” events in acrolein-mediated cell injury, we investigated the time dependence for the drug’s protective actions against allyl alcohol

hepatotoxicity. For this experiment, 200 $\mu\text{mol/kg}$ hydralazine was administered to mice either 0, 20 or 30 minutes after they received 90 mg/kg allyl alcohol. Four hours after receiving the initial dose of allyl alcohol, animals were sacrificed for the determination of plasma enzymes and hepatic GSH. We predicted that if interference with “early” adduction chemistry underlies hepatoprotection, hydralazine would be less protective when administered 30 minutes after allyl alcohol than at earlier time points (the 30 minute period was the latest time to which drug administration could be delayed since irreversible liver damage in the form of enhanced enzyme leakage was detected at latter time points - *data not shown*). The results in Figure 3 confirm that hydralazine was strongly hepatoprotective when either co-administered with allyl alcohol or following a 20 minute delay (Figure 3A). However, if a 30 minute period was allowed between the administration of allyl alcohol and hydralazine, the drug’s hepatoprotective efficacy was diminished (Fig. 3A, $p < 0.001$ relative to saline-treated control). No differences in the degree of hepatic GSH depletion were evident between these various dosing regimens (Figure 3B).

Western Blot Analysis Reveals Intense, Dose-Dependent Adduct-Trapping by Hydralazine in Mouse Liver. Rabbit antiserum raised against hydralazine- and acrolein-modified KLH was then used to determine whether “adduct-trapping” accompanies hepatoprotection by hydralazine in allyl alcohol-treated mice. The antiserum has high affinity for hydralazine-trapped acrolein adducts at lysine and histidine residues (Burcham et al., 2004). A Western blot depicting trapped adducts in proteins recovered from mouse liver 60 minutes after the administration of 90 mg/kg allyl alcohol with or without hydralazine (100 or 200 $\mu\text{mol/kg}$, *i.p.*) is shown in Figure 4. Lanes 1 to 4 reveal a lack of immunoreactivity in proteins from mice treated with either injection vehicle only (Lane 1), allyl alcohol only (Lane 2), or 100 (Lane 3) or 200 $\mu\text{mol/kg}$ hydralazine

only (Lane 4). The lack of signals in these lanes concurs with our previous finding that the antiserum is highly specific for hydralazine/acrolein-adducted proteins (Burcham et al., 2004). In sharp contrast, strong adduct-trapping by hydralazine was evident in the livers of two allyl alcohol-treated mice exposed to 100 $\mu\text{mol/kg}$ hydralazine (Lanes 5 and 6). Some 20 to 25 proteins can be distinguished as targets for hydralazine in Lanes 5 and 6, confirming that acrolein generates drug-reactive adducts in a diverse range of tissue proteins. Doubling the dose of hydralazine increased the intensity of adduct-trapping in two additional animals (Lanes 7 and 8), but due to signal saturation, bands corresponding to proteins with masses greater than 40 kDa are poorly resolved (Lanes 7 and 8). In the case of 2 small well-resolved protein targets (26 and 31 kDa, depicted with arrows on Figure 4), densitometric analysis revealed 2.6- and 2.4-fold elevations in signal intensity respectively in animals receiving 200 $\mu\text{mol/kg}$ hydralazine compared to the lower dose.

Adduct-Trapping Occurs Diffusely Throughout the Liver Lobule. To explore the spatial heterogeneity of trapping reactions within the liver, immunohistochemical analysis was used to detect hydralazine-stabilized acrolein adducts in sections of right medial liver lobe collected from animals 4 hours after they received allyl alcohol and hydralazine (Figure 5). Consistent with the results from the Western blot analysis (Fig. 4), the primary antibody did not detect antigens in mice that received injection vehicle only, 90 mg/kg allyl alcohol only or 300 $\mu\text{mol/kg}$ hydralazine only (Panel A of Fig. 5 shows a representative image obtained during analysis of vehicle-treated liver sections – images from allyl alcohol-only and hydralazine only-treated animals were comparable and are omitted due to space considerations). Likewise, no immunorecognition was evident in the livers of mice 4 hours following a large dose of (1E)-

acrylaldehyde 1-[1-phthalaziny]-hydrazone (300 $\mu\text{mol/kg}$), the main isolable product formed during reactions between free acrolein and hydralazine (Panel B). This confirms that residual tissue levels of this product could not account for any signals detected in allyl alcohol and hydralazine-treated animals.

In striking contrast, intense adduct-trapping was evident in the livers of mice that concurrently received allyl alcohol and 300 $\mu\text{mol/kg}$ hydralazine (Panel C). Analysis of these sections using non-immune rabbit serum instead of primary antibody yielded no signals (i.e. image resembled that shown in Panel A), indicating the strong signals in Panel C were due to specific antigen recognition by the antiserum raised against hydralazine/acrolein-modified KLH. Although it was difficult to identify cellular structures in tissues from allyl alcohol-treated animals that received 300 $\mu\text{mol/kg}$ hydralazine, analysis of liver from a mouse that received 100 $\mu\text{mol/kg}$ hydralazine yielded a clearer image (Panel D). Adduct-trapping reactions are most intense within the cytoplasmic and membrane regions of individual cells (Panel D). Also, while adduct-trapping is evident at nuclear membranes, intranuclear staining is conspicuously absent (Panel D). Since allyl alcohol toxicity is typically localized in periportal regions (1), we expected that immunoreactivity would be most intense in these areas. However, although occasional slices displayed increased staining in periportal sinusoidal cells (*data not shown*), little zonation of adduct-trapping reactions was evident (Panel D).

Since acrolein can target multiple nucleophilic amino acids during reactions with protein, we assessed the prevalence of adduct-trapping at acrolein-adducted lysine versus histidine residues in liver proteins *in vivo*. For this experiment, we treated liver sections from allyl alcohol- (90 mg/kg) and hydralazine (100 $\mu\text{mol/kg}$)-treated mice with primary antibody that had been pre-incubated with either hydralazine/acrolein-modified poly-L-lysine or

hydralazine/acrolein-modified poly-L-histidine (Panels E and F respectively). We have previously shown that these modified polyaminoacyl reagents block immunorecognition of hydralazine-stabilized acrolein adducts in a model protein, BSA (Burcham et al., 2004). Pre-incubation of the primary antibody with 2 mg/ml concentrations of both reagents prior to the immunoassay step strongly inhibited immunorecognition of antigens in liver slices from allyl alcohol and hydralazine-treated animals (Panels E and F).

Discussion

Our findings extend recent observations in our laboratory concerning a role for protein adduct-trapping in the protection afforded by hydralazine against allyl alcohol toxicity in mouse hepatocytes (Burcham et al., 2004). In this work, hydralazine provided strong hepatoprotection when administered in the early stages of allyl alcohol intoxication in mice, an effect that was accompanied by intense, dose-dependent trapping of a broad range of adducted proteins. These findings reinforce the idea that protein adduction plays a key role in the pathogenesis of acute acrolein toxicity, perhaps by generating reactive carbonylated adducts that further propagate cell damage. More work is needed to establish this hypothesis, but one possibility is that proteins containing electrophilic acrolein adducts are attacked by nucleophiles in adjacent macromolecules, generating cross-linked species that are lethal to the cell. A similar mechanism of toxic action involving a central role for cross-linking has been demonstrated for other xenobiotics with bifunctional reactivity (Palom et al., 2002). Evaluation of this possibility would require the development of new analytical tools to detect acrolein-linked macromolecules in biological systems, perhaps via similar approaches to those used to identify 4-hydroxynonenal-linked lysine residues in adducted proteins (Tsai et al., 1998; Xu et al., 2000). Such methodologies would facilitate study of such questions as whether cross-linking contributes to the molecular effects of acrolein in exposed cells, including protein kinase C activation (Maddox et al., 2003) or alterations in the activity of various transcription factors (e.g. AP-1, NF κ B or Nrf2) (Horton et al., 1999; Biswal et al., 2002; Tirumalai et al., 2002).

In addition to clarifying the chemistry underling the cross-linking reactivity of acrolein, the identity of the carbonylated residues participating in drug trapping reactions with hydralazine also awaits characterization. In Fig. 5 E and F, hydralazine- and acrolein-modified poly-L-lysyl

and poly-L-histidyl reagents both strongly inhibited the immunorecognition of drug-trapped adducts. While this might indicate that trapping reactions occurred equally at each of these residues, an alternative explanation could be that the main epitope for the rabbit antiserum is either the hydralazine moiety *per se* or the drug-acrolein portion of drug-acrolein-protein ternary complexes, irrespective of the amino acid involved in adduct formation. Ongoing work is aimed at developing analytical methods to identify the amino acids involved in drug-trapping reactions.

The availability of rabbit antiserum against hydralazine-derivatized, acrolein-adducted-proteins provided a powerful means of detecting trapping reactions in liver proteins at both the individual protein level (Western blotting) and the macroscopic level (immunohistochemistry). In the former instance, the detection of hydralazine-labeled adducts in dozens of cell proteins concurred with the expectation that as a reactive, promiscuous electrophile, acrolein would generate drug-reactive adducts throughout the hepatocellular proteome. The indiscriminate nature of protein adduction by acrolein is also consistent with the diffuse, global distribution of adduct-trapping reactions across the liver lobule revealed during immunohistochemical analysis. Such observations suggest that allyl alcohol bioactivation and protein adduction occur throughout the liver parenchyma, and that the typical localization of allyl alcohol necrosis to periportal regions presumably reflects zonal heterogeneity in cellular responses to macromolecular adduction by acrolein, rather than differences in metabolic activation across the lobule. The precise nature of these cellular responses await further investigation, but one factor that might influence the susceptibility to acrolein-mediated toxicity could be the higher oxygen tension in periportal regions (Badr *et al.*, 1986). If, as is discussed below, protein adduction by acrolein compromises the cell's ability to sequester redox-active transition metals, the elevated oxygen availability may ensure oxidative cell injury is maximized within periportal zones.

Hydralazine provided clear hepatoprotection despite having no effect upon the hepatic GSH depletion that accompanied allyl alcohol intoxication. This finding rules out any possibility that hydralazine interfered with the alcohol dehydrogenase-catalyzed conversion of allyl alcohol to acrolein, since this outcome would have attenuated GSH depletion. Hydralazine's lack of effect on GSH depletion is intriguing given that the drug is an efficient acrolein scavenger, readily forming novel hydrazones with the aldehyde in buffered solutions (Figure 1, Panel A).¹ If hepatoprotection was due to such trapping of free aldehyde, hydralazine administration would be expected to reduce the pool of acrolein available for conjugation with GSH, thereby attenuating hepatic GSH loss. Perhaps the most likely explanation for the drug's lack of effect on hepatic GSH levels lies in differences in the kinetics of acrolein trapping by hydralazine relative to GSH, since the rate of acrolein-scavenging by the latter is much faster than the corresponding reaction between acrolein and hydralazine. Consequently, instead of sequestration of free acrolein underlying the hepatoprotection elicited by hydralazine, the ability to interfere with downstream events such as proteome modification is likely of greater relevance to the protective mechanism. A future publication from our laboratory will provide confirmatory evidence that the acrolein-trapping reactivity of hydralazine can be dissociated from its primary cytoprotective actions in allyl alcohol-treated mouse hepatocytes.

Hydralazine's ability to prevent hepatocellular death despite pronounced GSH depletion mirrors the actions of various reducing compounds and antioxidants in cells exposed to acrolein or allyl alcohol (Miccadei et al. 1988; Rikans and Cai, 1994; Nardini et al., 2002). Whether hydralazine exerts antioxidant actions *in vivo* seems unlikely given that others have reported that hydralazine administration to rats has no effect on basal lipid peroxidation markers in various tissues including liver (Cabell et al., 1997; Viridis et al., 2002; Pu et al., 2003). An alternative

explanation for the similarities between hydralazine and antioxidants could be that the adduct-trapping properties of hydralazine block the molecular events that trigger oxidative stress in acrolein-exposed cells. Precisely how a powerful 2-electron acceptor such as acrolein triggers oxidative stress in exposed cells is unknown, although alkylation of key nucleophilic residues in proteins involved in redox control or antioxidant defense is one possible contributor to the phenomenon. For example, acrolein is thought to attack the iron-binding protein ferritin, promoting the release of redox-active metal ions and thereby triggering oxidative cell injury (Jaeschke et al., 1992). This mechanism is consistent with the ability of desferrioxamine to attenuate allyl alcohol toxicity in isolated hepatocytes (Miccadei et al., 1988). If transition metal dyshomeostasis plays a key initiatory role in acrolein-mediated hepatic injury, whether adduct-trapping drugs block the chemical events that trigger iron loss from iron-binding proteins would emerge as an important research question.

The present study provides clear experimental proof that nucleophilic drugs can target carbonylated adducts in tissue proteins *in vivo*, but work is needed to establish the dose-response relationships for these reactions under conditions of less severe acrolein exposure. In this study, we required a large dose of allyl alcohol (1800 $\mu\text{mol}/\text{kg}$) to reproducibly elevate plasma levels of liver enzymes in the Swiss mouse strain used. This necessitated the use of correspondingly high doses of hydralazine, with the top dose (300 $\mu\text{mol}/\text{kg}$) approaching the maximal tolerated acute dose in mice. Whether better-tolerated doses of hydralazine can be used to target carbonylated proteins that might form under conditions of lesser acrolein exposure should be explored in future studies. Under such conditions, questions surrounding the long term safety and fate of “drug-trapped adducts” within the proteome could be explored. Given that we previously observed clear, concentration-dependent adduct trapping in allyl alcohol-preloaded hepatocytes exposed to

low micromolar hydralazine concentrations (2 to 10 μ M), it is reasonable to predict that adduct-trapping will be demonstrable in animals exposed to lower doses of acrolein and nucleophilic drugs (Burcham et al., 2004)

In conclusion, these findings provide new insights into the role of protein adduction in the pathogenesis of allyl alcohol hepatotoxicity, a widely used model of bioactivation-dependent, chemically-induced liver injury. Also, given the growing body of literature incriminating acrolein and related bifunctional electrophiles in diverse disorders (Uchida, 1999; Baynes and Thorpe, 1999; Liu et al., 2003), by confirming that efficient adduct-trapping and cytoprotection can be achieved using low molecular weight nucleophilic drugs, these findings raise the possibility of novel chemico-pharmacological interventions in diseases involving carbonyl stress.

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Footnotes:

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Figure legends:

Figure 1: **Panel A.** Formation of (1*E*)- and (1*Z*)-acrylaldehyde 1-[1-phthalazinyl]-hydrazones during reactions between free acrolein and hydralazine. **Panel B.** Diversity of hydrazones formed during trapping reactions between hydralazine and an acrolein-modified, lysine-containing peptide (see Burcham et al. (2004) for details).

Figure 2: Protection against allyl alcohol hepatotoxicity in mice. Hydralazine prevents elevations in plasma SDH (Panel A) and GPT (Panel B) activities but not hepatic GSH depletion (Panel C) in mice 4 hours after concurrent dosing with 90 mg/kg allyl alcohol (AA, i.p). Hydralazine (HYD; 100, 200 or 300 $\mu\text{mol/kg}$) was co-administered as a single i.p. dose with AA. Control mice received PBS, AA or 300 $\mu\text{mol/kg}$ HYD (HYD300). Data are represented as mean \pm SEM of 6 to 8 animals per group. Data from treated animals was compared to controls via 1 way ANOVA followed by Dunn's (Panels A and B) or Dunnett's (Panel C) post-hoc tests. ** $p < 0.01$, *** $p < 0.001$ compared to vehicle control. † $p < 0.05$, †† $p < 0.01$ compared to AA-treated mice.

Figure 3: Loss of hepatoprotection with delayed hydralazine administration. Mice received AA (90 mg/kg, i.p.) followed either immediately [co], 20 or 30 minutes later by hydralazine (HYD; 200 $\mu\text{mol/kg}$, i.p.). Four hours after the initial injection, animals were sacrificed for the determination of plasma SDH (Panel A) and liver GSH (Panel B). Values are reported as mean \pm SEM of 5 to 9 animals per group. SDH data from treated animals was compared to controls (PBS-treated) by 1-way ANOVA with a Dunn's post-hoc test whereas GSH data from treated

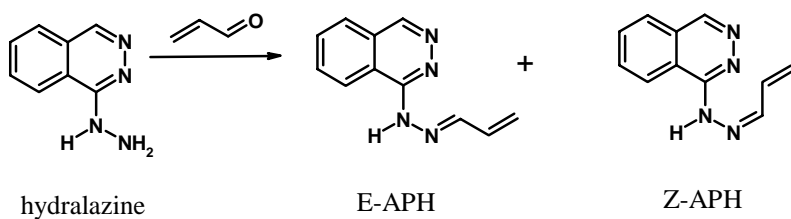
mice was compared to control by a 1 way ANOVA with a Dunnett's post-hoc test. ** $p < 0.01$, *** $p < 0.0001$ compared to vehicle control.

Figure 4: Strong adduct-trapping accompanies hepatoprotection by hydralazine. Western blot showing dose-dependent adduct-trapping in liver proteins (125 $\mu\text{g}/\text{lane}$) of mice 60 minutes after concurrent administration of allyl alcohol (AA, 90 mg/kg) and hydralazine (HYD; 100-200 $\mu\text{mol}/\text{kg}$). Drug-trapped adducts were detected using rabbit antiserum raised against hydralazine/acrolein-modified KLH (see Burcham et al., 2004). The location of MW Markers was determined using *Kaleidoscope* prestained markers from BioRad (Hercules, CA). Lanes correspond to: (1) vehicle-treated, (2) AA-treated, (3) 100 $\mu\text{mol}/\text{kg}$ HYD, (4) 200 $\mu\text{mol}/\text{kg}$ HYD, (5 & 6) AA plus 100 $\mu\text{mol}/\text{kg}$ HYD and (7 & 8) AA plus 200 $\mu\text{mol}/\text{kg}$ HYD. The arrows highlight two proteins (26 and 31 kDa) that were analyzed via densitometry as discussed in the text.

Figure 5: Immunohistochemical detection of adduct-trapping in mouse liver. Images depict the distribution of hydralazine-stabilized, acrolein-adducted proteins in the right medial liver lobe of mice treated with AA and hydralazine. The various panels represent the following: *Panel A* (200X magnification) - liver section from a control, vehicle-treated animal. *Panel B* (200X) - liver section from a mouse 4 hours after it received 300 $\mu\text{mol}/\text{kg}$ (1E)-acrylaldehyde 1-[1-phthalazinyl]-hydrazone. *Panel C* (200X) - section from mouse co-administered with AA and 300 $\mu\text{mol}/\text{kg}$ HYD; *Panel D* (400X) liver section from mouse co-administered AA and 100 $\mu\text{mol}/\text{kg}$ HYD (N = nucleus, CM = cell membrane); *Panels E and F* (200X) - slices as per *Panel*

C except the primary antibody was pre-incubated with 2 mg/mL hydralazine/acrolein-modified poly-L-lysine (*Panel E*) or hydralazine/acrolein-modified poly L-histidine (*Panel F*).

A. Reactions between hydralazine and free acrolein.



B. Reactions between hydralazine and protein-adducted acrolein.

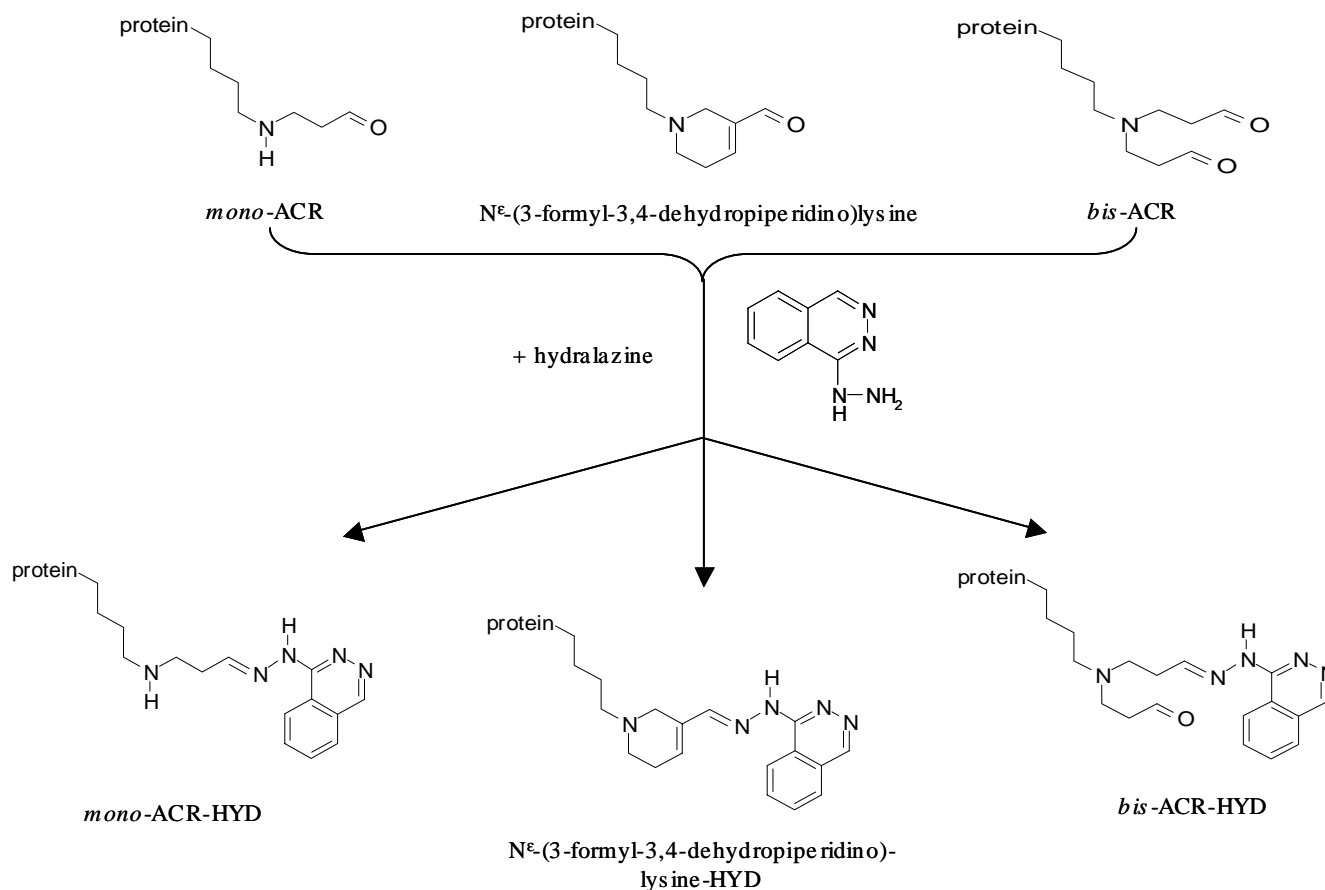


Fig 1 #67330

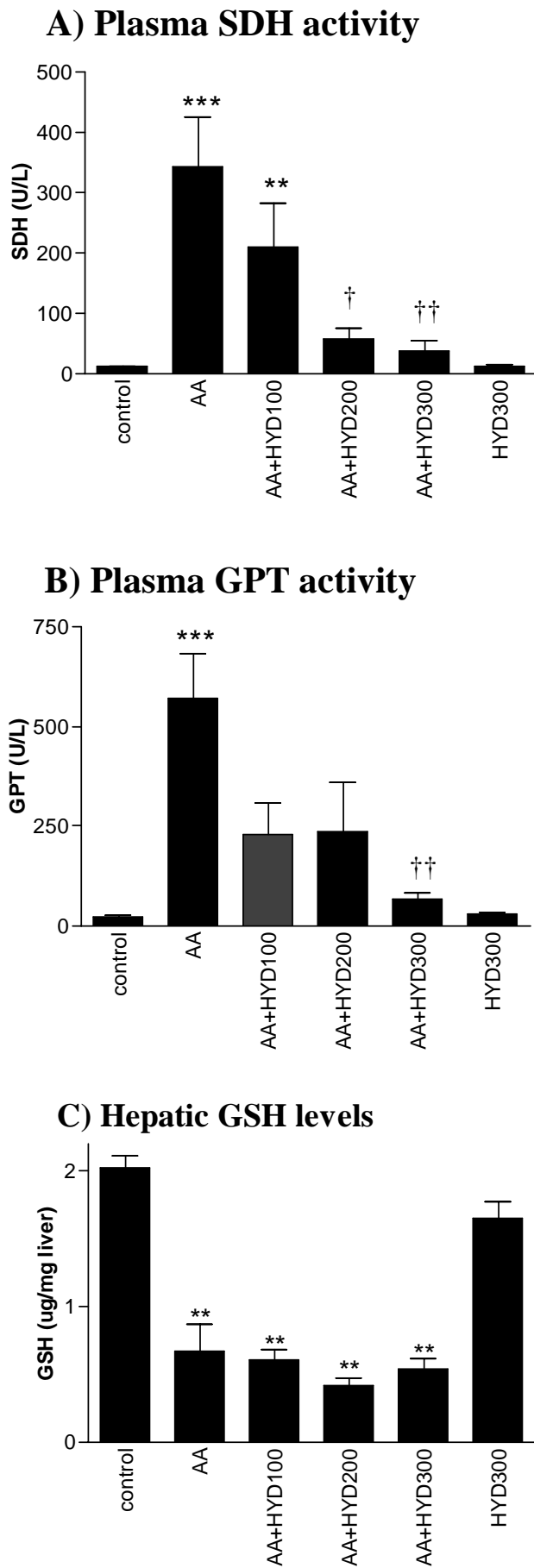
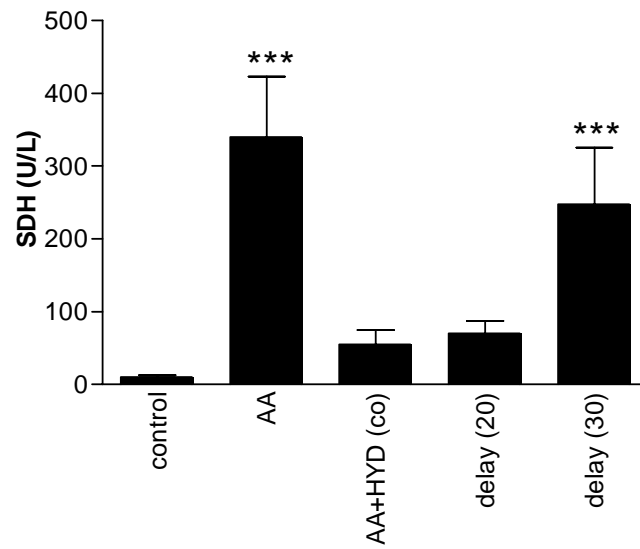


Fig 2 #67330

A) Plasma SDH Activity



B) Hepatic GSH Levels

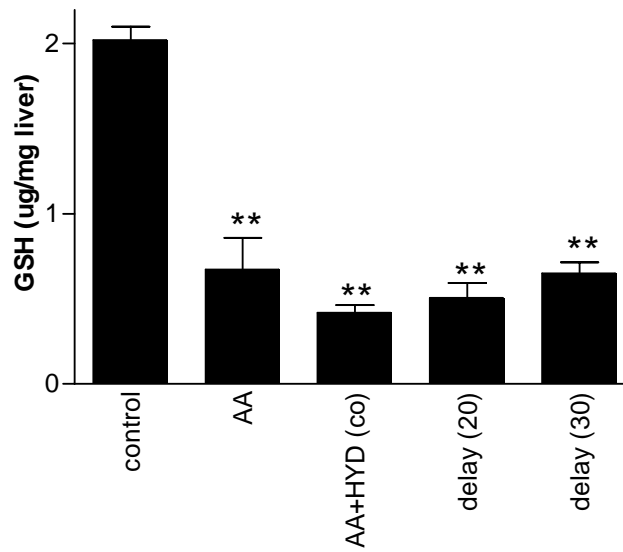


Fig 3 #67330

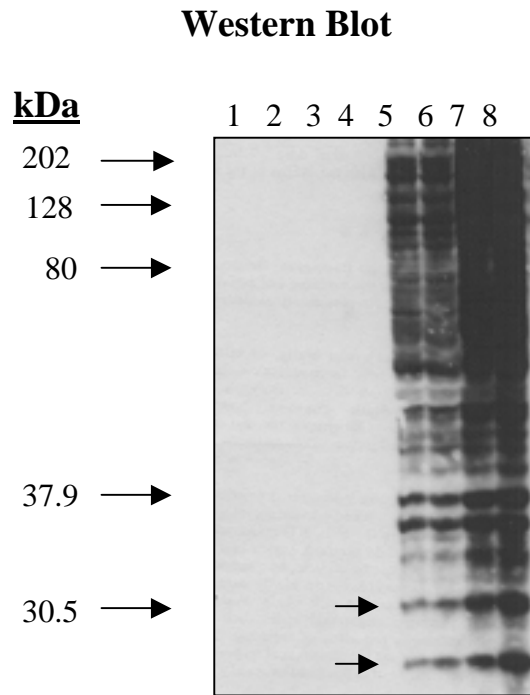


Fig 4 #67330

Immunohistochemical Analysis

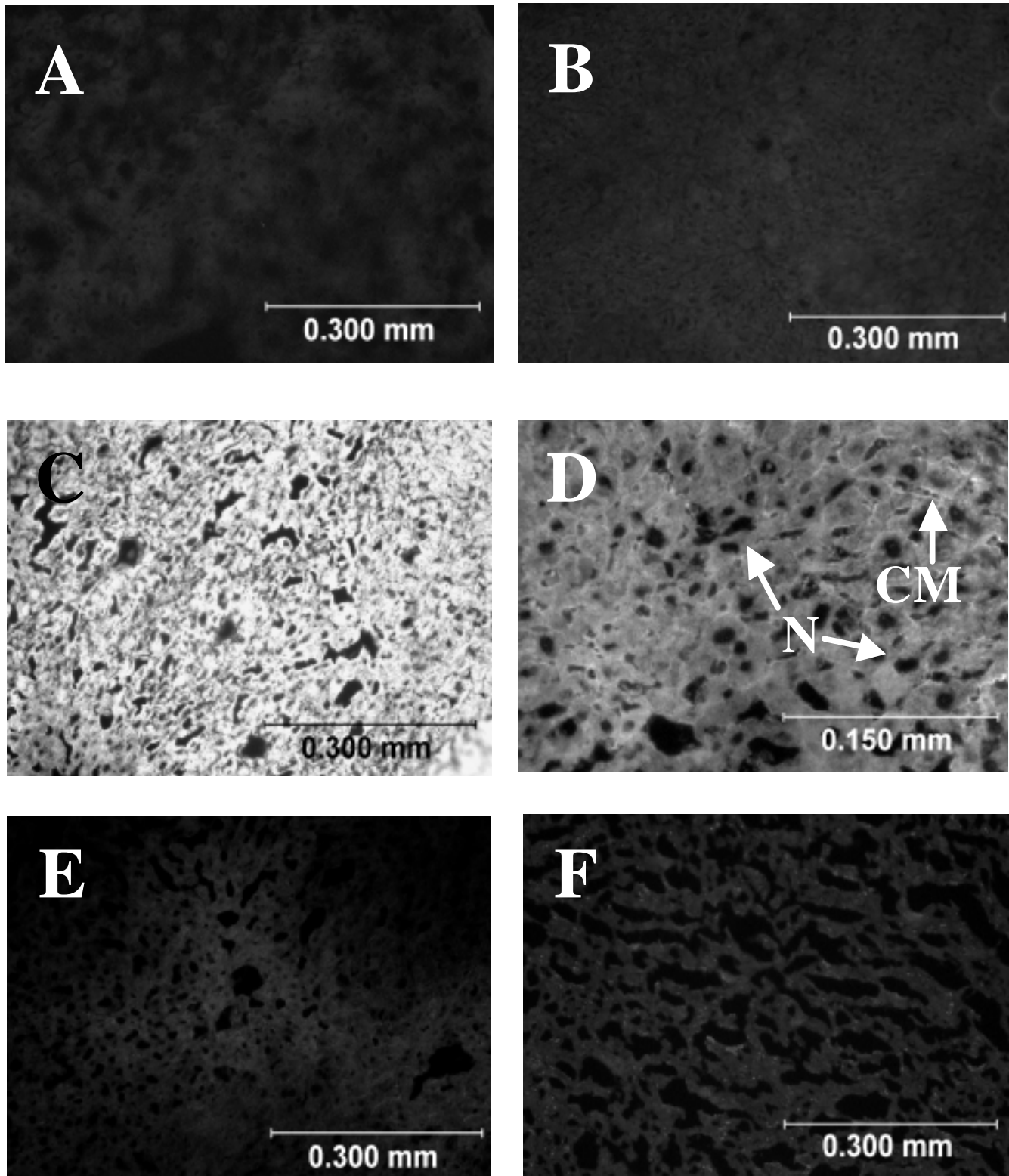


Fig 5 #67330