DISSERTATION

CHARACTERIZATION OF ATRAZINE INDUCED PROTEIN ADDUCTS

Submitted by

Gregory Dooley

Department of Environmental and Radiological Health Sciences

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY GREGORY DOOLEY ENTITLED CHARACTERIZATION OF ATRAZINE INDUCED PROTEIN ADDUCTS BE ACCEPTED AS FULFILLING IN PART REQUIRMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Ronald B. Tjalkens, PhD.

Marie E. Legare, PhD.

Kenneth F. Reardon, PhD.

William H. Hanneman, PhD.

Adviser, John D. Tessari, PhD.

Department Head, John D. Zimbrick, PhD.

ABSTRACT OF DISSERATION

CHARACTERIZATION OF ATRAZINE INDUCED PROTEIN ADDUCTS

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) (ATRA) is the most commonly applied herbicide in the U.S. and is frequently detected in drinking water at significant levels. ATRA metabolism yields diaminochlorotriazine (DACT), an electrophilic molecule that can react with nucleophilic protein residues forming a covalent adduct. We first demonstrated this interaction with hemoglobin from rats exposed to 30-300 mg/kg ATRA. Mass spectrometry (MS) analysis of hemoglobin tryptic digests indicated a 110 Da adduct on Cys-125 of the β-subunit. Based on *in vitro* incubations of 90 ug/ml DACT and hemoglobin yielding the identical adduct observed with *in vivo* ATRA exposures, adduct formation was by a nucleophilic substitution reaction between DACT and Cys-125. Albumin was then investigated since it contains an exposed Cys-34 that could be targeted by DACT. Again using MS, a 110 Da adduct was located on Cys-34 of albumin from rats exposed to 20-200 mg/kg ATRA and rat and human albumin exposed *in vitro* to 90 ug/ml DACT. Immunochemical detection using a DACT adduct antibody also detected the adduct in albumin samples from rats given 5-200 mg/kg ATRA and rat and human albumin exposed *in vitro* to DACT. No adducts were detected in control animals or in the *in vitro* controls with this method. These data support a novel immunochemical detection system that could provide a rapid screening methodology for the detection of ATRA in exposed human populations. Finally, we used the DACT antibody to located modified proteins in the pituitaries of ATRA exposed rats

and DACT exposed LβT2 rat pituitary cells. Since ATRA exposure suppresses the luteinizing Hormone (LH) surge, protein adducts in the pituitary may be involved in this mechanism of action. 2DE followed by Western blotting showed numerous spots (>30) that were not present in control from both exposed rats and LβT2 cells. Using MS analysis of matched protein spots, 8 unique proteins in the rats and 19 unique proteins in LβT2 cells were identified. Each of these proteins contained solvent exposed cysteine residues, making them targets for DACT. Future research will be necessary to elucidate the functional role of these adduct and their involvement in ATRA/DACT induced LH suppression.

Gregory Patrick Dooley
Department of Environmental and Radiological Health Sciences
Colorado State University
Fort Collins, CO 80523
Summer 2007

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DEDICATION

To my parents for their unconditional support in all my life's endeavors, Thank You!

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Literature Review

Introduction

Atrazine [CAS 1912-24-9], (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) (ATRA), is one of the most commonly used herbicides in North America with 57.4 million pounds applied in the U.S. in 2005 (USDA 2006). It provides control of broadleaf and grassy weeds by inhibiting photosynthesis, and is used primarily in corn but also in sorghum, sugarcane, citrus, bananas, asparagus, and recreational turf. It can be applied pre-emergence or post-emergence in dry flowable, flowable liquid, liquid, coated fertilizer granule, and wettable powder formulations. The upper Midwest of the U.S. sees the heaviest annual use of ATRA, especially in Indiana, Illinois, Iowa, and Nebraska (Figure 1). It is a designated at Restricted Use Pesticide by the U.S. Environmental Protection Agency (USEPA), which restricts ATRA to use by a certificated pesticide applicator or under the direct supervision of a certified applicator. ATRA is sold under the trade names Aatrex, Aktikon, Alazine, Atred, Atranex, Atrataf, Atratol, Azinotox, Crisazina, Farmco Atrazine, G-30027, Gesaprim, Giffex 4L, Malermais, Primatol, Simazat, and Zeapos (EXTOXNET 1996).

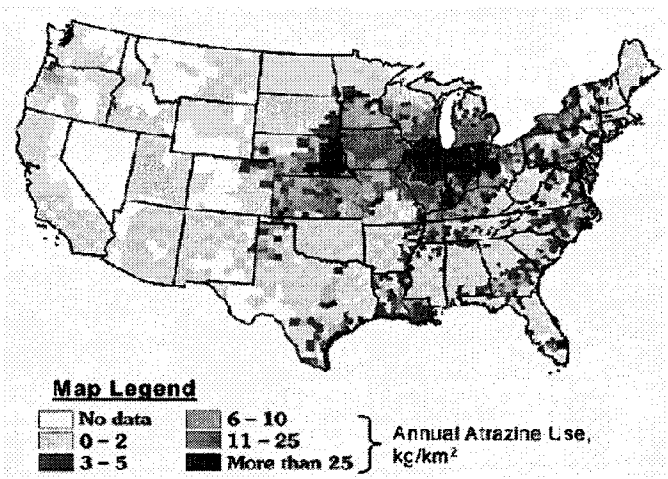

Figure 1. Annual use of ATRA in the United States, 1990-1991. (Adapted from Graziano et al. 2006)

Chemical and Physical Properties

ATRA is produced by reacting cyanuric chloride with ethylamine followed by isopropylamine forming an odorless white powder with a molecular weight of 215.7 g/mol, density of 1.23 g/cm^3 and a melting point of 178.5°C (Muller 2000, HSDB 2002). It is relatively stable with moderate water solubility (34.7 mg/L at 22°C, pH 7) and is very soluble in methanol, chloroform, diethyl ether, and dimethyl sulfoxide (Humberg 1989). ATRA has a low volatility with a vapor pressure of 3x10^{-7} mmHg and a Henry's Law constant of 2.63x10-9 atm-m^3/mol at 25 °C (USEPA 2006). The partition coefficients for ATRA are K_{ow} of 2.60 and a K_{oc} of 122 (USEPA 2006). These chemical properties contribute to ATRA's environmental fate following field application.

Environmental Fate and Occurrence

Once applied to a field, ATRA can degrade or remain active in the soils, taken up by crops, or enter ground and surface waters where it can persist for long periods. With its widespread heavy agricultural use and persistence, ATRA has become a common environmental contaminant in ground waters, both fresh and salt surface water, and drinking water sources in the U.S.

In soils, chemical hydrolysis and soil microorganisms accounts for most of the degradation of ATRA. Acidic or basic environments lead to rapid hydrolysis of ATRA to hydroxyatrazine, but this reaction is slower at neutral pH (Chan 1992). In aerobic environments, soil microorganisms degrade ATRA through the N-dealkylation of the ethyl and isopropyl side chains to desethylatrazine (DEA) and deisopropylatrazine (DIA) or dechlorination to hydroxyatrazine, while in anaerobic environments microbial

degradation is minimal leading to a longer half-life (Kruger et al. 1993). ATRA degrading bacterial cultures have been isolated from soil previously impacted by pesticide spills, and are capable of ring cleavage and deamination in addition to dealkylation and dechlorination (Radosevich et al. 1995). Degradation rates in soils are variable and depend on environmental conditions such as soil pH, temperature, moisture, organic matter, and use history of the field. In a sandy Florida field planted with sorghum, the estimated half-life for ATRA was 32 d in topsoil to 83 d in subsoil (Mbuya et al. 2001), while in a Colorado study the half-life of ATRA ranged from 3.5-7.2 days in loamy topsoil planted with corn (Shaner and Henry 2007). In anaerobic wetland soils from coastal Virginia, ATRA had a half-life of 38 d and was degraded to hydroxyatrazine and DEA, indicating ATRA can degrade under reducing conditions (Seybold 2001). ATRA is generally expected to have a soil half-life from 3-4 months (USEPA 2003) that can lead to significant soil accumulation and availability for runoff. In the Rio Grande Valley, Texas, it was the most frequently detected pesticide (60%) in soil samples at concentration up to 15.25 mg/kg (Garcia et al. 2001).

Once transported from soils as a result of runoff, ATRA is commonly detected in surface or groundwater were minimal degradation occurs (half-life of >200 days) (ASTDR 2003). From 1993-1995, ATRA was the most frequently detected pesticide in a U.S. groundwater survey, found in 38.2% of sampling sites throughout the U.S. (Kolpin et al. 1998). Thurman et al. (1991) focused only on the Midwest and found that immediately after application; the herbicide could be detected in 91% of surface water samples. ATRA levels exceeded the 3 μg/L maximum contaminant levels (MCL) set by the EPA in 52% of the samples. In another 1991 survey, ATRA was detected in 100% of

water samples collected at 8 locations from the Mississippi, Ohio and Missouri rivers and their tributaries with 27% of these samples containing ATRA concentrations greater than 3 μg/L (USGS 1991). In a similar study from 1999-2001, water samples were taken from four major junctures along the Mississippi River and analyzed for herbicide concentrations. ATRA was detected in 97% of the 119 samples, at concentrations up to 9.84 mg/L and accounted for 49% and 45% of the total herbicides in the Missouri and Ohio rivers, respectively (Rebich et al. 2004).

Runoff from agricultural zones has also contributed ATRA contamination in coastal estuaries of the US. ATRA was detected in 91% of sampling sites around the Biscayne Bay, Fl at concentrations that ranged from 0.9-108 ng/L with highest concentrations near corn production (Harmen-Fetcho et al. 2005). In the Patuxent River estuary of the Chesapeake Bay, which receives runoff from agricultural and urban land uses, ATRA was the most detected herbicide during a 4 month monitoring period in 1996. ATRA and its metabolites DEA and DIA were detected at maximum concentrations of 1.36, 1.1, and 0.76 μg/L respectively and it was estimated that 71 kg of ATRA entered the estuary during the monitoring period (McConnell et al. 2004). Runoff from sugarcane cultivation in southern Louisiana has lead to ATRA in excess of the 3 μg/L being detected in 22% of samples from the Vermilion-Teche Basin of the Gulf Coast with concentrations up to 18 μg/L measured during the months May and June (Southwick et al. 2002). In a similar region of the Gulf Coast, ATRA was detected in 93% of estuarine surface water samples collected from the mid-Texas coast at concentrations up to 62.5μg/L (Pennington et al. 2001).

ATRA contamination in drinking water systems has been documented within the US. In a 1985-1990 monitoring study entitled National Survey of Pesticides in Drinking Water Wells, the USEPA detected ATRA in 1570 (1.7%) community water system wells and 70, 800 (0.7%) rural domestic wells sampled, with maximum ATRA concentrations measured at 0.92 and 7.0 μg/L, respectively (USEPA 1990). More recently (1995-2001), a survey of 175 community water systems was conducted in 12 corn producing states. Of the 16,258 samples collected, 87% contained ATRA greater than 0.05 μg/L and 25 reservoirs exceeded the 3 μg/L MCL (Hackett et al. 2005). With the widespread occurrence of ATRA in drinking water, Jiang and Adams (2006) evaluated the effectiveness of conventional water treatment technologies for the removal of chloro-*s*-triazines (ATRA and it metabolites) from drinking water. They concluded that coagulation/flocculation/sedimentation with alum and iron salts, excess lime/soda ash softening, and disinfection by free chlorine did not remove chloro-*s*-triazines from drinking water and only activated carbons filters and ozonation could partially remove chloro-*s*-triazines. These studies clearly show the potential for human exposure to ATRA through drinking water.

TOXICOLOGY

Absorption, Distribution, Metabolism, and Elimination of ATRA

ATRA exposure in mammalian systems is most commonly through dermal or oral routes. Dermal absorption has been researched with flow-through *in vitro* diffusion skin models and controlled dermal patch studies in human volunteers. Several factors have been shown to modulate dermal ATRA adsorption with rodent *in vitro* models. When male rats were given ethanol for 6 weeks, skin absorption of ATRA increased by a factor

of 2.4, suggesting that ethanol consumption by occupationally exposed people may increase absorption (Brand et al. 2004). Age may also influence absorption as 32 day old Fisher 344 rats showed significantly greater percutaneous penetration of ATRA when compared to 82 day old rats (Shah et al. 1987). Also, a commercial formulation of ATRA (Aatrex), that included surfactants, was shown *in vitro* with mouse skin to increase cumulative penetration by a factor of 5 compared to pure ATRA penetration (Brand et al. 2002). To estimate absorption in human skin, Ademole et al. (1993) used flow-through *in vitro* diffusion of human skin to determine percutaneous absorption of [^{14}C]-ATRA. They found 16.4% of applied dose was absorbed, with 12% [^{14}C]-ATRA found in the skin supernants, 3.5% [^{14}C]-ATRA found in receptor fluid, and the remainder as [^{14}C]-DIA and [^{14}C]-DACT in the receptor fluid. Dermal absorption was also demonstrated as an exposure route for ATRA with human volunteers. Ten subjects were dermally exposed to low (0.167 mg) and high (1.98 mg) doses of [^{14}C]-ATRA for 24 h and urine was collected for 7 days. Analyzed urine contained 4% of the dose, primarily as [^{14}C]-DACT and [^{14}C]-mercapturate metabolites, while no parent [^{14}C]-ATRA was found indicating applied ATRA had been absorbed and metabolized (Buchholz et al. 1999).

Following oral exposure, ATRA is rapidly metabolized and eliminated from the body. Timchalk et al. (1990) measured radioactivity distribution in male Fisher 344 rats given 30 mg/kg [^{14}C]-ATRA via oral gavages. Within 24h, 57% of the administered dose was found in urine and 14% found in feces. After 72h, 66% of was found in urine and 19% in feces, while < 8% was found in skin, carcass, or blood. In a case of fatal human ingestion of a herbicide mixture containing ATRA, the kidneys showed the highest

concentration of ATRA (97.6 mg/g) with less amounts found in the lungs, small intestine, and liver (Pommery et al. 1993). Mathew et al. (1998) found no traces of [^{14}C]-ATRA in the liver, kidney, or hearts of male SD rats fed a diet of soybean and canola beans containing bound ATRA residues. Several sources have measured accumulation of radioactivity in plasma and red blood cells in rats exposed to [^{14}C]-ATRA (Thede et al. 1987, Timchalk et al.1990, Prentiss 2004). Pharmacokinetic modeling of suggested covalent binding of ATRA or metabolites to albumin in plasma or hemoglobin in RBCs contributed to this accumulation of radioactivity (McMullin et al. 2003). Dooley et al. (2006) showed that DACT forms a covalent adduct to Cys-125 of hemoglobin in SD rats given 30-200 mg/kg ATRA via oral gavages. Adduct formation showed a dose dependent increase, with a peak accumulation at 72h post-exposure that could account for the radioactivity seen in red blood cells. Adduct formation can also account for the radioactivity seen in plasma. Cys-34 of serum albumin has also been shown to form an identical covalent adduct with DACT both *in vivo* following ATRA exposure (200 mg/kg) in rats and *in vitro* with human albumin incubated with DACT (90 μg/L) (Dooley et al. 2007).

ATRA is generally metabolized to DEA, DIA, DACT, and glutathione mercapturates as shown in Figure 2. Phase I biotransformation of ATRA by cytochrome P-450 enzymes is the primary metabolic pathway observed in both *in vivo* and *in vitro* rodent studies. It first undergoes oxidative *N*-dealkylation via cytochrome P-450 (CYP) enzymes at either the ethyl or the isopropyl groups at the 4- or 6-amino substituent to form DIA or DEA. DACT is formed by an additional *N*-dealkylation, which removes the remaining alkyl group (Timchalk et al. 1990). Fisher 344 rats metabolized 30 mg/kg

[^{14}C]-ATRA to DACT (67%), DIA (5.1%), and DEA (<1.0%) as detected in urine after 72 h (Timchalk et al. 1990). Male C57BL/6 mice showed a similar pattern of metabolites in urine after single oral doses of ATRA ranging from 5 to 250 mg/kg. At 24 h, DACT was the most abundant metabolite at concentrations of 10 μM (5 mg/kg) to 1200 μM (250 mg/kg) and it was more than 30 fold higher than DEA or DIA. DACT was also detected at the highest concentrations in plasma at each time point and in all dose groups

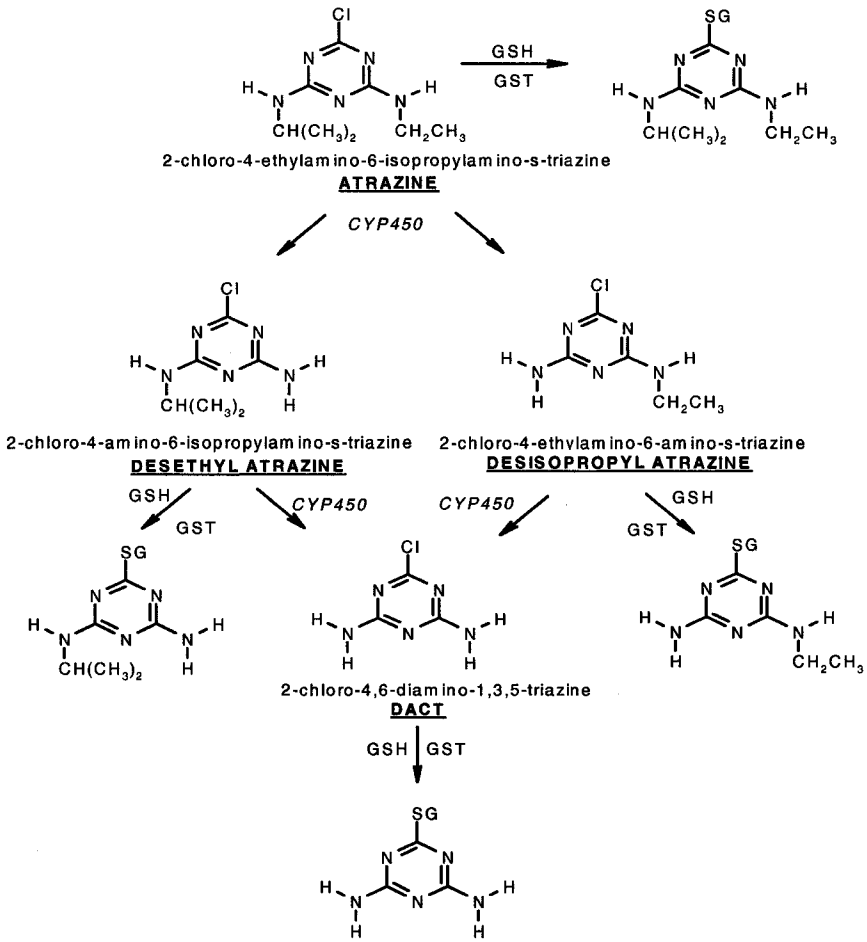

Figure 2. Proposed ATRA metabolism in the rat model (adapted from McMullin et. al 2003).

with significant quantities of DACT in the plasma within 2h (Ross and Filipov 2006). McMullin et al. (2003) showed DACT was the only metabolite present in plasma within 48h of a single 90 mg/kg ATRA dose in SD rats. These *in vivo* studies indicate DACT is the terminal metabolite following oral exposure in rats and mice, but these results are not observed with *in vitro* ATRA incubations. Primary hepatocytes from female SD rats incubated with 1.74 to 266 μM ATRA for 90 mins showed at low doses DACT and DEA were the primary metabolites, while at higher doses DEA was the primary metabolite followed by DIA and DACT (McMullin et al. 2006). Incubations of 100 μM ATRA with male and female SD rat liver microsomal fractions gave a different metabolic profile than observed with primary hepatocytes. There were no sex differences observed as male and female fraction had metabolite ratios of 80:13:7 and 77:16:7 (DIA: 1-OH- DEA: DEA) respectively, while no DACT was detected in either preparation (Hanoika et al. 1998). Also, no metabolites were formed without the addition of NADPH to the preparation. ATRA (50 μM) incubated in NADPH-fortified male C57BL/6 liver microsomes was metabolized to DIA and DEA in similar ratios and there was no DACT detected (Ross and Filipov 2006). In a subsequent study, Hanoika et al. (1999) used liver microsomal fractions treated with various P-450 inducers to determine which P-450 enzymes were responsible for each step in the ATRA metabolism pathway. They concluded CYP 1A1/2 was responsible for the dealkylation of ATRA to DEA and DIA, while CYP 2B1/2 was responsible for the formation of 1-OH-DEA. In contrast, CYP1A2 alone is the predominant enzyme involved in Phase I metabolism of ATRA in human liver microsomes (Lang et al. 1997).

Phase II biotransformation of ATRA to mercapturate metabolites by glutathione *S*-transferases also occurs in humans (Abel et al. 2004, Buchholz et al. 1999) and rodents (Abel et al. 2004, Timchalk et al. 1990). Abel et al (2004) demonstrated GSTP1-1 was the only glutathione *S*-transferase isoforms in both humans and CD1 mice with activity towards ATRA. Liver cytosolic fractions from CD1 mice showed a much higher activity towards ATRA (282 pmol AT-SG/min/mg protein) as compared to human cytosolic fractions (3.0 pmol AT-SG/min/mg protein) likely due to a higher expression of GSTP1-1 in the mouse liver. In humans dermally exposed to 1.98 mg [^{14}C]-ATRA, 90% of [^{14}C] in urine after 48 h was identified as DACT and mercapturate metabolites (Buchholz et al. 1999). Mercapturate metabolites were also detected in Fisher 344 rats exposed to 30 mg/kg [^{14}C]-ATRA, where they accounted for ~ 21% of radioactivity in urine after 24 h (Timchalk et al. 1990). From these *in vivo* metabolic studies, it is clear that urine is the primary excretion route of ATRA metabolites from both dermal and oral exposures in rodents and humans.

Health Concerns

The common use of ATRA, especially in the Midwestern US, puts the general population at risk for exposure to low levels in drinking water (USEPA 1990). Occupational exposure is greater risk for pesticide handlers and farmers, as direct contact may lead to higher levels of exposure through multiple routes (Garmmon et al. 2005). The primary adverse health effects associated with ATRA exposure are abnormalities of the reproductive system that target the developing organism. Abnormalities following ATRA exposure in animal models include delayed puberty, immunotoxicity, delayed mammary gland development and tumors, alterations of hormone levels, disruption of

sex steroid levels, and disruption of ovarian cycles. There is limited data available concerning the human heath effects of ATRA; as a result the majority of exposure data has come from studies using the rodent model.

Delayed puberty

Delays in the onset of puberty in both male and female rats exposed to ATRA or its metabolites have been documented in recent research. In male Wister rats, preputial separation (PPS), an indicator of progression of puberty, was delayed 1.7-3 days following ATRA exposure (12.5 to 200 mg/kg) from PND 23 to PND 53. Also, weights of ventral prostate, seminal vesicle, and epididymides were significantly reduced in the 200 mg/kg ATRA group (Stoker et al. 2000). Using the same exposure protocol, Stoker et al. (2002) documented a similar delay in PPS when the rats were exposed to DIA (1.2-3.4d), DEA (1-4d), and DACT (1-4.2d) at ATRA molar equivalent doses of 25-200 mg/kg. Significant weight reductions in ventral prostate occurred with exposures to DEA (100-200mg), DIA (50-200mg), and DACT (200mg); in the seminal vesicles with DEA (25,100-200mg), DIA (100-200mg), and DACT (100-200mg); and epididymides with exposures to DEA (200mg), DIA (200mg), and DACT (100-200mg). Recently, Rayner et al. (2007) demonstrated that gestational ATRA exposure could also delay puberty in male offspring. Pregnant Long Evans rats were given 100 mg/kg ATRA on gestational days 15-19 and males allowed to suckle from an ATRA exposed dam showed a 2.5 day delay in PPS.

In female rats, similar delays in onset of puberty have been observed. When female Wister rats were exposed to ATRA from PND 22-PND 44, vaginal opening (VO) was delayed an average of 3.4 days (50 mg/kg), 4.5 days (100 mg/kg), and > 6.8 days

(200 mg/kg) when compared to controls In the high dose group (200 mg/kg), VO did not occur at all in 4/15 animals and occurred only after the end of treatment on PND 44 in 8/15 animals. Also in the high dose group, weights of adrenal gland, kidney, pituitary, ovaries, and uterus were significantly reduced (Laws et al. 2000). Puberty delay following ATRA exposure was confirmed by Ashby et al. (2002). They observed delayed VO in female Wister rats exposed to ATRA (PND 21-46) at 100 mg/kg and in SD rats at 30 and 100 mg/kg. In a follow up study using ATRA metabolites, Laws et al (2003) repeated their experiments with exposures of peripubertal female Wister rats to DACT and hydroxyATRA (OH-ATZ). In these treatments, exposure to DACT delayed VO by 3.2 days (33.8 mg/kg), 4.8 days (67.5 mg/kg), and 7.6 days (135 mg/kg) compared to the controls, while OH-ATZ had no effect on progression of puberty. Gestational exposure to ATRA can also affect puberty in female offspring. Pregnant Long Evans rats were given 100 mg/kg ATRA on gestational days 13-19 and female offspring had VO delayed 2.1 days and mammary glands that were half the size of controls on PDN 25 (Rayner et al. 2005).

Immunotoxicity of ATRA

Several rodent studies indicate that ATRA may also target the immune system at various functional levels. One month old male C57BL/6 mice given ATRA at doses up to 250 mg/kg for 14 days showed several alterations in their immune system (Filipov et al. 2005). Thymus and spleen weights were decreased and cellular phenotypes were altered following ATRA exposure. In the thymus, all T cell populations were dose-dependently decreased. In the spleen, MHC-II+ and CD19+cells, T helper, and cytotoxic T cells were decreased, while the percentage of highly activated cytotoxic/memory T

cells increased. In circulation, ATRA exposure decreased CD4+ lymphocytes and MHC-II+ cells at the 250 mg/kg dose. Karrow et al. (2005) also noted dose-dependent decreases in thymus and spleen weight in female B6C3F1 mice given ATRA up to 500 mg/kg for 14 days. Treatment significantly increased the number of splenic CD8+ T cells, cytotoxic T cell and mixed leukocyte responses, and reduced total spleen cell numbers, fixed macrophage function, and the mice's resistance to B16F10 melanoma. Rooney et al. (2003) suggested that developmental ATRA exposure causes gender-dependent immunosuppression. They exposed pregnant Sprague-Dawley rats to 35 mg/kg from gestational day 10 through postnatal day 23. In male offspring only, ATRA significantly reduced primary (IgM) antibody response to sheep erythrocytes and decreased delayed-type hypersensitivity response when the animals were sensitized to bovine serum albumin.

Disruption of Estrous Cycles

ATRA has been shown to disrupt the normal 4-5 day estrous cycles observed in rats. In a short term study, female SD and Fisher 344 rats were given 100 and 300 mg/kg ATRA for two weeks and both showed dose related irregular and significantly longer estrous cycles, but for different reasons. The SD rats had a greater amount of time in estrus and reduction in time in diestrus, while the Fisher 344 rats spent less time in estrus and more time in diestrus (Eldridge et al. 1994a). This observation of extended time in diestrus was also seen in female Fisher 344 rats given 120 mg/kg for 7 days (Simic et al. 1994). Laws et al. (2000) exposed female Wister rats to 12.5-200 mg/kg ATRA via gavages on PND 22-41. They monitored estrous cycles from VO to PND 149 and found

100% of control females with regular 4-5 day cycles compared to only 46% in the 100 and 200 mg/kg exposures, with irregular cycles characterized by extended diestrus. Similar results were seen when 7 week old female SD rats were given 0-200 mg/kg ATRA via oral gavages for 42 days (Eldridge et al. 1999). At doses of 40 and 200 mg/kg, only 55% and 36% respectively of animals showed regular estrous cycles with extended diestrus the common irregularity. In a chronic feeding study, ATRA up to 400 ppm was provided in diet for 26 weeks to 7 week old female SD rats (Eldridge et al. 1999). Prolonged diestrus was seen for the first 14 weeks in rats fed 400 ppm ATRA, but for the rest of the study, persistent estrus was characteristic of all dose groups. This prolonged estrus was associated with aging in controls and increased with exposure to ATRA. During the 26 week study, control rats spent 43% of the days in estrus compared to 68% in the 400 ppm dose group. In another feeding chronic study (1 year), Eldridge et al. (1994b) demonstrated that estrous cycling is disrupted by 400 ppm ATRA exposure in female SD, but not Fisher 344 rats. SD female rats showed a significantly increased number of estrus days, both with aging and dosing and SD rats given ATRA for 9 months had twice as many days in vaginal estrus as controls. At 12 months, ATRA fed rats were in estrus 3 times per 5 days as compared to 1 per five days in controls. Fisher 344 females showed no signs of disrupted estrous cycling. In SD rats, plasma estradiol levels also showed a dose-dependent increase where Fisher 344 showed no effects. The authors concluded that ATRA exposure had altered estrous cycling activity in a way that caused increased estrogen production in SD but not in Fisher 344 rats and suggested this may related to the observance of mammary tumors in SD but not Fisher 344 rats.

Disruption of Hormone Levels in Female Rats

Studies cited in the previous section demonstrated that ATRA exposure can disrupt estrous cycles in female SD rats and increase estrogen production. Other hormones levels have also been shown to be disrupted by ATRA. In females, luteinizing hormone (LH) is necessary to stimulate ovarian follicular development and ovulation, while stimulating the ovaries to produce estrogen and progesterone. Cooper et al. (2000) tested whether ATRA was able to disrupt estrogen-induced surges of LH and prolactin in ovariectomized female SD and LE rats. They exposed the rats via gavage with 50–300 mg/kg for 1, 3, or 21 days and measured LH and prolactin in serum. One day of exposure to 300 m/kg ATRA was enough to significantly suppress the serum LH and prolactin surge in LE, but not SD rats. After 3 days of exposure, LE rats showed a dose-dependent decrease in serum LH and prolactin surge while SD rats showed no treatment effect. Not until after 21 days of treatment at doses of 75–300 mg/kg ATRA did SD rats show suppression of both LH and prolactin. They also showed that in rats exposed to 300mg/kg ATRA for 3 days, pituitaries were able to release LH in response to injection of synthetic GnRH. They suggest LH and prolactin levels were altered by deregulation of hypothalamic control of these hormones, since pituitary release of LH was seen in ATRA exposed rats following injection of GnRH. Stoker et al. (1999) showed that suppression of suckling induced prolactin release can also be seen in female Wister rats exposed to ATRA during lactation. Rats given ATRA at 0, 6.25, 12.5, 25, and 50 mg/kg twice daily on PND 1-4 had complete inhibition of suckling induced prolactin release in all animals at 50 mg/kg, 60% had inhibition at 25 mg/kg, and 40% at 12.5 mg/kg. McMullin et al. (2004) showed that ATRA can suppress LH surge in SD rats, contrary to the result presented by Cooper et al. (2000). Dose-dependent suppression of the estradiol

benzoate/progesterone LH surge in female SD rats was caused by exposure to 30-300 mg/kg ATRA for 5 days, with complete blockage of the LH surge at 300 mg/kg. They also showed that DACT exposure can have a similar effect. In estradiol benzoate/progesterone primed ovariectomized SD rats exposed to 300 mg/kg DACT for 5 days, total plasma LH and peak LH surge levels were suppressed by 60 and 58%, respectively. A 47% decrease in pituitary release of LH in response to added gonadotropin releasing hormone was also seen in animals treated with 200 mg/kg DACT.

Disruption of Hormone Levels in Male Rats

ATRA exposure has also been shown to alter hormone levels in male rats. Male Wistar rats exposed to 200 mg/kg ATRA from PND 23 to PND50 showed no affect on testosterone levels, but increased levels of estrone, estradiol, and T3 (Stoker et al. 2000). A follow-up study with the ATRA metabolites DACT, DIA, DEA also showed alterations in some hormone levels. DIA decreased testosterone at 100 and 200 mg/kg and DACT increased estrone levels at 100 and 200 mg/kg, while no significant differences were seen in estradiol or thyroid hormones (Stoker et al. 2002). Male SD rats have shown decreased in testosterone production with ATRA exposure at doses that did not affect Wister rats during a similar exposure regime (PND 22-47) (Trentacoste et al. 2001). Serum testosterone concentration was reduced by ATRA at doses of 100 and 200 mg/kg per day by 34% and 32% and in intratesticular testosterone 41% and 45% respectively, while no changes in testosterone levels were seen at doses less than 100 mg/kg. ATRA doses of 100 and 200 mg/kg also resulted in reductions in serum LH concentration by 17% and 20%. Since both serum and intratesticular testosterone levels and LH were reduced, it was suggested that reduced testosterone production by Leydig

cells was responsible for these decreases. Frideman (2002) later demonstrated that both *in vivo* and *in vitro* ATRA exposure could decrease testosterone levels in male SD rats given less than 100 mg/kg ATRA and that Leydig cells are affected. ATRA was given at 50 mg/kg by gavage from PND 46-48 in an acute study and on PND 22-48 in a chronic study. In the acute study, serum and intratesticular levels of testosterone were reduced by 43% and 42% respectively. The chronic study showed similar results with serum and intratesticular levels of testosterone were reduced by 38% and 53%. *In vitro* experiments were done with Leydig cells isolated from rats on PND 49 and exposed to 50 μg/ml ATRA with LH stimulation for 3 h. Cells showed a 35% reduction in testosterone production with ATRA exposure compared with LH alone.

Mammary Gland Tumors

The disruption of normal estrogen levels and ovarian cycling has been implicated in the development of mammary tumors in ATRA exposed rats. Increased mammary gland tumor incidence has been observed in intact female SD rats following chronic ATRA exposure up to 1000 ppm (Eldridge et al. 1994, Stevens et al. 1999). However, numerous studies have shown that ATRA is not genotoxic or mutagenic (Brusick 1994). Also, increases in tumor incidence have not been observed in male or female Fischer 344 rats, male or female CD-1 mice, or male SD rats fed ATRA for 24 months (Stevens et al. 1999). These results suggest female SD rats have a strain and sex specific response to ATRA making them more susceptible to mammary gland tumors. Stevens et al. (1999) observed no increase in tumors in ovariectomized female SD rats after 24 months of 400 ppm ATRA exposure. They concluded that increases in tumor incidence were due to an acceleration of reproductive aging resulting in increased tissue exposure to estrogen and

prolactin creating an endocrine environment promoting tumorigenesis. It has also been suggested that blockage of LH by high level ATRA exposure promotes mammary tumor formation since ovarian follicles that fail to ovulate resulting in abnormal levels of estrogen (Eldridge et al. 1999).

Epidemiologic Studies

Several epidemiological studies have been conducted to evaluate cancer risk in ATRA exposed populations, such as ATRA manufacturing workers, pesticide applicators, and those living in agricultural communities with ATRA contaminated drinking water. Prostate cancer incidence and ATRA exposure was evaluated in 2045 workers at a Louisiana plant that were involved with the manufacturing or formulation of triazine herbicides from 1985-1997. It was concluded that no epidemiologic or other information supports an association between ATRA and prostate cancer (Maclennan et al. 2002). Mortality patterns were also evaluated from 1970-1997 for 2213 workers at this same manufacturing plant (MacLennan et al. 2003). Standard mortality ratios were compared with mortality in the general industrial worker population and it was found that triazine workers had a possible increased (4:1) death rate from non-Hodgkin's lymphoma, but overall this study provided no specific evidence of increased mortality rates with triazine manufacturing. Rusiecki et al. (2004) evaluated cancer incidence in licensed ATRA applicators in Iowa and North Carolina from 1992-2001. Exposure was assessed in 36,513 applicators based on questionnaires concerning work practices to generate exposure metrics of lifetime, day of exposure and intensity weighted lifetime day of exposure. This study also concluded that ATRA exposure was not associated with increased incidence of numerous cancers. Hopenhayn-Rich et al. (2002) investigated a

possible association of ATRA exposure and incidence of breast and ovarian cancer in women living in agricultural areas of Kentucky from 1993–97. Using ATRA exposure indices from public water measurements, acres of corn planted, and pounds of ATRA sold and ovarian and breast cancer age-adjusted incidence rates from the Kentucky Cancer Registry, they found no evidence to support an association between ATRA exposure and either type of cancer.

Biomarkers of Exposure

ATRA Biomonitoring

Current methodologies for human ATRA biomonitoring have been validated in occupational exposures using field applicators handling ATRA. These include detection of ATRA or metabolites in urine, saliva, hand washes, and dermal patches following direct ATRA exposure.

Detection of ATRA metabolites in human urine of occupationally exposed people has been shown to be a potential biomarker of exposure. Lucas et al. (1993) measured dermal and inhalation ATRA exposures of a single applicator during a 10-day work period and compared them with urinary levels of ATRA metabolites. The worker's total ATRA exposures ranged from 1 mg to 21 mg/day. ELISA analysis detected DEA and DIA at low levels and ATRA mercapturate was the most prevalent metabolite. They suggested the use of ELISA for the mercapturate of ATRA as a method of biomonitoring. A larger study by Hines et al. (2003) confirmed these results of detectable levels of ATRA or metabolites in urine of occupationally exposed people. They collected urine samples from 15 pesticide applicators preseason, during a 6 week application period, and from a reference population and used ELISA to analyze for ATRA and metabolites. Only

2 of the 15 workers showed detectable levels of ATRA (12 and 19 nmol/L) and no workers had detectable levels of DIA, DEA or ATRA mercapturate in urine in pre-season measurements. During the spraying season, ATRA mercapturate levels were the highest of the ATRA metabolites in urine (83 nmol/L) and ranged from 1.3-2100 nmol/L, indicating it would be the most likely ATRA biomarker in urine.

Another biological matrix suggested for biomonitoring ATRA is saliva. This method was first demonstrated in field studies by Denovan et al. (2000) following validation in rats (Lu et al. 1997). Saliva was collected from 15 ATRA applicators during preseason and spraying season, and ATRA concentration was measured with ELISA. ATRA concentrations were significantly higher on spraying days compared to non spray days with 85% of samples at > 10 μg/L ATRA and 79% at < 10 μg/L ATRA, respectively. It was concluded that detecting ATRA in saliva could provide a simple and inexpensive analytical method for biomonitoring ATRA exposure.

Other methods of exposure monitoring for ATRA include air sampling, dermal patches, and hand wash sampling. Hines et al. (2006) collected these samples from 15 pesticide applicators during the pre-emergent spray season for at total 89 applicator-days. ATRA concentrations in air samples were near the limit of detection in 27% of samples and ranged from 1.3-75 $\mu g/m^{3.}$ Dermal patches showed the greatest levels of ATRA when applied to the left thigh (7.4-13,000 μg/sample) and the hat (7.5-7,800 μg/sample). Hand washes contained detectable levels of ATRA in 74% of samples and concentrations ranged from 24-18,000 μg/sample. These results indicate that an exposure assessment with multiple exposure indices is necessary to determine a workers total potential exposure.

These current methodologies are limited in that the measurement of ATRA or metabolites in biological fluids or environmental measurements do not show a biologically significant interaction indicating an internal dose has occurred. Using protein adducts of a chemical is an attractive methodology to indicate internal dose and may be a more accurate biomarker of exposure. Since blood proteins such as hemoglobin and albumin have relativity slow turnover rates, adducts to these proteins could be detected several weeks post exposure, a much longer detection window than other biomarker methodologies and provide a measure of internal exposure.

Protein Adducts as Biomarkers of Exposure

Covalent adducts formed when reactive metabolites interact with susceptible sites on a protein may either cause a protein to become nonfunctional or this binding may have no effect and serve a protective role by sequestering the reactive molecule (Cohen et al. 1997). The protective role that protein adducts may serve can be used to indicate and monitor exposure to specific metabolites, regardless of a functional effect on the protein. The use of protein adducts as biomarkers of chemical exposure can also provide estimates of actual internal dose instead of potential dose that is estimated with measurements of inhaled or ingestion concentrations of a certain chemical (Boogaard 2002). The two primary proteins that have served as biomarkers of chemical exposure are hemoglobin and albumin. The analysis of protein adducts may therefore provide an effective method for biomonitoring environmental exposure to chemicals such as ATRA. There has been no published research on the formation of ATRA adducts with hemoglobin or albumin.

Biomonitoring Using Hemoglobin Adducts

Erythrocytes are among the body's first cells to be exposed to exogenous chemicals that are ingested, inhaled, or injected (Rossi et al. 1998). Mammalian erythrocytes contain no organelles and very little protein other than hemoglobin (Miranda 2000). Therefore, hemoglobin provides a suitable protein for measurement of covalent adducts and exposure monitoring for several reasons. It is easily available in large amounts from blood samples, there are no repair mechanisms as with DNA, hemoglobin has a long life span (120 days in humans), adducts are chemically stable under biological conditions, and adducts do not affect the stability of the protein (Ehrenberg and Granath 1996; Skipper and Tannenbaum 1990). An extensive list of chemicals has been shown to form covalent adducts with hemoglobin such as the herbicides molinate (Zimmerman et al. 2002) and thiocarbamates (Zimmerman et al. 2004), as well as numerous industrial chemicals including 2-nitrotoluene (Jones et al. 2003), benzene oxide (Lindstrom et al. 1998), phosgene (Noort et al. 2000), acrylamide (Sumner et al. 2003, Fennell et al. 2003), butadiene monoxide (Moll et al. 2000) and styrene oxide (Basile et al. 2002). The use of hemoglobin adducts for biomonitoring of human exposure to hazardous chemicals has been used in several occupational settings to confirm such exposures.

Occupational exposures of workers in the polyacryl fiber industry to the common industrial solvent N,N-dimethylformamide (DMF) was determined by Angerer et al. (1998) by measuring hemoglobin adducts of the metabolite N-methylcarbamoyl. Tryptic digest of hemoglobin from exposed and unexposed workers was analyzed for adducts with LC/MS. Results showed that workers exposed to DMF during an 8-h shift possessed adduct levels 100 times higher than unexposed workers. This work indicated for the first

time that metabolism of DMF from occupational exposure can result in the formation of adducts at levels greater than the general population.

Another biomonitoring study based on hemoglobin adducts in workers exposed to styrene in the reinforced plastics industry was done by Fustinoni et al. (1998). They measured styrene adducts of cysteine and determined that exposed workers had adduct levels of 5.44 nmol/g as compared to 0.43 nmol/g in the unexposed control group. They concluded that background adducts in controls were due to cigarette smoking and only exposures at high concentrations would permit a conclusive relationship between styrene exposure and the formation of adducts.

Thier et al. (2001) monitored nitrobenzene exposure of eighty workers at an industrial nitrobenzene reduction plant by measuring adducts of aniline to hemoglobin. They routinely drew blood samples from workers and determined hemoglobin adducts using GC with a nitrogen-specific detector. They detected aniline-hemoglobin adduct levels in workers at about 1/20 of the exposure limits, which indicated that adduct formation could be used to monitor continuous low industrial aniline exposures in workers. Aniline exposure was also monitored with hemoglobin adducts at a rubber manufacturing plant in Niagara Falls, NY by Ward et al. (1996). They measured aniline and o-toluidine adducts to hemoglobin in 64 workers in the rubber department and 27 people working in other departments. Both adducts were identified in 72% of exposed and 52% of unexposed, but exposed workers had significantly higher levels of adducts. Post-shift urinary levels of aniline and o-toluidine were also significantly higher in exposed workers.

Begeman et al (2001) detected epoxybutene hemoglobin adducts in workers exposed to 1,3-butadine. Blood samples from workers at a petrochemical plant in Prague, Czech Republic, were collected after work shifts and hemoglobin adducts analyzed with GC/MS. Workers who were exposed to 440 $\mu g/m^3$ 1,3 butadiene showed significantly higher adduct levels (0.7 pmol/g) than controls (0.2 pmol/g).

Other industrial chemicals such as ethylene oxide and propylene oxide have been shown to form hemoglobin adducts in occupationally exposed workers (Boogaard et al. 1999). Workers at a glycol and glycol ether manufacturing plant wore personal air monitors during the work day and gave blood samples following maintenance activities that had potential for exposure to ethylene oxide and propylene oxide to determine internal and external exposure. A highly significant correlation was found between both ethylene oxide adducts ($P< 0.0001$) and propylene oxide adducts ($P=0.0004$) and the total inhalation exposure to these chemicals.

Biomonitoring Using Albumin Adducts

Albumin also provides a suitable protein for measurement of covalent adducts and chemical exposure biomonitoring. Like hemoglobin, large quantities of albumin can be easily obtained from blood samples, there is no repair mechanism, and the lifespan of albumin is about 16 days. The advantage of albumin adducts over hemoglobin adduct for biomonitoring is that human and rat albumin are nearly identical (Peters 1996) allowing results from *in vivo* rodent exposures to be extrapolated to human. Albumin has been shown to covalently bind xenobiotics such as nitrogen mustards (Noort et al. 2002), doxorubicin derivatives (Kratz et al. 2002), camptothecin derivatives (Warnecke and

Kratz 2003), acrylamide (Noort et al. 2003), auranofin (Roberts et al. 1996), chlorpyrifos oxon, dichlorvos, diisopropylfluorophosphate (Li et al. 2007), methyl parathion (Silva et al. 2004), pyridostigmine bromide, N,N-diethyl-*m*-toluamide , permethrin (Abu-Qare et al. 2002), and *cis*-dichlorodiammineplatinum (Gonais and Pizzo 1983). Albumin adducts have also been successfully used to monitor occupational exposure to industrial chemicals.

Sepia et al. (1995) obtained blood samples to measure albumin adducts in a group of 20 workers occupationally exposed to 4, 4'-methylenediphenyl diisocyanate vapor during the manufacture of polyurethane products. They measured albumin adducts with the metabolite 4, 4'-methylenedianiline using GC/MS and detected these adducts in 16 of the 20 workers at concentrations up to 120 fmol/mg.

Their et al. (2001) used albumin adducts to monitor nitrobenzene exposure in employees of a nitrobenzene reduction plant. Blood samples were taken from workers with potential skin contact to nitrobenzene and extracts of albumin hydrolysis lysates analyzed with GC for aniline. Although they found aniline-albumin adducts at detectable levels, they were significantly less than the hemoglobin adducts mentioned previously.

Yeowell-O'Connell et al. (1998) looked at the formation of albumin adducts with the benzene metabolite benzene oxide (BO) in occupational exposure worker in China. Benzene oxide was analyzed with GC-MS following acid cleavage of the adduct from albumin. Albumin adduct levels were significantly higher (2010 pmol/g) than controls (103 pmol/g) and adduct levels showed a significant correlation with levels of exposure. Rappaport et al. (2005) also used albumin adducts to investigate the production of BO and 1, 4-benzoquinone (1, 4-BQ) in workers exposed to benzene in China. Adducts were

measured in 160 benzene exposed workers who did not use respiratory protection. BO and 1, 4-BQ albumin adducts were measured in all subjects including controls, but levels of both adducts were 2.4-fold greater in those who did not wear respiratory protection.

Waidyanatha et al. (2004) investigated the utility of adducts formed by the reaction of the naphthalene metabolites naphthalene-1, 2-oxide, 1, 2-naphthoquinone (1, 2-NPQ), and 1, 4-naphthoquinone (1, 4-NPQ) with albumin as biomarkers of exposure to polycyclic aromatic hydrocarbons. Coke oven workers from China exposed via inhalation to PAHs were compared with steel industry workers (controls) and albumin adducts of 1, 2-NPQ and 1, 4-NPQ, but not of naphthalene-1, 2-oxide were detected in all workers. Only the adduct levels of 1, 2-NPQ were significantly higher in coke oven workers.

Protein Adduct Toxicity

Protein adducts may be of much more significance than being applied as a biomarker of exposure. Adduct formation could play a central in the observed toxicity of a reactive chemical. For all manifestations of chemical-induced toxicity, a series of cascading events must occur between ambient exposure and the observation of clinical disease. The formation of a covalent adduct with a protein following chemical exposure is a possible step in a chemical-induced toxicity, which could occur if the adduct disrupts the structure or function of the macromolecule. The list of xenobiotics known to form protein adducts is extensive and includes pesticides (Zimmerman et al. 2004), industrial chemicals (Boogaard 2002), and pharmaceuticals (Zhou et al. 2005). Metabolism of xenobiotics can generate electrophilic metabolites that can potentially react with numerous nucleophilic sites (cystiene, lysine, arginine, histidine, and methionine) on proteins forming a covalent bond. This bond is usually an irreversible modification and if

this occurs in a target cell, the protein adduct may be involved in the toxic mechanism of action associated with the xenobiotic. It has been suggested that covalent binding of a xenobiotic is the initiating event with some target organ toxicity (Hinson et al. 2000), but the mechanism of action is of the greatest importance when investigating the association between protein adducts and target organ toxicity. Protein adducts may result in toxicity by disrupting the normal function of the protein (Brame et al. 2004) , causing alterations in signaling pathways (LoPachin and Decaprio 2005), altering cellular localization of proteins (Hall et al. 1995), inhibition of enzymatic activity (Gupta et al. 1997), or altered immune recognition (Lung et al. 1990). A simplified sequence of events that could result in toxicity following exposure to an electrophilic xenobiotic is shown in Figure 3. Proteomic research can be applied to this scheme to gain data on which proteins are being adducted and which residues on these proteins are modified. Protein adducts may have no effect, but it is also possible that critical residues become modified. If so, this information could indicate which cellular pathways may be disrupted and be related to the observed toxicity. Therefore, identifying proteins that are modified by xenobiotic exposure may provide some insight to the mechanism of action associated with observed toxicity. The mechanism of action of ATRA with respect to reproductive dysfunction is not known, but the formation of ATRA based protein adducts may play a role.

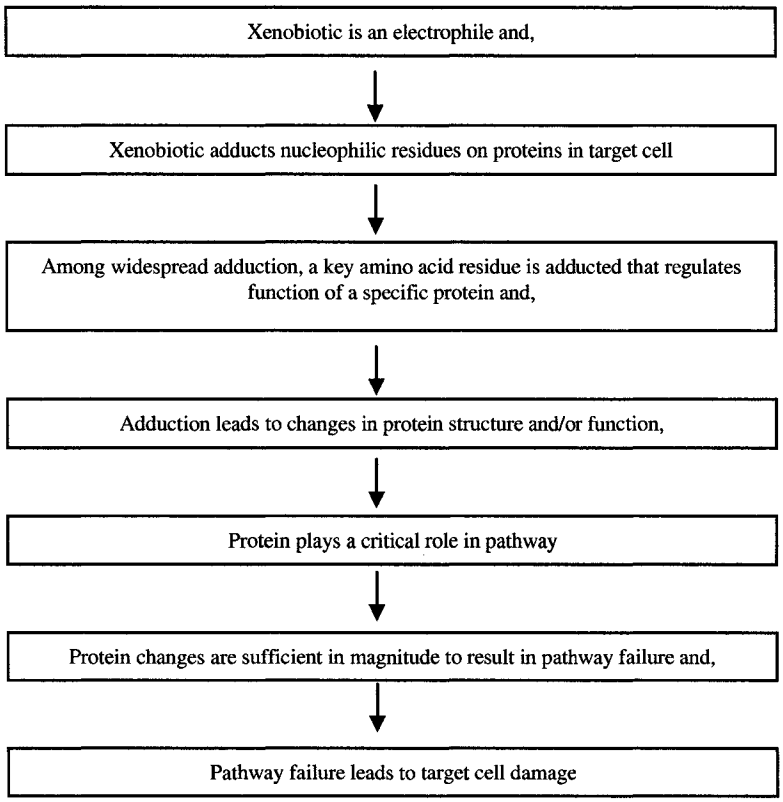

Figure 3. Possible sequence of events in chemical-induced toxicity resulting from covalent protein modification. (Adapted from LoPachin and DeCaprio 2005).

Proteomics and Mass Spectrometry

At any given time or condition, all proteins expressed by the genome of cells, tissues, or organisms are collectively termed the "proteome" (Wilkins et al. 1996). Proteomics is therefore defined as "any large scale protein based systematic analysis of the entire proteome or a defined sub-proteome from a cell, tissue, or entire organism" (Speicher 2004). This broad analysis can be very complicated as the proteome is constantly changing, unlike the genome, and is subject to changes due to developmental stage, disease state, or environmental conditions (Liebler 2002). Different cell types or

the same cells may express different proteomes at different times. No cell in any organism will contain all the proteins encoded by its genes together at one time, so the proteome represents a subset of all possible gene products.

In proteomics studies, analytical methods are used to identify proteins in a sample, develop protein expression profiles, map protein networks, and identify protein modifications. The two most commonly used tools in proteomics are 2-dimensional electrophoresis (2-DE) to separate proteins, and a mass spectrometer to generate peptide mass spectra data. A typical proteomics procedure would utilize 2-DE, excising spots of interest, enzymatic digestion of the proteins within the spots, MALDI-TOF MS analysis of the protein digest, and searching a protein database such as the National Center for Biotechnology Information (NCBI) with Mascot (Matrix Science search engine) to identify proteins within the spot. This general procedure is central to the field of proteomics as it can provide a snapshot of the proteins present in a specific proteome at a point in time and compared to the proteome under different conditions. From here, hypotheses may be generated as to the mechanisms of disease or toxicity as it relates to alterations in the proteome.

Central to the above mentioned proteomic procedure is the use of mass spectrometry to identify proteins. Beginning with the 1980's development of ESI and MALDI techniques, mass spectrometry has been widely applied to the characterization of primary structure in proteins. Among the early successful proteomic applications of mass spectrometry was the realization that by measuring molecular masses of the set of peptides produced by enzymatic digestion of a protein, one could produce a fingerprint suitable for use in identifying the original protein. Peptide Mass Fingerprinting has

become a powerful technique both for identifying proteins and for revealing the presence of post-translational modifications. Even more detailed sequence information became available by subjecting those peptides to tandem mass spectrometry (MS/MS). In a tandem mass spectrometer, selected peptide molecular ions are separated from all other peptides in a first stage mass analyzer and introduced into a region where they are allowed to fragment, usually by collision with neutral background gas molecules. The ionic fragments are extracted into a second stage analyzer where their masses are determined. It was soon confirmed that peptides fragment by predictable mechanisms leading to predictable series of ions, which are directly related to peptide amino acid sequence. Using the MS/MS spectra in a database search gives much more reliable protein identifications and facilitates homology searches for characterization of unknown proteins. MALDI generates peptide ions with a pulsed laser, producing packets of peptide ions. These ions are separated by mass as they traverse a short first stage time of flight analyzer and enter into a region of precisely timed gate pulses which deflect all ions except those of selected mass. Fragment ions stemming from the selected peptides are then re-accelerated into a high resolution second time of flight stage where their masses are measured with high accuracy. Fragments can be generated either by collisional activation or by metastable decay initiated during the MALDI process.

CHAPTER 2

Purpose and Scope

The objectives of this dissertation research was to determine if ATRA exposure causes the formation of covalent adducts with the intention of developing a biomarker of exposure and exploring the possibility the protein adduct may be responsible for ATRA toxicity. This research tested the hypothesis that **ATRA is metabolized to DACT, a reactive electrophile, which will react with nucleophilic protein residues such a Cys forming a stable covalent adduct with blood proteins that can be detected as a biomarker of exposure and can form adducts with proteins in the pituitary where ATRA causes suppression of LH release.** Each chapter of this dissertation addresses a specific aim in an attempt to answer the following questions related to ATRA induced protein adducts.

***SPECIFIC AIM 1*. Characterize and Identify ATRA Induced Hemoglobin Adduct**

Does ATRA exposure in rats cause the formation of a covalent adduct with hemoglobin?

Where is this adduct located?

Is ATRA or a metabolite responsible for the adduct formation?

***SPECIFIC AIM 2*. Characterize and Identify ATRA Induced Albumin Adduct as a Human Biomarker of Exposure**

Does ATRA exposure in rats cause the formation of a covalent adduct with albumin?

Where is this adduct located?

Is ATRA or a metabolite responsible for the adduct formation?

Can we develop a human biomarker of ATRA exposure based on albumin adducts?

***SPECIFIC AIM 3*. Identify Protein Adducts Within the Pituitary of ATRA Exposed Rats and DACT Exposed LβT2 Rat Pituitary Cells**

What proteins are modified in the pituitary of ATRA exposed rats?

What proteins are modified in LβT2 rat pituitary cells exposed to DACT?

CHAPTER 3

Identification of a Novel Hemoglobin Adduct in Sprague Dawley Rats Exposed to Atrazine

Greg P Dooley[†], Jessica E Prenni*, Pilar L Prentiss[†], Brian K Cranmer[†], Melvin E Andersen[‡], John D Tessari[†]

[1]Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO
*Macromolecular Resources, Colorado State University Core Laboratory, Fort Collins, CO
[‡]Chemical Industrial Institute of Toxicology, Research Triangle Park, NC

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Abstract

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) (ATRA) is one of the most commonly used herbicides in North America and is frequently detected in ground and surface waters. This research investigated possible covalent modifications of hemoglobin following *in vivo* exposures to ATRA in Sprague Dawley rats and *in vitro* incubations with diaminochlorotriazine. SD rats were exposed to 0, 10, 30, 100, and 300 mg ATRA/kg/day for 3 days via oral gavages and blood drawn at 0 h, 24 h, 72 h, 20 d, 1 m, and 2 m for globin analysis. Globin was purified from red blood cells, separated with high performance liquid chromatography, and analyzed with matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). An additional β globin peak was seen in exposed animals during the HPLC and MALDI-TOF MS analysis with a mass 110 Da greater than the normal β subunits. Tryptic digests of this β peak contained a peptide of 1449.9 m/z that corresponded to a modified peptide of amino acids 121-132. Mass spectrometry sequencing of this peptide indicated a 110 Da addition to Cys-125 of the major beta globin chain, which corresponds to a nucleophilic substitution reaction with a diaminochlorotriazine. *In vitro* incubations of SD globin and diaminochlorotriazine also resulted in a peptide of 1449.6 m/z that was identical in sequence to the modified peptide seen in the *in vivo* digest confirming the nucleophilic substitution mechanism of adduct formation. Exposures of SD rats to ATRA results in formation of an adduct that is easily detected and provides an analytical model for detection of triazine adducts in other macromolecules with sulfhydryl functional groups.

Introduction

ATRA (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) is one of the most commonly used herbicides in North America with 76.4 million pounds applied in the U.S. in 2002 (ATSDR 2003). It is used for control of broadleaf and grassy weeds, primarily in corn but also in sorghum, sugarcane, and recreational turf, by inhibiting photosynthesis. Environmental release of ATRA occurs with application to soils and subsequent runoff into surface or groundwater where ATRA and its metabolites are commonly detected (Colburn and Short 1999). From 1993-1995, ATRA was the most frequently detected pesticide in U.S. groundwater, found in 38.2% of sampling sites in the U.S. (Koplin et al. 1998). Thurman et al. (1991) focused only on the Midwest and found that immediately after application; the herbicide could be detected in 91% of water samples. ATRA levels exceeded the 3 ppb maximum contaminant levels set by the EPA in 52% of the samples. ATRA does not degrade significantly in groundwater and in surface waters has a half-life of >200 days (ATSDR 2003). ATRA also volatilizes into the atmosphere following the application to soil. Therefore, the general population is likely exposed to ATRA from drinking water or air, but increased occupational exposure risks exist for agricultural workers.

The primary health effects associated with ATRA exposure are reproductive and developmental abnormalities, while carcinogenesis data remain inconclusive. These abnormalities following ATRA exposure include delayed puberty in male Wistar rats (Stoker et al. 2002)), delayed mammary gland development in prenatal exposed Long Evans rat pups (Rayner et al. 2005), alteration of luteinizing hormone and prolactin serum level disrupting ovarian cycles in LE and Sprague Dawley rats (Cooper et al.

2000), and suppression of estradiol benzoate/progesterone induce LH surge (McMullin et al. 2004), neurotoxicity in dopaminergic systems in male LE rats (Rodriquez et al. 2005), and developmental immunotoxicity in male SD rats (Rooney et al. 2003).

ATRA is metabolized to diaminochlorotriazine (DACT), desethylatrazine (DEA), and desisopropylatrazine (DIA) as shown in Scheme 1. It first undergoes oxidative *N*-dealkylation via cytochrome P450 1A1/2 or 2B1/2 at either the ethyl or the isopropyl group at the 4- or 6-amino substituent. DACT is formed by an additional *N*-dealkylation, which removes the remaining alkyl group (Timchalk et al. 1990, Brzezicki et al. 2003). These intermediates may be capable of covalent binding to cellular macromolecules such as proteins.

An extensive list of chemicals have been shown to form covalent adducts with hemoglobin such as the herbicides molinate (Zimmerman et al. 2002) and thiocarbamates (Zimmerman et al. 2004), as well as numerous industrial chemicals 2-nitrotoluene (Jones et al. 2003), benzene oxide (Lindstrom et al. 1998), phosgene (Noort et al. 2000), acrylamide (Fennell et al. 2003) and styrene oxide (Basile et al. 2002). The analysis of protein adducts may provide an effective method for biomonitoring environmental exposure to chemicals and may provide estimates of internal dose of a xenobiotic. To our knowledge, there has been no research on the formation of ATRA adducts with macromolecules. This research is the first to investigate the possible reactivity of this widespread herbicide, using hemoglobin as a model for potential intracellular adduct formation. The purpose of this research was to investigate possible covalent modifications of hemoglobin following in vivo exposures to ATRA in Sprague Dawley

rats and to characterize adducts formed and specific amino acids modified both *in vivo* and *in vitro.*

Methods

Chemicals. ATRA (97.1% purity) was a gift from Syngenta (Research Triangle Park, NC). Acetonitrile, bromophenol blue, coomassie blue, dimethylsulfoxide, ethyl acetate, hexane, and ammonium bicarbonate were purchased from Fisher Chemical Company (Fair Lawn, NJ). Proteomic grade porcine trypsin, iodoacetamide, tris-HCl, glycerol, triflouroacetic acid, hydrochloric acid, and sodium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). 1-propanol was purchased from J.T Baker Chemical Co. (Phillipsburg, NJ). Methanol was purchased from Mallinckrodt Baker Inc. (Paris, KY). Polyacrylamide gels, sodium dodecylsulfate, dithiothreitol, and glycine were purchased from Bio-Rad Laboratories (Hercules, CA). Heparin was purchased from the Colorado State University Veterinary Hospital (Fort Collins, CO).

Animals. Eighteen jugular vein catheterized female Sprague-Dawley rats were purchased from Charles River Laboratories (Raleigh, NC), and housed in Colorado State University's central animal care facility, which is fully accredited by the American Association for Accreditation for Laboratory Animal Care. The rats were allowed to acclimate in ventilated cages for at least one week, during which time they were maintained on a 12-h light/dark cycle at a constant temperature of 25°C and humidity of 55%. All animals had free access to Teklad NIH-07 rodent diet and tap water.

In vivo exposure of Sprague Dawley rats to ATRA. The dosing experiment was carried out over a period of two weeks, with nine animals being treated per week. For each treatment set, there was one control rat and two rats in each dose group. Animals were

treated with 0, 10, 30, 100, and 300 mg ATRA/kg/day for 3d via oral gavages. Baseline control blood samples (0.3-0.5 ml) were collected through the indwelling jugular cannula of each animal the morning before dosing began. Following the first dose, blood samples (0.3-0.5 ml) were collected via the cannulae at 24h , 48h, and 72h. The catheter manipulation consisted of the following steps: 1) removing the cannula from the subcutaneous skin pocket, 2) removing the stainless steel plug, 3) withdrawing the heparinized glycerol lock solution, 4) drawing the blood sample into a 1-ml syringe prerinsed with 70 iu/ml heparin saline, 5) flushing the catheter with sterile saline, 6) replacing the glycerol lock and stainless steel plug, and 7) gently pushing the cannula back into the skin pocket. Disinfection measures were taken at all steps using 70% EtOH. The ten day, 1 month, and 2 month post-dose samples (0.5-ml) were collected by tail vein blood draw, as the cannulae were no longer patent. Tail vein draws required warming the animals under a heat lamp to increase blood flow, and then securing them in a plastic Decapicone restraint. Blood was drawn into a 1-ml syringe pre-rinsed with 70 iu/ml heparin saline. The animal was then released from the restraint and returned to its cage.

Purification of globin from whole blood. Samples were centrifuged at 1500 rpm for 10 minutes and the plasma pipetted off. RBC pellet was washed 3 times with 0.5 ml 0.9% NaCl, and lysed with 0.15 ml chilled DI water with gentle shaking for 1 min. 100 µl of each erythrocyte solution was added to eppendorf tubes containing 0.6 ml 50 mM HCl in propanol, and shaken gently. Samples were placed on ice for 30 minutes and then centrifuged for 10 minutes at 13,000*g* to remove cell components. Supernatant was removed and added to 400 µl of ethyl acetate, iced for ~60 mins, and centrifuged at 150*g* for 10 mins. To precipitate globin, samples were washed 3 times with 500 µl ethyl

acetate, centrifuged at 150*g* for 10 minutes and washed finally with 250 µl of *n*-hexane. Samples were then dried under nitrogen and stored at –20 °C.

Purified globin analysis with matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Globin samples were dissolved in water to make 100 µM solutions. One microliter of the globin solution was mixed with 1 µl of sinapinic acid matrix solution, which was then spotted on a MALDI target plate and allowed to air dry. MALDI-TOF MS analyses were performed on a Voyager DE-Pro (Perseptive Biosystems, Foster City, CA). Data were acquired in linear mode with delayed extraction and 25 kV acceleration voltage and external calibrations performed every 4 samples.

Purified globin analysis by HPLC. Globin samples at approximately 5 mg/ml (80% H_20 20% Acetonitrile (ACN) 0.1% TFA) were made fresh from frozen globin. Samples were analyzed on a Shimadzu LC-10AS HPLC equipped with a SPD-M10VP diode array detector. Forty microliters of sample were injected with an autosampler onto a reverse phase C_4 column (4.6 mm X 250 mm, 5 µm, 300 Å, Grace Vydac, Hesperia, CA). Globin subunits were eluted isocratically with 53.5 % H_20 46.5% ACN 0.1% TFA at a flow rate of 1 ml/min. Absorbance was measured at 203 nm and fractions were collected by hand. Shimadzu EZstart version 7.2.1 SPI software was used for data analysis.

Tryptic digestion of β subunits. Fractions collected from the HPLC analysis were concentrated to approximately 50 µl with a Speed Vac (Savant). Thirty microliters of each fraction was added to 20 µl of treatment buffer (10% Glycerol, 4% SDS, 0.25 M Tris-HCl, 0.2 mg/ml bromophenol blue) and boiled for 90 sec. Samples were loaded on a 12% Tris-HCl polyacrylmide gel (Bio-Rad) and run at 100 V for 1 hour. The gel was stained with coomassie blue for 15 mins and destained with 30% methanol. Bands were

excised with a razor blade and minced into 1 mm^2 pieces and placed in small Eppendorf tube. Gel pieces were washed twice for 15 mins (gentle vortex) with 100 µl 100 mM NH_4HCO_3/50% ACN, then 100 µl of 10 mM DTT added and incubated at 60°C for 30 mins. The supernatant was removed and add 100 µl of 55 mM iodoacetamide (IAA) added and incubate in the dark at room temperature for 30 mins to alkylate cysteine residues. Gel pieces were completely dried with a speed vac and 6 µl of 0.1 µg/µl trypsin solution and 34 µl 100 mM NH_4HCO_3 were added to the gel pieces and incubated overnight at 37 °C. Peptides were extracted from the gel pieces with 40 µl of 50% ACN (0.1% TFA) under a gentle vortex for 20 mins. This extraction step was repeated, and extracts were pooled and concentrated to approximately 2 µl. Peptides were further purified with Zip-tip (C_{18} Millipore) clean-up for mass spectral analysis. Briefly, tips were wetted with 50% ACN and equilibrated with 0.1% TFA in H_20. Peptides were bound by aspirating and dispensing the sample for 7-10 cycles and washed with 0.1% TFA in H_20. Peptides were eluted by aspirating and dispensing 5 µl of 50% ACN (0.1% TFA) three times into a clean vial.

Mass Spectrometry analysis of tryptic digest and peptide sequencing. One microliter of the tryptic peptide solution was mixed with 1 µl of matrix solution (10 mg/ml 50% ACN 0.1%TFA α-4-hydroxycinnamic acid) which was then spotted on a MALDI target plate and allowed to air dry. Mass spectrometry analysis was performed using an UltraFlex TOF/TOF (Bruker Daltonics, Billurica, MA). Spectra were acquired in reflector mode with a 25 kV acceleration voltage. External calibrations were performed every 4 samples using a Bruker Peptide Calibration Standard (Cat #22570, Bruker Daltonics, Billurica, MA)

In vitro exposure of purified globin to DACT. Sprague Dawley rat hemoglobin collected from control animals was used for the *in vitro* exposure to DACT. Ten milligrams of globin was dissolved in a degassed phosphate buffer (pH 7.4) and 10 mM DTT added to keep free thiols reduced. DACT was dissolved in DMSO and added to the globin solution for a final concentration of 90 ppm. Test tubes were sealed under nitrogen and solutions incubated at room temperature for 5 days. Globin was precipitated from the solutions as described in the whole blood purification section starting with the addition of 0.6 mL 50 mM HCl in propanol step. Purified globin was dissolved in water and subjected to the same in gel digestion procedure as described in the tryptic digestion of the β subunit section. Tryptic digests were analyzed with MALDI-TOF/TOF MS as described earlier. Control globin exposures were run under the same conditions with DMSO but without DACT.

Results

Purified globin analysis with HPLC. The chromatography of the purified globin effectively resolved the α (4.5 mins) and β subunits (7-11 mins), although the two alpha subunits were not resolved from each other (Figure 2). Total globin from rats 72 h following exposure to 300 mg/kg ATRA gives three β peaks that eluted at 7.4, 8.7, and 10.4 mins (Figure 2a), while total globin from control rats gives two β peaks at 8.8 and 10.4 mins (Figure 2b), The peak eluting at 7.4 mins was seen only in exposed rats prior to the two β subunits seen in both the controls and exposed rats. This subunit was suspected to be an ATRA modified β subunit. We investigated the nature of the 7.4 mins peak by collecting HPLC area data from all rats in the experiment at doses of 0 mg/kg, 10 mg/kg, 30 mg/kg, 100 mg/kg, and 300 mg/kg at 72 h post exposure and from rats exposed to 300

mg/kg at 0 h, 24 h, 48 h, 72 h, 10 d, 1 m, and 2 m post exposure. By measuring the area of each of the 3 β peaks and normalizing to percentage of the total area of the β peaks, we were able to identify two significant trends. The percentage of the 7.4 min. peak increases with dose as the percentage of the 10.4 mins decreases with dose (Figure 3). The 8.7 mins peak does not change with dose. Time course data (Figure 4) shows the 7.4 mins peak area increasing up to a maximum at 10 d and then decreasing to 0 h levels after 2 m. The 10.4 mins peak shows an inverse pattern decreasing from 0 h to 10 d and returning to 0 h levels after 2 months. These data also suggest the peak at 7.4 mins is a modified version of the peak at 10.4 mins that disappears as red blood cells are turned over after two months.

Mass spectrometry analysis of purified globin. The MS spectrum of control rat globin showed peaks corresponding to the α and β subunits at 15,205 m/z and 15,860 m/z respectively (Figure 5a). The spectrum of total globin from rats exposed to 300 mg/kg showed peaks corresponding to the α and β subunits at 15,213 m/z and 15,866 m/z respectively, as well as a peak at 15,974 m/z that was not seen in the control (Figure 5b). The difference between the β subunits peak and the 15,974 m/z peak in the exposed rats was ~ 109 m/z. The mass accuracy of the mass spectrometer at this mass range is ~1000ppm (+/- 16 Da).

Mass spectrometry analysis of β globin subunits. The MS spectrum of the 7.4 mins peak fraction in rats exposed to 300 mg/kg contains a peak at 15,993 Da (Figure 6b), while the spectrum of the same fraction in controls contains no peaks in the 16,000 Da range (Figure 6a.). The spectrum of the 8.8 min peak in control rats has a peak at 15,914 Da (Figure 6b), as compared to the spectrum of the 8.8 min peak in the rats exposed to 300

mg/kg which contains peaks at 15,851 Da (Figure 6e). The spectrum of the 10.4 min peak fraction in control rats contains a peak at 15,858 Da (Figure 6c), which is similar to the peak found in the spectrum of the 10.4 min fraction peak in rats exposed to 300 mg/kg at 15,869 Da (Figure 5f). The mass difference of the 7.4 and 10.4 min peaks in the exposed rats was 124 ± 32 Da, suggesting that the 7.4 min peak fraction may contain the same subunit as 10.4 min peak with an adduct similar to original data showing a 110 mass addition.

Tryptic digestion of β subunits. Tryptic digestion of the 7.4 and 10.4 min HPLC fractions from rats exposed to 300 mg/kg were analyzed by mass spectrometry (MS and MS/MS) followed by a MASCOT peptide mass fingerprint search (Perkins et al. 1999). Both fractions were significantly identified by MASCOT as *Rattus norvegicus* hemoglobin β chain major form, with protein sequence coverage of 83% and 91% for 7.4 and 10.4 min peaks, respectively. Further comparison with the known sequences of the β isoforms (Ferranti et al. 1993) indicated our sequence coverage matched exactly the sequence for the β_3 isoform for both fractions. These results indicate that the 7.4 and 10.4 min fractions most likely contain the same β_3 isoform subunit. The missed peptides in the 7.4 min peak digest were amino acids 60-65, 77-82, and 121-132, while the 10.4 digest missed only amino acids 60-65 and 77-82. The peptide corresponding to amino acids 121-132 contains the reactive Cys-125 residue and has a mass of 1340 Da (Ferranti et al. 1993). A comparison of the MS spectra of 7.4 and 10.4 min peak digests (Figure 7) shows the alkylated version of this peptide at 1397.7 m/z only in the10.4 min fraction. Unique to the 7.4 spectrum is a significant peak 1449.9 m/z, which corresponds to a 110 mass addition to the unmodified peptide (1340 Da) most likely from a triazine adduction.

This 1449.9 m/z peak is absent in all spectra of the control rats at all time points during the experiment. In the high dose groups (100 mg/kg and 300 mg/kg), the 1449.9 m/z peak was present from 24h post exposure to 1 month and absent at 0h and 2 months. The lower dose groups (10 mg/kg and 30 mg/kg) had more inconsistent data on the presence of the 1449.9 m/z peak. In the 10 mg/kg group, the peak was found in the spectrum of one rat at 48h, 72h, and 10d. In the 30 mg/kg dose group, the peak was found in the spectra for all three rats at 72h and 10d, and in 2 out 3 rats after 1 m.

Mass Spectrometry peptide sequencing. The amino acid sequence of the peak at 1449.9 m/z was confirmed by matching the experimental MS/MS fragmentation spectrum to the known sequence of the 121-132 peptide (Ferranti et al. 1993) of the *Rattus norvegicus* hemoglobin β_3 isoform (Figure 7c). Using an unmodified cysteine, the y-ions (charged carboxy-terminal fragments) and b-ions (charged amino terminal fragments) were matched in the experimental spectrum only up to Cys-125, which indicated a possible modification at this point in the sequence (data not shown). Adding the triazine adduct mass of 110 Da to Cys-125 resulted in a perfect match of the sequence to the experimental spectrum with complete coverage of both the y and b-ion series (Figure 8). This result is strong evidence that Cys-125 has a mass addition of 110 Da. In addition, the MS/MS spectrum of the peak at 1397.7 m/z from the control rats showed complete coverage of y and b-ions using the sequence of the 121-132 peptide with a standard alkylation (58 Da) on Cys-125 (Figure 7d and Figure 8).

Tryptic digestion and mass spectrometry of globin exposed to DACT in vitro. The tryptic digest of α and β globin exposed to 90 ppm DACT contained a 1449.6 m/z peak that was not observed in α and β globin exposed DMSO (Figure 9). The MS/MS spectrum of this

1449.6 m/z peak matched that obtained from *in vivo* exposures to ATRA. Additionally, using the triazine adduct mass of 110 Da to Cys-125 resulted in a perfect match of the 121-132 peptide sequence to the experimental MS/MS spectrum with complete coverage of both the y and b-ion series (Figure 9c).

Discussion

Environmental chemicals can be metabolized to reactive intermediates that are capable of forming covalent adducts with cellular macromolecules. Electrophilic metabolites can potentially react with numerous nucleophilic sites (cystiene, lysine, arginine, histidine, and methionine) on proteins forming a covalent adduct. The list of xenobiotics known to form protein adducts is extensive and includes pesticides (Zimmerman et al. 2004), industrial chemicals (Boogaard 2002), and pharmaceuticals (Zhou et al. 2005). In recent years, xenobiotic adducts of hemoglobin and albumin has become increasingly utilized in exposure assessments since samples are readily obtainable (Waidyanatha et al. 2004, Their et al. 2001, Begemann et al. 2001, Kafferlein and Angerer 2001, Van Sittert et al. 2000). We believe the analysis of protein adducts may provide an effective method for biomonitoring of environmental exposures to chemicals such as ATRA.

In our experiments, HPLC analysis of globin samples from Sprague Dawley rats exposed to ATRA showed a peak (7.4 mins) that eluted earlier than the two β peaks (8.8 and 10.4 mins) seen in control animals. This 7.4 peak was not seen in controls and was not seen at 0h or 2m for exposed animals. The peak presented itself in a dose dependent increase that coincided with a dose dependent decrease in the 10.4 min peak. This relationship was also seen over the course of the experiment. After 48h, the 7.4 min peak

was significantly increased from 0h, reaching a maximum at 10d and decreasing back to 0h levels after 2m. The 10.4 min peak showed a significant decrease to 10d and returned to 0h levels at 2m. These data indicate the 7.4 min peak is a modified version of the 10.4 min peak where the modification caused a shift in chromatographic behavior that decreased the area of the 10.4 min peak. The decrease in retention time of the 7.4 min peak may be due to the addition of a polar triazine adduct. The decrease in the7.4 min peak from 10d to 2m is likely due to red blood cell turnover (lifespan of 60 d in rats) causing the modified β peak to be replaced, and subsequent increase in the 10.4 min peak from 10d to 2m.

Mass spectrometry analysis of the 7.4 min peak showed a peak of 15,993 m/z consistent with mass addition modification of the 15,869 m/z 10.4 min peak. Tryptic digestion of the 7.4 and 10.4 min peaks yielded the expected protein sequence of the *Rattus norvegicus* hemoglobin β_3 isoform, however, the 7.4 min peak digest was missing the peptide corresponding to amino acids 121-132 of this β_3 chain. This peptide has a mass of 1340 Da and contains Cys-125, which as been shown to form a covalent adduct with xenobiotic chemicals (Zimmerman et al. 2002, Zimmerman et al. 2004, Erve et al. 2000, Hughes et al. 1981). Rossi et al (1998) also showed that Cys-125 in hemoglobin from Sprague-Dawley rats was approximately 4000 times more reactive towards DTNB (5,5-dithio-bis-2-nitrobenzoic acid) than glutathione. Examination of the 7.4 min peak digest spectrum in the region of 1400 m/z to 1500 m/z revealed a large peak at 1449.9 m/z that was not present in the 10.4 min peak digest spectrum and is suggestive of the 1340 Da peptide with a 110 Da modification. This was consistent with our MS data for the whole globin that showed a peak 108 m/z greater than the β subunits. Analysis of the

MS/MS spectrum of the 1449.9 m/z peak showed a fragmentation pattern which matched that of the known sequence for amino acids 121-132 with a 110 Da adduct on Cys-125. These results are very strong evidence that the 7.4 min peak is a modified β_3 isoform with a 110 Da adduct from the ATRA exposures. The 7.4 min peak digests from all time points and doses indicated that the 1449.9 m/z adduct peak is readily detected with 30 mg/kg exposures at 72h and 10 days, and the highest adduct levels are seen in the 300 mg/kg dose group. This peak was also seen in one rat with 10 mg/kg exposure at 48h-10d, but this may be due to variability in the dosing regime or metabolic variability. The lowest reliable dose that produced the ATRA adduct in this experiment was 30 mg/kg.

Phase I metabolism of ATRA is cytochrome P-450 mediated with N-dealkylation at the ethyl or the isopropyl group to desethylatrazine or desisopropylatrazine. Additional *N*-dealkylation removes the remaining alkyl group forming DACT (Timchalk et al. 1990, Brzezicki et al. 2003). Phase II metabolism is via glutathione conjugation resulting in nonchlorinated metabolites (Scheme 1). The principal metabolite DACT is thought to be responsible for the *in vivo* hemoglobin adduct seen in this study. The absence of ATRA adducts and adducts of desethylatrazine or desisopropylatrazine is likely due to rapid metabolism to DACT. McMullin et al. (2003) showed complete metabolism of ATRA to DACT within 48h of a single 90 mg/kg ATRA dose in SD rats. This adduct formation was confirmed with *in vitro* incubation of globin obtain from control rats and DACT. Tryptic digestion and MS/MS analysis of this globin indicated that Cys-125 is modified with a 110 Da mass addition following DACT incubation. The MS/MS fragmentation of the peak at 1449.6 m/z seen in the DACT exposed globin was identical to that of the 1449.9 m/z peak of the modified β subunit seen in the *in vivo* ATRA exposures. This

result clearly indicates the Cys-125-triazine adduct that forms *in vivo* can also be formed *in vitro* with exposure to DACT. The proposed mechanism of the triazine adduct formation (Scheme 2) is via nucleophilic aromatic substitution. Only the chlorinated metabolites would provide a carbon center with partial positive charge favoring this nucleophilic displacement. Since a dechlorinated diaminotriazine has a mass of 110 Da, the modified Cys-125 is hypothesized to have the structure shown in Figure 10. Although the chemical structure of this adduct was not positively identified, this is the most realistic option for an adduct of this size from ATRA metabolism. Typically NMR would be useful to determine the chemical structure, but the lack of protons would result in very low sensitivity and would not be practical. Further investigation is needed to conclusively identify the structure of this adduct.

Because humans lack Cys-125 in their hemoglobin, this adduct is not directly applicable to human biomonitoring, but it does provide evidence that adducts could be formed with other suflhydryl containing proteins such as albumin, which is directly applicable to human biomonitoring. McMulllin et al. (2003) measured radioactivity in rat blood after treatment with C^{14}-ATRA and found evidence of covalent binding to plasma proteins, possibly adduction of ATRA or metabolites to albumin. Human and rat albumin both contain a Cys-34 residue that is solvent accessible similar to Cys-125 in rat hemoglobin. The Cys-34 residue has been shown to covalently bind xenobiotics such as nitrogen mustards (Noort et al. 2002), doxorubicin derivatives (Kratz et al. 2002), camptothecin derivatives (Warnecke and Kratz 2003), acrylamide (Noort et al. 2003), auranofin (Roberts et al. 1996), and *cis*-dichlorodiammineplatinum (Gonias and Pizzo 1983). Since human and rat albumin are nearly identical and both contain this Cys-34,

results from measuring ATRA adducts at this Cys-34 residue in *in vivo* rodent exposures could be extrapolated to humans for developing a human biomarker of ATRA exposure.

Conclusions

These results show that ATRA metabolism in Sprague Dawley rats yields a product capable of forming a covalent adduct with cysteine residues of hemoglobin and possibly other proteins with sufhydryl functional groups. This stable adduct has a mass of 110 Da and is found on Cys-125 of the major beta subunit. The structure of this adduct is not known, but based on the mass the anticipated structure is a dechlorinated diaminotriazine. This research also provides an analytical model for detection of ATRA/protein adducts from blood. The analysis of other protein adducts with exposed cystiene residues, possibly albumin, may provide an effective method for biomonitoring environmental exposure to ATRA or other chlorinated xenobiotics. The adduct discovered in this study clearly indicates exposure to ATRA results in a chemical interactions with cellular macromolecules. Using protein adducts to measure exposure at the molecular level can provide a more applicable internal dose estimate than using ATRA concentrations from air, water, or soil for biomonitoring or risk assessments.

Acknowledgements

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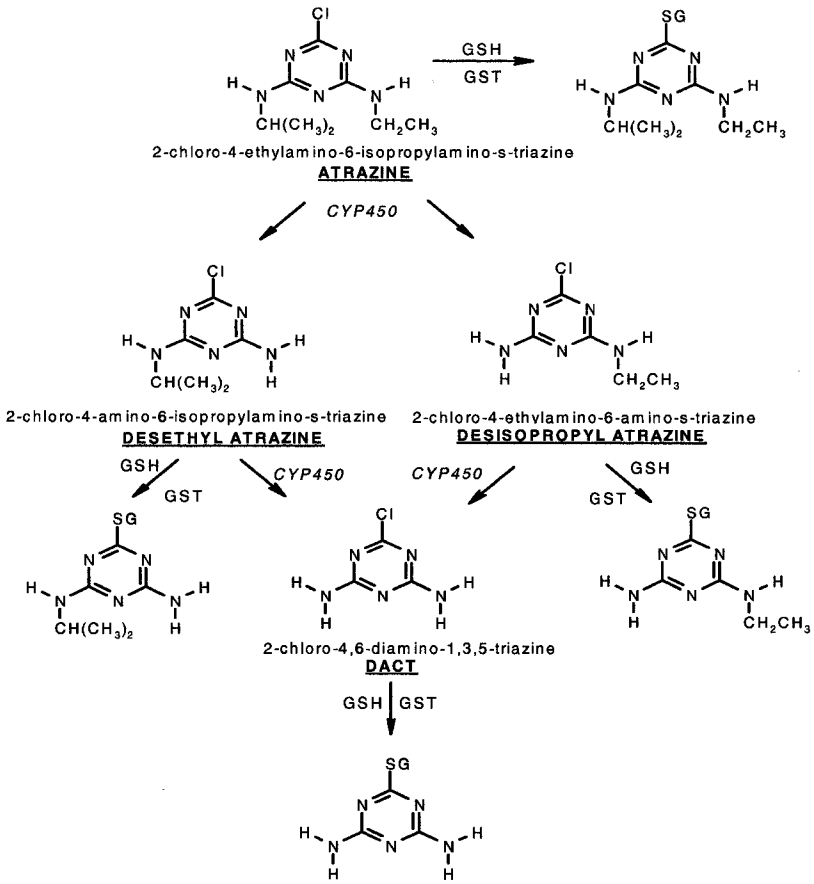

Figure 1. Proposed ATRA metabolism in the rat model (adapted from McMullin et. al 2003).

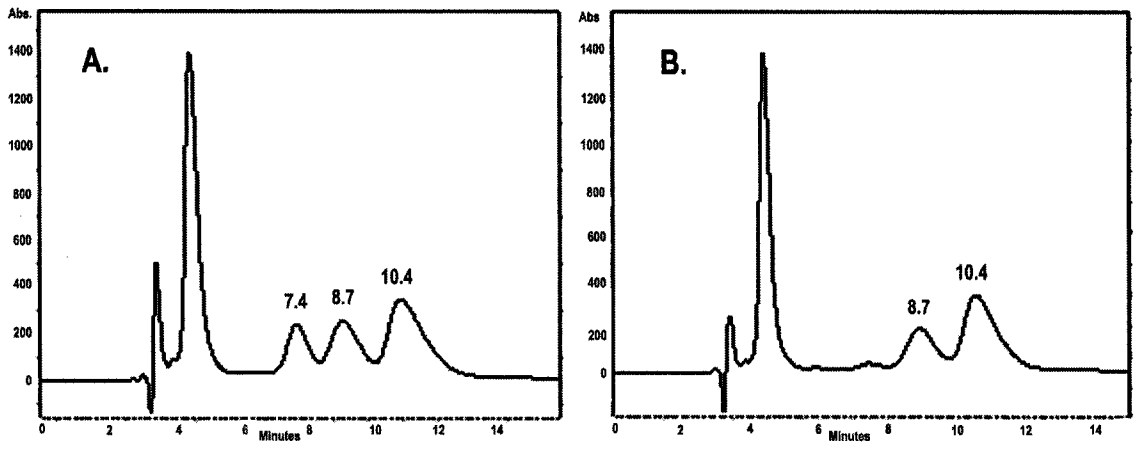

Figure 2. HPLC separation of Sprague Dawley rat globin with a Vydac C_4 reversed phase column and detection at 203 nm. The α subunits eluted at 4-5 mins and β subunits eluted 7-12 mins. (A.) 72h post-exposure to 300 mg/kg and (B.) control 72 h. *Subsequent MALDI-TOF-MS analysis of the β_1 peak retention time in the control samples showed no peak in the 16,000 Da range.

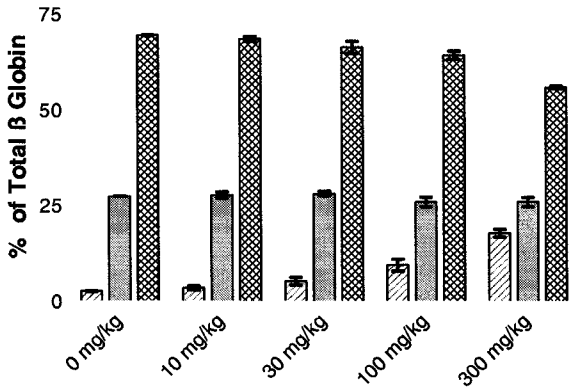

Figure 3. Relative percentage of each β subunit over the dose range of the experiment in Sprague Dawley rats 72h following exposure. (β_1), (β_2), (β_3)

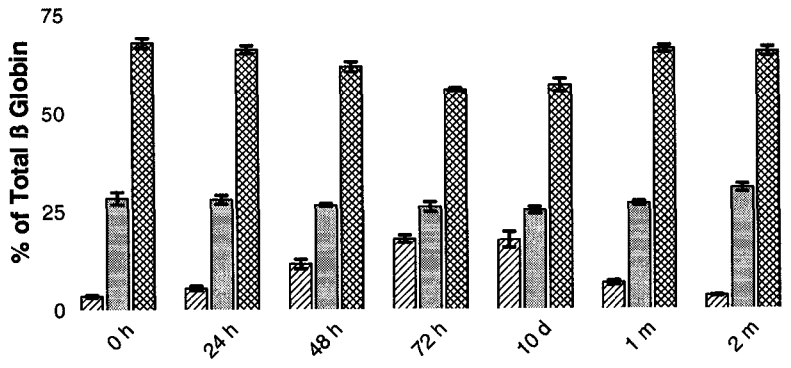

Figure 4. Relative percentage of each β subunit over the time course of the experiment in Sprague Dawley rats exposed to 300 mg/kg ATRA. (β_1), (β_2), (β_3)

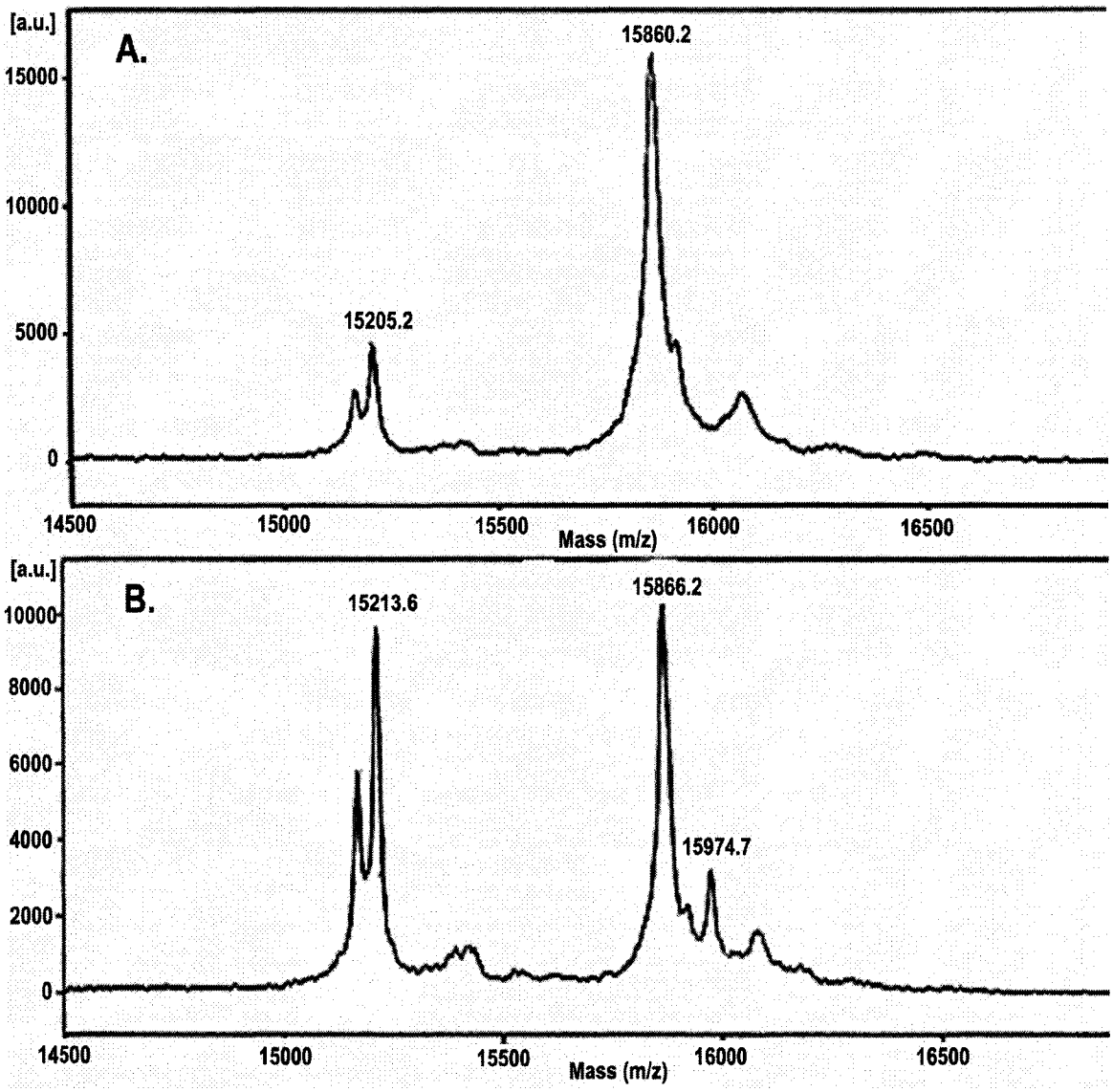

Figure 5. MALDI-TOF MS analysis of globin isolated from whole blood of female Sprague Dawley rats. (A. control, 72h) (B. 300 mg/kg ATRA, 72 h). The peaks in spectrum B at 15,974 Da corresponds to a modified β subunit with a mass addition of ~109 that was not seen in controls.

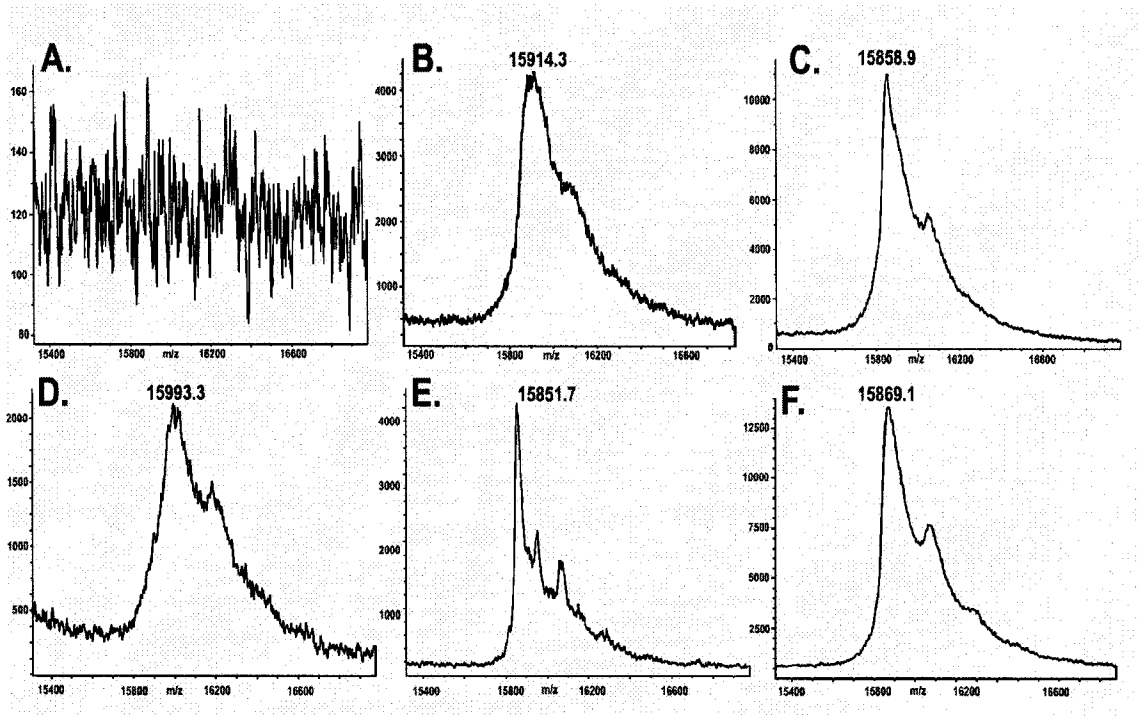

Figure 6. MALDI-TOF-TOF MS spectra of the β subunits from control Sprague Dawley rats at 72h. (A. β_1 retention time), (B. β_2), (C. β_3) and 72h post-exposure to 300 mg/kg. (D. β_1), (E. β_2), (F. β_3).

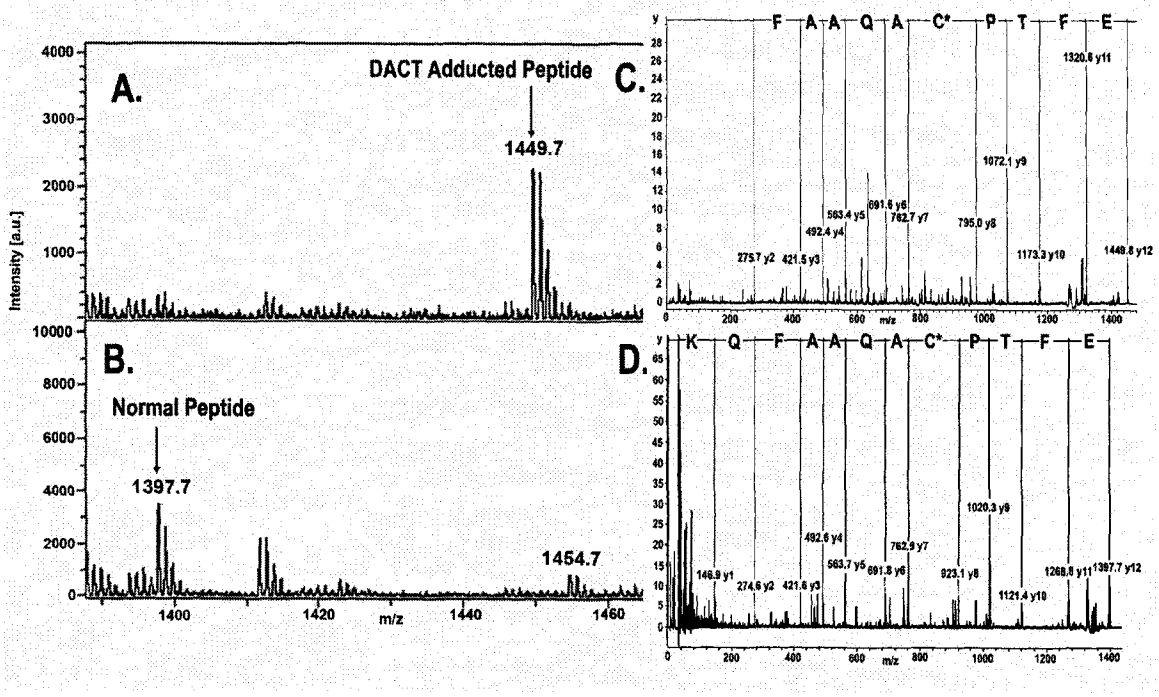

Figure 7. MALDI-TOF-TOF MS spectra of tryptic digest of selected β subunits from Sprague Dawley rats 72h post-exposure to 300 mg/kg. (A. β_1) and (B. β_3). MALDI-TOF-TOF MS/MS sequencing of the (C.)1449.9 Da peak and the (D.) 1397.7 Da peak gave identical sequences with a 110 Da addition to the Cys-125 in the 1449.9 Da peptide.

A.	y-ions sequence	EFTPC*AQAAF
	b-ions sequence	TPC*AQAAF
	Known sequence of 121-132	EFTPCAQAAFQK
B.	y-ions sequence	EFTPC#AQAA
	b-ions sequence	EFTPC#AQAAFQK
	Known sequence of 121-132	EFTPCAQAAFQK

Figure 8. Sequences determined by MALDI-TOF-TOF-MS/MS for the (A.) 1449.9 Da peak with * indicating a mass addition of 110 Da to Cys-125 and (B.) 1397.7 Da peak with # indicating a mass addition of 58 Da to Cys-125 from the acetylation reaction.

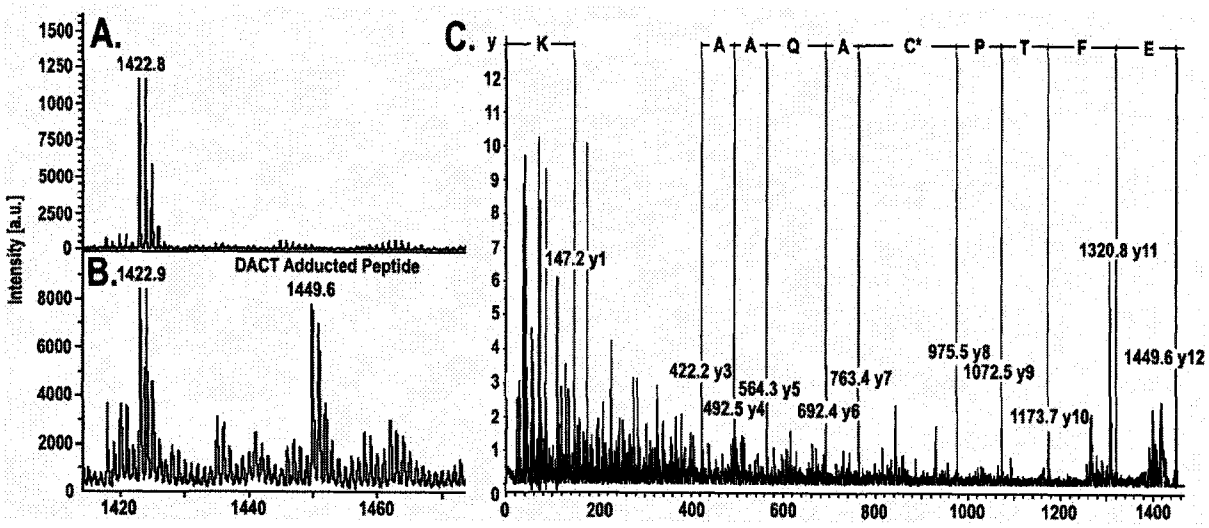

Figure 9. MALDI-TOF-TOF MS spectra of tryptic digest of Spraque Dawley rat globin from *in vitro* exposures (A. DMSO control) and (B. 90 ppm DACT). MALDI-TOF-TOF MS/MS sequencing of the 1449.6 Da peak from DACT exposured globin gave a sequence of EFTPC*AQAA with Cys-125* modified by a 110 Da triazine adduct.

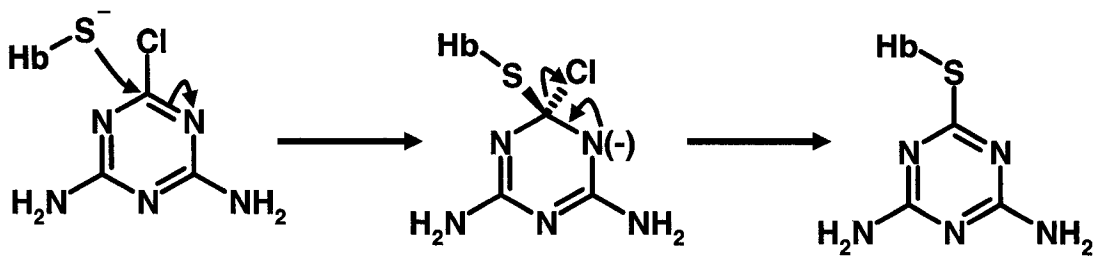

Figure 10. Proposed nucleophilic aromatic substitution reaction for adduct formation as the nucleophilic cysteine residue of hemoglobin attacks the chlorine of the diaminochlorotriazine.

CHAPTER 4

Development of an Immunochemical Detection Method for Atrazine-Induced Albumin Adducts

Greg P. Dooley[†], Melvin E. Andersen[‡], David L. Carbone[†], William H. Hanneman[†], Marie E. Legare[†], and John D. Tessari[†] *

Department of Environmental and Radiological Health Sciences,
Colorado State University, Fort Collins, CO
‡Chemical Industrial Institute of Toxicology, Research Triangle Park, NC

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Abstract

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) is one of the most commonly used herbicides in the United States. Exposures in rodent models have lead to a host of biological effects, most notably the suppression of luteinizing hormone surge. Previously, we have reported that diaminochlorotriazine (DACT), an ATRA metabolite, forms a covalent adduct with rat hemoglobin at Cys-125. In the present study, we investigated the formation of a similar covalent adduct at Cys-34 of rat and human albumin following ATRA exposure using MALDI-TOF-TOF MS and adduct specific immunochemical detection. Using mass spectrometry, a covalent adduct with a mass of 110 Da was located on Cys-34 of albumin from rats exposed to 20, 50, 100, and 200 mg/kg ATRA as well as rat and human albumin exposed *in vitro* to 90 ug/ml DACT. Based on the formation of the adduct *in vitro*, the adduct structure is a dechlorinated diaminochlorotriazine. To further study this unique protein adduction, we developed a polyclonal antibody specific for the DACT adduct and report its use for immunochemical detection. We detected adduct formation in purified serum albumin samples from rats given 5, 10, 20, 50, 100, and 200 mg/kg ATRA as well as rat and human albumin exposed *in vitro* to 90 ug/ml DACT by using immunochemical analysis. No adducts were detected in control animals or in the *in vitro* controls using our immunochemical detection method. In summary, these data report the development of a novel immunochemical detection system that could provide a rapid screening methodology for the detection of ATRA in exposed human populations.

Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) (ATRA) is one of the most commonly used herbicides in the U.S., with 57.4 million pounds applied in 2005 primarily to corn for control of broadleaf and grassy weeds (*1*). ATRA and its metabolites are commonly detected in surface or groundwater where it has a half-life of greater than 200 days (*2-4*). The general population is potentially exposed to ATRA from drinking water; however, occupational exposure among agricultural workers is increased (*5*). ATRA is rapidly metabolized in the body to diaminochlorotriazine (DACT) by P450 enzymes in the liver (12) and data from *in vivo* treatments of Sprague Dawley (SD) rats show high concentrations of DACT in the plasma and brain shortly after exposure (*13*). Although the human health effects associated with ATRA exposure are not fully characterized, ATRA exposure in rodent models has identified reproductive and developmental abnormalities. These abnormalities include: delayed puberty: (*6*), delayed mammary gland development (*7*), disruption of ovarian cycles (*8*), suppression of luteinizing hormone surge (*9*), neurotoxicity in dopaminergic systems (*10*), and developmental immunotoxicity (*11*).

We have previously shown that DACT is capable of forming covalent adducts with Cys-125 of rat hemoglobin via nucleophilic aromatic substitution (*14*). Since humans lack Cys-125 in their hemoglobin, the adduct we discovered is not directly applicable to human biomonitoring, but it does provide evidence that ATRA adducts could be formed with other proteins containing free thiol groups. Human and rat albumin have high sequence homology (73%) (*15*) and both contain a free thiol group (Cys-34) that is solvent accessible similar to Cys-125 of rat hemoglobin.

Albumin is the most abundant serum protein (60%) and serves as a circulatory transporter for several ligands including nitric oxide (*16*), long chain fatty acids (*17*), bilirubin and metal ions (*18*), and many exogenous drugs (*19*). Albumin contains 17 disulfide bonds and one free thiol (Cys-34) (*15*). This free thiol has a pK_{SH} of 5.0 (*19*) and under physiological conditions would be found as a thiolate anion and can act as a nucleophile forming covalent adducts with electrophilic molecules. Cys-34 has been shown to covalently bind xenobiotics such as nitrogen mustards (*20*), doxorubicin derivatives (*21*), cisplatin (*22*), camptothecin derivatives (*23*), acrylamide (*24*), auranofin (*25*), and *cis*-dichlorodiammineplatinum (*26*). McMullin et al. (*13*) measured radioactivity in rat blood after treatment with C^{14}-ATRAand found evidence of covalent binding to plasma proteins, suggesting adduction to albumin. Based on these reports and the modification Cys-125 of hemoglobin by DACT, Cys-34 is a good candidate for DACT adduct formation. We therefore propose that albumin Cys-34 in rats and humans will form a covalent adduct with DACT following exposure that can be detected as a biomarker of ATRA exposure. Current suggested biomarkers for ATRA exposure focus on detection of the parent compound or metabolites in biological fluids. However, by detecting protein adducts, we provide a better method for exposure because we can demonstrate that an internal dose of ATRA has occurred. Furthermore, this method provides the potential for monitoring long-term exposures.

The purpose of this research was to detect and locate a DACT adduct at the Cys-34 residue of albumin from rats exposed to ATRA and to validate the use of a DACT adduct specific antibody in a detection methodology. With characterization and detection of the adduct in the rodent model, it will be possible to develop biomarker protocols for

detecting this Cys-34 DACT adduct in human albumin from potentially exposed populations, thus allowing for the detection of an internal dose.

Methods

Chemicals and Materials. ATRA(97.1% purity) and diaminochlorotriazine (97.6% purity) were a gift from Syngenta (Research Triangle Park, NC). Unless otherwise stated, all reagents were purchased from Fisher Scientific (Fair Lawn, NJ). Proteomic grade porcine trypsin, thermolysin, iodoacetamide, Tris-HCl, glycerol, trifluoroacetic acid, rat and human albumin, and sodium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol was purchased from Mallinckrodt Baker Inc. (Paris, KY). Polyacrylamide gels, sodium dodecylsulfate, dithiothreitol, tris-base, and glycine were purchased from Bio-Rad Laboratories (Hercules, CA). Antibodies recognizing the DACT adduct were custom made by Strategic Biosolutions (Newark, DE) and the horseradish-peroxidase conjugated anti-rabbit secondary antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

Animals. Twenty four female Wistar rats (~250g) were purchased from Charles River Laboratories (Raleigh, NC), and housed in Colorado State University's central animal care facility, which is fully accredited by the American Association for Accreditation for Laboratory Animal Care. Animals' welfare was maintained following an Animal Care and Use Research Protocol approved by the Colorado State University Animal Care and Use Committee. The rats were allowed to acclimate in ventilated cages for at least one week and all animals were ovariectomized one week later. During this time, they were maintained on a 12-h light/dark cycle at a constant temperature of 25°C and humidity of 55%. All animals had free access to Teklad NIH-07 rodent diet and tap water.

***In vivo* exposure of Wister rats to atrazine.** Twelve animals were treated over a period of two weeks. For each treatment cohort, there were three animals in each dose group. Animals were treated with 0, 5, 10, and 20 mg atrazine/kg/day via oral gavage for 4 days and sacrificed via decapitation. A second cohort of animals were treated with 0, 50, 100, and 200 mg atrazine/kg/day via oral gavage for 4 days and sacrificed via decapitation.

Purification of Albumin from Whole Blood. Blood was collected in 50 ml Falcon tubes from ATRA exposed rats at all doses via decapitation. Albumin was purified from the plasma as described by Bechtold et al. (*27*). A 10 µg albumin solution was mixed 1:1 with loading buffer (10% Glycerol, 4% SDS, 0.25 M Tris-HCl, 0.2 mg/ml Bromophenol Blue), and boiled for 90 sec. Purity was confirmed through SDS-PAGE using a 12% Tris-HCl gel (Bio-Rad, Hercules CA) followed by staining with Coomassie blue for 15 mins and destaining with 30% methanol until clear.

Thermolysin Digestion of Albumin. Following SDS-PAGE, the albumin band was excised with a razor blade, minced into 1 mm^2 pieces, and placed in a microcentrifuge tube. Gel pieces were washed twice for 15 mins (gentle vortex) with 100 µl 100 mM NH_4HCO_3/50% acetonitrile (ACN), then 100 µl of 10 mM dithiothreitol (DTT) was added and tubes incubated at 60°C for 30 mins. The supernatant was removed and 100 µl of 55 mM iodoacetamide (IAA) added prior to incubation at room temperature for 30 mins to acylate free cysteine residues. Gel pieces were completely dried, 6 µl of 0.1 µg/µl thermolysin solution and 34 µl 100 mM TrisHCl/10 mM $CaCl_2$ added, and incubated at 65°C for 2h. Peptides were eluted from the gel pieces with 40 µl of 50% ACN (0.1% triflouroacetic acid, TFA) under a gentle vortex for 20 mins. Elutions were repeated, pooled, and concentrated to ~2 µl. Peptides were further purified with a Zip-Tip

(C_{18} Millipore) clean-up for mass spectral analysis. One microliter of the thermolysin peptide solution was added to 1 μl of a 10 μg/ml 50% ACN 0.1% TFA α-4-hydroxcinnamic acid matrix solution, spotted on a MALDI target plate, and allowed to air dry. MALDI-TOF/TOF MS and tandem MS/MS analyses were performed at the Macromolecular Resource Facility at Colorado State University on an UltraFlex TOF/TOF (Bruker Daltonics, Billurica, MA) and data acquired in the reflector mode with a 25 kV acceleration voltage.

***In vitro* Exposure of Rat and Human Albumins to DACT**. Rat and human albumin were used for separate *in vitro* exposures to DACT. Two milligrams of albumin was dissolved in 2 ml of degassed phosphate buffer (pH 7.4) with 10 mM DTT. DACT was dissolved in DMSO and added to the albumin solution for a final concentration of 90 ug/ml DACT and 5 μl/ml DMSO. Test tubes were sealed under nitrogen and solutions incubated at room temperature for 5 days. Twenty microliters of the *in vitro* solution was subjected to the same electrophoresis procedure and analyzed as described above. Control albumin exposures were run under the same conditions with DMSO but without DACT.

Immunization Protocol. The DACT antigen was created at Strategic Biosolutions by conjugating DACT to the carrier protein keyhole limpet haemocyanin (KLH) via a spacer linkage to the 6 position on the triazine ring. This is the expected orientation of the adduct generated *in vivo* (*14*). A polyclonal antibody against the DACT antigen was generated by immunizing 2 female New Zealand rabbits with the DACT-KLH conjugate by Strategic Biosolutions, (Newark DE). Each rabbit was initially injected subcutaneously with 200 μg antigen in complete Freund's adjuvant. Antigen boost of 250

μg antigen in incomplete Freund's adjuvant were given on days 14, 28, and 49. Production bleeds of 22.5 ml of serum were done on days 42, 56, 63, and at termination on day 70. All production bleeds were pooled and an antibody titer of 2,076,000 measured with ELISA. The antibody was affinity purified from pooled serum against a column matrix with DACT bound through the 6 position on the triazine ring.

Western Blot of Purified Albumin with DACT Adduct Antibody. Purified albumin (10μg) from each ATRA exposure dose was loaded on a 12% Tris-HCl polyacryamide gel (Bio-Rad, Hercules, CA) and electrophoresed for 1 h at 140 V. The albumin was then transferred to a PVDF membrane at 15V for 1h using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). Following transfer, the membrane was washed in TBS-T (10 mM Tris-HCl, 0.15 M NaCl, 0.05% tween-20, pH 8.0) 3 times for 10 mins and blocked in a 5% non-fat milk-TBS-T solution for 1h. The membrane was then incubated with a 1:1000 dilution of the DACT adduct antibody (Strategic Biosolutions) in 10 ml of 5% non-fat milk-TBS-T solution overnight at 4°C. The following day, the membrane was washed with TBS-T and then incubated with a Horseradish-peroxidase (HRP) conjugated anti-rabbit antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) at a 1:2000 dilution in a 5% non-fat milk-TBS-T solution for 1h at room temperature. The membrane was again washed with TBS-T and the DACT adduct antibody complex was detected by chemiluminescence with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) and visualized using a BioChemi Bioimaging system (UVP, Upland, CA). If the band was detected by chemiluminescence, it was considered positive for containing the DACT adduct.

Results

Mass Spectrometry Detection of Cys-34 DACT Adduct in ATRAExposed Rats. The thermolysin digests of albumin purified from control and ATRA exposed rats were analyzed by mass spectrometry and the spectra were searched for the digest product that contains Cys-34. The peptide that would contain Cys-34 in a thermolysin digest has a predicted mass of 1145.5 Da (MH+) and a sequence of LQKCPYEEH. During the digestion procedure, albumin is reduced with dithiothreitol (DTT) and the free thiols acylated with iodoacetamide (IAA). Assuming Cys-34 is a free thiol, acylation results in a peptide with a mass of 1203.5 Da. The MALDI analysis of the albumin digest showed a peak at 1203.6 m/z in both the control rats and ATRA exposed rats (Figure1a/b). MS/MS was used to sequence this 1203.6 m/z peptide using the y-series ions and the resulting sequence was LQKCPYEEH confirming this as the peptide containing Cys-34 with a standard alkylation (58 Da) on Cys-34 (Figure 1c.). Previous research with globin from ATRA exposed rats demonstrated adducts formation resulted in a 110 Da mass increase to cysteine (*14*). We therefore searched for a 1255.5 m/z peak as a possible DACT adducted version of the 1145.5 Da peptide. MALDI-TOF spectra from the ATRA exposed rats showed a peak at 1255.6 m/z that was not seen in control animals (Figure 1b). MS/MS sequencing using an unmodified cysteine gave a matching y-ions series only up to Cys-34, indicating a modification at this point in the sequence (data not shown). When the mass of the cysteine residue was changed to 212 Da instead of 102 Da corresponding to the 110 Da adduct, the experimental spectrum matched the LQKCPYEEH sequence with complete coverage of y-ion series (Figure 1d). This result confirms a 110 Da adduct with Cys-34 of albumin from ATRA exposed rats. This 1255.5

m/z peak was absent in all spectra from control rats, but was found in ATRA dose groups 20-200 mg/kg and confirmed with MS/MS. The 1255.6 m/z peak was not clearly distinguishable from baseline peaks in the 5 and 10 mg/kg doses.

Mass Spectrometry Detection of Cys-34 DACT Adduct in Purified Rat and Human Albumin Exposed to DACT. Thermolysin digest of control (DMSO) or DACT treated rat albumin resulted in a peptide of 1203.5 m/z in the MALDI spectra (Figure 2a). MS/MS analysis matched the spectra from the 1203.5 m/z peak seen with *in vivo* exposures with the expected sequence of LQKCPYEEH (Data not shown). Spectra from albumin exposed to 90 ug/ml DACT resulted in a mass-shift of 110 Da (1255.6 m/z) as seen in the *in vivo* exposures to ATRA (Figure 2b) that was not seen with the DMSO exposure. The MS/MS spectra of this peak was identical to the spectra from MS/MS (Figure 2c) from the *in vivo* exposure data (Figure 1b) confirming these two peptides were identical, containing a 110 Da adduct on Cys-34. Human albumin has a slightly different amino acid sequence than rat albumin around Cys-34. The theoretical thermolysin digest peptide containing Cys-34 is LQQCP and has a mass of 588.3 Da. When we searched the MS spectra of DACT exposed albumin for this peptide with a mass 698.3 Da from the addition of a 110 Da DACT adduct, a peak was not present. Since Cys-34 was near the site of cleavage by thermolysin, it was possible that the adduct caused a missed cleavage at this site. A missed cleavage result would generate the peptide sequence LQQCPFEDH, with a mass of 1115.5 Da or 1225.5 Da with the adduct. A peak at 1225.5 m/z was present in the MS spectra of DACT exposed albumin and not in the MS spectra of control DMSO exposed albumin (Figure 3a/b). MS/MS sequencing of the 1225.5 m/z peak (Figure 3c) gave the predicated sequence of LQQCPFEDH when

the mass of Cys was increased by 110 Da to account for the DACT adduct. This indicates DACT does form an adduct with Cys-34, which likely prevented cleavage by thermolysin.

Immunodetection of DACT-Albumin Adducts. The capability and specificity of the anti-DACT adduct antibody to detect proteins with the DACT modification was tested using purified albumin from rats exposed *in vivo* to ATRA along with human and rat albumin exposed *in vitro* to DACT. The mass spectrometry data shown previously demonstrated the presence of DACT adducts on albumin from rats exposed to 20-200 mg/kg ATRA and on rats and human albumin exposed *in vitro* to 90 ug/ml DACT. No adducts were detected with mass spectrometry on albumin from control and 5 or 10 mg/kg ATRA exposures or from rat and human albumin exposed to DMSO *in vitro* (controls). Western blot analysis demonstrated chemiluminescence signals in albumin from rats given 5, 10, 20, 50, 100, and 200 mg/kg atrazine, but no signal was from detected in untreated (0 mg/kg) samples indicating an absence of adducts (Figure 4). This signal also demonstrated a dose dependent increase from 5 to 200 mg/kg indicating the formation of more adducts at higher doses or a greater percentage of albumin adducted. Human and rat albumin that were exposed *in vitro* to DACT exhibited chemiluminescence signal by Western blot analysis indicating the presence of DACT adducts (Figure 5), while human and rat albumin exposed *in vitro* to DMSO as a control showed no chemiluminescence signal. Based on these results, the antibody is able to detect adducts formed both *in vivo* and *in vitro* and shows no reactivity with control samples.

Discussion

The reactivity of albumin Cys-34 and the formation of covalent adducts has been demonstrated with numerous xenobiotics *(20-26).* This research now shows that Cys-34 reacts with DACT, the terminal metabolite of the commonly used herbicide atrazine, to form a covalent adduct.

In rats exposed to atrazine, McMulllin et al. (*13*) demonstrated complete metabolism to DACT within 48 h following exposure, and found high levels of DACT in the plasma. This research also provided the initial indication that DACT may be binding to plasma proteins by measuring radioactivity in blood after rat were treated with C^{14}-ATRA(*13*). We later showed that DACT is capable of forming a covalent adduct with Cys-125 of rat hemoglobin following ATRA exposure. The Cys-125 residue is solvent exposed and reactive (*28*) much like the Cys-34 residue in albumin (*20-26*), providing a nucleophilic target.

Based on our DACT-hemoglobin adduct data, if a DACT adduct formed on Cys-34, the mass of that residue would increase by 110 Da with addition of a dechlorinated DACT molecule. Our results from *in vivo* ATRA exposure and *in vitro* DACT exposures provide clear evidence that an adduct with a mass of 110 Da does form on Cys-34. As we suggested with the DACT hemoglobin adduct (*14*), the triazine adduct likely forms via nucleophilic aromatic substitution (Figure 6). Only the chlorinated ATRA metabolites would provide a carbon center with partial positive charge favoring this nucleophilic displacement.

Albumin adducts provide an attractive target for biomonitoring chemical exposures since albumin has a slow turnover rate of ~ 15.9 day in humans (*29*), sequence

homology is generally conserved across species (*15*), and it is easily obtained in large quantities from small blood samples via a simple precipitation procedure (*27*). Human and rat albumin have high sequence homology (73%) (*15*) and both contain a free thiol group (Cys-34), which allows our adduct results from the rat *in vivo* ATRA exposures to be extrapolated to human for developing a human biomarker of ATRA exposure. To provide further evidence that Cys-34 adducts in rats are relevant to humans, we treated human albumin with DACT and followed the same procedures we used to identify the Cys-34 adduct with mass spectrometry. Our results demonstrated DACT does form a covalent adduct with Cys-34 of human albumin with an identical mass of the adduct observed in the rodent model. This evidence confirming the formation of a DACT adduct on Cys-34 of human albumin, although only *in vitro*, makes detection of ATRA induced protein adducts a viable target for human exposure biomonitoring.

The detection of protein adducts with MALDI-TOF-TOF MS/MS has not become a generally available technology. A more generally applied method is immunodetection of protein adduct using specific antibodies generated against xenobiotic adducts (*30-35*). To provide another more practical method of detecting the DACT-albumin adduct, we generated a polyclonal antibody which recognizes DACT adducts in the specific orientation that it would be found if formed *in vivo*. Using this antibody, we were able to detect albumin adducts via western blot from all *in vivo* ATRA dose levels and with both human and rat albumin exposed *in vitro* to DACT. A clear dose-response was also seen in the *in vivo* albumin as signal intensity increase from 5 mg/kg to 200 mg/kg samples and no signal detected in control samples. These immunodetection results were validated with the corresponding mass spectrometry detection of the DACT adduct, although the

immunodetection proved more sensitive as it was able to detect adducts at the 5 and 10 mg/kg doses. The observation that human albumin with a Cys-34 DACT adduct formed *in vitro* can be detected by this methodology suggests the possibility of developing a rapid assay for internal ATRA exposure.

Current methodologies for human ATRA biomonitoring have been validated in occupational exposure scenarios with workers handling atrazine. These include detection of ATRA or ATRA metabolites in urine with ELISA (*36-37*) and accelerator mass spectrometry (*38*), in saliva with ELISA (*39*), and detection in air, hand wash, and dermal patches with gas chromatography (*40*). If we can use immunodetection to show DACT-albumin adducts in similarly exposed peoples, it could provide a simple and sensitive analytical method that would provide a better detection method to show an internal dose has occurred. Since the Cys-34 DACT adduct can only form with exposure of DACT to albumin in blood, it provides us with conclusive evidence of that external exposure to ATRA has resulted in an internal dose. Measurement of ATRA or metabolites in biological fluids does not show a biologically significant interaction has occurred as detection of a protein adduct does. Also, since albumin has a relativity slow turnover rate, adducts could be detected several weeks post exposure, a much longer detection window than other biomarker methodologies. This research provides solid evidence for the potential of immunodetection methodology for the detection of DACT-protein adducts as a biomarker of ATRA exposure.

Acknowledgements

We thank Dr. Philip Ryan and Dr. Jessica Prenni for help with the MALDI-TOF-MS analysis and Dr. Ronald Tjalkens for use of his western blotting equipment. This research was supported by the HICAS Center Grant 1-846000545-A1 and the USEPA Star Grant R-828610-01-0.

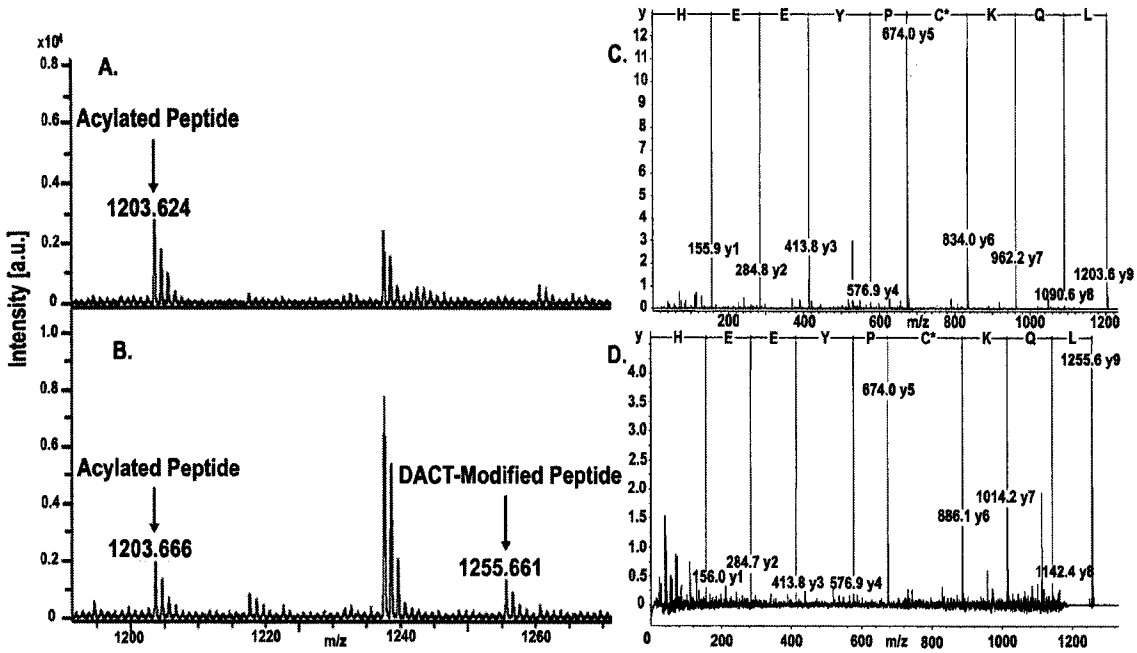

Figure 1. MALDI-TOF-TOF MS spectra of thermolysin digest of rat albumin from *in vivo* exposures (A. 0 mg/kg atrazine) and (B. 200 mg/kg atrazine). MS/MS sequencing of the (C.)1203.5 Da peak found in thermolysin digests of albumin from rats given 0 mg/kg and the (D.) 1255.6 Da peak found in thermolysin digests of albumin from rats given 200 mg/kg. The y-ions sequence is HEEYPCKQL for both peaks with Cys-34* modified by a 110 Da adduct on the 1255.6 Da peak.

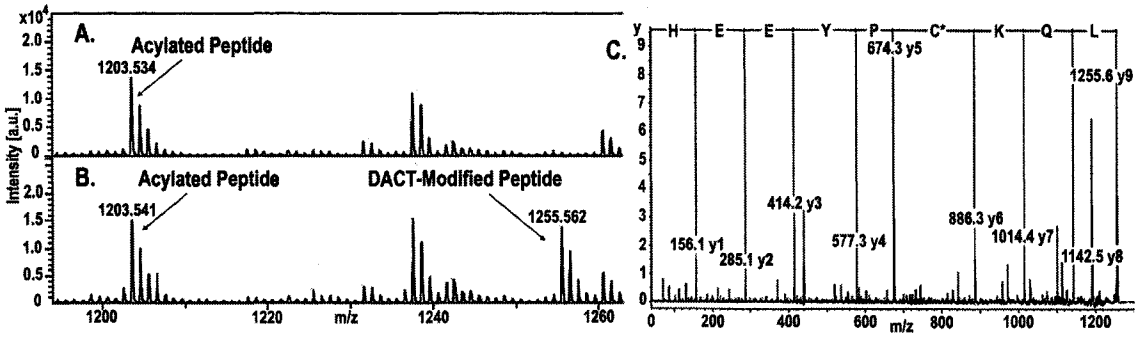

Figure 2. MALDI-TOF-TOF MS spectra of thermolysin digest of rat albumin from *in vitro* exposures (A. DMSO control) and (B. 90 ug/ml DACT). The MS/MS spectra of the (C.) 1255.5 Da peak from 90 ug/ml DACT exposure shows a y-ions sequence is HEEYPC*KQL with Cys-34* modified by a 110 Da adduct.

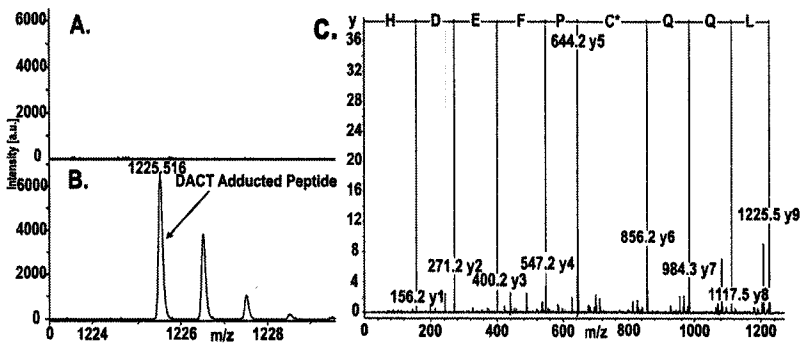

Figure 3. MALDI-TOF-TOF MS and MS/MS spectra of thermolysin digest of human albumin from *in vitro* exposures (A. DMSO control) and (B. 90 ug/ml DACT). The MS/MS spectra of the (C.) 1225.5 Da peak from 90 ug/ml DACT exposure shows a y-ions sequence is HDEFPC*QQL with Cys-34* modified by a 110 Da adduct.

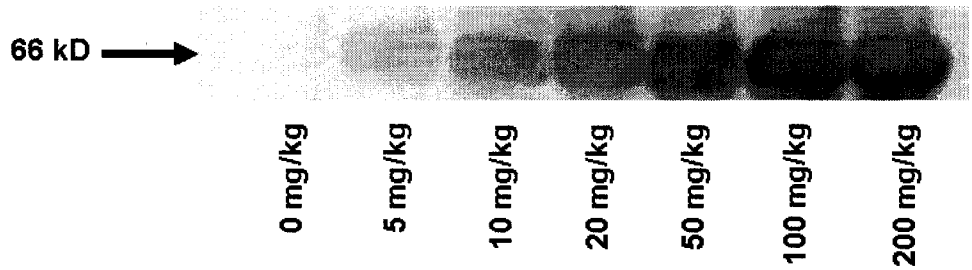

Figure 4. Western blot analysis of albumin for DACT adducts by probing with a DACT adduct antibody. Albumin was purified from rats exposed *in vivo* to 0, 5, 10, 20, 50, 100, and 200 mg/kg atrazine for 4 days and the 66 kD band analyzed.

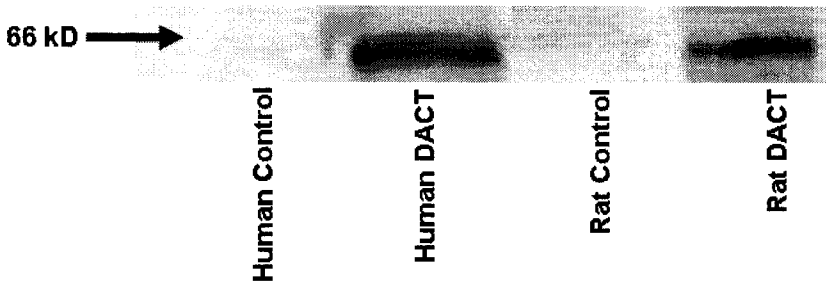

Figure 5. Western blot analysis of albumin for DACT adducts by probing with a DACT adduct antibody. Purified human or rat albumin was exposed to 90 ug/ml DACT or DMSO (control) *in vitro* for 4 days and the 66 kD band analyzed.

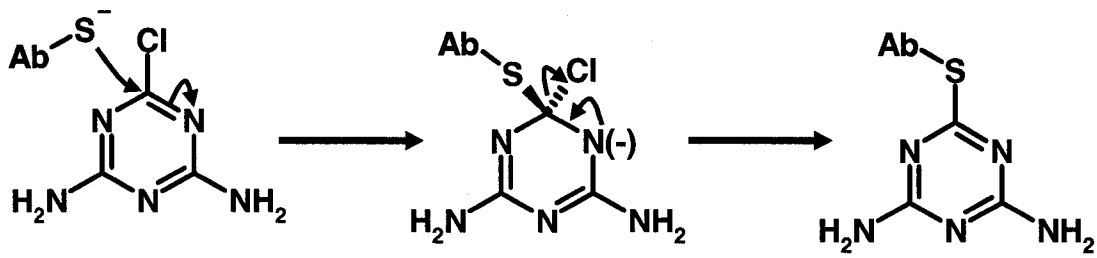

Figure 6. Proposed nucleophilic aromatic substitution reaction for adduct formation as the nucleophilic Cys-34 residue of albumin attacks the chlorine of the diaminochlorotriazine.

CHAPTER 5

Proteomic Analysis of Diaminochlorotriazine (DACT) Adducts in Wister Rat Pituitary Glands and LβT2 Rat Pituitary Cells

Dooley G, Reardon K†, Prenni J‡, Tessari J, and Hanneman W.

Department of Environmental and Radiological Sciences. †Department of Biological and Chemical Engineering, ‡Macromolecular Resources, Colorado State University
Fort Collins, CO 80523

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Abstract

Atrazine (ATRA) is the most commonly applied herbicide in the United States and is frequently detected in drinking water at significant levels. Following oral exposure, ATRA metabolism yields diaminochlorotriazine (DACT), an electrophilic molecule that has been shown to react with exposed protein cystiene residues to form a stable covalent adduct. Female rat exposure to ATRA results in the suppression of the luteinizing hormone (LH) surge, which is required for ovulation. Given the critical link between the pituitary gland and LH release, this research was designed to identify ATRA-induced protein adducts formed in the pituitary gland of ATRA exposed rats and in DACT exposed LβT2 rat pituitary cells. Protein targets in the water soluble fraction of lysates from both rat pituitaries and cell culture were analyzed with 2-dimensional electrophoresis, immunodetection, and MALDI-TOF mass spectrometry. Western blots from both exposed rats and LβT2 cells showed numerous spots (>30) that were not present in control animals. Protein spots were matched to concurrently run 2-DE gels stained with Sypro Ruby, excised, and in-gel digested with trypsin. Mass spectrometry analysis of digest peptides resulted in the identification of 19 spots and 8 unique proteins in the rats and 21 spots and 19 unique proteins in LβT2 cells. The identified proteins present in both sample types included proteasome activator complex subunit 1, ubiquitin carboxyl-terminal hydrolase isozyme L1, tropomyosin, ERp57, and RNA binding proteins (heterogeneous nuclear ribonucleoprotein A2/B1 and heterogeneous nuclear ribonucleoprotein 1). Each of these proteins contains active site or solvent exposed cysteine residues, making them viable targets for covalent modification by DACT.

Future research will be necessary to elucidate the functional role of these proteins as well as their involvement in ATRA/DACT induced LH suppression.

Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) (ATRA) is one of the most commonly used herbicides in the U.S., with 57.4 million pounds applied in 2005 primarily to corn for control of broadleaf and grassy weeds (USDA 2006). With its widespread use, ATRA is frequently detected in surface and drinking waters at levels greater than the 3 μg/L maximum contaminant levels (MCL) set by the U.S. EPA (Thurman et al. 1991, Rebich et al. 2004, USEPA 1990, Hackett et al. 2005).The general population is potentially exposed to ATRA from drinking water; however, occupational exposure among agricultural workers is increased (Gammon et al. 2005). Following oral exposure, ATRA metabolism by P450 enzymes in the liver (Timchalk et al. 1990) yields diaminochlorotriazine (DACT), a reactive electrophilic molecule capable of forming covalent adducts with cellular nucleophiles.

We initially showed that ATRA exposure in rodents results in the formation of covalent adduct to Cys-34 of albumin and Cys-125 of hemoglobin. Adduct formation was attributed to a nucleophilic aromatic substitution reaction between reduced cystiene residues and DACT. This mechanism was confirmed with *in vitro* exposures of hemoglobin and albumin to DACT, which resulted in adducts identical to those formed *in vivo* (Dooley et al. 2006, Dooley et al. 2007). It is not known if DACT forms covalent adducts with tissue specific proteins or if protein modification plays a role in the endocrine disrupting effects associated with ATRA exposure.

In female rats, luteinizing hormone (LH) is necessary to stimulate ovarian follicular development and ovulation, while stimulating the ovaries to produce estrogen and progesterone. Previous research with female Sprague Dawley rats exposed to ATRA has shown high concentrations of DACT in the plasma and brain shortly after exposure (McMullin et al. 2003) along with a suppression of the LH surge from the pituitary (McMullin et al 2004). A dose dependent suppression of the estradiol benzoate/progesterone LH surge in female SD rats was caused by exposure to 30-300 mg/kg ATRA for 5 days with complete blockage of the LH surge at 300 mg/kg. It was also showed that DACT exposure can have a similar effect. In estradiol benzoate/progesterone primed ovariectomized SD rats exposed to 300 mg/kg DACT for 5 days, total plasma LH and peak LH surge levels were suppressed by 60 and 58%, respectively. A 47% decrease in pituitary release of LH in response to added gonadotropin releasing hormone was also seen in animals treated with 200 mg/kg DACT (MuMullin et al. 2004). Currently there is little known about the mechanism of action of DACT and/or ATRA exposures with relation to suppression of LH release. Since we have shown DACT is capable of forming a covalent protein adducts and brain tissue is exposed to large quantities of DACT following ATRA exposure, it is possible that DACT is forming an adduct with proteins involved in the signal transduction pathway leading to LH release from the pituitary. This DACT-protein adduct could lead to alterations in function or premature degradation of the proteins leading to suppression of the LH release. Given the critical link between the pituitary gland and LH release, this research was designed to identify ATRA-induced protein adducts formed in the pituitary gland of ATRA exposed rats and in DACT exposed LβT2 rat pituitary cells.

Materials and Methods

Chemicals and Materials. ATRA (97.1% purity) was a gift from Syngenta (Research Triangle Park, NC). Acetonitrile, bromophenol blue, Tween, and ammonium bicarbonate were purchased from Fisher Chemical Company (Fair Lawn, NJ). Proteomic grade porcine trypsin, iodoacetamide, tris-HCl, glycerol, CHAPS, urea, triflouroacetic acid, agarose, and Pefabloc SC were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol and acetic acid were purchased from Mallinckrodt Baker Inc. (Paris, KY). Sypro Ruby, acrylamide, sodium dodecylsulfate, potassium chloride, magnesium chloride, dithiothreitol, tris-base, mineral oil, and glycine were purchased from Bio-Rad Laboratories (Hercules, CA). The DACT adduct antibody was generated by Strategic Biosolutions (Newark, DE) and the Horseradish-peroxidase conjugated anti-rabbit antibody purchased from Santa Cruz Biotechnology, Inc, (Santa Cruz, CA).

Rodent ATRA Exposures. Eight female ovariectomized Wistar rats were allowed to acclimate in ventilated cages for one week, during which time they were maintained on a 12-h light/dark cycle at a constant temperature of 25°C and humidity of 55%. All animals had free access to Teklad NIH-07 rodent diet and tap water. Following the acclimation period, rats (4 per treatment) were given via oral gavage doses of either 200 mg/kg ATRA (97.9% pure) in carboxymethylcellulose or equivalent mg/kg doses of carboxymethylcellulose as controls. These dose levels were chosen as they are similar to doses that produced covalent hemoglobin and albumin adducts (*8-9*) and LH surge suppression (*11*) in rats following ATRA exposure. On the 5th day, rats were anesthetized with isoflurane and sacrificed via decapitation. The pituitary glands were immediately removed, snap frozen with liquid nitrogen, and stored at -80°C until analysis.

Sample Preparation and 2-Demensional Electrophoresis. Pituitary was sonicated in ice cold lysis buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% SDS, 0.5 mM DTT, 0.5 mM Pefabloc SC), stored on ice for 1h, and centrifuged at 14,000 rpm for 10 mins. Supernant were removed and proteins precipitated with a 2-D Clean-up Kit (Bio-Rad, Hercules, CA) following manufacturer protocols. For the 1st dimension (isoelectric focusing), protein (200 µg) was dissolved by sonication in 400 µl rehydration buffer (8M urea, 0.3% w/v DTT, 2% w/v CHAPS, pH 3-10 buffer, bromophenol blue) and pipetted into a rehydrating tray. An immobilized pH gradient (IPG) strip, pH 3-10 (Bio-Rad, Hercules, CA), was laid gel side down through the solution and mineral oil pipetted over the IPG strip. The strip was allowed to rehydrate passively for at least 12 hr. Using the Multiphore II system (Pharmacia Biotech, Uppsala, Sweden), the protein mixture was isoelectricly focused in the voltage gradient: 0 to 500 V over 1 min, 500 V to 3500 over 5 hr, and at 3500 V for 17.5 hr. For the 2nd dimension (SDS-PAGE) separation, the IPG strip was removed from the focusing tray and reduced in 3 ml of 2% w/v DTT in equilibration buffer (6M urea, 30% v/v glycerol, 2% w/v SDS, 24 mM Tris-HCl) for 15 mins, followed by acetylation in 3 ml of 2.5% w/v IAA in equilibration buffer for 5 mins. The IPG strip was transferred onto a slab gel (12% polyacrylamide), overlayed with 1 ml of hot 0.5% agarose, and agarose allowed to solidify. The gel was run in a Protean II cell (Bio-Rad, Hercules, CA) for 2.5h at 3000V, 400W, and 40ma. 2-D gels were either stained SYPRO® Ruby (Bio-Rad, Hercules, CA) for protein detection or subjected to Western blotting for immunodetection of proteins with DACT adducts. For protein detection, the gel was fixed for 1 hr in 10% ethanol/7% acetic acid and then stained in

100 ml of SYPRO® Ruby stain overnight. The gel was destained in 10% ethanol/7% acetic acid water before imaging. Stained proteins were visualized with 320 nm UV light using a BioChemi Bioimaging system (UVP, Upland, CA) and the imaged saved for comparison with Western blotting results.

Immunodetection of DACT Modified Proteins. An unstained 2D gel, a PVDF membrane, and two extra thick fiber pads were soaked in transfer buffer (25 mM Tris-HCl, 0.2 M glycine, 0.2% SDS, 20% methanol) for 15 mins. A Western blot sandwich of filter paper, membrane, gel, filter paper was assembled and the gel electrophoresed at 15V for 1h using a Semi-Dry transfer unit (Bio-Rad, Hercules, CA). Following electrophoresis, the membrane was washed in TBST (10 mM Tris-HCl, 0.15 M NaCl, 8 mM sodium azide, 0.05% Tween-20, pH 8.0) for 30 mins and blocked in a 5% non-fat milk-TBST solution for 30 mins. The membrane was rinsed in TBST twice for 10 mins and incubated with a 1:500 dilution of DACT adduct antibody (Strategic Biosolutions, Newark, DE) in 10 ml of 5% non-fat milk-TBST solution overnight at 4°C. The membrane was then washed 3 times in TBST and incubated with a Horseradish-peroxidase (HRP) conjugated secondary rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:2000 dilution for 1h at room temperature. The membrane was again washed 3 times with TBST and the DACT-antibody complex was detected by chemiluminescence with an Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA) using a BioChemi Bioimaging system (UVP, Upland, CA) and the imaged saved for comparison with Sypro Ruby stained gels. Protein spots from the Western blot were matched to spots on Sypro stained gels using the DELTA 2D 3.4 (DECODON, Greifswald, Germany) gel analysis program by analyst at DECODON.

Pituitary Protein Identification using MALDI-TOF/TOF MS

Matched spots were excised from the Sypro Ruby stained gel with a spot picker and placed in an Eppendorf tube. Gel pieces were washed twice for 15 mins (gentle vortex) with 100 µl 100 mM NH_4HCO_3/50% ACN, 100 µl of 10 mM DTT was added and sample was incubated at 60°C for 30 mins. The supernatant was removed and 100 µl of 55 mM IAA added and incubated in the dark at room temperature for 30 mins to acylate cysteine residues. Gel pieces were completely dried, 6 µl of 0.1 µg/µl trypsin solution and 34 µl 100 mM NH_4HCO_3 added, and incubated at 37°C overnight. Peptides were extracted from the gel pieces with 40 µl of 50% ACN (0.1% TFA) under a gentle vortex for 20 mins. The extraction was repeated, extracts pooled, and concentrated to ~2 µl. Peptides were further purified with a Zip-Tip (C_{18} Millipore) clean-up for mass spectral analysis. For MS analysis, 1 µl of the tryptic peptide solution was added to 1 µl of α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% ACN, 0.1% TFA) was spotted on the MALDI target and allowed to dry. External calibration used an 8 peptide mixture on a spot adjacent to the sample. Analysis was performed with an UltraFlex-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA) in positive ion, reflector mode with a 25 kV acceleration voltage. Data were processed using the SNAP algorithm in the FlexAnalysis software (version 2.4, Bruker Daltonics). A monoisotopic list was generated using a signal-to-noise threshold of 6 for MS spectra and 3 for MS/MS spectra. The combined MS and MS/MS spectra for each sample were searched using the Mascot (version 2.1) database search engine against the Swiss Prot or NCBInr database using a taxonomy filter for a rat (*Rattus*, Taxonomy ID 101144) containing 5,446 and 40,152

sequence entries respectively. Parameters used in the database search are as follows: peptide mass tolerance of 0.15 Da, fragment ion mass tolerance of 0.8 Da, trypsin peptides only allowing for 1 missed cleavage, variable modifications of cysteine carbamidomethylation and methionine oxidation.

LβT2 Cell Culture DACT Exposures. LβT2 immortalized rat anterior pituitary cells were cultured in complete Dulbelco's modified essential medium (DMEM) containing 4.5 mg of glucose, supplemented with 10% fetal bovine serum and 1% Pen/Strep (Media Tech, Herndon VA). Cultures were grown in a humidified environment with 95% air and 5% CO_2 at 37° C to 80% confluency. They were then exposed to 0 or 300 μM DACT in DMEM for 24 hours. Following DACT incubation, media was removed, cells rinsed with DMEM and PBS, and scraped from flask into 15 ml conical tube. Tubes were centrifuged for 10 mins to form a tight cell pellet. The pellet was sonicated in lysis buffer and proteins precipitated as described for pituitary tissue samples. 2DE, western blotting, spot matching, and in-gel digestion were also performed as described for pituitary tissue samples.

LβT2 Cell Protein Identification using LC-MS. For MS analysis, 5 μl of the tryptic peptide solution was injected onto a reverse phase capillary column (1100 HPLC, 75 μmID x 15cm column). Peptides were eluted directly into the mass spectrometer using a 40 minute linear gradient from 2%-60% buffer B (80% ACN, 0.1% acetic acid) at a flow rate of 5 μl/min. Spectra were collected over a m/z range of 200-2000 Da (Thermo Scientific LTQ linear ion trap) using a dynamic exclusion limit of 2 MS/MS spectra of a given peptide mass for 30 s (exclusion duration of 90 s). Compound lists of the resulting spectra were generated using Bioworks 3.0 software (Thermo Scientific, Waltham, MA)

with an intensity threshold of 5,000 and 1 scan/group. The compound lists were searched against the NCBInr database using a taxonomy filter for a rat (*Rattus*, Taxonomy ID 101144) containing 40,120 sequence entries using the Sequest (Bioworks 3.0) database search engine. Parameters used in the database search were as follows: average mass, peptide mass tolerance of 1.8 Da, fragment ion mass tolerance of 0.8 Da, tryptic peptides only allowing for 1 missed cleavages, and variable modifications of cysteine carbamidomethylation and methionine oxidation.

Results

Identification of DACT Modified Proteins in ATRA Exposed Rats

2DE gels of water soluble pituitary proteins from Wistar rats exposed to 200 mg/kg ATRA generated a representative spot pattern as detected with Sypro Ruby staining shown in Figure 1, with consistent spot patterns observed between replicate exposures. 2DE gels from rats exposed to carboxymethylcellulose as a control showed a spot pattern identical to ATRA exposed rats in both number of observed spots and relative abundances (Data not shown). When 2DE gels were western blotted to a PVDF membrane and probed with a DACT adduct specific antibody 55 spots were detected in animals given ATRA (Figure 2), while no spots were detected in control animals (Figure 3). Some variance in spot number was seen in replicates of ATRA exposed animals, but the number of spots that were confidently matched to spots of the Sypro Ruby stained gels were consistent. Of the 55 spots detected, 44 spots were confidently matched to spots on Sypro Ruby stained gels and were picked for protein identification.

Identification of DACT Modified Proteins in DACT Exposed LβT2 Cells

2DE gels of water soluble pituitary proteins from LβT2 cells exposed to 300μM DACT generated a representative spot pattern as detected with Sypro Ruby staining shown in Figure 4. 2DE gels from cells exposed to carboxymethylcellulose as a control showed a spot pattern consistent to DACT exposed cells (Data not shown). Western blot analysis detected 89 spots in DACT treated cells (Figure 5) and no spots from control cells (Figure 6). Of the 89 spots detected, 54 spots were confidently matched to spots on Sypro Ruby stained gels and picked for protein identification.

Target Protein Identification in ATRA Exposed Rats. Peptides from tryptic digest solutions were analyzed with MALDI-TOF-TOF MS followed by tandem MS/MS of abundant peptides if available. Proteins were identified using a MASCOT search of combined MS and MS/MS data against the Swiss-Prot or NCBInr databases for the *Rattus* taxonomy. Sufficient protein material for MS analysis and subsequent significant protein identification was only obtained in 19 of the 42 matched spots. See Table 1 for Mascot protein ion scores and cutoffs, and sequence coverage for each identified protein. Significant protein ion scores (>23) with 2 or more matched peptide sequences were obtained for 12 spots, which were identified as 6 unique proteins. These proteins included serum albumin precursor, ERp57, heterogeneous nuclear ribonucleoprotein A2/B1, proteosome acitivator complex subunit 1 (PA28α), ubiquitin carboxy-terminal hydrolase isozyme L1 (UCHL1), and peroxiredoxin 6. Spots 10 and 15, identified as serum albumin precursor and tropomyosin respectively, matched 1 peptide sequence giving significant protein ion scores. The remaining spots were identified based on significant protein ion scores, since a sufficient amount of protein was not available for confident MS/MS analysis. This analysis only provided a new protein ID for Spot 12 as

heterogeneous nuclear ribonucleoprotein H. Overall, the analysis of DACT target proteins in the pituitary resulted in identification of 8 unique proteins.

Target Protein Identification in DACT Exposed LβT2 Cells. Peptides from tryptic digest solutions were analyzed with LC followed by tandem MS/MS. Proteins were identified using a Sequest search of MS/MS data against the Swiss-Prot or NCBInr databases for the *Rattus* taxonomy. Confident protein identifications were made for 21 of the 54 matched spots based on 2 or more matched peptides with Xcorr scores greater than 2 for each peptide. Sequence coverages ranged from 5-57%. Table 2 lists 19 unique proteins identified as DACT targets in LβT2 cells from the 21 spots analyzed. Comparison of this list to the DACT modified proteins in pituitaries of ATRA exposed rats showed 6 proteins that were detected an identified in both exposure scenarios. These included ERp57, heterogeneous nuclear ribonucleoproteins A2/B1 and H, PA28α, UCHL1, and tropomyosin.

Discussion

ATRA is metabolized via successive dealkylations to DACT by P450 enzymes in the liver (Timchalk et al. 1990). This metabolic pathway is rapid as ATRA is completely metabolized to DACT within 48 h following exposure (McMullin et al. 2003). We have previously demonstrated in rats exposed to ATRA, that DACT is reactive and forms covalent adducts with protein nucleophiles, specifically exposed cystiene residues on hemoglobin and albumin (Dooley et al. 2006, Dooley et al. 2007). The mechanism of adduct formation through a nucleophilic aromatic substitution with DACT and specific Cys residues was confirmed *in vitro*. We have not detected adducts of ATRA or mono-

dealkylated metabolites in either of these studies, indicating that DACT is the principle reactive metabolite present following ATRA exposure.

Protein adducts could play a central role in the observed toxicity associated with a reactive chemical such as DACT. Chemical-induced toxicity follows a series of cascading events between ambient exposure and the observation of clinical disease. The formation of a covalent protein adduct is a possible step in a chemical-induced toxicity, which could occur if the adduct disrupts the structure or function of the protein. It has been suggested that covalent binding of a xenobiotic is the initiating event with some target organ toxicity (LoPachin and Decaprio 2005)

One of the primary toxic affects associated with ATRA exposure in rodents is the suppression of the LH surge from the pituitary (McMullin et al. 2004). This observation has been seen in SD rats exposed separately to ATRA and DACT, as well as in cultured LβT2 cells exposed to DACT (unpublished data). These observations along with high levels of DACT in the brain following ATRA exposure (McMullin et al. 2003), suggesting that DACT, not ATRA, is responsible for this effect. Currently there is little known about the mechanism of action of DACT with relation to suppression of LH release. Since DACT is capable of forming covalent protein adducts and brain tissue is exposed to large quantities of DACT following ATRA exposure, specific modifications of proteins within the pituitary could cause aberrant function leading to the observed suppression of the LH surge. In this study, we identified DACT modified proteins in both pituitaries of ATRA exposed Wistar rats and DACT exposed LβT2 cells providing an initial point to further investigate the potential mechanistic role of proteins adduct with LH suppression in the rodent model.

Immunochemical detection of DACT modified proteins from the pituitaries of rats exposed to 200 mg/kg ATRA revealed numerous modified proteins. Although greater than 50 spots were detected, only 9 unique proteins could be definitively identified as target for DACT modification. Included in this list was serum albumin, which we previously demonstrated to form a covalent adduct with DACT at Cys-34 (Dooley et al. 2007). The other proteins detected each contain solvent exposed Cys residues that would provide a clear target for DACT modification. The include ERp57 (Cys-57, 60, 406, 409) (Urade et al. 1997), UCHL1 (Cys-90) (Larsen et al. 1996), heterogeneous nuclear ribonucleoprotein A2/B1 (Cys-38) (Burd et al. 1989), heterogeneous nuclear ribonucleoprotein H (Cys-34) (Honore et al. 1995), tropomyosin (Cys-190) (Perry 2001), PA28α (Cys-22) (Knowlton et al. 1997), and peroxiredoxin 6 (Cys-47) (Choi et al. 1998).

Proteins identified as DACT targets in Lβ2 cells can be grouped in several categories based on their physiologic functions including RNA binding proteins, cytoskeleton proteins, proteins involved in proteasomal degradation, chaperones, and metabolic enzymes. Heterogeneous nuclear ribonucleoproteins (hnRNPs) bind heterogeneous nuclear RNA via conserved RNA binding motifs and are important in nRNA processing and transport. Four different isoforms of hnRNPs were identified including hnRNP A2/B1, hnRNP H2, hnRNP L, and hnRNP D. The cytoskeleton proteins provide mechanical strength and shape to cells and are also involved with intracellular transport of organelles. B-tubulin, a major constituent of microtubules, and tropomyosin-3, which stabilizes actin filaments are cytoskeleton proteins targeted by DACT. The proteasome is a large multi-protein complex responsible for the degradation of a variety of cellular proteins. Several proteins related to this degradation system were

targeted by DACT including proteasome activator complex subunit 1, proteasome 26S subunit 9, 26S protease regulatory subunit 6a, and UCHL1. Chaperone proteins play a major role in the processing newly translated proteins by binding and refolding incorrectly folded proteins and forming correct disulphide bonds. ERp57, heat shock protein-60, and stress-70 protein are mitochondrial chaperones also modified with DACT exposure. Finally, enzymes involved in carbohydrate biosynthesis/metabolism (α-enolase, phosphoglycerate kinsasetriosephosphate isomerase), electron transfer (α-EFT and flavin reductase), and the hydrolase pyridoxal phosphatase were identified as DACT targets.

Of particular interest are the proteins that were identified from both *in vivo* ATRA exposed rats and *in vitro* DACT exposed Lβ2 cells. UCHL1 is cystiene protease that catalyzes the removal of ubiquitin from ubiquitin conjugated proteins by hydrolyzing peptide-ubiquitin bonds (Kim et al. 2003). Deubiquitination is essential for recycling of the ubiquitin monomers in the ubiquitin-proteasome system. This system serves to degrade ubiquitin tagged proteins that may be in damaged, misfolded, or in excess and is critical for cell growth and differentiation, development, and transcriptional regulation (Kim et al. 2003). The active site of UCHL1 is a catalytic triad of Cys-90, His-161, and Asp-176 (Larsen et al. 1996). Since these residues are solvent exposed, the cysteine is a potential target for a DACT adduct. Modification the active site Cys-90 with a covalent DACT adduct would inactivate the enzyme resulting in the potential disruption of the ubiquitin-proteasome system.

ERp57 is an oxidoreductase found in the endoplasmic reticulum of cells, where its exact function is not completely understood. It has been shown to be involved in

formation of disulphide bonds and folding glycoproteins when associated with calnexin and careticulin (Zapun et al. 1998, High et al. 2000), as well as in folding major histocompatability complex class I molecules (Lindquist et al. 1998). ERp57 contains two redox active Cys-Gly-His-Cys motifs that forms mixed disulfide bonds with substrate during folding. When these Cys residues are mutated, the reductase activity of ERp57 is completely inhibited (Hirano et al. 1995). These Cys residues are potential targets for DACT and it is possible that alkylation by DACT could mimic the effects of Cys mutation and inhibit the oxidoreducatse activity of this protein. Interference of normal glycoprotein folding in the ER through the calnexin/calreiculin cycle could result, as it is dependent on ERp57 binding (Williams 2006).

The proteins hnRNP A2/B1 are hnRNP H2 members of a family of ubiquitously expressed proteins called heterogeneous nuclear ribonucleoproteins (hnRNPs). These proteins bind pre-mRNAs and influence pre-mRNA processing, metabolism and, transport. The hnRNP proteins bind RNA with two repeats of a RNA recognition motif (RRM) (Dreyfuss et al. 1993). Within the first RRM is a Cys-38 (A2/B1) or Cys-34 (H2) that is available to react with DACT (Burd et al. 1989, Horone et al. 1995), but it is unknown if a DACT modification on these residues would influence the RNA binding capability of this RRM.

Tropomyosin can be found in all eukaryotic cells types as a component of the microfilament system. In non-muscle cells, it binds actin providing stability and can influence actin dynamics (Perry 2001). The tropomyosin unit consists of α-helical peptide dimer in a coiled coil structure with one disulphide bond between Cys-190 of each peptide. This is the only Cys residue present in the peptide, so it is possible DACT

may react with this Cys and disrupt proper dimeraztion leading to alteration in actin binding.

Proteosome activator complex subunit 1 (PA28α) forms a ring shaped heteromultimer with PA28β, which bind to and activates the hydrolytic function of the proteasome (Kuehn and Dalhmann 1997). This protein contains an exposed Cys-22, but it is not located in the proteasome binding sequence (Knowlton et al. 1997). It is unclear what potential effect, if any, DACT modification of this residue may have on the protein stability or function.

In summary, we have demonstrated the ATRA metabolite DACT forms covalent adducts with numerous proteins in the pituitary of rats exposed to ATRA. Also, *in vitro* exposure of LβT2 rat pituitary cells to DACT caused numerous proteins to be modified, several of which were common between both exposure scenarios. It is clear from these results that DACT is the primary active metabolite following ATRA exposure, likely acting as an alkylating agent to Cys residues. Most the targeted proteins contain exposed Cys residues, which we have previously demonstrated to be modified by DACT (8, 9). The functional effect of these modifications to the proteins is unknown at this time. Further research on the individually identified proteins will be necessary to determine these effects and if they play a role in the mechanism of LH suppression from the pituitary following ATRA exposure.

Acknowledgments

Amanda Ashley for help with cell culture, Carla Lacerda for help with 2DE setups, Chad Forodori for ovarectomizing the rats, and Christin Sonntag at DECODON for spot matching of western blots and stained gels.

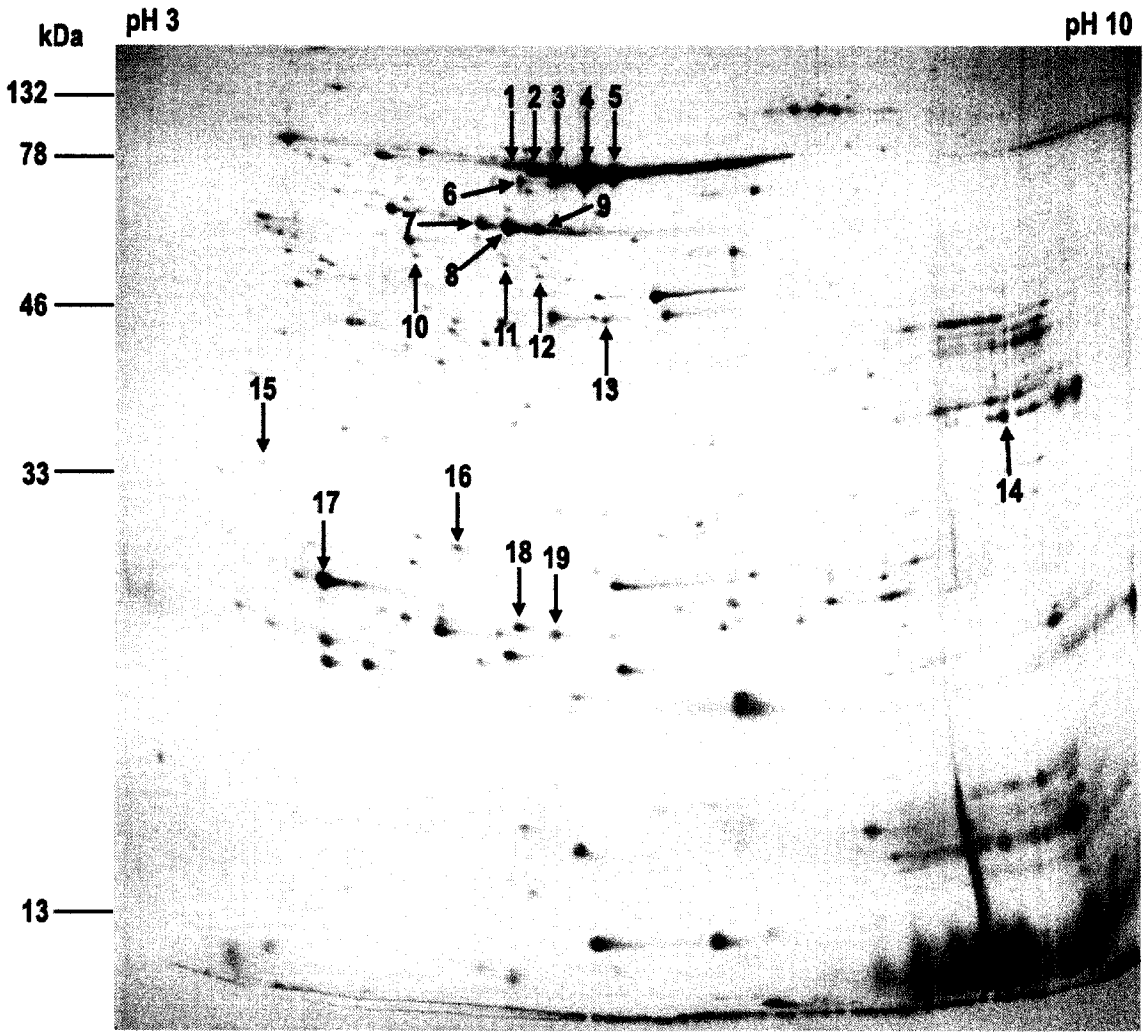

Figure 1. 2DE separated and Sypro Ruby stained water soluble proteins from pituitaries of female Wistar rats exposed to 200 mg/kg ATRA for 4 days

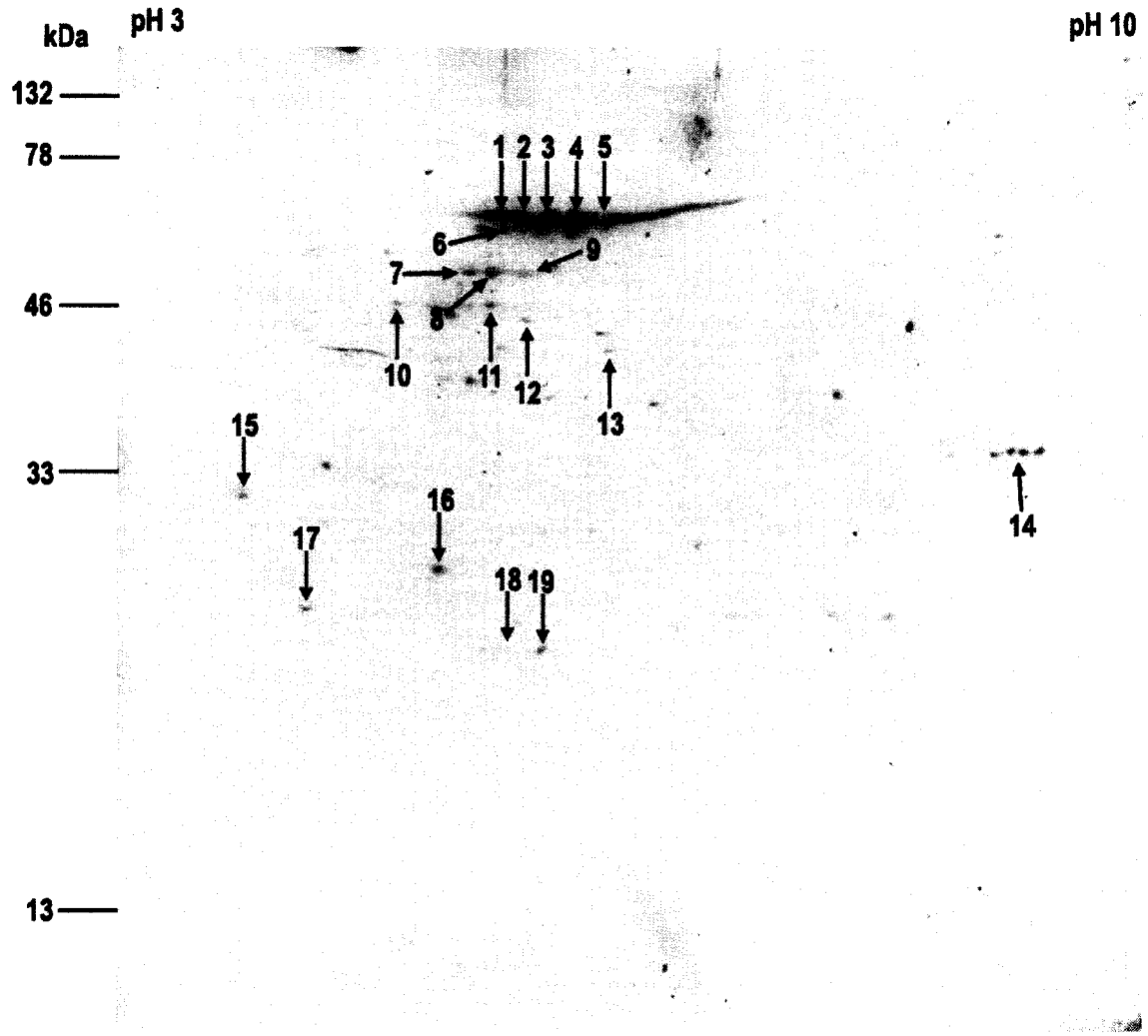

Figure 2. 2DE separated and Western blotted water soluble proteins from pituitaries of female Wistar rats exposed to 200 mg/kg ATRA for 4 days.

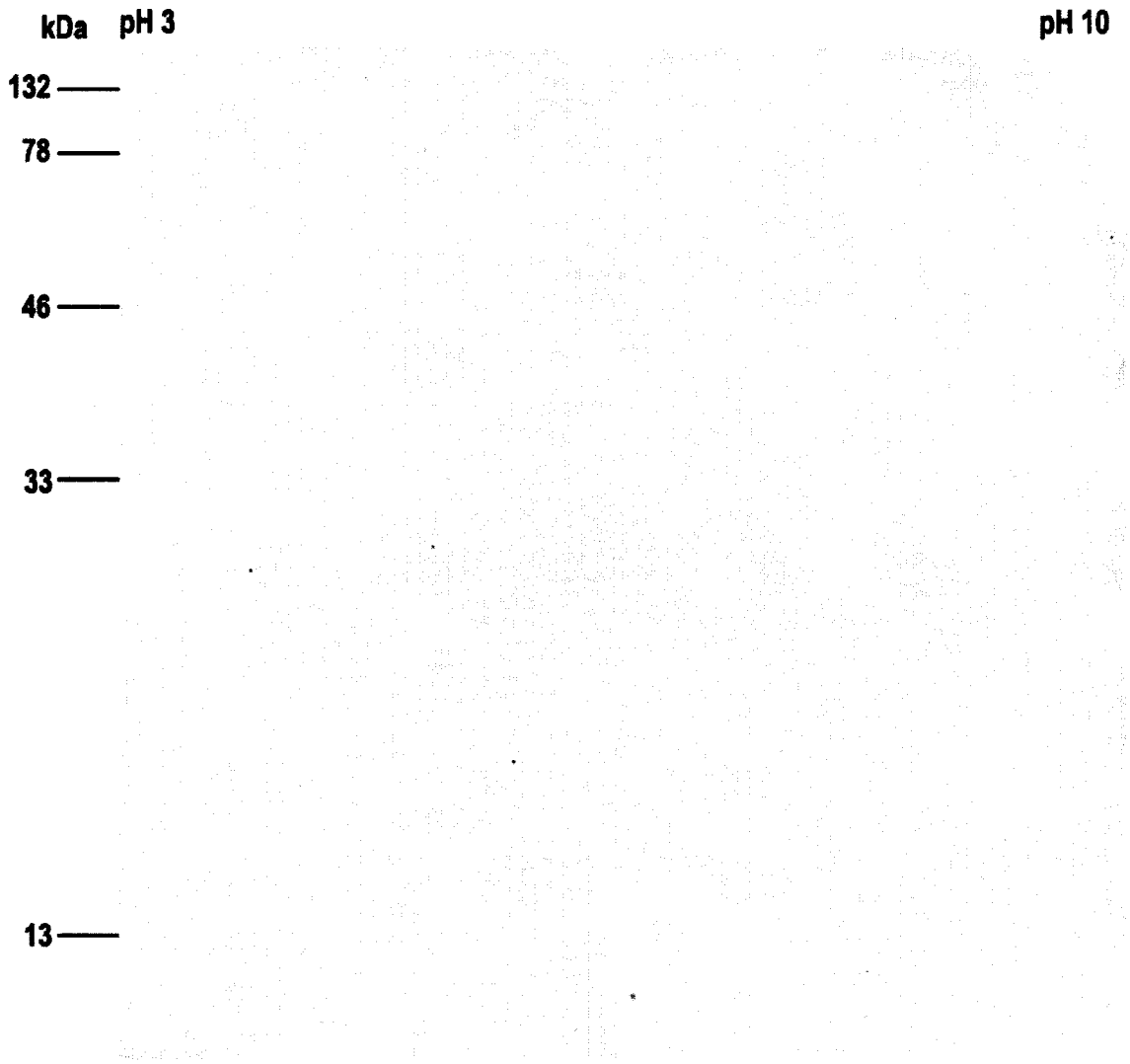

Figure 3. 2DE separated and Western blotted water soluble proteins from pituitaries of female Wistar rats exposed to 0 mg/kg ATRA for 4 days.

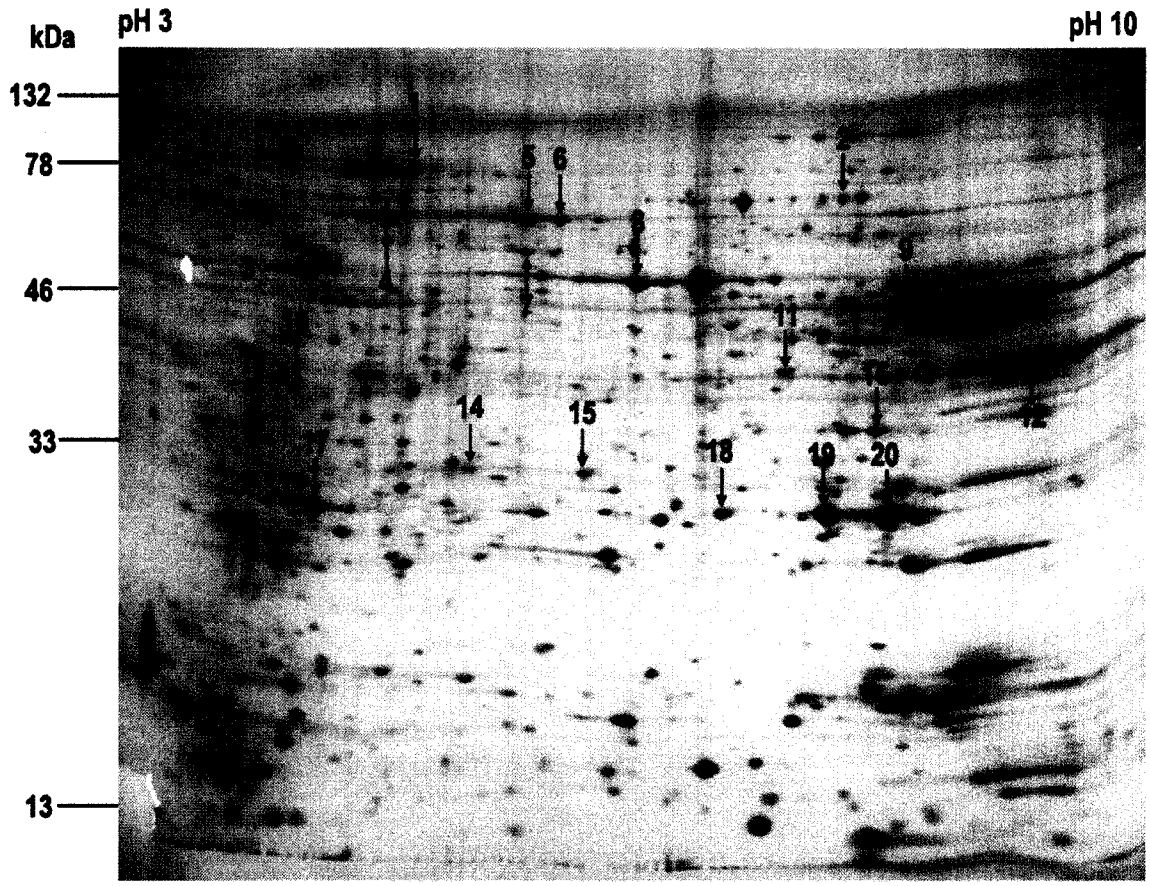

Figure 4. 2DE separated and Sypro Ruby stained water soluble proteins from LβT2 rat pituitary cells exposed to 300 μM DACT for 24 hours.

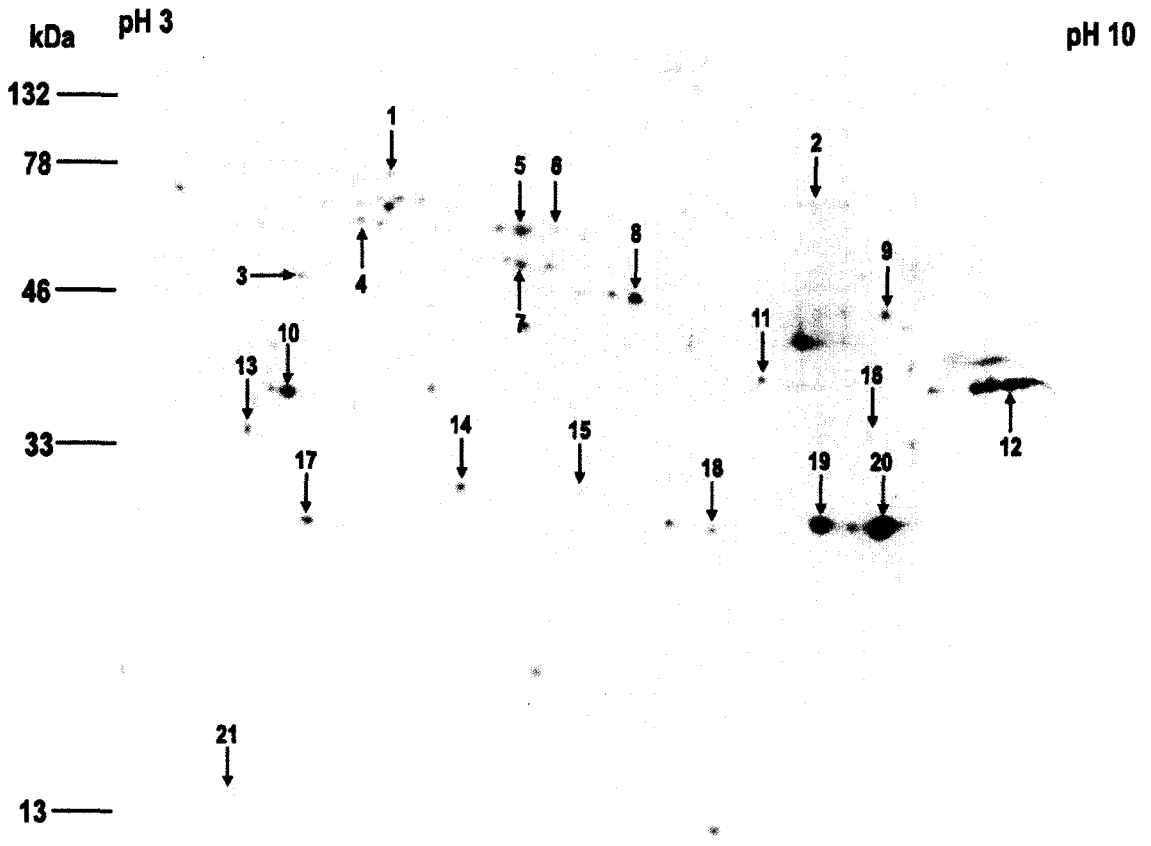

Figure 5. 2DE separated and Western blotted water soluble proteins from LβT2 rat pituitary cells exposed to 300 μM DACT for 24 hours.

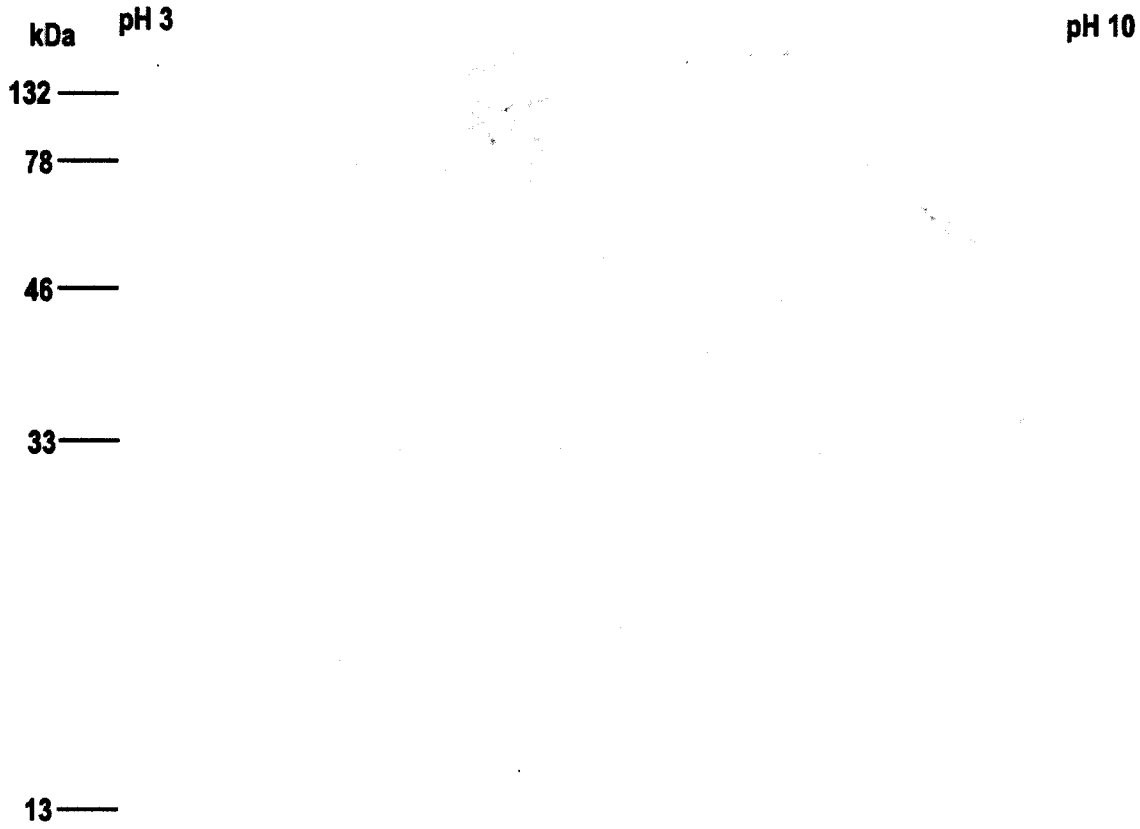

Figure 6. 2DE separated and Western blotted water soluble proteins from LβT2 rat pituitary cells exposed to 0 μM DACT for 24 hours.

Table 1. DACT adducted proteins in the pituitary of Wistar rats exposed to 200 mg/kg ATRA identified with MALDI TOF/TOF MS.

Spot	Protein Designation	Accession No.	MASCOT Protein Ion Score (Cutoff)	Peptides (MS) (Seq. Coverage)	Peptides (MS/MS)*
1	Serum albumin precursor	P02770	226 (50)	13 (24%)	2
2	Serum albumin precursor	P02770	187 (50)	15 (29%)	2
3	Serum albumin precursor	P02770	329 (50)	16 (34%)	4
4	Serum albumin precursor	P02770	541 (50)	21 (43%)	4
5	Serum albumin precursor	P02770	343 (50)	16 (35%)	3
6	Serum albumin precursor	P02770	344 (50)	13 (30%)	3
7	ERp57	P11598	231 (50)	20 (40%)	2
8	ERp57	P11598	495 (50)	24 (45%)	4
9	ERp57	P11598	94 (50)	13 (26%)	1
10	Serum albumin precursor	P02770	56 (50)	6 (12%)	1
11	Serum albumin precursor	P02770	70 (50)	10 (19%)	1
12	Heterogenous nuclear ribonucleoprotein H	120538378	89 (59)	7 (23%)	1
13	Serum albumin precursor	P02770	57 (50)	14 (33%)	-
14	Heterogenous nuclear ribonucleo protein A2/B1	14043072	178(59)	11 (27%)	3
15	Tropomyosin 1 alpha	P04692	84 (50)	7 (17%)	1
16	Proteasome activator complex subunit 1	Q63797	242 (50)	12 (57%)	4
17	Ubiquitin carboxyl-terminal hydrolase isozyme L1	Q00981	167 (50)	10 (62%)	2
18	Peroxiredoxin-6	O35244	72 (50)	7 (37%)	2
19	Peroxiredoxin-6	O35244	50 (50)	6 (33%)	-

* Individual peptide sequences and ion scores can be found in supplementary data.

Table 2. DACT adducted proteins in LβT2 cells exposed to 300μM DACT identified with LC MS/MS

Spot	Protein Designation	Accession No.	No. of Peptides (MS/MS)*	Seq. Coverage
1	Stress-70 Protein	116242506	11	21%
2	Heteronuclear nuclear ribonucleoprotein 1	55562839	3	17%
3	26 S Protease regulatory subunit 6A	2492523	9	25%
4	Heat shock protein 60	51702230	16	43%
5	ERp57	1352384	13	30%
6	ERp57	1352384	8	18%
7	Heteronuclear nuclear ribonucleoprotein H2	62078769	4	14%
8	Alpha Enolase	56757324	8	30%
9	Phosphoglycerate kinase 1	40254752	12	38%
10	PREDICTED: similar to pyridoxal phosphatase	109487833	4	12%
11	Heteronuclear nuclear ribonucleoprotein D-like	76096336	2	5%
12	Heteronuclear nuclear ribonucleoprotein A2/B1	14043072	7	24%
13	Tropomyosin 3	438882	5	15%
14	Proteasome activator complex subunit 1	18202600	9	32%
15	Proteaseome 26S non-ATPase Subunit 9	18426882	3	14%
16	Alpha ETF	57527204	5	23%
17	Ubiquitin carboxyl-terminal hydrolase isozyme L1	61098212	8	52%
18	Flavin reductase	34855391	2	22%
19	Triosephosphate isomerase 1	117935064	8	51%
20	Triosephosphate isomerase 1	117935064	10	57%
21	Beta-tubulin	40018568	3	10%

* Individual peptide sequences and Xcorr scores can be found in supplementary data.

Supplementary Data 1.

Spot No.	Protein designation (Accession number)	Database Searched	Mascot protein ion score (cutoff score)	Sequence coverage (No. of matched peaks)	Peptide sequences identified by MS/MS spectra	Mascot MS/MS score (cutoff score)
1	Serum albumin precursor (P02770)	Swiss-Prot	226 (50)	24% (13)	K.LGEYGFQNAVLVR.Y K.DVFLGTFLYEYSR.R	120 (23)
2	Serum albumin precursor (P02770)	Swiss-Prot	187 (50)	29% (15)	K.LGEYGFQNAVLVR.Y K.DVFLGTFLYEYSR.R	95 (23)
3	Serum albumin precursor (P02770)	Swiss-Prot	329 (50)	34% (16)	K.LGEYGFQNAVLVR.Y K.DVFLGTFLYEYSR.R R.LPCVEDYLSAILNR.L R.RPCFSALTVDETYVPK.E	203 (23)
4	Serum albumin precursor (P02770)	Swiss-Prot	541 (50)	43% (21)	K.LGEYGFQNAVLVR.Y K.DVFLGTFLYEYSR.R R.LPCVEDYLSAILNR.L R.RPCFSALTVDETYVPK.E	405 (23)
5	Serum albumin precursor (P02770)	Swiss-Prot	343 (50)	35% (16)	K.LGEYGFQNAVLVR.Y K.DVFLGTFLYEYSR.R R.LPCVEDYLSAILNR.L	176 (23)
6	Serum albumin precursor (P02770)	Swiss-Prot	344 (50)	30% (13)	K.LGEYGFQNAVLVR.Y K.DVFLGTFLYEYSR.R R.RPCFSALTVDETYVPK.E	244 (23)
7	Protein disulfide-isomerase A3 precursor (P11598)	Swiss-Prot	231 (50)	40% (20)	K.FVMQEEFSR.D R.ELNDFISYLQR.E	72 (23)
8	Protein disulfide-isomerase A3 precursor (P11598)	Swiss-Prot	495 (50)	45% (24)	K.FVMQEEFSR.D R.ELNDFISYLQR.E K.TFSHELSDFGLESTTGEIPVVAIR.T R.FLQEYFDGNLKR.Y	280 (23)
9	Protein disulfide-isomerase A3 precursor (P11598)	Swiss-Prot	94 (50)	26% (13)	R.FLQEYFDGNLKR.Y	17 (23)
10	Serum albumin precursor (P02770)	Swiss-Prot	56 (50)	12% (6)	K.LGEYGFQNAVLVR.Y	27 (23)
11	Serum albumin precursor (P02770)	Swiss-Prot	70 (50)	19% (10)	K.LGEYGFQNAVLVR.Y	21 (23)
12	Heterogeneous nuclear ribonucleoprotein H (gi|120538378)	NCBInr	89 (59)	23% (7)	K.HTGPNSPDTANDGFVR.L	24 (31)

13	Serum albumin precursor (P02770)	Swiss-Prot	57 (50)	33% (14)		
14	Heterogeneous nuclear ribonucleoprotein A2/B1 (gi|14043072)	NCBInr	178 (59)	27% (11)	R.GGNFGFGDSR.G R.GGGGNFGPGPGSNFR.G K.LFIGGLSFETTEESLR.N	110 (32)
15	Tropomyosin 1 alpha chain (P04692)	Swiss-Prot	84 (50)	17% (7)	R.IQLVEEELDR.A	61 (23)
16	Proteasome activator complex subunit 1 (Q63797)	Swiss-Prot	242 (50)	57% (12)	K.IVVLLQR.L K.QPHVGDYR.Q K.APLDIPVPDPVKEK.E R.QLVHELDEAEYQEIR.L	157 (23)
17	Ubiquitin carboxyl-terminal hydrolase isozyme L1 (Q00981)	Swiss-Prot	167 (50)	62% (10)	K.LGVAGQWR.F K.NEAIQAAHDSVAQEGQCR.V	75 (24)
18	Peroxiredoxin-6 (O35244)	Swiss-Prot	72 (50)	37% (7)	R.VVFIFGPDKK.L K.LSILYPATTGR.N	30 (22)
19	Peroxiredoxin-6 (O35244)	Swiss-Prot	50 (50)	33% (6)		

Supplementary Data 2.

Spot No.	Protein Designation (Accession No.)	No. Unique Peptides	Peptide Sequences	SEQUEST XCorr Score
1	Stress-70 protein (116242506)	11	DAGQISGLNVLR	3.24
			EQQIVIQSSGGLSK	2.8
			ERVEAVNMAEGIIHDTETK	3.48
			ETAENYLGHTAK	2.28
			LLGQFTLIGIPPAPR	2.93
			NAVITVPAYFNDSQR	2.13
			QAVTNPNNTFYATK	2.94
			STNGDTFLGGEDFDQALLR	4.42
			VEAVNMAEGIIHDTETK	3.06
			VINEPTAAALAYGLDK	4.09
			VQQTVQDLFGR	3.06
2	Heterogeneous nuclear ribonucleoprotein 1 (55562839)	3	AITHLNNNFMFGQK	3.2
			MGPPVGGHR	2.41
			SKPGAAMVEMADGYAVDR	3.65
3	26S Protease regulatory subunit 6A (2492523)	9	APSIIFIDELDAIGTK	3.97
			DSYLILETLPTEYDSR	2.78
			EKAPSIIFIDELDAIGTK	4.45
			FENLGIQPPK	3
			KMNVSPDVNYEELAR	4.9
			LKPGDLVGVNK	2.65
			QIQELVEAIVLPMNHK	2.97
			QTYFLPVIGLVDAEK	2.56
			VDILDPALLR	2.97
4	Heat Shock Protein-60 (51702230)	16	AAVEEGIVLGGGCALLR	3.82
			ALMLQGVDLLADAVAVTMGPK	4.81
			CEFQDAYVLLSEK	3.88
			DIGNIISDAMK	2.74
			ISSVQSIVPALEIANAHR	3.45
			KISSVQSIVPALEIANAHR	4.28
			LSDGVAVLK	2.47
			LVQDVANNTNEEAGDGTTTATVLAR	4.09
			NAGVEGSLIVEK	3.07
			QSKPVTTPEEIAQVATISANGDK	2.52
			TALLDAAGVASLLTTAEAVVTEIPK	4.38
			TLNDELEIIEGMK	3.21
			TVIIEQSWGSPK	3.61
			VGGTSDVEVNEK	2.92
			VGLQVVAVK	2.29
			VTDALNATR	3.52
5	ERp57 (1352384)	13	DASVVGFFR	2.6
			DLFSDGHSEFLK	3.01
			DLLTAYYDVDYEK	3.78
			EATNPPIIQEEKPK	2.55
			ELNDFISYLQR	2.76
			FISDKDASVVGFFR	2.68
			FVMQEEFSR	2.58

			GFPTIYFSPANK	2.87
			LAPEYEAAATR	2.27
			LNFAVASR	2.48
			MDATANDVPSPYEVK	3.56
			TADGIVSHLK	2.84
			YGVSGYPTLK	2.48
6	ERp57 (1352384)	8	DASVVGFFR	2.06
			DLFSDGHSEFLK	2.71
			EATNPPIIQEEKPK	2.49
			ELNDFISYLQR	2.9
			GFPTIYFSPANK	2.37
			LAPEYEAAATR	2.35
			QAGPASVPLR	2.07
			YGVSGYPTLK	2.55
7	Heterogeneous nuclear ribonucleoprotein H2 (62078769)	4	ATENDIYNFFSPLNPMR	4.71
			HTGPNSPDTANDGFVR	3.64
			STGEAFVQFASQEIAEK	4.37
			VHIEIGPDGR	2.33
8	Alpha-enolase (56757324)	8	AAVPSGASTGIYEALELR	3.52
			DATNVGDEGGFAPNILENK	3.71
			GNPTVEVDLYTAK	2.89
			GVSKAVEHINK	2.8
			HIADLAGNPEVILPVPAFNVINGGSHAGNK	6.61
			IGAEVYHNLK	2.65
			LAMQEFMILPVGASSFR	4.69
			YITPDQLADLYK	2.03
9	Phosphoglycerate kinase 1 (40254752)	12	AHSSMVGVNLPQK	3.22
			ALESPERPFLAILGGAK	3.91
			DVLFLK	2
			ELNYFAK	1.52
			FHVEEEGK	2.02
			ITLPVDFVTADK	2.25
			ITLPVDFVTADKFDENAK	4.43
			LGDVYVNDAFGTAHR	3.14
			VLNNMEIGTSLYDEEGAK	3.82
			VNEMIIGGGMAFTFLK	4.3
			VSHVSTGGGASLELLEGK	4.37
			YSLEPVAAELK	2.59
10	PREDICTED: similar to pyridoxal phosphatase (109487833)	4	AVVVGFDPHFSYMK	2.71
			GETAVPGAPETLR	2.1
			LGFITNNSSK	2.01
			TILTLTGVSSLEDVK	3.4
11	Heterogeneous nuclear ribonucleoprotein D-like (76096336)	2	DLTEYLSR	2.54
			YHQIGSGK	2.02
12	Heterogeneous nuclear ribonucleoprotein A2/B1 (14043072)	7	DYFEEYGK	2.24
			EESGKPGAHVTVK	2.47
			GFGFVTFDDHDPVDK	2.95
			GGGGNFGPGPGSNFR	2.13
			GGNFGFGDSR	2.48

			IDTIEIITDR	3.1
			LFIGGLSFETTEESLR	4.19
13	Tropomyosin 3 (438882)	5	EQAEAEVASLNR	2.48
			IQVLQQQADDAEER	3.69
			KIQVLQQQADDAEER	4.55
			KLVIIEGDLER	2.45
			LVIIEGDLER	2.66
14	Proteasome activator complex subunit 1 (18202600)	9	APLDIPVPDPVK	2.04
			EPALNEANLSNLK	2.45
			ISELDAFLK	2.6
			ISELDAFLKEPALNEANLSNLK	6.18
			KISELDAFLK	2.59
			LMVMEIR	2.13
			NAYAVLYDIILK	3.28
			QLVHELDEAEYQEIR	4.67
			TENLLGSYFPK	3.16
15	Proteasome 26S non-ATPase subunit 9 (18426882)	3	ADVDLYQVR	2.72
			ANYDVLESQK	2.97
			QVEEALHQLHAR	2.52
16	Alpha-ETF (57527204)	5	AAVDAGFVPNDMQVGQTGK	3.33
			GLLPEELTPLILETQK	3.05
			LGGEVSCLVAGTK	3.8
			LLYDLADQLHAAVGASR	4.35
			LNVAPVSDIIEIK	2.79
17	Ubiquitin carboxy-terminal hydrolase L1 (61098212)	8	FSAVALCK	2.54
			LEFEDGSVLK	2.18
			LGVAGQWR	2.16
			MPFPVNHGASSEDSLLQDAAK	4.19
			MQLKPMEINPEMLNK	2.6
			NEAIQAAHDSVAQEGQCR	4.54
			QTIGNSCGTIGLIHAVANNQDK	3.09
			VNFHFILFNNVDGHLYELDGR	5.79
18	Flavin reductase (NADPH) (34855391)	2	TGLTTLAQAVQAGYEVTVLVR	4.35
			YVAVMPPHIGDQPLTGAYTVTLDGR	5.53
19	Triosephosphate isomerase (109483205)	8	DLGATWVVLGHSER	4.08
			ELASQPDVDGFLVGGASLKPEFVDIINAK	2.97
			HVFGESDELIGQK	3.47
			IIYGGSVTGATCK	3.11
			RHVFGESDELIGQK	3.21
			TATPQQAQEVHEK	2.39
			VTNGAFTGEISPGMIK	3.48
			VVLAYEPVWAIGTGK	3.36
20	Triosephosphate isomerase (109483205)	10	DLGATWVVLGHSER	3.47
			ELASQPDVDGFLVGGASLKPEFVDIINAK	2.7
			FFVGGNWK	2.04
			HVFGESDELIGQK	2.42
			IIYGGSVTGATCK	2.75
			KFFVGGNWK	2.06
			RHVFGESDELIGQK	3.45
			TATPQQAQEVHEK	2.05

			VTNGAFTGEISPGMIK	1.6
			VVLAYEPVWAIGTGK	2.02
21	Tubulin, beta, 2 (40018568)	3	AVLVDLEPGTMDSVR	2.94
			GHYTEGAELVDSVLDVVR	3.56
			INVYYNEATGGK	3.16

CHAPTER 6

Conclusions

This research project started with the theory that ATRA may be reactive towards proteins *in vivo* leading to formation of covalent adducts. This theory was based on observations from studies of rats given [^{14}C]-ATRA that showed radioactivity in plasma and red blood cells and mass spectral analysis of hemoglobin from ATRA exposed rats indicating a mass shift in the β-subunit peak suggestive of an adduct. The initial objective of this research was to conclusively determine if ATRA exposure caused the formation of a covalent adduct with hemoglobin with the intention of developing a biomarker of exposure based on this interaction.

Using hemoglobin from SD rats given 10-300 mg/kg ATRA for 3 days, we identified a 110 Da adduct on Cys-125 of the β-subunit by mass spectrometry analysis of tryptic peptide. The adduct reached a maximum 10 days following the initial exposure and had disappeared by 2 months due to protein turnover. This modification was not seen in control exposures. The structure of this adduct was not known, but based on the mass, a dechlorinated DACT was thought to be responsible for the observed adduct. This was confirmed with *in vitro* exposures of hemoglobin with DACT that generated an identical adduct as observed with the *in vivo* ATRA exposures. The proposed mechanism of the DACT adduct formation is via nucleophilic aromatic substitution, since the chlorine would provide a carbon center with partial positive charge favoring this displacement. The absence of ATRA adducts and adducts of the metabolites desethylatrazine or desisopropylatrazine was likely due to rapid metabolism to DACT or steric influences of the alkyl groups. These results clearly showed that in SD rats ATRA metabolism to DACT caused the formation of a covalent adduct with a Cys residue of hemoglobin and

raised the possibly other proteins with sulfhydryl functional groups being modified in a similar reaction.

Because humans lack Cys-125 in their hemoglobin, this adduct was not directly applicable to human biomonitoring, but it did provide evidence that an adduct could form on other Cys residues, which may be directly applicable to humans. Human and rat albumin are nearly identical and both contain an exposed Cys-34 similar to hemoglobin Cys-125, therefore results from measuring ATRA adducts at this Cys-34 residue in *in vivo* rodent exposures could be extrapolated to humans for developing a human biomarker of ATRA exposure.

Since, albumin Cys-34 forms covalent adducts with many electrophilic xenobiotics and radioactivity has been observed in rat plasma after treatment with C^{14}-ATRA, we investigated the formation of a DACT adduct at the Cys-34 residue of albumin from rats exposed to ATRA. We also validate the use of a DACT adduct specific antibody in an immunodetection methodology for biomonitoring ATRA exposure.

Mass spectrometry analysis of trypsin digested albumin from Wistar rats given 20-200 mg/kg for 4 days showed a 110 Da adduct on Cys-34 that was not seen in control exposures. This adduct was again confirmed to result from a nucleophilic substitution reaction by *in vitro* incubations of rat albumin and DACT. Human albumin was also incubated with DACT resulting in an identical 110 Da adduct on Cys-34. This evidence confirming the formation of a DACT adduct on Cys-34 of human albumin, although only *in vitro*, makes detection of ATRA induced protein adducts a viable target for human exposure biomonitoring.

To provide more practical method of detecting the DACT-albumin adduct as a biomarker of exposure, we generated a polyclonal antibody which recognizes DACT adducts in the specific orientation that it would be found if formed *in vivo.* Using this antibody, we were able to detect albumin adducts via western blot from all *in vivo* ATRA dose levels and with both human and rat albumin exposed *in vitro* to DACT. These immunodetection results were validated with the corresponding mass spectrometry detection of the DACT adduct. The observation that human albumin with a Cys-34 DACT adduct formed *in vitro* can be detected by this methodology suggests the possibility of developing a rapid assay for internal ATRA exposure. Current suggested biomarkers for ATRA exposure focus on detection of the parent compound or metabolites in biological fluids, which does not show a biologically significant interaction has occurred as detection of a protein adduct does. By detecting protein adducts, we provide a better method for exposure because we can demonstrate that an internal dose of ATRA has occurred. Also, since albumin has a relativity slow turnover rate, adducts could be detected several weeks post exposure, a much longer detection window than other biomarker methodologies. This research provides solid evidence for the potential of immunodetection methodology for the detection of DACT-protein adducts as a biomarker of ATRA exposure.

The identification of these two DACT modified proteins caused us to broaden the scope of this research past using protein adducts as a biomarker of exposure to what potential toxicity protein adduct may induce such as LH suppression from the pituitary. Currently there is little known about the mechanism of action of DACT and/or ATRA exposures with relation to suppression of LH release from the pituitary. Since we have

shown DACT is capable of forming a covalent protein adducts and brain tissue is exposed to large quantities of DACT following ATRA exposure, it is possible that DACT is forming an adduct with proteins involved in the signal transduction pathway leading to LH release from the pituitary. This DACT-protein adduct could lead to alterations in function or premature degradation of the proteins leading to suppression of the LH release. Given the critical link between the pituitary gland and LH release, we investigated ATRA-induced protein adducts formed in the pituitary gland of ATRA exposed rats and in DACT exposed LβT2 rat pituitary cells.

Ovariectomized female Wistar rats were given 200 mg/kg ATRA for 4 days, while LβT2 rat pituitary cells were incubated with 300 μM DACT for 24 hours. DACT protein targets in the water soluble fraction of lysates from both the rat pituitaries and LβT2 cells were analyzed with 2-DE, immunodetection with the DACT adduct antibody, spot matching, and MALDI-TOF or LC mass spectrometry analysis of the trypsin digested protein spots. MS analysis resulted in the identification of 19 spots and 8 unique proteins in the rats and 21 spots and 19 unique proteins in LβT2 cells. The identified proteins present in both sample types included proteasome activator complex subunit 1, ubiquitin carboxyl-terminal hydrolase isozyme L1, tropomyosin, ERp57, and RNA binding proteins (heterogeneous nuclear ribonucleoprotein A2/B1 and heterogeneous nuclear ribonucleoprotein 1). Each of these proteins contains active site or solvent exposed Cys residues similar to albumin and hemoglobin, making them viable targets for covalent modification by DACT. Further research on the individually identified proteins is necessary to determine if their modification by DACT plays a role in the mechanism of LH suppression from the pituitary following ATRA exposure.

In summary, this research has demonstrated the ATRA metabolite DACT forms covalent adducts with numerous proteins following ATRA exposure, both cellular and extracellular. It is clear from these results that DACT is the primary active metabolite following ATRA exposure, likely acting as an alkylating agent to Cys residues. We have shown this to be true for albumin and hemoglobin and most the targeted proteins in the pituitary contain exposed Cys residues supporting this mechanism of action. The functional significance of this DACT modification to the proteins is unknown at this time, but this interaction provides us with a method of monitoring ATRA exposure as well as a future direction to investigate the toxicity of ATRA.

CHAPTER 7

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