LAWRENCE
LIVERMORE
NATIONAL
LABORATORY

LLNL-TR-695881

# Mechanism for Clastogenic Activity of Naphthalene

B. A. Buchholz

June 24, 2016

## Disclaimer



This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

MILITARY INTERDEPARTMENT PURCHASE REQUEST: 10567486

TITLE: Mechanism for Clastogenic Activity of Naphthalene

PRINCIPAL INVESTIGATOR: Bruce A. Buchholz

CONTRACTING ORGANIZATION: Lawrence Livermore National Laboratory

REPORT DATE: June 20, 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012



LLNL-TR-695881

## Disclaimer



## Auspices Statement

This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| 06/20/2016 | Final | 1 Sep 2014- 31 Mar 2016 |

**4. TITLE AND SUBTITLE**

Mechanism for Clastogenic Activity of Naphthalene

**5a. CONTRACT NUMBER**
10567486

**5b. GRANT NUMBER**
LC130820

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Bruce A. Buchholz

E-Mail: buchholz2@llnl.gov

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Lawrence Livermore National Lab
7000 East Avenue
Livermore, CA 94550

UC Davis
One Shields Avenue
Davis, CA 95616

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

Naphthalene incubations form DNA adducts in vitro in a dose dependent manner in both mouse and rat tissues. Rodent tissue incubations with naphthalene indicate that naphthalene forms as many DNA adducts as Benzo(a)pyrene, a known DNA binding carcinogen. The mouse airway has the greatest number of DNA adducts, corresponding to the higher metabolic activation of naphthalene in this location. Both rat tissues, the rat olfactory (tumor target) and the airways (non-tumor target), have similar levels of NA-DNA adducts, indicating that short term measures of initial adduct formation do not directly correlate with sites of tumor formation in the NTP bioassays.

**15. SUBJECT TERMS**

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| Unclassified | Unclassified | Unclassified | Unclassified | 12 | 19b. TELEPHONE NUMBER *(include area code)* |

## Table of Contents

**Page**

## 1. Introduction

There are two primary sources of naphthalene (NA) exposure to military personnel and to their families: the use of JP-8 as a common fuel for both aircraft and transport vehicles and mainstream and sidestream tobacco smoke. Jet fuels contain 1-3% naphthalene by weight, DoD uses an estimated 5.5 billion gallons of JP-8 annually. Naphthalene is the single most prevalent aromatic hydrocarbon in second hand smoke exceeding the levels of polycylic aromatic hydrocarbons like benzo(a)pyrene (BaP) nearly 250 fold (Witschi et al, Carcinogenesis 18, 2035, 1997). The EPA has listed NA as a probable human carcinogen based on the results of the cancer bioassay conducted in both mice and rats. However, there are many groups arguing that NA-induced respiratory tumors in rodents are not relevant to estimating the risks to exposed human populations (see Piccirillo et al, Regul Toxicol Pharmacol. 62: 433, 2012) based on the much slower rates of microsomal NA metabolism in primate compared to rodent respiratory tissue, on the finding that NA is not mutagenic in most *in vitro* short term assays and on epidemiologic data showing that nasal tumors in humans are very rare. This work is intended to provide solid evidence for or against a clastogenic mechanism of action for NA in rodent and non-human primate respiratory tissues. Determining the ability of fresh non-human primate tissues to generate NA metabolites that bind to DNA using ultrasensitive and highly specific assays would provide a mechanistic basis for assessing the risks of NA exposure. If these studies demonstrate binding of metabolites generated in primate tissues to DNA, either removal of NA from fuel sources or additional engineering controls could be established to further protect personnel.

## 2. Keywords

naphthalene, DNA adducts, clastogen, metabolite

## 3. Overall Project Summary

All IACUC and ACURO approvals were obtained quickly at the start of project and the subcontract between LLNL and UC Davis (UCD) was executed at the start of December 2014. During the reporting period all the rodent exposures were completed. Rhesus monkey exposures on animals culled from the colony at the UCD California National Primate Research Center (CNPRC) need to be conducted during the fall of 2015.

As proposed, all *in vitro* exposures were conducted using fresh micro-dissected tissues obtained at UCD as described in Van Winkle et al (1996). Tissues were incubated with 2.5, 25, or 250 μM $^{14}$C-NA, 25 or 50 μM $^{14}$C-benzo(a)pyrene (BaP), or unlabeled sham controls. The groups were treated with NA at 250 and 2.5 μM to test the upper and lower limits of exposure based on John Morris' published (2012) calculations of an upper limit for delivered dose at 250 μM as equivalent to the 10 ppm OSHA exposure limit for NA. Tissues were incubated for 60 minutes followed by 12-15 rinses with ethanol to remove unbound NA or BaP. Tissues were rinsed until the rinse no longer had excess $^{14}$C. DNA was isolated from tissues with Qiagen DNeasy kits according to the manufacturer protocols with modification. Two proteinase K digestions were done to assure complete removal of protein. DNA purity was assessed using UV absorbance at 260/280 nm. DNA samples were then prepared for graphitic carbon $^{14}$C-AMS analyses using standard procedures (Ognibene et al, 2003; Ognibene et al, 2015a). The NA-DNA adduct levels are depicted in Figures 1-5.

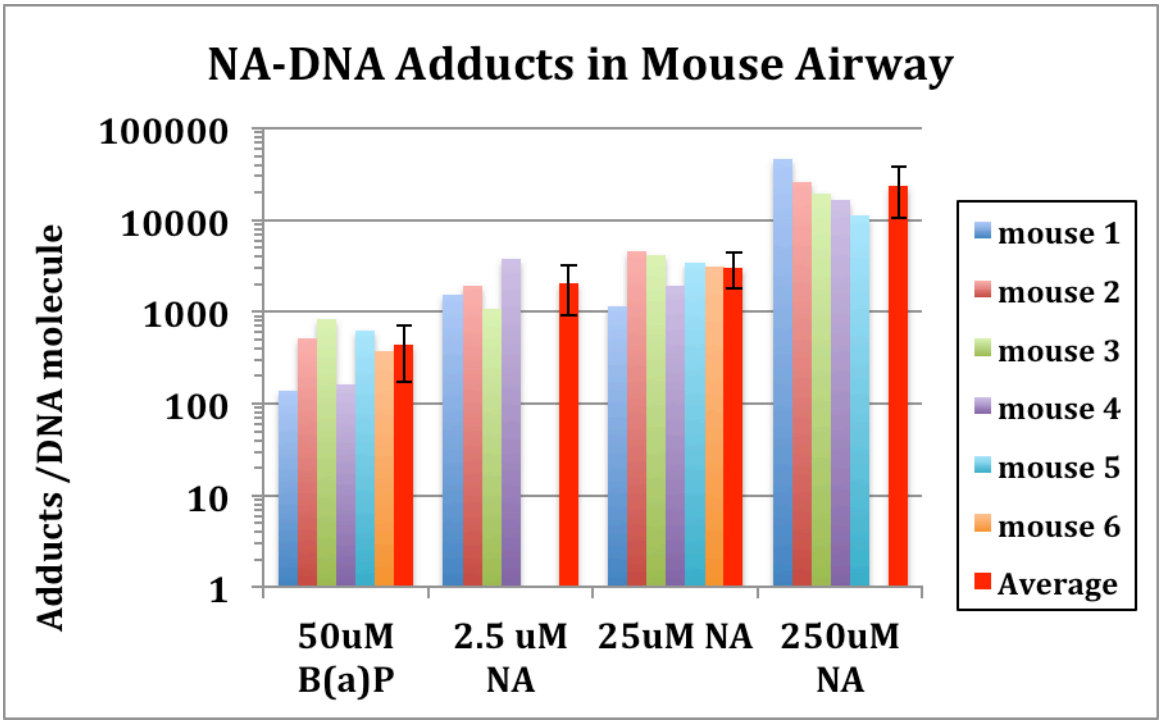



Figure 1. Adducts of BaP or NA per genome in mouse airway. Six replicates were prepared for each dose group with one sample failure at the 250 μM NA and two sample failures at the 2.5 μM NA dose. The average is shown for each dose group.

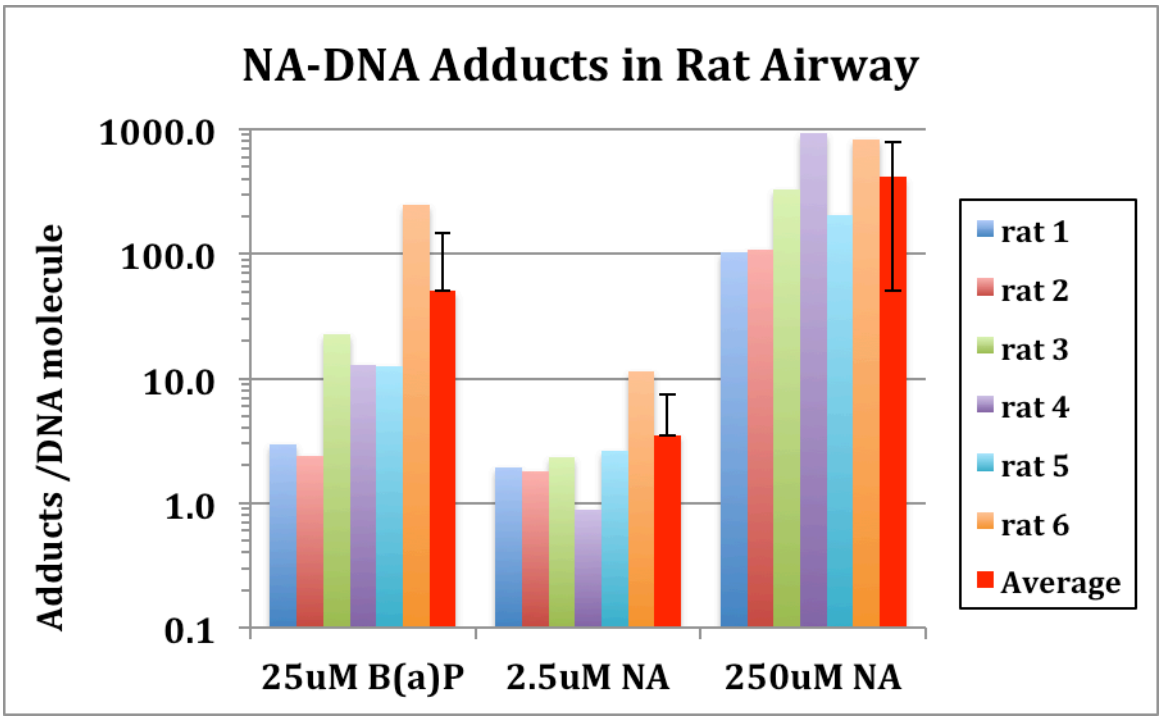



Figure 2. Adducts of BaP and NA per genome in rat airway. Six replicates were prepared and the average is shown for each dose group.

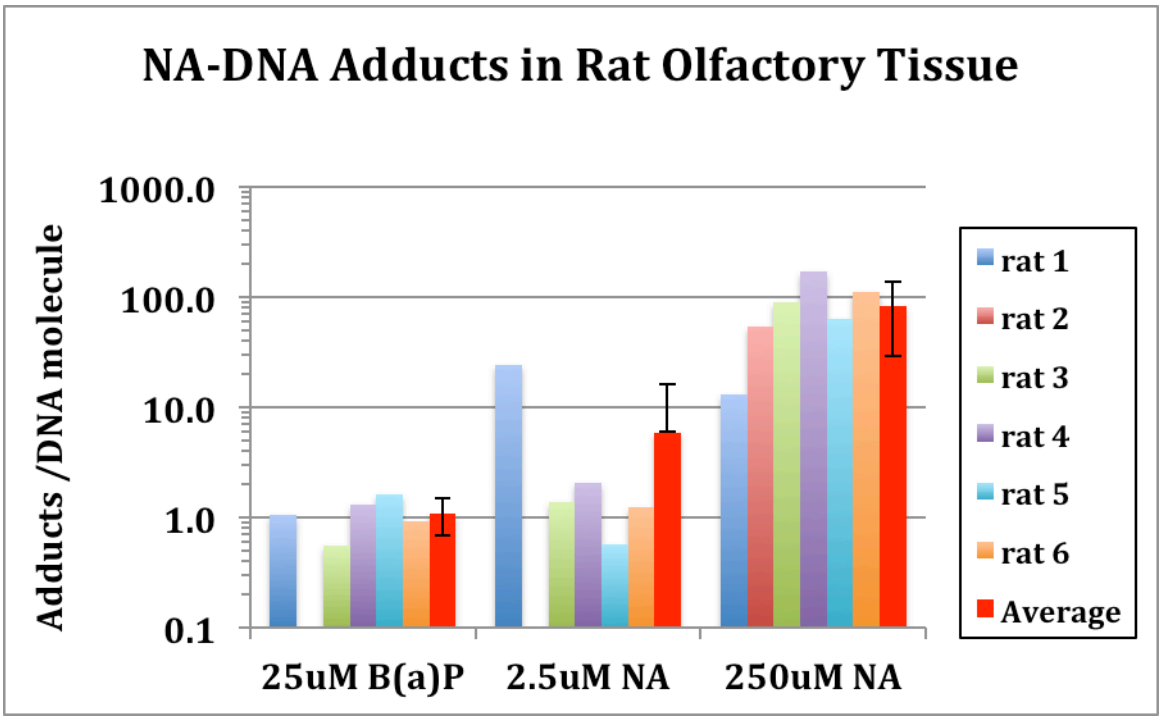



Figure 3. Adducts of BaP and NA per genome in rat olfactory tissue. Six replicates were prepared for each dose group with one sample failure at each the 25 µM BaP and the 2.5 µM NA dose. The average is shown for each dose group.

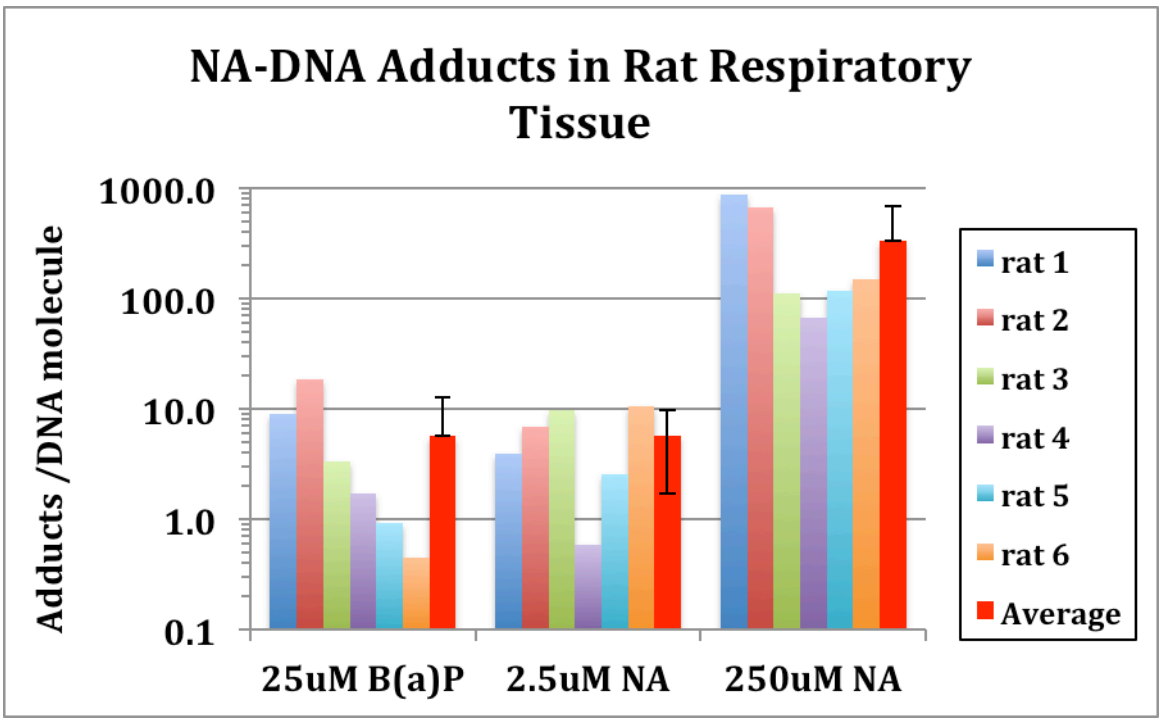



Figure 4. Adducts of BaP and NA per genome in rat respiratory tissue. Six replicates were prepared and the average is shown for each dose group.

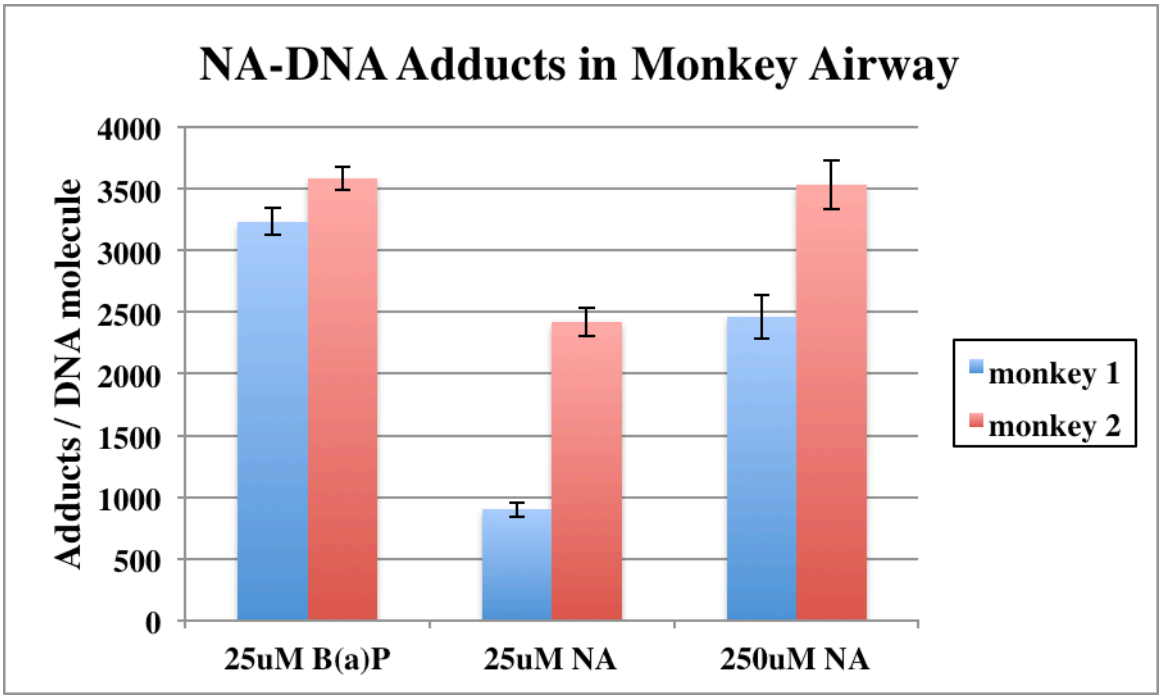



Figure 5. Adducts of BaP and NA per genome in rhesus monkey respiratory tissue. Error bars denote 1 standard deviation uncertainty in measurement.

Based on the National Toxicology Program (NTP) carcinogenesis bioassays (North et al, 2008), we expected to see the highest level of NA-DNA adducts correspond to the recorded cancer sites following NA exposures: bronchiolar alveolar carcinomas in the airways of female mice and neuroblastomas in the nasal epithelium of rats. Instead, we found the highest measured frequency of NA-DNA adducts in mouse airway, followed by rat airway. Rat olfactory tissue had low levels of adducts similar to rat respiratory tissue. Rat airway did not develop tumors in the NTP bioassay (North et al, 2008). The limited number of monkey airway tissues exposed indicated that NA-DNA adducts formed at a rate lower than B(a)P-DNA adducts. Our experiments did not examine adduct stability, only whether adducts formed upon exposure.

**DNA Adduct Identification**

The experiments aimed at determining which DNA bases were adducted by specific NA metabolites were more difficult to complete than anticipated. Producing adducted nucleosides of NA metabolites was impeded by the inability to obtain all the reactive NA metabolites. Our original plan was to perform *in vitro* exposures and purify DNA as described above, followed by DNA digestion and HPLC separation of adducted bases followed by AMS analyses of collected fractions (Buchholz et al, 1999). Adducted based would be identified by co-chromatography with synthetic standards. During the summer of 2015 LLNL acquired a Waters Xevo G2 XS QTOF instrument for qualitative and quantitative sample analysis that has been installed with the new liquid sample AMS interface ((Ognibene et al, 2015b). The QTOF enables accurate mass analyses for a variety of analytical applications, including metabolite profiling, identification, characterization, and quantification of both small and macromolecules. The QTOF is coupled to a Waters Acquity H Class HPLC system used to separate samples for introduction to the liquid sample AMS interface. A flow splitter is configured such that eluent from the HPLC flows to

the QTOF for qualitative and quantitative analysis, and to the AMS instrument for isotope ratio measurement. Mass spectra are aligned based on retention times to match AMS results to mass spectrometric analytes. Coupling QTOF and AMS measurements in this way constitutes a powerful improvement to our analytical capabilities since molecular ion mass identifies the analyte and AMS quantifies the adduct level.

**DNA Digestion Procedure:** DNA concentration was measured using a NanoDrop instrument. Digestion was performed following the procedure reported by Quinlivan and Gregory (2008). Digestion buffer was prepared by adding 200 U benzonase, 200 U alkaline phosphatase, and 300 mU phosphodiesterase I to 5 mL of 20 mM Tris-HCl with 100 mM NaCl and 20 mM $MgCl_2$, pH 7.9. Briefly, 2 micrograms of DNA (at a concentration of 40 ng/µL) were digested by addition of an equal volume (50 µL) of digestion buffer and incubation at 37° C for 6 hours.

**LC-MS-AMS Procedure:** DNA digests were initially analyzed by separation on a Waters Acquity H Class HPLC system with a Phenomenex Luna-2 C18 column, 250 x 4.6 mm, 5 µm particle size. The injection volume was 10 µL. The column temperature was 30° C. Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. A gradient elution was performed over 35 minutes as follows: Initial composition, 10% B; 1 minute, 10% B; 20 minutes, 60% B; 25 minutes, 100% B; 30 minutes, 100% B; 30.1 minutes, 10% B; 35 minutes; 10% B. The flow rate was 0.6 mL/minute, with flow split between the AMS moving wire interface (approximately 100 µL/min) and a Waters Xevo G2-XS mass spectrometer (500 µL/min). Detection was accomplished by AMS, UV detection at 254 nm, and positive mode electrospray ionization in resolution mode.

**LC-AMS Procedure:** Due to initial low detection of Carbon-14 signal, a smaller column was used to enable the analysis of the entire HPLC effluent by AMS without flow splitting. No mass spectral data was collected. Mobile phases, injection volume, column temperature, and UV detection were the same as the LC-MS-AMS procedure. The column was a Waters Atlantis T3 2.1 x 50 mm column with 5 µm particle size. The flow rate was 100 µL/min and the run time was 40 minutes. The gradient was as follows: Initial, 10% B; 1 minute, 10% B; 20 minutes, 60% B; 25 minutes, 100% B; 30 minutes, 100% B; 30.1 minutes, 10% B; 40 minutes, 10% B.

**Results:** Specific adducts were not identified, but carbon-14 signal indicates that it may be possible to measure and identify specific adducts with additional method development in samples containing sufficient material to detect by both AMS and mass spectrometry. Detection of adducts was limited by low carbon-14 signal and inadequate detection by mass spectrometry. Mass spectrometric detection and identification could be enhanced by optimization of flow split ratio and of detection parameters. Optimization of DNA digestion could also improve adduct detection. Figure 6 shows chromatographic separation with flow splitting of undigested (upper panel) and digested DNA (lower panel). Deoxyribonucleosides were not definitively identified, but based on peak retention times, probable peak identification is as follows: 1, Deoxycytidine; 2, Deoxyguanosine; 3, Thymidine; and 4, Deoxyadenosine. Figure 7 shows a chromatogram overlay of digested DNA analyzed by LC-AMS on a 50 mm column without flow splitting. Deoxynucleosides elute earlier on the 50 mm column than on the 250 mm column. The bulk of the carbon-14 signal elutes later than unmodified deoxynucleosides, which is indicative of

naphthalene adducts or incomplete digestion. Additional method development with adequate sample quantities could enable definitive identification of these putative adducts.

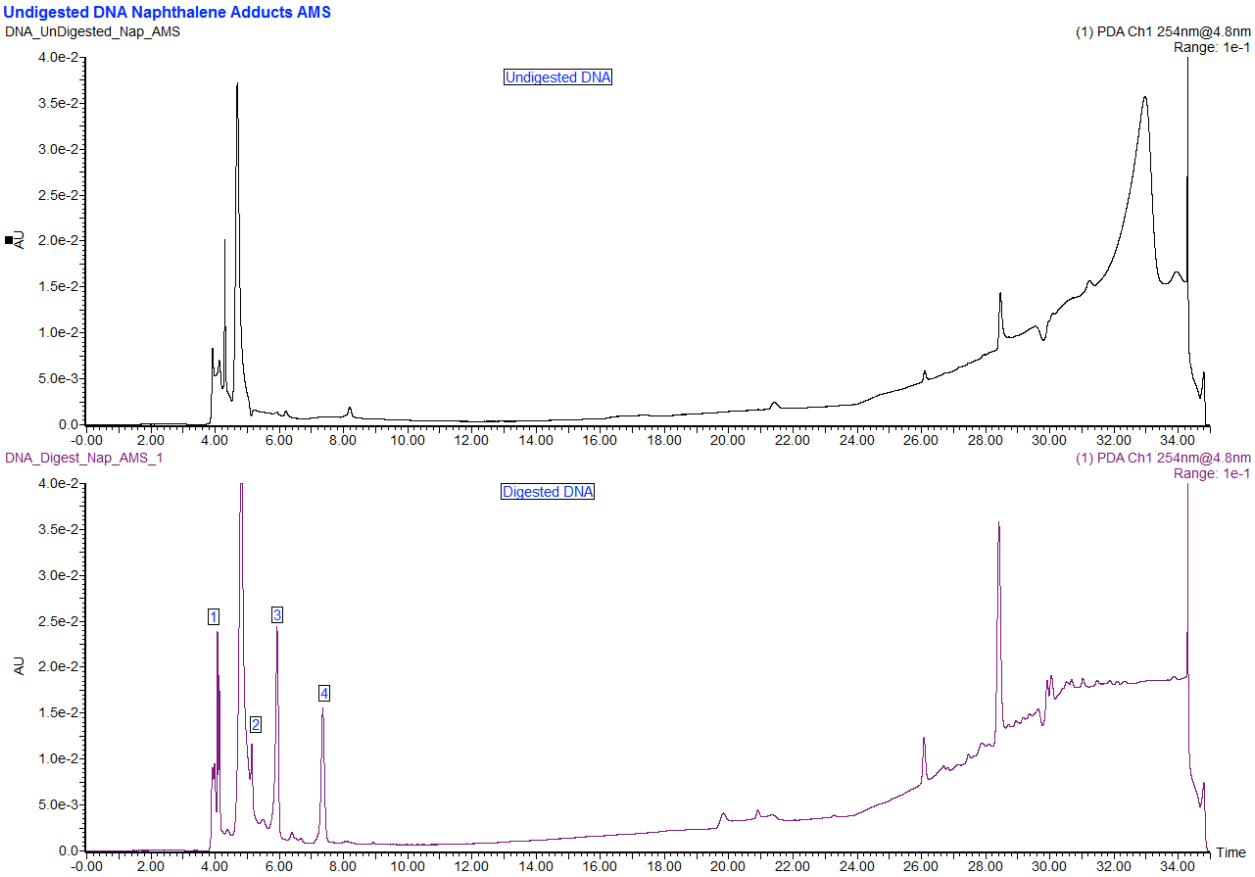



Figure 6. HPLC chromatograms of undigested and digested DNA.

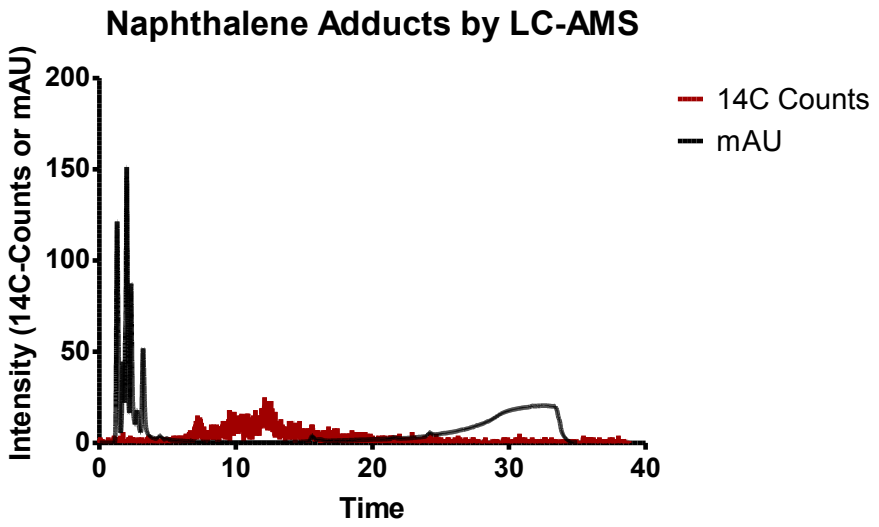



Figure 7. Overlay of HPLC (254 nm) and AMS chromatograms for digested DNA analyzed by LC-AMS on a 50 mm column.

## 4. Key Research Accomplishments

The key research accomplishments from the quantitation of NA-DNA adducts in mouse, rat, and monkey tissues are the following:

- Naphthalene incubations form DNA adducts in a dose dependent manner in both mouse and rat tissues
- Rodent tissue incubations with naphthalene indicate that naphthalene forms as many DNA adducts as Benzo(a)pyrene, a known DNA binding carcinogen, acutely.
- The mouse airway has the greatest number of DNA adducts, corresponding to the higher metabolic activation of naphthalene in this location.
- Both rat tissues, the rat olfactory (tumor target) and the airways (non-tumor target), have similar levels of NA-DNA adducts, indicating that short term measures of initial adduct formation do not directly correlate with sites of tumor formation in the NTP bioassays.
- NA-DNA adducts are formed in rhesus monkey airway at levels slightly below that of B(a)P adducts.
- NA-DNA adducts survive the DNA digest procedure used the low levels of 14C

## 5. Conclusion

The NTP bioassays following NA exposure produced neuroblastomas in the nasal epithelium of rats while rat airways did not grow tumors. If the number of initial NA-DNA adducts was the single key event in determining tumor formation at these sites we would expect more adducts in the rat olfactory epithelium than in the rat airway. However, our data indicates slightly higher formation of adducts in rat airway than rat olfactory tissues. Our experiments quantified NA-DNA adduct formation, but did not assess adduct stability. The lack of tumors in the rat airway of the NTP bioassays suggests the adducts we measured are not stable in the long term and are cleared shortly after formation. Furthermore, the high level of NA-DNA adducts we measured in mouse airway were 60-300x that measured in rat tissues at the 250 μM exposure. Yet, the NTP bioassays found rat nasal tumors more prevalent that mouse airway tumors. It appears as though the airway tissues clear a significant amount of NA-DNA adducts *in vivo*. The rhesus monkey tissue exposures indicated that NA-DNA adducts form at rates similar to B(a)P. When attempting to identify specific adducts, the bulk of the carbon-14 signal elutes later than unmodified deoxynucleosides, which is indicative of naphthalene adducts or incomplete digestion. Additional method development with adequate sample quantities could enable definitive identification of these putative adducts. The limited results from attempts to identify the specific DNA adducts of NA or its metabolites indicate the need of significant effort required for the development of a robust protocol.

## 6. Publications, Abstracts, & Presentations

An abstract was published and poster presented at the Society of Toxicology Meeting in spring 2016. The title of the presentation was "Naphthalene DNA Adduct Formation in *Ex-Vivo* Rodent Tissue." A paper is in preparation and planned to be submitted near September 2016.

## 7. Inventions, Patents, & Licenses

None.

**8. Reportable Outcomes**

NA-DNA adducts were detected after *ex-vivo* exposure in target tissues of three different species, mouse, rat and monkey. Additional work needs to be done to assess the stability of the adducts and specific identification of the adducts formed.

**9. Other Achievements**

None.

**10. References**

Buchholz BA, Fultz E, Haack KW, Vogel JS, Gilman SD, Gee SJ, Hammock BD, Hui X, Westr RC, Maibach HI (1999) HPLC-accelerator MS measurement of atrazine metabolites in human urine after dermal exposure. *Anal Chem* **71(6)**: 3519-3525.

Morris JB (2012) Biologically-based modeling insights in inhaled vapor absorption and dosimetry. Pharmacology & Therapeutics **136(3)**: 401-413.

North DW, Abdo KM, Benson JM, Dahl AR, Morris JB, Renne R, Witschi H (2008). A review of whole animal bioassays of the carcinogenic potential of NA. *Regul Toxicol & Pharmacol* **51**: S6-14. DOI: 10.1016/j.yrtph.2007.09.022

Ognibene TJ, Bench G, Vogel JS, Peaslee GF, Murov S (2003) A high-throughput method for the conversion of CO2 obtained from biochemical samples to graphite in septa-sealed vials for quantification of $^{14}C$ via accelerator mass spectrometry. *Anal Chem* **75(9):** 2192-2196.

Ognibene TJ, Haack KW, Bench G, Brown TA, Turteltaub KW (2015a) Operation of the ‘‘Small’’ BioAMS spectrometers at CAMS: Past and future prospects. *Nucl. Instr. Meth. B* [Epub ahead of print 26 May 2015] doi:10.1016/j.nimb.2015.05.019

Ognibene TJ, Thomas AT, Daley PF, Bench G, Turteltaub KW (2015b) An interface for the direct coupling of small liquid samples to AMS. *Nucl. Instr. Meth. B* [Epub ahead of print 28 May 2015] doi:10.1016/j.nimb.2015.05.018

Piccirillo VJ, Bird MG, Lewis RJ, Boyer WJ (2012) Preliminary evaluation of the human relevance of respiratory tumors observed in rodents exposed to naphthalene. *Regul Toxicol Pharmacol* **62(3)**: 433-440.

Quinlivan EP, Gregory JF (2008) DNA digestion to deoxyribonucleoside: a simplified one-step procedure. *Anal Biochem* **373(2)**: 383-385.

Van Winkle LS, Buckpitt AR, Plopper CG (1996) Maintenance of differentiated mutine Clara cells in microdissected airway cultures. *Am J Respir Cell Mol Biol* **14(6)**: 586-598.

Witschi, H, Espirit, I, Maronpot RR, Pinkerton KE, Jones AD (1997) The carcinogenic potential of the gas phase of environmental tobacco smoke. *Carcinogenesis* **18(11)**: 2035-2042.

**11. Appendices**

Quad chart attached.

Published abstract form the 2016 Society of Toxicology Meeting attached.

# Naphthalene DNA Adduct Formation in Ex-Vivo Rodent Tissues

Sarah A. Carratt, Bruce A. Buchholz, Xinxin Ding, Laura S. Van Winkle

Naphthalene (NA) is a respiratory toxicant and possible human carcinogen. Human epidemiology data are unclear on the long-term effects of NA exposure and risk assessment relies heavily on animal data. Recurrent cycles of cytotoxicity and proliferation are thought to be the driving force behind formation of mouse lung tumors and rat nasal tumors; however, the formation of DNA adducts from NA metabolites has not been ruled out and could impact the decision to evaluate NA as a genotoxic agent. This study evaluated DNA adduct formation in target tissues for carcinogenesis in the National Toxicology Program's chronic bioassays: female mouse airway epithelium and male rat nasal epithelium (respiratory, olfactory). Metabolically active epithelial tissue was isolated and incubated in explant culture with $^{14}$C-labeled NA (0, 2.5, 25, 250 uM) for 1 h. The tissue was then rinsed 12-15 times to remove unbound NA; DNA was isolated and prepared for $^{14}$C-AMS (accelerator mass spectrometry) analyses. Male rat airway, which is not susceptible to tumor formation by NA, was used as a negative control. Benzo(a)pyrene (25 uM), a known carcinogen and DNA adductor, was the positive control. We found the highest level of ex vivo formed NA-DNA adducts in mouse airway, followed by rat airway and nasal olfactory tissue, in a manner corresponding with the previously reported, differing rates of in vitro NA bioactivation at these sites. We conclude that NA is capable of forming DNA adducts in target tissues of NA carcinogenesis in mice and rats. Future experiments will determine adduct structure and examine adduct stability, both properties may impact the carcinogenic potential of the detected DNA adducts. (Funded by T32 ES007059, R01 ES020867, ES020867S1 and DOD LC130820) This work performed in part under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

# Mechanism for Clastogenic Activity of Naphthalene

LC130820
MIPR:10567486

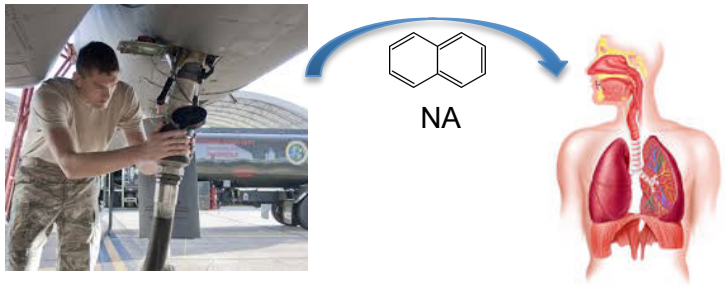
**PI:** Bruce Buchholz **Org:** Lawrence Livermore National Laboratory **Award Amount:** $165K

## Study/Product Aim(s)

- Identify the types of adducts naphthalene (NA) forms with DNA
- Determine whether adduct formation correlates with site selective tumor formation in defined subcompartments of the respiratory tract

## Approach

The location of lung and nasal tumors in rodents exposed to NA are highly specific to defined regions within the respiratory tract. We will utilize microdissection methods to isolate live tissue from these target areas. This approach combined with accelerator mass spectrometry (AMS) will determine whether DNA adducts are formed in the target tissue following incubation with $^{14}$C-NA.




Determine if naphthalene (NA) in jet fuel and cigarette smoke forms DNA adducts that can lead to cancer in respiratory tissues

Accomplishment: All rodent and some monkey in vitro exposures complete. Analyses of adducted DNA bases underway with procedural changes to improve yield..

## Timeline and Cost

| Activities /CY | 14 | 15 | 16 |
|---|---|---|---|
| Animal Protocol & Contract Complete | | | |
| In Vitro Studies for Aim 1 Complete | | | |
| In Vitro Studies for Aim 2 Complete | | | |
| Sample Analyses Complete | | | |
| Data Analyses and Reporting | | | |
| **Estimated Budget ($K)** | **$15** | **$150** | |

**Updated:** (1-April-2016)

**Goals/Milestones**

**Q1 Goals** – Approval of Animal Protocol and Subcontract Executed
☑ Animal protocols approved
☑ Subcontract with UCD executed
**Q2 Goal** – Begin Experimental Work
☑ Begin in vitro studies and sample analyses for aims 1& 2
**Q3 Goal** – Complete Experimental Work
☑ Finish all in vitro studies and sample analyses
**Q4 Goals** – Complete Data Analyses and Reporting
☐ Complete and submit peer-reviewed publication
☑ Complete and submit final report

**Comments/Challenges/Issues/Concerns**

- Fewer monkey samples obtained than planned.
- Low concentration and stability of DNA-adducts through digest and HPLC prevented specific metabolite identification.

**Budget Expenditure to Date**

Projected Expenditure: $165K
Actual Expenditure: $165K