West Virginia Chemical Spill: 5-Day Rat Toxicogenomic Studies
July 2016 NTP Update

Synopsis
The National Toxicology Program (NTP)[1] evaluated three chemicals spilled into the Elk River in West Virginia for their ability to cause toxicity or biological changes in a short-term toxicogenomic study in male rats. The primary goal of a toxicogenomic study is to monitor gene expression in selected organs as a sensitive indicator of a biological response to a foreign chemical. This type of study allows identification of the lowest dose that produces a change in any molecular biological process (MBP; a group of genes that work together to regulate a given cellular function). This study evaluated MBP changes in the liver and kidney produced by giving 4-methylcyclohexanemethanol (MCHM), propylene glycol phenyl ether (PPH), or the commercial mixture "crude MCHM" containing primarily MCHM and lesser amounts of other spilled chemicals to male rats. Groups of rats were given one of seven doses of the chemical orally once per day for five days. For MCHM and crude MCHM, the doses ranged from 0.1-500 mg/kg/day, and for PPH the doses ranged from 1-2000 mg/kg/day. The results from these studies were discussed in a previous NTP Update released in June 2015. Based on further review of the genomic and non-genomic data, NTP refined the statistical methods used to analyze the data that resulted in a few changes in the findings. This July 2016 NTP Update communicates the revised findings from the 5-day rat toxicogenomic studies and replaces the June 2015 NTP update.

MCHM had effects on MBPs in the liver starting at doses between 6-99 mg/kg/day. No MBPs were changed in the kidney following MCHM treatment. Crude MCHM had effects similar to MCHM and caused MBP changes in the liver starting at doses of 5-7 mg/kg/day. No MBPs were changed in the kidney following crude MCHM treatment. The changes in MBPs produced by PPH occurred at doses as low as 3-4 mg/kg/day in liver and 4-76 mg/kg/day in kidney. In addition to effects on MBPs, the chemical treatments at high doses resulted in a number of non-genomic effects. MCHM produced an increase in triglycerides at the highest dose level (500 mg/kg/day). Crude MCHM increased liver weight at the highest dose level (500 mg/kg/day) and changed several of blood chemistry measurements including increasing triglycerides at the second highest dose level (300 mg/kg/day). PPH caused morbidity, or a diseased or unhealthy state, at the highest dose level (2000 mg/kg/day) and changes in blood chemistry measurements indicative of liver damage at 1000 mg/kg/day.

5-Day Rat Toxicogenomic Studies

Background on Toxicogenomic Studies
A toxicogenomic study is conducted to identify effects of a chemical on toxicological or biological processes in the liver or other organs. A wide range of biological processes can be evaluated at the molecular level, that is, by evaluating changes in the expression of genes that are responsible for various aspects of cellular function. Genes that are expressed in a coordinated manner and enable cells to carry out a series of actions can be grouped together for analysis. Changes in the expression of these groups of genes provide information on the molecular biological processes (MBPs) that are changed by chemical exposure.

In a toxicogenomic study, these types of molecular changes can be compared to other traditional indications of toxicity such as changes in organ weight and structure, clinical measurements in the

[1] NTP is a federal, interagency program whose goal is to safeguard the public by identifying substances in the

blood,[2] or genetic damage. Changes at the molecular level in response to a chemical exposure often occur at lower dose levels and more quickly than other toxic effects. For this reason, such changes can give a more sensitive view of potential effects on biological activity. Furthermore, this means that toxicological effects are less likely to occur at doses below those producing changes in MBPs.

For this study, effects on gene expression were determined by measuring the levels of messenger RNA molecules in the liver and kidney. Liver and kidney were selected because previous toxicology studies identified them as target organs for effects of the spill chemicals. A wide range of doses of the chemicals was tested to allow identification of the MBPs that are most sensitive to chemical treatment.

In order to identify the dose level where effects on MBPs start to occur, a modeling approach, referred to as benchmark dose (BMD) analysis, is used. BMD analysis of gene expression data estimates the lowest dose level where significant effects on MBPs occur. An MBP benchmark dose lower confidence limit (BMD_L) is reported along with a BMD and it represents the lower 95% confidence limit for the BMD (i.e., it reflects the statistical variation in the BMD). Because there are many MBPs that regulate the diverse functions of the cell and they are changed at different dose levels, the BMD analysis for each chemical yields a range of MBP BMD values. In order to identify effects on MBPs from BMD analysis, chemical treatment had to produce a significant change in gene expression for at least 5 genes in that MBP ($p<0.05$, ≥1.5 fold change) and exhibit reliable dose-response behavior (i.e., the data had to fit a predefined dose-response curve). Both the mean and median BMD and BMD_L values are reported and collectively define the dose level range where effects of the spill chemicals on biological activity for a given MBP occurred.

Findings from the 5-Day Toxicogenomic Study

NTP conducted a 5-day toxicogenomic study in male rats to evaluate the toxicity of chemicals spilled into the West Virginia Elk River. The chemicals tested were the primary spill chemical 4-methylcyclohexanemethanol (MCHM), propylene glycol phenyl ether (PPH), and crude MCHM, a commercial mixture containing primarily MCHM along with lesser amounts of other spill chemicals.[3] Each chemical was mixed in corn oil and administered orally once daily for five days to separate groups of male rats at seven doses. Blood samples were obtained from the rats 24 hours after the fifth treatment for measurement of organ damage markers, biochemical changes, and DNA damage.[4,5] Results from this study for effects on genetic damage in the bone marrow were reported previously.[6]

[2] http://ntp.niehs.nih.gov/testing/types/clin/index.html
[3] Dipropylene glycol phenyl ether was also studied and those results will be reported in a later NTP Update.
[4] http://ntp.niehs.nih.gov/testing/types/clin/index.html
[5] http://ntp.niehs.nih.gov/testing/types/genetic/invivo/mn/index.html
[6] http://ntp.niehs.nih.gov/ntp/research/areas/wvspill/micronucleus_wvfeb2015_508.pdf

Table 1. Substances Studied in the 5-Day Toxicogenomic Study

CASRN*	Chemical Name	Dose Levels (mg/kg/day)	Notes
34885-03-5	4-Methylcyclohexanemethanol (MCHM)	0, 0.1, 1, 10, 100, 300, 500	a
NA	Crude 4-Methylcyclohexanemethanol (crude MCHM)	0, 0.1, 1, 10, 100, 300, 500	b
770-35-4	Propylene glycol phenyl ether (PPH)	0, 1, 10, 100, 500, 1000, 2000	c

*CASRN = Chemical Abstract Registry Number; [a]Major or minor constituent of the spilled liquid; [b]A commercial mixture containing >70% MCHM along with lesser amounts of five other chemicals; [c]Minor constituent of the spilled liquid.

MCHM

MBP Findings. Statistical analysis of the gene expression data indicated a small number of genes in the liver (16) and kidney (5) were significantly changed ($p<0.05$, ≥ 2-fold change) and showed reliable dose-response behavior in response to MCHM treatment. Following MCHM treatment, a total of 22 MBPs were considered changed in liver and none were considered changed in kidney. The MBP showing the greatest statistical enrichment[7] was related to fatty acid metabolism, which is likely related to the changes in triglyceride levels that were observed. In liver, the MBP with the lowest BMD_L was related to cholesterol homeostasis. The cholesterol homeostasis MBP in liver following MCHM treatment had a median BMD and BMD_L of 13 mg/kg/day and 6 mg/kg/day, respectively, and a mean BMD and BMD_L of 151 and 99 mg/kg/day, respectively (Table 2). In previous studies on MCHM, including the NTP Prenatal Toxicity studies,[8] the lowest dose associated with toxicological effects in rats was about 100 mg/kg/day. The gene expression findings here are consistent with previous studies with other chemicals[9] showing that effects on gene expression occur at doses within an order of magnitude below the dose where toxicity is observed.

Other Findings. There was no effect of MCHM on body weight at any dose level. No significant changes in organ weight were observed. An increase in triglycerides, a type of fat found in the blood, in the 500 mg/kg/day dose group was the only significant effect on clinical chemistry and hematology attributable to MCHM administration. The change in triglycerides was considered minor.

[7] MBP that possessed the lowest p-value as measured by statistical test referred to as a two-tail Fischer exact test.

[8] http://ntp.niehs.nih.gov/ntp/research/areas/wvspill/prenatal_wvupdate_june2015_508.pdf and http://ntp.niehs.nih.gov/ntp/research/areas/wvspill/prenatal_wvupdate_dec2014_508.pdf

[9] Thomas, R. S., et al. (2013). "Temporal concordance between apical and transcriptional points of departure for chemical risk assessment." Toxicol Sci 134(1): 180-194.

Table 2. Number of Changed MBPs and the Lowest Median and Mean MBP BMD in Liver and Kidney Following MCHM Treatment

Liver					Kidney				
# Changed[1]	BMD[2] (Median)	BMD_L[2] (Median)	BMD (Mean)	BMD_L (Mean)	# Changed	BMD (Median)	BMD_L (Median)	BMD (Mean)	BMD_L (Mean)
22	13	6	151	99	0	NA[3]	NA	NA	NA

[1]Number of MBPs that were identified as changed following chemical treatment.
[2]BMD and BMD_L values are reported as mg/kg/day.
[3]NA = No changed MBPs.

Crude MCHM.
MBP Findings. Statistical analysis of the gene expression data indicated a small number of genes in the liver (14) and kidney (4) were significantly changed ($p<0.05$, ≥ 2-fold change) and showed reliable dose-response behavior in response to crude MCHM treatment. Following crude MCHM treatment, a total of 28 MBPs were considered changed in liver and none were considered changed in kidney. Of the 28 changed MBPs in liver following crude MCHM exposure, 9 (22%) were also found changed in liver following MCHM treatment. The MBP in liver showing the greatest enrichment was related to carboxylic acid metabolism, an MBP very closely related to fatty acid metabolism. The overlap of changed MBPs and the shared effect on fatty acid metabolism suggest MCHM and crude MCHM have similar overall effects on MBPs. In liver following crude MCHM treatment, the MBP with the lowest BMD_L was related to ribosome biogenesis, suggesting that cellular protein synthesis was sensitive to treatment with crude MCHM. The ribosome biogenesis MBP in liver following crude MCHM treatment had a median BMD and BMD_L of 10 and 5 mg/kg/day, respectively, and a mean BMD and BMD_L of 35 and 7 mg/kg/day, respectively (Table 3).

Other Findings. There was no effect of crude MCHM on body weight at any dose level. An increase in liver weight was observed in the groups of rats receiving the two highest dose levels. The only significant effects on clinical chemistry and hematology measurements attributable to crude MCHM all occurred at the highest dose levels. They include an increase in triglycerides (300 mg/kg/day), increase in creatinine (500 mg/kg/day), decrease in blood glucose (500 mg/kg/day), increase in albumin (500 mg/kg/day), increase in mean red blood cell volume (500 mg/kg/day), and decrease in eosinophils (500 mg/kg/day). All significant clinical chemistry and hematology findings were considered minor.

Table 3. Number of Changed MBPs and the Lowest Median and Mean MBP BMD in Liver and Kidney Following Crude MCHM Treatment

Liver					Kidney				
# Changed[1]	BMD[2] (Median)	BMD_L[2] (Median)	BMD (Mean)	BMD_L (Mean)	# Changed	BMD (Median)	BMD_L (Median)	BMD (Mean)	BMD_L (Mean)
28	10	5	35	7	0	NA[3]	NA	NA	NA

[1]Number of MBPs that were identified as changed following chemical treatment.
[2]BMD and BMD_L values are reported as mg/kg/day.
[3]NA = No changed MBPs.

PPH
MBP Findings. Statistical analysis of the gene expression data indicated a small number of genes in the liver (26) and kidney (32) were significantly changed ($p<0.05$, ≥ 2-fold change) and showed reliable dose-response behavior in response to PPH treatment. Following PPH treatment a total of 24 MBPs were considered changed in liver and 32 were considered changed in kidney. The MBP in liver showing the

greatest enrichment following PPH treatment was related to cholesterol metabolism, a biological process predominantly controlled by the liver. In liver following PPH treatment, the MBP with the lowest BMD_L was related to peptide metabolism, suggesting PPH may have an effect on protein metabolism in the liver. The peptide metabolism MBP in liver following PPH treatment had a median BMD and BMD_L of 5 and 3 mg/kg/day, respectively, and a mean BMD and BMD_L of 32 and 4 mg/kg/day, respectively (Table 4). The MBP in kidney showing the greatest enrichment was related to cell division, suggesting PPH may have effects on cell proliferation in the kidney. In kidney, the MBP with the lowest BMD_L was related to the regulation of chromosome segregation, suggesting that PPH produced effects on kidney cell proliferation. The regulation of chromosome segregation MBP in kidney following PPH treatment had a median BMD and BMD_L of 8 and 4 mg/kg/day, respectively, and a mean BMD and BMD_L of 104 and 76 mg/kg/day, respectively (Table 4).

Other Findings. The group of rats administered PPH at the top dose level of 2000 mg/kg exhibited signs of acute toxicity and were removed from the study before it ended. In addition, one death occurred in the 1000 mg/kg/day dose group, and there was a slight decrease in body weight in the group of rats receiving 1000 mg/kg/day. Decreased thymus weights were found in groups of rats receiving 1000 mg/kg/day and are likely attributable to general toxicological stress. The only significant effects on clinical chemistry and hematology measurement that was attributable to PPH was a slight increase in the levels of the enzyme ALT, a marker of liver damage, in the 500 and 1000 mg/kg/day dose groups.

Table 4. Number of Changed MBP and the Lowest Median and Mean MBP BMD in Liver and Kidney Following PPH Treatment

Liver					Kidney				
# Changed[1]	BMD[2] (Median)	BMD_L[2] (Median)	BMD (Mean)	BMD_L (Mean)	# Changed	BMD (Median)	BMD_L (Median)	BMD (Mean)	BMD_L (Mean)
24	5	3	32	4	32	8	4	104	76

[1]Number of MBPs that were identified as changed following chemical treatment.
[2]BMD and BMD_L values are reported as mg/kg/day.

Next Steps

This update reflects results from a re-analysis of the 5-day toxicogenomic studies of MCHM, crude MCHM, and PPH. These studies were designed to evaluate potential toxicity and identify the level at which molecular biological effects occurred in liver and kidney are finished. NTP will consider the findings in an overall assessment of the spilled chemicals.