Mode Of Action Research, Multi-species Pharmacokinetic Modeling and Risk Assessment For The Carcinogenesis Of Hexavalent Chromium in the Small Intestine

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ToxStrategies, Inc. and Summit Toxicology

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Cr(VI) MOA Study Research Team

Universities
- George Washington University Medical Center
- Michigan State University
- University of Cincinnati Medical Center
- Duke University Medical School

Risk Assessors and Modelers
- Summit Toxicology
- ToxStrategies

Analytical Laboratories
- Applied Speciation
- Brooks Rand Laboratory
- Environmental Standards

Research Laboratories
- Experimental Pathology Laboratories
- Southern Research Institute
- National Center for Toxicological Research
- ThermoFisher
National Toxicology Program (NTP) Study Results for Cr(VI) and Cr(III)

NTP Cr(VI) drinking water study

- Mice and rats consuming 5,000 – 180,000 µg/L (ppb) Cr(VI) as sodium dichromate dihydrate (SDD) for 2-years
- Rare tumors appeared late in the study
  - Mice: adenomas and carcinomas of small intestines
  - Rats: squamous cell carcinoma in oral cavity

NTP Cr(III) oral feeding study

- No significant effects observed in either species
The Cr(VI) MOA research project was developed using EPA Cancer Risk Assessment guidance (2005)

- Provides information as to why tumors occurred in rodents
- Provides information on the differences between rodents and humans with regard to internal dose
- Develops the models and data needed to do a State-of-the-Art Risk Assessment
**Mode of Action for Intestinal Cancer**

- **Cr(VI) Exposure**
  - Detoxification by Conversion to Cr(III) in the Stomach
  - Intestinal Absorption
  - Oxidative Stress and Chronic Toxicity in Intestinal Villi
  - Prolonged Repair Response in Intestinal Crypt
  - Expansion of Spontaneous Mutations

- Elimination
  - Reduced to Cr(III) in the Gastric and Intestinal Lumen

Detoxification by Conversion to Cr(III) in the Stomach

Intestinal Absorption

Oxidative Stress and Chronic Toxicity in Intestinal Villi

Prolonged Repair Response in Intestinal Crypt

Expansion of Spontaneous Mutations

Reduced to Cr(III) in the Gastric and Intestinal Lumen
Stomach Reduction Kinetics
Stomach Reduction Capacity is Exceeded At Carcinogenic Doses in Rodents

Data by Applied Speciation
Oxidative Stress and Chronic Toxicity in Intestinal Villi
Biochemical and Genomic Responses to Oxidative Stress

- Significant decreases in reduced to oxidized glutathione in mouse duodenum and jejunum
- Activation of genomic response to oxidative stress

mg/L (ppm) = 1,000 μg/L (ppb)

Toxicogenomics by Michigan State; GSH/GSSG data by University of Cincinnati
Toxicity in Villus & Expansion of Crypt

At High Doses:
- Expanded Crypt Area, Blunted Villi
- Damage at villi tips

Control
Total number of Epithelial Cells in the Duodenal Crypt (Mouse Day 90)

![Graph showing the relationship between Cr(VI) concentration and enterocytes per crypt with p < 0.01 significance level.]

Experimental Pathology Laboratories
Does Cr(VI) Cause DNA Mutations in the Crypt or Do Tumors Occur by Spontaneous Replication Error?
Toxicity and DNA Damage to Cells in the Duodenal Crypt

- **Mitotic Index**: Percentage of Cells Undergoing Division

- **Apoptotic Index**: Percent of cells undergoing apoptosis (programmed cell death)

- **Micronuclei**: Total number of cells with an extra smaller nucleus indicating broken chromosomes

Measured in 10 fully intact crypts per animal, 5 animals per dose
No Toxicity to Cells in the Duodenal Crypt  
(Mice Day 91)

<table>
<thead>
<tr>
<th>Cr(VI) Drinking Water (mg/L)</th>
<th>Mitotic Index (%)</th>
<th>Apoptotic Index (%)</th>
<th>Total Number of Micronuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.43 ±1.17</td>
<td>0.47 ±0.22</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>2.28 ±1.07</td>
<td>1.0 ±0.47</td>
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<tr>
<td>1</td>
<td>2.36 ±0.684</td>
<td>0.5 ±0.44</td>
<td>0</td>
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<tr>
<td>5</td>
<td>3.08 ±0.46</td>
<td>0.7 ±0.45</td>
<td>0</td>
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<tr>
<td>20</td>
<td>2.46 ±0.76</td>
<td>0.5 ±0.32</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>2.72 ±0.97</td>
<td>0.84 ±0.96</td>
<td>0</td>
</tr>
<tr>
<td>180</td>
<td>2.11 ±1.09</td>
<td>0.67 ±0.33</td>
<td>0</td>
</tr>
</tbody>
</table>

Values represent total number of aberrant nuclei in 15 sections (3 slides per animal; 5 animals per treatment group, except only 4 animals for 4 mg/L SDD treatment group at day 91).

* Significantly different from control group by Poisson regression.

**No Effect on Normal Cell Generation or Cellular Death**

Purple for Carcinogenic Doses
Mitotic and apoptotic indices are percent of mitotic and apoptotic cells per total cells evaluated
Data represent total number of cells evaluated in 10 fully intact crypts per animal, 5 animals per dose group

Experimental Pathology Laboratories
### No DNA Damage in Duodenal Crypt
(Mice Day 91)

<table>
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<tr>
<th>Cr(VI) Drinking Water (mg/L)</th>
<th>Mitotic Index (%)</th>
<th>Apoptotic Index (%)</th>
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*Significantly different from control group by Poisson regression.*

**Purple for Carcinogenic Doses**
Mitotic and apoptotic indices are percent of mitotic and apoptotic cells per total cells evaluated.
Data represent total number of cells evaluated in 10 fully intact crypts per animal, 5 animals per dose group.

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No Evidence of DNA damage in Target Crypt Cells with Proliferation Response
We looked for a specific mutation in K-Ras in mouse intestinal tissue with a very sensitive method, Allele-specific Competitive blocker (ACB) PCR at doses that cause hyperplasia

K-Ras codon 12 is commonly mutated in intestinal cancers

K-Ras codon 12 GAT mutation is also a “reporter gene” for mutations in other parts of the DNA sequence

K-RAS mutations occur early in carcinogenesis

Mutation data, such as this, are EPA’s highest tier of data for assessing whether a chemical acts by a mutagenic MOA
K-Ras Codon 12 GAT Mutations
(Mouse Duodenum, Day 91)

- K-Ras commonly mutated in intestinal cancers
- No dose-related trend with Cr(VI) exposures
- No increase at carcinogenic doses

Log_{10} Cr(VI), mg/L

Log_{10} MF
K-Ras Mutations: Comparison with Benzo(a)pyrene (Mouse Duodenum, Day 91)

- K-Ras Codon 12 GAT mutations increased with BaP dose and adduct formation in mouse lung (Meng et al. 2010)
- Evidence for a Mutagenic MOA for BaP in lung
- High background rate of K-Ras mutations in mouse small intestine as compared to lung and other tissues (Mutant fraction of ~10^{-3} in intestine and ~10^{-6} in lung)

From Meng et al. 2010. Environ Mol Mutagen 51, 146-155
Lack of Early DNA Damage or Mutations

Cr(VI) Exposure

Detoxification by Gastric Reduction of Cr(VI) to Cr(III)

Intestinal Absorption

Oxidative Stress and Chronic Villous Cytotoxicity

Prolonged Repair Response in Crypt

Expansion of Spontaneous Mutations

Reduced to Cr(III) in the Gastric and Intestinal Lumen
## MOA Study Findings (Mice)

<table>
<thead>
<tr>
<th>Significant change</th>
<th>Cr6 Drinking Water Concentration (mg/L)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Day 91 Duodenum</strong></td>
<td></td>
</tr>
<tr>
<td>Cr in duodenum</td>
<td>-</td>
</tr>
<tr>
<td>Oxidative Changes</td>
<td>-</td>
</tr>
<tr>
<td>Gene Changes</td>
<td>-</td>
</tr>
<tr>
<td>Villus toxicity</td>
<td>-</td>
</tr>
<tr>
<td>Crypt proliferation</td>
<td>-</td>
</tr>
<tr>
<td>Crypt DNA damage</td>
<td>-</td>
</tr>
<tr>
<td><strong>K-Ras mutation</strong> (Codon 12 GAT)</td>
<td>-</td>
</tr>
</tbody>
</table>

Underlined checks indicate significant changes at day 8 as well, Cr concentrations not measured at day 8.
PBPK Modeling For Oral Exposure to Chromium in Rodents and Humans
Model Development/Application

• Problem Formulation
• PBPK Model Goals
• Conceptual Model
• PBPK Model Development
• Model Application to Risk Assessment
PBPK Models Can Predict Internal Dose by Intestinal Segment

Tumor Incidence in NTP Study in the Duodenum, Jejunum and Ileum (male and female mice)

Administered Dose (mg/kg-day)

- Duodenum
- Jejunum
- Ileum
Conceptual Model For Cr in the GI Tract

**Competing Rates**
- R1 = GI transit
- R2 = Reduction
- R3 = Transport to epithelium
- R4 = Absorption into blood
- R5 = Blood flow
- R6 = Sloughing of epithelium

Cr(VI)

Lumen

Cr(VI) -> Cr(III)

Epithelium

Cr(VI) -> Cr(III)

Portal Plasma

Cr(VI) -> Cr(III)

R1

R2a

R2b

R2c

R3

R4

R5

R6
PBPK Model Goals

Primary

- Estimates of the lifetime average daily internal dose for Cr(VI) in the small intestines to support dose-response assessment for the NTP cancer bioassay
- Identify model parameters that are important with respect to target tissue dose and species differences

Secondary

- Estimate internal doses for systemic tissues
  - Strong consideration was given to lumping all systemic into a single compartment in the model
**PBPK Model Structure**

- **Oral Dose**
- **Gavage Dose**

- **Fecal Excretion**
- **Urinary Excretion**

- **Systemic RBC**
- **Portal RBC**

- **Bone**
- **Other**
- **Kidney**

- **Portal Plasma**
- **Systemic Plasma**

- **Liver**

- **Other**
- **Kidney**
- **Liver**

- **Oral Dose**
- **Gavage Dose**

- **Fecal Excretion**
- **Urinary Excretion**
General Approach to Model Parameterization

Step 1: Develop Model for Cr(III)

Step 2: Ex Vivo Reduction Data

Step 3: Develop Model for Cr(VI)

3 Steps performed for mice, rats, and humans.
Step 1: Cr(III) Model Development

Example of Model Fits to Rodent Data for Cr(III)
Step 1: Cr(III) Model Development
Example of Model Fits to Human Data for Cr(III)

(A) Plasma

(B) Urine
Step 2: Ex Vivo Cr(VI) Reduction Data
Indicate 2\textsuperscript{nd} Order, Capacity-limited Reaction
Step 2: Ex Vivo Cr(VI) Reduction Data

pH-Dependence

$k = 44.5 \exp(-pH) \ (L/mg\text{-}hr)$

Graph showing the relationship between $k$ and pH.
Step 3: Cr(VI) Model Development
Example of Model Fits to Cr(VI) Data in Rodents

(A) Erythrocytes
(B) Liver
(C) Stomach
(D) Plasma
(E) Kidney
(F) Urine
Step 3: Cr(VI) Model Development
Example of Model Fits to Cr(VI) Data in Humans

(A) Plasma and erythrocytes

(B) Urine
Possible Dose Metrics for Risk Assessment

Total Cr in tissue (mass/mass)
Model predicted Cr(VI) conc in lumen
Model predicted Cr(VI) conc in SI tissues
Model-predicted Cr(VI) flux (mg CrVI/mass SI/day)

- Causal relation with tumor formation
- Can be estimated with confidence in rodents and humans
- Correlated with SI tissue concentration
- Demonstrates excellent dose-response concordance across SI tissue sections
Cr(VI) SI Flux

**Competing Rates**
- R1 = GI transit
- R2 = Reduction
- R3 = Transport to epithelium
- R4 = Absorption into blood
- R5 = Blood flow
- R6 = Sloughing of epithelium
Model Application to Dose-Response Assessment (Mouse Data)

- Flux (mg Cr\(^6\)/kg SI/day)
- PBPK-Derived Internal Dose Measure

- Diffuse hyperplasia
- Adenomas
- Carcinomas

Incidence

SI Section Response
Because Cr(VI) reduction is pH-dependent, exposure events A & B will result in different internal doses even if external doses are the same.
Model Application to Risk Assessment

**Stomach pH**

- Y-axis: pH
- X-axis: Hour

**Reducing Equivalents Regeneration**

- Y-axis: mg/L
- X-axis: Hour

**Gastric Transit Rate Constant**

- Y-axis: 1/hr
- X-axis: Hour
Rodent and Human PBPK Models

• Model provides a good description of the toxicokinetic behavior of Cr in target & nontarget tissues
• Model predictions appear “useful” in its ability to characterize mouse tumor dose-response data for duodenum, jejunum, and ileum on a single curve
• Model will be able to address key factors (i.e., diurnal variation in gastric pH, age differences) that may impact internal dose and risk for human exposures
Risk Assessment for Intestinal Carcinogenesis
Overview of Dose-Response Analysis

- **Applied Doses (mg Cr/kg BW)**
  - **Mouse PBPK Model**
  - **Intestinal Tissue Doses (mg Cr/kg SI)**
    - BMD Modeling
    - Global nonlinear regression
  - **Mouse POD (mg Cr/Kg SI)**
    - Use human PBPK model
  - **Human LADD (mg Cr/kg BW)**
    - Apply UFs
  - **RfD (mg Cr/kg BW)**
    - **ACCOUNT FOR HUMAN VARIABILITY & SUSCEPTIBILITY**
  - **MODEL MOUSE DATA (TWO APPROACHES)**
    - **CONVERT APPLIED DOSE TO INTERNAL TISSUE DOSE**
    - **CONVERT MOUSE POD TO HUMAN INTERNAL POD, AND THEN TO APPLIED DOSE**

SI = small intestine
BW = bodyweight
Create a Robust Dataset (Part 1): Combine Data for Both Male and Female Mice
Create a Robust Dataset (Part 2): Convert Applied Doses to Tissue-Specific Doses

Mouse PBPK Model of GI

Absorption Reduction and Transport are Competing kinetic Rates
### Table 1

<table>
<thead>
<tr>
<th>Segment</th>
<th>Sex</th>
<th>Dose (mg/kg SL/d)</th>
<th>N</th>
<th>Effect</th>
<th>Segmen</th>
<th>Sex</th>
<th>Dose (mg/kg SL/d)</th>
<th>N</th>
<th>Effect</th>
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</thead>
<tbody>
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<td>0</td>
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<td>m</td>
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<td>m</td>
<td>0.04</td>
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<td>i</td>
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**Table 3**

<table>
<thead>
<tr>
<th>Segment</th>
<th>Sex</th>
<th>Dose (mg/kg SL/d)</th>
<th>N</th>
<th>Effect</th>
<th>Segment</th>
<th>Sex</th>
<th>Dose (mg/kg SL/d)</th>
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<th>Effect</th>
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<tr>
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<td>m</td>
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<td>d</td>
<td>f</td>
<td>8.7</td>
<td>50</td>
<td>42</td>
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</tbody>
</table>

**Adenomas/Carcinomas**

<table>
<thead>
<tr>
<th>Segment</th>
<th>Sex</th>
<th>Dose (mg/kg SL/d)</th>
<th>N</th>
<th>Effect</th>
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<tbody>
<tr>
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<td>m</td>
<td>1.6</td>
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<td>f</td>
<td>29.30</td>
<td>50</td>
<td>29.30</td>
</tr>
</tbody>
</table>

**Notes:**

- Based on applied dose (mg/kg bodyweight/day)
- Based on combined incidence of adenomas and carcinomas
- Data points were dropped to achieve BMD model fits (see text for discussion)
- d, duodenum; j, jejunum; i, ileum; m, male; f, female

---

*a* based on applied dose (mg/kg bodyweight/day)

*b* based on combined incidence of adenomas and carcinomas

*c* data points were dropped to achieve BMD model fits (see text for discussion)
Visualization of Robust Dose-Response Dataset

Flux (absorption) of Cr(VI) into mouse small intestinal tissue for all three segments of the small Intestine
Example BMD Plot: Tumors

- Uses incidence data from 24 intestinal dose levels
- Each point represents n=48-50 animals (i.e. intestinal segments)

Tumor Incidence vs. Flux (mg/kg SI/day)

p-value > 0.1
Example BMD Plot: DEH

Hyperplasia (Incidence) vs. Flux (mg/kg SI/day)

Why does the model over predict these points?

dropped 3 highest doses
Hyperplasia in Jejunum is Likely Underestimated in NTP Study

Tumor Assessment

(~20 cm)

DEH Assessment
(5 µm)
BMD Modeling of DEH (Duodenum & Ileum)

- p-value > 0.1
- dropped 3 highest doses
BMD Modeling of DEH (Duodenum Only)

Dropped 3 highest doses

P-value > 0.1

Flux (mg/kg SL/day) vs. Diffuse Hyperplasia
### Example BMDL$_{10}$ Values for Intestinal Tumors and Diffuse Hyperplasia (Based on Flux)

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>BMDL$_{10}$ (flux)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal Tumors</td>
<td>5.8</td>
</tr>
<tr>
<td>DEH (Duodenum, Jejunum, Ileum)</td>
<td>1.9</td>
</tr>
<tr>
<td>DEH (Duodenum &amp; Ileum)</td>
<td>1.1</td>
</tr>
<tr>
<td>DEH (Duodenum Only)</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Examples Using Global Nonlinear Regression

- **No Shared Parameters**
- **Shared Slope**
- **Shared Slope & Top**

### Endpoint | $ECL_{10}$ (flux)
--- | ---
Intestinal Tumors | 3-6
DEH (Duodenum and Ileum) | 0.6-0.7
Use Human PBPK Model for Interspecies Extrapolation

Graph showing the relationship between flux and extra risk with data points and curves indicating hyperplasia and tumor.

Diagram illustrating the flow through the SI lumen and SI tissue with numbered steps 1 and 2.
Account for State-Dependent Reduction and Age-Dependent Changes in Parameters (e.g. Gastric pH)
Calculation of a Reference Dose Protective of Intestinal Cancer

\[ \text{RfD} = \frac{\text{LADD}}{\text{UF}} \]

- **RfD** = Reference Dose (mg/kg-day)
- **LADD** = Lifetime Average Daily Dose (mg/kg-day) in Human
- **UF** = Uncertainty Factors

Accounts for pH variability at all life stages

- People on Proton Pump Inhibitors have ~3-fold higher dose
- Variations in Water Consumption can result in up to a higher dose by ~2-fold
RfD Can Be used To Calculate a DWEL

Drinking Water Equivalent Level (DWEL) = (RfD x BW) ÷ IR

DWEL = Drinking Water Equivalent Level
RfD = Reference Dose for a Lifetime exposure including sensitive subpopulations
IR = Ingestion Rate (2 L/day)
BW = Body Weight (70 kg)

Preliminary results suggest that the DWEL is supportive of the current MCL (~100 ppb)
Risk Assessment of Cr(VI) in Drinking Water

- Background is ~1-5 ppb in CA and US
- DWEL is higher than background
- Current Standards are protective
- No risk at normal background exposures, even for sensitive subpopulations

Data from CDHS 2001-2003 drinking water concentrations by county, does not include non-detects
Summary Conclusion

• Weight of Evidence supports a cytotoxic MOA
  • At non-cytotoxic doses, this MOA is not operable

• This MOA is consistent with the notion that there is an exposure level that does not pose an increased cancer risk

• This MOA is consistent with other small intestinal carcinogens (e.g. captan, folpet)

• PBPK models allow for development of a robust dataset based on intestinal tissue doses

• The BMDL for DEH is several fold lower than the BMDL for intestinal tumor formation
  • Prevention of DEH should protect against cancer
Questions and Discussion