HOW CAN MECHANISTIC DATA FROM SYSTEMS APPROACHES IMPROVE OUR UNDERSTANDING OF CARCINOGENIC RISK FOR MIXTURES?

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Environmental and Molecular Toxicology Department
Superfund Research Center
CASE STUDY WITH POLYCYCLIC AROMATIC HYDROCARBONS (PAHS)

- What are PAHs? common sources, exposure, diversity of chemical class, toxicity, mechanism(s) of action

- PAH risk assessment and regulation of PAHs as mixtures
  - Current considerations and future needs for evaluating risk

- Whole mixture approach for predicting carcinogenic risk of PAH mixtures in vitro
  - Predictive model based on short-term bioactivity profiles
  - Needs and future directions
WHAT ARE PAHS?
POLYCYCLIC AROMATIC HYDROCARBONS (PAHS): SOURCES AND USES

- Ubiquitous contaminants occurring naturally (crude oil) or created from incomplete combustion and released from both natural (forest fires) or anthropogenic (burning of fossil fuels)

- Natural
  - Forest fires
  - Oil seeps
  - Volcanos

- Anthropogenic
  - Wood burning
  - Internal combustion engine (vehicle exhaust)
  - Cigarette smoke
  - Roofing/coal tar products
  - Electric power generation
  - Petroleum
POLYCYCLIC AROMATIC HYDROCARBONS (PAHS): SOURCES AND USES

- PAHs are not synthesized for industrial purposes, but can be used as intermediaries in manufacturing (examples below)
  - Acenaphthene: manufacture of dyes, plastics, pigments, pharmaceuticals and pesticides
  - Anthracene: manufacture of dyes and pigments; diluent for wood preservatives;
  - Fluoranthenne: manufacture of dyes, pharmaceuticals and agrochemicals.
  - Fluorene: manufacture of dyes, pigments, pesticides, thermoset plastic and pharmaceuticals;
  - Phenanthrene: manufacture of pesticides and resins
  - Pyrene: manufacture of pigments
POLYCYCLIC AROMATIC HYDROCARBONS (PAHS): CHEMICAL CHARACTERISTICS

- Two or more aromatic rings with a pair of carbon atoms shared, highly lipophilic

- 16 priority EPA PAHs (ATSDR, 2005)
  - Toxicity
  - Potential for human exposure
  - Frequency of occurrence at hazardous waste sites
  - Available information
  - Include probable and known human carcinogens
POLYCYCLIC AROMATIC HYDROCARBONS (PAHS):
CHEMICAL CHARACTERISTICS

- Broader class of polycyclic aromatic compounds
  - >1500 chemicals total
  - Diverse structural features
  - Includes both unsubstituted and substituted forms
  - O-, N-, S-, CH3-
  - Little data available on sources, exposure, toxicity, mechanisms

- Consideration of PAH mixtures
  - Diesel exhaust
  - Coal tar
  - Cigarette smoke

9,10-Phanthrenequinone
1,6-Dinitropyrene
2,3-Dimethylanthracene
POLYCYCLIC AROMATIC HYDROCARBONS (PAHS): TOXICITY

- Carcinogenicity – Include Class I known human carcinogens (benzo[a]pyrene, diesel exhaust, occupational exposure to coal/coke PAH mixtures) and Class 2A/B probable/possible carcinogens

- Teratogenicity – animal and epidemiology studies

- Genotoxicity – via metabolism in animal and human cells

- Immunotoxicity – suppression

- Reproductive toxicity – e.g. reduced ovarian size
HOW ARE PAHS REGULATED?
ASSESSING RISK TO PAHS: RELATIVE POTENCY FACTORS (RPF)

- EPA 1993, 2002 & 2010 guidances
  - Based on tumor studies comparing >1 PAH
  - Should be able to estimate carcinogenic potency for various PAHs by comparison to a standard
  - Recommend BaP as a standard
  - Estimates of individual slope factors could be calculated as a percentage of the slope factor for BaP
- Apply approach to Group B2 probable PAH carcinogens
- Evaluation of PAHS as complete carcinogens in skin was most comprehensive and recommended for use

### Table 7.1. Final RPFs based on tumor bioassay data

<table>
<thead>
<tr>
<th>PAH</th>
<th>Average RPF</th>
<th>Range of RPF</th>
<th>Number of datasets</th>
<th>Exposure routes tested</th>
<th>Species tested</th>
<th>Sex tested</th>
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<tbody>
<tr>
<td>Anthracene</td>
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<td>Mouse, rat, Female</td>
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<td>0</td>
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<td>Dibenzo[a,fl]pyrene</td>
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<td>Dibenzo[a,h]anthracene</td>
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<td>7 (nonpositive)</td>
<td>Dermal, intraperitoneal</td>
<td>Mouse</td>
<td>Female, male</td>
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</table>

Benzo[a]pyrene (BAP) = RPF 1.0
ASSESSING RISK TO PAHS: RELATIVE POTENCY FACTORS (RPF)

- Guidance for mixtures
- 1993 Guidance for RA of PAHs
  - Recommendations for a component-based RPF approach assuming additivity of priority PAHs scaled for concentration in mixture
  - Based on 7 PAHs
- 2002 Working Group Consultation
  - Expanded RPFs to 15 unsubstituted PAHs
  - Discussion of alternative approaches
- 2010 IRIS RPF Approach for PAH Mixtures
  - Provided rationale for component-based RPF approach
  - Expanded data for 26 PAHs

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<tr>
<td>Anthracene</td>
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ASSESSING RISK TO PAHS: RELATIVE POTENCY FACTORS (RPF)

Risk Assessment of PAH Mixtures:
Dose Additivity

\[ B[a]P_{eq} = \sum_{i=1}^{n} (C_{PAH_i} \times RPF_i) \rightarrow \]

EPA/635/R-08/012-A
ASSUMPTIONS AND CHALLENGES OF RPF APPROACH FOR PAH MIXTURES

- Applies to cancer endpoints only
  - Assumes cancer is most sensitive endpoint

- Assumes priority PAHs are representative of the mixture
  - Calculated for a small subset of (unsubstituted) PAHs (limited data available)
  - Lack of knowledge about mixture components

- Large range in reported RPF values depending on test system
  - Differs depending on studies included

- Assumes additivity of component PAHs
  - Evidence for inhibitory interactions reported in literature
  - Other interactions unknown

- Estimation of the carcinogenic potency relative to benzo[a]pyrene (BAP)
  - Assumes common mechanism for all PAHs

Leads to uncertainty
Many actions of PAHs are known to be mediated through interaction with the arylhydrocarbon receptor (AhR)

e.g. benzo[a]pyrene
POLYCYCLIC AROMATIC HYDROCARBONS (PAHS): MECHANISM OF ACTION

Metabolism of PAHs to reactive intermediates e.g. benzo[a]pyrene

POLYCYCLIC AROMATIC HYDROCARBONS (PAHS): MECHANISM OF ACTION

Genotoxic and nongenotoxic actions of PAHs

General overview:

Genotoxic and nongenotoxic actions of PAHs
KNOWLEDGE GAPS FOR ASSESSING HEALTH EFFECTS OF PAH MIXTURES

- **Exposure assessment**
  - Do 16 commonly monitored PAHs represent the class of 1500+ compounds?
  - Priority PAHs may not be most appropriate indicator compounds (lack diversity)
  - Complex environmental mixtures are unlikely to be characterized

- **Hazard identification**
  - Bulk of PAH research focuses on relative few PAHs (unsubstituted)
  - Endpoint most likely assessed is carcinogenicity
  - Majority of PAHs are uncharacterized (cancer and noncancer endpoints)

- **Risk characterization**
  - Risk assessments focus on carcinogenicity of commonly monitored unsubstituted PAHs and have conformed to RPF approach
  - Relies on prior assumptions

---

1983 NRC Red Book
Current guidance from 2010 asserts assumptions are reasonable
  - Emphasizes this may only be true for some PAH mixtures

Some studies report discrepancies between calculated RPFs for certain mixtures and individual PAHs and measured carcinogenic potential in animal models

Alternative approaches:
  - Mechanistic based approaches
  - Tox21 high-throughput screening approaches (includes broader group of PAHs)
  - Evaluation of noncancer endpoints
  - Component versus whole mixture or comparative mixture approaches
ALTERNATIVE APPROACHES?

EPA Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures, February 2000. EPA/630/R-00/002
CASE STUDY: PATHWAY-BASED PREDICTION OF PAH-INDUCED SKIN CANCER

OBJECTIVE

Develop a whole mixture approach for predicting carcinogenic potential of PAHs and mixtures to overcome challenges of component-based analysis.

HYPOTHESIS

Hypothesize that short-term bioactivity profiles are prognostic of cancer outcome for individual PAHs and complex mixtures.
Component-based v. Whole mixture approach for assessing risk of environmental mixtures

**Component-based approach**
- Requires toxicity data for individual chemicals within mixture
- Dose addition with RPFs
- Assumes common mechanism for all PAHs/PACs

**Whole mixture approach**
- Requires toxicity data on whole mixtures
- Can rely on mechanistic data
- Lack of accepted approach
- Current lack of adequate data on mixtures
TOX21 PARADIGM FOR RISK ANALYSIS

Traditional

Risk Assessment
1. Hazard Identification
2. Risk Characterization
3. Exposure Assessment

Risk Management
1. Dose-Response Assessment
2. Control Options
3. Non-Risk Analyses

New 21st century

Emphasis on
- Pathway/mechanistic data
- Short-term endpoints
- In vitro (human) responses

1. Hazard identification
2. Exposure assessment
3. Risk characterization
4. Dose-response assessment

Krewski et al., 2011
CASE STUDY: PATHWAY-BASED PREDICTION OF PAH-INDUCED SKIN CANCER

GOALS

- Identify bioactivity profiles for PAHs and PAH mixtures short-term after exposure that act as a chemical fingerprint
- Utilize a pathway-driven approach to classify PAH exposures based on carcinogenic potential
- Identify the key biological pathways that predict tumor outcome
- Evaluate mechanisms likely contributing to carcinogenic MOA
CASE STUDY: PATHWAY-BASED PREDICTION OF PAH-INDUCED SKIN CANCER

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EVALUATING CARCINOGENIC POTENTIAL OF PAHS: CURRENT PARADIGM

Key Events in the Mode of Action for PAH Carcinogenicity

- Exposure
  - PAH
- Metabolism
  - DNA Adduct
  - Mutations
  - Binding to Ah Receptor
  - Upregulation of genes related to biosynthesis, growth, and differentiation
- Initiation
  - DNA Adduct
  - Mutations
  - DNA Adduct and oxidative base damage
- Promotion
  - Proliferation of initiated cells
  - Mutations (deamination, oxidative damage, and strand scission)
  - Proliferation of initiated cells
- Progression
  - Neoplasms
  - Inflammatory response
  - Cytotoxicity
Instead of making a priori decisions about what attributes of PAHs are important for determining carcinogenicity (DNA adducts, receptor binding, CYP450 induction, structure),
EVALUATING CARCINOGENIC POTENTIAL OF PAHS: NEW PARADIGM

Instead of making a priori decisions about what attributes of PAHs are important for determining carcinogenicity (DNA adducts, receptor binding, CYP450 induction, structure),

We evaluate global response post-exposure and model which processes are associated with carcinogenesis for both individual and mixture PAHs.
**CASE STUDY: PATHWAY-BASED PREDICTION OF PAH-INDUCED SKIN CANCER**

**GOAL**: Identify pathways predictive of PAH and PAH-mixture carcinogenic potential and tumor outcome

**MOUSE STUDY DESIGN**

2-Stage skin tumor model in 6-week old FVB/N mice:

- **Time (weeks)**: 0, 2, 25
- **(12 hr)**: PAH Initiation, Skin samples for microarray analysis
- **2**: TPA Promotion
- **Tumor samples for histology**

## Case Study: Pathway-Based Prediction of PAH-Induced Skin Cancer

2-Stage skin tumor model in 6-week old FVB/N mice:

### PAH Treatments:

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Components</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>200 μl toluene</td>
<td>Control</td>
</tr>
<tr>
<td>B[α]P</td>
<td>200 μl toluene 400 nM B[α]P</td>
<td>PAH Initiation</td>
</tr>
<tr>
<td>DBC</td>
<td>200 μl toluene 4 nM DBC</td>
<td>Skin samples for microarray analysis</td>
</tr>
<tr>
<td>Mix1</td>
<td>200 μl toluene 1 mg DPE</td>
<td>TPA Promotion</td>
</tr>
<tr>
<td>Mix2</td>
<td>200 μl toluene 1 mg DPE 1 mg CTE</td>
<td>Tumor samples for histology</td>
</tr>
<tr>
<td>Mix3</td>
<td>200 μl toluene 1 mg DPE 1 mg CTE 2 mg CSC</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> PAH – Polycyclic aromatic hydrocarbon

B[α]P – Benzo[α]pyrene
DBC – Dibenzod[def,p]chrysene
DPE – Diesel particulate extract
CTE – Coal tar extract
CSC – Cigarette smoke condensate

UNIQUE SKIN TUMOR PROFILES FOR PAHS AND PAH MIXTURES
UNIQUE SKIN TUMOR PROFILES FOR PAHS AND PAH MIXTURES

**Graph**: Mean tumors/TBA and Incidence per tumor type (%).

- **CON B[a]P DBC Mix 1 Mix 2 Mix 3**

- **Bars**: Mean tumors/TBA.

- **Line Graph**: Incidence per tumor type (%).

- **Tumor Types**: Hyperplasia, Papilloma, Carcinoma in situ, Squamous cell carcinoma.

**Significance**: ***P<0.001, *p<0.05**
LACK OF CORRELATION BETWEEN TUMOR RESPONSE, RPFS AND OTHER TRADITIONAL ENDPOINTS

Comparison with tumor incidence

A. Relative potency factor

B. DNA adducts and Cyp1a1

C. DNA adducts v. RPF

- Actual, $r^2=0.09$, $R=0.5$ ($p=0.45$)
- Predicted (linear)

- Adducts, $r^2=0.14$, $R=0.70$ ($p=0.23$)
- Cyp1a1, $r^2=0.004$, $R=-0.30$ ($p=0.68$)
- Actual, $r^2=0.95$, $R=0.90$ ($p=0.083$)
- Predicted (linear)
**CASE STUDY: PATHWAY-BASED PREDICTION OF PAH-INDUCED SKIN CANCER**

**GOAL:** Identify pathways predictive of PAH and PAH-mixture carcinogenic potential and tumor outcome

Classification of PAH/Mixture treatments based on tumor outcome

<table>
<thead>
<tr>
<th>Tumor Outcome:</th>
<th>DBC</th>
<th>$\gg \gg \gg$</th>
<th>$[\text{B[a]P} = \text{Mix2} = \text{Mix3} \gg \text{Mix1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classification:</td>
<td>High</td>
<td>Moderate</td>
<td>Low</td>
</tr>
</tbody>
</table>

**CASE STUDY: PATHWAY-BASED PREDICTION OF PAH-INDUCED SKIN CANCER**

**GOAL:** Identify pathways predictive of PAH and PAH-mixture carcinogenic potential and tumor outcome

Pathways significantly enriched ($p<0.05$) in skin post-initiation by PAH/Mixtures

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>BAP</th>
<th>DBC</th>
<th>Mix1</th>
<th>Mix2</th>
<th>Mix3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulation of apoptosis</td>
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<tr>
<td>Protein targeting</td>
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<td>Interphase</td>
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<td>Proteasomal ubiquitin-dependent protein catabolic process</td>
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<tr>
<td>Response to DNA damage stimulus</td>
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<tr>
<td>DNA metabolic process</td>
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<tr>
<td>Positive regulation of DNA metabolic process</td>
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<tr>
<td>Nucleosome assembly</td>
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<tr>
<td>Chromatin modification</td>
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<tr>
<td>M phase</td>
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<tr>
<td>Xenobiotic metabolic process</td>
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<tr>
<td>Cellular response to chemical stimulus</td>
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<tr>
<td>Fat-soluble vitamin metabolic process</td>
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<tr>
<td>Glutathione metabolic process</td>
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<tr>
<td>Interferon gamma signaling</td>
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</tbody>
</table>

Bayesian integration of pathways using K-nearest neighbors statistical learning algorithm with leave-one-out cross validation improves classification accuracy of PAH treatments based on tumor outcome.

Integration of 4 pathways predicts tumor outcome ~100% classification accuracy indicating their importance for the carcinogenic potential of PAHs during initiation.

CASE STUDY: PATHWAY-BASED PREDICTION OF PAH-INDUCED SKIN CANCER

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Integration of 4 pathways predicts tumor outcome ~100% classification accuracy indicating their importance for the carcinogenic potential of PAHs during initiation.
CASE STUDY: PATHWAY-BASED PREDICTION OF PAH-INDUCED SKIN CANCER

Transcription factor analysis and network analysis of pathways predictive of PAH carcinogenic potential during initiation

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>B[a]P</th>
<th>DBC</th>
<th>Mix 1</th>
<th>Mix 2</th>
<th>Mix 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARNT</td>
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<tr>
<td>NRF2</td>
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<tr>
<td>SP1</td>
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<td>P53</td>
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<tr>
<td>C-MYC</td>
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****p<0.00001, ***p<0.0001, **p<0.001, *p<0.05
**CASE STUDY: PATHWAY-BASED PREDICTION OF PAH-INDUCED SKIN CANCER**

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**p<0.00001, ***p<0.0001, **p<0.001, *p<0.05

In particular, DBC displays unique gene expression and regulation compared to B[a]P and the PAH-mixtures.
Proof-of-concept take home points:

- Pathways predictive of carcinogenic potential of PAHs and mixtures were used to appropriately classify chemicals based on outcome
- PAHs function through different mechanisms of action
- PAHs in mixtures are not strictly additive
- Potential for application to whole mixture assessments based on molecular endpoints
- Assessment of whole mixtures without complete knowledge of components or potential interactions
NEEDS AND FUTURE DIRECTIONS

- For inclusion of genomics and mechanistic data into PAH risk assessments
  - Expanded signatures and pathways for PAHs and mixtures
  - Integration of traditional endpoints
  - In vivo validation of carcinogenic potential for novel PAHs and mixtures
  - Dose-response data for quantitative endpoints (i.e. threshold response)

- For consideration of whole mixture approach
  - Expanded data on toxicity and carcinogenicity of mixtures
    - Evaluate sufficient similarity approach
    - Expand diversity of PAHs and mixtures tested
  - Compare complex whole mixtures to more simplistic mixtures
    - Defined mixtures (based on primary chemical make-up)
    - Chemical fractionation
  - Consider noncancer modes of action
ACKNOWLEDGEMENTS

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