Applying High Throughput Bioassays as Monitoring Tools in AWT/DPR Facilities

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Introduction

• First two talks address methods, applications and validation of bioassays relevant to health effects
• The present talk addresses issues related to the use and interpretation of results in water analyses
  – How is a metric to be applied to a response per unit of water sampled?
  – Are these bioassays for imputed health effects?
  – Water industry or state regulators to develop a process for validating the measure for water analysis?
Outline

• Role of in vitro bioassays in health effects testing
• Approaches to water testing with bioassays
• Adverse outcome pathways (AOP)
• Review of decision logic in health effects testing
• History of bioassays applied to water
• Illustrate the role of pharmacokinetics
• Validation that is needed beyond activities of that of EPA programs for monitoring of water
• What decision logic will be applied to results of AWT/DPR/Drinking water testing?
Role of in vitro bioassays in health effects testing

• To detect an biological activity that causes or contributes to the development of toxicity/disease – e.g. mutagenicity & cancer

• To explore possible mechanisms/modes of action, e.g.:
  – Provided evidence that chemical could induce mutation is used to support the use of linear, low-dose extrapolation of *in vivo* data

• The introduction of HTP has facilitated the measurement of many more “effects”.
Conventional Bioassay Decision-Tree used in Health Effects Testing of Chemicals

Tier 1 – Screening

- Produce and market product (if there is confidence in the negative result)

Tier 2 – Confirmation (or discard product & avoid development cost)

- Produce and market product (if there is confidence in the negative result)

Tier 3 – Risk Assessment

The missing piece!
A key difference in approaches

• ToxCast & related programs:
  – Are built on the expectation of having results from many bioassays to characterize a compound’s toxic potential
  – A “big data” approach

• Applications to water have proposed fielding individual or at most a few bioassays
  – A conclusion of safety depends on results of single bioassays
  – A “small data” approach.
Monitoring of water with bioassays

• How is the response to be scaled so that a result has a quantitative meaning?
  – A requirement for any monitoring tool.
  – Each bioassay has to have a specific purpose and the dose-response scaled to that purpose.
  – Very different from chemical monitoring, where a criterion has been developed by considering the dose-response for a chemical to induce a critical health effect in vivo.

• Is the intent to be a surrogate for possible health effects?
  – First step requires relating the bioassay result to an adverse outcome pathway (AOP).
  – Second, the dose-response of the bioassay with the dose-response for adverse health outcomes produced in vivo (animals or humans).
    • Pharmacokinetic analyses will be necessary to normalize doses (usually blood plasma vs. media concentrations).
    • Pharmacodynamic modeling (i.e., pathway analysis and linkage to adverse outcome) is also being pursued within EPA.
Alternative AOPs

Parent chemical or metabolite

- Direct cell stress
  - Severe hepatocyte injury
    - JNK, bim, bax

- Direct mitochondrial inhibition
  - Mitochondrial permeability transition
    - Bid, Ceramide
    - Cytochrome c release
      - Massive ATP depletion
        - Necrosis
        - Remaining ATP production
          - Apoptosome
            - Activation of Caspase 9
              - Effector caspases 3, 6, 7
                - Apoptosis

- Specific immune reaction
  - TNFα/FasL
    - Initiator caspase 8
History of bioassays in water

- Uses:
  - Screening followed by identification of responsible chemical(s).
  - Mutagenesis assays were employed in water testing world wide
    - Positives not followed up (except in the Netherlands)
      - Virtually all disinfected water is mutagenic
      - Decision logic would be to test for carcinogenicity OR discard product
    - There is no relationship between mutagenic potency \textit{in vitro} and carcinogenic potency \textit{in vivo} across chemical classes.
      - Therefore, there is no basis for translation of bioassay result into a limit on exposure without the in vivo data
  - Decision logic violated!
  - What is the basis for a meaningful numerical value for mutagenesis bioassays?
    - Such a relationship has been pursued, but simply does not work across compound classes
  - Reason that mutagenesis assays have never been adopted/required by state or federal governments
Change in decision logic?

- ToxCast is EPA’s database that is used to explore predictive ability of HTP data. Within these data sets, HTP results are compared with toxic endpoints *in vivo* with the same compound
  - Developed by applying virtually any HTP bioassay that was available
  - Generates a lot of data, much of which has no obvious utility in conventional risk assessment
- Application of a specific bioassay for health effects testing requires that the results be associated with an adverse outcome pathway (AOP)
- The AOP is likely to be one of several than can contribute to an given adverse health effect
  - Therefore, a negative bioassay does not allow the conclusion that a particular health effect will not be induced by the chemical (or water sample)
- Many chemicals are associated with multiple AOPs, especially *in vitro* (usually addressed by comparing dose response relationships)
- The EPA system is still focused on prioritization for further evaluation in an apical test
- The question is how to structure *an in vitro* systems such that is equivalent to the apical test
**Example of a problem**

**KEAP1**

![KEAP1 diagram]

**Figure 2:** Domain structure of human KEAP1. NTR: N-terminal region (amino acids 1–60); BTB: broad complex, Tramtrack, Bric-à-brac (amino acids 61–179). KEAP1 forms a homodimer through the BTB binding domain; IVR: intervening region (amino acids 180–314); Kelch domain (amino acids 315–598). The Kelch domain is the binding site with NRF2; CTR: C-terminal region (amino acids 599–624). The positions of the cysteine residues are indicated with yellow bars. The most commonly modified cysteine residues by sulfhydryl-reactive small molecules are shown in red.

Dinkova-Kostova, A.T. 2012 The role of sulfhydryl reactivity of small molecules for the activation of the KEAP1/NRF2 pathway and the heat shock response. Scientifica Article ID 606104.
KEAP1/NRF2 (AREc32) Receptors

Disinfectant Residuals

- References indicating chlorine and chloramine concentrations used for residual disinfection will trigger KEAP1/Nrf2 assays
Direct genomic action of classic receptors

Non-genomic (second messenger-mediated) action of classic receptors

Non-genomic action of non-classical receptors

Steroid

Non-classic steroid receptor

Classic Steroid receptor

Coactivator

Polymerase

AC

Adenyl cyclase

PKA

Protein kinase A

PKC

Protein kinase C

DNA, mRNA

Nucleus

Steroid-induced protein synthesis

Ras/Raf-1 MAPK

MAPK

PI3K

Ca++

cAMP

ATP

IP3

DAG

PKC

CREB

pCREB

PKA

PLC

Calcium

Non-genomic (second messenger-mediated) action of classic receptors
Are HTPs useful in drinking water testing?

• Bioassay must address an established AOP
• A response level of the bioassay that can be related to risk of the adverse outcome must be established
• Knowledge of the chemical’s pharmacokinetics
• Consideration of populations unusually sensitive to the AOP can be mechanism-based
• Given the data described above, risk assessment could be as straight-forward as use of conventional human/animal data


Importance of pharmaco-kinetics

In vivo

In vitro data

In vivo

Bromate Concentration—nM/L

Lowest Active Concentration in Study
Unknown mixtures of variable composition

• Testing/monitoring of water is very different than testing identified chemicals or specified products
  – How are doses used in HTP bioassays to be compared to *in vivo* doses (in pharmacology and toxicology thought of as concentration at the affected cell or receptor)?
  – What is the value of a negative result (i.e., negative result is only applicable to the AOP measured)?
  – How many HTPs would have to be fielded to say the water is safe?
Rules that should apply to testing of water

- No bioassay can be said to relate to adverse health effects unless there is a clearly established AOP
- Activation of an AOP could predict a health effect, likely more than one
- A negative result predicts nothing related to health effects as the health effect may be produced by another AOP
  - Broader detection with bioassays is mostly a myth
  - Additive or synergistic risk will not be detected without assays for alternative AOPs
- There must be a consensus interpretation of individual bioassay results
  - Qualitative (adverse effect can be associated with the AOP)
  - What is not being measured? (other AOPs)
  - Quantitative (risk assessment for AOP activation detected)
Validating the applications of health bioassay for water monitoring

• Panel/board should be established to review proposed bioassay applications for drinking water (and source waters)

• Requires:
  – Association of AOP with adverse health outcome
  – Establish a dose-response curve for producing at least one adverse outcome via AOP
  – Was the identified AOP critical to the outcome (i.e., the most sensitive) for individual chemicals?

• Would seem essential for regulatory and public acceptance

• Should make use of the EPA databases
Expertise

• Signaling pathway and outcome analyses (known as pharmacodynamics or toxicodynamics in the field)
• Expert on reporter assay constructs
• Non-receptor-mediated toxicity
• Pharmacokinetics
• Statistician/epidemiologist
• Risk Assessment
• Utility professional
Assuming single assays with Dose-Response validated AOPs are to be employed

• Few HTP bioassays ready, e.g.:
  – Selected steroid hormone receptor-based constructs
  – AhR receptor reporters – some caveats
    • How do these compete with chemical analyses?
Limits on the validation

- Receptor-mediated modifications apply only to receptor-mediated effects. Modifications of an AOP at a non-receptor site may not be recognized/detected.
  - Endocrine effects of Dichloroacetic acid and bromate have been identified as acting through such mechanisms.
  - Does not apply to other causes (or AOPs) that produce the same adverse effect.
  - This has to be clearly communicated to the public
- Demonstration that a test can be run consistently in water samples across laboratories is not sufficient validation.
Potential advantages of bioanalytical methods

• In selected cases they may be easier/less expensive to employ than analytical chemistry
• May have greater sensitivity than chemical analyses? (only if the “right AOP” has been tested)
• Will capture compounds that act through the tested AOP – but quantitation of risk will be seriously in error if it is not the appropriate receptor for the compound, e.g.:
  – Bisphenol A may not act through the same receptor as EE2 (ERRγ) Its affinity for this receptor is 500X its affinity for classical ERα or β
Disadvantages HTP Assays

- Have been largely limited to receptor-reporter constructs (a result of tests being developed by the pharmaceutical industry)
- There are non-receptor-mediated ways of affecting an AOP to produce adverse health effects
- Poor at detecting target cell-specific effects
  - Lack of ability of non-differentiated cells to metabolically activate toxicants
  - Response of AOP activation does vary among differentiated cell types, e.g., see history of estrogen/anti-estrogen effects in different primary cell types
- In some cases, activation of AOP tightly associated with adverse effects, but most HTP assays have no AOP
- As currently presented/proposed do not provide a broader assessment of water quality than chemical analyses.
Independent validation of bioassays applied to water is essential!