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

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Specialty Seminars

Bioanalytical Tools

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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

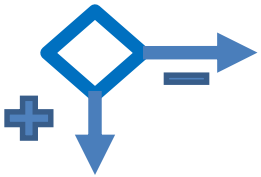
- Role of in vitro bioassays in health effects testing
- Approaches to water testing with bioassays
- Adverse outcome pathways (AOP)
- Review of decision logic in health effects testing
- History of bioassays applied to water
- Illustrate the role of pharmacokinetics
- Validation that is needed beyond 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”.

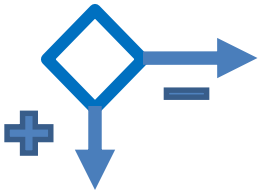
Conventional Bioassay Decision-Tree used in Health Effects Testing of Chemicals

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

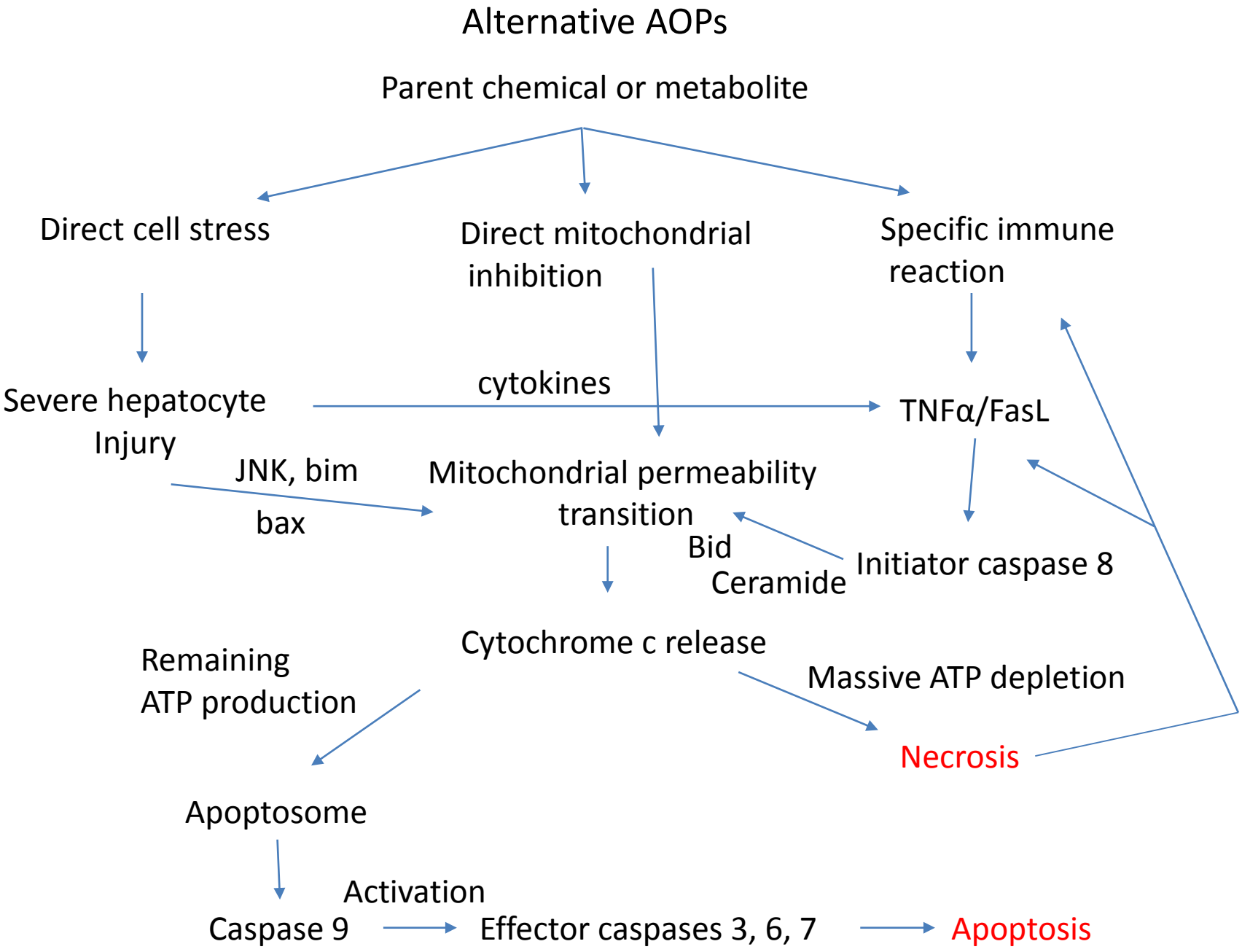
The missing piece!

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
Direct mitochondrial inhibition
Specific immune reaction
Severe hepatocyte Injury
cytokines
TNFα/FasL
JNK, bim
bax
Mitochondrial permeability transition
Bid
Ceramide
Initiator caspase 8
Cytochrome c release
Remaining ATP production
Massive ATP depletion
Necrosis
Apoptosome
Activation
Caspase 9
Effector caspases 3, 6, 7
Apoptosis

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 *in vitro* and carcinogenic potency *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

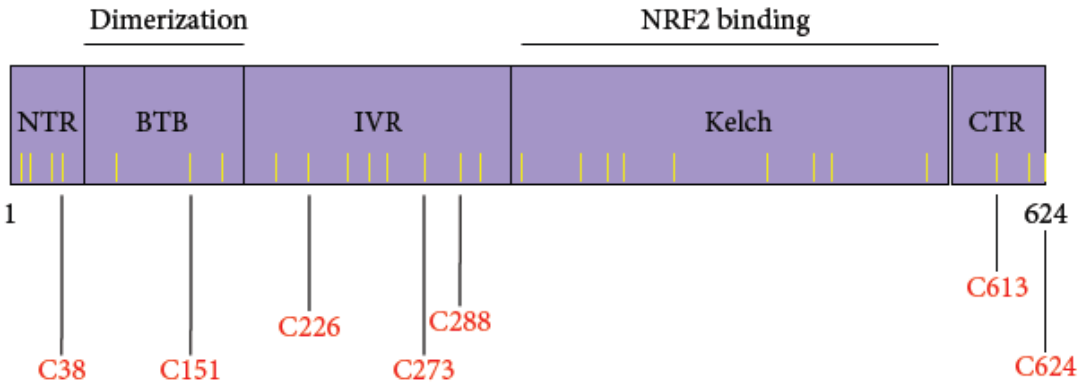
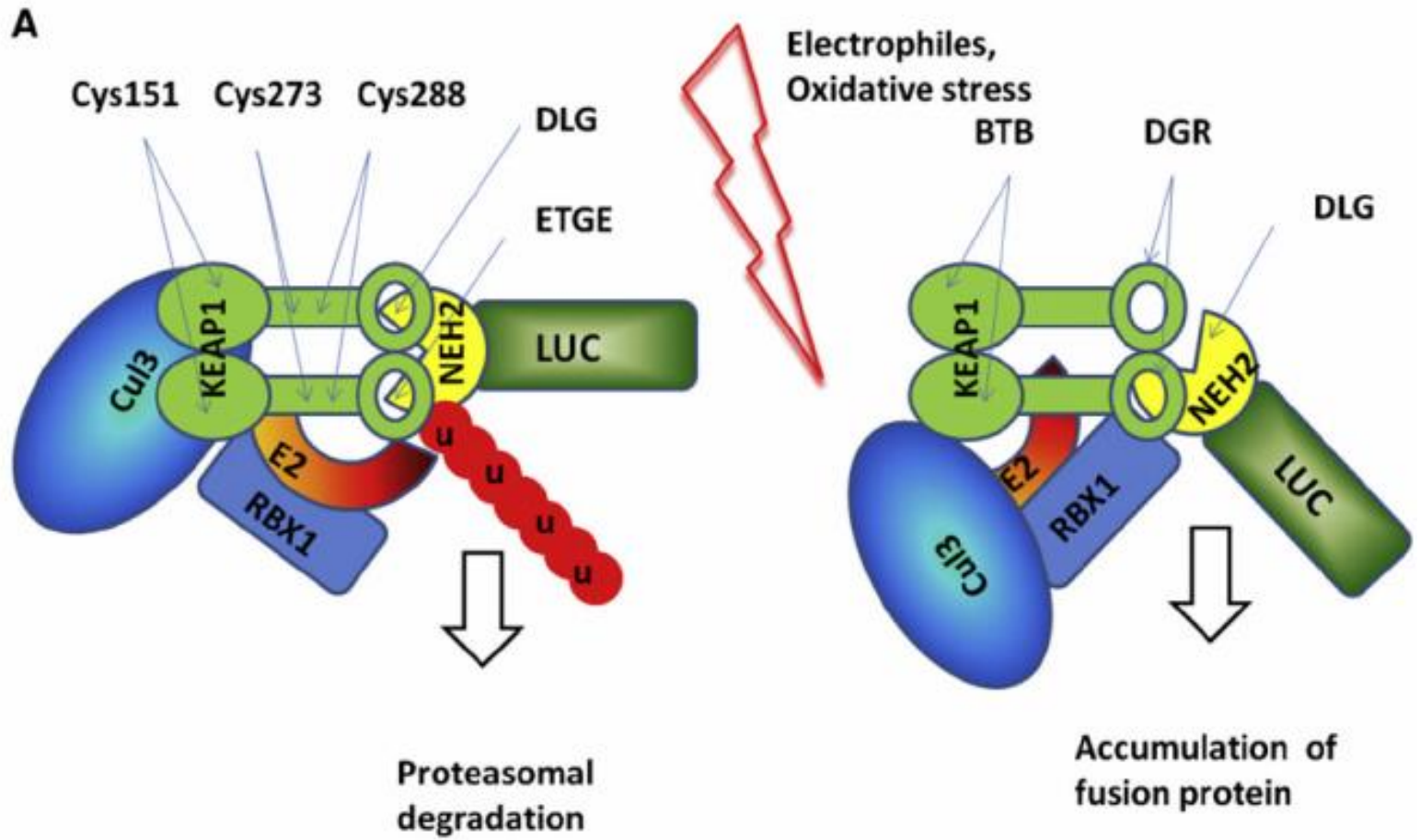


FIGURE 2: Domain structure of human KEAP1. NTR: N-terminal region (amino acids 1–60); BTB: broad complex, Tramtrack, Bric-á-brac (amino acids 61–179). KEAP1 forms a homodimer through the BTB binding domain; IVR: intervening region (amino acids 180–314); Kelch domain (amino acids 315–598). The Kelch domain 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.

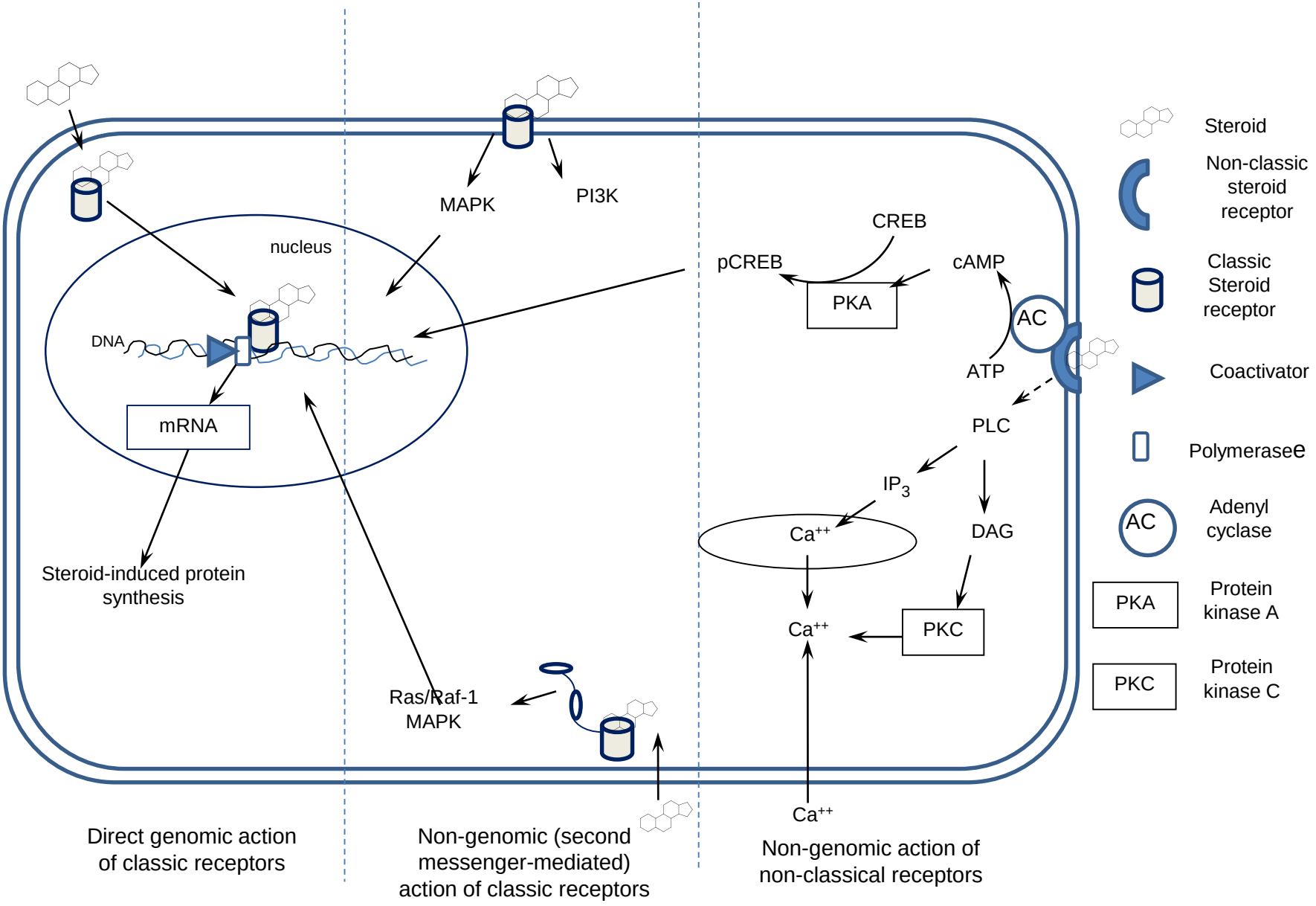
KEAP1/NRF2 (AREc32) Receptors



From: Smirnova et al. 2011. Development of Neh2-Luciferase reporter and the application for high throughput screening and real-time monitoring of Nrf2 activators. Chem. Biol. 18(6):752-765

Disinfectant Residuals

- References indicating chlorine and chloramine concentrations used for residual disinfection will trigger KEAP1/Nrf2 assays
  - Woods et al. 2009. Dose-dependent transitions in Nrf2-mediated adaptive response and related stress responses to hypochlorous acid in mouse macrophages. Toxicol. Appl. Pharmacol. 238(1):27-36
  - Wei et al., 2009. Hypochlorous acid-induced heme oxygenase 1 gene expression promotes human endothelial cell survival. Am. J. Physiol. Cell Physiol. 297:C907-C915
  - Zhu et al., 2008. Identification of Nrf2-dependent airway epithelial adaptive response to proinflammatory oxidant-hypochlorous acid challenge by transcription profiling. Am. J. Physiol. Lung Cell Mol. Physiol. 294:L469-K477.
  - Jang et al., 2009. Taurine chloramine activates Nrf2, increases HO-1 expression and protects cells from death caused by hydrogen peroxide. J. Clin. Biochem. Nutr. 45:37-43.


MAPK
PI3K
nucleus
DNA
mRNA
Steroid-induced protein synthesis
Ras/Raf-1
MAPK
CREB
pCREB
PKA
cAMP
AC
ATP
PLC
IP3
Ca++
DAG
PKC
Ca++
Ca++
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
Adenyl cyclase
Protein kinase A
Protein kinase C

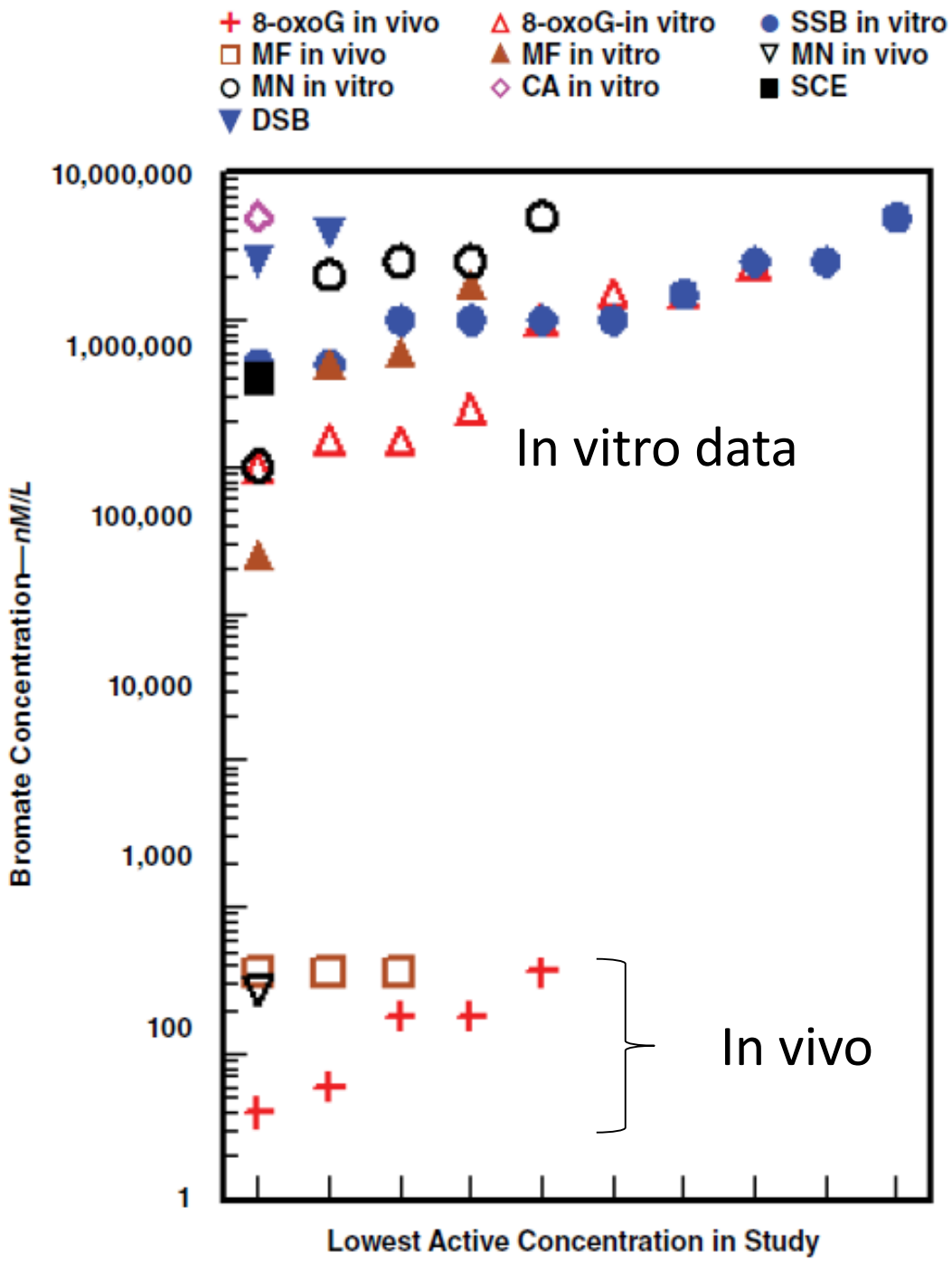
Are HTPs useful in drinking water testing?

- 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

Crump et al. The future of in vitro data in risk assessment to set exposure standards; Challenging problems and familiar solutions. Environ. Health Perspect. 118(10):1350-1354)

Yoon et al. 2012. Quantitative in vitro to in vivo extrapolation of cell-based toxicity assay results. Crit. Rev. Toxicol. Doi: 10.3109/10408444.2012.692115.)

Importance of pharmaco-kinetics



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 pubic 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!