In Vitro Bioactivity in ToxCast™ Assays for Fruit and Vegetable Juices

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Abstract

The ToxCast and Tox21 programs have generated *in vitro* screening data for over 1000 chemicals to aid in hazard identification and setting chemical testing priorities. These data, together with high-throughput pharmacokinetic data, are used to infer possible toxic responses and external concentrations required to elicit these effects. There is only limited experience in evaluating dose-response for natural products in these assays. In this study, juices were extracted from 30 organically grown fruits and vegetables. These juices were screened in concentration-response format across primary human cell and co-culture assays (BioMAP systems) to assess similarities of pathway responses of the extracts with those of database compounds. Bioactivities noted with unpeeled potato juice were immunomodulatory and tissue remodeling activities across endothelial, peripheral blood mononuclear and fibroblast cells. This pattern of response was similar to quercetin, a plant-derived flavonoid. Broccoli juice initiated anti-proliferative effects on endothelial, fibroblast and smooth muscle cells with significant similarity to mitomycin C (374 ng/mL; Pearson, r=0.806) and the fungicide mancozeb (40 µM; Pearson, r=0.776). To relate *in vitro* concentrations to administered dose, the filtered juice yield per g item was used with the plasma volume of a 70kg adult to approximate % juice present in systemic circulation after eating. This value was used as a surrogate for target tissue concentration. The activity described for broccoli was elicited at 0.5%, the amount of juice anticipated in the circulation following consumption of 2 cups of broccoli. Importantly, the bioactivities noted do not necessarily lead to adverse effects. These data provide context for assessing the *in vivo* relevance of *in vitro* concentration-response and bioactivity data generated in ToxCast and similar screening programs. *This abstract does not necessarily reflect EPA policy.*

Background

- ToxCast HT screening data have been generated for >1000 chemicals to aid in hazard identification.
- Many of these assays probe biological and therapeutic activities that span multiple processes and pathways.
- Evaluation of natural products in these systems may aid in 1) ToxCast data interpretation and 2) relating *in vitro* bioactivity to external exposure.

Experimental Design

- **Fruit and vegetable juices were prepared from 30 organically grown items.**
- **Juices were assessed for osmolality, pH and presence of pesticide residues, heavy metals and mycotoxins.**
- **Juices were screened in BioMAP Predictive Tox Panel; activity profiles were compared against ToxCast Phase I and BioMAP reference compounds.**

Fruits and Vegetables Assessed

Test Material	Activity Reported[a]
Bean Sprouts	-Cytotoxicity; +Chromosomal Abs (+/-S9); +/-Limb bud; ++ER activation
Beets, Red	++Cytotoxicity
Broccoli	+++Cytotoxicity; +++Chromosomal Abs (+/-S9); +/- Limb bud; +++ER activation
Carrots	-Cytotoxicity; -Chromosomal Abs (+/-S9); -Limb bud; +ER activation
Cauliflower	+Cytotoxicity
Celery	+Cytotoxicity
Garlic	+Cytotoxicity
Ginger	+++Cytotoxicity
Potatoes, peeled	NT in Charles *et al.*
Potatoes, unpeeled	Developmental toxicity (Renwick *et al.)*
Soybeans	-Cytotox; Non-monotonic proliferation below cytotox; +Chromosomal Abs (+/-S9); ++Limb bud; +++ ER activation
Edamame	NT in Charles *et al.*
Sweet Potatoes	++Cytotoxicity; Non-monotonic proliferation below cytotoxicity

Test Material	Activity Reported
Green Beans	+++Cytotoxicity
Spinach	++Cytotoxicity; -Chromosomal Abs (+/- S9); +/- Limb bud; +ER activation
Kale	NT in Charles *et al.*
Cabbage	+Cytotoxicity; +Chromosomal Abs(-S9)
Peppers, Red bell	++Cytotoxicity
Onions, Vidalia	++Cytotoxicity
Parsley	+Cytotoxicity; non-monotonic proliferation below cytotoxicity
Snow peas	+Cytotox, non-monotonic proliferation below cytotox;++Chromosomal Abs (-S9); ++Limb bud, +ER activation
Tomatoes, Red	-Cytotoxicity
Apples	-Cytotoxicity
Blueberries	NT in Charles *et al.*
Grapefruit	+Cytotoxicity
Grapes, Green	-Cytotoxicity; +ER activation
Orange	+Cytotoxicity
Pineapple	++Cytotoxicity, non-monotonic proliferation below cytotoxicity
Strawberries	-Cytotoxicity, non-monotonic proliferation below cytotoxicity

[a]As reported in Charles *et al.*, 2002, Food Chem. Toxicol., 40:1391-1402; Renwick *et al.*, 1984, Teratol., 30:371-381.

Experimental Design

BioMAP Diversity 8 Panel

BioMAP System	3C	4H	LPS	SAg	BE3C	CASM3C	HDF3CGF	KF3CT
Primary Human Cell Types	Vascular endothelial cells	Vascular endothelial cells	Peripheral blood mononuclear cells + Endothelial cells	Peripheral blood mononuclear cells + Endothelial cells	Bronchial epithelial cells	Coronary artery smooth muscle cells	Fibroblasts	Keratinocytes + Fibroblasts
Stimuli	IL-1β + TNF-α + IFN-γ	IL-4+Histamine	TLR4	TCR	IL-1β + TNF-α + IFN-γ	IL-1β + TNF-α + IFN-γ	IL-1β + TNF-α + IFN-γ + EGF + FGF2+SB	IL-1β + TNF-α + IFN-γ + TGFβ1
# of Endpoints	13	7	11	10	11	14	12	9
Endpoint Types: Acute Inflammation	E-selectin, IL-8		E-selectin, IL-1α, IL-8, TNF-α, P0E2	IL-8	IL-1α	IL-8, IL-6, SAA	IL-8	IL-1α
Endpoint Types: Chronic Inflammation	VCAM-1, ICAM-1, MCP-1, MIG	VCAM-1, Eotaxin-3, MCP-1		VCAM-1, MCP-1	IP-10, MIG, HLA-DR	MCP-1, VCAM-1,MIG, HLA-DR	VCAM-1, IP-10, MIG	MCP-1, ICAM-1, IP-10
Endpoint Types: Immune Response	HLA-DR		CD40, M-CSF	CD38, CD40, CD69, P3NKC, Cytotox, T cell Proliferation	HLA-DR	M-CSF	M-CSF	
Endpoint Types: Tissue Remodeling					uPAR, MMP-1, PAI-1, TGFb-1, SRB, EPA, uPA	uPAR,	Collagen III, EGFR, MMP-1, PAI-1, Fibroblast Proliferation, SRB, TIMP-1	MMP-9, SRB, TIMP-2, uPA, TGFb1
Endpoint Types: Vascular Biology	TM, TF, uPA-R, EC Proliferation, SRB, VCa	VEGFR9, uPAR, P-selectin, SRB	Tissue Factor, SRB	SRB		TM, TF, LDLR, SMC Proliferation, SRB		
Disease / Tissue Relevance	Vascular Biology, Cardiovascular Disease, Chronic Inflammation	Asthma, Allergy, Oncology, Vascular Biology	Cardiovascular Disease, Chronic Inflammation, Infectious Disease	Autoimmune Disease, Chronic Inflammation, Immune Biology	COPD, Respiratory, Epithelial Biology	Vascular Biology, Cardiovascular Inflammation, Restenosis	Tissue Remodeling, Fibrosis, Wound Healing	Skin Biology,Psoriasis, Dermatitis

Relating *In Vitro* Concentrations to External Dose

Given:

- A 75 kg adult has 5 L total blood
- Plasma comprises 55% of the total blood volume…
  - Juice would be present in a total blood volume of 2.75L plasma.
    - 12.5% juice in systemic circulation = 344 mL / 2.75 L
    - 4.17% juice = 114 mL
    - 1.39% juice = 44.8 mL
    - 0.46% juice = 12.75 mL

Relating to vegetable consumption…

1 g broccoli yields 85 µL juice.
150 g = 12.75 mL juice → 0.46% concentration

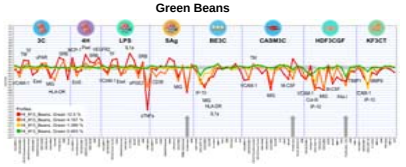
Results

Pesticide, Heavy Metal and Mycotoxin Assessment of Produce

All but two items confirmed to be pesticide-free
Exceptions:
Kale – Azoxystrobrin (<LOQ of 0.015 mg/kg)
Green Grapes – Imidacloprid (<LOQ of 0.015 mg/kg)

Fruit and Vegetable BioMAP Activity Profiles

Green Beans

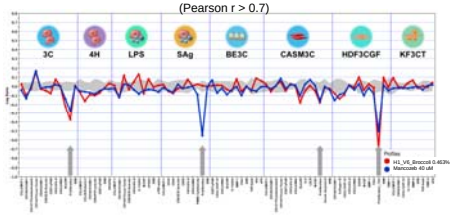


Strawberries

Sweet Potato Activity Similar to JAK 1/2 Kinase Inhibitor

Results

Broccoli Activity Profile Similar to Fungicide Mancozeb
(Pearson r > 0.7)



Unsupervised Similarity Search Database Hits (Pearson >0.7)

Test Agent	Hit	Hit Information
Broccoli (0.46%)	Mitomycin C (370 ng/mL)	alkylating agent, DNA cross-linker
Broccoli (0.46%)	Mancozeb (40 µM)	Fungicide; anti-proliferative effects in multiple cell types
Red Beets (0.46%)	Tannic acid (4.1 µM)	polyphenol, component in anti-allergen sprays
Kale (1.39%)	ZM449829 (3.3 µM)	JAK3 inhibitor tool compound binds to ATP site on enzyme
Sweet Potatoes (4.17%)	INCB-018424 (10 µM)	Ruloxitinib, JAK 1/2 kinase inhibitor; Phase III trial, myelofibrosis
Unpeeled Potatoes (4.17%)	Quercetin (22.22 µM)	plant-derived flavonoid found in fruits vegetables, leaves, grains
Unpeeled Potatoes (4.17%)	Fucoidan (7 ng/mL)	sulfated polysaccharide in brown algae/seaweed; dietary supplement
Unpeeled Potatoes (4.17%)	ODN2395 (1 µM)	synthetic oligonucleotides with TLR 9 activity (immunostimulatory)

Mechanistic Hits Using BioSeek SVM Model Prediction Tool

Test Agent	Mechanism
Butternut Squash (12.5%)	HDAC (1.11 µM)
Steamed Edamame (12.5%)	HDAC (1.11 µM)
Sweet Potatoes (4.17%)	JAK Inhibitor (1.11 µM)

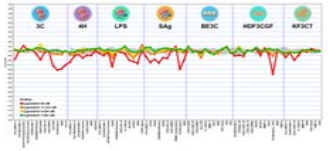
Comparing Apples to Azoxystrobin…

Test Agent	Minimum Concentration >10% LEC hits	Oral Equivalent (OE)		Maximal Pesticide Exposurea (mg/kg produce)	Kg Produce Consumed to Achieve OE / Minimum Concentration
		mg/kg/day	mg/day 75 kg adult		
Azoxystrobin	13.3 µM (9)	6.38	479	9.0	53
Cyprodinil	40 µM (20)	0.08	6	2.2	2.7
Fludioxonil	13.3 µM (15)	0.18	14	3.1	4.5
Pyraclostrobin	1.48 µM (29)	0.29	22	8.0	2.75
Apples	0.463% (28)			NA	0.059 (0.5 cups)
Green Beans	0.463% (21)			NA	0.083 (0.8 cups)
Spinach	0.463% (25)			NA	0.177 (1.8 cups)
Blueberries	0.463% (16)			NA	0.122 (0.8 cups)

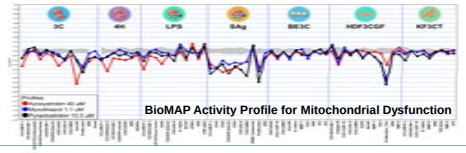
ToxCast Phase I Chemical Activities in BioMAP

Phase I Chemicals typically exhibited lower activity; on an LEC basis fruits and vegetables typically had 4-fold more hits per test agent.

Cyprodinil



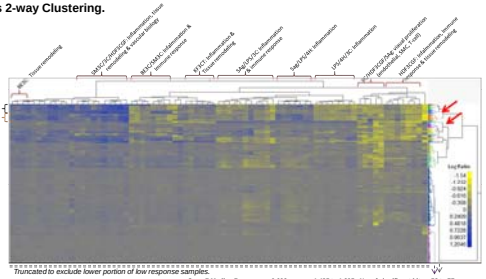
Azoxystrobin, Pyraclostrobin



BioMAP Activity Profile for Mitochondrial Dysfunction

Clustering of Fruit, Vegetable, and Phase I Chemical BioMAP Activities Distinct groupings revealed, but some similarities exist

Ward's 2-way Clustering.



Truncated to exclude lower portion of low response samples

Log Ratio data centered by rows (individual sample's values scaled to mean of zero). Assays with toxic hits in any sample excluded (77 assays retained for clustering).

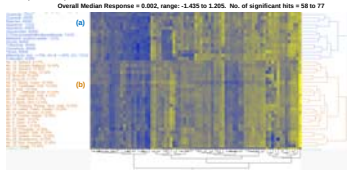
Cluster order corresponds to the dendogram in the original heat map, as indicated by red arrows.

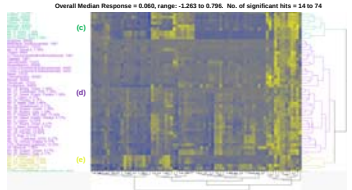
Significant Log Ratios based on a 0.95 CI envelope.

Median response calculated across all 77 assays.

(a) Median response = -0.013
(b) Median response = 0.008

(c) Median response = 0.167
(d) Median response = 0.054
(e) Median response = 0.112





Summary & Conclusions

- Fruit and vegetable juices elicited extensive bioactivities across the BioMAP Diversity 8 Panel.
- When compared to Phase I chemicals, the produce elicited ~4-fold more LEC hits per test agent.
- Clustering revealed that while chemicals and produce typically grouped with their own, similarities in activity were also identified (clades c and d).
- The ability to relate external exposures to internal concentrations for produce and ToxCast chemicals provides critical context.

Acknowledgments

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