Does Predictive Toxicology Live Up to its Promise?

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Main Points

- Predictive toxicology should generate function from structure
  - *In silico* modeling predicts biological activity, reducing need for in vitro and in vivo testing

- Proper prediction requires accurate assays and integration of results into model refinement

- Current models and approaches work to some degree, depending on how stringently you evaluate results
  - "Predictive models" for most chemicals perform poorly
  - "Predictive models" for obesogens have many limitations
  - Accuracy can be improved by adding additional screens
    - E.g., siRNA knockdown of putative AOP members

- Predictive toxicology must be grounded in biology

Screening for EDC activity

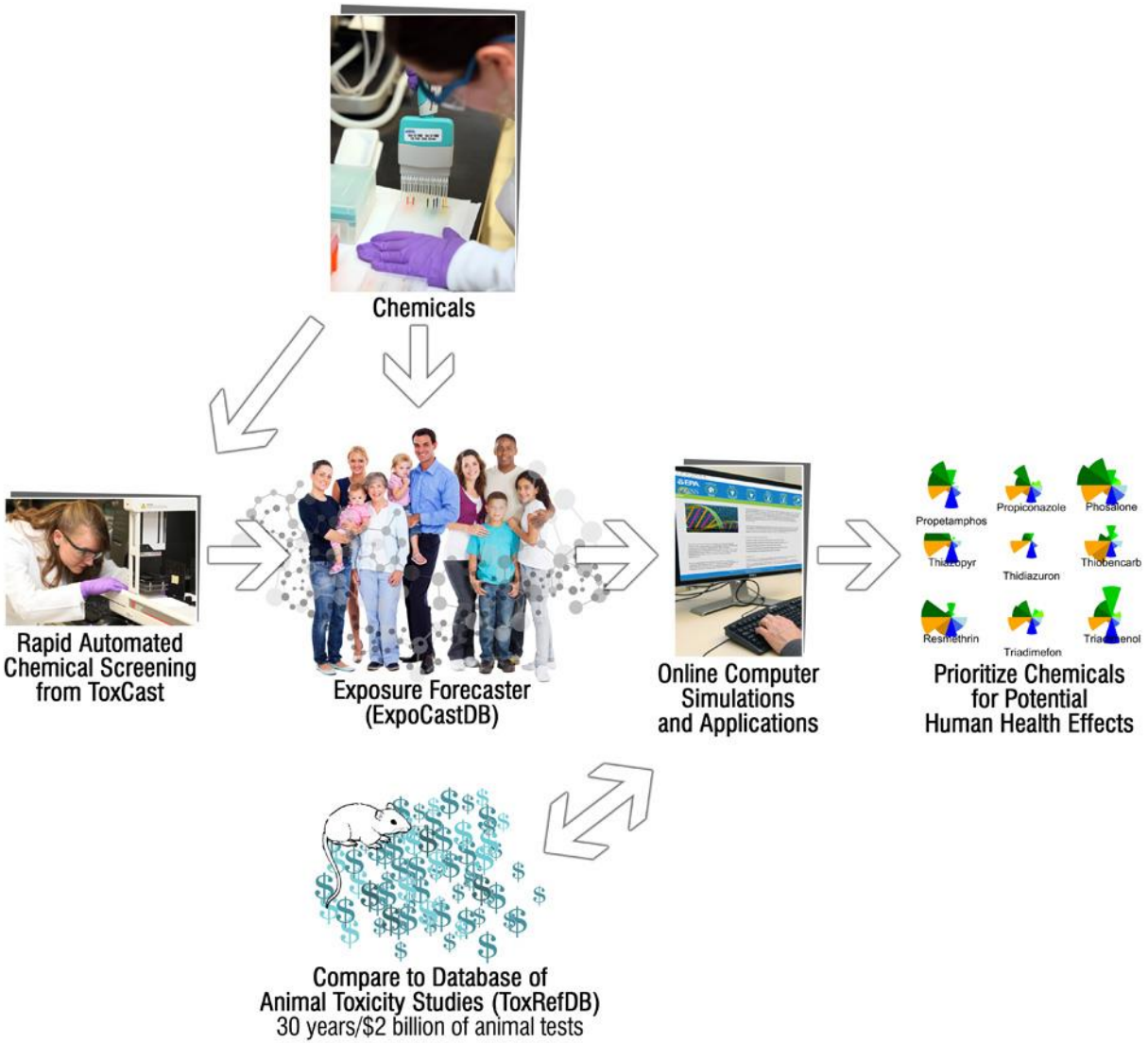
- The 1996 Food Quality Protection Act
  - directed EPA to develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have hormonal effects in humans

- 1996 amendments to the Safe Drinking Water Act authorized EPA to screen for potential EDCs in drinking water.

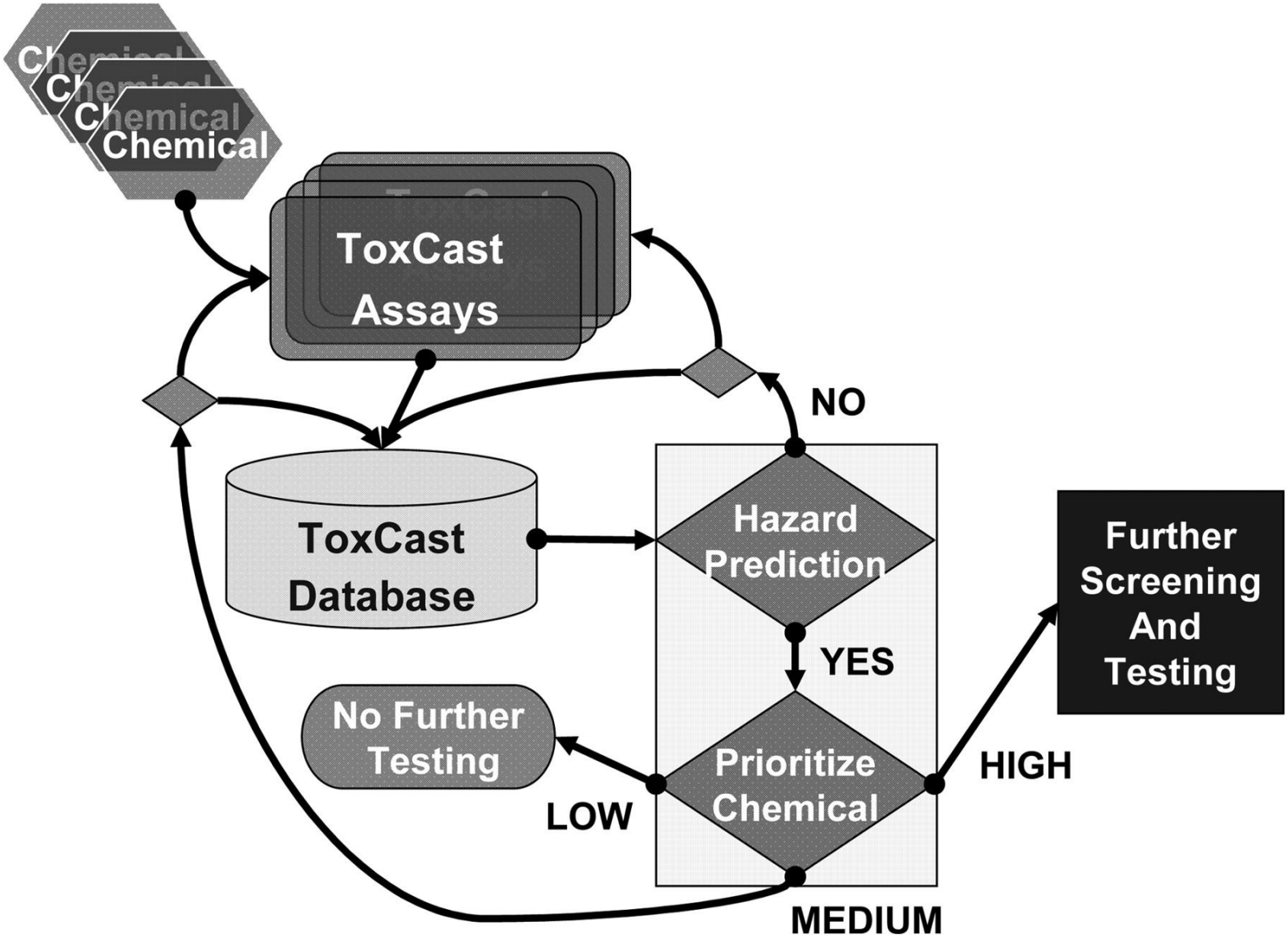
- Federal Register: October 21, 2009 , Volume 74, Number 202, 54422-5
  - First orders issued for tier 1 screening!

EPA's Approach to Assess Chemical Risk

Screen and prioritize chemicals for potential human toxicity using in vitro assays and in silico approaches.



ToxCast – EPA's Approach to Assess Chemical Risk



ToxCast – EPA's Approach to Prioritization

- ToxCast reframed as hazard assessment, prioritization tool rather than the basis for risk assessment
- ToxCast and Tox21 are continuing. Will screen 10,000 chemicals using a variety of in vitro assays to prioritize those requiring further testing in depth.
- EPA states that this approach is good enough to prioritize estrogens for screening
  - Estrogen defined as active in 2 of 18 estrogenicity assays
  - They did not feel the need to consult experts in estrogen signaling or ER biology before making this determination

Can we use ToxCast to predict adipogenic activity?

- PPARγ is key regulator of adipogenesis
- ToxCast has PPARγ assays in its collection
  - Attagene cis/trans reporter assay
  - Standard reporter assay (NCGC) (GeneBLAzer)
  - Direct ligand binding assay (NCGC)
- Are ToxCast results valid (no one had tested yet - 2010)
- Can they predict adipogenic activity of test chemicals?

- Test activity on PPARγ
- Test adipogenic action
  - 3T3-L1 and MSC differentiation assays

Testing ToxCast Predictions of Activity - PPARγ

	Toxcast Assay (AC50 Values in μM)				
ToxCast Chemical We Retested	ATG reporter assay	Standard reporter assay	Ligand binding assay	Results of Our Retesting on PPARγ	Adipogenesis assay results
Fentin	0.02		0.54	ACTIVE	ACTIVE
Fluazinam	0.51		0.3	ANTAGONIST	INACTIVE
Nidosamide	0.56			INACTIVE	INACTIVE
Pyradostrobin	0.76			INACTIVE	INACTIVE
Zoxamide	1.5			ACTIVE	ACTIVE
Acetochlor	1.7			ANTAGONIST	INACTIVE
Butachlor	2.1			INACTIVE	INACTIVE
Triflumizole	2.8		25.3	ACTIVE	ACTIVE
Prochloraz	3.5			INACTIVE	INACTIVE
Spirodidofen	3.6	2.78		ACTIVE	ACTIVE
Alachlor	3.7		37.8	ANTAGONIST	INACTIVE
Tebufenpyrad	4.9			INACTIVE	INACTIVE
Dimethenamid	5.6			INACTIVE	INACTIVE
Tebufenozide	6.3			INACTIVE	INACTIVE
Quinoxyfen	6.6	6.7		Borderline active	Active
Indoxacarb	8.5			INACTIVE	INACTIVE
(Z,E)-Fenpyroximate	8.6			INACTIVE	INACTIVE
S-Bioallethrin	9.2			INACTIVE	INACTIVE
Cyazofamid	11			INACTIVE	INACTIVE
Dimethomorph	11			INACTIVE	INACTIVE
Chlorothalonil			0.54	INACTIVE	INACTIVE

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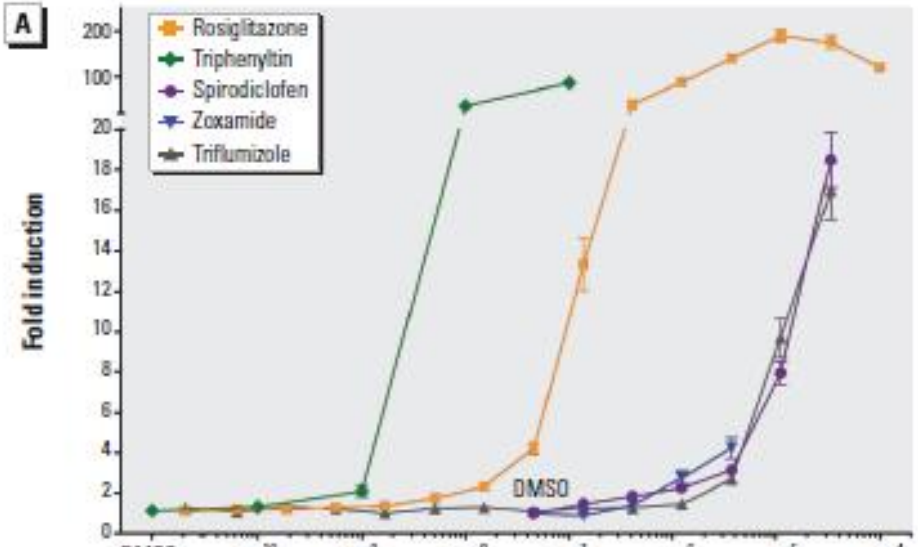
Testing ToxCast Predictions of Activity - PPARγ



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PPARγ agonists			
Chemical	Spirodiclofen		
Assay	Fig 1A	ATG	NCGC
EC50	12.76 µM	1.85 µM	1.64 µM
EC10	7.27 µM	0.10 µM	0.17 µM
Chemical	Zoxamide		
Assay	Fig 1A	ATG	NCGC
EC50	1.31 µM	1.27 µM	NA
EC10	0.31 µM	0.18 µM	NA

- Most favorable interpretation:
  - 5/16 EPA tested actives are active
  - 3/16 EPA tested actives are antagonists
  - 8/16 EPA tested actives are inactive

- Why are so many of top 16 tested (not predicted) actives inactive or antagonists?

- The answer? Substandard assays….

¿Cómo saboroso es un ToxPI?

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ToxPI Generation

- Participants at 2011 NIEHS meeting on chemical effects on obesity and diabetes nominated assays that predicted
  - Adipogenesis (2 groups)
  - Feeding behavior (rodents and *C. elegans*)
  - Peripheral insulin sensitivity
  - β-cell function
  - Islet cell function

- David Reif (then of EPA) generated ToxPIs (**Tox**icological **P**riority **I**ndex) for each endpoint and selected chemicals predicted to be
  - Strongly active
  - Medium active
  - Inactive - negative across all of the assays

- Recipients received chemicals from NTP, testing began
  - Unknown to most of the participants, the "predictive" models changed MANY times between 2011 and 2015
  - Study became unpublishable as originally conceived

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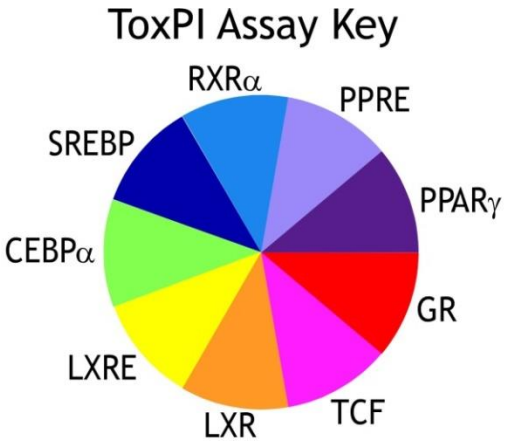
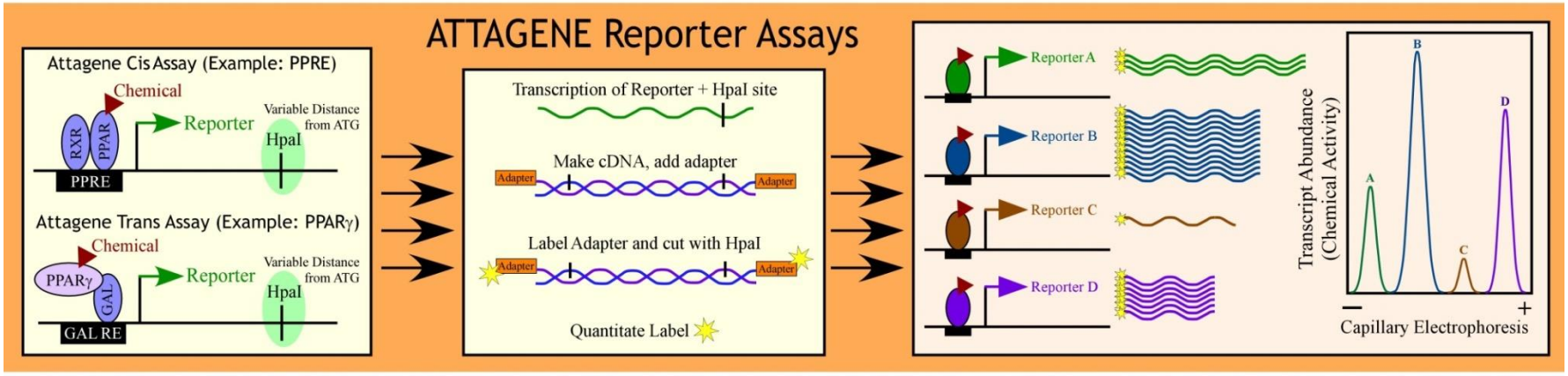
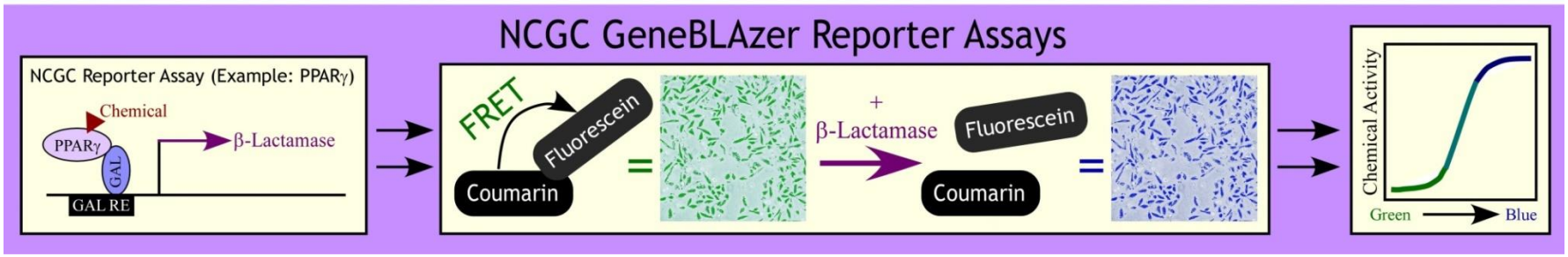
ToxPI for Adipogenesis



PPARγ -- ATTAGENE Trans & NCGC Reporter Assays (Gal-PPARγ), NovaScreen Direct Binding
PPRE -- ATTAGENE Cis Assay for PPARα, PPARδ, and PPARγ (Direct Repeat 1 Element)
RXRα -- ATTAGENE Trans & NCGC Reporter Assays for Gal-Retinoid-X-Receptor
GR -- ATTAGENE Trans & NCGC Reporter Assays (Gal-GR), NovaScreen Direct Binding
TCF -- ATTAGENE Cis Assay for TCF-1/β-Catenin Family/Wnt Pathway
LXR -- ATTAGENE Trans & NCGC Reporter Assays (Gal-LXR), NovaScreen Direct Binding
LXRE -- ATTAGENE Cis Assay for Liver X Receptor (Direct Repeat 4 Element)
CEBPα -- ATTAGENE Cis Assay for CCAAT-Enhancer Binding Element
SREBP -- ATTAGENE Cis Assay for Sterol Regulatory Element Binding





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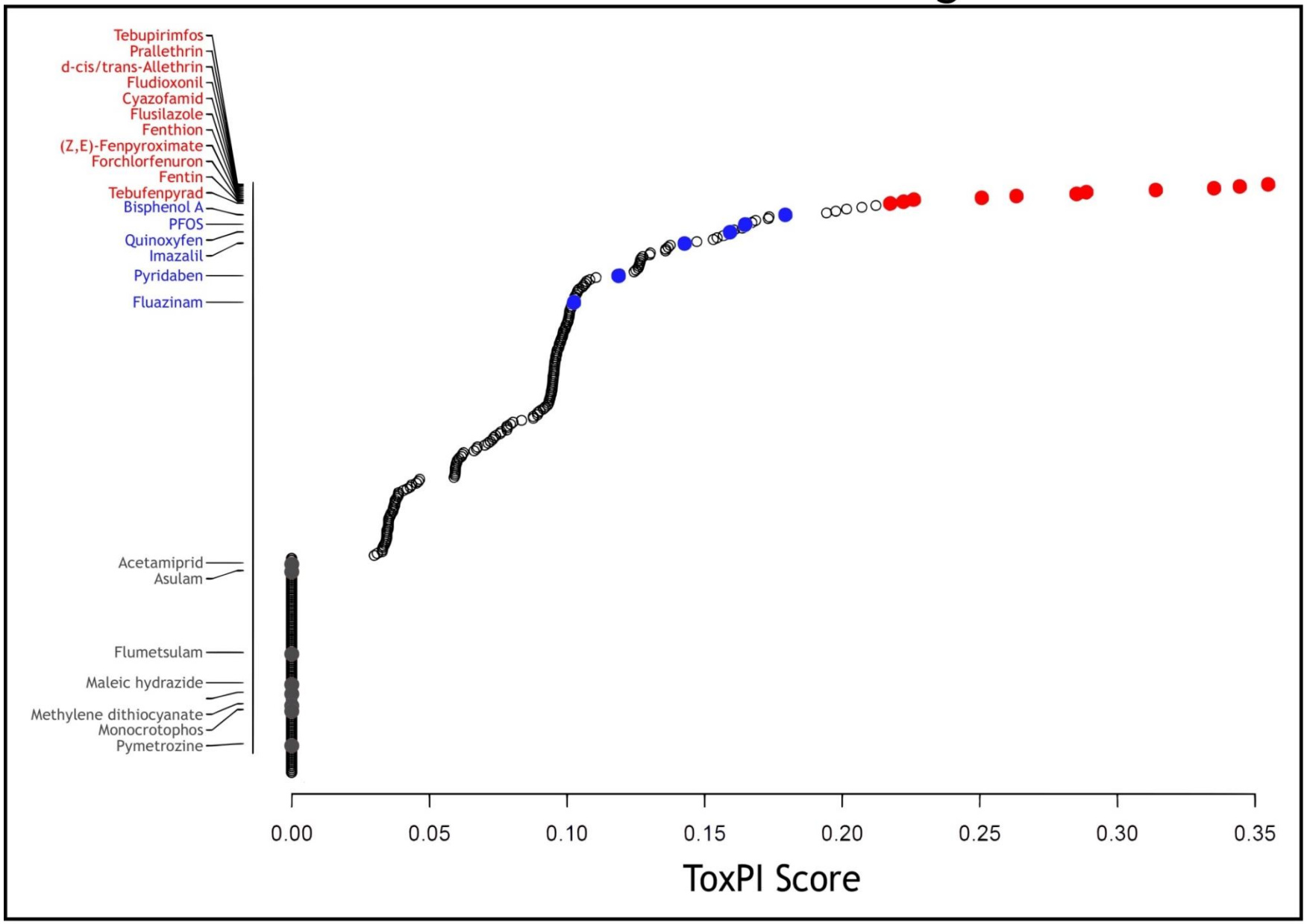
ToxCast Phase I Chemicals Ranked According to ToxPI Scores



- Sample chemicals provided by NTP to 7 laboratories for testing

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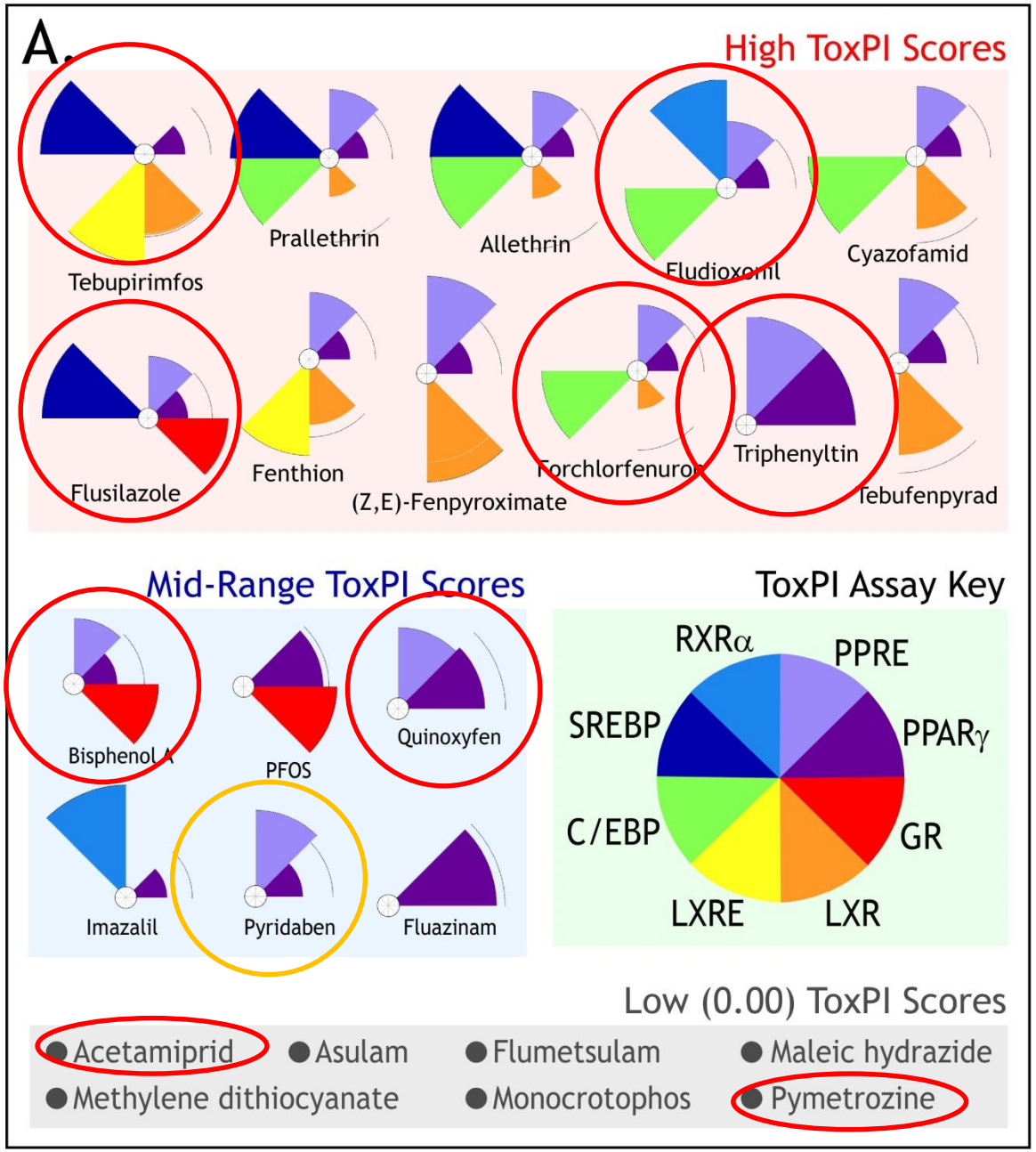
Adipogenesis ToxPI chemicals



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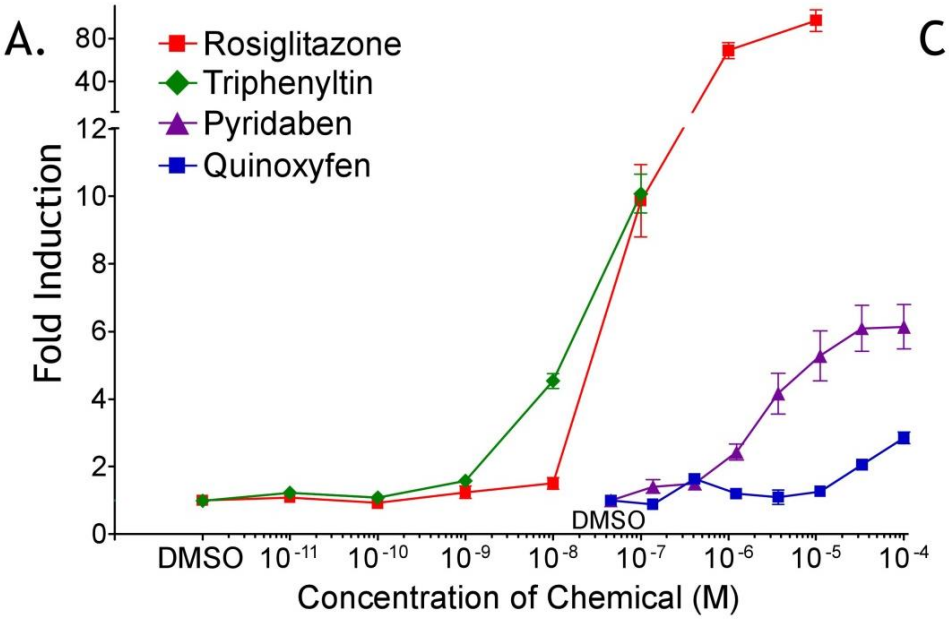
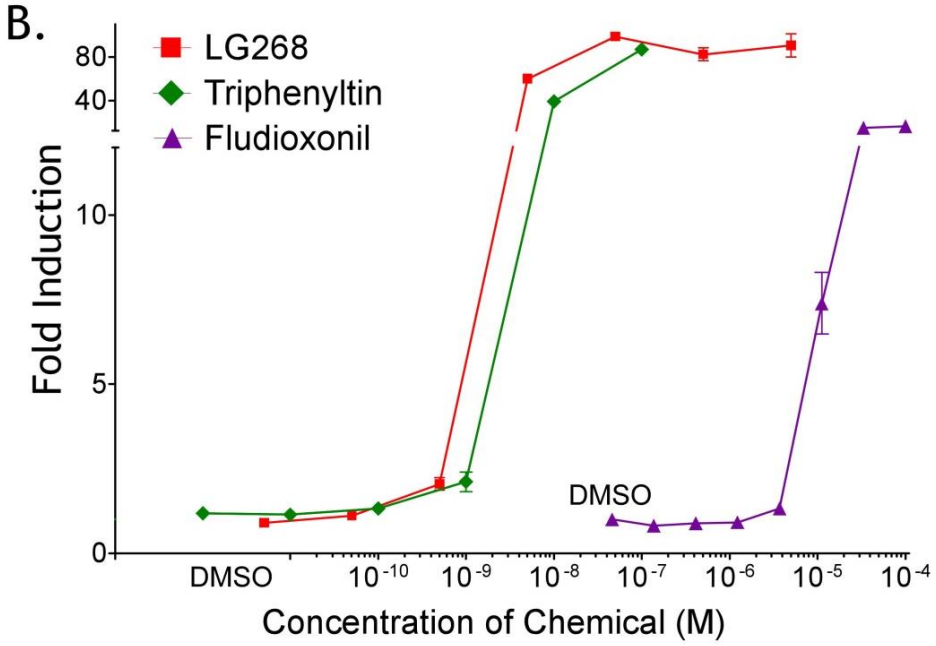
Activity of ToxPI chemicals on PPARγ and RXRα





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PPARγ Agonists			
Chemical	**Pyridaben**		
Assay	Fig 4A	ATG	NCGC
EC50	2.98 μM	10.08 μM	NA
EC10	0.53 μM	2.57 μM	NA
Chemical	**Quinoxyfen**		
Assay	Fig 4A	ATG	NCGC
EC50	33.4 μM	5.12 μM	40.55 μM
EC10	14.24 μM	3.98 μM	2.44 μM

RXRα Agonist			
Chemical	**Fludioxonil**		
Assay	Fig 4B	ATG	NCGC
EC50	14.3 μM	23.59 μM	2.24 μM
EC10	5.42 μM	17.93 μM	1.20 μM

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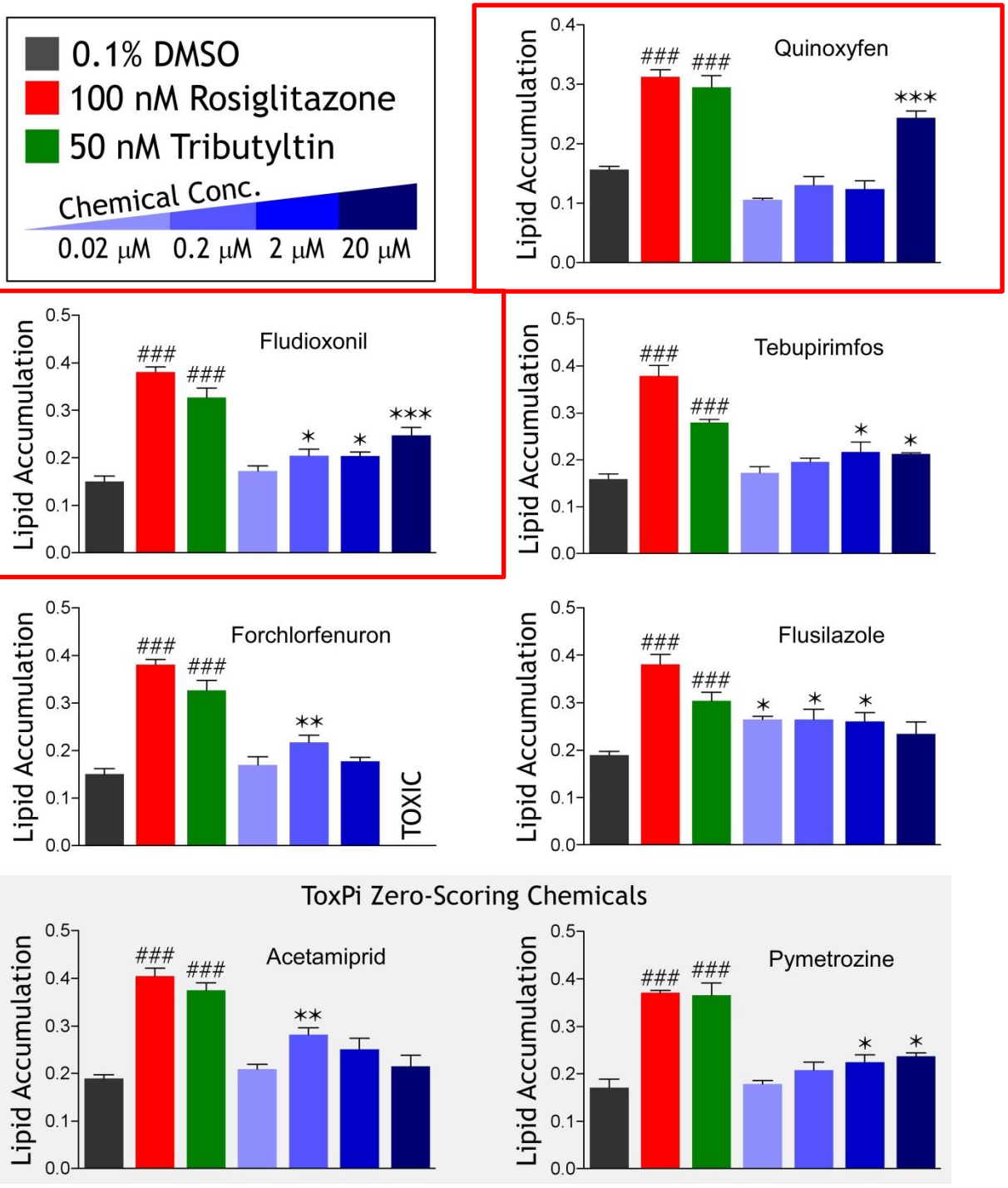

3T3L1

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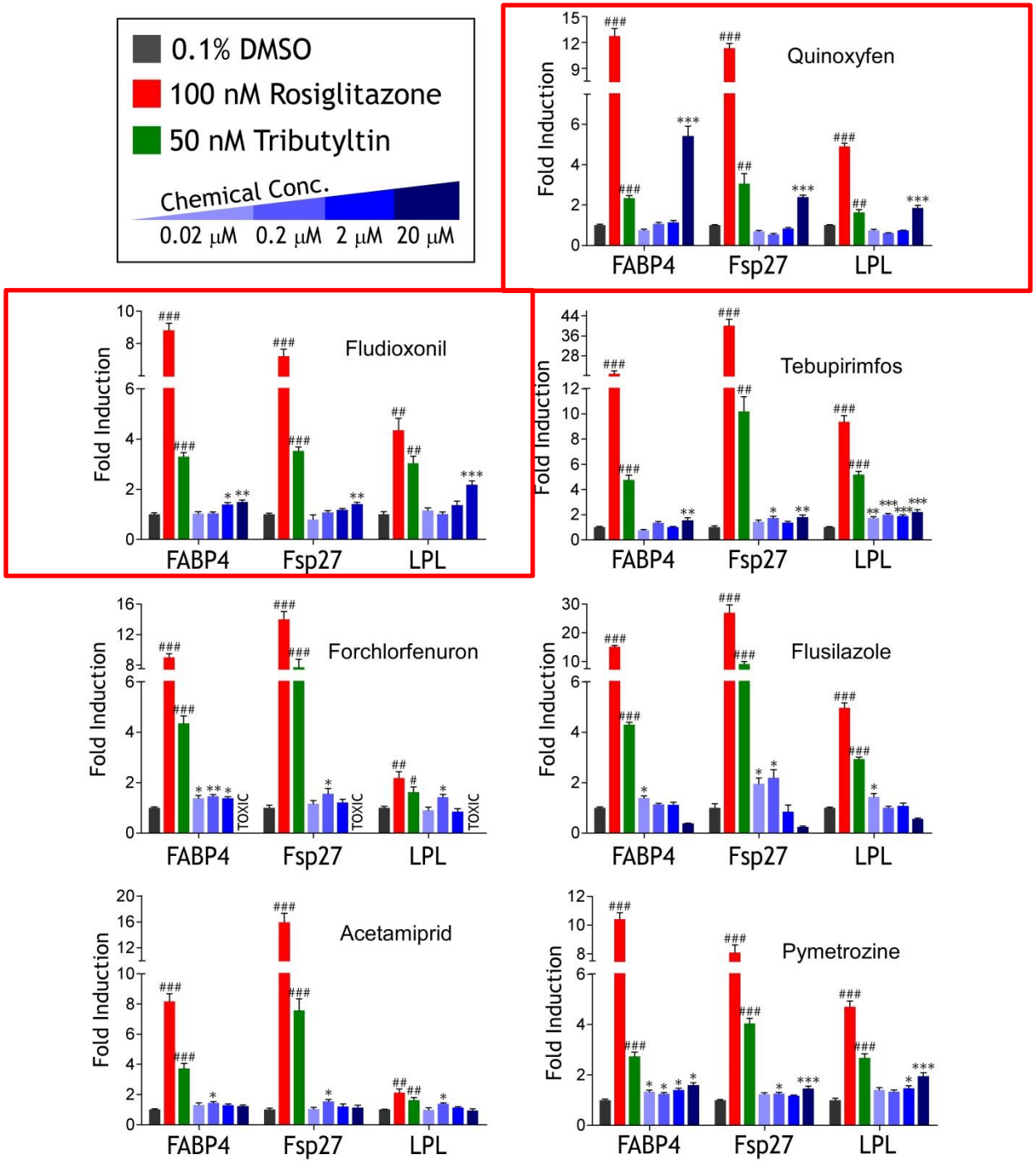

3T3L1

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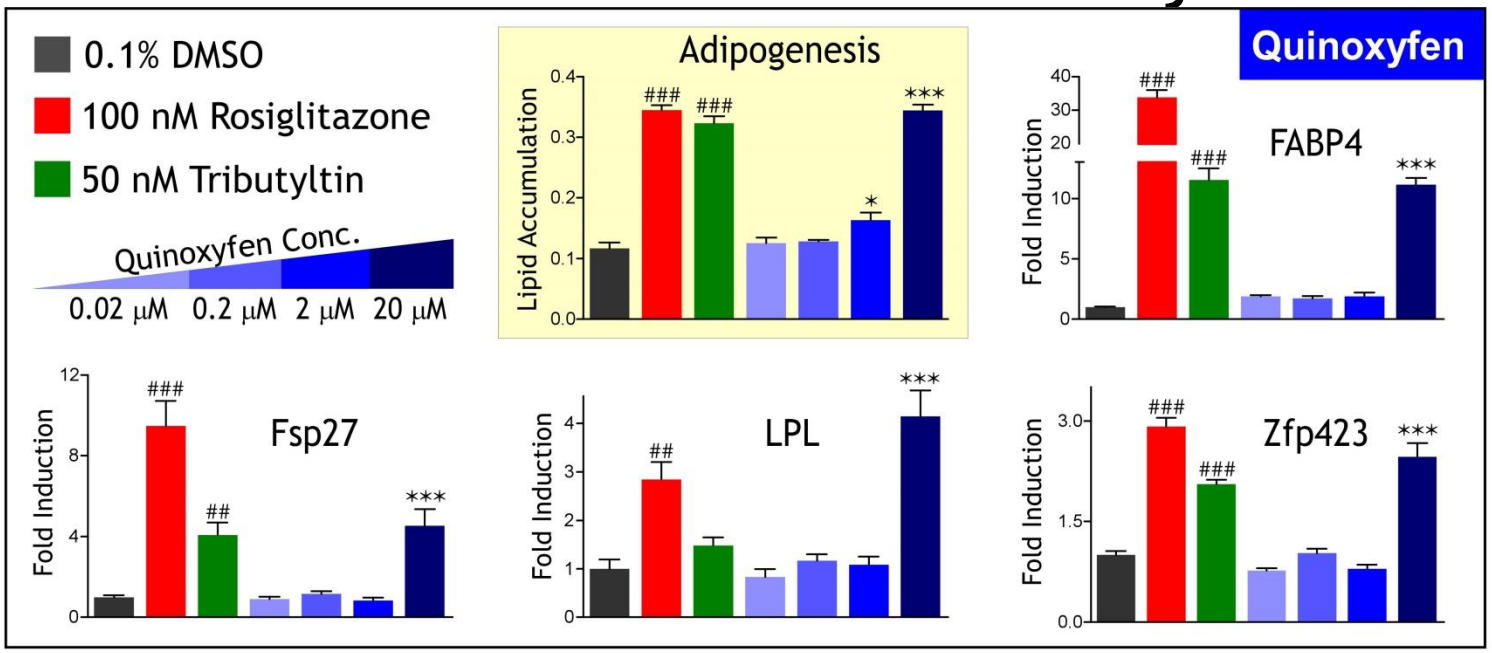
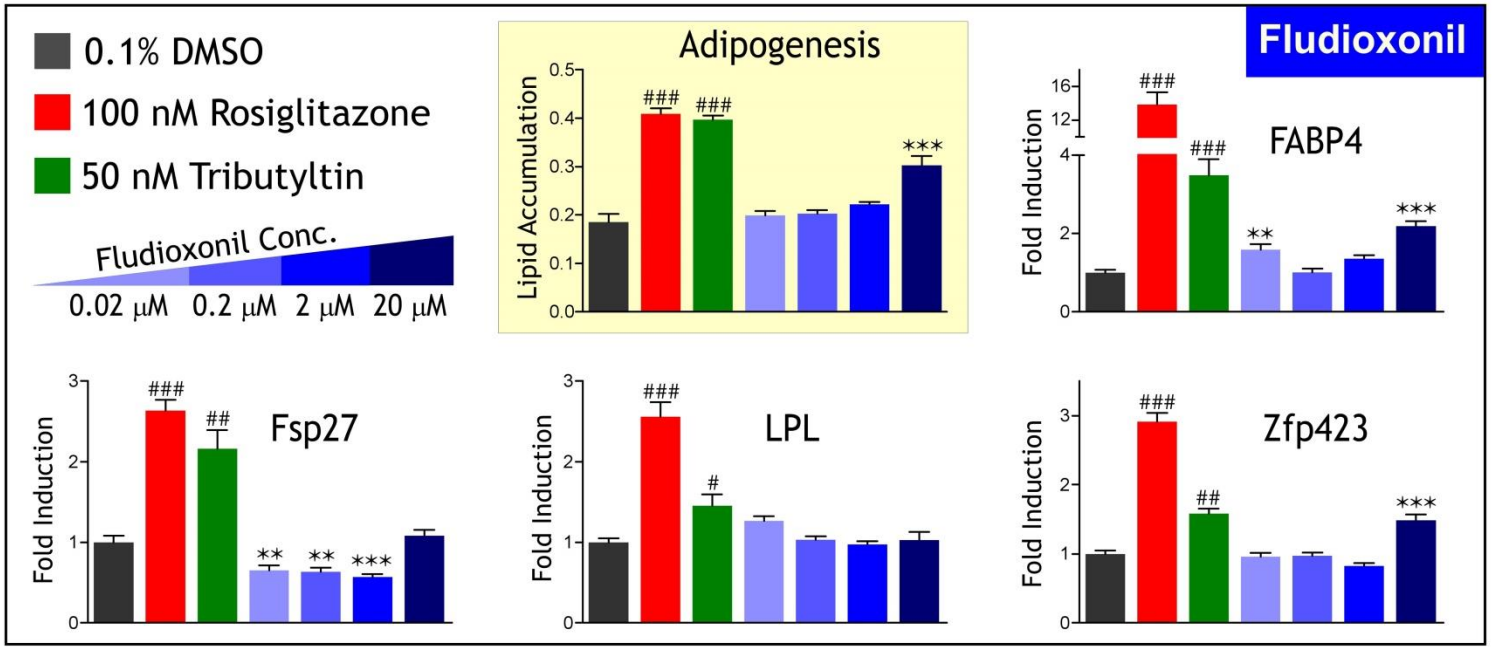
MSC Differentiation Assay





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Summary of our ToxPI Testing Results

TOXPI	Prioritization	Adipogenesis	Activation		PPARγ Activation Assays AC50 Values, 2011 Release			PPARγ Activation Assays AC50 Values, 2014 Release			
Chemical Name	ToxPI Score	3T3-L1	COS7		ATG Agonist	NVS Agonist	NCGC Agonist	ATG Agonist	NVS Agonist	NCGC Agonist	NCGC Antag.
Tebupirimfos	HIGH	Positive	Inactive		43	NA	NA	36.38	NA	NA	NA
Prallethrin	HIGH	Negative	Inactive		44	NA	NA	27.33	NA	NA	NA
d-cis/trans Allethrin	HIGH	Negative	Inactive		36	NA	NA	11.93	NA	NA	61.16
Fludioxonil	HIGH	Positive	RXRα Activator	14.3	28	NA	NA	19.28	NA	NA	27.43
Cyazofamid	HIGH	Negative	Inactive		11	NA	NA	10.12	9.57	NA	32.34
Flusilazole	HIGH	Positive	Inactive		51	NA	NA	46.40	NA	NA	71.04
Fenthion	HIGH	Negative	Inactive		36	NA	NA	39.44	NA	NA	NA
Fenpyroximate (Z,E)	HIGH	Negative	Inactive		8.6	NA	NA	2.40	NA	NA	35.93
Forchlorfenuron	HIGH	Positive	Inactive		46	NA	NA	48.15	NA	NA	23.19
Triphenyltin	HIGH	Positive	PPARγ Activator	0.02	0.02	NA	NA	0.02	0.28	21.64	0.02
Tebufenpyrad	HIGH	Negative	Inactive		4.9	NA	NA	2.64	NA	NA	23.05
Bisphenol A	MEDIUM	Positive	Inactive		27	NA	NA	NA	NA	NA	2.40
PFOS	MEDIUM	Negative	Inactive		26	16	NA	26.67	5.94	NA	252.98
Quinoxyfen	MEDIUM	Positive	PPARγ Activator	33.4	6.6	NA	6.7	5.12	NA	40.55	NA
Imazalil	MEDIUM	Negative	Inactive		73	NA	NA	59.91	NA	NA	NA
Pyridaben	MEDIUM	Inhibitor	PPARγ Activator	2.98	14	NA	NA	10.08	NA	NA	23.37
Fluazinam	MEDIUM	Negative	PPARγ Antagonist	7.2	0.51	0.28	NA	1.21	0.26	0.07	1.27
Methylene dithiocyanate	NEGATIVE	Negative	Inactive		NA	NA	NA	NA	NA	104.14	25.67
Maleic hydrazide	NEGATIVE	Negative	Inactive		NA	NA	NA	NA	NA	NA	NA

- 7/17 (41%) predicted actives are active in 3T3-L1 adipogenesis assays
- 2/17 (8.5%) predicted actives were active in MSC adipogenesis assays
- 1/17 predicted active (and a PPARγ activator) inhibited adipogenesis
- 2/7 chemicals predicted negative in all assays induced adipogenesis

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Prioritizing Environmental Chemicals for Obesity and Diabetes Outcomes Research: A Screening Approach Using ToxCast™ High-Throughput Data

Scott Auerbach,[1] Dayne Filer,[2] David Reif,[3] Vickie Walker,[1] Alison C. Holloway,[4] Jennifer Schlezinger,[5] Supriya Srinivasan,[6] Daniel Svoboda,[7] Richard Judson,[2] John R. Bucher,[1] and Kristina A. Thayer[1]

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Auerbach vs. Janesick Comparison

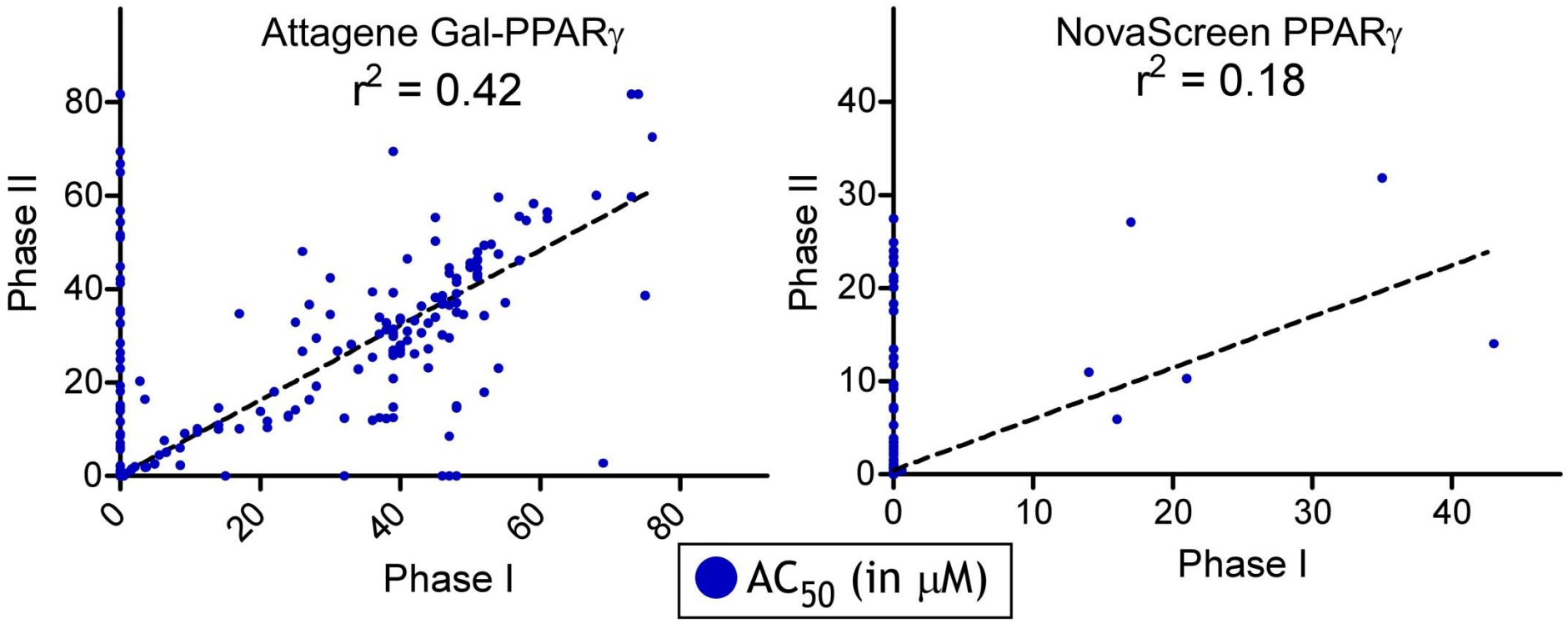
- 1890 ToxCast Phase II chemicals vs 309 Phase I chemicals used to generate our list
- EPA ranked chemicals from 1-810 based on a compilation of activity measurements, using their "latest and greatest" model
- #810 comprised 1050 chemicals predicted to have no activity

- Prioritized 30 chemicals for each assay
  - none of our tested actives appeared on prioritized list
  - Only 3 chemicals on both lists – all tested inactive
  - Some obvious actives on prioritized list
    - Tetrabutyltin, tributyltin benzote, farglitazar,
  - Obvious inactives as well
    Acrylamide, aspirin, 3 inactives, etc
  - No testing was performed due to so many changes in model and exhaustion of budget by participants.

- Our tested actives ranked 48, 71 (TPT, the most potent of all tested), 96, 150, 223, 290, 444, 490, 525, 663 and 2 scored 810 (zero)

- What is source of problem? Experience suggests it is the assays….

ToxCast Phase I vs. Phase 2 on same endpoints



- ToxCast assays for the same chemical, in the same labs are not very reproducible
- Why?

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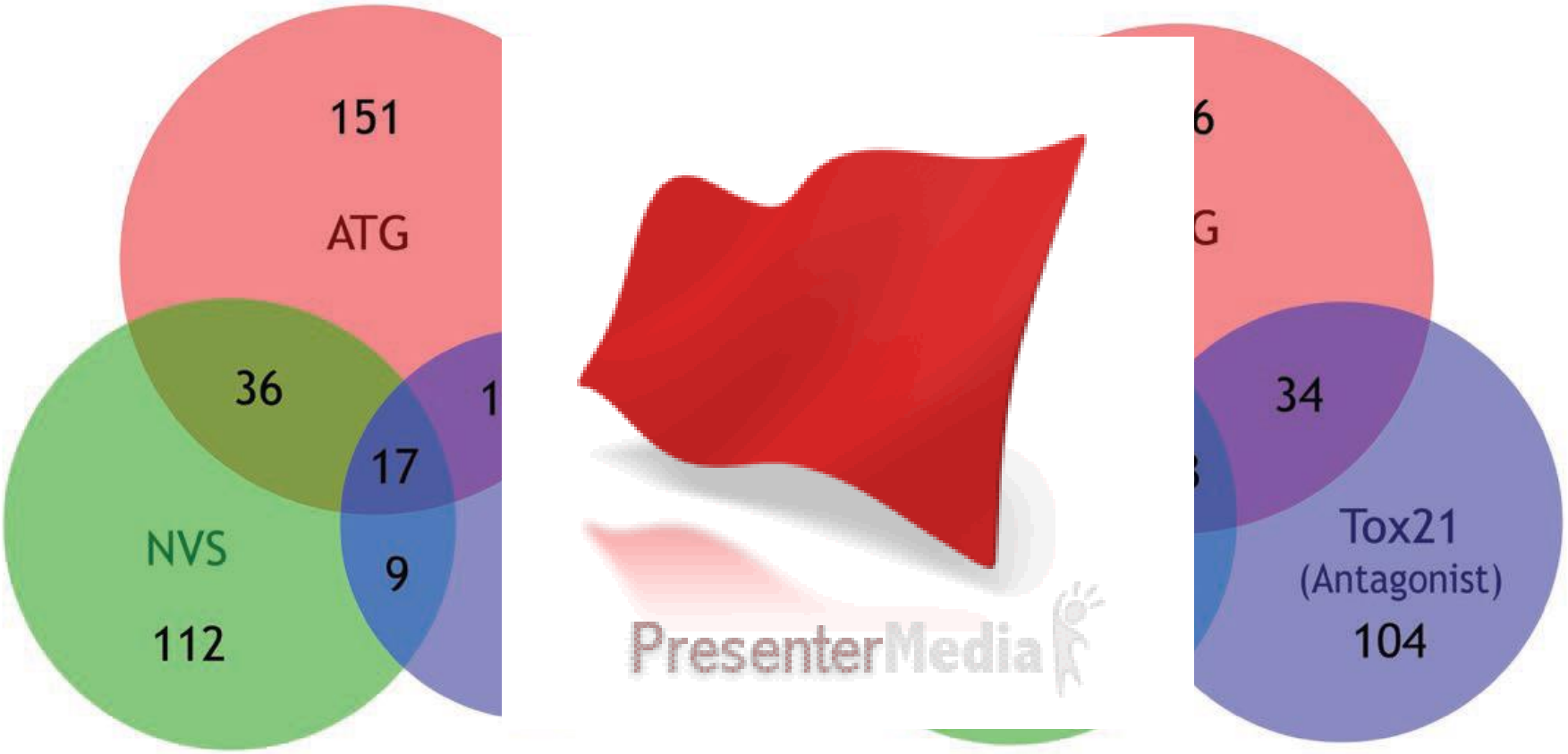
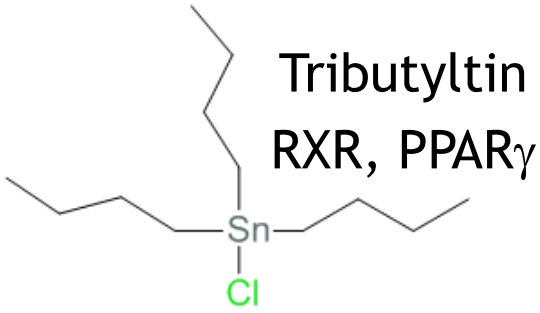
Unreproducible results of PPARγ assays

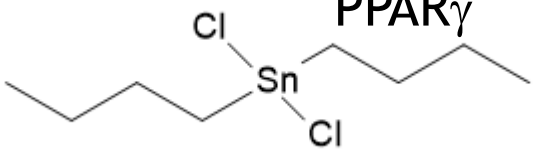


- ToxCast PPARγ assays are not congruous
  - Ligand binding (NVS) and agonist or antagonist assays should be highly congruent
- Why do ATG activation assays overlap more with Tox21 antagonist 52/309 vs agonist 34/309 assays?

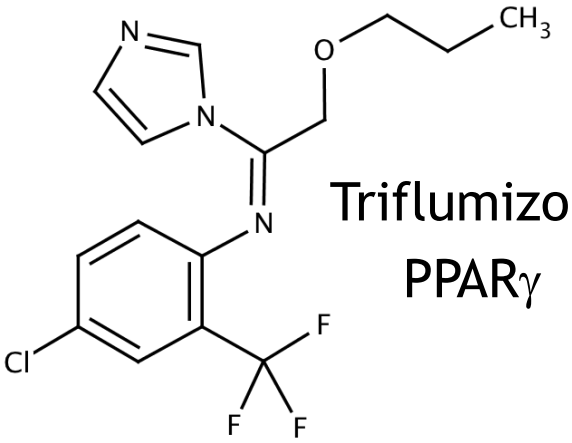
Many Fungicides May Be Obesogens



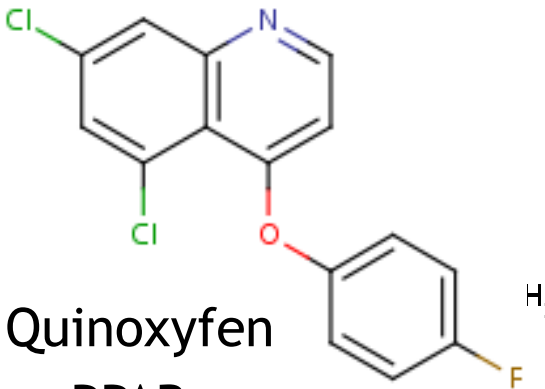
Tributyltin
RXR, PPARγ



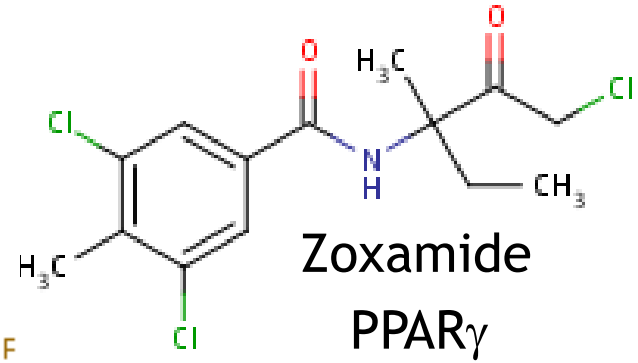
Dibutyltin
PPARγ



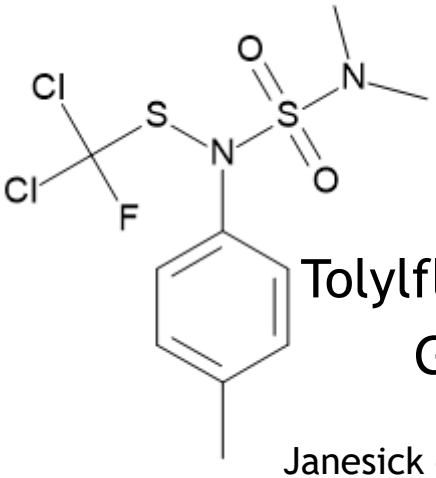
Triflumizole
PPARγ



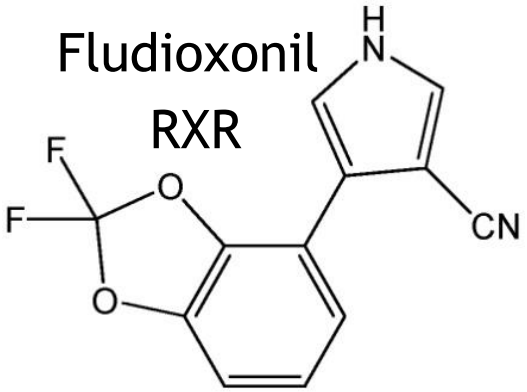
Quinoxyfen
PPARγ



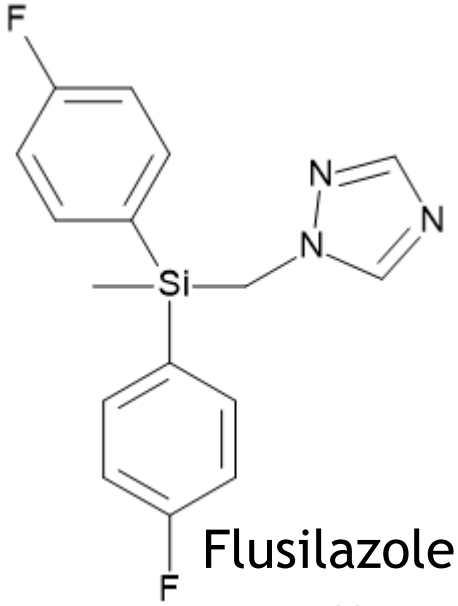
Zoxamide
PPARγ



Tolylfluanid
GR



Fludioxonil
RXR



Flusilazole
??

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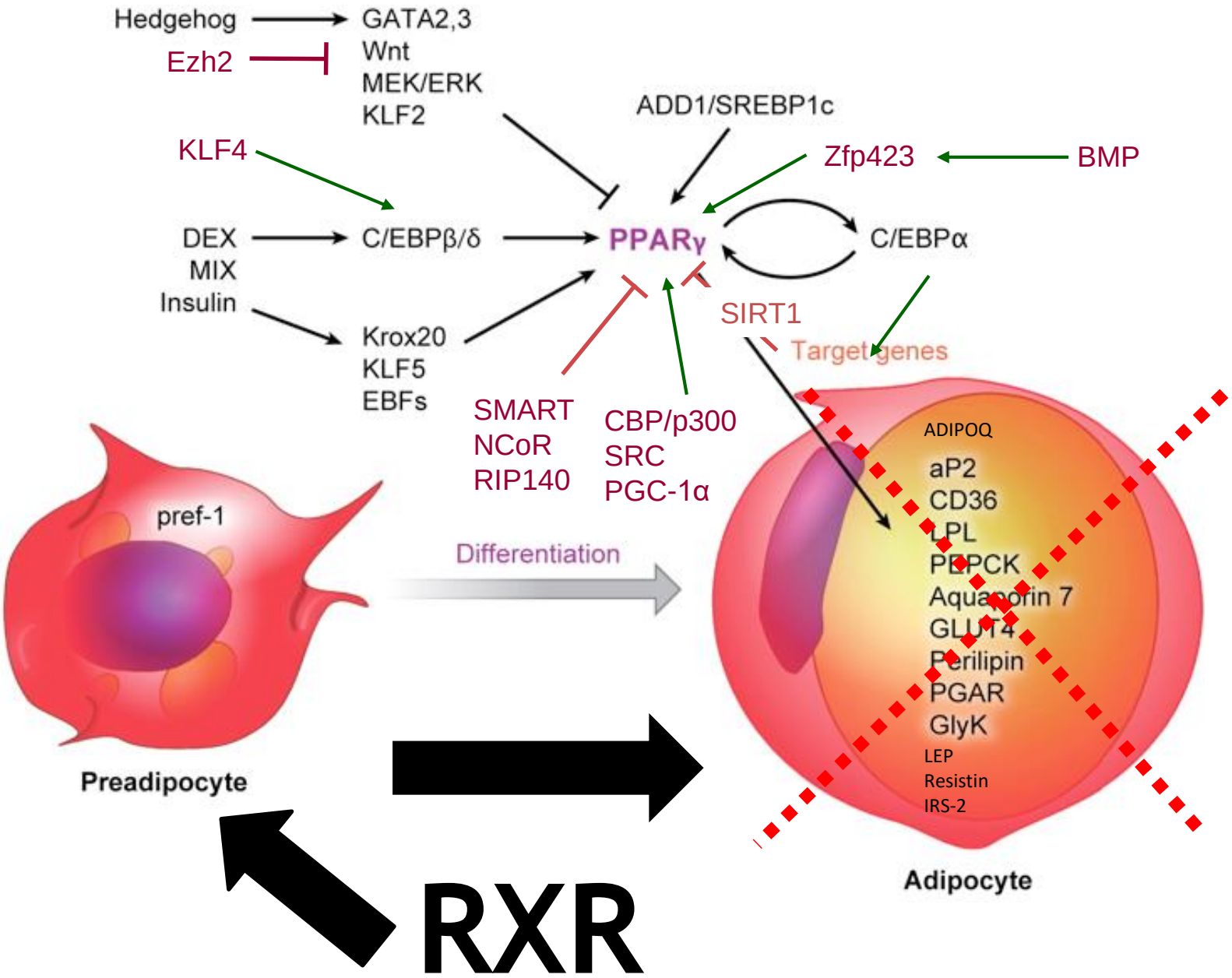
Research article

Screening ToxCast Prioritized Chemicals for *PPARG* Function in a Human Adipose-Derived Stem Cell Model of Adipogenesis

Briana Foley,[*] Daniel L. Doheny,[*,†] Michael B. Black,[*,†] Salil N. Pendse,[*,†] Barbara A. Wetmore,[*,†] Rebecca A. Clewell,[*,†] Melvin E. Andersen,[*,†] and Chad Deisenroth[*,†,1]

- Tested 60 prioritized ToxCast PPARg chemicals,
  - 49 agonists, 11 antagonists
  - Evaluated 7 different PPARγ dependent endpoints
- 14/49 (29%) prioritized PPARγ actives were moderately to strongly adipogenic

Are All Obesogens PPARγ regulators?



Summary

- Screening for PPARγ and RXR transactivation appears to identify a good number of bona fide or potential obesogens
  - But ToxCast HTS assay results less reliable than expected
  - Many obesogens activate neither receptor
- Combination of receptor activation and adipogenesis assays is a good predictor of activity in vivo (n=4)
- A fair number of obesogens remain to be identified
- Toxcast assays for RXR and PPARγ activity are highly suspect
  - Attagene often gives 5x more positives than other assays
  - Why do HTS assays work so poorly as predictors of PPAR, RXR activity and adipogenesis? Tox21, too….
- Main problem is uncritical reliance on HTS assays with post facto adjustment of models
- "21st Century Toxicology" has problems that need to be addressed to maximize utility of the approach

One Possible Solution

- EU issued Horizon 2020 call for grants that will generate robust, reproducible, validated and standardized assays for EDC testing that can become OECD guideline assays
- 8 consortia likely to be funded, almost all comprised of academic experts in particular fields
  - GOLIATH *Beating Goliath: Generation of novel, integrated and internationally harmonised approaches for testing metabolism disrupting compounds*
  - FREIA *Female reproductive toxicity of EDCs: a human evidence-based screening and identification approach*
  - ATHENA *Assays for the identification of thyroid hormone axis-disrupting chemicals: elaborating novel assessment* strategies
  - OBERON *An integrative strategy of testing systems for identification of EDs related to metabolic disorders*
  - ENDpoiNTs *Novel testing strategies for endocrine disruptors in the context of developmental neurotoxicity*
  - ERGO *Breaking down the wall between human health and environmental testing of endocrine disrupters: endocrine guideline optimisation*
  - SCREENED *A multistage model of thyroid gland function for screening endocrine-disrupting chemicals in a biologically sex-specific manner*
  - EDCMET *Metabolic effects of endocrine disrupting chemicals: novel testing methods and adverse outcome pathways*