Targeted high throughput screening for nuclear receptor activation, cell stress, and immunosuppressive bioactivities with 147 perfluoroalkyl substances

Presentation to FLUOROS

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Outline of this talk

Concept	Publication(s)	DOI
Chemical library of PFAS screened	Patlewicz et al. 2019	10.1289/EHP4555
Tiered in vitro screening strategy	Thomas et al. 2019 (general)	10.1093/toxsci/kfz058
	EPA's PFAS Action Plan	https://www.epa.gov/pfas/epas-pfas-action-plan
Targeted screening for nuclear receptor activation and cell stress	Houck et al. (2021)	10.1016/j.tox.2021.152789
Targeted screening for immunosuppressive bioactivity signature	Houck et al. (in prep)	Short preview today of work in progress

Chemical library of PFAS screened

How to select PFAS for tiered screening?

Brief Communication

A Section 508-conformant HTML version of this article is available at https://doi.org/10.1289/EHP4555.

A Chemical Category-Based Prioritization Approach for Selecting 75 Per- and Polyfluoroalkyl Substances (PFAS) for Tiered Toxicity and Toxicokinetic Testing

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Summary: Per- and polyfluoroalkyl substances (PFASs) are a group of fluorinated substances of interest to researchers, regulators, and the public due to their widespread presence in the environment. A few PFASs have comparatively extensive amounts of human epidemiological, exposure, and experimental animal toxicity data (e.g., perfluorooctanoic acid), whereas little toxicity and exposure information exists for much of the broader set of PFASs. Given that traditional approaches to generate toxicity information are resource intensive, new approach methods, including *in vitro* high-throughput toxicity (HTT) testing, are being employed to inform PFAS hazard characterization and further (*in vivo*) testing. The U.S. Environmental Protection Agency (EPA) and the National Toxicology Program (NTP) are collaborating to develop a risk-based approach for conducting PFAS toxicity testing to facilitate PFAS human health assessments. This article describes the construction of a PFAS screening library and the process by which a targeted subset of 75 PFASs were selected. Multiple factors were considered, including interest to the U.S. EPA, compounds within targeted categories, structural diversity, exposure considerations, procurability and testability, and availability of existing toxicity data. Generating targeted HTT data for PFASs represents a new frontier for informing priority setting. https://doi.org/10.1289/EHP4555

Introduction

Per- and polyfluoroalkyl substances (PFASs) are a group of fluorinated substances that have generated increased public attention due to their potential health hazard and widespread presence in the environment (Wang et al. 2017; Xiao 2017; Ross et al. 2018). The U.S. Environmental Protection Agency (EPA) Office of Research and Development (ORD) in partnership with the National Toxicology Program (NTP) are currently engaged in producing toxicity information to facilitate human health assessments for PFASs. A few PFASs have comparatively extensive amounts of toxicity data (e.g., perfluorooctanoic acid), but little toxicity information exists for much of the broader set of PFASs identified from preliminary exposure studies that capture potential occurrence in the environment. The hundreds of untested PFASs provide a scenario in which traditional one-by-one toxicity testing would consume tremendous resources and useful toxicity information would not be available for decades. The U.S. EPA's ToxCast program and the multi-federal agency Tox21 program (which includes the NTP and the U.S. EPA as major partners) have developed the capacity to screen hundreds to thousands of chemicals for bioactivity through *in vitro* high-throughput toxicity (HTT) testing. Data generated from these assays are already being used to inform hazard identification and prioritize chemicals for further *in vivo* testing (U.S. EPA 2012, 2014a, 2014b, 2015; Judson et al. 2010, 2015; Kleinstreuer et al. 2017). Within the U.S. EPA, generating such data to inform agency and partner decision making regarding potential human health hazard and risk across the broad landscape of PFASs represents a real-world challenge that HTT coupled with cheminformatic approaches is uniquely designed to address.

This article describes, in brief, the development of the PFAS screening library and the process by which a subset of 75 PFAS substances were selected for HTT screening and tiered toxicity testing, along with mention of the toxicity and toxicokinetic experiments currently underway.

Discussion

Development of the PFAS Screening Library

Since there are no specific chemical catalogs for PFASs, an initial scoping for potentially procurable PFAS substances relied on the use of candidate PFAS structure lists generated from the U.S. EPA's Distributed Structure-Searchable Toxicity (DSSTox) chemical database. DSSTox currently exceeds 760,000 substances, each of which has undergone some level of chemical structure curation prior to registration (Williams et al. 2017). The largest registered list of PFAS chemicals available at the time this study was initiated was the KEMI PFAS list in DSSTox (named PFASKEMI and available for download at https://comptox.epa.gov/dashboard/chemical_lists/pfaskemi). Approximately 1,200 structures from this list were provided to the chemical contractor for scoping purposes, from which approximately 600 substances were identified as potentially procurable but likely to require on-demand synthesis and exceed standard costs. Based on this preliminary scoping, U.S. EPA funds were secured for the purchase and processing of approximately 400 substances to create a PFAS testing library.

The first procurement phase considered the feasibility of procuring substances of interest to the U.S. EPA. A U.S. EPA workgroup was formed to identify PFASs of interest to U.S. EPA programs and regions and to include PFASs with associated toxicity data that would inform human health risk assessment. The final set of 31 PFASs recommended for further study by this workgroup (list denoted as EPA PFAS WG 31) identified PFASs whose review may support risk evaluation. Also included in the

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Patlewicz et al. 2019

- A few PFAS (e.g., PFOA, PFOS) have extensive information whereas many PFAS have little to no information.
- Select the original 75, extended to select ~150 for screening activities.

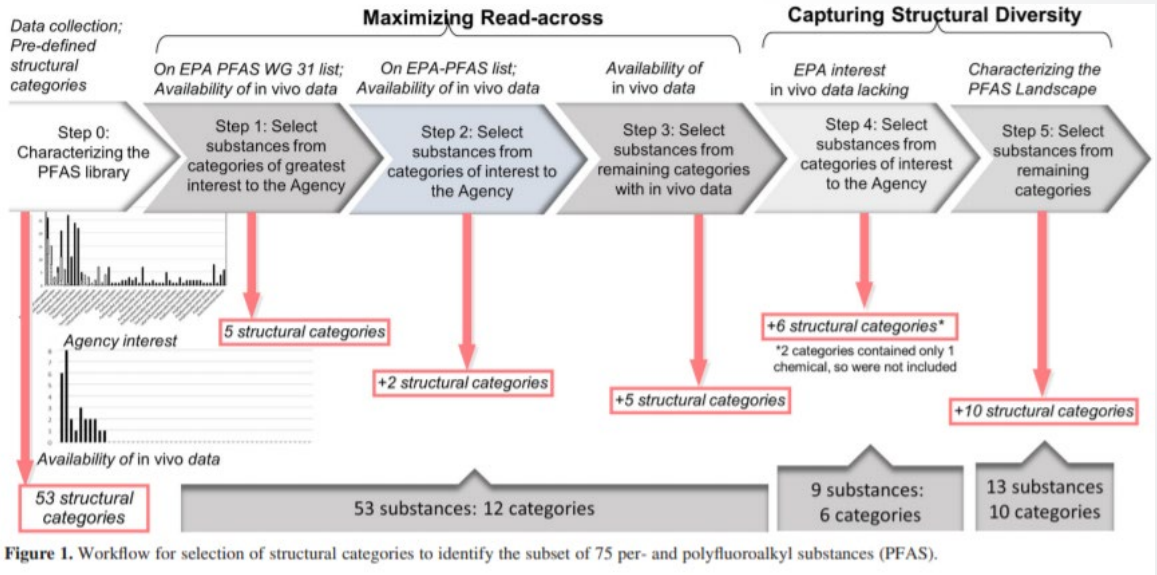
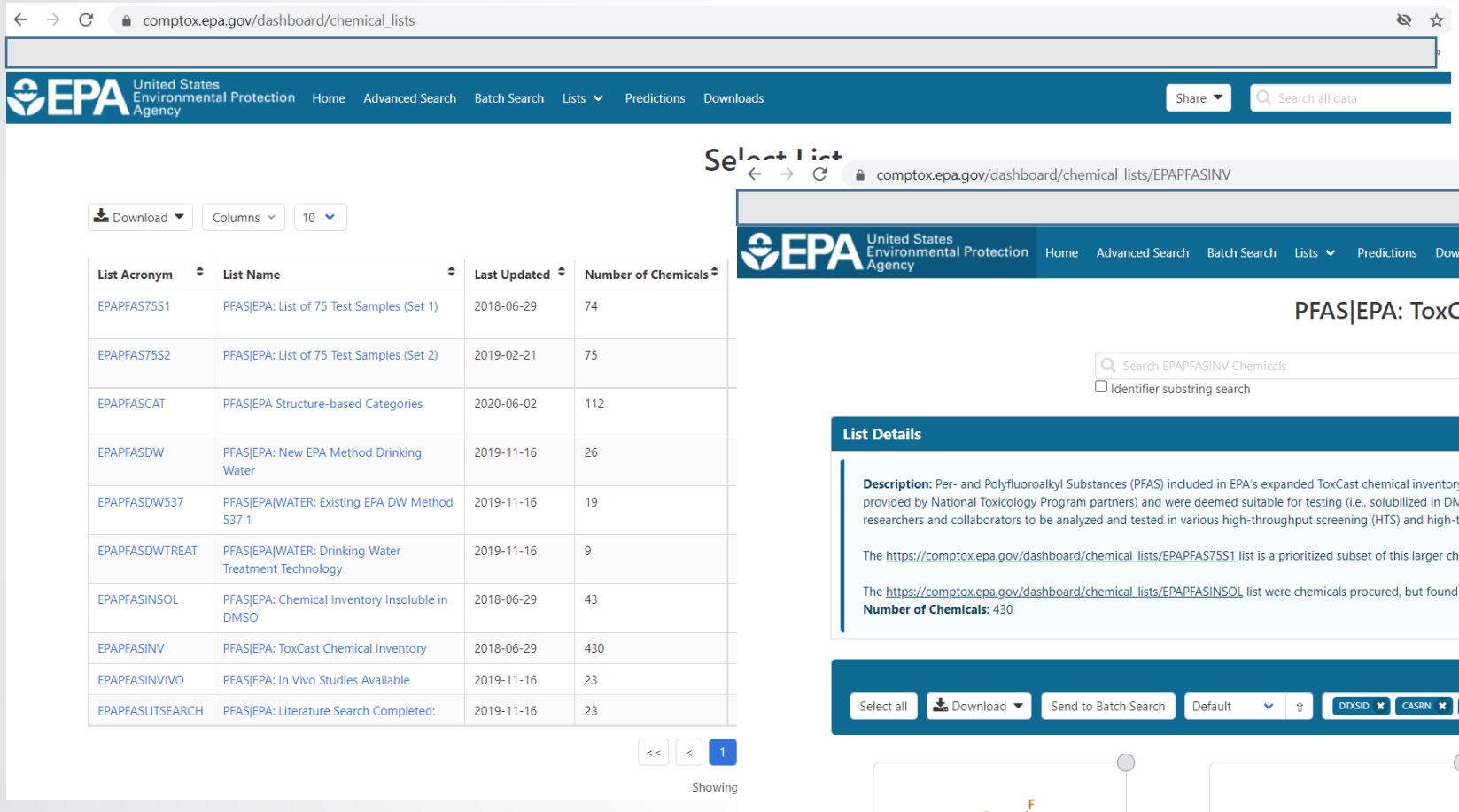


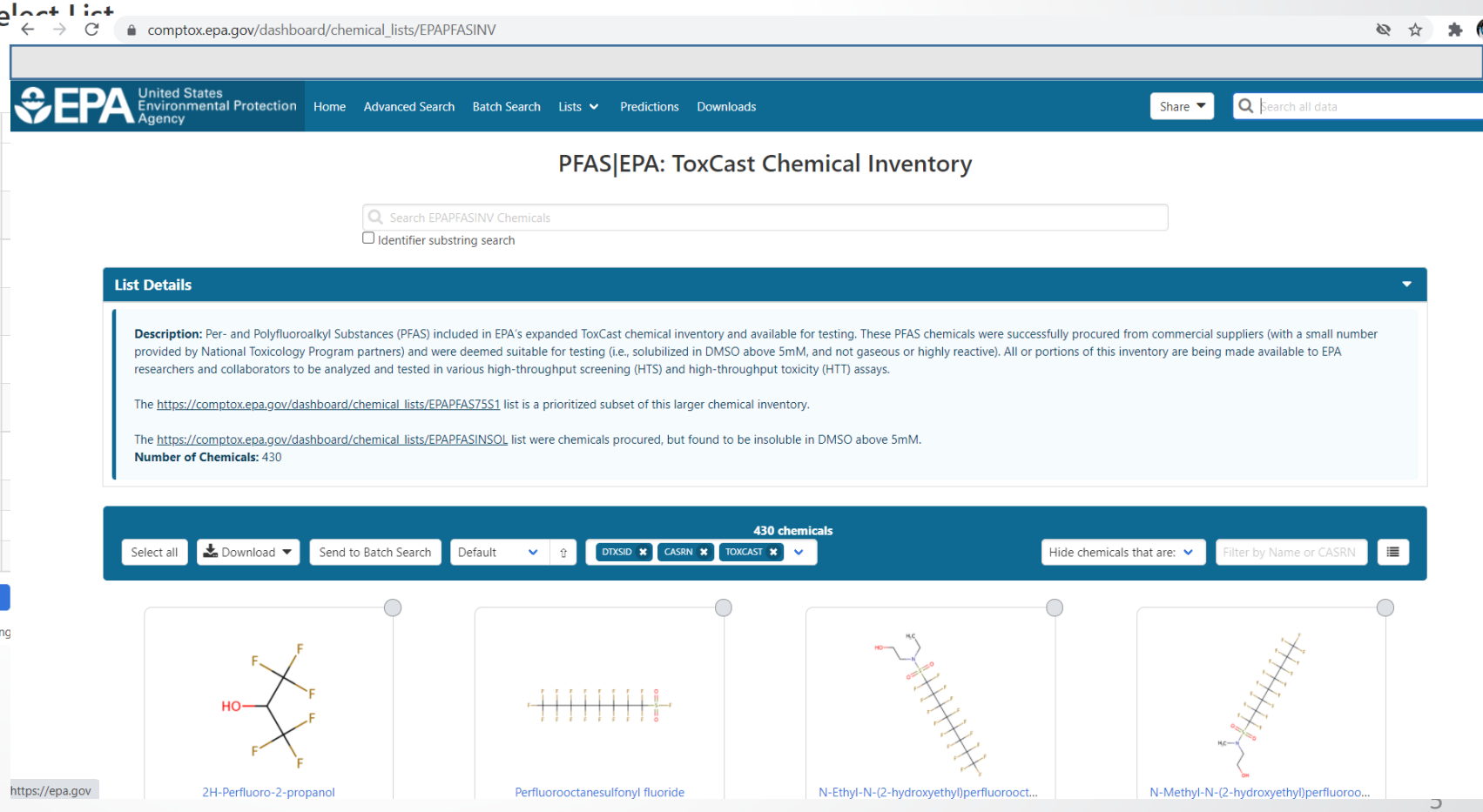
Figure 1. Workflow for selection of structural categories to identify the subset of 75 per- and polyfluoroalkyl substances (PFAS).

https://comptox.epa.gov/dashboard/chemical_lists/?search=PFAS

You can search our lists of chemicals on our public CompTox Chemicals Dashboard, and from there link to data resources

https://comptox.epa.gov/dashboard/chemical_lists





Tiered *in vitro* screening strategy

Executing the Next Generation CompTox Blueprint to inform putative chemical hazard

OXFORD SOT Society of Toxicology
www.toxsci.oxfordjournals.org

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doi: 10.1093/toxsci/kfz058
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Forum

FORUM

The Next Generation Blueprint of Computational Toxicology at the U.S. Environmental Protection Agency

Russell S. Thomas,*,1 Tina Bahadori,† Timothy J. Buckley,‡ John Cowden,* Chad Deisenroth,* Kathie L. Dionisio,‡ Jeffrey B. Frithsen,§ Christopher M. Grulke,* Maureen R. Gwinn,* Joshua A. Harrill,* Mark Higuchi,¶ Keith A. Houck,* Michael F. Hughes,¶ E. Sidney Hunter, III,¶ Kristin K. Isaacs,‡ Richard S. Judson,* Thomas B. Knudsen,* Jason C. Lambert,‖ Monica Linnenbrink,* Todd M. Martin,‖ Seth R. Newton,‡ Stephanie Padilla,¶ Grace Patlewicz,* Katie Paul-Friedman,* Katherine A. Phillips,‡ Ann M. Richard,* Reeder Sams,* Timothy J. Shafer,¶ R. Woodrow Setzer,* Imran Shah,* Jane E. Simmons,¶ Steven O. Simmons,* Amar Singh,* Jon R. Sobus,‡ Mark Strynar,‡ Adam Swank,‡ Rogelio Tornero-Valez,‡ Elin M. Ulrich,‡ Daniel L. Villeneuve,‖ John F. Wambaugh,* Barbara A. Wetmore,‡ and Antony J. Williams*

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High-throughput transcriptomics and high-throughput phenotypic profiling

High-throughput targeted screening (ToxCast)

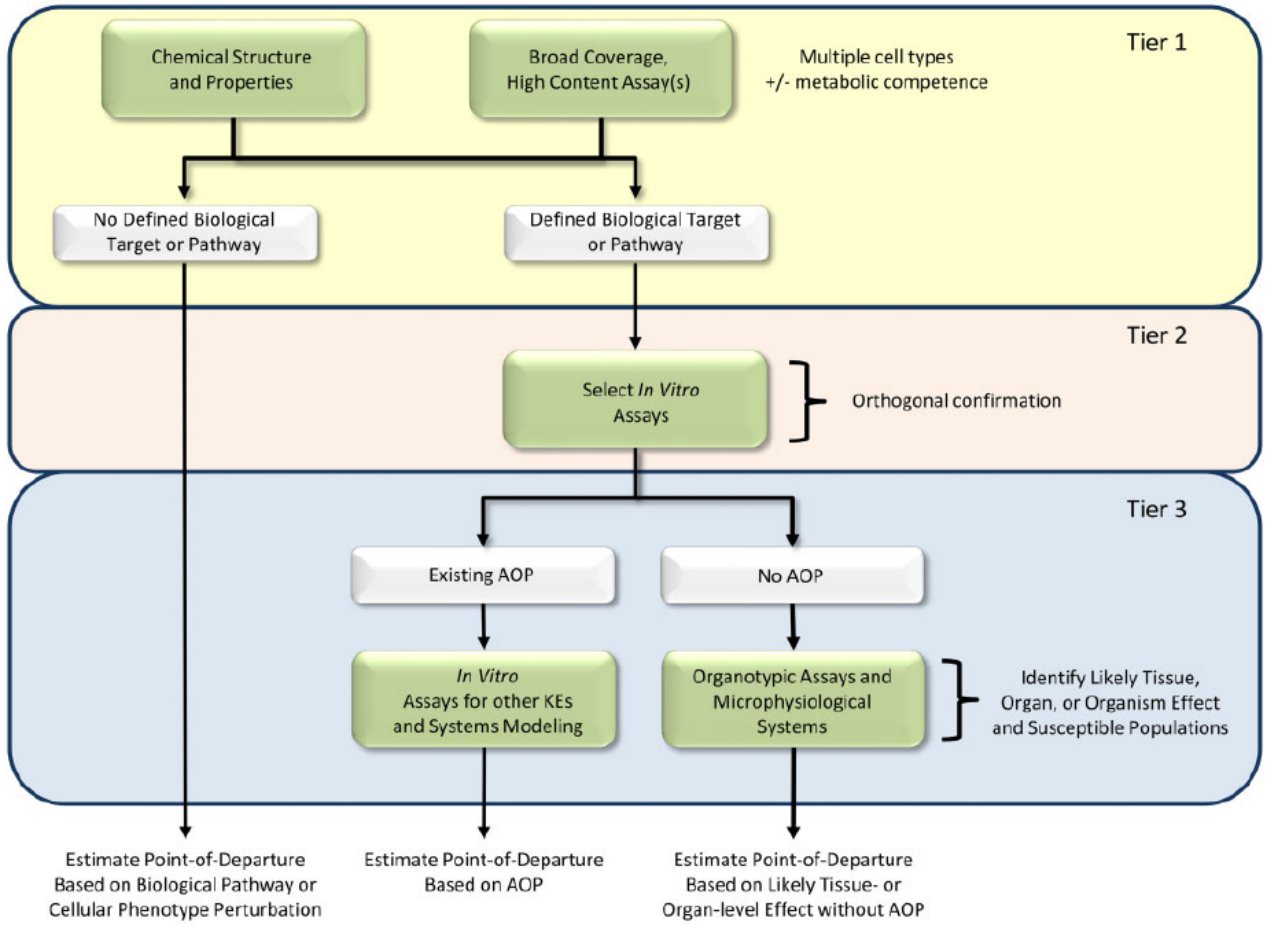
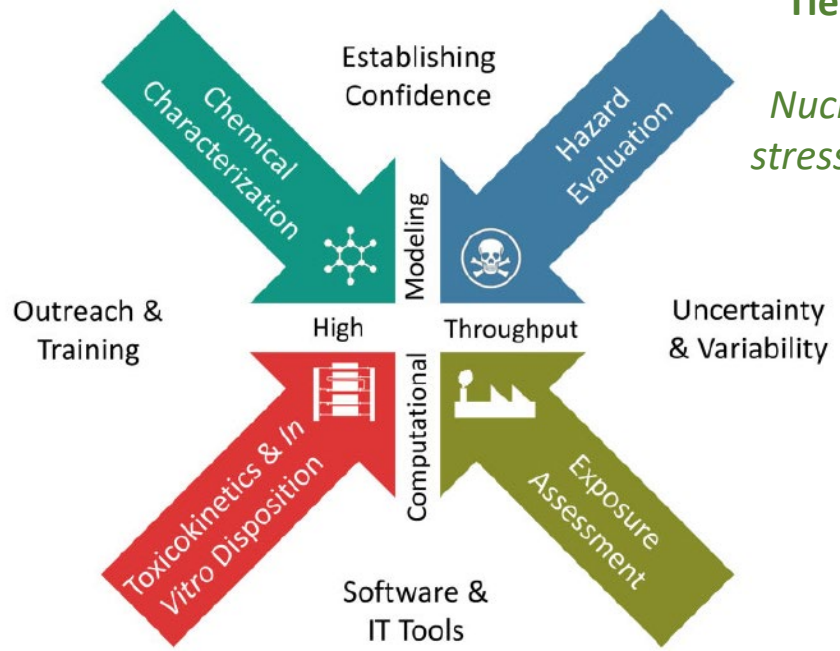


Figure 2. Tiered testing framework for hazard characterization. Tier 1 uses both chemical structure and broad coverage, high content assays across multiple cell types for comprehensively evaluating the potential effects of chemicals and grouping them based on similarity in potential hazards. For chemicals from Tier 1 without a defined biological target / pathway, a quantitative point-of-departure for hazard is estimated based on the absence of biological pathway or cellular phenotype perturbation. Chemicals from Tier 1 with a predicted biological target or pathway are evaluated Tier 2 using targeted follow-up assays. In Tier 3, the likely tissue, organ, or organism-level effects are considered based on either existing adverse outcome pathways (AOP) or more complex culture systems. Quantitative points-of-departure for hazard are estimated based on the AOP or responses in the complex culture system.

Major elements of the EPA CompTox Blueprint can be applied to PFAS screening

Analytical quality control (QC)



Tier 1, 2 and 3 new approach methodologies

Nuclear receptor pathways; cell stress; immunosuppression; other bioactivity signatures

Public and soon to be public Tier 2 data are the focus of today's talk

Toxicokinetic screening data:

Hepatocellular clearance; renal transport; fraction unbound in plasma

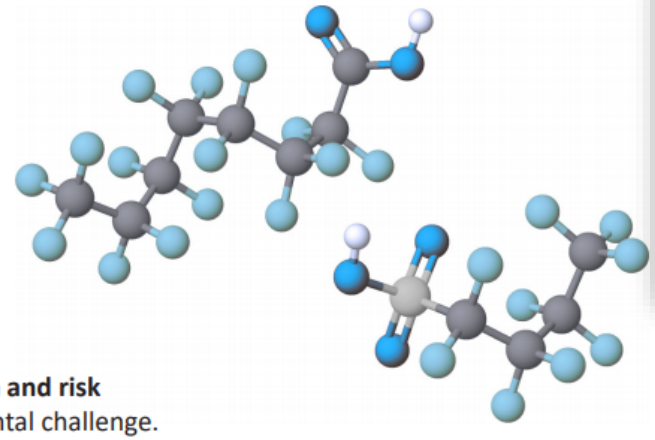
This talk includes some of the publicly available data from research on PFAS

EPA's PFAS Action Plan: A Summary of Key Actions

EPA's PFAS Action Plan outlines concrete steps the agency is taking to address PFAS and to protect public health.

EPA's Per- and Polyfluoroalkyl Substances (PFAS) Action Plan:

- Demonstrates the agency's critical national leadership **by providing both short-term solutions and long-term strategies** to address this important issue.
- Provides a **multi-media, multi-program, national research and risk communication plan** to address this emerging environmental challenge.
- Responds to the extensive public input the agency has received over the past year during the PFAS National Leadership Summit, multiple community engagements, and through the public docket.



EPA is taking a proactive, cross-agency approach to addressing PFAS. The key actions EPA is taking to help provide the necessary tools to assist states, tribes, and communities in addressing PFAS are summarized below.

RESEARCH

EPA is rapidly expanding the scientific foundation for understanding and managing risk from PFAS.

Improved detection and measurement methods, additional information about PFAS presence in the environment and drinking water, better understanding of effective treatment and remediation methods, and more information about the potential toxicity of a broader set of PFAS will help EPA, states, and others better manage PFAS risks.

EPA's PFAS Action Plan: A Summary of Key Actions

Targeted screening for nuclear receptor activation and cell stress

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Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Bioactivity profiling of per- and polyfluoroalkyl substances (PFAS) identifies potential toxicity pathways related to molecular structure

Keith A. Houck [a,*], Grace Patlewicz [a], Ann M. Richard [a], Antony J. Williams [a], Mahmoud A. Shobair [a], Marci Smeltz [a], M. Scott Clifton [a], Barbara Wetmore [a], Alex Medvedev [b], Sergei Makarov [b]

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ARTICLE INFO

Keywords:
Perfluoroalkyl substances
PFAS
Transcription factors
Chemical safety
Nuclear receptors

ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are a broad class of hundreds of fluorinated chemicals with environmental health concerns due to their widespread presence and persistence in the environment. Several of these chemicals have been comprehensively studied for experimental toxicity, environmental fate and exposure, and human epidemiology; however, most chemicals have limited or no data available. To inform methods for prioritizing these data-poor chemicals for detailed toxicity studies, we evaluated 142 PFAS using an *in vitro* screening platform consisting of two multiplexed transactivation assays encompassing 81 diverse transcription factor activities and tested in concentration-response format ranging from 137 nM to 300 μM. Results showed activity for various nuclear receptors, including three known PFAS targets–specifically estrogen receptor alpha and peroxisome proliferator receptors alpha and gamma. We also report activity against the retinoid X receptor beta, the key heterodimeric partner of type II, non-steroidal nuclear receptors. Additional activities were found against the pregnane X receptor, nuclear receptor related-1 protein, and nuclear factor erythroid 2-related factor 2, a sensor of oxidative stress. Using orthogonal assay approaches, we confirmed activity of representative PFAS against several of these targets. Finally, we identified key PFAS structural features associated with nuclear receptor activity that can inform future predictive models for use in prioritizing chemicals for risk assessment and in the design of new structures devoid of biological activity.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a class of man-made chemicals that have been in use since the 1940s and are found in a broad array of industrial and consumer products (Glüge et al., 2020). Their common usage as non-stick surface repellants, in fire-fighting foams, in fluoropolymer manufacturing, and in other applications, coupled with a tendency of some members of the class to bioaccumulate and be resistant to biodegradation, has led to a high level of concern for their contamination of the environment (Wang et al., 2017). There are well documented, widespread, human and wildlife exposure to some of these chemicals, the best known being perfluorooctanoic acid (PFOA; DTXSID8031865) and perfluorooctane sulfonic acid (PFOS; DTXSID3031864) (Kelly et al., 2009; Poothong et al., 2020; Hansen et al., 2002; Noorlander et al., 2011). These two chemicals are no longer manufactured in the U.S. and their international manufacturing has declined, but other PFAS chemicals have been developed to replace their commercial utility (REACH, 2014; OECD, 2015; Stockholm Convention, 2017; EPA, 2000; EPA, 2017). While the toxicities of PFOA and PFOS have been extensively studied by many researchers, numerous other PFAS have little to no toxicity or environmental fate information available. The lack of data and potential environmental impact of this class of chemicals led the U.S. Environmental Protection Agency (EPA) and the National Institute of Health's National Toxicology Program (NTP) to collaborate on conducting PFAS toxicity testing to facilitate PFAS human health assessments (Patlewicz et al., 2019). A targeted selection of 430 PFAS (https://comptox.epa.gov/dashboard/chemical_lists/EPAPFASINV) designed to be representative of the range of

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Available online 20 April 2021

Houck et al. 2020

Gathering information on nuclear receptor and cell stress pathways via transcription factor activity profiling (TFAP)

>3800 ToxCast chemicals have been screened in concentration response in the Attagene transcription factor profiling system

- HepG2 HG19 subclone for elevated xenobiotic metabolic capacity
- “CIS” assays: endogenous transcription factors that regulated transfected reporters (nuclear receptor promoter elements, cell stress)
- “TRANS” assays: exogenous receptor-reporter system is transfected in (xenobiotic nuclear receptors)
- Used for environmental mixtures and single chemical screening

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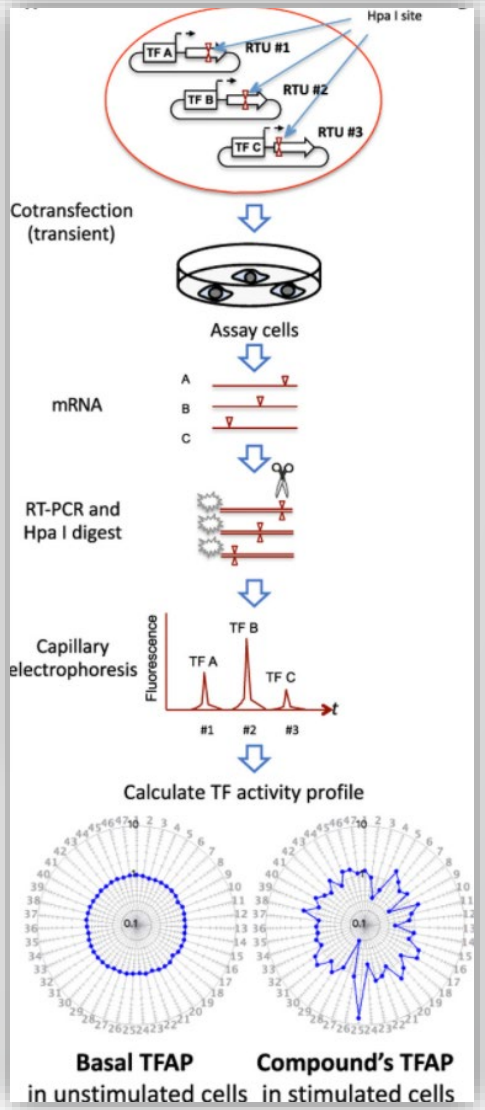


Number	Endpoint	Go Process
1	GAL4_TRANS	control
2	M_06_CIS	
3	M_06_TRANS	
4	M_19_CIS	
5	M_19_TRANS	
6	M_32_CIS	
7	M_32_TRANS	
8	M_61_CIS	
9	M_61_TRANS	
10	TA_CIS	
11	TAL_CIS	
12	CMV_CIS	
13	E_Box_CIS	cell proliferation
14	E2F_CIS	
15	EGR_CIS	
16	Ets_CIS	
17	Pax6_CIS	
18	AR_TRANS	reproduction
19	ERa_TRANS	
20	ERE_CIS	
21	THRa1_TRANS	
22	VDR_TRANS	
23	VDRE_CIS	
24	ISRE_CIS	immune system process
25	NF_kB_CIS	
26	IR1_CIS	lipid metabolic process
27	FXR_TRANS	
28	DR4_LXR_CIS	
29	LXRa_TRANS	
30	LXRb_TRANS	
31	PPARa_TRANS	
32	PPARd_TRANS	
33	PPARg_TRANS	
34	PPRE_CIS	
35	SREBP_CIS	
36	Ahr_CIS	xenobiotic metabolic process
37	CAR_TRANS	
38	PBREM_CIS	
39	PXR_TRANS	
40	PXRE_CIS	

Number	Endpoint	Go Process
41	AP_1_CIS	response to stress
42	HIF1a_CIS	
43	HSE_CIS	
44	MRE_CIS	
45	NRF1_CIS	
46	NRF2_ARE_CIS	
47	Oct_MLP_CIS	
48	p53_CIS	
49	Xbp1_CIS	
50	CRE_CIS	biosynthetic process
51	ERRa_TRANS	
52	ERRg_TRANS	
53	GR_TRANS	
54	GRE_CIS	
55	DR5_CIS	cell differentiation
56	RARa_TRANS	
57	RARb_TRANS	
58	RARg_TRANS	
59	RXRa_TRANS	
60	RXRb_TRANS	
61	NURR1_TRANS	
62	RORb_TRANS	
63	RORg_TRANS	
64	RORE_CIS	
65	Sox_CIS	
66	AP_2_CIS	anatomical structure development
67	BRE_CIS	
68	C_EBP_CIS	
69	FoxA2_CIS	
70	FoxO_CIS	
71	GATA_CIS	
72	GLI_CIS	
73	HNF4a_TRANS	
74	HNF6_CIS	
75	Myb_CIS	
76	Myc_CIS	
77	NFI_CIS	
78	Sp1_CIS	
79	STAT3_CIS	
80	TCF_b_cat_CIS	
81	TGFb_CIS	

There are differences in assay sensitivity by mode and receptor, based on expression and design differences.

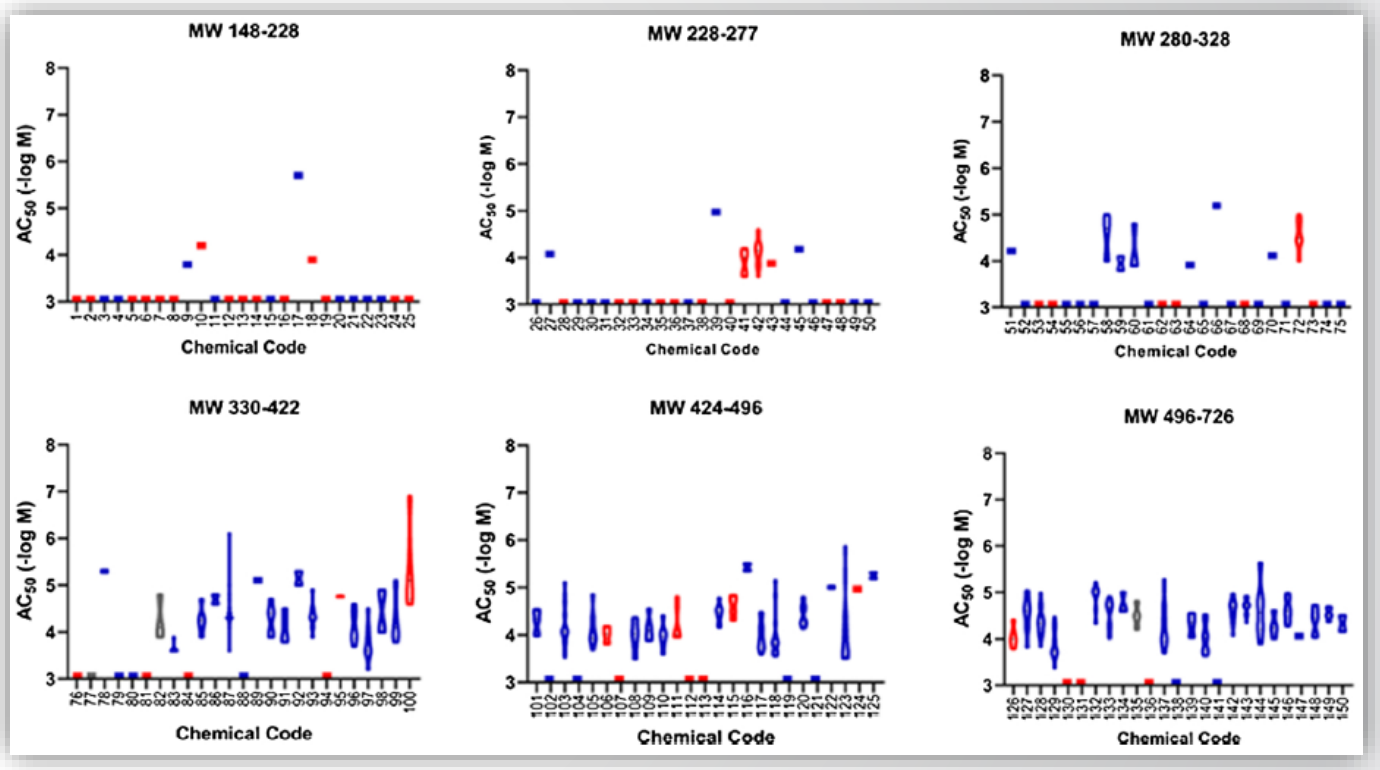
Table 1
Nuclear receptors included in FACTORIAL-TRANS assay.

#	Abbreviation	Receptor Name	Nomenclature	Reference Agonist (Fold-Increase)	*cis*-Factorial Assay (Fold-Increase)	Receptor Expression in HepG2[1]
1	FXR	Farnesoid X receptor	NR1H4	Lithocholic acid (3.5)	IR1 (1.9)	Moderate
2	AR	Androgen receptor	NR3C4	Testosterone propionate (44.1)	NA	Very low
3	RARγ	Retinoic acid receptor-γ	NR1B3	All-trans retinoic acid (3.9)	DR5 (20.2)	Moderate (RAR subfamily)[2]
4	GAL4	Yeast GAL4, negative control	GAL4	NA	NA	NA
5	RXRα	Retinoid X receptor-α	NR2B1	Bexarotene (18.5)	DR5 (8.3)	Moderate (RXR subfamily)[2]
6	GR	Glucocorticoid receptor	NR3C1	Betamethasone (29.1)	GRE (4.6)	Moderate
7	RARβ	Retinoic acid receptor-β	NR1B2	All-trans retinoic acid (1.6)	DR5 (20.2)	Moderate (RAR subfamily)[2]
8	RARα	Retinoic acid receptor-α	NR1B1	All-trans retinoic acid (5.5)	DR5 (20.2)	Moderate (RAR subfamily)[2]
9	PPARγ	Peroxisome proliferator-activated receptor-γ	NR1C2	Rosiglitazone maleate (44.8)	PPRE (3.8)	High
10	ERRγ	Estrogen-related receptor-γ	NR3B3	4-Nonylphenol, branched (2.7)	NA	NA
11	RORβ	RAR-related orphan receptor-β	NR1F1	SSR69071 (7.8)	RORE (5.9)	NA
12	ERα	Estrogen receptor-α	NR3A1	17β-Estradiol (22.6)	ERE (19.1)	Very low; full-length human ERα co-expressed in FACTORIAL-CIS
13	LXRα	Liver X receptor-α	NR1H3	Lynestrenol (13.9)	DR4 (2.3)	High (LXR subfamily)[2]
14	ERRα	Estrogen-related receptor-α	NR3B1	4-Nonylphenol, branched (2.7)	NA	NA
15	PXR	Pregnane X receptor	NR1I2	Rifampicin (3.8)	PXRE (9.1)	Moderate; full-length human PXR co-expressed in FACTORIAL-CIS
16	TRα	Thyroid hormone receptor-α	NR1A1	3,5,3′-Triiodothyronine (33.0)	NA	High
17	LXRβ	Liver X receptor-β	NR1H2	Lynestrenol (8.7)	DR4 (2.3)	High (LXR subfamily)[2]
18	CAR	Constitutive androstane receptor	NR1I3	p,p'-DDT (3.5)	PBREM (1.0)	Very low
19	PPARα	Peroxisome proliferator-activated receptor-α	NR1C1	Pirinixic acid (14.1)	PPRE (2.4)	Moderate
20	RORγ	RAR-related orphan receptor-γ	NR1F3	SSR69071 (14.2)	RORE (5.9)	NA
21	RXRβ	Retinoid X receptor-β	NR2B2	Bexarotene (15.2)	DR5 (8.3)	Moderate (RXR subfamily)[2]
22	HNF4α	Hepatocyte nuclear factor-4-α	NR2A1	NA	NA	High
23	NURR1	Nuclear receptor related 1	NR4A2	Bexarotene (24.6)	NA	NA
24	VDR	Vitamin D receptor	NR1I1	Ergocalciferol (32.6)	VDRE (1.2)	Very low
25	PPARδ	Peroxisome proliferator-activated receptor-δ	NR1C3	12-Hydroxyoctadecanoic acid (9.3)	PPRE (2.9)	NA

- Low- to negligible-expression in HepG2 cells of ERα and PXR was overcome by cotransfection of full-length receptors in the TRANS assay
- CAR and VDR have very low sensitivity to ligands due to reliance only on endogenous receptor expression in the host cell.

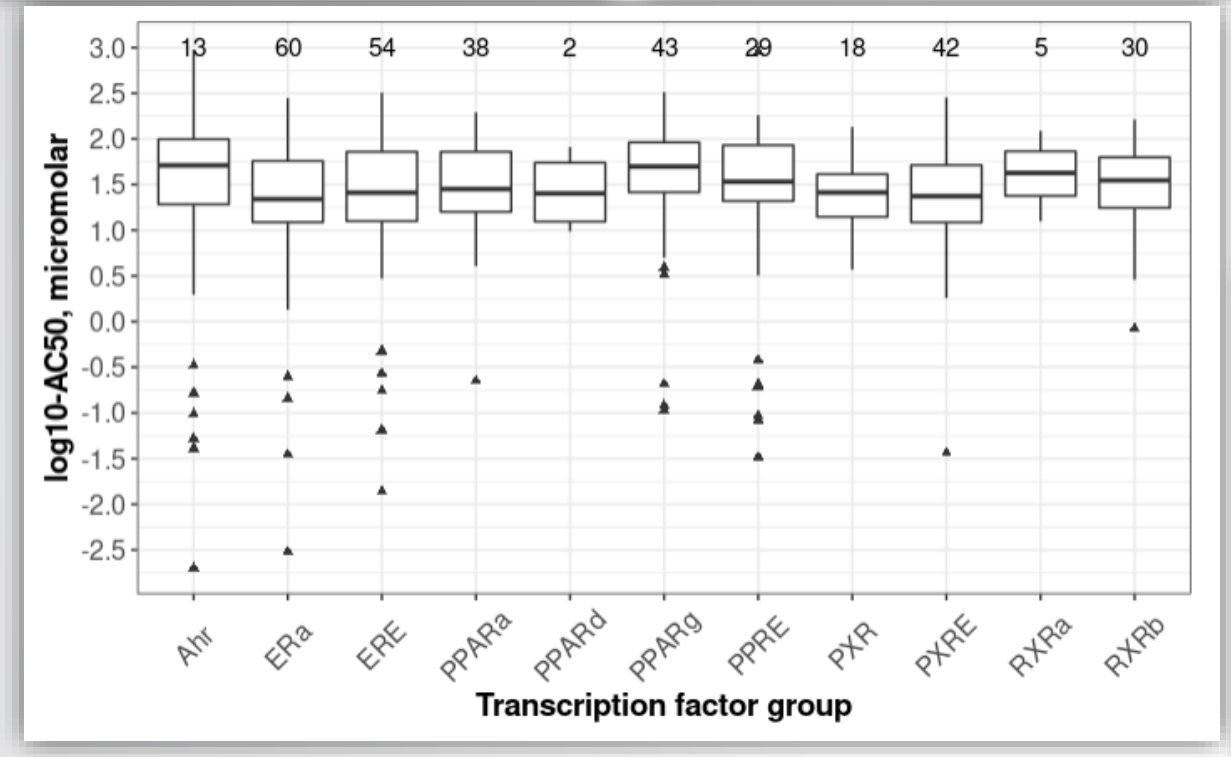
As with other assay platforms screened, lower MW often corresponded to more limited bioactivity, but there may be more than one reason.

- PFAS with molecular weight less than 330 g/mol appeared less likely to be active in the Attagene assays and more likely to "fail" analytical QC (defined as parent structure not detected).
- Activity was not detected for 76 distinct structures, of which 55 % failed analytical QC.
- 67% of the "failed" samples had predicted vapor pressures in excess of 100 mmHg, suggesting that chemical volatilization may have played a role in limited bioactivity of some of these samples.
- The specific acid form of PFAS may also be important, as the free acid form of the chemical known as "GenX" (perfluoro-2-methyl-3-oxahexanoic acid (DTXSID70880215) did not have a high vapor pressure (was unlikely to have volatilized), but the ammonium salt form of this chemical (DTXSID40108559) showed activity as a PPARα agonist when solubilized in water (rather than DMSO).



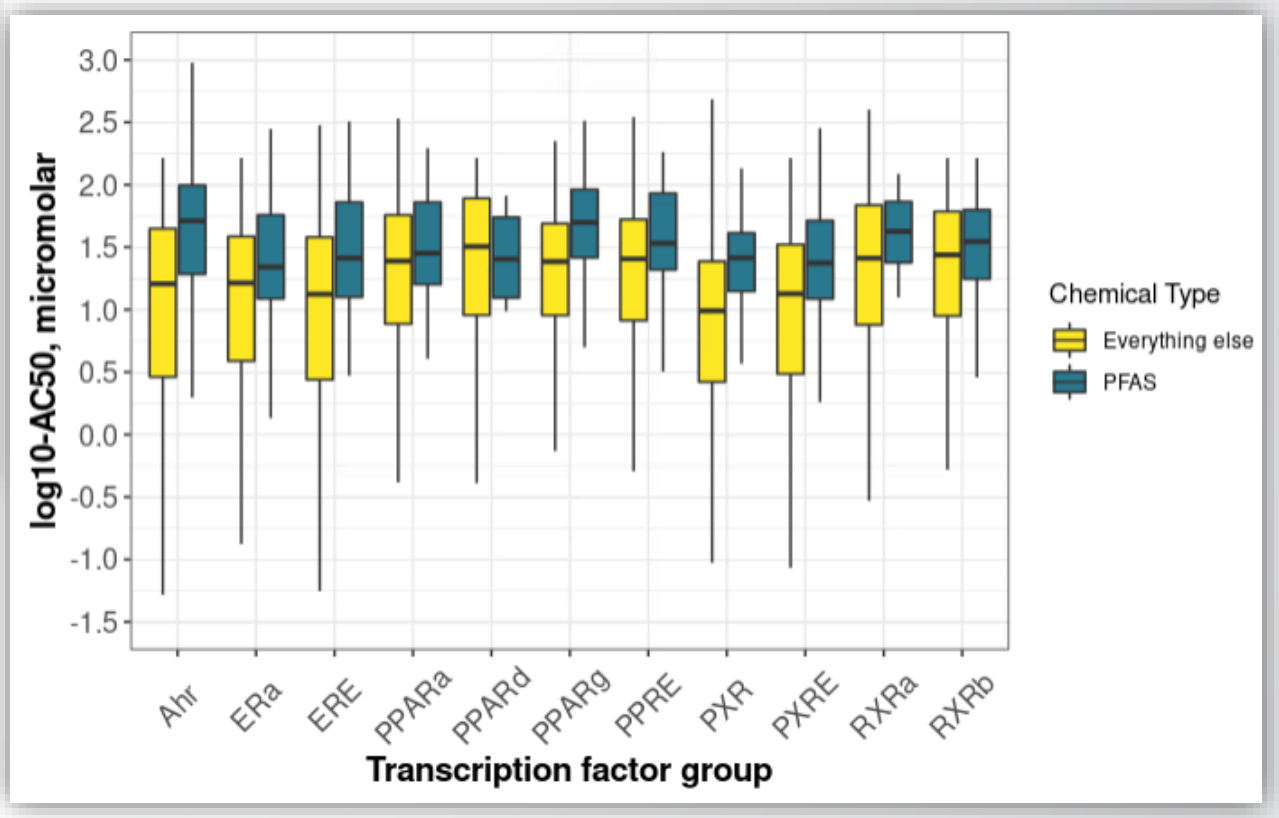
Houck et al. 2020, Fig1B.

Potency for the PFAS that were positive at key transcription factor targets tended to be somewhat left-shifted from the rest of the ToxCast library

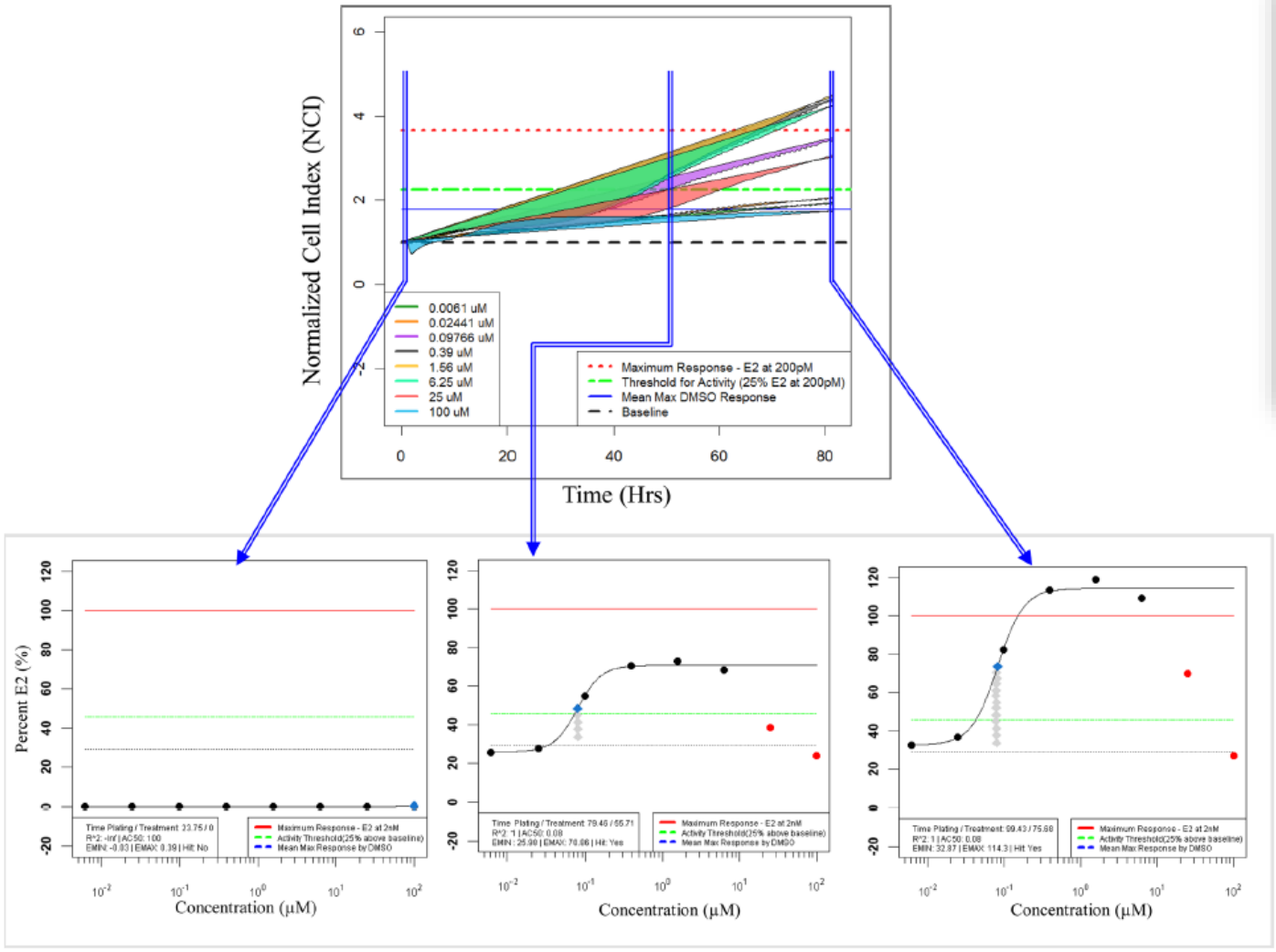


- Many PFAS were negative in the transcription factor activity screening
- Aryl hydrocarbon receptor (AhR), estrogen receptor alpha (ERa), PPAR alpha, delta, and gamma (PPARa,d,g), the pregnane X receptor (PXR), and RXR alpha and beta (RXRa,b) emerged as targets.

- The number of chemicals that simply hit one or more relevant assays for a particular transcription factor group can be examined in more depth for confirmation.



Estrogen receptor activity can be confirmed with orthogonal assays including ACEA: Real Time Cell Analysis Based on Electrical Impedance



Chemical Research in Toxicology

Article

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Real-Time Growth Kinetics Measuring Hormone Mimicry for ToxCast Chemicals in T-47D Human Ductal Carcinoma Cells

Daniel M. Rotroff,[†,‡] David J. Dix,[‡] Keith A. Houck,[‡] Robert J. Kavlock,[‡] Thomas B. Knudsen,[‡] Matthew T. Martin,[‡] David M. Reif,[‡] Ann M. Richard,[‡] Nisha S. Sipes,[‡] Yama A. Abassi,[§] Can Jin,[§] Melinda Stampfl,[§] and Richard S. Judson*[,‡]

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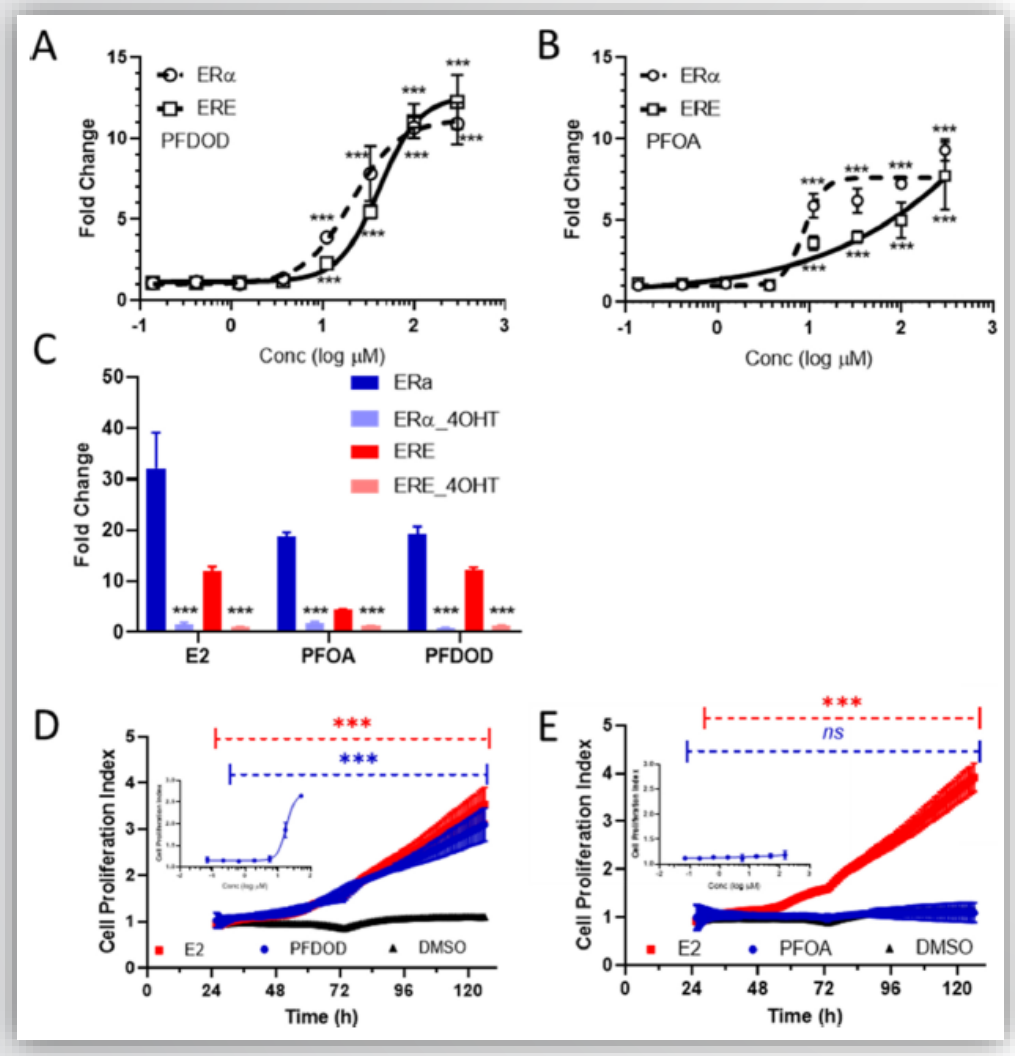
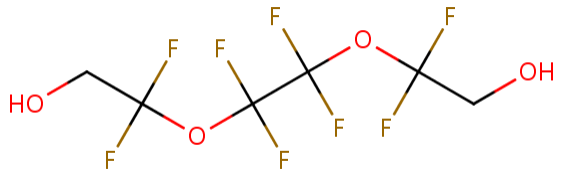
- Can measure cell proliferation or cytotoxicity depending on the direction
- Electrical impedance measured over 80 hr
- ACEA ER assay uses T-47D breast cancer cells

Confirmation of transcriptional responses with functional activity is an important strategy for ER bioactivity

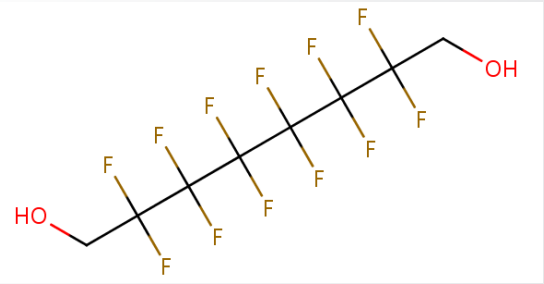


Houck et al. 2020, Fig5.

- 40-60 PFAS demonstrated some activity in the ATG ERa TRANS or ERE CIS assays; viewing these assays as orthogonal reduces the set to <10.
  - All of these were less potent than 17β-estradiol.
  - Acrylates and N-akyl perfluoroalkyl (linear) sulfonamide structural categories were significantly associated with ER activity.
- Adding in ACEA as another orthogonal assay to confirm specificity leads indicates few PFAS with transcription factor *and* functional ER-dependent cell proliferation.



1H,1H,8H,8H-Perfluoro-3,6-dioxaoctane-1,8-diol



1H,1H,8H,8H-Perfluorooctane-1,8-diol

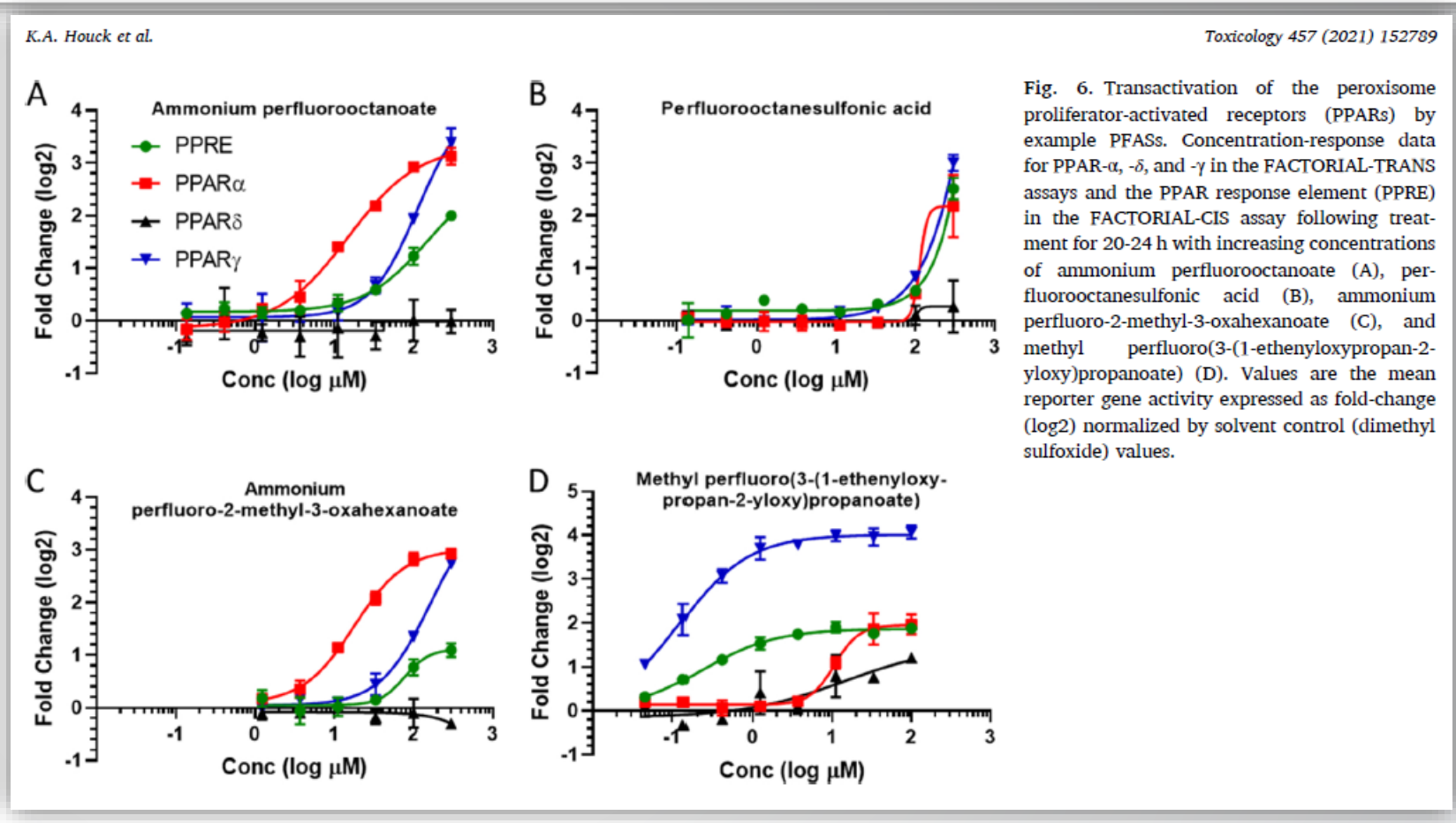
- PFOA activated ATG_ERa_TRANS and ERE_CIS but failed to produce functional ER-dependent cell proliferation in ACEA.

As expected PPAR activity was observed for a subset of PFAS.

- The TRANS assay contained endpoints for all three human PPARs (α,δ,γ) whereas the CIS assay contained a reporter gene controlled by a PPAR-response element that responds to all three PPARs endogenously expressed in the HepG2 host cells.

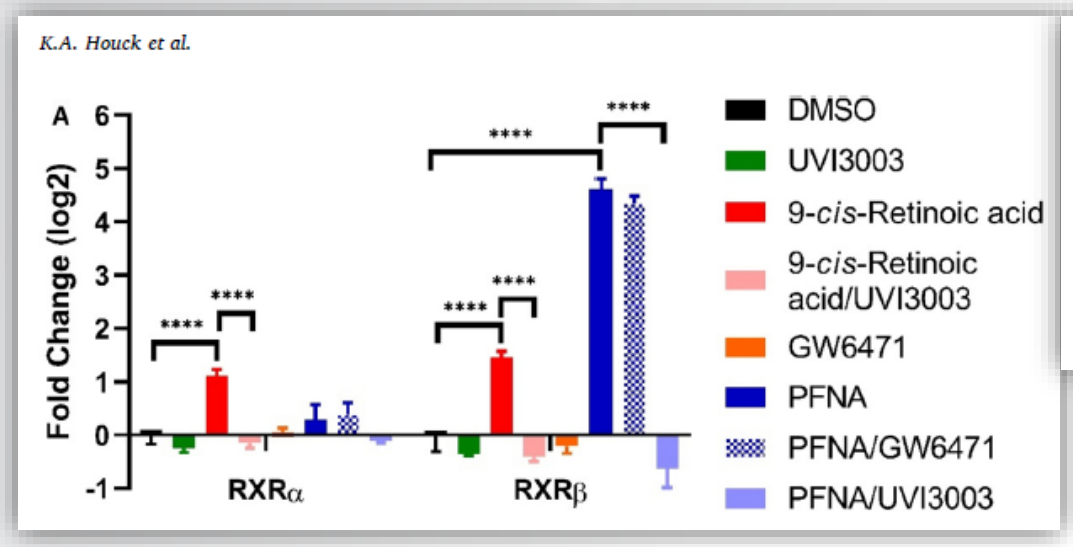
- Functional groups enriched within the actives were mostly carboxylates along with sulfonates, sulfonamides and a thenoylketone, which all have a negative ionic charge at physiological pH, consistent with known critical components for ligand-binding.

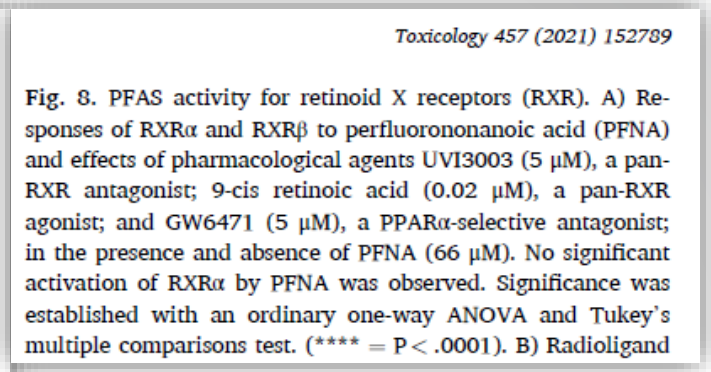
- Not much activity at PPARδ (smaller binding pocket?).



Houck et al. 2020, Fig6.

~17 PFAS activated RXRβ, with two of these active at RXRα



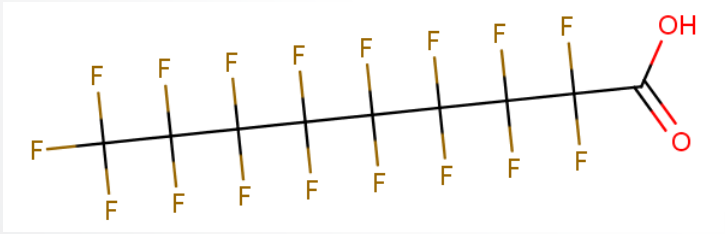


Toxicology 457 (2021) 152789

Fig. 8. PFAS activity for retinoid X receptors (RXR). A) Responses of RXRα and RXRβ to perfluorononanoic acid (PFNA) and effects of pharmacological agents UVI3003 (5 μM), a pan-RXR antagonist; 9-cis retinoic acid (0.02 μM), a pan-RXR agonist; and GW6471 (5 μM), a PPARα-selective antagonist; in the presence and absence of PFNA (66 μM). No significant activation of RXRα by PFNA was observed. Significance was established with an ordinary one-way ANOVA and Tukey's multiple comparisons test. (**** = P < .0001). B) Radioligand

Houck et al. 2020, Fig8A.

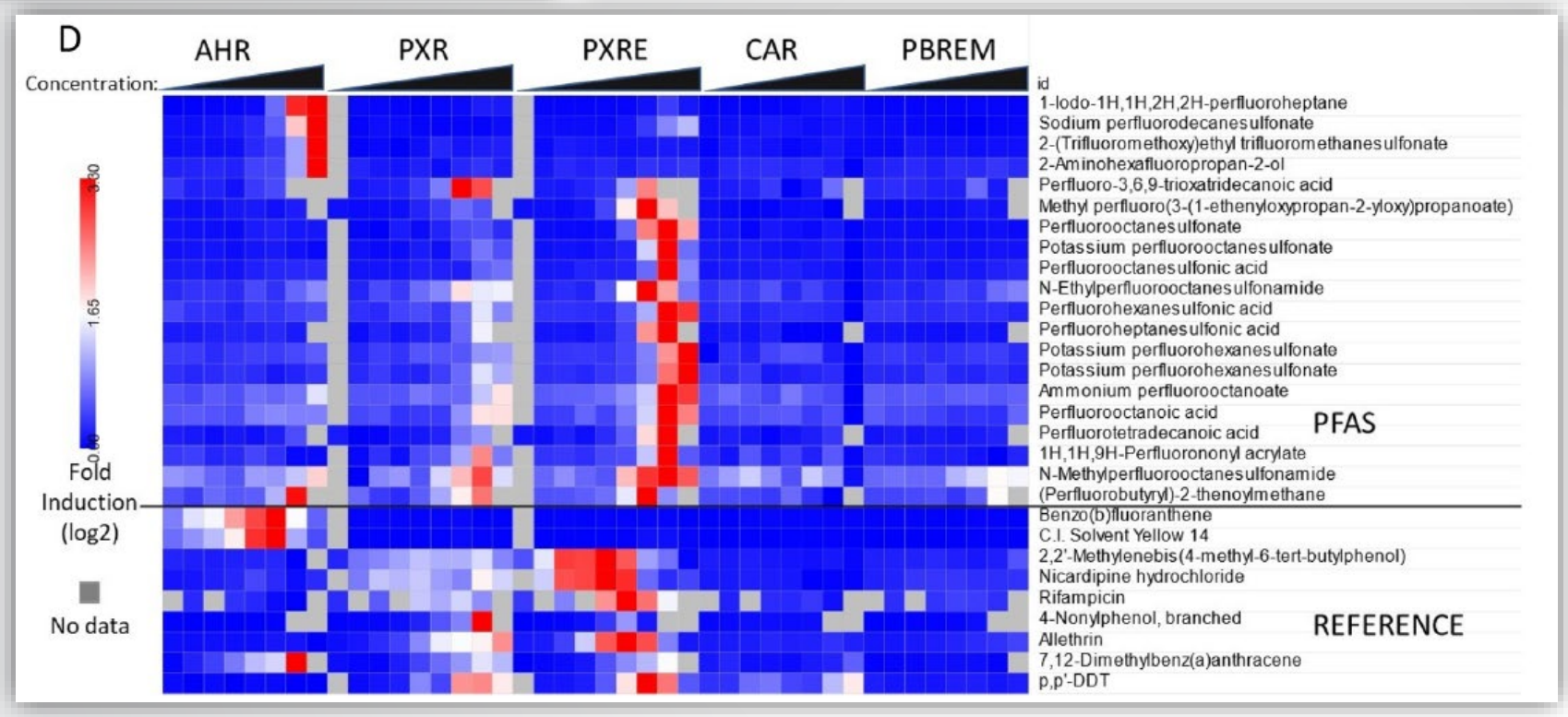
PFNA appears to work through RXR specifically: an RXR-selective antagonist, UVI3003 (DTXSID501024375), completely blocked PFNA activation of RXR, whereas the PPARα antagonist GW6471 was ineffective.



PFNA

- Seventeen of the PFAS, mostly linear, fluorinated carboxylic acids, showed a novel finding of activation of RXRβ.
- Most also activated PPARα, PPARγ and NRF2, with varying levels of selectivity. Only two activated RXRα; however, NURR1 was activated, presumably through agonist effects on RXRβ .
- All are structurally related perfluorinated carboxylic acids and meet defined ligand structural requirements for RXR.

Xenobiotic nuclear receptor responses associated with hepatic metabolism may also be important targets to screen for PFAS bioactivity.



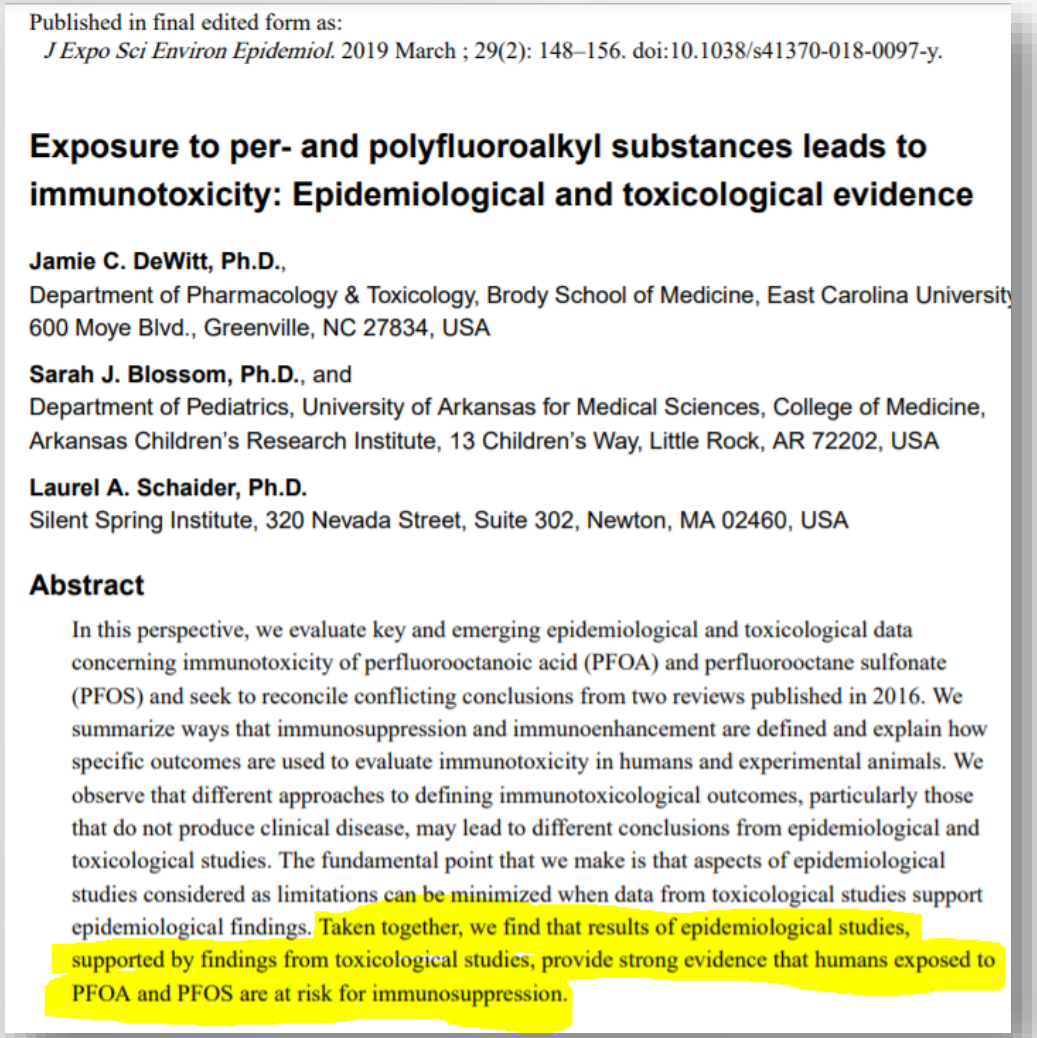
Houck et al. 2020, Fig3B.

- Many of the PFAS modulated the xenobiotic response, particularly PXR.
- Responses were generally modest with respect to potency and efficacy relative to prototypical PXR inducers.
- None of the PFAS were determined to be CAR activators, recognizing limitations in the FACTORIAL-CIS assay for CAR, likely due to negligible expression of CAR in HepG2 cells.
- Several PFAS structures activated the AhR, somewhat surprising in that all were linear fluoroalkyl molecules while the protypical activator is a polycyclic aromatic hydrocarbon. Except for sodium perfluorodecanesulfonate and 1-Iodo-1H,1H,2H,2H-perfluoroheptane, the responses were very weak with unknown *in vivo* relevance.

Immunosuppressive activity of the PFAS150 in an *in vitro* assay suite

Houck KA, Paul Friedman K, Feshuk M, Patlewicz G, Smeltz M, Clifton MS, Wetmore BA, Velichko S, Berenyi A, Berg EL. (*In internal review*). Evaluation of 147 Perfluoroalkyl Substances for Immunosuppressive and Other Activities through Phenotypic Screening of Human Primary Cells.

PFOA and PFOS are suspected of being immunosuppressive



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Exposure to per- and polyfluoroalkyl substances leads to immunotoxicity: Epidemiological and toxicological evidence

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Abstract

In this perspective, we evaluate key and emerging epidemiological and toxicological data concerning immunotoxicity of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) and seek to reconcile conflicting conclusions from two reviews published in 2016. We summarize ways that immunosuppression and immunoenhancement are defined and explain how specific outcomes are used to evaluate immunotoxicity in humans and experimental animals. We observe that different approaches to defining immunotoxicological outcomes, particularly those that do not produce clinical disease, may lead to different conclusions from epidemiological and toxicological studies. The fundamental point that we make is that aspects of epidemiological studies considered as limitations can be minimized when data from toxicological studies support epidemiological findings. Taken together, we find that results of epidemiological studies, supported by findings from toxicological studies, provide strong evidence that humans exposed to PFOA and PFOS are at risk for immunosuppression.

- A 2016 NTP review of PFOA/PFOS had concluded that suppression of antibody responses in animals had the most evidence.
- Chang et al. 2016 had concluded that the available evidence was insufficient to make a conclusion about causality; could cause immunosuppression in animals, but inconsistencies were present across species and strain.
- DeWitt et al. 2019 attempts to reconcile these opinions and concludes what the NTP concluded, that PFOA and PFOS may be associated with immunosuppression based on available data.
- The limited data landscape, focused on PFOA and PFOS, is complex, with differences by species, strain, sex, endpoints measured, and doses used.

BioMAP is a suite of primary and primary co-culture models for assessing phenotypes, including some elements of immunosuppression

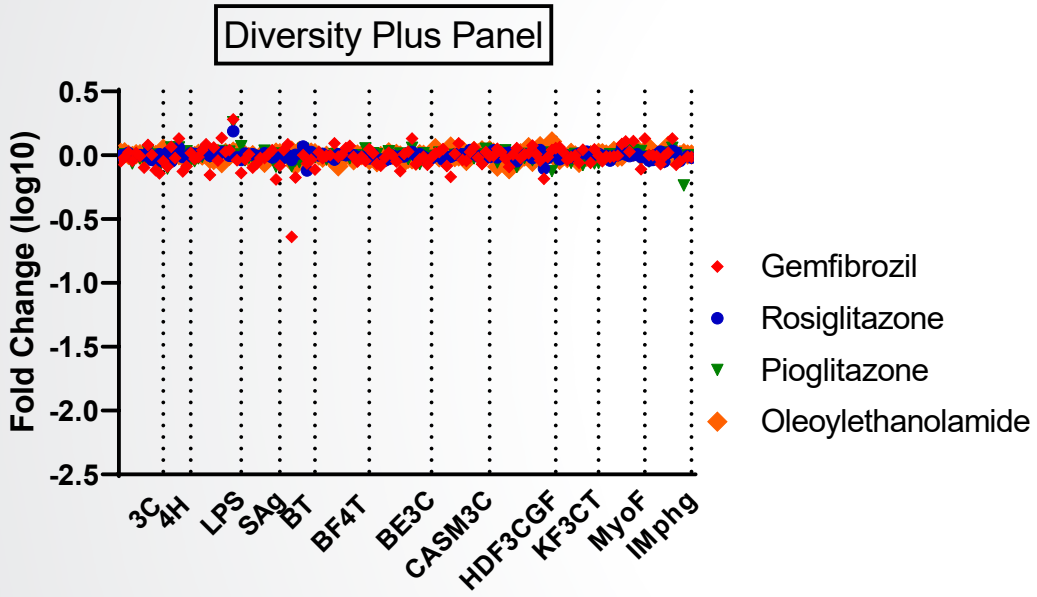
System	Icon	Cell Type	Disease Relevance	Biomarker Readouts	Description
SAg		Peripheral blood mononuclear cells + Venular endothelial cells	Autoimmune Disease, Chronic Inflammation	MCP-1, CD38, CD40, E-selectin, CD69, IL-8, MIG, PBMC Cytotoxicity, Proliferation, SRB	The SAg system models chronic inflammation of the Th1 type and T cell effector responses to TCR signaling with costimulation. This system is relevant to inflammatory conditions where T cells play a key role including organ transplantation, rheumatoid arthritis, psoriasis, Crohn's disease and multiple sclerosis.
BT		Peripheral blood mononuclear cells + B cells	Asthma, Allergy, Oncology, Autoimmunity	B cell Proliferation, PBMC Cytotoxicity, Secreted IgG, sIL-17A, sIL-17F, sIL-2, sIL-6, sTNFα	The BT system models T cell dependent B cell activation and class switching as would occur in a germinal center. This system is relevant for diseases and conditions where B cell activation and antibody production are relevant. These include autoimmune disease, oncology, asthma and allergy.
Mphg		Venular endothelial cells + Macrophages	Cardiovascular Inflammation, Restenosis, Chronic Inflammation	MCP-1, MIP-1α, VCAM-1, CD40, E-selectin, CD69, IL-8, IL-1α, M-CSF, sIL-10, SRB, SRB-Mphg	The Mphg System models chronic inflammation of the Th1 type and macrophage activation responses. This system is relevant to inflammatory conditions where monocytes play a key role including atherosclerosis, restenosis, rheumatoid arthritis, and other chronic inflammatory conditions.

**Does not cover every type of cell involved in immune suppression nor every marker*

A subset of BioMAP includes biomarkers in relevant cell types with measures of specific immune cells and interleukins known to be immune-relevant.

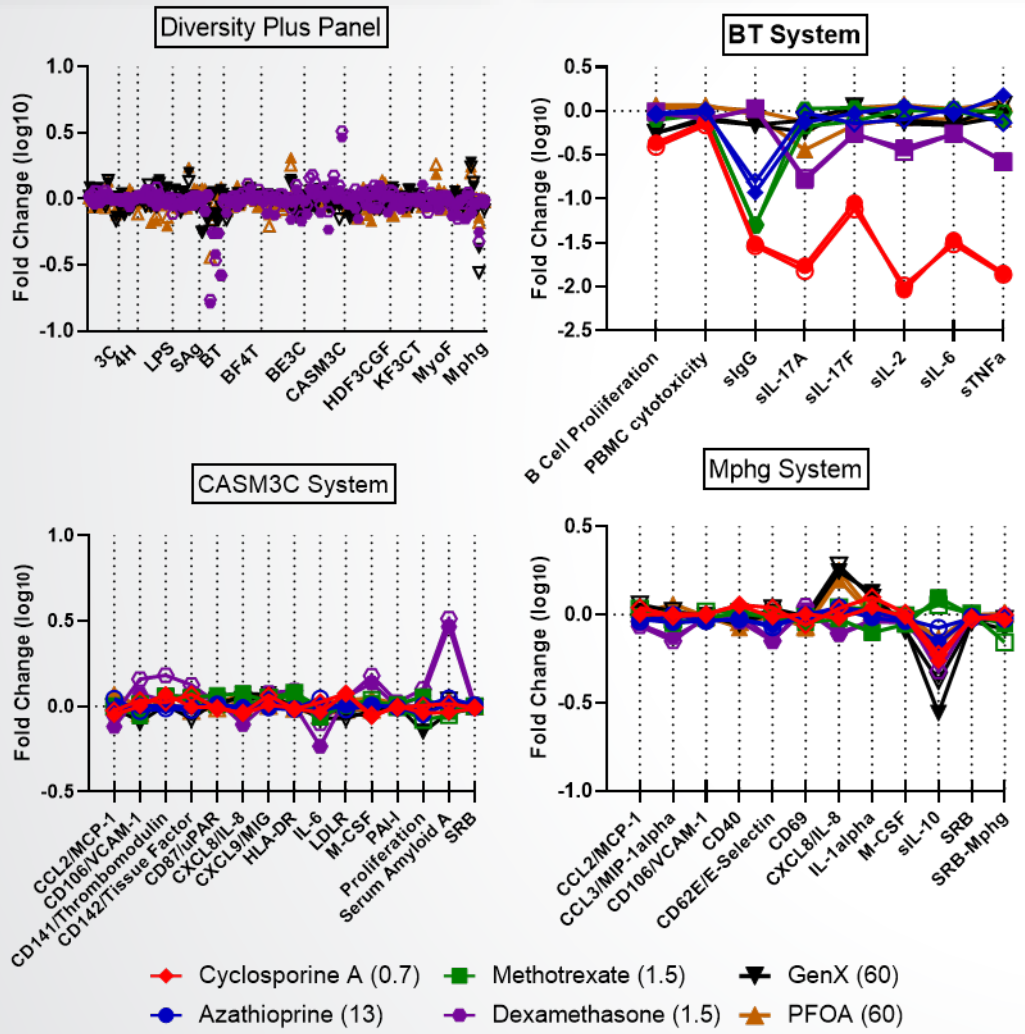
- A TDAR will measure T-cell dependent IgM antibody response, can also IgG if modified.
- SAg: markers of decreased T-cell proliferation or specifically cytotoxic to PBMCs.
- BT: Decreased IgG and B cell proliferation or specifically cytotoxic to PBMCs.
- Mphg: Decreased IL-10 (like dexamethasone)
- Reference chemicals used: cyclosporin A, azathioprine, methotrexate [these 3 strongly suppress IgG production in BT], and dexamethasone [affected IL-10 in Mphg]

It's unlikely that PPAR is related to any immunosuppressive activity in the BioMAP assay suite as PPAR agonists have little activity



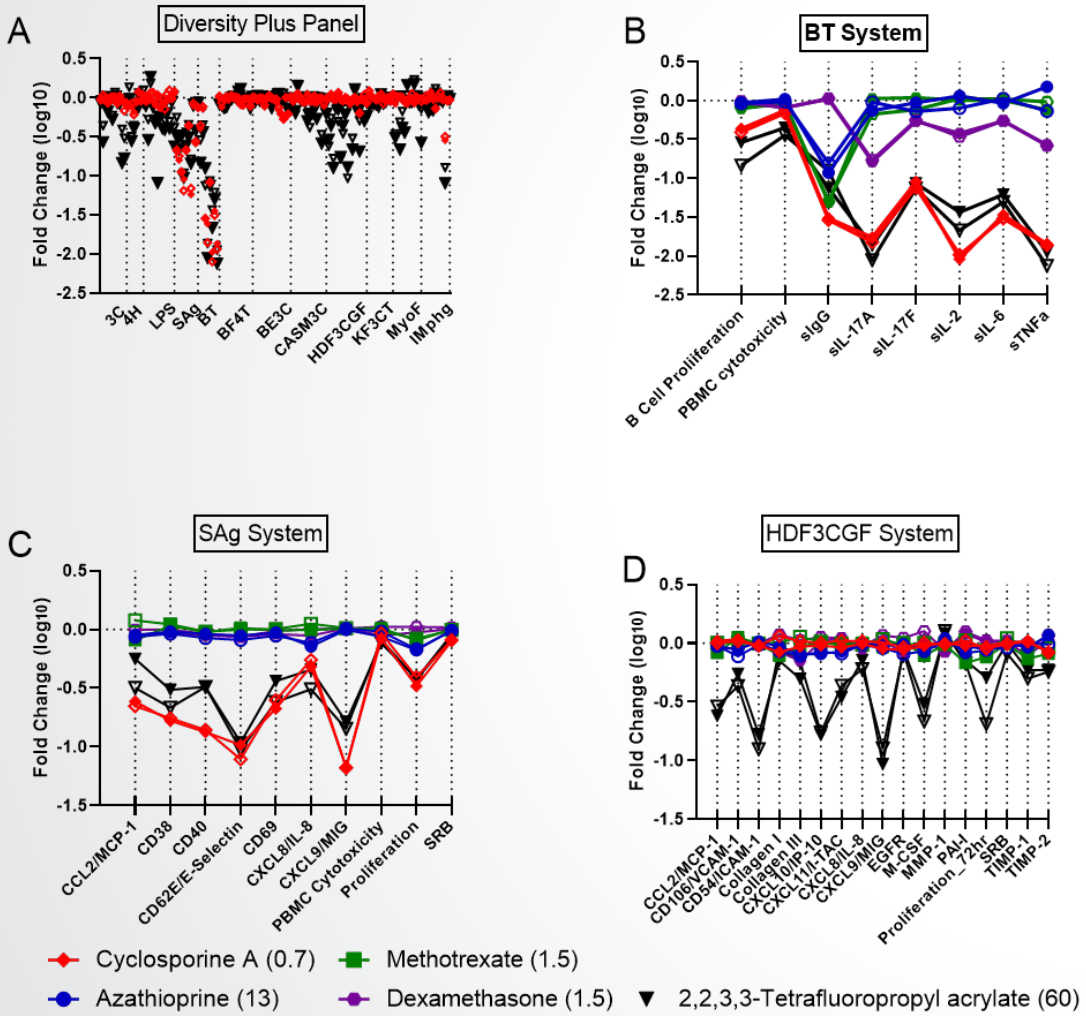
BioMAP Diversity Plus profiles of PPARa and PPARg reference chemicals. Profiles for PPARg agonists rosiglitazone (3.7 µM), pioglitazone (10 µM) and PPARa agonists gemfibrozil (200 µM) and oleoylethanolamide (1.1 µM) are shown for the 12 assay systems of the BioMAP Diversity Plus platform. Concentrations were selected from the database to exceed reported *in vitro* EC50's for the corresponding receptor targets by 5- to 40-fold.

PFOA (and PFOS) failed to provide support for suppression of IgG in BioMAP at the screened concentration range

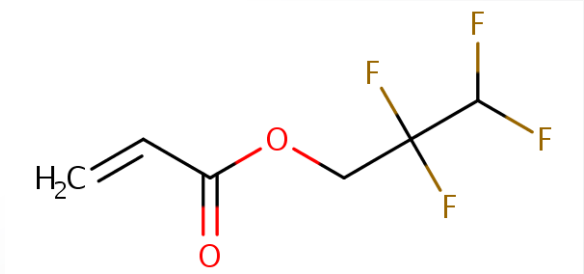


- Overall, neither PFOA nor PFOS seemed to show activity similar to the reference immunosuppressants except perhaps PFOA at its highest testing concentration of 60 μM where it clustered with dexamethasone.
- PFOA and PFOS decreased IL-10 in a co-culture system (Mphg assay) that detects macrophage responses. IL-10 is a cytokine that promotes B cell IgG production, but human relevance of this in vitro finding is unknown.
- Several other PFAS, *i.e.* 3-Bis(trifluoromethyl)-2-propenoic acid, 3H-perfluoro-2,2,4,4-tetrahydroxypentane and perfluoropinacol, have activities similar to the reference immunosuppressants in some of the cell systems, including suppression of IgG secretion.

A single PFAS at its highest screened concentration associated closely with cyclosporin



- Suppression of multiple endpoints in the BT system and the SAg system is similar for both chemicals with strong reduction in secreted IgG and the cytokines IL-17A, IL-2, IL-6 and TNFα in the BT assay.
- Notably, while cyclosporine A was very selectively active for these two assay systems, 2,2,3,3-tetrafluoropropyl acrylate was also active in others, in particular the wound healing and inflammation model (HDF3CGF).



2,2,3,3-Tetrafluoropropyl acrylate

Overall conclusions of these high-throughput screening data for a PFAS library

- PFAS with MW >330 g/mol tend to be more active in vitro in the current aqueous media, cell-based assays.
- Analytical quality control is exceedingly important particularly for PFAS which may include substances that volatilize. Additionally the specific salt form of a PFAS greatly impacts its bioactivity.
- In general, the PFAS tend to be similarly or less potent than the rest of the ToxCast chemical library for any of the targets screened to date.
- Subsets of PFAS have activity for various nuclear receptor targets, and the use of orthogonal assays *in vitro* can further inform interpretation of these transcription factor targets. Screening for AhR, ER, PPAR, PXR, and RXR may be important.
- Work in progress on research-based screening models of immunosuppression fails to support PFOA and/or PFOS induced IgG suppression.

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