Evaluation of toxic activities of selected nitrogen-containing polycyclic aromatic hydrocarbons PANH compared with their PAH analogues using *in vitro* cell-based bioassays

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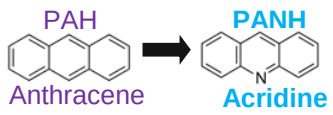
INTRODUCTION

Polycyclic Aromatic Hydrocarbons (homocyclic PAHs) are known ubiquitous environmental contaminants. Humans are exposed from various sources including food. Because of their carcinogenic properties, PAHs have been widely studied and are currently strictly regulated. Recently, PAH nitro-derivatives, also called PANHs or Azaarenes, have been reported to occur at low levels in food materials. These molecules are structurally very similar to PAHs (carbon substituted by a nitrogen) (Figure 1) and have been anticipated to exhibit similar or higher toxic potential. However, very little information is available on their toxic properties as compared to PAHs. It is currently very difficult to establish the level of safety concern associated with the presence of PANHs in food. In this context, the main objective of the present work was to compare the *in vitro* toxicity properties of PANHs with their respective PAH structural analogues.



METHODOLOGY

Several PANHs and PAHs structural counterparts were selected (Table 1). They were studied using a battery of *in vitro* toxicology bioassays (Table 2 & 3). Benzo(a)pyrene (B(a)P) was used as positive/prototypical control. Based on literature, the most relevant *in vitro* bioassays endpoints were the following: (1) nuclear receptors activation/inhibition (Estrogen receptor α (ERα), Estrogen receptor β (ERβ), Androgen receptor (AR), Aryl Hydrocarbon Receptor (AhR)) using CALUX assays, (2) genotoxicity potential in absence and presence of metabolic activation (S9) (Gadd45α using the Bluescreen (Gentronix), p53 induction using the CALUX assay and Histone phosphorylation (H2AX) (ToxInsight-ThermoFisher)), and (3) cell viability in absence and presence of S9 (mitochondrial, lysosomal activity and protein synthesis effect) from Xenometrix.

Table 1: List of the PANHs and PAHs counterpart tested

Type	Rings	Compounds	Structure	CAS
PAH	3R	Anthracene		120-12-7
PANH		Acridine		260-94-6
PAH	4R	Benz(a)anthracene		56-55-3
PANH		Benz(a)acridine		225-11-6
		Benz(c)acridine		225-51-4
PAH	5R	Dibenz(ah)anthracene		53-70-3
PANH		Dibenz(ah)acridine		226-36-8
		Dibenz(aj)acridine		224-42-0
		Dibenz(ch)acridine		224-53-3
PAH		Benzo(a)pyrene *		50-32-8

RESULTS

Dose-response curves for each molecule were obtained in each bioassays. In house and CRO's data were concordant.

The results obtained for the reference compound B(a)P were in agreement with reported data from the literature confirming the suitability of the battery of tests:

Cytotoxicity: there was no specific tendency in cytotoxicity potency with respect to the ring number or PANHs *vs* PAHs (Table 2).

Genotoxicity: there was no simple trends in genotoxicity with respect to the ring number (although the most potent were amongst the highest number of ring) or PANHs vs PAHs. Metabolic activation was a pre-requisite step (Figure 1 and Table 2).

Nuclear receptor-mediated effects:

- AhR was the most sensitive parameter with a direct correlation between AhR activation potency and the ring number (Fig. 2a).
- Inverse correlation between anti-AR activity and ring number (Fig.2b)
- Only one compound with anti-ERα activity (Fig. 2c).
- Inverse correlation between ERα-activation potency and the ring number (Fig 2d).

Figure 1: Genotoxicity (Gadd45α) induction

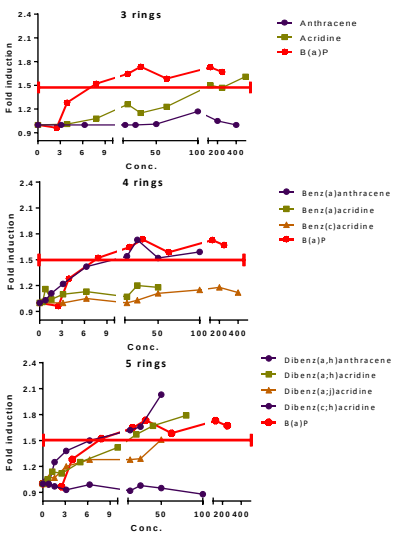


Figure 2. Nuclear receptor activation/inhibition

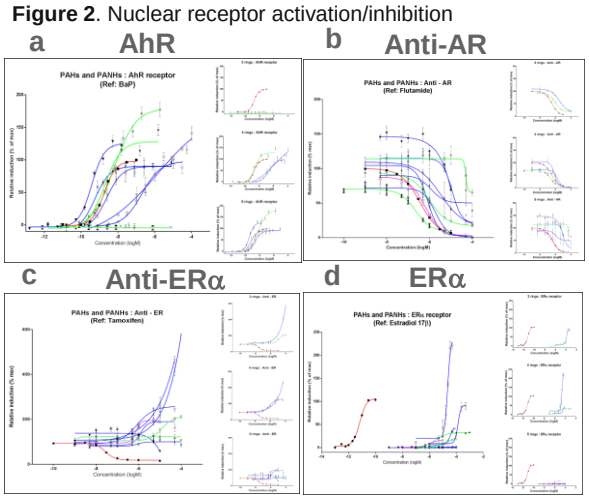


Table 2: Genotoxicity induction expressed as the lowest effective concentration (LEC)

CYTOTOXICITY / GENOTOXICITY (LEC in µM)										
Sample				(-S9)			(+S9)			
Type	Rings	Compound	Structure	Cytotox	Gadd45α	p53	Cytotox	Gadd45α	p53	H2AX
PAH	5R	B(a)P*		-	-	-	100	6.25	41	2.0
PANH	5R	Dibenz(ch)acridine		-	-	-	-	12.5	10	1.6
PAH	4R	Benz(a)anthracene		12.5	-	-	100	12.5	10	12.5
PANH	5R	Dibenz(ah)acridine		-	-	-	-	20	-	1.3
PANH	5R	Dibenz(aj)acridine		25	-	-	-	50	49	-
PANH	3R	Acridine		125	250	34	125	500	71	
PAH	5R	Dibenz(ah)anthracene		-	-	-	-	-	-	5.0
PANH	4R	Benz(c)acridine		25	-	-	-	-	-	
PANH	4R	Benz(a)acridine		50	-	4.3	-	-	18	200
PAH	3R	Anthracene		-	-	-	-	-	210	

* PAH Reference

Table 3: Nuclear receptor activation/inhibition ranked from stronger to weaker activator

Nuclear receptors							
Sample				Nuclear receptors (EC50) in µM			
Type	Rings	Compound	Structure	AhR	ER a	Anti ER	Anti AR
PANH	5R	Dibenz(ah)acridine		0.0004	-	16.6	-
		Dibenz(ch)acridine		0.0006	-	-	1.19
PAH		B(a)P*		0.0021	0.97	-	0.63
PANH		Dibenz(aj)acridine		0.0025	-	-	9.56
PAH		Dibenz(ah)anthracene		0.006	-	-	49
PAH	4R	Benz(a)anthracene		0.002	3.0	-	0.22
PANH		Benz(c)acridine		0.203	35	-	0.82
		Benz(a)acridine		1.42	23	-	1.91
PAH	3R	Anthracene		-	16	-	0.97
PANH		Acridine		-	142	-	3.40

Strong activator

Weak activator

CONCLUSION

The data did not identify any correlation between cytotoxicity, genotoxicity and receptor-mediated effects. PAHs and their derivatives seem to act mainly as ligands for the AhR receptor. Data allowed concluding that compared to PAH analogues, the tested PANHs exhibit similar toxicological profiles and are likely to raise similar toxicological concern. However, PANHs may not bring significant additional risk burden since exposure seems much lower than for PAHs. This would need further analytical confirmation and proper exposure assessment to better estimate the level of safety concern. Further work should involve the evaluation of additional PANHs and PAHs, in isolation and in mixtures, as well as the investigation of the role of metabolism on the effects on nuclear receptors.

Finally, the current data show the applicability of an *in vitro* battery to compare structurally similar chemicals and to set priorities for further work. Such an approach is also aligned to the 3Rs initiative for reduction of animal testing.