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Anti-androgenic activity of novel flame retardants in mixtures: Newly identified contribution from tris(2,3-dibromopropyl) isocyanurate (TDBP-TAZTO)

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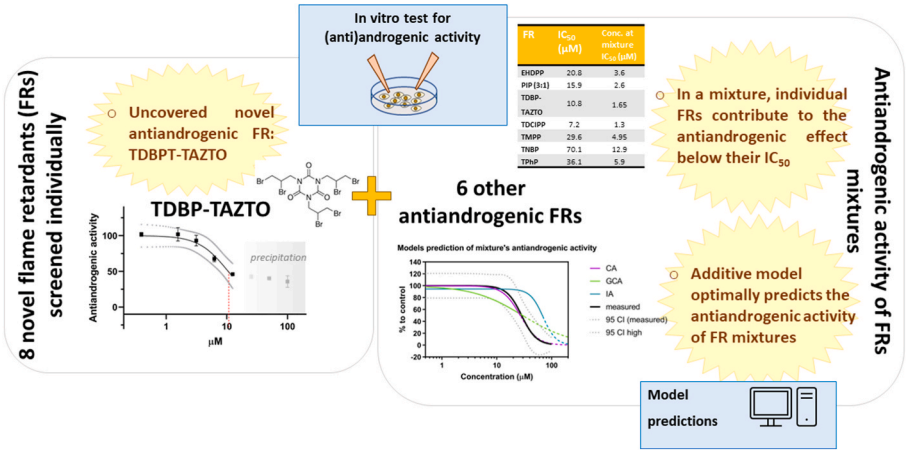
HIGHLIGHTS

- Androgen receptor (AR) agonist and antagonist activities were explored for eight novel flame retardants (FRs)
- TDBP-TAZTO (tris (2,3-dibromopropyl) isocyanurate) is anti-androgenic
- Four mixtures with different ratios of six anti-androgenic FRs, with or without TDBP-TAZTO, have anti-androgenic activity
- The concentration of each FR at the mixture's inhibiting concentrations (IC) is several times lower than the individual ICs
- The mixture effect is best predicted by the additivity model, with no clear evidence of synergy or antagonism

GRAPHICAL ABSTRACT



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ABSTRACT

In recent decades, male infertility has been on the rise, largely attributed to exposure to chemicals with endocrine-disrupting properties. The adverse effects of disrupting androgen actions on the development and reproductive health of children and adolescents have been extensively studied. Flame retardants (FRs), used in consumer products to delay flammability, have been identified as antagonists of the androgen receptor (AR), potentially leading to adverse outcomes in male reproductive health later in life. This study examined the interaction of eight novel FRs with the AR, employing an in vitro AR-dependent luciferase reporter gene assay utilizing MDA-kb2 cells. The investigation revealed the anti-androgenic activity of tris(2,3-dibromopropyl) isocyanurate (TDBP-TAZTO), a frequently detected FR in the environment. Furthermore, TDBP-TAZTO contributed to anti-androgenic activity when combined with six other anti-androgenic FRs. The mixture effects were predicted by three commonly employed models: concentration addition (CA), generalized CA, and independent action, with the CA model showcasing the highest accuracy. This suggests that all FRs act through a similar mechanism, as further confirmed by in silico molecular docking, indicating limited synergy or

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antagonism. Importantly, in the mixtures, each FR contributed to the induction of anti-androgenic effects at concentrations below their individual effective concentrations in single exposures. This raises concern for public health, especially considering the co-detection of these FRs and their potential co-occurrence with other anti-androgenic chemicals like bisphenols. Therefore, our findings, along with previous research, strongly support the incorporation of combined effects of mixtures in risk assessment to efficiently safeguard population health.

1. Introduction

Male infertility is a significant public health concern (Agarwal et al., 2015; Sharlip et al., 2002; WHO, 2020). The observed decline in semen quantity and quality over the past decades is believed to contribute to approximately half of all infertility cases, affecting around 15% of couples worldwide (Agarwal et al., 2015; Jørgensen et al., 2012). Along with genetic, epigenetic, and lifestyle factors, exposure to environmental chemicals with endocrine-disrupting properties, which interfere with the hormonal system, has been associated with detrimental effects on male reproductive health (Nordkap et al., 2012; Sharma et al., 2020). Specifically, the disruption of androgen actions can impact male sex differentiation during fetal development, resulting in adverse outcomes later in life (MacLeod et al., 2010; Schwartz et al., 2019; Sharpe, 2006). Therefore, identifying and prioritizing chemicals with anti-androgenic potential is critical for public health. However, this task is challenging due to the overwhelming number of chemicals present in the environment. To address this issue, the United States Environmental Protection Agency (US EPA) has launched the Toxicity Forecaster (ToxCast) and Toxicity Testing in the 21st Century (Tox21) programs. These programs employ high-throughput screening methods to evaluate numerous chemicals using a wide array of in vitro assays, including several reporter assays for androgen receptor (AR) activities (https://comptox.epa.gov/dashboard/) (Judson et al., 2009). Furthermore, considering the large number of chemicals detected in the environment, the co-occurrence of multiple chemicals may alter the overall activity of their mixtures. In practical terms, the dose at which a chemical contributes to inducing an effect within a mixture can often be different, in many cases lower, than its effective dose in a single exposure scenario (Carvalho et al., 2014; Conley et al., 2018, 2021). Such “mixture effects” have been observed in cases of co-exposure to several anti-androgenic chemicals (Ermler et al., 2011; Kjærstad et al., 2010; Kjeldsen et al., 2013; Orton et al., 2012, 2014). This poses particular concern for risk assessment, which currently relies mainly on effective doses derived from single-chemical experiments (European Commission, 2012).

Flame retardants (FRs) are industrial chemicals added to consumer products and building materials, such as electronics and textiles, to delay flammability (Melymuk et al., 2020). In recent years, numerous novel FRs have been introduced as replacements for the long-standing polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD), which were included in the Stockholm Convention in 2009 and 2013, respectively (The Stockholm Convention). These novel FRs exhibit highly diverse chemical structures and properties, including brominated, chlorinated, and organophosphate (OPFRs) FRs. They tend to migrate from products and are frequently detected in dust, food, as well as in human blood, urines, and milk (Demirtepe et al., 2019; Gbadamosi et al., 2021; Mitro et al., 2016; Rantakokko et al., 2019; Saillenfait et al., 2018; Schyff et al., 2023; Shi et al., 2016). Limited toxicological data for some of these novel FRs indicate that they may be associated with health outcomes (Bajard et al., 2019; Blum et al., 2019). In particular, a few rodent experimental and human epidemiological studies point to the impact of some OPFRs on male reproductive health (Carignan et al., 2018; Chen et al., 2015; Hales and Robaire, 2020; Meeker et al., 2013; Meeker and Stapleton, 2010). Moreover, in vitro studies published as peer-reviewed papers or reported in the ToxCast program and predictions from computational models have highlighted the anti-androgenic potential of many FRs (Bajard et al., 2021; Kojima et al., 2013; Rosenmai et al., 2021; Suzuki et al., 2013; Young et al., 2021). This raises concerns about potential combined effects on anti-androgenic activity when multiple FRs co-occur in exposed individuals. However, for most novel FRs, data on their effects on AR activity are currently lacking. In this paper, we investigate the androgenic and anti-androgenic activity of eight novel FRs, for which little or no information is available, as well as we examine FR mixture effects.

2. Material and methods

2.1. Chemicals

Bisphenol A bis(diphenyl phosphate) (BPA-BDPP), dechlorane A (DDC-CO), ethylenebistetrabromophthalimide (EBTEBPI), 2,3,4,5,6-pentabromophenol (PBP), phenol, isopropylated, phosphate (3:1) (PIP (3:1)), tris(2,3-dibromopropyl) isocyanurate (TDBP-TAZTO), triethyl phosphate (TEP), tri-n-butyl phosphate or phosphoric acid tributyl ester (TnBP), Phosphoric acid tripropyl ester (TnPP), tris(1,1,3-tribromo-2,2-dimethylpropyl)phosphate (TTBNPP), 2,4,6-tris (2,4,6-tribromophenoxy)-1,3,5-triazine (TTBP-TAZ), 2,2-bis(chloromethyl)trimethylenebis[bis(2-chloroethyl) phosphate] (V6) were purchased from Toronto Research Canada (TRC). 2,4,6-tribromophenol (246-TBP), decabromodiphenylethane (DBDPE), 2-ethylhexyl diphenyl phosphate (EHDPP), tris(1,3-ichloropropyl)phosphate (TDCIPP) and tricresyl phosphate (TMPP) were purchased from Tokyo Chemical Industry (TCI) and triphenyl phosphate (TPhP) from Sigma Aldrich. The full name, abbreviation, CAS number, source (including catalog and lot numbers), and some physico-chemical properties of the FRs are provided in Supplementary Table S1.

Stock solutions of chemicals under investigation were prepared in dimethyl sulfoxide (DMSO). In vitro assays, the final concentration of DMSO was maintained at 0.5% for all chemicals, except for TTBNPP, where the final DMSO concentration in the solution was 2%. This variation was necessary due to solubility issues encountered with the stock solution, which required a more diluted form. For all tests conducted throughout our study, we consistently utilized 0.5–2% DMSO as the solvent control, ensuring the absence of cytotoxicity (data not shown). Dihydrotestosterone (DHT) and hydroxyflutamide (Flu) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), and 200× concentrated stock solutions were prepared in DMSO.

2.2. Cell culture

We used MDA-kb2 cells (American Type Culture Collection, ATCC #CRL-2713), which are derived from a breast cancer cell line MDA-MB-453, stably transformed with the MMTV. luciferase.neo reporter gene construct (Wilson et al., 2002). Maintenance and handling of the cells were done as described previously by Bittner and colleagues (Bittner et al., 2012). Briefly, cells were cultivated at 37 °C without CO_2 in an L-15 medium with 10% fetal bovine serum (FBS, Biosera, Nuaille, France). For the assays, charcoal-stripped FBS (Sigma-Aldrich) was used to minimize the level of hormones. Cells were seeded in 96-well plates (Greiner Bio-One black/clear bottom plate for cytotoxicity assay and white/clear bottom plate for (anti)androgenicity assays, Kremsmünster, Austria) at approximately 25,000 cells/well. After 24-h incubation, chemicals were added to the wells, and the cells were incubated for another 24 h.

2.3. Cytotoxicity assay

To define the non-cytotoxic range for further testing, cytotoxicity was measured using 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM, Invitrogen, Waltham, Massachusetts, USA). After 24-h exposure, cells were washed with PBS (10 mM phosphate-buffered saline, pH 7.2), incubated with CFDA-AM (4 μM) in serum-free medium without phenol red for ~ 1 h, and fluorescence at 485/520 nm was measured following manufacturer's instructions using Biotek Synergy 4 (BioTek, Winooski, Vermont, USA).

2.4. Androgenic and anti-androgenic assays

The androgenic and anti-androgenic assays were calibrated using DHT as a reference agonist of the AR and Flu as its reference antagonist. After 24-h exposure, luciferase activity was measured as described previously (Bittner et al., 2012). Since both androgen and glucocorticoid receptors (AR and GR) are present in these cells and both can activate the MMTV reporter, MDA-kb2 cells are a reporter for both activities. To distinguish between AR and GR, chemicals inducing reporter activity were co-incubated with synthetic non-steroidal antiandrogen Flu (20 μM), a prototypical antagonist of AR. To test for anti-androgenic activity, FRs were co-incubated with 0.3 nM DHT (active androgenic steroid hormone). Calibration was conducted systematically on the same plate as the tested chemicals using a concentration series of DHT (for androgenicity testing) or Flu (for anti-androgenicity testing). In our experimental setup, the average EC_{50} for DHT was determined to be 0.20 nM [95% CI: 0.16; 0.23], while the average IC_{50} for Flu was found to be 1.51 μM [95% CI: 1.33; 1.69]. The dose-response curves illustrating these findings are presented in Supplementary Fig. S1.

2.5. Absorbance measurements to detect the precipitate formation

To evaluate how well the chemicals were dissolved and detect potential precipitation, chemical solutions were prepared exactly as for the other assays in transparent 96-well plates (TPP, Trasadingen, Switzerland) by diluting 200× concentrated stock solutions in DMSO into a culture medium. Absorbance (400 nm–650nm spectrum) was measured immediately after preparing the solution (0' time point, T0) and after 24-h incubation at 37 °C (24-h time point, T24) with the Biotek Synergy 4.

2.6. Measuring chemical concentration in the exposure medium

Samples of stock solutions in DMSO or exposure medium containing FR mixtures or individual chemicals were diluted in DMSO or medium, respectively, and finally in 50% vol methanol in water containing the isotopically labeled internal standards. The isotopically labeled internal standards employed for quantification were TDCIPP-D15, TnBP-D27, and TPHP-13C13. Specifically, TDCIPP and TPHP were quantified using their respective labeled analogs, while the remaining analytes were quantified using TnBP-D27. Samples were taken immediately after adding chemicals (T0) or after 24-h exposure (T24). Typical recoveries were between 93 and 105%, with variability (calculated as RSD, relative standard deviation) lower than 10%.

TDBP-TAZTO was analyzed using an Agilent 1290 Infinity liquid chromatography (HPLC) system. Chromatographic separation was accomplished using the Acquity BEH C-18 analytical column (100 × 2.1 mm, 1.7 μm particle size). The mobile phases for the gradient separation of the analytes were a 1 mM water solution of ammonium acetate (component A) and an acetonitrile solution with an addition of 1 mM ammonium acetate (component B). The flow rate was 0.25 mL·min-1, and the injection volume was 5 μL. A linear gradient began at an initial composition of A/B of 50:50 (v/v), ran to 10:90 over 6 min, where it was held for 8 min. The column was equilibrated for 5 min at the initial composition of the mobile phase. Analyte detection was performed by means of tandem mass spectrometer AB Sciex Qtrap 5500 operating in negative electrospray ionization mode at 450 °C with N2 as a nebulizer gas and an entrance potential of -4kV. Scheduled MRM was used to monitor the signal from *m/z* 727.4 → 79.0 for quantification and 727.4 → 80.9 for qualification. TDBP-TAZTO was quantified using 13C-labeled α-HBCD (hexabromocyclododecane). The quantitation limits (S/N 10:1, peak-to-peak method) were 0.02 ng/mL for a limit of detection (LOD) and 0.05 ng/mL for a limit of quantification (LOQ) (Supplementary Table S2).

Organophosphorus flame retardants (EHDPP, ip-TPP, TMPP, TnBP, TPHP, TDCIPP) were analyzed using the method previously described (Negi et al., 2021). The limit of detection (LOD) and quantification (LOQ) are provided in Supplementary Table S2. Phenol isopropylated phosphate (PIP) (3:1) was characterized using the LC-MS/MS technique, using the same HPLC method and ionization parameters as mentioned above. The mass spectrometer was operated in single ion monitoring (SIM) mode scanning for *m/z* 327.1 (triphenyl phosphate), 369.1 (isopropylphenyl diphenyl phosphate/tricresyl phosphate), 411.6 (diisopropylphenyl diphenyl phosphate) and 453.2 (trisisopropylphenyl phosphate). The composition of PIP (3:1) was estimated using the peak area ratio after solvent blank subtraction. Isopropylphenyl diphenyl phosphate (IPPDPP) and TPhP were the two main components, while diisopropylphenyl phenyl phosphate (DIPPPP) and tris (isopropylphenyl) phosphate (ip-TPP) were represented in small amounts (Supplementary Table S3). In a recent publication by Witchey et al. (2023), a similar finding was reported, demonstrating that the compound with CAS No. 68937-41-7, obtained from a different supplier (AmplaChem, Carmel, Indiana), contained IPPDPP, TPhP, DIPPPP, and ip-TPP. Consistent with our findings, IPPDPP was identified as the major component, while ip-TPP was present in smaller quantities.

Due to missing labeled internal standards for IPPDPP and DIPPPP, the total measured concentration of PIP (3:1) was inferred from measuring TPhP and ip-TPP in single exposures, considering that they represent 40% of the mix, based on the full scan analysis (Supplementary Table S3). In combined exposures, the nominal concentration of ip-TPP was calculated considering that it is approximately 1.1% of the PIP (3:1) nominal concentration.

2.7. Data and statistical analysis

Measurements were performed in triplicates and results (mean or median ± standard deviation (SD) or 95% confidence interval (CI)) were obtained from at least three independent repeats. Statistical significance was estimated with the Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunnett's Method, using the Sigma Plot 12.3 software. Graphs were prepared in GraphPad Prism 8.

2.8. Curve fitting, ICxs calculations, and mixture effect modeling

Seven different sigmoidal 2-4 parametric (IC_{50}/location parameter, Hill coefficient/slope parameter, top, bottom) functions (Hill, Weibull, and Logit models) (Supplementary Table S4) were used to model both individual chemicals and their mixtures. The equations were described previously (Zhu and Chen, 2016). After starting parameter estimation, we fitted all models and estimated CIs (Dybowski and Roberts, 2001) and goodness of fit statistics (coefficient of determination (R^2), the bias-corrected coefficient of determination (R^2_{adj}), root mean squared error (RMSE), mean absolute error (MAE), Akaike information criterion (AIC), the bias-corrected AICc, and Bayesian information criterion (BIC)) (Liao and McGee, 2003). We set the top parameter to 100 (0% effect) for both values, minimum and maximum and the bottom parameter to 0 (100% effect) as a minimum in the case when the model was 3- or 4-parameter. The Hill three model was shown as the best model for most dose-response curves and was reverse-queried to estimate IC_{50} and IC_{01} values (the concentrations of chemical triggering 50 or one, respectively, percentage of the maximum effect) presented in the

results section.

We predicted mixture effects/concentrations using three mathematical models for analyzing data and predicting the effect of mixtures: concentration addition (CA), generalized CA (GCA), and independent action (IA) model (a description of the functions and equations is provided in Appendix in Supplementary Material) (Faust et al., 2001; Howard and Webster, 2009). For each predicted data set, the IC_{01}, IC_{50}, and 95% CI values were estimated in the same way as for the original data. The analysis was performed using R version 4.2.1 (The R Project for Statistical Computing) and the Mixtox R package (https://cran.r-project.org/package=mixtox) (Zhu and Chen, 2016) modified for our purposes (Appendix in Supplementary Material).

2.9. Molecular docking

Molecular docking was performed using AutoDock Vina in PYMOL, as described previously (Negi et al., 2021). In brief, we used the UniProt database (https://www.uniprot.org/uniprot) to identify the active binding sites of the AR, the AutoDock Vina plugin in PyMOL 2.3 (Schrödinger LLC, NY, USA) for docking calculation, and the Docking pose was visualized using Discovery Studio Visualizer (BIOVIA, Dassault Systèmes, CA, USA). The X-ray crystallographic structures of human AR, Protein Data Bank (PDB) code 3L3X (DHT-bound AR in complex with the first motif of steroid receptor coactivator 3), and PDB code 1Z95 (AR Ligand-binding Domain W741L Mutant Complex with R-bicalutamide, AR antagonist) were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) PDB database (https://www.rcsb.org/).

3. Results and discussion

3.1. Assessment of androgenic and anti-androgenic activities of eight novel FRs

This study was prompted by a previous comprehensive investigation into the endocrine-disrupting activities of novel FRs (Bajard et al., 2021), which revealed shared anti-androgenic properties among several FRs and highlighted a critical lack or insufficiency of information for the majority of FRs to which individuals may be exposed. Consequently, we decided to employ the MDA-kb2 cell line to explore the androgenic and anti-androgenic characteristics of twelve novel FRs. These FRs were selected based on limited or insufficient data regarding endocrine-disrupting activities but with indications of potential exposure (e.g., registration in REACH – Registration, Evaluation, Authorisation and Restriction of Chemicals, and/or identification in human biomonitoring studies). Unfortunately, four out of these, namely DBDPE, DDC-CO, EBTEBPI, and TTBP-TAZ, could not be dissolved in DMSO or MeOH for further investigation, possibly due to their high hydrophobicity, as evidenced by their elevated logKow values (Supplementary Table S1) (Zhang et al., 2016). This partly explains the dearth of in vitro data for these chemicals, despite substantial evidence for their widespread use (DBDPE, for instance, is registered in REACH with 10,000–100,000 tonnage). Given the increasing reliance on alternative approaches in toxicological testing, it becomes particularly crucial to overcome this challenge and efficiently conduct in vitro testing for all chemicals involved.

Ultimately, we evaluated eight novel FRs that could be successfully dissolved in DMSO, namely TDBP-TAZTO, V6, TTBNPP, BPA-BDPP, TnPP, TEP, 246-TBP, and PBP. The results are summarized in Table 1.

We initiated our study by evaluating the cytotoxicity of the eight novel FRs on MDA-kb2 cells using the 5-CFDA-AM dye. In the case that substantial cytotoxicity was observed, characterized by a decrease in signal intensity, we employed a non-linear regression curve fitting to determine the IC_{50} value (Table 1). Notably, TEP, TTBNPP, and TnPP exhibited no cytotoxic effects even at the highest concentration tested. However, BPA-BDPP, 246-TBP, V6, PBP, and TBDP-TAZTO demonstrated some degree of cytotoxicity, with IC_{50} values ranging from 35 μM (PBP) to 241 μM (246-TBP). Subsequently, the androgenic and anti-androgenic activity assays were conducted at the concentrations below the respective IC_{50} values for cytotoxicity to ensure cellular viability and minimize cytotoxic effects.

Table 1
Summary of cytotoxic, androgenic, and anti-androgenic activities of eight novel flame retardants (FRs) in MDA-kb2 cells after 24-h exposure.

Flame retardant	Cytotoxicity	Androgenic activity		Anti-androgenic activity	
	IC_{50} [a] (μM)	LOAEC [b] (μM)	% of max. DHT effect	IC_{50} [a] (μM)	IC_{01} [a] (μM)
246-TBP	241	50	5.2 [c] [4.6; 5.7]	ND [e]	ND
BPA-BDPP	202	ND	ND	ND	ND
PBP	35	15	4.8 [c] [3.3; 6.3]	ND	ND
TBDP-TAZTO	62.9	ND	ND	10.8 [d] [7.4; 15.7]	0.8 [d] [NA; 4.2]
TEP	>300	ND	ND	ND	ND
TnPP	>250	ND	ND	ND	ND
TTBNPP	>200	ND	ND	ND	ND
V6	167	ND	ND	ND	ND

[a] IC_{50} or IC_{01}, the concentration causing 50% or 1%, respectively, inhibition of activity compared to the control.
[b] LOAEC the lowest adverse effect concentration – the lowest tested concentration, at which a significant effect was observed.
[c] Mean [lower; upper 95% confidence interval of the mean] of the effect at 50 μM for 246TBP and 22.5 μM for PBP.
[d] IC_{50}/IC_{01} value [lower; upper 95% confidence interval].
[e] ND, not determined (no effect was detected up to the highest non-cytotoxic concentration).

Among the tested FRs, V6, TNPP, BPA-BDPP, TDBP-TAZTO, and TEP did not significantly activate the AR receptor at the non-cytotoxic concentrations, as compared to DHT (Table 1). In the case of 246-TBP (at concentrations of 50 μM and above) and PBP (at concentrations of 15 μM and above), a weak yet statistically significant increase in the response of approximately 5% of the maximum level of activation by DHT was observed (Table 1 and Supplementary Fig. S2). This finding aligns with previous observations of weak activation in a ToxCast assay for both PBP and 246-TBP, falling below the automatically set cutoff of 20% activity (https://comptox.epa.gov/dashboard/) (Williams et al., 2017). Importantly, the observed effects of 246-TBP and PBP were specific to the AR receptor, as demonstrated by their loss when co-incubated with the FR and AR inhibitor flutamide (data not shown). While this weak AR activation is significant, its potential impact on health remains unclear.

To investigate the potential inhibitory effects of FRs on androgenic activity, MDA-kb2 cells were co-exposed to various concentrations of the tested FR and 0.3 nM DHT. The chosen DHT concentration slightly exceeded its EC_{50} value of 0.2 nM determined in our experimental setup. Among the eight novel FRs examined, only one exhibited inhibition of DHT-induced AR receptor activity: TBDP-TAZTO (Table 1, Fig. 1). The anti-androgenic activity of TDBP-TAZTO reached a plateau at concentrations above approximately 10 μM (Fig. 1), which may be attributed to chemical precipitation at concentrations surpassing its water solubility (Matsukami et al., 2021). Supporting this notion, we observed an increase in the absorbance (400 nm-650 nm) in the exposure medium starting at concentrations exceeding 12.5 μM. This effect was observed at both the initial (0') and 24-h time points, with a more pronounced increase in absorbance at 650 nm after 24-h incubation (as depicted in Supplementary Fig. S3). To assess the total TBDP-TAZTO levels in the DMSO stock solution (2 mM) and the culture medium (10 μM) at the start of exposure (T0) and after a 24-h exposure period (T24), measurements were conducted in these matrices. The results reveal the absence of significant contamination by the other examined FRs, with

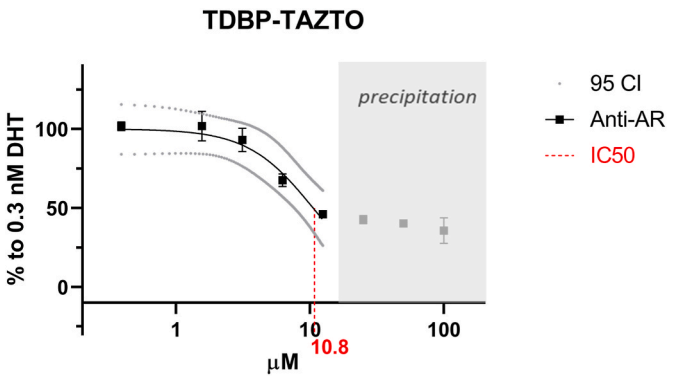

Fig. 1. Anti-androgenic activity of TDBP-TAZTO in MDA-kb2 cells after a 24-h exposure, with an IC_{50} value of 10.8 μM. The dose-response curve illustrates the inhibitory effect on androgen receptor activation, presented as a percentage of activation by 0.3 nM DHT. The dots represent the mean ± SD, the black lines depict the non-linear regression fit, and the grey lines indicate the 95% confidence interval. The precipitation of TDBP-TAZTO was confirmed through spectroscopic analysis (Supplementary Fig. S3). The X-axis is presented on a logarithmic scale.

the measured concentrations exhibiting negligible deviation from the nominal values and maintaining stability throughout the 24-h exposure period (Supplementary Fig. S4, Supplementary Tables S5–S7).

No significant anti-androgenic activity was observed for PBP at concentrations below its IC_{50} value of 35 μM for cytotoxicity. This finding contrasts with the results of two ToxCast assays that reported its antagonist activity (Williams et al., 2017). This inconsistency could potentially be attributed to the utilization of different experimental setups, including variations in agonist type and concentration. However, it is worth noting that the IC_{50} values reported in both ToxCast assays (29.2 and 73.9 μM) may have cytotoxic effects, as indicated by viability tests conducted within the ToxCast program (12.4 and 74.2 μM for the respective assays) (https://comptox.epa.gov/dashboard/chemical/invitrodb/DTXSID9022079). Therefore, the inhibitory effect reported in ToxCast may be attributed to a non-specific disruption of cellular metabolism rather than a specific inhibition of AR.

In our comprehensive in vitro testing for androgen receptor (AR) and anti-AR activities of eight novel FRs, we made several noteworthy observations. First, five FRs (V6, TNPP, BPA-BDPP, TTBNPP, and TEP) showed no significant activity at non-cytotoxic concentrations, indicating their inactivity in relation to the AR. Secondly, two FRs (246-TBP and PBP) demonstrated weak androgenic activity, albeit to a limited extent. Thirdly, we discovered that one FR (TBDP-TAZTO) exhibited clear anti-androgenic activity. It is worth noting that the anti-androgenic potential of TBDP-TAZTO had been predicted by the structure-based predictive model CoMPARA (Mansouri et al., 2020). Moreover, previous in vitro studies (Cao et al., 2018; Krivoshiev et al., 2016) have indicated estrogenic activity for TBDP-TAZTO, suggesting its potential acting as an endocrine disruptor. These findings contribute to our understanding of the AR and endocrine-disrupting activities of these novel FRs and emphasize the need for further investigation into their potential health implications.

In terms of anti-androgenic activity, TBDP-TAZTO exhibited an IC_{50} value of 10.8 μM, which is higher but still within the same order of magnitude (approximately seven times higher) when compared to the IC_{50} value of 1.51 μM for the positive control, Flu, which is a well-established antiandrogen with known in vivo activity. Flu has demonstrated the ability to induce intersex gonads in male medaka (Kang et al., 2006), highlighting its potency. Therefore, the relatively low IC_{50} value of TBDP-TAZTO suggests a significant potential for further anti-androgenic effects. This notion is further supported by an in vivo study conducted in zebrafish, which reported a range of potential reproductive and endocrine toxic effects associated with TBDP-TAZTO exposure (Zhang et al., 2011). These findings indicate the need for further investigation into the potential impacts of TBDP-TAZTO on reproductive and endocrine systems.

Moreover, several other in vivo and in vitro studies have indicated additional potential hazards associated with this emerging chemical. These investigations have identified adverse effects such as hepatotoxicity, neurotoxicity, and lung toxicity (Bar and Szychowski, 2022; Dong et al., 2015; Li et al., 2015; Szychowski et al., 2021; Zhang et al., 2011). Notably, TBDP-TAZTO is registered in REACH with an annual production volume ranging from 10 to 100 tons, and it has been frequently detected in soils, water, and dust in China, raising concerns about its potential to bioaccumulate and associated risk (Bar and Szychowski, 2022; Feng et al., 2021; Ruan et al., 2009).

3.2. *Effect of combined exposure to six anti-androgenic FRs, with or without TDBP-TAZTO*

Given that anti-androgenic activity has been previously reported for various other FRs, we sought to investigate whether TDBP-TAZTO could influence anti-androgenic activity when co-exposed with other anti-androgenic FRs. Specifically, we selected six FRs known to exhibit anti-androgenic activity: TDCIPP, TPhP, TMPP, EHDPP, PIP (3:1), and TnBP (Bajard et al., 2021; Kojima et al., 2013; Reers et al., 2016; Rosenmai et al., 2021; Suzuki et al., 2013). In our experimental setup using MDA-kb2 cells, we confirmed that all six FRs inhibited DHT-induced AR activation at non-cytotoxic concentrations. The dose-response curves followed a sigmoidal regression, as depicted in Supplementary Fig. S5. Table 2 includes the derived IC_{50} and IC_{01} values, which are also compared with values reported in the literature and from ToxCast for reference. Overall, the IC_{50} values obtained in our

Table 2
Summary of anti-androgenic activity obtained in this study for six flame retardants (FRs) in MDA-kb2 cells after a 24-h exposure, compared with their previously reported anti-androgenic activity. The mean of IC_{50} and IC_{01} values (μM) with 95% CI are provided unless stated otherwise.

Flame retardant	Cytotoxicity determined in this study	Anti-androgenic activity determined in this study		Anti-androgenic activity reported in literature [a]	Anti-androgenic activity reported in ToxCast assays [b]
	IC_{50} (μM)	IC_{50} (μM)	IC_{01} (μM)	IC_{50} or RIC_{20} (μM)	AC_{50} (μM)
EHDPP	61.2 [51.4; 72.8]	20.8 [9.3; 46.2]	4.4 [NA; 16.7]	6 (IC_{50})	inactive
PIP (3:1)	56.6 [47.6; 67.3]	15.9 [12.2; 20.7]	2.9 [NA; 7.9]	NT	31.7–49.9
TDCIPP	106.7 [103.1; 110.5]	7.2 [5.9; 8.8]	0.4 [NA; 1.9]	1.9–7 (IC_{50}); 1.9 (RIC_{20})	17–75.5
TMPP	100.6 [75.1; 134.9]	29.6 [21.9; 40.1]	7.3 [NA; 18.7]	4.1–10 (IC_{50}); 24 (RIC_{20})	57.3–253
TNBP	307 [296.5; 317.9]	70.1 [53.8; 91.4]	10.1 [NA; 29.3]	10–24 (RIC_{20})	inactive
TPhP	102.1 [97.3; 107.1]	36.1 [21.1; 61.8]	7.4 [NA; 25]	5.8–9 (IC_{50}); 17 (RIC_{20})	25.3

[a] Kojima et al. (2013); Rosenmai et al. (2021); Suzuki et al. (2013); Williams et al. (2017), IC_{50} or IC_{01}, the concentration causing 50% or 1%, respectively, inhibition of activity compared to the control, NT, not tested, RIC_{20}, 20% relative inhibitory concentration.

[b] https://comptox.epa.gov/dashboard/; more detailed information can be found in Bajard et al. (2021), AC_{50}, the concentration at which half of maximal activity is observed.

experimental setup align with the range of values previously documented in the literature (Bajard et al., 2021; Kojima et al., 2013; Rosenmai et al., 2021; Suzuki et al., 2013; Williams et al., 2017), whereas the AC_{50} values from ToxCast assays tend to be higher or indicate inactivity for some FRs (https://comptox.epa.gov/dashboard/).

There are two equally important approaches for assessing mixture toxicity and safety: (1) the whole mixture-based approach, which involves studying and evaluating the entire mixture as a single entity, and (2) the component-based approach, which assesses the individual substances and uses mathematical modeling to predict their combined effect. While testing real-life mixtures is crucial, our work focuses on investigating the overall mixture effect and evaluating the performance of different predictive models. In this context, determining proportions based on distinct fixed ratios is particularly suitable. These ratios are carefully selected to ensure a balanced contribution of each individual compound to the overall mixture effect, as utilized in similar studies (Ermler et al., 2011; Orton et al., 2012).

We evaluated four different mixtures comprising selected anti-androgenic FRs. These mixtures consisted of six OPFRs (TDCIPP, TPhP, TMPP, EHDPP, PIP (3:1), and TNBP) with (MixTD) or without (Mix) TDBP-TAZTO. The mixtures were tested at two distinct fixed ratios, referred to as MixTD50 or Mix50 (with proportions based on IC_{50} values) and MixTD01 or Mix01 (with proportions based on IC_{01} values), as shown in Table 3. It is worth noting that the proportions of TPhP, TMPP, EHDPP, and PIP (3:1) were relatively higher in Mix01 or MixTD01, while the proportions of TDCIPP, TnBP, and TDBP-TAZTO (for MixTD) were comparatively lower in Mix01 or MixTD01 than in Mix50 or MixTD50. This approach has proven valuable in designing mixtures and enhancing the assessment of mixture activity, with previous studies reporting significant differences in the activities of mixtures based on IC_{50} and IC_{01} values (Ermler et al., 2011; Orton et al., 2012).

None of the four mixtures exhibited agonistic activity on AR/GR receptors (Fig. 2). However, they displayed dose-dependent inhibition of DHT-induced androgenic activity, with IC_{50} values ranging between 28 and 39 μM. These values were lower than the IC_{50} value of 70.1 μM for the FR with the lowest activity, TnBP (Fig. 2, Table 4). To determine the concentration of each FR in the mixtures at the IC_{50}, we multiplied the IC_{50} of the mixture by the proportion of the chemical in the mixture (pi) (Table 3). This calculation revealed that when co-exposed with other anti-androgenic FRs, each FR contributes to the induction of anti-androgenic activity at concentrations several times lower than their individual IC_{50} values (Tables 2 and 3). Notably, the addition of TDBP-TAZTO, another AR antagonist, to the mixtures enhanced this effect (Table 3), demonstrating its similar contribution to the overall mixture effect, along with the other FRs.

The concentrations of chemicals in the cell culture medium were measured immediately after adding them to the culture medium (T0) and 24 h after starting exposure (T24) for both single exposures and mixtures. The measured levels of most chemicals remained relatively stable throughout the entire exposure period and fell within the range of nominal concentrations for both single and combined exposures (Supplementary Tables S5–S8, detailed description of the results in Supplementary Text S1). However, there were a few exceptions. The concentration of TMPP was approximately five times lower than expected, even at T0 (Supplementary Tables S5–S8). Additionally, the concentrations of TnBP and TDCIPP were substantially higher than expected in the mixtures, likely due to cross-contamination from other sources (Supplementary Tables S7 and S8, Supplementary Text S1).

Currently, hazard associated with chemical exposure is standardly assessed individually, deriving reference doses from single chemical-exposure experiments. To ensure the derived values are protective, uncertainty factors are generally applied to account for interspecies and interindividual variability, as well as extrapolation from laboratory bioassay or data quality (WHO, 2021). However, our findings that FRs when combined in mixtures induce anti-androgenic activity below their individual effective doses strongly emphasize the need to include these mixture (combined) effects in risk assessment schemes. One potential approach is to incorporate a mixture assessment factor (MAF) into single chemical risk assessments (Bopp et al., 2019; Swedish Government, 2019). Nonetheless, determining the appropriate value for this factor, which neither over nor underestimates risk, poses a significant challenge (Bopp et al., 2019; Swedish Government, 2019). Assuming additivity, a more rigorous approach involves summing the hazard index or relative potency factor adjusted for exposure (ECHA, 2013; Meek et al., 2011). However, this approach necessitates demonstrating additivity and consistently deriving individual reference doses for the same endpoint, as exemplified in studies on PBDEs and their impact on semen quality (Ermler and Kortenkamp, 2022) or neurodevelopmental toxicity (Martin et al., 2017). Regrettably, attaining the required high-quality evidence for each constituent of a mixture is a challenging task. In this context, computational models offer valuable potential for predicting the impact of co-exposures and combined effects (Backhaus, 2016).

3.3. Modeling the mixture effect

In the subsequent step, we employed various mathematical models capable of quantitatively predicting the dose-response relationship for the FR mixtures. The three commonly used models to represent the interactions between individual chemicals in co-exposure scenarios are the IA, CA, and GCA models (Backhaus, 2016; Kim et al., 2022). The IA (response addition, effects addition) model assumes that each chemical in the mixture acts independently of the others through different mechanisms. In this scenario, the expected effect of the mixture is based on the probabilistic risk associated with each chemical calculated, following the law of statistically independent events. The CA model, on the other hand, assumes that chemicals share the same mechanism but differ in potency. Therefore, it predicts that the effect of the mixture is the sum of all chemicals weighted by their individual potency. This model has been validated in numerous mixture studies (Martin et al., 2021) and is often considered as a reference. Deviations from this model may indicate antagonism (lower effect of the mixture than predicted by the CA model) or synergies (greater effect). The GCA model was developed to overcome the limitations of CA and IA models, particularly for chemicals with low (partial) efficacy. It is primarily used to predict the effects of mixtures containing diverse receptor ligand types, including partial agonists/antagonists, which are theoretically not suited for the CA or IA models (Hadrup et al., 2013; Howard and Webster, 2009). The appropriateness of the GCA, CA, and IA models for predicting the anti-androgenic effects of the FR mixtures was compared

Table 3
Proportions and concentrations of flame retardants (FRs) in four mixtures. The table presents the proportion of individual flame retardants (FRs) in each of the four mixtures, along with their corresponding single concentrations (Ci) within the mixture at 50% inhibition of androgen receptor (AR) activation. The IC_{50} values of the mixtures are as follows: Mix50 – 39 μM, Mix01 – 37 μM, MixTD50 – 33 μM, and MixTD01 – 28 μM.

Flame retardant	Mix50		Mix01		MixTD50		MixTD01	
	pi	Ci in IC_{50} mix [a]	pi	Ci in IC_{50} mix [a]	pi	Ci in IC_{50} mix [a]	pi	Ci in IC_{50} mix [a]
EHDPP	0.11	4.3	0.16	5.9	0.11	3.6	0.16	4.3
PIP (3:1)	0.09	3.5	0.11	4.1	0.08	2.6	0.11	3.1
TDBP-TAZTO	–	–	–	–	0.05	1.65	0.03	0.8
TDCIPP	0.04	1.6	0.02	0.7	0.04	1.3	0.02	0.4
TMPP	0.16	6.2	0.26	9.6	0.15	4.95	0.25	7.0
TNBP	0.41	16.0	0.18	6.7	0.39	12.9	0.18	4.9
TPhP	0.19	7.4	0.27	10.0	0.18	5.9	0.27	7.4

[a] The concentration of each chemical at the IC_{50} for a given mixture: IC_{50} (in μM) of the mixture multiplied by the proportion of the given FR in the mixture (pi).

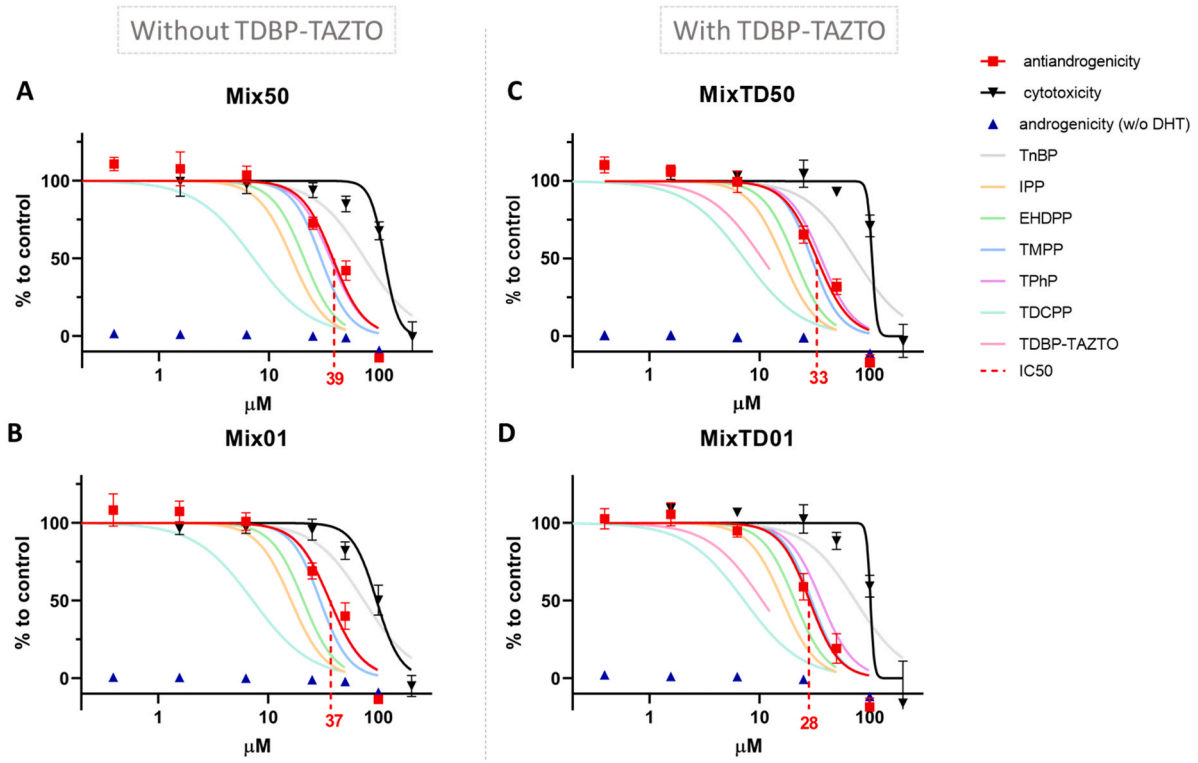

Fig. 2. Effects of four flame retardant (FR) mixtures on androgen receptor (AR) activity and cell viability in MDA-kb2 cells after 24-h exposure. As a control, 0.3 nM DHT was used for androgenic activity assessment and a solvent control for cytotoxicity assessment. The IC_{50} values for the anti-androgenic activity of each mixture: 39 μM for Mix50, 31 μM for Mix01, 33 μM for MixTD50, and 28 μM for MixTD01. Panels (**A**) and (**B**) show the cytotoxic, androgenic, and anti-androgenic activities of mixtures without TDBP-TAZTO, while panels (**C**) and (**D**) depict the same activities for mixtures containing TDBP-TAZTO. The dose-response curves are presented for the mixtures, with individual data points included, and the proportions of individual chemicals in the mixtures are based on their IC_{50} values in panels (**A**) and (**C**), or IC_{01} values in panels (**B**) and (**D**). Additionally, the dose-response curves for the anti-androgenic activity of individual FRs are shown. The X-axis is displayed on a logarithmic scale.

Table 4
Measured anti-androgenic activity of four flame retardant mixtures, along with their corresponding predictions by three computational models. The anti-androgenic activity is expressed as IC_{50} (inhibitory concentration 50%) and IC_{01} (inhibitory concentration 01%).

	Mix50		Mix01		MixTD50		MixTD01	
	IC_{50} (μM)	IC_{01} (μM)	IC_{50} (μM)	IC_{01} (μM)	IC_{50} (μM)	IC_{01} (μM)	IC_{50} (μM)	IC_{01} (μM)
Measured	39	8.2	37	7	33	7	28	7
IA	75	ND	67	ND	70	ND	68	ND
CA	30	4.4	28	5.5	28	3.3	27	4.6
GCA	31	0.3	28	0.3	32.5	0.2	29	0.25
MDR for CA	0.77	0.54	0.76	0.79	0.85	0.47	0.96	0.66

CA, concentration addition; GCA, generalized concentration addition; IA, independent action; MDR, model deviation ratio, for the CA model (the ICx value predicted by the CA model divided by the ICx value measured); ND, not determined – IC_{01} for IA could not be derived because the dose-response curve dose goes up to 99% of DHT activity.

for all four different mixtures of FRs.

For all four mixtures, the CA model exhibited the closest correspondence to the experimental measurements, as evidenced by the predicted dose-response curves (Fig. 3, Table 4). The accuracy of the model was assessed by calculating the model deviation ratio (MDR), which is obtained by dividing the predicted effect by the observed effect (Table 4). A MDR greater than two would indicate some level of synergy, while a MDR lower than 0.5 would suggest antagonism (Cedergreen, 2014). In the case of all four mixtures, the MDR values calculated based on IC_{50}s ranged between 0.5 and 1, indicating that additivity provides the best prediction for the mixture effect, albeit slightly overestimating it.

This observation holds true for Mix01 and Mix50, where all FRs in these mixtures were chosen based on a similar mode of action (anti-AR) and exhibit full antagonistic properties. Consistently, molecular docking predictions comparing the binding energy between binding sites of the AR support this finding, as they indicate that the FRs have a higher affinity for the same binding pocket or demonstrate a similar affinity for both (TDCIPP and TnBP) (Supplementary Table S9).

Notably, the CA model provided a more accurate prediction than the GCA model, even for MixTD50 and MixTD01, which is somewhat surprising considering that TDBP-TAZTO does not exhibit the full inhibitory effect within the tested concentration range. This suggests that, under the experimental conditions employed, where precipitation does not occur, TDBP-TAZTO behaves as a full antagonist, similar to the other six FRs in the mixture. The comparable effects observed in mixtures with different proportions of chemicals (Mix01 vs. Mix50 and MixTD01 vs. MixTD50) further support this conclusion. The applicability of the GCA model may not be universal for all chemicals with incomplete dose-response curves, as it was originally designed (Howard and Webster, 2009). In cases like TDBP-TAZTO, where the plateauing is attributed to solubility issues rather than partial activity, the CA model is likely to be the most appropriate choice.

3.4. Study limitations

This study focuses on exploring a specific molecular endpoint, representing the molecular initiating event or key event at the molecular level, without examining the downstream events. As a result, it remains uncertain whether the additive effects observed at the molecular level

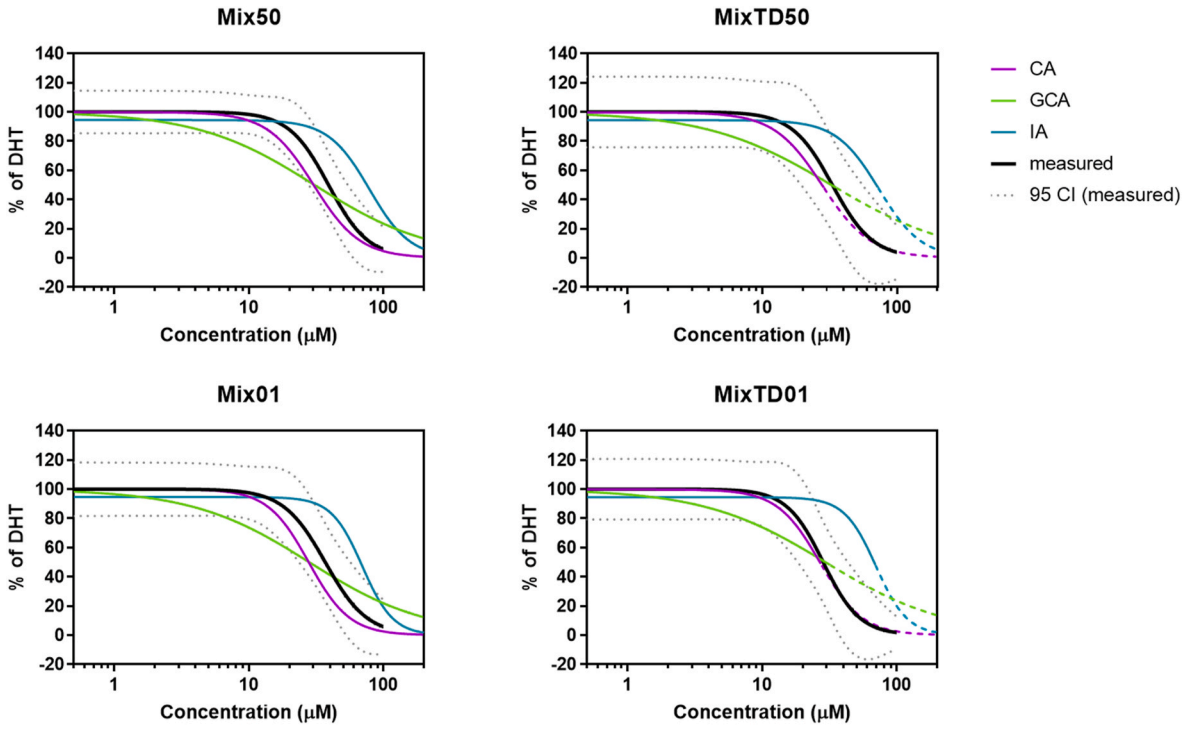

Fig. 3. Measured vs. predicted dose-response curves for the anti-androgenic activity of four mixtures of flame retardants in MDA-kb2 cells after 24-h exposure. The solid lines depict the measured or predicted dose-response curves, representing the actual anti-androgenic activity. The dashed part of the solid lines shows the predicted activity of the MixTD50 and MixTD01 mixtures beyond the highest activity achieved by the chemical with limited efficacy (TDBP-TAZTO). This emphasizes the restricted predictions of the IA (independent action) and CA (concentration addition) models, particularly in the presence of TDBP-TAZTO. The X-axis represents the logarithmic scale of the dose concentrations. GCA, generalized CA.

would also manifest at the apical endpoint. However, previous evidence supports the impact of novel FR mixtures on male reproductive health, with studies linking aberrant DNA methylation in sperm cells of men to higher levels of TPhP, EHDPP, TDCPP, or PIP (3:1) metabolites, particularly with multiple exposures (Soubry et al., 2017).

Another limitation is that the dose-response curve for MixTD50 and MixTD01 could only be partially predicted using the CA and IA models. This is because TBDP-TAZTO has limited efficacy, and its dose-response curve is incomplete, reaching only up to approximately 50% of DHT due to its precipitation at higher concentrations (above 12.5 µM) (Fig. 1, Supplementary Fig. S3). Nevertheless, the concentrations of TBDP-TAZTO in MixTD50 or MixTD01 mixtures did not surpass the precipitation limit, with concentrations up to 5 µM. Additionally, we confirmed that there was no precipitation within the tested concentration range for MixTD50 and MixTD01 (data not shown). Therefore, we present the complete dose-response curves for the CA and IA models, and we indicate the extrapolated part of the predicted lines with dashed lines, where the curve falls below 50% of DHT (Fig. 3).

Lastly, it is important to note that while our study measured the actual concentrations in a medium, we did not address the fraction of biologically available FRs (such as intracellular levels). Furthermore, without information on toxicokinetics and in vitro-in vivo extrapolation (IVIVE), we cannot conclusively determine the environmental relevance of the concentrations and ratios studied.

4. Conclusions

In conclusion, our study provides experimental evidence for the anti-androgenic properties of TBDP-TAZTO, with an IC_{50} value of 10.8 µM. Considering the emerging evidence of toxicity and exposure (Bar and Szychowski, 2022; Zhang et al., 2011), further investigation into its toxic potential and endocrine-disrupting properties, particularly its impact on the androgen receptor, is crucial.

Our results also reinforce previous studies indicating that TDCIPP, TPhP, TMPP, EHDPP, PIP (3:1), and TnBP are anti-androgenic chemicals. Notably, TDCIPP appears to be the most potent anti-androgenic FR, consistent with other reports of IC_{50} values below 10 µM (Kojima et al., 2013; Rosenmai et al., 2021; Suzuki et al., 2013).

For the first time, we demonstrate the additivity of anti-androgenic activity when these FRs are simultaneously exposed. As a result, each FR contributed to inhibiting the AR at concentrations substantially lower than their individual effective doses. Considering the frequent co-detection of these FRs or their metabolites in environmental and human samples, simultaneous exposure to these chemicals is a realistic scenario (Carignan et al., 2018; Cequier et al., 2014; He et al., 2018; Rantakokko et al., 2019; Saillenfait et al., 2018). Moreover, the co-occurrence of FRs with other anti-androgenic chemicals, such as phthalates, bisphenols, and pesticides, which have also been associated with mixture effects, could potentially amplify the overall mixture potency (Ermler et al., 2011; Kjærstad et al., 2010; Kjeldsen et al., 2013; Orton et al., 2014). It is important to note that accurate predictions were obtained using the CA model for several, but not all, of the mixtures in the previous studies, possibly increasing the impact on their mixture potency.

This study emphasizes the need to consider the mixture effect in chemical regulation schemes (Kienzler et al., 2016; Kortenkamp and Faust, 2018). Currently, these schemes focus on individual chemicals and should incorporate the risks associated with combined exposures. Approaches such as using the MAF or summing hazard index or relative potency factors, assuming additivity, can be employed to address this concern (ECHA, 2013; Kienzler et al., 2016; Meek et al., 2011). Our results confirm that assuming additivity for predicting the effects of combined exposures effects seems to be a pragmatic, efficient, and protective approach in the majority of the cases (Martin et al., 2021).

Author contribution statement

Lola Bajard: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing- Reviewing and Editing, Visualization Hana Vespalcova: Formal analysis, Writing- Reviewing and Editing Chander K. Negi: Investigation, Formal analysis, Writing- Reviewing and Editing Jiri Kohoutek: Investigation, Formal analysis, Writing- Reviewing and Editing Ludek Blaha: Conceptualization, Funding acquisition, Supervision, Writing- Reviewing and Editing Iva Sovadinova: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Writing – original draft, Writing- Reviewing and Editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.140004.

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