A Conflicted Tale of Two Novel AR Antagonists In Vitro and In Vivo: Pyrifluquinazon Versus Bisphenol C

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ABSTRACT

Chemicals that disrupt androgen receptor (AR) function in utero induce a cascade of adverse effects in male rats including reduced anogenital distance, retained nipples, and reproductive tract malformations. The objective of this study was to compare the in vitro and in utero activities of two novel AR antagonists, bisphenol C (BPC) and pyrifluquinazon (PFQ). In vitro, BPC was as potent an AR antagonist as hydroxyflutamide. Furthermore, BPC inhibited fetal testis testosterone production and testis gene expression ex vivo. However, when BPC was administered at 100 and 200 mg/kg/d in utero, the reproductive tract of the male offspring was minimally affected. None of the males displayed reproductive malformations. For comparison, in utero administration of flutamide has been shown to induce malformations in 100% of males at 6 mg/kg/d. In vitro, PFQ was several orders of magnitude less potent than BPC, vinclozolin, or procymidone. However, in utero administration of 12.5, 25, 50, and 100 mg PFQ/kg/d on GD 14–18 induced antiandrogenic effects at all dosage levels and 91% of the males displayed reproductive malformation in the high dose group. Overall, BPC was ~380-fold more potent than PFQ in vitro, whereas PFQ was far more potent than BPC in utero. Incorporating toxicokinetic and toxicodynamic data into in vitro to in vivo extrapolations would reduce the discordance between the in vitro and in utero effects of PFQ and BPC and combining in vitro results with a short-term Hershberger assay would reduce the uncertainty in predicting the in utero effects of antiandrogenic chemicals.

Key words: antiandrogen; risk assessment; male rat reproductive tract; pyrifluquinazon; bisphenol C.

Exposure to androgen receptor (AR) antagonists, such as the pesticides vinclozolin or procymidone, or the drug flutamide, during sexual differentiation disrupts development of the male rat reproductive system. Metabolites of these chemicals bind the AR and disrupt androgen-induced gene expression (Rosen et al., 2005) and protein synthesis causing agenesis/aplasia of

several androgen-dependent tissues among other effects. Male offspring display reduced anogenital distance (AGD), retained female-like nipples/areolae, reduced accessory sex gland size or agenesis, and reproductive tract malformations including the presence of a vaginal pouch, cleft phallus, hypospadias, epididymal agenesis, and undescended testes (Gray et al., 1994, 1999;

Fussell et al., 2015; McIntyre et al., 2001; Miyata et al., 2002; Ostby et al., 1999). In utero, flutamide is significantly more potent than either vinclozolin or procymidone, although these two pesticides are approximately equipotent.

The current study was designed to compare the effects of pyriproxyfen (PFQ) with bisphenol C (BPC also known as dihydroxy-methoxychlor olefin or BPC2) in vitro and in utero. As compared with the aforementioned well-studied AR antagonists, there is only a single published study on the effects of PFQ on androgen-dependent tissues weights in males in the Hershberger assay (Yasunaga et al., 2013) and none on BPC.

BPC is used in the production of plastic polymers. These polymers have mechanical and thermal characteristics equivalent to or better than other bisphenol A analogs, and they are more ignition resistant making them useful for aircraft interior materials. BPC is a bisphenol analog that has been shown to bind AR with high affinity (Fang et al., 2003) and act as an AR antagonist in vitro at concentrations orders of magnitude below all other bisphenol analogs (Conley et al., 2015; Wang et al., 2014) and other environmental antiandrogens. Although several studies have shown that BPC is a very potent AR antagonist in vitro, the antiandrogenic activity and toxicokinetics (TK) of BPC have not been studied in vivo. If the in vitro potency can be used to predict the potency in utero then BPC would be as potent as flutamide and far more potent than any other environmental chemical studied to date. Wang et al. (2014) also reported that BPC inhibited steroidogenesis in H295R cells. For this reason, in addition to evaluating the antiandrogenic potential of BPC in vitro and in utero, we also evaluated the ability of BPC to reduce fetal rat testosterone production and inhibit testis mRNA expression for genes involved in steroid synthesis, transport, and sexual determination.

PFQ is a new active ingredient insecticide used for the control of sucking insects (eg, aphids, thrips, whiteflies), which activates the transient receptor potential vanilloid channel complex, which in turn overstimulates stretch receptors in joint in the legs and antennae, disturbing insect locomotion and feeding. PFQ was initially registered in Japan in 2007 and was registered for use on horticultural crops in greenhouses in the United States by the Environmental Protection Agency (U.S. EPA) in 2013 <https://www.regulations.gov/document?D=EPA-HQ-OPP-2011-0971-0008>. At present, EPA is considering an application proposing to register the first food and outdoor uses for PFQ in the United States <https://www.federalregister.gov/documents/2018/11/26/2018-25690/pyriproxyfen-pesticide-tolerances>.

Yasunaga et al. (2013) reported that PFQ displayed AR antagonism in HEK293 cells transfected with rat AR (rAR) but it did not inhibit androgen-induced luciferase expression using MDA-Kb2 cells, which constitutively express human AR (hAR). They also reported that PFQ did not bind rat prostatic AR (rAR). They proposed that the rAR antagonism was induced because of a decline in cellular AR protein levels rather than via AR binding and that PFQ interacted differently with rAR than hAR. They also reported that PFQ inhibited androgen-dependent tissue growth in the Hershberger assay (Yasunaga et al., 2013). The current study re-evaluated these hypotheses using a chimpanzee (*Pan troglodytes*) AR (chAR) binding assay and AR transcriptional activation assays (ARTA) with either rAR, chAR, or hAR. The objectives were to determine if PFQ was antiandrogenic with rAR but not with hAR or chAR, and if PFQ did or did not bind chAR. The chAR binding assay and the CV-1 ARTAs were developed and validated by Hartig et al. (2008). Both assays have high-throughput potential and the CV-1 transduction assays have

high fold inductions, low background, and high Z scores. The chAR was first sequenced by Choong et al. (1998) and they found that the protein encoded by this sequence is 99.8% homologous to the hAR. The only substitutions in the amino acid sequence were (1) asparagine 233 was replaced with serine and (2) serine 494 was replaced with glycine.

In addition to the above in vitro studies with PFQ and BPC, we conducted one-generation reproduction studies with BPC and PFQ. Pregnant rats were exposed to varying doses of BPC and PFQ during sexual differentiation on gestational days (GD) 14–18 and reproductive development of the male offspring was monitored through full maturity. Exposure to antiandrogens during this window induces malformations in androgen-dependent tissues, reduces AGD, and induces retained female-like nipples/areolae (Wolf et al., 2000). The effects of in utero BPC have not been studied and the detailed results of a PFQ reproduction study, conducted in support of registration, are unpublished. A summary of the PFQ reproduction study (discussed in <https://www.federalregister.gov/documents/2018/11/26/2018-25690/pyriproxyfen-pesticide-tolerances>; last accessed January 28, 2019) reports that PFQ reduced AGD in male rats and altered some reproductive tissues. However, comprehensive life-long analyses of the dose-related effects (such as NOAELs and ED50s) of PFQ on F1 male rat reproductive development have not been published.

As compared with many endocrine signaling pathways, a variety of robust, precise in vitro, and short-term in vivo assays to identify antiandrogenic chemicals have been developed and validated. As highlighted by the results of the current study with BPC and PFQ, what is lacking are TK and toxicodynamic data (TD) that would enhance the quantitative extrapolation from in vitro assays of AR antagonism to the adverse effects of BPC and PFQ in vivo. By themselves, in vitro assays are limited because they do not account for in vivo TK factors affecting internal exposures (adsorption, distribution, metabolism, and excretion) or key TD tissue-specific responses to antiandrogenic chemicals (eg, AR levels, cofactors, and androgenic hormone concentrations).

METHODS AND MATERIALS

Chemicals. PFQ (CAS no. 337458-27-2) was purchased from Chem Services, Inc. (lot no. 2110000, cat no. RPN-13158-5G, purity = 99%) and purity was verified by the supplier. The vehicle used to deliver PFQ was laboratory-grade corn oil (Sigma; CAS no. 8001-30-7) in 2.5 ml/kg body weight (PFQ cost from the supplier is >\$3500/g as of August 26, 2018). BPC [also referred to as dihydroxy-methoxychlor olefin, CAS 14868-03-2] was purchased from TCI America (lot no. WH66A-LQ, cat no. 03223; purity = 98%) and purity was verified by the supplier. Inert methyltriene (R1881) (CAS no. 965-93-5 and 3H-R1881, 3089.5 GBq/mM cat# NET-590) was obtained from NEN Life Science Products, Inc. (Boston, Massachusetts). Dihydrotestosterone (CAS no. 521-18-6, >97.5% pure) was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). Hydroxyflutamide (CAS no. 52806-53-8) was provided by R.O. Neri (Schering Corp., Bloomfield, New Jersey) and purity was determined by the vendor.

Animals. Timed pregnant Sprague Dawley (SD) rats, approximately 90 days old, were purchased from Charles River Laboratories (Raleigh, North Carolina) and shipped to EPA on GD 2 (date of sperm plug positive = GD 1). Animals were housed individually in clear, polycarbonate cages (20 × 25 × 47 cm) lined with laboratory-grade heat-treated pine shavings (Northeastern

Products, Warrensburg, New York), with a 12:12 light: dark photoperiod (lights off at 18:00) at 20°C–22°C and 45%–55% humidity. Dams and offspring were provided with NIH07 rat chow during gestation and lactation and offspring were provided NTP 2000 rodent diet after weaning. Rats had access to filtered (5 micron) municipal tap water ad libitum which is tested every 4 months for a subset of heavy metals, pesticides, and other chemical contaminants and tested monthly for *Pseudomonas*. These studies were conducted under protocols approved by the National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee at a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

In vitro methods assessing androgen receptor antagonism of pyrifluquinazon and bisphenol C. In the *in vitro* studies with PFQ we assessed the ability of PFQ to inhibit the binding of DHT to α AR binding in a cell-free assay and determined the antiandrogenic activity of PFQ in CV-1 cells transfected with rAR and hAR. The final study examined the antiandrogen potencies of BPC, PFQ, flutamide, and vinclozolin in ARTA assays with CV-1 cells transduced with α AR. These experiments were designed to re-examine previously reported antiandrogenic activity, or lack thereof, of PFQ and BPC.

Assessment of pyrifluquinazon as an androgen receptor ligand in a competitive binding assay with chimpanzee AR. In the first experiment, the ability of PFQ to displace radiolabeled R1881 (2 nM) from α AR was examined in a cell-free competitive binding assay (Hartig et al., 2008) and the K_i value for PFQ was calculated using the Cheng-Prusoff equation as follows:

$$K_i = EC_{50} / \left(1 + ([L]/K_d) \right)$$

Where EC_{50} is the Prism value for PFQ, L is the concentration of R1881 used in the assay (2 nM), and K_d is the dissociation constant for R1881 (1 nM) in this assay.

Androgen receptor transcriptional activation assessment of pyrifluquinazon as an androgen receptor antagonist with rat (rAR) and human (hAR) androgen receptors. The second *in vitro* experiment examined the antiandrogenicity of PFQ using rAR and hAR to determine if we could replicate the observation of Yasunaga et al. (2013). They reported that PFQ was antiandrogenic with a cell line that expressed rAR (HEK293R) but not in a different cell line that expressed hAR (MDA-Kb2). Our experiment was conducted in CV-1 cells transfected (FuGENE HD, Promega, Madison, Wisconsin, according to manufacturer's protocol) with one of the two receptors (rAR or hAR) and the MMTV-Luciferase reporter-promoter construct. There were four independent runs with each receptor competed against 30 pM DHT. K_i values were calculated as above except that the reference ligand was DHT which has a K_d value of 0.028 nM in this assay.

Androgen receptor transcriptional activation assessment of the antiandrogenic activity of pyrifluquinazon in CV-1 cells transduced with chimpanzee AR. The third experiment used CV-1 cells that were adenovirus transduced with chimpanzee AR (α AR) and the MMTV-luciferase promoter-reporter construct (Hartig et al., 2002, 2007, 2008). PFQ was competed against 0.1 and 1 nM DHT and K_i values were calculated as above except the equation was adjusted to the K_d value of DHT and the concentration of DHT used in the assay. CV-1 cells were treated with DHT and varying

concentrations of PFQ to determine if PFQ was antiandrogenic and if the concentration-response curve shifted downward as the DHT concentration was reduced from 1 to 0.1 nM. The ARTA assays in this study included an assessment of cytotoxicity using the MTT assay to separate specific from nonspecific effects on luciferase expression (Hartig et al., 2008). K_i values were calculated as above with DHT as the ligand with a K_d in this assay of 0.0537 nM (based upon 31 runs with DHT).

Androgen receptor transcriptional activation comparison of pyrifluquinazon, bisphenol C, hydroxyflutamide, and vinclozolin as AR antagonists. A fourth *in vitro* experiment also used CV-1 cells that were adenovirus transduced with α AR and the MMTV-luciferase promoter-reporter construct. This experiment was designed to compare the antiandrogenic activity of PFQ, BPC, hydroxyflutamide, and vinclozolin against 100 pM DHT (2–4 independent runs per chemical). The objective was to directly compare the relative potencies of these chemicals *in vitro* to their *in vivo/in utero* effects on male rat reproductive tract development. The data on the *in utero* effects of flutamide effects on F_1 male rat reproductive development were taken from three dose response publications that taken together used dosage levels ranging over more than 5 orders of magnitude (Fussell et al., 2015; McIntyre et al., 2001; Miyata et al., 2002).

Prenatal assessment of the effects of bisphenol C administered on GD 14–18 on fetal testis function. The prenatal assessments of the effects of BPC on testis testosterone production (T Prod) and mRNA expression were conducted in three blocks, with approximately 15 dams per block. The dams in each block were ranked by body weight and assigned to groups (three dams per group) with each group having similar mean weights and variances. We did not conduct a similar fetal study with PFQ because the expense of the pure chemical limited the number of *in vivo* studies we could conduct as this time.

Timed pregnant rats were dosed by oral gavage with vehicle (corn oil) or BPC from GD 14–18, the period that covers the critical window of sex differentiation in the male rat fetus. On GD 18, dams were euthanized by decapitation within a 2-h time frame between 08:00 and 10:00 am Eastern Standard Time to avoid any potential confounding effects of fetal growth or time of day on the fetal endpoints. T Prod was measured in 36 males from 12 litters, 34 males from 12 litters, 33 males from 11 litters, 27 males from 9 litters, and 27 males from 9 litters in the 0, 50, 100, 200, and 300 mg/kg/d dose groups, respectively.

Fetal testes were removed (one testis from three males per litter where possible) and immediately transferred to a single well in a 24-well plate (one testis per well) containing 500 μ l M-199 media without phenol red (Hazelton Biologics, Inc., St. Lenexa, Kansas), supplemented with 10% dextran-coated charcoal-stripped fetal bovine serum (GE Healthcare Life Sciences, HyClone Laboratories, Logan, Utah). Testes were incubated in a humidified atmosphere for 3 h. at 37°C on a rotating platform. Following incubation, media were removed and stored at –80°C until analyzed for testosterone (T). The level of T in the media was measured by radioimmunoassay (RIA) according to the manufacturer's instructions (Diagnostic Products Corporation Coat-A-Count kits, Siemens Corp., Los Angeles, California) and as outlined in Hannas et al. (2012). The intra-assay coefficient of variation was 3.1% (based on variability of the standard curve) and the inter-assay coefficient of variation was 13.7%. Cross-reactivity of the T antibody with dihydrotestosterone (DHT) was 3.2%. The limit of detection was 0.2 ng/ml for T.

In addition to measuring ex vivo fetal testis testosterone production, we measured mRNA expression levels in the fetal rat testis using targeted, custom-designed qPCR 96 gene arrays (arrays described in detail by Hannas et al. [2012]) to detect BPC-induced alterations in gene expression. The ΔC_T value for each gene was determined by dividing the gene C_T value by the mean C_T value of three housekeeping genes. The $2^{-\Delta\Delta C_T}$ method was used to analyze data and change in gene expression levels were reported as fold change or the ratio of the BPC-treated groups to the respective control group. Changes in mRNA expression were considered as statistically significant when the F-value from the ANOVA model was significant at $p < .01$ level or less.

Postnatal experiment with pyrifluquinazon administered on gestational days 14–18. Twenty-five pregnant rats were randomly assigned to one of 5 treatment groups and gavaged (orally) daily on GD 14–18 with 0, 12.5, 25, 50, or 100 mg PFQ/kg/d in corn oil (5 dams/dose group). Doses were adjusted daily based upon maternal body weights. Dams gave birth naturally (day of birth = PND 0), offspring were weighed, and AGD was measured using a dissecting microscope on postnatal day (PND) 2 as described previously (Gray et al., 1999). AGD was defined as the distance between the rostral end of the anal opening and the base of the genital papilla. On PND 13, male offspring were assessed for areola or nipple retention and examined for genital malformations. At PND 23, male and female offspring were weaned and housed in unisexual groups of two per cage with a sibling where possible. Following weaning, dams were euthanized and the number of uterine implantation sites was recorded. The number of fetal/F₁ offspring mortality was determined as the number of uterine implants for a single litter minus the number of live pups at different ages. Beginning on PND 41, male offspring were examined 3 days each week for the onset of puberty (Monday, Wednesday, and Friday) until PND 52, and the age and body weight at puberty (defined as complete preputial separation) were recorded. This assessment did not include males with gross genital malformations.

Female offspring were necropsied at approximately 6 months of age and body and ovarian weights were recorded. F₁ females were also examined for gross external abnormalities and reproductive tract malformations (including uterine or vaginal aplasia or agenesis, and hydrometrocolpos).

Male offspring were necropsied at about 9 months of age and body and reproductive organ weights were recorded, and gubernacular ligaments measured. F₁ males were also observed for retained nipples, hydronephrosis, and bladder stones and examined for internal and external reproductive tract malformations.

Postnatal experiment with bisphenol C administered on gestational days 14–18. The BPC experiment was executed similar to the PFQ experiment described above, with the following exceptions. The BPC postnatal study was intended as a preliminary to a larger study with larger sample sizes and several lower dosage levels if it was indeed as potent as one would predict from the in vitro data. Given the minimal effects seen in the current study at these dose levels, we do not intend to conduct a larger follow-up study. In this experiment, BPC was administered at 0, 100 and 200 mg/kg/d. Higher dosage levels were not used because of the toxicity of this chemical. If BPC was as potent in vivo as it is in vitro, it should behave like flutamide which causes reproductive tract malformations in a majority of the F₁ males at dose levels ≥ 6 mg/kg/d (Fussell et al., 2015; McIntyre et al., 2001; Miyata et al., 2002).

Antandrogenic effects of Hydroxyflutamide, BPC, PFQ and Vinclozolin using Chimpanzee AR in an ARTA transduction assay with 100pM DHT

Figure 1. Androgen receptor transcriptional activation (ARTA) assay competing varying concentrations of pyrifluquinazon (PFQ), vinclozolin (VIN), bisphenol C (BPC), and hydroxyflutamide (OHF) with 100 pM dihydrotestosterone (DHT) to determine the EC₅₀ for inhibition of DHT-induced luciferase expression in CV-1 cells transduced with chimpanzee androgen receptor (chAR).

Statistics. Data analysis was performed using one-way ANOVA through the General Linear Model procedure (PROC GLM) in the Statistical Analysis System (SAS, SAS Institute, Cary, North Carolina). If the overall ANOVA was significant at $p < .05$, the significant differences between control and treated groups were determined by a post hoc two-tailed t test (LSMEANS in SAS) between litter means. Maternal data were analyzed using individual values whereas F₁ data were analyzed using litter means. Litter mean AGD data were analyzed with and without the cube root of body weight as a covariate. Categorical data (0 or 1; yes or no affect) were converted to percent affected in each litter, and litter means analyzed using logit transformed and untransformed values.

Dose-response curves from all experiments were analyzed using untransformed and normalized data (to percent of control) in a nonlinear four-parameter regression analysis (sigmoidal fit with variable slope Prism GraphPad v5.01, GraphPad Software, Inc., La Jolla, California). Percentage data based upon litter means was analyzed after logistic transformation. For logistic regression analyses, the control dose value was set to 1 mg/kg/d rather than 0 mg/kg/d so the control data would be included in the analysis as Prism converts the dose to log 10 values (the log of dose zero does not exist whereas the log value of the control at 1 of is now zero).

RESULTS

Pyrifluquinazon and Bisphenol C In vitro Results

BPC was as potent, or slightly more potent, as hydroxyflutamide in inhibiting DHT-induced luciferase activity. Both BPC and hydroxyflutamide were orders of magnitude more potent than vinclozolin, PFQ (Figure 1) and other bisphenol analogs (Conley et al., 2015). In contrast, PFQ was the weakest antagonist studied herein, displaying AR antagonist activity at concentrations that also induced some cytotoxicity and were near the limit of solubility. The log 10 K_i values in the chAR antagonist assay were -5.0 for PFQ, -7.3 for vinclozolin, -8.1 for BPC, and -7.7 for hydroxyflutamide (Figure 1). The binding assay indicated that

PYRIFLUQUINAZON CHIMP AR BINDING

Figure 2. Competitive chAR cell-free binding assay with different concentrations of R1881 and pyrifluquinazon (PFQ) competed against 2 nM radiolabeled R1881. K_i values are the disassociation constants for R1881 and PFQ with chAR.

PFQ-bound chAR with a log $10 K_i$ value (absolute disassociation constant) of about -3.59 , which is ~ 6 orders of magnitude higher than the log $10 K_i$ value for R1881, the positive control (Figure 2). When PFQ was run in the chAR transduction assay against two concentrations of DHT, the EC_{50} shifted downwards, as expected for an AR antagonist, when the DHT concentration was reduced from 1 to 0.1 nM (Figure 3).

Because it had been hypothesized that PFQ only displayed antiandrogenic activity with rAR, but not hAR, we transfected CV-1 cells with rAR or hAR and assessed the ability of PFQ to compete with 30 pM DHT. In this experiment, there were no differences in the antiandrogenicity displayed with rAR or hAR (Figure 4). The log $10 K_i$ values in this study were -4.74 and -4.65 for the rAR and hAR, respectively. Neither BPC nor PFQ displayed any androgenic activity in the chAR ARTA assay (data not shown).

Pyrifluquinazon In Vivo Results: Postnatal Study

Maternal (F_0) and F_1 male and female offspring effects. Overt maternal and fetal toxicity was evaluated by measurement of maternal body weight gain during dosing and pup or fetal survival, respectively. Administration of PFQ for 5 days (GD 14–18) reduced maternal weight gain during dosing, but only in the 100 mg/kg/d dose group, the effect was not statistically significant (27.3 g vs 42.3 g in controls; $p = .093$). PFQ did not reduce postnatal viability at 2, 13, or 23 days of age (data not shown) but male and female pup weight was significantly reduced at PND 2 from 7.63 g in females and 8.24 g in males to 6.52 g ($p < .004$) in females and 7.0 g ($p < .005$) in males in the 100 mg PFQ/kg/d dose groups, but this effect was attenuated by PND 13 (data not shown).

In utero PFQ exposure significantly reduced AGD in a dose-linear manner on PND 2 ($ED_{50} = 74$ mg/kg/d) in male offspring exposed in utero to doses of 25, 50, and 100 mg/kg/d ($p < .001$; Figure 5) and induced significant male nipple retention by PND 13 in all dose groups ($p < .001$; Figure 5). Neither effect display a clear threshold. Furthermore, 23.4% and 88% of the males in the 50 and 100 mg/kg/d dose groups, respectively, displayed genital abnormalities at this age. Excluding males with gross genital abnormalities, age and weight at PPS were not significantly affected (data not shown). Other effects in adult F_1 males included

Pyrifluquinazon Antiandrogen Luciferase Inhibition RUN AGAINST 2 DHT Concentration (3 runs/concentration) chAR in ARTA transduction assay using CV-1 cells

Null hypothesis	LogEC50 same for all data sets
Alternative hypothesis	LogEC50 different for each data set
P value	0.0052
Conclusion (alpha = 0.05)	Reject null hypothesis
Preferred model	LogEC50 different for each data set
F (Dfn, Dfd)	8.708 (1, 42)

Figure 3. Pyrifluquinazon inhibits 0.1 and 1 nM DHT-induced luciferase activity in CV-1 cells transduced with chAR.

Pyrifluquinazon antiandrogenicity using hAR versus rAR 4 INDEPENDENT RUNS

	hAR DHT PFQ 4 runs	rAR DHT PFQ 4 runs
LogEC50	-4.654	-4.736
HillSlope	-1.254	-0.8397
EC50	2.220e-005	1.838e-005
Ki	1.637e-005	1.126e-005

Figure 4. Pyrifluquinazon (PFQ) inhibits 30 pM DHT-induced luciferase activity in CV-1 cells transfected with rat (rAR) or human (hAR) similarly. The antiandrogenic activity of PFQ was not greater using rAR than hAR. Also shown, are the results of the MTT cytotoxicity assay.

significant reductions in reproductive organ weights at 50 and 100 mg/kg/d (Table 1) and there were dose-related increases in reproductive tract malformations and variations at 25 mg PFQ/kg/d and above (Table 2).

Adult F_1 female body and ovarian weights were unaffected by in utero PFQ treatment (data not shown) and no external or reproductive tract alterations were displayed in any dose group.

Bisphenol C Prenatal Results

Maternal and fetal male on gestational day 18. Administration of BPC from GD 14 to 18 significantly reduced maternal weight gain during dosing from 34.1 g (± 0.54) in controls to 20.1 g (± 3.8), 5.4 g (± 4.7), 9.9 g (± 6.1), and 1.8 g (± 10.8) in the 50 (NS, $p < .1$), 100 ($p < .01$), 200 ($p < .01$), and 300 ($p < .01$) mg/kg/d dose groups, respectively. However, maternal and fetal viability at GD 18 were not affected by BPC in any dose group.

Fetal T Prod, measured ex vivo, was significantly reduced at all dosage levels ($p < .05$) by about 23%, 28%, 27%, and 44% versus the control value in the 20, 100, 200, and 300 mg BPC/kg/d

Figure 5. The effects of pyrifluquinazon (PFQ) on F₁ neonatal male rat AGD on PND 2 following in utero exposure from GD 14–18 (upper left panel). PFQ also induced the retention of female-like nipples/areolae at 13 days of age. The upper right panel shows the litter means of the number of nipples (out of 12 maximum) in each male. The lower left panel displays the percent of males per litter that displayed any nipples (no nipples = 0%; 1–12 nipples = 100%). The lower right panel shows the percentage of males that displayed grossly malformed genitalia at 13 days of age. Data points represent litter means ($\pm$ SE) of 4–5 litters.

dose groups, respectively (Figure 6). The logistic regression model for TProd plateaued at about 35% of control (down maximally by 65% of control). In addition, maternal BPC administration significantly (overall F -value $p < .01$) altered testis mRNA expression of six genes, some of which are involved in hormone synthesis and sexual differentiation, as assessed on our qPCR custom arrays. For example, *StAR*, *Insl3*, *Dhcr7* and *Cyp17A1* were significantly reduced and *Fgf8* and *PPAR α* were increased (Figure 7).

Bisphenol C Postnatal Results

Maternal and F₁ male and female effects. In the current study, BPC reduced maternal weight gain from 37.6 g in controls to 27.5 g (NS) and 10.6 g ($p < .003$) in the 100 and 200 mg/kg/d dose groups, respectively. However, viability and growth of the F₁ offspring were not affected by GD 14–18 oral administration of BPC in any dose group (data not shown). In addition, neonatal AGD was not significantly affected in either male or female offspring, being 3.75, 3.69, and 3.65 mm in males in the control, 100 and 200 mg/kg/d dose groups, respectively. Although not statistically

significant, males in the 200 mg BPC/kg/d dose group had slightly higher number of female-like nipples than in the control and 100 mg/kg/d dose groups (0.04, 0, and 1.21 nipples per male, respectively, with 4.7, 0, and 37.5% [NS] of the males displaying any nipples [0–12]).

The ages and body weights at puberty in F₁ females (vaginal opening) and F₁ males (PPS) were not significantly altered by BPC treatment. In fact, males in the 200 mg BPC/kg/d group attained full PPS slightly earlier and at a lighter weight (both NS) than did controls rather than being delayed in the attainment of these pubertal landmarks (data not shown). The necropsy of adult F₁ females included only the measurement of body weight and an examination for external malformations because no prior effects were noted in this sex, and our interest was focused on disruption of the male reproductive tract.

When adult F₁ males were necropsied, no internal or external reproductive tract malformations or variations were detected in any group and there were few statistically significant alterations in reproductive organ weights. Several of the

Table 1. Effects of Oral Gavage Administration of the Antiandrogenic Pesticide Pyrifluquinazon from Gestational Days 14–18 on Body and Reproductive Organ Weights, Gubernacular Lengths (Left and Right Combined) Permanent Nipple Retention in F1 Male Rat Offspring Necropsied in Adulthood

Pyrifluquinazon															
Dose	0			12.5			25			50			100		
Weight	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N
Body (g)	819.3	38.8	5	810.3	73.9	4	842.7	20.5	5	816.4	18.7	5	807.3	31.8	5
Glans penis	138.6	1.1	5	132.9	2.4	4	131.7	5.4	5	133.9	7.9	4	137.1	.	1
Ventral prostate	493.1	67.2	5	500.9	77.9	4	491.1	28.8	5	323.4	41.9	5	134.3	41.4	5
Seminal vesicle (g)	2.16	0.10	5	2.07	0.11	4	2.08	0.07	5	2.18	0.17	5	1.48	0.26	5
Paired testes (g)	4.11	0.14	5	4.14	0.09	4	4.29	0.21	5	4.09	0.07	5	3.47	0.24	5
Paired epididymides	1430.6	44.7	5	1448.5	15.7	4	1430.7	53.5	5	1392.4	40.6	5	1192.3	89.0	5
Cauda epididymis	329.3	14.0	5	332.6	4.0	4	315.1	20.3	5	318.0	10.3	5	265.2	21.9	5
Caput epididymis	385.9	13.4	5	397.7	8.8	4	383.0	28.9	5	364.1	18.9	5	350.5	29.4	5
LABC (g)	1.59	0.05	5	1.52	0.04	4	1.48	0.05	5	1.32	0.12	5	1.31	0.46	5
Cowper's glands	264.2	33.9	5	192.4	19.2	4	222.0	12.6	5	181.6	11.1	5	116.3	24.9	5
Paired gubernaculum (mm)	18.4	1.1	5	16.3	1.0	4	17.5	0.8	5	17.4	0.7	5	17.6	0.6	5
Number of permanent nipples	0	0	5	0	0	4	0.91	0.32	5	2.64	0.75	5	5.3	0.64	5
Percent of males with nipples	0	0	5	0	0	4	25	7.3	5	59.3	16.8	5	89.1	6.8	5

Values are percent affected (based upon litter means), standard errors, and the number of litters examined at each dose level. Values highlighted in gray are statistically significantly increased versus the control values.

Table 2. Effects of Oral Gavage Gestational Administration of the Antiandrogenic Pesticide Pyrifluquinazon from Gestational Days 14–18 on the Display of Reproductive Tract Malformations in F1 Male Rat Offspring Necropsied in Adulthood

Pyrifluquinazon															
Dose	0			12.5			25			50			100		
Abnormalities	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N
Gubernaculum	0	0	5	0	0	4	0.0	0.0	5	0.0	0.0	5	0.0	0.0	5
Vas deferens	0	0	5	0	0	4	0.0	0.0	5	0.0	0.0	5	4.0	4.0	5
Bladder	0	0	5	0	0	4	0.0	0.0	5	0.0	0.0	5	4.0	4.0	5
Hydronephrosis	0	0	5	0	0	4	0.0	0.0	5	0.0	0.0	5	6.9	4.3	5
Seminal vesicle	0	0	5	0	0	4	2.2	2.2	5	4.0	4.0	5	6.9	4.3	5
Undescended testes	0	0	5	0	0	4	0.0	0.0	5	3.3	3.3	5	10.9	7.8	5
Epididymis	0	0	5	0	0	4	5.0	5.0	5	6.7	6.7	5	13.7	5.8	5
Ventral prostate	0	0	5	0	0	4	0.0	0.0	5	7.3	4.5	5	14.9	7.4	5
Ectopic testes	0	0	5	0	0	4	0	0	5	3.3	3.3	5	16.6	0.08	5
Testis	0	0	5	0	0	4	10.0	10.0	5	9.5	6.6	5	31.4	10.7	5
Vaginal pouch	0	0	5	0	0	4	0.0	0.0	5	13.0	5.7	5	66.3	18.3	5
Hypospadias	0	0	5	0	0	4	5.0		5	43.1	18.6	5	85.1	4.0	5
Retained nipples	0	0	5	0	0	4	20.6	6.5	5	56.0	17.6	5	85.1	7.4	5
Major repro malfs	1	0	5	1	0	4	10.8	9.8	5	53.8	17.7	5	91.4	4.7	5
Any reproductive effect	0	0	5	0	0	4	27.8	8.2	5	61.7	19.6	5	92.0	4.9	5

Any reproductive effect = Variations or malformations

Variations = Retained nipples or inflamed ventral prostate or asymmetric seminal vesicle or coagulating gland detached from seminal vesicle

Major repro malfs = Any male with a malformation (agenesis or aplasia) of any reproductive tract

Values are percent affected (based upon litter means), standard errors, and the number of litters examined at each dose level. Values highlighted in gray are statistically significantly increased versus the control values.

tissues were lighter in the BPC-treated groups than in controls but by only a few percent (Table 3). Two endpoints were significantly affected in the 200 mg BPC/kg/d group included a decrease in cauda epididymal weight and an increase in gubernacular length.

DISCUSSION

In the current study, we found that BPC antagonized the action of DHT in transcriptional activation assay using chAR at concentrations similar to hydroxyflutamide and at least two orders

of magnitude below that of PFQ. PFQ did bind AR and PFQ inhibited androgen-induced luciferase expression using AR from three mammalian species (hAR, chAR, and rAR). In contrast to the potencies of BPC and PFQ in vitro, the potencies of BPC to PFQ were reversed in vivo. PFQ caused a plethora of severe reproductive abnormalities, whereas BPC only produced subtle postnatal alterations of the male reproductive tract.

The results of the current study with BPC and PFQ provide a striking example of the limitations of attempting to predict the in vivo effects of chemicals from in vitro assays of AR antagonism. When evaluated in vitro, BPC was one of the most potent

Figure 6. The effects of bisphenol C (BPC) on ex vivo fetal testicular testosterone production (T Prod) following a maternal oral BPC administration from GD 14–18. The upper panel shows the logistic regression analysis of these data, with the top of the model constrained to 100% and unconstrained at the bottom. The lower panel displays the absolute values in ng testosterone produced per testis during the 3-h incubation period. Data points represent litter means ($\pm$ SE).

environmental antagonists described to date. However, BPC produced minimal effects on F₁ male rat reproductive development at dosage levels at least 80-fold above those expected to induce hypospadias and other malformations based upon the in vitro potency. Furthermore, BPC (EC₉₅ = 202 mg/kg) was roughly 20 and 400-fold less potent in demasculinizing F₁ male rat AGD at birth than are PFQ (EC₉₅ = 9.3 mg/kg) and flutamide (EC₉₅ = 0.5 mg/kg) (Fussell et al., 2015; McIntyre et al., 2001; Miyata et al., 2002), respectively. In contrast, PFQ is a very weak in vitro AR antagonist as compared with BPC and other chemicals (Figure 1) whereas PFQ was quite potent in vivo.

We found that BPC antagonized the action of DHT in the transcriptional activation assays using chAR (log₁₀ K_i = -8.0 M) at concentrations similar to hydroxyflutamide (K_i = -7.6 M) and at least two orders of magnitude below that of PFQ (K_i value = -5.0 M) (Figure 1). These results are consistent with previous publications with BPC that described AR binding at low concentrations (relative binding affinity = 0.0487% vs R1881 = 100%) and inhibition of androgen-induced gene expression in the U2OS AR CALUX cell line (IC₅₀ of 4.3E-08 vs 200 pM DHT) (Wang et al., 2014). In addition, we observed that BPC reduced fetal T

Prod and mRNA for some of the genes in the steroid synthesis pathway, which is consistent with BPC-induced alterations of steroidogenesis in H295R cells (Wang et al., 2014). Given that BPC potentially disrupts the androgen signaling pathway via two independent molecular initiating events (AR antagonism and reduced fetal T Prod), it was quite surprising that BPC only induced minimal alterations of male reproductive tract development. None of the F₁ males exposed to 100 or 200 mg BPC/kg/d displayed any reproductive malformations, and the only statistically significant effects were a 12% reduction in cauda epididymal weight and a 4-mm elongation of the paired gubernacular ligaments in the 200 mg BPC/kg/d dose group. However, in previous investigations of the relationship between fetal testis T Prod and postnatal reproductive abnormalities in F₁ male rat we found that reductions less than 50% of control were only associated with subtle changes in reproductive organ weights and low incidences of reproductive tract malformations (Gray et al., 2016; Howdeshell et al., 2015).

BPC is not only an AR antagonist it is also an ER agonist (Conley et al., 2016). In an in vitro ER transcriptional assay, BPC was more potent than other bisphenol analogs with a log₁₀ EC₅₀ = -8.28 M versus -7.54, -6.19, -6.92 for BPAF, BPS, and BPA, respectively. For comparison, the log₁₀ EC₅₀ for E2 is -11.73. In the ovariectomized adult female rat uterotropic assay, oral administration of BPC for 4 days increased uterine tissue weight with an ED₅₀ of about 75 mg/kg (vs E2 of 0.053 mg/kg/d), a NOEL of 13.5 mg/kg, and LOEL of 45 mg/d. At 150 mg/kg/d, BPC stimulated uterine tissue by more than 70% of maximum (where the curves for BPC and E2 plateau). The difference in ER affinity for BPC with E2 is about 3.5 log units, whereas the difference in affinity for AR is 2.5 log units. Taken together, these results demonstrate that dosage levels at or above 100 mg/kg/d result in sufficient levels of free BPC in the tissue to induce a large estrogenic stimulation of uterine tissue weight in the ovariectomized female rat, but not an antiandrogenic effect in a fetal male rat after maternal dosing. These data suggest that the BPC levels in the fetus may be lower than in the dam.

If BPC metabolism and distribution is similar to BPA then approximately 95% of the BPC would be rapidly metabolized by the dam to BPC-glucuronide (BPC-G) and excreted rapidly (Domoradzki et al., 2003) and levels of BPC-G in the fetal tissues would be about 3.5% of maternal plasma levels and free BPC would be about 4-fold lower than in the dam. Chuchwell et al. (2014) (Figure 4) reported that oral administration of 100 mg BPA/kg/d resulted in BPA (aglycone) C_{max} serum levels of about 120 nM (log₁₀ = -6.92) in 80-day old female rats, a value that is similar to the AR K_i value for BPC. We suspect that the potency of BPC in a Hershberger assay (Gray et al., 2005; Owens et al., 2006, 2007) for antiandrogenicity would be far more concordant with the in utero results than are the in vitro results.

PFQ also displayed AR antagonism with chAR, hAR, and rAR, but at concentrations more than three orders of magnitude higher than BPC. We found that PFQ bound chAR with relatively low affinity and inhibited androgen-induced luciferase expression using AR from three mammalian species (hAR, chAR, and rAR) but only at relatively high concentrations. PFQ did not appear to interact differently with rAR than hAR as had been hypothesized (Yasunaga et al., 2013). Our binding and ARTA results differ from those previously reported for PFQ that suggested that PFQ did not bind AR and failed to inhibit DHT-induced luciferase activity in cells expressing hAR.

When administered during sexual differentiation, PFQ was far more potent than expected from the in vitro results and it was far more potent, not less potent, than was BPC in utero,

Figure 7. GD 14–18 bisphenol C (BPC) exposure significantly altered fetal testicular gene expression of six genes using custom QPCR arrays (F -value < 0.01). Each data point represents the litter mean $\pm$ SE. The mRNA levels shown here are expressed as fold versus control and includes mRNA coding for genes and ultimately proteins involved in steroid hormone synthesis, steroid hormone transport, and Ins13 hormone synthesis. * p < .05; ** p < .01.

being as potent or slightly more potent than is vinclozolin in utero (Gray Jr et al., 1999). At 100 mg/kg/d, PFQ induced reproductive tract malformations in >90% of the F_1 males with 85% displaying hypospadias (litter mean values), whereas vinclozolin is about 100-fold more potent in vitro than PFQ. Our in utero post-natal study results are consistent with the effects reported by the Agency from studies submitted in support of registration (discussed in: EPA-HQ-OPP-2011-0971-0023). The EPA document reports that PFQ reduced F_1 male rat AGD in a two-generation reproduction study, decreased body weights and AGD in the F_2 pups were observed at the LOAEL of 11.6 mg/kg/day.

Reproductive effects in adult males in a high-dose group (51.9 mg/kg/d) included effects on mating, sexual development, and sperm measures.

In the current study, no NOEL was established. PFQ induced reproductive changes in all dose groups that increased in severity with increasing dose. This includes retained female-like nipples/areolae at 12.5 mg PFQ/kg, and reduced AGD in males at 25 mg PFQ/kg, and reductions in reproductive organ weights and malformations at 25 mg PFQ/kg and above. PFQ was as potent, or slightly more potent, than the well-characterized anti-androgen vinclozolin (Gray Jr et al., 1999; Hellwig et al., 2000;

Table 3. Bisphenol C effects on adult male body and organ weights.

Dose Weight	0			100			200		
	Mean	SE	N	Mean	SE	N	Mean	SE	N
Body	650.9	41.8	3	675.5	11.9	3	631.3	10.9	4
Glans penis	124.8	2.3	3	127.5	6.4	3	118.5	3.7	4
Ventral prostate	634.7	11.0	3	593.2	73.4	3	586.0	45.0	4
Seminal vesicle	1.76	0.07	3	1.71	0.11	3	1.83	0.07	4
Paired testes	3.93	0.12	3	3.84	0.05	3	3.78	0.08	4
Paired epididymis	1341.4	36.4	3	1288.5	24.9	3	1251.6	54.9	4
Cauda epididymis	291.0	3.4	3	293.2	9.0	3	256.8*	12.7	4
Caput epididymis	383.9	13.4	3	351.8	7.2	3	371.1	14.4	4
Whole epididymis	666.5	20.1	3	643.5	14.8	3	623.7	30.6	4
LABC	1.40	0.07	3	1.35	0.02	3	1.38	0.05	4
Cowper's glands	161.2	9.4	3	137.8	15.5	3	166.9	7.9	4
Gubernacular (mm)	14.2	0.5	3	12.9	0.9	3	18.4*	0.8	4
Number of nipples	0	0	3	0	0	3	0	0	4

Values highlighted in gray differ significantly from control.
 $p < .05$ different from control value.

Monosson et al., 1999; Schneider et al., 2011) and procymidone (Ostby et al., 1999) in disrupting sexual differentiation in male rats. One hypothesis for the enhanced effects of PFQ during sexual differentiation versus the weak activity in vitro is that, like flutamide and vinclozolin, the in vivo activity is the result of metabolic activation of PFQ to a more potent antiandrogenic metabolite. In contrast to the discordance between the in vitro and in utero potencies, PFQ reduced androgen-dependent organ weights in the Hershberger assay (Yasunaga et al., 2013) and in utero (Table 1) at similar dosage levels.

In summary, we found that BPC antagonized the action of DHT in transcriptional activation assays using either hAR or rAR at concentrations similar to hydroxyflutamide and at least two orders of magnitude below PFQ. PFQ did bind chAR and inhibited androgen-induced luciferase expression using AR from three mammalian species (hAR, chAR, and rAR), but only at high concentrations. When administered in utero during sexual differentiation, the relative potency of BPC to PFQ was reversed as compared with the in vitro results. PFQ caused a plethora of severe reproductive abnormalities, whereas BPC was minimally effective in this regard. BPC was roughly 20- and 400-fold less potent than PFQ and flutamide (Fussell et al., 2015; McIntyre et al., 2001; Miyata et al., 2002), respectively, in demasculinizing F₁ male rat AGD at birth. Administration of flutamide at 6 mg/kg/d in utero induced hypospadias in most male offspring (McIntyre et al., 2001) and 100 mg/kg/d causes agenesis of all the androgen-dependent reproductive tract organs in male offspring (Miyata et al., 2002), whereas administration of BPC at 100 and 200 mg/kg/d failed to induce agenesis or malformations of any reproductive tissues.

The discordance between the in vitro and in vivo extrapolation of antiandrogenic activity (IVIVE-antiAR) reported herein for BPC and PFQ is not limited to these two chemicals. In a review of the utility of IVIVE-antiandrogenicity for about 65 chemicals, including pesticides, bisphenol analogs, triclosan, other toxic substances, and drugs, we found that a significant percentage of the chemicals that display antiandrogenic activity in vitro display no such activity in the Hershberger assay, a short-term in vivo assay of antiandrogenicity, or when administered in utero (Gray, 2017, in preparation).

It is possible that the pregnancy-IVIVE TK models for BPA could be modified to attempt predict the fetal concentrations of BPC and to improve the prediction of the in vivo antiandrogenic

effects of BPC (Kawamoto et al., 2007; Sharma et al., 2018). However, this assumes that the TK behavior of BPC is similar to BPA. Alternatively, the TK behavior of BPC may more closely parallel to that of the estrogenic and antiandrogenic methoxychlor metabolite 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) which is metabolized by CYP enzymes (Bulger et al., 1978; Hu and Kupfer, 2002) to a less active antiandrogen (Gaido et al., 2000). In contrast to the bisphenols and the methoxychlor metabolite HPTE, rather extensive studies would need to be conducted to determine TK/ADME and TD parameters for PFQ because there are little or no published data on PFQ or a similar chemical that would enable one to develop a TK-TD models with any certainty.

To date, IVIVE-TK models have been used with several endocrine active chemicals, however attempts to model the antiandrogenic activity of flutamide have not been very successful. For example, Fabian et al. (2018) used reverse dosimetry to compare in vitro effect concentrations to in vivo doses causing toxic effects related to endocrine disruption with ten compounds including flutamide and the lowest observed effect concentrations from the in vitro assays were extrapolated to an oral LOELs in vivo using an eight-compartment physiologically based pharmacokinetic model. When the predicted LOELs for the ten chemicals were compared with the LOELs actually observed in corresponding in vivo studies, the LOELs were predicted within an order of magnitude for six of the ten chemicals. However, they concluded that the “applied methods were insufficient” for flutamide. The LOELs for flutamide and the androgen agonists methyltestosterone and trenbolone “were not well predicted, with more than an order of magnitude difference between the modeled and observed in vivo values.” Similarly, when investigators used high-throughput data on flutamide in an attempt to “translate in vitro bioactivity concentrations to oral equivalent doses (OED) that would achieve these levels internally” they found that “the predicted value deviated from observed by 160-fold” (Wetmore et al., 2015). Needless to say, BPC and PFQ have not been included in IVIVE model assessments for AR antagonism.

It should also be noted that even if TK models enabled one to predict fetal concentrations of antiandrogenic chemicals, this information alone would not be sufficient to predict the tissue concentrations that induce an adverse effects in utero. Antiandrogenic chemicals compete with the natural hormones dihydrotestosterone (DHT) and/or testosterone (T) for the AR. The levels of DHT, T, and AR, among other factors within the cell, also need to be included in TK-TD models for antiandrogens along with the concentration of the antiandrogenic chemical. These factors vary significantly among male reproductive tract tissues and as a consequence the ED₅₀s for different male reproductive tissues are not the same. For example, when vinclozolin is administered in utero the ED₅₀s of affected tissues in the male offspring varies by almost 20-fold (Gray et al., 1999). In addition, in the OECD Hershberger assay, the ED₅₀s for the five androgen responsive tissues varies by 3–4-fold with 10 days of oral flutamide administration (Owens et al., 2006). In summary, the application of existing data on BPA or HPTE TK models might reduce some of the uncertainty in the IVIVE extrapolation for BPC, but there would far more uncertainty in the application of IVIVE models for PFQ given the dearth of TK/ADME and TD data on PFQ. Taken together, these results clearly demonstrate that in the absence of validated TK (ADME) and TD models, extrapolating the effects of AR antagonists from in vitro to in vivo is an uncertain process, as best. In contrast, combining validated in vitro (eg, AR binding and transcriptional activation) assays and short-term in vivo Hershberger assay for

antiandrogenic activity can provide a relatively accurate prediction of the antiandrogenic potential of chemicals in utero.

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