Triazine Herbicides and Their Chlorometabolites Alter Steroidogenesis in BLTK1 Murine Leydig Cells

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The triazine herbicides, atrazine (ATR), simazine (SIM), propazine (PRO), terbuthylazine (TBA), and their chlorinated metabolites have been implicated in the etiology of testicular dysgenesis by altering steroidogenesis. To further investigate their effects on testosterone biosynthesis, BLTK1 cells were used to evaluate steroid hormone levels and genome-wide gene expression. BLTK1 cells are a novel murine Leydig cell line possessing an intact steroidogenic pathway with constitutive low basal testosterone levels that can be induced by recombinant human chorionic gonadotropin (rhCG). Triazines (ATR, SIM, PRO, and TBA) and their chlorometabolites (DEA, DIA, and DACT) induced concentration-dependent (1, 3, 10, 30, 100, 300, and 600 µM) increases in progesterone (P) and T levels relative to solvent control at 24 h. Temporal analysis (300 µM at 1, 2, 4, 8, 12, 24, or 48 h) elicited comparable P and T profiles by all compounds with varying efficacies (ATR > TBA > PRO > DEA > DIA > DACT > SIM) that were similar to rhCG. ATR and TBA elicited time- and concentration-dependent induction of Star, Hsd3b6, and Hsd17b3 mRNA levels, whereas Hsd3b1, Cyp17a1, and Srd5a1 mRNA expression was repressed. PRO elicited similar albeit weaker effects, whereas SIM had negligible effects consistent with their induction of P and T levels. Whole-genome microarrays identified 797 differentially regulated genes elicited by 300 µM ATR, occurring primarily at later time points (> 12 h) with overrepresented functions associated with steroidogenesis and cholesterol metabolism. These results indicate that changes in P and T levels can be partially attributed to triazine-elicited alterations in steroidogenic gene expression.

Key Words: testosterone; Leydig; triazine; gene expression; microarray.

Chloro-s-triazines are widely used broad-spectrum herbicides that block photosynthesis by inhibiting electron transfer at the reducing site of photosystem II in the chloroplasts of broadleaf and grassy weeds (Good, 1961; Tischer and Strotmann, 1977). Atrazine (ATR), simazine (SIM), propazine (PRO), and terbuthylazine (TBA) are triazine herbicides registered for use in the United States: ATR for agricultural crops and grasses, SIM for grasses, fruit, and agricultural crops, PRO for indoor greenhouse use, and TBA as a slimicide for ornamental fountains (USEPA, 1995, 2006a–c). PRO and TBA use is restricted in the United States and therefore human exposure is negligible (USEPA, 1995, 2006d). In contrast, ATR and SIM are widely distributed agricultural herbicides with potential exposure to both the parent triazine and its major metabolites (USEPA, 2006d). ATR and SIM are biotransformed in the environment by microbial and fungal degradation, as well as photodegradation, to form three major chlorinated metabolites (chlorometabolites): des-ethyl-s-Atrazine (DEA), des-isopropyl-s-Atrazine (DIA), and diaminochlorotriazine (DACT; Fig. 1) (Evgenidou and Fytianos, 2002; Kaufman and Blake, 1970). Furthermore, cytochrome P450 metabolism produces the same chlorometabolites detectable in urine, serum, and various tissues of exposed animals (Barr et al., 2007; Hanioka et al., 1999; Ross et al., 2009).

ATR, SIM, DEA, DIA, and DACT persist in the environment and are mobile in soil and ground water (Thurman et al., 1992). ATR and SIM have been reported above the U.S. maximum contaminant levels in groundwater (Thurman et al., 1991; USEPA, 2006d). For example, ATR and its chlorometabolites were the most common pesticide contaminant detected in water, found in 75% of the streams and 40% of the groundwater samples tested in agricultural and urban areas in the United States between 1992 and 2001 (Gilliom et al., 2006). Groundwater contamination resulted in the cancelation of ATR and SIM regisrations and cessation of their use in most of Europe (European Commission, 2003a, b; Sass and Colangelo, 2006), with the European Union approving the use of TBA as an agricultural alternative (EFSA, 2011). In contrast, the Environmental Protection Agency has approved the continued use of triazines in the United States (USEPA, 2006a).

ATR, SIM, PRO, and their major chlorometabolites (DEA, DIA, and DACT) are categorized as a Common Mechanism Group (CMG) based on their disruption of the
hypothalamic-pituitary-gonadal (HPG) axis (USEPA, 2002, 2006d). CMGs are defined for cumulative risk assessment as two or more chemicals that cause a common toxic effect(s) using the same sequence of major biochemical events, interpreted as mode of action (USEPA, 2012a). Moreover, their major chlorometabolites are classified as toxicologically equipotent to the parent triazines by the CMG (USEPA, 2006d). Therefore, all triazines and their major chlorometabolites require further examination regarding potential endocrine-disrupting activities.

The endocrine-disrupting effects of triazines and their chlorometabolites have been extensively studied in mammalian models and aquatic species. In amphibians, reptiles, and fish, ATR reduced reproductive capacity, disrupted steroidogenesis, and altered gonadal development resulting in hermaphroditism and male feminization (Crain et al., 1997; Hayes et al., 2002, 2010; Spanò et al., 2004; Tillitt et al., 2010). Effects on steroidogenesis in amphibians and fish suggest that disruption of testosterone (T) biosynthesis may underlie the effects of ATR in males (Hayes et al., 2002, 2010; Sanderson, 2006). Rodent studies also report that ATR disrupts male and female reproductive tract development and function by altering steroid levels that affect estrus, androgen-dependent tissue development, puberty onset, reproductive senescence, and overall fertility (Cooper et al., 2007; Laws et al., 2000; Narotsky et al., 2001; Stoker et al., 2000). PRO, SIM, DEA, DIA, and DACT elicit similar effects in rodents that are generally characterized as ATR-like with a limited number of studies examining TBA (Fraites et al., 2009; Laws et al., 2003; Stoker et al., 2002; Zorrilla et al., 2010). Collectively, these results

FIG. 1. Structures of the chloro-s-triazines and their primary chlorometabolites. (A) SIM, ATR, and PRO are metabolized by cytochrome P450 enzymes predominantly into the active metabolites DIA, DEA, and DACT. (B) TBA is used as an ATR alternative approved for use in the European Union.
sugar, and their chlorometabolites target the HPG axis (Cooper et al., 2007; Laws et al., 2009).

This study examines the effects of ATR, SIM, PRO, TBA, and their primary chlorometabolites (DEA, DIA, and DACT) on steroidogenesis in BLTK1 murine Leydig cells. Leydig cells are the primary site for T biosynthesis required for proper male development and reproduction. Briefly, luteinizing hormone (LH) stimulation induces the expression and activation of steroidogenic enzymes, which increases progesterone (P) levels and metabolism to T (Dufau et al., 1984; Payne and Youngblood, 1995). BLTK1 cells, a subclone of a murine Leydig cell line isolated from a testicular tumor in a inhibin α/SV40Tag transgenic mouse, produce low basal P and T levels (Forgacs et al., 2012; Rahman and Huhtaniemi, 2004). Unlike other murine Leydig cell lines such as BLTC-1 (Kananen et al., 1996), MA-10 (Ascoli, 1981), and mLTC-1 (Rebois, 1982), which do not proceed beyond P biosynthesis, BLTK1 cells produce appreciable basal levels of T, which can be induced by rhCG, an LH analog (Forgacs et al., 2012).

In this study, we have evaluated the time- and concentration-dependent effects of ATR, SIM, PRO, TBA, and their major chlorometabolites on P and T production and gene expression in BLTK1 cells. Our results confirm that changes in P and T levels can be partially attributed to triazine-elicited alterations in steroidogenic gene expression.

MATERIALS AND METHODS

Cell culture and treatment. BLTK1 cells were maintained in phenol red-free Dulbecco’s modified Eagle’s medium nutrient mixture F-12 (DMEM/F-12 media; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, UT), 100 U/ml penicillin, and 100 ng/ml streptomycin (Invitrogen) and incubated under standard conditions (5% CO₂, 37°C). Cells were seeded at 80% confluency in 24-well tissue culture plates (Sarstedt, Newton, NC) for measurement of P and T levels in media. Concentration-response studies at 24 h were conducted with dimethyl sulfoxide (DMSO; vehicle; Sigma, St Louis, MO); 0.1, 0.3, 1, 3, 10, 30, or 100 ng/ml rhCG (obtained from A.F. Parlow, NIDDK’s National Hormone & Peptide Program, Harbor-UCLA Medical Center, Torrance, CA); or 1, 3, 10, 30, 100, 300, or 600 μM of ATR (Sigma), DEA, DIA, DACT, PRO, SIM, or TBA (Accustandard, New Haven, CT). Time course studies were conducted with DMSO (vehicle), 300 μM ATR, DEA, DIA, DACT, PRO, SIM, or TBA, and rhCG (3 ng/ml) as a positive control. The entire volume of media from wells was collected at 1, 2, 4, 8, 12, 24, or 48 h, with different wells used for each time point, such that cumulative steroid production was evaluated for the time points indicated. Cells were grown to 80% confluency in T-25 flasks (Sarstedt) for RNA isolation.

MTT cytotoxicity assay. BLTK1 cells, plated into 96-well tissue culture plates (Sarstedt), were treated with 1, 3, 10, 30, 100, 300, or 600 ng/ml rhCG or 1, 3, 10, 30, 100, 300, or 600 μM of ATR, PRO, SIM, TBA, DEA, DIA, or DACT. After 24 h, media were aspirated and 50 μl of freshly prepared MTT reagent (5 mg/ml thiazolyl blue tetrazolium bromide (Sigma) in PBS) was added to each well. Cells were incubated for 3 h, and then MTT reagent was aspirated and 150 μl DMSO was added to each well for 2 h before spectrophotometric measurement of product at 595 and 650 nm (A595-A650) using an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA). Results are reported as percentage of control calculated from relative absorbance of treated versus controls, where 100% indicates no cytotoxicity. MTT assay results are presented in Supplementary figure S1.

Enzyme immunoassays. T (Cayman Chemical Company, Ann Arbor, MI) and P (ALPCO Diagnostics, Salem, NH) enzyme immunoassay (EIA) kits were used following manufacturer’s instructions, as previously described (Forgacs et al., 2012). Briefly, conjugated hormone and sample compete for antibody on 96-well precoated plates. Following incubation, the plates were washed, and a reaction was initiated to yield a spectrophotometrically detectable product (412 nm for T, 450 nm for P) using an Emax precision microplate reader (Molecular Devices). A standard curve (3.9–500 pg/ml T and 0–60 ng/ml P) was generated for P and T quantification. All chemicals were confirmed to have no cross-reactivity with the EIA kits (data not shown).

RNA isolation and quantitative real-time PCR. Treated cells (T-25 flasks) were trypsinized (Invitrogen) and stored as pellets at −80°C. RNA was extracted using RNaseasy Mini Kits (QIAGEN, Valencia, CA) according to the manufacturer’s protocol with an additional RNase-free DNase (QIAGEN) digestion. RNA was spectrophotometrically quantified at 260 nm (A260), and purity was assessed using the A260/A280 ratio and denaturing gel electrophoresis.

RNA (1 μg) was reverse transcribed by SuperScript II (Invitrogen) using an anchored oligo-dT primer (Invitrogen) as described by the manufacturer. Quantitative real-time PCR (QRT-PCR) reactions consisted of 30 μl reaction volumes that included 1 μl of cDNA template, 0.1 μM forward and reverse gene-specific primers, 3 mM MgCl₂, 1 mM dNTPs, 0.052 IU AmpliTaq Gold, and 1× SYBR Green PCR Buffer (Applied Biosystems, Foster City, CA) were carried out in 96-well plates and amplified using an Applied Biosystems PRISM 7500 Sequence Detection System. Dissociation curve analysis assessed single product amplification. To control for differences in RNA loading, quality, and cDNA synthesis, samples were standardized to the geometric mean of three housekeeping genes (Actb, Gapdh, and Hprt) (Vandesompele et al., 2002). Primer sequences for all genes evaluated are available in Supplementary table 1. Results were quantified using a standard curve generated on the same 96-well plate used for samples by amplifying a purified cDNA template specific for each gene (serial 10x dilutions from 10⁶ to 10² copies). Standard curve slope was used to assess amplification efficiency, as described by the manufacturer, with all amplification efficiencies between 80 and 100%. Fold changes were calculated relative to time-matched vehicle. Relative expression levels were scaled such that the expression level of the time-matched vehicle control group equaled one for graphing purposes.

Microarray analysis. The temporal changes in gene expression elicited by 300 μM ATR were evaluated using Agilent mouse 4×44K oligonucleotide microarrays according to the manufacturer’s protocol (Agilent Manual: G4140-90050 v5.0.1; array product number: G4122F; Agilent Technologies, Santa Clara, CA). Briefly, 1 μg total RNA from ATR-treated BLTK1 cells and time-matched DMSO controls was isolated independently with Cy3 and Cy5 dye and cohybridized in an independent reference design. This design involves comparing treated samples to controls with two independent labelings per sample (dye swap) for a total of 14 arrays per replicate (seven time points in total). Three replicates of this design were performed for a total of 42 arrays. Microarrays were scanned at 532 nm (Cy3) and 635 nm (Cy5) using a GenePix 4000B scanner (Molecular Devices, Union City, CA), using GenePix Pro 6.0 to extract features and background intensities. All images and data were loaded into the TIMS dbZach data management system (Burgoon and Zacharewiski, 2007). All microarray data passed our quality assurance protocols (Burgoon et al., 2005) and were normalized in SAS v9.1 (SAS Institute, Cary, NC) using a semiparametric approach (Eckel et al., 2005). Posterior probabilities were determined on a per gene and per time point basis (Eckel et al., 2004) to determine model-based t-values (SAS v9.1), which were then used to determine P(t) values by an empirical Bayes method using R (R, Institute for Statistics and Mathematics, WU Wien). Data were filtered and prioritized in a MySQL v5.1.40 database using a filtering criteria of fold change > 1.5 and P(t) > 0.999 to identify differentially expressed genes. The complete microarray data set including fold change and P(t) values is available in Supplementary table 2.

Functional annotation was carried out using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA; www.ingenuity.com) and the Database for Annotation, Visualization and Integrated Discovery (DAVID;
http://david.abcc.ncifcrf.gov/}. Using IPA, genes were evaluated on a per time point basis to identify significantly enriched (p value < 0.05) functions. The IPA transcription factor tool was used to identify predicted activation or inhibition of transcription factors. K-means clustering was conducted using the TM4 MultiExperiment Viewer v.4.6.0 (Saeed et al., 2003). DAVID was used to identify significantly enriched (p value < 0.05) Gene Ontology (GO) biological process functional annotation terms (GOTERM_BP_FAT).

**Statistical analyses and dose-response modeling.** EIA and qRT-PCR concentration-response data were analyzed using SAS v9.1 (SAS Institute) by ANOVA followed by Dunnett’s post hoc test. Differences between groups were considered significant when p < 0.05. EIA and qRT-PCR data from time course studies were also analyzed by SAS v9.1 by ANOVA, but followed by Tukey’s post hoc test, using a significance cutoff of p < 0.05. Dose-response modeling was carried out using a particle swarm optimization algorithm (ToxResponse modeler (Burgon and Zacharewski, 2008)) to identify the best fit across five model classes (sigmoidal, exponential, linear, quadratic, and Gaussian). All EC₅₀ values included in this study were determined from sigmoidal concentration-response curves.

**RESULTS**

**Effect of Triazines and Their Chlorometabolites on P and T Levels**

The effect of triazine herbicides and their primary chlorometabolites on P and T levels in media was assessed by EIA at 24 h. ATR, PRO, SIM, or TBA and DEA, DIA, and DACT elicited concentration-dependent increases in P levels at 24 h albeit with lower efficacy and potency relative to rhCG (Fig. 2). Each triazine and chlorometabolite induced P at 100–600 μM, with a maximum induction of ~3.5-fold (90 ng/ml) in response to 600 μM ATR relative to vehicle controls. In comparison, P levels were induced up to 170 ng/ml (~sixfold) following treatment with 100 ng/ml rhCG. The EC₅₀ for P induction by rhCG was 0.8 ng/ml, whereas triazine and chlorometabolite EC₅₀s were ~33 μM. Differences in efficacy were more evident for T, where 100 ng/ml rhCG induced levels ~fourfold (170 pg/ml) and ATR only elicited a ~twofold (75 pg/ml) increase. SIM was the least efficacious with a modest 1.3-fold induction of T. EC₅₀ values for T induction were 0.7 ng/ml for rhCG and 30–45 μM for the triazines and chlorometabolites. DACT only had T induction at 600 μM, and therefore, an EC₅₀ value could not be determined using the ToxResponse modeler. Overall, each compound elicited concentration-dependent induction of P and T levels, with ATR exhibiting the greatest efficacy.

Based on the 24-h concentration-response analysis, 300 μM was used to examine the time-dependent effects of the parent triazines and their primary chlorometabolites on the induction of P and T levels. ATR, TBA, and PRO elicited comparable temporal P and T profiles albeit with lower potency compared with rhCG (Fig. 3) with efficacy similar to levels in the concentration-response studies. Like rhCG, triazines and their chlorometabolites induced maximum P levels by 2 h, which subsequently decreased, whereas rhCG maintained P levels for 2–4 h before decreasing. Concomitantly, T increases were inversely proportional to the time-dependent decreases in P levels. However, rhCG-mediated efficacy was significantly greater than the levels induced by any triazine or chlorometabolite.

Although SIM and DACT elicited comparable temporal profiles, the effects on P and T levels were not statistically significant (Supplementary Table 4). Overall, ATR, SIM, PRO, TBA, DEA, DIA, and DACT elicited comparable temporal effects on P and T levels with differences in efficacy.

**Triazine Effects on Steroidogenic Enzyme Gene Expression Profiles**

The expression of steroidogenic enzymes involved in the metabolism of cholesterol to progestagens, androgens, and estrogens was evaluated by qRT-PCR for parent triazines and rhCG. ATR, PRO, and TBA elicited concentration-dependent differential expression of several steroidogenic genes at 24 h (Fig. 4). Compared with the ~20-fold rhCG-mediated induction of Star, only ATR, PRO, and TBA induced Star mRNA levels (4-, 3-, and 3.5-fold, respectively), whereas SIM had negligible effects. Meanwhile, Hsd3b6 mRNA induction was comparable (~threefold) for ATR, PRO, and TBA, similar to rhCG. Interestingly, Hsd3b1 was repressed 10-, 10-, and 4-fold in response to 600 μM ATR, TBA, and PRO, respectively, whereas rhCG had no significant effect on the expression of this gene. Although Cyp17a1 was also repressed (eight-, seven-, and fourfold decrease by ATR, PRO, and TBA, respectively), rhCG and SIM elicited negligible effects. Srd5a1 was induced ~threefold by rhCG but repressed ~twofold by ATR, PRO, and TBA. Hsd17b3 mRNA levels were induced ~sixfold by both rhCG and ATR treatment, whereas PRO, SIM, and TBA had negligible effects. Only TBA repressed Cyp19a1 (fivefold). None of the treatments altered Cyp11a1 expression.

The temporal effects of ATR, PRO, SIM, and TBA on steroidogenic mRNA levels were also examined (Fig. 5). Despite some differences in triazines- versus rhCG-elicited concentration-response profiles at 24 h, time-dependent ATR-, PRO-, and TBA-elicited steroidogenic gene expression profiles were comparable to rhCG albeit with differing efficacies. In general, Cyp11a1 and Cyp19a1 were unaffected by treatment, whereas Star, Hsd3b6, Hsd17b3, and Cyp17a1 were induced by rhCG and triazines, in agreement with the 24-h dose-response profiles. For example, Star was induced 2- to 4-fold by ATR, PRO, SIM, and TBA and 10-fold by rhCG with maximum expression achieved at 8–12 h. Similarly Cyp17a1 also showed peak induction at ~8 h with repression evident by 24–48 h for both rhCG and ATR. Furthermore, Srd5a1 exhibited divergent regulation with rhCG inducing expression fivefold from 8 to 24 h, whereas ATR, PRO, and TBA elicited > 80% repression from 24 to 48 h. Overall, SIM elicited negligible effects on the expression of the steroidogenic genes examined.

**Temporal Whole-Genome Evaluation of Atrazine-Mediated Differential Gene Expression**

Due to the similarities among the triazines and chlorometabolites, only ATR was investigated for genome-wide differential gene expression as it had greater efficacy.
Agilent microarrays contain ~41,000 features, representing 21,308 unique genes, with 797 differentially expressed (l-fold change > 1.5 and \( P_{(0.05)} > 0.999 \)) by 300 \( \mu \)M ATR (Fig. 6A). Differential gene expression increased over the course of the study with 23 genes at 1 h, 18 at 2 h, 117 at 4 h, 134 at 8 h, 284 at 12 h, 377 at 24 h, and 493 at 48 h (Fig. 6B). Star induction measured by microarray was validated by QRTPCR, demonstrating comparable time-dependent profiles and levels of induction from 4 to 48 h (Fig. 6C). K-means analysis of the 797 differentially expressed genes identified three distinct clusters with 317 induced, 362 repressed, and 118 exhibiting induction from ~4 to 24 h that tapered by 48 h (Fig. 7). The Ingenuity transcription factor tool predicted enrichment of androgen receptor (AR) activity (24 and 48 h, \( p \) value 7.59e−03) based on 19 differentially expressed AR target genes at 24 and 48 h, of which 10 were repressed and 9 exhibited induction. The AR target genes contributing to the prediction of AR signaling activation were all differentially expressed at later time points, coinciding with increasing testosterone levels detected at 24 and 48 h (Fig. 3). However, AR mRNA expression levels were not altered by ATR, suggesting that the activation of AR signaling was a result of androgen binding rather than increased expression.

Functional annotation was conducted using DAVID and IPA to identify enriched GO terms and overrepresented functions, respectively (Table 1). Enriched GO terms were associated with hormone metabolism and regulation (including steroidogenic gene expression evaluated by QRTPCR) as well as cholesterol.
biosynthesis and metabolism. Examples included cholesterol de novo biosynthesis-related genes such as *Hmcgr*, *Hmgcs1*, *Lss*, *Sqle*, and *Cyp51* (all showing 1.5- to 2-fold induction at 8–12 h), cholesterol transport and uptake (*Star* up to 3-fold from 4 to 48 h and *Ldlr* 2-fold at 8 and 12 h), and metabolism into steroids (as demonstrated by QRTPCR of steroidogenic enzyme expression, Fig. 5). IPA transcription factor analysis also predicted the activation of *Srebf1* (Table 2) and *Srebf2* (p value 1.70e-05), which regulate gene expression associated with sterol homeostasis. The predicted activation of Sreb1 and Sreb2 was consistent with the intermediate upregulation of their target genes as identified in K-means clustering. In addition, *Lhcgr* the cell surface receptor for LH/rhCG was upregulated 1.6-fold at 24 h.

IPA also identified functions associated with reproduction as overrepresented. This included the induction of *Rad18*, *Ins16*, and *Prnp* (1.5-fold at 48 h) required for spermatogenesis, as well as the repression of *Srd5a1* and *Srd5a3* (2-fold at 48 h) required for male reproductive development. Tissue development and morphology were also overrepresented and included several actin regulatory proteins genes such as *Avil* (induced twofold at 48 h), *Fmn1* (induced ~twofold at 8–12 h), and *Cald1* (downregulated ~twofold at 8–48 h). The predicted activation of Creb1 and *Atf4* (Table 2) suggests cAMP-mediated gene expression and included the induction of *Vegfa*, *Gadd45a*, and *Bcat1* consistent with changes in cell morphology and growth. Induction of the phosphodiesterase (PDE) genes *Pde4b* (up 1.8-fold at 12–48 h), *Pde4 dip* (up 1.6-fold at 24 h), and *Pde10a* (up to 3-fold at 4–48 h) is also indicative of cAMP signaling. The predicted activation of CCAAT/enhancer-binding proteins (Cebp), *Cebpa* (p value 4.38e−05), and *Cebp* (Table 2), as well as ATR-mediated differential expression of *Crebd* (repressed 1.5-fold at 12 h) and *Cebpg* (upregulated 1.5-fold at 24 h) further suggest ATR-mediated changes in cellular growth and differentiation. Interestingly, BLTK1 cells exhibited morphological changes (e.g., contracted cell bodies, increased pseudopod length) in

**FIG. 3.** Time-dependent effects of triazines and chlorometabolites on P and T levels. BLTK1 cells were treated with 3 ng/ml rhCG or 300 μM of ATR, PRO, SIM, TBA (A and B), or chlorometabolite (i.e., DEA, DACT, or DIA) (C and D) and evaluated for effects on P and T levels relative to time-matched DMSO (vehicle control). P (A and C) and T (B and D) levels were measured at each time point by EIA. P levels in media increased within 2 h of treatment followed by a gradual decrease over time. In contrast, T levels exhibited a concomitant time-dependent increase following treatment. Data were plotted as mean ± standard error. Statistical significance was determined using ANOVA followed by Tukey’s post hoc test, n = 3 (See Supplementary table 4 for concentration values and statistics).
response to rhCG at 24h (Fig. 8). MTT (Supplementary fig. S1) and trypan blue staining (data not shown) indicated no significant rhCG- or ATR-elicited cytotoxicity with no effects on cell growth or doubling time over the 48-h time course based on cell counting (data not shown).

FIG. 4. Dose-response evaluation of triazines- and rhCG-mediated gene expression. The dose-dependent effects of rhCG (black striped bars), PRO (medium grey bars), ATR (white bars), SIM (light grey bars), or TBA (black solid bars) on steroidogenic enzyme gene expression examined 24h posttreatment using QRTPCR. Data are plotted as mean ± standard error of three independent replicates, *p < 0.05 versus DMSO controls.

DISCUSSION

The endocrine-disruptive effects of triazines have been extensively studied in vivo (Cooper et al., 2007; Fraites et al., 2009; Hayes et al., 2010; Laws et al., 2003; Pogrmic et al.,...
2009; Stoker et al., 2002, 2000; Zorrilla et al., 2010); however, their mechanism remains unresolved. ATR, SIM, PRO, TBA, and DACT all induce mammary gland tumors in female Sprague Dawley rats, which was the common mode of action used to define them as a CMG (Cooper et al., 2007; USEPA, 2002). The CMG also includes the chlorometabolites DEA, DIA, and DACT, which are found in drinking water and food and elicit comparable HPG axis disruption (Laws et al., 2003, 2009; USEPA, 2002). At the time, only ATR, SIM, and PRO were deemed to have uses that result in exposure to the general public, thus excluding TBA from the CMG. ATR and SIM exposure was of greatest risk due to widespread use and persistence in ground and surface water (Gilliom et al., 2006). Estimations of human ATR exposure based on urine levels of turf applicators are ~2 μM ATR equivalents (including metabolites), whereas acute exposure is approximately half this amount (Barr

FIG. 5. Time-dependent effects of triazines- and rhCG-mediated steroidogenic gene expression. Temporal gene expression profiling of steroidogenic enzymes in response to 3 ng/ml rhCG (black striped bars) or 300 μM of PRO (medium grey bars), ATR (white bars), SIM (light grey bars), or TBA (black solid bars), respectively, was examined by QRT-PCR. Data are plotted as mean ± standard error of three independent replicates, *p < 0.05 versus time-matched DMSO controls.
et al., 2007). ATR metabolite profiles in human urine consist of 50% DACT, 30% DEA, and 5% DIA (Barr et al., 2007). Rodent studies suggest similar ATR metabolism, primarily by CYP1A1/2 (Hanioka et al., 1999; Ross et al., 2009). Maximum plasma levels in mice are achieved within 1 h, with maximum DACT plasma concentrations within 2 h postdose (Ross et al., 2009). Chlorometabolite concentrations of ~2 μM are reported in serum with less than 1 μM of parent compound following administration of 200 mg/kg ATR or an equimolar SIM or PRO dose in male Wistar rats (Laws et al., 2009). Although these human and rodent urine and serum levels encompass the concentrations used in our concentration-response studies, the effects on steroidogenesis appear to occur at triazine levels not encountered by humans, even in occupational environments.

Responses in BLTK1 cells are consistent with in vivo studies indicating that ATR, PRO, DEA, DIA, and DACT induce dose-dependent increases in serum P levels in male Wistar rats within 15 min to 3 h with no significant induction in response to SIM (Laws et al., 2009). SIM treatment for 41 days delayed vaginal opening and the onset of puberty in female rats (Zorrilla et al., 2010), suggesting more modest disruption of steroidogenesis compared with other triazines and chlorometabolites in agreement with its effects in BLTK1 cells. ATR and DIA were reported to be equally efficacious on eliciting changes in plasma P levels in male Wistar rats (Laws et al., 2009), consistent with their equipotent induction of P and T levels in BLTK1 cells. Additionally, DACT was described to be the least efficacious in male Wistar rats (Laws et al., 2009), also consistent T induction in the current study where it had no effects at concentrations < 600 μM. In addition, the CMG categorization of ATR, PRO, SIM, DEA, DIA, and DACT is in agreement with their P and T induction effects in BLTK1 cells as demonstrated by their similar EC50 values (Laws et al., 2003). Furthermore, TBA elicits comparable effects including P and T induction and differential steroidogenic gene expression. Moreover, the effects of all triazines and their chlorometabolites, including TBA, are similar to effects reported in rat primary Leydig cells at doses as low as 20 μM ATR (Pogrmic-Majkic et al., 2010). Despite differences in the differential expression of specific steroidogenic genes, the effects on P and T levels were comparable across all treatments, suggesting ATR, PRO, SIM, and TBA as well as their major chlorometabolites alter the regulation of steroidogenesis using a common mode of action.

Activation of Leydig cell steroidogenesis exhibits (1) an acute phase initiated by gonadotropin binding to LHCGR and
induction of intracellular cAMP and PKA signaling events and (2) a longer term effect involving differential gene expression and increased steroidogenic enzyme expression that affects the steroidogenic maintenance (Chen et al., 2007). Although the acute phase induces immediate T levels, sustained production requires both phases. ATR alters basal T levels in BLTK1 cells and alters Star, Hsd3b, Cyp17a1, and Hsd17b expression in rat primary Leydig cells (Pogrmic et al., 2009). The current study extends the effects on P and T levels to other triazines and their major chlorometabolites to include time- and dose-dependent effects on the long-term phase of steroidogenesis. Most ATR-elicited differential gene expression occurred after 12 h, suggesting effects on long-term steroidogenesis. For example, although Star and Cyp17a1 showed maximum induction at ~8 h, repression at later time points (24–48 h) is consistent with the feedback inhibition exerted by androgens mediated by the AR (Hales et al., 1987; Houk et al., 2004). These genes contributed to the prediction of AR activation at later time points (24–48 h), most likely due to increasing T levels at these time points. In addition, the induction of several cholesterol biosynthetic genes and predicted activation of Srebf1 and Srebf2 are consistent with steroid biosynthesis and longer term maintenance of steroidogenesis.

The early induction of Star also coincides with immediate increases in P and T levels. Star mediates the rate-limiting transport of cholesterol to the inner mitochondrial membrane required for steroidogenesis and is one of the few genes with significant induction at earlier time points. Star expression is largely cAMP mediated, with cAMP levels regulated not only by production but also by phosphodiesterase (PDE) turnover (Tsai and Beavo, 2011). ATR is reported to inhibit PDE4 in rat Leydig and anterior pituitary cells (Kucka et al., 2012), human recombinant PDE4A1 (USEPA, 2012b), and cAMP-specific PDEs in swine (Roberge et al., 2004, 2006). Biologically Multiplexed Activity Profiling (BioMAP; a primary, human, cell-based assays covering a diverse array of regulatory networks with a broad range of target mechanisms relevant to human toxicity) also suggests that ATR, SIM, and PRO increase cAMP levels, consistent with PDE inhibition (Houck et al., 2009). These data suggest that the late induction of Pde10a, Pde4b, and Pde4dip in BLTK1 cells at 48 h may be a compensatory response to PDE inhibition that warrants further investigation.

Collectively, the current study demonstrates the effects of the CMG triazines, as well as TBA, and their primary chlorometabolites on steroidogenesis in BLTK1 Leydig cells. Our data suggest that altered gene expression may underlie the long-term steroidogenic effects of triazines and their chlorometabolites although additional studies are needed to determine the specific cellular processes involved.
elucidate their acute effects on P and T levels. Although our results are consistent with other reports of triazines inducing P and/or T levels (Fraites et al., 2009; Laws et al., 2009; Pogrmic-Majkic et al., 2010; Tinfo et al., 2011), other in vivo studies show triazines antagonize serum T levels (Friedmann, 2002; Jin et al., 2013; Trentacoste et al., 2001; Victor-Costa et al., 2010). Preliminary studies suggest that triazines antagonize rhCG-stimulated steroidogenesis ( Forgacs et al., 2012), and ongoing cotreatment studies with triazine and rhCG are examining potential triazine targets that underlie the disruption of steroidogenesis in BLTK1 Leydig cells.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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**REFERENCES**


**TABLE 2**

*IPA-Predicted Transcription Factor Activation*

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>p Value</th>
<th>Time points enriched</th>
<th>Predicted activation* (time point in h)</th>
<th>Number of genes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atf4</td>
<td>1.68e−12</td>
<td>4, 8, 12, 24, 48</td>
<td>Activated (4, 12, 24, 48)</td>
<td>25</td>
</tr>
<tr>
<td>Foxo4</td>
<td>1.54e−07</td>
<td>4, 8, 12, 24, 48</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cebpb</td>
<td>5.31e−07</td>
<td>4, 8, 12, 24, 48</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Creb1</td>
<td>5.29e−06</td>
<td>4, 8, 12, 24, 48</td>
<td>Activated (4, 48)</td>
<td>27</td>
</tr>
<tr>
<td>Srebf1</td>
<td>4.87e−06</td>
<td>4, 8, 12</td>
<td>Activated (8, 12)</td>
<td>14</td>
</tr>
</tbody>
</table>

*Note.* *p* Value, *p* value; *Number of genes,* number of genes contributing to prediction.

**FIG. 8.** BLTK1 cell morphology. Cells were photographed using a phase/contrast microscope at 10× magnification 24 and 48 h after treatment with DMSO, 3 ng/ml rhCG, or 300 µM ATR.


