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Development of a combined method to assess the complex effect of atrazine on sex steroid synthesis in H295R cells

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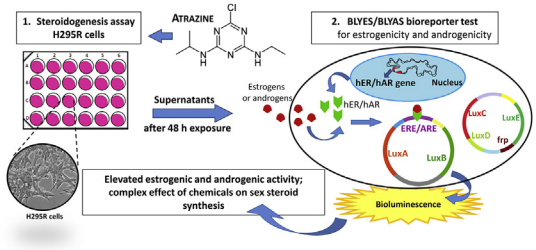
HIGHLIGHTS

- New, combined method with H295R cells and bioluminescence bioreporter assay.
- Inductive effect on both estrogen and androgen synthesis by atrazine in H295R cell.
- Rapid and simple detection of the complex, indirect effect of chemicals.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of the study was to develop a rapid, cost-effective combined testing method to assess the indirect effect of compounds interfering with sex steroid synthesis and to determine complex effects of atrazine on estrogen and androgen synthesis *in vitro* on H295R human cell line. Steroidogenic assay was performed on H295R human adrenocortical carcinoma cell line. Instead of standard analytical methods, bioluminescence bioreporter assays (*Saccharomyces cerevisiae* BLYES and BLYAS) were used to measure estrogenic and androgenic effects of sex steroid hormones released by human cells in response to atrazine. Atrazine resulted in elevated estrogen production presumably due to its well documented inductive effect on aromatase on H295R cell line, detected by BLYES. Interestingly, results of BLYAS test showed concentration-dependent increase of androgen production in H295R cells. That indicates that atrazine can not only increase estrogen level via aromatase induction, but may interfere in androgen synthesis as well. The combined method allows us to assess the androgenic and estrogenic effect of sex steroids produced by human cells in increased or decreased quantity as a result of the different chemicals, without determining specific analytical measurement endpoints, by using the yeast based bioluminescent bioreporter test.

1. Introduction

Endocrine disrupting chemicals (EDCs) interfere with hormone signalling in a variety of ways depending on the chemical and the hormonal system. Many of these chemicals interact with sex

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hormone receptors (androgenic-antiandrogenic-, estrogenic-antiestrogenic effects); others affect key enzymes involved in steroid synthesis and breakdown.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a member of chlorinated *s*-triazine group of herbicides having been widely used worldwide over the last 50 years. European Union has banned atrazine since 2004, but it is one of the most extensively used herbicide in North America (and several other countries) for pre- and post emergence control of broadleaf and grassy weeds in corn, sorghum, sugarcane and other crops (Sass&Colangelo, 2006; Ghosh&Philip, 2006).

Although atrazine does not bind to androgen and estrogen receptors (Kojima et al., 2003; Roberge et al., 2004; Suzawa&Ingraham, 2008), it has been associated with various endocrine disrupting effects including reduction of number and motility of sperm (Kniewald et al., 2000), hermaphroditism and demasculinization in frogs (Hayes et al., 2002, 2006, 2010, 2011), change in sex- and estrogen-androgen ratio in fish and amphibian (Oka et al., 2008; Suzawa&Ingraham, 2008). Furthermore, atrazine has been reported to up-regulate aromatase activity *in vitro* in H295R human adrenocortical carcinoma cells (Sanderson et al., 2000, 2002). Aromatase enzyme is responsible for the conversion of androgens to estrogens; therefore, atrazine causes elevated estrogen production due to its inductive effect on aromatase. Moreover, atrazine induces tumours of the mammary gland and reproductive organs (Pintér et al., 1990). According to the World Health Organization International Agency for Research on Cancer (IARC) there is sufficient evidence in experimental animals for the carcinogenicity of atrazine, however, in humans, atrazine is not classifiable (Group 3) since 1999 (IARC, 1999).

In 1996, EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), charging the Committee to develop a screening program that would provide necessary information to make regulatory decision about the endocrine effects of approximately 87 000 existing and new chemicals that require screening for endocrine disrupting activity. EDSTAC created a tiered Conceptual Framework consisting three major activities: i. Priority setting to obtain and evaluate existing data and to identify chemical substances and mixtures requiring further testing; ii. Tier 1 Screening to detect chemicals and mixtures capable of interacting with estrogen, androgen, or thyroid (EAT) hormone system; iii. Tier 2 Screening to precisely determine and identify the mode of action of the endocrine disrupting ability of chemicals and mixtures selected by Priority setting and/or Tier 1 (EDSTAC, 1998).

There are various techniques and methods available to analyze endocrine disrupting effects of EDCs. However, many of these tests focus on certain endpoints, e.g., estradiol and testosterone concentration measured by ELISA, or specific receptor-binding and gene-expression assays, while the complexity of multiple biological effects of EDCs on endocrine system is poorly investigated.

Herein we report a novel test method and a concentration-response study for determine the complex effects of atrazine on sex steroid synthesis in H295R human adrenocortical carcinoma cells.

The human H295R adrenocortical carcinoma cell line expresses most of the key enzymes required for steroidogenesis (Gazdar et al., 1990; Rainey et al., 1993, 1994). Therefore, the cell line is a useful *in vitro* model to investigate steroidogenic pathways and processes (Hecker & Giesy, 2008; USEPA, 2011).

Sanseverino et al. (2005) and Eldridge et al. (2007) developed a low cost and rapid bioassay, measuring estrogenic-, androgenic potential and toxicity with the purpose to create an assay that is applicable for Tier 1 high-throughput screening in EDSTAC and for monitoring endocrine-disrupting activity of unknown chemicals. *Saccharomyces cerevisiae* strains (BLYES and BLYAS) were constructed by inserting human estrogen and androgen receptor genes in the chromosome, a series of estrogen and androgen response elements, and the prokaryotic lux-genes of *Photorhabdus luminescence*. When an estrogenic or androgenic compound enters the cell of *Saccharomyces cerevisiae* BLYES and BLYAS, it is bound by the human estrogen and androgen receptor, a complex is formed, activating the lux-genes, and resulting in bioluminescence. The constitutive control strain (*S. cerevisiae* BLYR) is used to assess the toxicity of samples with a decrease in bioluminescence.

These bioluminescence bioreporters are appropriate tools to measure effects of estrogens and androgens. Here, we have combined *in vitro* steroidogenesis assay with bioreporters to reveal indirect and complex effects of atrazine on sex steroid synthesis.

2. Materials and methods

2.1. H295R cell line and culture conditions

H295R human adrenocortical cell line was obtained from the CLS Cell Line Service GmbH (Germany, NCI–H295R, no. 300483). Cells were cultured as described in OCSPP Guideline 890.1550. Briefly, upon arrival, cells were propagated in a complete growth medium consisting of 1:1 mixture of DMEM:F12 medium supplemented with ITS + Premix (insulin, transferrin, selenium, bovine serum albumin, and linoleic acid; final dilution 1:100; BD) and fetal calf serum FCS (final concentration 2.5% v/v) for at least five passages. Aliquots were regularly frozen in liquid N_2. Cells were harvested between passages 5 and 10 by 0.5% trypsin and 2×10^5 cells were plated into 24 wells' plates resulting in 60–70% confluency by 24 h. H295R cells were exposed to test compounds or medium and solvent (1 μl DMSO/ml medium) controls for 48 h. For steroidogenesis assay, cells were treated with the known aromatase inducer forskolin (CAS 66575-29-9, purity >99%), inhibitor prochloraz (CAS 67747-09-5, purity 98.6%) and atrazine. Forskolin is also an adenylyl-cyclase activator chemical and results in an elevated cAMP level. That is a second messenger and plays an essential role in the adrenal steroidogenesis by activating the protein kinase A (PKA) signalling pathway (Seamon et al., 1981; Hecker et al., 2006) and resulting in increased estrogen and androgen level at the same time. Prochloraz acts as an androgen and estrogen receptor antagonist and inhibit steroidogenic enzymes such as CYP17A1 (Vinggaard et al., 2006) and CYP19A1 (aromatase) (Andersen et al., 2002) decreasing the level of testosterone and estrogens. Chemicals were purchased from Sigma-Aldrich Ltd. Cells in 24-well culture plates containing 1 ml medium per well, were exposed to various concentrations of atrazine (1, 3, 10, 30, 100 and 300 μM), forskolin (0.03, 0.1, 0.3, 1, 3 and 10 μM) and prochloraz (0.01, 0.03, 0.1, 0.3, 1 and 3 μM) dissolved in 1 μl of dimethyl-sulfoxide (DMSO; CAS67-68-5, purity ≥99.7%, Sigma-Aldrich). In parallel with atrazine, treatments with at least two different concentrations of positive control chemicals (forskolin and prochloraz) were run as quality controls at the same time, according to the recommendations of the OCSPP Guideline. At the end of the experiment, culture supernatants were collected and frozen at −20 °C. Cells were rinsed 3 times with PBS and the plate was frozen after adding 200 μl of TRISol into each well. All treatments were carried out in duplicate in three independent experiments.

2.2. BLYES, BLYAS and BLYR test

S. cerevisiae strains BLYES, BLYAS and BLYR (The University of Tennesse, Knoxville) harbouring plasmids with leucine and uracil selective markers were stored at −80 °C and were grown overnight at 30 °C and 200 rpm to an OD_{600} of 1.0 in a modified minimal medium (YMM) without leucine and uracil (Routledge & Sumpter,

1996).

Bioluminescence (detected in photon counts per second, CPS) was measured after 5 h by a VictorX Multilabel Plate Reader (Perkin-Elmer Inc., USA). BLYES, BLYAS and BLYR tests have been carried out in triplicates for each supernatant sample originated from the steroidogenesis assay and pure compounds (atrazine, prochloraz, forskolin) and have been repeated three times using *S. saccharomyces* cells from different stocks each time. 17β-estradiol (E2) and 5α-dihydrotestosterone (DHT) (concentration range from 2E-05 to 3.5E-02 μM) dissolved in 20 μl methanol have been used as positive controls in each assay. Negative controls included wells with YMM + yeast cells and YMM + yeast cells + methanol.

2.2.1. Cross-reaction between BLYAS and BLYES tests

In order to assess the extent of possible cross-reaction, we evaluated the E2 and DHT responses detected in BLYAS and BLYES tests, respectively. 20 μl of E2 (concentration range tested from 1.8E + 02 μM to 3.7E + 05 μM) and DHT (from 5.4E + 01 μM to 1.1E + 05 μM) dissolved in methanol were placed into wells of black, sterile, flat bottom, 96-well microplate (Grenier Bio-one Gmbh, Germany), then after evaporation of the solvent 200 μl BLYES and BLYAS was added to the DHT and E2 containing wells, respectively.

2.2.2. Estrogenicity and androgenicity of the pure test compounds

To determine the estrogenic, androgenic and cytotoxic effects of the pure test compounds, BLYES, BLYAS and BLYR tests have been carried out with atrazine (1, 3, 10, 30, 100 and 300 μM), forskolin (0.03, 0.1, 0.3, 1, 3 and 10 μM) and prochloraz (0.01, 0.03, 0.1, 0.3, 1 and 3 μM) dissolved in 20 μl DMSO. Wells with YMM + yeast cells and YMM + yeast cells + DMSO were applied as negative controls.

2.2.3. Measuring estrogenicity and androgenicity of the steroid hormones produced by H295R cells

Supernatants of H295R human cells after 48 h exposure to various concentrations of atrazine, forskolin and prochloraz have been examined for their estrogenic and androgenic hormone activity. Supernatants of H295R cells treated with aromatase inducer forskolin and inhibitor prochloraz have been used as positive controls which chemicals also have inductive and inhibitory effects on testosterone production. Negative controls for the basal hormone production of H295R (Medium + H295R) and solvent controls (SC = Medium + H295R + DMSO) were also included. 20 μl samples were placed appropriate wells. Subsequently 200 μl of cultures (BLYES, BLYAS and BLYR) were placed into each well, respectively. Bioluminescence values of supernatants of H295R treated with atrazine, forskolin and prochloraz have been compared to the solvent control (SC).

2.2.4. Data analyses

Statistical analyses of bioluminescence data have been performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA). All data are expressed as means and standard deviations. Differences among all treatments have been determined using analysis of variance (ANOVA) followed by the Tukey's post hoc test, *differences with* $p \leq 0.05$ were considered significant. For curve fitting and EC_{50} calculations, normalized bioluminescence values (CPS) versus the log of chemical concentrations (μM) were plotted using the four-parametric logistic equation (Hill-equation or variable slope sigmoidal equation) as a non-linear regression model to generate concentration-response curves.

Bioluminescence intensification has been determined as calculated in Krifaton et al. (2013). Briefly:

$$\text{Bioluminescence intensification } (\%) = -1 \times [(SC - S)/SC] \times 100$$

where SC is the arithmetic mean of the bioluminescence values of parallel solvent controls after the incubation time and S represents the bioluminescence average value of parallel samples, determined at the time of contact.

3. Results

To examine endocrine disrupting effects of atrazine on steroid synthesis in H295R cells, a combined method with BLYES, BLYAS and BLYR bioreporters was developed. The literary EC_{50} values are 6.3 ± 2.4E-04 μM for E2 and 1.1 ± 0.5E-02 μM for DHT as positive control chemicals, using BLYES and BLYAS test, respectively (Sanseverino et al., 2009). The EC_{50} values (means with standard deviations from the independent bioreporter assays) were 6.46 ± 3.7E-04 μM for E2 and 3.91 ± 2.0E-03 μM for DHT measured in our study, thus the sensitivity of the bioreporter tests was sufficient.

3.1. Cross-reactivity between BLYES and BLYAS tests

Cross-reactivity was detected between the BLYES and BLYAS tests; the androgenic activity of E2 measured in the BLYAS test is nearly 30 times weaker than the effect of DHT; the total estrogenic activity of DHT when assessed in the BLYES test is almost 600 times weaker than the effect of E2. The estrogenic and androgenic effects of the estrogens and androgens produced by human cells in response to atrazine were approximately 60 000 times and 7000 times weaker, respectively than that of E2 and DHT (Table 1). The atrazine concentrations in the steroidogenesis assay, due to which the supernatant of human cells causes 50% of the maximum estrogenic and androgenic effects in the bioreporter tests (EC_{50}), are largely the same (Fig. 3). Therefore, it can be concluded that the increased estrogenic and androgenic effects in supernatants after exposure to atrazine, detected in the BLYES and BLYAS tests, are indeed due to the increased level of both the androgen and estrogen hormones and not to the cross-reaction of estrogens with the androgen receptor and the androgens with the estrogen receptor.

3.2. Estrogenicity and androgenicity of the pure test compounds

In order to evaluate whether by attaching to the human estrogen and androgen receptors, forskolin, prochloraz and atrazine, directly are able to cause increase in bioluminescence, the test chemicals were examined in BLYES, BLYAS and cytotoxicity assessing BLYR tests (the concentration range was identical to the one used in the steroidogenesis assay). The bioluminescence values evoked by the different compounds and expressed in % compared to the solvent control value are shown in Fig. 1. None of the chemicals resulted in an increase in bioluminescence as measured in the BLYES and BLYAS tests. They were not cytotoxic in the BLYR test in the highest concentration applied: forskolin, prochloraz and atrazine resulted in 4.3–12.1%, 6–12.6% and maximum 14.7% decrease in bioluminescence, respectively, and these values were not found significantly different from the solvent control. However, a significant decrease in bioluminescence was detected in case of pure prochloraz in BLYES and atrazine and forskolin in BLYAS tests, respectively (Fig. 1). Forskolin does not have cytotoxic effect, up to the best of the authors' knowledge. Although the decrease in the bioluminescence caused by forskolin is statistically significant, it is small-scale, especially as BLYES and BLYAS have only a modest bioluminescence (hence their role is to respond with elevated luminescence in presence of chemicals that are able to bind to the estrogen and androgen receptors), whereas the basic bioluminescence of BLYR strain is two orders of magnitude larger. Prochloraz is also an imidazole-type fungicide inhibiting the ergosterol

Table 1
Cross-reactivity between BLYES and BLYAS tests and relative estrogenic and androgenic activity of the compounds compared to the corresponding assay standard.

Compound	BLYES test		BLYAS test	
	Estrogenic activity[a]	EC_{50} (μM)	Androgenic activity[a]	EC_{50} (μM)
E2	1	2,740E − 04	1/27	1,290E − 01
DHT	1/571	1,570E − 01	1	4,730E − 03
H295R supernatant after atrazine exposure	1/61350	1,678E + 01	1/7256	3,438E + 01

[a] Ratio of the EC_{50} of the corresponding assay standard divided by the EC_{50} of a compound.

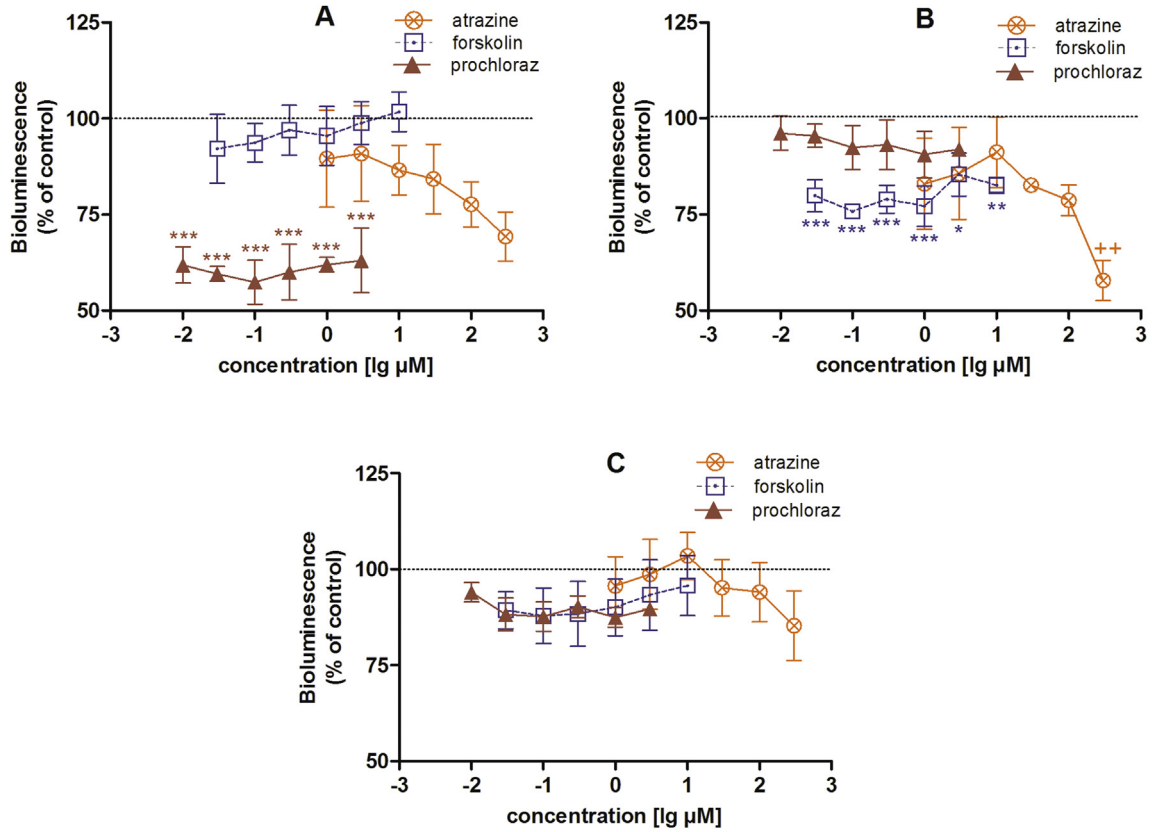

Fig. 1. Estrogenic, androgenic and cytotoxic effects of the pure compounds on *S. cerevisiae* bioluminescence bioreporter tests measured by BLYES (A), BLYAS (B) and BLYR (C) after 5 h contact time. Bioluminescence data are expressed in percentage of control (100%). One-way ANOVA followed by the Tukey's post hoc test was used. Data are presented as mean ± SD of three independent experiments. (*) significantly different from control ($p < 0.05$), (**, ++) significantly different from control ($p < 0.01$), (***, +++) significantly different from control ($p < 0.001$). Test compounds were tested three times with BLYES/BLYAS/BLYR tests in triplicate.

biosynthesis in fungi (Paranjape et al., 2014), thus this chemical can cause a decrease in bioluminescence in the bioreporters due to its anti-fungal activity. According to Sanseverino et al. (2009), atrazine has a cytotoxic effect on the bioreporters in relatively high dose (IC_{20}: 5.3E + 02 μM in BLYR) corresponding with our results (highest decrease in bioluminescence was measured at 3E + 02 μM of atrazine).

Our conclusion, based on these results, is that test chemicals alone cannot cause an increase in bioluminescence in the bioreporter test. Furthermore they cannot attach either to the estrogen or the androgen receptors directly. Therefore, when examining the supernatants of human cells originating from the steroidogenesis assay, we excluded that the pure test chemicals cause an increase in bioluminescence in the bioreporter tests.

3.3. Hormone effect of supernatants of H295R cells after exposure to test compounds

After testing pure test compounds, the supernatants originated from the steroidogenesis assay have been examined.

Supernatant samples of H295R cells treated with positive controls (forskolin and prochloraz) or atrazine did not show cytotoxic effect in BLYR test.

The effects of positive control inducer forskolin and inhibitor prochloraz, elevating and reducing estradiol and testosterone production in cells at the same time, have been measured in BLYES/BLYAS assays. In both cases, the relative responses of estrogen and androgen hormones produced by H295R cells after exposing them to forskolin and prochloraz have met expectations. BLYES and BLYAS assays produced increased bioluminescence after exposing them to supernatants of forskolin treated H295R cells, while bioreporters showed decreased values after exposing them to supernatants of prochloraz treated H295R cells. These results indicate that BLYES and BLYAS are able to detect inductive and inhibitory effects of forskolin and prochloraz in a concentration-dependent manner (Fig. 2).

Lowest observed effect concentrations, 0.3 and 0.1 μM of forskolin and 0.3 and 0.03 μM of prochloraz, significantly elevated and

abated estrogenic and androgenic effects in the supernatants of H295R cells, respectively. At a concentration 10 μM of forskolin, increases in levels of estrogen and androgen hormones resulted in 38% bioluminescence intensification of BLYES and 67% bioluminescence intensification of BLYAS strains compared to the solvent (DMSO) control (SC). 3 μM of prochloraz completely blocked the basal aromatase activity (i.e. estrogen production), thus BLYES detected 92% decrease in bioluminescence compared to SC, while decreased androgenicity resulted in 76% less bioluminescence (Fig. 2). The EC_{50} values measured by the BLYES and BLYAS tests were 0.609 μM and 0.83 μM for forskolin and 0.14 μM and 0.0358 μM for prochloraz, respectively (Table 2). These values fall within the expected hormone effective ranges of the positive control chemicals recommended by the OPSCC Guideline. These data, meeting the Guideline's requirements regarding the sensitivity of the hormone measurement system, help the validation and are confirming the efficiency of yeast based bioreporter tests in the combined method.

Atrazine induced both estrogen and androgen production in H295R cells concentration-dependently after 48 h exposure and the elevated estrogenic and androgenic effects were detected by strains of *S. cerevisiae* BLYAS and BLYES after 5-h contact time. Lowest observed effect concentrations of atrazine were 1 and 3 μM resulting in significant increase in estrogenic and androgenic effects in the supernatants measured by BLYES and BLYAS, respectively (Fig. 3).

Over 10 μM of atrazine H295R cells produced elevated estrogen level that caused more than 20% bioluminescence intensification in the BLYES test, at concentrations of 100 and 300 μM atrazine the intensifications were 40 and 52%, respectively. BLYAS test showed 30, 100 and 300 μM atrazine was able to increase androgen production in H295R cells. This caused 23, 31 and 48% bioluminescence intensification in the yeast cells compared to the solvent control (Fig. 3).

Elevated hormone activity of estrogens and androgens produced by H295R cells resulted in 50% increase in maximal bioluminescence (EC_{50}) after exposure to 16.78 μM (BLYES) and 34.38 μM (BLYAS) atrazine, respectively (Fig. 3).

4. Discussion

In this study, a combined assay has been developed to analyze endocrine disrupting effect resulting from compounds with non-direct receptor binding. For that reason, *S. cerevisiae* BLYES and BLYAS were adapted to H295R cell lines. The aromatase inducer and adenylyl-cyclase activator forskolin has to elevate both estrogen and androgen level according to the OCSPP guideline for steroidogenesis (USEPA, 2011), showed increased estrogenic and androgenic effects when supernatant of the H295R cells have been tested. Moreover, the aromatase inhibitor prochloraz is also known as an antiandrogenic chemical (Vinggaard et al., 2002), resulted in decreased level of estrogens and androgens having been detected

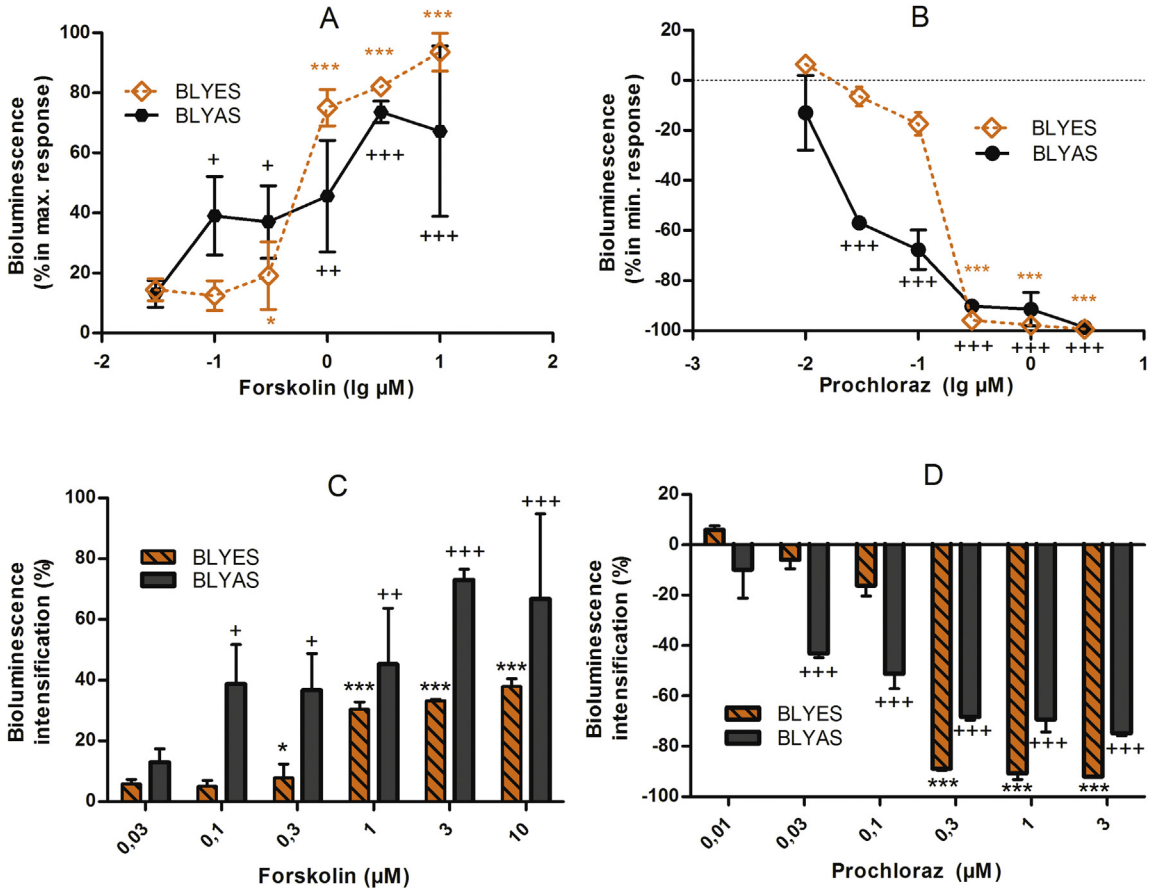

Fig. 2. Estrogenic and androgenic effects in supernatants of H295R cells measured by BLYES and BLYAS strains after 5 h contact time. Cells were exposed for 48 h to the known inducer forskolin (A) and inhibitor prochloraz (B). Data are expressed as percentage of the maximum and minimum bioluminescence values (CPS) (maximum induction = 100%; maximum inhibition = −100%). Bioluminescence intensification data (C, D) have been calculated as described in the Methods from CPS values compared to the solvent control (SC) (0%). One-way ANOVA followed by the Tukey's post hoc test was used. Data are presented as mean ± SD of three independent experiments. (*, +) Significantly different from control ($p < 0.05$), (++) significantly different from control ($p < 0.01$), (***, +++) significantly different from control ($p < 0.001$).

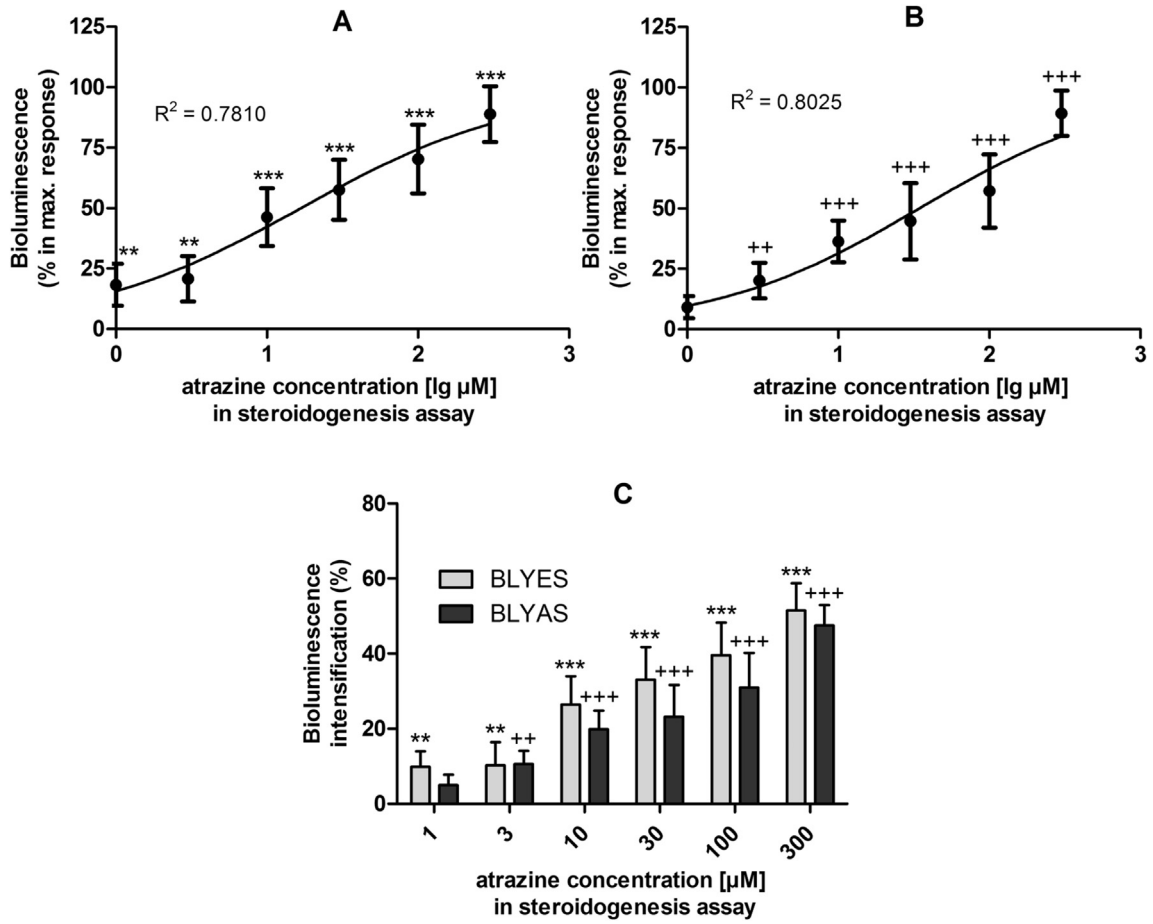

Fig. 3. Concentration-response curves show concentration-dependent elevation of estrogenic (A) and androgenic (B) effect in the supernatants of H295R adrenocortical carcinoma cells after 48 h exposure to atrazine. Data are expressed in percentage of the maximum bioluminescence (CPS) measured by BLYES and BLYAS at 5 h contact time. Each concentration of atrazine from 1 to 3 µM resulted in significantly elevated estrogenic and androgenic activity in the supernatants of human cells, respectively and was measured by BLYES and BLYAS compared to the solvent control (SC). Bioluminescence intensification data (C) in the bioreporter assays caused by increased estrogenic and androgenic effects were calculated as described in the Methods comparing the bioluminescence values of the supernatants to the solvent control (SC) (0%). One-way ANOVA followed by the Tukey's post hoc test was used. Data are presented as mean ± SD from three independent experiments. (**, ++) Significantly different from control ($p < 0.01$), (***, +++) significantly different from control ($p < 0.001$).

by the BLYES and BLYAS bioreporters.

The aromatase-inductive effect of atrazine has been widely examined in several *in vivo* and *in vitro* studies. According to previous results, atrazine caused increased estrogen level in plasma, demasculinization in frogs and increased incidence of estrogen-dependent tumours that indicated the investigation of inductive effects of atrazine on aromatase. In previous *in vivo* studies, decreased testosterone level was observed in plasma in parallel with increased estrogen production (Friedmann, 2002; Trentacoste et al., 2001; Hayes et al., 2006). However, the regulation of steroidogenic genes is very difficult and the effects of various EDCs are highly complex and poorly understood.

Aside from the expected estrogenic effect, our study has shown that atrazine is able to induce the synthesis of androgen hormones in H295R human adrenocortical cells, as well.

The main subset of androgens, known as adrenal androgens, is composed of 19-carbon steroids synthesized in the *zona reticularis*, the innermost layer of the adrenal cortex. Beside testosterone, being the main target of androgens in steroidogenesis assays, androgens include other hormones i.e. dehydroepiandrosterone, androstendione, androstendiol, androsterone and dihydrotestosterone. Atrazine showed inhibitory effect of 5α-reductase in earlier studies. This enzyme is most known to convert testosterone into the more potent androgen, dihydrotestosterone (Babić-Gojmerac et al., 1989; Kniewald et al., 1979). However, increased androgenic effect detected in BLYAS test can be attributed to neither aromatase inductor nor to 5α-reductase inhibitory effect. Atrazine may induce other enzyme activity and/or gene expression involved in the synthesis of androgenic hormones in H295R cells causing androgenicity. The *in vivo* and *in vitro* aromatase inducer effect of atrazine is well documented in the literature, but the increased androgen production induced by the chemical, up to the best of the authors' knowledge, has been only recorded in a couple

Table 2
Comparison of the expected EC_{50} ranges and the measured EC_{50} values regarding the positive control forskolin and prochloraz.

Inducer/Inhibitor	EC_{50} (µM) values			
	E2[a]	T[a]	BLYES[b]	BLYAS[b]
Forskolin	0.3–3.0	0.2–2.0	0.609	0.830
Prochloraz	0.03–0.03	0.01–0.1	0.140	0.0358

[a] Expected EC_{50} ranges for forskolin and prochloraz using 17β-estradiol (E2) and testosterone (T) as measuring end-points (OCSPP Guideline 890.1550).

[b] EC_{50} values for forksolin and prochloraz using yeast based bioreporter tests to measure hormone activity in supernatants of H295R cells (BLYES for estrogenicity and BLYAS for androgenicity) in this study.

of experiments. Pogrmic-Majkic et al. (2010) detected elevated androgen production induced by short-term atrazine treatment in rat Leydig-cells *in vitro* and *in vivo* and it was found that atrazine has a transient stimulatory action on steroidogenesis. When studying H295R cells, Higley et al. (2010) demonstrated that in response to atrazine the cells produce more estradiol and testosterone. The result of the combined method we have developed seems to support this; in response to atrazine the androgen and estrogen synthesis in H295R human adrenocortical carcinoma cells increased, and this resulted in an increased androgenic and estrogenic effect in the bioreporter test.

The H295R cell line combined with BLYES/BLYAS bioreporter system applied in the present study has been proven useful in identifying the cumulated biological effect of atrazine in sex steroid synthesis *in vitro*.

One of the advantages of the semi-quantitative method, it does not require measurement of pre-specified analytical endpoints demanding various kits and methods, since the BLYES/BLYAS system is suitable for the detection of molecules being able to attach directly to the human estrogen and androgen receptors without individual measurement of the different components.

Furthermore, the combined test allows for rapid and simple detection of the complex, indirect effect of chemicals, even combination of chemicals, certain metabolites on sex steroid synthesis without the need for costly chemical and/or immunoanalytical evaluations and related preparation and extraction procedures. Additionally, the method is also applicable in cases when the effect of the test substance(s) on steroid synthesis, hence the analytical endpoints are not possible to pre-specify accurately. By using the complex effect evaluation the false negative results due to inadequate selection and use of measurement endpoints can be avoided and the method can be a useful tool to determine the most suitable further tests and assays more efficiently.

These properties of the combined method meet many of the recommendation proposed by EDSTAC (1998) considering the scope of the Tier 1 screening and testing strategy, such as that the assays should: i. be inexpensive, quick, easy to perform; ii. capture multiple endpoints. Moreover, it fulfils the need that the test should be predictive across species; hence most of the genes and enzymes involved in adrenal steroidogenesis are present and serve the identical biochemical role in several different species and classes. After further validation and standardization, the developed combined assay can be a supplementary method assessing the indirect endocrine disrupting ability of chemicals and mixtures.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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