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More than just hormones: H295R cells as predictors of reproductive toxicity☆

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ABSTRACT

Many of the commonly observed reproductive toxicities associated with therapeutic compounds can be traced to a disruption of the steroidogenic pathway. We sought to develop an *in vitro* assay that would predict reproductive toxicity and be high throughput in nature. H295R cells, previously validated as having an intact and functional steroidogenic pathway, were treated with 83 known-positive and 79 known-negative proprietary and public-domain compounds. The assay measured the expression of the key enzymes *STAR*, 3*βHSD2*, *CYP17A1*, *CYP11B2*, *CYP19A1*, *CYP21A2*, and *CYP11A1* and the hormones DHEA, progesterone, testosterone, and cortisol. We found that a Random Forest model yielded a receiver operating characteristic area under the curve (ROC AUC) of 0.845, with sensitivity of 0.724 and specificity of 0.758 for predicting *in vivo* reproductive toxicity with this *in vitro* assay system.

1. Introduction

Toxicology is in the middle of a profound change, from a descriptive science to a predictive science, mediated by the visionary Toxicity Testing in the 21st Century publication [1]. This approach has certainly found fertile ground in pharmaceuticals [2]. Some form of safety evaluation often occurs very early in the development a of compound series. Selection of key compounds for further development uses data from multiple assays, each for an individual pathway of toxicity or key biological process [3,4]. The body depends on steroid hormones to regulate or influence the immune system, response to stress, gluconeogenesis, components of behavior, and the many complex parts of male and female reproduction. Based on the importance of this pathway in many physiological processes, it would be benificial to be able to choose between two candidate drug molecules, one of which showed an unwanted impact on steroidogenesis, and the other of which did not. To accommodate the needs of a screening program early in the candidate selection process, such an assay should require minimal amount of test compound (*de novo* synthesized drug candidate molecules), be at least relatively high-throughput, and not require extended exposures.

In steroidogenesis, cholesterol is first shuttled to the inner mitochondrial membrane in a rate-limiting step by steroidogenic acute regulatory protein (*StAR*) [5] (Fig. 1). Cholesterol is then converted to pregnenolone by side chain cleavage enzyme (*CYP11A1*). Progesterone is produced by 3 beta-hydroxysteroid dehydrogenase (*3βHSD2*) action on pregnenolone. *CYP17A1* catalyzes the 17-hydroxylation of pregnenolone and progesterone to 17α-hydroxy intermediates and the 17,20 lyase reactions leading to DHEA and along with 17beta-hydroxysteroid dehydrogenase (*17βHSD*) activity, to testosterone. Cortisol is synthesized from the 17α-hydroxy intermediates by the enzymes 3βHSD, 21-hydrolase (*CYP21A2*), and 11-beta hydroxylase (*CYP11B1*). Estradiol is converted from testosterone by the enzyme aromatase (*CYP19A1*). Estradiol can alternatively be converted by *17βHSD* from estrone, a hormone produced by aromatase activity on androstenedione.

H295R cells are a transformed human adrenal cell line which secretes all the steroid intermediates of the steroidogenesis pathway, and has been found useful for studying steroidogenesis [6–9]. These cells are zonally undifferentiated, *i.e.*, they produce the steroids of each of the three zones normally segregated in the adult adrenal cortex [10–12]. Because H295R cells uniquely express all of the enzymes in the steroidogenesis pathway, they allow the

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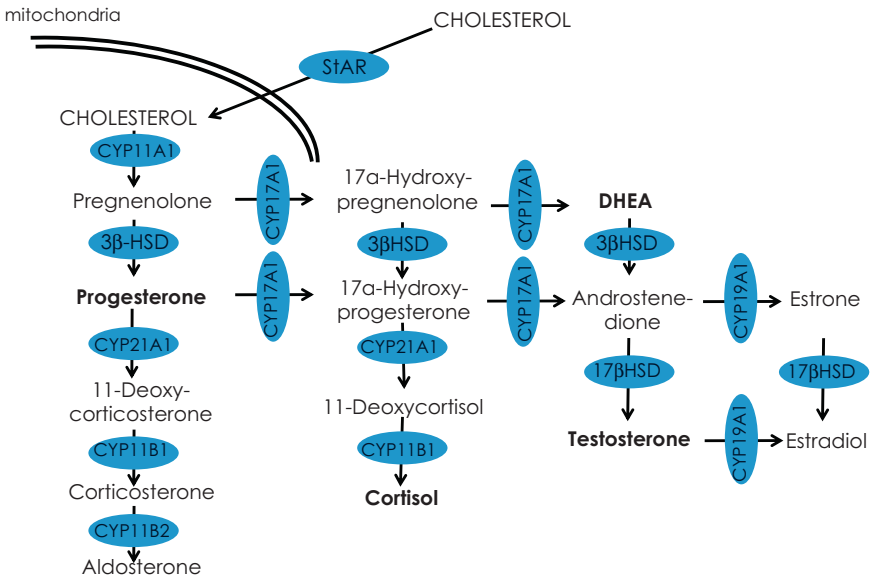

Fig. 1. The steroidogenesis pathway. Enzymes and transport proteins are represented in ovals. Measures hormone endpoints are presented in bold.

simultaneous testing of all components of steroidogenesis to the test exposure *in vitro*. These cells have been widely used in other testing efforts: the US EPA endocrine disruptor screening program (EDSP), ToxCast, European REACH, as well as the global Organization for Economic Cooperation and Development (OECD) [13–15] have all employed hormone measurement in the H295R cell model for identification of endocrine disrupting chemicals.

The objective of our work reported here was to develop a multi-parameter assay to detect chemical disruption of the steroidogenesis pathway. The assay would measure quantitative differences in the expression of enzymes in the steroidogenic pathway and levels of hormones secreted into the media following treatment with known *in vivo* toxicants and non-toxic compounds. This approach then allowed us to explore whether there was any relationship between changes in these H295R endpoints and male or female reproductive system pathology in rats in preclinical safety studies. Thus, we posed the question "how well does one or a combination of steroidogenic endpoints *in vitro* predict any male or female reproductive toxicity *in vivo*?"

2. Materials and methods

2.1. Compound selection

Compounds that had previously been evaluated *in vivo* for their adverse effects on reproductive tissues were selected from Pfizer's internal library and from published *in vivo* studies (Table 1). We took an unbiased approach and chose compounds that displayed reproductive toxicity in either male or females, and incorporated all reproductive findings, not just those considered to be hormonally driven. Not requiring a known mechanism of action for the *in vivo* reproductive toxicity was necessary to ensure sufficient power for the planned model building. It also allowed for us to determine if an assay focusing on steroidogenic regulation could play a role in predicting more general reproductive toxicity especially since most toxicity testing ends at that level of pathological resolution. The vast majority of these findings were of structural abnormalities (*i.e.*, pathology or lesions) noted in reproductive organs after dosing for periods ranging from 2 weeks to 6 months. We compiled 83 compounds with adverse reproductive findings and 79 compounds with no *in vivo* adverse finding. Forskolin (Sigma) and prochloraz (Sigma) served as positive controls for the assay. All compounds were dissolved in DMSO and DMSO (1%) was used as vehicle control.

2.2. Cell culture and toxicity assay

H295R (ATCC) cells were cultured at 37 °C in 5% CO_2 in DMEM:F12 (Invitrogen) medium supplemented with 2.5% Nu-Serum (BD Biosciences), 1% ITS+ Premix (BD Biosciences), L-glutamine (Invitrogen), and penicillin-streptomycin (Invitrogen). Exposures were conducted in 2 phases: dose-range finding, and the response phase. For dose-range finding, cells were plated at 10×10^4 per well in 96 well plates for 48 h before treating with compounds dissolved in DMSO for 30 h in 8 point curves (2-fold dilutions, starting at 300 μM). Compound effect on cell viability was determined by using the Cell Titer Glo (Promega) assay kit on compound-treated cells and results were graphed using the IDBS *XLFit* version *4.2.1* plug-in for Microsoft Excel to determine a TC_{10} (toxic concentration at which 10% cell death is observed) for the concentration used in the response experiment. If no cytotoxicity was observed, the cells were treated at 300 μM. The response phase exposure was run with cells plated at 10×10^4 cells per well in 96-well plates for 48 h before compound exposure at the TC_{10} for an additional 30 h.

We wanted to determine if the readout from our assay was correlated with cytotoxicity. We employed an assay which is commonly used in our lab as a general readout of cytotoxicity: the transformed human liver epithelium cell line [16,17]. THLE-2 cells (ATCC: CRL2706) were cultured at 37 °C in 5% CO_2 in BEBM (Lonza) supplemented with 10% HI-FBS, 5 ng/ml hEGF, 70 ng/ml phosphoethanolamine, and the supplied BEBM bullet kit (Lonza). Cells were plated at 2.5×10^3 cells/well in 384-well plates for 24 h before compound treatment. Compounds were added dissolved in DMSO in 10-point curves with 300 μM as the highest concentration. After a 72-h incubation, Cell Titer Glo reagent (Promega) was added. Luminescence values were read on a luminometer and graphed using the IDBS *XLFit* version *4.2.1* plug-in for Microsoft Excel to determine an IC_{50} values for cytotoxicity.

2.3. RNA isolation and quantitative RT-PCR analysis

At time of harvest, the cell supernatants were removed and frozen, and 100 μl lysis buffer was added to each well in the 96-well plate. The RNA was then extracted using the SV96 RNA kit (Promega) according to manufacturer's instructions. cDNA was generated using HiCapacity RT kit (ABI #4368813) according to