A novel *in vitro* toxicological approach to identify chemicals with a prostate-mediated effect on male reproduction

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Abstract

Prostate, an overlooked target in** in vitro **alternative methods, is critical for male fertility. Within the EU project ReProTect, the LNCaP cell line was used as a model system to screen chemicals affecting prostate by a tiered approach integrating two toxicological endpoints: cell viability and PSA secretion. A* ReProTect training set *of (anti) androgenic chemicals and a* ReProTect feasibility set *of blinded chemicals affecting reproductive tissues were used. Androgens, and unexpectedly glufosinate ammonium, markedly increased PSA, whereas anti-androgens also increased PSA, but at a much lower magnitude than androgens. Our tiered approach properly discriminated androgenic compounds as well as yielded no false positives, as based on available toxicological evidences. The PSA secretion assay is directly linked to the prostate physiological function and it may integrate the information provided by mechanistic-based assays (i.e., AR binding and gene expression).

The EU Integrated Project ReProTect

The identification and screening of reproductive toxicants is a main scientific challenge into the risk-to-benefit and safety assessment of chemicals [1]. So far, to assess reproductive toxicity, only *in vivo* studies are accepted by the regulatory authorities, although within the EU regulatory framework REACH (Registration, Evaluation and Authorization of several thousands of existing and new Chemicals) an impressive number of animals are required to fit the currently authorized tools for hazard assessment as well as the respect of the 3Rs principle of refinement, reduction and replacement for animal experimentation [2-4]. The EU Integrated Project ReProTect - *Development of new in vitro tests to replace animal experimentation in reproductive toxicology* - focused to provide an array of *in vitro* tests to study different phases of the mammalian reproductive cycle with the goal to contribute to the development of intelligent testing strategies for the compilation of reliable and valid safety information [5]. With the new *in vitro* testing strategy developed by ReProTect, it will be possible to screen and prioritize reproductive toxicants providing valuable information for hazard identification of chemicals [6]. Overall, ReProTect covered the reproductive cycle (Fig. 1) by three main research areas (workpackages/WPs) - (I) *fertility*, (II) *implantation* and (III) *prenatal development* - plus an extra research area – (IV) *cross-cutting technologies* – devoted to support the development of cell- and tissue-based models implemented in the other areas by developing assays based either on shared critical mechanism (*i.e.*, receptor interaction, biotransformation) or on high-tech methodologies (*i.e.*, toxicogenomics, QSARs). WP (IV) provided mechanism-based assays that can be used i) independently for replacement of animal experiments (*i.e.*, to test endocrine active substances/EASs), and ii) as building blocks in a modular testing battery [5, 6]. Within the WP (IV), dedicated also to toxicogenomics, a cell-based assay was employed to provide a phenotypic anchoring to gene expression profiling data [7]: since human prostate cell lines have been used as cellular model to investigate androgen receptor (AR)-dependent signaling, the selected cell-based, cell-specific, clinically assay used has been the prostate-specific antigen (PSA) secretion assay. Besides to be a supportive tool for the toxicogenomic approach (Fig. 2), the PSA secretion assay has been implemented as an independent tool to investigate prostate-mediated effects on male reproduction [8].

Prostate as a novel in vitro toxicological target

The evaluation of reproductive toxicology requires the development and optimization of an integrated *in vitro* testing strategy that will provide detailed information on the hazard of chemicals covering all main phases of the mammalian reproductive cycle [9], thus including all potential toxicological targets contributing to human fertility impairment [10]. Regarding male reproductive functions, spermatogenesis, semen quality of the ejaculate and function of specific testicular cell types (*i.e.*, Leydig and Sertoli cells) represent the main toxicological endpoints [11, 12]. However, the main male accessory sex gland, prostate, has received so far limited attention as a target in reproductive toxicity assays although it is essential for male fertility (Fig. 3) since secretes the prostatic fluid that constitutes ~30% of the whole ejaculate. Indeed, sperm

functional competence depends on prostatic fluid that provides proteins (e.g., PSA), trace elements (e.g., zinc) and other molecules (e.g., citrate) essential to sperm cell activation and capacitation [13 - 15]. PSA has a central role in semen liquefaction [16, 17], an event required for sperm motility toward the oviduct: a reduced PSA secretion would clearly impact on the sperm fertilization potential (Fig. 1) whereas increased PSA secretion is an established prostate cancer/PCa biomarker [16, 17]. Noteworthy, from a toxicological point of view human-derived cell lines may be considered more representative for hazard assessment since rodent prostate is not physiologically overlapping to humans due to differences in the ejaculation process as evidenced by the lack of KLK2 and PSA/KLK3, proteins regulating human liquefaction of the clot in the prostatic fluid [18].

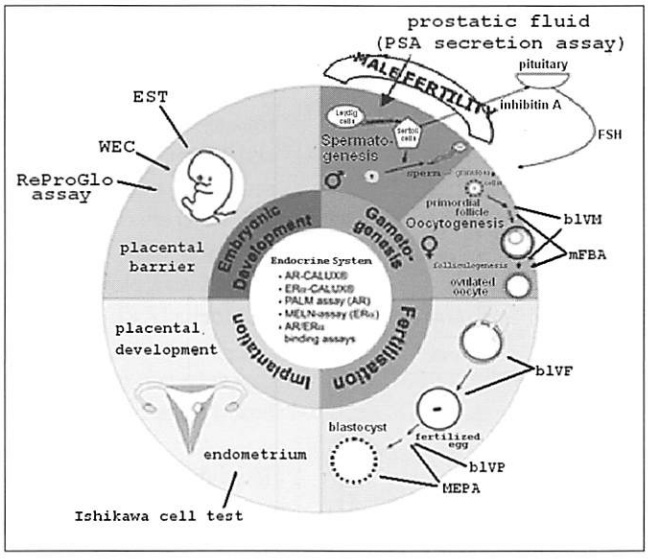

Figure 1. Mammalian reproductive cycle and ReProTect feasibility assays: PSA secretion assay and its contribution to alternative methods in male fertility. http://www.reprotect.eu//files/internal/Document%20Exchange/Figure/Download/ReProCycle%.

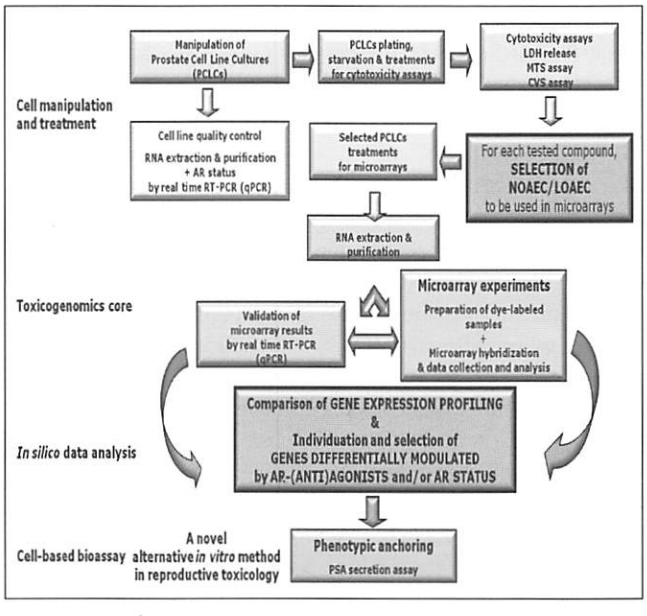

Figure 2. Implementing PSA secretion assay: from phenotypic anchoring to a novel in vitro alternative method in reproductive toxicology.

Androgens strongly regulate PSA production and secretion (Fig. 3) within the prostatic fluid [19]: under pathological conditions (*i.e.*, PCa, benign prostatic hyperplasia/BPH) androgens loose their ability to regulate the AR-mediated signaling pathway leading to a constitutive activation of AR-signaling target genes (e.g., PSA), by other proliferative signals [16, 20]; PSA secretion becomes also partially mistargeted and reaches the blood flow rather than being fully addressed to the prostatic ducts [14, 16].
Most PSA in human serum is complexed to proteins, although a significant fraction is free: the role of free PSA is not known but its concentration is apparently higher in BPH than in PCa [21]. Thus, ratio of free to total PSA might be detecting factors that predispose or promote malignant transformation in prostate cells. PSA secretion is maintained by a few established human cell lines, among them LNCaP (Fig. 3A) that, although of tumor origin, has features of normal prostate epithelial cells, such as AR expression, androgen sensitivity and PSA-secretory capability [22, 23 and refs therein].

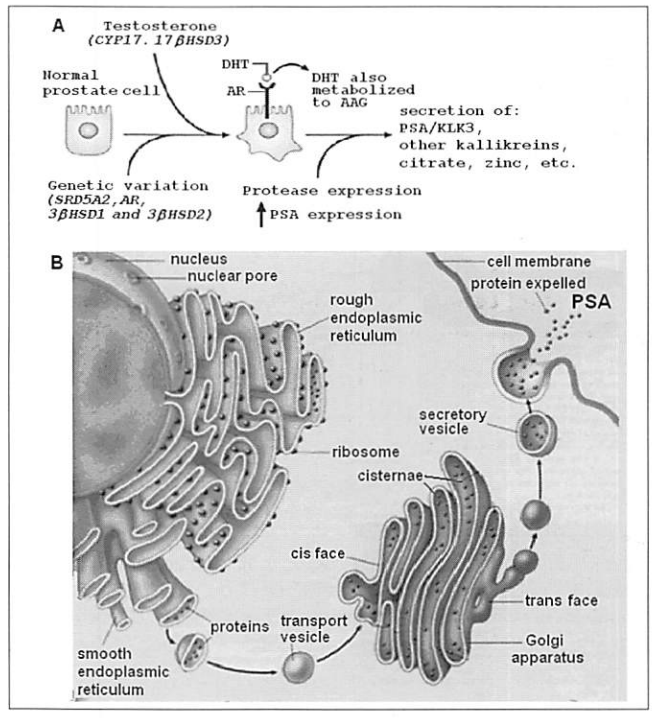

Figure 3. Overview of the functional features of the LNCaP model system (A) and of the PSA secretion process (B): http://www.carolguze.com.

Principle of endocrine disruption

As defined in 1996 [24], "*an endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function*": furthermore, it was also agreed that "*a potential endocrine disrupter is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism*". The developmental and/or reproductive function in wildlife, experimental animals and humans can be indeed adversely affected by many EASs: both natural and man-made environmental chemicals [25, 26 and refs therein] have the potential to disrupt the

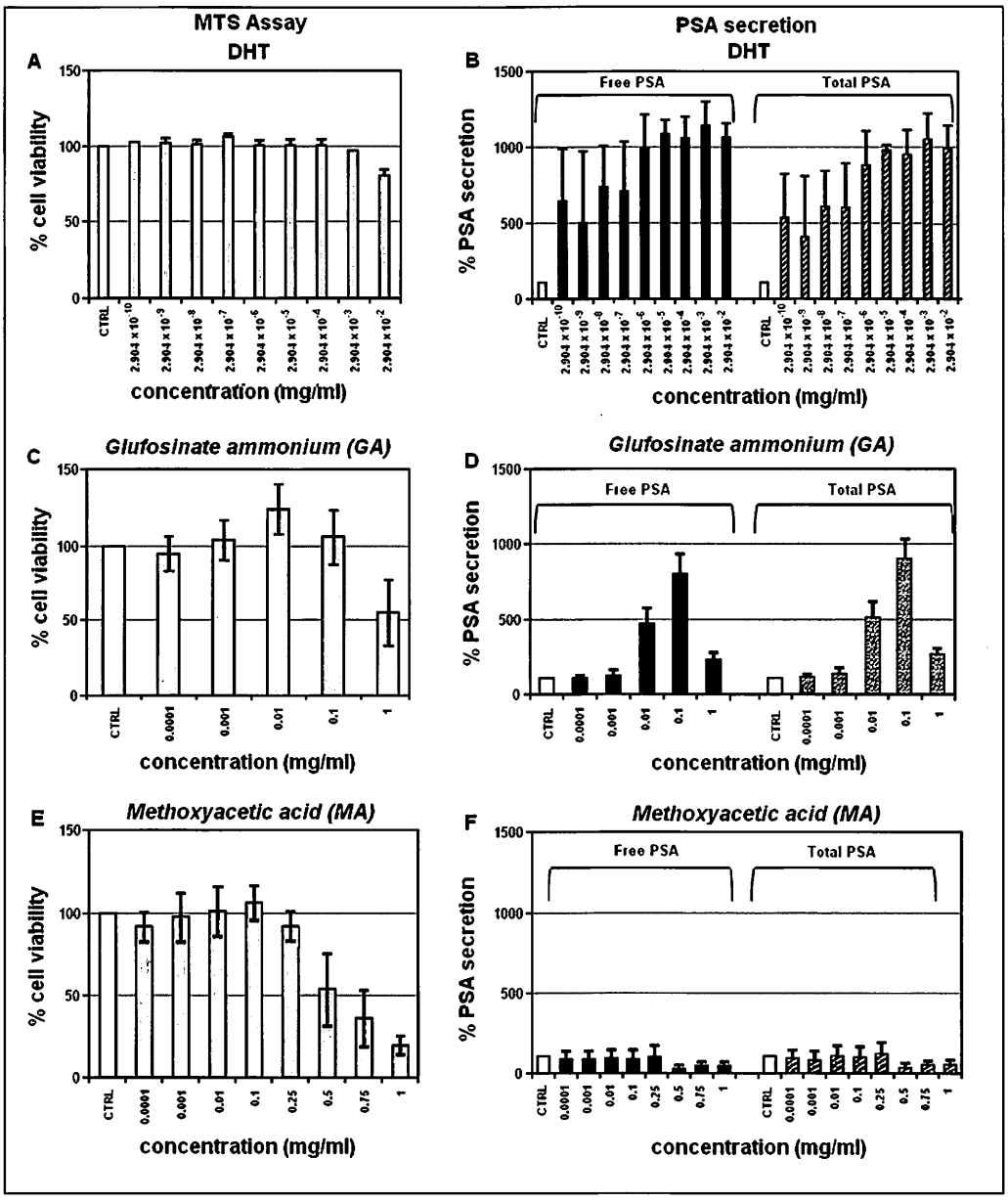

Figure 4. Effects of androgens on prostate in vitro toxicological endpoints. LNCaP cell viability after treatment with the ReProTect training set of androgens like DHT (A) and after treatments with the ReProTect feasibility set of blinded chemicals like Glufosinate Ammonium (C) and MethoxyAcetic Acid (E). LNCaP free and total PSA secretion after exposure with DHT (B), Glufosinate Ammonium (D) and MethoxyAcetic Acid (F). Adapted from [8].

endocrine system by mimicking or inhibiting endogenous hormones such as estrogens and androgens.
EASs either inhibit the biosynthesis or metabolism of endogenous ligands to indirectly modulate endocrine function (non receptor-mediated disruptors) or interfere with the ligand-dependent transcriptional function (receptor-mediated disruptors) [25, 26]. Nuclear receptor/NR ligands can be classified as NR-(anti)agonists. As NR ligands, EASs can behave either as a (non)competitive antagonist or as a NR-agonist. Among the many biological mechanisms that can result in endocrine disruption, an important one is the expression of an (anti)androgenic response as demonstrated for instance for many man-made chemicals [25, 26]. EASs with (anti)androgenic activity may exert their regulatory action on the AR by direct binding to AR or acting on its regulated signaling pathway. *In vivo* assays for the detection of (anti)androgenic action are time-consuming, costly, and labor intensive, which makes them impractical for routine screening and testing of a large number of chemicals. Thus, a novel *in vitro* approach was implemented to discriminate EAS-like chemicals not yet identified with an (anti) androgenic activity able to affect functionality of the prostate epithelium: the proposed *in vitro* alternative method takes advantage from the availability of commercial human prostate cell lines where both a general toxicity assay (cell viability and indirect proliferation assay) and a cell-specific functional effect (PSA secretion assay) can be used as toxicological endpoints [7, 8].

PSA secretion as a cell based assay

To study the reproductive toxicology, an useful alternative to *animal* testing methods are cell lines derived from specific tissues maintaining their basic functional features: indeed, to monitor the cellular behaviour of protein secretion may represent a feasible parameter (biomarker). Hence, monitoring the effect(s) of a certain EAS by a specific biomarker in a cell-based assay may be used to evaluate either a general biological status (*i.e.*, cell viability, cell proliferation, ATP levels) or a cell-specific endpoint typically or exclusively representing a function of the cell of interest. Thus, the availability of a cell-specific biomarker of effect might indirectly provide information on the patho-physiological role of EASs, above all when the selected cell-specific bioassay is already applied in the clinical field [27].

A prostate cell-specific assay

Since PSA blood levels is a recognized PCa biomarker [15-17], we implemented it as an *in vitro* toxicological biomarker in human prostate cell lines to test and compare the role of both a well-known (anti)androgen set of EASs (*ReProTect training set*) and a *ReProTect feasibilty set* of blinded chemicals whose androgen-like activity had to be

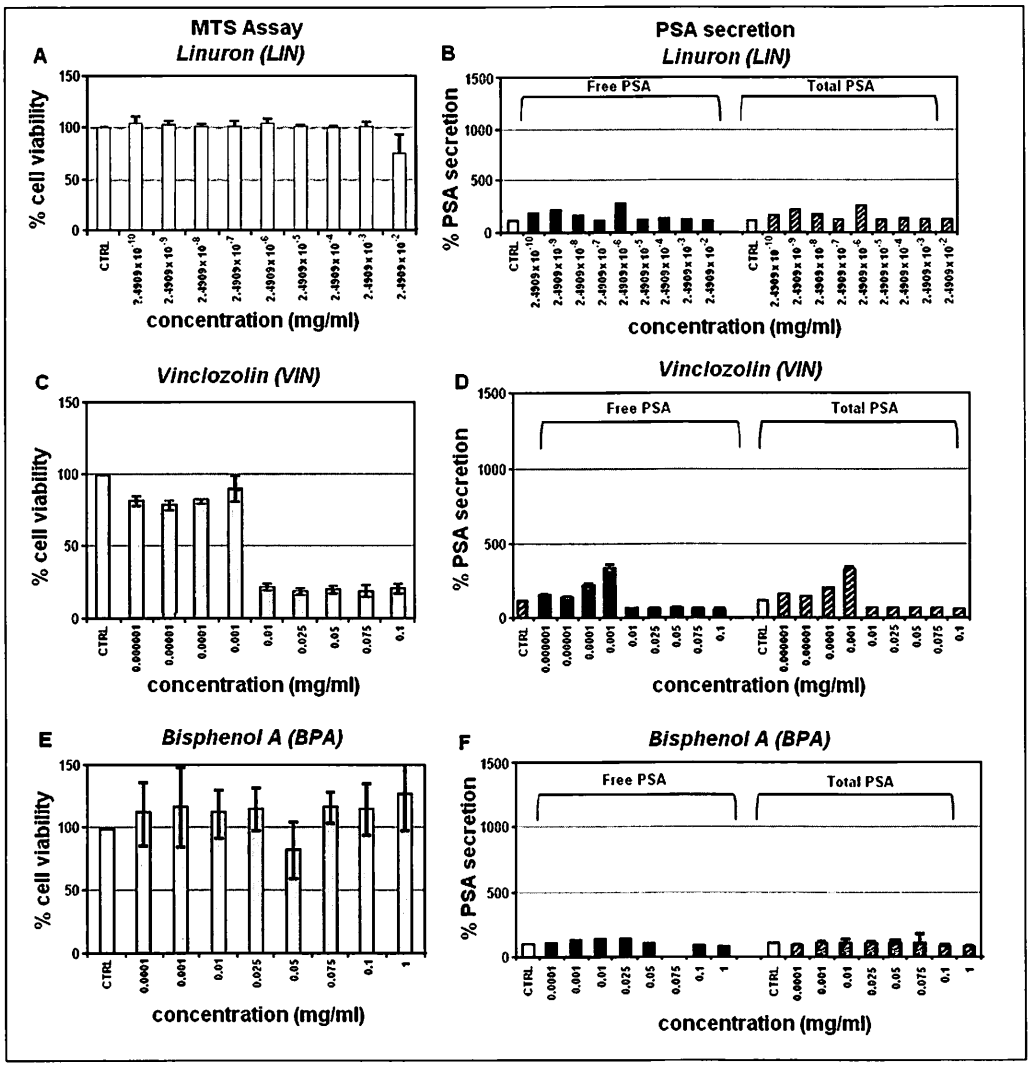

Figure 5. Effects of anti-androgens on prostate in vitro toxicological endpoints. LNCaP cell viability after treatment with the ReProTect training set of anti-androgen like linuron (A) and after treatments with the ReProTect feasibility set of blinded chemicals like vinclozolin (C) and bisphenol A (E). LNCaP free and total PSA secretion after exposure with linuron (B), vinclozolin (D) and bisphenol A (F). Adapted from [8].

established [8]. Specifically, the cell line LNCaP was used as a model system of prostate epithelial cell line, in which, the analysis of cell viability and indirect proliferation (MTS assay) and the changes in free and total PSA secretion (DELFIA assay) were used to evaluate, respectively, general and cell-specific toxicity of EAS [8 and refs therein]. Thus, PSA secretion has been shown to be applicable in *in vitro* toxicology and - without to detect any false positive or negative - may represent a valuable toxicological biomarker for the screening of EASs that may interfere with prostate-mediated male fertility.

Feasibility study: screening of androgen like-chemicals

Using LNCaP as an *in vitro* model system, a tiered approach integrating the two mentioned endpoints allows to investigate chemicals affecting prostate epithelium functionality by the contemporary measurement of both toxicological endpoints in the same cell cultures distinguishing changes due to a direct effect on PSA secretion or due to a secondary effect subsequent to cytotoxicity. Since androgens tightly regulate PSA secretion, it may be used in reproductive toxicology to detect EASs with a regulatory role on androgen-regulated pathways, which in turn affect PSA secretion itself. To evaluate the feasibility and applicability of the novel approach, as well to obtain a set of reference profiles of both free and total PSA secretion [8], both assays were firstly applied on the *ReProTect training set* (5α-dihydro testosterone/DHT, 17α-methyl testosterone/MT, 2-hydroxy-flutamide/2-OH-FTA, linuron/LIN and di-*n*-butyl phthalate/DBP) and afterwards used to screen the *ReProtect feasibility set* (listed in Table 2 of refs 8). As shown in Fig. 4 (adapted from 8), the AR-agonist DHT (Fig. 4B) and MT [8] increased both free and total PSA secretion, whereas - within the *ReProTect feasibility* (double-blinded) *set* - glufosinate ammonium/GA (Fig. 4D) [28] unexpectedly resulted to induce both free and total PSA secretion at 0.1 mg/ml, suggesting weak androgen-like properties of GA by comparison to DHT and MT. The effect on PSA secretion after GA treatment in LNCaP was clearly detectable also after normalization for cell viability/indirect proliferation confirming its target cell-specific effect. Interestingly, the PSA secretion profiles of the environmental contaminants LIN and DBP [8] - a herbicide and a plasticizer, respectively - that were used as recognized anti-androgens, overlapped with a blinded chemical that, upon un-blinding [1], resulted to be another environmental contaminant, the fungicide vinclozolin/VIN (Fig. 5D), evidencing the feasibility of the PSA secretion assay in correctly determining the AR-mediated role of the tested chemicals [8]. Furthermore, another chemical within the *ReProTect feasibility set* was recognized as a specific modulator of PSA secretion [8]: the plasticizer bisphenol A/BPA, an estrogen-like compound, showed a significant decrease in PSA secretion (Fig. 5F) although cell viability increased (Fig. 5E). The PSA secretion profile of BPA also overlapped to those ones of the known

anti-androgens LIN and DBP, although it was previously shown that BPA cannot recognize the wild-type form of the AR [29]. We hypothesized that BPA shows its anti-androgenic properties only in presence of a mutated AR: indeed, LNCaP expresses a point mutated AR (AR^{T877A}) that alters the ligand binding specificity [30].
On the contrary, 7 out of 10 chemicals within the ReProTect feasibility set [8] behaved similarly to one of them, methoxyacetic acid (MA): their free and total PSA secretion profiles changed on the basis of cell viability and indirect proliferation changes, evidencing that their role in decreasing PSA secretion (Fig. 4F) was directly proportional to the number of viable cells (Fig. 4E).

Conclusions

Overall, our tiered approach (see above Feasibility study: screening of androgen like-chemicals) used as a mean to screen androgen like-chemicals with a prostate-mediated effect, constitutes a reliable and feasible integrated *in vitro* toxicological assessment to detect chemicals affecting the male reproductive system at a commonly overlooked target, thus adding a further piece of information to male fertility issue. In particular, our integrated approach [8] to the prostate-mediated male reproductive toxicity allowed to: i) individuate a putative endocrine disrupter (GA), whose role as AR-interfering chemical has yet to be characterized; and ii) by comparison to LIN and DBP PSA secretion profiles, detect both a blinded anti-androgen as VIN and a blinded estrogen-mimicking chemical as BPA. Finally, to implement the described *in vitro* approach on prostate-mediated toxicity, molecular assays have to be included: a) to assess if the EAS of interest is an AR ligand, an AR binding assay [29] has to be performed to define the mechanism of action as receptor-mediated or not; and b) to completely characterize the mechanism of action, the modulation of gene expression of AR and of its direct molecular target PSA has to be proven as well.

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