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Evaluation of (Anti)androgenic Activities of Environmental Xenobiotics in Milk Using a Human Liver Cell Line and Androgen Receptor-Based Promoter-Reporter Assay

Himanshu Agrawal,# Keshav Thakur,# Shreyasi Mitra, Debarghya Mitra, Chetan Keswani, Debabrata Sircar, Suneel Onteru, Dheer Singh, Surya P. Singh, Rakesh K. Tyagi,* and Partha Roy*

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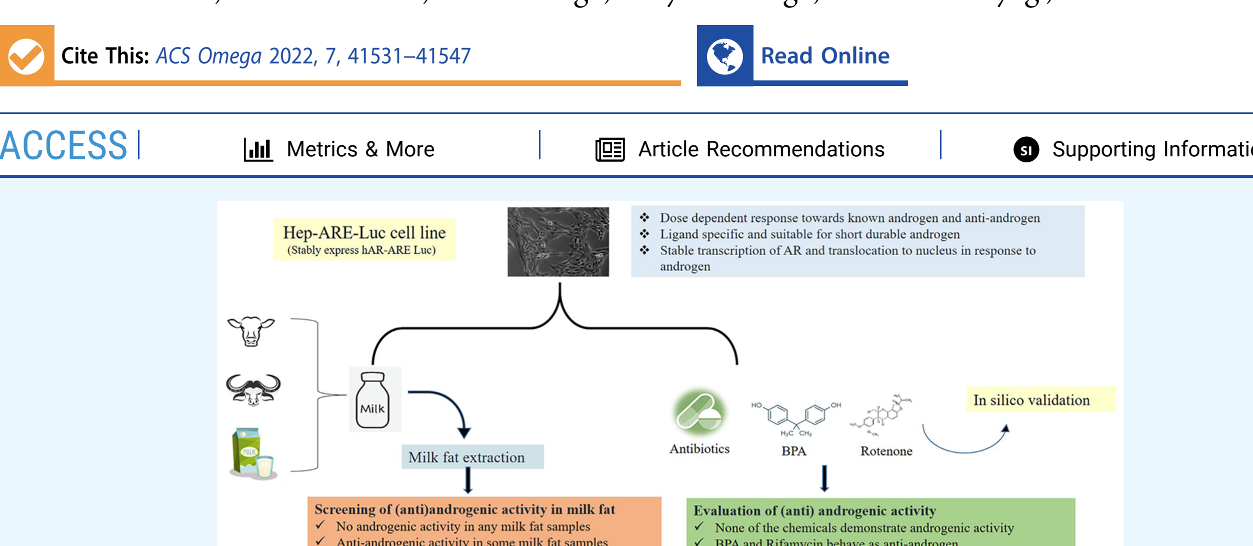

ABSTRACT: The recent reports on milk consumption and its associated risk with hormone related disorders necessitates the evaluation of dairy products for the presence of endocrine disrupting chemicals (EDCs) and ensure the safety of consumers. In view of this, we investigated the possible presence of (anti)androgenic contaminants in raw and commercialized milk samples. For this purpose, a novel HepARE-Luc cell line that stably expresses human androgen receptor (AR) and the androgen responsive luciferase reporter gene was generated and used in the present study. Treatment of this cell line with androgens and corresponding antiandrogen (flutamide) stimulated or inhibited expression of reporter luciferase, respectively. Real time polymerase chain reaction and immunostaining results exhibited transcription response and translocation of AR from the cytoplasm to the nucleus in response to androgen. Observations implied that a cell-based xenobiotic screening assay *via* AR response can be conducted for assessing the (anti)androgenic ligands present in food chain including milk. Therefore, the cell line was further used to screen the (anti)androgenic activity of a total of 40 milk fat samples procured as raw or commercial milk. Some of the raw and commercial milk fat samples distinctly showed antiandrogenic activities. Subsequently, some commonly used environmental chemicals were also evaluated for their (anti)androgenic activities. Initial observations with molecular docking studies of experimental compounds were performed to assess their interaction with AR ligand binding domain. Furthermore, (anti)androgenic activities of these compounds were confirmed by performing luciferase assay using the HepARE-Luc cell line. None of the test compounds showed androgenic activities rather some of them like Bisphenol A (BPA) and rifamycin showed antiandrogenic activities. In conclusion, our results provide a valuable information about the assessment of (anti)androgenic activities present in milk samples. Overall, it is proposed that a robust cell-based CALUX assay can be used to assess the (anti)androgenic activities present in milk which can be attributed to different environmental chemicals present therein.

■ INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are the category of environmental contaminants that interfere with the function of the endocrine system.[1] The discovery of nuclear receptors (NRs) and their interactions with a number of synthetic compounds/xenocompounds with the potential to deregulate the endocrine homeostasis among animals and humans have brought EDCs to the focal point of endocrine disorder related research.[2] The omnipresence of EDCs in the environment and food chain makes both animals and humans susceptible to exposure to EDCs and the health risks associated with them. Once inside the physiological system, these synthetic chemicals selectively bind to NRs and modulate the function of their

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target genes, thus in turn impairing the physiological functions of an organism.[3] These undesirable changes lead to alteration in the hormonal profile of the body and may cause the occurrence of diverse health-threatening hormone-related disorders such as cryptorchidism, oligospermia, endocrine-related cancers, metabolic disorders, etc.[4]

The food chain is generally considered as one of the vital routes of EDCs exposure among humans and animals.[5] Milk, due to its high nutritional value, is the most consumed food by the people of all age groups across the globe. Studies have demonstrated that milk possesses endogenous estrogen as well as its allied metabolites.[6,7] The level of these metabolites depends upon animals' physiological stage (estrous cycle, gestation, lactation) and diet.[2] Recently, a few studies have established a contentious link between the milk consumption and its associated risk with hormone-related diseases such as prostate cancer.[8−10] Apart from this, an increased intake of dairy products is also closely related to increased risk of testicular cancer,[11] and altered maturation of prepubertal children.[12] In a study, Afeiche et al.[13] determined an inverse relationship between the dairy food intake and certain semen parameters like sperm morphology and progressive motility among humans.

Milk producing animals are broadly exposed to drugs (antibiotics and veterinary medicines) and the hazardous environmental chemicals having lipophilic properties such as pesticides and insecticides (through consumption of contaminated feed and fodder, grazing on polluted soil, or usage for the cleaning of milk processing area and animal sheds).[14,15] Exposure to such lipophilic chemicals over an extended period may lead to a magnified accumulation of their residues in fat-enriched tissues, which are then excreted through milk.[16] Some hazardous chemicals enter the dairy products during the collection and processing.[17] Bisphenol A (BPA), a well-known EDC, is widely used in the production of certain packaging materials like polycarbonate plastics and epoxy resin (as internal coating in canned food). It has the tendency to leach out from food containers even under the normal conditions and thus may contaminate the packaged food materials.[18] Additionally, at farms, BPA may be introduced in milk from plastic parts (plastic resin, PVC tubes) of milking machines.[19] Thus, altogether environmental pollution may result in the contamination of milk in numerous ways.

In recent years, novel biological nontargeted techniques have been established to complement the targeted chemical analytical techniques. Currently, estrogenic compounds in food samples are detected using instrument-based methods,[20] which are unable to recognize the chemically synthesized small compounds, particularly at low concentrations.[7] Additionally, the utility of analytical methods for large scale screening is negatively affected by their relatively high cost and labor involvement.[7] In contrast, mammalian cell-based reporter assays appear to be an efficient and cost-effective tool to detect the total hormonal activity of samples.[21] Though these techniques cannot determine the exact amount of hormone active compounds found in a sample, they do provide valuable information about the biological response of food samples, including synergetic, additive and antagonistic effects that are expected to occur in the physiological system.[7] From this perspective, cell-based reporter assays are quite helpful in understanding and estimating the impact of any food or chemical sample on living individuals.[2]

Among the different reporter assays, the mammalian cell-based reporter assay is becoming an efficient technique for the detection of (anti)androgenic activity as compared to the yeast cell-based assay.[22,23] Yeast-based assays are convenient due to easy culture conditions and low cost. However, they suffer from limitations such as expression of different androgen receptor (AR) coregulators and variation in chemical permeability as compared to mammalian cells.[24] The mammalian cell-based assays involve genetically modified mammalian cells that stably express human AR along with the reporter luciferase genes under the control of hormone responsive elements. These cells respond to (anti)androgenic compounds in a dose-dependent manner through AR transcription function determined by the expression of the corresponding reporter gene.[25,26] These methods rapidly detect the presence of EDCs and define the inclusive toxicity of the sample. A large number of studies have enumerated the estrogenic potency of milk samples using the analytical or reporter-based assays.[20,7] However, except for a few reports, little is known about the androgenic contaminants in milk and there is need to consider this effect for environmental risk assessment.[27−29] Androgens are crucial male hormones and mediate critical functions in the male reproductive system. However, irregular exposure of androgenic compounds may result in several types of disorders or even may progress to malignancies in androgen relevant tissues.[30] A recent study has shown the presence of antiandrogenic substances in breast milk.[31] Therefore, an imperative need for efficient screening of dairy products for (anti)androgenic compounds to ensure their safety is evident. The primary aim of the current study is to evaluate the (anti)androgenic properties of individual and conglomerates of diverse xenobiotics (like pesticides, plasticizers, contaminated food/fodder, antibiotics, and other pharmaceuticals) that may be present in commercial and raw milk samples. Based on the advantages ascribed by mammalian cell-based assays, we developed a human liver (HepG-2) cell-based transactivation assay in which the cells were stably integrated with AR and its promoter-reporter gene constructs. The most responsive clones were selected for screening (anti)androgenic xenobiotics in milk and other relevant environmental samples.

■ MATERIALS AND METHODS

Chemicals. Fetal bovine serum (FBS) and an antibiotic-antimycotic solution (100×) were purchased from the GIBCO (GIBCO, BRL, Inchinnman UK). Escort IV reagent, Dithiothreitol (DTT), anti-Rabbit-IgG-cy-3, Hoechst 33258, TRI-Reagent, testosterone, dihydrotestosterone (DHT), *β*-estradiol, and flutamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrocortisone, MTT (3-(4,5-dimethyl-2-thiazolyl) 2,5diphenyl-2H-tetrazolium bromide), and cell culture media were purchased from Himedia (Mumbai India). DNaseI was purchased from NEB, England. Oxytetracyclin (catalog number-75966), chloramphenicol (catalog number-C0378), oxacillin (catalog number-28221), streptomycin (catalog number-S6501), tetracyclin (catalog number-T3258), rifamycin (catalog number-R3501), ciprofloxacin (catalog number-17850), rotenone (catalog number-R8875), and BPA (catalog number-239658) purchased from Sigma-Aldrich (St. Louis, MO, USA) were a kind gift from Prof. S. P. Singh, Banaras Hindu University, Varanasi, India.

Cell Culture and Stable Cell Line Generation. The HepARE-Luc stable cell line was developed by stable

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Table 1. Primers Used for PCR

Oligo name	Primer Sequence (5′−3′)	Annealing Temperature (°C)	Product Size (bp)	Accession number	PCR type
AR	FP: CGCTGAAGGGAAACAGAAGTA RP: CGAAGACGACAAGATGGACAA	60	100	NM_000044.6	Reverse transcription-PCR
β-actin	FP: CTGGCACCCAGCACAATG RP: GCCGATCCACACGGAGTACT	60	129	NM_001101.5	Reverse transcription-PCR
AR	F-TGCCCATTGACTATTACTTTCC R-TACTTCTGTTTCCCTTCAGCG	60	139	M23263.1	Real time q-PCR
β-actin	F-GCATGGGTCAGAAGGATTCCTA R-TGTAGAAGGTGTGGTGCCAGAT	60	138	NM_001101.5	Real time q-PCR

transfection of p-tk-Luc-ARE (ARE-tk-Luc), (WT) pSG5-AR, and ptk-Hygromycin plasmid (selection marker for mammalian cells) in HepG2 human liver cells. HepG2 cells (AT HB-8065) were purchased form National Centre for Cell Science (NCCS), Pune, India. The cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin. Transient DNA transfections ARE-tk-Luc, Wt-AR, and pkt-Hygromycin plasmid in HepG2 cells were performed with Escort IV reagent as per user instructions. After a transfection period of 16−20 h in minimum essential medium (MEM), the medium was replaced with complete medium, and HepG2 cells were further incubated for 24 h. Postincubation, the transfected cells were trypsinised and seeded in three 100 mm culture plates at volumes of 7.2×10^5, 3.6×10^5, and 1.2×10^5 cells, respectively. The selection of the transfected HepG2 cells was done by antibiotic hygromycin at concentrations from 200 to 400 μg/mL. The selection media was changed three times a week following a PBS wash, until individual colonies were visible in culture plates. Colonies of single clones were picked up and tested for their integrated transcriptional function. As per the experimental requirements, the clones used for testing were seeded and grown in steroid free conditions until 70% confluency was achieved. Subsequently, the cells were treated with AR's cognate ligand DHT for 24 h, before doing cell lysis using the reporter lysis buffer (for 5× composition: 125 mM Tris−HCl (pH= 7.8), 10 mM DTT, 50% glycerol, and 5% Triton X) and performing the transcriptional assay. Clones expressing the optimum activity with their natural ligand DHT at 10^{-8} M were selected for further testing and validation. The final selected clones were further validated by biochemical and molecular biology tools such as immunocytochemistry, immunoblotting, and polymerase chain reaction (PCR). The most active clone used in this study was named as HepARE-Luc. The HepG2 and HepARE-Luc cells were routinely grown in the high glucose DMEM media supplemented with 10% FBS and a 1% antibiotic-antimycotic solution. The cells were maintained in a 5% CO_2 incubator under humidified conditions.

Immunostaining for HepARE-Luc Cell Line Characterization. For indirect immunodetection, the cells (HepG2, HepAR (cell line stably expressing (WT) pSG5-AR, used as a positive control as shown earlier in Kumar et al.,[32] and HepARE-Luc) were cultured on sterile glass coverslips. After the confluency of 70% was achieved, the cells were treated with solvent alone and AR ligand DHT alone for 24 h in steroid free conditions. Following treatment, the medium was decanted and the cells were washed twice with PBS to remove traces of the medium. The cells were then fixed with chilled methanol and kept on ice for 20 min. After the incubation period, the coverslips were frozen at −20 °C for 1 h. Subsequently, cells were transferred from −20 °C to a humid chamber at room temperature and were incubated for 30 min. Post-incubation, cells were washed thrice with PBS and blocked with 2% bovine serum albumin-phosphate buffered saline (BSA-PBS; BSA prepared in PBS) for 30 min at room temperature. Subsequently, the coverslips were probed with the Rabbit Anti-human AR primary antibody at a dilution of 1:300, appropriately diluted in 2% BSA-PBS and incubated in a humid chamber at room temperature for 1 h. After incubation, the cells were washed thrice with PBS to remove unbound primary antibody and incubated with the appropriate anti-Rabbit-IgG-cy-3 conjugated secondary antibody at a dilution of 1:400 (Sigma, St. Louis, MO, USA) combined with Hoechst 33258 (0.5 μg/mL) (Sigma, St. Louis, MO, USA) in a humid chamber for 1 h at room temperature. The cells were again washed thrice with PBS and mounted on glass slides with 40% glycerol. The edges and corners of coverslips were sealed with transparent nail polish, allowed to air-dry and observed using the Olympus inverted fluorescence microscope (model IX71) (magnification 60×, scale bar 10 μm).

Immunoblotting for HepARE-Luc Cell Line Characterization. For characterization of HepARE-Luc by the immunoblotting method, the cells (COS-1 cells transiently transfected with fluorescently tagged-AR(GFP-AR) (COS-1 were purchased form National Centre for Cell Science (NCCS), Pune, India) HepG2, HepAR, and HepARE-Luc) were cultured in complete media (HepAR and GFP-AR transfected COS-1 cells were used as positive controls). Later the cells were lysed using a cell lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 0.5 mM DTT, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% NP-40 and protease inhibitor cocktail) and incubated on ice for 30 min with intermittent tapping. Protein quantification in the whole cell lysate was performed using the Bradford method. Based on protein quantification, the cell lysate volume containing equal amounts of proteins (from the above-mentioned different cell lines) mixed with sodium dodecyl sulfate (SDS) dye were resolved on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel. Resolved protein samples were transferred onto a polyvinyl difluoride (PVDF) membrane (freshly charged with methanol) using a wet transfer system (Invitrogen, USA). The size-fractionated proteins were then transferred to a PVDF membrane, which was blocked with 5% nonfat milk in PBS for 2 h at room temperature. Rabbit antihuman AR primary antibody at a dilution of 1:5000, diluted in PBS (pH 7.2), against the respective protein, was incubated with the membrane overnight at 4 °C. Post-incubation, the membrane was washed with PBS containing 0.05% Tween-20. After three wash treatments with PBS, the membrane was incubated with the horseradish

peroxidase (HRP) conjugated secondary antibody at room temperature for 1 h. The membrane was again washed three times, and immune complexes were detected by enhanced chemiluminescence. Blots were treated with 10 mL 100 mM Tris−Cl, pH 8.5, containing 22 μL of *p*-coumaric acid (90 mM), 50 μL of luminol (250 mM), 3 μL of H_2O_2, and monitored until bands fluoresced. Bands of the desired protein were observed using the ChemiDoc MP System from Bio-Rad make CA, USA.

RNA Isolation and Semiquantitative Reverse transcription-PCR. Total RNA was isolated from HepG2 cells, HepAR, and experimental cell line HepARE-Luc using TRI-Reagent. Genomic DNA contamination in the sample was removed using DNaseI. After the total RNA concentration was determined, 5 μg of RNA was used as a template for the synthesis of cDNA using a cDNA synthesis kit from Takara, Japan. cDNA synthesized from RNA of test cell lines was used as a template along with a 250 μM dNTP mix, 10pmol target gene specific (AR and β-actin) primers (forward and reverse) and 2.5 U of Taq polymerase in taq buffer to examine and characterize the integration of AR in HepARE-Luc cells. Target gene amplification was achieved in 22 cycles at a primer annealing temperature of 60 °C (Table 1) and final extension at 72 °C. The PCR products were resolved in a 1% gel prepared in a TAE buffer.

Preparation of Test Chemicals. Oxytetracycline, chloramphenicol, BPA, oxacillin, streptomycin, DHT, testosterone, β-estradiol, and hydrocortisone were prepared as a stock solution (1 mM) in ethanol. Flutamide was prepared as stock solution (100 mM) in ethanol. Stock solutions (1 mM) of tetracycline, rifamycin, ciprofloxacin, and rotenone were prepared using acetonitrile. The stock solutions of test compounds were further diluted in required solvent to achieve desired concentrations in such a way that the final concentrations of solvent never exceeded more than 0.1% in the cell culture media.

Collection of Milk Samples and Extraction of Milk Fat. A total of 40 milk samples were collected in the month of June−July 2020, which belonged to two categories, i.e., raw milk directly from vendors and commercially processed packed milk samples. Raw milk samples from individual cows (n = 10) and buffaloes (n = 10) were collected from different farms located in the surrounding villages of Roorkee, district Haridwar Uttarakhand, India. Commercial packed samples produced by two different companies (6% fat) were from the local milk vendors of Roorkee, district Haridwar Uttarakhand, India. To ensure the different pool of samples, raw and commercial packet milk samples were collected on different dates. Milk fat was extracted following the dichloromethane extraction method described previously.[33] Then 10 mL of the homogenized milk sample was collected in a 50 mL sterile tube, followed by addition of 16 mL of a dichloromethane-ethanol solution (2:1). The resulting mixture was then vortexed vigorously for 90 s, afterward centrifuged at a speed of 2500*g* for 8 min at 4 °C. Next, the aqueous phase was cautiously discarded and 10 mL of dichloromethane-ethanol (2:1) was transferred into the centrifuge tube. The mixture was again vortexed for 60 s followed by centrifugation at 2500*g* for 15 min. An upper organic phase containing milk fat and a precipitate of milk protein were observed. Milk fat was removed cautiously and kept for drying in a hot air oven overnight. The tube was then weighed and milk fat was dissolved in 1% dimethyl sulfoxide (DMSO) with a gentle vortex (1 min) to obtain the final concentration of milk fat as 20 mg/mL. This 20 mg/mL was the highest amount of fat that dissolved completely in 1% DMSO. Buffalo and cow milk fat samples were designated as BF and CF respectively, whereas commercial packed milk fat samples from companies, i.e., commercial vendor 1 and commercial vendor 2, were designated as CV and CS, respectively. Milk fat samples from each category are denoted in the order of 1−10; for example, buffalo milk samples as BF1−BF10.

Cell Viability Assay. To determine the effect of different concentrations of milk fat and test compounds on cell viability, the MTT assay was performed. The cells were seeded in 96 well plates at the initial seeding density of 5×10^3 cells per well. The cells were allowed to attach to the plate for 24 h followed by treatment with different concentrations of milk fat samples and test compounds for the next 24 h. The cells were grown and treated in the DMEM along with charcoal stripped FBS (CSFBS). Impact on cell viability was examined by measuring the ability of cells to convert yellow dye MTT to purple formazan crystals. These crystals after decanting the culture media, were solubilized using DMSO. The absorbance of colored formazan crystals was measured at 570 nM using the Fluostar optima (BMG Labtech, Germany) multiwell plate reader. The percentage cell viability was calculated by the following formula: Percentage Cell Viability = (Mean OD of treated cells/Mean OD of untreated cells) × 100.

Luciferase Assay. For performing the luciferase assay, the cells were cultured in high glucose DMEM supplemented with 10% CSFBS (DMEM-CSFBS) for 24 h to suppress the concentration of contaminating steroids in the serum. Afterward, HepARE-Luc cells were seeded at an initial density of 5×10^4 cells/well (24 well plate) in DMEM-CSFBS. After 24 h, the cells were washed with PBS and incubated with different concentrations of test compounds and milk fat samples for 24 h in DMEM-CSFBS. For all the milk fat experiments, the cells were exposed to optimized fat concentration, i.e., 1 mg/mL of culture media. After the completion of the incubation period, the cells were lysed using the 1× Glo lysis buffer (Promega, USA) according to the manufacturer's instructions. The luciferase activity was measured using the luciferase reaction assay buffer that contained tricine (40 mM, pH 7.8), $MgSO_4$ (10 mM), EDTA (0.5 mM), DTT (10 mM), coenzyme A (0.5 mM), ATP (0.5 mM), and D-luciferin sodium salt (0.5 mM). The multilabel plate reader (BMG Labtech Germany) was used to measure the light produced in the reaction.

Real Time Quantitative PCR. To determine the expression level of AR, the cells were grown for 24 h in a 6 cm culture plate (1×10^6 cells per plate) followed by different treatment for 24 h in DMEM-CSFBS. The total RNA was extracted using the RNA-XPress (Himedia Mumbai, India) following the manufacturer's instructions. The quantity and purity were determined using the Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Equal quantity of total RNA from different treatment groups (1000 ng) was reverse transcribed using the MMLV reverse transcriptase and oligo dT primers (New England Biolab, USA). The difference in transcript level was quantified using real time PCR (quant studio 3, applied biosystem USA) with the SYBR green master mix (Applied Biosystem, USA). β-Actin was used as a housekeeping gene for the normalization, and the $2-\Delta\Delta Ct$ method was used to determine the difference in transcript level.[34] Details of primers are provided in Table 1.

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Immunostaining. HepARE-Luc cells were grown in 24 wells (5×10^4 cells/well) for 24 h in DMEM-CSFBS followed by treatment with ethanol, DHT (10 nM), milk fat (1 mg/mL), and milk fat (1 mg/mL) spiked with DHT (10 nM) for 24 h. Afterward, the cells were fixed in a 4% paraformaldehyde solution for 20 min at room temperature and permeabilized by incubating cells in a 0.5% Triton X-100 solution for 30 min at room temperature. Then, the cells were incubated in 3% BSA for 1.5 h at room temperature for blocking. Afterward, cells were incubated overnight with the rabbit antihuman AR antibody as described earlier[32] at a dilution of 1:100 followed by the FITC labeled antirabbit antibody (1:1500) for 1.5 h. The cell nucleus was visualized with a DAPI solution. The cells were washed four times with PBS after completion of each step. Images were captured using confocal laser scanning microscopy (Zeiss confocal microscope, LSM 780, Germany) at 200× magnification.

Molecular Docking Analysis. Existing literature indicates the availability of X-ray structures of the ligand binding domain of AR (AR-LBD) in complex with the DHT and hydroxyflutamide molecule deposited in the RCSB Protein Data Bank (PDB) as PDB IDs 2AMA and 2AX6, respectively. However, 2AX6 AR LBD has a point mutation at the ligand binding site that makes the structure biased if considered for docking of the compounds onto the AR. Therefore, the docked poses generated in the case for DHT and hydroxyflutamide bound to AR-LBD were verified by superimposing the existing PDB structures, 2AMA and 2AX6. Next, the EMBL PISA platform was used to identify the interface residues across the 2AMA and 2AX6 with DHT and hydroxyflutamide. This was validated from SITE header data deposited in the PDB format itself in each case. The following residues were identified as primary binding site for DHT and hydroxyflutamide on 2AMA and 2AX6, respectively. 2AMA: L701, L704, N705, L707, G708, Q711, W741, M742, M745, V746, M749, R752, F764, M780, M787, L873, F876, T877, L880, F891, M895. 2AX6: L701, L704, N705, L707, G708, Q711, W741, M742, M745, V746, M749, R752, F764, M780, M787, L873, F876, T877, L880, F891, M895, I899. Since interacting residues were almost identical and 2AX6 has a point mutation, 2AMA was chosen for molecular docking analysis. Desired ligands were retrieved from several other PDB IDs (with the existing structure) and PubChem, which included these ligands complexed with the corresponding targets. UCSF Chimera was utilized to separate the ligands in each case. DHT and hydroxyflutamide structures were also retrieved using 2AMA and 2AX6 after separation through UCSF Chimera.[35] Prior to docking, UCSF Chimera was utilized to remove all water molecules and any other cofactor molecules that were attached to the receptor in PDB ID: 2AMA. A similar predocking approach was carried out for all the ligands over UCSF Chimera.[36] The Haddock 2.4 web server[37] was utilized to carry out local docking on the AR-LBD with the screened compounds. Haddock allows for a semiflexible docking between the ligand and the receptor. Docking results were analyzed through the Protein Contact Atlas,[38] for determination of contacts formed between docked ligands on the receptor, and the FireDock server,[39] for determination of the global energy values that is representative of the docking score in each docked pose.

Experimental Design. **In experiment 1**, the HepARE-Luc cell line was generated using the HepG2 cells. The selected clone cells stably expressing AR and reporter construct (ARE-tk-Luc) was characterized by luciferase assay, immunostaining, immunoblotting, and RT-PCR. Further, to assess the dose−response of HepARE-Luc cell line in the presence of its cognate ligands (agonist/antagonist), the cells were treated with increasing concentrations (0.01−50 nM) of androgen agonist, i.e., testosterone and DHT and antagonist, i.e., flutamide (0.1−100 μM) for 24 h. To examine the suitability of the assay for short durable androgens, the DHT (10 nM) mediated response in HepARE-Luc was analyzed in a time dependent manner (0−24 h). For antagonistic activity, the cells were incubated with flutamide in the presence of 0.3 nM DHT as shown in earlier studies.[25,40] Additionally, to assess the ligand specificity of HepARE-Luc, the cell line was treated with nonandrogenic steroids like β-estradiol and hydrocortisone in a concentration range (0.1−10 nM) for 24 h.

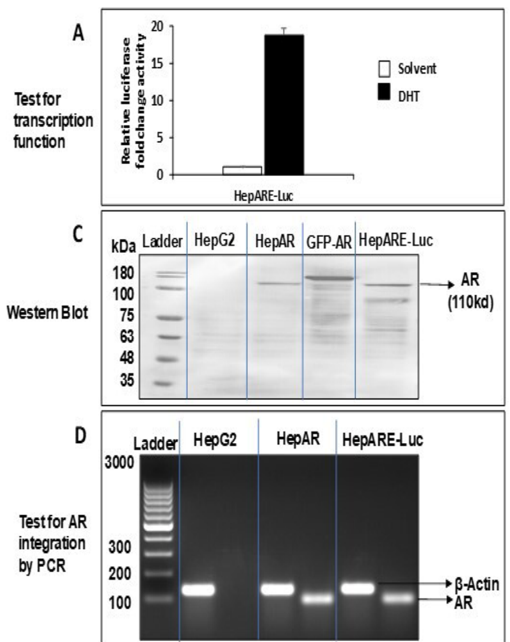
In experiment 2, the fat was extracted from raw and commercial milk samples. Afterward, Hep-ARE-Luc cells were incubated with different fat samples (1 mg/mL) for 24 h to evaluate the possible (anti)androgenic activities. For determining the antiandrogenic activity, the cells were incubated with fat samples in the presence of 0.3 nM DHT. To reconfirm androgenic activity results, HepARE-Luc cells were incubated with different concentrations of DHT (0.01−10 μM) spiked milk fat (1 mg/mL) for 24 h. Subsequently, the expression of AR was examined in HepG2 and HepARE-Luc cells. For the next experiment, HepARE-Luc cells were divided into four groups and cultured in the presence of (1) vehicle control (ethanol); (2) 10 nM DHT dissolved in ethanol; (3) milk fat (1 mg/mL) alone, and (4) DHT (10 nM) spiked milk fat (1 mg/mL). After this, the expression and translocation of AR was examined through real time PCR and immunostaining, respectively.

In experiment 3, we determined the (anti)androgenic activities of various environmental chemicals (antibiotics, insecticide and food contacting material). For this, molecular docking studies of these chemicals were performed to determine their binding interaction and affinity with the LBD of AR. Further, luciferase assay was performed to ascertain their role as an AR agonist/antagonist. HepARE-Luc cells were cultured in the presence of different concentrations (0.1−10 μM) of environmental chemicals for 24 h for androgenic activity, whereas for antiandrogenic activity, the cells were incubated for various concentrations of these chemicals (0.1−10 μM) in the presence of 0.3 nM DHT for 24 h.

Statistical Analysis. The values were presented as mean ± standard error of mean (SEM) from three independent experiments. Data was analyzed through one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test or Student's t test (as applicable) using the Graphpad Prism (version 5.0, Graphpad software Inc., San Diego, CA). The level of significance is considered as $p < 0.05$. The half-maximum effective concentration (EC50) and half-maximum inhibitory concentration (IC50) were calculated following the procedure of dose response stimulation or inhibition through Graphpad Prism. For agonist and antagonist activity, treatments were compared with vehicle control groups and 0.3 nM DHT (positive control group), respectively.

■ RESULTS

Experiment 1: Generation and Characterization of an (Anti)Androgenic Responsive Promoter-Reporter Cell Line, HepARE-Luc. The liver is the principal site of xenobiotic metabolism. Human hepatocellular carcinoma



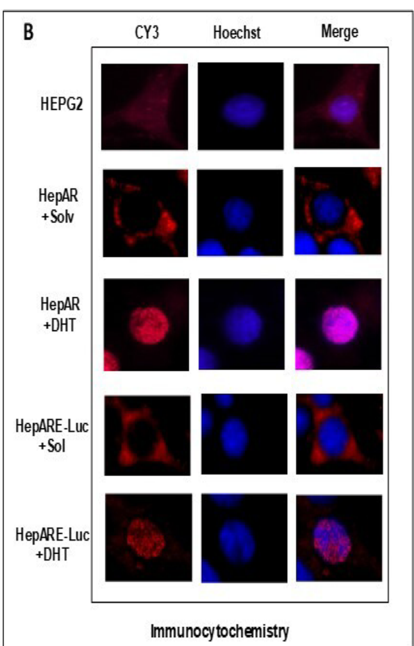


Figure 1. Generation and characterization of a stable cell line HepARE-Luc. HepG2 cells were used as a parent cell line to generate a stable cell line. Wild type AR ((WT) pSG5-AR), with its hormone response element (ARE-tk-Luc) and selection marker plasmid (pKT-Hygromycin) were cotransfected into the HepG2 cells in a ratio of 1:6:1. The selection was done using the antibiotic hygromycin at concentrations from 200 to 400 μg/mL. Single clones were picked up and tested for their integrated transcriptional function. (A) Clones expressing optimum activity with their natural ligand DHT at 10 nM were selected for further testing and validation. The selected clone was characterized by biochemical and molecular analysis tools such as (B) immunocytochemistry (C) immunoblotting, and (D) RT-PCR.

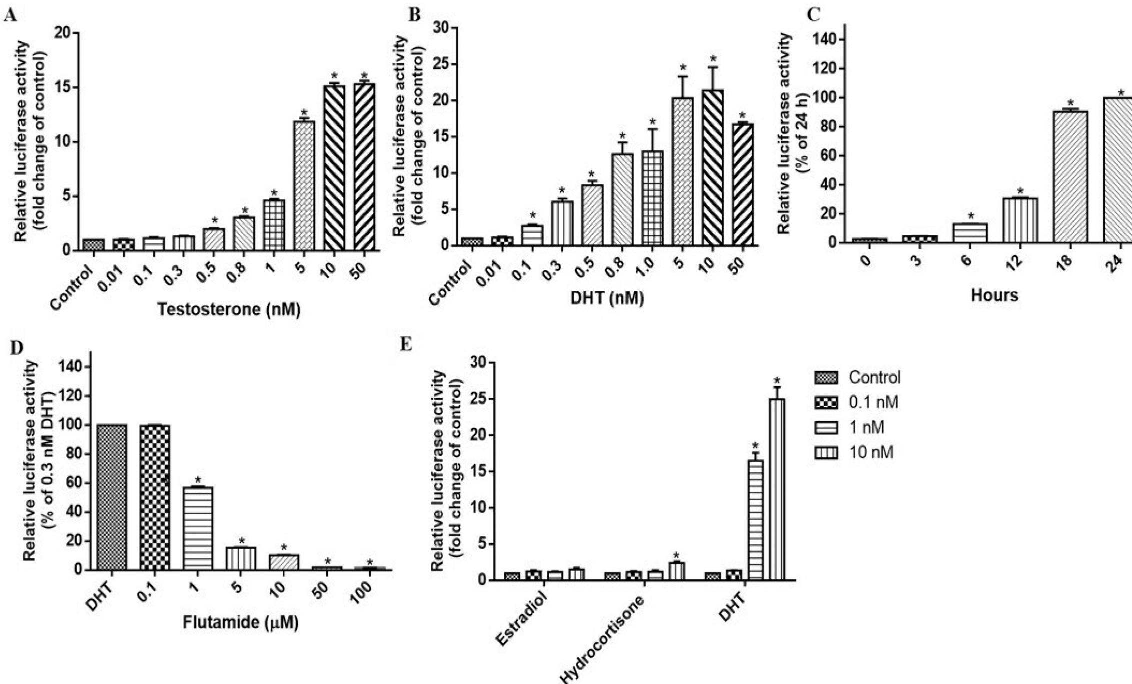


Figure 2. Characterization of transactivation assay in HepARE-Luc cell line. (A) Dose dependent induction of luciferase activity by increasing concentrations of testosterone and (B) DHT after 24 h of treatment. (C) Induction of luciferase activity by DHT (10 nM) at different time points. (D) Inhibition of DHT mediated activity (at 0.3 nM) by verifying concentrations of flutamide. (E) Determination of ligand specificity by measuring luciferase activity in response to varying concentrations of different steroids after 24 h of treatment. Data are the mean ± SEM of three independent experiments performed in triplicates. The histogram represents the relative luciferase activities expressed as (i) fold change over the vehicle treated cells (A, B, and E), (ii) transcription values expressed in percentage with 10 nM DHT from 0 to 24 h (C), and (iii) transcription values expressed in percentage with DHT at 0.3 nM and varying concentration of flutamide (D). *, $p < 0.05$ with corresponding control.

(HepG2) cells display the morphology and biochemical activities of healthy hepatocytes and widely used for *in vitro* xenobiotic studies. Hence, HepG2 cells were transfected with human nuclear receptor AR and its promoter-reporter

constructs. Several clonal cell populations were obtained using dilution cloning method in selective media as described earlier by Negi et al.[41] Clones obtained were subjected to screening by transcriptional assay, immunostaining (Figure 1B), immunoblotting (Figure 1C), and PCR (Figure 1D). The final selected clone was stored in liquid nitrogen until further use (Figure 1).

Determination of Sensitivity of HepARE-Luc Cell Line. HepARE-Luc cells were treated with an increasing concentration of two known androgen agonists/cognate ligands, testosterone and DHT. A gradual rise in luciferase activity in response to increasing concentrations of testosterone and DHT was observed. The first detectable response of luciferase expression was observed at 0.5 and 0.1 nM in the case of testosterone (Figure 2A) and DHT (Figure 2B), respectively ($p < 0.05$). A significant ($p < 0.05$) increase in luciferase activities at such a lower concentration of androgen indicates a high specificity and responsiveness of the HepARE-Luc cell line toward androgenic ligands/chemicals. The EC50 values of testosterone and DHT were found to be 2 and 1.44 nM, respectively. Subsequently, the response of HepARE-Luc cells with DHT (10 nM) in a time dependent manner was examined. The primary transcription response was observed at the 6 h time point, followed by a steady increase of transcriptional activity up to the 24 h time point (Figure 2C). These results demonstrated that this cell line is useful for examining the androgenic activities of compounds with high sensitivity.

Effects of a Potent Antiandrogen on Luciferase Activity in HepARE-Luc Cell Line. To further illustrate the application of the HepARE-Luc cell line for the screening of antiandrogenic compounds, HepARE-Luc cells were treated with increasing concentrations of a nonsteroidal antiandrogenic compound, flutamide in the presence of 0.3 nM DHT. As demonstrated in Figure 2D, flutamide inhibited the DHT induced AR transactivation by almost 50 and 85% at 1 and 5 μM concentrations, respectively.

Determination of Specificity of HepARE-Luc Cell Line. As some members of the nuclear steroid receptor family display cross-talk through binding to other nonspecific steroids, determination of specificity of a reporter cell line was desirable. For this, we incubated the HepARE-Luc cells with 0.1, 1, and 10 nM concentrations of β-estradiol and hydrocortisone. Estradiol was unable to induce transcriptional activation at all the examined concentrations, whereas hydrocortisone showed a marginal luciferase activity ($p < 0.05$) (about 2-fold over vehicle control) at 10 nM concentration, but no significant transcriptional activity was observed at 0.1 or 1 nM (Figure 2E). This observation may be explained by the fact that response elements of androgen receptor and glucocorticoid receptor share a similar consensus sequence with inverted repeats of "TGTTCT".[42] Hence, both the receptors may recognize and bind to the response elements in the presence of certain ligands. Additionally, it is believed that HepG2 has an endogenous expression of GR as well. Since the level of this increase was approximately 10 times lower than the luciferase activity observed with DHT at the same concentration, i.e., 10 nM, this minor nonspecific effect could be ignored as shown with hydrocortisone. Together, these results suggest a distinct selectivity of the HepARE-Luc cell line toward the androgenic steroids.

Experiment 2: Determination of Possible (Anti)-androgenic Activity in Milk Fat Samples and Expression Analysis of AR. *Cell Viability Assay.* To determine the nontoxic concentration of fat, for luciferase assay, the MTT-based viability assay was performed in HepARE-Luc cells. Milk fat in the range of 0.25−1.0 mg/mL showed no significant ($p < 0.05$) influence on the cell viability (Figure 3). A nominal but significant ($p < 0.05$) decrease in cell viability was noticed when cells were incubated with a higher fat concentration (2−4 mg/mL). Based on these observations, we decided to use the fat concentration (1 mg/mL) to assess the (anti)androgenic activities of different milk fat samples.

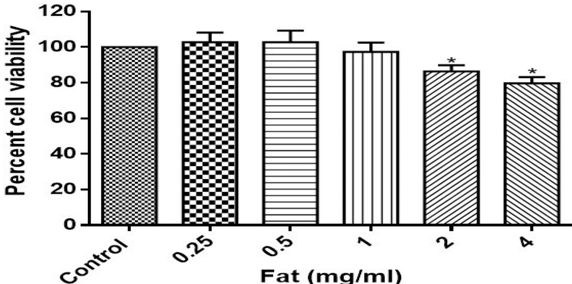


Figure 3. Milk fat at 0.25−1 mg/mL in medium added to HepARE-Luc cell line does not show cell toxicity. Varying concentrations of milk fat were added to the HepARE-Luc cells as indicated in the figure for 24 h. A cell viability assay was performed and expressed as relative cell viability over the vehicle treated control (given a value of 100%). Data are the mean ± SEM of three independent experiments performed in triplicates. *, $p < 0.05$ versus vehicle treated control.

Androgenic Activity of Milk Fat Samples. The androgenic activity in samples were analyzed using the CALUX assay protocol developed herein for milk fat samples. Experiments were performed with the milk fat extracted from the commercial and raw milk samples as described in "Materials and Methods". Fat extracted from different milk samples did not show androgenic activity, as demonstrated in Figure 4. Subsequent to milk fat treatments of HepARE-Luc cells, the levels of luciferase activities were observed to be comparable to that of the vehicle treated control cells. This data indicated the complete absence of any androgenic contaminants in the tested milk samples irrespective of their sources of collections ($p < 0.05$). However, as expected, a 15−20-fold increase in luciferase activity was observed in the case of cells treated with 10 nM DHT. This confirmed the functionality of the assay and was used to analyze the milk samples. Based on this data, it could be inferred that androgenic compounds are not present at detectable limit in the tested milk fat samples.

Estimation of Response of Different Concentrations of DHT Spiked in Milk Fat. Since we did not find androgenic activity in any of the analyzed milk fat samples, to further reconfirm and exclude the possibility of any opposing effect of milk fat on androgen mediated luciferase activities, different concentrations of DHT (0.01−10 nM) spiked milk fat (1 mg/mL) were used for the treatment of HepARE-Luc cells. Our data showed that the increase in luciferase activities of HepARE-Luc in response to various concentrations of DHT spiked milk fat (Figure 5) ($p < 0.05$) was in parallel to the response as shown by DHT alone (Figure 2B). Altogether, our results indicated that (i) the current assay may detect the presence of androgen(s) in milk fat if any, with similar responsiveness toward the free DHT; (ii) the milk fat matrix does not influence the (anti)androgenic sensitivity of the HepARE-Luc cell-line, as no modulation of transcription

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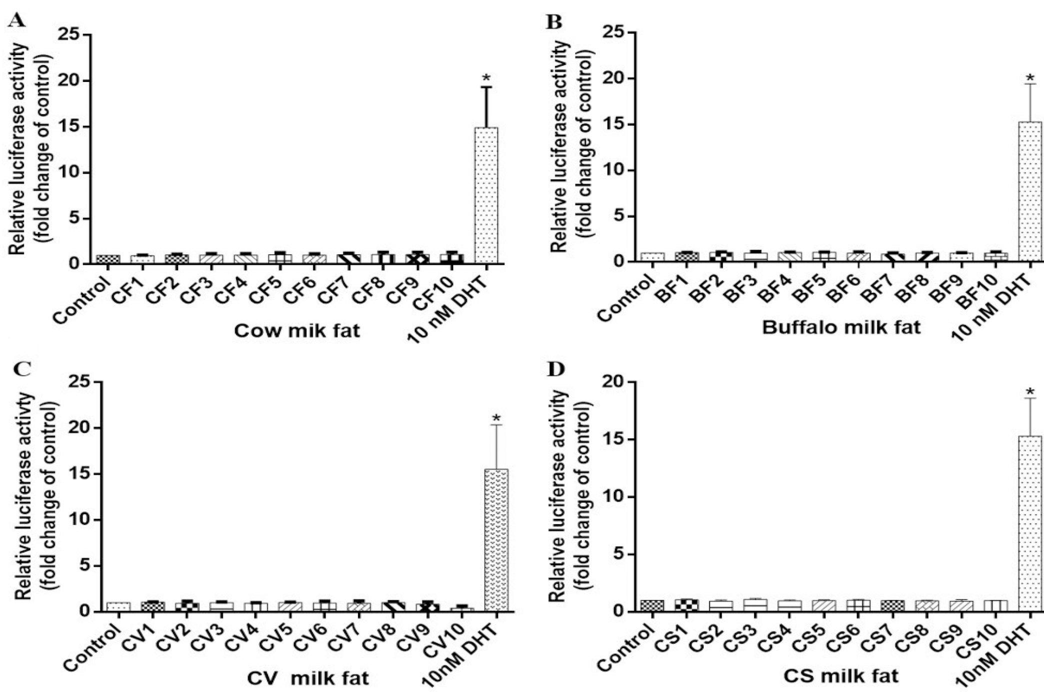

Figure 4. Effect of different milk fat samples on luciferase activity in HepARE-Luc cells after 24 h of treatment. HepARE-Luc cells were treated with fat extracted from milk samples collected from different sources. (A) Cow milk fat, (B) buffalo milk fat, (C) CV milk fat, (D) CS milk fat for 24 h in steroid-free conditions. Luciferase activities are expressed as fold changes over the vehicle treated control (given a value of 1). Data are the mean ± SEM of three independent experiments performed in triplicates. *, $p < 0.05$ versus vehicle treated control. CF, cow milk fat; BF, buffalo milk fat; CV, commercial vendor 1; CS, commercial vendor 2.

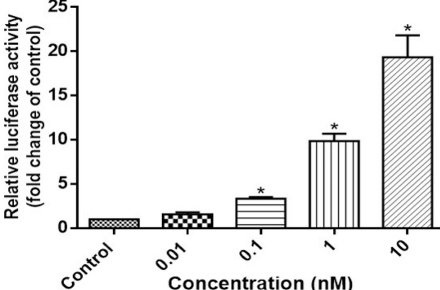

Figure 5. DHT spiked milk fat (1 mg/mL) induce transcription function in HepARE-Luc cells. Effect of different concentrations of DHT spiked milk fat on luciferase activity in HepARE-Luc cells after 24 h of treatment. Luciferase activities are expressed as the fold changes over the vehicle treated control (given a value of 1). Data represent the mean ± SEM of three independent experiments performed in triplicates. *, $p < 0.05$ versus vehicle treated control.

function of AR in HepARE-Luc cells were observed in the presence of milk fat alone (Figure 4) and DHT spiked milk fat (Figure 5) when compared to organic solvent controls. Therefore, the results of this assay reconfirmed the absence of androgenic contaminants in milk samples as shown in an earlier experiment (Figure 4). However, the presence of antiandrogenic ligands in milk fat could not be ruled out.

Antiandrogenic Activity of Milk Fat Samples. To assess the antiandrogenic activity of collected milk samples (pasteurized $n = 20$ and raw milk $n = 20$), the HepARE-Luc cells were cotreated with milk fat and 0.3 nM DHT. As shown in Figure 6A, none of cow milk fat samples ($n = 10$) exhibited significant ($p < 0.05$) antiandrogenic activities. In contrast to the cow milk fat samples, the milk fat samples obtained from buffalo ($n = 10$), 5 samples significantly ($p < 0.05$) suppressed the DHT (0.3 nM) induced luciferase activities (Figure 6B). In addition, the milk fat samples extracted from pasteurized milk were also tested separately for the presence of antiandrogenic activities. Out of the 10 pasteurized milk fat samples from CS, 8 samples showed significant ($p < 0.05$) antiandrogenic activities (Figure 6D), whereas no significant ($p < 0.05$) antiandrogenic activities were observed in any of the milk fat samples belonging to CV (Figure 6C). Since we did not observe any cytotoxic effect of milk fat samples at this dilution (1 mg/mL) (Figure 3), the current data implies that the antiandrogenic activities exhibited in some of the tested milk samples can be attributed to the presence of antiandrogenic EDCs. These observations were further corroborated with our results from chromatographic analysis of milk fat samples. The preliminary observations suggested the presence of certain chemicals which may be contributing to the antiandrogenic activities (not shown).

Expression Analysis of AR in Hep-ARE Luc Cell Line. The untransfected HepG2 and stably transfected HepARE-Luc cell lines were analyzed for the expression of human AR through quantitative PCR. As expected, the expression of AR was significantly augmented (even in basal condition) in HepARE-Luc cells as compared to the parent HepG2 cell line (that express AR very weakly) (Figure 7A). Subsequently, we determined the effect of milk fat and DHT on AR expression level in Hep-ARE Luc cells. The relative abundance of AR in HepARE-Luc cells were found to be constant following incubation in either ethanol, DHT, milk fat or milk fat spiked with DHT (Figure 7B). This observation indicates that AR expression is persistent in HepARE-Luc cells and not altered in response to milk fat or DHT. Exposure of androgen has been found to upregulate the AR expression in some cell types.[43,44] However, in HepARE-Luc cells, we did not observe such effect

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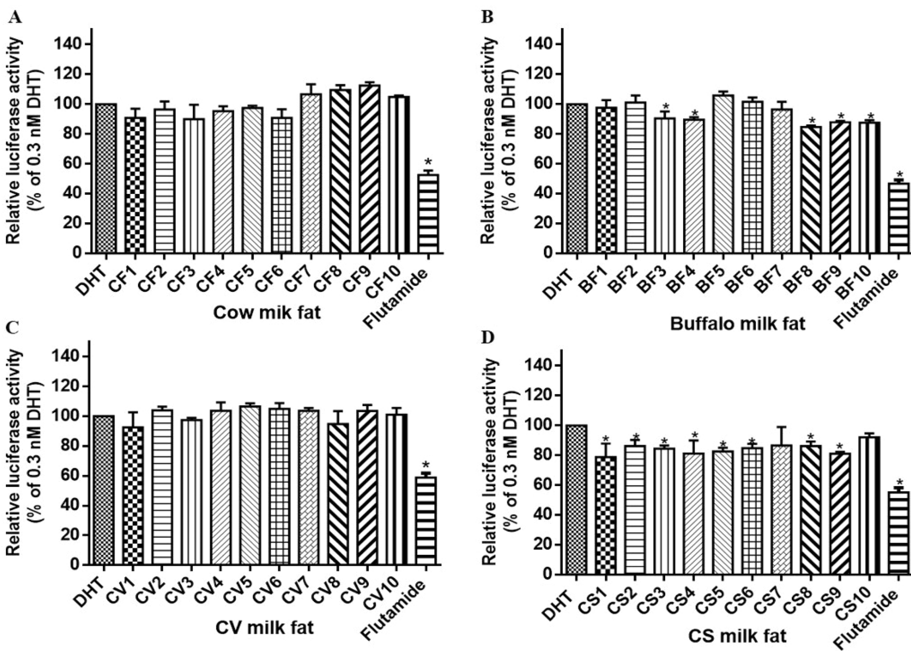

Figure 6. Determination of antiandrogenic activities of milk fat samples. HepARE-Luc cells were treated with different milk fat samples (1 mg/mL) in the presence of 0.3 nM DHT for 24 h. (A) Cow milk fat, (B) buffalo milk fat, (C) CV milk fat, (D) CS milk fat. Luciferase activities obtained post-treatment are expressed as percentage of 0.3 nM DHT (given a value of 100%). Data are the mean ± SEM of three independent experiments performed in triplicates. *, $p < 0.05$ versus 0.3 nM DHT. CF, cow milk fat; BF, buffalo milk fat; CV, commercial vendor 1; CS, commercial vendor 2.

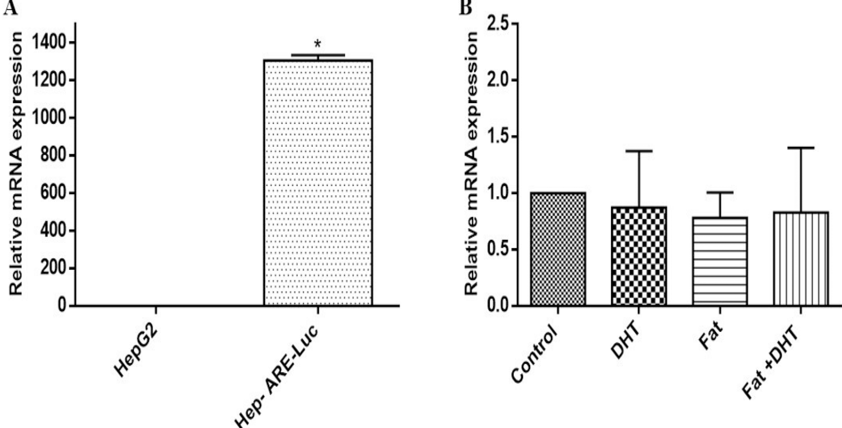

Figure 7. HepARE-Luc stably expresses abundant levels of AR. HepARE-Luc cells treated with solvent/DHT/Fat/Fat+DHT were used for mRNA expression assessment of AR. (A) Relative m-RNA expression of AR in parent HepG2 and HepARE-Luc cell line. (B) Effect of different treatments on mRNA expression level of AR in HepARE-Luc cells after 24 h of treatment. Data are the mean ± SEM of three independent experiments performed in triplicates. *, $p < 0.05$ versus HepG2 (A) and vehicle treated cells control (B).

of DHT. This observation can be explained by the fact that HepARE-Luc cells stably express a significant level of AR. Treatment with androgenic compound or cognate ligand of AR will enhance the transcription function of AR, which in turn will express the target androgenic gene. Hence, no change in the mRNA expression of AR was observed after treatment of HepARE-Luc with DHT alone, fat alone, and fat with DHT (Figure 7).

Further, we determined the cellular localization of AR in HepARE-Luc through immunostaining (Figure 8). Unliganded AR is predominantly cytoplasmic; however, after binding with respective ligand, it translocates to the nucleus to regulate the gene expression. In the absence of DHT, AR was found to be primarily located in the cytoplasm. After the treatment of cells with 10 nM DHT for 24 h, AR was found to be translocated into the nucleus. Similarly, in the presence of milk fat alone, the AR was localized predominantly in the cytoplasm. However, in the presence of milk fat spiked with DHT, AR was observed to be translocated to the nucleus. The observations from cell imaging assay were in sync with our previous results where assessment of nonspiked milk fat transcriptional potency of AR in HepARE-Luc was performed. Together, the translocation of AR from the cytoplasm to the nucleus in response to DHT confirmed the functionality of the stable cell line for testing any (anti)androgenic contaminants.

Experiment 3: Determination of (Anti)androgenic Activities of Various Environmental Chemicals Using HepARE-Luc Cell Line. Environmental chemicals belonging

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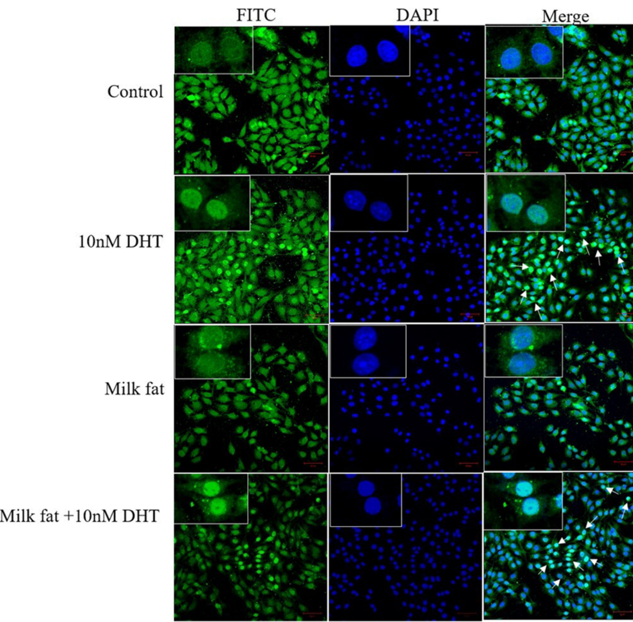

Figure 8. Nuclear translocation of AR in the presence of DHT. Representative immunofluorescence images of HepARE-Luc cells with different treatment conditions for 24 h. Green and blue fluorescence represent the AR immunoreactivity and nucleus, respectively. All the images are captured at 200× magnification; scale bar indicates 50 μm.

to diverse categories were also analyzed for their (anti)-androgenic activities to assess their endocrine disrupting properties. Oxytetracycline, chloramphenicol, oxacillin, streptomycin, tetracycline, rifamycin, and ciprofloxacin belong to the antibiotic category and are often used as veterinary medicines. Rotenone and BPA are insecticides and a food contacting chemical, respectively. Molecular dockings of these chemicals were performed to determine their binding interaction and affinity with the LBD of AR. Deposited structures of docked AR-LBD with DHT and hydroxyflutamide were considered as the mainstay in the experiment. DHT has already been reported through innumerable literatures (including ours) as a potent agonist for AR-LBD. Upon docking, the amino acid residues forming the major contacts with the DHT were found to be L701, N705, Q711, R752, L873, F876, and T877. The polar residues contribute toward the formation of a major number of contacts with DHT and result in a docking score of −46.90. On comparing the docking scores of the other environmental chemicals, screened against AR-LBD, we found that none of the compounds showed docking scores even higher than −40 (Figure 9 and Table 2). Moreover, the majority of the chemicals screened did not exhibit binding to the similar site as DHT across the AR-LBD, rather they occupied a secondary site (Supplementary Figure S1).

Subsequently, we compared the docking score and interacting residues of these compounds with hydroxyflutamide, a well-known AR antagonist. Among the screened compounds, oxytetracyclin, chloramphenicol, oxacillin, rifamycin, and ciprofloxacin had lower docking scores compared to hydroxyflutamide and also occupy a site distinct from hydroxyflutamide. However, docking scores of some compounds, i.e., rotenone, streptomycin, and tetracycline, were observed to be higher than that of hydroxyflutamide, but they also do not occupy the same site as the latter. The higher docking scores of these chemicals might have been achieved due to the presence of significantly increased contacts between the docked ligand and the AR-LBD. Rotenone and streptomycin interact with residues 13 and 10 respectively across AR-LBD in comparison to hydroxyflutamide (7 residues), indicating a possible explanation for the higher docking scores for the former compounds. Tetracycline has the same number of contacts as hydroxyflutamide, but the higher docking score can be traced to the number of polar residues like S884, S888, and D890 making contact with docked tetracycline, unlike that of hydroxyflutamide, in which polar contacts are restricted to T877 and Q711. Interestingly, BPA showed a higher docking score than hydroxyflutamide and also occupied the same binding site on AR-LBD as that of hydroxyflutamide. We found that BPA shares the contact with hydroxyflutamide through L704, Q711, M745, and T877 residues. *In silico* analysis suggested that a few of the analyzed environmental chemicals had an interaction with LBD of AR, and therefore, may bind with AR to alter receptor function. Furthermore, to understand the functional relevance of *in silico* interaction data; we performed a luciferase reporter assay to confirm whether these environmental chemicals can act as either agonist or antagonist for AR.

Next, these test compounds were used in concentrations ranging from 0.01 to 10 μM for analyzing their androgenic as well as antiandrogenic activities through luciferase assay. When HepARE-Luc cells were treated alone with these compounds, none of the compounds showed androgen agonistic activities at any of the examined concentrations (Table 3).

Conversely, 2 out of 9 chemicals were found to behave as AR antagonists as they significantly ($p < 0.05$) inhibited the DHT induced luciferase activities (Table 4 and Figure 10). According to the inhibitory property, BPA and rifamycin might be classified as moderate and potent antiandrogenic compounds, respectively. BPA can be considered as a potent antiandrogenic compound as it was found to inhibit approximately 50% of the DHT induced promoter-reporter activity even at a 1 μM concertation. At a 10 μM concentration, BPA was found to inhibit more than 80% of the activities. Rifamycin could be considered as a moderate

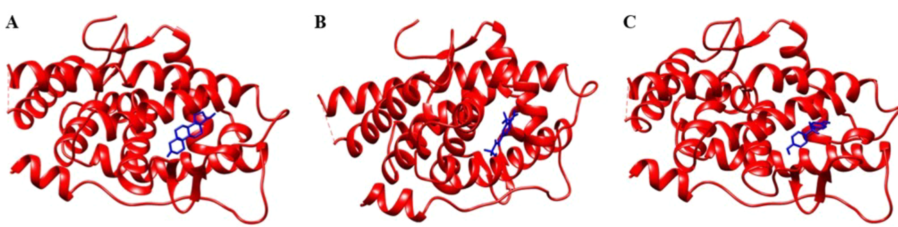

Figure 9. Docked poses of (A) DHT, (B) HFT, (C) BPA with LBD of AR protein. Red and blue colors represent AR-LBD and ligand, respectively.

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Table 2. Chemical Formula, 2-D Structure, and Docking Scores of Tested Environmental Chemicals with 2AMA along with the Interacting Residues

Name	Chemical formula	2-D structure	Interacting residues	Docking Score
Dihydrotestosterone	$C_{19}H_{30}O_2$		L701, N705, Q711, R752, L873, F876, T877	-46.90
Hydroxyflutamide	$C_{11}H_{11}F_3N_2O_4$		L704, L707, G708, Q711, M745, M780, T877	-33.81
Oxytetracline	$C_{22}H_{24}N_2O_9$		L881, S884, H885, S888, V889, D890, F891	-31.67
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$		E681, P682, G683, V684, V685, Q711, M745, A748, R752	-30.79
Oxacillin	$C_{19}H_{21}N_3O_5S$		E681, G683, V685, Q711, R752, T755, N756	-33.20
Streptomycin	$C_{21}H_{39}N_7O_{12}$		A687, H689, S702, S703, N705, E706, D890, F891, P892, E893	-38.42

Table 2. continued

Name	Chemical formula	2-D structure	Interacting residues	Docking Score
Tetracyclin	$C_{22}H_{24}N_2O_8$		L881, S884, H885, S888, V889, D890, F891	-34.41
Rifamycin	$C_{37}H_{49}NO_{12}$		E681, P682, G683, A748, W751, R752, T755, N756, F804,	-30.09
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$		E681, G683, V684, V685, Q711, R752, P766	-33.43
Rotenone	$C_{23}H_{22}O_6$		E681, P682, G683, V684, V685, Q711, A748, W751, R752, T755, N756, F764, F804	-37.92
Bisphenol A	$C_{15}H_{16}O_2$		L704, N705, Q711, M745, F876, T877, M895	-36.17

antiandrogen since it was found to inhibit about 50% DHT induced activity at 10 μM ($p < 0.05$). Rifamycin did not show any significant antiandrogenic potency at a lower concentration range, i.e., 0.01−1.0 μM. The IC_{50} values for BPA and rifamycin were found to be 1.75 and 15.4 μM, respectively. No cytotoxicity of any of the tested compounds (except rotenone) was observed in the concentration range (0.01−10 μM) (data not shown). The overall observations confirmed that BPA and rifamycin specifically inhibit the transactivation of AR without exhibiting any cytotoxic effects.

■ DISCUSSION

Milk is one of the most consumed-nutritive-complete food sources, specifically for infants. However, milk producing animals might be exposed to various EDCs either through consumption of polluted feed or *via* inhalation or dermal exposure.[14,15] Lipophilic chemicals can easily transport through lipid bilayers and get deposited in lipid rich tissues such as adipose tissue, kidney, liver, and, in the case of lactating animals, in mammary glands.[45] These lipid soluble chemicals are transported from adipose tissue through circulation and are stored into the milk fat, followed by elimination from the body through milk.[46] Due to the relative permeability of the blood/milk barrier, lipophilic hormones circulating in blood plasma may also be transferred to the milk.[45] Recently, Collet et al.[31] have confirmed the existence of antiandrogenic compounds in human breast milk. However, there is limited information about the existence of (anti)androgenic compounds in milk obtained from dairy animals. To address this important issue, the current study was performed to determine the total

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Table 3. Environmental Chemicals Analyzed for Their Androgenic Activities Using the HepARE-Luc Cell Line

Name of Compound	Concentration (μM) 0.01	0.1	1	10
Antibiotic				
Oxytetracycline	–[a]	–[a]	–[a]	–[a]
Chloramphenicol	–[a]	–[a]	–[a]	–[a]
Oxacillin	–[a]	–[a]	–[a]	–[a]
Streptomycin	–[a]	–[a]	–[a]	–[a]
Tetracycline	–[a]	–[a]	–[a]	–[a]
Rifamycin	–[a]	–[a]	–[a]	–[a]
Ciprofloxacin	–[a]	–[a]	–[a]	–[a]
Insecticide				
Rotenone	Cytotoxic	Cytotoxic	Cytotoxic	–[a]
Food contacting material				
BPA	–[a]	–[a]	–[a]	–[a]

[a]No effect.

Table 4. Environmental Chemicals Analyzed for Antiandrogenic Activities Using HepARE-Luc Cell Line

Name of Compound	Concentration (μM) 0.01	0.1	1	10
Antibiotic				
Oxytetracycline	–[a]	–[a]	–[a]	–[a]
Chloramphenicol	–[a]	–[a]	–[a]	–[a]
Oxacillin	–[a]	–[a]	–[a]	–[a]
Streptomycin	–[a]	–[a]	–[a]	–[a]
Tetracycline	–[a]	–[a]	–[a]	–[a]
Rifamycin	–[a]	–[a]	–[a]	+[b]
Ciprofloxacin	–[a]	–[a]	–[a]	–[a]
Insecticide				
Rotenone	Cytotoxic	Cytotoxic	Cytotoxic	
Food contacting material				
BPA	–[a]	–[a]	+[b]	++[c]

[a]No effect. [b]Weak antiandrogenic. [c]strong antiandrogenic.

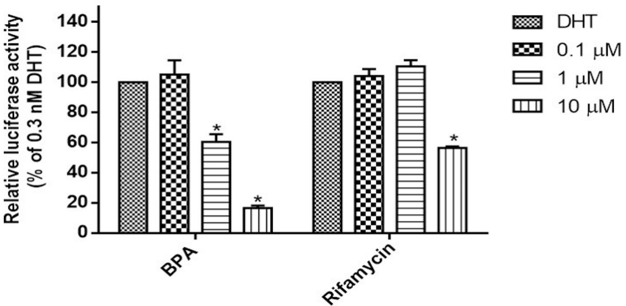

Figure 10. Assessment of antiandrogenic activities of BPA and rifamycin. HepARE-Luc cells were treated with varying concentrations of BPA and rifamycin for 24 h. Luciferase activities are expressed as percentage of 0.3 nM DHT (given a value of 100%). Data shown are the mean ± SEM of three independent experiments performed in triplicates. *, $p < 0.05$ versus 0.3 nM DHT.

(anti)androgenic activity of raw as well as processed cow and buffalo milk. In a couple of earlier reports, the concentration of free testosterone in milk was found to be in the range of 20–120 pg/mL as determined by radioimmunoassay.[27,28] In a recent report, Goyon et al.[47] determined the occurrence of various steroid hormone in milk samples (n = 103) using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. Testosterone was not detected at its limit of detection (30 pg/mL) in all the samples except one sample. In view of this, a cell-based screening protocol, which can detect the presence of (anti)androgenic xenobiotics in milk is warranted. Owing to such timely needs, there have been attempts to develop various other methods including cell-based transactivation assays.[48,49] One of the major advantages of the current cell-based assay is that it not only detects the presence of these contaminants but also indicates its bioactivity. Hence, the cell-based assay may serve as a critical tool to assess the endocrine disruptive potential of these chemicals and the health hazards caused by them.

In the current study, we used a human liver (HepG2) cell line to generate the HepARE-Luc cell line stably expressing a human AR and AR-responsive ARE-tk-luciferase promoter-reporter construct. The stable cell line successfully served as a potent tool for the screening of various androgenic or antiandrogenic compounds including their presence in spiked and nonspiked milk fat. The biggest advantages of this cell-based assay are (i) it is an AR-based transactivation assay indicating the (in)activation of AR by test samples and (ii) the reporter genes are stably integrated into the genome, giving consistent results and needing no repeated transfections. Initially, we observed that milk fat at concentrations of 2–4 mg/mL showed cytotoxicity in the HepARE-Luc cells and significantly inhibited their growth. Therefore, the a milk fat concentration of 1 mg/mL was included in the cell-based assay protocol. Overall, we observed that the current assay detected androgen with high sensitivity. However, the raw and the commercial milk samples collected from the diverse areas did not exhibit any AR transcriptional response, possibly due to the absence of significant levels of AR-activating ligands. It is conceivable that the androgenic activity in milk samples may be suppressed by the copresence of antiandrogenic chemicals. Previous reports suggested that milk may have contamination of environmental pollutants that possess estrogenic and antiandrogenic activities, such as pesticide residues and compounds leaching from food packages.[50] Considering these facts, in subsequent parts of our studies, we determined the antiandrogenic activity of the milk fat samples. Interestingly, we observed a significant antiandrogenic activity in some milk fat samples belonging to the buffalo and CS categories. BPA is a well-recognized xenoestrogen, with both estrogenic and antiandrogenic properties. It may be introduced into milk from food containers or during the collection, processing, and storage of dairy products. Various reports have documented the presence of BPA in milk, powdered milk, and infant formulas.[51,52] Additionally, animal diet derived phytoestrogens (flavonoids, isoflavonoids, stilbenes and lignans) have also been identified in milk samples. A number of reports have recognized the interaction of phytoestrogen with androgen receptors, thus exhibiting antiandrogenic activities.[53]

Pesticides that are generally used for the management of agricultural and indoor pests are widely suspected as endocrine disruptors. Dichloro-diphenyl trichloroethane and hexachlorocyclohexane pesticides were extensively used pesticides in India until late 1990.[54] As such, there are no established pesticides that are found recurrently in the milk. However, certain studies performed to determine milk quality collected from different regions of India have reported their findings otherwise. In a recent report, Gill et al.[15] analyzed the presence of pesticide residue in milk samples collected from five different cities of India. Their result reflected the occurrence of

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pesticide residue belonging to organochlorines (hexachlorocyclohexane, endosulfan, dichloro-diphenyl trichloroethane) organophosphates (ethion, profenofos, chlorpyrifos), and synthetic pyrethroids (cyhalothrin, cypermethrin, permethrin) in some milk samples. Similarly, Kumari et al.[55] also confirmed the presence of organochlorine, synthetic pyrethroid, and organophosphate pesticide residues in butter and ghee (clarified butter fat) samples collected across various part of Northern India. Numerous studies have documented the antiandrogenic properties of pesticides that belong to the aforementioned families of the chemicals.[56−58,25,41] Food contamination by environmental pollutants (such as pesticides, plasticizers) can vary by the geographical region. This in turn also influences the physiological concentrations of such pollutants in both animals and humans of that region. Several reports have shown the presence of diverse concentrations of these environmental pollutants or xenobiotics in body fluids such as milk, serum, or urine.[59−76] Hence, the milk quality in terms of contaminants may vary from region to region. The current cell-based assay as reported in this paper may provide valuable information about the (anti)androgenic properties of milk samples. Taken together, weak antiandrogenic activity in some of our milk fat samples might be attributed to the presence of certain environmental pollutants such as pesticides/BPA or phytoestrogens.

In addition to plasticizers, insecticides, and pesticides, the broad category of the environmental toxicants/EDCs also covers pharmaceuticals and antibiotics. Various studies have revealed that a substantial amount of these antibiotics (30−70%) are released unaltered into the environment.[77] Current conventional veterinary practice commonly uses multiple antibiotics for promoting the growth and prevention of infectious diseases in livestock.[78] Antibiotic residues have been observed in the milk due to their imprudent usage during the treatment of animals against infectious diseases.[79] Since various antibiotics belong to different chemical categories, endocrine disruption by them may involve different modes of actions. Hence, in addition to milk fat alone, the androgenic and antiandrogenic potencies of some antibiotics, insecticide (rotenone), and food packaging material (BPA) were also evaluated using HepARE-Luc cells. The *in vitro* observations were compared to the binding affinities of selected chemicals with AR-LBD using an *in silico* method. None of the compounds were found to possess androgenic properties at different analyzed concentrations. However, BPA and rifamycin displayed antiandrogenic properties. The results of the docking study also suggest that rifamycin interacts differently with AR-LBD as compared to hydroxyflutamide Thus, based on the result of *in vitro* and *in silico* experiments, we could further confirm that these compounds fail to modulate androgen-dependent gene expression. The observations made with docking scores complimented with our cell-based screening assays. Based on current study, it is apparent that the physiological impact of (anti)androgens present in milk cannot be disregarded completely. However, subsequent animal studies would be helpful to validate the physiological inferences proposed with the use of cell-based assays.

■ CONCLUSIONS

In conclusion, it is apparent that EDCs pose serious health risks and hence need to be addressed suitably due to their deleterious impact on developmental and other physiological functions. In addition to biophysical-principled molecule detection techniques, mammalian cell-based assays reinforce the laboratory-based screening protocols to determine the safety of numerous chemically synthesized xenocompounds. Due to the endocrine disrupting potential of such xenocompounds *via* NRs, the cell-based promoter-reporter assays are being projected as a valuable tool to assess the interaction potential between NRs and xenocompounds. From the current study, xeno-ligand-based transcriptional modulation of NRs appears to provide an integrated platform for the concrete assessment of the impact at the cellular and genetic levels, and, thus in turn, helps to speculate the influence of NR-EDC interaction on the health of both humans and animals alike. The current *in vitro* assays utilizing a stable cell line are a reliable and cost-efficient platform. Stable integration of genetic components into the central chromatin environment of the model cell provides a reliable, reproducible, and physiological approach. In this effort, the current study shows the suitability of a stable live cell line HepARE-Luc *via* the CALUX assay, for the screening of general environmental chemicals with androgenic and antiandrogenic properties concealed in milk samples.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c05344.

Docked poses of screened chemicals (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Rakesh K. Tyagi − *Special Center for Molecular Medicine, Jawaharlal Nehru University, New Delhi 110067 Delhi, India*; Phone: +91 1126 738741; Email: rktyagi@yahoo.com

Partha Roy − *Department of Bioscience and Bioengineering, Indian Institute of Technology Roorkee, Roorkee 247667 Uttarakhand, India*; orcid.org/0000-0003-1943-3079; Phone: +91 1332 285686; Email: patha.roy@bt.iitr.ac.in

Authors

Himanshu Agrawal − *Department of Bioscience and Bioengineering, Indian Institute of Technology Roorkee, Roorkee 247667 Uttarakhand, India*

Keshav Thakur − *Special Center for Molecular Medicine, Jawaharlal Nehru University, New Delhi 110067 Delhi, India*

Shreyasi Mitra − *Department of Bioscience and Bioengineering, Indian Institute of Technology Roorkee, Roorkee 247667 Uttarakhand, India*

Debarghya Mitra − *Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai 400076 Maharashtra, India*

Chetan Keswani − *Department of Biochemistry, Banaras Hindu University, Varanasi 221005 Uttar Pradesh, India*

Debabrata Sircar − *Department of Bioscience and Bioengineering, Indian Institute of Technology Roorkee, Roorkee 247667 Uttarakhand, India*

Suneel Onteru − *Division of Animal Biochemistry, National Dairy Research Institute, Karnal 132001 Haryana, India*; orcid.org/0000-0002-2834-314X

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Dheer Singh − *Division of Animal Biochemistry, National Dairy Research Institute, Karnal 132001 Haryana, India;* orcid.org/0000-0003-2686-0730
Surya P. Singh − *Department of Biochemistry, Banaras Hindu University, Varanasi 221005 Uttar Pradesh, India*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.2c05344

Author Contributions

The stable cell line was developed and characterized by K.T. and R.K.T. The data of cell response to milk fat and/or presence of environmental chemicals were conducted at IIT-Roorkee (H.A., S.M., and P.R.). The bioinformatics analysis as reported in the study were performed by the D.M. at IIT Bombay. The main draft of the paper was prepared by H.A. and P.R. The paper was further edited and approved with inputs by all the contributing authors (including K.T., R.K.T., C.K., S.P.S., S.O., and D.S.) as part of this joint interinstitutional research project.

Author Contributions

[#]H.A. and K.T. are equal first authors.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

EDCs, endocrine disrupting chemicals; AR, androgen receptor; DHT, dihydrotestosterone; BPA, Bisphenol A; CSFBS, charcoal stripped FBS; AR-LBD, ligand binding domain of AR; PXR, Pregnane X receptor; CALUX, Chemical activated luciferase gene expression

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