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We have established highly sensitive and specific androgen and estrogen reporter cell lines which we have named AR (androgen receptor) and ERα (estrogen receptor alpha) CALUX© (Chemically Activated LUCiferase eXpression), respectively. Both bioassays are member of a panel of CALUX reporter cell lines derived from the human U2-OS osteosarcoma cell line, all using highly selective reporter constructs based with a basal promoter element linked to multimerized response elements, allowing efficient and specific measurement of compounds interfering with androgen, estrogen, progesterone, and glucocorticoid receptors. The AR CALUX bioassay contains the human androgen receptor and a luciferase reporter construct containing three androgen-responsive elements coupled to a minimal TATA promoter. This cell line was characterized by its stable expression of AR protein, its highly selective response to low levels of different natural and synthetic androgens, and its insignificant response to other nuclear hormone receptor ligands such as estrogens, progestins, and glucocorticoids. The EC50 of dihydrotestosterone (DHT) was found to be 0.13 nM, consistent with the high affinity of this ligand to the human AR. Flutamide, cyproterone acetate, and the environmental contaminants vinclozolin, DDT, methoxychlor, its metabolite HPTE, and penta-BFR showed clear antagonistic activity in the AR CALUX bioassay, competitively inhibiting DHT-mediated transactivation. The established AR CALUX bioassay proved to excel in terms of easy cell line maintenance, high fold induction range (typical 30 times over solvent control), low minimal detection limit (3.6 pM), and high androgen selectivity. Potential applications such as testing the androgenic or estrogenic activity of pure chemicals and pharmaceuticals and complex mixtures (environmental, food, feed, and clinical) are discussed.

Key Words: androgen; estrogen; receptor; CALUX; luciferase; bioassay.

Steroid hormones are essential in most reproductive processes and can influence many other physiological processes as well. Due to the relatively simple chemical structure and lipophilic nature of steroids, their regulatory pathways can be readily modified by pharmacological, environmental, and dietary agents. The mechanism of action of steroids allows the development of straightforward screening methods, making use of the fact that steroid receptors are transcription factors that induce transcription of target genes after binding to specific DNA sequences in their promoter. When these DNA sequences are linked to the gene of a readily measurable protein (the so-called reporter gene; e.g., firefly luciferase) and introduced into a suitable cell line, a steroid-responsive reporter cell line can be generated. By fusing multiple copies of a hormone response element to a minimal promoter containing the TATA box only, we have developed a series of highly sensitive and specific reporter cell lines. These bioassays form a group of the so-called CALUX (Chemically Activated LUCiferase eXpression) bioassays. These systems are exemplified by the estrogen receptor ER CALUX bioassay consisting of the human T-47D breast tumor cell line expressing estrogen receptors (ER) endogenously together with an ER-specific 3×ERE-TATA-Luciferase construct (Legler et al., 1999). This approach, using a minimal reporter construct circumvents that signaling pathways other than the signaling pathway of the steroid receptor of interest regulate promoter activity, and luciferase expression, and thereby avoids nonspecific responses. Similarly, we have developed doubly transfected cell lines expressing estrogen receptors (ERα or ERβ) as well as the 3×ERE-TATA-Luciferase construct. The ERα and ERβ CALUX bioassays have the advantage of even more selective responses toward ER interacting ligands (Lemmen et al., 2002; Quaedackers et al., 2001).

Because of the many possible applications of steroid bioassays, we were interested in developing a panel of assays using the same cellular background, in which the activity of all major classes of steroid hormones can be determined specifically and sensitively. In particular we were interested in generating a selective and sensitive bioassay for androgens, in response to the recent interest in environmental androgens and anti-androgens (ICCVAM, 2003; Kelce et al., 1995), together with the paucity of good assay systems for this class of hormones.
Androgens are a major class of steroid hormones that have critical roles in the development and maintenance of the male reproductive system and other physiological targets, predominantly in males. Through their anabolic effects, androgens are used to promote muscle strength in athletes and meat quantity in farm animals (Evans, 2004; Meyer, 2001). It has also been found that environmental chemicals can interfere with androgen action, thereby possibly contributing to disruption of the endocrine system in wildlife and humans (Andersen et al., 2002; Kelce and Wilson, 1997). Therefore, identification of androgen active compounds is important in a variety of fields, ranging from pharmacological and clinical screening, food and feed manufacturing, to toxicological monitoring and risk assessment. Traditionally, monitoring strategies focus on two extremes: (1) sophisticated, detailed chemical analysis and (2) determination of biological effects using whole-animal assays and epidemiology. With these two methods a correlation can be made between in vivo or environmental levels of a chemical and the effect seen in organisms (exposure and effect determinations). Rapid advances in molecular biology and biotechnology have allowed identification of mechanisms of action of toxicants and facilitated development of simple assays based on these signaling mechanisms. These assays have the potential to be used as monitoring tools for chemical contaminants interfering with these signaling mechanisms, but also have the potential to replace, in part, animal experimentation for effect determination by offering prescreens to identify chemicals that impact major toxicological endpoints. In the case of androgens, the main mode of signaling is well established.

The effects of androgens in target cells are mediated by the androgen receptor (AR), a member of the nuclear hormone receptor superfamily that also includes receptors for other steroid hormones like progestins and glucocorticoids, retinoids, and thyroid hormones (Mangelsdorf et al., 1995; McKenna and O’Malley, 2002). AR is a ligand-dependent transcription factor that regulates specific gene expression by binding to specific hormone response elements (HREs) within the regulatory DNA sequences of androgen-responsive genes (Claessens et al., 2001). The enhancer region of the mouse mammary tumor viral long terminal repeat (MMTV-LTR) promoter is the viral long terminal repeat (MMTV-LTR) promoter is the most widely used enhancer to study AR function, although it was originally isolated as a progesterone and glucocorticoid-responsive enhancer (Di Croce et al., 1999). This can be explained by the fact that the four inverted repeats of the core sequence 5’-TGGTCT-3’ within the MMTV-LTR enhancer are recognized by: AR, glucocorticoid receptor (GR), progesterone receptor (PR), and mineralocorticoid receptor (MR; Glass, 1994), now classified as the members of the 3C group within the nuclear receptor family (Nuclear Receptors Nomenclature Committee; 1999). The MMTV promoter also contains several enhancer regions that can be addressed by transcription factors that may respond to other hormonal and cellular stimuli, thereby modulating steroid responses (Aurrekoetxea-Hernandez and Buetti, 2004; Uchiumi et al., 1998).

Several stable reporter gene assays have been described for androgens. However, these systems still have several drawbacks, since they either have a low responsiveness, use slowly growing prostatic cell lines, or are not selective in their response because of expression of other nuclear hormone receptors of the C3 class, activating the transfected reporter gene through non-AR-mediated mechanisms (Blankvoort et al., 2001; de Gooyer et al., 2003; Paris et al., 2002a; Terouanne et al., 2000; Wilson et al., 2002). We decided to generate a new androgen reporter cell line that combines high specificity, sensitivity, and ease of handling. To attain this we selected a cell line, the human bone cell line U2-OS, in which the stably introduced human androgen receptor was highly active, while expression of other C3 class receptors is insignificant. In this line we cotransfected a highly specific reporter construct, containing three HREs and a minimal promoter linked to luciferase, and selected a stable highly responsive clone. The AR CALUX cell line combines rapid growth and levels of high specificity and inducibility so far unreported. We studied its basal response characteristics, as well as its potential to serve in a variety of applications. The AR CALUX cell line is a member of a panel of reporter cell lines with the same cellular background (the U2-OS cell line), allowing efficient and convenient measurement of not only androgen-, but also estrogen-, progesterone-, and glucocorticoid-receptor interacting compounds (Quaedackers et al., 2001; Sonneveld et al., manuscripts in preparation). Besides describing the characteristics and applications of the AR CALUX cell line, additional data are provided for the ERα CALUX cell line as a complimentary bioassay in the group of CALUX reporter cell lines.

**MATERIALS AND METHODS**

**Chemicals.** Androstenedione, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), corticosterone, dehydroepiandrosterone (DHEA), dexamethasone, diethylstilbestrol (DES), 5α-dihydrotestosterone (DHT), 17β-estradiol, 17β-estradiol (E2), estradiol, estrone, 17α-ethinyl-estradiol (EE2), flutamide, genistein, hydrocortisone, methoxychlor (MX), methyl testosterone (MT), mifepristone (RU486), prednisolone, progesterone, testosterone, 4-OH-tamoxifen, and tamoxifen citrate were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). The synthetic androgen receptor agonist methyltrienolone (R1881) was obtained from Packard (Packard BioScience, Groningen, The Netherlands). Bethamethasone, cyproterone acetate (CA), medroxyprogesterone acetate (MPA), 19-nor-testosterone (nandrolone), pregnenolone, 17-hydroxy-pregn-5-en-3β-ol (17OH-pregnenolone), and testosterone glucuronide were obtained from Steraloids Inc. (Newport, RI). IC1 164.384, 19-Nor-17α-methyl-testosterone (MENT), Org 2058 and raloxifene (RAL) were kind gifts from W. Schoonen (N.V. Organon, Oss, The Netherlands). Vinclozolin was purchased from Riedel-de Haën (The Netherlands). a; a'-dichlorodiphenyl tri-chloroethane (a; a'-DDT), and p; p'-dichlorodiphenyl trichloroethane (p; p'-DDT) were kindly provided by J. Legler (Institute for Environmental Studies, VU, Amsterdam, The Netherlands). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was purchased from Cambridge Isotope Laboratories (Andover, MA). Octabromodiphenyl ether (Octa-LM) and pentabromodiphenyl ether (DE-71) technical mixtures were gifts from A. Bergman (Stockholm University, Sweden). All chemicals were diluted in either ethanol or dimethylsulphoxide (DMSO; Acros, Geel, Belgium) and stored at –20°C. Neomycin (G418) was purchased from Life Technologies (Breda, The Netherlands).
Sera. Fetal calf serum was obtained from Invitrogen (Breda, The Netherlands). A pooled human serum batch was a gift from B. Hendriks-Stegeman (University Medical Centre, Utrecht, The Netherlands). In short, blood from 15 healthy adult volunteers (male/female ratio 8:7) was collected in silica-coated tubes (Capiject, Terumo Medical Corp.). After centrifugation serum was removed, all collected sera were pooled and stored at −20°C.

DNA constructs. A blunt-ended 3050 bp SalI fragment from pSV0-hAR (obtained from A. Brinkmann, Rotterdam, The Netherlands) containing the full-length human androgen receptor (AR) (Brinkmann et al., 1989) was inserted into the blunt-ended Xhol fragment from pSG5-neo (Sonneveld et al., 1998) containing the neomycin resistance gene, resulting in the expression plasmid pSG5-neo-hAR. An 1800 bp EcoRI fragment from pSG5-hERα (HEGO) (obtained from P. Chambron, Strasbourg, France) containing the full-length human estrogen receptor alpha (Green et al., 1986) was inserted in the EcoRI fragment from pSG5-neo, resulting in the expression plasmid pSG5-neo-hERα. The reporter construct pMMTVLuc was described earlier (Hartig et al., 2002). The reporter construct 3× HRE-TATA-Luc was constructed as follows: three tandem repeats of ARE oligos AACCTTAAACCTTTGTCAC and GACCTCCGATCT were inserted in the multiple cloning site of the promoter less luciferase reporter construct pLuc (Folkerks et al., 1995). The reporter construct 3× ERE-TATA-Luc was described earlier (Legler et al., 1999).

Cell culture. The human osteoblastic osteosarcoma cell line U2-OS (ATCC) was cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (DF, Gibco) supplemented with 7.5% fetal calf serum. AR and ERα CALUX cells were cultured in DF medium supplemented with 7.5% FCS and 200 μg/ml G418.

Transient transfections. For transient transfections, cells were plated in 24-well tissue culture plates. After culturing for 1 day, cells were transfected with 1 μg reporter plasmid (3× HRE-TATA-Luc, pMMTVLuc, or 3× HRE-TATA-Luc), 200 ng SV2-lacZ, and 200 ng expression plasmid (pSG5-neo-hERα, pSG5-neo-hPR, pSG5-neo-hGR, or pSG5-neo-hAR) or empty vector DNA (pSG5-neo), using the calcium phosphate coprecipitation method. Luciferase activity was corrected for transfection efficiency by measuring LacZ expression as a result of SV2-lacZ co-transfection (Kalkhoven et al., 1994).

Establishment of stable AR and ERα CALUX cell lines. U2-OS cells were transfected with 3× HRE-TATA-Luc and pSG5-neo-hAR, using calcium phosphate precipitation to generate AR CALUX cells. G418-resistant clones were tested for their response to dihydrotestosterone (DHT). Eight clones showed consequent high response. One of these (clone 568) responding to the lowest concentration of DHT (10 pM) was selected for further investigation. ERα CALUX cells (Quaedackers et al., 2001) transfected with 3× ERE-TATA-Luc and pSG5-neo-hERα were regenerated in our laboratories, since the original clones showed bell-shaped dose-response curves and relatively high backgrounds, making them less suitable for routine applications (data not shown).

AR and ERα CALUX bioassays. AR and ERα CALUX cells were plated in 96-well plates (6000 cells/well) with phenol red-free DF medium supplemented with 5% dextran-coated charcoal-stripped FCS (DCC-FCS; van der Burg et al., 1988) at a volume of 200 μl per well. Two days later, the medium was refreshed, and cells were incubated with human or fetal serum (0–10% [v/v]) or the compounds to be tested (dissolved in ethanol or DMSO) in triplicate at a 1:1000 dilution. In case of serum incubation, final serum concentration was 10% (v/v), and lower percentages of the tested sera were supplemented with DCC-FCS. After 24 h the medium was removed, cells were lysed in 30 μl Triton-X buffer and measured for luciferase activity using a luminometer (Lucy2; Anthos Lattec Instruments, Wals, Austria) for 0.1 min/well.

Western blotting. Whole-cell extracts were prepared as described previously (Sonneveld et al., 1998). 20 μg of protein was run on an 8% (w/v) SDS–polyacrylamide gel and transferred electrothermally to nitrocellulose sheets. Membranes were treated with blocking buffer containing 4% (w/v) nonfat powdered milk in TBST (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.2% (v/v) Tween-20) and then incubated for 2 h with anti-hAR mouse monoclonal antibody Ab-1 (clone AR441) (NeoMarkers, Fremont, CA), diluted at 1:200 in TBST buffer. After washing with TBST, the membranes were immunostained using the ECL Western blotting system (Amersham).

Immunofluorescence. Cells were grown on coverslips and fixed on ice for 15 min with 3.6% (v/v) formaldehyde in ethanol. Subsequently, the cells were washed three times with phosphate buffered saline (PBS), permeabilized with 0.1% Triton X-100/PBS, incubated with 1% (w/v) BSA/PBS, and washed three times with PBS. Cells were incubated with anti-hAR mouse monoclonal antibody Ab-1 (1:80) in 10% (v/v) normal goat serum/PBS for 1 h, washed three times with PBS, incubated with GAM-Cy3 (second) antibody (1:250) in 10% (v/v) normal goat serum/PBS for 1 h, washed three times with PBS, and mounted in Moviol.

Data analysis. Luciferase activity per well was measured as relative light units (RLUs). Fold induction was calculated by dividing the mean value of light units from exposed and nonexposed (solvent control) wells. Luciferase induction as a percentage of maximal DHT (AR CALUX) or E2 (ERα CALUX) activity was calculated by setting the highest fold induction of DHT (AR CALUX) or E2 (ERα CALUX) at 100%. When assessing for anti-androgenic effects, the fold induction at the EC50 concentration of DHT was set at 100%. Data are represented as mean values ± SEM from at least three independent experiments, with each experimental point performed in triplicate. Dose-response curves were fitted using the sigmoidal fit (y = a0 + a1/(1 + exp(−(x − a2)/a3))) in GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego, CA), which determines the fitting coefficients by an iterative process minimizing the c2 merit function (least squares criterion). The EC50 and 100-times EC50 values were calculated by determining the concentration by which 50 or 100% of maximum activity was reached using the sigmoidal fit equation. The relative transactivation activity (RTA) of each compound tested was calculated as the ratio of maximal luciferase reporter gene induction values of each compound and the maximal luciferase reporter gene induction value of reference compound DHT (AR CALUX) or E2 (ERα CALUX). The transactivation activity of DHT or E2 was arbitrarily set at 100.

RESULTS

Establishment of a Panel of Steroid-responsive CALUX Cell Lines

Based on earlier observations (Quaedackers et al., 2001) and transient transfections using a panel of steroid receptors, steroid reporter plasmids, and different cell lines (HEK293, T-47D, U2-OS, HeLa, CHO), the osteoblastic osteosarcoma U2-OS cell line was selected as the best candidate to serve as the basis of androgen-, estrogen-, glucocorticoid-, and progestin-responsive reporter cell lines. This selection was mainly based on the observation that the U2-OS cell line showed little or no endogenous reporter activity using reporter plasmids only, while it supported strong hormone-mediated responses when cognate receptors were transiently introduced (Fig. 1 and data not shown; Quadackers et al., 2001). We were particularly interested in the activation through possible endogenous members of the 3C group of nuclear receptors, since not only AR but also progesterone (PR) and glucocorticoid receptors (GR) can also activate the HRE-containing constructs. In transient transfection assays, no evidence for significant endogenous activity of AR, PR, or GR upon ligand stimulation (DHT, Org 2058, and dexamethasone, respectively) was found when the selective
3× HRE-TATA-Luc construct or the more conventional pMMTV-Luc construct was used. Cotransfection of the appropriate receptors, however, resulted in high reporter activity upon ligand treatment (Fig. 1). Stable transfectants were selected from U2-OS cells transfected with the hAR (this study), hPR and hGR (Sonneveld et al., manuscripts in preparation) and the 3× HRE-TATA-Luc reporter construct, or with the hERα in combination with the 3× ERE-TATA-Luc reporter construct (this study; Quadackers et al., 2001). In this manner, distinct steroid reporter cell lines with the same cellular background (U2-OS) and comparable minimal promoter reporter constructs (multimerized response elements coupled to the TATA box and the luciferase reporter gene) were generated. Table 1 shows a summary of the basic properties of these lines, characterized by high levels of induction (fold induction ranging between 30 and 80), high stability (usually more than 40 passages), high sensitivity (picomolar to nanomolar range), and high selectivity. We next determined the characteristics of the U2-OS cells stably transfected with hAR and 3× ARE-TATA-Luc, which we have named the AR CALUX cell line.

**Characterization of AR CALUX Cells**

Figure 2 shows the expression of human AR as determined by Western analysis (Fig. 2A) and whole-cell immunofluorescence in parental U2-OS cells (Fig. 2C), and U2-OS-derived AR CALUX cells (Figs. 2D and 2E). As shown by both assays, maternal U2-OS cells do express small amounts of AR, while the stable AR CALUX clone clearly expresses AR. The human breast cancer cell line T-47D (Sutherland et al., 1988) was used as a positive control, expressing AR endogenously at moderate levels (Figs. 2A and 2B; Blankvoort et al., 2001). Both cytoplasmic and nuclear expression of AR in AR CALUX cells was observed using immunofluorescence (Fig. 2D). Since ligated steroid receptors have a nuclear

### TABLE 1

Performance Characteristics of U2-OS-based CALUX Bioassays for Detection of Steroidal Activity

<table>
<thead>
<tr>
<th>Reference compound</th>
<th>ERα CALUX&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>ERβ CALUX&lt;sup&gt;c&lt;/sup&gt;</th>
<th>AR CALUX&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PR CALUX&lt;sup&gt;c&lt;/sup&gt;</th>
<th>GR CALUX&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference compound (nM)</td>
<td>E2</td>
<td>E2</td>
<td>DHT</td>
<td>Org 2058</td>
<td>Dex</td>
</tr>
<tr>
<td>EC50 reference compound (nM)</td>
<td>0.02 ± 0.004</td>
<td>0.06 ± 0.01</td>
<td>0.13 ± 0.02</td>
<td>0.09 ± 0.01</td>
<td>0.37 ± 0.08</td>
</tr>
<tr>
<td>Fold induction typical</td>
<td>30</td>
<td>80</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Inter-assay CV (%) reference compound</td>
<td>25</td>
<td>17</td>
<td>22</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td>LOD reference compound (pM)</td>
<td>0.8</td>
<td>nd</td>
<td>3.6</td>
<td>1.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Selectivity</td>
<td>high</td>
<td>high</td>
<td>high</td>
<td>high</td>
<td>high</td>
</tr>
<tr>
<td>Stability passages</td>
<td>&gt;52</td>
<td>nd</td>
<td>&gt;36</td>
<td>&gt;48</td>
<td>&gt;62</td>
</tr>
</tbody>
</table>

Note. Nd = not determined.
<sup>a</sup>Quadackers et al., 2001.
<sup>b</sup>Present study.
<sup>c</sup>Sonneveld et al., manuscripts in preparation.
localization we also treated cells with DHT (1 nM) (Fig. 2E). The observed shift to nuclear staining confirmed the specificity of the signal in the immunofluorescence and the normal cellular distribution of the AR expressed in AR CALUX cells (Avances et al., 2001). Furthermore, the stable homogeneous expression of AR is demonstrated as well by immunofluorescence, as 100% of the AR CALUX cells stained positive (Figs. 2D and 2E).

**Maintenance, Responsiveness, and Stability of AR CALUX Cells**

The selected AR CALUX cell line was found to be robust and easily maintainable in standard culture media with a population doubling time of 24 h, comparable with U2-OS parental cells (data not shown). Freeze–thaw procedures had no significant influence on the viability of the cells, again showing the robustness of the selected cell line. The cells showed a remarkably strong response (typically between 15- and 50-fold induction) upon DHT treatment, relative to low background values in control cells treated with solvent alone. This response has been shown to be stable over 36 passages conducted to date (Table 1). The range of the fold inductions was fairly wide (15 to 50 fold), mainly due to relatively small changes in the low background activity having a large influence on the fold induction (data not shown). The range in EC50 values measured with different ligands over time, including the positive control DHT, however, was small (Table 2), showing that changes in fold induction did not influence quantification of the potency of the ligands. The interassay CV was determined using EC50 values (n = 12) obtained by various persons within two independent laboratories using different batches of AR CALUX cells and was 22% (Table 1).

**Sensitivity and Selectivity of the AR CALUX Bioassay**

The sensitivity of the AR CALUX cells was assessed by measuring the luciferase activity induced by a series of natural steroids and precursor molecules compared to solvent control. The most potent androgen was dihydrotestosterone, activating these cells with an EC50 of 0.13 nM (Fig. 3A and Table 2). The AR CALUX cells showed high sensitivity toward all natural androgens tested, with the following range of potencies (EC50 values in nM): DHT (0.13), testosterone (0.66), and androstenedione (4.5) (Fig. 3A and Table 2). The AR CALUX cell line was remarkably selective for androgens, showing no substantial agonistic response to the (androgen) precursors DHEA (no EC50 reached; 11% relative transactivation activity [RTA] to DHT) and pregnenolone (no response; Table 2), the PR ligand progesterone (no EC50 reached; RTA = 36%), the ER ligand 17β-estradiol (EC50 = 3090 nM; RTA = 93%; Table 2), and the GR ligand hydrocortisone (no EC50 reached; RTA = 9%). Figure 3B shows that the selectivity for androgens was confirmed using a panel of synthetic ligands. Only ligands with affinity for the AR, such as MENT (19-nor-7-alpha-methyltestosterone), nandrolone, the synthetic progesterin MPA (reported to possess androgenic activity as well; Bentel et al., 1999), and R1881, strongly induced luciferase activity in AR CALUX cells, with MENT acting as an even more potent AR activator than DHT (EC50 = 78 pM; RTA = 121%; Table 2). The strong synthetic GR-activating ligand dexamethasone did induce a response at high concentrations (0.1 μM) that was 8% of the maximum of that reached by DHT. Other GR-activating ligands such as prednisolone, corticosterone, and bethamethasone showed similar responses at even higher concentrations (10 μM; Table 2), consistent with a minor GR-mediated effect at high levels of ligand. No cross-reactivity was observed with the specific PR-agonist Org 2058 (Fig. 3B) and the ER-agonist ethynyl estradiol (EE2; Table 2), showing the absence of PR- and ER-mediated responses in these cells, respectively. The known AR antagonist flutamide (Fig. 3C) repressed DHT-induced reporter gene activity. While flutamide at 10 μM effectively repressed transactivation by DHT at EC50 values, this repression was reversed by excess DHT, confirming the competitive nature of the antagonistic effect.

**Determination of (Anti-) Androgenicity of Pure Compounds Using AR CALUX Cells**

It has been found that a variety of environmental chemicals mimic androgens or interfere in an antagonistic fashion with
TABLE 2

EC50, RTA, and IC50 Values of Agonistic and Antagonistic Compounds in AR and ERα CALUX Reporter Cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>AR CALUX</th>
<th></th>
<th>ERα CALUX</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agonism LogEC50 (M)</td>
<td>RTA (%)</td>
<td>Agonism LogIC50 (M)</td>
<td>Antagonism LogIC50 (M)</td>
</tr>
<tr>
<td>MENT</td>
<td>−10.1 ± 0.1</td>
<td>121</td>
<td>&gt; −5.0</td>
<td>−7.6 ± 0.0</td>
</tr>
<tr>
<td>R1881</td>
<td>−9.9 ± 0.0</td>
<td>69</td>
<td>&gt; −5.0</td>
<td>−6.1 ± 0.2</td>
</tr>
<tr>
<td>DHT</td>
<td>−9.9 ± 0.1</td>
<td>100</td>
<td>&gt; −5.0</td>
<td>&gt; −6.0</td>
</tr>
<tr>
<td>Nandrolone</td>
<td>−9.5 ± 0.0</td>
<td>92</td>
<td>&gt; −5.0</td>
<td>−6.7 ± 0.2</td>
</tr>
<tr>
<td>Testosterone (T)</td>
<td>−9.2 ± 0.0</td>
<td>94</td>
<td>&gt; −5.0</td>
<td>&gt; −6.0</td>
</tr>
<tr>
<td>Methyl testosterone (MT)</td>
<td>−9.1 ± 0.1</td>
<td>108</td>
<td>&gt; −5.0</td>
<td>nd</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>−8.4 ± 0.3</td>
<td>82</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>MPA</td>
<td>−8.2 ± 0.1</td>
<td>75</td>
<td>&gt; −5.0</td>
<td>&gt; −6.0</td>
</tr>
<tr>
<td>17-beta-estradiol (E2)</td>
<td>−5.5 ± 0.2</td>
<td>93</td>
<td>nd</td>
<td>−10.8 ± 0.1</td>
</tr>
<tr>
<td>Cyproterone acetate (CA)</td>
<td>−5.4 ± 0.1</td>
<td>50</td>
<td>−8.2 ± 0.3</td>
<td>&gt; −6.0</td>
</tr>
<tr>
<td>Progesterone</td>
<td>&gt; −5.0</td>
<td>36</td>
<td>nd</td>
<td>&gt; −6.0</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>nr</td>
<td>8</td>
<td>&gt; −5.0</td>
<td>&gt; −6.0</td>
</tr>
<tr>
<td>RU486</td>
<td>&gt; −5.0</td>
<td>&lt;5</td>
<td>−7.6 ± 0.1</td>
<td>&gt; −6.0</td>
</tr>
<tr>
<td>HPTE</td>
<td>&gt; −5.0</td>
<td>&lt;5</td>
<td>−6.6 ± 0.1</td>
<td>&gt; −7.1</td>
</tr>
<tr>
<td>Vinclozolin</td>
<td>&gt; −5.0</td>
<td>&lt;5</td>
<td>−6.0 ± 0.0</td>
<td>&gt; −5.0</td>
</tr>
<tr>
<td>α-DDT</td>
<td>&gt; −5.0</td>
<td>&lt;5</td>
<td>−6.0 ± 0.1</td>
<td>&gt; −6.0</td>
</tr>
<tr>
<td>Flutamide</td>
<td>&gt; −5.0</td>
<td>&lt;5</td>
<td>−5.9 ± 0.1</td>
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<tr>
<td>ICI 164.384</td>
<td>&gt; −5.0</td>
<td>&lt;5</td>
<td>−5.8 ± 0.2</td>
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<tr>
<td>Penta-BDE</td>
<td>&gt; −5.0</td>
<td>&lt;5</td>
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<tr>
<td>p,β-DDT</td>
<td>&gt; −5.0</td>
<td>&lt;5</td>
<td>−5.6 ± 0.2</td>
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<td>Methoxychloride</td>
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<td>&lt;5</td>
<td>−5.1 ± 0.3</td>
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<tr>
<td>Octa-BDE</td>
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<td>&lt;5</td>
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<tr>
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<td>&gt; −5.0</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

Note. EC50 values (average ± SD) and relative transactivation activity (RTA) of various agonistic compounds and IC50 values of various antagonistic compounds in AR and ERα CALUX reporter cells. Nd = not determined. nr = EC50 not reached.

androgen action, thereby possibly contributing to negative health effects in humans and wildlife (Andersen et al., 2002; Kelce and Wilson, 1997). Particularly for the latter purpose, screening of large numbers of chemicals is planned to be undertaken (ICCVAM, 2003). This necessitates the use of cost-effective screening of pure chemicals, preferably with in vitro assays. While only a small number of environmental compounds are currently known AR agonists, some of them are quite potent antagonists. Figure 4 shows that the AR CALUX bioassay readily classifies chemicals according to their antiandrogenic properties when tested in the presence of EC50 concentrations of DHT. The well-known and widely used AR antagonists flutamide (IC50 = 1.3 μM), vinclozolin (IC50 = 1.0 μM), and cyproterone acetate (IC50 = 7.1 nM) clearly show antagonistic properties, with the latter being a partial agonist also, showing agonism for the AR at relatively high concentrations (EC50 = 4.0 μM) (Fig. 4A and Table 2). In addition, we tested a set of environmental chemicals for their agonistic and
antagonistic properties (Fig. 4B and Table 2). This set of chemicals consisted of the environmental pesticides o,p′DDT, p,p′DDT, methoxychlor, and HPTE, as well as the penta and octa technical mixtures of brominated flame retardants (penta-BFR and octa-BFR). None of the above-mentioned compounds showed agonistic properties (Table 2). As shown in Figure 4B, the compounds o,p′DDT and p,p′DDT were able to completely antagonize DHT-mediated AR activity with IC50 values of 1.1 µM and 2.8 µM, respectively (Table 2). The DDT-related pesticide methoxychlor (IC50 = 8.5 µM) was a less potent antagonist than DDT, but its metabolite HPTE was a 30-times more potent antagonist (IC50 = 0.3 µM), being the strongest environmental AR antagonist found so far in this bioassay. The penta-BFR mixture showed antagonistic activity to the AR (IC50 = 2.1 µM), although complete antagonism was not reached (60% inhibition) (Fig. 4B). The octa-BFR mixture was not able to antagonize the AR (Fig. 4B). Another environmental contaminant, dioxin (TCDD), did not show agonistic or antagonistic activity toward AR (Table 2). As a control for nonspecific inhibition of reporter gene activity, putative antagonistic effects of the compounds shown in Figure 4 were tested.

FIG. 3. Dose-response curves for different androgen-receptor-activating compounds in the AR CALUX bioassay. AR CALUX cells were plated in 96-well plates and treated with (A) the androgen progenitors progesterone (●), DHEA (★), androstenedione (▲), the natural glucocorticoid hydrocortisone (○), or the natural androgens DHT (▲) and testosterone (●), or (B) the synthetic androgens MENT (●), R1881 (▲), and nandrolone (●), the synthetic progestins MPA (●) and Org 2058 (★), and the synthetic glucocorticoid dexamethasone (○) for 24 h using DF medium containing 5% DCC-FCS. (C) Luciferase induction by DHT (●) and repression of this induction by flutamide (○) (10 µM). Each point represents the mean of at least three independent experiments ± SEM.

FIG. 4. Repression of AR activity by AR antagonists in the AR CALUX bioassay. AR CALUX cells were plated in 96-well plates and treated with 0.13 nM DHT (EC50) and (A) the standard AR antagonists flutamide (●), vinclozolin (○), and cyproterone acetate (★), or (B) the environmental compounds o,p′DDT (●), p,p′DDT (▲), methoxychlor (○), HPTE (★), penta-BFR (▲), and octa-BFR (●) for 24 h using DF medium containing 5% DCC-FCS. Each point represents the mean of three independent experiments ± SEM.
for their reversibility by adding excess of the agonist DHT. The antagonistic effects of all of the compounds tested were reversed by coincubation with excess DHT (100 times the EC50 value), showing the specificity of the response (data not shown). In contrast, the inhibitory effects of high levels of a number of individual BFR congeners (not present in the mixtures used in this study) could not be reversed by excess DHT (Hamers et al., manuscript in preparation). This coincided with cytotoxicity of these ligands, as assessed through inhibition of expression of a constitutively expressed reporter gene and a positive response in the MTT assay (Hamers et al., manuscript in preparation). The AR CALUX line is a clearly efficient tool to screen for agonistic and antagonistic effects of compounds toward the androgen receptor.

**Determination of Estrogenicity of Pure Compounds Using ERα CALUX Cells**

Since several environmental chemicals with anti-androgenic activity have been shown to possess estrogenic activity as well (Paris et al., 2002a; Sohoni and Sumpter, 1998; Willemse et al., 2004), we decided to test the panel of pesticides and BFR-mixtures in the estrogen-specific ERα CALUX bioassay. In a similar manner as for (anti-) androgens in the AR CALUX cell line, the ERα cell line is an effective tool to screen for agonistic and antagonistic effects of compounds acting at the ERα. ERα CALUX cells are also U2-OS based with the same basal characteristics as other CALUX bioassays (ERβ, AR, PR, and GR CALUX), being robust, easily maintainable, highly stable, highly responsive, and highly selective to estrogens (Tables 1 and 2). U2-OS cells transfected with 3× ERE-TATA-Luc and hERα were described earlier (Quaedackers et al., 2001), but bell-shaped dose-response curves and relatively high backgrounds made this original cell line less suitable for routine applications (data not shown). For this reason we stably transfected U2-OS cells with 3× ERE-TATA-Luc and pSG5-neo-hERα, producing the ERα CALUX bioassay. Like AR CALUX cells, ERα CALUX cells showed a strong response (typically between 20- and 60-fold induction) upon E2 treatment, relative to low background values in control cells. This response to date is stable over 52 passages (Table 1). The range in EC50 values measured with different ligands over time, including the positive control E2, was small (Table 2), reflected by an interassay CV of 25% for E2 (n = 15; Table 1).

![FIG. 5. Dose-response curves for various estrogens and environmental compounds with estrogenic activity in the ERα CALUX bioassay. ERα CALUX cells were plated in 96-well plates and treated with (A) the natural estrogens E2 (●), estriol (▼), estrone (○), and 17α-estradiol (▲) and the synthetic estrogens EE2 (▲) and DES (▲), or (B) the pesticides o.p’DDT (●), p.p’DDT (▲), methoxychlor (○), and HPTE (▼), and the brominated flame retardants penta-BFR (▲) and octa-BFR (●) for 24 h using DF medium containing 5% DCC-FCS. Each point represents the mean of three independent experiments ± SEM.](https://doi.org/10.1093/toxsci/kfp408)
bioassay, dioxin did not show agonistic or antagonistic activity toward ERα (Table 2; Sonneveld et al., 2003). These experiments clearly show that certain environmental pesticides with anti-androgenic activity possess estrogenic activity as well.

**Determination of Estrogens and Androgens in Serum Using AR and ERα CALUX Cells**

In addition to testing pure compounds (steroids as well as environmentally relevant compounds with endocrine disrupting potency) for estrogenic and androgenic activity, the need to determine steroid bioactivity status in a wide range of pediatric as well as adult clinical conditions is indicated. As a potential clinical application, human serum was applied directly to the AR and ERα CALUX bioassays (Fig. 6). Increasing amounts of human serum resulted in increasing luciferase activity (Fig. 6A) in both AR and ERα CALUX cell types, indeed showing the presence of androgenic as well as estrogenic compounds in human serum comparable with plasma levels found in humans, as shown recently by other ER and AR bioassays (Paris et al., 2002b,c). The results show that these CALUX bioassays can potentially be used in a clinical setting, thereby potentially having the advantage of demanding only very small serum volumes (maximally 30 µl), making them applicable for pediatric purposes as well. In addition to human serum we also tested fetal calf serum for estrogenic and androgenic activities. As shown in Figure 6B, FCS showed estrogenic activity, but no AR-activating compounds.

**DISCUSSION**

We have developed a panel of stable human cell lines that specifically respond with compounds interacting with human AR, PR, GR, ERα, or ERβ, allowing efficient screening of hormonal activity of chemicals alone or in complex mixtures. Of particular note is the AR CALUX cell line expressing an androgen-responsive luciferase reporter gene and an androgen receptor expression construct. The AR CALUX cells combine rapid growth, high stability, high selectivity, and high inducibility, which is, in our experience, extraordinary for an androgen-responsive line. With its unique properties, this cell line is potentially suitable for a wide variety of applications, some of which we have illustrated here.

To generate an androgen reporter line superior to ones currently available, we chose not to use yeast cells, but rather mammalian cells with an origin close to the organism of main concern in the field of endocrine disruption (i.e., fish and mammals, including humans). Yeast-based reporter cells, although convenient in their use (Sohoni and Sumpter, 1998) can have notably different quantitative and qualitative response to hormonally active substances, mainly due to poor transport across the yeast cell membrane, and are therefore not recommended as screening models for endocrine disruptors (ICCVAM, 2003). Our objective was therefore to construct a mammalian, preferably human, reporter cell line with characteristics superior to the ones available. To avoid interference of signal transduction pathways other than AR-mediated signals, we choose to use a minimal AR-responsive promoter element coupled to a very minimal promoter containing a TATA box only. This approach has been shown to be successful in generation of both in vitro (Legler et al., 1999; Lemmen et al., 2002) and in vivo (Legler et al., 2000; Lemmen et al., 2004) models for selective measurement of estrogen effects. We show here that this approach can also be successfully used to generate a highly selective androgen reporter cell line in U2-OS cells, the AR CALUX cell line.

Previously, the full length MMTV promoter has been used to generate a number of androgen-responsive reporter cell lines. Although this promoter is quite selective to AR, PR, and GR, it also contains a number of regulatory sites that can be targeted by different agents other than steroids (Ouatas et al., 2002;
Spangenberg et al., 1998; Uchiumi et al., 1998). MDA-kb2 is a derivative of a human breast cancer cell line named MDA-MB-453, containing such a stably integrated MMTV-luciferase reporter (Wilson et al., 2002). In addition to responding to androgens, this cell line responds very strongly to glucocorticoids acting through the GR that is present endogenously, making it unsuitable as a selective screening tool. Much better androgen specificity was obtained by stable transfection of human prostate PC-3 cells with hAR and the MMTV-luciferase reporter, named PALM cells (Terouanne et al., 2000), CHO-hAR-MMTVluc cells (de Gooyer et al., 2003), and COS-hAR-MMTVluc cells (Paris et al., 2002c). So far, the only cell line that uses a simpler reporter construct, thereby avoiding influences by nonsteroidal regulatory pathways is derived from the human breast cancer cell line T-47D, stably transfected with a luciferase reporter under transcriptional control of the PB-ARE2 androgen response element (Blankvoort et al., 2001). This stable cell line shows additional hormone class specificity, as it mainly responds to progestins, due to the known over-expression of PR in T-47D cells, and relatively low endogenous AR levels (this study, Sonneveld et al., unpublished results; Sutherland et al., 1988), making it less suitable as a selective screening tool.

Due to the known problems of transcriptional interference between C3 group nuclear receptors, we choose to systematically select a line with an extremely low baseline of PR and GR while supporting an optimal androgen response when the cognate receptor was transiently introduced. This led to selection of the U2-OS cell line, which has the additional advantage of being robust, genetically stable, and of fast proliferation compared to most prostate cell lines. Through the introduction of a highly selective and responsive reporter gene, we generated the AR CALUX cell line.

Our results with the AR CALUX cell line show that it readily classifies the activities of pure chemicals, including natural and synthetic steroids. The EC50 values obtained with these compounds (partly listed in Table 2) correlate very well with corresponding EC50 values obtained with another established AR reporter cell line, the CHO-hAR-MMTVluc (de Gooyer et al., 2003; van der Burg et al., manuscript in preparation). These data are also consistent with binding affinities to the AR of these chemicals and the in vivo Hershberger assay (van der Burg et al., manuscript in preparation). Not all tested androgens reached the maximal induction level of DHT (Table 2). For example, R1881 only reached a relative transactivation activity of 69% compared to DHT. The reason for this lower maximal response is not clear, but could be due to differences in ligand-dependent AR stabilization as a result of different rates of androgen metabolism (Spangenberg et al., 1998). R1881 previously (Kuill et al., 1996) might also be an explanation for the lower maximal induction level of R1881. On the other hand, the synthetic androgen MENT was able to induce a supramaximal response (RTA = 121%). This supra-induction was also observed for genistein and o,p’DDT on ERα (this study; Legler et al., 1999) and an explanation for this phenomenon could be ligand-dependent differences in the ability of receptor to bind coactivators, such as TIF2 and SCR-1a as shown recently for ER by xenoestrogens (Routledge et al., 2000).

Weak activation of reporter gene activity was obtained at high concentrations of the strongest synthetic glucocorticoid dexamethasone only, while other high-affinity GR ligands such as hydrocortisone and corticosterone had little or no effect (Table 2). This data correlates with the affinity of these compounds to the GR and their response in the GR CALUX bioassay, with the latter showing EC50 values of 0.5 nM for dexamethasone, 5 nM for hydrocortisone, and 15 nM for corticosterone (Sonneveld et al., manuscript in preparation). Accordingly, GR-mediated activity is insignificant in the AR CALUX bioassay, since only weak effects can be observed with high concentrations of the strongest glucocorticoids. Such activities are very unlikely to be present in chemicals not designed to be glucocorticoids.

While active androgens bind directly to the androgen receptor and induce luciferase activity in AR CALUX cells, androgen precursors need metabolic activation. Androstenedione is a very weak binder to AR (0.1% compared to DHT; van der Burg et al., manuscript in preparation), but is a potent androgen in the AR CALUX bioassay, suggesting the presence of the metabolic enzyme 17β-HSD type 5 (with 17β-ketosteroid reductase activity), converting androstenedione to testosterone in AR CALUX cells. DHEA is a very weak transactivator of AR (EC50 > 10 μM; RTA = 11%), indicating absence or low activity of the 3β-HSD enzyme responsible for the conversion of DHEA to androstenedione. Preliminary PCR data show that 17β-HSD (type 5), but not 3β-HSD (type 1 and 2) is expressed in U2-OS cells (data not shown). On the other hand, the precursor progesterone shows induction of luciferase in AR CALUX cells (EC50 = 8.7 μM; RTA = 36%). This could mean that the enzyme CYP17 is present in AR CALUX cells, converting progesterone via OH-progesterone (17α-hydroxylase activity) to androstenedione (17, 20 lyase activity). Indeed, PCR experiments showed the expression of CYP17 in U2-OS cells (data not shown). Alternatively, since progesterone can bind AR (2% compared to DHT), this possibly results in direct AR transactivation (van der Burg et al., manuscript in preparation). Cross-talk with PR is not an issue in the bioassay, since the PR specific synthetic ligand Org 2058 did not show activity in the AR CALUX bioassay.

Figure 4 shows that the AR CALUX bioassay readily picks up antagonistic effects of known environmental anti-androgens. Pesticides from the DDT family clearly can antagonize the AR with IC50 values around 1 μM, while the metabolite HPTE was even 30 times more potent than its parental compound methoxychlor, making this compound one of the strongest environmental AR antagonists found so far in the AR CALUX bioassay. The antagonistic activity of vinclozolin (IC50 = 1 μM) is

A PANEL OF STEROID-RESPONSIVE BIOASSAYS 145
rather potent compared to another AR reporter cell line, the MDA-kb2 cells (IC50 = 10 μM; Wilson et al., 2002). In the latter cell line, metabolites of vinclozolin, M1 and primarily M2, are far more potent than the parental compound, suggesting that in the AR CALUX cells vinclozolin can be metabolized to more active AR antagonistic compounds like M1 and M2.

Compounds, particularly at μM levels or higher, can occasionally nonspecifically repress responses in reporter gene assays. This can be due to overall cytotoxicity, ultimately leading to cell death, but can also be due to more specific effects such as inhibition of protein synthesis or mRNA transcription. In our experience the latter effects precede the more general cytotoxic effects, with overt cell death as the least sensitive parameter. Therefore, controls should be assessing nonspecific repression of reporter gene activity rather than overall cytotoxicity and cell death. ‘Constitutively’ expressed reporter genes that often are used as controls have the drawback that no bona fide constitutive promoters have been identified so far; therefore the use of these controls should be avoided. In the case of steroid-receptor-mediated responses, the best control for nonspecific inhibition is considered the determination of the effect of the test compound on the reporter gene activation by an excess of high-affinity agonist. This approach worked well with the ligands tested, and all inhibitory responses were reversed by co-incubation with excess DHT, demonstrating the specificity of the response. Squelching of common cofactors by other nuclear (hormone) receptors is a well-known mechanism of interference and might therefore produce false-negative results. An example for this type of mechanism is the interference between PR and ER (Kraus et al., 1995). However, squelching seems not to be prominent in U2-OS derived CALUX bioassays, since they do not express high levels of steroid receptors other than the stably introduced receptor of interest. This is shown by the fact that progestins and glucocorticoids do not interfere with DHT- or E2-induced luciferase activity in the AR and ERα CALUX bioassays, respectively, while androgens do not show reduced expression of functional AhR (Legler et al., 1999; Sonneveld et al., 2003). The use of such low volumes makes usage of these CALUX bioassays for human serum even potentially applicable to infants, where low-volume sample taking is desired. In fetal calf serum, high estrogenic activity was measured. For this reason, charcoal-stripped serum is used in the CALUX bioassays to remove all steroids present. However, we could not demonstrate androgenic activity in fetal calf serum, suggesting either the presence of low levels of active androgens in fetal calf serum, or the presence of inactive precursors in the fetal serum which may be converted to active androgens in target tissues.

The high sensitivity and high selectivity of the AR and ERα CALUX bioassays allowed direct measurements in nonextracted biological samples. As a potential clinical application, very low volumes of human serum were applied directly to the CALUX bioassays. The presence of androgens and estrogens in human serum was shown by the AR and ERα CALUX bioassays, respectively, demonstrating that these bioassays can be used to measure low levels of bioavailable hormones directly in very small amounts of human serum (approximately 30 μl), as demonstrated recently for glucocorticoids (Sonneveld et al., manuscript in preparation; Vermeer et al., 2003). The use of such low volumes makes usage of these CALUX bioassays for human serum even potentially applicable to infants, where low-volume sample taking is desired. In fetal calf serum, high estrogenic activity was measured. For this reason, charcoal-stripped serum is used in the CALUX bioassays to remove all steroids present. However, we could not demonstrate androgenic activity in fetal calf serum, suggesting either the presence of low levels of active androgens in fetal calf serum, or the presence of inactive precursors in the fetal serum which may be converted to active androgens in target tissues.

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profiling” system will greatly improve by expanding the number of cell lines used. This will, however, lead to increased handling and accordant additional costs. Auto-motion of the handling will therefore be an important future step in an efficient “effect profiling” system. With this in mind, the use of a single robust parent cell line, such as the U2-OS cells, with identical culture and handling conditions greatly facilitates the possibilities for automation.

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