Application Note

Cell Analysis

Agilent

Trusted Answers

Identifying and Characterizing Endocrine Disruptors Using a Cell Panel-based Real-time Assay

Authors

Diana Guimet, Can Jin, David Santa Ana, and Yama Abassi

Agilent Technologies, Inc.

San Diego, CA, USA

Abstract

This application note describes a cell-based assay for quantitative, noninvasive, and continuous monitoring of cellular responses to chemicals of interest, using an Agilent xCELLigence real-time cell analysis (RTCA) instrument. Three mammalian cell lines, each responsive to modulators of ER, AR, or TR, were analyzed for their real-time responses to reference agonists and antagonists. The unique specificity and sensitivity of each cell line were then used as standards for evaluating unknown compounds. The validity of this assay was confirmed using molecular and cell biology techniques.

Introduction

Endocrine-disrupting chemicals (EDCs) are substances that interfere with the ability of endogenous hormones to regulate homeostasis through their cognate nuclear receptors.[1] By either mimicking ligands (agonists) or inhibiting ligand binding activity (antagonists),[3,4] EDCs produce adverse reproductive, neurological, proliferative, and immunological disorders.[5] EDC exposure can occur directly through the use of consumer products. Alternatively, because many constituents of consumer products, pesticides, and pharmaceuticals biodegrade poorly, they accumulate in the environment[6] and can cause EDC exposure through dermal, inhalation, embryonic, and oral routes[2] in both humans and wildlife.

EDC-laden wastewater causing intersex characteristics in fish and the correlation between breast cancer and bisphenol A are just two examples that highlight the severity of the EDC problem. There is a necessity to develop more efficient means of identifying these compounds before they are included in consumer products or used openly in the environment.

The estrogen, androgen, and thyroid hormone systems are primary regulators of a broad array of critical physiological functions and are targets of numerous EDCs. Historically, various *in vivo* and *in vitro* assays have been used to detect EDCs that interact with the estrogen receptor (ER), androgen receptor (AR), or thyroid hormone receptor (TR). Among these approaches, cell-based assays are especially powerful because:

- The receptor targets are presented within their normal biochemical milieu (maximizing physiological relevance).
- The hormone receptor targets remain linked with their downstream responses (including transcriptional activation, cell cycle regulation, and proliferation[1,4,15,16]), providing multiple readouts of EDC activity.

However, a major limitation of traditional *in vitro* cell-based assays is that they only generate end-point data – representing mere snapshots in the dynamic continuum of a cell's response to a treatment/exposure. Working with such a limited data set can lead to spurious conclusions and poor estimations of how a compound will behave *in vivo*.

Using celluar impedance to study endocrine disruptors

The functional unit of the Agilent xCELLigence RTCA impedance assay is a set of gold biosensors fused to the bottom surface of a microplate well (Agilent E-Plate, Figure 1). When submersed in an electrically conductive solution (such as buffer or growth medium), the application of a weak electric potential across these electrodes causes electric current to flow between them. Because this phenomenon depends on the electrodes interacting with bulk solution, the presence of adherent cells at the electrode-solution interface impedes current flow. The magnitude of this impedance is dependent on the number of cells, the size of the cells, and the cell-substrate attachment quality. Significantly, numerous studies have demonstrated that cell health and behavior are not affected by the gold biosensor surfaces, nor the electric potential (22 mV applied intermittently, at a user-defined frequency).

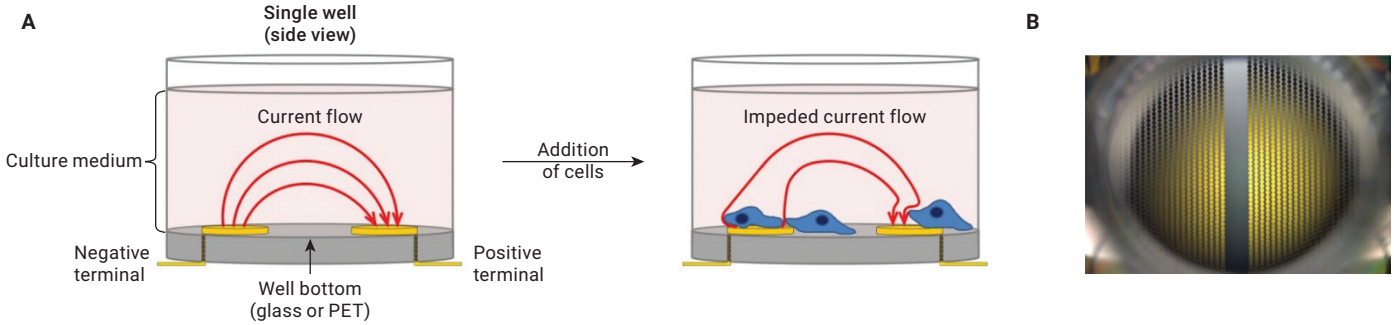

Figure 1. Overview of cellular impedance apparatus. (A) A side view of a single well from an Agilent xCELLigence electronic microplate (E-Plate) is shown before and after cells have been added. (Biosensors and cells are not to scale.) In the absence of cells, electric current flows freely through culture medium, completing the circuit between the biosensors. As cells adhere to and proliferate on the biosensors, current flow is impeded, providing a sensitive readout of cell number, cell size, and cell-substrate attachment quality. (B) Photograph looking down into a single well of an Agilent E-Plate. In contrast to the simplified scheme in part A, the electrodes are actually an interdigitated array that covers >70% of the well bottom. Though cells can also be seen on the gold biosensor surfaces, the biosensor-free region in the middle of the well facilitates microscopic imaging.

Because the estrogen, androgen, and thyroid hormone signaling axes each control many of biochemical pathways that have an impact on cell number, cell size, or cell-substrate attachment quality, compounds that interfere with these axes can readily be detected and characterized using cellular impedance.

Protocol

xCELLigence instrument and data plotting

All impedance experiments in this study were conducted using Agilent xCELLigence 96-well electronic microplates (E-Plate 96) on an xCELLigence real-time cell analysis (RTCA) multiple plates (MP) instrument. The instrument was housed in a standard tissue culture incubator, set to maintain 37 °C and a 5% CO_2 atmosphere. All tests were performed in biological duplicate, and were repeated in at least two independent experiments. Impedance values recorded by the xCELLigence instrument were reported using the unitless parameter Cell Index (CI), which is defined as $(Z_n-Z_b)/15$, where Z_n and Z_b are the impedance values in the presence and absence of cells, respectively. Data were typically plotted as the Normalized Cell Index (NCI), where $NCI = (CI_A)/(CI_B)$, where A = at time point of interest and B = at time point immediately before compound addition. All EC_{50} values were calculated using the RTCA 2.0 software.

Cells, media, and assay details

T-47D cells were maintained in RPMI 1640 with 10% regular FBS. Four days before conducting an assay these cells were preconditioned in phenol red-free RPMI 1640 with charcoal stripped 10% FBS. This estrogen-free media was used in all assays.

LNCAP cells were maintained in RPMI 1640 with 10% regular FBS. The media used for assays was phenol red-free RPMI 1640 with charcoal stripped 10% FBS.

GH3 cells were maintained in F12k containing 2.5% FBS and 15% horse serum. The media used for assays was DMEM, containing charcoal stripped 4% FBS.

For all cell types, after overnight growth in E-Plates, cells were treated and responses were monitored using the xCELLigence instrument every hour for a total of 120 hours.

For shRNA knockdown of the ERα gene, lentiviral transduction particles were used. Cells were seeded in growth media on E-Plates, allowed to grow overnight, and the next day virus particles were added directly to the cells. E-plates were immediately placed in the xCELLigence instrument for monitoring cell growth throughout the entire experiment, including treatment and monitoring of the knockdown mediated response. At the end of the experiment, cells were harvested to check for knockdown efficiency through qPCR.

Results and discussion

Time-dependent response of cell panel to reference compounds

Three mammalian cell lines derived from different tissues were exposed to reference agonist against the ER, AR, and TR (Figure 2). Rat pituitary GH3 cells, which express TR, and have been used extensively to study thyroid system function,[17,18] were sensitive not only to the thyroid hormone T3, but also to the ER agonist, E2, and the AR agonist, R1881 (Figure 2, Panel A). The stimulated proliferation of E2 and R1881 is consistent with previous reports of ER and AR signaling in rat pituitary cells.[20,21] T-47D is a human mammary gland epithelial cell line, which expresses both ER and AR.[19] Consistent with this, T-47D cells displayed sensitivity to both E2 and R1881. The impedance profiles in the presence of these ligands were dramatically different (Figure 2, panel B). Similar to GH3 cells, proliferation of the human prostate cancer cell line LNCaP was stimulated by all three agonists (Figure 2, panel C). While prostate cancer cell sensitivity to E2 and R1881 is expected, sensitivity to T3 is less intuitive but is consistent with the fact that TR expression has been demonstrated in LNCaP cells.[22]

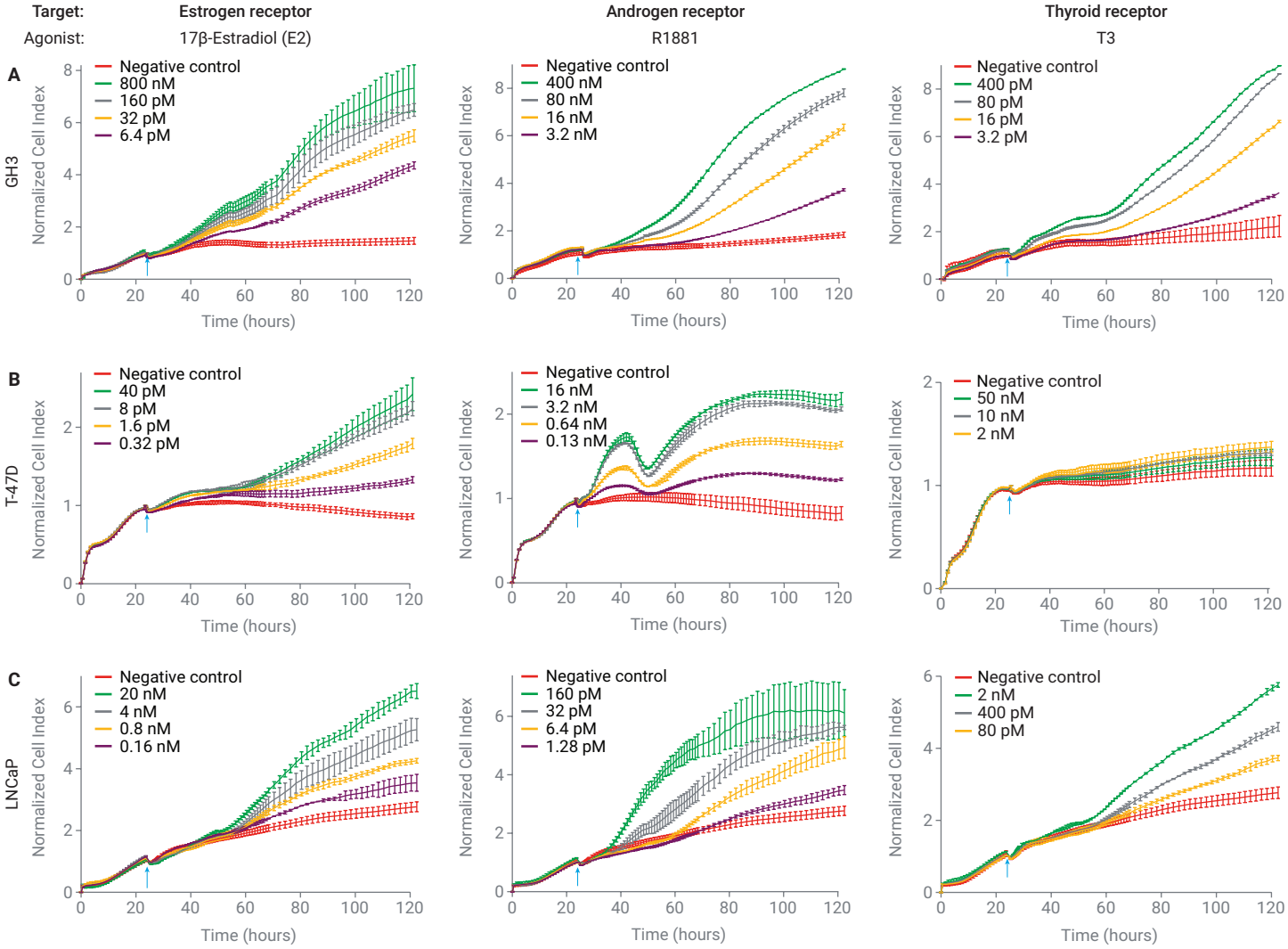

Figure 2. Time-dependent cellular responses to different agonists of the estrogen, androgen, and thyroid receptors. GH3 (panel A), T-47D (panel B), and LNCaP cells (panel C) were cultured in Agilent E-Plates overnight, followed by treatment with representative agonists of the estrogen receptor (BPA), androgen receptor (testosterone or DHT), or thyroid receptor (T3). CI was then monitored for 96 hours and normalized against the CI at the time of treatment (indicated by the arrow). Cells without compound treatment were used as a negative control. Note the different scales in each graph.

If RTCA traces are to be useful for identifying EDCs and characterizing their mechanism of action, the stimulation of a single receptor by different ligands should produce similar impedance traces (for example, "unknowns" should behave similarly to the reference compound). To assess this, additional well-characterized compounds were tested in GH3, T-47D, and LNCaP cell lines. Figure 3 shows that, in all three cell lines, the stimulation of ER by the plastic-associated toxin BPA results in impedance traces very similar to those produced by E2 stimulation in Figure 2. Similarly, testosterone or dihydrotestosterone (DHT) treatment gives rise to curve shapes similar to R1881 treatment, and T4 treatment recapitulates the curves shapes induced by T3 treatment (Figures 2 and 3). Collectively, the data suggests that the dynamic impedance response of reference cell lines to reference endocrine receptor agonists can serve as standards for identifying endocrine disrupting activity in "unknown" samples, and determining which nuclear receptor they target.

Beyond their distinctive impedance curve shapes, the different sensitivity that each

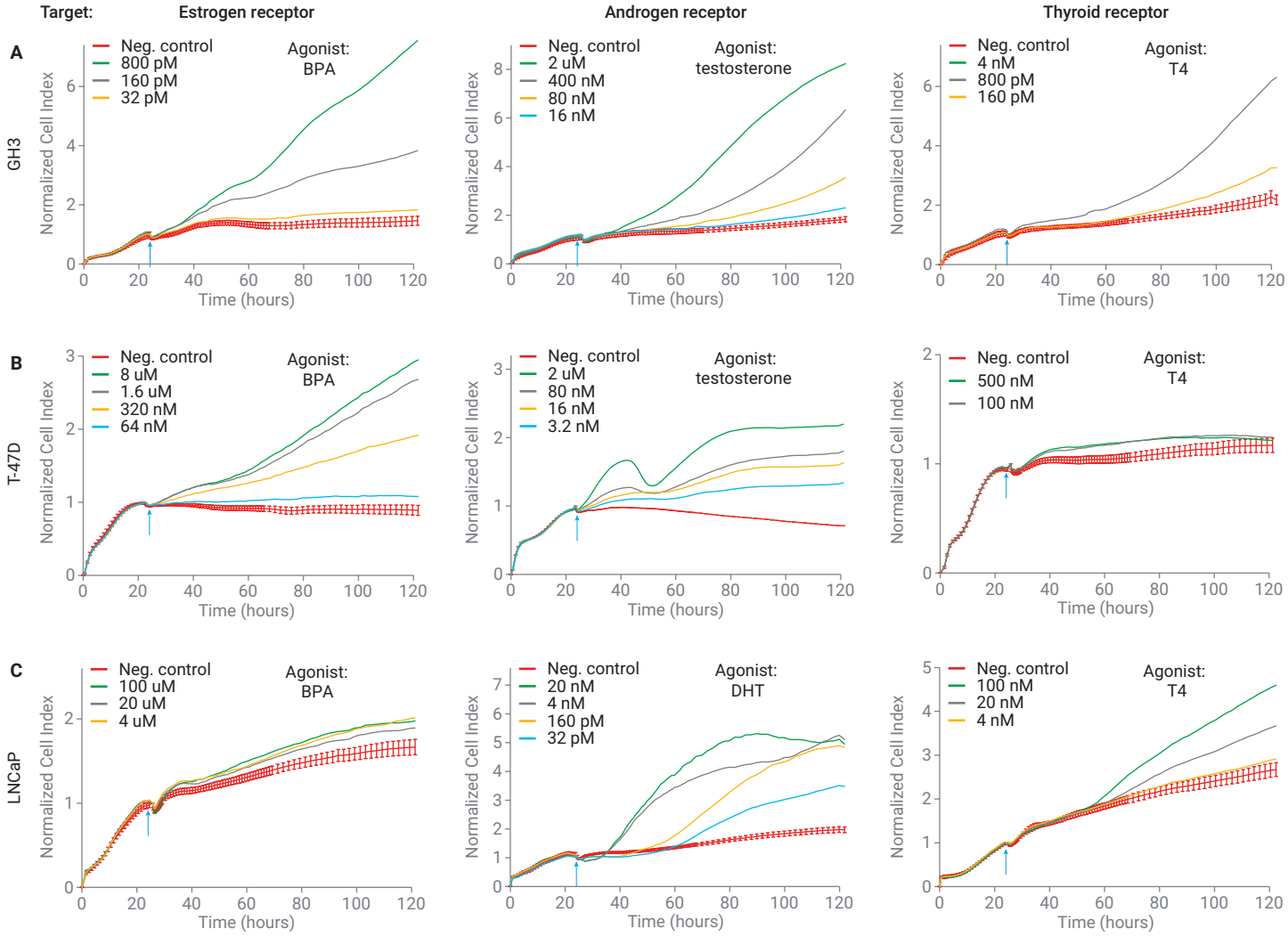

Figure 3. Time-dependent cellular responses to different agonists of the estrogen, androgen, and thyroid receptors. GH3 (panel A), T-47D (panel B), and LNCaP cells (panel C) were cultured in Agilent E-Plates overnight, followed by treatment with representative agonists of the estrogen receptor (BPA), androgen receptor (testosterone or DHT), or thyroid receptor (T4). CI was continuously monitored for 96 hours and normalized against the CI at the time of treatment (indicated by the arrow). Cells without compound treatment were used as a negative control. Note the different scales in each graph.

cell line displays for a given class of agonist (Table 1) provides an extra point of differentiation for identifying which endocrine receptor an EDC is acting on. For example, the androgen receptor agonists R1881, testosterone, and DHT each stimulate proliferation in all three cell lines, but their potency in each cell line varies dramatically. For all three of these agonists the relative sensitivity displayed by the cell lines is LNCaP > T-47D > GH3, where LNCaP is 1 to 2 orders of magnitude more sensitive than T-47D, and T-47D is 1 to 2 orders of magnitude more sensitive than GH3. Combining this type of relative sensitivity data with the distinctive impedance curve shapes provides a more rigorous means of deciphering which receptor an EDC is stimulating.

Receptor specificity of the cell panel-based RTCA assay

The receptor responses observed in the assays discussed are consistent with previous reports.[17,18,21,22] But confirmation was needed that the distinctive features of impedance traces recorded by RTCA correlate with specific receptor-ligand interactions. To this end, antagonists that targeted the different hormone receptors were used. Figure 4 shows that T-47D cells exhibit specific proliferative responses to ER and AR agonists, which can only be inhibited by each receptor's respective antagonist. Specifically, the proliferation that is stimulated by E2 is abolished by the ER antagonist ICI 182,780[23] (Figure 4A), but not by the anti-androgen compound bicalutamide[24] (Figure 4B). Similarly, the proliferation that is stimulated by the androgen DHT is effectively blocked by bicalutamide, but not by ICI 182,780 (Figure 4C and D). Further testing with additional hormone receptor-specific antagonists, such as the ER antagonist tamoxifen and the AR antagonist vinclozolin (data not shown), confirmed that the impedance readouts of the xCELLigence assay are specific to which hormone receptor is being stimulated or blocked.

How specific RTCA impedance responses are to stimulation of a particular receptor was further probed using shRNA-mediated knockdown of estrogen receptor alpha (ERα).

T-47D cells grown on E-Plates were infected with lentivirus expressing either

Table 1. Cell panel sensitivity to agonist derivatives in RTCA assays. Reference compounds were tested for agonistic effects on GH3, T-47D, and LNCaP cells. EC50 was calculated using NCI values 72 hours post compound addition. nd = not determined since the compound did not have a proliferative effect. The cell line that shows the most sensitive response to each category of agonist is highlighted in pink.

Receptor Type	Reference Compound	GH3 EC50 (M)	T-47D EC50 (M)	LNCaP EC50 (M)
Estrogen receptor	E2	5.9×10^{-12}	2.8×10^{-12}	3.7×10^{-12}
	BPA	1.7×10^{-7}	1.9×10^{-7}	nd
Androgen receptor	Testosterone	6.8×10^{-7}	2.9×10^{-9}	1.3×10^{-10}
	R1881	1.3×10^{-8}	1.1×10^{-9}	1.2×10^{-11}
	DHT	3.9×10^{-7}	2.9×10^{-9}	4.5×10^{-11}
Thyroid receptor	T3	2.0×10^{-11}	nd	2.6×10^{-10}
	T4	6.3×10^{-10}	nd	2.7×10^{-8}

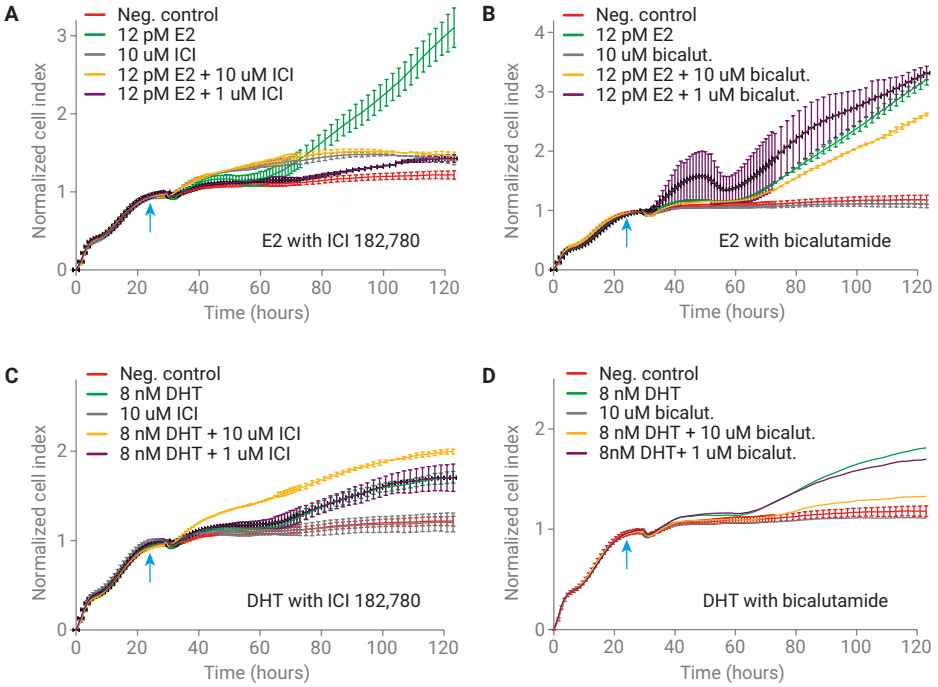

Figure 4. Time-dependent cellular responses of T-47D cells to specific agonist and antagonist combinations. T-47D cells were cultured in Agilent E-Plates overnight. Following treatment with the ER antagonist ICI 182,780 (A and C) or AR antagonist bicalutamide (B and D), cells were immediately treated with E2 or DHT. CI was continuously monitored for 96 hours and normalized against the CI at the time of treatment (indicated by the arrow). Cells without compound treatment or treated with antagonists alone were used as a control and cells treated with E2 alone or DHT alone were used as a reference for the level of inhibition by the antagonist.

a nontargeting control (NTC) shRNA or an ERα-specific shRNA (Figure 5A), followed by treatment with E2. While E2 induced cellular proliferation in the NTC shRNA cells (Figure 5B), this was abolished in ERα knockdown cells (Figure 5D). In contrast, the proliferation profile induced by R1881 was not affected by ERα knockdown (Figure 5C and 5E). These data suggest the unique kinetic response profiles elicited by estrogen-active compounds are a consequence of ER activation. This knockdown study also demonstrates the efficacy of using T-47D cells to determine whether a compound is acting as an EDC by binding ER versus AR.

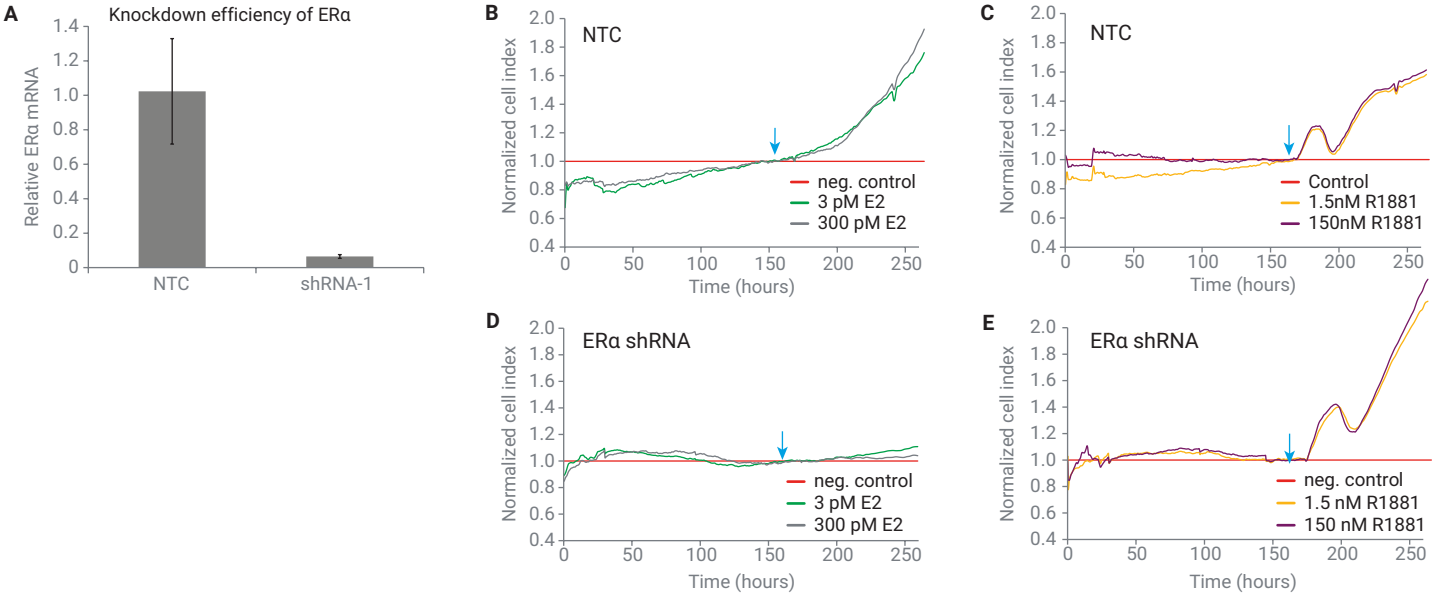

Figure 5. Probing the receptor specificity of RTCA proliferation profiles using shRNA-mediated knockdown of estrogen receptor alpha (ERα). T-47D cells growing on Agilent E-Plates were infected with lentivirus expressing either ERα-specific shRNA or NTC shRNA. Infected cells were treated with E2 or R1881 and growth was monitored by impedance. (A) ERα knockdown efficiency. Growth profiles of NTC shRNA infected cells treated with E2 (B) or R1881 (C). Growth profiles of ERα shRNA infected cells treated with E2 (D) or R1881 (E). All graphs are normalized to NTC shRNA values, set to NCI = 1.

Conclusion

There is a steady increase in the number of synthetic chemicals that are being used in products ranging from cosmetics and sunscreens to food additives and building materials. With this, there is a growing need for assays that can efficiently evaluate whether a compound has EDC activity. Using xCELLigence RTCA, such an assay has been developed. The workflow, shown in Figure 6, involves growing three different cell lines (GH3, T-47D, and LNCaP) in E-Plates for 24 hours, adding reference compounds, which have known activity against either the ER, AR, or TR, and tracking the proliferation response for three to five days using continuous impedance recording. The resulting growth curves have distinctive shapes that are specific to the cell line being used and the receptor being stimulated. These data can then be used as the standard by which the data from "unknown/test" compounds are compared. Beyond the impedance curve shape, the relative sensitivity of each cell line to the test compound helps to elucidate which of the endocrine receptors is being stimulated. If no agonistic activity is observed for a test compound, it can subsequently be evaluated for antagonistic activity (Figure 6).

As well as the efficiency gained from identifying EDC activity and target receptor identity in a single assay, other benefits of the RTCA approach include: (1) The ability to detect low EDC activity with late onset. For example, when LNCaP cells were exposed to 1.28 pM R1881, a proliferative effect was only observed after 90 hours of exposure (Figure 2C). Using traditional *in vitro* assays that lack real-time kinetic data would lead to subtle and delayed effects going undetected.

(2) The ability to differentiate cytotoxicity from EDC activity. A drawback of traditional cell-based assays is that, because cytotoxicity can interfere with EDC activity, a separate cytotoxicity experiment has to be carried out in parallel. With RTCA assays, cytotoxic effects are readily identified by impedance (CI) values dropping lower than the values displayed by negative controls.

The use of three cell lines in our assay provides a multifaceted view of how a test compound behaves, increasing the sensitivity and predictivity of the assay. The inclusion of extra cell lines from different tissues or different species could help elucidate tissue-specific effects. The approach shown for endocrine receptors can just as easily be applied to other families of receptors, with one caveat being that, to be observable by RTCA, the activity of a receptor needs to have an impact on cell number, cell size, or cell-substrate attachment quality over time. Because most receptors have an impact on at least one of these three parameters, the breadth of applications/targets that are accessible through RTCA is substantial.

In addition to enabling the rapid and facile identification of EDC activity in previously uncharacterized compounds, this method allows elucidation of the identity of the endocrine receptor being agonized or antagonized. Use of the RTCA approach with multiple cell lines provides a multifaceted view of a potential EDC, improving the predictive value of the assay. This approach can be expanded to study other types of nuclear receptor activation, given that the downstream effects involve changes in cell proliferation, morphology, or cell attachment quality.

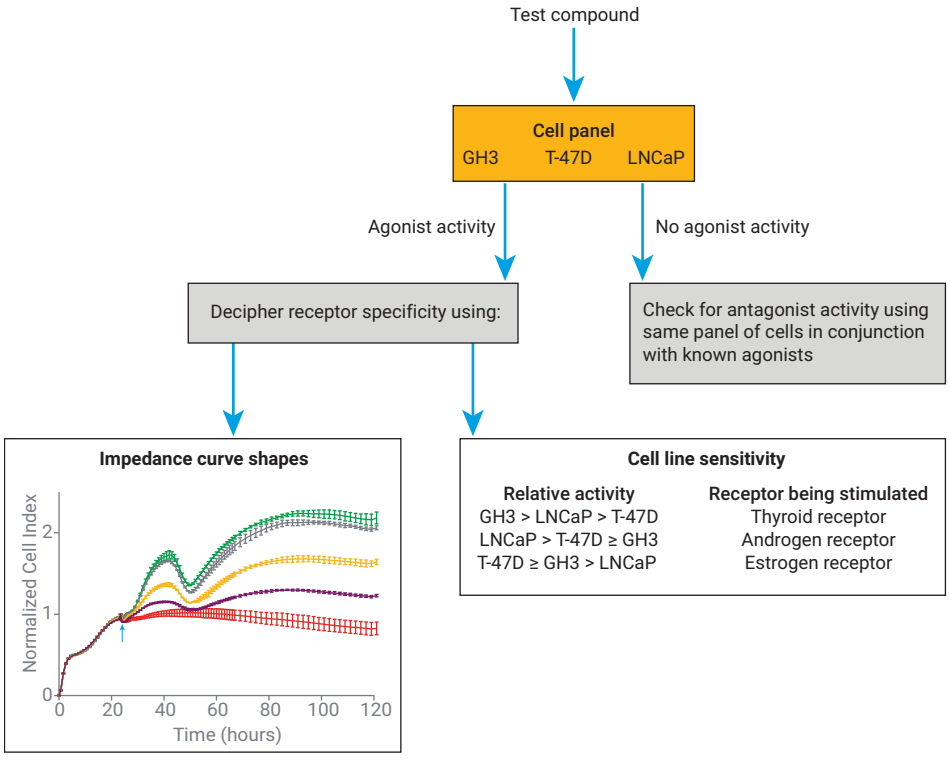

Figure 6. Workflow for EDC identification and characterization using Agilent xCELLigence real-time cell analysis. See text for details.

References

1. Yang, O. *et al.* Endocrine-Disrupting Chemicals: Review of Toxicological Mechanisms Using Molecular Pathway Analysis. *J. Cancer Prev.* **2015**, *20(1)*, 12–24.
2. Grimaldi, M. *et al.* Reporter Cell Lines for the Characterization of the Interactions Between Human Nuclear Receptors and Endocrine Disruptors. *Front. Endocrinol.* **2015**, *6(62)*, doi: 10.3389.
3. Swedenborg, E. *et al.* Endocrine Disruptive Chemicals: Mechanisms Of Action And Involvement In Metabolic Disorders. *J. Mol. Endocrinol.* **2009**, *43*, 1–10.
4. Hotchkiss, A. K. *et al.* Fifteen Years After "Wingspread"-Environmental Endocrine Disrupters and Human and Wildlife Health: Where We Are Today and Where We Need to Go. *Toxicol. Sci.* **2008**, *105*, 235–259.
5. Sharpe, R. M.; Irvine, S. How Strong is the Evidence of a Link Between Environmental Chemicals and Adverse Effects on Human Reproductive Health? *BMJ* **2004**, *328*, 447–451.
6. Gronemeyer, H.; Gustafsson, J.-Å.; Laudet, V. Principles for Modulation of the Nuclear Receptor Superfamily. *Nat. Rev. Drug Discov.* **2004**, *3*, 950–964.
7. Bovee, T. F.; Pickkemaat, M. G. Bioactivity-Based Screening of Antibiotics and Hormones. *J. Chromatogr. A.* **2009**, *1216*, 8035–8050.
8. Doisneau-Sixou, S. F. *et al.* Estrogen and Antiestrogen Regulation of Cell Cycle Progression in Breast Cancer Cells. *Endocr. Relat. Cancer* **2003**, *10*, 179–186.
9. Schriks, M. *et al.* T-Screen to Quantify Functional Potentiating, Antagonistic and Thyroid Hormone-Like Activities of Poly Halogenated Aromatic Hydrocarbons (PHAHs). *Toxicol. In Vitro.* **2006**, *20*, 490–498.
10. Ghisari, M. *et al.* Effects of Currently Used Pesticides and Their Mixtures on the Function of Thyroid Hormone and Aryl Hydrocarbon Receptor in Cell Culture. *Toxicol. Appl. Pharmacol.* **2015**, *284*, 292–303.
11. Caporali, S. *et al.* Distinct Signaling Pathways Mediate Stimulation of Cell Cycle Progression and Prevention of Apoptotic Cell Death by Estrogen in Rat Pituitary Tumor PR1 Cells. *Mol. Biol. Cell.* **2003**, *14*, 5051–5059.
12. O'Hara, L. *et al.* Pituitary Androgen Receptor Signalling Regulates Prolactin but Not Gonadotrophins in the Male Mouse. *PLoS One* **2015**, *10 (3)*, e0121657.
13. Wang, S. *et al.* Proliferation Assays for Estrogenicity Testing with High Predictive Value for the In Vivo Uterotrophic Effect. *J. Steroid. Biochem. Mol. Biol.* **2012**, *128*, 98–106.
14. Hsieh, M.-L.; Juang, H.-H. Cell Growth Effects on Triiodothyronine and Expression of Thyroid Hormone Receptor in Prostate Carcinoma *Cells. J. Androl.* **2005**, *26 (3)*, 422–428.
15. Osborne, C.; Wakeling, A.; Nicholson, R. Fulvestrant: an Oestrogen Receptor Antagonist with a Novel Mechanism of Action. *Br. J. Cancer.* **2004**, *Suppl 1*, S2–S6.
16. Osguthorpe, D. J.; Hagler, A. T. Mechanism of Androgen Receptor Antagonism by Bicalutamide in the Treatment of Prostate Cancer. *Biochemistry* **2011**, *50 (19)*, 4105–4113.

www.agilent.com/chem

For Research Use Only. Not for use in diagnostic procedures.

This information is subject to change without notice.

Printed in the USA, November 1, 2019
5994-1064EN
AN12
DE.5668287037

Agilent
Trusted Answers