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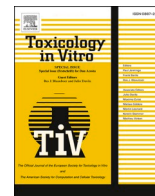
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## Evaluation of an imaging-based in vitro screening platform for estrogenic activity with OECD reference chemicals

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### ABSTRACT

Estrogen receptor alpha (ER $\alpha$ ) is often a primary target of endocrine disrupting chemicals (EDCs) and therefore several biochemical and cell-based assays for the detection of chemicals with estrogenic properties have been developed in the past. However, the current approaches are not suitable for the monitoring of pathway activation dynamics, and they are mostly based on expression constructs that lack physiological promoter regulation. We recently developed MCF7 fluorescent reporter cell lines of 3 different green fluorescent protein (GFP)-tagged ER $\alpha$  target genes: *GREB1*, *PGR* and *TFF1*. These reporters are under control of the full physiological promoter region and allow the monitoring of dynamic pro-proliferative pathway activation on a single cell level using a live-cell imaging set-up. In this study, we systematically characterized the response of these reporters to a full reference compound set of known estrogenic and non-estrogenic chemicals as defined by the Organization for Economic Co-Operation and Development (OECD). We linked activation of the pro-proliferative ER $\alpha$  pathway to a potential adverse outcome by additionally monitoring cell cycle progression and proliferation. The correct classification of the OECD reference compounds showed that our reporter platform has the same sensitivity and specificity as other validated artificial ER $\alpha$  pathway reporters, such as the ER $\alpha$  CALUX and VM7 Luc ER TA assay. By monitoring several key events (i.e. ER target activation, cell cycle progression and proliferation), and subsequently determining Point-of-Departure (POD) values, our reporter panel can be used in high-throughput testing for a physiologically more relevant, quantitative temporal endocrine modulation analysis to improve human carcinogen risk assessment.

### 1. Introduction

Non-genotoxic carcinogens can increase the risk to develop cancer through many different mechanisms such as immune suppression, inflammation, and cytotoxicity, along with receptor-mediated endocrine modification (Hernández et al., 2009; Beatriz, 2000). Both experimental and epidemiological studies have highlighted the association between endocrine disrupting chemicals (EDCs) (i.e. polychlorinated bisphenyls, bisphenol A, cadmium, butyl benzyl phthalate, dioxins, diethylstilbestrol and several pesticides and herbicides) and breast, uterine, ovarian and prostate cancer (Gore et al., 2015; De Coster and Sam and van Larebeke, 2012; Diamanti-Kandarakis et al., 2009). Exposure to EDCs was also shown to result in other diseases such as

diabetes, obesity, metabolic syndromes, thyroid disruption and diseases of the reproductive system (Gore et al., 2015). The estrogen receptor alpha (ER $\alpha$ ) is often a primary target of these EDCs and several estrogenic compounds are in the International Agency for Research on Cancer (IARC) list of proven human carcinogens (IARC group 1) (Hernández et al., 2009; Schug et al., 2011). ER $\alpha$  is a ligand-dependent transcription factor belonging to the family of nuclear hormone receptors (NHRs). Upon binding of ligand, the receptor will dimerize and translocate to the nucleus where it modulates the expression of several genes involved in cell proliferation, including cell cycle control, apoptosis and transcriptional regulation (Welboren et al., 2009; Jia et al., 2015).

The gold standard for the carcinogenic assessment of chemical compounds is a 2-year bioassay in rodents (von Wittenau, 1983).

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However, these rodent bioassays are expensive, time-consuming, not able to describe the molecular mechanism and have poor translatability to humans (Krewski et al., 2010; van der Laan, 2016; Van Oosterhout et al., 1997). Also for ethical reasons, toxicological risk assessment techniques are undergoing a shift from animal-based approaches to human tissue culture-based approaches capable of providing detailed dose-response profiles (Dix et al., 2007; van der Laan, 2017). These techniques mainly focus on chemical-receptor interactions and the consequent cascades of events taking place during and after the signal transduction. Cell-based techniques can be employed in a high-throughput fashion and their output can be used for the development of computational system biology models. Once developed, such models allow an even more efficient compound screening system firmly based on human biology (Krewski et al., 2010).

In contrast to mutagenic chemicals, the main mode of action of carcinogenic EDCs is non-genotoxic and carcinogenic risk can therefore not be assessed by mutagenicity studies such as the Ames mutagenicity test in bacteria or comet assays (Sauer, 2004; Luijten et al., 2016; Lilienblum et al., 2008). For this reason, efforts are being made to develop new cell-based systems. As mentioned above, ER $\alpha$  has high affinity for several EDCs, and given the involvement of estrogen signaling in breast cancer progression, this receptor was employed for the development of several cell-based assays (van Vugt-Lussenburg, 2018; Dreier et al., 2015; van der Burg et al., 2010; Huang et al., 2011; Ashcroft et al., 2011). However, these approaches are not suitable for the monitoring of pathway activation dynamics, and they are mostly based on non-physiological constructs. We recently created fluorescent reporter cell lines by stably transfecting the breast adenocarcinoma cell line MCF7 with bacterial artificial chromosomes (BACs) containing 3 different green fluorescent protein (GFP)-tagged ER $\alpha$  target genes: *GREB1*, *PGR* and *TFF1*. These target genes are inducible by the non-genotoxic carcinogen and ER $\alpha$  agonist 17 $\beta$ -estradiol (E2) in an ER $\alpha$ -dependent manner and are essential for ER $\alpha$ -dependent cell cycle progression and proliferation (Duijndam et al., 2021). These reporters allow the monitoring of dynamic pro-proliferative pathway activation on a single cell level using a live-cell imaging set-up. We demonstrated the different activation dynamics upon exposure to E2, and a small set of estrogenic

compounds with varying potencies. In this study, we further characterize the response of these reporters to a set of known estrogenic and non-estrogenic reference chemicals as defined by the Organization for Economic Co-Operation and Development (OECD) (OECD, 2016). We link activation of the pro-proliferative ER $\alpha$  pathway to a potential adverse outcome by monitoring cell cycle progression and proliferation. The correct classification of the OECD reference compounds shows that our reporter platform has the same sensitivity and specificity as the recombinant ER $\alpha$  pathway reporters, while allowing high-throughput measurement of additional physiologically relevant key events. These features are expected to be instrumental for weight-of-evidence-based risk assessment of EDCs.

## 2. Results

To further validate our fluorescent MCF7 ER $\alpha$  pathway reporter lines, we employed a reference chemical set of agonists described in OECD guideline 455 "Performance-based test guideline for stable transfected transactivation in vitro assays to detect estrogen receptors agonists and antagonists". For this study, we selected the 16 ER agonists and 6 non-estrogenic compounds (Table 1) which were also used in the "me-too" test validation of the ER $\alpha$  CALUX assay (OECD, 2016). First, we performed systematic concentration-range finding (CRF) runs to determine possible cytotoxic concentrations and to obtain a refined concentration-range for the second phase. The second phase consisted out of 3 biologically independent definitive runs to classify the test chemicals (positive or negative response) and to characterize the potency and magnitude of the positive responses.

### 2.1. MCF7 BAC-GFP reporters identify positive and negative estrogenic compounds in a systematic screening set-up

In the CRF run we aimed at defining starting concentrations for each separate compound with the lowest non-cytotoxic concentrations and with the highest ER $\alpha$  target activation. During the first CRF run, the MCF7 GREB1-GFP, PGR-GFP and TFF1-GFP reporter cell lines were exposed to a wide concentration range of the test chemicals. After 48 h,

**Table 1**

Classification of reference chemicals based on validated assays (table adapted from OECD TG-455 (OECD, 2016)).

Substance	CASno.	Product class <sup>a</sup>	IARC <sup>b</sup>	STTA	VM7Luc ER TA	ER $\alpha$ CALUX	Other ER TAS <sup>c,d</sup>	ER binding <sup>c</sup>
17 $\beta$ -estradiol	50-28-2	Hormone	Group 1	POS	POS	POS	POS(227/227)	POS
17 $\alpha$ -estradiol	57-91-0	Pharmaceutical	–	POS	POS	POS	POS (11/11)	POS
17 $\alpha$ -ethinylestradiol	57-63-6	Pharmaceutical	Group 1	POS	POS	POS	POS (22/22)	POS
19-nortestosterone	434-22-0	Pharmaceutical	–	POS	POS	POS	POS (4/4)	POS
4-cumylphenol	599-64-4	Chemical intermediate	–	POS	POS	POS	POS (5/5)	POS
4-tert-octylphenol	140-66-9	Chemical intermediate	–	POS	POS	POS	POS (21/24)	POS
Bisphenol A	80-05-7	Chemical intermediate	–	POS	POS	POS	POS (65/65)	POS
Butyl benzyl phthalate	85-68-7	Plasticizer, Industrial chemical	Group 3	POS	POS	POS	POS (12/14)	POS
Coumestrol	479-13-0	Natural product	–	POS	POS	POS	POS (30/30)	POS
Diethylstilbestrol	56-53-1	Pharmaceutical	Group 1	POS	POS	POS	POS (42/42)	POS
Ethyl paraben	120-47-8	Pharmaceutical, Preservative	–	POS	POS	POS	POS	–
Genistein	446-72-0	Natural product, Pharmaceutical	–	POS	POS	POS	POS (100/102)	POS
Kaempferol	520-18-3	Natural product	Group 3	POS	POS	POS	POS (23/23)	POS
Kepone	143-50-0	Pesticide	Group 2B	POS	POS	POS	POS (14/18)	POS
Meso-hexestrol	84-16-2	Pharmaceutical	–	POS	POS	POS	POS (4/4)	POS
Methoxychlor	72-43-5	Pesticide	Group 3	POS	POS	POS	POS (24/27)	POS
Norethynodrel	68-23-5	Pharmaceutical	Group 1	POS	POS	POS	POS (5/5)	POS
Atrazine	1912-24-9	Herbicide	Group 3	NEG	NEG	NEG	NEG (30/30)	NEG
Corticosterone	50-22-6	Pharmaceutical	–	NEG	NEG	NEG	NEG (6/6)	NEG
Ketoconazole	65277-42-1	Pharmaceutical	–	NEG	NEG	NEG	NEG (2/2)	NEG
Linuron	330-55-2	Pesticide	–	NEG	NEG	NEG/POS	NEG (8/8)	NEG
Reserpine	50-55-5	Pharmaceutical	Group 3	NEG	NEG	NEG	NEG (4/4)	NEG
Spironolactone	52-01-7	Pharmaceutical	Group 3	NEG	NEG	NEG	NEG (4/4)	NEG

POS = positive estrogenic agonist, NEG = negative compound.

<sup>a</sup> Based on (ICCVAM, 2003).

<sup>b</sup> Classification based on the IARC Monographs, Volumes 1–129 (International Agency for Research on Cancer and World Health Organization, 2019).

<sup>c</sup> Classification based on ICCVAM Background Review Documents for ER binding and TA test methods.

<sup>d</sup> Number in parenthesis represents the test results classified as positive of negative over the total number of referenced studies.

GFP intensity levels and the propidium iodide (PI) positive fraction were measured to determine ER $\alpha$  target activation levels and possible cytotoxicity (Fig. 1A). In cases where the lowest concentration tested still showed ER $\alpha$  target activation, the CRF run was repeated using higher serial dilutions of the test chemical. Each plate contained several reference compounds for quality control and normalization purposes: reference compound 17 $\beta$ -estradiol (100 fM- 10  $\mu$ M), weak positive control 17 $\alpha$ -methyltestosterone (3  $\mu$ M), solvent control DMSO (0,1%), experimental medium (vehicle control) and negative control corticosterone (10 nM) (Supplementary Fig. 1A-B). All three reporters showed similar responses upon exposure to the different reference compounds and test chemicals (Fig. 1B), except for the PGR-GFP reporter which only showed a very weak response to the weak positive control 17 $\alpha$ -methyltestosterone (Supplementary Fig. 1A-B). Cytotoxicity, as indicated by an increased PI positive fraction, was only observed for the highest concentrations (100  $\mu$ M) of 5 compounds: 4-cumylphenol, 4-tert-octylphenol, diethylstilbestrol, kepone and meso-hexestrol (Fig. 1B). Increased numbers of PI positive cells were accompanied by decreased GFP responses (Fig. 1B, C). Conversely, a drop in GFP intensity was not always linked to increased PI staining, as seen after exposure to for instance genistein or butyl benzyl phthalate (Fig. 1B, D). In addition, PI positive cells were not detected upon exposure to the negative test compounds. The highest concentrations of coumestrol and reserpine were excluded from the definitive runs since these compounds showed autofluorescence, thereby hampering proper segmentation of the nuclei during image analysis (Supplementary Fig. 1C). This CRF run resulted in a good overview of the cytotoxic potential of the test chemicals as well as the ER $\alpha$  target activation level and provided us with a refined concentration range for each test chemical in order to perform the comprehensive runs for classification and characterization of the response (Supplementary Table 1). Only for bisphenol A, we had to adjust the concentration range in the definitive runs, due to high GFP signals in the single CRF run which could not be replicated in the three biologically independent definitive runs.

In the definitive runs, we evaluated the response of the MCF7 GREB1-GFP, PGR-GFP and TFF1-GFP reporter cells after multiple timepoints (i.e. 24, 48 and 72 h), to evaluate temporal pathway activation dynamics. In general, the reporters showed similar responses upon exposure to the test chemicals (Fig. 2A), and no cell death was observed at any tested concentration during the comprehensive runs (Supplementary Fig. 2A). Clear differences in temporal activation dynamics were noticed between the three GFP reporters. After exposure to positive estrogenic compounds, GREB1-GFP reached maximum expression after 48 h and PGR-GFP only after 72 h, while TFF1 already reached maximum expression after 24 h (Fig. 2B). These temporal differences in reporter activation are in line with effects of estrogenic signaling on transcription of endogenous *GREB1*, *PGR* and *TFF1* (Supplementary Fig. 1D). No concentration-dependent response was observed after exposure to the negative test chemicals, except for the glucocorticoid receptor agonist corticosterone (weak GREB1-GFP activation only) and the antifungal compound with anti-androgenic activity ketoconazole (Supplementary Fig. 2B). This could indicate crosstalk between different nuclear hormone receptor pathways, resulting in GREB1-GFP expression, but no PGR-GFP or TFF1-GFP expression. Kaempferol, kepone and methoxychlor induced very weak responses, especially in the GREB1-GFP reporter (Supplementary Fig. 2C).

To further quantify the magnitude and potency of the positive responses, we performed a non-linear curve fit (Hill equation with variable slope) (Fig. 2B). Besides differences in efficacy and potency, the concentration response curves also showed different slope factors (Hill slope). When we performed a hierarchical clustering based on magnitude of the response i.e. the maximum response ( $E_{max}$ ) of the GFP reporters, we did not observe a clear distinction between the negative and positive test chemicals (Supplementary Fig. 2D). Very weak positive test chemicals (kepone, butyl benzyl phthalate, genistein, kaempferol and methoxychlor) clustered with the negative test chemicals. As reported

by the OECD, several other in vitro ER transactivation assays were also not able to positively identify some of these very weak agonists. For instance, kepone was classified as a positive ER agonist 14 out of 18 times, butyl benzyl phthalate 12 out of 14 times, and methoxychlor 24 out of 27 times (Table 1). Next, we evaluated the potencies of the responses by comparing EC<sub>50</sub> values. Similar as the clustering based on the  $E_{max}$  values, we were not able to clearly separate positive from negative test chemicals based on EC<sub>50</sub> values (Supplementary Fig. 2E). However, a clear separation of the strong estrogenic compounds (diethylstilbestrol, norethynodrel, 17 $\alpha$ -estradiol, meso-hexestrol and 17 $\alpha$ -ethinylestradiol) and reference standard was observed. When combining both the EC<sub>50</sub> and  $E_{max}$  values, we detect four clusters: strong activators and the reference standard (cluster I), weak/moderate activators (cluster II), negative compounds (cluster III) and very weak activators (cluster IV) (Fig. 2C). Only ketoconazole remained clustered with the very weak activators. Although it was not possible to qualify the compounds solely on the potency and magnitude of the GFP response, we did observe similar sensitivity of our reporters compared to other validated OECD assays, as indicated by the high R square values (Fig. 2D, Table 2).

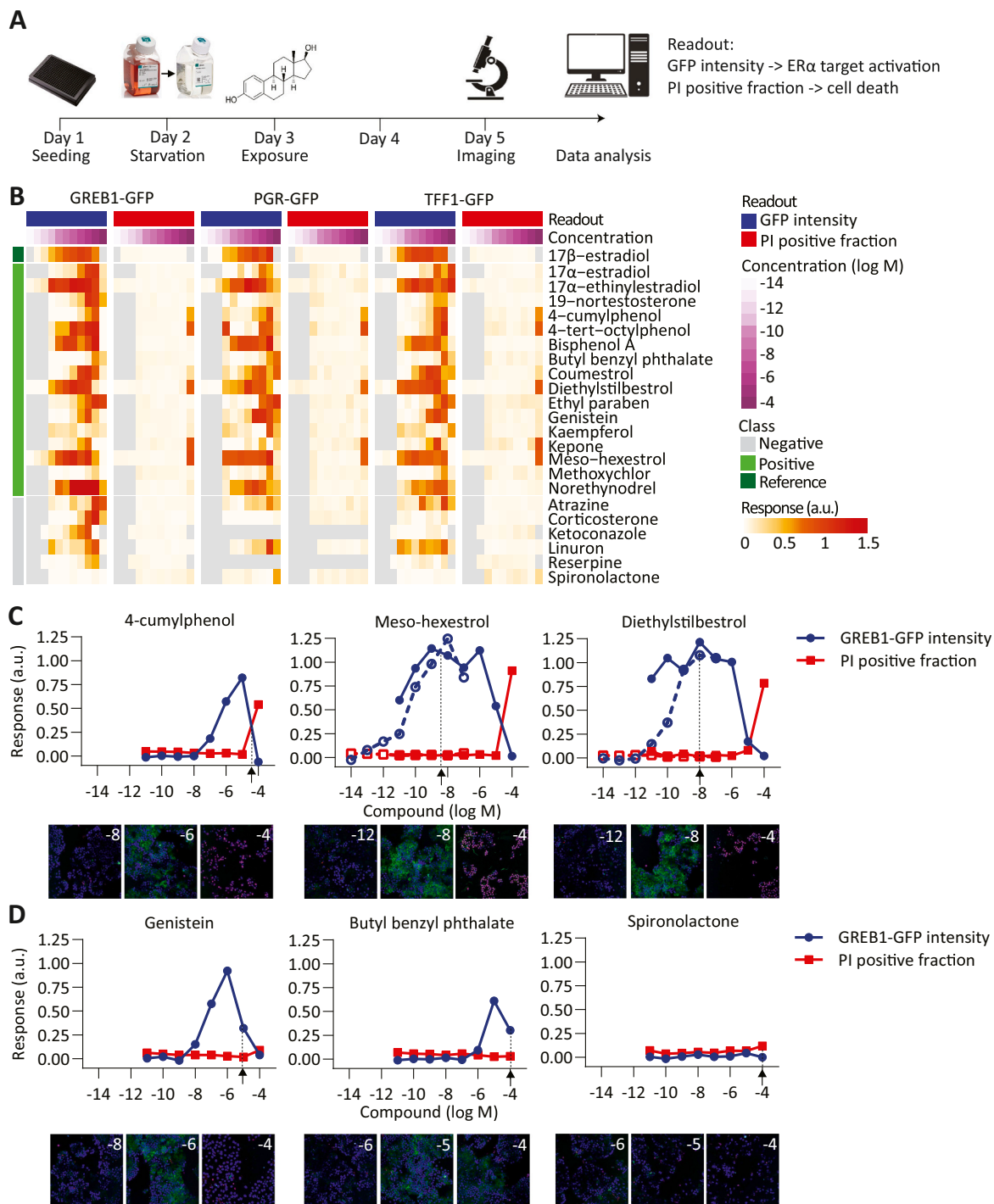
## 2.2. Incorporating data on subsequent key events improves further qualification of estrogenic compounds

As we were not able to fully qualify the chemicals on the potency and magnitude of the GFP response alone, we wanted to incorporate information on other key events in the pathway leading to a potential adverse outcome. For this purpose, we added a Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) reporter line to our definitive runs to determine cell cycle distribution. In addition, we determined the effects of compound exposures on the proliferation rates of the cells. In this way, we were able to monitor several key events (i.e. ER $\alpha$  target activation, cell cycle progression and proliferation) in our reporter platform (Fig. 3A).

We have demonstrated in a previous study that the MCF7 FUCCI-H2B reporter line is able to monitor ER $\alpha$ -dependent cell cycle progression (Duijndam et al., 2021). The FUCCI sensor consists of fluorescently labeled Geminin-GFP and Cdt1-RFP to mark different stages of the cell cycle and the iRFP-labeled Histone 2B (H2B) for nuclear segmentation (Fig. 3A). We evaluated the response of the MCF7 FUCCI-H2B reporter after 24, 48 and 72 h exposure. After exposure to the reference standard E2, over time we observed an increase in the amount of cells in the G<sub>1</sub> and G<sub>1</sub>-S transition phase fraction associated with a decrease of cells in the early G<sub>1</sub> and S-G<sub>2</sub>-M phase at low concentrations (Supplementary Fig. 3A). The effects on cell cycle distribution were most obvious after 72 h of exposure. Since any change in a single fraction will affect other fractions, the EC<sub>50</sub>/IC<sub>50</sub> values of the different fractions are similar. We therefore used the EC<sub>50</sub> values of the early G<sub>1</sub> fraction curves as a representative value of cell cycle progression, as these curves are more consistent compared to the curves of the S-G<sub>2</sub>-M phase (Fig. 3B).

Following cell cycle progression, we included data on actual cell proliferation. For this purpose, we determined the amount of cells (nuclei count) after 24, 48 and 72 h of all our reporters compared to control conditions (DMSO) (Supplementary Fig. 3B). To compare the sensitivity of the different readouts, we performed a non-linear curve fit (Hill equation with variable slope), identical to the GFP reporter data. Since the response in nuclei count was similar between all reporter lines (Supplementary Fig. 3C), we averaged the response and compared it to the results of the SRB assay after 72 h (Fig. 3C, Supplementary Fig. 3D). Like the effects on cell cycle progression, the effects on proliferation were clearest after 48 and 72 h. The EC<sub>50</sub> values of the early G<sub>1</sub> fraction and nuclei count were similarly sensitive readouts (Fig. 3D).

To qualify and quantify the effect of the test chemicals on ER $\alpha$  pathway activation in relation to proliferation, we clustered the compounds based on the EC<sub>50</sub> values of the different readouts (i.e. GFP intensity, FUCCI fractions and nuclei count) of our reporter platform (Fig. 3E). Together with ER target activation, incorporation of data on



**Fig. 1.** CRF runs for OECD reference compounds in fluorescent MCF7 ERα pathway reporter lines. A) Workflow of each CRF run. MCF7 GREB1-GFP, PGR-GFP and TFF1-GFP reporter cells were cultured at least one week before plating in 384-well plates. The following day, the culture medium was replaced with experimental medium containing charcoal-stripped serum to remove estrogenic stimuli. After 24 h, cells were stained with Hoechst for 2 h and subsequently exposed to the compounds in experimental medium containing PI. Plates were imaged after 48 h. B) Non-clustered heatmap summarizing the normalized response (GFP intensity and PI positive fraction) of the reporter cells after 48 h. Grey: condition not tested. C) Normalized response of GREB1-GFP reporter cells demonstrating cytotoxicity at highest tested concentration. In case the lowest concentration tested still showed ERα target activation, the CRF run was repeated using higher serial dilutions (dashed curve). Dotted line with arrow indicates starting concentration of definitive runs. Nuclei are visualized in blue, GFP response in green, PI positive cells in red. D) Normalized response of GREB1-GFP reporter cells demonstrating a drop in GFP response in the absence of cell death. Dotted line with arrow indicates starting concentration of definitive runs. Nuclei are visualized in blue, GFP response in green, PI positive cells in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

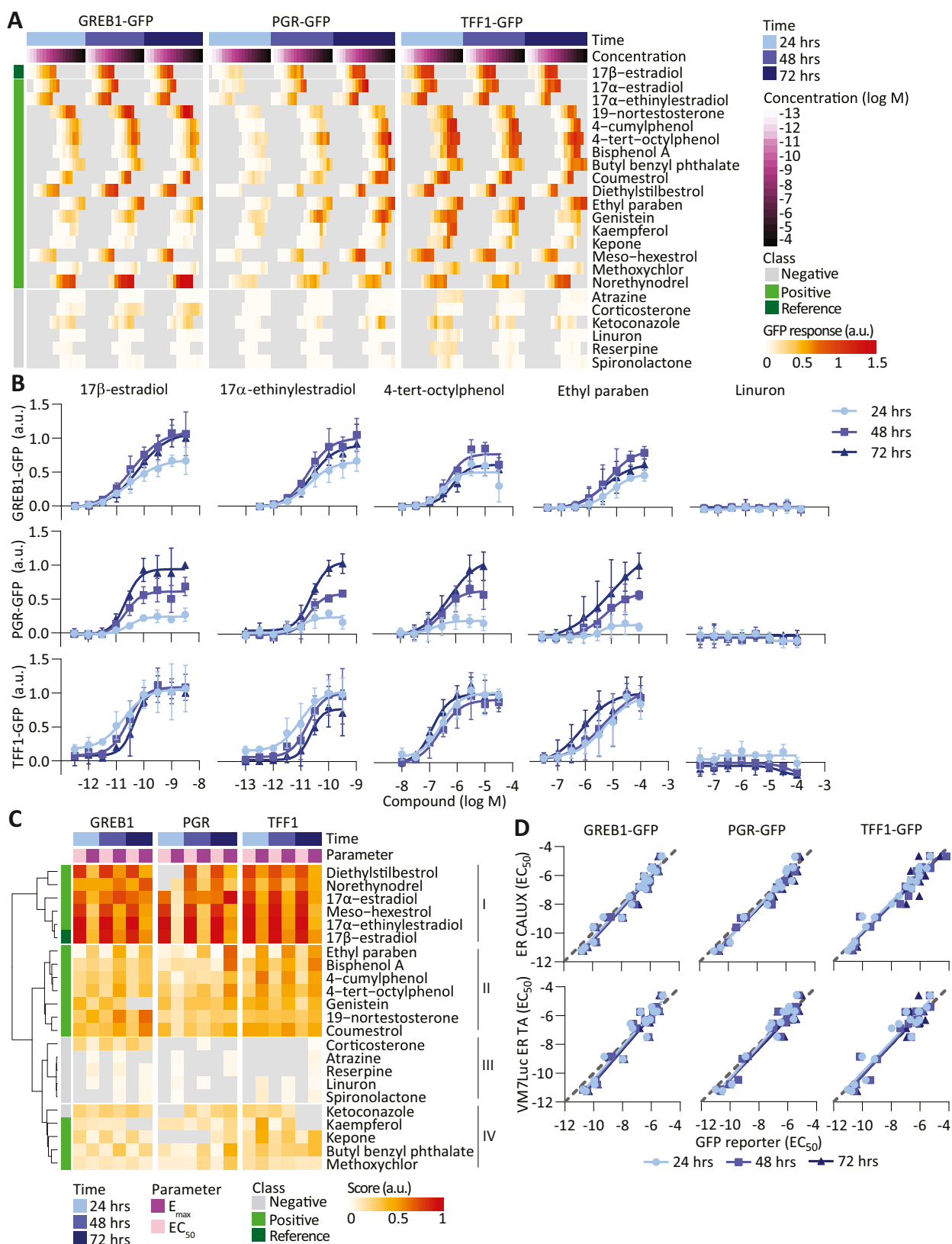


Fig. 2. MCF7 ERα pathway reporters identify positive and negative estrogenic compounds in a systematic screening set-up.

A) Non-clustered heatmap summarizing the normalized GFP response of the reporter cells after exposure up to 72 h. Grey: condition not tested. B) Normalized response of GFP reporters with non-linear curve fit (Hill equation with variable slope). C) Heatmap with hierarchical clustering based on the E<sub>max</sub> and EC<sub>50</sub> values of the GFP reporters after 24, 48 and 72 h. E<sub>max</sub> and EC<sub>50</sub> values were transformed to score. Grey: parameter could not be determined. D) EC<sub>50</sub> values of the GFP reporters compared to other validated OECD assays.

Table 2

Comparison of EC<sub>50</sub> (logM) values of our MCF-GFP reporters (GFP intensity) to different validated OECD assays.

Substance	STTA <sup>a</sup>	VM7Luc <sup>a</sup>	CALUX <sup>a</sup>	GREB1-GFP			PGR-GFP			TFF1-GFP		
	24 h	24 h	24 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
17β-estradiol (reference)	< -11	-11.25	-11.00	-10.54	-10.46	-10.33	-10.52	-10.65	-10.64	-10.74	-10.57	-10.35
17α-estradiol	-9.19	-8.85	-8.89	-9.27	-8.74	-9.04	-8.92	-8.76	-8.75	-10.02	-10.17	-10.19
17α-ethinylestradiol	< -11	-11.14	-11.23	-10.74	-10.75	-10.62	-11.02	-10.72	-10.63	-11.02	-10.77	-10.66
19-nortestosterone	-6.57	-5.74	-6.80	-6.75	-6.76	-6.71	-6.30	-6.65	-6.71	-6.96	-7.02	-6.74
4-cumylphenol	-5.80	-6.94	-6.46	-6.38	-6.33	-6.10	-6.48	-6.17	-6.07	-6.27	-6.21	-6.00
4-tert-octylphenol	-7.13	-7.50	-6.94	-6.32	-6.19	-6.11	-6.75	-6.49	-6.23	-6.59	-6.62	-6.91
Bisphenol A	-6.53	-6.27	-6.37	-6.09	-6.01	-6.15	-5.59	-6.06	-5.40	-6.59	-6.61	-6.27
Butyl benzyl phthalate	-5.39	-5.70	-5.72	-5.59	-5.49	-5.39	-6.00	-5.38	-5.19	-6.11	-6.24	-6.65
Coumestrol	-7.70	-6.88	-8.02	-7.30	-7.09	-6.80	-7.30	-6.98	-7.07	-7.97	-7.37	-6.97
Diethylstilbestrol	-10.69	-10.48	-9.60	-9.96	-9.86	-9.77	-	-9.44	-9.49	-9.78	-9.45	-9.47
Ethyl paraben	-	-4.61	-4.69	-5.20	-5.29	-5.44	-5.38	-5.24	-5.01	-5.29	-5.33	-6.06
Genistein	-7.61	-6.57	-7.47	-7.46	-7.25	-	-7.13	-7.03	-6.81	-7.47	-6.80	-6.06
Kaempferol	-5.92	-5.40	-5.53	-5.97	-5.67	-	-5.92	-5.48	-5.44	-5.54	-5.29	-
Kepone	-5.11	-6.31	-6.05	-6.42	-6.44	-6.47	-	-	-5.71	-6.06	-6.10	-5.63
Meso-hexestrol	-10.56	-10.78	-10.64	-9.93	-9.76	-9.71	-9.87	-10.01	-10.08	-10.55	-10.47	-10.39
Methoxychlor	-	-5.72	-5.45	-5.84	-5.87	-5.88	-5.51	-5.49	-5.49	-6.06	-5.88	-5.72
Norethynodrel	-8.82	-9.03	-8.95	-7.98	-7.94	-7.86	-	-9.14	-8.97	-9.15	-8.97	-8.99
Atrazine	-	-	-	-	-	-	-	-	-	-	-	-
Corticosterone	-	-	-	-6.50	-6.68	-6.79	-	-	-	-	-	-
Ketoconazole	-	-	-	-6.56	-6.63	-6.70	-	-6.50	-6.49	-6.54	-6.54	-
Linuron	-	-	-4.70	-	-	-	-	-	-	-	-	-
Reserpine	-	-	-	-	-	-	-	-	-	-	-	-
Spirolactone	-	-	-	-	-	-	-	-	-	-	-	-
R square values compared to the ER CALUX assay				0.9529	0.9522	0.9287	0.9632	0.9731	0.9698	0.9678	0.9430	0.8789
R square values compared to the VM7Luc assay				0.9256	0.9310	0.9235	0.9483	0.9516	0.9386	0.9169	0.9135	0.8762

<sup>a</sup> EC<sub>50</sub> values as reported in OECD TG-455 (OECD, 2016).

cell cycle progression and proliferation significantly improved the classification of the compounds, reflected by the formation of four distinct clusters: negative compounds (cluster I), very weak/weak activators (cluster II), weak/moderate activators (cluster III) and the very potent estrogenic compounds and the reference standard (cluster IV).

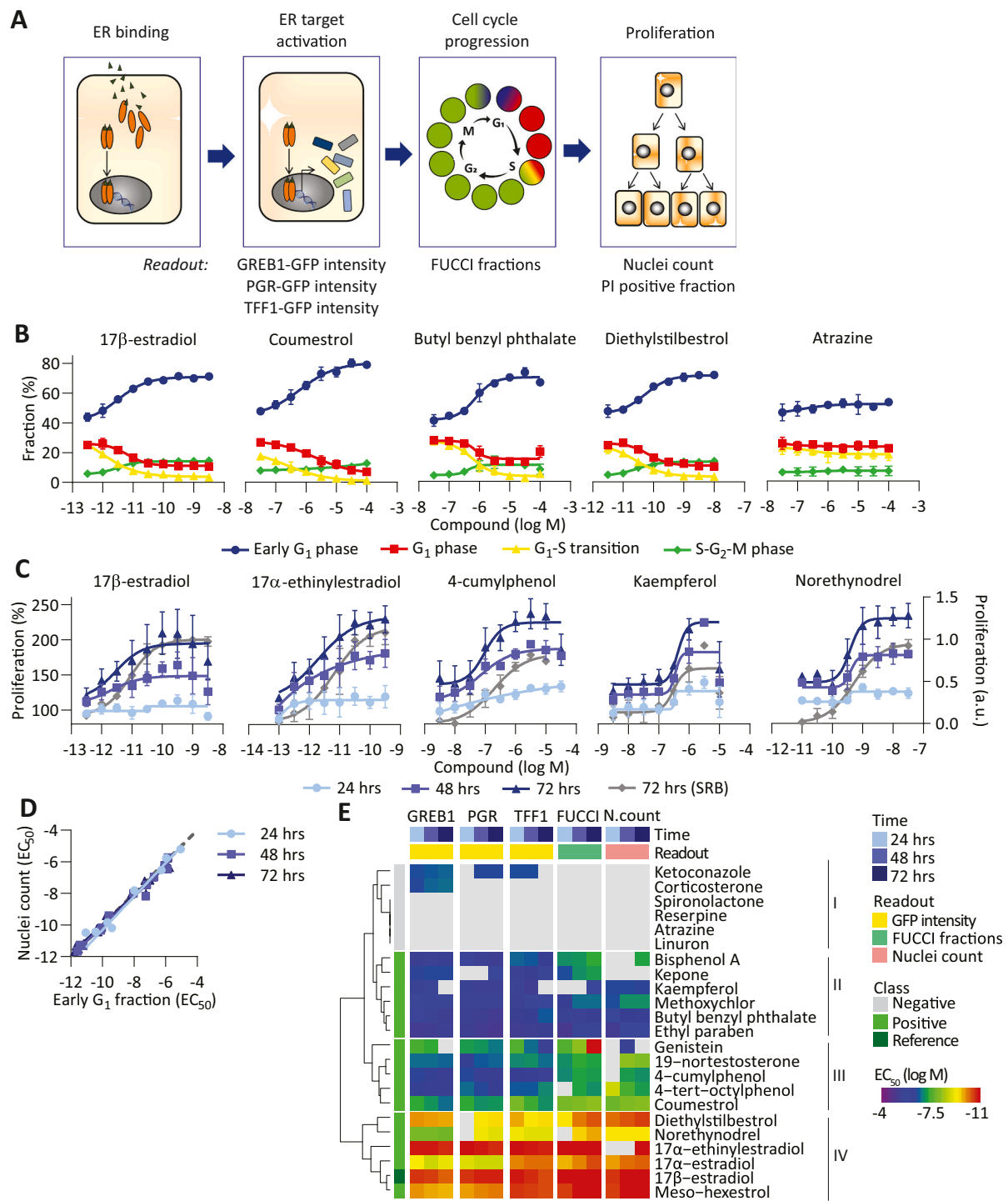
### 2.3. Data generated by our ER pathway activation reporter platform are suitable for point-of-departure modelling

As demonstrated above, our reporter platform is capable of identifying estrogenic compounds with similar sensitivity as other validated assays. In addition, it can produce data which are suitable for point-of-departure (POD) modelling. In human risk assessment, PODs can be used to derive human health guidance values, such as acceptable daily intake (ADI) or derived no-effect level (DNEL). To determine these PODs, the more data-driven benchmark dose/concentration (BMD/BMC) analysis is considered favorable over the use of No-Observed (Adverse) Effect Levels (NO(A)ELs) (Davis et al., 2011; Sand et al., 2017; Hernández et al., 2013). To determine BMCs, we used the software of the US National Toxicology Program “BMDExpress2” (Phillips et al., 2019), originally developed for transcriptomic dose-response data in toxicology. We determined the benchmark response (BMR) and related BMC per test chemical and readout (Fig. 4A-B). The software fits the concentration-response data to different models (linear, exponential, Hill, polynomial, power), and chooses the model that best describes the data (i.e. the best fit model). Since our concentration response curves consist of continuous data and are properly fitted by the Hill equation, as previously demonstrated for the determination of the EC<sub>50</sub> values, we selected the BMC values based on the Hill model. For most conditions, there was a good correlation between the Best BMC and Hill BMC values (Fig. 4C). When we clustered the positive agonists based on the BMC values, we observed smaller clusters with compound analogs (i.e. phenols in cluster IV) showing also a separation between the very strong and strong activators (cluster I and II) (Fig. 4D). In conclusion, our reporter platform can detect and quantify physiologically relevant ER pathway activation, enabling the determination of PODs which can be used for the derivation of guidance values in human risk assessment.

### 3. Discussion

In this study, we demonstrate the performance of our mechanism-based reporter screening platform for ERα pathway activation. By using a systematic approach, our technology was able to classify estrogenic and non-estrogenic compounds from a pool of 22 OECD “Performance-based Test Guideline for Stably transfected transactivation *in vitro* assays to detect estrogen receptor agonists and antagonists” (TG-455) reference chemicals. Furthermore, our ERα target GFP reporters can quantify the potency of positive compounds with a similar performance as other validated assays. By monitoring several key events (i.e. ER target activation, cell cycle progression and proliferation), and subsequently determining POD values, our reporters can be used in a more mechanistic approach to improve human carcinogen risk assessment.

Several biochemical and cell-based assays for the detection of chemicals with estrogenic properties have been developed in the past. Amongst the first developed assays, there are the MCF7-based E-screen proliferation assay (Soto et al., 1995) and the recombinant yeast cell bioassay (RCBA). The RCBA includes yeast cells transformed with plasmids encoding hERα and an estrogen responsive promoter linked to a reporter gene encoding β-galactosidase (Coldham et al., 1997). A major limitation of the RCBA is the transport step of compounds across the yeast cell membrane, which is not very effective, and therefore this assay is not recommended as screening model for EDCs (ICCVAM, 2003). However since the principle behind this technology is very robust, it has been employed in more recent OECD validated assays in mammalian cells, like the Stably Transfected Transactivation Assay (STTA) or the VM7Luc ER TA. Both assays are sensitive luciferase-based reporter systems, where the luciferase enzyme transforms luciferin substrate to a luminescent measurable product. The STTA makes use of the human cervical adenocarcinoma HeLa-9903 cell line ectopically expressing human ERα. In contrast, the VM7Luc ER TA is based on MCF7 cells which endogenously express both ERα and ERβ (OECD, 2016). Multiple studies have shown a good performance on both assays compared to a panel of other frequently used assays (Lee et al., 2012; Huang et al., 2014; Dreier et al., 2015). More recently, the ERα Chemically Activated Luciferase Expression (CALUX) assay was added to the OECD “Conceptual Framework for the Testing and Assessment of



**Fig. 3.** Incorporating data on subsequent key events improves further qualification of estrogenic compounds.

A) Simplified schematic overview of ER $\alpha$  pathway activation and corresponding readouts in our reporter platform. The FUCCI sensor consists of fluorescently labeled Geminin-GFP (green) and Cdt1-RFP (red) to mark different stages of the cell cycle. All cells are labeled with a Histone 2B-iRFP marker, which is expressed in all stages of the cell cycle (blue). Cdt1-RFP is expressed in G<sub>1</sub> phase, and Geminin-GFP is expressed in S-G<sub>2</sub>-M phase. Both markers are expressed during G<sub>1</sub>-S transition. Directly after mitosis, neither Cdt1-RFP nor Geminin-GFP is expressed. Image adapted from (Duijndam et al., 2021). B) Different fractions of FUCCI-H2B reporter upon 72 h exposure to the test chemicals. C) Proliferation based on nuclei count (left Y-axis) and SRB assay (left Y-axis) after exposure to test chemicals. D) EC<sub>50</sub> values based on nuclei count vs EC<sub>50</sub> based on the early G<sub>1</sub> fraction. E) Heatmap with hierarchical clustering based on the EC<sub>50</sub> values of the GFP and FUCCI reporters after 24, 48 and 72 h. Grey: parameter could not be determined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



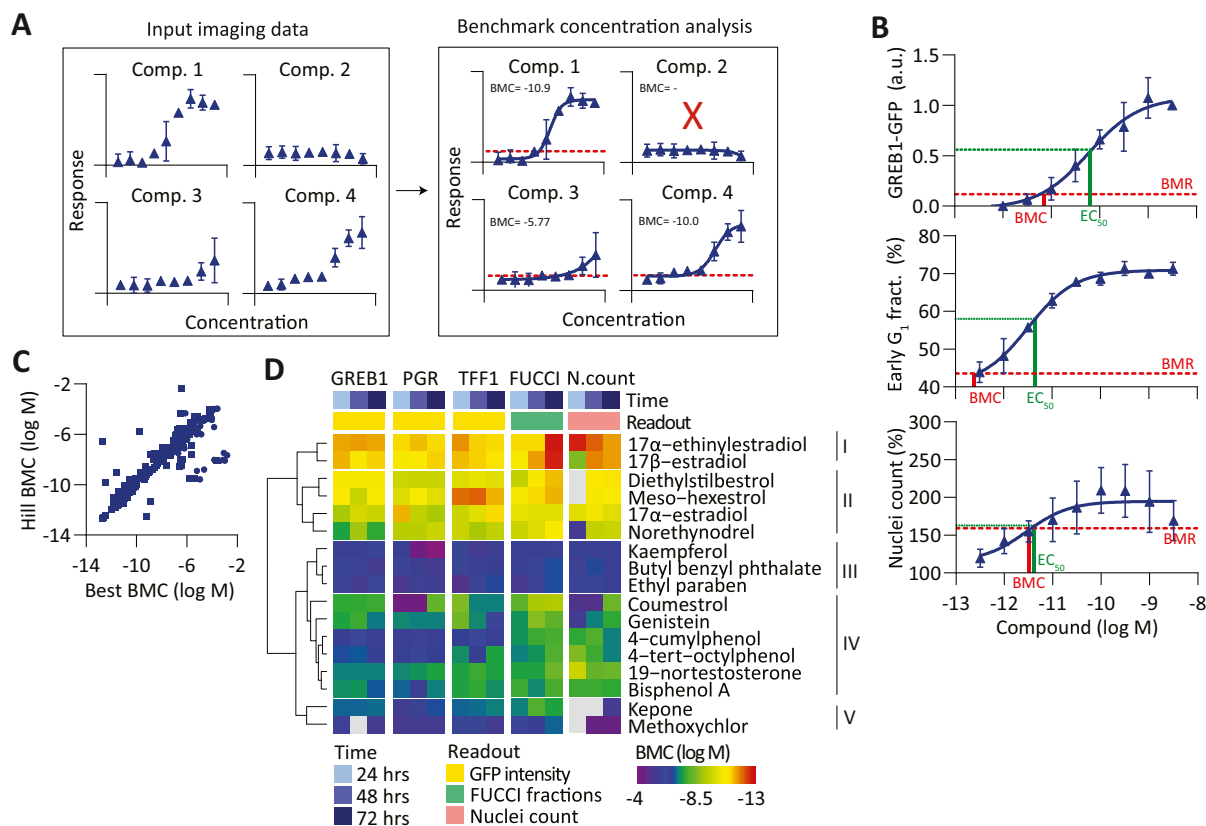


Fig. 4. Data generated by our ER $\alpha$  pathway reporter platform are suitable for Point-of-Departure modelling.

A) Workflow of BMC analysis. B) Examples of BMC graphs after 72 h E2 exposure. C) Best BMC compared to Hill BMC. D) Heatmap with hierarchical clustering based on the BMC values of the GFP and FUCCI reporters after 24, 48 and 72 h. Grey: parameter could not be determined.

Endocrine Disrupting Chemicals" Level 2 *in vitro* assays, after completing validation as a "me-too" test (OECD, 2016). The ER $\alpha$  CALUX transcriptional bioassay is based on the human osteoblastic osteosarcoma U2-OS cell line stably transfected with hER $\alpha$  (Sonneveld et al., 2005; van der Burg et al., 2010). Although these assays, and other *in vitro* assays to assess the estrogenic activity of EDCs, have been proven to be more efficient and molecularly more insightful than *in vivo* assays (Krewski et al., 2010), they are characterized by intrinsic limitations. For instance, the E-Screen assay solely quantifies cell proliferation, which can be mediated through other pathways and is therefore not recommended (ICCVAM, 2003). Several cell-free (i.e. comparative receptor-binding assays (Akahori et al., 2008) and cell-based systems (i.e. STTA, VM7Luc ER TA, ER $\alpha$  CALUX) are proven to be specific for ER $\alpha$  pathway activation, but solely measure ligand-binding or transcriptional activity, respectively, but not the physiological outcome. In addition, the lack of endogenous regulation remains a deficiency. By employing overexpressing constructs in non-physiologically relevant cell lines, thereby eliminating crosstalk with other NHRs for instance, high specificity can be obtained. However, this approach is less reflective of the complex regulation of the ER pathway in humans.

In contrast, our MCF7 fluorescent protein reporter panel enables the monitoring of several key events, i.e. transcriptional activation, cell cycle progression and proliferation, leading towards a potential adverse outcome. In line with the adopted Bradford-Hill considerations for assessing the weight of evidence of key events, key event-relationships and overall adverse outcome pathways (AOPs) (Becker et al., 2015), we clearly demonstrated the requirement of GREB1, PGR and TFF1 expression for ER $\alpha$ -directed cell proliferation using RNA interference approaches (Duijndam et al., 2021). We employed BAC reporter technology (Poser et al., 2008) in the human MCF7 cell line, endogenously expressing ER $\alpha$ . These fluorescent protein reporters were elaborately

characterized based on the induction and localization of the GFP fusion protein, correct fusion and size of the fusion protein and the proliferation rate compared to the parental MCF7 WT cells. We did not observe any interference of the BAC-GFP incorporation with the regulation of the ER $\alpha$  signaling pathway (Duijndam et al., 2021). The BACs we used contain large regions upstream and downstream from the reporter genes, resulting in the presence of the full endogenous promoter and most other intronic regulatory elements. These aspects help to better represent the endogenous molecular environment of the cell and to normalize the sensitivity of the receptor, compared to TAs which employ overexpressing constructs and multiple response elements to enhance sensitivity. Since the MCF7 cell line also endogenously expresses multiple other NHRs, crosstalk with these other NHRs cannot be excluded and this can reduce specificity. This may explain the GFP response to the non-estrogenic compounds corticosterone and ketoconazole, mainly by the GREB1-GFP reporter. However, when connecting the transcriptional response to cell cycle progression and proliferation, these compounds classified as non-estrogenic chemicals. Since pathway activation does not necessarily result in an adverse outcome, monitoring multiple subsequent key events in ER $\alpha$  pathway activation has added value. Solely measuring a critical endpoint such as proliferation is a quick and solid approach but lacks specificity for endocrine modulation. By combining data on the expression of the three different ER $\alpha$  target genes with data on cell cycle progression and proliferation, this reporter panel provides substantial mechanistic insight with a sensitivity comparable to other validated assays, like the ER $\alpha$  CALUX and VM7 Luc TA assay.

Just recently, the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) has released the ICH S1B(R1) addendum (ICH, 2021), describing an additional approach for assessing carcinogenic risk of small molecule pharmaceuticals, where emphasis is given to the human relevance of the

potential carcinogenicity. In this weight-of-evidence approach, primary pharmacologic mechanisms of the compounds and related receptor biology are key contributors. Non-genotoxic pro-proliferative ER $\alpha$  signaling is recognized as one of the mechanisms of carcinogenicity which represents a major risk in human tumor development. Therefore, the *in vitro* assay described in this study is expected to provide essential information for this weight-of-evidence approach, by identifying PODs of key events in pro-proliferative ER $\alpha$  pathway activation. Considering the need for novel high-throughput screening platforms, we believe that our technology represents a valuable asset in the field. In addition, this reporter platform can provide spatial and temporal pathway activation dynamics on a single cell level, which can be incorporated in a quantitative AOP modelling framework, improving carcinogen risk assessment with a more mechanistic approach.

## 4. Materials and methods

### 4.1. Cell culture

MCF7 wildtype (WT), MCF7 GREB1-GFP, MCF7 PGR-GFP, MCF7 TFF1-GFP and MCF7 Fucci-H2B cells (Duijndam et al., 2021), were routinely maintained in RPMI-1640 medium modified with L-glutamine, HEPES and phenol red (#22400089, Gibco, ThermoFisher Scientific) and supplemented with 10% fetal bovine serum (FBS) (#10270106, Gibco, ThermoFisher Scientific), 25 U/ml penicillin and 25  $\mu$ g/ml streptomycin (#15070063, Gibco, ThermoFisher Scientific) (further referred to as “complete medium”) at 37 °C under 5% CO<sub>2</sub> atmosphere. Cells were plated in complete medium and after 16–24 h the medium was replaced by phenol red-free RPMI1640 medium modified with L-glutamine (#11835105, Gibco, ThermoFisher Scientific) and supplemented with 5% charcoal/dextran-treated fetal bovine serum (cdFBS) (#SH30068.03, HyClone, GE Healthcare), further referred to as “experimental medium”.

### 4.2. Chemicals

All reference chemicals were kindly provided by BioDetection Systems b.v. (Table 1) as 100 mM stock solutions in DMSO (3 mM for ketoconazole and reserpine due to solubility). Serial dilutions in DMSO (VWR International) were freshly prepared per independent run and further diluted in experimental medium with a maximum concentration of 0.1% (v/v) DMSO.

### 4.3. Screening workflow

Cells were cultured at least on before they were seeded in a Cell-Carrier black 384-well imaging plates (#6007550, PerkinElmer). The following day, the culture medium was replaced with experimental medium to remove estrogenic stimuli present in the culture medium. After 24 h, and 2 h prior to exposure, cells were loaded with 100 ng/ml Hoechst 33342 (ThermoFisher Scientific) to visualize the nuclei. Hoechst-containing medium was removed before exposure to avoid Hoechst phototoxicity. To evaluate potential cytotoxicity, the experimental medium also contained 100 nM propidium iodide (PI) (P4170, Merck). In the concentration-range finding (CRF) run, a broad concentration range (10-fold dilution steps) per test chemical was used to determine the GFP response and possible cytotoxicity and plates were imaged after 48 h exposure. A refined concentration-range (half log dilutions steps) for the definitive runs was determined based on the results of the CRF run (Supplementary Table 1). In the definitive runs, plates were imaged after 24, 48 and 72 h.

### 4.4. Live cell imaging and image analysis

Hoechst, GFP, RFP and iRFP levels were detected using a Nikon TiE2000 confocal laser microscope (lasers: 408 nm, 488 nm, 561 nm,

647 nm), equipped with an automated stage, perfect focus system and climate chamber (at 37 °C under 5% CO<sub>2</sub> atmosphere). Imaging was done with a Nikon Plan Apo 20 $\times$  magnification objective lens using NIS elements software (Nikon). Quantitative image analysis was done with CellProfiler version 2.1.1. with an in house developed pipeline as previously described (Duijndam et al., 2021; Wink et al., 2017).

### 4.5. Sulforhodamine B (SRB) colometric proliferation assay

To compare the nuclei count imaging readout of the definitive runs to a well-established method, MCF7 WT cells were seeded in a 96-well plate (#3599, Corning). The following day, the culture medium was replaced with experimental medium to remove estrogenic stimuli present in the culture medium. After 24 h, cells were exposed to the test chemicals in experimental medium. After 72 h exposure, the cells were fixed and the sulforhodamine B (SRB) colometric assay was performed as previously described by our group (Zhang et al., 2011).

### 4.6. Data analysis

Raw integrated GFP intensity values were min-max normalized per plate-cell line combination as previously described (Wink et al., 2017). The response at the lowest and highest tested concentration of the reference compound E2 after 72 h were used for the minimum and maximum values, respectively. Graphpad Prism 8 software was used for concentration response curve fitting (nonlinear regression with variable Hill slope) to determine EC<sub>50</sub> and E<sub>max</sub> values. This software was also used for linear regression between assays to perform correlation analysis. Benchmark dose/concentration modelling was done using EPA BMDS Models (parametric) in the BMDExpress2 software (Phillips et al., 2019) with the following settings: max 250 iterations, confidence level of 0.95, standard deviation (SD) as BMR type and 1 SD as BMR factor. The best model was selected based on the lowest AIC. Hierarchical clustering was done by calculating the mean euclidean-based distance from all parameters using R functions “dist” and “hclust” and subsequently plotted as heatmap using the R package “pheatmap”.

### 4.7. Transcriptomics

Cells were seeded in a 96-well plate (#3599, Corning). The following day, the culture medium was replaced with experimental medium to remove estrogenic stimuli present in the culture medium. After 24 h, cells were exposed to E2 in experimental medium. After 24, 48 and 72 h exposure, the cells were washed once with PBS (D8537, Sigma) and lysed using TempO-Seq lysis buffer (BioClavis) for 15 min at room temperature. Samples were stored at –80 °C until shipment for TempO-Seq analysis at BioClavis (Yeakley et al., 2017). TempO-Seq analysis was performed using the Human Whole Transcriptome v2.0 panel with standard attenuators. Expression data were returned by BioClavis as counts per probe, and subsequently CPM (counts per million) normalized with an in-house R script.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The views expressed in this article are personal views of the authors and may not be understood or quoted as being made on behalf of, or reflecting the position of the Medicines Evaluations Board or one of its committees.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tiv.2022.105348>.

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