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Considerations when assessing antagonism *in vitro*: Why standardizing the agonist concentration matters

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Abstract

There is increasing recognition of the importance of assessing both agonism and antagonism in parallel for environmental samples. Cell-based *in vitro* assays have the advantage over receptor binding assay that they are able to differentiate between agonist and antagonist activity, but at present there is no standardized approach to assess antagonism *in vitro*, and in particular the competing agonist concentration can vary in the literature anywhere from half maximal to maximal effect concentrations. In this study, we investigated the influence of changing agonist concentrations in the estrogen receptor alpha (ERα), progesterone receptor (PR) and glucocorticoid receptor (GR) assays run in antagonist mode. The antagonistic effect varied by over two orders of magnitude when using the range of agonist concentrations applied in the literature, clearly indicating the need for standardization. By comparing antagonist EC_{50} values with background agonist concentrations, an EC_{80} background agonist concentration is recommended when assessing antagonism *in vitro* to optimise both assay sensitivity and reproducibility.

Keywords: Antagonist, estrogen receptor, glucocorticoid receptor, *in vitro*, progesterone receptor

1. Introduction

Environmental waters, such as wastewater and surface water, can contain countless chemical contaminants with different modes of toxic action. Test batteries of *in vitro* bioassays focusing on endpoints relevant for both human and environmental health are increasingly applied for water quality monitoring and to assess treatment efficiency (e.g. van der Linden et al., 2008; Escher et al., 2014). For a more accurate assessment of effect, it is important to evaluate agonism and antagonism in parallel for receptor mediated endpoints, such as estrogen and progesterone receptor assays. This is because the presence of antagonists in environmental samples may decrease the agonist response, as demonstrated recently by Ihara et al. (2014) for wastewater. Cell-based bioassays can detect antagonism when run in a so-called "antagonist mode", where a potent competing agonist is added at a constant concentration and the suppression of the agonist effect indicates antagonism (Soto et al., 2006).

At present, there is no standard protocol to measure antagonism, and in particular the agonist concentration added can range from the concentration causing 50% effect (EC_{50}) to the maximal effect (EC_{100}) (e.g. van der Linden et al., 2008; Ihara et al., 2014). Further, the applied concentration is often not reported. However, competitive antagonists will compete with agonists for the receptor sites; hence the observed effects are likely to change depending on the concentration of agonists and antagonists in the assay.

In this short communication we investigated the implications of changing agonist concentration in estrogen receptor alpha (ERα), progesterone receptor (PR) and glucocorticoid receptor (GR) assays and suggest a standardized approach for assessing antagonism using *in vitro* reporter gene assays.

2. Materials and Methods

The study was conducted using GeneBLAzer® ERα-UAS-bla, PR-UAS-bla and GR-UAS-bla assays (Life Technologies, Mulgrave, Australia). All assays are based on the HEK 293T cell line. The cells were grown in DMEM with GlutaMAX™ with 10% dialysed fetal bovine serum (FBS), while phenol red-free DMEM with 2% charcoal stripped FBS was used for the assay media. 17β-Estradiol and tamoxifen were used as the agonist and antagonist in the ERα assay, respectively, while levonorgestrel and RU486 were used as the agonist and antagonist in the PR assay, respectively. RU486 was also the antagonist in the GR assay, with dexamethasone as the agonist (Leusch et al., 2014). The chemical stocks were prepared in methanol (HPLC grade, Fisher Scientific, Scoresby, Australia) and the maximum solvent concentration in the assay was 0.2%. Standard curves with 1:4 serial dilutions for each reference compound were prepared in phenol red-

free DMEM media in separate 96 well plates, with the agonists serially diluted across the plate and the antagonists serially diluted down the plate. Fifty microliters from both the agonist and antagonist plates for each assay were mixed together in a separate 96 well plate. The final concentration of the reference compounds was 2.4×10^{-14} to 2.5×10^{-8} (log -13.6 to -7.6) M for 17β-estradiol, 1.2×10^{-8} to 1.3×10^{-5} (log -7.9 to -4.9) M for tamoxifen, 9.5×10^{-14} to 1.0×10^{-7} (log -13.0 to -7.0) M for levonorgestrel, 2.5×10^{-13} to 2.6×10^{-7} (log -12.6 to -6.6) M for dexamethasone and 3.9×10^{-10} to 1.0×10^{-7} (log -9.4 to -7.0) M for RU486 (same concentration range in both PR and GR assays). The cells were seeded in black clear bottom 384 well plates with densities of 20,000 cells per well for ERα and GR and 15,000 cells per well for PR. Eight microliters of sample was added to the cells in duplicate and incubated overnight for 16 h at 37 °C in a 5% CO2 incubator. Standard curves of the agonist and antagonist (with EC_{80} agonist constant concentration) were included on each plate for assay validation, along with a methanol standard curve with a maximum final solvent concentration of 0.2%, which was added to cells to ensure that the solvent itself was not having an effect. The next day 8 µL of LiveBLAzer™-FRET B/G substrate mixture was added to each well and the plate was incubated at room temperature for 2 h. Fluorescence at 460 and 520 nm was measured using a Fluostar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The results were expressed as % maximum response relative to the reference agonist compound.

3. Results and Discussion

All studied assays demonstrated that changing the concentration of either the antagonist (in classical agonist mode) or the competing agonist (in antagonist mode) altered the dose-response curves.

3.1. Impact of the presence of an antagonist on a classical "agonist mode" assay

As the antagonist concentration increased the agonist curves shifted to the right, increasing the agonist EC_{50} value and consequently decreasing effect (Figure 1). Within the studied antagonist range, the agonist EC_{50} values increased by over two orders of magnitude for the ERα and PR assays and over one order of magnitude for the GR assay. A reduction in agonistic activity in the presence of antagonists has also been previously reported by Ihara et al. (2014) in both human and medaka ERα assays with 17β-estradiol and 4-hydroxy-tamoxifen. Further, Barkhem et al. (1998) also observed a concentration-dependent shift in 17β-estradiol binding to a human ERα and ERβ reporter assays in the presence of antagonists ICI 164 384 and raloxifene. The observed result was not unexpected as tamoxifen and RU486 are both competitive antagonists. While the percent maximum response dropped to zero in the PR and GR assays, up to 20% effect was observed with increasing tamoxifen concentrations in the ERα assay. This is not surprising: as well as being an antagonist, tamoxifen is known to be a weak ERα agonist *in vitro* (Gutendorf and Westendorf,

2001). While tamoxifen is a widely used anti-estrogen in *in vitro* assays, it may not be the most suitable reference antagonist and other alternatives, such as fulvestrant (ICI 182, 780) (Wilson et al., 2004), may be more appropriate.

3.2. Impact of the concentration of agonist on an assay run in "antagonist mode"

When operated in antagonist mode, different competing agonist concentrations resulted in a shift in the reported antagonistic effect (Figure 2). As previously stated, competing agonist concentrations range from EC_{50} to EC_{100} in the literature, but this can translate into over a 100 fold difference in antagonist EC_{50} value in the same assay. For example, the RU486 EC_{50} values decreased by approximately two orders of magnitude in the PR assay (log -6.9 to -8.9 M) and the GR assay (-log 6.9 to -8.8 M) with decreasing constant agonist concentrations. In the ERα assay the EC_{50} value for tamoxifen ranged from log -6.4 to -4.9 M with agonist concentrations from EC_{50} to EC_{100}. This will obviously hamper comparisons of potency between laboratories that utilise different competing agonist concentrations. In all assays, the antagonists only caused minimal reduction of response at the highest agonist concentration tested, which cautions against the use of excessive agonist background concentrations.

The results clearly show that selecting a lower agonist concentration (*e.g.*, EC_{50}) increases the sensitive of the assay to antagonist compounds. This is an advantage for assessing environmental samples, where compounds may only be present at trace concentrations. However, the downside is that the EC_{50} is the area of the concentration-effect curve that is most influenced by a minimal change in agonist concentration. This is demonstrated in Figure 3, where the rate of change of the response (Δy) as a function of concentration (Δx) is greatest at the EC_{50} concentration. In practical terms, this means that small errors in dosing will have a 1.6 times stronger effect on the variability of the results at EC_{50} compared to EC_{80} (based on the rate of change at the two agonist concentrations, Figure 3).

To find the optimal agonist concentration for assessing antagonism in reporter gene assays, a balance has to be found between assay sensitivity and reproducibility. The antagonist EC_{50} values were compared with the background agonist concentration for each assay, and plotted alongside $\Delta y/\Delta x$ (Figure 3). In all assays, the antagonist EC_{50} value decreased with decreasing agonist concentration; however, there was little difference in sensitivity at the EC_{50} and EC_{80} agonist concentrations. Given that the EC_{80} concentration is less susceptible to variability compared to EC_{50}, we recommend the use of EC_{80} background agonist concentration when assessing antagonism *in vitro*.

A standardization of the competing agonist concentration to EC_{80} in antagonist mode will allow better comparison between laboratories and hopefully reduce the variability in EC_{50} values reported for antagonists in the literature.

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Figure Captions

Figure 1: Changes in agonist standard curve in the presence of different antagonist concentrations for A) ERα assay with 17β-estradiol (E2) and tamoxifen (TMX), B) PR assay with levonorgestrel (LVG) and RU486 and C) GR assay with dexamethasone (DXM) and RU486 (all concentrations presented as log units).

Figure 2: Changes in antagonist standard curve in the presence of different agonist concentrations for A) ERα assay with tamoxifen (TMX) and 17β-estradiol (E2), B) PR assay with RU486 and levonorgestrel (LVG), and C) GR assay with RU486 and dexamethasone (DXM) (all concentrations presented as log units).

Figure 3: Changing antagonist EC_{50} values at different agonist concentrations (left axis, symbols) and derivation ($\Delta y/\Delta x$, blue line) of the agonist dose-response curve (right axis) for A) ERα assay with tamoxifen (TMX) and 17β-estradiol (E2), B) PR assay with RU486 and levonorgestrel (LVG), and C) GR assay with RU486 and dexamethasone (DXM). The agonist EC_{50}, EC_{80} and EC_{99} concentrations are shown as dashed lines (all concentrations presented as log units).

Figure 1

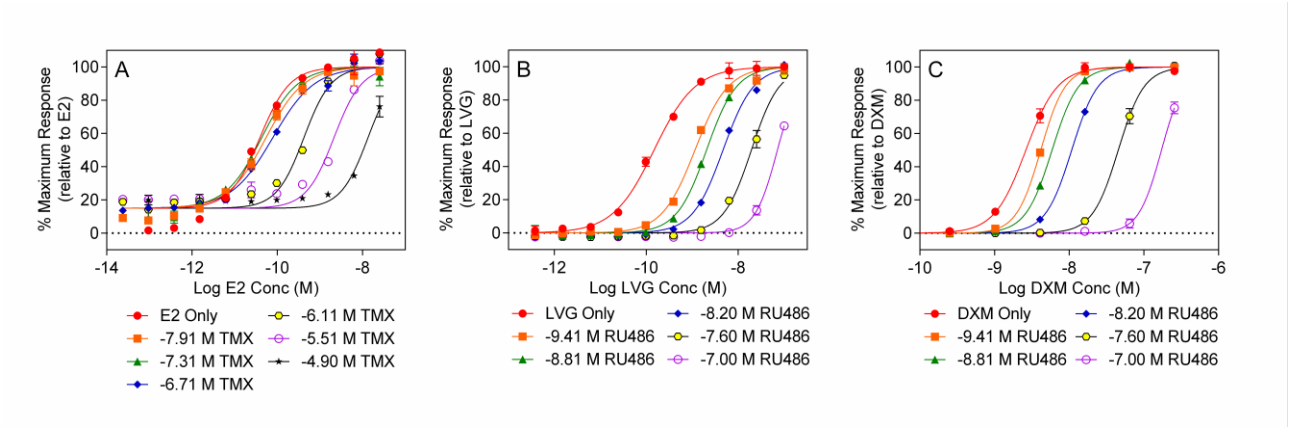

A
% Maximum Response (relative to E2)
Log E2 Conc (M)
E2 Only
-7.91 M TMX
-7.31 M TMX
-6.71 M TMX
-6.11 M TMX
-5.51 M TMX
-4.90 M TMX
B
% Maximum Response (relative to LVG)
Log LVG Conc (M)
LVG Only
-9.41 M RU486
-8.81 M RU486
-8.20 M RU486
-7.60 M RU486
-7.00 M RU486
C
% Maximum Response (relative to DXM)
Log DXM Conc (M)
DXM Only
-9.41 M RU486
-8.81 M RU486
-8.20 M RU486
-7.60 M RU486
-7.00 M RU486

Figure 2

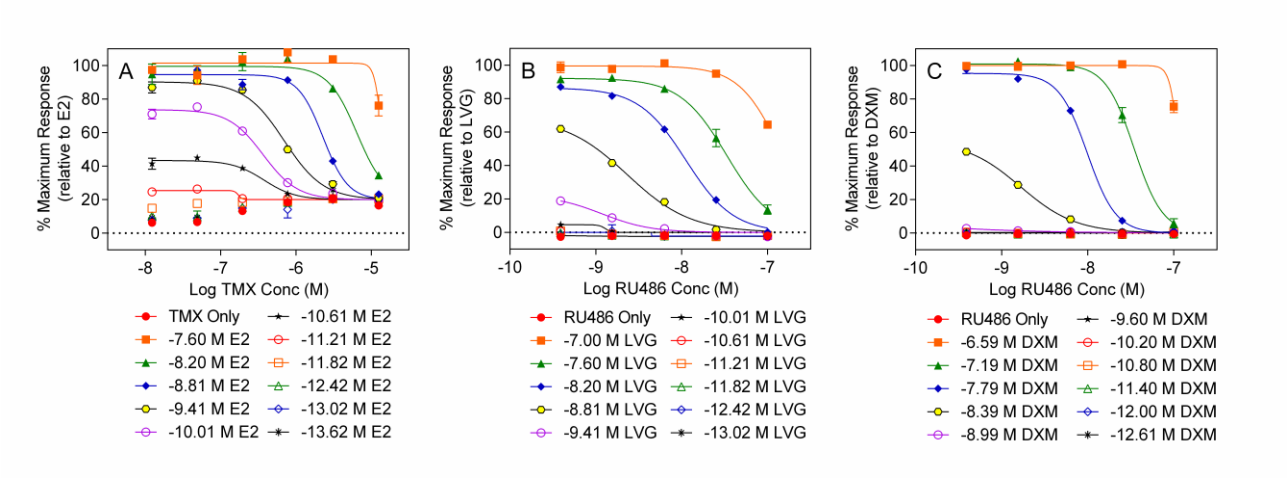
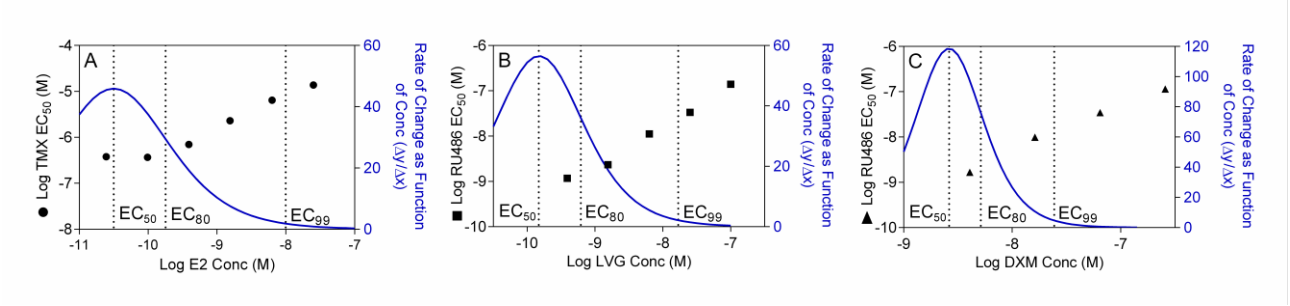

A
% Maximum Response (relative to E2)
Log TMX Conc (M)
TMX Only
-7.60 M E2
-8.20 M E2
-8.81 M E2
-9.41 M E2
-10.01 M E2
-10.61 M E2
-11.21 M E2
-11.82 M E2
-12.42 M E2
-13.02 M E2
-13.62 M E2
B
% Maximum Response (relative to LVG)
Log RU486 Conc (M)
RU486 Only
-7.00 M LVG
-7.60 M LVG
-8.20 M LVG
-8.81 M LVG
-9.41 M LVG
-10.01 M LVG
-10.61 M LVG
-11.21 M LVG
-11.82 M LVG
-12.42 M LVG
-13.02 M LVG
C
% Maximum Response (relative to DXM)
Log RU486 Conc (M)
RU486 Only
-6.59 M DXM
-7.19 M DXM
-7.79 M DXM
-8.39 M DXM
-8.99 M DXM
-9.60 M DXM
-10.20 M DXM
-10.80 M DXM
-11.40 M DXM
-12.00 M DXM
-12.61 M DXM

Figure 3


A
Log TMX EC50 (M)
Rate of Change as Function of Conc (Δy/Δx)
EC50
EC80
EC99
Log E2 Conc (M)
B
Log RU486 EC50 (M)
Log LVG Conc (M)
C
Log DXM Conc (M)