

Correlation of functional and radioligand binding characteristics of GPER ligands confirming aldosterone as a GPER agonist

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Abstract

Aldosterone exerts some of its effects not by binding to mineralocorticoid receptors, but rather by acting via G protein-coupled estrogen receptors (GPER). To determine if aldosterone binds directly to GPER, we studied the ability of aldosterone to compete for the binding of [³H] 2-methoxyestradiol ([³H] 2-ME), a high potency GPER-selective agonist. We used GPER gene transfer to engineer Sf9-cultured insect cells to express GPER. We chose insect cells to avoid interactions with any intrinsic mammalian receptors for aldosterone. [³H] 2-ME binding was saturable and reversible to a high-affinity population of receptors with $K_d = 3.7$ nM and $B_{max} = 2.2$ pmol/mg. Consistent with agonist binding to G Protein-coupled receptors, [³H] 2-ME high-affinity state binding was reduced in the presence of the hydrolysis-resistant GTP analog, GppNHp. [³H] 2-ME binding was competed for by the GPER agonist G1, the GPER antagonist G15, estradiol (E2), as well as aldosterone (Aldo). The order of potency for competing for [³H] 2-ME binding, namely $2ME > Aldo > E2 \geq G1$, paralleled the orders of potency for inhibition of cell proliferation and inhibition of ERK phosphorylation by ligands acting at GPER. These data confirm the ability of aldosterone to interact with the GPER, consistent with the interpretation that aldosterone likely mediates its GPER-dependent effects by direct binding to the GPER.

Significance statement: Despite the growing evidence for aldosterone's actions via G protein-coupled estrogen receptors (GPER), there remains significant skepticism that aldosterone can directly interact with GPER. The current studies are the first to

Abbreviations: 2-ME, 2-Methoxyestradiol <https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=12058>; Aldo, Aldosterone [aldosterone](#) | [Ligand page](#) | [IUPHAR/BPS Guide to PHARMACOLOGY](#); B_{max} , Total receptor density; E2, Estradiol [17β-estradiol](#) | [Ligand page](#) | [IUPHAR/BPS Guide to PHARMACOLOGY](#); EdU, 5-ethynyl-2'-deoxyuridine; ERK, Extracellular signal-regulated kinase [ERK subfamily](#) | [Enzymes](#) | [IUPHAR/BPS Guide to PHARMACOLOGY](#); G1, Selective GPER agonist. 1-94-(6-bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-[3H]-cyclopenta[c]quinolin-8-yl)-ethanone, 1-[[[3aS,4R,9bR-rel]-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-[3H]-cyclopenta[c]quinolin-8-yl)-ethanone [G-1](#) | [Ligand page](#) | [IUPHAR/BPS Guide to PHARMACOLOGY](#); G15, GPER-selective agonist. (3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone [G15](#) | [Ligand page](#) | [IUPHAR/BPS Guide to PHARMACOLOGY](#); GPER, G protein-coupled estrogen receptors [G protein-coupled estrogen receptor](#) | [G protein-coupled receptors](#) | [IUPHAR/BPS Guide to PHARMACOLOGY](#); K_d , Dissociation constant; MR, Mineralocorticoid receptor [Mineralocorticoid receptor](#) | [3C](#). 3-Ketosteroid [receptors](#) | [IUPHAR/BPS Guide to PHARMACOLOGY](#); Sf9, Spodoptera frugiperda cells.

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demonstrate directly that aldosterone indeed is capable of binding to the GPER and thus likely mediates its GPER-dependent effects by direct binding to the receptor.

KEYWORDS

aldosterone, GPCR, GPER, mineralocorticoid

1 | INTRODUCTION

For many years, the action of the mineralocorticoid hormone **aldosterone** was thought to be adequately explained by activation of the classic **mineralocorticoid receptor** (MR), a member of the steroid receptor superfamily. Further, the actions of aldosterone were thought to be primarily due to transcriptional activation.¹⁻³ However, some actions of aldosterone, including a range of vascular effects, are too rapid to be mediated by the MR.⁴⁻⁶ The receptor mechanism for these effects remained obscure.

We previously reported that at least some of the rapid effects of aldosterone are not mediated by mineralocorticoid receptors, but instead are mediated via **G protein-coupled estrogen receptors** (GPER). These studies were performed in both rat aortic vascular smooth muscle cells and rat vascular endothelial cells.⁷⁻⁹ Subsequently, other laboratories confirmed these findings using a range of in vivo and in vitro models.¹⁰⁻¹⁶

Despite the growing evidence for aldosterone's actions via GPER, there remains significant skepticism that aldosterone directly interacts with GPER. This skepticism has persisted, even though GPER activation by aldosterone has been reported in models devoid of detectable MR.⁹ Alternate mechanisms for the effects of aldosterone to activate GPER-dependent pathways have been suggested, including transactivation of GPER by MR activation.¹⁷ Reports from two laboratories using radioligand techniques to assess aldosterone binding to GPER using [³H] aldosterone or aldosterone competition for [³H] **estradiol** did not find evidence for interaction with the sites identified by these radioligands.^{18,19} However, neither of these manuscripts demonstrated unambiguous evidence that the radioligand binding reported was to a physiologically relevant GPER, i.e., fulfilling the long-established criteria for the identification of GPCRs using radioligand binding techniques: (i) saturable, (ii) specific, with an order of potency for binding consistent with ligand potency for the receptor's functional effects, and (iii) reversible over a time course consistent with physiological reversibility.²⁰ Additionally, radiolabeled agonist binding to GPCR should be reduced by **GTP** (or an analog).^{21,22} Neither of the prior radioligand binding reports fully fulfilled these criteria.

Radioligand techniques for determining GPER binding are, at least conceptually, problematic. The radioligands available for GPER binding have a relatively low potency for the receptor and so have relatively rapid off-rates. This makes it nearly impossible to trap bound radioligand using vacuum filtration separation techniques.²³ The biological material used in radioligand binding assays also is problematic: GPER residence time in the plasma membrane is limited and GPER appears to have a more long-lived residence in the endoplasmic reticulum,²⁴⁻²⁷

so isolated plasma membrane preparations would underestimate the GPER capacity in a cell or tissue preparation. However, utilizing whole cell lysates to assess GPER binding is confounded by competing binding to classic steroid receptors and other lower affinity steroid binding sites. Further, GPER ligands are relatively lipophilic compared to most other GPCR ligands resulting in high proportions of non-receptor-related "non-specific" binding.

The present studies address whether or not aldosterone can bind to GPER using GPER-transduced Sf9 cultured insect cells as the cellular host. We chose to utilize an Sf9/baculovirus system transduced with GPER since its high transduction efficiency and rapid protein transcription rates allowed us to create cells with a large number of receptors for study in the absence of competing mammalian steroid-binding receptors.^{28,29} [³H] **2-methylestradiol** (2-ME) was utilized as the radioligand for identifying GPER, as this ligand manifests the highest potency of available radioligands for GPER binding—especially as compared to E2.^{30,31} The present data demonstrate that [³H] 2ME binding in GPER-transduced Sf9 cell lysates fulfills the criteria for binding to a physiological GPCR. Further, we demonstrate that previously characterized GPER agonist ligands, including **G1**, E2 as well as aldosterone, demonstrate high-affinity binding to this receptor.

2 | MATERIALS AND METHODS

2.1 | Sf9 cell culture

Spodoptera frugiperda cells (Sf9 cells- obtained from Life Technologies Inc. Burlington ON, Canada) were cultured in Grace's insect medium (Invitrogen, Mississauga, ON) supplemented with penicillin, streptomycin, and 10% fetal calf serum in a humidified atmosphere at 27°C. To express recombinant GPER for radioligand binding studies, Sf9 cells were grown to 70% confluency and 50 µl of recombinant GPER baculovirus was added into the medium and cultured for an additional 48h.

2.2 | Generation of GPER baculovirus

GPER baculovirus was generated using Bac-to-Bac™ Baculovirus Expression System (Invitrogen, Mississauga, ON) according to the protocol provided by the manufacturer. Briefly, GPER cDNA was cloned into the p FastBac-1 vector to construct a donor plasmid. The donor plasmid containing GPER insert was then transformed into DH10 Bac™ E coli to generate recombinant bacmid DNA, followed

by transfection of bacmid DNA into Sf9 cells for 96 h. The supernatant of transfected Sf9 cells (P0 virus) was collected. High titers of recombinant baculovirus were generated by infection of P0 virus of Sf9 cells for another 48 h. For research involving recombinant DNA, containment facilities and guidelines conformed to those of the National Institutes of Health.

2.3 | Assessment of GPER expression

GPER expression in Sf9 cells was assessed by western blot analysis and immunoblotting. Forty-eight hours following infection, Sf9 cells were collected in lysis buffer (20 mM Tris, pH 8.0, 1% NP-40, 0.1% SDS, 140 mM NaCl and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were then resolved by 10% SDS-PAGE and transferred electrophoretically onto Immun-Blot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked for 1 hour at room temperature in a blocking buffer (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.1% Tween 20, and 5% skim milk). The membranes were then incubated overnight with an anti-GPER antibody (ABCAM, Toronto, ON, 1:1000). Blots were washed in Tris-buffered saline (3 × 10 minutes) and then incubated in HRP-conjugated secondary anti-rabbit (Cat#, A0545, Sigma-Aldrich, St. Louis, MO, 1:5000 dilution). Immunoreactive proteins were detected by chemiluminescence, as described by the manufacturer's protocol (DuPont NEN, Boston, MA).

2.4 | Assessment of GPER agonist-mediated effects proliferation

2-ME-, G1-, E2-, or aldosterone-mediated effects on proliferation were assessed by measuring the rate of incorporation of 5-ethynyl-2'-deoxyuridine (EdU).⁹ Briefly, after 24 h of serum starvation in serum-free Grace's insect medium, wild-type Sf9 cells or cells infected with GPER baculovirus were treated with the GPER agonist 2-ME (10 pM–10 nM) or G1 (0.001–1 μM), E2 (0.1–100 nM), aldosterone (0.01–10 nM), or vehicle (control) for 2 h, after which cells were restimulated with 10% fetal bovine serum for 18 hours and then incubated with EdU, (10 μmol/L) for 2 h. EdU incorporation was assessed using Click-iT EdU flow cytometry assay kit (Lifetech, Carlsbad, CA).

2.5 | Assessment of GPER agonist-mediated effects on ERK phosphorylation

Wild-type Sf9 cells or cells infected with GPER baculovirus were serum-starved in Grace's insect medium for 24 h and then incubated with increasing concentrations of 2-ME (1 pM–1 nM), G1 (0.001–1 μM) or E2 (0.1–100 nM) or aldosterone (0.01–10 nM) for 15 min at room temperature. After being washed twice with PBS, cells were resuspended in lysis buffer. The effects of G1, E2, or aldosterone on phospho-ERK and total ERK were assessed by immunoblotting

using antiphospho ERK (cat# 9101, Cell Signaling, Danvers, MA) and anti-total ERK (cat# 9102, Cell Signaling, Danvers, MA) respectively. Bands corresponding to phospho ERK or total ERK were quantified by densitometry. The expression of phospho ERK was normalized to the total ERK expression. To assess the effects of GPER antagonism on the agonist-mediated inhibition of ERK phosphorylation, G15 (1 μM) was added to cells 30 min prior to the addition of agonists.

2.6 | Sf9 lysate preparation

Cells were harvested and resuspended in 1 ml ice-cold 50 mM Tris-HCl, pH 7.4, and then kept on ice for 15 min, followed by homogenization by a Polytron homogenizer. Particulate fractions used as the biological preparations in radioligand binding studies were prepared by centrifugation at 1000 × g for 10 min, followed by isolation of the supernatant and subsequent centrifugation of this particulate fraction at 20000 × g for 20 min in binding buffer (see below), followed by isolation of the supernatant and a final dilution in the binding buffer to a protein concentration of 4–12 μg/ml.

2.7 | GPER radioligand binding assays

Binding studies were performed with [³H] 2-methoxyestradiol, [³H] 2-ME, (60 Ci/mmol, 1 mCi/ml, 99% purity) from American Radiolabeled Chemicals Inc (<http://www.arc-inc.com>), St Louis, MO., according to modifications of the methods of Karamyan et al.^{30,32} Briefly, [³H] 2-ME (2 nM) dissolved in 50 μl binding buffer (50 mM Tris-HCl, pH 7.4, 120 nM NaCl, 4 mM KCl, 1 nM CaCl₂, 10 μg bacitracin, 0.25% BSA and 2 mg/ml dextrose) was added to binding buffer (450 μl) containing 10–30 μg of the particulate preparation and unlabeled 2-ME (3 nM–30 μM) or aldosterone (0.3 nM–10 μM) or E2 (30 nM–100 μM) or G1 (30 nM–100 μM) or G15 (30 nM–100 μM) or hydrocortisone (30 nM–100 μM) to final volume of 500 μl. Following an incubation of 120 min at 15°C in a shaking water bath, 5 ml of ice-cold binding buffer was added to dilute and chill the incubation, terminating the binding reaction. The reaction mixture was then rapidly passed through a cellulosic nitrate membrane filter (Whatman, Buckinghamshire, UK) presoaked in binding buffer mounted on a Millipore vacuum filtration unit (Millipore, Etobicoke, ON). After 2 × 5 ml rapid washes with ice-cold binding buffer, the individual filters were transferred into scintillation counting vials and 10 ml scintillation liquid was added (CytoScint, Solon, OH). Counts per minute (cpm) were determined with a Beckman scintillation spectrometer.

2.8 | Defining non-specific binding in the GPER radioligand binding assays

Analyses of competition binding studies fitted total binding, with nonspecific binding defined by the bottom plateau of the curve. In those studies, where specific (total-nonspecific) binding was

assessed (saturation binding assays, dissociation studies, guanine nucleotide sensitivity studies) nonspecific binding was determined as binding seen in the presence of 100 nM cold 2-ME, added 12 min prior to the addition of [³H] 2-ME.

Saturation binding studies were performed by incubation with increasing concentrations of [³H] 2-ME (0.5–10 nM) with and without 100 nM unlabeled 2-ME.

2.9 | Assessing the dissociation rate

Incubation of particulate fractions with 2 nM [³H] 2-ME radioligand was allowed to occur at 15°C for 120 min. Association was terminated by the addition of 5 ml of ice-cold binding buffer to immediately slow the reaction by reducing the temperature and diluting the radioligand concentration tenfold, effectively terminating detectable continuing radioligand association. The rate of dissociation was then examined at varying time points from 5 to 120 s.

2.10 | Determining the guanine nucleotide sensitivity of [³H] 2-ME binding to high-affinity binding sites

The hydrolysis-resistant GTP analog GppNHp (100 μM, Sigma-Aldrich, St Louis, MO) was added to reaction mixtures containing [³H] 2-ME (0.5–2 nM). In these studies, a Mg⁺⁺ concentration of 5 mM was utilized to promote the high-affinity state of the receptor for agonists.³³

2.11 | Statistical analyses

For multiple group comparisons, initial analysis by one-way ANOVA was followed by Dunnett's multiple comparison tests. Data are expressed as mean ± SD or mean with a 95% confidence interval. Nonlinear regression was done with GraphPad Prism (version 9.4). For the concentration-response curves of Figures 2, 4, and 6, a single curve was fit to the pooled normalized results of multiple experiments. The top of the curve was fixed to 100 (because the data are normalized to control binding in each experiment), and the slope factor was fixed to the standard value of -1.0 (because there are too few concentrations to define the slope). Nonlinear regression fit the bottom plateau (nonspecific binding) and the EC₅₀, which we report along with its 95% confidence interval computed by the profile likelihood method (which reports asymmetrical CIs).

2.12 | Reagents

The GPER-selective agonist G1 (1-94-(6-bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-[³H]-cyclopenta[c]quinolin-8-yl)-ethanone,1-[(3aS,4R,9bR-rel)-4-(6-bromo-1,3-benzodioxol-5-yl)-

3a,4,5,9b-tetrahydro-[³H]-cyclopenta[c]quinolin-8-yl)-ethanone was purchased from Calbiochem-Novabiochem (San Diego, CA). The GPER antagonist, G15 (3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-(cyclopenta[c]quinolone) was obtained from TOCRIS (Bristol, UK). G1 and G15 were dissolved in DMSO with a working dilution in assays of 1:1000 DMSO: H₂O. DMSO at a 1:1000 dilution was also included in all control conditions tested. All other chemical reagents were purchased from Sigma-Aldrich.

2.13 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY³⁴ and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.³⁵

3 | RESULTS

3.1 | GPER expression is readily detected in baculoviral GPER-transduced Sf9 cells but is not detected in wild-type Sf9 cells

As shown in Figure 1, an approximately 55 kDa protein was identified by immunoblotting using an anti-GPER antibody in GPER-transduced Sf9 cells (Figure 1), consistent with the molecular size of GPER as detected in mammalian cells.³² No such band was seen in wild-type Sf9 cells.

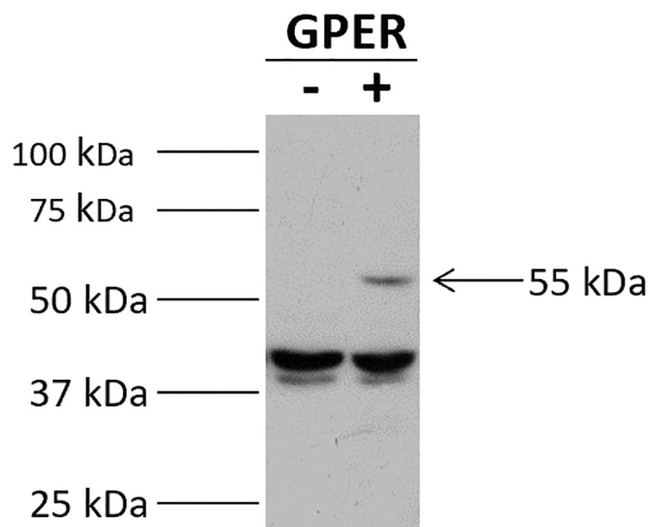


FIGURE 1 Representative immunoblot of GPER expression in baculoviral GPER-transduced Sf9 cells vs in wild-type Sf9 cells. A protein was expressed in GPER-transduced Sf9 cells (lane marked with +) migrating at 55 kDa on SDS-PAGE, not expressed in non-transduced (WT) cells (lane marked with -), consistent with migration of GPER.

3.2 | GPER-mediated inhibition of proliferation and ERK phosphorylation in GPER-transduced Sf9 cells shows an order of potency of 2-ME > Aldosterone > E2, G1 fully blocked by the GPER antagonist G15

Prior to rigorously determining the binding of GPER ligands in GPER-transduced Sf9 cells, we first examined whether there was any functional response detectable upon GPER expression in Sf9 cells not

observed in wild-type Sf9 cells. We assessed the effects of GPER ligands on cellular proliferation and ERK phosphorylation- as a read-out of an earlier step in the signaling pathway linking GPER activation and regulation of cell growth. Proliferation was examined using EdU incorporation (a measure of DNA synthesis). Aldosterone, 2-ME, E2, and G1 all inhibited EdU incorporation (22–30%) with an order of potency of 2-ME > Aldosterone > E2 > G1 (Figure 2). Preincubation with the GPER antagonist G15 led to negligible effects of all of the drugs (G1, 2-ME, aldosterone, and E2) on EdU incorporation (Figure 3).

FIGURE 2 GPER agonists inhibit the proliferation of GPER-transduced Sf9 cells. GPER transduced Sf9 cells were incubated with increasing concentrations of either 2-ME (A), aldosterone (B), E2 (C), G1 (D), DMSO (vehicle) as control. Proliferation was assessed as EdU incorporation normalized to a control in that experiment (which defines 100%). Shown are the mean (and SD) of the normalized proliferation from three experiments with a dose-response curve fit by nonlinear regression constraining the top plateau to 100% and the Hill Slope to -1. The EC_{50} s are tabulated in Table 1.

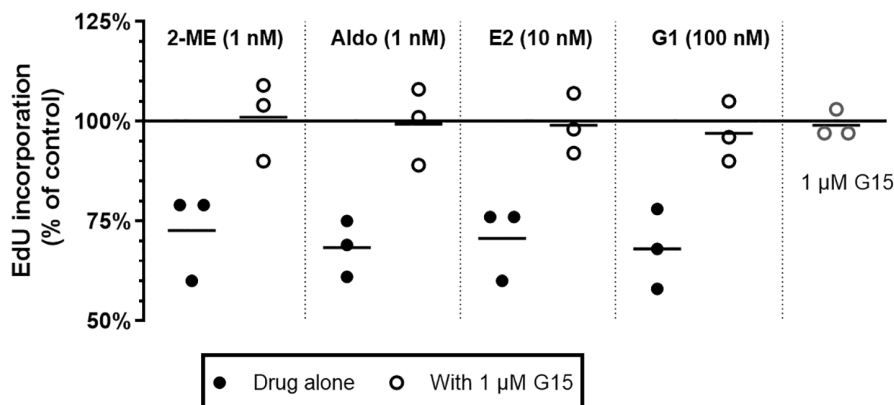
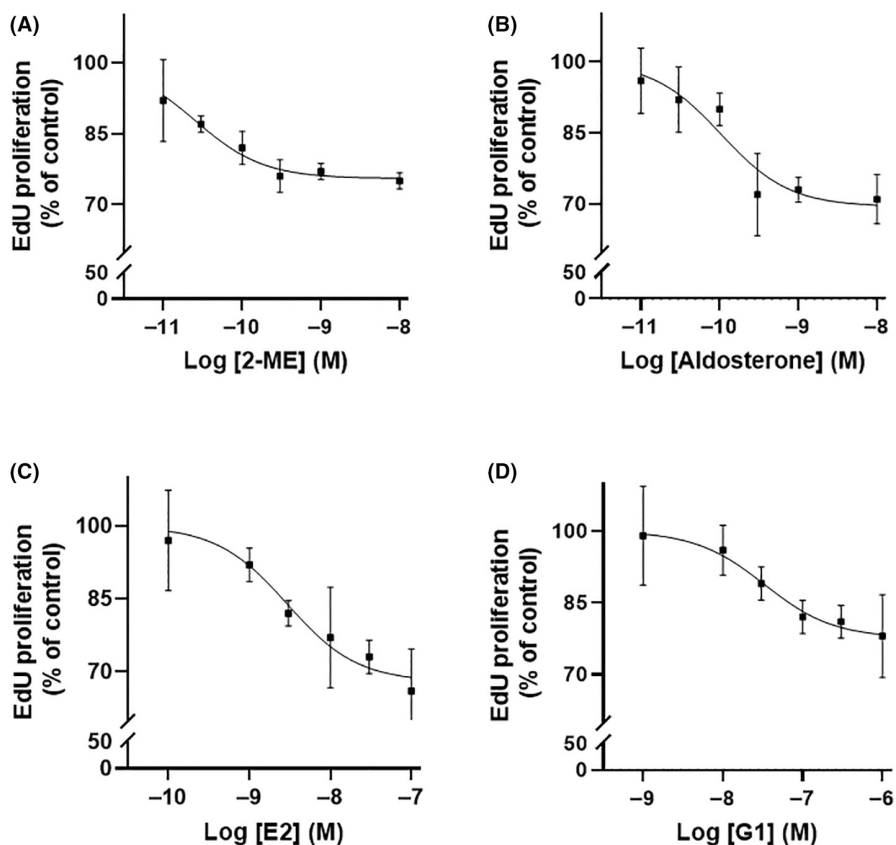


FIGURE 3 The GPER antagonist, G15, attenuates inhibition of proliferation by aldosterone as well as known estrogen agonists. GPER transduced Sf9 cells were preincubated with G15 (1 μM, open circles) or vehicle (closed circles) for 30 min, followed by treatment with 2-ME (1 nM), aldosterone (1 nM), E2 (10 nM), or G1 (100 nM) for 15 min. Cell proliferation was determined by assessing EdU incorporation (a measure of DNA replication). Shown are individual data (with mean) from three separate experiments.

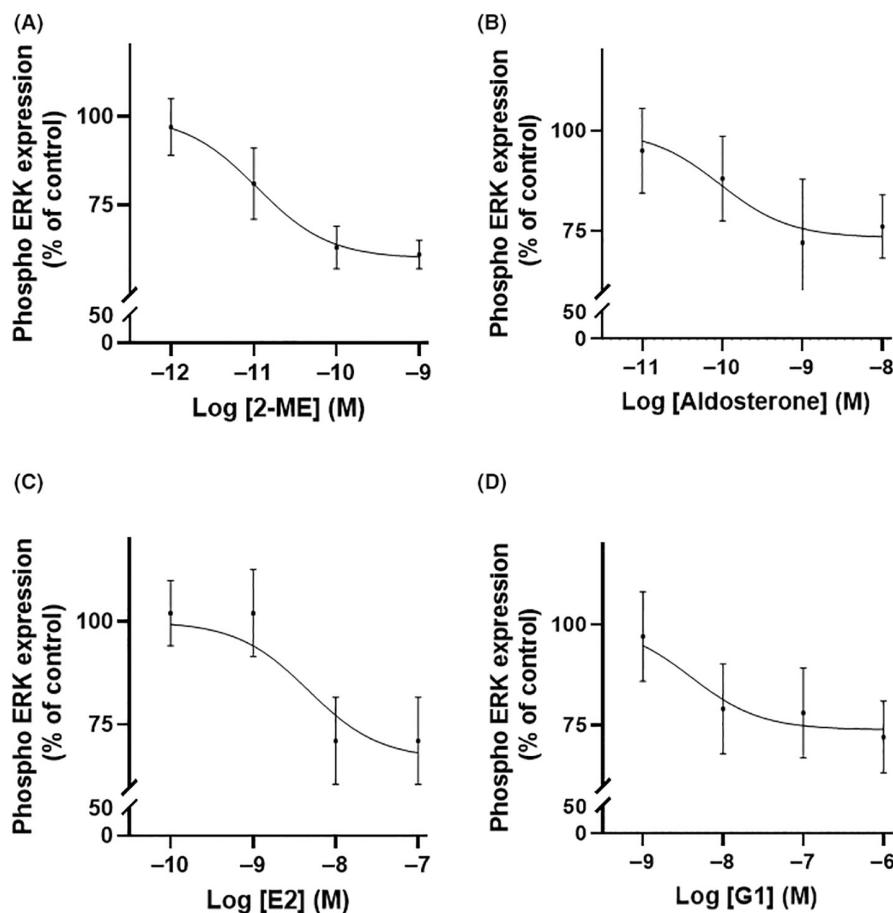


FIGURE 4 GPER agonists inhibit ERK phosphorylation. GPER transduced Sf9 cells were incubated with increasing concentrations of either 2-ME (A) or aldosterone (B) or E2 (C) or G1 (D) or DMSO (vehicle) as control. The effects on ERK phosphorylation were determined by immunoblotting. Shown are the mean (and SD) of 3–5 experiments with a dose-response curve fit by nonlinear regression constraining the top plateau to 100% and the Hill Slope to -1 . The EC_{50} s are tabulated in [Table 1](#).

There were no detectable effects of any of these agents on proliferation in the wild-type Sf9 cells used as control (data not shown).

ERK activation is an upstream marker of receptor activation. In GPER-infected Sf9 cells, the GPER agonists 2-ME, aldosterone, E2, and G1 all mediated a concentration-dependent inhibition of ERK phosphorylation with an order of potency of 2-ME > aldosterone > E2 \approx G1 ([Figure 4](#)), similar to the order of potency of these agents in suppressing serum-mediated proliferation ([Figure 2](#)). The inhibition of ERK phosphorylation by 2ME (1 nM), aldosterone (1 nM), E2(10nM), or G1(100nM)-mediated were all blocked by pre-incubation with the GPER antagonist G15 (1 μ M) ([Figure 5](#)), just as G15 had blocked GPER agonist-induced proliferation ([Figure 3](#)). In wild-type Sf9 cells (non-transfected) aldosterone, 2-ME, G1 or E2 did not inhibit ERK phosphorylation (data not shown).

3.3 | GPER ligands competed for [3 H] 2-ME binding with an order of potency that mimics their effects on suppressing proliferation and ERK phosphorylation

To determine the GPER-specificity of binding of radiolabeled 2-ME, we assessed the competition of the [3 H] 2-ME binding by the GPER agonists 2-ME, aldosterone, E2, and G1 ([Figure 6](#)). The order of potency of the GPER agonists for competition for the 2-ME binding site was 2-ME > aldosterone > E2, G1 approximating their order of

potency for inhibition of proliferation and inhibition of ERK phosphorylation (Summarized in [Table 1](#) and [Figure 8](#)).

In contrast to the specificity of competition of the GPER ligands for high-affinity [3 H] 2-ME binding and only in GPER-transduced Sf9 cells, hydrocortisone displaced [3 H] 2-ME binding and did so with similar K_i s, in wild-type, and GPER-transduced cells with $K_i = 1.2 \mu$ M (CI: 0.17 to 5.6) and 4.4 μ M (CI: 0.68 to 21), respectively ($n = 3$).

3.4 | Specific High-Affinity Binding of [3 H] 2-ME GPER in GPER-transduced Sf9 cells is both saturable and readily reversible

[Figure 7A](#) shows that specific [3 H] 2-ME high-affinity site binding was saturable, with a calculated B_{max} of 2.2 pmol/mg (95% CI: 1.9 to 2.6) and a calculated K_d of 3.7 nM, (95% CI: 2.3 to 6.0), similar to the K_i of the high-affinity site in GPER-transduced Sf9 cells determined from competition binding assays [$K_i = 1.5$ nM (CI: 0.65 to 3.1), $n = 4$].

High-affinity [3 H] 2-ME binding dissociated completely and rapidly, with a $K_{off} = 1.97 \text{ min}^{-1}$ ($n = 4$; 95% CI: 1.26 to 3.03), corresponding to a half-life of 21 s. The saturability and ready reversibility of this radioligand binding are consistent with expectations of binding to a physiologically relevant receptor.

To assess the guanine nucleotide sensitivity of the high-affinity site [3 H] 2-ME binding site in GPER-transduced cells, we compared

FIGURE 5 The GPER antagonist, G15, attenuates inhibition of ERK phosphorylation by aldosterone as well as known estrogen agonists. GPER transduced Sf9 cells were preincubated with (open circles) or without (closed circles) G15 (1 μ M) for 30 min, followed by treatment with 2-ME (1 nM), aldosterone (1 nM), E2 (10 nM), or G1 (100 nM) for 15 min. ERK phosphorylation was determined by immunoblotting. Shown are individual data (with mean) from four experiments normalized to the mean of the cells preincubated with G15. The inset shows a representative immunoblot demonstrating attenuation of agonists-mediated inhibition of ERK phosphorylation by G15.

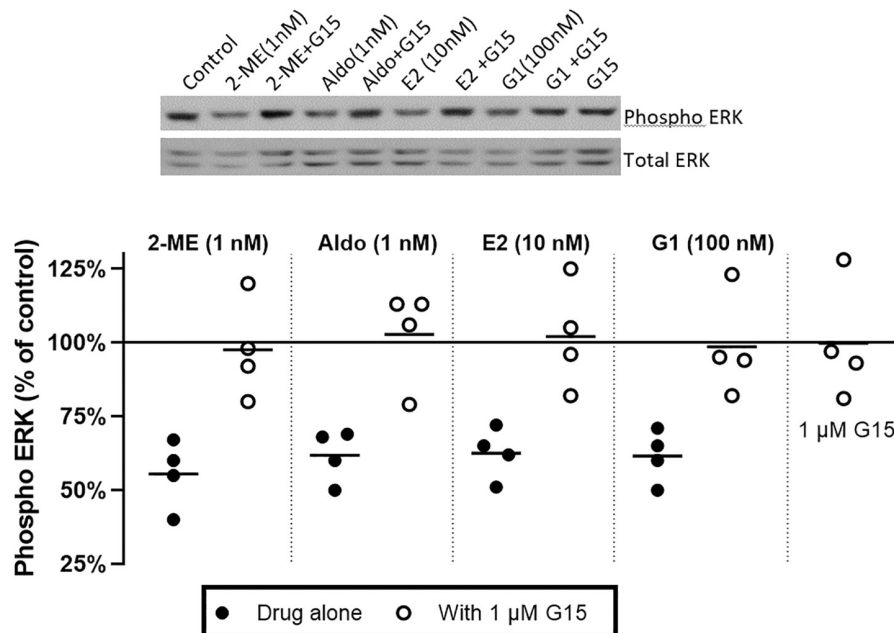


TABLE 1 Comparison of GPER agonists' potencies on functional responses (cell proliferation), signaling pathways (MAPK regulation), and GPER radioligand binding competition. This table summarizes Figures 2, 4, and 6. Legends to those figures give the experimental details

	Cell proliferation		MAPK regulation		Binding	
	IC ₅₀ (nM)	95% CI (nM)	IC ₅₀ (nM)	95% CI (nM)	K _i (nM)	95% CI (nM)
2-ME	0.026	0.013 to 0.048	0.01	0.005 to 0.024	1.5	0.63 to 3.2
Aldosterone	0.104	0.043 to 0.226	0.09	0.015 to 0.362	24	4.1 to 100
E2	2.91	1.01 to 7.84	4.6	1.72 to 12.02	41	15 to 94
G1	33.1	10.1 to 97.9	4.0	0.8 to 17.7	82	27 to 217

binding in the presence and absence of the hydrolysis-resistant GTP analog, GppNHp. Addition of GppNHp (100 μ M) to the binding assay reduced high-affinity [³H] 2-ME (0.5–2 nM) binding by 46 \pm 23%, 19 \pm 7%, 53 \pm 24 (mean and SD; $n = 3$) at [³H] 2-ME concentrations of 0.5, 1 and 2 nM, respectively (all $p < .05$ for an effect greater than control and $p > .05$ for comparisons of one concentration vs. another). These data are consistent with the interpretation that the [³H] 2-ME binding detected indeed is due to interaction with a GTP-binding protein-coupled receptor.

4 | DISCUSSION

Despite multiple reports of the very potent effects of aldosterone to mediate GPER-dependent effects, including in models devoid of detectable mineralocorticoid receptors (reviewed in Feldman and Limbird),⁶ the ability of aldosterone to directly bind to GPER has remained a point of contention. The current studies demonstrate that utilizing [³H] 2-methoxyestradiol ([³H] 2-ME) as a GPER-selective radioligand and expressing GPER in Sf9 cells, it can be demonstrated that aldosterone directly binds to GPER and with greater potency than that of E2, estradiol.

Our interpretation that [³H] 2-ME can identify a physiologically relevant GPER is based on the results of assays that, in aggregate, fulfill the criteria for establishing that a given radioligand is binding to a GPCR, i.e., assays demonstrating the saturability, reversibility and order of potency of ligand competition for [³H] 2-ME binding consistent with their functional effects. It is especially notable that the K_d for 2-ME determined by saturation binding assays (3.7 nM) was quite close to the K_i determined by competition binding assays (1.5 nM). Further supporting the conclusion that high-affinity site [³H] 2-ME binding was to a physiological GPER was the finding that the order of potency of GPER ligands for binding to the receptor exactly paralleled their order of potency for GPER-dependent inhibition of proliferation and 2-ME, aldosterone, E2, and G1. Additionally, supporting the conclusion that high-affinity site [³H] 2-ME binding was to a GPER receptor population was the finding that high-affinity [³H] 2-ME binding was regulated by guanine nucleotides. It has been recognized, since the initial appreciation of the ternary complex model for GPCR binding, that the addition of excess nonreversible/slowly reversible guanine nucleotides mediated the reversion of GPCRs to their low-affinity state for agonist binding.²¹ With agonist radioligands (like 2-ME) this would be reflected by an apparent

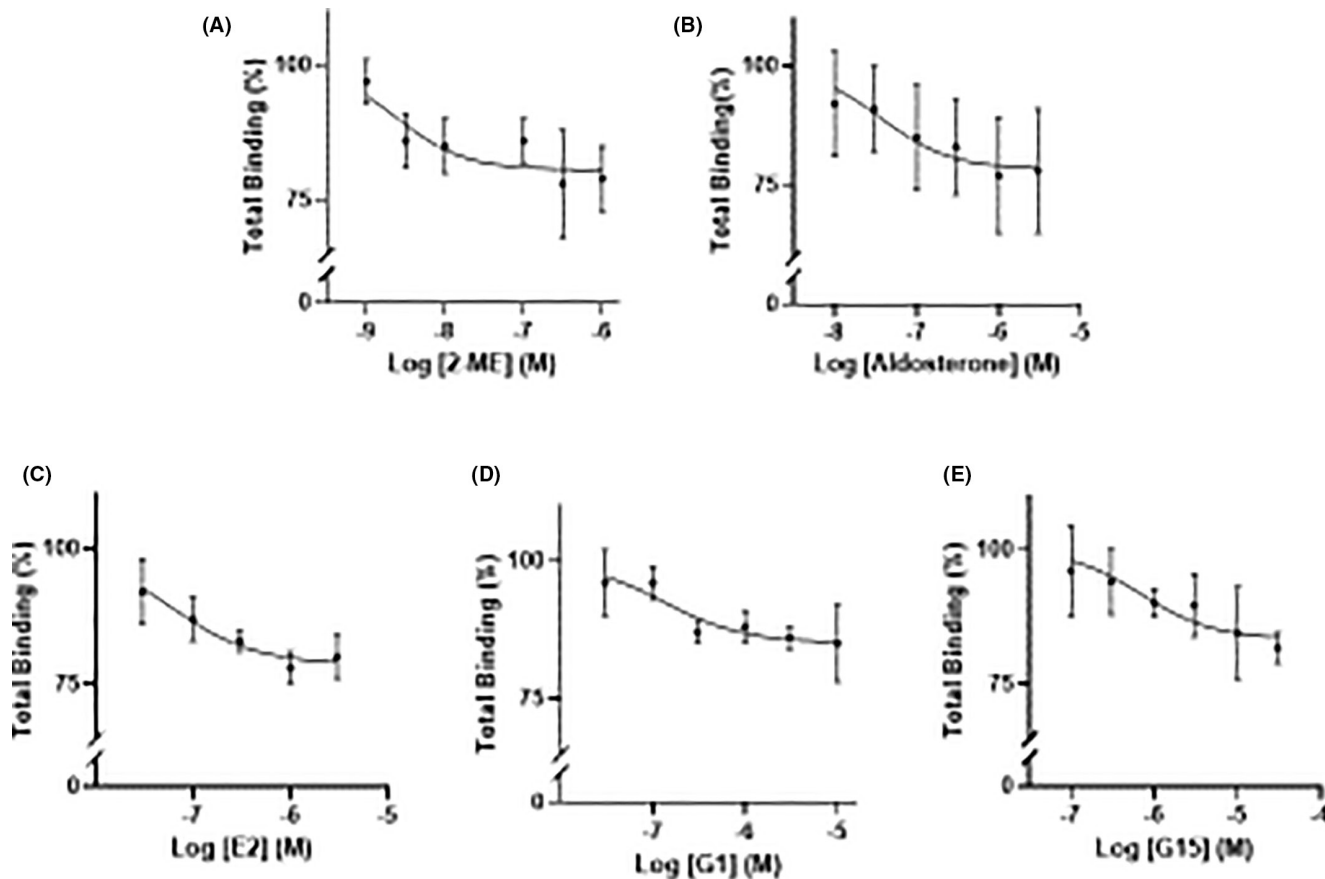


FIGURE 6 ^3H 2-ME competition binding assays in GPER-transduced Sf9 cells. 2ME (A) or Aldosterone (B) or E2 (C) or G1 (D) or G15(E). Shown are the mean (and SD) of 3–5 experiments with a concentration-response curve fit to the pooled data by nonlinear regression constraining the top plateau to 100% and the Hill Slope to -1 . The K_i values were calculated from the EC_{50} s by the method of Cheng and Prusoff using the K_d for ^3H 2-ME determined in Figure 7. The K_i s are tabulated in Table 1.

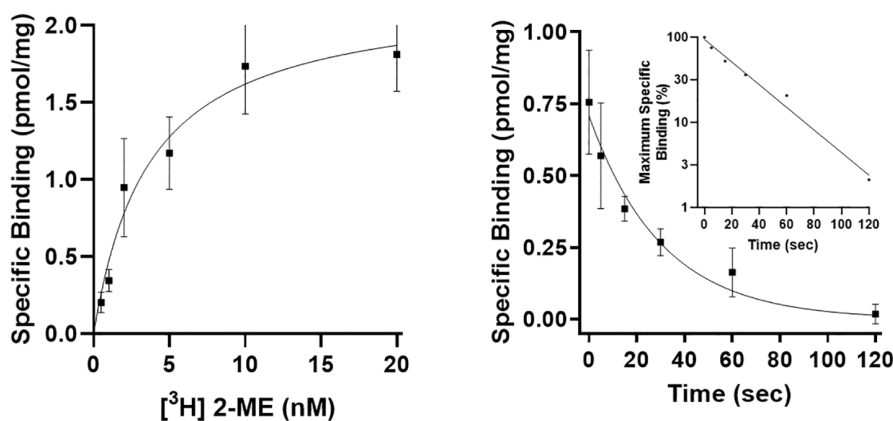


FIGURE 7 Saturability and Rapid Dissociation of ^3H 2-ME binding in GPER-transduced Sf9 cells. Left. Saturation binding assays of high-affinity site binding. Data are depicted as the mean \pm SD ($n = 4$) of high-affinity specific binding defined as total binding minus nonspecific binding in the presence of 100 nM unlabeled 2-ME. Right. After 2 nM ^3H 2-ME was incubated for 120 min at 15°C, the binding reaction was slowed by adding 5 ml of ice-cold binding buffer. High-affinity site-specific binding was measured during the next 5 to 120 s. Data are depicted as the mean \pm SD ($n = 4$). A monoexponential model was fit by nonlinear regression, forcing the curve to plateau at $Y = 0$ (since the graph shows specific binding). The inset shows the same data with a logarithmic vertical axis.

reduction in trappable binding in the presence of a nonreversible guanine nucleotide (like GppNHp).

Considering the off-rate kinetics for 2 ME binding, our studies would suggest that ^3H 2-ME is probably the only feasible radioligand

currently available for performing GPER radioligand binding studies utilizing vacuum filtration techniques. As demonstrated in prior studies, ^3H 2 ME is the most potent GPER radioligand commercially available, with a K_d in the low nanomolar range (i.e., almost 100 times

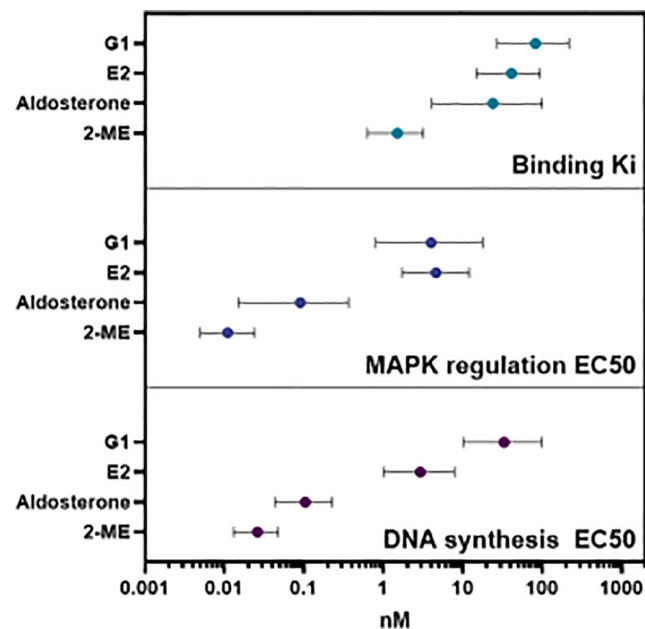


FIGURE 8 Summary of EC_{50} s and K_i s. This graph summarizes the EC_{50} values obtained from Figures 2 and 4 and the K_i values from Figure 6. Table 1 shows the values. The symbols show the fit value of the K_i or EC_{50} ; the lines show the 95% CIs.

more potent than estradiol) as determined from saturation binding studies, displacement studies, and dissociation assays.^{30,31} However, as potent as this radioligand is, it is evident from the examination of its off-rate kinetics that even at assay temperatures of 15°C, [³H] 2-ME binding dissociates with a half-life of 21 s. Given the generalized property that for GPCR ligands, their K_d s are primarily predicted by their K_{off} values, it would not be expected that GPER binding assays using lower potency radioligands (like estradiol or aldosterone) would be able to detect physiological GPER binding- at least utilizing vacuum filtration techniques.²⁰

Aldosterone's functionality as a GPER agonist is evident from the proliferation and ERK phosphorylation studies. Notably, and consistent with prior studies assessing both ERK phosphorylation and proliferation, aldosterone demonstrates greater potency for mediating GPER-dependent effects than estradiol.^{8,9} Amplification of the binding to subsequent responses, where maximal functional responses can occur at very low receptor occupancy rates, is characteristic of signal transduction pathways of G protein-coupled receptors.^{36,37} Thus, the potencies of GPCR agonists for functional effects (e.g., stimulation of proliferation) is greater than their affinities for receptor binding (i.e., their K_i s in competition binding assays). This property of signal amplification also is evident in the current studies, where the EC_{50} for the function was much lower than the K_i for binding for all of the GPER agonists testing, including those with the highest potency (i.e., 2ME and aldosterone). Nonetheless, the orders of the potency of competition for binding and functional effects are practically indistinguishable (cf Figure 8).

In other cell systems reported to date, GPER activation has been described to mediate EITHER stimulation or inhibition of

proliferation.^{6,38-41} Similarly, both stimulatory and inhibitory effects of GPER agonists on ERK phosphorylation have been described, that in specific systems seem to parallel their effects on proliferation,⁴²⁻⁴⁵ as is the case in the current studies. It is of interest that GPER activation in Sf9 cells solely inhibited ERK phosphorylation and proliferation; thus, this model might be of value in identifying the specific molecular events that are causal for GPER-mediated suppression of proliferation.

We chose the Sf9 system to obtain high protein translation efficiency which was needed for our primary goal of identifying GPER receptor binding with radioligands in a readily detectable fashion. However, in some cases, proteins expressed using the Sf9/baculovirus system have been reported *not* to undergo the post-translation modifications seen in mammalian systems which for GPER could, theoretically, affect binding characteristics.⁴⁶ However, this discrepancy in post-translational modification of expressed proteins in Sf9 models has not been universally the case- especially for transmembrane proteins.⁴⁷ The molecular size of GPER transduced in Sf9 cells (Mr-55 kD; Figure 1) approximates its molecular size in mammalian systems³² and is higher than its calculated molecular weight, which may suggest that some post-translational modification of GPER is occurring in Sf9 cells. Most importantly, the order of potency for the functional effects of GPER ligands parallels their effects reported in mammalian systems.^{8,9} Taken together, the data demonstrate that Sf9 cells are a reasonable model for studying GPER.

The limitations in utilizing radioligand binding should be noted. Even under the most optimal experimental conditions we could design, the very high nonspecific binding seen makes radioligand binding techniques using vacuum filtration of only limited utility in the characterization of GPER-ligand interactions- beyond establishing the receptor's affinity for that ligand as we have done in the current study.

In summary, these studies establish that [³H] 2ME binding to baculovirus-transduced Sf9 cells is a valid model to study GPER binding directly and to interrogate the molecular events linking receptor binding to inhibition of cellular proliferation. The data also provide conclusive evidence that aldosterone directly interacts with GPER. It should be emphasized that these studies leave open the possibility that some actions of aldosterone working via GPER-dependent pathways may be indirect (i.e., via mineralocorticoid receptor activation). However, these studies do establish that at least some effects of aldosterone are due to direct interaction with the GPER receptor, and that aldosterone is the most potent endogenous physiological hormone for GPER activation currently identified.

AUTHOR CONTRIBUTIONS

Qingming Ding: Participated in research design, conducted experiments, performed data analysis, and wrote or contributed to the writing of the manuscript. **Jozef Chorazyczewski:** Conducted experiments and performed data analysis. **Robert Gros:** Participated in research design and wrote or contributed to the writing of the manuscript. **Ross D Feldman:** Conceived the project and had a

major role in designing the experiments and writing the manuscript.

Harvey J Motulsky: Helped with analyzing the data and writing the manuscript. **Lee E Limbird:** Gave editorial feedback concerning the manuscript.

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

There are no relevant conflicts of interest to disclose for any of the authors listed.

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