Establishment of a CaCC-based Cell Model and Method for High-throughput Screening of M3 Receptor Drugs

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Abstract
Muscarinic acetylcholine receptor subtype 3 (M3 receptor) is a G Protein-Coupled Receptor (GPCR) that mediates many important physiological functions. Currently, most M3 receptor drugs also have high affinity for other subtypes of muscarinic acetylcholine receptors (mAChRs) and produce the risk of side effects. Therefore, in order to find M3 receptor drugs with high specificity, high activity and low side effects, we established a cell model and method for efficient and sensitive screening of M3 receptor based on calcium-activated chloride channels (CaCCs), and this method is also suitable for the screening of other GPCR drugs. This screening model consists of Fischer rat thyroid follicular epithelial (FRT) cells that endogenously express M3 receptors, CaCCs, and the indicator YFP-H148Q/I152L. We verified that the model can sensitively detect changes in intracellular Ca2+ concentration using fluorescence quenching kinetics experiments, confirmed the screening function of the model by applying available M3 receptor drugs, and also evaluated the good performance of the model in high-throughput screening.

Keywords CaCC · High-throughput screening · M3 receptor · Cell model · YFP-H148Q/I152L

Introduction
The muscarinic acetylcholine receptors (mAChRs) are very important members of the human G protein-coupled receptor (GPCRs) family [1]. Molecular cloning studies have revealed the presence of five subtypes of mammalian mAChRs, namely M1-M5 [2]. Of these, muscarinic acetylcholine receptor subtype 3 (M3 receptor) is predominantly distributed in smooth muscle in the vasculature, as well as in the pulmonary respiratory system. Activation of the M3 receptor by acetylcholine activates downstream Gq proteins and mediates an increase in intracellular Ca2+ levels, leading to smooth muscle contraction [3].

Because of its important physiological function in vivo, the M3 receptor has been an important drug target. For example, tolterodine, a drug used clinically to treat overactive bladder syndrome, works by blocking M3 receptor on the bladder’s detrusor muscle [4]. Then there are the bronchodilators ipratropium Bromide and tiotropium, which work through M3 receptor to inhibit glandular secretion and reduce respiratory mucus production [5]. However, most of these drugs are non-selective in that they not only inhibit M3 receptor, but also block M1 receptor distributed in gastric wall cells and M2 receptor that affect myocardial function. Thus, the lack of specificity is a common feature of most M3 receptor drugs, and they can produce adverse effects such as dry mouth, dry eyes, palpitations and dyspepsia while treating [6]. Therefore, new drugs targeting the M3 receptor with greater potency are urgently needed.

The main method of drug screening for GPCRs is to detect changes in intracellular second messenger Ca2+ or
cAMP concentrations. One of the mainstream methods used for intracellular Ca\(^{2+}\) detection is the use of fluorescent molecular probes [7]. The limitations of such methods in terms of price and experimental manipulation have hindered their application in high-throughput drug screening. Based on this, we established a high-throughput screening cell model and method for targeting M3 receptors based on calcium-activated chloride channels (CaCCs). This screening strategy is applicable to screen most of the GPCRs drugs that mediate intracellular Ca\(^{2+}\) elevation.

CaCCs are ion channels that can be activated by trace amounts of intracellular Ca\(^{2+}\) and open to transport anions such as Cl or I into the cell. Chlorine anions are present in large amounts in the cell, while iodine anions are present in very low amounts in the cell, so demonstrating the validity of the model by changes in I has the advantage of accurate assay results and is less susceptible to interference. Anoctamin-1 (ANO1) is the molecular basis of CaCCs, responding to intracellular Ca\(^{2+}\) concentrations at nanomolar levels [8, 9]. At the same time, it is a powerful I transporter, capable of transporting 10\(^{-6}\) anions per second in a single channel [10]. In combination with it, YFP-H148Q/I152L is a double mutant of yellow fluorescent protein (YFP) that can be quenched by trace amounts of I\(^-\) [11]. After extensive experiments, we found that the M3 receptor is endogenously expressed in Fischer rat thyroid (FRT) cells. FRT cells can be grown on relatively inexpensive media with good adhesion to commonly used cell culture plates while allowing stable structures expression of the transfected protein. Therefore, we applied FRT cells as vectors to stably transfect ANO1 and YFP-H148Q/I152L and successfully validated that the screening model we constructed could perform high-throughput screening of M3 receptor drugs. These results provide support for the future development of M3 receptor drugs as well as GPCRs drugs.

### Materials and Methods

#### Materials

FRT cells were donated by Prof. Tong-hui Ma from Northeast Normal University (Jilin, China); The primers, cut-gel recovery kits, PLVX/G-418-pEGFP-ANO1 plasmid and PLVX/puromycin-YFP-H148Q/I152L plasmid, were purchased from Sangon Biotech (Shanghai, China); 10% fetal bovine serum (FBS) was purchased at Thermo Fisher Scientific (Waltham, Massachusetts, USA); Lipofectamine 2000 liposomes, TRizol reagent, reverse transcription reagents, Geneticin (G-418), puromycin, rabbit anti-rat M3 polyclonal antibody, rabbit anti-rat \(\beta\)-actin monoclonal antibody, and goat anti-rabbit IgG purchased from Invitrogen (Carlsbad, California, USA); F-12 nutrient medium, Eact, niflumic acid (NFA), carbachol, acetylcholine, bethanechol, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), and tiotropium purchased from Sigma-Aldrich (St. Louis, Missouri, USA); RT-PCR reagents and whole protein extraction kits were purchased from TransGen Biotech (Beijing, China), PVDF membranes were purchased from Millipore Sigma (Darmstadt, Germany); ECL chemiluminescence assay kits were purchased from Solebro (Beijing, China).

#### Detection of M3 Receptor Endogenous Expression on FRT Cells

Total RNA from FRT cells was extracted, total RNA concentration and purity were measured by Nanodrop 2000 (Thermo Fisher, Waltham, Massachusetts, USA), and then reverse transcribed into cDNA. We designed and synthesized six pairs of primers: M1 to M5 receptors and \(\beta\)-actin, and the primer sequences and gene bank numbers are

| Primers   | Sequences (5’ to 3’) | Base number | Product length (bp) | Gene bank no.     |
|-----------|----------------------|-------------|---------------------|-------------------|
| \(\beta\)-actin(Forward) | GTCGTCGACAACCGGCTCC | 18          | 260                 | NM_001101.5       |
| \(\beta\)-actin(Reverse) | AGGTCTCAACATGATCTGGGT | 22          |                     |                   |
| M1(Forward)   | GTTACACCGAGCTCAGG    | 20          | 343                 | NM_080773.1       |
| M1(Reverse)   | TTGCCAGAAGGAGATGG    | 20          |                     |                   |
| M2(Forward)   | TGAGGGAAGGAGCACAGGT  | 20          | 680                 | NM_031016.2       |
| M2(Reverse)   | GCCATTTTTGCTGTCGGCTT | 20          |                     |                   |
| M3(Forward)   | CAGCAGAAGCGGATGTAGA  | 20          | 300                 | NM_012527.2       |
| M3(Reverse)   | TGACGCAAATGAGAGCTT   | 20          |                     |                   |
| M4(Forward)   | CGGACCTCCTGCTTGTTC   | 19          | 537                 | NM_031547.1       |
| M4(Reverse)   | TGCCACTTATAGTGGCCGG  | 20          |                     |                   |
| M5(Forward)   | GTGCTAATATGGCCACGGG  | 20          | 781                 | NM_017362.5       |
| M5(Reverse)   | TCTGGCAGTACATCAGGTT  | 20          |                     |                   |
shown in Table 1. Amplification was performed using a PCR instrument (ABI, Waltham, Massachusetts, USA), and the products were subjected to agarose gel electrophoresis and imaged using a gel imager (Bio-Rad, Hercules, California, USA), then sequenced.

Detection of M3 Receptor Expression by Western Blot

The total protein of FRT cells was extracted according to the whole protein extraction kit. The protein concentration was determined using the BCA protein assay kit. Add 1× electrophoresis solution to the electrophoresis tank, add samples, electrophoresis at a constant voltage of 80 V for 30 min, and then change the voltage to 120 V for 60 min until the blue bands run out of the bottom of the gel plate. The gel was transferred to the PVDF membrane and then placed in 5% skimmed milk powder for closure. The membrane was washed with Phosphate Buffered Saline (PBS) to finish adding rabbit anti-rat antibodies to M3 and β-actin as primary antibodies overnight at 4 °C. The primary antibody was recovered and the membrane continued to be washed. After washing, the protein bands were observed using the ECL chemiluminescence kit. Three sets of parallel experiments were set up for this experiment.

Establishment of a Cell Model Co-Expressing ANO1 and YFP-H148Q/I152L

Cell Culture

FRT cells were cultured in modified F12 nutrient medium supplemented with 10% fetal bovine serum (FBS) in a 37 °C, 5% CO2 incubator. Cells were passaged more than 3 times and kept in good cell condition prior to the experiment.

Transfections

The ANO1 plasmid, transfection reagent, and F-12 basic culture medium were added to the EP tubes according to the Lipofectamine 2000 instructions, incubated at room temperature for 20 min, and then added to the cell culture medium. After 48 h, observed green fluorescence of EGFP on the cell membrane indicates successful transfection. Transfected cells were screened using antibiotic G418. After 2 weeks of screening, the obtained cell lines were subjected to limited dilution to obtain cloned lines with high expression. The YFP-H148Q/I152L plasmid was transfected with FRT cells stably expressing ANO1 in the same manner. The transfected cells were screened using the antibiotic puromycin. That is, a FRT cell line with high expression of ANO1-YFP-H148Q/I152L was obtained.

Detection of Cell Model Functions

Whole-cell Patch-clamp Detection of ANO1 Currents

FRT-ANO1 cells in good condition were taken and placed under an inverted fluorescence microscope and perfused with the electrode outer solution. The resistance of the electrode to water was approximately 4–6 MΩ, and after applying negative pressure to form a 6 GΩ high impedance seal, the cell membrane at the tip of the electrode was ruptured by rapid negative pressure to form a whole-cell recording pattern. The initial clamp voltage was 0 mV, and after 10 ms of recording, the cells were given step stimulation (−80 mV to 80 mV in steps of 20 mV). Each step stage was recorded for 1000 ms. The experiment was divided into 3 groups: Ca2+ group, Ca2+-NFA group, and control group.

The electrode outer solution (bath solution) was 140 mmol/L NMDG-Cl, 2 mmol/L MgCl2, 5 mmol/L CaCl2, and 10 mmol/L HEPES. The inner electrode solutions (pipette solution) contained zero calcium, 0.6 mmol/L calcium solution, 100 μmol/L NFA (ANO1 antagonist), and 0.6 mmol/L calcium solution, respectively.

Fluorescence Quenching Kinetic Assay to Detect ANO1 and YFP-H148Q/I152L Function

FRT cells stably co-transfected with ANO1-YFP-H148Q/I152L were inoculated into black-walled clear-bottomed 96-well plates and then cultured for 36 h. The experiment was divided into three groups: agonist group, antagonist group, and control group. Each group of experiments was conducted 3 times. To the wells of the antagonist group, 40 μmol/L ANO1 antagonist NFA was added and incubated for 10 min and the supernatant containing unbound antagonist NFA was aspirated. Nal-PBS was added to the pump of the FLUOstar microplate reader (BMG, Offenburg, Germany), and the relative fluorescence intensity was calculated automatically every 0.2 s. The first 2 s was used as the baseline, and after 2 s the instrument added 120 μL of Nal-PBS containing 20 μmol/L ANO1 agonist Eact to the wells of the agonist and antagonist groups at a rate of 100 μL/s. To the wells of the control group, 120 μL of Nal-PBS was added. Changes in fluorescence slope values were analyzed by macro-calculation of the raw data using Excel software.

Detection of Cell Model to Screen for M3 Receptor Drugs

Fluorescence Quenching Kinetics Experiments

The experimental method and microplate reader parameters were set as in “Fluorescence quenching kinetic
assay to detect ANO1 and YFP-H148Q/I152L function”. The cells were divided into 5 groups: 3 agonist groups and 2 antagonist groups. The three agonist groups were added with M3 receptor agonist 10 μmol/L carbachol, 20 μmol/L acetylcholine, and 35 μmol/L bethanechol, respectively, and the change of fluorescence slope value was analyzed by adding 120 μL of NaI-PBS after 2 s of detection with a microplate reader. The supernatant containing unbound antagonist was aspirated after incubation for 10 min with the M3 receptor antagonist 20 μmol/L 4-DAMP and 40 μmol/L tiotropium for the 2 groups of antagonists, respectively, and the microplate reader was also set to add another 120 μL of NaI-PBS containing 10 μmol/L carbachol after 2 s of detection. The changes in the fluorescence slope values were analyzed by macro calculation of the raw data using Excel software. Then different concentrations of the drug were applied to ANO1-YFP-H148Q/I152L cells respectively, and the trends of fluorescence slope values of different concentrations of the drug were observed.

Z-factor Assessment

The Z-factor is an important parameter for evaluating the stability of high-throughput screening methods. It is calculated as follows: 
\[
Z\text{-factor} = 1 - 3 \times \frac{(SD_{\text{positive}} + SD_{\text{negative}})}{(\text{Mean}_{\text{positive}} - \text{Mean}_{\text{negative}})}
\]  
[12]. 10 μmol/L carbachol was added to 6 columns (48 wells) of the 96-well plate as a positive control, while PBS was added to the other 6 columns (48 wells) as a negative control. The same microplate reader was set up to add NaI-PBS for another 120 μL after 2 s of detection. Calculate the Z-factor values.

Statistical Analyses

Graphical and statistical analysis was performed using GraphPad Prism, using a T-test, one-way analysis, with \( P < 0.05 \) being considered a statistically significant difference. The non-linear curve fitting analysis was performed to calculate the half effective concentration (EC\(_{50}\)) and half inhibitory concentration (IC\(_{50}\)) for the concentration-dose dependence of the different agonists and antagonists.

Results

M3 Receptors are Endogenously Expressed in FRT Cells

To construct a drug screening model for M3, we found that FRT cells endogenously express M3 receptors by RT-PCR and Western blot assays.

RT-PCR results showed specific bands for the M3 receptor and \( \beta \)-actin, respectively. (A) Electrophoresis results. RT-PCR results show specific bands at 300 and 260 bp for M3 receptor and \( \beta \)-actin, respectively. (A2) Sequencing results of the M3 receptor on FRT cells. (A3) Comparison of the sequencing results of Fig. A2 with the sequences of the M3 gene included in the GenBank database, with 100% similarity. (B) Western blot results. \( \beta \)-actin relative expression is 42 kDa and M3 receptor protein relative expression is 66 kDa.
Fig. 2 Results of cell modeling. A Fluorescence microscopy results after transfection of FRT cells with ANO1 and YFP-H148Q/I152L transfected with FRT-ANO1 cells. B Analysis of FRT-ANO1 cell results by flow cytometry FL1-A channel and FRT-ANO1-YFP-H148Q/I152L cell results by flow cytometry FL2-A channel.
and the similarity was 100%, indicating that the cloned DNA fragment was the M3 gene fragment (Fig. 1A3). The RT-PCR results showed that FRT cells endogenously expressed the M3 receptor at the mRNA level. Analysis of the three sets of Western blot results showed (Fig. 1B) that FRT cells expressed M3 receptor at the protein level.

Fig. 3 Cell model functional validation results. A Current changes in FRT-ANO1 cells in response to 600 nmol/L Ca\textsuperscript{2+} stimulation were recorded using a whole-cell patch-clamp. B Current changes in FRT-ANO1 cells after incubation with 100 μmol/L NFA stimulated by 600 nmol/L Ca\textsuperscript{2+} were recorded by a whole-cell patch-clamp. C Whole-cell patch-clamp recording of current changes in FRT-ANO1 cells in the absence of Ca\textsuperscript{2+} stimulation. D Current-voltage relationships for groups A, B, and C. E Current values for each group in Fig. D at 80 mV, bars represent mean±standard deviation, \( n = 3 \), statistically significant for Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-NFA groups, **\( P < 0.01 \), T-test. F Homogenization results of experimental data on fluorescence quenching kinetics with the application of ANO1 drugs, values obtained are the change of each point from the first recorded value in the experiment difference (\( \Delta F \)) divided by the first recorded value (\( F \)). G The fluorescence slope values of each group in Fig. F. The agonist and antagonist groups were statistically significant, **\( P < 0.01 \), T-test.
Successful Establishment of a Cell Model of FRT-ANO1-YFP

When observed by inverted fluorescence microscopy with a blue excitation slice (475/35 nm) at ×400 magnification, green fluorescence was visible on the FRT cell membrane, demonstrating that ANO1 was expressed in the cell membrane; after subsequent transfection with YFP-H148Q/I152L, also at ×400 magnification, green fluorescence was found to be visible in the FRT cell cytoplasm, demonstrating that YFP-H148Q/I152L was expressed in the cytoplasm (Fig. 2A). Cell transfection efficiency was analyzed by applying flow cytometry (BD, Franklin Lakes, New Jersey, USA). The excitation wavelength was 488 nm. For best results, the FL1-A channel (533/30 nm) was analyzed for FRT-ANO1, and the FL2-A channel (585/40 nm) for FRT-ANO1-YFP-H148Q/I152L, with transfection efficiencies of 93.5 and 80.5%, respectively (Fig. 2B). That is, a stable transfection model of FRT cells with high expression of ANO1 and YFP-H148Q/I152L was successfully established.

ANO1 Regulation and Transporter Function

ANO1 Activation by Ca\(^{2+}\) Generates Currents in Classical CaCCs

In the patch-clamp experiment, the current increased with increasing stimulation voltage in the Ca\(^{2+}\) group after +20 mV (Fig. 3A). At the same concentration of calcium activation, NFA effectively inhibited the open current of ANO1 (Fig. 3B). The control group showed no significant current changes in the absence of Ca\(^{2+}\) stimulation (Fig. 3C). The I-V curve showed a clear outward rectification feature (Fig. 3D), we analyzed the current values of the above three groups at 80 mV (Fig. 3E). The results showed that ANO1 can be activated by Ca\(^{2+}\) and generate currents of classical CaCCs, i.e., these results taken together demonstrate Ca\(^{2+}\) regulates ANO1 function. And NFA can act as an effective antagonist to inhibit the open current of ANO1.

Demonstration of ANO1 I- Transport and Associated YFP-H148Q/I152L Sensitivity

The results of fluorescence quenching kinetics experiments showed that the relative fluorescence intensity of the agonist group decreased significantly after the addition of Eact and I\(^-\), indicating that ANO1 opened and transported I\(^-\) into the cell, resulting in the quenching of YFP-H148Q/I152L. The antagonist group was incubated with NFA, and then the relative fluorescence intensity did not change significantly after the addition of activator and I\(^-\), indicating that ANO1 was not opened. There was no significant change in relative fluorescence intensity in the control group, indicating that ANO1 was not opened (Fig. 3F). The fluorescence slope values of the agonist, antagonist, and control groups are shown in Fig. 3G. That is, we confirmed that ANO1 can be activated by Eact and transport I\(^-\) intracellularly in a short period. And NFA can effectively inhibit the activation effect of Eact. Meanwhile, experiments showed that YFP-H148Q/I152L is extremely sensitive to I\(^-\) and can be rapidly quenched by encountering I\(^-\).

Cell Models with the Ability to Screen M3 Receptor Drugs in High Throughput

Principles of Cellular Models

To demonstrate that the cell model has the function of screening M3 receptor drugs, we applied the M3 receptor
existing drugs for validation. The agonist principle of action
is represented by Carbachol as shown in Fig. 4A, and the
antagonist is represented by 4-DAMP as shown in Fig. 4B.

Cell Models Can Screen for M3 Receptor Drugs

The experimental results showed that the relative fluo-
rescence intensity decreased significantly after the addition
of the M3 receptor agonist and I⁻ in the agonist group,
indicating that the agonist bound to the M3 receptor,
causing a biological cascade reaction, Ca²⁺ concentration
increase, ANO1 channel opening, I⁻ inward flow and fluo-
rescence quenching. The antagonist group added M3 receptor
antagonist also binds to M3 receptor, but plays the opposite
role, after adding I⁻, there is no significant change in rela-
tive fluorescence intensity, ANO1 channel is not opened,
and there is no significant change in fluorescence signal
value (Fig. 5A). The fluorescence slope values of the ago-
nist and antagonist groups are shown in Fig. 5B. The above
results suggest that FRT cells co-expressing ANO1-YFP-
H148Q/I152L can screen M3 receptor drugs.

The fluorescence signal detected in the agonist group
showed different changes after the addition of different
concentrations of the M3 receptor agonist. The EC₅₀ of
carbachol, acetylcholine, and bethanechol were 5.477, 7.863,
and 14.75 µmol/L, respectively. In the antagonist group, the
fluorescence signal decreased as the concentration of the
antagonist increased, i.e. the greater the concentration of the
antagonist, the stronger the inhibitory effect, showing a dose-
dependent relationship. The IC₅₀ of 4-DAMP and tiotropium
were 10.74 and 18.49 µmol/L, respectively (Fig. 5C).

Cell Models Suitable for High-throughput Screening

The results of the software analysis showed that the SDpo-
sitive value was 4.648, the SDnegative value was 4.758, the
Mean_positive value was 89.64 and the Mean_negative value
was 14.01. The Z-factor was calculated according to the formula
to be 0.627 and the signal-to-noise ratio was 6.398:1 (Fig. 5D).
It is generally considered that the model stability is high when
the Z-factor is above 0.5 [13]. Therefore, this cell model is
suitable for high-throughput screening of M3 receptor drugs.

Discussion

Like other GPCRs, mAChRs are characterized by having
seven transmembrane helices [2]. The five MR isoforms
share 82–92% homology in the transmembrane region and 64–82% sequence similarity overall [14]. MACHRs drugs cause significant patient distress due to side effects caused by low selectivity [15]. Therefore, the design of studies to investigate ligands selective for a particular isoform of mACHRs is of great clinical importance.

To obtain a specific drug requires two conditions: a suitable screening method and an abundant and diverse screening library. Screening methods for M3 receptor-specific drugs focus on testing for changes in intracellular Ca\(^{2+}\) concentration. Nowadays, the commonly used methods to detect Ca\(^{2+}\) concentration such calcium ion indicator, fluorescent probe, patch-clamp technique, etc [7, 16–18], and new methods for screening based on the affinity of the drug to the receptor such as cell membrane chromatography (CMC) method [19]. The reagents or equipment used in these methods are more expensive and require high technical skills of the experimenter. For example, Fluo-4 AM, a calcium ion fluorescent probe, can only screen 400~500 times with a nearly one hundred dollars reagent and requires tedious loading of the fluorescent probe [20]. More importantly, the extremely low intracellular content of Ca\(^{2+}\) determines the difficulty of its direct detection [21, 22]. Nowadays, there is an increasing variety of various screening libraries, and they even exceed 10\(^{15}\) members [23, 24]. With the increasing number of members in the library over time, there is an urgency for an economical, sensitive, and convenient way to screen a massive number of libraries.

We propose a high-throughput screening model based on ANO1 GPCRs. It has the following advantages. First, only a trace amount of Ca\(^{2+}\) is required to activate ANO1 and it can transport approximately 10\(^{6}\) I\(^{-}\) per second into the cell [8, 10]. YFP-H148Q/I152L fluorescent protein has a strong optical signal, is easily captured, and can be rapidly quenched upon encountering I (11). The combination of the two components allows for the detection of linear changes in fluorescence signal intensity. Secondly, our designed method only requires 13.8 s to complete the single-well assay, and it takes less than 25 min to screen nearly 100 drugs, and the model can be repeatedly passed for more than 20 generations, which is economical, convenient, and technically simple. In conclusion, this model is advantageous for the high-throughput screening of GPCRs drugs that can cause intracellular Ca\(^{2+}\) elevation.

Because the assay is indirect, false positive results will occur when small molecules acting on upstream and downstream targets of M3 receptors are encountered during the screening, which may screen for agonists of ANO1 channels or other endogenous Ca\(^{2+}\) channels on FRT cells. However, this does not affect our initial screening experiments for a large number of drugs, which we can subsequently validate by other methods for detecting Ca\(^{2+}\) concentration as mentioned previously.

In summary, this experiment successfully constructed a cellular screening model for drugs based on CaCC targeting the M3 receptor. This method is a milestone breakthrough for the high-throughput screening of M3 receptor drugs, and is also applicable to the screening of other GPCRs and other drugs with Ca\(^{2+}\) signaling-related targets. It lays a solid foundation for the development of drugs targeting GPCRs and opens up new ideas in many fields such as basic research and drug development, which has a rather broad application prospect.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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