PMA induces androgen receptor downregulation and cellular apoptosis in prostate cancer cells

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Abstract

Phorbol 12-myristate 13-acetate (PMA) induces cellular apoptosis in prostate cancer cells, the growth of which is governed by androgen/androgen receptor (AR) signaling, but the mechanism by which PMA exerts this effect remains unknown. Therefore, in this study, we investigated the mechanistic action of PMA in prostate cancer cells with regard to AR. We showed that PMA decreased E2F1 as well as AR expression in androgen-dependent prostate cancer LNCaP cells. Furthermore, PMA activated JNK and p53 signaling, resulting in the induction of cellular apoptosis. In LNCaP cells, androgen deprivation and a novel anti-androgen enzalutamide (MDV3100) augmented cellular apoptosis induced by PMA. Moreover, castration-resistant prostate cancer (CRPC) C4-2 cells were more sensitive to PMA compared with LNCaP cells and were sensitized to PMA by enzalutamide. Finally, the expression of PKC, E2F1, and AR was diminished in PMA-resistant cells, indicating that the gain of independence from PKC, E2F1, and AR functions leads to PMA resistance. In conclusion, PMA exerted its anti-cancer effects via the activation of pro-apoptotic JNK/p53 and inhibition of pro-proliferative E2F1/AR in prostate cancer cells including CRPC cells. The therapeutic effects of PMA were augmented by androgen deletion and enzalutamide in androgen-dependent prostate cancer cells, as well as by enzalutamide in castration-resistant cells. Taken together, PMA derivatives may be promising therapeutic agents for treating prostate cancer patients including CRPC patients.

Key Words
- androgen receptor
- E2F1
- prostate cancer
- phorbol 12-myristate 13-acetate

Introduction

Prostate cancer is the most common non-cutaneous cancer and the second leading cause of cancer-related mortality in males in developed countries. For growth and survival, prostate cancer cells characteristically require androgens, which bind to their cognate receptor androgen receptor (AR), and transactivate AR, resulting in the modulation of its target genes. Thus, the gold standard for the treatment of recurrent or advanced prostate cancer has been androgen-deprivation therapy, which reduces androgen production in the testes, or interference of AR function with anti-androgen agents (Sharifi et al. 2010). Although most prostate cancers are initially dependent on androgens for growth and exhibit apparent response to androgen-deprivation therapy, most eventually recur in a castration-resistant manner during androgen-deprivation therapy; these are defined as castration-resistant prostate cancer...
(CRPC). Even after castration resistance is achieved, cell proliferation and survival are dependent on AR signaling by aberrant augmentation under a milieu of suppressed circulating androgens via various mechanisms (Sadar 2011, Shiota et al. 2011a,b,c). Hence, inhibition of aberrant AR signaling is a promising strategy for treating androgen-dependent prostate cancer as well as CRPC patients (Snoek et al. 2009, Tran et al. 2009, de Bono et al. 2011, Yamashita et al. 2012). Therefore, we developed various novel small-molecule inhibitors that suppress AR expression and function, such as antioxidant N-acetylcysteine (Shiota et al. 2012a), methyltransferase inhibitor adenosine dialdehyde (Shiota et al. 2012b), HMG-CoA reductase inhibitor statin (Yokomizo et al. 2011), protein kinase A inhibitor H89 (Kashiwagi et al. 2012), and histone acetyltransferase inhibitor procyanidin B3 (Choi et al. 2011).

Previously, phorbol 12-myristate 13-acetate (PMA) has been shown to induce apoptosis in prostate cancer cells such as LNCaP (Gonzalez-Guerrico & Kazanietz 2005, Gavrielides et al. 2006, Chen et al. 2011), C4-2 (Yin et al. 2005), and 22Rv1 (Truman et al. 2005). Its action is thought to be mediated by various pathways including TRAIL/tumor necrosis factor-α (TNFα; Gonzalez-Guerrico & Kazanietz 2005), p38/JNK (Gonzalez-Guerrico & Kazanietz 2005, Yin et al. 2005), p53 (Vigorito et al. 1999), and ROCK (Xiao et al. 2009), but inhibited by ERK and NF-κB signaling (Chen et al. 2011). However, the pharmacological effect of PMA on AR expression is unknown. Furthermore, the effect of anti-androgen agents on PMA-induced apoptosis and the sensitivity of androgen-dependent prostate cancer and CRPC cells to PMA have not been clarified. Recently, E2F1 (Sharma et al. 2010) and p53 (TP53) (Alimirah et al. 2007) have been shown to transcriptionally regulate AR expression in prostate cancer cells. Therefore, in this study, we focused on PMA treatment as a therapeutic approach for prostate cancer by downregulating AR expression.

**Materials and methods**

**Cell culture**

Human prostate cancer LNCaP and C4-2 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and charcoal-stripped serum (CSS) respectively and were maintained in a 5% CO₂ atmosphere at 37 °C. LNCaP cells were obtained from the American Type Culture Collection and propagated ~10–40 times. C4-2 cells were kindly provided by Dr Martin Gleave (Vancouver Prostate Centre, Vancouver, BC, Canada). PMA-resistant derivatives of LNCaP cells (LNCaP/PMA cells) were established by long-term culture in an appropriate medium containing gradually increasing concentrations of PMA and maintained in a medium containing 5 µM of PMA.

**Antibodies and reagents**

Antibodies against E2F1 (sc-193) and AR (sc-815) were purchased from Santa Cruz Biotechnology. Antibodies against acetyl-p53 (#2525), p53 (#9282), JNK (#9258), phosphorylated JNKThr183/Tyr185 (p-JNK; #4668), cleaved caspase 3 (#9664), caspase 3 (#9662), cleaved PARP (#9541), and PARP (#9542) were purchased from Cell Signaling Technology (Cambridge, MA, USA). Anti-PKC (SAB4502356) and anti-β-actin (A3854) antibodies were obtained from Sigma. PMA, JNK inhibitor SP600125, and enzalutamide (MDV3100) were purchased from Sigma, Calbiochem (Gibbstown, NJ, USA), and Selleck Chemicals (Houston, TX, USA) respectively.

**Knockdown analysis using siRNAs**

The following double-stranded RNA 25 bp oligonucleotides were commercially generated (Invitrogen): 5'-UGACGUGUCAGGACCUUGCAU-3' (sense) and 5'-AUGCUACGAAGGUCCUGACGUCA-3' (antisense) for E2F1 and 5'-CCAGUGAUACUCCUGGAA-3' (sense) and 5'-UUCGUCCAGUAGAUUACCACUGG-3' (antisense) for p53. LNCaP cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

**RNA isolation, RT, and quantitative real-time PCR**

RNA isolation and RT were performed as described previously (Shiota et al. 2011d,e). Quantitative real-time PCR (qRT-PCR) was performed using TaqMan Gene Expression Assays for AR (Hs00907244_m1), E2F1 (Hs00153451_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs03929097_g1) (Applied Biosystems) and TaqMan Gene Expression Master Mix (Applied Biosystems) on a 7900HT PCR system (Applied Biosystems). The transcript levels of AR and E2F1 were normalized to the corresponding GAPDH transcript levels. All values represent the results of three independent experiments.

**Western blot analyses**

Whole-cell extracts were prepared as described previously (Shiota et al. 2011d,e). Briefly, the concentration of the
preparation of protein extracts was quantified using the Bio-Rad Protein Assay, which is based on the Bradford method. Aliquots of 30 μg of protein were separated by 4–20% SDS–PAGE and transferred onto PVDF microporous membranes (GE Healthcare Bio-Science, Piscataway, NJ, USA) using a semi-dry blotter. The membranes were then incubated with the primary antibodies described above for 1 h at room temperature and then incubated with peroxidase-conjugated secondary antibodies for 40 min at room temperature. The bound antibodies were visualized using an ECL Kit (GE Healthcare Bio-Science), and the membranes were exposed to X-ray films (GE Healthcare Bio-Science).

Cytotoxicity analyses

Cytotoxicity analyses were carried out as described previously (Shiota et al. 2011f). Briefly, prostate cancer cells (2.5 × 10^4) that were transfected and not transfected with 40 nM of the indicated siRNAs were seeded in 96-well plates. On the following day, various concentrations of the agents in the indicated media were applied. After the indicated duration, the surviving cells were stained by the alamarBlue assay (TREK Diagnostic Systems, Cleveland, OH, USA) at 37 °C for 180 min. The absorbance of each well was measured using the ARVO MX plate reader. The results are representative of three independent experiments.

Flow cytometry

Flow cytometry was performed as described previously (Shiota et al. 2011d, e). Briefly, transfected prostate cancer cells (2.5 × 10^4) were seeded in six-well plates. On the following day, the cells were treated with the indicated concentrations of the agents. After 72 h, the cells were harvested, washed twice with ice-cold PBS with 0.1% BSA, and resuspended in 70% ethanol. After washing twice with ice-cold PBS, the cells were resuspended in PBS with 0.1% BSA, incubated with RNase (Roche Molecular Biochemicals), stained with propidium iodide (Sigma), and analyzed using a FACSCalibur (BD Biosciences, San Jose, CA, USA). The results are representative of three independent experiments.

Statistical analysis

All data were assessed using Student’s t-test. Levels of statistical significance were set at P < 0.05.

Results

PMA downregulates E2F1 as well as AR expression and induces cellular apoptosis in prostate cancer cells

To investigate the effect of PMA on AR expression, we incubated AR- and WT p53-expressing prostate cancer LNCaP cells with 10 and 100 nM PMA for various durations and then quantified their AR mRNA expression levels. As shown in Fig. 1A, PMA reduced AR transcript levels in a time- and dose-dependent manner. Similarly, PMA downregulated AR protein expression in a time- and dose-dependent manner (Fig. 1B) after the suppression of PKC expression, indicating that it functions as a PKC down-regulator at a late phase as shown previously (Parker et al. 1995). As E2F1 has been shown to be inhibited by PMA and induce AR transcription (Cartee et al. 2001, Cozzi et al. 2006, Sharma et al. 2010, Shiota et al. 2011a), we focused on the E2F1 transcription factor as an AR regulator mediating the effects of PMA. Expectedly, E2F1 knockdown reduced AR mRNA and AR protein expression levels (Fig. 1C). Accordingly, the effect on E2F1 expression after PMA treatment was investigated. As expected, PMA decreased E2F1 transcript levels as well as E2F1 protein levels in a time- and dose-dependent manner (Fig. 1A and B).

As AR is a critical factor for cell survival and proliferation in prostate cancer, we next investigated the effect of PMA on cell survival. Treatment with PMA for 48 h reduced the viability of LNCaP cells in a dose-dependent manner (Fig. 2A). To delineate the mechanism of decreased cell viability induced by growth suppression or cell death, we carried out a cell-cycle analysis using flow cytometry. The results revealed that PMA increased the levels of the sub-G0/G1 fraction, which represents cell death, in a dose-dependent manner (Fig. 2B); this was accompanied by increased levels of cleaved PARP, indicating the occurrence of cellular apoptosis (Fig. 2C).

PMA activates JNK and p53, resulting in cellular apoptosis

To determine the mechanism by which PMA, JNK, and p53 induce cellular apoptosis, apoptotic signals (Zuckerman et al. 2009, Chen 2012) were investigated after the exposure of cells to PMA. As shown in Fig. 3A, PMA induced JNK phosphorylation and p53 expression as well as p53 acetylation. Furthermore, JNK and p53 were inhibited by small-molecule inhibitor and siRNA respectively. The inhibition of JNK by SP600125 increased the cell viability after PMA treatment (Fig. 3B). Similarly, the inhibition of p53 by p53-specific siRNA augmented the
These results were supported by decreased caspase 3 cleavage and resulting PARP cleavage after JNK inhibition and p53 knockdown (Fig. 3C), indicating that JNK and p53, at least in part, mediate and cause apoptotic signals induced by PMA.

AR blockade enhances the cytotoxic effect of PMA in LNCaP cells

As androgens are critical for prostate cancer cell growth and survival, we investigated the role of androgens in cellular apoptosis induced by PMA. As shown in Fig. 4A, incubation of LNCaP cells in CSS media lacking androgens reduced their viability after exposure to PMA, which was partially restored by the addition of dihydrotestosterone (DHT), which was in agreement with the results of the cell-cycle analysis indicating that cell death induced by PMA was enhanced by androgen deprivation and partially reversed by DHT addition (Fig. 4B). Furthermore, the cleavage of caspase 3 and PARP was inhibited by DHT (Fig. 4C), indicating that androgen/AR signaling also, at least in part, mediates apoptotic stimuli induced by PMA.

Based on the effect of androgens on PMA-induced apoptosis, the inverse effect of an anti-androgen agent in combination with PMA was investigated. We used enzalutamide as an anti-androgen as it has been reported to have superior anti-androgen and anti-tumor effects (Scher et al. 2012). As shown in Fig. 4D, enzalutamide treatment enhanced the cytotoxic effect of PMA. Cell death represented by the sub-G0/G1 fraction in the cell-cycle analysis was also enhanced by enzalutamide treatment (Fig. 4E). Furthermore, cellular apoptosis induced by PMA was enhanced by enzalutamide treatment, indicated by increased cleaved caspase 3 and cleaved PARP levels (Fig. 4F).
C4-2 cells are sensitive to PMA and are sensitized to PMA by enzalutamide

The growth of CRPC cells is also known to be dependent on AR signaling. Next, we investigated the cytotoxic effect of PMA on CRPC cells. C4-2 is a CRPC cell line that expresses AR protein at levels comparable to those expressed by its parental LNCaP cells. Curiously, PMA significantly decreased the viability of C4-2 cells compared with LNCaP cells (Fig. 5A), which was confirmed by the increased levels of the sub-G0/G1 fraction in C4-2 cells, compared with LNCaP cells (Fig. 5B). Similar to LNCaP cells, AR transcript levels in C4-2 cells were suppressed by PMA treatment (Fig. 5C). Furthermore, cellular apoptosis represented by cleaved caspase 3 and cleaved PARP was more prominent in C4-2 cells (Fig. 5D).

Enzalutamide has been shown to exert anti-tumor effects even in CRPC cells (Scher et al. 2012). Thus, similar to LNCaP cells, the cytotoxic effect of enzalutamide in combination with PMA was investigated, where 25 μM enzalutamide was used as the minimum plasma enzalutamide concentrations in the 150 mg cohort were found to be about 22 μM in the phase I/II study (Scher et al. 2010). Enzalutamide alone exhibited anti-proliferative effects on C4-2 cells (Fig. 5E). This synergy was confirmed by increased cell death and apoptosis represented by increased levels of the sub-G0/G1 fraction and increased levels of cleaved caspase 3 and cleaved PARP respectively (Fig. 5F and G).

PMA-resistant cells express less amounts of PKC, E2F1, and AR than parental cells

Finally, to further investigate the mechanism by which PMA induces cellular apoptosis, we established a PMA-resistant LNCaP (LNCaP/PMA) cell line, the resistance of which to PMA was increased by ~150-fold (Fig. 6A). In LNCaP/PMA cells, E2F1 and AR transcript levels were all reduced compared with those in parental LNCaP cells (Fig. 6B), which was in agreement with decreased protein expression of E2F1 and AR (Fig. 6C). Furthermore, PKC expression was suppressed, while JNK activation and p53 expression were elevated in LNCaP/PMA cells, indicating that cellular resistance to PMA was achieved by gaining independence from PKC, E2F1, and AR functions (Fig. 6C). In addition, cellular sensitivity to androgen deprivation and enzalutamide in LNCaP/PMA cells was investigated. As shown in Fig. 6D, LNCaP/PMA cells were cross-resistant to androgen deprivation as well as enzalutamide, indicating that the viability of LNCaP/PMA cells was independent of androgen/AR signaling.

Discussion

PMA has been shown to induce suppressive effects on cancer cell proliferation and survival in various prostate cancer cells including LNCaP (Gonzalez-Guerrico & Kazanietz 2005, Gavrielides et al. 2006, Chen et al. 2011)
PMA activates JNK and p53, resulting in cellular apoptosis. (A) LNCaP cells were treated with 10 or 100 nM PMA for the indicated duration. Whole-cell extracts were subjected to SDS–PAGE, followed by western blot analyses for the indicated proteins. (B, left panel) LNCaP cells were seeded in 96-well plates. On the following day, the cells were pre-incubated with 30 μM SP600125 for 1 h and treated with various concentrations of PMA. (B, right panel) LNCaP cells were transfected with 40 nM of the indicated siRNAs and seeded in 96-well plates. On the following day, 0, 25, or 50 nM PMA was applied. After 48 h, the cell survival rates were determined by cytotoxicity analyses. Cell survival in the absence of PMA was defined as 1. Boxes, mean; bars, S.D.*P<0.05 (compared with cells not treated with SP600125 or transfected with the control siRNA). (C, left panel) LNCaP cells were pre-treated with 30 μM of SP600125 for 1 h before incubation with 25 or 50 nM PMA for the indicated durations. (C, right panel) LNCaP cells were transfected with 40 nM of the indicated siRNAs. After 24 h, LNCaP cells were treated with 25 or 50 nM PMA for the indicated durations. Whole-cell extracts were subjected to SDS–PAGE, followed by western blot analyses for the indicated proteins.

and C4-2 (Yin et al. 2005) as well as 22Rv1 (Truman et al. 2005). In agreement with this finding, this study showed that PMA suppressed cell viability and induced cellular apoptosis via caspase activation in LNCaP and C4-2 cells.

To determine the mechanism by which PMA exerts suppressive effects on prostate cancer cell proliferation, we focused on AR because it is known to play a critical role in the development and progression of prostate cancer through its downstream targets. Furthermore, inhibition of AR by various means such as androgen deprivation, anti-androgen agents, and AR-targeting siRNA has been shown to retard prostate cancer cell proliferation (Ryan & Tindall 2011). Interestingly, this study clearly showed that PMA reduced AR expression at the transcriptional level, indicating that PMA downregulated AR expression via a transcription factor that regulates AR expression. At present, several transcription factors are known to regulate AR transcription (Shiota et al. 2011a). Among these transcription factors, we focused on E2F1 because PMA has previously been shown to decrease E2F1 activity and expression (Cartee et al. 2001, Cozzi et al. 2006). E2F1 knockdown led to a decrease in AR expression, indicating that the E2F1 transcription factor mediates AR downregulation induced by PMA. These findings are supported...
LNCaP cells were treated with 100 nM PMA for the indicated durations. After incubation for 48 h, the cell survival rates were determined by cytotoxicity analyses. After 48 h, the cells were stained with propidium iodide and analyzed by flow cytometry. The percentage of cell-cycle fraction is shown. Boxes, mean; bars, ±S.D.* *P < 0.05 (compared with cells incubated under FBS or CSS + DHT conditions).

Cell survival in the absence of drugs was defined as 1. Boxes, mean; bars, ±S.D. *P < 0.05 (compared with cells not treated with enzalutamide). Whole-cell extracts were subjected to SDS–PAGE, followed by western blot analyses. (D) LNCaP cells were seeded in 96-well plates and treated with 25 μM enzalutamide and 0, 25, 50, or 100 nM PMA on the following day. After 48 h, the cells were stained with propidium iodide and analyzed by flow cytometry. The percentage of cell-cycle fraction is shown. Boxes, mean; bars, ±S.D.* *P < 0.05 (compared with cells not treated with enzalutamide). (E) LNCaP cells were treated with 25 μM enzalutamide and 100 nM PMA. After incubation for 48 h, the cells were stained with propidium iodide and analyzed by flow cytometry. The percentage of cell-cycle fraction is shown. Boxes, mean; bars, ±S.D. *P < 0.05 (compared with cells not treated with enzalutamide). (F) LNCaP cells were treated with 25 μM enzalutamide and 0 or 100 nM PMA for the indicated duration. Whole-cell extracts were subjected to SDS–PAGE, followed by western blot analyses for the indicated proteins.

by the result that PMA immediately reduced E2F1 expression, followed by sequential downregulation of AR expression, as shown in Fig. 1. Furthermore, E2F1 itself is known to be involved in cell proliferation independently of AR signaling. CDKs phosphorylate RB and the other pocket proteins on multiple sites during cell-cycle progression. These phosphorylation events regulate the interactions of RB with co-repressors and E2F family members to modulate the transcription of various genes involved in cell proliferation and other processes. Thus, E2F1 is a critical transcription factor involved in the regulation of the expression of genes involved in the cell cycle, such as cyclin A, cyclin E, CDC2 (CDK1), PCNA, and thymidine kinase (DeGregori et al. 1995). Thus, it is considered that E2F1 as well as AR contributes to the suppression of prostate cancer cell growth in a coordinated manner. In addition, cellular apoptosis has been shown to be induced by PMA via various signaling pathways including TRAIL/TNFα (Gonzalez-Guerrico & Kazanietz 2005), p38/JNK (Gonzalez-Guerrico & Kazanietz 2005, Yin et al. 2005), p53 (Vigour and others 1999), and ROCK (Xiao et al. 2009). Consistently, this study also showed that JNK and p53 signaling mediated apoptosis signaling, as represented by caspase activation. In addition, JNK...
inhibition reduced the induction of p53 by PMA, indicating that p53 facilitated JNK-apoptosis signaling. This notion is supported by a well-known interaction between JNK and p53 (Wu 2004). In addition, p53 has been shown to negatively regulate AR expression at the transcriptional level (Alimirah et al. 2007), supporting that PMA inhibits AR expression through E2F1 suppression as well as p53 induction. Taken together, JNK/p53 and E2F1/AR are suggested to mediate apoptotic and growth-suppressive signals induced by PMA.

PMA has been shown to affect both classical and novel PKC isoforms (MacDonald et al. 1994). PKCα has been shown to be involved in the pro-apoptotic effect of PMA using knockdown and pharmacological methods (Garcia-Bermejo et al. 2002, Yin et al. 2005). As well, the pro-apoptotic effect of PMA may be mediated by novel PKC isoforms as PKCδ (PRKCD) knockdown has been shown to crucially suppress the induction of cellular apoptosis by PMA (Afrasiabi et al. 2008). Moreover, recently PKD overexpression and novel PKC isoform (PKCδ and PKCd (PRKCE)) knockdown have been shown to counteract cellular apoptosis induced by PMA (Chen et al. 2011). As reviewed by Kazanietz’s group (Gonzalez-Guerrico et al. 2005), these findings indicate that classical as well as novel PKC and PKD exert pro-apoptotic and anti-apoptotic effects on PMA respectively, although it remains unclear which isoform mediates the effects of PMA on AR expression, which should be elucidated in future.
Considering PMA as a potential therapeutic agent for treating prostate cancer patients, we investigated the combinational effect of PMA and androgen-deprivation therapy, which is a gold standard for recurrent or advanced prostate cancer treatment. This study revealed that PMA in combination with androgen deletion increased the therapeutic effect of PMA, indicating that PMA with castration may be a favorable therapeutic combination. In addition, we utilized a new-generation anti-androgen, enzalutamide, which intriguingly also augmented the therapeutic effects of PMA in androgen-dependent cells. Based on these results, PMA appears to support androgen-deprivation therapy in androgen-dependent prostate cancer cells. Compared with androgen-dependent prostate cancer, the therapeutic options for CRPC are limited, and castration resistance in prostate cancer presents a difficult challenge to be overcome in the future. Surprisingly, PMA also downregulated AR expression in CRPC cells, as well as exerted a more prominent therapeutic effect compared with that in androgen-dependent cells. Furthermore, the favorable combination with enzalutamide used for CRPC treatment...
indicates that PMA may be a promising agent for treating CRPC patients. These results are consistent with the finding that CRPC cells remain dependent on AR signaling, similar to androgen-dependent prostate cancer cells. However, it is well known that PMA induces skin cancer through the PKCδ isoform by direct chronic exposure (Aziz et al. 2007). On the other hand, the PKCδ isoform plays an important role in the induction of cellular apoptosis caused by PMA (von Burstin et al. 2010). Considering these isoform-specific roles of PKC, the development of a PKCδ isoform-specific activator as a lead chemical PMA would be an ideal therapeutic option for treating prostate cancer patients.

In conclusion, PMA exerted anti-cancer effects through apoptotic and tumor-suppressive signals induced by JNK/p53 and E2F1/AR on prostate cancer cells including CRPC cells. The therapeutic effects of PMA were augmented by androgen deletion and enzalutamide in androgen-dependent prostate cancer cells, as well as by enzalutamide in CRPC cells. Taken together, PKCδ isoform-specific PMA derivatives may be promising therapeutic agents for treating prostate cancer patients including CRPC patients.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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