Dieckol exerts anticancer activity in human osteosarcoma (MG-63) cells through the inhibition of PI3K/AKT/mTOR signaling pathway

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A B S T R A C T
Background: Osteosarcoma (OS) is the most common malignant bone cancer with more metastasis and increased occurrence in children and teen-agers and being responsible for more number of morbidity and mortality worldwide.

Objective: The current exploration was planned study the in vitro anticancer actions of dieckol against human OS MG-63 cells via PI3K/AKT/mTOR signaling inhibition.

Methodology: The cytotoxicity of dieckol was scrutinized by MTT assay. Effects of dieckol on the ROS accumulation, apoptotic cell death, and MMP level in the MG-63 cells were studied by respective fluorescence staining assays. The levels of proliferative, inflammatory, and apoptotic markers in the dieckol treated MG-63 cells were scrutinized by marker specific kits. The expressions of PI3K, AKT, and mTOR was assayed by RT-PCR.

Results: The MTT assay revealed that the dieckol dose dependently prevented MG-63 cells viability and the IC50 was found at 15 μM. Dieckol treatment effectively reduced the MMP level and improved the ROS generation and apoptosis in MG-63 cells. Dieckol also regulated the proliferative (cyclin D1), inflammatory (COX-2, IL-6, TNF-α, and NF-κB), and apoptotic (caspase-3, Bax, Bcl-2) markers in the MG-63 cells. The PI3K/AKT/mTOR signaling in the MG-63 cells were effectively inhibited by the dieckol treatment.

Conclusion: In conclusion, our findings from this study recommends that the dieckol could be a talented anticancer candidate for the OS management in the future.

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1. Introduction
Cancer is a multifaceted disease that bangs in a heterogeneous environment, which is exceptionally adaptive (Park et al., 2014). Osteosarcoma (OS) signifies the most common malignant bone cancer that represents more metastatic capacity and increased occurrence in children and teen-agers (Wang et al., 2018). OS can emerge in any bone, but frequently tumors were originated from the long bones of the metaphyseal growth plates, appendicular skeleton, proximal humerus and tibia. The skull, pelvis, and jaw are reported to be the other potential parts of OS (Yan et al., 2015). The characteristic symptoms and signs of OS include pain, swelling, and joint movement problems (Hashimoto et al., 2020). The precise causes of OS is not fully understood yet, though it is apparent that numerous risk factors were connected with the instigation and progression of OS for instance, gender, age, and genetic reasons (Ottaviani and Jaffe, 2009).

Reactive oxygen species (ROS) are continuously accumulated in the aerobic species as a oxygen metabolic by-products (Liang et al., 2018). ROS play an imperative functions in the triggering of numerous signaling axis in cells in response to the alterations in cellular environments. Though, more ROS accumulation are concerned in various pathological conditions, for instance aging, inflammation, and other chronic ailments (Reuter et al., 2010).

The accumulation and elimination of ROS are regulated and balanced by the oxidant and antioxidant systems under the physiological conditions. ROS are triggered by both exogenous and endogenous factors, and contribute to regulating the biological...
functions like cell multiplication and apoptosis (Sies and Jones, 2020). Apoptosis is specified as a programmed cell death, in which a
array of molecular mechanisms directs to the removal of malignant
or anomalous cells. This event is whichever activated by extrinsic
signals like triggering of cell death receptors or by intrinsic cas-
cades that was mitochondrial dependent. Tumor cells uses a array
of molecular processes to evade apoptosis which accelerates the
atypical cell proliferation and its resistance towards anticancer
treatments (Pistritto et al., 2016). Therefore, the documentation
of apoptosis enhancing therapeutic targets is a vital approach to
cancer management.

The PI3K/mTOR cascades is a intracellular signaling pathway
necessary for controlling the cell cycle. Hence, this pathway
unswervingly connected to the cellular proliferation, longevity,
survival. In most cancers, this cascade became overactive that
eventually directs to unrestrained proliferation through inhibiting
the apoptosis. The most components of the PI3K/mTOR cascade are
underwent to the rapid alterations or genetic modifications during
the tumor progressions (Zhang et al., 2019a). PI3K pathway could
mediate numerous cellular activities via triggering the AKT protein
and enhancing the cell cycle. The stimulated AKT directs to the
downstream proteins phosphorylation that is participated in the
cell proliferation, and growth (Botter et al., 2014). AKT/mTOR sig-
naling was highlighted to be participated in the metastatic action
of OS (Egas-Bejar et al., 2014). The PI3K/Akt/mTOR signaling target-
ing could signify an hopeful therapeutic option to prevent and treat
the OS (Zhang et al., 2015). Consequently, the development of
PI3K/mTOR axis inhibitor could be a advancement in the OS
management.

OS was primarily treated with the chemotherapy and surgery.
The overall survival rate of OS was augmented in past years with the
remarkable improvements in the chemotherapy (Boon et al.,
2017). Nonetheless, the currently available chemotherapeutic agents like methotrexate, cisplatin, ifosfamide, and etoposide were
restricted by their remarkable side effects for instance, neural and
hepatic toxicity. Additionally, a group of patients were poorly
responded to these chemotherapeutic agents (Luetke et al.,
2014). Although the integration of these multimodality therapies,
30–40% of OS victims still develop the local reappearance and/or
distant metastasis, which makes OS as a specially challenging dis-
ease with increased mortalities (Meazza and Scanagatta, 2016).

*Ecklonia cava* is brown marine alga from Lessoniaceae family
profusely occurs in Korea and Japan, which contains manifold
bioactive ingredients, for instance phlorotannins, carotenoids,
and fucoidans (Lee et al., 2010). Dieckol is a phlorotannin exten-
sively found in the *E. cava*. Numerous preceding studies already
uncovered the therapeutic potentials of dieckol like antioxidant
(Li et al., 2009), anti-inflammatory (Li et al., 2011), anti-diabetic
(Kang et al., 2013a), hepatoprotective (Kang et al., 2013b), and
anti-tumor properties (Sadeshkumar et al., 2016) of dieckol. Nev-
evertheless, there are no scientific evidences for the anticancer role of
dieckol against OS. As a result, the current exploration was planned study the in vitro anticancer actions of dieckol towards OS MG-63
cells via PI3K/AKT/mTOR signaling pathway inhibition.

2. Materials and methods

2.1. Chemicals

Dieckol, 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium
bromide (MTT), Dulbecco’s Modified Eagle’s Medium (DMEM) and
other chemicals and reagents were purchased from Sigma-Aldrich,
USA. All assay kits for biochemical assays were procured from the
MyBiosource, USA and Thermofisher Scientific, USA, respectively.

2.2. Maintenance of MG-63 cells

The human OS MG-63 cells was acquired from ATCC, USA and
the same was grown in DMEM with 10% FBS. The MG-63 cells were
sustained at 37 °C in humidified and 5% CO2 supplied incubator.
The grown cells with 80% confluency were trypsinized and used for
additional investigations.

2.3. Cytotoxicity assay

The MTT cytotoxic test was done to evaluate the cytotoxic
upshots of dieckol towards MG-63 cells. Cells was placed onto the
96-wellplate containing DMEM medium at 5 × 103 cell popula-
tion per well maintained for a night at 37 °C to permit the cell
adhesion. After that, the dieckol at diverse dosages (5–100 μM)
was administered to the medium containing MG-63 cells and sus-
tained for 24 h at 37 °C. Later, the 20 μl of MTT with 100 μl of
DMEM was mixed to a plate and sustained for further 4 h at 37
°C. The 100 μl of DMSO was mixed in order to liquefy the
formed formazan crystals. Lastly, the absorbance were taken at
570 nm.

2.4. Dual staining

The AO/EB dual staining were performed to investigate the
apoptosis inducing ability of dieckol in MG-63 cells. Cells was placed onto the
24-wellplate at the 5 × 105 cells density per well and sustained at 37 °C for 24 h. Later, the dieckol at 20 μM dose was supplemented to the MG-63 cells for 24 h at 37 °C. The dox-
orubicin DOX (2 μg) was utilized as standard. After that, 100 μg/ml of AO/EB (1:1) dye were mixed to every wells and sustained for 5 min to stain the cells. Lastly, treated cells were investigated using fluorescent microscope for identifying the apoptosis.

2.5. Measurement of ROS accumulation

ROS generation in the control and treated MG-63 cells were
investigated using DCFH-DA staining. Cells were placed onto the
24-wellplate containing DMEM medium at 1 × 105 cell density per well and sustained at 37 °C. Later, MG-63 cells was adminis-
tered with 20 μM of dieckol and 2 μg of DOX and sustained for
further for 24 h. Later, DCFH-DA (10 μl) stain were mixed to each well
and sustained for 1 h. At end, ROS content in the treated MG063
cells were studied using fluorescent microscope.

2.6. Mitochondrial membrane potential (MMP)

Effect of dieckol on the MMP status of MG-63 cells was exam-
ined by Rh-123staining. MG-63 cells were placed onto the 24-
well plate with DMEM at 1 × 106 cell population and preserved at
37 °C for 24 h. Later, the 20 μM of dieckol and 2 μg of DOX
was administered to the medium containing MG-63 cells and pre-
served for further 24 h at 37 °C. Afterward, 10 μg/ml of Rh-123 was
mixed to each well for 30 min to stain the MG-63 cells. Lastly,
MMP status of treated MG-63 cells monitored using fluorescence
microscope.

2.7. Propidium iodide (PI) staining

The apoptotic cell morphology of MG-63 cells were scrutinized by PI staining. MG-63 cells was placed onto the 24-wellplate with
DMEM at 1 × 106 cell population per well and preserved at 37 °C
for 24 h. Afterward, the 20 μM of dieckol and 2 μg of DOX was sup-
plemented to the MG-63 cells for 24 h at 37 °C. Later, PI stain
(5 μl) was exposed to every well for 20 min in a dark to stain the
cells. At end, dieckol induced cell death was observed using fluorescent microscope.

2.8. Quantification of apoptotic markers

The cellular apoptotic markers i.e. cyclin D1, Bax, Bcl-2, and caspase-3 in control and dieckol treated MG-63 cells was detected with the aid of marker specific assay kits (Mybiosource, USA), as per the suggestions given by kit’s manufacturer. All assays were done triplicates.

2.9. Measurement of inflammatory markers

The inflammatory marker levels like TNF-α, NF-kB, COX-2, and IL-6 in both control and dieckol treated MG-63 cells were investigated using respective assay kits as suggested by the manufacturer’s guidelines (Thermofisher Scientific, USA).

2.10. RT-PCR analysis

The inhibitory effects of dieckol on the PI3K/AKT/mTOR cascade in the MG-63 cells were inspected using RT-PCR study. For this, the complete RNA was separated from cells using TRizol reagent as per the guidelines of manufacturer (Thermofisher, USA). Later, the cDNA was prepared by using the isolated RNA with the aid of cDNA kit as recommended by the manufacturer’s guidelines. mRNA expression of target genes were studied by RT-PCR assay using PCR kit according to the suggestions of manufacturer (Takara Bio, Japan). The primers utilized for PI3K forward: 5'-GTATCCCCAGAG CAGGATTAG-3', reverse: 5'-CAGAGAGAGGATCTCGTGTAGA-3', AKT forward: 5'-GTGCTGAGGACAATTAGA-3', reverse: 5'-AG CAGCCCTGAAAGCAAGGA-3', and mTOR forward: 5'-GTGGAACAG GACCCATGAA-3', reverse: 5'-CATTCCAGCCGACTTCTT-3'. The relative expressions of target genes were inspected using 2-ΔΔCT technique and outcomes were normalized with the internal control gene GADPH expression.

2.11. Statistical analysis

All the outcomes were examined statistically using SPSS software ver.17.0. Values are portrayed as mean ± SD of triplicates. Values are investigated by one-way ANOVA and Tukey post doc assay and significance was fixed at p < 0.05.

3. Results

3.1. Effect of dieckol on the MG-63 cells viability

The cytotoxic effects of dieckol on the MG-63 cells were examined using MTT test and the findings were represented in the Fig. 1. Dieckol administration potentially inhibited the cell viability and prevented the viability of MG-63 cells at dose dependent mode. Dieckol was supplemented at diverse doses i.e. 5–100 µM and the viability was appreciably inhibited at dose dependently. The increased dose significantly reduced the MG-63 cell viability. The 15 µM of dieckol was opted as IC50 dose due to the inhibition of 50% of cell growth and utilized for additional assays.

3.2. Effect of dieckol on the apoptotic cell death in MG-63 cells

Fig. 2 demonstrates the apoptotic cell death in the control and dieckol supplemented MG-63 cells, which is evidenced by dual staining. Control cells displayed the more green fluorescence, which shows higher MMP levels. Conversely, the MG-63 cells treated with 15 µM of dieckol demonstrated the augmented yellow and orange fluorescence due to the presence of early/late apoptotic cells. This finding proved the apoptosis triggering ability of dieckol in MG-63 cells. The standard drug DOX treatment also improved the apoptosis in MG-63 cells that was similar with dieckol.

3.3. Effect of dieckol on the ROS accumulation in the MG-63 cells

As represented in the Fig. 3, control cells exhibited the reduced green fluorescence that represents the less ROS generation. Whereas, MG-63 cells supplemented with 15 µM of dieckol displayed the intense green fluorescence that evidenced the increased ROS accumulation. The DOX treatment also substantially elevated the ROS production in MG-63 cells. The outcomes of dieckol and DOX were found similar with each other.

3.4. Effect of dieckol on the MMP level of MG-63 cells

Effects of dieckol on the MMP status in the MG-63 cells was detected by Rh-123 staining and the outcomes were depicted in the Fig. 4. As represented in the Fig. 4, control cells displayed increased green fluorescence, which shows higher MMP levels. Conversely, the 15 µM of dieckol supplemented cells displayed less fluorescence when compared with control. the dull fluorescence evidences the reduced MMP status in the dieckol supplemented cells. DOX treatment also declined the MMP levels in the MG-63 cells. Both dieckol and DOX exhibited the similar kind of results.

3.5. Effect of dieckol on the apoptotic cell morphology in MG-63 cells

MG-63 cells supplemented with dieckol subjected to PI staining to detect the apoptotic cell death and the outcomes were presented in the Fig. 5. The control cells exhibits the reduced red fluorescence due to the absence of apoptosis. Conversely, the MG-63 cells treated with 15 µM of dieckol demonstrates the increased red fluorescence when compared with control. The higher red fluorescence proves the presence of more amount of apoptotic cells by the dieckol treatment. The DOX treated MG-63 cells also demonstrates the increased red fluorescence due to the increased apoptosis. The outcomes of dieckol and DOX were found similar with each other.

3.6. Effect of dieckol on the apoptotic marker levels in the MG-63 cells

Fig. 6 demonstrated that the dieckol treatment effectively reduced the proliferative marker cyclin D1, caspase-3, and apoptotic protein Bax when compared with control. Dieckol also diminished the level of anti-apoptotic Bcl-2 protein in the MG-63 cells than control. DOX treated MG-63 cells also exhibited reduced
**Fig. 2.** **Effect of dieckol on the apoptotic cell death in MG-63 cells.** The results dual staining (AO/EB) demonstrated the increased early/late apoptotic cell deaths (yellow arrows) in 15 μM of dieckol treated MG-63 cells, which is evidenced by increased yellow/orange fluorescence.

**Fig. 3.** **Effect of dieckol on the ROS generation in the MG-63 cells.** The 15 μM of dieckol supplemented cells demonstrated the improved ROS accumulation (yellow arrows), which is confirmed by intense green fluorescence than the control cells analyzed by DCFH-DA staining.

**Fig. 4.** **Effect of dieckol on the MMP level of MG-63 cells.** The results of Rh-123 staining demonstrates the dull green fluorescence (yellow arrows) in the 15 μM of dieckol supplemented cells than control, which proves the reduced MMP level.

**Fig. 5.** **Effect of dieckol on the apoptotic cell morphology in MG-63 cells.** The PI stained cells administered with 15 μM of dieckol demonstrated the strong red fluorescence (yellow arrows) than the control that confirms the more number of apoptotic cells.
levels of cyclin D1, caspase-3, and Bax and elevated the Bcl-2 level. DOX and dieckol treatment showed similar kind of outcomes.

3.7. Effect of dieckol on the inflammatory markers in the MG-63 cells

Effects of dieckol on the inflammatory markers i.e. TNF-α, NF-κB, COX-2, and IL-6 were studied and the outcomes were illustrated in the Fig. 7. When compared with control, 15 μM of dieckol supplemented cells exhibited the decreased TNF-α, NF-κB, COX-2, and IL-6 status. These levels also decreased by the DOX treatment when compared with control, both DOX and dieckol exhibited the analogous outcomes with each other.

3.8. Effect of dieckol on the PI3K/AKT/mTOR pathway in MG-63 cells

The inhibitory potentials of dieckol against the PI3K/AKT/mTOR pathway in MG-63 cells was identified by RT-PCR and outcomes were depicted in the Fig. 8. As illustrated in the Fig. 8, the PI3K, AKT, and mTOR expressions was appreciably down-regulated by the 15 μM of dieckol supplementation. This finding unveiled that the dieckol potentially suppressed the PI3K/AKT/mTOR pathway. DOX treatment also diminished the PI3K/AKT/mTOR expressions, which is similar to the dieckol treatment.

4. Discussion

OS is a common malignant tumor occurs in children as well as adolescents with a reduced prognosis. The currently existing treatments for OS leftovers unacceptable because of the higher side effects and development of resistance towards chemo and radio-therapy (Kong et al., 2019; Goudarzi et al., 2014). Furthermore, continued administration of the same medical interventions could directs to the tumor resistance. Hence, the exploration of novel and efficient treatment options with lesser side effects has emerged greatly to manage the OS (Robertson and Orrenius, 2002). The current exploration was aimed to uncover the in vitro anticancer potentials of dieckol against MG-63 cells. The unrestrained cell proliferation are the vital factor of tumor initiation and progression. Hence, the restriction of irregular cell multiplication signals was an promising approach to possess anticancer property (Wang et al., 2014). The unrestricted proliferation and escaping from apoptosis are the crucial events that ultimately promotes the tumor progressions. Many explorations has uncovered that the natural herbal based bioactive compounds exerts their anticancer actions through triggering the apoptosis in tumor cells (Majed et al., 2015). In this exploration, we discovered that the dieckol effectively prevented the MG-63 cell growth.

Apoptosis is a well established cellular cytotoxic event in that order anticancer agents exhibits antitumor actions (Xie et al., 2020). Apoptosis is an multiple gene controlled event and categorized by nuclear fragmentation, shrinkage, membrane blebbing, DNA damage, chromatin condensation, and development of apoptotic bodies. Apoptosis performs an imperative function in chemotherapeutics against almost all kinds of tumors (Burgess, 2013; Zimmermann et al., 2001). Apoptosis inhibition plays a vital function in the tumor progression (Jan and Chaudhry, 2019). Over the last few decades, many scientists have motivated on the apoptotic-mediated cell death regulation processes associated in a numerous human ailments (Mi et al., 2016; Tang et al., 2016). In this exploration, our findings from dual staining and PI staining demonstrated that the dieckol treatment enhanced the apoptosis.
in the MG-63 cells. These findings suggested that the dieckol has the ability to trigger the apoptosis in MG-63 cells.

Accumulation of ROS is a crucial factor that affects many cellular events like proliferation, redox balance, and responsible for cellular damage. Tumor cells are extremely vulnerable to the elevated ROS accumulation and enhances the more apoptosis than in normal cells (He et al., 2017). Nonetheless, excessive ROS could injure the cancer cells, and this strategy has regarded as a hopeful approach for the huge amount of drug handlings (Zhang et al., 2019b). Additionally, ROS mediate the cancer activities via PI3K/AKT/mTOR axis, for instance proliferation, metabolism, and apoptosis (Wu et al., 2020). In recent times, more number of anticancer drugs being developed which is capable of regulate the apoptosis, which is of great importance for additional explorations chemotherapeutic purposes (Lee et al., 2018). Zhu et al. (2020) suggested that the over ROS generation restrict the PI3K/AKT/mTOR axis and leads to the apoptosis initiation. Similarly, our findings from this study also proved that the dieckol supplemented MG-63 cells demonstrated augmented ROS accumulation, which could directs to a initiation of apoptosis.

Apoptosis is regulated by highly multifaceted mechanisms in that stimulation of caspases plays a major function (Nagata, 2018). Caspases are the critical players of apoptosis in that caspase-3 is a effector unit occur in the apoptotic cells (Masumura et al., 2000). Our findings clearly proved that the MG-63 cells treated with dieckol exhibited the elevated caspase activity. This could due to the initiation of apoptosis in the dieckol administered MG-63 cells.

The activated caspase-3 could directly trigger the apoptosis and mediate the Bcl family protein like cleaving the Bcl-2. The cleaved products further enhances the caspase stimulation and cell necrosis via the mitochondrial cascades (Zhong et al., 2020). Furthermore, Bcl-2 a anti-apoptotic protein restricts mitochondria from delivering of cytochrome-c, and hold cells viable. Bcl-2 is a apoptosis inhibitor gene and Bax disturbs the reserving property of Bcl-2 on apoptosis through merging with Bcl-2, consequently, enhance the apoptosis (Kroemer, 1997).

The Bcl-2 is a key regulator of cellular apoptosis and has concerned to be altered in array of tumors. Many investigations has highlighted that the Bcl-2 was over-expressed and Bax are less expressed in several human tumors, includes OS to evade the apoptosis. The Bcl-2 cross talks with Bax in order to prevent the dimerization of Bax to inhibit the apoptosis (Khan et al., 2014). More similarly, our findings from this investigation demonstrated the elevated pro-apoptotic protein Bax status and diminished Bcl-2 status in the dieckol administered MG-63 cells. This findings evidenced that dieckol could initiate the apoptosis in MG-63 cells.

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**Fig. 7. Effect of dieckol on the inflammatory markers in the MG-63 cells.** The levels of TNF-α, NF-κB, Cox-2, and IL-6 were appreciably decreased by the dieckol treatment. Values were presented as mean ± SD of triplicates. Data are analyzed by one-way ANOVA and Tukey post doc assay using SPSS software. *p < 0.05 compared with control.

**Fig. 8. Effect of dieckol on the PI3K/AKT/mTOR pathway in MG-63 cells.** Values were presented as mean ± SD of triplicates. Data are analyzed by one-way ANOVA and Tukey post doc assay using SPSS software. **p < 0.05 compared with control.
through triggering pro-apoptotic and hindering anti-apoptotic proteins. PI3K/AKT/mTOR signaling axis is an imperative intracellular pathway that regulate the cell multiplication and metabolism (Engelman et al., 2006). Stimulation of the PI3K/AKT/mTOR cascade participates in the tumor progression and resistance towards anti-cancer therapeutics (Martini et al., 2014). The non-intervention of mTOR expression is connected with various type of cancers. Oncogenic stimulation of mTOR signaling axis via PI3K/AKT phosphorylation potentially participates in the commencement and progression of cancers. The over-stimulation of mTOR expression maintains the growth of tumors via enhancing cell cycle, cell proliferation, and stopping apoptosis via its effect on protein synthesis (Xu et al., 2014).

The PI3K inhibition could lead to both reduced cell multiplication and elevated cell deaths (Hennesy et al., 2005). Similarly, the findings from this study also discovered that the dieckol treatment potentially down-regulated the PI3K/AKT/mTOR pathway. Besides, an earlier report by Perry et al. (2014) has highlighted that the OS cells are reactive to PI3K/mTOR axis inhibition and this cascade is a critical target for the anticancer candidates (Perry et al., 2014). Our current findings were coincides with this previous report. NF-κB is a universally expressed factor capable of controlling manifold of target gene expressions. The NF-κB signaling axis is abnormally stabilized in several human cancers, for instance colon, prostate, breast, thyroid, and bone cancers (Myant et al., 2013; Park et al., 2011). Additionally, NF-κB signaling cascade also seems to cross talk with other signaling axis like AKT signaling (Sizemore et al., 1999). We found that the dieckol treatment also decreased the NF-κB level in the MG-63 cells.

5. Conclusion

In conclusion, our findings provided clear evidence that dieckol effectively inhibited the human OS MG-63 cells growth by triggering the ROS accumulation and apoptosis via down-regulating the PI3K/AKT/mTOR pathway. Dieckol also regulated the proliferative, inflammatory, and apoptotic markers in the MG-63 cells. Our findings recommend that the dieckol could be a possible chemotherapeutic candidate for the OS management in the future. The additional investigations still needs in the future to improve the knowledge on the therapeutic role of dieckol against OS.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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