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The influence of TSA and VPA on the in vitro differentiation of bone marrow mesenchymal stem cells into neuronal lineage cells: Gene expression studies*

Wpływ TSA i VPA na różnicowanie w warunkach in vitro szpikowych mezenchymalnych komórek macierzystych do komórek linii neuronalnej – analiza ekspresji genów

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Epigenetic mechanisms regulate the transcription of genes, which can affect the differentiation of MSCs. The aim of the current work is to determine how the histone deacetylase inhibitors TSA and VPA affect the expression of neuronal lineage genes in a culture of rat MSCs (rMSCs).

Materials and Methods: We analyzed the expression of early neuron marker gene (*Tubb3)*, mature neuron markers genes (*Vacht*, *Th*, *Htr2a)* and the oligodendrocyte progenitor marker gene (*GalC*). Moreover, changes in the gene expression after three different periods of exposure to TSA and VPA were investigated for the first time.

Results: After six days of exposition to TSA and VPA, the expression of *Tubb3* and *GalC* decreased, while the expression of *Th* increased. The highest increase of *VAChT* expression was observed after three days of TSA and VPA treatment. A decrease in *Htr2a* gene expression was observed after TSA treatment and an increase was observed after VPA treatment. We also observed that TSA and VPA inhibited cell proliferation and the formation of neurospheres in the rMSCs culture.

Discussion: The central findings of our study are that TSA and VPA affect the expression of neuronal lineage genes in an rMSCs culture. After exposure to TSA or VPA, the expression of early neuronal gene decreases but equally the expression of mature neuron genes increases. After TSA and VPA treatment ER of the oligodendrocyte progenitor marker decreased. TSA and VPA inhibit cell proliferation and the formation of neurospheres in rMSCs culture.

Keywords: **trichostatin A • valproic acid • mesenchymal stem cells • differentiation, QRT-PCR**

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Abbreviations: **BDNF** – brain derived neurotrophic factor; **ER** – expression ratio; **HDAC** – histone deacetylase; **HDACi** – inhibitors HDAC; **NPCs** – neural progenitor cells; **NSCs** – neural stem cells; **OPCs** – oligodendrocyte progenitor cells; **QRT-PCR** – quantitative reverse transcription polymerase chain reaction; **rMSCs** – rat MSCs; **TSA** – Trichostatin A; **VPA** – valproic acid.

Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that have the ability to differentiate into cells from all three germ layers under appropriate environmental conditions. However, the molecular mechanisms that control the process are not yet fully understood. Identifying the regulators that control differentiation is essential for the development of MSC-based cell replacement therapies for human neurodegenerative diseases and CNS injuries. Epigenetic control has been shown to play an important role in the differentiation of MSCs.

Histones can take one of two antagonist forms – acetylated or deacetylated – and both of these have been shown to be important means of gene regulation [7,8]. Acetylation of the histone tail disrupts the electrostatic interaction between positively charged amino acids from the histone tail and negatively charged phosphatase group in DNA, which leads to nucleosomal relaxation. As a result, the access of transcription factors is easier and transcription is activated [8]. On the other hand, histone deacetylases (HDACs) can stabilize the nucleosomal structures and repress transcription [7]. A previous study demonstrated that histone acetyltransferases can also acetylate transcription factors, thereby signaling proteins and microtubular α-tubulin [20]. The acetylation of proteins is involved in interactions between proteins and protein stability, which regulate a diverse set of functions [15]. HDACs have been shown to regulate many biological processes such as proliferation, differentiation and development by forming complexes with various transcription factors and transcriptional coregulators [9].

Trichostatin A (TSA) is an antifungal antibiotic that is a more specific HDAC inhibitor than VPA [6]. TSA has been shown to block oligodendrocyte differentiation, morphological maturation and myelin gene expression [4]. Moreover, TSA *in vitro* can increase neuronal production from embryonic NSCs [1].

Valproic acid (VPA) is an established drug that is used in the long-term treatment of epilepsy [2]. VPA is a broad-spectrum inhibitor of HDACs that has been shown to mediate neuronal protection through the activation of signaling transduction pathways [5] and through the inhibition of proapoptotic factors [19]. A previous study showed that VPA suppresses the growth and increases the differentiation of different tumor cell lines [2] and induces neural progenitor cells to differentiate into neurons [11].

In our experiment we investigated how TSA and VPA affect the expression of neuronal lineage genes in a culture of rat MSCs (rMSCs). We analyzed the expression of early and mature neuron markers and the oligodendrocyte progenitor marker. Moreover, changes in the expression after three different periods of exposure to VPA and TSA were investigated for the first time.

Materials and methods

Culture of rat MSCs (rMSCs)

rMSCs were isolated from the femurs and tibias of 25 adult Wistar rats (180 - 220 g), as was previously described [25], and then maintained in a Dulbecco's modified Eagle's medium – nutrient mixture F-12 (DMEM/F12; PAA) supplemented with 10% fetal bovine serum (FBS; PAA) and a 1% Antibiotic Antimycotic Solution (100X) (Sigma-Aldrich). Non-adherent cells were removed for the first time after 24 hours and adherent cells were cultured in a 10 ml fresh culture medium. Non-adherent cells were centrifuged at 400 G for 10 min at 4°C and plated in new 25cm^2 tissue-culture flasks in a 10 ml culture medium. Non-adherent cells were removed from both flasks after 24 hours and adherent cells were cultured to the confluent stage. The media were replaced every two to three days. All of the protocols related to the use of animals were approved by the Local Animal Experimentation Ethics Committee in Katowice (Resolutions No 26/2009 and 40/2010).

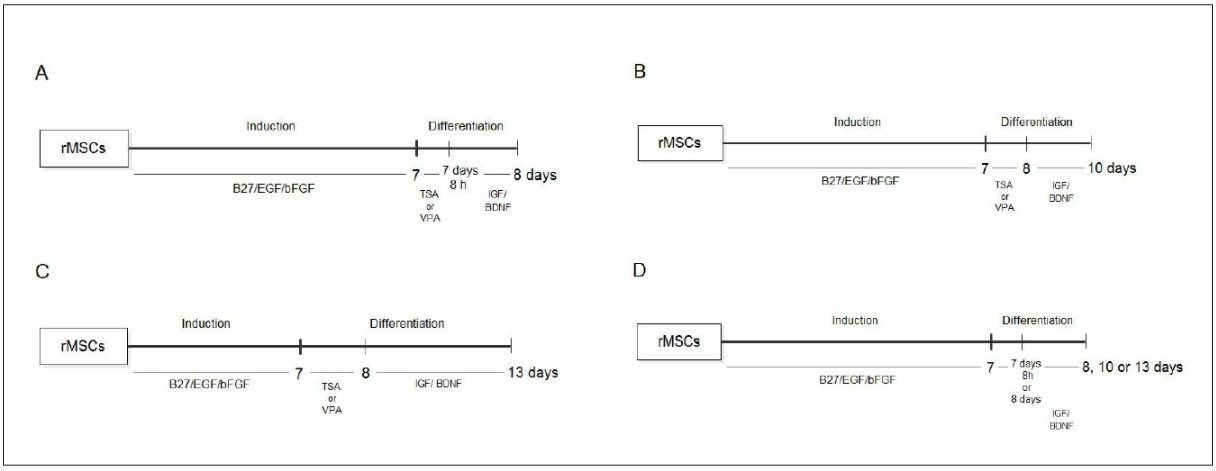

Fig. 1. TSA and VPA treatment schemes (A, B, C). Scheme of culture for control samples (D)

Cell culture conditions

The adherent cells were cultured in a growth medium containing DMEM/F12 supplemented with 10% fetal bovine serum (FBS; PAA), 1% Antibiotic Antimycotic Solution (100X) (Sigma-Aldrich). The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. When the cells reached 80-90% confluency, the cultures were harvested with a Trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA; Sigma-Aldrich). Experiments were performed before the third passage.

Neuronal induction (Sphere stage)

The MSCs were harvested using a Trypsin-EDTA solution and plated in a 25 cm^2 plastic flask at a concentration of 1×10^5 cells/cm^2 in an induction cocktail that contained a DMEM/F12 medium supplemented with 2% B-27 (Gibco), 20 ng/ml recombinant human basic fibroblast growth factor (rhbFGF, Sigma-Aldrich), 20 ng/ml recombinant human epidermal growth factor (rhEGF, Sigma-Aldrich) and 1% Antibiotic Antimycotic Solution (100X). Fifty percent of the induction medium was removed three times a week. After seven days, both non-adherent and adherent sphere structures could be observed. Both types of spheres were dissociated to single cells using a NeuroCult™ Enzymatic Dissociation Kit for Adult CNS Tissue (Mouse and Rat) (StemCell Technologies) according to the manufacturer's protocol.

Neuronal differentiation

Single cells from the spheres were plated on poly-l-ornithine and fibronectine coated (Sigma-Aldrich) 35 mm culture dishes (350,000 per dish). Cells were cultured in a basic differentiation promoting medium that was composed of NeuroCult® NS-A Basal Medium Rat (StemCell Technologies) with a 1% Penicillin Streptomycin Solution (Sigma-Aldrich).

At the beginning of the differentiation process, one of the HDACi (TSA or VPA) was added to the culture. Each HDACi was removed after 8 h from 24 hours' culture (Figure 1A) or was removed after 24 h when the culture takes three days (Figure 1B) or six days (Figure 1C). The final concentrations of HDACi were 10 ng/ml TSA (Sigma-Aldrich) or 0.5 mM VPA (Sigma-Aldrich) and were chosen based on previous reports [6, 13, 16, 18, 21]. Each HDACi affected 8 h or 24 h and after culture medium was supplemented by 20 ng/ml IGF (R&D), 10 ng/ml BDNF (R&D) and 10% differentiation supplement (StemCell Technologies). In the six days' culture, the medium was changed only once (after 3 days). Controls were cultured under the same conditions but without HDACi (Figure 1D). Each experimental protocol was replicated twice in three separate experiments.

Reverse transcription polymerase chain reaction

When cultures were finished, total RNA was extracted from the cells. RNA was extracted using TRIzol® (Invitrogen, Life Technologies) according to the manufacturer›s protocol. The RNA concentration was evaluated using spectrophotometry and the integrity was checked using 1% agarose gel electrophoresis. The QRT-PCR was carried out using 50 ng RNA at a total volume of 20 µl. The PCR cocktail contained 10 µl TaqMan2X Master Mix, 0.5 µl 40X MultiScribe and RNAse Inhibitor Mix and 7.5 µl DEPC-treated water. PCR was performed with 1 µl of the appropriate 20X Target Primers and Probes (Table 1) with the following cycling parameters: 48°C for 30 min. (1 cycle), 95°C for 10 min. (1 cycle) and 40 cycles at 95°C for 30 sec. and 60°C for 30 sec. An additional five minutes of incubation at 72°C after the completion of the last cycle was also done. All components of the PCR cocktail were produced by Applied Biosystems, Life Technologies. QRT-PCR was performed in duplicate for all of the samples from three independent experiments. Moreover, a negative control (without RNA) and a positive control (total RNA isolated from adult rat hippocam-

pus) were performed. The β-actin (housekeeping gene) expression level was analyzed in each RNA extract The amplimers were electrophoresed through a 6% polyacrylamide gel stained with ethidium bromide and analyzed and registered under ultraviolet light using a gel documentation system. QRT-PCR was carried out using an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Life Technologies). mRNA expression is presented as the expression ratio (ER), which was calculated as follow: The ΔCt of each sample equals the Ct of the target gene minus the Ct of the endogenous control. ΔΔCt equals the ΔCt of the sample for the target gene minus the ΔCt of the control sample [17]. ER was calculated as $2^{-\Delta\Delta Ct}$.

Table 1. List of the primers that were used in QRT-PCR

Gene name	Accession number	ID Primers (Life Technologies)	Product size [bp]
Tubb3 (tubulin, beta 3 class III)	NM_139254	Rn01431594_m1	68
GalC (Galactosylceramidase)	NM_001005888	Rn01517759	71
VAChT (Vesicular acetylcholine transporter)	NM_031663	Rn01450312_s1	91
Th (Tyrosine hydroxylase)	NM_012740	Rn00562500_m1	60
Htr2A (5-hydroxytryptamine (serotonin) receptor 2A)	NM_017254	Rn00568473_m1	71
β-actin	NM_031144	Rn00667869_m1	91

Statistical analysis

Each treatment was replicated on three 35 mm Perti dishes from three separate experiments. QRT-PCR was performed in duplicate. Data are presented as the means ± SEM of separate experiments. All of the results were statistically analyzed using MS Excel 2007 software and the Graph Pad Prism version 4.0 software tool. The relative expression data that was obtained from the QRT-PCR were normalized to β-actin using the comparative method of relative quantification ($2^{-\Delta\Delta Ct}$) [17]. The statistical significance of any changes in the ER values (an average fold-change in gene expression) for the mRNA of each gene compared to the control cultures was evaluated with one-way ANOVA using the post hoc Newman-Keuls test. The differences were accepted as statistically significant at $p < 0.05$.

Results

Neuronal lineage gene expression level in cells that were treated with 10 ng/ml TSA

The *Tubb3* and *GacC* expression were up-regulated after three days and down-regulated after six days of TSA treatment (ER for *Tubb3*: 0.7 at day 1, 1.4 at day 3 and 0.3 at day 6; ER for *GalC*: 1.4 at day 1, 2.0 at day 3 and 0.2 at day 6). *Th* was detected for the first time at day 3 (ER = 0.5) and slightly increased until day 6 (ER = 0.8). *VAChT* increased between day 1 (ER = 0.6) and day 3 (ER = 3.0) and remained at the level of ER = 3.0 until day 6. A down-regulation of the expression level of Htr2a mRNA from day one (ER = 1.4), through day 3 (ER = 1.3) to day 6 (ER = 0.7) of the exposition was observed (Figure 2).

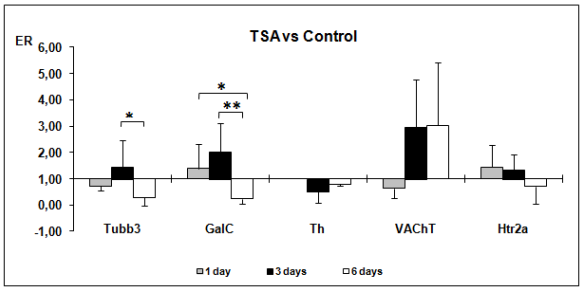

Fig. 2. Expression ratio of neuronal lineage markers after three different schemes of TSA treatment. Early neuronal marker (Tubb3), immature oligodendorcytes marker (GalC) as well as markers for dopaminergic neurons (Th), cholinergic neurons (Vacht) and serotoninergic neurons (Htr2A) were analyzed by QRT-PCR. Th gene expression was not noticed after one day of differentiation. The data are presented as a expression ratio of three independent experiments (each in triplicate). Error bars indicate standard error of the mean; *statistically significant $p < 0.05$; **statistically significant $p < 0.01$; ANOVA followed by post hoc Newman-Keuls test

Neuronal lineage gene expression level in cells that were treated with 0.5 mM VPA

The *Tubb3* expression level gradually decreased (3.0 at day 1, 2.5 at day 3 and 0.7 at day 6). The expression level of the oligodendrocyte progenitor marker *GalC* decreased insignificantly on the third day of culture in the differentiation promoting conditions (0.9 at day 1 and 0.8 at day 3) compared to the control culture. On the sixth day of culture in the differentiation promoting conditions, we noted a statistically significant decrease in the expression level of *GalC* (ER = 0.1). The expression of *Th* increased from the first day of culture in the differentiation promoting conditions compared to the control cultures. There was a statistically significant increase between the first (ER = 0.3) and the sixth (ER = 0.8) day of culture in the differentiation promoting conditions, but the expression level of *Th* did not reach the values that were observed in the control. The expression level of *VAChT* increased on day 3 (ER = 2.6; $p < 0.05$) and decreased on day 6 of the exposition (ER = 0.9). The expression level of *Htr2a* gradually increased from the first to the sixth day of culture in the differentiation promoting conditions (1.5 at day 1, 1.8 at day 3 and 1.7 at day 6) (Figure 3).

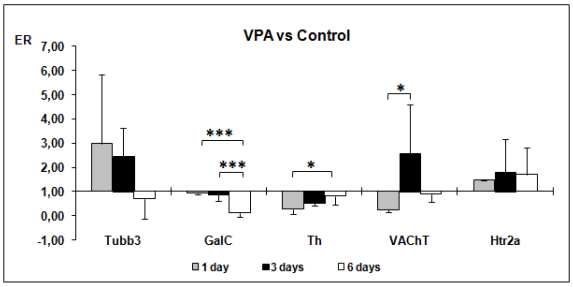

Fig. 3. Expression ratio of neuronal lineage markers after three different schemes of VPA treatment. Early neuronal marker (Tubb3), immature oligodendorcytes marker (GalC) as well as markers for dopaminergic neurons (Th), cholinergic neurons (Vacht) and serotoninergic neurons (Htr2A) were analyzed by QRT-PCR. The data are presented as a expression ratio of three independent experiments (each in triplicate). Error bars indicate standard error of the mean; *statistically significant $p < 0.05$; **statistically significant $p < 0.01$; ANOVA followed by post hoc Newman-Keuls test

Discussion

The first extremely important stage of our experiment was the neuronal induction, which should have reprogrammed the MSCs and regulated their pluripotency. In a previous experiment, we investigated whether the induction stage is the key to directing the maximal number of cells to a specific type of differentiation [3] but this method is not an efficient way to achieve functional neurons.

In order to strengthen the induction and differentiation efficiency, we tried to modify the epigenetic mechanism and inhibit histone deacetylase by using two HDACi, in the differentiation. In the previous study Sun et al. (2011) investigated that HDACi enhances the efficiency of reprogramming and plays an important role in inducing pluripotent stem cells [24]. HDACi induced neuronal differentiation and upregulated neuronal-specific genes, such as *NeuroD* [1,11]. Overexpression of *NeuroD* resulted in the induction of neuronal differentiation but the suppression of glial differentiation [11].

In this study, the oligodendrocyte progenitor marker *GalC* was up-regulated after three days and down-regulated after six days of TSA treatment and it was increasingly down-regulated after VPA treatment. A statistically significant reduction of *GalC* expression level was observed after six days of treatment with each HDACi. Our results confirm the results of a previous study conducted by Marin-Husstege et al. (2002), where HDACi blocked oligodendrocyte differentiation. Previous reports investigated whether TSA prevents the progression of progenitors into mature oligodendrocytes in rats [18] and humans, although human oligodendrocyte progenitor cells (OPCs) appear to be more sensitive to HDAC inhibition than their rodent equivalents [4]. TSA-treatment is responsible for the exit of cells from the cell cycle [18], blocked oligodendrocyte cell differentiation, a reduction of oligodendrocyte morphological maturation and the downregulation of myelin basic protein mRNA [4]. The authors concluded that histone deacetylation is a necessary component of oligodendrocyte [18] and astrocyte differentiation although the role of HDACs on oligodendrocyte development may differ between species [4]. A previous study found that activating HDAC1 and HDAC2 promoted oligodendrocyte development [22, 26], while the activation of HDAC3 repressed oligodendrocyte differentiation [4]. We determined that VPA down-regulated the *GalC* gene expression more strongly. Our result is compatible with an existing study that found that among the HDAC inhibitors, VPA showed the most potent effect and enhanced reprogramming efficiency more than 100-fold [12].

In our experiment the *Tubb3* expression was up-regulated after three days, down-regulated after six days of TSA treatment and gradually decreased in the differentiation process after VPA treatment. *Tubb3* is *inter alia* an early neuronal marker that is highly expressed in MSCs after induction [3]. In the process of differentiation, the expression of early neuronal markers (e.g., *Tubb3*) should decrease while the expression of mature neuron markers (e.g., *Gad65/67*, *Th*, *VAChT*, *Htr2a*) should increase. The following dependence was observed in some cases in this experiment. In the differentiation process *Th* gene expression slightly increased after TSA treatment until day 6. *VAChT* also increased between day 1 and day 3 and remained at a similar level until day 6. Furthermore, the expression of *Th* increased from the first day of culture in the differentiation promoting conditions and the expression of *Htr2a* gradually increased from the first to the sixth day of the exposition after VPA treatment. Dependences that were slightly different than expected were observed after TSA treatment when the *Htr2a* expression gradually decreased and after VPA treatment when the expression level of *VAChT* increased at day 3 and decreased at day 6 of exposition.

In a previous experiment in which adult subventricular zone precursor cells were treated with second-generation iHDAC, an increase of β-III-tubulin positive neurons was observed [23]. The range of the increase was from 258% - 431% and was dependent on the iHDAC concentration. The most pronounced increase in neuronal differentiation was found after 24 h and 48 h of treatment [23]. In our experiment we observed the same dependence. The *Tubb3* gene expression was higher after three days than after long-term exposure to each HDACi. Hsieh et al. (2004) showed a large increase in Tuj1 and MAP2ab positive neurons in a VPA-treated culture of neural progenitor cells [11]. Neuronal differentiation by VPA appeared to be dose-dependent. After a 0.3 mM VPA treatment, the authors observed ~20% Tuj1 (+) cells and ~38% MAP2ab (+) cells but after a 1 mM VPA treatment ~55% Tuj1 (+) cells and ~60% MAP2ab (+) cells were observed. Higher VPA concentrations (> 3 mM) resulted in increased cell death [11]. In the same experiment, the authors found that neural progenitors that overexpres-

sed *NeuroD* differentiated into > 75% Tuj1 and MAP2ab positive neurons, respectively, and reduced their ability to differentiate into oligodendrocytes and astrocytes under appropriate conditions [11]. Treatment with 100 nM TSA promoted the neural differentiation of neural progenitor cells. After two days of culture, ~60% cells were MAP2ab positive [11]. Another study found that rat forebrain stem cells increased the number and percentage of β-III-tubulin immunopositive neurons by up to fivefold, increased neurite outgrowth and decreased the number of astrocytes by fivefold without changing the number of cells after six days of VPA exposure. Most of the newly formed neurons were GABA-ergic, as was shown by a 10-fold increase in the neurons that were immunostained for GABA and the GABA- synthesizing enzyme GAD65/67 [16].

We observed that a 10 ng/ml TSA treatment promotes an increase in the *VAChT* expression and a previous report showed that TSA promotes the cholinergic differentiation of mouse neural stem cells (NSCs) as well. The above-mentioned report found that the relative expression of *ChAT* mRNA was 2 times higher after 24 h of TSA treatment. The researchers determined that less than 1% of the mouse NSC-derived neurons showed a GABA-ergic or dopaminergic phenotype with or without TSA exposure. However, 15% of NSC-derived neurons were cholinergic and this number increased to 25% when the dosage of TSA was increased from 1 ng/ml to 10 ng/ml [1]. The same report showed that the inhibition of HDACs by TSA increased neuronal differentiation and induced electrophysiological maturation derived neurons. TSA not only affected the percentage of neuronal differentiations but also the morphology of the neurons. The TSA-treated cultures developed significantly longer dendrites and significantly more branching points, thereby resulting in a larger dendritic area [1]. Kim et al. (2003) reported that treatment with TSA can induce Th promoter activity in both neural and non-neural cell lines [14]. After TSA treatment, ER for *Th* decreased in comparison with the control. Other researchers were unable to detect dopaminergic neurons after histone hyperacetylation in the mouse embryonic NSCs as well [1].

In order to strengthen the efficiency of differentiation HDACi 20 ng/ml BNDF and 20 ng/ml IGF were added to the culture. In the experiment of Laeng et al. (2004), the addition of 25 ng/ml BDNF approximately doubled the number of neurons [16] and the addition of IGF induced oligodendrocyte differentiation [10].

Conclusions

The central findings of our study are that 1) TSA and VPA affect the expression of neuronal lineage genes in an rMSCs culture, 2) after exposure to TSA or VPA, the expression of early neuronal gene decreases but equally the expression of mature neuron genes increases 3) after TSA and VPA treatment ER of the oligodendrocyte progenitor marker decreased. Our differentiation protocols were not so efficient but these results suggest that after further optimization, HDACi may be useful in the preparation of rMSCs for transplantation.

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The authors have no potential conflicts of interest to declare.