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Original Article

Vapreotide regulates neuronal ATPase activity

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ABSTRACT

Vapreotide is a somatostatin peptide analogue, which is commonly used for the treatment of oesophageal varicose veins in liver diseases. We study the effect of different concentrations of vapreotide on the ATPase activity of the cerebral and cerebellar membranes. Vapreotide 10^{-6} M produced the inhibition of Ca^{2+}-ATPase activity, but not of other enzyme activities, suggesting a selective response according to the brain area and the preparation used as an enzyme source. Vapreotide at 10^{-9} to 10^{-6} M concentrations produced an inhibitory effect on Na^+, K^+-ATPase, resulting in an inhibitory concentration 50 (IC50) equal to 5.7×10^{-9} M and no changes in Mg^{2+}-ATPase activity. Commercial enzymatic preparation of the pig cerebral cortex used as an enzyme source also produced an inhibitory enzyme effect. The vapreotide inhibitory effect was abolished by the addition of cSSTA, a somatostatin receptor antagonist. Besides, in crude membranes of cerebral cortex vapreotide produced [^{3}H]-ouabain binding inhibition by direct interaction with the ouabain site (close to K^+ site) in the enzyme Na^+, K^+-ATPase. The previous administration of the NOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME), increased [^{3}H]-ouabain binding. In this experimental condition, vapreotide could not displace many ouabain molecules bound with high affinity to its site. All these experiments provided evidence that ATPase could be one of the target sites for the effects of vapreotide at the neuronal level.

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KEYWORDS: ATPases, Na^+, K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase, vapreotide, somatostatin analogue, somatostatin system.

INTRODUCTION

Vapreotide is a somatostatin analogue with higher metabolic stability than somatostatin, the parent hormone. Vapreotide reduces the splanchnic blood flow, which is a benefit to patients affected by liver disease. Like somatostatin, vapreotide inhibits growth hormone release and the release of peptides and vasoactive compounds from neuroendocrine tumours [1]. Vapreotide is a neurokinin-1 receptor antagonist producing a therapeutic analgesic effect [2]. Immuno-cytochemical, electrophysiological and biochemical studies have suggested neurotransmitter and neuromodulator behaviours at CNS. Vapreotide can interact with somatostatin receptors, mainly subtypes 2 and 5, inducing actions in the central and peripheral nervous systems [3].

Na^+, K^+-ATPase activity is involved in membrane potential and cell volume regulations, transmembrane fluxes of Ca^{2+} and excitatory neurotransmitters, normal cell cycle, and CNS differentiation. Na^+, K^+-ATPase extrudes 3 Na^+ and accumulates 2 K^+ per molecule of ATP hydrolysed, constituting the most relevant active transport system in the mammalian organisms. This enzyme activity is responsible for the maintenance of the electrochemical gradient essential in the synaptic transmission [4]. ATPase activities are determined by measuring ATP hydrolysis [5]. The difference between total ATPase activity and Mg^{2+}-ATPase activity corresponds to Na^+, K^+-ATPase activity.

The roles of sodium and potassium ions have been studied and are perfectly defined in the Na^+, K^+-ATPase catalytic cycle. Magnesium interactions have also been studied using fluorescence techniques to determine Mg^{2+}-ATPase functioning [6]. Ca^{2+}-ATPase regulates the amount of intracellular calcium to remove calcium ions from the cell. There is an electrochemical gradient across the plasma membrane that facilitates calcium entry into the cells. Therefore, Ca^{2+}-ATPase activity results principally in the maintenance of low calcium concentrations required for adequate cell signalling [7].

Different types of membrane preparations were used to achieve a better understanding of peptide and enzyme interactions. We performed the study of vapreotide effects on ATPase activity in synaptosomes. These structures contain a complete presynaptic terminal along with the postsynaptic membrane and postsynaptic density. In general, synaptosomes are derived from axosomatic or axodendritic synapses [8]. Enzyme assays were also carried out on the synaptosomal membranes. They were obtained from a purification process of synaptosomes and composed by the pre- and post-synaptic membranes [9]. Besides, adenosine 5'-triphosphatase is a lyophilized commercial enzyme from porcine cerebral cortex used to evaluate Na^+, K^+-ATPase activity. The crude membranes were used for [^{3}H]-ouabain binding assays because the radionuclide shows a high affinity for the ouabain site. Therefore, the preparation of an excessively purified membrane fraction was not necessary. Ouabain site (close to K^+ site) in Na^+, K^+-ATPase is a regulatory site for enzyme activity [10].

Nitric oxide (NO) is involved in the central noradrenaline and dopamine release, memory and learning processes, and pathologies such as schizophrenia, depression, or bipolar disorder [11]. The relationships between nitric oxide and ouabain are studied in a heart failure model, these results showing that low doses of ouabain increases nitric oxide production by PI3/Akt-dependent pathways. Since nitric oxide is a gaseous molecule, the feasible way to understand its effects is the nitric oxide synthase (NOS) inhibition, an enzyme involved in NO synthesis. L-NAME (Nω-Nitro-L-arginine methyl ester) behaves as a NOS inhibitor, but in this case, L-NAME is a substance involved in the increase of the ouabain binding and a useful tool to study the interactions between vapreotide and the ouabain site [12].

MATERIALS AND METHODS

Reagents

Vapreotide, Nω-nitro-L-arginine methyl ester (L-NAME), cyclo-(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr [BZL] (cSSTA), ouabain, disodium ATP (grade I) and Adenosine 5'-triphosphatase of cerebral cortex from porcine were obtained from Sigma Chemical Co., St. Louis, MO, USA. BCS. The biodegradable counting scintillant liquid was from Amersham Biosciences, UK, and [^{3}H]-ouabain (Specific radioactivity 0.932 TBq/mmol) was from New England Nuclear, Du Pont, Boston Ma, USA.

Animals

Male Sprague-Dawley rats (120-150 g) were used in this work. The studies described were conducted according to the Guide for Care and Use of Laboratory Animals provided by the National Institutes of Health, EE. UU and the Committee on Animal Experimentation, Universidad de Buenos Aires.

All animals were housed in a light-controlled and temperature-controlled facility, with free access to food and water.

Crude membrane preparation

Cerebral cortices were pooled in 0.32 M sucrose (neutralized to pH 7.0 with Tris base) and centrifuged at 900 g for 10 min; the supernatant was spun down at 100,000 g for 30 min in an L90-Beckman ultracentrifuge. After this procedure, the supernatant discarded, and sediment was stored in the freezer at -70 °C. Before use, the sediment was resuspended in redistilled water to achieve 10 mg protein per ml concentration and used for [3H]-ouabain binding assay.

Isolation of synaptosomes and synaptosomal membranes

Cerebral cortices were pooled in 0.32 M sucrose (neutralized to pH 7.0 with Tris base) and centrifuged at *900 g* for 10 min; supernatants

were spun down at *11500 g* for 20 min. Crude mitochondrial pellet was resuspended in 5 ml of sucrose and layered on top of a gradient containing Percoll 8% and 18%. This gradient was centrifuged at *60000 g* for 30 min in a rotor SW 40 of L90 Beckman ultracentrifuge. Synaptosomes were separated by aspiration, diluted with 0.32 M sucrose, and spun down at *100000 g* for 40 min in a rotor 70.1 Ti of L40 Beckman ultracentrifuge. The crude mitochondrial pellet was resuspended in 5 ml of sucrose 0.32 M to prepare synaptosomal membranes and layered on the top of a gradient containing 0.8, 0.9, 1.0, 1.2 M sucrose. Then, the fraction at 1.0 M sucrose level was separated, diluted with 0.16 M sucrose, and spun down at *100000 g* for 30 min in a rotor 70.1 Ti of L40 Beckman ultracentrifuge.

Enzyme assays

ATPase activity was measured as was described by Albers (1965) [13]. This test consists of a pre-incubation period of 10 min at 37 °C during the vapreotide or 0.20 M Tris-HCl buffer (pH 7.4) (controls) is added, followed by an incubation period at the same temperature. Further experiments required the previous addition of 10^{-8} M cSSTA. during the pre-incubation period. Total ATPase activity was assayed in a medium containing 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 0.20 M Tris-HCl buffer (pH 7.4) and 4 mM ATP. The Mg^{2+}-ATPase activity was determined in a similar medium but with the addition of 1 mM ouabain. The difference between total ATPase and Mg^{2+}-ATPase activities corresponds to Na^+, K^+-ATPase. This activity was monitored by the spectrophotometric determination of orthophosphate released [5]. Ca^{2+}-ATPase was assayed in a medium containing 2.6 mM $CaCl_2$, 0.16 M Tris-HCl buffer (pH 7.4) and 4 mM ATP [14].

Specific [^{3}H]-ouabain binding assay

[^{3}H]-ouabain binding was carried out by a filtration assay [15]. The binding was performed in a medium (0.5 ml final volume) consisting of 3 mM $MgCl_2$, 2 mM H_3PO_4, 0.25 mM sucrose, 0.25 mM EDTA, 30 mM imidazole-HCl buffer (pH = 7.4), 250 μg cerebral cortex membrane protein, 45 nM [^{3}H]-ouabain, and 10^{-6} M somatostatin. After incubation at 37 ºC for 60 min, samples were filtered under vacuum using GF/B filters positioned in a Millipore multifilter and rinsed twice with 2 ml of ice-cold 30 mM imidazole-HCl buffer, pH 7.4. The filters were transferred to vials containing 10 ml of counting scintillant liquid. The radioactivity was quantified by a Beckman Coulter-LS 6500 scintillation counter with 64% efficiency.

Specific binding is calculated as the difference between total binding and non-specific binding determined in the presence of 100 μM unlabelled ouabain. Non-specific binding accounted for less than 10% of total membrane-bound radioactivity.

Protein content in the membrane fractions was determined by the method of Lowry *et al.* [16], using bovine serum albumin as standard.

Data analysis

Data for ATPase activities were expressed as μmoles Pi released per mg protein per hour or the percentage of enzyme activity (mean ± SD). The membrane preparation without peptide addition represented control membranes. The comparisons between experimental data obtained with control and drug-treated membranes were performed using the one-sample or two-tailed Student 't' test.

A probability level of $*P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Since ATPase activity plays the principal role in neurotransmission, many substances can regulate this activity. Thus, this work aimed to study somatostatin analogue modulation on neuronal ATPase activity. Different mechanisms may inhibit Na^+, K^+-ATPase activity, such as an excess of sodium content or a direct interaction with the ouabain site. [17]. Both neuropeptides and classical neurotransmitters have a high affinity for their specific plasma membrane receptors close to targets where ATPase is inserted. These macromolecules are most likely localized contiguously and may well interact in the synaptic membranes. Thus, the vapreotide interaction with somatostatin receptors could produce an allosteric modulation that induced NA^+, K^+-ATPase inhibition.

Herein, "*ex vivo*" and "*in-vitro*" assays were performed to characterize vapreotide effects on neuronal ATPase activity. The synaptosomes or

"pinched-off nerve endings," are formed when lipid bilayers naturally reseal together after the physical shearing force of homogenization tears off the axon terminals. Synaptosomes contain the complete presynaptic terminal, the postsynaptic membrane, and the postsynaptic density [18]. We used them to evaluate Na^+, K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase activities of rat cerebral cortex since the synaptosomes have molecular machinery for the release, uptake, and storage of neurotransmitters, processes in which ATPase activity is involved. Vapreotide slightly inhibited Na^+, K^+-ATPase, but this analogue did not modify Ca^{2+}-ATPase or Mg^{2+}-ATPase activities on rat cortical synaptosomes. However, the addition of vapreotide 10^{-6} M to synaptosomes from rat cerebellum produced a significant inhibition of Ca^{2+}-ATPase activity suggesting that vapreotide effect on this enzyme activity could depend on the brain area used as an enzyme source (Figure 1).

Vapreotide at the range of concentration 10^{-9} to 10^{-6} M produced inhibition of 2-26% on Na^+, K^+-ATPase activity, with an inhibitory concentration 50 (IC50) equal to 5.7×10^{-9} M. Vapreotide failed to modify Mg^{2+}-ATPase activity using rat cortical synaptosomal membranes, as the enzyme source. Basal values recorded in the absence of vapreotide were 49.2 ± 6.6 and 12.10 ± 6.26 μmoles Pi released per mg protein per hour for Na^+, K^+-ATPase and Mg^{2+}-ATPase activities (means ± SD, n = 6), respectively (Figure 2).

We subsequently conducted experiments in the presence of the partial agonist cSSTA in a concentration in which cSSTA behaves as an antagonist. Vapreotide inhibitory effect on Na^+, K^+-ATPase activity was prevented by cSSTA after 5 minutes of preincubation in buffer containing cortical synaptosomal membranes. Since somatostatin receptors are sites of high affinity for peptide binding, it was feasible that the vapreotide interaction with them led to conformational changes in the enzyme that result in Na^+, K^+-ATPase inhibition (Figure 3).

Adenosine 5'-triphosphatase is a lyophilized commercial enzyme from porcine cerebral cortex used to assess Na^+, K^+-ATPase activity regulated

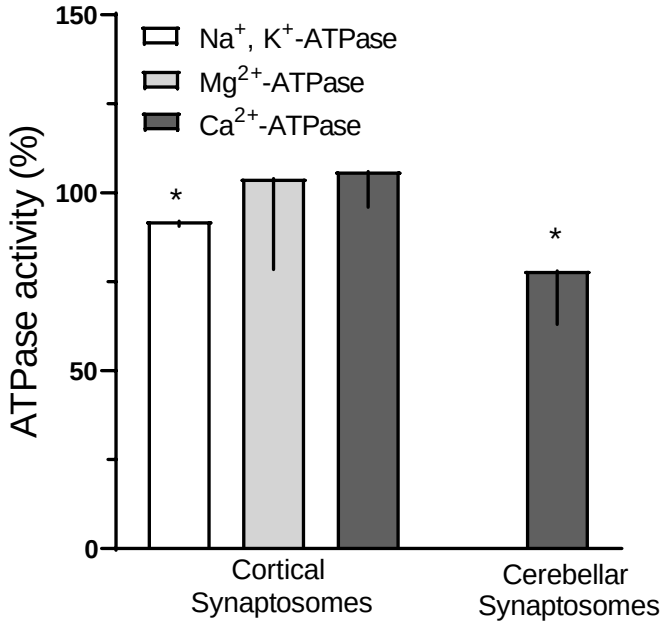

Figure 1. Effect of vapreotide on Na^+, K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase activities. Cortical and cerebellar synaptosomes were preincubated in the absence (control) or presence of the vapreotide 1.0×10^{-6} M in the test for ATPases. Results are expressed as a percentage of enzyme activity taking 100% values obtained for each assay in the absence of vapreotide. SD of five experiments performed in triplicate is indicated within the bars. *$P < 0.05$ with respect to the control synaptosomes using one-sample Student 't' test.

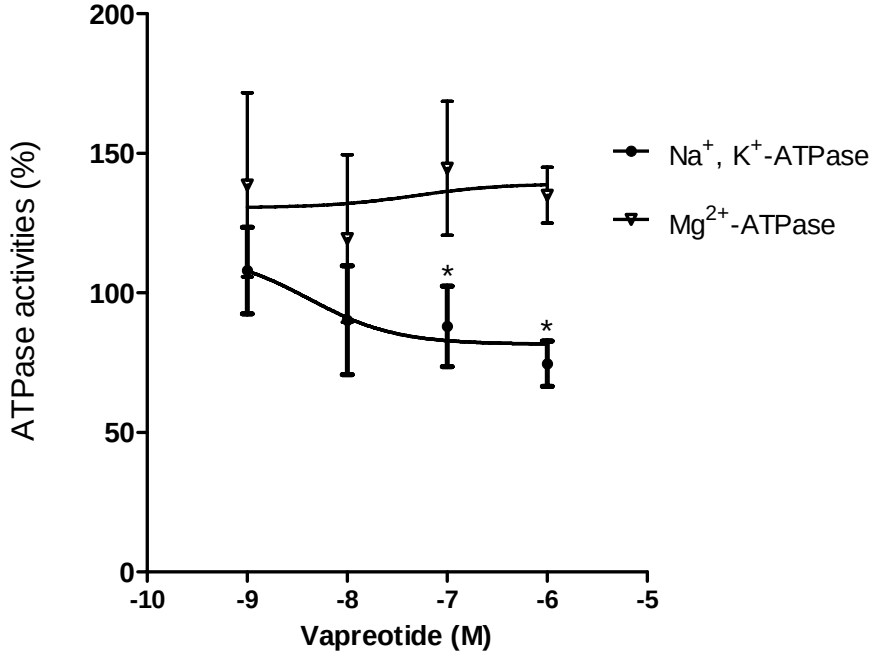

Figure 2. Vapreotide effect on A) Na^+, K^+-ATPase activity B) Mg^{2+}-ATPase activity. Cortical synaptosomal membranes were preincubated in the absence (control) or presence of 1.0×10^{-9} M to 1.0×10^{-5} M vapreotide and assayed for ATPases. Results are expressed in activity percentage (±SD) of five experiments performed in triplicate. *$P < 0.05$ with respect to the control membranes, by one sample Student *'t'* test.

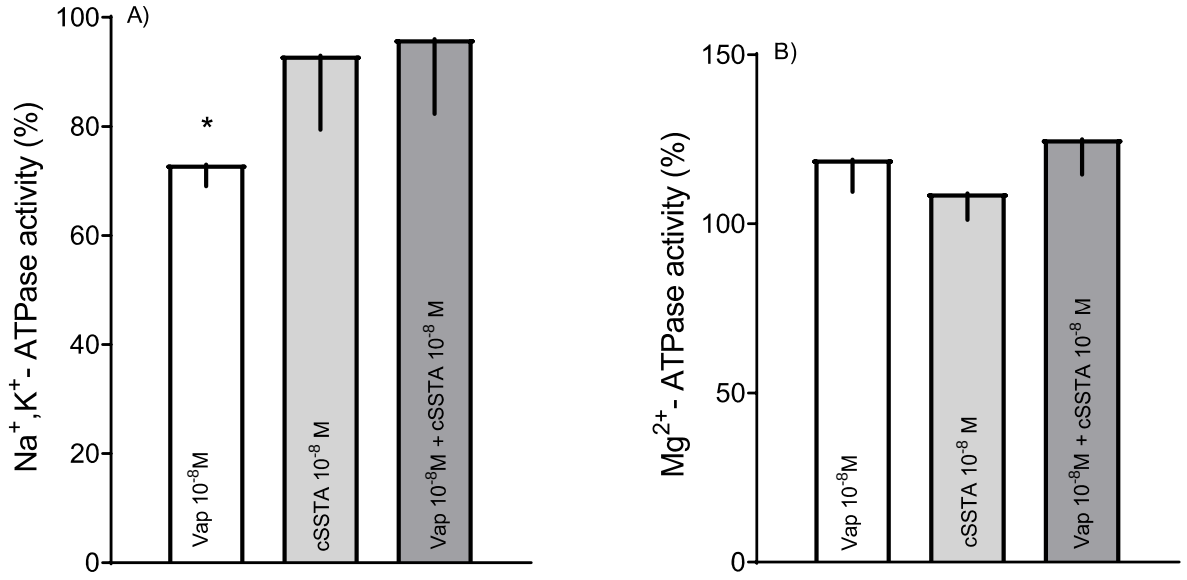

Figure 3. A) Na^+, K^+-ATPase activity and B) Mg^{2+}-ATPase activity were recorded in the absence (control) or presence of vapreotide and cSSTA, a somatostatin receptors antagonist. Synaptosomal membranes from the cerebral cortex of adult male rats were preincubated for 5 min with or without cSSTA, followed by incubation with 10^{-8} M vapreotide, for further a 5-min period. Values for four experiments performed in triplicate are expressed as the percentage of enzyme activity (±SD), taking 100% data obtained without peptide addition. *$P < 0.05$ with respect to the control, using one-sample Student 't' test.

for different substances. In this case, the addition of 1.0×10^{-6} M vapreotide produced a 27.5% Na^+, K^+-ATPase inhibition (data not shown). Considering that this preparation contains a purified enzyme, vapreotide could directly interact with Na^+, K^+-ATPase; for this reason, it was of interest to assay [^{3}H]-ouabain binding as a method to study the vapreotide interaction with the enzyme.

Rats injected with 10 mg/kg L-NAME or saline solution (control), were used to prepare crude cortical membranes for [^{3}H]-ouabain binding. The crude membranes are enough for the binding assay considering the high affinity that ouabain has for its site in Na^+, K^+-ATPase enzyme. In the control membranes, the presence of vapreotide 1.5×10^{-6} M produced [^{3}H]-ouabain binding reduction of roughly 30%, but at the same concentration, the peptide analogue did not modify the [^{3}H]-ouabain binding in the membranes from animals acute administered with L-NAME. It is known that the ouabain site exists in equilibrium in two conformational states [10]. Acute treatment with L-NAME could displace the balance towards a high-affinity state for ouabain, favouring its binding during the assay. In this experimental condition, vapreotide could not compete with many ouabain molecules bound to its site, which would suggest a low-affinity interaction with the ouabain site (Figure 4).

In this work, we have tried to demonstrate the importance of carrying out enzymatic tests in different membrane preparations to achieve a better characterization of vapreotide effects on neuronal ATPase activity.

CONCLUSION

The different membrane preparations made it possible to evaluate vapreotide effects on neuronal ATPase activity. Thus, synaptosomes were useful to test Ca^{2+}-ATPase activity in the cerebellum.

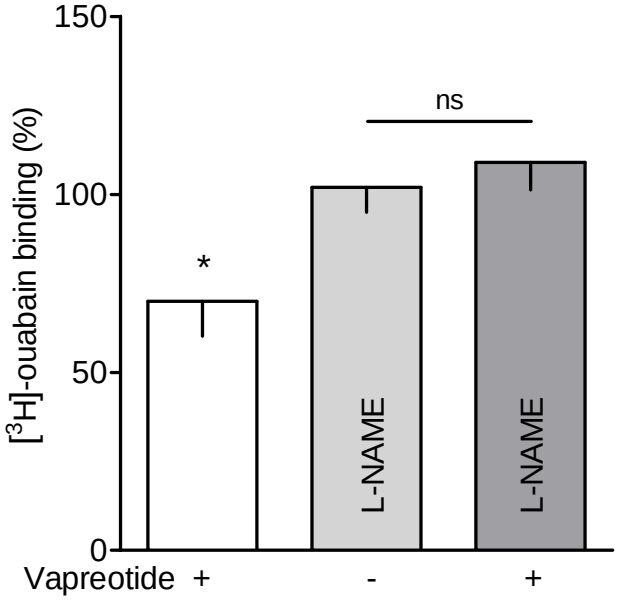

Figure 4. White bar shows [^{3}H] ouabain binding performed in the presence of vapreotide 1×10^{-6} M added to crude membranes. Results are expressed as percentage [^{3}H] ouabain binding taking 100% values obtained for each assay without vapreotide addition. SD of five experiments performed in triplicate is indicated within the bar. *$P < 0.05$ with respect to the membranes without the vapreotide addition, using one-sample Student *'t'* test.

In the *ex-vivo* experiments, represented using grey bars, 10 mg/kg L-NAME was administered intraperitoneally to adult rats before preparing the crude membranes. [^{3}H] ouabain binding was performed in the presence or absence of vapreotide 1×10^{-6} M added to membranes. SD of four-five experiments performed in triplicate is indicated within bars. *$P < 0.05$ with respect to the membranes without vapreotide addition, using two tailed Student *'t'* test, ns: non-significant difference.

The synaptosomal membranes were helpful to evaluate Na^+, K^+-ATPase activity that was inhibited by the vapreotide addition. Neuropeptides and classical neurotransmitters have a high affinity for their specific plasma membrane receptors probably located very close to sites where ATPase is inserted. The vapreotide binding to somatostatin receptors could produce an allosteric effect that inhibited ATPase activity. Finally, the ouabain binding made possible the knowledge about the direct interaction of Na^+, K^+-ATPase with the vapreotide.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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