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Kava and its Kavalactones Inhibit Norepinephrine-induced Intracellular Calcium Influx in Lung Cancer Cells

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ABSTRACT

Kava, the extract of the roots of *Piper methysticum*, has been traditionally consumed in the South Pacific islands for its natural relaxing property. Epidemiological data suggests that kava consumption may reduce human cancer risk, and *in vitro* and *in vivo* models suggest chemopreventive potential against carcinogen-induced tumorigenesis. Therefore, knowledge about its molecular mechanisms and responsible ingredient (s) for these beneficial properties will better guide kava's use for the management of these disorders. Psychological stress typically results in increased production of stress hormones, such as norepinephrine (NE), which activate adrenergic receptors (ARs). Psychological stress has also been associated with increased cancer incidence and poor clinical outcomes in cancer patients. Mechanistically, binding of NE to ARs induces intracellular calcium influx, which activates downstream signaling pathways involved in both stress and cancer development. In this study, we characterized the effect of kava and its components, 3 fractions and 6 major kavalactones, on NE-induced intracellular calcium influx in H1299, a human non-small cell lung carcinoma cell line. Results show that kava extract effectively inhibits NE-mediated intracellular calcium influx in H1299 cells, potentially through antagonizing β-AR signaling. This inhibitory activity is recapitulated by the major kavalactones in kava. Among the 6 major kavalactones, DHK demonstrated the best potency. Taken together, our study suggests a novel mechanism through which kava and its ingredients potentially offer the anxiolytic and cancer-preventive activity.

Introduction

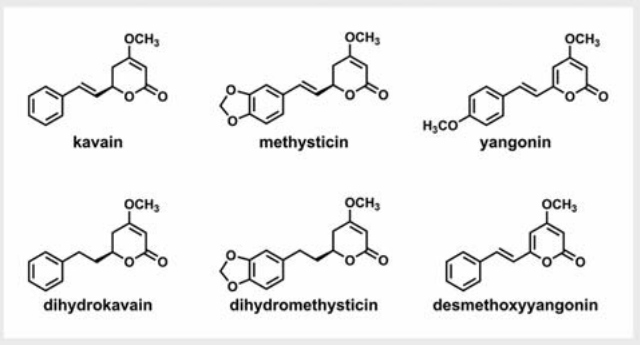
Lung cancer is the leading cause of cancer-related deaths worldwide and in the U. S. A. with a 5-year survival rate of less than 20 % for decades [1, 2]. Therefore, development of novel avenues that can prevent this deadly disease is of urgent need. In addition to tobacco exposure, psychological stress has been associated with increased lung cancer risk [3, 4]. Psychological stress was also found to be a significant predictor of lung cancer mortality [5]. Furthermore, epidemiological studies indicated extended lung cancer survival associated with antidepressant use [6]. These observations overall suggest the potential causality link between psychological stress and lung cancer risk/outcome, which has not been extensively explored in lung cancer management.

Psychological stress is best known to trigger the release of neuronal hormones, such as NE, that mediate the "fight-or-flight" response of the sympathetic nervous system [7]. The released NE, upon binding to the ARs, induces intracellular calcium influx and activates various oncogenic signals, which have been implicated in cancer development and progression, including enhanced proliferation, resistance to therapies, and development of metastasis [8–11]. Therefore, agents that can inhibit NE-mediated intracellular calcium influx represent potential opportunities for the management of mental stress and cancer.

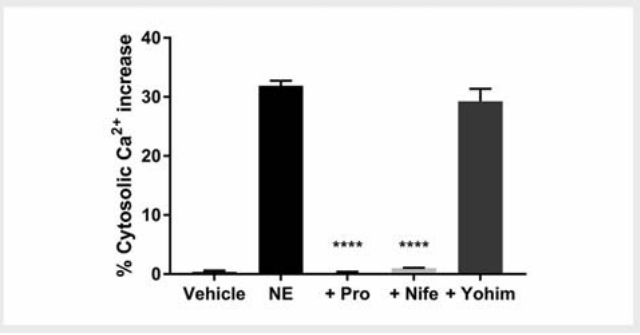
Kava, an extract of the roots of *Piper methysticum* G. Forst (Piperaceae) has a long history of traditional use as a beverage because of its relaxing properties. Interestingly, kava consumption is epidemiologically associated with lower cancer incidence [12].

ABBREVIATIONS

AR	Adrenergic receptor
DHK	Dihydrokavain
DHM	Dihydromethysticin
DMY	Desmethoxyyangonin
K	Kavain
M	Methysticin
NE	Norepinephrine
Nife	Nifedipine
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
Pro	Propranolol
Y	Yangonin
Yohim	Yohimbine



▶ **Fig. 1** The structures of 6 major kavalactones in kava.



▶ **Fig. 2** Norepinephrine induces intracellular calcium influx in H1299 cells via β-ARs and L-type calcium channels, not α-ARs. Error bars represent standard deviation of 3 technical replicates (n = 3).

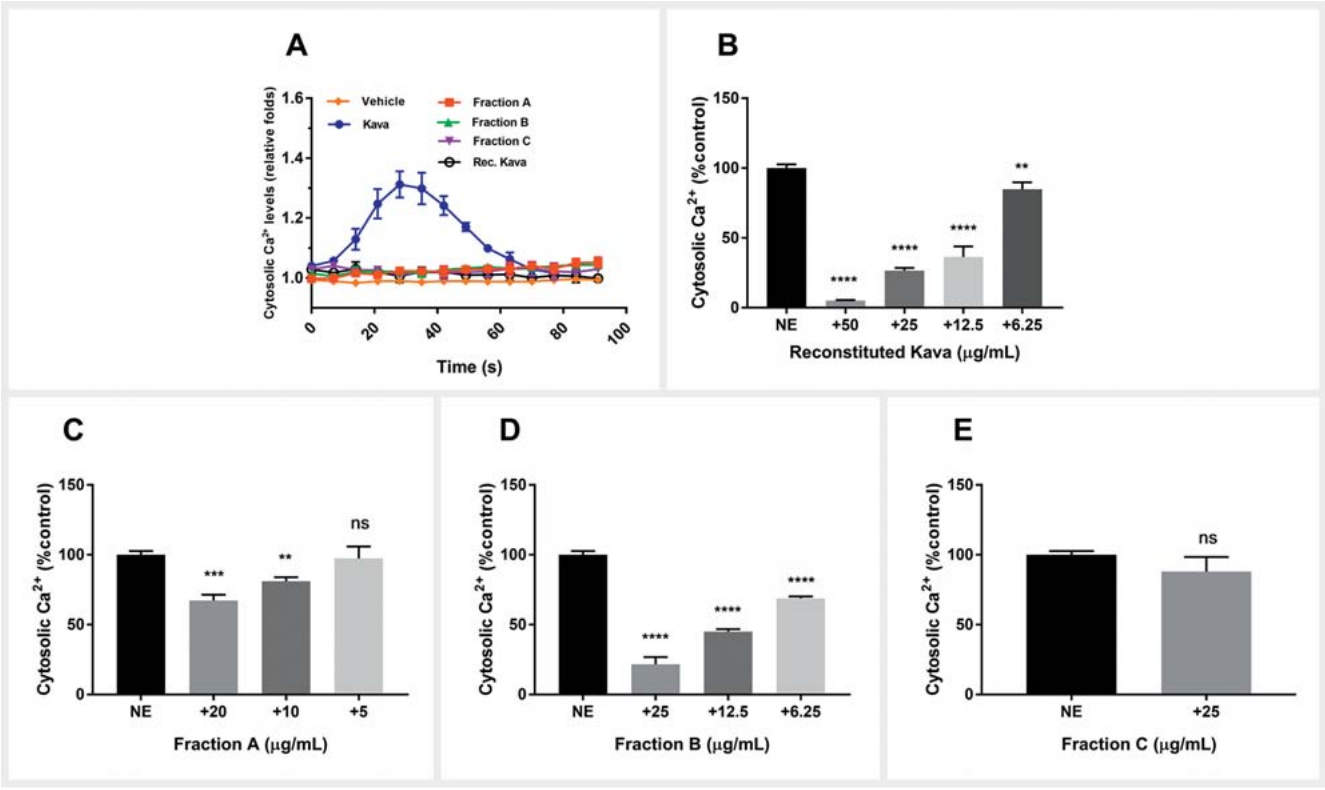
Because of its relaxing property, kava has also been marketed as a dietary supplement in the U.S. market for decades. Recently, kava and its components have demonstrated chemopreventive activity against carcinogen-induced tumorigenesis *in vivo* [13–17]. Yet, the associated mechanisms remain to be fully characterized. Although kava and its ingredients have been reported to modulate $GABA_A$ receptor [18], dopamine receptors [18], opioid receptors [18], MAO-B [19], or ion channels [20], its function on the adrenergic system has never been evaluated. Given the importance of the adrenergic system in mental stress and cancer, we characterized the effect of kava and its components on stress hormone NE-mediated intracellular calcium influx in a non-small cell lung cancer cell line (H1299) in this study.

Our results show that NE effectively induces intracellular calcium influx through the activation of β-ARs and L-type calcium channels in H1299 cells while kava extract dose-dependently inhibited this calcium signaling. To identify the components in kava responsible for this activity, we evaluated different kava fractions and demonstrated that this activity was mediated by kavalactones, the ingredients responsible for its anti-stress and cancer chemopreventive activity [21,22]. A preliminary structure-activity relationship study of 6 major kavalactones (▶ **Fig. 1**) shows that all 6 major kavalactones inhibit NE-induced calcium influx in H1299 cells with slightly different potencies, with DHK being the most potent. These results suggest a previously unstudied mechanism through which kava extract and its components may offer anti-stress and chemopreventive activity. Such knowledge provides molecular insight to guide future studies and the use of kava in stress reduction and cancer chemoprevention.

Results

We first characterized the effect of NE on intracellular calcium homeostasis in H1299 cells. NE at 1 μM effectively raised intracellular calcium levels in H1299 cells (▶ **Fig. 2**). Because stress hormones can increase intracellular calcium through various ARs [8], we next used pharmacological probes to explore whether the intracellular calcium increase was mediated through α- or β-ARs. As shown in ▶ **Fig. 2**, NE elevates intracellular calcium levels in H1299 cells via β-ARs not α-ARs, because such an increase was abolished by the pre-treatment of pro (a β-AR inhibitor) but not by yohim (an $\alpha_{1,2}$-AR inhibitor). As expected, the increase in intracellular calcium is mediated by L-type calcium channels, which was blocked by nife (an L-type calcium channel blocker). These results suggest that NE binds to β-ARs to induce calcium influx via L-type calcium channels in H1299 cells.

Given kava's anti-stress activity and its chemopreventive potential, we attempted to characterize the effect of kava on NE-induced calcium signaling in H1299 cells. We first evaluated the effect of an ethanolic kava extract alone on calcium homeostasis in H1299, which would serve as the background. Surprisingly, treatment with the ethanolic kava extract, in concentrations as low as 6.25 μg/mL, elicited strong calcium influx responses in H1299 cells (▶ **Fig. 3A**). Interestingly, treatment with any of its fractions obtained via the normal-phase silica gel chromatography did not result in any calcium influx signals, at concentrations as high as 50 μg/mL (▶ **Fig. 3A**). Given that treatment with the ethanolic kava extract induced intracellular calcium influx and treatment with individual fractions did not, we characterized whether a combination of components in different fractions were responsible for our observation. Therefore, we reconstituted kava extract from its fractions based on their relative abundance (40% Fraction A, 55% Fraction B, and 5% Fraction C) [14] and followed by treating H1299 cells. Interestingly, reconstitution of kava ex-



▶ **Fig. 3** Kava and its fractions inhibit NE-induced intracellular influx in H1299 cells. Kava extract induces intracellular calcium movements in H1299 cells, while individual fractions and reconstituted kava extract do not (**A**). Reconstituted kava extract and Fractions A and B inhibit NE-induced calcium influx, while Fraction C does not (**B**–**E**). Error bars represent standard deviation of 3 technical replicates (n = 3).

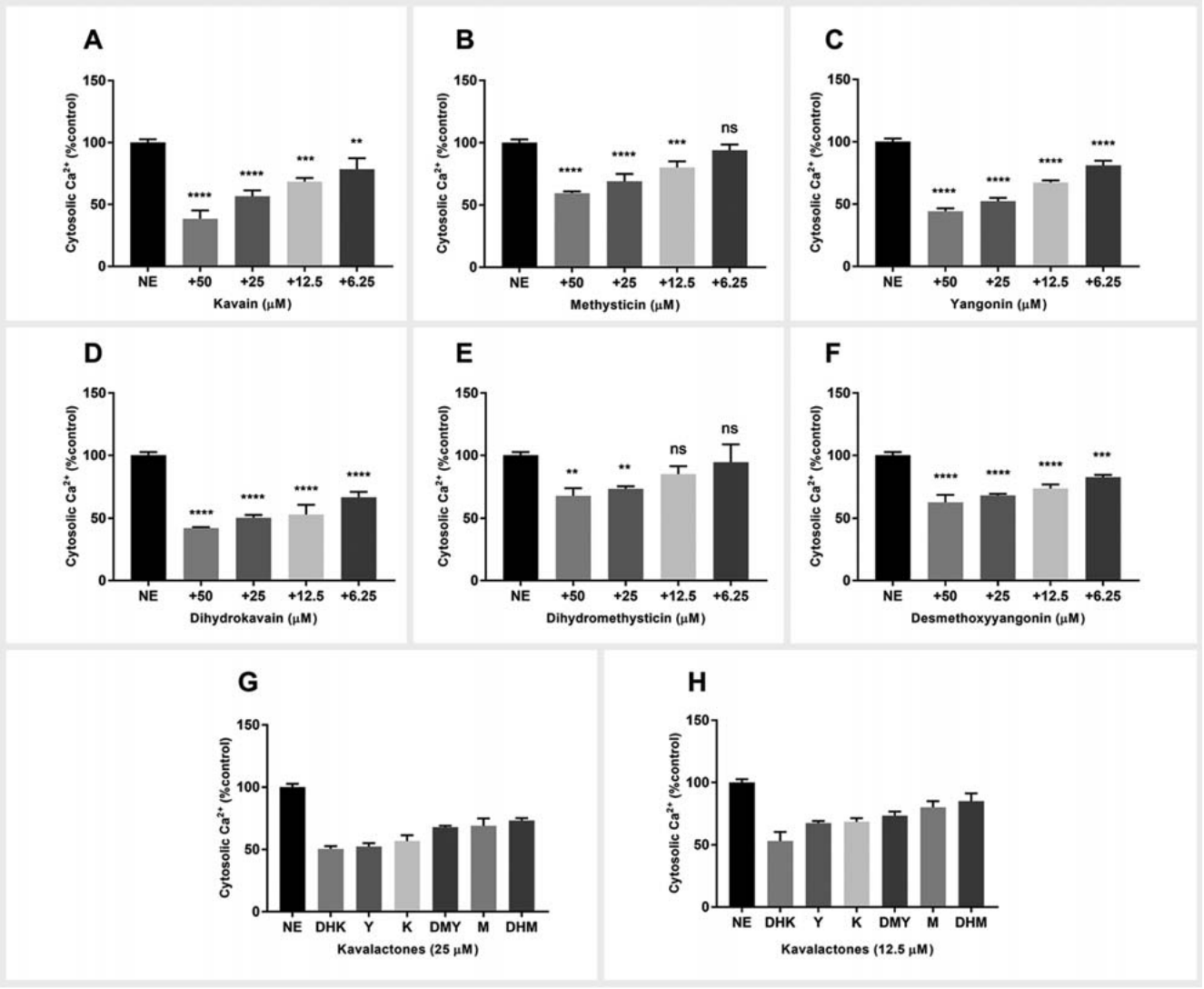
tract from its fractions did not elicit calcium signaling in H1299 cells (▶ **Fig. 3A**). These data suggest that the component(s) in the original ethanolic kava extract that elicited the calcium influx response were lost during the normal-phase chromatography fractionation. In fact, a previous report by Turner et al. suggested that the water-soluble component(s) in kava extract were responsible for the induction of intracellular calcium influx [23], which is consistent with our data because the water-soluble components are not recovered via our normal phase chromatography.

As the reconstituted kava extract does not perturb intracellular calcium homeostasis while it has no composition difference relative to the ethanolic kava extract except the polar components [14], we used the reconstituted kava to evaluate its effect on NE-induced calcium influx. As shown in ▶ **Fig. 3B**, pre-treatment of H1299 cells with reconstituted kava effectively and dose-dependently inhibited calcium influx induced by NE treatment. In order to determine the component(s) in kava responsible for this effect, we pre-treated H1299 cells with various concentrations of Fractions A, B, and C, followed by NE treatment. As shown in ▶ **Fig. 3C, D**, Fractions A and B exhibited inhibitory effects on NE-induced calcium responses with Fraction B being more potent. Fraction C, on the other hand, had no significant effect at a concentration as high as 25 μg/mL (▶ **Fig. 3E**). Since Fraction B contains most of the major kavalactones [14], these data suggest that kavalactones may be the responsible components. Therefore, we characterized the effects of the major kavalactones in kava Fraction B on NE-induced calcium influx in a similar manner.

The 6 major kavalactones in kava extracts, particularly Fraction B, include K, DHK, M, DHM, Y, and DMY (▶ **Fig. 1**). We first confirmed that none of these 6 major kavalactones elicited calcium responses by themselves (data not shown). All 6 kavalactones demonstrated inhibitory effects against NE-induced calcium influx (▶ **Fig. 4A–F**). To preliminarily explore the structure-activity relationship, their inhibitory activities at 25 and 12.5 μM were compared (▶ **Fig. 4G, H**). It appeared that DHK was the most potent one while DHM was the least active one. The overall potency rank of these 6 kavalactones in inhibiting NE-induced calcium responses are as follows: DHK, Y, K, DMY, M, DHM.

Discussion

Kava has been consumed as a beverage and dietary supplement for its relaxing property. There is also an increasing interest in its anticancer and chemopreventive potential, partly stimulated by the epidemiological observations. For instance, we have recently demonstrated that kava can completely block tobacco-specific carcinogen (NNK)-induced lung carcinogenesis in an A/J mouse model [14] while it also showed chemopreventive potential against prostate [15] and colon [16] tumorigenesis. However, its associated mechanisms remain largely unknown [22]. Even its anxiolytic mechanism is not firmly established [18–20, 24–26]. In-



▶ **Fig. 4** The 6 major kavalactones in kava dose-dependently inhibit NE-induced intracellular influx in H1299 cells. Dose-response of individual kavalactones on NE-induced calcium influx (**A**–**F**). Comparison of individual kavalactone potencies at 25 and 12.5 μM (**G**, **H**). Error bars represent standard deviation of 3 technical replicates (n = 3).

terestingly, psychological distress has long been suggested as a risk factor for lung cancer [27] and has been demonstrated to predict lung cancer mortality [5]. Mechanistically, perturbing intracellular calcium homeostasis by stress hormones via the activation of adrenergic signaling has been implicated in the onset and progression of lung cancer [9, 28], which may account for the cancer-promoting effect of psychological stress. Consistent with these, retrospective studies have demonstrated a clinical association between calcium channel blocker usage and reduced cancer mortality, particularly lung cancer [6, 29, 30]. Therefore, kava's anti-stress properties may contribute to its anti-cancer potential. Given the potential link of stress hormones and adrenergic activation in stress and cancer, this study aims to explore the potential impact of kava on this signaling pathway, which may reveal its anti-stress and anti-cancer mechanisms.

The results for the first time demonstrate the effects of kava and its components on inhibiting NE-disrupted calcium homeostasis. Specifically, while the whole ethanolic kava extract itself induces intracellular calcium influx in H1299 cells, such an activity is likely due to the highly polar components as the fractions collected via the normal phase chromatography and the reconstituted kava failed to do so. This is consistent with the results reported by Turner et al. [23]. The polar components responsible for the intracellular calcium increase are likely physiologically less relevant given its polar nature, potentially leading to minimal bioavailability. Nonetheless, the reconstituted kava from the normal phase chromatography effectively inhibited intracellular calcium influx induced by the treatment with NE. This inhibitory effect was better recapitulated by Fraction B, which contains the major kavalactones. All of the 6 major kavalactones in Fraction B demonstrate inhibitory activity against NE-induced calcium influx with slight differences in their potency. DHK, being the most potent inhibitor, is of particular interest because it has been identified as the most potent anxiolytic agent in kava in 2 *in vivo* studies [31,

32], although K has been traditionally proposed as the anxiolytic ingredient in kava. It remains to be determined whether kavalactones induce their anxiolytic activity via interaction with adrenergic signaling processes. DHM, on the other hand, was found to be the least potent kavalactone in suppressing NE-induced calcium influx although it was identified as the active ingredient in kava against NNK-induced lung tumorigenesis in A/J mice [13]. It should be noted that psychological stress has not been designed in the NNK-induced lung tumorigenesis A/J mouse model, which likely does not recapitulate the promoting effect of mental stress in human lung cancer development. Such a model, therefore, may not be capable to identify anti-stress based cancer chemopreventive agents.

Given the mixed potencies of kavalactones, such as DHK and DHM, in perturbing NE-induced calcium influx and in blocking NNK-induced lung tumorigenesis, the kavalactone mixtures may offer advantages over individual natural kavalactones in managing stress and lung cancer risk/development. Further studies are needed to evaluate the communications and regulations between kava's anti-stress and anti-cancer properties, particularly its perturbation of NE-mediated intracellular calcium influx and associated signaling. It is also possible that structural modifications of kavalactones may lead to candidate(s) capturing both activities.

Materials and Methods

Chemicals and reagents

(−)-NE and pro were obtained from Sigma-Aldrich. Yohim and nife were obtained from Alfa Aesar. Fluo-4 AM Direct Calcium Assay Kits were purchased from Invitrogen. An ethanolic kava extract was purchased from Gaia Herbs. All reagents were purchased from vendors and used without further purification. All reagents used were 95% purity or greater.

Cell culture

NCI-H1299 cells (human non-small cell lung cancer) were purchased from ATCC. The cell line was authenticated by Genetica Cell Line Testing prior use. The cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C with an atmosphere of 5% CO_2 and 95% humidity. The cells were passaged every 2 to 3 days. Cells were used for a maximum of 5 passages.

Kava preparation

Kava fractions and the 6 major kavalactones were isolated from the ethanolic kava extract by following our published procedures [14].

Detection of intracellular calcium influx

For intracellular calcium influx measurements, Fluo-4 AM Direct Calcium Assay Kits were used. Briefly, H1299 cells were plated in 96-well plates with 10% FBS-based RPMI-1640 (10^4 cells/well). After attachment, H1299 cells were cultured overnight in medium supplemented with 0.5% FBS-based RPMI-1640. Cell media was removed and HBSS buffer (50 µL/well, 1.3 mM Ca^{2+} and 20 mM HEPES) was added followed with Fluo-4 AM solution supplemented with 2.5 mM probenecid (50 µL/well). Cells were incubated at 37 °C for 60 min. Compound solutions, prepared in the same buffer, were added (50 µL/well) for a total volume of 150 µL/well. Fluorescence signals (excitation 485 nm and emission 525 nm) were collected using a Synergy H1 (BioTek) microplate reader.

Data analysis

The data are presented as mean ± SD. All experiments were independently performed at least twice with 3 technical replicates, and a representative result was presented. Data were graphed using GraphPad Prism 7 (GraphPad Software Inc.). Intracellular calcium levels represent percent increase over baseline levels. Calcium curves represent an average of 3 technical replicates with standard deviation. Statistical significance between 2 groups was analyzed with a Student's t-test, and statistical significance among multiple groups was analyzed with one-way ANOVA.

$p < 0.05$ was considered to be statistically significant.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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