ANTIHYPERTENSIVE EFFECT OF RUTIN: PHARMACOLOGICAL AND COMPUTATIONAL APPROACH

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ABSTRACT

Objective: Phenolic compounds, such as flavonoids, have aroused great scientific interest due to their diverse pharmacological activities. Several studies suggested that flavonoids act as antihypertensive by inhibiting angiotensin-converting enzyme (ACE). In the present study, rutin, which is a citrus flavonoid, was evaluated for its antihypertensive activity using in vivo and in vitro models. Rutin was screened for in vitro assay procedures such as diphenylpicrylhydrazyl and nitroblue tetrazolium (NBT) for its antioxidant activity.

Methods: Its antihypertensive effect was investigated in Nω-Nitro-l-arginine methyl ester hydrochloride-induced hypertensive rats, and various parameters such as blood pressure and heart rate were measured; in vitro ACE inhibitory activity was carried out against ACE, aiming at a better understanding of the interaction of this flavonoid with the enzyme. To understand its binding affinity with the angiotensin-converting enzyme, molecular docking studies were carried out using ligand fit of Maestro 9.1 (Schrodinger Software Inc.). An in silico study of rutin was performed for the prediction of Absorption, distribution, metabolism and elimination (ADME) by utilizing a web-based program (www.swissadme.ch). This software computes physicochemical descriptors as well as predicts pharmacokinetic properties and drug-like nature of one or multiple small molecules (blood-brain barrier, cytochromes P450, and P-glycoproteins).

Results: Rutin at different dose levels of 200 and 400 mg/kg was tested, and the results have shown its antihypertensive, hypotensive, and negative chronotropic effects. Its antihypertensive activity might be mediated through angiotensin-converting enzyme inhibition (half maximal inhibitory concentration=66.01 µg/ml). In vitro studies also revealed the antioxidant activity of rutin, thus playing a major role in reducing oxidative stress associated with hypertension. The rutin showed optimum binding affinity with a molecular target (angiotensin-converting enzyme) with the binding energy of −9.0 kcal/mol as compared to the standard (−6.3 kcal/mol). These results indicated that rutin is one of the potential ligands to treat hypertension. ADME results revealed the three violations of rutin (such as molecular mass, hydrogen donor, and acceptors) of five, and the standard captoril has got zero violations which clearly indicated the probability for its higher oral bioavailability.

Conclusion: From the above, it is concluded that rutin possesses antioxidant and antihypertensive activities.

Keywords: Nω-Nitro-l-arginine methyl ester hydrochloride, Antioxidant, Hypertension, Angiotensin-converting enzyme, Rutin, ADME, Molecular docking

INTRODUCTION

The compounds such as flavonoids are found in plants and are consumed in the form of fruits, nuts, vegetables, and derivative foods such as wine and brumette. The diet consumed by the western countries mostly comprises quercetin [1]. Quercetin is an example of a flavonoid group which is found in nutrients having sugars, chiefly as β-glycosides. Rutin, also called rutoside, is the glycoside linking the flavonol quercetin and the disaccharide rutinose. This citrus flavonoid is found in a wide diversity of plants. Rutin is a nutritional flavonoid which has established prodigious consideration due to their pharmacological properties, including antimicrobial, anti-inflammatory, anticancer, anti-diabetic, and inter alia [2].

Although flavonoids are lacking typical nutritive value, they are gradually more regarded as valuable dietary constituents that act as probable defenders contrary to human diseases such as coronary heart disease, cancers, and inflammatory bowel disease. Rutin turns out to be a quercetin releaser to the gut; moreover, quercetin is widely broken down in the gut and released from rutin, and/or its colonic metabolites might play a vital role [3].

Hypertension induced by Nω-Nitro-l-arginine methyl ester hydrochloride (L-NAME), a nitric oxide synthase (NOS) inhibitor, is a frequently used model in higher mammals. L-NAME-treated rats have downregulated eNOS protein expression in blood vessels and exhaustion of plasma NO levels, which leads to systemic vasoconstriction, increased vascular resistance, and high blood pressure. There is an association between L-NAME-induced hypertension and oxidative stress indicators. L-NAME at a high dose (40 mg/kg/day) has been stated to raise the levels of oxidative stress markers such as vascular superoxide (O2−), plasma malondialdehyde, and plasma protein carbonyl. It has been accepted that oxidative stress contributes to the etiology of hypertension in animals and humans as categorized by the augmented bioavailability of reactive oxygen species (ROS) in hypertension [4]. Raised levels of ROS stimulate vascular smooth muscle cell propagation and increase arterial resistance due to reducing NO availability, leading to the damage of vascular relaxation [5]. There is an indication that it is the ROS-producing enzyme, β-Nicotinamide adenine dinucleotide phosphate oxidase (NOX), that is responsible for increased vascular O2− production in L-NAME hypertensive rats via upregulation of the NOX subunit p47 [6].

To understand the ligand-binding properties of rutin with the angiotensin-converting enzyme (ACE), the test compound rutin was subjected to molecular docking studies. These studies act as a computational tool to expect the probable interactions between rutin and protein. An in silico study of rutin was performed by SwissADME to calculate its pharmacokinetics, drug-likeness, and medicinal chemistry friendliness of trivial molecules to support drug discovery [7]. Different rutin preparations could fetch this encouraging flavonoid to become the leader of nutraceuticals for the deterrence and/or management of numerous chronic human diseases.
MATERIALS AND METHODS

Animals
Healthy Sprague Dawley rats, weighing approximately 150–200 g, were selected for the study. They were retained in a workroom with a 12 h light/dark cycle. During the experiment, the room temperature was maintained at 25°C. The rats were kept on a standard chow diet and water ad libitum prior to dietetic management. They were trained for the 1st week to become familiarized with the technique of indirect blood pressure measurement. All the experimental works with the animals were carried out after obtaining approval from the institutional animal ethics committee (Reg. No. 1175/ac/08/CP/CSEA).

In vitro antioxidant assays
Diphenylpicrylhydrazyl (DPPH) radical scavenging activity
The ability of rutin to donate hydrogen was observed in the occurrence of DPPH stable radical. 1 mL of 0.3 mM DPPH solution was mixed with 2.5 mL of test compound of various concentrations and permitted to react at room temperature. The absorbance values were measured at 517 nm after half an hour. Methanol (1.0 mL) and the test substance rutin (2.5 mL) were kept as blank, DPPH solution (1.0 mL and 0.3 mM), and methanol (2.5 mL) as a negative control. Vitamin C was used as standard [9].

Nitroblue tetrazolium reduction assay
A reaction mixture of 3 mL was prepared with 1.4 mL of 50 mM KH₂PO₄·KOH at pH 7.4 containing 1 mM Methyleneediaminetetraacetic acid, 0.5 mL of 100 µM hypoxanthine, and 0.5 mL of 100 µM NBT. The reaction was initiated by adding 0.066 units per tube of xanthine oxidase diluted in 100 µL of phosphate buffer freshly and 0.5 mL of rutin in saline solution. The reduction rate of NBT was determined by spectrophotometric method at 560 nm. Gallic acid was used as standard. The outcomes were expressed as the percentage inhibition of NBT [8].

In vitro ACE inhibitory activity
In vitro ACE inhibitory activity was measured using the hippuryl-histidyl-leucine (HHL), as substrate, ACE (EC 3.4.15.1) obtained from rabbit lung. Rutin at different concentrations (40 µL) was incubated with 100 µL of 0.1 M borate buffer (pH 8.3) containing 5 mM HHL and 0.3 M NaCl and with 20 µL of ACE (2 µg) at 37°C for 30 min. The reaction was terminated with 150 µL of 1 M HCl. The hippuric acid formed was extracted with ethyl acetate (1000 µL) and centrifuged at 1500 rpm for 10 min, and 750 µL of the organic phase was evaporated. The residue was made up to 800 µL with distilled water, and the absorbance at 228 nm was measured. Triplicates were performed for each sample. Inhibitory activity was expressed as the protein concentration (Pierce, Rockford, IL, USA) using bovine serum albumin as standard needed to inhibit 50% of ACE activity (half maximal inhibitory concentration (IC₅₀)) [9].

In vivo study of rutin on hypertensive rats
Thirty healthy Sprague Dawley rats weighing 150–200 g were used for the study. They were divided into 5 groups of 6 animals each. Hypertension is induced in Sprague Dawley rats by administering the L-NAME hydrochloride (50 mg/kg, p.o.) consecutively for 21 days. L-NAME hydrochloride at a dose of 50 mg/kg was given to all groups except the control group. Group I received 0.5 mL/kg p.o. of normal saline. Group II received only L-NAME at a dose of 50 mg/kg. Group III and Group IV received rutin a dose of 200 and 400 mg/kg p.o. Group V received the standard drug captopril at a dose of 60 mg/kg after 60–90 min of L-NAME hydrochloride administration. Systolic blood pressure (SBP) and heart rate (HR) were measured by the noninvasive blood pressure amplifier system (PowerLab, ADInstruments, Australia) on days 0, 7, 14 and 21 [10].

Docking studies
The crystallographic structure of the enzymatic target ACE was obtained from the Protein Data Bank (PDB) database (PDB: 1O86). The molecular docking study was performed using Schrodinger software 5.6. The docking analysis of the compounds with ACE was carried out by ligand fit of Maestro 9.1 (Schrodinger Software Inc.). The software allows us to virtually screen a database of compounds and predict the strongest binders based on various scoring functions. The collection of enzyme-substrate complexes was identified via docking, and their relative stabilities were evaluated using their binding affinities. Ligand fit was used for accurately docking ligands into protein active sites employing a cavity detection algorithm. A high-throughput screening study applied to the ACE receptor is also presented in which ligand fit when combined with LigScore, an internally developed scoring function, yields very good hit rates for a ligand pool seeded with known actives [11].

Docking protocol

Protein preparation
The crystal structure of ACE 1086 was prepared, and the active site was identified. The ligands and crystallographic water molecules were removed from the protein, and the chemistry of the protein was corrected for missing hydrogen. Crystallographic disorder and unfilled valence atoms were corrected using alternate conformations and valence monitor options. Following the above steps of preparation, the protein was subjected to energy minimization using the CHARMM force field.

Ligand preparation
The three-dimensional structure of compounds were downloaded in structure data file format from PubChem and ChemDraw database. Hydrogen bonds were added and the energy was minimized using CHARMM force field. The parameters like G score, hydrogen bond, rotatable bond penalty and a lipophilic term derived from the hydrophobic grid for the rutin and captopril are reported in Table 1.

Docking studies
The active site of the protein was first identified and it is defined as the binding site. The binding sites were defined based on the ligands already present in the PDB file (i.e., ACE-binding site region) which were followed by site sphere definition. The determination of the ligand-binding affinity was calculated using LigScore and dock score to estimate the ligand-binding energies. Apart from these, other input parameters for docking were set as default options.

ADME studies
An in silico pharmacokinetic study of rutin was performed for the prediction of ADME properties like molecular weights, topological polar surface area (TPSA), miLog P, number of rotatable bonds, number of hydrogen donor and acceptor atoms (Lipinski 2001). In the present study, ADME was done by utilizing a web-based program (www.swissadme.ch). This software computes physicochemical descriptors as well as predicts pharmacokinetic properties and drugs such as nature of one or multiple small molecules (blood-brain barrier [BBB], cytochromes P450, and P-glycoproteins). The compounds with positive values can cross readily in the BBB, while compounds with negative values are poorly distributed to the brain [12].

Statistical analysis
All the values were expressed as the mean±standard error of mean. The data were statistically analyzed by one-way analysis of variance,
followed by Dunnett’s test, and p<0.05 was considered to be statistically significant.

RESULTS

In vitro antioxidant activity of test compound rutin
Rutin was screened for its antioxidant activity using DPPH and NBT assays.

DPPH radical scavenging assay
Rutin was tested for free radical scavenging activity using the DPPH method. The concentrations and percentage inhibition values were recorded, and from the percentage inhibition, the IC$_{50}$ value of rutin and Vitamin C are calculated and is reported in Table 2. Vitamin C (standard) was tested at different dose levels and found to be linear, which substantiates the use of ascorbic acid for comparison of the test doses. IC$_{50}$ of rutin was 16.5 µg/mL and Vitamin C was 3.89 µg/mL.

The assay was performed in triplicate. The above results showed that rutin and Vitamin C have antioxidant activity.

The DPPH radical was widely used as the model system to investigate the scavenging activity on several natural compounds such as phenolics and anthocyanins. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm.

Radical scavenging activity increased with an increased percentage of free radical inhibition. DPPH is a relatively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants toward the stable radical DPPH. These radicals react with suitable reducing agents, the electrons become paired off, and the solution loses color depending on the number of electrons taken up [13]. The results revealed the definite scavenging activity of the rutin toward DPPH radical in comparison with standard Vitamin C.

NBT inhibition assay
Rutin was tested for superoxide scavenging activity using NBT method. The concentrations versus percentage inhibition values were recorded, and from the determination of free radical scavenging activity and antitumor activity of some Myanmar herbal plants, IC$_{50}$ value of rutin and gallic acid was calculated and is reported in Table 3. The standard gallic acid was tested at different dose levels and found to be linear, which substantiates the usefulness of gallic acid for comparison of the test doses. IC$_{50}$ of rutin was 7.2 µg/mL and gallic acid was 2.5 µg/mL.

The assay was performed in triplicate. The above results showed that rutin and gallic acid have antioxidant activity.

Superoxide dismutase (SOD) is an antioxidant enzyme that neutralizes the free radicals in the cell; it dismutates superoxide anion (O$_2^−$) into H$_2$O and protects the cells from damage by cleaning up O$_2^−$. The level of SOD activity represents the intracellular antioxidant ability. Superoxide anion radical is one of the strongest ROS among the free radicals that are generated [14]. Superoxide is produced from molecular oxygen by oxidative enzymes as well as via non-enzymatic reaction such as autoxidation by catecholamines. In the present study, superoxide radical reduces NBT to a blue-colored formazan that is measured at 560 nm.

The above results suggested that rutin has antioxidant activity (DPPH: IC$_{50}$=16.5 µg/mL and NBT: IC$_{50}$=7.2 µg/mL). Hence, rutin was further proceeded for in vivo antihypertensive activity in L-NAME-induced hypertension in Sprague Dawley rats.

Table 2: Effect of rutin on DPPH radical scavenging assay

| Rutin       | Percentage inhibition (Mean±SEM) | IC$_{50}$ (µg/mL) |
|-------------|----------------------------------|-------------------|
| Concentration (µg/mL) |                             |                   |
| 25          | 63.09±0.42                       | 16.5              |
| 12.5        | 51.80±0.21                       |                   |
| 6.25        | 34.51±0.96                       |                   |
| 3.12        | 20.64±0.42                       |                   |

Table 3: Effect of rutin on NBT inhibition assay

| Rutin       | Percentage inhibition (Mean±SEM) | IC$_{50}$ (µg/mL) |
|-------------|----------------------------------|-------------------|
| Concentration (µg/mL) |                             |                   |
| 25          | 82.80±0.37                       | 7.2               |
| 12.5        | 64.63±0.17                       |                   |
| 6.25        | 43.10±0.88                       |                   |
| 3.125       | 25.18±0.10                       |                   |

Table 4: Effect of rutin on ACE inhibition assay

| Rutin       | Percentage inhibition (Mean±SEM) | IC$_{50}$ (µg/mL) |
|-------------|----------------------------------|-------------------|
| Concentration (µg/mL) |                             |                   |
| 100         | 77.25±0.22                       | 66.01             |
| 50          | 35.50±0.09                       |                   |
| 25          | 19.50±0.87                       |                   |
| 12.5        | 10.25±0.19                       |                   |

IC$_{50}$: Half maximal inhibitory concentration, SEM: Standard error of mean
Rutin was also screened for in vitro ACE inhibition assay for antihypertensive activity. Inhibitory activity of ACE plays a major role in the treatment of L-NAME-induced hypertensive animals. Hence, the test compound and standard drug are screened for ACE inhibition assay at different concentrations, and the IC_{50} value is calculated.

**In vitro ACE inhibition assay**
Rutin was tested for ACE inhibitory activity using the ACE inhibition assay method. The concentrations and percentage inhibition of rutin and standard drug captopril were recorded. From the percent inhibition, IC_{50} values were calculated and are reported in Table 4. The standard drug captopril was tested at different dose levels and found to be linear, which substantiates the usefulness of captopril for comparison of the test doses. IC_{50} of rutin was 66.01 µg/mL and captopril was 20.31 µg/mL.

The assay was performed in triplicate. The above results show that the test extract rutin and standard compound captopril have antihypertensive activity.

Rutin has antihypertensive activity by ACE inhibitory action (IC_{50}=66.01 µg/mL). In vitro studies have revealed the antioxidant activity of rutin and the role of rutin in reducing oxidative stress associated with hypertension. Hence, the test compound is preceded for in vivo antihypertensive activity in L-NAME-induced hypertension in Sprague Dawley rats using captopril.

**In vivo studies of rutin on SBP and HR in Sprague Dawley rats**
Effect of rutin on SBP

The test compound rutin was tested for its effect on SBP of L-NAME-induced hypertension in Sprague Dawley rats. SBP was recorded on a weekly basis, i.e., on day 0, day 7, day 14, and day 21. SBP (mmHg) was measured using the NIBP system for all groups of animals. The results are reported in Table 5.

Animals treated with L-NAME at 50 mg/kg bd. wt showed a significant increase in the SBP as compared to a normal control group, and this might be due to the inhibition of NO synthesis. Oral administration of rutin at a dose of 200 mg/kg and 400 mg/kg bd. wt significantly decreased the SBP in treatment groups when compared to the L-NAME control group, and this might be due to the inhibition of ACE activity [15]. The standard drug captopril at a dose of 60 mg/kg bd. wt has shown a significant reduction in SBP.

Effect of rutin on HR
Rutin was tested for its effect on the HR of L-NAME-induced hypertension in Sprague Dawley rats. HR was recorded on a weekly basis, i.e., on day 0, day 7, day 14, and day 21. HR (beats per minute) was measured using the NIBP system for all groups. The results are reported in Table 6.

Animals treated with L-NAME at 50 mg/kg bd. wt showed a significant increase in the HR as compared to the normal control group, and this might be due to compensatory baroreceptor reflex mechanism [16] which shows a relationship between HR and blood pressure variability in rats. Oral administration of rutin at a dose of 200 mg/kg and 400 mg/kg bd. wt significantly decreased the HR in treatment groups when compared to the L-NAME control group, and this might be due to ACE inhibitory activity [17]. The standard drug captopril at a dose of 60 mg/kg bd. wt has shown a significant decrease in HR.

Regulation of body weight is also one of the parameter studied in hypertension like in obese rats [18]. Hypertension is a metabolic syndrome which affects the body weight also, so the test compound

### Table 5: Effect of rutin on SBP of L-NAME-induced hypertensive Sprague Dawley rats

| Groups | Treatment                  | SBP (mm/Hg)     | Day 0 | Day 7 | Day 14 | Day 21 |
|--------|----------------------------|-----------------|-------|-------|--------|--------|
| I      | Normal                     | 108.05±0.43     | 107.27±0.51 | 102.47±0.92 | 110.41±0.08 |
| II     | L-NAME+Vehicle             | 106.67±0.46**   | 137.03±0.43** | 150.92±0.04** | 161.33±0.92** |
| III    | L-NAME+Rutin (200 mg/kg bd. wt) | 108.93±0.25***  | 145.75±0.27*** | 136.13±0.14*** | 131.22±0.98*** |
| IV     | L-NAME+Rutin (400 mg/kg bd. wt) | 108.09±0.22**   | 137.97±0.24** | 129.47±0.59** | 120.34±0.53** |
| V      | L-NAME+Captopril (60 mg/kg bd. wt) | 108.48±0.51***  | 103.13±0.69**  | 110.39±0.62**  | 111.92±0.44**  |

Values are expressed as mean±SEM (n=6). The test groups are compared to L-NAME control group. Significant values are expressed as (*p<0.05, **p<0.01) compared to control, (p < 0.01, p < 0.001) to standard (=non significant), L-NAME: N'-Nitro-l-arginine methyl ester hydrochloride, SBP: Systolic blood pressure

### Table 6: Effect of test compound rutin on the HR of L-NAME-induced hypertensive Sprague Dawley rats

| Groups | Treatment                  | HR (BPM)        | Day-0 | Day-7 | Day-14 | Day-21 |
|--------|----------------------------|-----------------|-------|-------|--------|--------|
| I      | Normal                     | 305.49±0.12     | 308.02±0.29 | 314.25±0.65 | 312.06±0.54 |
| II     | L-NAME+vehicle (50 mg/kg bd. wt)  | 308.8±0.55***   | 352.41±0.54*** | 374.6±0.51*** | 404.57±0.13*** |
| III    | L-NAME+Rutin (200 mg/kg bd. wt) | 302.42±0.15***  | 356.30±0.05*** | 349.9±0.21*** | 324.6±0.96*** |
| IV     | L-NAME+Rutin (400 mg/kg bd. wt) | 302.8±0.15***   | 350.1±0.65***  | 324.10±0.82*** | 312.33±0.25*** |
| V      | L-NAME+Captopril (60 mg/kg bd. wt) | 303.12±0.02**  | 354.52±0.98**  | 326.7±0.74**  | 314.8±0.73*** |

Values are expressed as mean±SEM (n=6). The test groups are compared to L-NAME control group. Significant values are expressed as (*p<0.05, **p<0.01) compared to control, (p < 0.01, p < 0.001) to standard (=non significant), L-NAME: N'-Nitro-l-arginine methyl ester hydrochloride, HR: Heart rate, BPM: Beats per minute

### Table 7: ADME profile of rutin and captopril

| Compound name | Physiochemical properties | Lipophilicity | Water Solubility |
|---------------|---------------------------|---------------|------------------|
|               | Mol wt g/mol | TPSS Å³ | No rot b | No H bond Acceptors | No H bond donors | BBB | Log S/Solubility |
| Rutin         | 610.52       | 269.43 | 6 | 16 | 10 | No | 2.43 | −0.29 Soluble |
| Captopril     | 217.29       | 96.41  | 4 | 3 | 1 | No | 1.46 | −0.38 Soluble |

BBB: Blood–brain barrier, TPSS: Topological polar surface area
Docking studies of rutin

Rutin (total score: -9) demonstrated hydrogen-bonding interactions with Glu 384, Arg 124, and Ala 356. Docking results are depicted in Figs. 1 and 2.

Captopril (total score: -6.03) demonstrated hydrogen-bonding interactions with Glu 376, Gly 2000, Gln 281, and Lys 511.

ADME properties

Lipinski's rule of five is to evaluate drug-likeness or determine if a chemical compound with a certain pharmacological or biological activity has chemical properties and physical properties that would make it an orally active drug in humans. In the present study, rutin has three violations (such as molecular mass, hydrogen donor, and acceptors) of five. Captopril got zero violations which clearly indicated the probability for its higher oral bioavailability. Lipinski violations of rutin and zero violations of standard captopril were depicted in Figs. 3 and 4.

TPSA allows the prediction of transport properties of drug candidates in the intestines and BBB [19]. Rutin has TPSA of 269.43, and this high score of TPSA suggested that this molecule preferentially acts as hydrophilic in nature and cannot easily transport through the BBB when compared to captopril which has TPSA score of 96.41 clearly indicating lipophilic in nature.

The three violations of rule five for rutin indicated the probability for its less oral bioavailability [20]. In this study, there is no BBB score but found to have a high TPSA of 269.43, and this high score of TPSA suggested that this molecule was preferentially found to be hydrophilic in nature and cannot easily transport through the BBB. The ADME profile of rutin and captopril is given in Table 7.

CONCLUSION

From the above, it was concluded that the test compound rutin has antioxidant and antihypertensive activity. Docking studies of the rutin with target protein showed that this is a promising candidate which docks well with the target enzyme related to hypertension. Although rutin has less oral bioavailability in spite of being pharmacologically active, it might be transformed into sulfates or glucuronides to enhance its bioavailability. Thus, rutin can be considered for developing into a potent nutraceutical in the treatment of cardiovascular disorders, especially hypertension.

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AUTHORS' CONTRIBUTIONS

Dr. M. Ganga Raju designed the study. Prem Prasad Goud carried out the experimental work; Dr. M. Ganga Raju and Dr. NVL Suvarchala Reddy drafted the manuscript, and they were involved in manuscript editing and finalization. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

All authors have no conflicts of interest to declare.

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