Anti-proliferative effect of rhein, an anthraquinone isolated from *Cassia* species, on Caco-2 human adenocarcinoma cells

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Abstract

In recent years, the use of anthraquinone laxatives, in particular senna, has been associated with damage to the intestinal epithelial layer and an increased risk of developing colorectal cancer. In this study, we evaluated the cytotoxicity of rhein, the active metabolite of senna, on human colon adenocarcinoma cells (Caco-2) and its effect on cell proliferation. Cytotoxicity studies were performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red (NR) and trans-epithelial electrical resistance (TEER) assays whereas 3H-thymidine incorporation and Western blot analysis were used to evaluate the effect of rhein on cell proliferation. Moreover, for genoprotection studies Comet assay and oxidative biomarkers measurement (malondialdehyde and reactive oxygen species) were used. Rhein (0.1–10 μg/ml) had no significant cytotoxic effect on proliferating and differentiated Caco-2 cells. Rhein (0.1 and 1 μg/ml) significantly reduced cell proliferation as well as mitogen-activated protein (MAP) kinase activation; by contrast, at high concentration (10 μg/ml) rhein significantly increased cell proliferation and extracellular-signal-related kinase (ERK) phosphorylation. Moreover, rhein (0.1–10 μg/ml): (i) did not adversely affect the integrity of tight junctions and hence epithelial barrier function; (ii) did not induce DNA damage, rather it was able to reduce H2O2-induced DNA damage and (iii) significantly inhibited the increase in malondialdehyde and reactive oxygen species (ROS) levels induced by H2O2/Fe2+. Rhein was devoid of cytotoxic and genotoxic effects in colon adenocarcinoma cells. Moreover, at concentrations present in the colon after a human therapeutic dosage of senna, rhein inhibited cell proliferation via a mechanism that seems to involve directly the MAP kinase pathway. Finally, rhein prevents the DNA damage probably via an anti-oxidant mechanism.

Keywords: rhein • human colon adenocarcinoma cells • mitogen-activated protein kinase • genoprotection • antioxidant

Introduction

Constipation, a complaint conceptually regarded as disordered movement of stool through the large intestine, afflicts many people in the Western countries [1, 2]. The first approach for the treatment of constipation consists in lifestyle changes including increased fibre (about 30 g/day), and water (about 2 l/day) intake and physical exercise. If these measures are ineffective, laxative therapy has to be considered. Among laxatives, anthranoids are the oldest used drugs in clinical practice and as self-medication [3]. Senna is the most known anthranoid laxative; it is obtained from the dried leaves and pods of *Cassia acutifolia* Delile and *Cassia angustifolia* Vahl (Caesalpinaceae family). Senna contains sennosides that are not absorbed in the small intestinal tract; in the colon, sennosides are metabolized by the bacterial β-glucosidase and reductase to the pharmacologically active compound, rhein anthrone [4, 5]. Rhein anthrone is poorly absorbed and produces a laxative effect throughout two independent mechanisms: (i) changing of colonic motility leading to an accelerated large intestinal transit and (ii) alterations in colonic adsorption and secretion of water and electrolytes resulting in fluid accumulation [6].

In the last years, there has been the concern that therapeutic or chronic use of anthranoid laxatives can cause structural damage...
to colonic tissue and induce colonic neuropathy and colorectal cancer. However, human and animal data in this area are inconsistent. Three clinical studies have evaluated the potential side effects of long-term use of anthraquinone laxatives on colonic nerve tissue [7–9]. One study found a direct association between stimulant laxative use and anatomic changes in the colon [7] while two clinical trials did not support the hypothesis that anthraquinone containing laxatives are able to provoke relevant degenerative changes in the colonic nerve tissue [8, 9]. van Gorkom and colleagues reported three clinical studies showing increased cell proliferation in colonic tissue of patients treated with a single high dose of sennosides [10–12]. Most epidemiological studies failed to find any association between anthranoid laxative use and colorectal cancer [13–17], although a prospective study showed an increased cell proliferation (interpreted as higher risk for developing colorectal cancer) in patients using anthranoid laxatives [18]. Finally, two single case reports identified an increased cell proliferation in patients using anthranoid laxatives [19, 20]. Similar conflicting data have been reported on preclinical studies. As a consequence of these data in 1995, the German Federal Institute for Drugs and Medical Devices revised the product information of all medicinal products containing anthranoids and in 1998 the Food and Drug Administration (FDA) classified the stimulant laxatives (including senna) from category I (generally recognized as safe and effective) to category III (further testing is required). In 2002, the FDA re-classified senna, but not aloe and cascara, to category II. Although there is no conclusive clinical evidence that senna is dangerous, there are still doubts and unresolved questions on the safety of this drug and its components. On this basis, physicians often undergo anthraquinones laxatives and are more willing to use far more expensive drugs that have a much shorter track record concerning potential long-term consequences to the constipated patient.

Therefore, the aim of this study was to investigate the potential damaging effects of the active anthranoid metabolite of senna, rhein, on a human colon adenocarcinoma cell line, Caco-2.

The cytotoxicity of rhein on proliferating and differentiating cells has been evaluated as well as its effects on cell proliferation, genotoxicity and epithelial integrity, all of which are thought to be associated with colorectal carcinogenesis.

Materials and methods

Chemicals

The 4,5-Dihydroxyanthraquinone-2-carboxylic acid (rhein), deoxycolic acid (DCA), 3,4,5-trimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1,1,3,3-tetramethoxypropane (malonaldehyde bis dimethyl acetal, standard MDA) were purchased from Sigma Aldrich S.r.l. (Milan, Italy). The 4,5-Dihydroxyanthraquinone-2-carboxylic acid (rhein), deoxycolic acid (250 g/ml) at 37°C used in the study was selected on the basis that a g/ml (0.35–3.5 μM) used in the study was selected on the basis that a g/ml (35 μM). The results are expressed as percentage of cell viability.

Neutral red (NR) assay

The NR assay system, one of the most used and sensitive cytotoxicity test, is a mean of measuring living cells via the uptake of the vital dye neutral red. Viable cells will take up the dye by active transport and incorporate the dye into lysosomes, whereas non-viable cells will not take up the dye. An increase or decrease in the number of cells or their physiological state results in a concomitant change in the amount of dye incorporated by the cells in the culture [22]. Caco-2 cells (passage between 26 and 29) were seeded in 96-wells plates at a concentration of 3 × 10^4 cells/well. After 48 hrs of culturing, the medium was removed and the cells were treated with rhein (0.1–10 μg/ml) at 37°C for 24 hrs. Following treatment, cells were washed and fresh medium was replaced. After 48 hrs of culturing at 37°C, 15 μl MTT dye solution were added to each well for 4 hrs. Finally, 100 μl of solubilization/stop solution were added to dissolve the purple crystals; absorbency of formazan was measured at a wavelength (λ) of 570 nm using a multwell reader (Rainbow, SLT, Austria). Treatments were compared with a positive control, deoxycolic acid (250 μM). Experiments were repeated independently three times. The results are expressed as percentage of cell viability.

Cell culture

Caco-2, A-431 cells (American Type Culture Collection, LGC Promochen, Italy) and human fibroblasts were cultured in 75 cm² polystyrene flasks (Falcon, Microglass Heim, Naples, Italy) as monolayers in modified Eagle medium (MEM) containing 10% foetal bovine serum (FBS), 100 Units/ml penicillin, 100 μg/ml streptomycin and 1% non-essential amino acids. Cells were cultured at 37°C in a humidified 5% CO₂ and 95% filtered air and the culture medium was replaced every 2 days. After washing in phosphate buffered saline (PBS), cells were trypsinized with 0.25% trypsin-EDTA at 37°C for 5 min., centrifuged at 1000 × g for 3 min. and then re-suspended in the appropriate medium. Cell viability was determined by trypan blue staining.

Cytotoxicity assay

MTT assay

The effect of rhein on cell survival was measured using the CellTiter 96® proliferation assay (MTT assay) (Promega, Madison, WI, USA) [21]. Caco-2 cells (passage between 26 and 29) were seeded in 96-wells plates at a concentration of 3 × 10^4 cells/well. After 48 hrs of culturing, the medium was removed and the cells were treated with rhein (0.1–10 μg/ml) at 37°C for 24 hrs. Following treatment, cells were washed and fresh medium was replaced. After 48 hrs of culturing at 37°C, 15 μl MTT dye solution were added to each well for 4 hrs. Finally, 100 μl of solubilization/stop solution were added to dissolve the purple crystals; absorbency of formazan was measured at a wavelength (λ) of 570 nm using a multwell reader (Rainbow, SLT, Austria). Treatments were compared with a positive control, deoxycolic acid (250 μM). Experiments were repeated independently three times. The results are expressed as percentage of cell viability.

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Trans-epithelial electrical resistance (TEER) assay

TEER was monitored as an indication of tight junction formation and epithelial monolayer integrity as previously described [23]. Falcon® Transwell inserts (0.4 μm pore size; BD Bioscience, Bucincozis, Italy) were coated with 0.01% type I rat-tail collagen (Sigma) and left to dry overnight under ultraviolet (UV) lights in 6-well plates. Caco-2 cells (passage between 57 and 63) were seeded into these inserts in 2.5 ml aliquots at a concentration of 2.5 × 10⁴ cells/ml. Culture medium (1.5 ml) was added to the basolateral compartment of each well. The cells were grown on these inserts and the medium was changed every 2 days. After 7 days of culturing TEER, readings (expressed as ohms/cm²) were taken using an EVDOM epithelial volthometer with chopstick electrodes (World Precision Instruments Inc., Stevenage, UK). Readings were taken every 24 hrs until they stabilized (at days 14–16). At this point the cells, maintained at 37°C in a humidified 5% CO₂ and 95% filtered air, were deemed fully differentiated. The culture medium was replaced every other day for 21 days. A final TEER reading was taken immediately before adding rhein (0.1–10 μg/ml) and after 24 and 48 hrs. Treatments were compared with a positive control, deoxycholic acid (250 μM). The results are expressed as percentage change in TEER compared with the TEER value at the start of the experiment (time 0). Three independent experiments were conducted.

Paracellular permeability assay

Caco-2 monolayers in 6-well plates were incubated with rhein (0.1–10 μg/ml) in presence of 10 μM fluorescein (Sigma). Samples (200 μl) were removed from the apical compartment at the beginning of the experiment (time 0) and from the basolateral compartment after 24 and 48 hrs. The samples fluorescence content was measured using a microplate reader (Tecan SPECTRA Rainbow, PAA Ltd, UK) (488 nm excitation and 510 nm emission). The paracellular permeability represents the amount of fluorescein in the basolateral compartment expressed as percentage of the amount added to the apical compartment at the start of the experiment, to give a measure of the relative amount of fluorescein travelling through the Caco-2 monolayer tight junctions (percentage of monolayer permeability). Three independent experiments were conducted.

Proliferation assay

³H-Thymidine incorporation

Caco-2 cells (passage between 28 and 34) were seeded in 24-well plates at a density of 1.0 × 10⁴ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. After 48 hrs of incubation, cells were treated with rhein (0.1–10 μM) in the presence of ³H-thymidine (1 μCi/well) for 24 hrs. Medium was removed by aspiration, and after washing in PBS, cells were scraped in 1 M NaOH (100 μl/well) and collected on glass fibre filter mats using an LKB (Bromma, Sweden) automatic cell harvester prior to liquid scintillation counting. The effect of rhein on cell proliferation was expressed as count per minute/mg of protein (CPM/mg protein) of incorporating ³H-Thymidine cells. The treatments were carried out in triplicate and three independent experiments were performed.

Preparation of cytosolic fractions and Western blot analysis

Caco-2 cytosolic extracts were prepared as previously described [24]. Briefly, after rhein (0.1–10 μg/ml) incubation for 24 hrs, the medium was removed and cells were washed with ice-cold PBS. The cells were collected by scraping for 10 min. at 4°C with lysis buffer (50 mM Tris-HCl pH = 7.4, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 1% NP-40, 1mM PMSF, 1 mM Na3VO4 containing complete protease inhibitor cocktail [Roche Diagnostics, Mannheim, Germany]). After centrifugation at 16,200 g for 15 min. at 4°C, the supernatants were collected and protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad, Milan, Italy). For Western blot analysis, lysate aliquots containing 70 μg of proteins were separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Protran®). Protran Nitrocellulose Transfer Membrane Schleicher & Schuell Bioscience, Dassel, Germany) using a Bio-Rad Transblot (350 mA, 3 hrs). Proteins were visualized on the filters by reversible staining with Ponceau-S solution (Sigma) and de-stained in PBS containing 0.1% Tween 20. Membranes were blocked at 4°C in milk buffer (5% non-fat dry milk in PBS/Tween 0.1%) and then incubated overnight at 4°C with mouse monoclonal antibodies for pERK1/2 and ERK2 (Santa Cruz, USA). The mouse monoclonal anti-pERK1/2 and anti-ERK2 were used at 1:1000 dilution in milk buffer (5% non-fat dry milk in PBS/Tween 0.1%). Subsequently, the membranes were incubated for 1 hr at room temperature with 1:2000-dilution of antimouse IgG-HRP-conjugated secondary antibody (Amersham Biosciences, UK). After washing in PBS/Tween 0.1%, the membranes were analysed by enhanced chemiluminescence’s (ECL: Amersham Biosciences). The optical density of the bands on autoradiographic films was determined by an image analysis system (GS 700 Imaging Densitometer, Bio-Rad) equipped with a software Molecular Analyst (IBM, Milan, Italy). The effect of rhein on the mitogen-activated protein (MAP) kinase activation was expressed as ratio of densitometric analysis of pERK1/2/total ERK bands.

Genotoxicity assay

DNA damage was examined by single-cell gel electrophoresis (Comet assay) [25]. Caco-2 cells (passage between 33 and 37) were seeded in 25 cm² polystyrene flasks (Falco) and grown to ~70% confluence. Then, the cells were incubated with rhein (0.1–10 μg/ml) for 24 hrs and subsequently they were trypsinized to obtain a suspension of 1.5 × 10⁵ cells/ml. Aliquots of cell suspension were incubated with and without H₂O₂ challenge (75 μM for 5 min. on ice) and then centrifuged at 1000 g for 5 min. at 4°C. The supernatants were discarded and the pellets were mixed with 85 μl of 0.85% low melting point agarose in PBS. Cells were added to previously prepared gels of 1% normal melting point agarose in PBS. The gels on frosted slides were mounted on a gel electrophoresis apparatus (ECL, Amersham Biosciences) and allowed to set at 4°C. The gels were immersed in 0.4 M Tris-HCl, pH 7.5 (3 × 5 min. washes) to neutralize the alkaline pH, and stained with 20 μl of ethidium bromide (2 μg/ml) before scoring. Images were analysed using a fluorescence microscope (Nikon, Florence, Italy) interfaced with a computer. DNA damage (percentage of tail DNA) was quantified using Komet 5.0 image analysis software (Kinetic Imaging, Liverpool, UK). Positive (H₂O₂: 75 μmol/L) and negative (PBS) controls were included for all experiments. The mean percentage of tail DNA was calculated from 100 cells per gel (each sample in triplicate) and the mean of each independent experiment (n = 3) was used in the statistical analysis.

Antioxidant assay

TBARS assay

Lipid peroxidation products from Caco-2 cells were measured by the thiobarbituric acid colorimetric assay [26]. The reaction of lipid peroxides...
In the MTT assay, rhein in the concentration range of 0.1–10 μg/ml had no significant cytotoxic effect on Caco-2 cells after 24 hrs exposure (control: 100 ± 0.0; rhein 0.1 μg/ml: 94.69 ± 3.45; rhein 1 μg/ml: 95.47 ± 2.49; rhein 10 μg/ml: 93.70 ± 0.42). Deoxycholic acid (DCA, 250 μM), a secondary bile acid used as positive control, significantly (P < 0.001) reduced the cell viability in proliferating Caco-2 cells (control: 100 ± 0.0 and DCA 250 μM: 69.04 ± 2.21). These data have been confirmed by the NR assay (control: 100 ± 0.0; rhein 0.1 μg/ml: 102.5 ± 2.02; rhein 1 μg/ml: 93.26 ± 7.14; rhein 10 μg/ml: 91.21 ± 5.07; DCA 250 μM: 50.5 ± 0.32).

### Western blot analysis

The possible molecular mechanism of rhein on cell proliferation was investigated by studying its effect on the MAP kinase (MAPK)
signalling pathways. The MAPK pathway involves two closely related kinases, known as ERK 1 (ERK1, p44) and 2 (ERK2, p42) that come from dimerization of total cytosolic ERK. The modulation in ERK1 and ERK2 phosphorylation after 24 hrs rhein exposure was consistent with the observed effect of rhein on cell proliferation. At concentrations of 0.1 and 1 \( \mu \text{g/ml} \), a significant \( (P < 0.001) \) reduction in the expression of phosphorylated ERK1 (pERK1) and ERK2 (pERK2) was observed (Fig. 3). By contrast, rhein at the higher concentration (10 \( \mu \text{g/ml} \)) significantly \( (P < 0.001) \) increased pERK1 and pERK2 expression, resulting in MAP kinase activation (Fig. 3).

**Genotoxicity assay**

The comet assay is a sensitive and valid method to evaluate the potential genotoxic effect of a substance. Rhein (0.1–10 \( \mu \text{g/ml} \)) alone did not significantly affect DNA damage after 24 hrs exposure suggesting the absence of a genotoxic effect. Exposure of Caco-2 cells to hydrogen peroxide (75 \( \mu \text{M} \)) produced a significant \( (P < 0.001) \) increase in the percentage of DNA in the comet tail, indicating an increase in single-strand breaks (Fig. 4). Pre-treatment Caco-2 cells with rhein for 24 hrs reduced, in a concentration-dependent manner, the \( \text{H}_2\text{O}_2 \)-induced DNA damage. The decrease was significant \( (P < 0.05) \) at a rhein concentration of 10 \( \mu \text{g/ml} \) (Fig. 4).

**MDA and intracellular ROS measurement**

Fenton’s reagent, hydrogen peroxide (1 mM) in the presence of iron (II) ions (1 mM), induced an oxidative stress in Caco-2 cells after 3 hrs exposure, resulting in malondialdehyde production \( (P < 0.001) \) (Fig. 5). Pre-incubation of Caco-2 cells for 24 hrs with high concentration of rhein (10 \( \mu \text{g/ml} \)) significantly \( (P < 0.001) \) reduced the increase of \( \text{H}_2\text{O}_2/\text{Fe}^{2+} \)-induced malondialdehyde cytosolic levels (Fig. 5). Rhein at the concentrations of 0.1 and 1 \( \mu \text{g/ml} \) was inactive.

The exposure of the Caco-2 cells to \( \text{H}_2\text{O}_2/\text{Fe}^{2+} \) (2 mM) produced a significant \( (P < 0.0001) \) increase in ROS formation (Fig. 6). A pre-treatment for 24 hrs with rhein (10 \( \mu \text{g/ml} \)) reduced significantly \( (P < 0.05) \) the ROS formation as measured by the inhibition of DCF fluorescence intensity (Fig. 6). Consistent with the results obtained in the MDA assay, rhein at the concentrations of 0.1 and 1 \( \mu \text{g/ml} \) was not active (Fig. 6).

**Discussion**

Gastrointestinal tract mucosa shows a rapid cell turnover rate determined by cell proliferation and cell loss (apoptosis) processes [29]. The integrity of the epithelial cell layer is maintained by intercellular junctional complexes composed of tight
junctions, adherent junctions and desmosomes. Tight junctions are essential structures for the physiological functions of epithelial and endothelial cells, and have been suggested to have both barrier and defence functions, including the regulation of paracellular pathways. Changes in epithelial cell turnover and alterations of the epithelial monolayer permeability can occur during many gastrointestinal diseases such as colon cancer. In fact, colon cancer cells exhibit both abnormal cell turnover and impaired tight junction morphology, leading to increase of paracellular permeability [30]. Furthermore, in the latter phases of carcinogenesis, the disruption of cell-cell junctions with concomitant changes in the junctional proteins expression represents a hallmark of cancer cell invasion and metastasis [31].
sine-5’-triphosphate (ATP) production or via an intercalation into uncontrolled proliferation of mucosa cells in the terminal portion of intestine. Conflicting results have been reported on the effect of rhein on cell proliferation. Two pre-clinical in vivo studies reported no effect of rhein on intestinal cell proliferation [41, 42] whereas other authors showed a stimulatory effect [40], and an inhibitory effect [43]. In this paper, for the first time we report a modulatory action of rhein on cell proliferation, depending on the concentration used. In fact, our experiments showed that rhein, at the concentrations of 0.1 and 1 μg/ml (rhein concentrations present in the colon after a human therapeutic dosage of senna) reduced the cell proliferation of human colon adenocarcinoma cells; whereas, rhein at the high concentration (10 μg/ml, concentration corresponding to rhein present in the colon after a dosage of senna ten-fold higher then therapeutic dosage) produced a significant increase of the human colon adenocarcinoma cell proliferation. Interestingly, long-term in vivo studies have shown that senna, used at laxative dosage, reduced the appearance of preneoplastic lesions and tumours, whereas a diarrhogenic dose increases the appearance of tumours induced by a carcinogenic agent [44, 45]. Whether or not the effect of rhein is time dependent has been not determined in this study. Also, it would be interesting to investigate in future studies if the effect of rhein involves c-Jun N-terminal kinase (JNK) or related pathways.

Using the normal human fibroblast cells and an epidermoid carcinoma cell line from human skin (A-431), we have demonstrated that the effect of rhein on cell proliferation is tumour and colon specific, respectively.

One of the most important pathway in cell proliferation is represented by the MAP kinases. MAPKs encompass a group of enzymes recruited in response to cellular stress, including heat and osmotic stress, cytokines and UV irradiation [46, 47]. The accepted role of MAPKs is to produce cellular response to those stresses by integrating multiple stimuli and activating transcription factors. In most cell lines, MAPKs have been implicated in regulating mitogenic responses and in the synthesis of stress response proteins [42]. The three major MAPK families have been recognized as p38 MAPK, ERK1 and ERK2 and stress-activated protein kinase (SAPK/JNK). Although the molecular mechanisms inducing MAPKs pathways activation are not totally clear, it is well known that ERK1,2 phosphorylated proteins stimulate the cell growth and inhibit the apoptotic processes [48, 49]. In this study, we observed that rhein at concentrations of 0.1 and 1 μg/ml reduced ERK1 and ERK2 phosphorylated proteins expression. By contrast, rhein at the concentration of 10 μg/ml determined an increase of ERK1 and ERK2 activation. These findings suggest that rhein modulates cell proliferation via an involvement of the MAPK pathway either inhibiting or stimulating the phosphorylation of ERK proteins. Our results are consistent with a recent study showing that rhein modulates cell proliferation of articular chondrocytes by acting on ERK and JNK-AP-1-dependent pathways [50]. Interestingly, other compounds isolated from wild plants, such as Saxifraga stolonifera and rhubarb have been reported to inhibit the proliferation of cancer cells by apoptosis induction or ERK phosphorylation inhibition [51, 52].

Studies in human beings and animals implicate oxidative DNA damage as an important factor in mutagenesis and carcinogenesis.
In our experiments, rhein did not induce DNA damage in Caco-2 cells suggesting that it is not a genotoxic agent. In fact, rhein exerted a protective effect on hydrogen peroxide-induced DNA damage in human colon carcinoma cells at the concentration of 10 \( \mu \text{g/mL} \). This is consistent with a previous report showing a rhein-mediated inhibition of DNA damage induced by Trp-P-1 in human lymphocytes [56].

In order to investigate the mechanism responsible for the genoprotective effect of rhein on \( \text{H}_2\text{O}_2 \)-induced DNA damage, we investigated MDA and ROS cytotoxic levels in Caco-2 cells. In accordance with other papers, exposure of Caco-2 cells to hydrogen peroxide and ferrous sulphate (Fe\(^{2+}\)) resulted in oxidative damage, assessed as increased cytotoxic levels of MDA and ROS [57, 58]. Pre-treatment with rhein significantly reduced oxidative damage, assessed as increased cytosolic levels of MDA and ROS [57, 58].

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Competing interest

None.

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