Research Article

Bitter Tastant Responses in the Amoeba *Dictyostelium* Correlate with Rat and Human Taste Assays

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Summary

Treatment compliance is reduced when pharmaceutical compounds have a bitter taste and this is particularly marked for pediatric medications. Identification of bitter taste liability during drug discovery utilizes the rat *in vivo* brief access taste aversion (BATA) test which, apart from animal use, is time consuming with limited throughput. We investigated the suitability of using a simple, non-animal model, the amoeba *Dictyostelium discoideum,* to investigate taste-related responses and particularly identification of compounds with a bitter taste liability. The effect of taste-related compounds on *Dictyostelium* behavior following acute exposure (15 minutes) was monitored. *Dictyostelium* did not respond to salty, sour, umami or sweet tasting compounds, however, cells rapidly responded to bitter tastants. Using time-lapse photography and computer-generated quantification to monitor changes in cell membrane movement, we developed an assay to assess the response of *Dictyostelium* to a wide range of structurally diverse known bitter compounds and blinded compounds. *Dictyostelium* showed varying responses to the bitter tastants, with IC_{50} values providing a rank order of potency. Comparison of *Dictyostelium* IC_{50} values to those observed in response to a similar range of compounds in the rat *in vivo* BATA test showed a significant ($p = 0.0132$) positive correlation between the two models and, additionally, a similar response to that provided by a human sensory panel assessment test. These experiments demonstrate that *Dictyostelium* may provide a suitable model for early prediction of bitterness for novel tastants and drugs. Interestingly, a response to bitter tastants appears conserved from single-celled amoebae to humans.

Keywords: BATA test, bitter tastants, *Dictyostelium discoideum*, taste aversion, replacement

1 Introduction

The ability to detect bitter substances is considered to have evolved to enable the recognition of toxic substances, which often present with a strong bitter taste (Mennella et al., 2013). Thus, there are clear survival advantages to the rejection of bitter tasting foods and the induction of learned aversion in the wild (Glendinning, 1994). However, when such effects are induced by therapeutic agents, many of which have a bitter taste, they can have a negative impact on compliance with treatment, leading to sub-optimal therapy. For example, around 40% of children worldwide are likely to not follow prescriptions due to the bitter taste of a medicine, leading to suboptimal dosing and preventable potential therapeutic failure (Mennella et al., 2013). Whilst reduced compliance is particularly acute in children, it is also a well-recognized factor in treatment regimens in adults (Clapham et al., 2012; Mennella et al., 2013).

In the development of new pharmaceuticals, unpleasant taste liability may not be apparent until initial clinical trials are undertaken (Clapham et al., 2012). If a strongly aversive taste is identified there may be a need to repeat studies with a taste matched placebo or to undertake taste masking of the active pharmaceutical ingredient (API) (where this is possible). In some cases it may be necessary to identify a different salt version of the API or even to change the API for another candidate, with clear implications for progression to the market and delay of patient access to a new therapeutic. It is also possible, at this stage, that the studies could be unblinded because

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of taste effects. Therefore, it is highly important to identify bitter taste effects early in the drug discovery process so that competing compounds and/or salt versions of compounds without such liability can be selected for development. In addition to acting on the tongue, bitter substances have also been shown to act in the pharynx, gut and airways (Bachmanov and Beauchamp, 2007). In airways, bitter tastants increase the beat frequency of cilia (Shah et al., 2009) with the potential to increase airway clearance and hence reduce the therapeutic effect of inhaled drugs. These increasingly complex effects and tissue specificity of bitter substances further emphasize the importance of identification of such substances early in the drug discovery process.

There is no universally applicable chemical approach to identifying compounds that will trigger a bitter taste response, although alkaloid structures have been associated with bitterness (Drewnowski and Gomez-Carneros, 2000). Since taste responses are based in the peripheral gustatory system along with a central nervous system recognition component, most research in this area employs animals or human-based tests. Currently, one technique employed to assess the palatability of drugs, including novel chemical entities (NCE), is the brief access taste aversion (BATA) model using the rat (Rudnitskaya et al., 2013) or mouse (Devantier et al., 2008). Although this assay is not considered harmful to the animals and has demonstrable translation to humans for identification of bitter tastants (Rudnitskaya et al., 2013), it is potentially unpleasant for the animal (due to the aversive nature of some of the substances tested), is relatively expensive, time consuming and has a limited throughput capacity. Thus, there is a need for a non-animal, higher throughput assay that can reliably establish the potential of a chemical to trigger a bitter taste.

The social amoeba *Dictyostelium discoideum* is a simple model system used for a range of pharmacological projects. It is a eukaryote with a haploid genome (Williams et al., 2006), and exhibits a bi-phasic life cycle, divided into unicellular and multicellular stages. In the unicellular stage, starvation induces individual cells to undergo directional movement (chemotaxis) to coalesce and form a multicellular fruiting body. It is at this stage, that *Dictyostelium* has been extensively utilized to investigate a range of fundamental biological processes such as cell migration and signal transduction, as well as a range of pharmacological studies. These include identifying the molecular targets of flavonoids (Waheed et al., 2014), bipolar disorder treatments (Williams et al., 2002; Williams, 2005) and epilepsy treatments including the MCT ketogenic diet (Xu et al., 2007; Chang et al., 2012). In all these cases, discoveries in *Dictyostelium* have been successfully translated to humans or other mammals (Xu et al., 2007; Chang et al., 2013, 2014). Finally, *Dictyostelium* was able to identify pungent (e.g., capsaicin) and bitter (quinine, denatonium, phenylthiourea) tastants (Robery et al., 2013; Otto et al., 2015) and led to the discovery of a novel human receptor implicated in detection of the bitter tastant phenylthiourea (Robery et al., 2013). These wide-ranging studies demonstrated the potential utility for *Dictyostelium* in the identification of novel pharmaceutical compounds with a bitter taste liability.

Here we investigated the effect of representative compounds from the five basic taste sensations, i.e., bitter, sweet, sour, salty and umami (Drewnowski and Gomez-Carneros, 2000; Wooding et al., 2010; DeSimone et al., 2012; Bachmanov and Beauchamp, 2007; Kawai et al., 2009; Uneyama et al., 2009) on *Dictyostelium* cell behavior. We found that only bitter tastants rapidly and strongly affected cell behavior, and developed an approach to quantify these changes. We then investigated a range of compounds with diverse chemical structures and bitterness (including compounds to which the investigators were blind) tailored to test if the model system is able to predict the bitterness of those compounds assessed in the *in vivo* rat BATA test and a human sensory panel (Clapham et al., 2012; Rudnitskaya, et al., 2013). Analysis of each compound in *Dictyostelium* provides concentration-response curves and IC_{50} values (the concentration of substance producing 50% inhibition), enabling comparisons of potency between compounds and comparison with rat and human data in addition to objective assessment of the potential for *Dictyostelium* to replace the rat BATA assay in identification of bitter taste liability of NCEs.

2 Animals, materials and methods

Chemicals

The following chemicals were obtained from Sigma Aldrich Co. Ltd (Dorset, UK): azelastine hydrochloride (4-((4-chlorophenyl)methyl)-2-(1-methylazepan-4-yl)phthalazin-1-one hydrochloride; A7611), chlorhexidine digluconate (1,1′-hexamethylenebis(5-(p-chlorophenyl) biguanide; C9394), caffeine anhydrous (1,3,7-trimethylxanthine; W222402), quinine hydrochloride dihydrate (Q1125), acetaminophen (paracetamol, 4′-hydroxyacetanilide; A7085), ibuprofen sodium salt (α-methyl-4-(isobutyl) phenyl acetic acid; I1892), potassium nitrate (KNO_3; P8394), adenosine 3′,5′-cyclic monophosphate (3′,5′-cyclic AMP; A9501 - 200mM stock solution), glucose (D(+)-glucose; G8270), sucrose (D(+)-saccharose; S1888) and glutamate (L-glutamic acid monosodium salt monohydrate; 49621). Compounds labelled "GSK" were provided by our industrial collaborators, GlaxoSmithKline, and due to intellectual property protection, full names and structures have been withheld.

Dictyostelium random cell movement

Dictyostelium cells were maintained in Axenic medium (Formedium Co. Ltd, Norfolk, UK) for at least 48 h prior to harvesting in mid-log phase growth (2-5 x 10^6 cells/ml). Cells (1 x 10^7) were washed with phosphate buffer (16.5 mM KH_2PO_4, 3.8 mM KH_2PO_4, pH 6.2), resuspended in 6 ml phosphate buffer and pulsed for 5 h with 30 nM cAMP at 6 min intervals at 120 rpm. Cells were resuspended in 4 ml phosphate buffer and diluted 1:9, and 250 µl aliquots of cells were transferred into Nunc Lab-Tek chambered cover glass (Thermo Fisher, Leicestershire, UK), and allowed to adhere for 10-15 min. In each experiment 250 µl of drug stock solution (to produce the desired final concentration) was added at the 225th second of the time lapse recording to investigate the effects on cell movement.

Osmolarity, acidity and vehicle control experiments

To investigate whether the changes in cell behavior were due

to osmolarity and pH variation, cells were exposed to increasing salt concentrations and different pH values. Phosphate buffer was prepared 10X (ten times the standard buffer concentration) by using 22 g of KH_2PO_4 and 7 g of KH_2PO_4 (total volume 1 l) and diluted for the experiments to 3.3X and 5X. For experiments regarding pH changes, a buffer solution was prepared using 2.72 g of K_2HPO_4 in 800 ml of water and the pH was adjusted with 1M KOH and made up to 1 l to obtain a final solution with a pH of 5. For the buffer with pH 7, 6.81 g of K_2HPO_4 and 291 ml of 0.10 M NaOH were made up to 1 l. Solvent only controls (DMSO at 1.5% (220 mM) or ethanol at 4.5% (770 mM)) were carried out for all experiments to establish that they did not significantly alter cell behavior (see Fig. S1 at http://dx.doi.org/10.14573/altex.1509011s1).

Live cell microscopy

To assess the suitability of *Dictyostelium* as a non-animal model for the investigation of bitter substances, a standardized assay was developed (Otto et al., 2015). Cell behavior was monitored in cells undergoing random movement by taking images every 15 sec over a 15 min period, with 3 min and 45 sec recorded prior to, and 11 min and 15 sec after compound addition. A minimum of three independent experiments for each drug concentration were used with at least 10 cells quantified per experiment. From these series of images, parameter protrusion formation was quantified with Fiji (Schindelin et al., 2012) using an image analysis software plugin, QuimP 11b software (Warwick University, Warwick, UK). Prior statistical analysis data were analyzed and formatted using MATLAB (Mathworks, Cambridge, UK).

Statistical analysis of cell movement

Data derived from membrane protrusions of cells during random movement was extracted from videos into a GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) spreadsheet as time versus number of protrusions formed. Data was normalized by defining zero as the smallest value in each data set and one hundred as the largest value in each data set, and the data expressed as a fraction. Mean and standard error was calculated for each set of results at all concentrations. To assess whether there was a significant change in protrusion formation, an unpaired, two tailed t-test (95% confidence interval) was used, comparing the mean of the last 8 min (from minute 4 min 30 sec to minute 12 min 30 sec) against the control conditions for all concentrations tested. To calculate the IC_{50} (the concentration required to produce a 50% reduction in cell movement) for each compound, the mean of the last 8 min of protrusion formation and the standard error were selected and plotted against their Log (concentration), and IC_{50} values with 95% confidence intervals were obtained by non-linear regression Log (inhibitor) vs. normalized response-variable slope equation.

Rat brief access taste aversion (BATA) assay

All the experiments were reviewed by an ethics committee, authorized by the UK Home Office and performed in accordance with the Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals.

Male rats weighing 250 to 350 g (age 8-10 weeks) Crl: CD (SD) strain (Charles River, UK) were used in the study of the five GSK compounds (12 rats per compound) to which the *Dictyostelium* investigators were blinded. Animals were kept under controlled environmental conditions (19-23°C; 45-65% humidity; 12 h light/dark cycle) with free access to food (Labdiet 5LF2 EURodent Diet 14%) and animal grade water (reverse osmosis filtered and UV treated) between test sessions. BATA tests were performed between 09.00 and 13.00. The BATA assay (see Devantier et al., 2008; Clapham et al., 2012 for additional details) employed an automated apparatus (MS-160 Davis Rig gustatory behavior apparatus, DiLog Instruments, Tallahassee, FL, USA) to measure the number of licks in response to water, a calibration compound or the test compound. The percentage inhibition at various concentrations of the test substance presented on multiple occasions in random order was used to calculate the IC_{50} (curve fitting with a four parameter logistic curve restrained to zero; SAS) for test substances with 12 rats used per group to test each substance. Tests were conducted using a one-week standardized protocol and analysis of welfare indicators showed that the rats were not adversely affected by these tests (Clapham et al., 2012). The protocol taken from (Clapham et al., 2012) is briefly as follows: Day 1. Withdrawal of water; Day 2. Shutter open (30 min), 1 bottle, water only. Train drinking. Rehydration; Day 3. Standard sequence (30-45 min), water only. Acclimatize to protocol. Rehydration; Day 4. Presentation sequence one. Controls and concentration response. Rehydration; Day 5. Presentation sequence two. Controls and concentration response. The same rats can be used over an extended period of testing (many months) with no loss of sensitivity of response. When required, rats were killed humanely (intraperitoneal overdose of pentobarbitone).

3 Results

3.1 *Dictyostelium* cell behavior responds to acute application of bitter tastants

Initial analysis of the effect of compounds representing the five basic taste groups (bitter, salty, sweet, sour and umami) on *Dictyostelium* used cells in the aggregation phase of development (showing active polarized movement), with cell images recorded before (Fig. 1A) or after treatment (Fig. 1B). To mimic salty taste, cells were exposed to increasing salt concentrations (potassium phosphate), from standard buffer conditions (24.2 mM potassium) up to a 3.3-fold increase (80 mM), and no gross change was observed in *Dictyostelium* cell shape following treatment. This is consistent with that reported by Robery et al. (2011), where *Dictyostelium* does not respond to the salty tasting central nervous system depressant lithium chloride (Schiffman and Erickson, 1971) under equivalent conditions. To mimic sour taste (Da Conceicao Neta et al., 2007), cells were transferred to a buffer at pH 5 (from pH 6.3). No gross change was observed in *Dictyostelium* cell shape following treatment. Similarly, treatment of cells with umami-related tastant, glutamate (6 mM; Fig. 1B) (Kawai et al., 2009; Uneyama et al., 2009), and sweet tasting glucose (10 mM; Fig. 1B) (Welcome

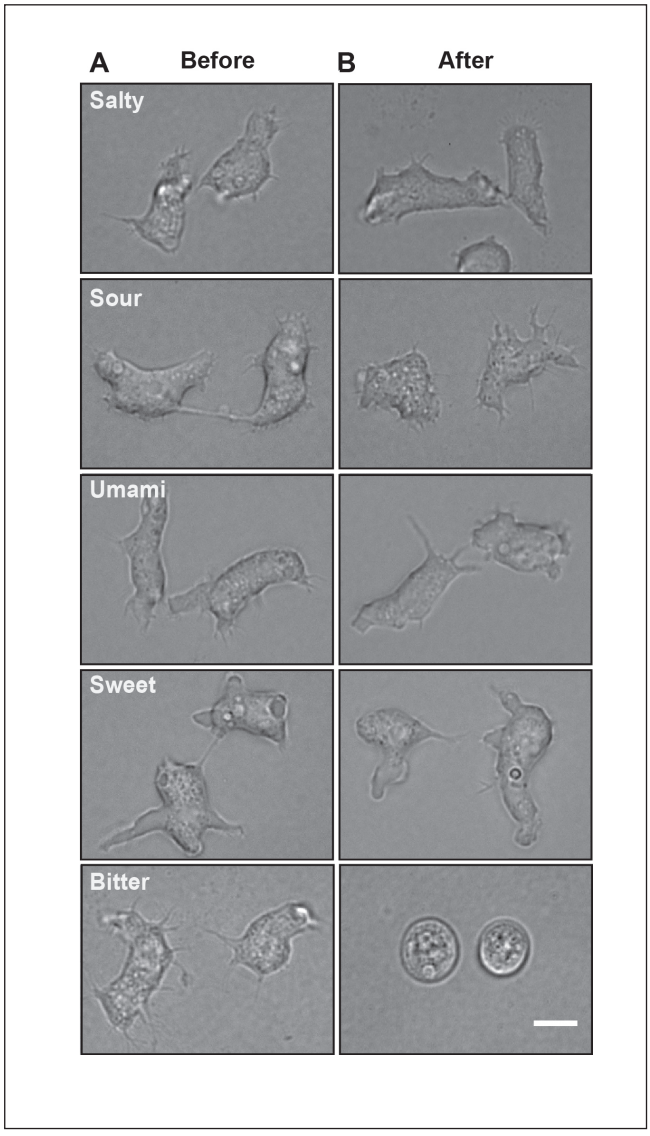

Fig. 1: *Dictyostelium* response to salty, sour, umami, sweet and bitter tastants

A. Images of *Dictyostelium* cells taken before the administration of the substances that represent each taste.

B. Responses in cells exposed to salty (3.3-fold higher salt content, 80 mM), sour (pH reduced from pH 6.3 to pH 5), umami (glutamate 6 mM), sweet (glucose 10 mM), or bitter (chlorhexidine 0.025 mM) tastants respectively.

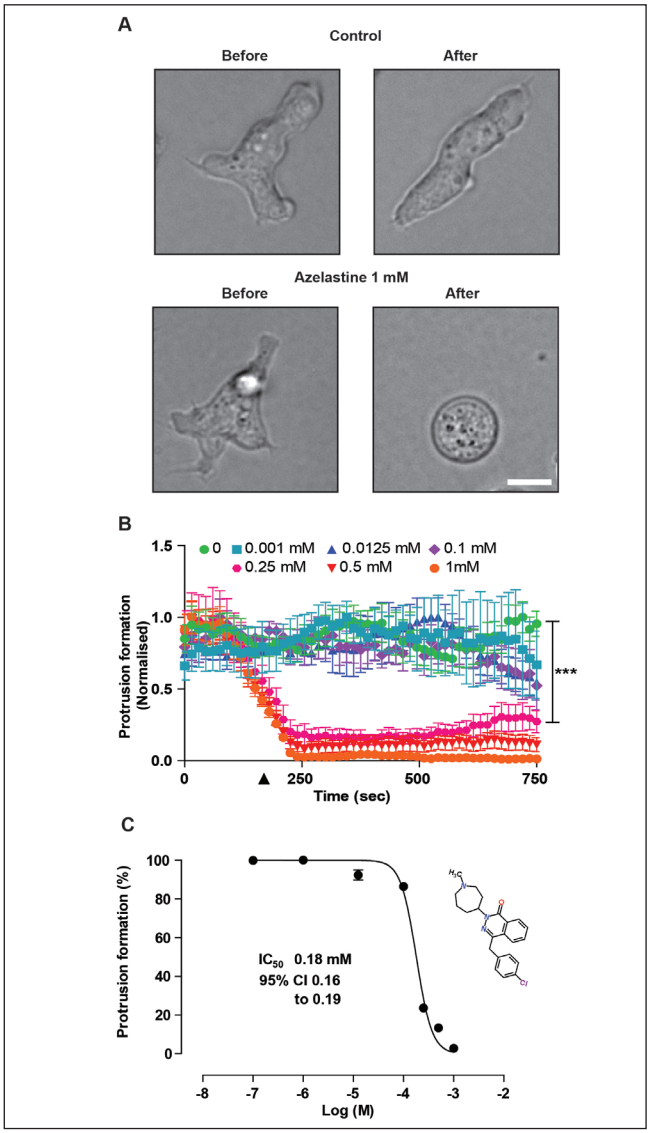

Fig. 2: Quantifying *Dictyostelium* response to bitter tastants

A. Images of individual *Dictyostelium* cells prior to and 15 minutes after addition of solvent only (control) or a bitter tastant (azelastine at 1 mM) showed that the bitter tastant caused a block in cell behavior (membrane protrusions) enabling quantification of compound effect. Scale bar is 12 µm.

B. Time-dependent changes in *Dictyostelium* cell behavior (membrane protrusions) was recorded over a 15 minute period for triplicate experiments (± SEM) at increasing concentrations of azelastine; addition of different concentrations of azelastine at 210 seconds (▲). Data is presented normalized to control conditions. Analysis with one-way ANOVA of the reduction of protrusion formation caused by azelastine showed a significant difference between control condition (vehicle) and 0.25 mM azelastine ($p < 0.05$ ***).

C. Concentration dependent response is illustrated as the normalized reduction of cell behavior (protrusion formation) against the Log (concentration) of azelastine, enabling calculation of an IC_{50} of 0.18 mM with a 95% confidence interval of 0.16 to 0.19 mM. The chemical structure of azelastine is provided as an insert.

et al., 2015) did not alter cell shape. However, treatment of cells with the standard bitter tasting substance, chlorhexidine (25 μM), caused a rapid loss of cell behavior leading to cell rounding. These results suggest that, under the conditions examined here, only the representative bitter tastant caused an effect on *Dictyostelium*.

Since *Dictyostelium* responded to chlorhexidine and earlier studies showed a response to the bitter tastants phenylthiourea and denatonium (Robery et al., 2013), we then sought to develop an approach to quantify cell behavior changes using another bitter substance, azelastine (Clapham et al., 2012) (250 μM; Fig. 2A). By recording time lapse images of cells over a 15 minute period, including baseline (prior to substance addition) and post addition (see also Fig. S2 at http://dx.doi.org/10.14573/altex.1509011s1 and Movie at http://dx.doi.org/10.14573/altex.1509011s2), and using computer-aided image analysis (Tyson et al., 2014), we monitored acute change in normalized cell behavior (protrusion formation) following tastant exposure (Fig. 2B). In this assay, cells maintained constant behavior over the test period following compound vehicle treatment alone. Addition of azelastine (1 μm-1 mM; Fig. 2C) did not affect cell behavior up to 100 μM, but caused a dose-dependent reduction in cell behavior at higher concentrations reaching near maximal effect at 250 μM. To extract comparative data from these results, cell responses during the last 8 minutes of treatment were averaged and plotted against compound concentration (28-32 cells per concentration) (Fig. 2D), with non-linear regression used to calculate the IC_{50} value for the compound. To examine if the effect of this compound was due to induction of cell death, we also exposed cells to azelastine (0.5 mM) for 10 minutes, washed off the azelastine with phosphate buffer and recorded cell behavior after 1 h, to show cells restored untreated behavior (see Fig. S2 at http://dx.doi.org/10.14573/altex.1509011s1).

3.2 Structurally diverse bitter tastants affect *Dictyostelium* cell behavior

Since bitter substances represent a wide chemical space with a range of different potencies in taste models, we then analyzed a broad group of chemical structures with different bitterness

Tab. 1: List of bitter substances with different mechanisms/receptor targets examined
A range of concentrations were tested for each compound, spanning concentrations used in human or rat studies.

Name	Mechanism / Target	Effects on *Dicty*	Conc. range examined (mM)	Human effect range (mM)	Rat effect range (mM)	Reference
Chlorhexidine Digluconate	Antimicrobial agent	Y	0.001 - 1	0.13 - 2.3	0.01 - 30	Clapham et al., 2012; Rudnitskaya et al., 2013; Trufello et al., 2014
Azelastine Hydrochloride	2nd-Gen, selective, histamine H_1-receptor antagonist; Rhinitis	Y	0.001 - 1	0.74 - 2.4	0.002 - 1	Uneyama et al., 2009; Clapham et al., 2012; Rudnitskaya et al., 2013; El-Shaheny and Yamada, 2014
Ibuprofen Sodium	NSAID - COX inhibitor; Analgesic	Y	0.001 - 10	n/a	0.1 - 30	Van et al., 1995; Clapham et al., 2012; Rudnitskaya et al., 2013
Quinine Hydrochloride	Crystalline alkaloid; Anti-malarial; Bitterness standard	Y	0.001 - 2	0.0028 - 0.016	0.01 - 5	Uneyama et al., 2009; Robery et al., 2011
Caffeine Anhydrous	Stimulant drug; Bitterness standard	Y	0.001 - 100	3.1 -16	1 - 100	Boughter, Jr. and Whitney, 1997; Zanger et al., 2008; Clapham et al., 2012; Rudnitskaya et al., 2013
Potassium Nitrate	Ionic salt ($K^+ NO_3^-$); Toothpaste ingredient	Y	0.001 - 500	89 - 500	0.1 - 3 M	Clapham et al., 2012; Rudnitskaya et al., 2013
Paracetamol (Acetaminophen)	COX-2 inhibitor; Analgesic	Y	0.001 - 75	6 - 33	0.1 - 30	Kis et al., 2005; Clapham et al., 2012; Rudnitskaya et al., 2013
GSK1C	H_1 receptor antagonist	Y	0.001 - 1	n/a	n/a	n/a
GSK0A	p38 inhibitor	Y	0.001 - 1	n/a	n/a	n/a
GSK7B	p38 inhibitor	Y	0.001 - 2	n/a	n/a	n/a
GSK9A	H_1 receptor antagonist	Y	0.001 - 1.65	n/a	n/a	n/a
GSK7L	p38 inhibitor	Y	0.001 - 5	n/a	n/a	n/a

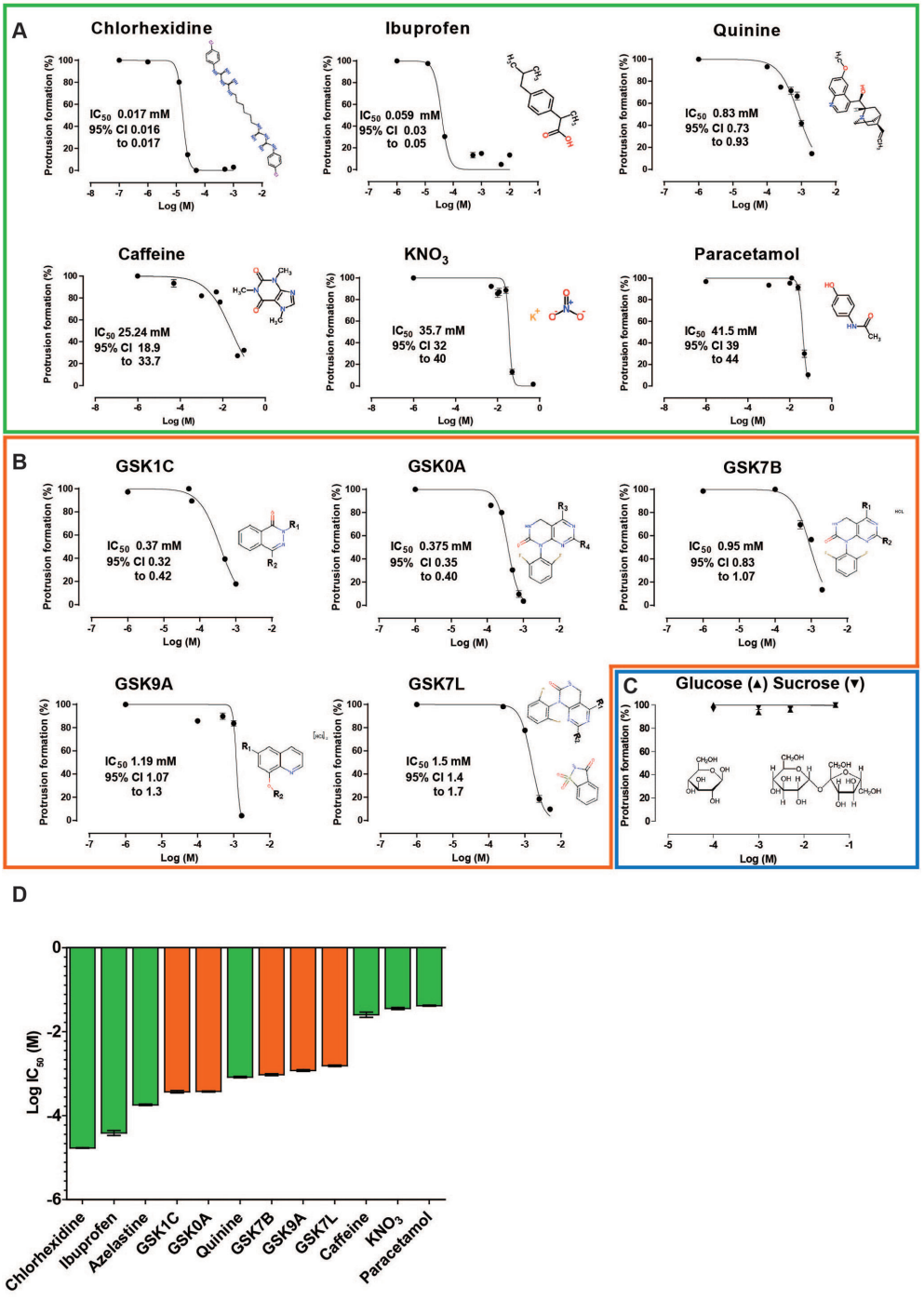

Fig. 3: Sensitivity of *Dictyostelium* to a range of bitter tastants

A. Using a range of substances with known variation in bitterness, concentration dependent responses were determined for *Dictyostelium* cell behavior (protrusion formation), and illustrated as the normalized reduction in response against the Log (concentration) of each compound (shown with errors based on the 95% confidence intervals), enabling calculation of an IC_{50} value and 95% confidence intervals for each compound. The graphic formula for each compound is provided as an insert to highlight the diversity of examined chemicals.

B. This analysis was repeated using five blinded compounds, provided by the industrial collaborator, again providing IC_{50} values and 95% confidence intervals. The core structure of each molecule is shown, with the side chains illustrated as R 1-4 due to intellectual property considerations.

C. The analysis was repeated with two non-bitter substances, sucrose and glucose.

D. Rank order of potency is provided for all tested compounds, based upon IC_{50} values.

ranking as described in the literature (Clapham et al., 2012). Selecting compounds that have established activities in the rat *in vivo* BATA test, we repeated the *Dictyostelium* cell behavior analysis experiments with these compounds (Fig. 3A). These compounds were: chlorhexidine digluconate, azelastine, ibuprofen, quinine, caffeine, potassium nitrate and paracetamol (acetaminophen). These compounds include organic and inorganic structures, with widely varying chemical composition (Tab. 1), and with a diverse range of known (and unknown) cellular effects. Compounds were tested over 3 to 4 log scale units of concentration for effects on *Dictyostelium* cell behavior, again with cell shape recorded prior to and after addition of each compound at each concentration. All compounds caused a change in cell behavior (reduced protrusions) at increasing concentrations (and see also Fig. S3 at http://dx.doi.org/10.14573/altex.1509011s1), and non-linear kinetic analysis enabled IC_{50} values to be determined with 95% confidence intervals, providing an activity for each compound in this model. Repetition of the behavioral tests using two compounds at two concentrations two months after the first experiments showed comparable responses not significantly different from each other (see also Fig. S4 at http://dx.doi.org/10.14573/altex.1509011). These experiments show that *Dictyostelium* can be used to reliably and reproducibly distinguish between the effects of a range of compounds associated with a bitter taste.

Since behavioral tests – even at a cellular level – may give rise to user-dependent outcome bias, a range of blinded compounds provided by the industrial partner were also examined (Fig. 3B). The structures and taste characteristics of these compounds were unknown to those conducting the *Dictyostelium* studies prior to calculation of the IC_{50} values. The compounds provided could have included any substance (i.e., including non-bitter tastants) studied in the rat BATA assay so that values from the rat and *Dictyostelium* could be compared.

The compounds provided by the industrial partner have different core chemical structures and variable functional side groups (represented by R1-R4) providing large chemical differences in overall structure (Fig. 3B). Again, analysis of the effect of the compounds on *Dictyostelium* cell behavior enabled IC_{50} values to be calculated as previously (Fig. 3B), indicating a range of different potencies for these compounds. In contrast, two non-bitter compounds (glucose and sucrose) were also assessed at multiple concentrations without effect (Fig. 3C and see also Fig. S5 at http://dx.doi.org/10.14573/altex.1509011s1). Comparison of IC_{50} values from the known bitter compounds (Fig. 3A) and the blinded compounds (Fig. 3B) enabled a potency ranking of compounds in the *Dictyostelium* model (Fig. 3D).

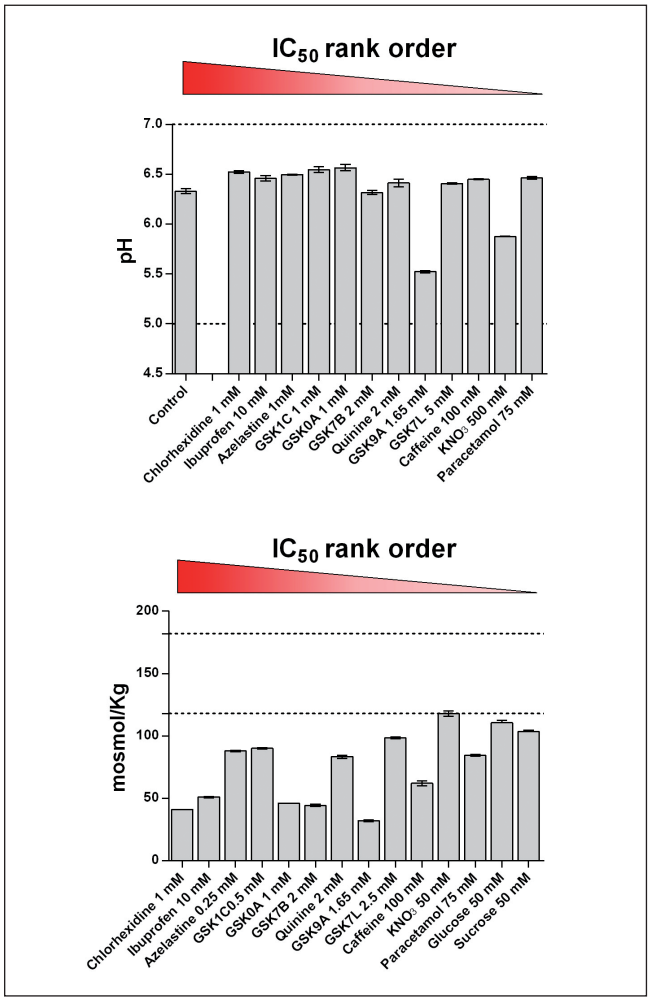

Fig. 4: Physicochemical properties of bitter molecules do not block *Dictyostelium* cell behavior

A. Comparison of cell behavior at pH 5 and at pH 7. Control condition represents the pH of the phosphate buffer used to resuspend cells for the random movement assay. Results show no effect in protrusion inhibition due to pH changes in this range. The range of compounds studied had a pH of *circa* 6.5 when in solution, with two exceptions: GSK9A (pH 5.5) and KNO_3 (pH 5.9).
B. Assessing the effects of osmolarity on cell behavior. Cell behavior was not inhibited by an osmolarity of 118 mosmol/kg (lower dotted line), which is the case of KNO_3. The osmolarity levels of test conditions for all the other compounds where below 118 mosmol/kg. At higher osmolarity levels (182 mosmol/kg, upper dotted line), cells were arrested in movement.

3.3 Physiochemical variables are not responsible for *Dictyostelium* movement inhibition

We next investigated a potential role for the compounds in altering *Dictyostelium* behavior through changing pH or osmolarity (Fig. 4). Firstly, exposing cells to pH conditions ranging from 5 to 7, on either side of the control buffer (pH 6.3), caused no changes in cell behavior (Fig. 4A). Measurement of the buffer pH at the concentration of each compound that blocked cell movement indicated that the compounds did not change buffer pH outside the range 5.5 and 6.6 (Fig. 4A), showing that these compounds did not alter cell behavior through pH changes. Secondly, exposing cells to increased osmolarity using elevated buffer concentration (Fig. 4B) caused no change in cell behavior up to 118 mosmol/kg, with affected behavior at 182 mosmol/kg (Fig. 4B). For all compounds and concentrations tested, buffer osmolarity did not exceed 118 mosmol/kg (Fig. 4B) even at the concentration of each compound that blocked cell movement,

demonstrating that these compounds did not alter cell behavior through osmolarity changes. In addition, maximal solvent concentrations did not alter cell behavior (Fig. S1). Overall these findings support the hypothesis that *Dictyostelium* cell behavior responses are due to properties of the compounds related to their ability to induce a bitter taste in mammals.

3.4 *Dictyostelium*, rat BATA test and human taste panel comparison reveals similarities in predicting bitterness

To evaluate whether the IC_{50} values calculated for each of the compounds using *Dictyostelium* cell behavior inhibition were predictive of perceived bitterness, we compared our data to results obtained in the established rat brief access taste aversion (BATA) assay (Fig. 5A), which has been shown to have predictive value for identification of bitter tasting substances in humans (Clapham et al., 2012; Rudnitskaya et al., 2013). This data was defined here for the five blinded GSK compounds, and for the other six compounds reported earlier in validation studies of the BATA assay using a total of 192 animals employing identical methodology (Clapham et al., 2012; Rudnitskaya et al., 2013). Data from these two sets were compared using a radar plot, where the similarities between the IC_{50} values for multiple compounds can be easily identified (Fig. 5A,B). Both *Dictyostelium* and rat models show an overall similar response to the known compounds, reflected by the conserved shape of the plot. Some compounds, chlorhexidine, ibuprofen and KNO_3 were more tolerable in the rat model, whereas quinine

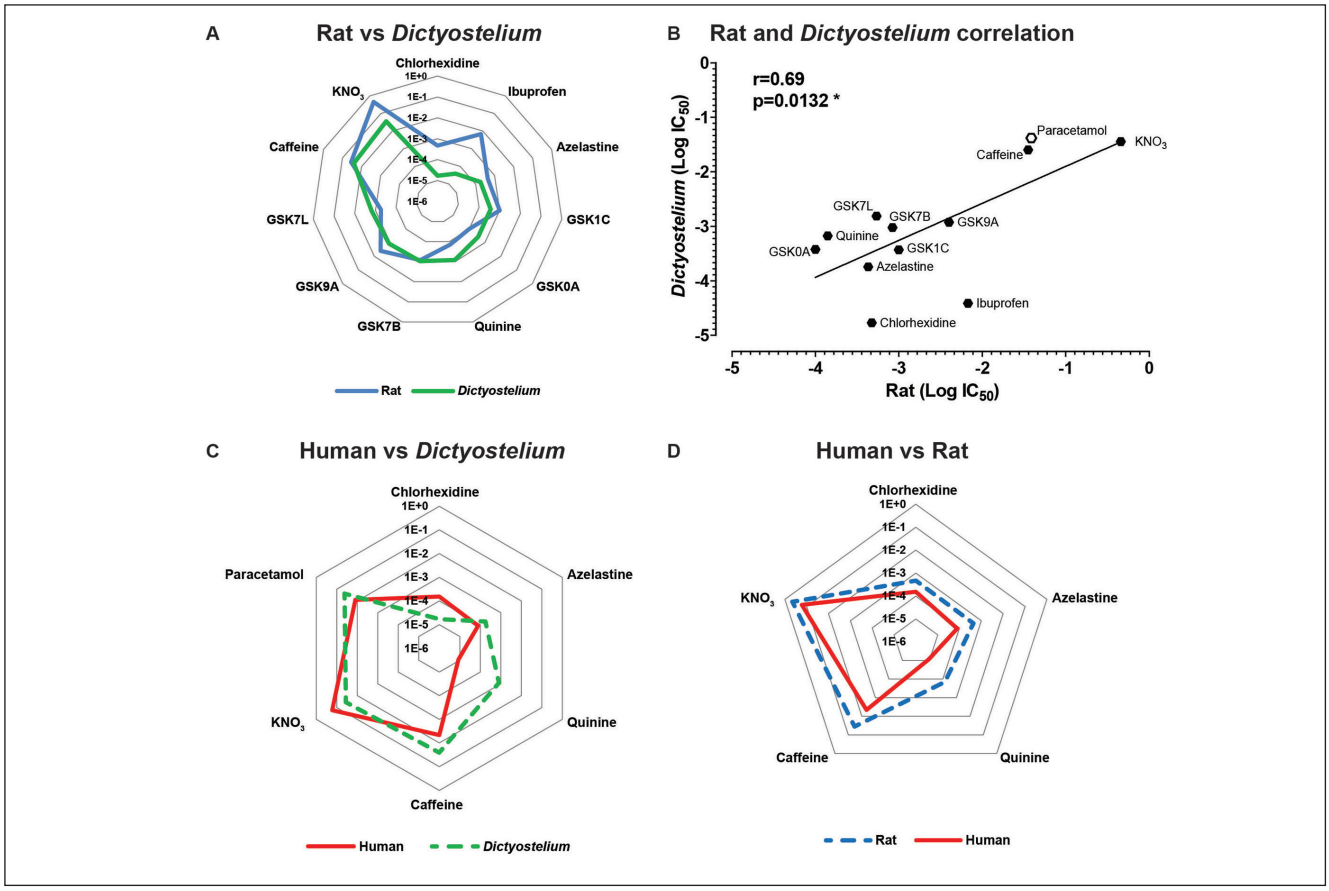

Fig. 5: *Dictyostelium* behavior model shows similarity to rat and human bitter taste models

A. Comparison of IC_{50} data derived from the *Dictyostelium* cell behavior model (green) and the rat BATA test model (blue). The radar plot provides the IC_{50} value of each compound on each corner of the polygon in Log scale. The ranking of potency starts with chlorhexidine and proceeds clockwise. The closer to the center the IC_{50} value is, the more potent the compound. The overlapping of the lines generated by connecting the values for all compounds of the two models show a similar trend.

B. The correlation graph (Log IC_{50} in M) was obtained by comparing the IC_{50} values of rat (values for GSK compounds from this study; other values from Clapham et al., 2012 and Rudnitskaya et al., 2013) and *Dictyostelium* models, and results show a significant correlation ($p = 0.0172^*$). The value for paracetamol is predicted (from the human data) using the constant difference between human and rat of one Log scale.

C. Human (red) and *Dictyostelium* data analysis shows that azelastine, caffeine, KNO_3 and paracetamol have a similar score prediction in the two models, whereas quinine and chlorhexidine have a different output. Data from humans taken from Clapham et al. (2012).

D. Rat and human comparison shows similar sensitivity with regard to chlorhexidine, azelastine, caffeine and KNO_3, and rat model is less susceptible to quinine by a Log unit.

(used at comparable levels in several studies (Soto et al., 2015; Clapham et al., 2012) was more tolerable in the *Dictyostelium* model. With regard to the blind compounds tested, the rat and *Dictyostelium* models also showed similar responses, with all compounds showing a similar potency to within half a Log unit. To determine whether the two data sets were comparable, we performed a Pearson correlation test, which showed a significant level of correlation between the *Dictyostelium* and rat models ($p = 0.0132$) (Fig. 5B). This analysis demonstrates that the *Dictyostelium* response to a wide range of chemical structures correlates with the data from the rat BATA test using the same range of bitter tastants.

Animal taste perception models may show considerable variation in response compared to that observed in human taste tests. Few published human taste response tests with IC_{50} values are available, thus providing scant data for direct comparison between *Dictyostelium* and human responses. However, we were able to compare the responses in *Dictyostelium* with results obtained previously for six compounds studied in a human sensory panel using established taste assessment methodology (Fig. 5C) (Rudnitskaya et al., 2013). In these standardized tests, human volunteers are asked to score the bitterness of solutions with each testing session including a fixed concentration of quinine (5 μM) as a calibration to ensure inter-session consistency (Rudnitskaya et al., 2013). Both *Dictyostelium* and humans show an overall similar order of response to compounds, reflected by the conserved shape of the plot, although the human response to quinine was stronger and to chlorhexidine was weaker than that observed for *Dictyostelium*. Although the data is limited, the *Dictyostelium* response to a range of chemical structures of varying bitter taste correlates with the taste responses in humans.

To explore the difference between the rat and human taste models, we also compared responses between these two models using the same radar plot analysis (Fig. 5D). Such a comparison also allows us to draw more valid conclusions regarding the relationship between *Dictyostelium* and human assessment. This analysis showed that the rat BATA response was consistently less sensitive to the range of bitter tastants examined than the human sensory panel, with this change varying between a half and one log unit. This analysis shows that the rat BATA response to a range of chemical structures of varying bitter taste is similar to that observed in humans, although with a lower sensitivity to all compounds. This difference in magnitude of response is to be expected since the rat response is driven by thirst whereas the human response is not. Thus, the rat is somewhat more tolerant of bitterness than the human subjects. Importantly the offset in IC_{50} values between the rat and human for these compounds is relatively consistent, allowing a good prediction of the human response to the particular tastant. Encouragingly, it would appear that a similar situation exists for the *Dictyostelium* response, at least for the majority of the compounds tested, suggesting that the amoeba model is likely to be predictive of the human response.

4 Discussion

In this paper, we investigated the suitability of employing a simple model system, *Dictyostelium*, in taste perception studies. Exposing *Dictyostelium* to substances that evoke the five basic tastes in humans showed that it was only affected by the bitter tastants. The response of *Dictyostelium* to bitter tastants is to lose the typical amoeboid shape and round up, and in so doing, block membrane protrusion formation. There are many potential mechanisms behind this effect. *Dictyostelium* is a well-studied model for cell movement (Dang et al., 2013; Artemenko et al., 2014) and has been explored in a range of pharmacological studies for identifying chemical targets (Robery et al., 2013; Waheed et al., 2014; Lockley et al., 2015). Indeed, a large number of studies have identified changes in cell behavior (particularly in movement) caused by deletion of individual proteins (Chattwood et al., 2014; Fets et al., 2014; Wessels et al., 2014). Thus it is likely that pharmacological regulation of several proteins by bitter tastants may result in altered cell behavior observed in this study. Each of the targets thus controls protrusion formation with a dose dependent effect, where protrusions formed is inversely proportional to the concentration administered. The broad and varied chemical structures examined here suggest that the bitter targets are distinct, but modification of each target gives rise to an imbalance of cell function resulting in a common behavioral phenotype.

Current opinions of the molecular mechanisms of strongly bitter compounds are that these compounds show activity via TAS2 receptors (Meyerhof, 2005; Ji et al., 2014), but there is little understanding of the molecular mechanisms regarding moderate or weakly bitter compounds. It is therefore surprising that *Dictyostelium*, lacking proteins related to the large family of TAS2 receptors, is sensitive to bitter compounds. Furthermore, *Dictyostelium* also responds to moderately bitter compounds. This suggests that, although TAS2 receptors are involved in bitter taste perception in mammals, perhaps other molecular targets may also be involved in *Dictyostelium* and mammalian systems. An example of this is provided by a recent study investigating novel targets of a standard strong bitter tastant, phenylthiourea (PTU), in *Dictyostelium*, where the bitter tastant inhibited cell movement (Robery et al., 2013), and a genetic screen identified a PTU-sensitive receptor with homology to a poorly characterized human GABAB protein, where the human protein restored the sensitivity to PTU in *Dictyostelium* (Robery et al., 2013). Another study, again in *Dictyostelium*, identified an ion channel (PDK2) to be targeted by a bitter taste-related compound, naringenin (Glendinning, 1994), a flavonoid found in high levels in citrus fruit (Waheed et al., 2014). This study also confirmed the conserved flavonoid-PDK2 interaction in mammalian (kidney) cells and proposed a therapeutic treatment for genetic mutations in the target through naringenin treatment. These combined data suggest that bitter tastants are likely to act via a wide range of targets in mammals, in addition to the well-characterized TAS2 receptors. Pharmacogenetic studies in *Dictyostelium* (Williams, 2005), including for example the analysis of cell behavior in the absence of functional

G proteins (Robery et al., 2013), may identify the molecular mechanism underlying the responses evoked by the structurally diverse bitter tastants in this study.

Can *Dictyostelium* be developed as an early, non-animal model, to inform academic and industrial researchers about the potential for adverse taste of a new compound? Our data show a significant positive correlation between *Dictyostelium* response and the rat BATA test (Fig. 5A,B), suggesting that the model may be useful in this role. The *in vivo* rat BATA test, although widely accepted, has limitations including slow throughput, significant economic costs of testing and the use of animals (albeit a relatively small number per compound, typically 6-12 (Clapham et al., 2012; present study) and 10 in a more recent study using a novel analytical method aimed to improve the robustness of the rat BATA model (Soto et al., 2015), in a relatively benign regimen). Comparison of the responses of *Dictyostelium* and humans suggest a similar pattern of response, although fewer compounds were available to compare between the models. We propose that the *Dictyostelium* assay described here could be developed as a validated early screening platform for the identification of bitter taste liability of novel pharmaceutical agents with the additional benefit of reducing animal experimentation.

In addition to suggesting a potential new non-animal model for provisional bitter tastant screening, our data provides an interesting insight into this field from an evolutionary perspective, since rat bitter taste perception is considered an evolutionary conserved mechanism used to avoid toxic food chemicals (Meyerhof, 2005; Mennella et al., 2013). The ability to recognize and/or respond to bitter tastants is shared amongst phylogenetically diverse groups, including mammals (Stern et al., 2011), amphibians (Go, 2006; Mashiyama et al., 2014), fishes (Ishimaru et al., 2005), cephalopods (Darmaillacq et al., 2004), decapod crustacea (Aggio et al., 2012), insects and nematodes (Hilliard et al., 2004; Gordesky-Gold et al., 2008; Apostolopoulou et al., 2014). However, data presented here suggest a conserved response from the unicellular *Dictyostelium* to primates; the last common ancestor of *Dictyostelium* and multicellular animals existed about a billion years ago (van Egmond and Van Haastert, 2010).

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Conflict of interest

The authors declare that they have no conflicts of interest.

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