Developing *Dictyostelium discoideum* as a model for the investigation of structurally diverse tastants

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Abstract

The activity of many tastant compounds is poorly understood at a cellular level due to limited understanding of the mechanisms of taste. The multiple cellular effects and complex pharmacology of tastants make challenging the characterisation of their biological activities through conventional approaches. In addition, taste research often employs animal models that are difficult to manipulate at a molecular level and have associated ethical concerns. The potential of employing *Dictyostelium discoideum* as an animal replacement model for the study of poorly characterised natural and synthetic tastants was investigated in this thesis. Initially, an assay was developed using *D. discoideum* to monitor the effects of tastants at a cellular level employing the pungent alkaloid capsaicin. In this assay, live cell imaging was employed to monitor cell behaviour for 15 minutes including pre (5 minutes) and post (10 minutes) addition of capsaicin and computer-aided cell tracking was used to monitor behavioural changes. This study suggests that capsaicin may modulate *D. discoideum* behaviour through a novel mechanism involving Guanine Nucleotide Exchange Factors proteins (Rac-GEF). The method was then applied to the analysis of eight known and five blinded bitter compounds with diverse chemical structures. Data obtained were compared to the rat *in vivo* Brief Access Taste Aversion (BATA) assay and human taste panels. This analysis showed a positive linear correlation between the three models, suggesting that *D. discoideum* may provide a new model for the investigation of bitter tastants. The same approach was also employed to investigate changes in *D. discoideum* cell behaviour after exposure to the natural polyphenol curcumin and a range of structurally related derivatives. These compounds were also tested on cell growth and development to obtain a more in-depth understanding of their chronic cellular effects. Indeed, results have demonstrated that curcumin and related compounds have distinct effects on acute cell behaviour, growth and development. A mutant screen was then carried out to identify potential molecular targets of curcumin and structurally related molecules. This screen identified the Protein Phosphatase 2A regulatory subunit and the Presenilin B protein which controlled resistance to curcumin and a synthetic analogue, respectively. These studies demonstrate that *D. discoideum* may be employed as an animal reduction model to investigate the cellular effects of poorly characterised complex tastants and propose novel targets and mechanisms of action.

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Abbreviations

5-HT	5-Hydroxytryptamine
AD	Alzheimer's disease
ADF	Actin-Binding Proteins
APIs	Active Pharmaceutical Ingredients
APP	Amyloid Precursor Protein
ATP	Adenosine Triphosphate
BATA	Brief Access Taste Aversion
BLAST	Basic Local Alignment Search Tool
CAM-K	Calmodulin-Dependent Protein Kinase
cAMP	Cyclic Adenosine Monophosphate
cAR	cAMP receptor
CCK	cholecystokinin
cDNA	complementary DNA
cGMP	cyclic Guanosine Monophosphate
COX	Cyclooxygenase enzyme
CRAC	Cytosolic Regulator of Adenylyl Cyclase
DAG	diacylglycerol
DHE	Dihydroethidium
DMSO	Dimethylsulphoxide

dNTPs	deoxynucleotide triphosphates
EGF	Epidermal Growth Factor
ERK	Extracellular Signal-Regulated Kinases
FAK	Focal Adhesion Kinase
FAD	Familial Alzheimer's Disease
FRAP	Ferric Reducing Ability of Plasma
GABA	Gamma-Aminobutyric Acid
GABAB	Gamma-Aminobutyric Acid Receptor B
GAP	Guanine Activating Protein
gDNA	genomic DNA
GDP	Guanosine Diphosphate
GEF	Guanine Exchange Factor
GFP	Green Fluorescent Protein
GPCRs	G Protein-Coupled Receptors
GSK3	Glycogen Synthase Kinase-3
GTP	Guanosine Triphosphate
hT1R2	Human Taste Type 1 Receptors 2
hT1R3	Human Taste Type 1 Receptors 3
IC_{50}	50% Inhibitory Concentration
IGF-1	Insulin Growth Factor 1

IP3	Inositol Triphosphate
IP3R	Inositol Triphosphate Receptor
MAPK	Mitogen-Activated Protein Kinase
MCAO	Middle Cerebral Artery Occlusion
MCT	Medium-Chain Triglyceride
MMPs	Matrix Metalloproteinases
NCEs	New Chemical Entities
NF-κB	Nuclear Factor Kappa-B
NMDA	N-Methyl-D-Aspartate
NSAID	Non-Steroidal Anti-Inflammatory Drug
PCR	Polymerase Chain Reaction
PGH_2	Prostaglandin H2
PhdA	Pleckstrin Homology Domain Containing Proteins
PI3K	Phosphatidylinositol-3-Kinase
PIP2	Phosphatidylinositol-4,5-Phosphate
PIP3	Phosphatidylinositol-3,4,5-Phosphate
PKB/Akt	Protein Kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PP2A	Protein Phosphatase 2a

PTEN	Phosphatase and Tensin Homologue
PTU	Phenylthiourea
REMI	Restriction Enzyme-Mediated Integration
SCAR	Suppressor of cAR
SDS	Sodium Dodecyl Sulphate
TAS2Rs	Taste-Sensing Type 2 Receptors
TORC2	Target of Rapamycin Complex 2
TRPM5	Transient Receptor Potential Melastatin 5
TRPs	Transient Receptor Potential Channels
TRPV1	Transient Receptor Potential Vanilloid 1
WASP	Wiskott-Aldrich Syndrome Protein

Chapter I

Introduction

1.1 Research context

Elements of the physiology and biology of taste will be examined in this introduction to provide the basis for considering the use of the simple eukaryotic organism *Dictyostelium discoideum* as a potential model for the replacement and reduction of animals for the investigation of tastant compounds. Prevailing models employed in taste research will then be described, before examining *D. discoideum* as an established biomedical model organism. Ultimately, the use of *D. discoideum* as a potential model for the assessment of the cellular effects and molecular targets of tastants will be discussed.

1.2 Taste perception

Tastants are taste-eliciting molecules that are dissolved in ingested liquids or saliva. A tastant represents the correct ligand for receptor proteins or ion channels located on the plasma membrane of taste receptor cells. Initially, the ingested tastant comes in contact with the oral tissues and, subsequently, receptors situated on the taste cells on the tongue are selectively activated by the tastant and trigger downstream signalling pathways (Roper, 2013). This signalling cascade leads to the release of neurotransmitters by taste cells to activate primary sensory afferent nerve fibres, and then the signal is processed by the central nervous system (CNS) (Figure 1). There is evidence implicating a

number of neurotransmitters involved in taste transduction including adenosine triphosphate (ATP), 5-hydroxytryptamine (5-HT), gamma-aminobutyric acid (GABA), noradrenaline and acetylcholine (Roper, 2013). However, they do not all meet the criteria to be accepted as afferent neurotransmitters (must be present in the presynaptic cell, released in response to presynaptic membrane depolarisation, able to activate postsynaptic receptors on afferent nerve fibres, and is degraded or transported from the extracellular space); ATP is the only candidate that meets all the requirements (Kinnamon and Finger, 2013).

The primary sensory apparatus is represented by the taste buds, which contain taste receptor cells (Bradbury, 2004). Each taste bud contains ~100 taste cells that possess some neuronal properties. Taste receptors are expressed on the apical membrane of microvilli. Anatomically, taste buds are arranged in the form of fungiform papillae in the anterior part of the tongue, whereas in the posterior part they form a trench-like structure known as foliate papillae. Finally, the dorsal area of the tongue contains the circumvallate papillae with a moat-like pattern.

1.3 Current knowledge of the primary human taste qualities

The current understanding of human taste (gustation) perception is based on five primary tastes, sweet for carbohydrates, a major source of calories (Kochem, 2017), sour for spoiled and acidic foods (Ugawa, 2003), salty for minerals (Roper, 2015), umami for proteins and amino acids (Ninomiya, 2016), and bitter for potentially harmful compounds which can induce aversive responses (Mennella et al., 2013). In addition, the gustatory system also includes pungent (sensed through chemesthesis) (Roper, 2014) and astringent (major source are tannins) tastes (Jiang et al., 2014; Loper et al., 2015; Schobel et al., 2014). Taste perception is thought to play an evolutionary role in nutrition and avoidance of

hazardous food chemicals (Breslin, 2013). Indeed, current understanding of how taste works is moving towards a more complete and realistic representation of the human taste perception, including the identification of a wider range of tastes (e.g. pungent, astringent, fat taste and metallicness), which increases the complexity of a previously oversimplified system (Chandrashekar et al., 2006).

1.4 Human taste qualities

1.4.1 Sweet

Sweet taste detection enables the discrimination of foods rich in calories. The receptors responsible for the detection of sweet substances are the G-Protein-Coupled Receptors (GPCRs) (Figure 1); in particular, the human taste type 1 receptors 2 and 3 (hT1R2 + hT1R3 heterodimer) (Assadi-Porter et al., 2010; Li et al., 2002), which are situated in the apical membrane of the taste buds. When a sweet tasting ligand binds the receptor, this activates a G-protein which in turn triggers the activation of phospholipase C (PLC-ß2). PLC generates inositol triphosphate (IP3) and diacylglycerol (DAG). This signalling cascade results in the activation of the transient receptor potential cation channel subfamily M member 5 (TRPM5) leading to cell depolarisation which causes the influx of calcium (Ca^{2+}) that triggers the release of the neurotransmitter ATP (Sclafani and Ackroff, 2015). Thus, this mechanism provides a basic understanding of sweet taste transduction.

1.4.2 Salty

The detection of salty compounds is required to control the body salt equilibrium (Bradbury, 2004). Salt perception is based on sodium chloride (NaCl) receptors, thought to be epithelial-type sodium (Na^{+}) channels (ENaC) present on the apical membrane of the taste buds (Kim et al., 2014). Na^{+} ions enter the receptor cells

via Na-channels, which causes membrane depolarisation, generating Ca^{2+} influx through voltage-sensitive Ca^{2+} channels which leads to neurotransmitter ATP release (Figure 1).

1.4.3 Sour

The sour taste allows acid detection and is triggered by protons (H^+) present in acidic foods (Figure 1). Therefore, sour taste is essential to avoid the ingestion of acids in excess, as well as to maintain physiological electrolytic and pH levels in humans (Melis and Tomassini Barbarossa, 2017). The current view of sour taste transduction is that it is generated by intracellular acidification, which stimulates acid-sensitive membrane proteins (Roper, 2007). However, the molecular mechanisms underlying the transduction of sour taste are not fully characterised.

1.4.4 Umami

The taste of umami is triggered by the amino acid L-glutamate, a cleavage product of all proteins, conferring the ability to taste protein-rich foods (Lindemann et al., 2002). Umami taste was first identified and characterised in 1909, and it was initially thought that the metabotropic glutamate receptor 4 (mGluR4) was involved in the detection of the umami taste (Chaudhari and Roper, 2010; Stanska and Krzeski, 2016). However, recent studies have demonstrated that the T1R1 and T1R3 (heterodimer) (Li et al., 2002) receptors mediate umami taste (Figure 1). In fact, similarly to the sweet taste signal transduction, the binding of L-glutamate to the T1R1 and T1R3 receptor activates G-proteins that lead to the increase of the intracellular Ca^{2+}concentration, causing the release of the neurotransmitter ATP (Nelson et al., 2002). In addition, there are ionotropic receptors in the brain, including the N-methyl-D-aspartate (NMDA) receptors, which are activated by neurotransmitter L-glutamate (Vandenbeuch et al., 2010).

1.4.5 Bitter

Bitter taste perception is essential for the detection of harmful compounds and enables humans to avoid ingesting potentially deadly toxins (Greene et al., 2011; Margolskee, 2002). The currently accepted model of bitter taste perception and signal transduction of bitter tastants is through a specific sub-group of GPCRs, Taste-Sensing Type 2 Receptors (TAS2Rs), which are expressed in the apical membrane of the receptor cells present in a taste bud (Mennella et al., 2013) (Figure 1). Signalling of bitter compounds is achieved via the α-subunit of gustducin (Gulbransen et al., 2008). This G protein subunit triggers a phosphodiesterase which hydrolyses cAMP, and this reduction leads to the increase of intracellular Ca^{2+}. In addition, the βγ-subunit of gustducin mediates taste signal by activating phospholipase C which generates inositol triphosphate (IP3) and diacylglycerol (DAG). As a result, these messengers activate and open gated ion channels or may generate the release of calcium ions from intracellular stores. This signalling cascade triggers the release of the neurotransmitter ATP, which generates the taste perception in the brain (Margolskee, 2002).

Interestingly, the number of bitter tastants that can be recognised by mammals is greater than the number of taste-sensing type 2 receptors (~ 25 TAS2Rs) (An and Liggett, 2017; Meyerhof et al., 2010; Meyerhof et al., 2005), suggesting the presence of multiple molecular pathways involved in the transduction of the bitter taste stimulus. Indeed, there are many bitter molecules which also have a wide range of molecular structures. For example, the Bitter DB database holds around 700 bitter tastants, with widely diverse chemical structures and biochemical characteristics (Wiener et al., 2012). These compounds include fatty acids, peptides, amino acids, amines, amides, carbamides, esters, lactones, phenols, terpenoids, alkaloids, glycosides, flavonoids, steroids and metal ions (Meyerhof

et al., 2010). Therefore, it is still a complicated task to predict the bitterness of novel chemical entities.

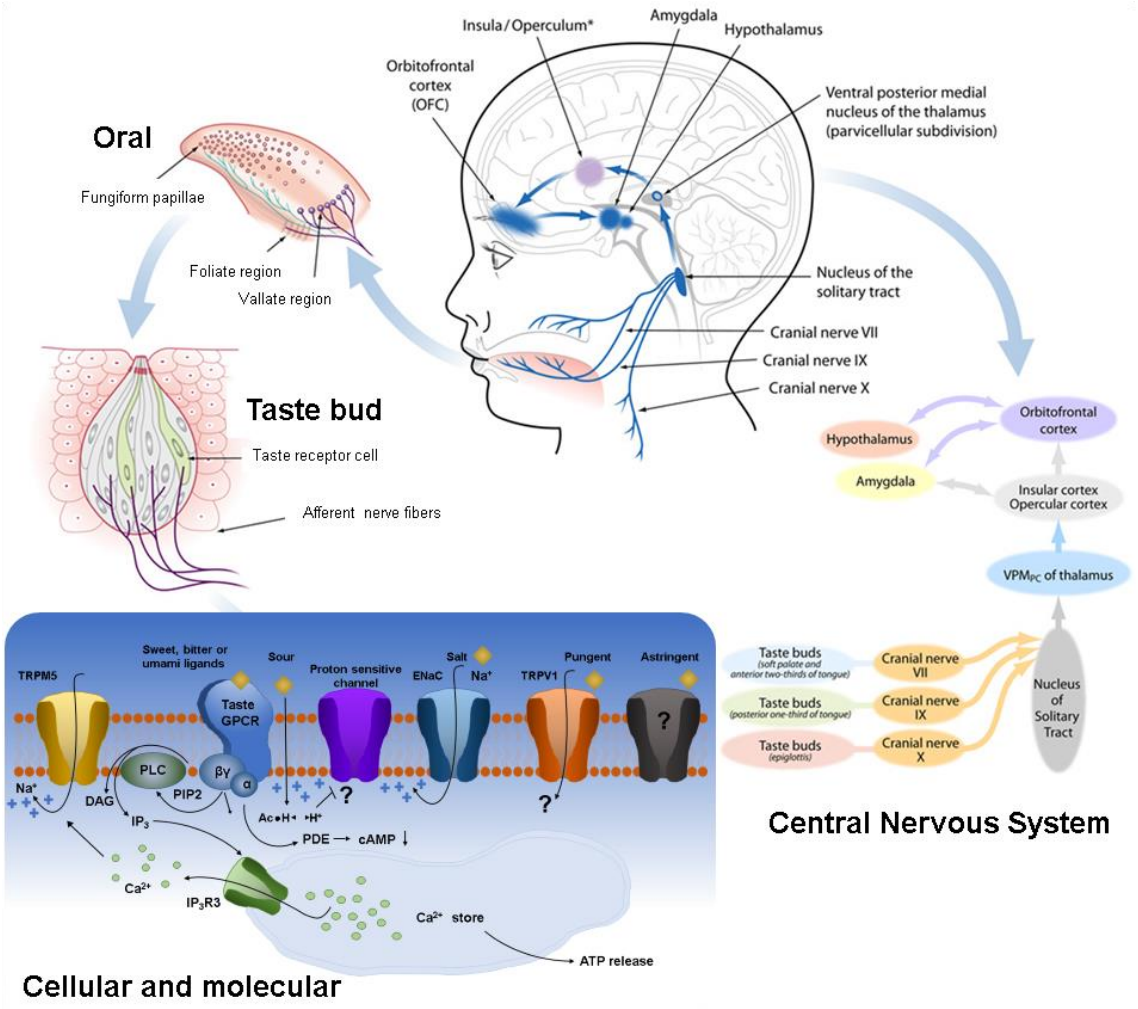

Figure 1. Accepted mechanisms by which the primary taste qualities are transduced (adapted from(Chaudhari and Roper, 2010; Mennella et al., 2013)**.** In taste receptor cells, sweet (T1R2 + T1R3), umami (T1R1 + T1R3) and bitter (TAS2R) ligands bind to GPCRs to activate a phosphoinositide pathway that elevates cytoplasmic Ca^{2+} and depolarises the membrane via a cation channel, TRPM5. The combined action of elevated Ca^{2+} and membrane depolarisation leads to the release of the neurotransmitter ATP. Organic acids (HAc) pass through the plasma membrane where they dissociate causing an increase in cytoplasm acidity. It is hypothesised that intracellular H^+ is able to block a proton-sensitive K^+ channel causing the depolarisation of the membrane. Calcium channels increase cytoplasmic Ca^{2+} which trigger the neurotransmitter ATP release. The salty taste of Na^+ is detected by direct diffusion of Na^+ ions through membrane ion channels, (e.g. ENaC), which depolarise the membrane, leading to neurotransmitter ATP release.

Pungent taste is transduced via TRPV1, which responds to pungent tastants such as capsaicin. Astringent taste transduction is not fully understood.

It is increasingly accepted that there are other molecular pathways involved in the transduction of bitter taste. One example is represented by the generation of gustducin knockout mice, which does not fully inhibit the sensitivity to bitter compounds, suggesting a redundant mechanism for bitter signal transduction (Wong et al., 1996). The one hypothesised mechanism for gustducin-independent bitter tasting is through ion channel (K^+) interaction with particular bitter ligands (denatonium), which is similar to the ion channel interaction for the recognition of sour and salty stimuli (Gulbransen et al., 2008; Spielman, 1998). Hence, the molecular mechanisms underlying bitter taste transduction are not fully characterised.

1.4.6 Pungent

Hot/pungent taste provides another taste type, independent of the five core tastes. This is detected and transduced via the Transient Receptor Potential Cation Channel Subfamily V Member 1 (TRPV1), which responds to chemical stimuli such as capsaicin and several irritants (chemesthesis) (Kawakami et al., 2015) (Figure 1). It has not been yet elucidated whether TRPV1 is present in the taste buds and directly transduces taste. However, it is known that it is expressed in non-gustatory sensory afferent fibres and keratinocytes of the oronasal cavity. In many sensory fibres and epithelial cells lining of these cavities, Transient Receptor Potential Ankyrin 1 (TRPA1) is also co-expressed with TRPV1 and transduces a wide variety of stimuli triggered by noxious chemicals in combination with TRPV1. TRP channels also include TRPM8, TRPV3, and TRPV4, which have less significant roles in chemesthesis and unknown functions

in taste (Roper, 2014). Although capsaicin is able to elicit a response in sensitive neurons containing TRPV1 receptors, a recent study has shown that this molecule is also able to induce a feedback in non-sensitive neurons at high concentration (Yang et al., 2014). Hence, other mechanisms are involved in the sensation of pungent compounds.

Interestingly, a study has also shown that the bitter taste of quinine sulphate and the burning sensations of capsaicin can be perceived similarly or even confused (Lim and Green, 2007). Hence, there may be a link between pungent and bitter taste signal transduction, which has been suggested to derive from a common function as sensory signals of potentially harmful substances.

1.4.7 Astringent

Astringent is an additional taste quality, which is described as a dry or rough sensation (Figure 1). This perception is caused by some chemicals (e.g., tannins or calcium oxalate), which produce a contraction of the mucous membrane of the mouth. Foods that produce an astringent taste include tea, red wine, rhubarb, some unripe fruits like persimmons and bananas. A recent study has shown that black tea infusion contains flavanol-3-glycosides besides some catechins, which contribute to the taste of black tea. The results form sensory studies demonstrated that the flavanol-3-glycosides confer the astringent taste sensation and also increase the bitter taste of tea infusions by amplifying the bitterness of caffeine (Scharbert and Hofmann, 2005). However, it is still unclear whether astringent taste is transduced by mechanoreceptors, chemoreceptors, or a combination of both. Moreover, the receptor(s) that recognise astringents and downstream signalling have not been identified (Jiang et al., 2014).

1.4.8 Transient potential receptor and taste transduction

Transient receptor potential channels (TRPs), situated downstream from the taste receptors (i.e., TAS2R, T1R1, T1R2 and T1R3), are expressed in taste buds, nerve fibres and keratinocytes in the oronasal cavity (Roper, 2014), and play a pivotal role in taste transduction. In particular, the TRPM5 is essential for transduction of bitter, sweet and umami tastes. TRPM5 operates downstream of taste receptors in the taste transduction pathway, converting taste-elicited intracellular Ca^{2+} release into membrane depolarisation to generate taste transmitter secretion (Mennella et al., 2013; Roper, 2014). Furthermore, TRPM5 channel activity is strongly regulated by voltage, phosphoinositides and temperature, and is blocked by acidic pH (Liman, 2007).

1.5 Drug development process

The development of a new medicine from the initial concept to the finalised product and commercialisation is a time-consuming and expensive process, which currently takes up to 15 years and cost over £1 billion (DiMasi et al., 2016). The first step in developing a novel drug is to identify and validate its target(s), which include proteins, genes and RNA involved in the etiopathogenesis of the condition. The second step focuses on the assessment of efficacy and safety of candidate drug compounds. While much of this can be achieved using *in vitro* techniques, the use of animal models is considered a heritage of the early pharmacological approaches and it is still considered the gold standard for drug development in preclinical studies (Hughes et al., 2011; McGonigle and Ruggeri, 2014). One example is the use of transgenic animals where a genetic manipulation to ablate a gene responsible for a disease can lead to embryonic lethality and therefore is challenging to accomplish (Hughes et al., 2011). Recent

studies have shown that the implementation of a multi-system approach, which includes different disciplines such as bioinformatics, proteomics, RNAi, genetic manipulation, modelling, mass spectrometry, and live cell microscopy can lead to an increase in target identification and validation (Hughes et al., 2011). Another crucial factor to account for when developing new drug formulations is the assessment of its palatability. Mice and rats are used for the investigation of the bitterness (acceptability/aversiveness) of medicines. Even though *in vivo* animal models are the closest to humans concerning the taste apparatus, they differ in the ability to detect and react to the administration of bitter compounds. Humans share some similarities in taste preferences with mice and rats in relation to acceptance and avoidance responses to a tastant compound and oromotor reflexes to a chemical stimulus (Spector 2002). However, there are still exceptions in the response to different bitter compounds such as phenylthiocarbamide (PTC) and phenyl-β-D-glucopyranoside which are known to be bitter to humans, but mice do not respond to them (Mueller et al., 2005). Moreover, these types of study involve ethical issues related to animal suffering (Holmes et al., 2009).

1.5.1 Medicines from plants

Another critical aspect of drug screening is the fact that many drugs commercially available have natural origins, hence can elicit an aversive taste response. Indeed, around 60% of the pharmaceutically active molecules introduced in the past 35 years are derived from plant sources, therefore playing a central role in the treatment of many conditions (Newman and Cragg, 2007). In addition, medicinal plants have been used for thousands of years to treat many ailments. For instance, cardiac glycosides to treat heart failure (Radford et al., 1986), taxanes (Jimenez et al., 2011) and vinca alkaloids (Moudi et al., 2013) to treat

cancer, and hyoscine to treat motion sickness (Spinks and Wasiak, 2011). Plants have co-evolved with animals and pests, producing chemicals to repel predators or against disease and infection. Many drugs are not taste-friendly, possibly due to our evolutionary ability to recognise and avoid toxins present in food. However, this does not always occur as many unpleasant tasting molecules are not toxic (Mennella et al., 2013). Furthermore, a recent study reported that there is a strong correlation between the taste of medicines and their curative properties (Gilca and Barbulescu, 2015).

1.5.2 The importance of understanding the taste of drugs

Taste is one of the critical factors in the development of orally administered drugs regarding patient compliance and acceptability, thus influencing the commercial success of a new drug, particularly in the paediatric sector (Anand et al., 2007). In fact, many pharmaceutically active ingredients have an unpleasant taste, causing aversive reactions in children and many adults, resulting in some cases in severe reactions such as nausea and vomiting (Holmes et al., 2009). For example, the unpleasant taste of oral antibiotics is considered the primary hindrance to patient compliance. However, antibiotics are the first line of treatment for a wide range of paediatric conditions (Baguley et al., 2012). It is currently estimated that the absence of more taste-friendly medicines puts 40% of children at risk of preventable adverse effects (i.e. suboptimal dosing) worldwide (Mennella et al., 2013). Therefore, the assessment and prediction of the taste of medicines is an essential requirement in the drug development process. Hence, it is crucial to understand the biology of taste and its molecular mechanisms.

Oral administration is the primary route of drug delivery, as it is the most convenient and economical solution, as well as being the safest in terms of

infection risks and side effects. Despite the benefits, the oral route is very challenging and unpredictable, due to the unpleasant taste of many drugs and the requirement for digestive-tract absorption. Higher bioavailability and absorption of certain drugs can be achieved through the sublingual route of administration. Nevertheless, the taste characteristics of the drugs is still an obstacle to sublingual delivery as they can still trigger the taste responses. For these reasons, researchers have put significant effort in the development of taste-masked formulations such as tablet coating, the addition of sweeteners or making the formulation insoluble by changing its pH. An additional masking approach is the mixing of the formulation with food and beverages, mainly when medicines are used to treat children or elderly patients (Gittings et al., 2014).

1.6 Taste research

1.6.1 Human taste panels

The use of a human taste panel for the assessment of the taste characteristics of pharmaceutical formulations represents the most widely accepted method (Anand et al., 2007). For these types of study, a panel of healthy volunteers is recruited and trained to evaluate the taste of active pharmaceutical ingredients (APIs) using the appropriate ranking on which a specific quality of the tastant is determined. However, human taste panels have several disadvantages, especially when assessing New Chemical Entities (NCEs). Moreover, this type of investigations can only be carried out when there is sufficient toxicological data, which is extremely rare to have in the initial stages of the drug development process (Cram et al., 2009). In addition, taste panels are expensive and time-consuming, due to the training of adult volunteers required and the overall cost for running and setting up a panel. The wide variability of taste perception

between individuals is another limitation of taste research studies. Thus, the use of human panels is problematic due to ethical reasons, liability issues and toxicity risks. In addition, this type of test is based on subjective preferences in adults; hence, it is questionable whether the data obtained can be used to predict preferences in the paediatric population (Gittings et al., 2014).

1.6.2 Brief Access Taste Aversion - BATA

The second most accepted method for the assessment of active pharmaceutical ingredients is the *in vivo* rat Brief Access Taste Aversion assay (BATA). In the BATA assay, mice or rats undergo a period of 16 to 24 hours in which they are deprived of water to induce them to drink when exposed to the substances to assess. The animals are then placed into a lickometer which records the number of licks taken, and this is repeated for all the concentrations tested. Animals are exposed to the solutions for limited time periods (5-10 seconds), and solutions/concentrations are usually presented in a random order. The less aversive the substance, the higher the number of licks. Whereas, when licks are almost completely abolished this suggests that the substance is aversive. However, this methodology allows only the evaluation of the palatability of drugs but not their taste quality (Soto et al., 2015).

1.6.3 Electrophysiological methods (*in vivo*)

In addition to human panels and BATA assays, research also employs electrophysiological methods to study taste stimuli. In these experiments, animals such as mice, bullfrogs or gerbils are anaesthetised, then electrodes are implanted in the chorda tympani nerve bundle and the glossopharyngeal nerve, before administering the tastants on the tongue (Anand et al., 2007). Electrophysiological recordings allow the assessment of the temporal profiles or

dose-response curves of taste stimuli. Although these procedures can determine the physiological mechanisms of taste stimuli, the underlying molecular mechanisms of taste remain diverse and not fully characterised.

1.6.4 Electrochemical methods (*in silico*)

Electrochemical taste-sensing systems, like the electronic tongue, are able to detect a range of substances with varying intensities by mimicking the interaction between the taste ligand and the receptor cell (Latha and Lakshmi, 2012). This interaction generates changes in current, which are detected by the computer and compared with physiological action potentials. Solubility represents the major limitation of this model as test drugs must be completely solubilised.

The assessment of the effects of tastants and investigation of the pathways involved in taste transduction is of primary importance. Therefore, implementing less complicated models like *D. discoideum* could be highly advantageous in improving the prediction of tastants, as well as reducing the number of animals used in this research and the cost of taste research. Indeed, the investigation of acute responses to chemicals and genetic manipulations are less challenging in this simple eukaryotic organism, thus, providing vital information regarding the effects of tastants and unknown mechanisms underlying taste transduction. The second section of the introduction will describe *D. discoideum* as a valuable biomedical model and examine its potential use in taste research.

1.7 *Dictyostelium discoideum* as a biomedical model organism

D. discoideum is a single- and multicellular eukaryotic organism, which belongs to the Protozoa Kingdom, and is present in the soil of the forest floor and moist leaf litter, and feeds on naturally occurring microflora (i.e. bacteria) (Williams et al., 2006). In the vegetative stage, single cells move towards bacteria attracted by their folic acid secretion and consume them through phagocytosis (Pan et al., 2016; Segota et al., 2013). In favourable conditions of a humid and warm environment (22 ºC), cells can double in number approximately every 8 h. Upon nutrient exhaustion, cells start a developmental program leading to the formation of fruiting bodies and the production of spores that are able to survive in hostile conditions (Fey et al., 2007). Starving cells produce a chemo-attractant, cyclic adenosine monophosphate (cAMP), serving as a signal for around 1 x 10^5 neighbouring cells to aggregate, which then develop into a mature fruiting body over a 24-hour period, where 20% of the cells form a stalk that supports a spore head incorporating 80% of the remaining cells as spores. When conditions become favourable, and cells come in contact with nutrients, the spores germinate into amoebae, completing the life cycle (Figure 2).

D. discodeum has been extensively utilised to investigate a range of fundamental biological processes such as cell migration, chemotaxis, signal transduction, phagocytosis and signalling during morphogenesis and cell differentiation (Insall and Andrew, 2007; King and Insall, 2009; Nichols et al., 2015; Stephens et al., 2008). The fully sequenced genome contains 34 Mb of DNA packed in 6 chromosomes (84-fold smaller than the human genome) (Eichinger et al., 2005). Genome sequencing has led to the identification of 22% *D. discoideum* genes that encode for proteins that are homologues of those present in humans (~60%), making this organism a valuable model for the investigation of drug targets and

molecular pathways involved in human disease (Eichinger et al., 2005; Williams et al., 2006). Therefore, it is possible to characterise the primary role of these proteins to understand their related disease function in this simple organism (Annesley and Fisher, 2009; Eichinger et al., 2005). *D. discoideum* brings several advantages in comparison to mammalian cell cultures and animal models such as the ease of genetic manipulation, behavioural and developmental investigations and laboratory maintenance (Annesley et al., 2014; Eichinger et al., 2005; Escalante and Vicente, 2000; King and Insall, 2009; Manstein et al., 1989; Montagnes et al., 2012). New developments have seen the rise of techniques capable of generating libraries of mutants that can be used to screen for compounds to identify their molecular targets. Therefore, this model can be employed as an innovative platform for molecular pharmacology research, to accelerate the compound validation prior to animal studies (Williams, 2005).

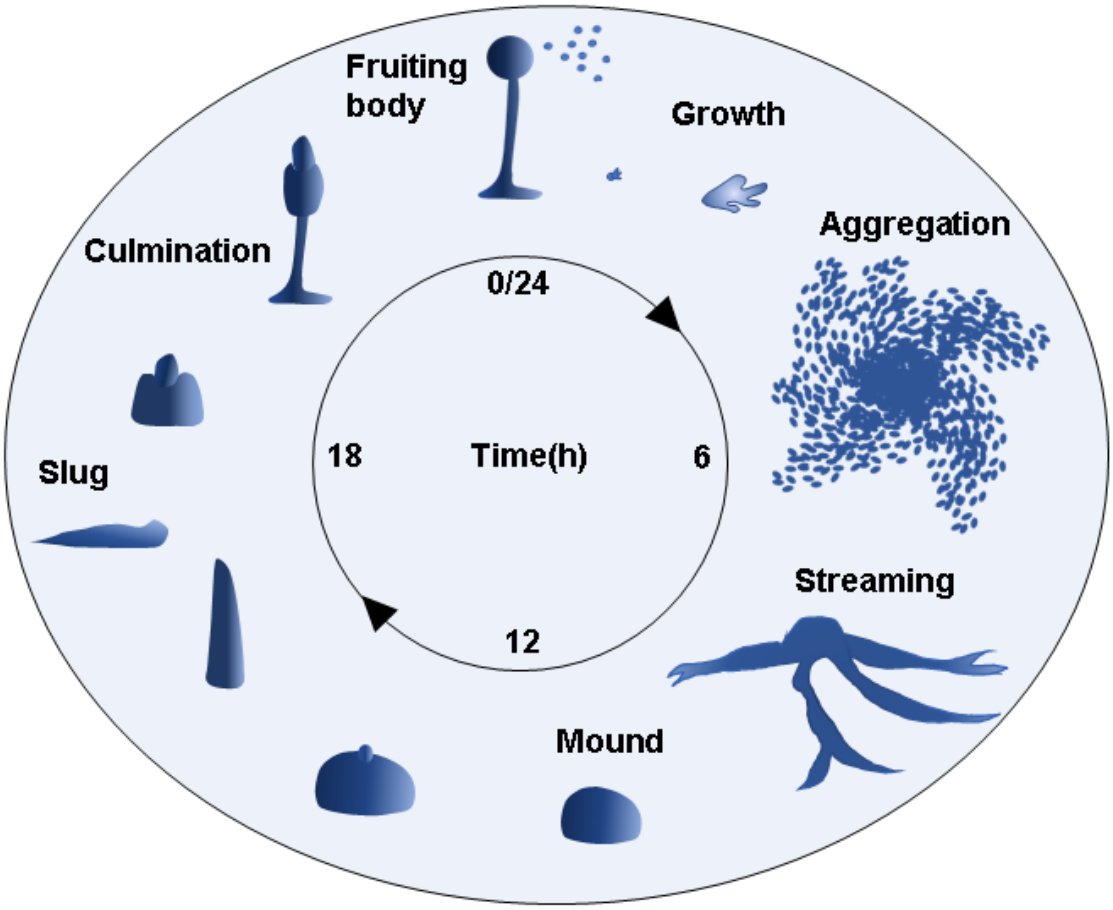

Figure 2. *D. discoideum* life cycle. *D. discoideum* develops from a single vegetative amoeba (0hrs) through to the generation of the mature fruiting body (24hrs). Aggregation is caused by the chemotaxis of cells toward cAMP waves to give rise to a multicellular aggregate. Aggregation results in the creation of a mound, then a tipped mound and, as development proceeds, the tip elongate and forms a finger. Ultimately, the finger collapses to form a slug or continues to form a fruiting body. During the final stage of development, the cells differentiate into vacuolated stalk cells that sustain a spore head containing spores which can tolerate a broader range of environmental conditions. The full developmental process from starvation of vegetative cells to the formation of a mature fruiting body is accomplished over a 24-hour period (Fey et al., 2007).

Recent studies using *D. discoideum* as a simple model system for bitter taste research have shown the utility of using this approach for the reduction of animals in testing bitter and emetic compounds (Robery et al., 2011). Furthermore, using *D. discoideum*, researchers were able to study the effects and identify the

molecular targets of bitter tastants (e.g. quinine, denatonium, phenylthiourea) and this led to the discovery of a novel human receptor implicated in the detection of the bitter substance phenylthiourea (Robery et al., 2013). These studies demonstrated the potential utility for *D. discoideum* in the identification of novel pharmaceutical compounds with a bitter taste liability. In addition, research studies employing *D. discoideum* lead to the identification of the molecular targets of flavonoids (Waheed et al., 2014), epilepsy therapy including the medium-chain triglyceride (MCT) diet (Chang et al., 2012; Eickholt et al., 2005; Xu et al., 2007), bipolar disorder treatments (Williams, 2005; Williams et al., 2006; Williams et al., 2002), and investigation of the molecular mechanism of Alzheimer's disease (Ludtmann et al., 2014; McMains et al., 2010). In all these cases, discoveries in *D. discoideum* have been successfully translated to humans or other mammals (Chang et al., 2013; Chang et al., 2014; Xu et al., 2007).

Furthermore, *D. discoideum* does not possess genes encoding homologues to TAS2R proteins associated with bitter compounds detection. However, the molecular mechanism responsible for the detection of the bitter alkaloid phenylthiourea (PTU) has been identified. A library screen was used to identify PTU resistant mutants, leading to the identification of GrlJ^{-}, a putative G-protein coupled receptor mutant (Robery et al., 2013) (Figure 3). Translation of this discovery to human context identified an uncharacterised human gamma-aminobutyric acid (GABA) type B-receptor isoform, with a relatively weak homology to GrlJ. The expression of the human GABA-B receptor restored GrlJ^{-} sensitivity to PTU, implicating this human protein as a novel receptor for PTU. In addition, GrlJ only partially controls PTU detection but not detection for all bitter substances (Robery et al., 2013).

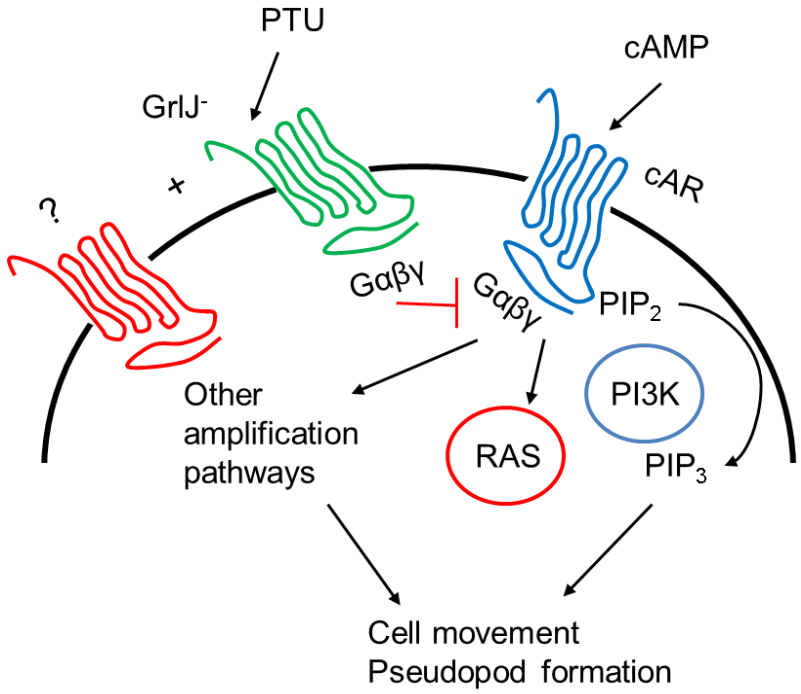

Figure 3. PTU pathway regulation in *D. discoideum*. Under experimental conditions during cell movement, cAMP triggers the cAR receptor, causing the activation of Ras through the dissociation of G-protein. This signalling cascade also increases the PI3K activity and therefore the production of PIP_3, leading to cell movement. PTU can block this pathway through GrlJ partially affecting cell behaviour (additional pathways involved) (Robery et al., 2013).

1.7.1 Underlying mechanisms of *D. discoideum* cell movement

The cyclic AMP receptor (cAR) is a seven-transmembrane receptor which is activated when cells start secreting cAMP due to starvation and initiate chemotactic movement towards an aggregation centre (Chung and Firtel, 2002). In addition, cARs are uniformly expressed on the cell membrane and modulate differentiation and developmental processes (McMains et al., 2008; Verkerke-Van Wijk et al., 1998). Random movement is a mechanism that initiates when cells are in either vegetative or starving states, which leads to profound changes in gene transcription (Goury-Sistla et al., 2012). Cells start secreting cAMP and responding to it, leading to the generation of random oscillating movement, achieved by the directional formation of membrane protrusions, which in turn

increases the probability to detect cAMP (Bosgraaf and Van Haastert, 2009; Li et al., 2008).

When cAMP binds to its G-Protein Coupled Receptor cAR1 (Insall et al., 1994), this generates the dissociation of the Gα2 and Gβγ sub-units and activate downstream signalling (Figure 4). *D. discoideum* possesses 14 Gα- subunits, but only one Gβ- and one Gγ- subunit, and all play a central role in cell polarisation (Chung and Firtel, 2002; Prabhu and Eichinger, 2006). When Gβγ dissociate, Ras-guanosine triphosphates (GTPases), like RasC and RasG, are activated through the binding with guanosine triphosphate (GTP), which is mediated by Ras-guanine exchange factors (GEFs). Subsequently, GTPases become inactivated when binding to guanosine diphosphate (GDP) mediated by Ras-guanine activating proteins (GAPs) which converts GTP to GDP (Jin et al., 2008; Kae et al., 2007). This complex machinery is involved in the movement of chemotactically competent cells, which leads to the migration of cells towards a cAMP gradient (Chung and Firtel, 2002; Jin et al., 2008; King and Insall, 2009).

D. discoideum also contains an actin cytoskeleton that allows cells to generate movement. The cytoskeleton consists of a network of F-actin filaments, which allow pseudopod extension at the leading edge of the cell and retraction of uropods at the rear (Bagorda et al., 2006). During random cell movement, the polymerisation of F-actin filaments is arbitrary as cells are not driven by external stimuli; whereas, in the presence of a chemotactic gradient, cell movement is driven by external signals towards the chemoattractant (Choi et al., 2013). For instance, chemotactic gradients activate Ras which is a monomeric G protein that functions as a molecular switch that activates downstream effectors such as PI3K, at the leading edge of the cell membrane (Cheng and Othmer, 2016;

Kortholt et al., 2011). These ultimately lead to the polymerisation of F-actin filaments.

The activation of Rho-GTPases (or Rac), which are regulated by RacGAPs and RacGEFs, is part of the cellular mechanisms that lead to F-actin polymerisation. Moreover, Rac activation is a main determinant of Arp2/3 complex activation, which modulated actin polymerisation during pseudopods formation (Pollard and Borisy, 2003; Veltman et al., 2012). Arp2/3, together with SCAR (suppressor of cAR complex)/WAVE complex are key factors in pseudopod formation. However, *D. discoideum* cells in which SCAR is ablated, Rac activation is not greatly modified, and migration and chemotaxis can still occur through the Arp2/3 complex. Furthermore, when the SCAR complex is not present, Wiskott-Aldrich syndrome protein (WASP) is recruited in areas in which Rac is active and can regulate pseudopod formation (Veltman et al., 2012). RasC and RasG are also involved in the cellular chemotactic signalling amplification pathway, which includes the phosphatidylinositol-3-kinase (PI3K) pathway (Bolourani et al., 2006; Hoeller and Kay, 2007). When cells detect cAMP, this leads to Ras stimulation, which activates PI3Ks, generating the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3), resulting in PIP3 waves followed by F-actin polymerization (Asano et al., 2008; Huang et al., 2003; Ueda et al., 2003). PIP3 also binds to myosin I, as well as PhdA (pleckstrin homology domain containing proteins), CRAC (cytosolic regulator of adenylyl cyclase) and PkB/Akt (protein kinase B), where each protein has a specific role in modulating cell chemotaxis (Chen et al., 2012; Funamoto et al., 2001; Lilly and Devreotes, 1994; Meili et al., 1999). PIP3 production is modulated by phosphatase and tensin homologue (PTEN), which dephosphorylates PIP3 into PIP2 at the uropod, and controls cell polarity spatially and temporally,

allowing the formation of PIP3 waves at the leading edge, which sustains the amplification and directionality of the chemotactic movement (Iijima and Devreotes, 2002; Iijima et al., 2002). In turn, PTEN is modulated by phospholipase C, activated by the dissociation of G-proteins, which catalyses the conversion of PIP2/DAG and inositol triphosphate (IP3) at the leading edge, promoting the translocation of PTEN to the rear end of the cell (Kae et al., 2007).

Ras, PIK3 and PTEN are activated independently of GPCRs decoupling in case of random cell movement (Sasaki et al., 2007), where Ras and PIK3 play a vital role in the signalling loop. When PIP3 gradients are removed by the ablation of PI3K and PTEN genes, cell motility during random cell movement is partially disrupted (Bosgraaf and Van Haastert, 2009; Hoeller and Kay, 2007). Furthermore, the target of rapamycin complex 2 (TORC2) is another crucial factor in the regulation of *D. discoideum* chemotaxis, and its activation is modulated by RasC, which controls PIP3 and PKB/Akt regulation (Kamimura et al., 2008; King and Insall, 2008). In the presence of cAMP gradients, F-actin assembles a complex of scaffolding proteins such as Sca1, RasGEF and PP2A and is enriched at the leading edge of chemotaxing cells where it modulates F-actin dynamics and signal transduction by controlling the activation of RasC and the downstream TORC2-Akt/protein kinase B (PKB) pathway (Charest et al., 2010). In addition, glycogen synthase kinase-3 (GSK3), a conserved human protein homologue, is also involved in regulation of these pathways. In fact, the ablation of the GSK3-A gene leads to the disruption of both chemotaxis and cAMP signalling mediated by both PIP3 and TORC2, which inhibits PKB/Akt and PKBR1phosphorylation (Teo et al., 2010).

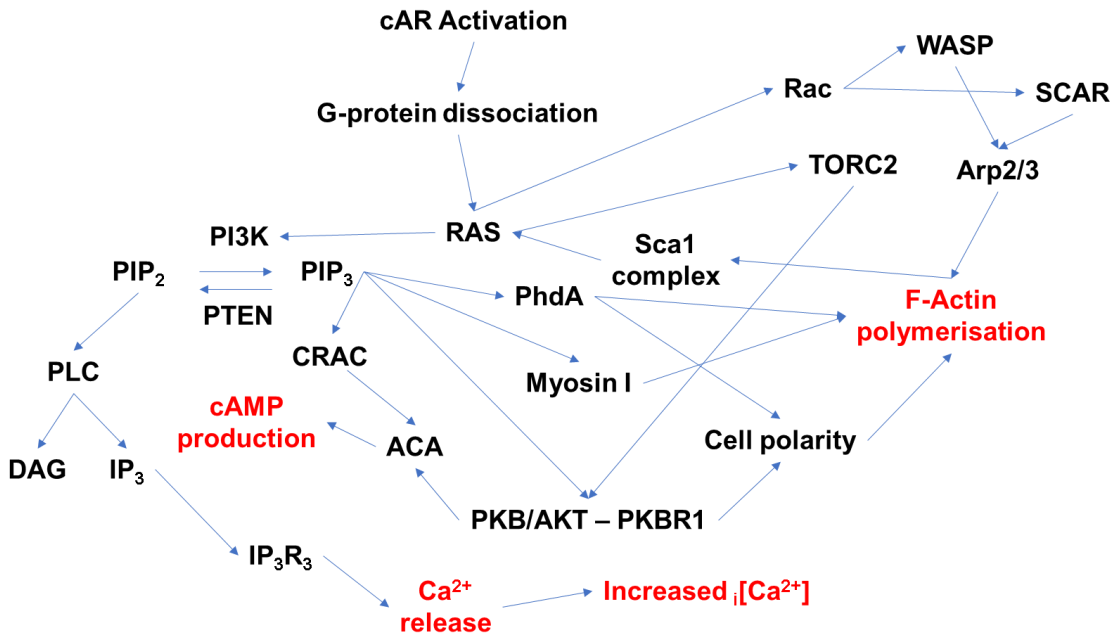

Figure 4. Schematic representation of *D. discoideum* chemotactic movement. Upon cAMP detection by cAR, G-proteins dissociate, which in turn leads to the activation of Ras. Ras activates PI3K, which in turn converts PIP2 to PIP3. PI3K activity results in PIP3 binding to myosin I, involved in F-actin recruitment. PhdA is implicated in maintenance of cell polarity and F-actin recruitment. PKB/Akt and CRAC signalling transduction pathways are involved in adenylyl cyclase A (ACA) activation, which controls cell polarity. In addition, PIP3 is dephosphorylated to PIP2 by PTEN, and PIP2 is subsequently split into diacylglycerol (DAG) and inositol triphosphate (IP3). This signalling cascade ultimately leads to calcium release through the inositol triphosphate receptor (IP3R3). Ras activation is also controlled by the scaffold protein complex consisting of Sca1, RasGEF and protein phosphatase 2A (PP2A), which is formed upon F-actin recruitment. In addition, activation of Ras leads to the initiation of the TORC2 complex, which in turn triggers PKB/Akt and PKBR1 activity.

Finally, *D. discoideum* possesses many signalling pathways controlling chemotaxis and random cell movement, where the inhibition of individual pathways does not entirely block chemotaxis. However, chemotactic behaviour can be severely hindered when multiple pathways are disrupted. These pathways are crucial for the study of cell movement as they are comparable to those

present in leukocytes (Veltman et al., 2008) and essential for the investigation of the fundamental molecular pathways involved in disease mechanisms and chemosensory responses (Carnell and Insall, 2011). Thus, *D. discoideum* represents an excellent model organism to study the behavioural changes and molecular pathways controlling cell movement.

Chapter II

Materials and methods

2.1 Cell culture

D. discoideum stocks were stored in freezing medium (7% dimethylsulfoxide - DMSO, horse serum) at -80ºC. Every four weeks, frozen stocks were scraped onto selective media (SM) agar plates, on which 300µL *Raoultella planticola* were previously spread. Colonies were allowed to form at 22ºC over 3-4 days, where cells from the growth zone of colonies were then transferred to liquid plates containing axenic medium and 100µg/mL penicillin/streptomycin. Liquid plates were washed three times per week and maintained in log phase (1-4x10^6 cells/mL) in shaking flasks (120rpm). Cell concentrations were determined using a Neubauer improved haemocytometer.

2.2 *D. discoideum* random cell movement assay

D. discoideum cells were maintained in axenic medium (Formedium Co. Ltd, Norfolk, UK) for at least 48 hours 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 K_2HPO_4, pH 6.2), resuspended in 6 mL phosphate buffer and pulsed for 5 hours with 30 nM cAMP at 6 minutes intervals at 120 rpm. Cells were then resuspended in 4 mL phosphate buffer and diluted 1:9, and 250 µL aliquots of cells were transferred into Nunc Lab-Tek chamber (Thermo Fisher, Leicestershire, UK), and allowed to adhere for 10-15 min (Figure 5). In each

experiment, 250 µL of drug stock solution (to produce the desired final concentration) was added at the 225th second of the image recording to assess the effects of the compounds on cell movement. To investigate the suitability of *D. discoideum* as a non-animal model for the investigation of bitter substances, a standardised assay was developed (Otto et al., 2015). Cell behaviour was monitored in cells undergoing random movement by taking images every 15 seconds over a 15-min period, with 3.75 minutes recorded prior to, and 11.25 minutes after compound addition. A minimum of three independent experiments for each drug concentration were carried out with at least 10 cells quantified per experiment. From these series of images, parameter protrusion formation was quantified using an image analysis software Fiji (Schindelin et al., 2012), and its plugin QuimP 11b (Warwick University, Warwick, England). Prior statistical analysis data were analysed and formatted using MATLAB (Mathworks, Cambridge, England).

2.3 Statistical analysis of random cell movement

Data obtained from membrane protrusions of cells during random movement were extracted from videos into GraphPad Prism spreadsheet as time versus the number of protrusions formed. Data were normalised by defining 100 as the highest value and 0 as the lowest value in each data set and the data expressed as a fraction. The mean and standard error was calculated for each set of results at all concentrations. To assess whether there was a significant difference in protrusion formation, an unpaired, two-tailed t-tests (95% confidence interval) was employed, comparing the mean of the last 8 minutes (from minute 4.5 to minute 12.5 - after drug addition) against the control conditions for all concentrations tested. To determine the IC_{50} (the concentration required to achieve a 50% reduction in cell movement) for each molecule, the mean of the

last 8 minutes of pseudopod 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.

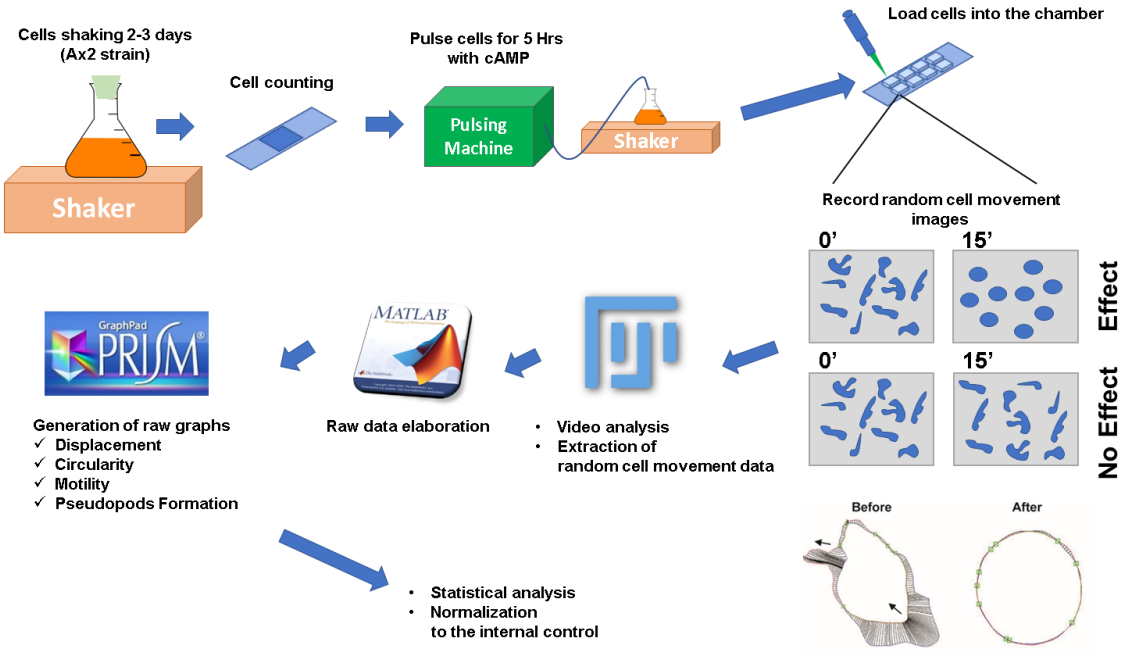

Figure 5. Representation of the random cell movement workflow. Cells are harvested from confluent plates and shaken for 2-3 days prior the experiment. Cells are then counted, diluted to the desired number and pulsed for 5 hours with cAMP in shaking suspension. Subsequently, cells are washed twice with KK2 and loaded into the chambers. Cell movement is recorded every 15 seconds over a 15 minutes period. Videos are then analysed using Fiji image software. Cells are outlined using the Fiji plugin Quimp 11b, which can determine cell displacement, motility, circularity and pseudopod formation. Data are elaborated using MATLAB and subsequently used to generate graphs and statistical analysis using GrapPad Prism.

2.4 *D. discoideum* growth assay

D. discoideum cells were harvested and diluted in axenic medium to $2x10^4$ cells/ml. Aliquots of cells (500 µL) were transferred to 24 wells plates containing consistent concentrations of solvent (DMSO) in addition to investigated

compounds. Cells were grown in 24 well plates at 22ºC and cell density calculated over a seven-day period.

2.5 Growth inhibition assay.

Cells were grown in shaking suspensions and harvested in exponential phase (1.5-2.5 x 10^6 cells/ml). Cells were divided into aliquots and shaken for 24 hours in the presence of solvent only or compound at a concentration which blocked growth by ~50% in a final volume of 2 ml axenic medium. Each condition tested was carried out at least in triplicate.

2.6 Development assays

1x10^7 *D. discoideum* cells were harvested from cells in shaking suspension, washed and resuspended in 1mL KK2 (16.2mM KH_2PO_4, 4mM K_2HPO_4 – pH=6.3). Cells were then evenly distributed onto 1% agar plates containing either solvent (DMSO) only to define the control conditions or a compound plus vehicle at a specific concentration. Cells were then incubated at 22ºC for twenty-four hours.

2.7 Ferric reducing ability of plasma (FRAP) assay.

The reducing ability of the compounds was assessed by using the ferric reducing ability of plasma (FRAP) assay. FRAP solution was prepared by combining 2 ml of TPTZ solution (10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl), 2 ml of $FeCl_3$ (10 mM) and 20 mL acetate buffer (300 mM, pH 3.6). The assay was carried out by combining 800 µL FRAP solution with 25 µL of positive control (1mM ascorbic acid) or curcumin-related compounds (to obtain a final concentration of 31.2 µM), and the absorbance measured at 595 nm. Measurements were obtained in triplicate.

2.8 Protein-ligand docking.

Protein sequences were obtained from dictybase.org and the tertiary structures of the *D. discoideum* proteins were predicted using Phyre^2 (Protein Homology/analogy Recognition Engine V 2.0). Docking analysis was performed using SwissDock to identify the possible binding sites in psrA and UFCS Chimera was used to display the results obtained from SwissDock. Results are expressed as ΔG (Gibbs free energy - where a negative value indicates a spontaneous interaction). Results obtained with the molecular docking analysis is based on the prediction of the most accurate computer modelling, taking as template other proteins with a similar tertiary structure. In addition, the prediction of the binding site is also based on the calculation of the most favourable interaction between the compound and the protein.

2.9 REMI screen.

Two libraries of insertional mutants containing 5,000 mutants (psrA) and 11,000 mutants (psenB) were used to identify *D. discoideum* mutants resistant to curcumin and its analogues. Cells were incubated with different concentrations of each compound over fourteen days. The disrupted genes of the mutants growing in the presence of each compound were identified using DNA purification and Inverse Polymerase Chain Reaction (iPCR).

2.9.1 Recapitulated mutants

psrA$^-$ (DDB_G0280469) was kindly provided by Dr Lou Kim (Department of Biological Sciences, Florida International University), whereas psenB$^-$ (DDB_G0292310) was readily available from our lab.

2.9.2 5k mutant library

Balint Stewart kindly provided this library (Department of Genetics, Evolution and Environment, University College London). The insertional vector used was pBSR1. Primers to identify the disrupted genes are T7-iPCR (CCCTATAGTGAGTCGTAT TA) and T7-iPCR reverse (CTGCACTACCAATCGCAATGG).

2.9.3 11k mutant library

Amy Baldwin kindly provided this library (Neuroscience and Mental Health Research Institute, Cardiff University). The insertional vector used was pREMI. Primers to identify the disrupted genes are AJBiPCR009-reverse (TGTATGCTA TACGAAGTTATCC) and AJBiPCR010-forward (GTAGAAGTAGCGACAGAGA AG) or AJBiPCR011-reverse (CCATTCGAAACTGCACTACC) and AJBiPCR013-forward (TGAATGGTGAAGATAAATATATGC).

2.10 Extraction of DNA from potential isogenic mutant cells

Cells were grown in liquid medium on a 10cm dish until confluent, washed from the plate and spun down (1000xg) for 3 minutes, and washed twice with KK2. DNeasy Blood & Tissue Kit was used to extract the DNA.

2.11 Polymerase Chain Reaction (PCR)

Amplification of DNA using polymerase chain reaction (PCR) was achieved under the following conditions for standard reactions: 2-5µL DNA, 2µL 2mM dNTPs, 2µL NH4 BIOTAQ reaction buffer, 1µL MgCl2, 0.2-0.5µL BIOTAQ DNA polymerase (5U µL-1), 2µL 5’ primer (10pmol), 2µL 3’ primer (10pmol) and double distilled water up to 20µL (total volume). The PCR was carried out using the following cyclical conditions for standard PCR reactions: initial denature, 10

minutes at 95ºC followed by 30 cycles as follows: denature 30 seconds, 95ºC, anneal 30 seconds, 50-60ºC (dependent on primer melting points), extension 4 minutes, 68ºC (or 1-minute 72ºC depending on the primer). The PCR was completed with a final extension of 10 minutes at 68ºC and samples were then stored at 4ºC.

2.12 Restriction digest of genomic DNA

All restriction digests were prepared according to the following conditions: 5µL DNA, 0.2-0.5µL each restriction enzyme (100µL-1), 1µL appropriate buffer (according to the manufacturer's instructions). Samples were incubated at 37ºC for 20 minutes to 2 hours, depending on the type of restriction enzyme and FastDigest enzymes were used in shorter incubations.

2.13 Agarose gel electrophoresis

To separate and visualise DNA, 1% agarose gels containing ethidium bromide and 1x TBE buffer were used. 5-10µL DNA was added to 1-2µL 5xDNA loading dye (10 mM Tris-HCl (pH 7.6) 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol 60 mM EDTA). 100bp plus or 1kb DNA ladders (Thermo Fisher Scientific) were used as a molecular marker to determine DNA fragment size on the agarose gels. Gels were run in an electrophoresis tank for 30 minutes at 110V and visualised with UV light using a GeneFlash gel documentation system (Syngene Bio Imaging).

2.14 Compounds

The following chemicals were obtained from Sigma Aldrich Co. Ltd (Dorset, UK): Capsaicin (8-Methyl-N-vanillyl-trans-6-nonenamide; M2028), 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 (KNO3; 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). Curcumin (E,E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; C1386), Demethoxycurcumin (E,E)-1-(4-Hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione; D7696), Bisdemethoxycurcumin (1E,6E)-1,7-bis(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione; B6938), EF-24 (3E,5E)-3,5-bis[(2-fluorophenyl)methylene]-4-piperidinone; E8409), FLLL31 (E,E)-1,7-Bis(3,4-dimethoxyphenyl)-4,4-dimethyl-1,6-heptadiene-3,5-dione; F9057), Tetrahydrocurcumin (1,7-Bis(4-hydroxy-3-methoxyphenyl)-3,5-heptanedione; 50202) were also obtained from Sigma Aldrich Co. Ltd (Dorset, UK). Curcumin Pyrazole ((E)-3,5-bis[β-(4-hydroxy-3-methoxyphenyl)-ethenyl]-1H-pyrazole-SL-318) was obtained from Syninnova, and Enaminestore supplied UBS109 (3,5-Bis(2-pyridinylmethylidene)-1-methyl-4-piperidone-Z46034963). Compounds labelled ‘GSK’ were provided by the industrial collaborator GlaxoSmithKline and, due to intellectual property protection, full names and structures have been withheld. All compounds were dissolved in DMSO.

2.15 Reagents

2-Propanol, bacteriological agar, sodium dodecyl sulphate (SDS), Luria-broth (LB) tablets, magnesium chloride, Nonidet-P40 (NP40). Agarose,

deoxynucleotide triphosphates (dNTPs) (Bioline, London, UK); Ethidium bromide (Bio-Rad Laboratories, Hemel Hampstead, UK); axenic (Ax) medium, SM medium, LoFlo medium (ForMedium, Hunstanton, UK); ethanol, dipotassium hydrogen phosphate (K_2HPO_4), chloroform, isopropyl β-D-1-thiogalactopyranoside (IPTG), methanol, paraformaldehyde (PFA), potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (VWR International Ltd., Lutterworth, UK).

2.16 Antibiotics

Ampicillin (Sigma Aldrich Co. Ltd, Dorset, UK); Blasticidin, penicillin/streptomycin (Pen/Strep) solution x100 (PAA Laboratories Ltd. Yeovil); Hygromycin, Geneticin (G418) (Invitrogen, part of ThermoFisher Scientific, Loughborough, UK).

2.17 Molecular weight standards

GeneRuler 100 bp and 1 kb DNA Ladder (0.1 µg/µL); (ThermoFisher Scientific, Loughborough, UK).

2.18 Restriction enzyme

The RSAI restriction enzyme was obtained from ThermoFisher Scientific (Loughborough, UK).

2.19 Other enzymes

RNase A, DNase (Sigma Aldrich Co. Ltd, Dorset, UK); BIOTaq polymerase (Bioline, London, UK).

2.20 Kits

MiniElute PCR purification kit (Qiagen Ltd, Crawley, West Sussex, UK); TOPO® TA Cloning kit (ThermoFisher Scientific, Loughborough, UK); DNeasy Blood & Tissue Kit (Qiagen Ltd, Crawley, West Sussex, UK).

2.21 Bacterial strains

TOP10 chemically-competent E.coli, chemically-competent JM107 E.coli (Invitrogen, part of ThermoFisher Scientific, Loughborough, UK); 10β-competent cells (New England Biolabs (UK) Ltd., Hitchin, UK); Raoultella planticola (lab-made stock).

2.22 Primers

Custom-made oligonucleotides were purchased from MWG-Biotech AG (Eurofins) (Germany) or Sigma Aldrich Co. Ltd (Dorset, UK).

2.23 Equipment

505Di peristaltic pump (Watson Marlow); centrifuge (Biofuge 13, Jencons); microcentrifuge (ThermoFisher Scientific, Loughborough, UK); Olympus IX71 microscope (U-RFP-T laser, 482nm emission, QImaging RetegaExi Fast1394 digital camera (Olympus, Southend-on-sea, UK); Neubauer improved haemocytometer; Centrifuge (Biofuge 13, Jencons) 47mm black nitrocellulose filters (Millipore, Oxfordshire, England); LabTek 8-well chambered coverglass (ThermoFisher Scientific, Loughborough, UK).

2.24 Software

Microscopy and quantification of chemotaxis assays were analysed using ImagePro Plus (Media Cybernetics). Random cell movement and global stimulation microscopy experiments were quantified in Fiji software, using the QuimP 11b plugin (Warwick University, Warwick, UK) and MATLAB software (Mathworks, Cambridge, UK). The graphical and statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc). Protein-ligand docking was visualised using UCSF ChimeraX.

2.25 Websites

Dictyostelium gene and protein information:

http://dictybase.org/

http://www.uniprot.org/

2.26 BLAST

http://www.ncbi.nlm.nih.gov/BLAST/

http://dictybase.org/tools/blast

2.27 Protein prediction and docking

Phyre2: http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index

SwissDock: http://www.swissdock.ch/docking

Chapter III

Rationale of the investigation and strategy employed

This study focuses on three significant milestones:

1. The use of *D. discoideum* as a model to investigate how taste perception works at the molecular level and to assess the effects of tastants;
2. Investigate this model to predict the aversiveness of active ingredients present in medicines and the correlation with results obtained in mammalian models;
3. Employ *D. discoideum* to assess the active components of tastant chemicals responsible for their biological activities and to identify their molecular targets.

The first stage was the assessment of the effects of capsaicin on cellular movement, which helped in the development of the compound screening methodology. In the second part of the project, the aforementioned methodology was employed to assess a wide range of bitter-tasting compounds, which included a set of known bitter therapeutic agents and a group of blinded compounds. Analysis of compounds with known bitter taste in humans and activity in the rat BATA assay enabled the comparison of the results obtained in *D. discoideum* with these models (Cocorocchio et al., 2015). The third stage was carried out to better understand the fundamental structural characteristics of a range of structurally related compounds to the bitter polyphenol curcumin on cell movement, growth and development.

3.1 Development of a model for the assessment of tastants

The first step in developing *D. discoideum* as a model for the investigation of tastants involved the assessment of behavioural changes in the amoeba as a readout to characterise the effects of the pungent molecule capsaicin. In our study, capsaicin was able to inhibit *D. discoideum* cell behaviour, even though the amoeba does not have a TRPV1 receptor homologue, suggesting that it may function through a TRPV1-independent mechanism (Otto et al., 2016). The acute response to capsaicin of previously identified mutants that showed growth resistance to capsaicin has also been characterised. These mutants contain disrupted RacGEF proteins (gxcP- and gxcKK), which are Guanine exchange factor for Rac GTPases. Studies have shown that in *D. discoideum* actin organisation, chemotaxis and endocytosis are processes all regulated by Rho GTPases, which are comparable to those mechanisms presents in higher organisms (Rivero and Xiong, 2016; Vlahou and Rivero, 2006). These proteins activate monomeric GTPases provoking the release of guanosine diphosphate (GDP) to allow the binding of guanosine triphosphate (GTP) (Bos et al., 2007). GTPases act as molecular switches in cell signalling pathways and have several downstream targets, which are involved in several cellular processes such as cell differentiation and proliferation, cytoskeletal organisation, vesicle trafficking and nuclear transport. It has been shown that *D. discoideum* possesses many of these processes that are important for its chemotaxis, migration, organisation of the cytoskeleton and the pseudopod formation. Thus, *D. discoideum* may provide a valuable model for the characterisation of novel mechanisms of action modulated by capsaicin.

3.1.1 Capsaicin

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) (Fig. 6) is an alkaloid present in plants that belong to the Capsicum genus and is the active ingredient in chilli peppers. Capsaicin and its analogues are natural molecules called capsaicinoids, which give the characteristic pungent flavour to chillies (Sharma et al., 2013).These chemicals are produced as a natural defence by the plant against predators and parasites. Capsaicin is a highly volatile, pungent, hydrophobic, colourless and odourless, white, crystalline powder in its pure form. The effects of capsaicin on the human body are a burning sensation, increase in blood flow, the rise in salivation and gastric secretion as well as increased intestinal contractile activity (Sharma et al., 2013). Research studies have shown that capsaicin possesses anti-cancer (Garufi et al., 2016; Park et al., 2014; Ramachandran and Srivastava, 2017), cardioprotective (Fragasso et al., 2004; Sharma et al., 2013), pain relief and anti-obesity properties (Anand and Bley, 2011; Mason et al., 2004; Narang et al., 2017).

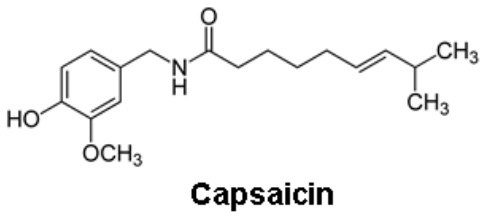

Figure 6. Structure of capsaicin.

The transient receptor potential vanilloid subfamily member 1 (TRPV1) cation channel, present in nociceptive neurons in the oral cavity, is responsible for the taste transduction of capsaicin in mammals, and is also present in skin, eyes, and gastrointestinal mucosa and in non-neuronal cells including muscle, keratinocytes, and endothelium (Sharma et al., 2013). The initial painful sensation after capsaicin ingestion arises from the selective activation of TRPV1,

mainly expressed in sensory neurons (Yang et al., 2014). However, a recent study has demonstrated that a low concentration (sub-micromolar) of capsaicin blocked capsaicin-sensitive (CS) neurons, while a high concentration of capsaicin (> 10 μM) blocked transient current (I_A) in both CS and capsaicin-insensitive (CIS) neurons (Yang et al., 2014). Therefore, this suggests the existence of two mechanisms through which capsaicin may function; a TRPV1-dependent and a TRPV1-independent mechanism.

3.1.2 Employing *Dictyostelium* as an Advantageous 3Rs Model for Pharmacogenetic Research

Chapter 9

Employing *Dictyostelium* as an Advantageous 3Rs Model for Pharmacogenetic Research

Grant P. Otto, Marco Cocorocchio, Laura Munoz, Richard A. Tyson, Till Bretschneider, and Robin S.B. Williams

Abstract

Increasing concern regarding the use of animals in research has triggered a growing need for non-animal research models in a range of fields. The development of 3Rs (replacement, refinement, and reduction) approaches in research, to reduce the reliance on the use of animal tissue and whole-animal experiments, has recently included the use of *Dictyostelium*. In addition to not feeling pain and thus being relatively free of ethical constraints, *Dictyostelium* provides a range of distinct methodological advantages for researchers that has led to a number of breakthroughs. These methodologies include using cell behavior (cell movement and shape) as a rapid indicator of sensitivity to poorly characterized medicines, natural products, and other chemicals to help understand the molecular mechanism of action of compounds. Here, we outline a general approach to employing *Dictyostelium* as a 3Rs research model, using cell behavior as a readout to better understand how compounds, such as the active ingredient in chilli peppers, capsaicin, function at a cellular level. This chapter helps scientists unfamiliar with *Dictyostelium* to rapidly employ it as an advantageous model system for research, to reduce the use of animals in research, and to make paradigm shift advances in our understanding of biological chemistry.

Key words *Dictyostelium*, Mechanism of action, Pharmacogenetics, Random cell movement

1 Introduction

Dictyostelium discoideum is a soil amoeba that consumes bacteria, but when starved it initiates multicellular development, a morphogenetic process that involves chemotaxis, intercellular signaling, and cell type differentiation; these are also the attributes of its life cycle that are most studied. However, *Dictyostelium* has becoming increasingly useful as a simple model organism to dissect the molecular mechanism of action of poorly characterized chemicals with therapeutic benefits, including new drugs and natural products. It is easy and cheap to grow and maintain, its small 34 Mb genome has been sequenced, and it is simple to manipulate genetically to

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either ablate or overexpress a gene of interest, all attributes that make it ideal as a pharmacogenetic model [1].

Drug discovery and development is a costly, lengthy process of identifying chemicals that are not only specific and effective, but are also well tolerated by patients. Many drugs have nausea as a side effect, which can lead to vomiting (emesis), and this adversely impacts patient compliance. Thus, investigating the emetic liability of new drugs is important, but it is expensive and has animal welfare implications because it typically involves vomiting or food avoidance experiments in sentient animals like dogs and ferrets. We have used *Dictyostelium* to study the emetic liability of compounds previously reported to be emetic or aversive in in vivo animal studies [2], and identified a G-protein-coupled receptor and its likely mammalian counterpart as the molecular target of the bitter compound phenylthiourea [3]. We have also used *Dictyostelium* to study the molecular mechanism of the action of the dietary flavonoid naringenin, a component of citrus fruits that has antiproliferative and chemopreventive actions *in vitro* and in animal models of carcinogenesis. Our studies in *Dictyostelium* identified a Ca^{2+}-permeable nonselective cation channel, TRPP2 or polycystin-2, as the target of naringenin [4]. This channel is implicated in the development of autosomal dominant polycystic kidney disease (ADPKD) [5, 6], and accounts for about 15 % of the mutations in ADPKD sufferers. The channel is the target of naringenin in mammalian kidney cells too, and represents a potential novel treatment for the 85 % of ADPKD patients where TRPP2 is present and can be activated by naringenin.

These studies used a simple, rapid assay based on random cell movement of chemotactically-competent amoebae as a phenotypic readout, but growth and development have also been used to dissect the cellular uptake mechanism for the bipolar disorder drug valproic acid [7]. Here we describe the random cell movement assay in *Dictyostelium* cells treated with the pungent alkaloid capsaicin, which is responsible for the spicy taste of chilli peppers of the *Capsicum* genus, and is of biomedical interest because of its potential therapeutic effects for the treatment of a variety of human conditions, including pain relief, weight reduction, anti-carcinogenic properties, and cardiovascular, gastric, urological, and dermatological conditions (reviewed in [8]). The gustatory effects of capsaicin in mammals are through its action as an agonist of a cation channel, transient receptor potential vanilloid subfamily member 1 (TRPV1), which is present in nociceptive neurons in the oral cavity, skin, eyes, and gastrointestinal mucosa, but is also present in non-neuronal cells including muscle, keratinocytes, and endothelium (reviewed in [9]). Prolonged exposure to capsaicin causes TRPV1 receptor desensitization [10]; yet extended effects on cellular signaling suggest that capsaicin can act independently of TRPV1. Evidence of these effects includes its cocarcinogenic effect

in TRPV1 knockout mice [10], that it alters sucrose preference in TRPV1 knockout mice [11], and that capsaicin inhibition of collagen-induced aggregation of platelets is unaffected by a TRPV1 antagonist [12]. Additionally, capsaicin has a significant acute effect on *Dictyostelium* cell behavior, including loss of velocity, cell shape, and angular movement [2], even though clear homologues of human, mouse, or worm TRPV1 proteins have not been identified in the *Dictyostelium* genome. This result suggests that capsaicin is exerting these behavioral effects in *Dictyostelium* by a TRPV1-independent mechanism, making this organism a useful model to identify the molecular identity of this alternate pathway.

2 Materials

2.1 Equipment

A small-volume peristaltic pump (e.g., Watson Marlow 505Di, Cornwall, UK) is required to deliver cAMP to the cells to make them chemotaxis-competent.

2.2 Cell Culturing and Preparation

1. *Dictyostelium* cells (*see* **Note 1**) are grown axenically in HL5 medium (Formedium) in sterilized glass conical flasks with aeration on a temperature-controlled platform shaker (21 °C, 120 rpm). Medium is supplemented with streptomycin and penicillin (100× stock of 5000 U/ml, Gibco) and either blasticidin S (10 μg/ml; Apollo Scientific) or G418 (10 μg/ml; Invitrogen).
2. KK2 buffer: 20 mM Potassium phosphate buffer, pH 6.1.
3. cAMP: A 200 mM stock solution is prepared in water; adjust to pH 6.3 with NaOH. Store in aliquots at −20 °C.

2.3 Preparation of Capsaicin

1. A 50 mM stock solution of capsaicin is prepared in the solvent dimethyl sulfoxide (DMSO). Store in aliquots at −20 °C.

2.4 Microscopy

1. Lab-tek 8-well chambered coverglasses (Thermo Scientific Nunc).
2. Olympus IX71 inverted microscope with 40× objective.
3. QImaging RetigaExi Fast 1394 digital camera.

2.5 Time-Lapse Video Analysis

The following four software programs are used to capture images, outline individual cells, quantify cell behavior parameters, and perform statistical analysis.

1. Images are captured using ImagePro software (Media Cybernetics, Buckinghamshire, UK).
2. The QuimP11 plug-ins for the Fiji distribution of ImageJ are used to outline individual cells and to quantify cell behavior parameters (available for download at http://go.warwick.ac.uk/quimp).

3. Two custom scripts in MATLAB (MathWorks, USA) are used to first read the output generated by Fiji cell segmentation and then combine the individual cell values for each parameter (displacement, motility, circularity, and protrusion formation) (described in [13]). The final MATLAB output is an Excel file containing all the information of cell segmentation for each video analyzed (individual values, mean and standard deviation).
4. Graphpad Prism v5.02 (GraphPad Software Inc, San Diego, USA) is used to perform statistical analysis (data normalization, one-way ANOVA with Bonferroni posttest) and to plot the data for the generation of graphs.

3 Methods

3.1 Preparation of Cells and Compounds

1. *Dictyostelium* strains are grown for at least 2 days on a shaking platform at 22 °C.
2. Cells are harvested during exponential growth (between 1 and 5×10^6 cells/ml) by centrifugation at $500 \times g$ for 3 min, and washed twice with KK2 buffer.
3. Cells (2×10^7) are resuspended in 6 ml KK2 buffer in a 50 ml conical flask, and pulsed with 20 nM cAMP every 6 min for 5 h at 22 °C (giving a final cAMP concentration of 1 μM).
4. These cells are chemotactically-competent, and can be used to monitor acute changes in cell behavior.

3.2 Imaging Cell Behavior

1. Starved, chemotactically-competent cells are diluted tenfold with KK2, and 250 μl of this dilution is added to individual wells of 8-well Lab-tek chambers. Allow 15 min for the cells to adhere before imaging.
2. Cell behavior is visualized using a 40× objective on an inverted bright-field microscope, and images are captured every 15 s with an attached CCD camera.
3. As an internal control for each experiment, images are captured every 15 s for 5 min before the addition of the compound to be analyzed (a 2× stock is prepared in 250 μl KK2 and added gently to the well), or vehicle alone for control experiments (*see* **Note 2**).
4. The camera shutter is closed for compound/vehicle addition, to provide one black image to indicate compound addition in the resultant movie.
5. Images are then captured for a further 10 min every 15 s to record cell behavior.

3.3 Analysis of Random Cell Movement

1. Cell outlines are established by hand on the first image of each series, and cells tracked through the series using Fiji. Two custom scripts in MATLAB are used to first read the output generated by Fiji and then combine the individual cell values for each of the four cell behavior parameters: cell shape (circularity), motility, protrusion number, and displacement.
2. The MATLAB output is transferred to Graphpad Prism for statistical analysis and graph production. The data for each compound concentration may be normalized to the highest value which is defined as 100 %. Data can be presented as an average cell response (in velocity, displacement, cell shape, or protrusion formation) during the time period assessed (e.g., 15 min) (*see* Fig. 1a, b), with a dose-dependent effect on all cell behavior parameters. Alternatively, a change in cell behavior can be visualized by plotting cell response over a period after addition of compound (Fig. 1c, d). In regard to capsaicin, cells treated with 2–3 μM changed shape and produced fewer protrusions, with the result that both displacement and motility were reduced.

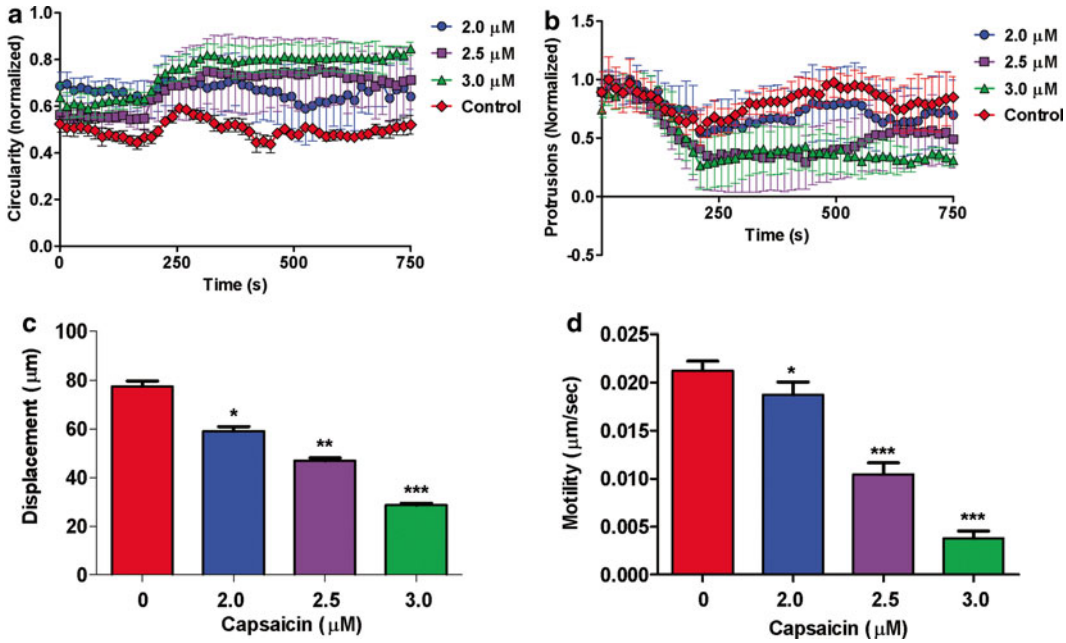

Fig. 1 The effect of capsaicin on random cell movement parameters in parental *Dictyostelium* cells. Adherent Ax2 cells in Lab-Tek chambers were imaged every 15 s for 5 min before the addition of capsaicin (2.0 μM, 2.5 μM, or 3.0 μM), followed by a further 10-min imaging in the presence of the compound. The effect of capsaicin on four parameters of cell behavior was analyzed. There was a dose-dependent effect on the degree of rounding up or circularity of the cells (**a**), the number of protrusions formed (**b**), displacement (**c**), and motility (**d**). Note that displacement and motility are the averages for the final 288 s of imaging, and statistical analysis was between control and each capsaicin concentration. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Measurements were derived from an average of 30 cells analyzed from triplicate experiments. Error bars represent ±S.E.M.

3. A two-tailed paired student t-test is performed to compare between the 5 min preceding addition of compound and the final 5 min of image capture for all four parameters.
4. A one-way analysis of variance (ANOVA) with subsequent Bonferroni test is conducted to determine whether there is any statistical difference in cell behavior between different concentrations of compound or between wild-type and mutant cells at the same compound concentration.
5. Several factors should be taken into consideration regarding the selection of concentrations of a given compound to be analyzed (*see* **Note 3**).

3.4 Identifying the Molecular Identity of Chemical Detection Pathways

1. To identify the molecular basis of the action of a chemical, a library of mutants is screened for resistance to the effects of the compound on growth, development, or cell behavior, as described elsewhere [3, 4, 7] (*see* **Note 4**).
2. Mutants identified in one type of screen (e.g., growth) can then be analyzed for resistance to a compound in other assays (e.g., development or behavior), thus providing additional information on the molecular pathway affected by each compound. Alternatively, a range of published mutants are widely available if a candidate approach is taken (*see* **Note 1**).
3. A recent library screen to characterize the molecular mechanism of the bitter tastant phenylthiourea [3] identified two RacGEF mutants that were partially resistant to the action of capsaicin on cell behavior: *gxcP*– (dictybase ID: DDB_G0285859) and *gxcKK*– (dictybase ID: DDB_G0293340) (*see* Fig. 2).

4 Notes

1. Strains: Many *Dictyostelium* strains are available from the searchable *Dictyostelium* strain database run by Dictybase (www.dictybase.org; *see* ref. [14]) and can be obtained for a nominal fee and the cost of shipping.
2. Vehicle for drugs/compounds: Commonly used vehicles include DMSO, DMF, ethanol, methanol, and acetic acid. We have conducted experiments to determine the highest concentrations that can be used that do not significantly disrupt cell behavior in the random cell movement assay (e.g., up to 1 % DMSO, 3 % EtOH).
3. The choice of concentrations to be analyzed is based on those used in published studies on whole animals or tissue culture cells, and two- to fivefold lower and higher concentrations can also be tested. If there is no published data for a chemical, then

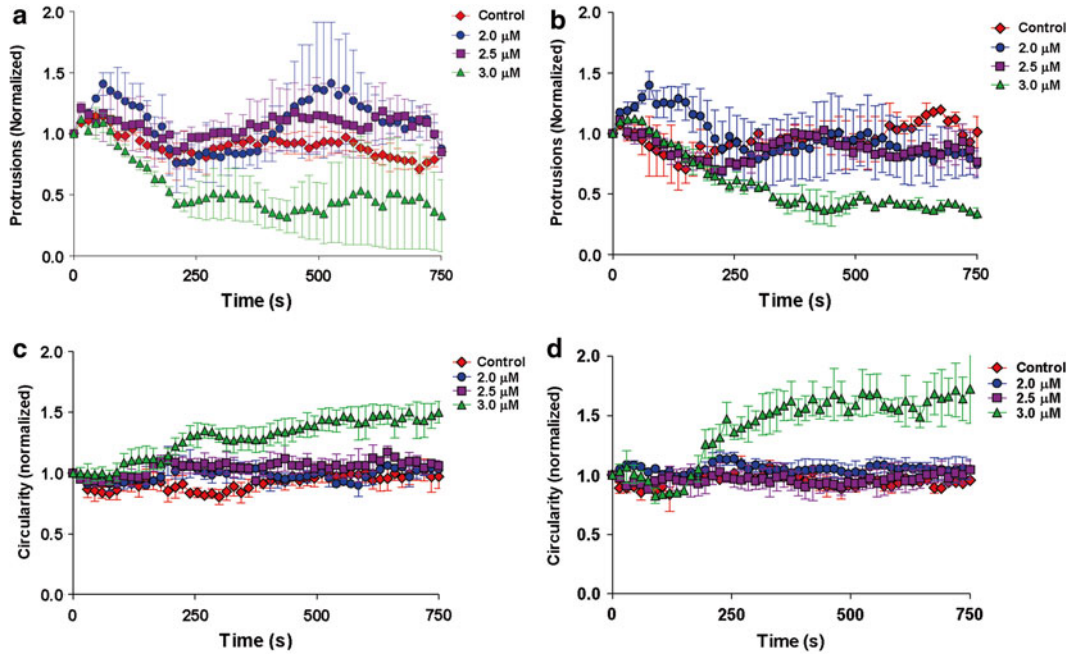

Fig. 2 Behavior of *Dictyostelium gxcP*– and *gxcKK*– cells in random cell movement assays. Both *gxcP*– and *gxcKK*– display partial resistance to 2 and 2.5 μM capsaicin, both in the number of protrusions formed (**a**, **b**, respectively) and the circularity of cells (**c**, **d**, respectively). Measurements were derived from 50 cells analyzed from a minimum of triplicate experiments. Error bars represent ±S.E.M.

a concentration range for related compounds is used, as long as it is within the solubility range of the chemical and does not have acute effects on cell viability (e.g., cell lysis). This can be simply tested by visual inspection under a microscope.

4. Where possible, it is advantageous to use appropriate controls during these experiments. For example, when screening a library of mutants for resistance to a compound, the wild-type parental strain containing the antibiotic resistance cassette but without gene ablation should be used. When studying individual mutants, at a minimum the immediate parental strain should be employed because variability in compound sensitivity is found between laboratory wild-type strains.

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Figure 1. N=30 cells analysed per condition from 3 independent technical repeats.

Figure 2. N=50 cells analysed per condition from ≥ 3 independent technical repeats.

3.2 Application of the methodology for the investigation of a range of structurally diverse bitter tastants

Early studies have shown that several bitter tasting compounds such as denatonium, phenylthiourea and naringenin were capable to affect distinct stages of *D. discoideum* life cycle (e.g. chemotaxis, random cell movement, growth and development) (Robery et al., 2011; Robery et al., 2013; Waheed et al., 2014). Therefore, to better understand the acute effects of bitter tasting substances on *D. discoideum* cell behaviour, changes in random cell movement were monitored before and after the exposure to a range of known and unknown bitter compounds with widely diverse molecular structures. The list of compounds was tailored to understand if the model system could predict the bitterness of those substances and, ultimately, obtain a ranking order comparable to the *in vivo* rat BATA assay and human sensory panel models (Rudnitskaya et al., 2013). The set of substances of both organic and inorganic origins included: azelastine hydrochloride, caffeine, chlorhexidine digluconate, potassium nitrate (KNO_3), paracetamol (acetaminophen), quinine, sumatriptan succinate as well as five blinded substances provided by the industrial partner GlaxoSmithKline (Cocorocchio et al., 2015) (Figure 7). Our results have shown a positive correlation between *D. discoideum* and the *in vivo* rat BATA test, suggesting that this approach may be used as an early screening platform for the assessment of bitter compounds and that the response to aversive chemicals is evolutionarily conserved (Cocorocchio et al., 2015).

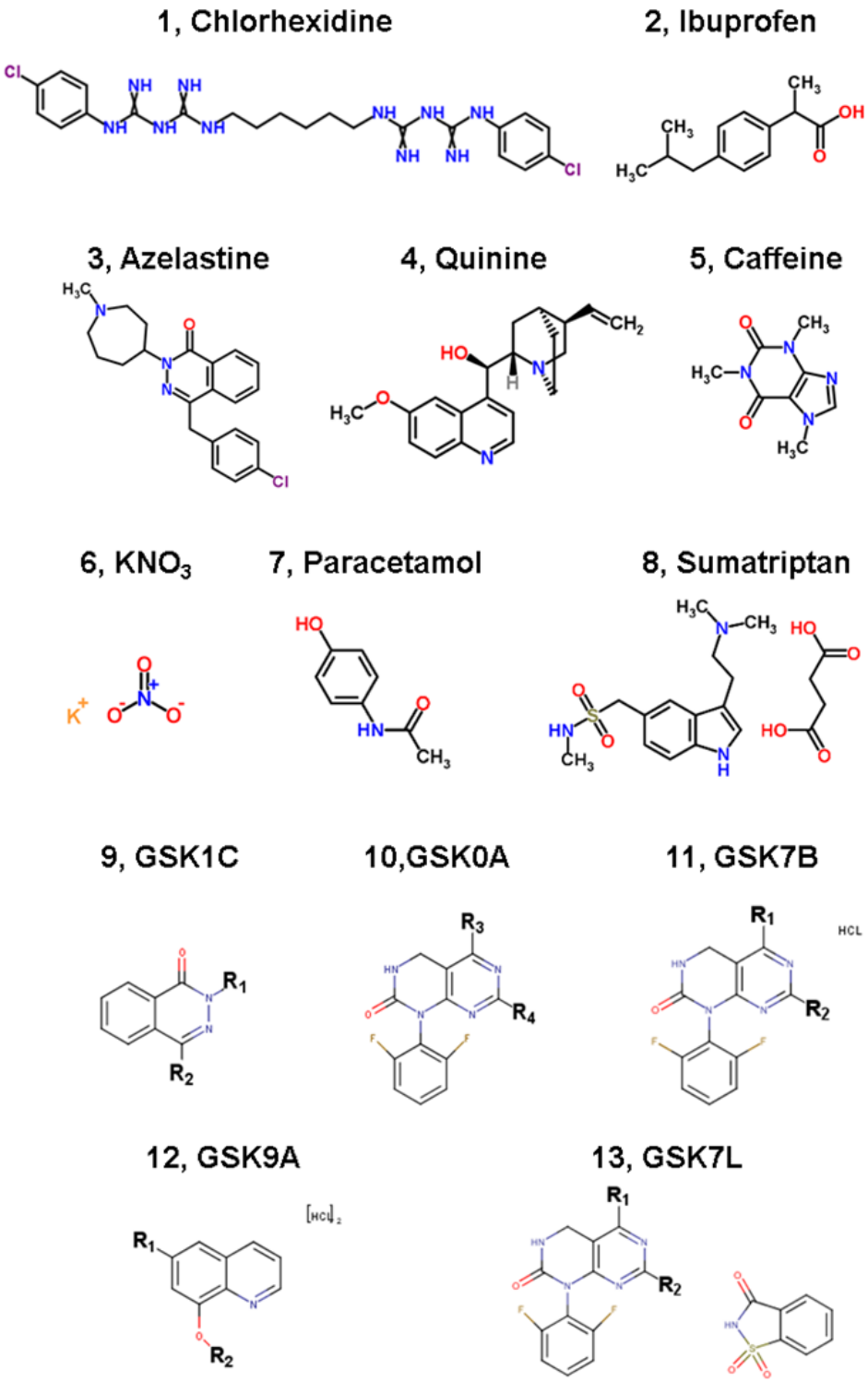

Figure 7. Structure of known (1-8) and blinded (9-13) bitter tasting substances tested in *D. discoideum* cell behaviour.

3.2.1 Azelastine hydrochloride (AZL)

Azelastine is a selective, non-sedating H_1-receptor antagonist, structurally similar to other anti-histaminic molecules and prevents the release of inflammatory mediators from mast cells (Baba et al., 2016). This bitter-tasting compound is pharmacologically classified as a second-generation antihistamine, with relative lack of central nervous system activity and is utilised for the treatment of allergic conditions, such as rhinitis and conjunctivitis (El-Shaheny and Yamada, 2014). Particularly, *in vitro* studies have shown that azelastine possesses higher affinity for H_1 receptors as compared to chlorpheniramine, a first-generation H_1-receptor antagonist (Slack et al., 2011). Finally, *in vivo* studies in a guinea pig model have demonstrated that both histamine-related and histamine-independent bronchoconstriction were inhibited by azelastine (Williams et al., 2010).

3.2.2 Chlorhexidine (CHX)

Chlorhexidine is a broad spectrum antimicrobial agent with a bitter taste (E Frank et al., 1995). It is frequently used in dental-care to inhibit bacterial growth and in periodontal disease prophylaxis. It is classified as an antibacterial, but it can also interfere with the proteolytic activity of some periodontal pathogens and therefore reduces caries progression in humans (Garcia et al., 2009; Trufello et al., 2014). This inhibitory effect is associated with its cation-chelating properties on matrix metalloproteinases (MMPs) (Gendron et al., 1999).

3.2.3 Quinine

Quinine is the active ingredient of the cinchona bark and is a naturally occurring bitter alkaloid, which possesses diverse medicinal properties and is also used as a flavouring ingredient (Sanchez et al., 2014). Although it is employed as an antimalarial (Achan et al., 2011) and babesiosis's treatment (Dorman et al.,

2000), quinine is poorly tolerated and has low patient compliance. Quinine is also used in some muscular disorders, especially nocturnal leg cramps and myotonia congenita (Mandal et al., 1995; Pusponegoro et al., 1991). However, the mechanisms of action responsible for its antimalarial and muscular disorder effects have not been fully understood. In addition, research studies have shown that quinine can activate multiple hTAS2Rs (Upadhyaya et al., 2016).

3.2.4 Caffeine

Caffeine is a bitter, white, crystalline, xanthine alkaloid and a stimulant drug (Nehlig et al., 1992). In humans, caffeine acts as a central nervous system (CNS) stimulant, temporarily warding off drowsiness and restoring alertness (Cappelletti et al., 2015). It is the most widely consumed psychoactive compound (Sajadi-Ernazarova and Hamilton, 2017). Studies have found that caffeine is also able to stimulate 4 hTAS2Rs in HEK293T cells (Ueda et al., 2003). In addition, it acts as a nonselective antagonist of adenosine receptors reducing the effects of adenosine, because of its structural similarity to adenosine (Ribeiro and Sebastiao, 2010). In physiological conditions, adenosine acts as an inhibitory neurotransmitter that suppresses neuronal activity in the CNS (Fisone et al., 2004). Caffeine is metabolised in the liver by the cytochrome P450 oxidase enzyme system, in particular by the CYP1A2 isozyme, into paraxanthine, theobromine, and theophylline (Zanger et al., 2008).

3.2.5 Ibuprofen

Ibuprofen also possesses a bitter taste and is classified as a non-steroidal anti-inflammatory drug (NSAID). It is widely used for relieving pain, helping with fever, and reducing inflammation (Van Esch et al., 1995). Nonsteroidal anti-inflammatory drugs such as ibuprofen work through the inhibition of the

cyclooxygenase enzyme (COX) which converts arachidonic acid to prostaglandin H2 (PGH_2). Subsequently, PGH_2 is converted into different prostaglandins (which are mediators of pain, inflammation, and fever) and thromboxane A2 (which stimulates platelet aggregation, leading to the formation of blood clots) (Karlsson and Fowler, 2014).

3.2.6 Paracetamol

Paracetamol is one of the most widely utilised antipyretic and analgesic drugs worldwide. The bitter taste of this drug is believed to originate from its hydroxyl group (Hejaz et al., 2012). Pharmacologically, paracetamol possesses weak inhibitory effects on COX-1 and 2 and is metabolised to AM404 (N-arachidonoyl-aminophenol). AM404 is able to inhibit the reuptake of the endogenous cannabinoid/vanilloid anandamide by neurons. Anandamide re-uptake lowers synaptic levels of anandamide, resulting in an increased activation of nociceptors in the body, such as the TRPV1 or vanilloid receptors (Bertolini et al., 2006). By blocking anandamide reuptake, levels in the synapse remain high and are able to desensitise the TRPV1 receptor much like capsaicin (Kis et al., 2005).

3.2.7 Potassium nitrate

KNO_3 is an ionic salt of potassium and nitrate ions. It naturally occurs as a mineral, is a solid source of nitrogen and possesses a bitter taste (Alfano et al., 1999). It has a wide range of applications, such as the manufacture of fertilisers, rockets (as propellant) and fireworks and is also used as a desensitising agent in some toothpaste for sensitive teeth (Hong and Lim, 2016; Sharma et al., 2012). Potassium nitrate is also one of the main constituents of gunpowder (black powder) and has been utilised as a food preservative since the 5th century C.E. A recent study has also shown that the oral administration of potassium nitrate

improved the response to a hypoxic challenge by increasing the number of perfused vessels (Kolb et al., 2017).

3.2.8 Sumatriptan succinate

Sumatriptan is a synthetic drug that belongs to the triptan class, with a strong bitter taste, used for the treatment of migraines (Munjal et al., 2017). Structurally, it is an analogue of the naturally occurring neuro-active alkaloids dimethyltryptamine (DMT), bufotenine, and 5-methoxy-dimethyltryptamine, with an N-methyl sulfonamide group at position C-5 on the indole ring (Razzaque et al., 1999). Sumatriptan is structurally similar to 5-Hydroxytryptamine and is a 5-HT (type 5-HT_{1D} and 5-HT_{1B}) receptor-agonist (Razzaque et al., 1999). Acting as an agonist of these receptors, sumatriptan reduces the vascular inflammation associated with migraines in the cranial and basilar arteries (Evans et al., 2012). These effects are achieved through vasoconstriction of those dilated arteries (Gulati et al., 2013). In addition, it has been shown that sumatriptan can decrease the activity of the trigeminal nerves, through its agonistic activity on TRPV1 receptors present in the trigeminal nociceptive system, which is responsible for sumatriptan's efficacy in treating cluster headaches (Evans et al., 2012).

3.2.9 Bitter tastant responses in the amoeba *Dictyostelium* correlate with rat and human taste assays

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

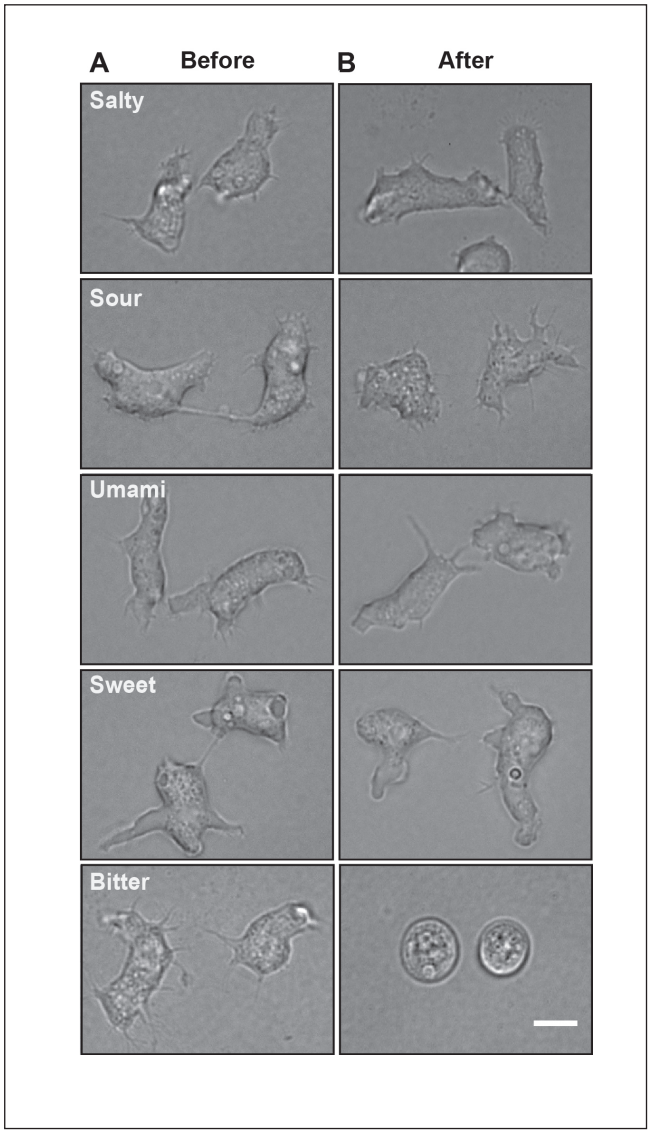

Fig. 1: *Dictyostelium* response to salty, sour, umami, sweet and bitter tastants

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.

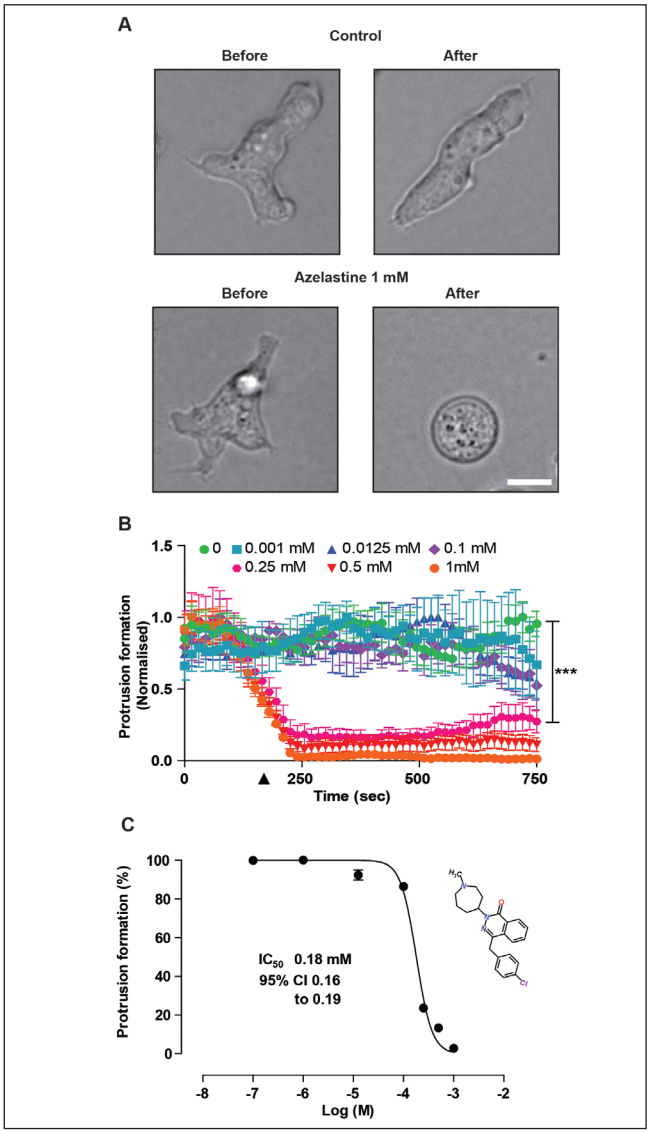

Fig. 2: Quantifying *Dictyostelium* response to bitter tastants

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

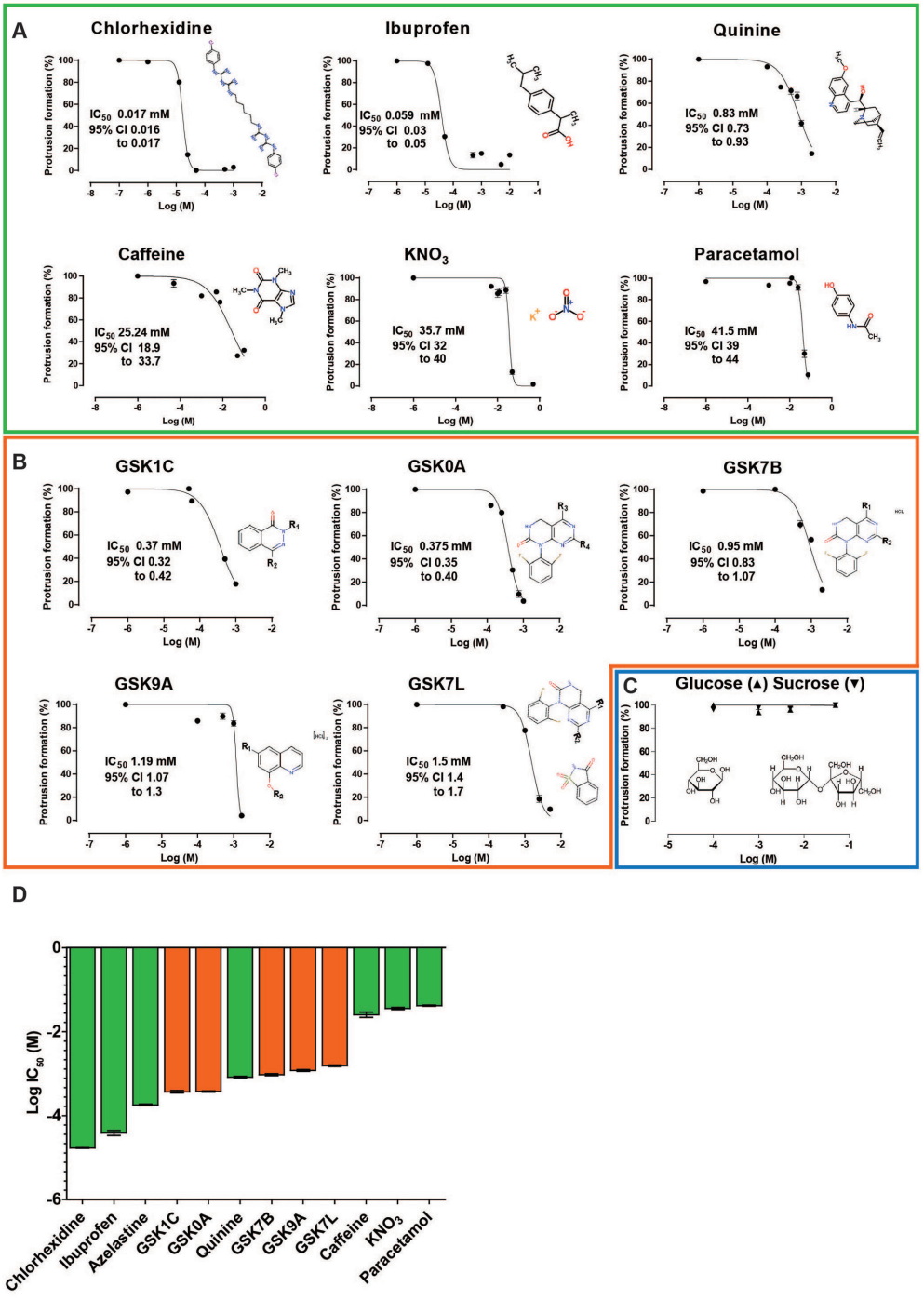

Fig. 3: Sensitivity of *Dictyostelium* to a range of bitter tastants

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).

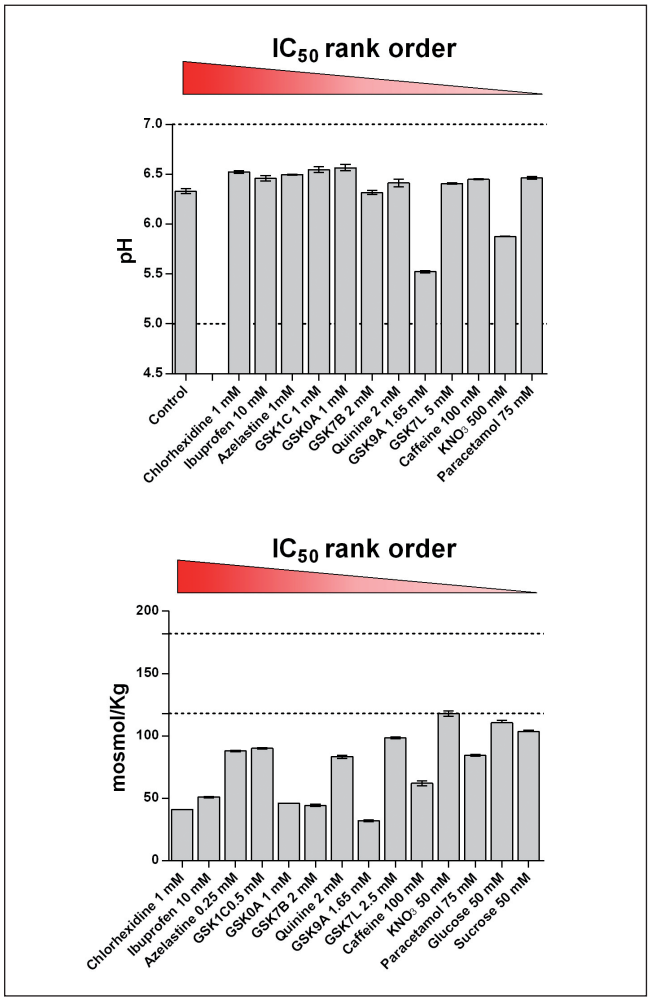

Fig. 4: Physicochemical properties of bitter molecules do not block *Dictyostelium* cell behavior

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

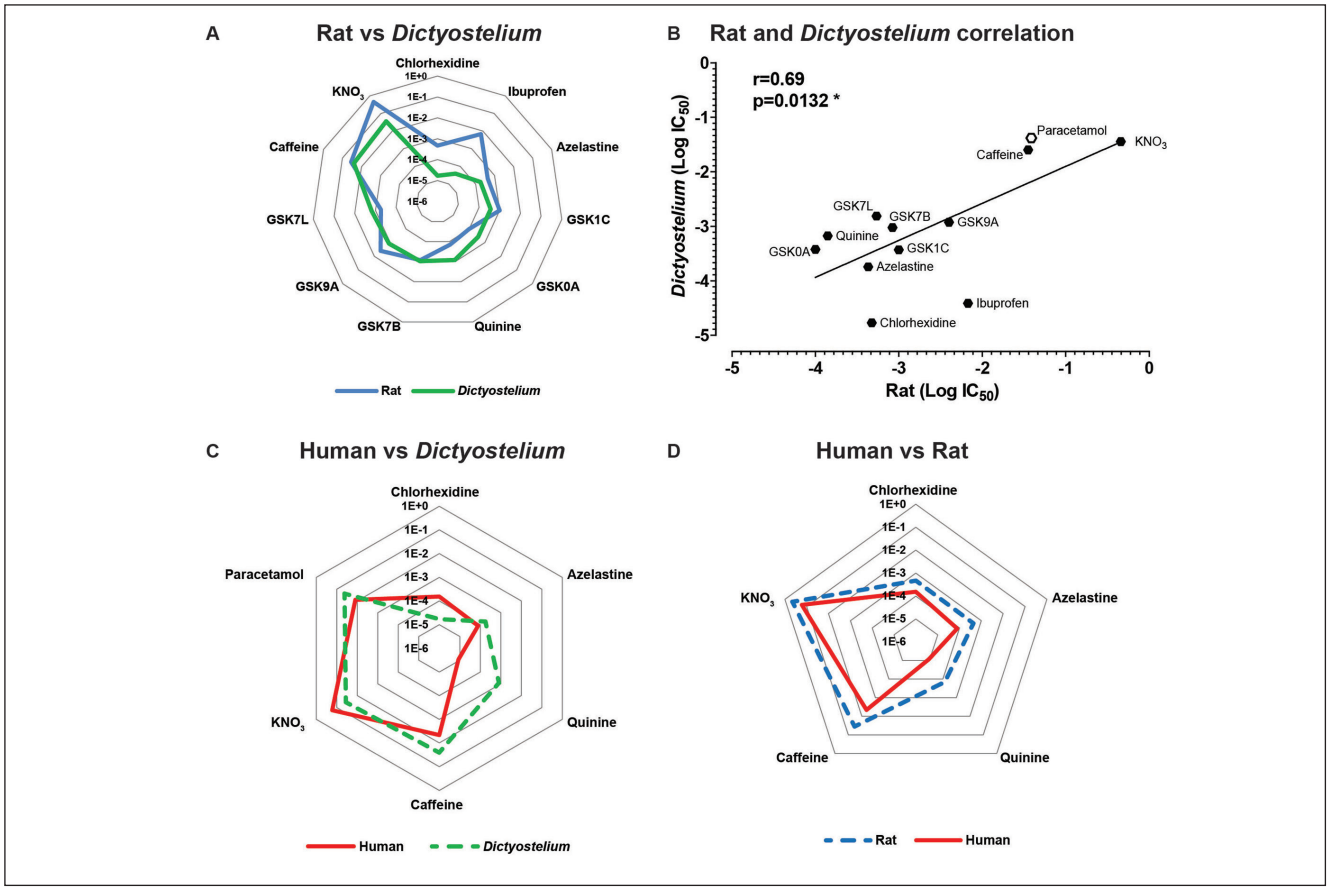

Fig. 5: *Dictyostelium* behavior model shows similarity to rat and human bitter taste models

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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Cocorocchio et al.:

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

Supplementary Data

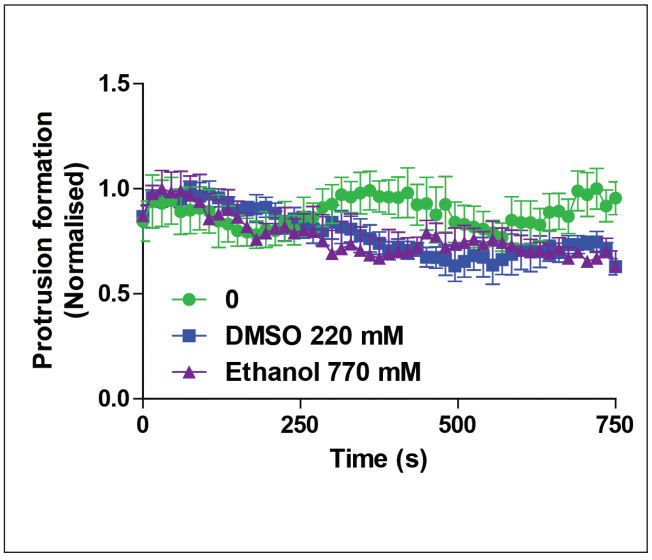


Fig. S1: Solvents do not affect *Dictyostelium* cell behavior
Addition of maximal concentrations of solvent: DMSO at 1.5% (220 mM) or ethanol at 4.5% (770 mM) did not significantly affect cell behavior. Data represent the number of protrusions formed (mean of at least three experimental repeats) after the compound addition (last 8 minutes). Analysis with one-way ANOVA showed no significant difference between control conditions and treatment with either solvent.

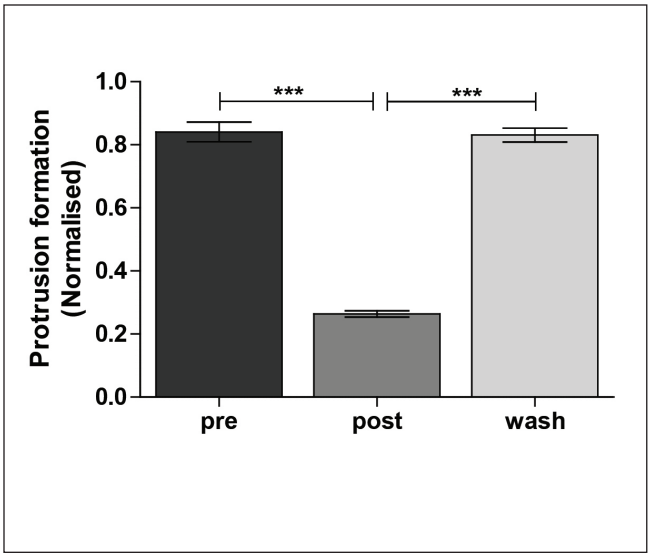


Fig. S2: Bitter tastant effects are reversible
After the addition of the bitter tastant (azelastine 0.5 mM), there is a strong inhibition of protrusion formation, with a full recovery of behavior following removal of the tastant (1 h post tastant addition, one-way ANOVA, $p < 0.001$ ***).

http://dx.doi.org/10.14573/altex.1509011s1

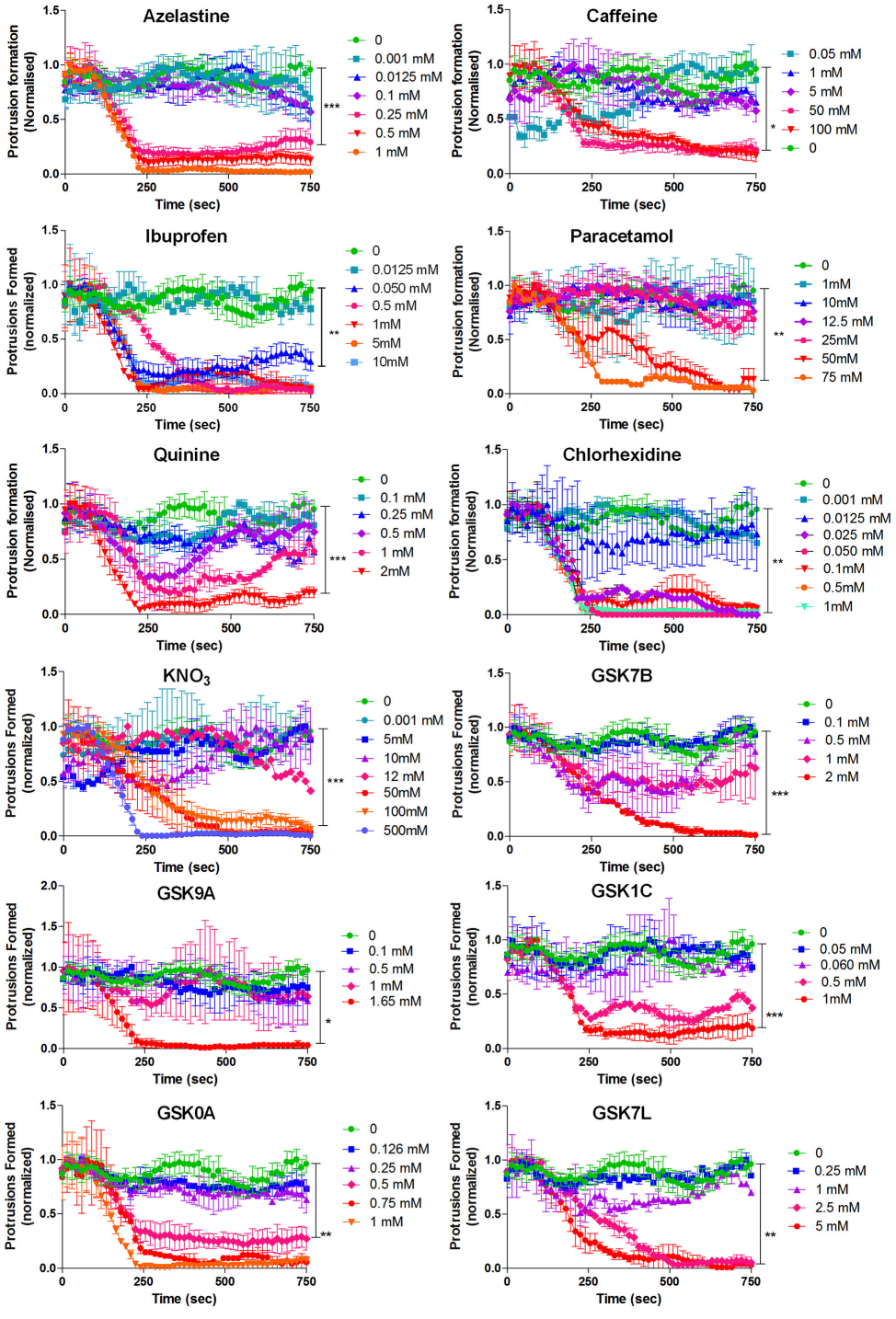


Fig. S3: Raw data of *Dictyostelium* response to standard bitter compounds

Time-dependent changes in *Dictyostelium* cell behavior (membrane protrusion) were recorded over a 15 minute period for triplicate experiments (± SEM) at increasing concentrations of seven different compounds to assess their ability to inhibit cell behavior. The addition of different concentrations of each compound at 210 seconds caused a reduction in protrusion formation. Data is presented as normalized to control conditions. Analysis with one-way ANOVA showed significant changes after the treatment with: azelastine 0.25 mM ($p < 0.001$ ***); caffeine 5 mM ($p < 0.05$ *); chlorhexidine 0.025 mM ($p < 0.01$ **); ibuprofen 0.050 mM ($p < 0.01$ **); paracetamol 50 mM ($p < 0.01$ **); potassium nitrate 50 mM ($p < 0.001$ ***); quinine 2 mM ($p < 0.001$ ***); GSK7B 2 mM ($p < 0.001$ ***); GSK9A 1.65 mM ($p < 0.05$ *); GSK1C 1 mM ($p < 0.001$ ***); GSK0A 0.5 mM ($p < 0.01$ **); GSK7L 2.5 mM ($p < 0.01$ **).

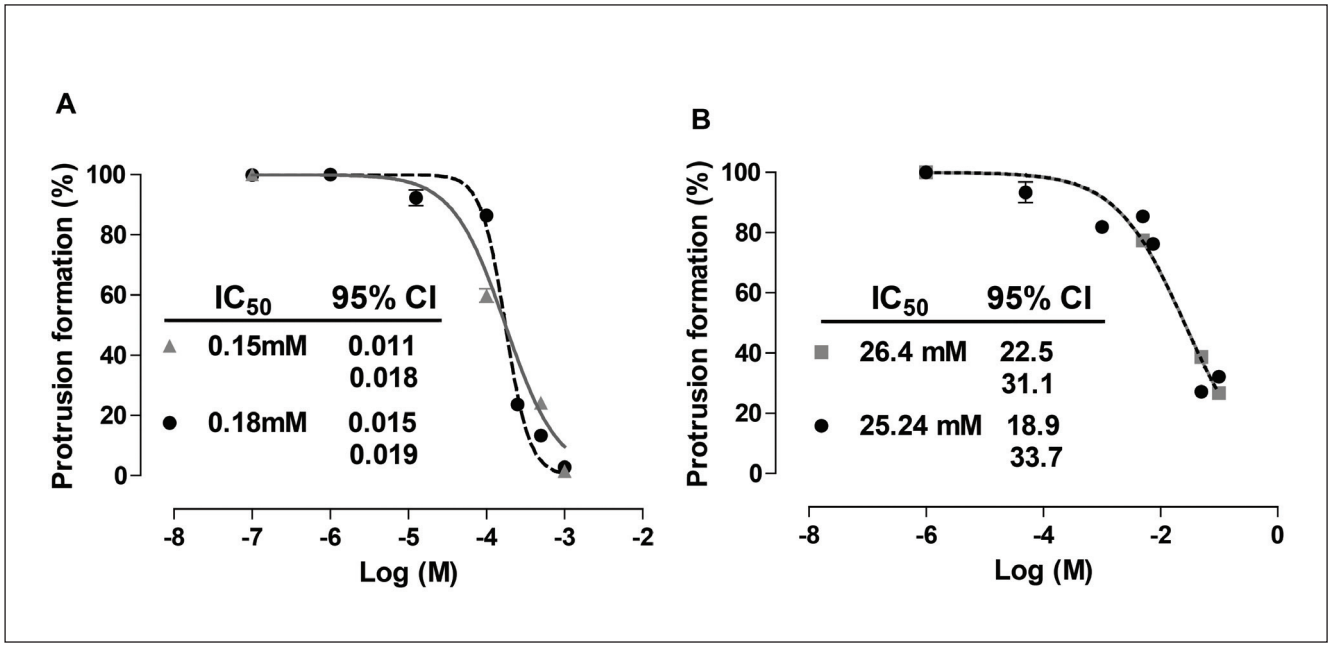


Fig. S4: Effects of bitter compounds are reproducible

To assess the reproducibility of the assay a set of repeat experiments were carried out one month after the initial experiments. (A) Comparison of data obtained from the azelastine shows that IC_{50} values are the same for both data sets. (B) The effects of caffeine show a similar response in both data sets.

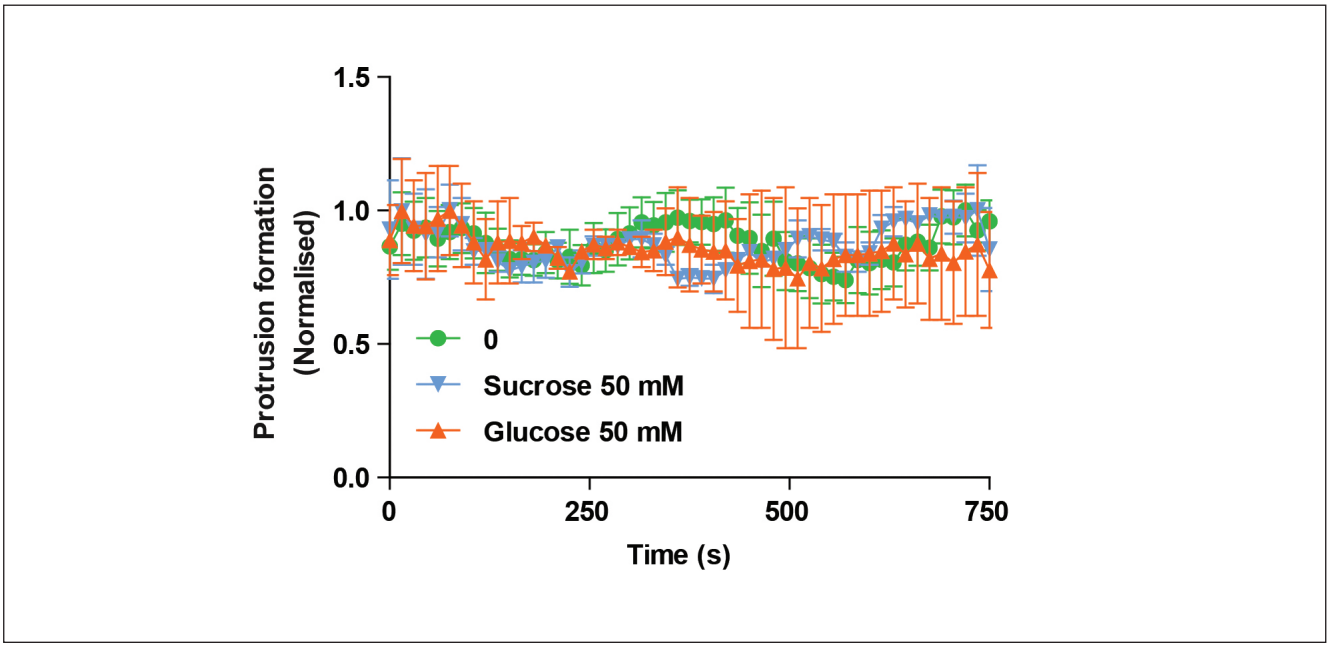
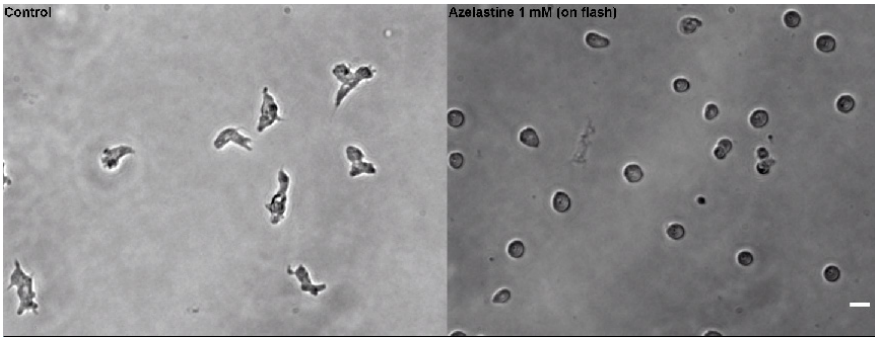


Fig. S5: Raw data of *Dictyostelium* response to non-bitter compounds, glucose and sucrose

Addition of glucose 50 mM and sucrose 50 mM did not affect cell behavior. Data represent the number of protrusions formed (mean of at least three experimental repeats) after the compound addition (last 8 min). Analysis with one-way ANOVA showed no significant difference between control conditions and treatment with either compound.



Supplementary Movie: Bitter tastants regulate *Dictyostelium* cell behavior
Wild type *Dictyostelium* cells were monitored by time-lapse photography to record cell behavior every fifteen seconds over a fifteen minute period. At 5 minutes (black frame) either solvent (Left) or bitter substance (Right) (azelastine, 1 mM) was added to cells and images were recorded for a further 10 minutes. Subsequent computer generated outlines enabled protrusion formation to be quantified. Bitter tastants gave rise to a loss in protrusion. View movie at http://dx.doi.org/10.14573/altex.1509011s2

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

Figure 2. (B) N≥30 cells analysed per condition from ≥3 independent technical repeats.

Figure 4. 3 independent technical repeats for each compound.

Figure S1. N=30 cells analysed per condition from 3 independent technical repeats.

Figure S2. N=30 cells analysed per condition from 3 independent technical repeats.

Figure S3. N≥30 cells analysed per condition from ≥3 independent technical repeats.

Figure S5. N=30 cells analysed per condition from 3 independent technical repeats.

3.3 Employing *D. discoideum* for the characterisation of a family of structurally related compounds

After the screening of a wide range of diverse bitter tastants, a more in-depth approach was employed for the investigation of the cellular effects and molecular targets of curcumin and a family of structurally related compounds (Figure 8). Curcumin (diferuloylmethane) is a natural hydrophobic phenol, the main component of the turmeric root, and has a bitter, astringent and pungent taste, with a distinctive yellow colour. The health benefits of curcumin have been proven by several clinical trials (Lopresti et al., 2014; Panahi et al., 2015; Panahi et al., 2017) and include anti-oxidant, anti-inflammatory, anti-viral and anti-bacterial properties as well as anti-cancer, neuroprotective and hypoglycaemic activities (Priyadarsini, 2014). In fact, curcumin is able to affect many different signalling pathways such as transcription factors, tumour suppressor genes, chemokines, cytokines, adhesion molecules, and microRNAs (Gupta et al., 2012; Prasad et al., 2014). In addition, natural phenols like curcumin have no severe side effects, are inexpensive and readily available (Goel et al., 2008). Therefore, curcumin might be an alternative to conventional drugs for the management of many diseases upon resolution of problems with solubility and bioavailability. For this reason, there has been an increasing interest in the design and testing of curcumin-structurally-related analogues of natural or synthetic origins. An example of this approach is represented by EF31 and UBS109 which in a recent study demonstrates an increased potency in inhibiting methylation of DNA methyltransferase 1 (DNMT-1), Nuclear Factor-kappa-B (NF-κB), and heat shock protein 90 (HSP90) (Nagaraju et al., 2013). In addition, these analogues have better solubility and bioavailability compared to curcumin, making them promising lead compounds for the treatment of cancer.

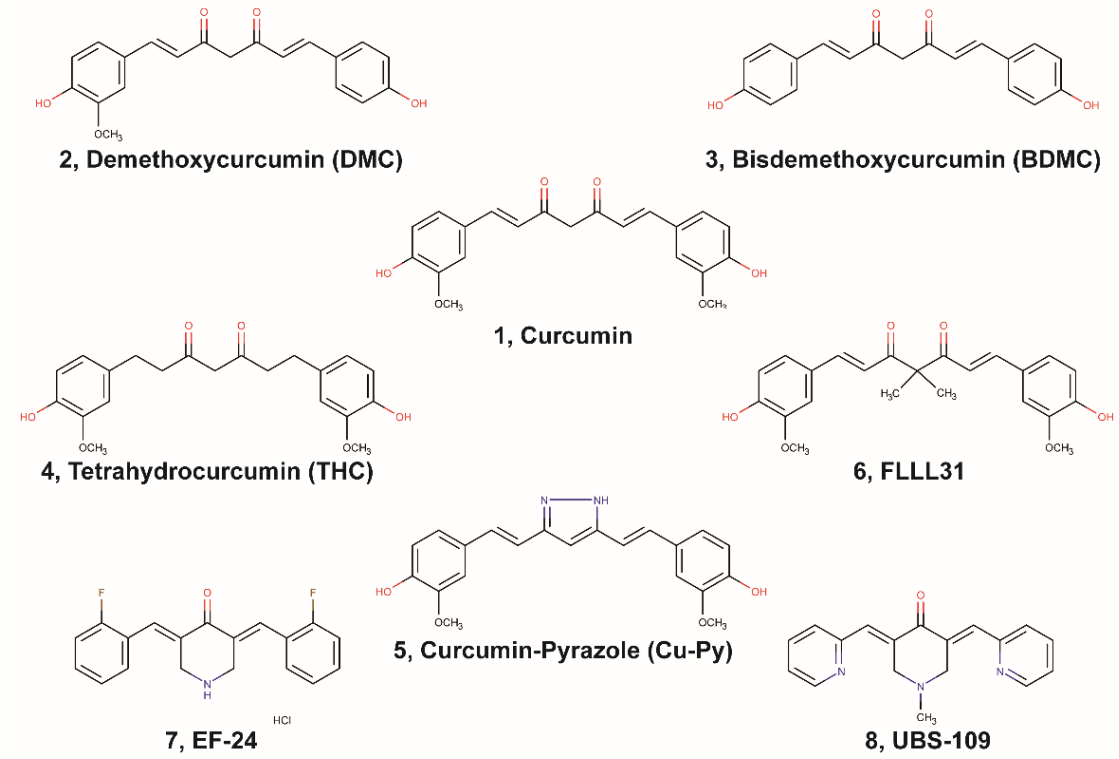


Figure 8. Structures of natural and artificial curcumin derivatives used in a quantitative structural analysis in *D. discoideum*.

The study presented in this section has shown that *D. discoideum* cellular functions like growth, development and cell behaviour are differentially modulated by structural modifications of the molecules. These results highlighted the importance of active functional groups and that the effects seen in *D. discoideum* are likely to be indipendent of the antioxidant activity of these compounds. Moreover, several mutants were identified through a genetic screen, in particular, one curcumin-resistant mutant lacking the protein phosphatase 2A regulatory subunit (PsrA) and an EF24-resistant mutant is lacking the Presenilin 1 orthologue (PsenB). These mutants were recapitulated to confirm their resistance to curcumin and its synthetic analogue EF24, respectively. In addition, using molecular docking analysis, this study has shown that curcumin may directly bind to a key regulatory region of PsrA to regulate its function. The identified molecular

targets have been associated with the pathogenesis of cancer and Alzheimer's disease (AD) in animal models and patients, making them clinically relevant. These results suggest that this model may be useful in the investigation of natural compounds with complex molecular structure and multiple cellular targets supporting the development of curcumin-based drugs for the treatment of a wide range of conditions. These findings reveal novel cellular and molecular mechanisms for the function of curcumin and related compounds.

3.3.1 Curcumin

Curcumin is a polyphenolic diketone and main curcuminoid of the turmeric root, in which are also present two naturally occurring curcumin analogues (curcuminoids), demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC). Ayurvedic medicine has been using curcumin for thousands of years, as a treatment for inflammatory diseases and wounds. Many studies reported that curcumin possesses pleiotropic activities, as it can modulate multiple signalling pathways including enzymes, receptors, growth factors, transcription factors, cell-cycle proteins, protein kinases, protein reductases, carrier proteins, cell survival proteins, drug resistance proteins, adhesion molecules, inflammatory molecules, chemokines, DNA, RNA, and metal ions (Aggarwal and Harikumar, 2009; Aggarwal and Sung, 2009; Ghosh et al., 2015; Zhou et al., 2011). It has been also shown that curcumin possesses antibacterial, anti-inflammatory, anti-oxidant, pro-apoptotic, anti-cancer, neuroprotective, wound healing, anti-nociceptive, anti-parasitic, and anti-malarial properties. (Goel et al., 2008; Gupta et al., 2012; Oliveira et al., 2015).

3.3.2 Demethoxycurcumin (DMC)

Demethoxycurcumin (DMC) is a β-diketone like curcumin in which one of the methoxy groups is replaced by a hydrogen and represents one of the major curcuminoids in turmeric, accounting for 15–20%. It has been recently shown that demethoxycurcumin is more potent than curcumin in inhibiting proliferation and inducing apoptosis in human colorectal carcinoma cells (HCT116) treated for 48 h. These effects were likely due to the lower rate of degradation of demethoxycurcumin (-70%) in comparison to curcumin (~100%) in 48 h, showing that the former compound is more stable *in vivo* (Tamvakopoulos et al., 2007).

3.3.3 Bisdemethoxycurcumin (BDMC)

Bisdemethoxycurcumin (BDMC) is also a natural demethoxy derivative of curcumin, accounting for 3-5% of the total curcuminoids. It has been reported to suppress the proliferation of several cancer cells and has a higher stability in physiological media as compared to curcumin (Luo et al., 2015). BDMC is also a potent activator of macrophage phagocytosis and, through the interaction with vitamin D3, stimulates amyloid-β clearance by macrophages (Cashman et al., 2008; Fiala et al., 2007; Masoumi et al., 2009). Thus, this compound may be employed in the immunoprophylaxis of AD.

3.3.4 Tetrahydrocurcumin (THC)

Tetrahydrocurcumin (THC) is a white curcumin metabolite which possesses pharmacological activities comparable to curcumin. THC is a product of bacterial or intestinal metabolism of curcumin (via the bacterial enzyme NADPH-dependent curcumin reductase). THC lacks α,β dienes and is therefore unable to form Michael adducts (nucleophilic addition of a carbanion or another nucleophile to a α,β-unsaturated carbonyl compound) with intracellular proteins,

but is more stable than curcumin in phosphate buffer solutions at various pH ranges (Aggarwal et al., 2014).

3.3.5 FLLL31

FLLL31 is a newly developed curcumin synthetic analogue which selectively binds to Janus kinase 2 (JAK2) and the STAT3 Src homology-2 (SH2) domain, suppressing the activation of STAT3 in both *in vitro* and *in vivo* studies. FLLL31 significantly decreases inflammatory cell recruitment and the onset of asthma *in vitro* and *in vivo*. Therefore it may be useful as an adjuvant therapy for asthma patients (Yuan et al., 2014).

3.3.6 Curcumin Pyrazole

Curcumin pyrazole is a curcumin derivative N-(3-Nitrophenylpyrazole), which shows great potency in arresting α-synuclein fibrillization and, disrupting preformed fibrils as well as preventing the formation of A11 conformation in the protein that imparts toxic effects in Parkinson's disease. Therefore, curcumin pyrazole may be a useful inhibitor for the treatment of α-synuclein amyloidosis and toxicity in Parkinson's disease and other synucleinopathies (Ahsan et al., 2015).

3.3.7 UBS109

UBS109 is a novel synthetic curcumin analogue which has demethylating properties and, due to the importance of methylation in pancreatic cancer, this compound can be used as a therapeutic agent. A recent study has shown that cancer cell lines MiaPaCa-2 and PANC-1 treated with UBS109 exhibited a more potent inhibition in cell proliferation and cytosine methylation compared to curcumin. Thus, UBS109 may be further developed for the treatment of

pancreatic cancer, alone or in combination with chemotherapeutic agents (Nagaraju et al., 2013).

3.3.8 EF24

EF24, 3,5-bis(2-fluorobenzylidine)-4-piperidone is a synthetic chalcone curcumin derivative, which participates in nucleophilic addition in the cellular environment due to the presence of α,β-unsaturated carbonyl functionality (Michael acceptor), able to undergo 1,4-additions with thiols. Many synthetic and naturally occurring Michael acceptors are known to exhibit anti-inflammatory activity via the inactivation of NF-κB by mediated by their interaction with cysteine residues in either IκB kinase (IKK) or the DNA binding domain of NF-κB. It has been shown that EF24 directly disrupt the catalytic activity of IkappaB kinase (IKK) which is essential for the phosphorylation of NF-kB inhibitory protein IkappaB. By inhibiting IKKβ, EF24 also reduces the phosphorylation-dependent proteasomal degradation of IkappaB which ultimately results in inhibition of the activation and nuclear translocation of NF-kB for its transcriptional activity (Vilekar et al., 2015).

3.3.9 Characterisation of the proteins regulated by curcuminoids

In this study, a Restriction Enzyme Mediated Insertional (REMI) mutagenesis screen was carried out to identify possible targets of curcumin and its derivatives in *D. discoideum*. These screens led to the identification of two gene products, psrA which is regualated by curcumin and Presenilin B targeted by the synthetic analogue EF24.

3.3.9.1 Protein Phosphatase 2A regulatory subunit

One of the main serine/threonine phosphatases present in cells is the protein phosphatase 2A (PP2A). This protein consists of a catalytic subunit (C), a structural subunit (A), and a regulatory/variable subunit (B). PP2A plays a pivotal

role in the regulation of cellular homeostasis and in particular the modulation of major cell signalling pathways through its phosphatase activity. Moreover, PP2A is involved in a wide range of cellular processes such as DNA replication, transcription, translation, cell cycle, development and programmed cell death (Magnusdottir et al., 2009; Seshacharyulu et al., 2013). PP2A is known to regulate the activity of more than 30 different kinases, including AKT, PKC, p70 S6 kinase, cAMP-dependent kinases, CAM-kinases and ERK/MAP kinase. It also modulates a range of proteins during cell cycle progression and apoptosis, which include Cdc25, Cdc6, Wee 1, DNA polymerase-primase, TAU and cyclin G2 (Seshacharyulu et al., 2013). PP2A represents 1% of total cellular proteins and together with PP1 accounts for >90% of all Ser/Thr phosphatase activity (Cho and Xu, 2007). In eukaryotes, the PP2A amino acid sequence is highly conserved (78-93%) amongst yeast, *Drosophila* and mammals (Seshacharyulu et al., 2013). Alterations in the PP2A holoenzyme assembly, activity and substrate specificity can lead to disease states and is responsible for the initiation and maintenance of cancer (Kiely and Kiely, 2015; Sangodkar et al., 2016).

The core dimer of the PP2A holoenzyme consists of a 65 kDa scaffolding subunit (A) and a 36 kDa catalytic subunit (C). However, the full activity of the protein, subcellular location and substrate target are conferred by the B regulatory subunit, which leads to the formation of a heterotrimeric holoenzyme (Cho and Xu, 2007; Sangodkar et al., 2016) (Figure 9). Studies have identified 26 different B subunit variants in humans, which represent alternative transcript and splice variants encoded by 15 different genes, that allow 96 possible combinations of the assembled holoenzyme. Both cell and tissue types determine the expression levels and the variant of these subunits.

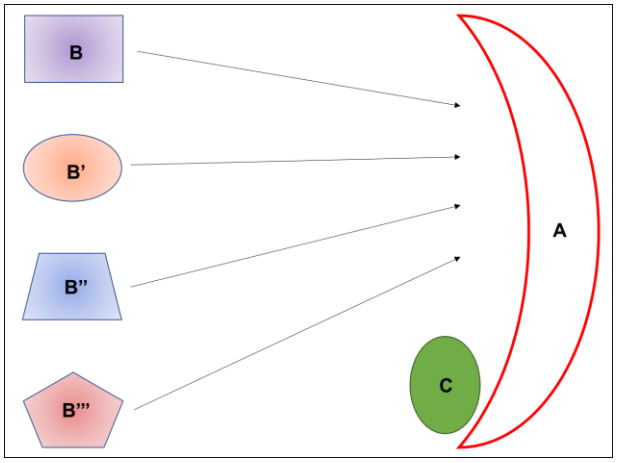


Figure 9. PP2A holoenzyme assembly. Core dimer of the PP2A enzime consists of a scaffolding subunit (A), a catalitic subunit (C) and number of different regulatory subunits (B) which determine tissue and cellular specificity.

In addition, the PP2A holoenzyme is dynamic as it has the ability to swap B subunits, allowing PP2A to react rapidly to specific environmental stimuli. The variable regulatory B subunits are divided into four subfamilies: B-B53-PR55, B'-B56-PR61, B''-B72-PR72 and B'''-PR93(SG2NA)-PR110. Amongst these subunits variants, the PP2A-B56 is the most studied regulatory subunit subfamily, with PP2A-B56α, β and ε expressed in the cytoplasm, γ1-3 isoforms in the nucleus and δ in both cellular compartments. PP2A also plays an essential role in the regulation of growth factor signalling. For instance, if cells are stimulated with epidermal growth factor (EGF), insulin or insulin growth factor 1 (IGF-1), this leads to the downregulation of the PP2A activity and upregulation of the MAP kinase pathway, which results in an increased cellular proliferation. The PP2A-B56 subunit has been implicated in the dephosphorylation of Akt (Thr308), which is responsible for the negative regulation of the insulin signalling pathway (Kiely and Kiely, 2015).

However, the biological function of PP2A seems to be controversial as it does not only behave as a tumour suppressor but has also anti-apoptotic abilities. Presumably, it plays its dual role by enabling both pro- and anti-apoptotic signalling pathways depending on the type of holoenzyme formation. An example is the interaction between Bcl-2 and PP2A, where PP2A can lead to apoptosis by dephosphorylating Bcl-2 on Ser70 in the mitochondria, and can inhibit programmed cell death by dephosphorylating Bcl-2 on Ser87 in cancer cells (not seen in healthy tissue) (Kiely and Kiely, 2015; Van Hoof and Goris, 2003). Furthermore, p53, one of the major and most studied tumour suppressors, is also regulated by PP2A. A recent study has shown that PP2A downregulates p53 by dephosphorylation on Ser15 and activates AKT-1 as well as Plk-1 in glioblastoma models. This, in turn, increases cell proliferation which can enhance the activity of chemotherapeutic drugs that work by damaging DNA, leading to accumulation of DNA mutations and therefore genomic instability resulting in cell death (Kiely and Kiely, 2015). On the other hand, the inhibition of PP2A in ovarian cancer cells stabilises and activates the overexpressed p53, leading to cell cycle arrest and programmed cell death, by increasing Bax and p21 expression (Kiely and Kiely, 2015). In addition, PP2A can modulate cancer signalling pathways such as PI3K/Akt, mammalian Target of Rapamycin (mTOR), Focal Adhesion Kinase (FAK) and Extracellular Signal-Regulated Kinase (ERK) to enable cancer growth. However, the exact mechanisms of action remain to be elucidated (Van Hoof and Goris, 2003).

PP2A is also involved in brain neurodegeneration, where its dysfunction can lead to an increase in TAU hyperphosphorylation, which plays a pivotal role in the aetiology of Alzheimer's disease (AD). Remarkably, PP2A mediates about 71% of total TAU phosphatase activity in the brain (Sontag and Sontag, 2014).

Moreover, studies have shown that reduction in PP2A mRNA, protein expression levels and phosphatase activity levels were detected in the brains of Alzheimer's patients (Sangodkar et al., 2016). It has been demonstrated that *in vivo* dysregulation of PP2A leads to deregulation of a range of Ser/Thr kinases involved in AD, including GSK3β, cdk5, ERK and JNK. It has also been linked to cytoskeleton abnormalities (microtubules and neurofilaments), alteration in the amyloid precursor protein (APP) phosphorylation and processing, as well as neurogenesis impairment, alteration in synaptic plasticity and induction of programmed cell death (Sontag and Sontag, 2014).

Interestingly, it has been shown that the curcumin-dependent activation of Mitogen-Activated Protein Kinases (MAPK) pathways is linked to the inhibition of the PP2A and PP5 proteins. Curcumin can induce apoptosis by activating the MAPKs as well as c-Jun N-terminal kinase (JNK), and ERK1/2 independently of p53. In fact, the inhibition of JNK (with SP600125) or ERK1/2 (with U0126), the downregulation of ERK1/2 or expression of dominant negative c-Jun partially inhibited the pro-apoptotic effects of curcumin. In addition, overexpression of PP2A or PP5 partially reduced the activation of JNK and Erk1/2 phosphorylation as well as cell death induced by curcumin (Han et al., 2012). Furthermore, a recent proteomics study has shown that curcumin is able to maintain PP2A subunit B expression levels in middle cerebral artery occlusion (MCAO) animal models. In particular, curcumin treatment prevented injury-induced reductions in PP2A subunit B levels, suggesting that curcumin mantains the levels of PP2A subunit B in response to cerebral ischemia, which exerts the neuroprotective function of curcumin in cerebral ischemic injury (Shah et al., 2015).

Finally, *D. discoideum* encodes for psrA, the protein phosphatase 2A regulatory subunit B56 orthologue (Murphy et al., 1999). PsrA is able to regulate cell

differentiation by downregulating glycogen synthase kinase 3 (GSK3) function (Lee et al., 2008). Ablation of psrA showed an inefficient activation of multiple Ras species, a reduction in random cell movement and impaired chemotaxis toward cAMP and folic acid (Rodriguez Pino et al., 2015). In addition, psrA$^-$ cells exhibited elevated basal and poststimulus phosphorylation levels of protein kinase B (PKB) family member PKBR1 and PKB substrates. Mutants in which Ras is constitutively expressed or GSK3 inhibition in psrA$^-$ cells leads to increased activity of both PKBR1 and PKBA (Rodriguez Pino et al., 2015). However, under identical conditions only the PKBR1 activity was intensified in wild-type cells, demonstrating that B56- or GSK3-mediated suppressive mechanism is insufficient in preserving low PKBA activity, but both mechanisms are essential for the inhibition of PKBR1. Furthermore, RasD or RasC ablation and GSK3 inhibition results in a normal PKBR1 modulation, indicating that RasC or RasD are critical factors for GSK3-mediated PKBR1 inhibition (Rodriguez Pino et al., 2015).

3.3.9.2 Presenilin protein

Alzheimer's disease (AD) is a neurodegenerative condition, affecting around 30 million people worldwide and accounts for 50%-75% of all cases of dementia (Alzheimer's Association, 2017). The disease etiopathology is characterised by intraneuronal neurofibrillary tangles composed of hyperphosphorylated TAU, and extracellular senile plaques induced by amyloid aggregation in the brain (Lakey-Beitia et al., 2017b). These processes can lead to chronic brain inflammation of the microglia, astrocytes, and neurons, which lead to overexpression of pro-inflammatory mediators, such as tumour necrosis factor- (TNF) and interleukin (IL)-6. Furthermore, Presenilin 1 (PS-1), a protein encoded by the PSEN1 gene and a component of the four core proteins in the γ-secretase complex, plays a

key role in generation of amyloid beta (Aβ) from Amyloid Precursor Protein (APP) (Giri et al., 2017; Schellenberg et al., 1992) (Figure 10). Presenilin is a protein with 9 transmembrane domains, possessing an extracellular C-terminus and a cytosolic N-terminus (Laudon et al., 2005; Spasic and Annaert, 2008). Presenilin is cleaved in ~27-28 kDa N-terminal and ~16-17 kDa C-terminal fragments in humans and is present in the cell primarily as a heterodimer of the C-terminal and N-terminus fragments (Thinakaran et al., 1996).

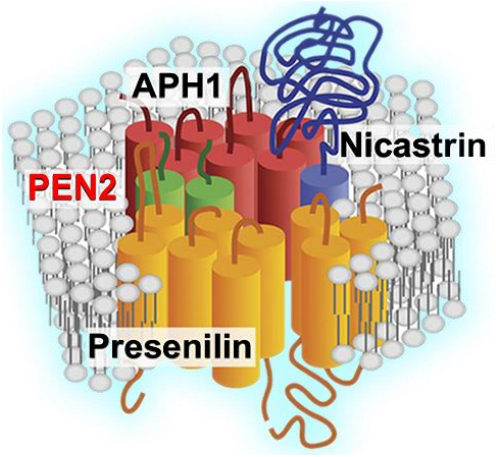


Figure 10. Gamma secretase complex. The γ-secretase complex is composed of four components: presenilins (PS), nicastrin, anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (PEN-2) (Parks and Curtis).

Studies have shown that the accumulation of amyloid beta is associated with the onset of Alzheimer's disease (Selkoe, 1994). Indeed, mutations in the PSEN1 gene are the most common cause of familial Alzheimer's disease (FAD) (Giri et al., 2017). The Presenilin 1 protein represents the catalytic subunit of the γ-secretase complex, which is a protease that cleaves a range of type 1 transmembrane proteins, including the APP and Notch (De Strooper et al., 2012). The cleavage of APP induced by γ-secretase generates β-amyloid (Aβ) peptides of varying lengths. The non-pathogenic form of Aβ is represented by Aβ40

peptides which account for ~90% of all Aβ production. However, when there is an aberrant Presenilin 1 function, this leads to the formation of the Aβ42 peptide, which is more hydrophobic and is thought to cause Aβ aggregation and plaque deposition in the brain of AD patients (Kelleher and Shen, 2017). It has been recently demonstrated that Presenilin 1 phosphorylation induces the pathogenic conformational shift in the living mouse brain, making these phosphorylation sites potential new targets for AD treatment (Maesako et al., 2017).

Recent *in vitro* studies have demonstrated that curcumin inhibits amyloid-aggregation, the activity of the enzymes β-secretase and acetylcholinesterase, and β-amyloid-induced inflammation (Lakey-Beitia et al., 2017b). Moreover, curcumin is also able to block the production of pro-inflammatory cytokines and enzymes, like COX-2 and the inducible nitric oxide synthase (NOS), and downregulates the expression of chemokines such as the Monocyte Chemoattractant Protein-1 (MCP-1) and interferon-inducible protein (Lakey-Beitia et al., 2017b). In addition, curcumin is able to inhibit amyloid oligomerisation, deposition, and TAU phosphorylation in Alzheimer's disease in *in vivo* animal models. In addition, *in vivo* studies have shown that dietary curcumin is able to cross the blood-brain barrier and significantly decrease amyloid deposition and plaque burden in AD transgenic mice, as well as preventing TAU phosphorylation. It is also capable of reducing inflammation and oxidative damage as well as decreasing genomic instability events (Zhang et al., 2010; Zheng et al., 2017). Curcumin also inhibits amyloid formation by blocking β-secretase (BACE-1) and glycogen synthase kinase-3β-(GSK-3β)-mediated phosphorylation of Presenilin 1 and γ-secretase proteolytic activity (Di Martino et al., 2016; Xiong et al., 2011).

Presenilin and other γ-secretase components are evolutionary conserved. Interestingly, highly diverged orthologs for each γ-secretase component have been identified in *D. discoideum*, but the amoeba does not possess APP, Notch and other characterised PS/γ-secretase substrates (McMains et al., 2010). It has also been shown that *D. discoideum* can process human APP to generate amyloid-β peptides Aβ40 and Aβ42, and the ablation of γ-secretase inhibits the production of Aβ peptides, leading to the accumulation of APP intermediates (McMains et al., 2010). Furthermore, PS is essential for *D. discoideum* phagocytosis and cell-fate. The ablation of presenilins leads to the inhibition of *D. discoideum* development, and this phenotype is rescued by the expression of human Presenilin 1. This role is not linked to its proteolytic activity because the mutation of both catalytic aspartates does not influence presenilin ability to rescue development, as well the ablation of nicastrin, crucial for proteolytic activity, does not inhibit cell development (Ludtmann et al., 2014; McMains et al., 2010; Smolarkiewicz et al., 2013). These results suggest that presenilin proteins and the γ-secretase complex have roles independent to Notch and form a highly conserved process that emerged prior to metazoan radiation, making *D. discoideum* a potential model system for the investigation of specific pathways involved in AD (Ludtmann et al., 2014; McMains et al., 2010).

3.3.10 Curcumin and derivatives function through protein phosphatase 2A and presenilin orthologues in *Dictyostelium discoideum*

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RESEARCH ARTICLE

Curcumin and derivatives function through protein phosphatase 2A and presenilin orthologues in *Dictyostelium discoideum*

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ABSTRACT

Natural compounds often have complex molecular structures and unknown molecular targets. These characteristics make them difficult to analyse using a classical pharmacological approach. Curcumin, the main curcuminoid of turmeric, is a complex molecule possessing wide-ranging biological activities, cellular mechanisms and roles in potential therapeutic treatment, including Alzheimer's disease and cancer. Here, we investigate the physiological effects and molecular targets of curcumin in *Dictyostelium discoideum*. We show that curcumin exerts acute effects on cell behaviour, reduces cell growth and slows multicellular development. We employed a range of structurally related compounds to show the distinct role of different structural groups in curcumin's effects on cell behaviour, growth and development, highlighting active moieties in cell function, and showing that these cellular effects are unrelated to the well-known antioxidant activity of curcumin. Molecular mechanisms underlying the effect of curcumin and one synthetic analogue (EF24) were then investigated to identify a curcumin-resistant mutant lacking the protein phosphatase 2A regulatory subunit (PsrA) and an EF24-resistant mutant lacking the presenilin 1 orthologue (PsenB). Using *in silico* docking analysis, we then showed that curcumin might function through direct binding to a key regulatory region of PsrA. These findings reveal novel cellular and molecular mechanisms for the function of curcumin and related compounds.

KEY WORDS: *Dictyostelium discoideum*, Curcumin, Presenilin, PP2A, Cancer, Alzheimer's disease

INTRODUCTION

Natural products obtained from plants have been used for thousands of years as medicines (Butler, 2004; Newman and Cragg, 2007; Gurib-Fakim, 2006). However, the active compound(s) often have complicated pharmacology, with multiple cellular targets and effects, making traditional pharmacological approaches insufficient to understand their biological activity. These factors preclude standard approaches to investigate mechanism(s) of action.

Curcumin (diferuloylmethane) is a flavonoid derived from turmeric, and provides a good example of a natural product with potential therapeutic activity (Aggarwal and Harikumar, 2009; Ghosh et al., 2015). Currently, ∼120 clinical trials have sought to demonstrate its efficacy in the treatment of various diseases (Gupta et al., 2013), yet an analysis published in 2017 reported that only 17 trials have shown positive outcomes (Heger, 2017). To improve our understanding of this compound, it is crucial to identify potential therapeutic targets, and to test related compounds that have improved chemical characteristics (e.g. solubility) (Oliveira et al., 2015) that can focus research on relevant therapeutic outcomes. Curcumin has diverse cellular effects, including the modulation of transcription and growth factors regulating cell growth and cell death, and as an anti- or pro-oxidant (Goel et al., 2008; Prasad et al., 2014; Priyadarsini, 2014; Gupta et al., 2012, 2013; Zhou et al., 2011). Curcumin has also been extensively investigated for the treatment of Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), cardiovascular diseases, cancer, allergy, asthma, rheumatoid arthritis, diabetes and inflammation (Yang et al., 2017; Lakey-Beitia et al., 2017; Jurenka, 2009; Srinivasan, 1972; Chougala et al., 2012; Zhang et al., 2013; Tang and Taghibiglou, 2017; McClure et al., 2017).

The main limitations of the therapeutic use of curcumin are its poor bioavailability and limited understanding of the cellular effects in relation to its molecular structure (Gupta et al., 2013). Its structure consists of two aromatic rings containing o-methoxy phenolic groups, with a seven-carbon linker consisting of an α,β-unsaturated β-diketone (Priyadarsini, 2014; Ruby et al., 1995; Selvam et al., 1995). Thus, modification of these groups, and analysis of distinct cellular effects and targets, could help with understanding the potential use of curcumin and its derivatives in medicinal roles.

Dictyostelium discoideum has been used as a tractable model system for the analysis of compounds with potential therapeutic function. It is a eukaryote, with a unique lifecycle including single-celled and multicellular stages, and contains a range of orthologues to disease-linked proteins (Müller-Taubenberger et al., 2013). It has also been used to investigate the molecular actions of structurally and pharmacologically diverse compounds from bitter tastants (Cocorocchio et al., 2015; Robery et al., 2011, 2013), to flavonoids (Waheed et al., 2014), to drugs used in the treatment of bipolar disorder (Williams et al., 1999, 2002) and epilepsy (Chang et al., 2012; Xu et al., 2007; Elphick et al., 2012; Boeckeler et al., 2006). Several of these studies have been successfully translated to *in vitro* and *in vivo* animal models (Chang et al., 2015, 2016; Chang et al., 2013, 2014). In *D. discoideum*, distinct cellular processes, including growth, acute cell behaviour and development provide valuable tools for the analysis of compound function. Numerous studies using *D. discoideum* have employed chemical genetic approaches to

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identify genes controlling the cellular effects of compounds through screening mutant libraries to identify potential molecular mechanisms of compounds (Williams et al., 1999, 2002; Waheed et al., 2014; Robery et al., 2013). Several recent papers have also developed an approach to monitor the acute effects of compounds by measuring changes in cell behaviour (Cocorocchio et al., 2015; Robery et al., 2011). *D. discoideum* is also widely used as a model to investigate development, where cells during starvation aggregate and differentiate to form multicellular fruiting bodies (Marée and Hogeweg, 2001). Thus, using *D. discoideum* provides an advantageous system to analyse the cellular and molecular effects of complex natural products.

In this study, we employ *D. discoideum* to investigate the cellular and molecular targets of curcumin, for which previous studies have demonstrated sensitivity (Garige and Walters, 2015; Swatson et al., 2017). We initially corroborated the effects of curcumin on cell growth and development and further showed an effect on acute cell behaviour (Garige and Walters, 2015; Swatson et al., 2017). To differentiate these cellular effects and mechanisms, we then employed a range of complex natural and synthetic curcumin derivatives to highlight key functional groups of curcumin and differentiate these effects from antioxidant activity. A chemical genetic approach was then used to investigate the molecular targets of curcumin and a synthetic derivative. From this approach, two proteins were identified – the protein phosphatase 2A regulatory subunit PsrA (Lee et al., 2008), associated with cancer onset (Kiely and Kiely, 2015), and the presenilin 1 orthologue PsenB (Ludtmann et al., 2014), implicated in AD (De Strooper and Annaert, 2010) – which partially control the effects of these compounds. *In silico* modelling of curcumin binding sites was then used to predict a binding site on PsrA.

RESULTS

Curcumin shows distinct effects on acute cell behaviour, growth and development in *D. discoideum*

To investigate the cellular effects of curcumin on *D. discoideum*, we initially assessed acute cell behaviour changes following compound exposure (Fig. 1). In these experiments, rapid cell movement was induced through starvation in the presence of pulsatile cyclic AMP (cAMP), leading to the expression of a discrete set of genes (Santhanam et al., 2015). Cell behaviour was recorded using time-lapse microscopy for a period of 15 min including pre- (5 min) and post- (10 min) curcumin addition and computer-aided cell tracking was used to analyse changes in membrane protrusions, normalised to average protrusions pretreatment (Fig. 1B). From this analysis, acute cell behaviour was unaffected at concentrations ≤2 µM but showed a concentration-dependent reduction at increasing concentrations, with a complete block at 3 µM (****P<0.0001; Fig. 1B). By plotting average cell behaviour following treatment against curcumin concentration, a nonlinear regression analysis was used to calculate an IC_{50} for the effect of curcumin on acute cell behaviour as 2.3 µM [95% confidence interval (CI) 2.0-2.6 µM] (Fig. 1C). These data show an acute effect of curcumin on cell behaviour in *D. discoideum*, suggesting the presence of rapidly modified target(s) involved in cellular behaviour.

We then examined the effect of curcumin treatment on *D. discoideum* cell growth. In these assays, growth was assessed in the presence of increasing concentrations of curcumin over 7 days at concentrations ranging from 0 to 100 µM (Fig. 1D). By plotting the rate of exponential growth at each concentration, an IC_{50} value was calculated by nonlinear regression curve fitting. Curcumin completely blocked cell growth at 100 µM with an IC_{50} of 45.7 µM (95% CI 41.7-50 µM) (Fig. 1E). These data indicate an effect of curcumin on *D. discoideum* cell growth, suggesting cellular target(s) for the flavonoid involved in this process.

Furthermore, the role of curcumin in regulating multicellular development was also assessed. When *D. discoideum* are starved, this causes cells to aggregate and differentiate to ultimately form multicellular fruiting bodies over 24 h. The resulting fruiting body consists of a spore head, containing dormant spores held above the substratum by dead, vacuolated stalk cells (Williams et al., 2006). In this process, a further subset of proteins, partially distinct from those of growth and early development, are employed to enable development. In these experiments, cells were plated onto nutrient-deficient solid media, in the absence or presence of curcumin, at a concentration shown above to block growth, and

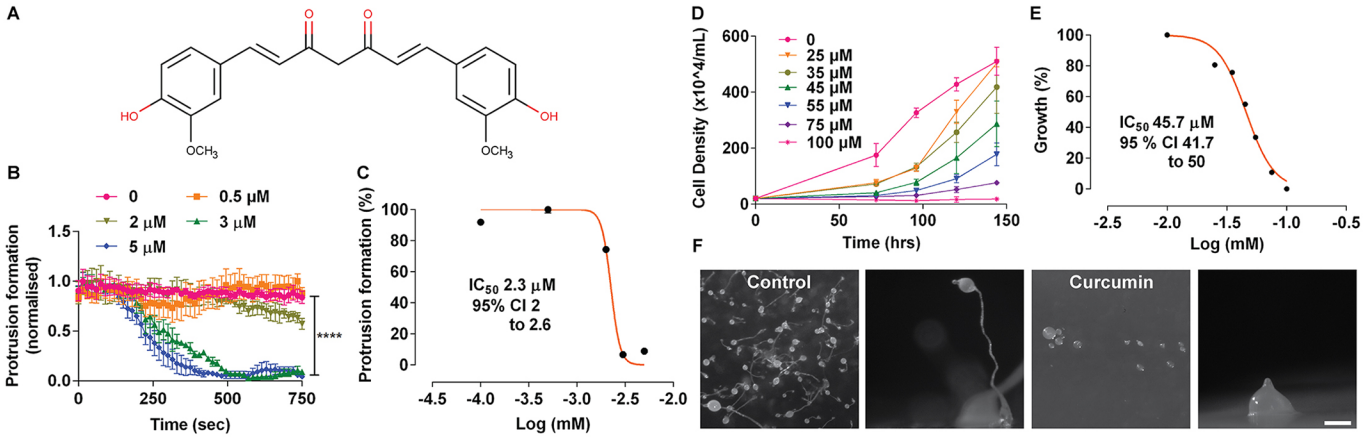

Fig. 1. Acute cell behaviour, growth and developmental effects of curcumin on *D. discoideum*. (A) Curcumin, a diferuloylmethane, was used to assess multiple roles using *D. discoideum* as a model. (B) Time-dependent changes in *D. discoideum* cell behaviour (membrane protrusion) were recorded over a 15-min period (±s.e.m.) at increasing concentrations of curcumin. Data are presented normalised to control conditions, showing a significant difference between control condition (vehicle) and 3 µM (****P<0.0001) using one-way ANOVA. (C) The concentration-dependent response is illustrated as the normalised reduction of cell behaviour (protrusion formation) against the Log (concentration) of curcumin, enabling calculation of an IC_{50} with a 95% CI. (D) *D. discoideum* cells were grown with increasing concentration of curcumin, causing a complete block at 100 µM, with (E) normalised concentration-dependent response shown plotted against Log curcumin (mM) concentration, providing an IC_{50} with 95% CI. (F) Cells were developed on agar over 22 h in control conditions (vehicle) and in the presence of 100 µM curcumin. Scale bar: 0.25 mm for both side view images. All experiments were carried out in triplicate.

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fruiting body structure was recorded after 24 h. In the absence of curcumin, a field of fruiting bodies was formed, with individual fruiting bodies consisting of spore heads elevated by stalks (Fig. 1F). In the presence of curcumin (100 µM), cells were able to form a reduced number of aggregates and were unable to develop into mature fruiting bodies (Fig. 1F). This suggests an effect of curcumin on *D. discoideum* late development, regulating cellular target(s) involved in differentiation.

Identifying active moieties in curcumin responsible for distinct effects on acute cell behaviour, growth and development

To improve our understanding of the distinct effects of curcumin in *D. discoideum*, we employed a range of compounds structurally related to curcumin (both natural and synthetic) to identify structural components of the curcumin molecule that are necessary for distinct effects (Fig. 2A). Analysis of the curcumin-related compounds on acute cell behaviour was carried out as described earlier, with cell behaviour recorded prior to and following the addition of each compound, with data describing a loss of membrane protrusions postaddition (Fig. S1). Secondary plots illustrated dose-dependent effects and provided an IC_{50} value specific to each compound (Fig. S2). From this approach (Fig. 2B), modulation of the phenolic groups through loss of one (demethoxycurcumin; DMC), or both (bisdemethoxycurcumin; BDMC), methoxy groups caused a step-wise reduction in potency in controlling acute cell behaviour (3.5- and 14-fold change, respectively). Similarly, a major metabolite of curcumin, tetrahydrocucrumin (THC), lacking the α,β-unsaturated carbonyl moiety on the seven-carbon linker, leading to loss of the planar structure of the compound, also showed a reduced potency (5-fold change). In addition, loss of the diketone group through formation of the pyrazolic ring (Jadhav et al., 2015) eliminated the effect on acute cell behaviour. Furthermore, FLLL31 (Yuan et al., 2014), which has two hydrogens on the central carbon replaced by methyl groups, and two extra methoxy groups, also showed a reduction in potency (5-fold). Lastly, two structurally distinct compounds, EF24 and UBS109, were investigated, with both compounds considered to be curcumin derivatives (Vilekar et al., 2015; Yamaguchi et al., 2014), where EF24 showed enhanced activity (1.8-fold), and UBS109 showed reduced activity (11-fold), in this model. These data suggest that the diketone moiety is essential for triggering curcumin-dependent inhibition of cell behaviour, and that the presence of the methoxy groups and planar nature of the molecule (lost in THC), and numerous changes to the basic flavonoid chemical composition in the structurally distinct compounds, provided opposite cellular effects on acute cell behaviour.

The assessment of curcumin derivatives on *D. discoideum* cell growth provided insight into chemical moieties of curcumin necessary for this effect. Analysis was carried out as described earlier, with cell growth recorded over 144 h (Fig. S3), and secondary plots illustrating dose-dependent effects and providing IC_{50} values specific to each compound (Fig. S4). For this cellular effect (Fig. 2C), modulation of the phenolic groups through loss of one or both methoxy groups (DMC and BDMC), caused a step-wise increase in potency in controlling growth (2.5- and 14-fold change, respectively). Loss of the planar nature of the compound (THC) also increased potency (1.8-fold change), and the addition of two methyl and methoxy groups in the synthetic analogue FLLL31 increased potency (1.5-fold). Loss of the diketone group through the formation of the pyrazole ring (CuPy) eliminated the effect on acute cell behaviour (and growth). Both structurally distinct compounds, EF24 and UBS109, showed a significant increase (14- and 26-fold change, respectively) in potency in this model. These data suggest that the diketone moiety is essential, and that the phenolic groups play key roles in curcumin activity in regulating cell growth.

Development assays were also carried out to determine which structural components of curcumin control potency in delaying multicellular development in *D. discoideum*. Here, cells were again plated on non-nutrient agar containing curcumin derivatives at concentrations that block growth, and allowed to develop over 24 h, after which fruiting body morphology was recorded (Fig. 2D). In these studies, loss of one or both methoxy groups (DMC and BDMC), loss of the planar structure of the compound (THC), or substitution of the central hydrogens caused delayed and aberrant fruiting body morphology (FLLL31). By contrast, loss of the diketone group through the formation of the pyrazole ring (CuPy) eliminated the developmental effect, and no effect was seen for both divergent structures (UBS109 and EF24). These data suggest that the key moiety of curcumin involved in developmental regulation is the central diketone group.

Analysis of antioxidant activity of curcumin-related compounds

Because curcumin has been widely proposed to function as an antioxidant (Sandur et al., 2007; Gordon et al., 2015), reducing free radicals, we then assessed this activity for curcumin and related compounds. Here, we employed the ferric-reducing ability of plasma (FRAP) assay, monitoring rapid (0 min) and extended (60 min) antioxidant function (Fig. 3). The strong antioxidant, ascorbic acid was used as a control. Of the curcumin-related compounds, THC provided the strongest rapid antioxidant activity, with CuPy also providing an immediate strong effect. Curcumin demonstrated time-dependent antioxidant activity, increasing over the experimental period. Loss of one or two methoxy groups reduced this effect (DMC and BDMC), and the addition of two methoxy groups plus two methyl groups (FLLL31), and modification of the β-diketone moiety or major structural change (EF24 and UBS109), eliminated antioxidant activity (Fig. 3).

Identification of the molecular targets of curcumin and analogues using a chemical genetic approach

To investigate distinct molecular targets and mechanisms for these compounds, we employed a mutant screen using curcumin and related compounds (Table 1). In these experiments, a library of *D. discoideum* insertional mutants was grown at a concentration of each compound giving an 80-90% reduction in growth over 21 days. Using this approach, a mutant was isolated, showing resistance to curcumin, with the mutagenic cassette inserted into the open reading frame of the protein phosphatase 2A regulatory subunit gene (*psrA*; DDB_G0280469) (Rodriguez Pino et al., 2015) (Fig. 4A). In addition, a mutant was isolated showing resistance to EF24, with the mutagenic cassette inserted immediately downstream of the start codon of presenilin B (DDB_G0292310) (Ludtmann et al., 2014) (Fig. 4A). To confirm that the encoded proteins regulate sensitivity to the compounds, recapitulated mutants were used to assess the rate of exponential growth for each mutant in the presence of the screening compound and a range of related structures over 24 h (Fig. 4B,C; Figs S5 and S6). In the presence of curcumin, psrA$^-$ showed significant resistance compared to wild-type cells (P<0.001), in addition to resistance to EF24 and DMC (P<0.05) (Fig. 4B). Similarly, in the presence of EF24, psenB$^-$ showed significant resistance compared to wild-type cells (P<0.01), in addition to resistance to UBS109 (P<0.01), but not curcumin or BDMC (Fig. 4C).

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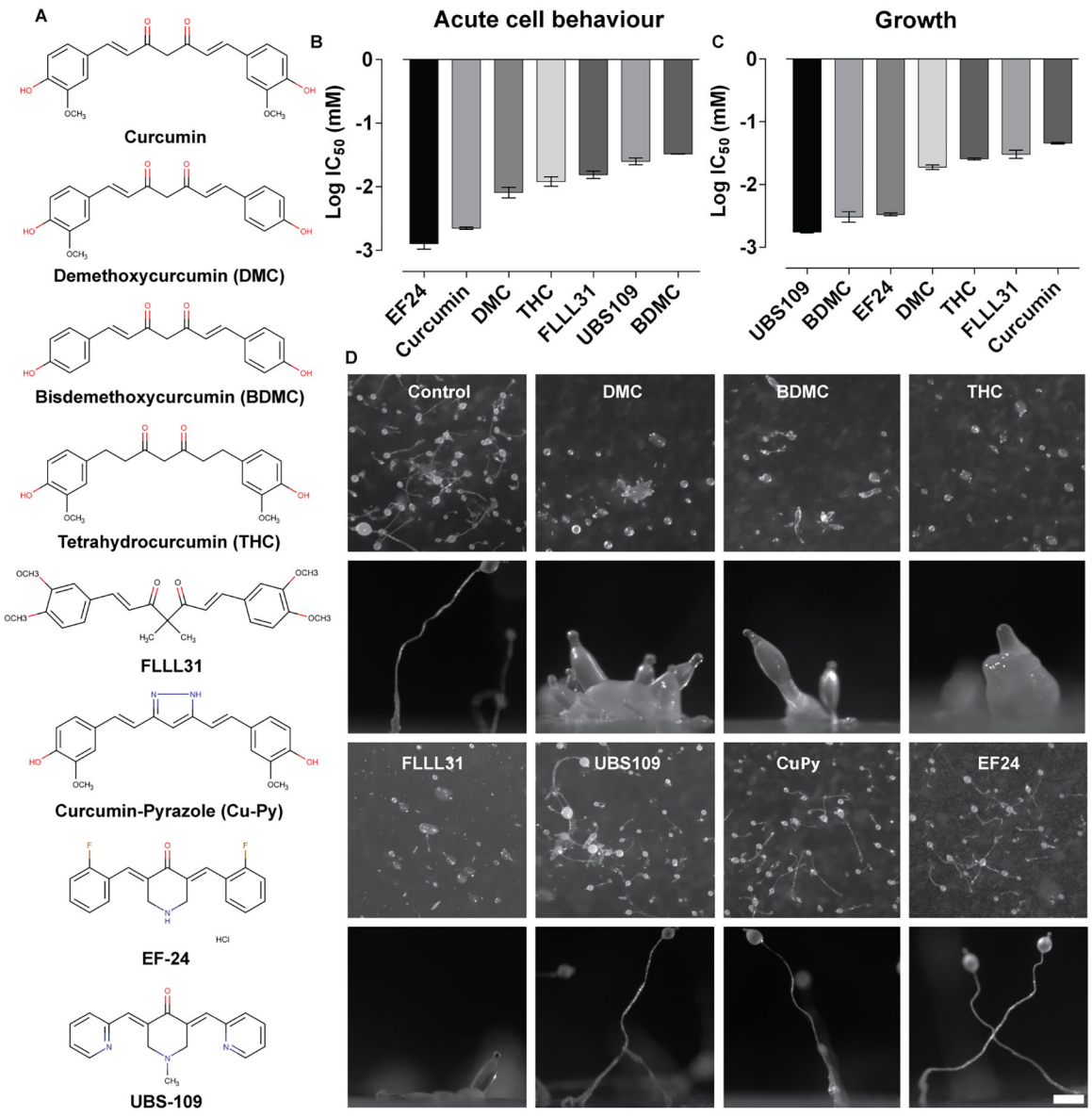

Fig. 2. Quantification of the acute cell behaviour, growth and developmental effects of curcumin derivatives on *D. discoideum*. (A) Structure of natural and artificial derivatives used in a quantitative structural analysis of curcumin effects in *D. discoideum*. (B) Concentration-dependent responses were determined for cell behaviour (protrusion formation) and illustrated as the IC_{50} for each compound with errors shown as 95% CIs (Figs. S1 and S2). (C) Concentration-dependent responses were determined for cell growth and illustrated as the IC_{50} for each compound with errors shown as 95% CIs (Figs. 3 and 4). Data from B and C are presented as mean±s.e.m. of triplicate experiments. (D) Cells were developed on agar over 22 h in the absence of compounds (vehicle only), or the presence of curcumin derivatives at concentrations shown to block growth (100 µM FLLL31, 25 µM DMC, 20 µM BDMC, 100 µM THC, 6 µM EF24, 5 µM UBS109 and 100 µM CuPy). All images are representative of triplicate experiments. Scale bar: 0.25 mm for all side view images.

Because curcumin and EF24 showed effects on both growth and cell behaviour, we also assessed the resistance of psrA$^-$ and psenB$^-$ to the cell behaviour effects of compounds. In these assays, both mutants were not resistant to curcumin and related compounds (EF24 and DMC) (Fig. S7). These data highlight the distinct mechanisms underlying the effects of curcumin and related compounds on growth and acute cell behaviour.

Ligand-protein docking prediction of curcumin with PsrA

Molecular docking analysis was used to propose an interaction site for curcumin on PsrA. Here, a tertiary structure of the protein was based upon the mammalian orthologue, PP2A. This tertiary structure was then used to calculate the most stable binding site of curcumin and related compounds, showing the lowest energetic expenditure. This approach identified a common site, on the interface between the regulatory subunit B and the scaffold subunit A (based on crystallography studies of the human PP2A enzyme) (Cho and Xu, 2007) of the protein, which is predicted to bind to curcumin (deltaG −7.39), EF24 (deltaG −7.23) and DMC (deltaG −7.54), but is not targeted by CuPy (deltaG −6.62) (Fig. 5), consistent with the resistance phenotype shown by the psrA$^-$ mutant.

DISCUSSION

Improving our understanding of the potential therapeutic roles of curcumin (Heger, 2017; Gupta et al., 2013) might facilitate its use in medicine. To do this, it is critically important to identify potential therapeutic targets, and to test related compounds that have improved

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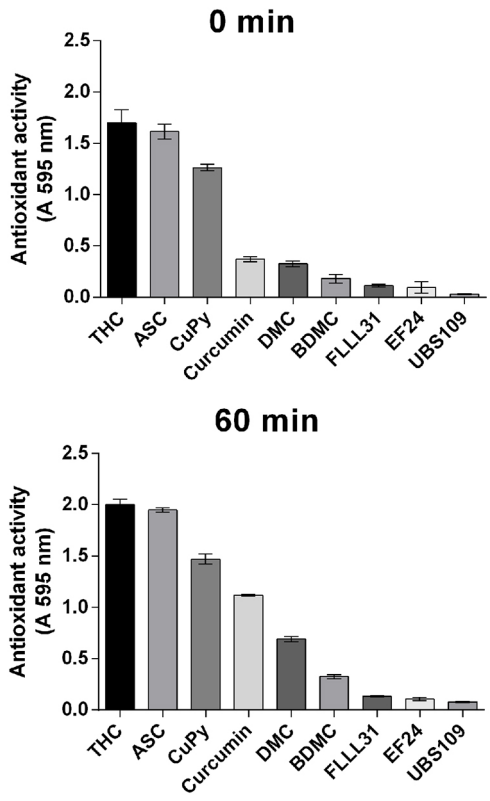

Fig. 3. Antioxidant activity of curcumin and related structures. Reducing activity was monitored using the FRAP assay. Initial rapid (0 min) and sustained (60 min) activity was measured, using ascorbic acid as an antioxidant standard. Data are presented as mean±s.e.m. of triplicate experiments.

chemical characteristics (e.g. solubility) (Oliveira et al., 2015) that can focus research on relevant therapeutic outcomes. The present study employed a novel system, the social amoeba *D. discoideum*, which enables the dissection of discrete acute, growth and developmental effects of curcumin. Utilising curcumin-related structures identified key chemical moieties responsible for the effects of curcumin, and eliminated an antioxidant mechanism for these effects. The study then identified two novel targets related to disease conditions that might aid in the investigation of its role in therapeutic function.

In this study, we initially quantified the potency of curcumin to regulate distinct aspects of acute cell behaviour, growth and development in *D. discoideum*. We show that curcumin provides the strongest inhibitory effect against acute cell behaviour (with an IC_{50} of 2.3 µM), with reduced potency against cell growth (with an IC_{50} of 44 µM) and development (>100 µM) (Fig. 1). These effects support and extend a previous study (Garige and Walters, 2015), and provide a platform for a comparative study of related chemicals. From these combined data, it is likely that curcumin has more than one molecular target in *D. discoideum* that play distinct cellular roles.

We then adopted a quantitative structure activity relationship approach (QSAR), employing a range of natural and artificial curcumin-related compounds to assess common and distinct cellular effects dependent upon the specific chemistry of the compound (Fig. 2). These studies showed that loss of the diketone group (in CuPy) blocked activity in all three functional roles (acute cell behaviour, growth and development), and modification by substitution of central hydrogens (FLLL31) reduced activity (in acute cell behaviour and growth), highlighting the key role of this group in curcumin function. For the remaining compounds, in acute cell behaviour, curcumin and EF24 showed the most potent activity, with any change in curcumin structure leading to a reduction of activity. By contrast, effects of curcumin-related compounds on growth showed that curcumin is the least potent of all compounds analysed. In development, a common delay was seen for the structures most related to curcumin, with no effect from the divergent artificial analogues (EF24 and UBS109). Independent of the central role of the diketone group, the remaining curcumin-related structures analysed here identified key regions of the chemical structure associated with distinct cell effects. In acute cell behaviour, the most important moieties were represented by the methoxy groups and the planar structure adjacent to the diketone groups, where loss of one or two methoxy groups (DMC and BDMC) strongly and incrementally reduced potency, and loss of the central double bonds (in THC) also reduced potency. However, the two synthetic analogues have a variable potency in blocking cell behaviour. A similar trend is present in growth inhibition, but reversed, where the same molecular substitutions that reduced potency in acute cell behaviour enhanced potency in growth. Interestingly, curcumin and its closely related analogues delayed development, but this was not evident for the synthetic analogues. These data support that curcumin has distinct targets related to acute cell behaviour, growth and development.

Many of the cellular roles for curcumin have been associated with the scavenging of reactive oxygen species (ROS) as an antioxidant. Through the mechanism, antioxidants quench free radicals to inhibit cellular damage (Nimse and Pal, 2015). In this role, specific

Table 1. Genes encoding putative targets for curcumin and related compound

Chemical	Gene ID	Gene Product	Orthologues	Identity
Curcumin	DDB_G0281669	LMBR1 family protein	Q68DH5	27%
	DDB_G0280469	**Protein phosphatase 2A regulatory subunit psrA**	Q15172	46%
	DDB_G0293904	NADPH-cytochrome-P450 oxidoreductase	ENSP00000393527	33%
	DDB_G0288103	Type A von Willebrand factor (VWFA) domain-containing protein		
BDMC	DDB_G0267524	Translocon-associated protein TRAP gamma subunit	Q9UNL2	33%
	DDB_G0289907	EGF-like domain-containing protein C-type lectin domain-containing protein		
	DDB_G0291722	UNC93-like protein MFSD11	O43934-1	31%
	DDB_G0272684	Dihydropteridine reductase	P09417	41%
EF24	DDB_G0276169	AAA ATPase domain-containing protein	Q8NBU5-1	39%
	DDB_G0292310	**Presenilin B**	P49768-1	32%
	DDB_G0279417	LRRK family protein kinase Roco6		
UBS109	DDB_G0287861	Glycine cleavage system H-protein	P23434	25%

Insertions in 11 genes that were found to convey resistance to curcumin, BDMC, EF24 and UBS109. For each insertion, Dictybase gene ID, product, orthologues and % identity to human protein have been indicated.

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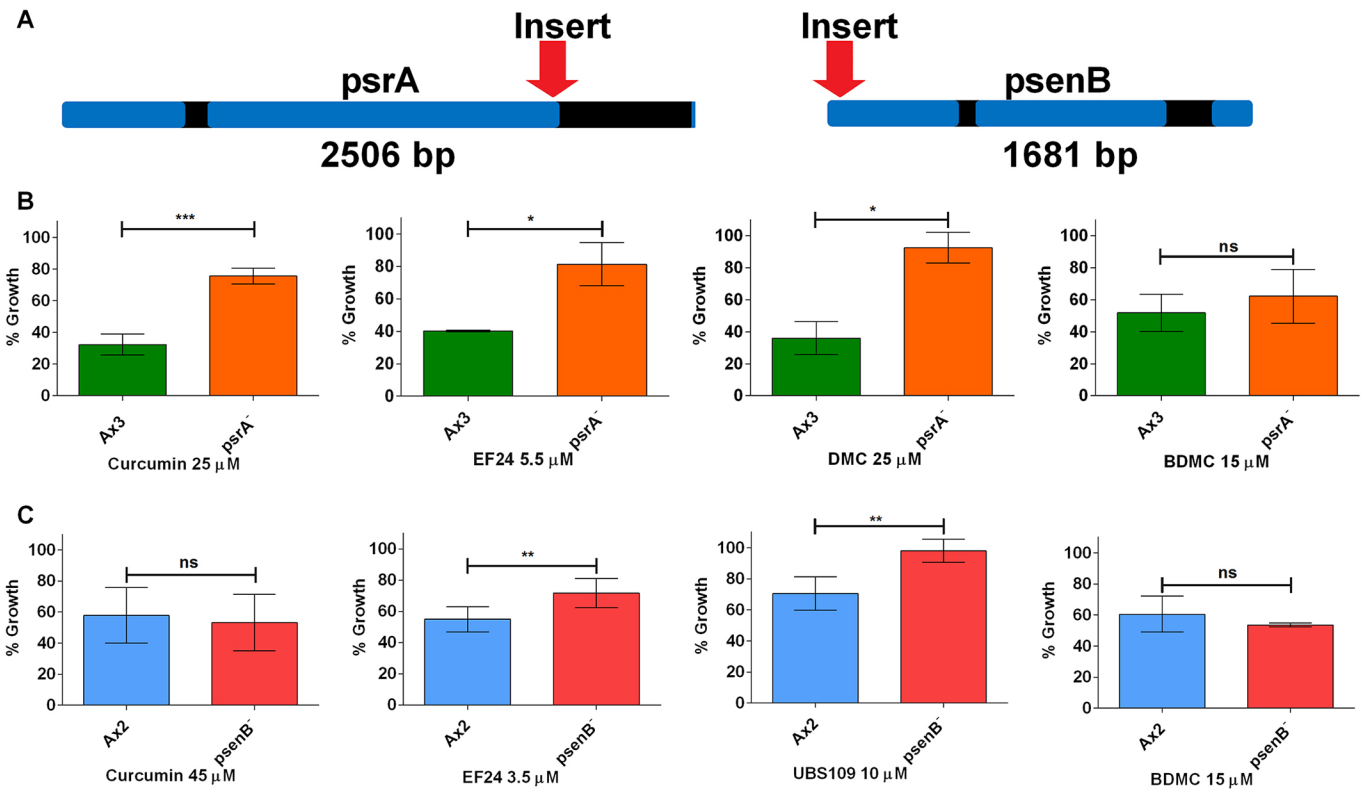

Fig. 4. Loss of *psrA* and *psenB* genes provides partial growth resistance to curcumin or its derivatives. (A) Through screening a *D. discoideum* mutant library, a mutant showing resistance to curcumin was identified showing an insertion into the protein phosphatase 2A regulatory subunit gene (*psrA*), and a mutant showing resistance to EF24 was identified showing an insertion into the presenilin B gene (*psenB*) (blue exons and black introns). (B) Analysis of wild-type (Ax3) and recapitulated psrA^{-} mutant growth rate confirmed that the psrA^{-} mutant was resistant to curcumin, and additionally to EF24 and DMC, but not BDMC (Fig. S5). (C) Analysis of wild-type (Ax2) and recapitulated psenB^{-} mutant growth rate showed that PsenB was not resistant to curcumin, but showed confirmed resistance to EF24, in addition to UBS109 (Fig. S6). Data are presented as mean±s.e.m. of triplicate experiments. **P*<0.05; ***P*<0.01; ****P*<0.001; ns, nonsignificant.

regions within the curcumin structure (the diketone moiety and two phenolic groups) can undergo oxidation by electron transfer and hydrogen abstraction (Priyadarsini, 2014), and the methoxy groups of curcumin are necessary for antioxidant effects in a range of models (Yang et al., 2017). To investigate whether the effects of curcumin and related compounds on *D. discoideum* were related to this effect, a time-dependent assay was used to assess antioxidant activity (Fig. 6). Surprisingly, THC and CuPy provided the largest rapid-onset activity, with both these compounds and curcumin providing strong activity over an extended period. The remaining compounds showed greatly reduced or no significant antioxidant function, consistent with a crucial role for the diketone and methoxy groups in antioxidant function, but not supporting this effect in the modulation of *D. discoideum* acute cell behaviour, growth or development roles. Similar outcomes for curcumin and its derivatives, shown in anti-inflammatory and antiproliferative

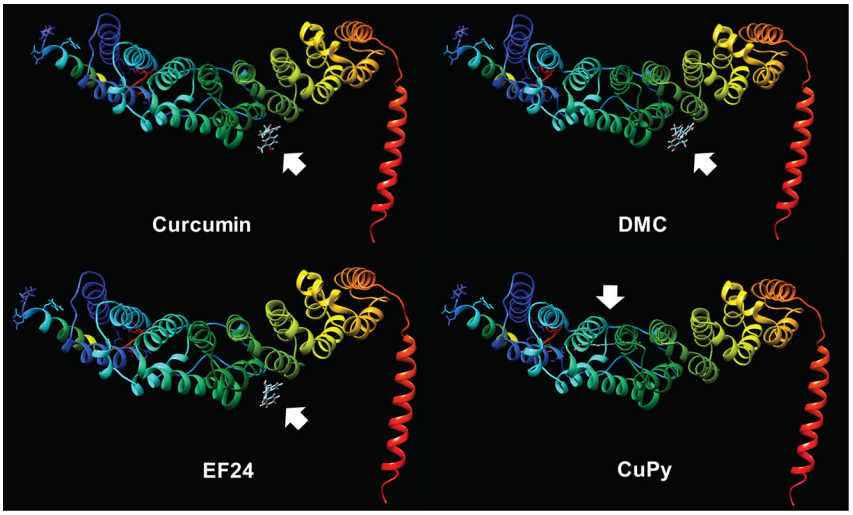

Fig. 5. Molecular docking prediction of PsrA and curcumin analogues. Tertiary protein structures were generated with Phyre2, with docking prediction performed by SwissDock to provide the most stable binding site (deltaG; Gibbs free energy). Using this approach, curcumin, DMC and EF24 are predicted to bind to the same site on PsrA that is not shown for CuPy.

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effects of human-derived cancer cell lines also did not relate to their ability to modulate ROS (Sandur et al., 2007). Thus, in our study, we have shown that the antioxidant properties of curcumin and its derivatives are not related to the cellular effects in *D. discoideum* observed here, and these effects are therefore likely to occur through other mechanisms.

Studies in animal models or animal-derived cells provide an insight into the potential of natural products as therapies in humans. However, limiting research to these models precludes a range of experiments that could provide important step changes in investigating molecular mechanisms. For example, in *D. discoideum*, a range of novel targets for therapies and natural products have been proposed by using a chemical biology approach (Cocorocchio et al., 2015; Robery et al., 2011, 2013; Waheed et al., 2014; Williams et al., 1999, 2002; Chang et al., 2012; Xu et al., 2007; Elphick et al., 2012; Boeckeler et al., 2006). To apply this approach to curcumin and its derivatives, we identify one gene product, PsrA, regulating the function of curcumin, and a second gene product, presenilin B, regulating the function of the synthetic analogue EF24.

The *D. discoideum psrA* gene encodes the orthologue of the mammalian protein phosphatase 2A (PP2A) regulatory subunit B56 (PPP2R5A). In *D. discoideum*, this protein has been shown to regulate cell chemotaxis and differentiation by negatively modulating glycogen synthase kinase 3 (GSK3) protein function (Rodriguez Pino et al., 2015; Lee et al., 2008). PP2A is a major Ser/Thr phosphatase expressed ubiquitously in eukaryotic cells. It is a heterotrimeric enzyme, consisting of a core dimer formed by the scaffolding subunit (A) and a catalytic subunit (C) (Sangodkar et al., 2016). The dimer complexes with one of the several regulatory subunits. In fact, there are >80 different combinations of the PP2A holoenzyme, which regulates the activity and cellular localization of PP2A (Magnusdottir et al., 2009). PP2A regulates a wide variety of cellular processes, including translation, transcription, inflammation, differentiation and apoptosis (Van Hoof and Goris, 2003; Codreanu et al., 2006). PP2A plays a pivotal role in numerous cellular processes, such as cell proliferation, signal transduction and apoptosis, and its deregulation is associated with multiple cancers, AD and increased susceptibility to pathogen infections (Cho and Xu, 2007). It has been shown that in many cancers, there is an abnormal function of the PP2A scaffold and regulatory subunits, supporting its role as a tumour suppressor (Seshacharyulu et al., 2013; Van Hoof and Goris, 2003). In cancer treatment, data are conflicting; patients with a range of cancers improve upon restoring PP2A activity (Kiely and Kiely, 2015), but, conversely, inhibition of PP2A also leads to programmed cell death in many tumour cells. Interest in curcumin as an anticancer treatment is due to a large number of *in vitro* and *in vivo* studies reporting growth arrest of different types of cancer, such as brain, breast, head and neck, liver, pancreas, colon, prostate, ovary and skin cancers (Anand et al., 2008; Kunnumakkara et al., 2008; Ghosh et al., 2015; Klinger and Mittal, 2016; Di Martino et al., 2017; Panda et al., 2017; Singh and Aggarwal, 1995; Bharti et al., 2003). In addition, curcumin has been demonstrated to exert neuroprotective effects by maintaining the levels of PP2A subunit B, leading to tau protein dephosphorylation and/or GSK3β inhibition, which prevents tau hyperphosphorylation (Shah et al., 2015; Sontag and Sontag, 2014; Di Martino et al., 2016).

In our study, we demonstrate that loss of PsrA markedly reduces growth sensitivity to curcumin, EF24 and DMC, suggesting that these compounds might function to regulate cellular activity through this protein. We further propose a potential direct binding of these compounds to PsrA through molecular docking analysis, in which these molecules bind to PsrA but related (inactive) compounds do not. Interestingly, the region of interaction identified in this approach is responsible for interaction with the scaffolding subunit, which might regulate this function. Thus, binding of curcumin and derivatives to the PP2A core regulatory dimer might influence the rate or specificity of binding to the scaffolding subunit and subsequent cellular function. Therefore, this approach has provided a novel insight into a mechanism of curcumin in regulating PP2A activity, with potential impact on therapeutic use.

The *D. discoideum* presenilin B gene encodes one of two presenilin proteins, as part of the γ-secretase complex (Ludtmann et al., 2014). Recent results in *D. discoideum* have shown that presenilin proteins play a conserved noncatalytic role which is independent of proteolytic activity (Otto et al., 2016b). This activity is conserved between human and *D. discoideum* proteins, because the expression of human presenilin 1 in *D. discoideum* restores γ-secretase complex function (Ludtmann et al., 2014). In addition, it has been demonstrated that *D. discoideum* presenilin/γ-secretase activity is required for both phagocytosis and cell-fate determination. Thus, presenilin function and γ-secretase activity are ancient processes that arose prior to metazoan divergence (McMains et al., 2010). The human presenilin 1 (PS1) protein, as a key component of the γ-secretase complex, plays a pivotal role in amyloid precursor protein (APP) cleavage to generate Aβ, where aggregates of Aβ provide a hallmark of AD pathology. In addition, PS1 is a substrate for GSK3β, which is also involved in the pathology of AD (Otto et al., 2016b). Curcumin has been proposed to decrease Aβ production by inhibiting GSK3β-mediated PS1 activation (Zhang et al., 2010; Di Martino et al., 2016), and curcumin downregulates presenilin 1 protein in a dose-dependent manner to regulate γ-secretase function (Yoshida et al., 2011). As a result, curcumin might have neuroprotective effects by inhibiting the generation of Aβ and tau fibrils, but the mechanisms of action remain unknown. In AD, animal models have shown that curcumin reduces amyloid levels and protein oxidisation, which are involved in the cognitive decline process (Baum and Ng, 2004). Furthermore, in patients with AD, macrophages unable to phagocytose Aβ show restored/enhanced activity following curcumin treatment (Zhang et al., 2006). Our study identified and validated a mutant lacking the presenilin B (PS1 homologue) resistant to curcumin-related EF24 and UBS109, and these compounds could provide interesting analogues for further study in the treatment of AD.

Numerous targets and effects have been proposed for curcumin that have led to its investigation for the treatment of several diseases. Targets include transcription and growth factors, cytokines, and regulators of cell growth and death (Goel et al., 2008). Furthermore, curcumin interacts with P-glycoprotein, glutathione, protein kinase C (PKC; PRKC proteins), ATPase, nuclear factor-κB (NFKB1), epidermal growth factor receptor (EGFR), phosphatidylinositol 3 kinase (PI3K; PIK3CA), AKT proteins, mTOR and many other cellular targets (Goel et al., 2008; Prasad et al., 2014; Priyadarsini, 2014; Gupta et al., 2012, 2013; Zhou et al., 2011). In addition, several studies have examined the heptadienedione moiety, which possesses two thiol-reactive α,β-unsaturated carbonyl groups (Fuchs et al., 2009; Ou et al., 2013), that might function to covalently modify cysteine residues of target proteins to regulate cellular functions. These results have stimulated many studies to investigate these targets and effects in a wide range of chronic illnesses such as AD, PD, MS, cardiovascular diseases, cancer, allergy, asthma, rheumatoid arthritis, diabetes and inflammation (Yang et al., 2017; Lakey-Beitia et al., 2017; Jurenka, 2009; Srinivasan, 1972;

Chougala et al., 2012; Zhang et al., 2013; Tang and Taghibiglou, 2017; McClure et al., 2017). It remains to be examined if the targets identified in this paper function as upstream modulators or downstream effectors for these curcumin-regulated effects.

In this study, we demonstrated the use of a chemical biology approach to highlight active moieties of curcumin with cellular function using the model system *D. discoideum*. Based upon identified effects, we further employed a chemical genetic approach to identify two possible molecular targets for curcumin and its derivatives, which have been associated with the pathogenesis of cancer and AD in animal models and patients. The study therefore proposes curcumin-related compounds with improved chemical characteristics, which might provide beneficial therapeutic approaches for treating a range of diseases that have been proposed to be curcumin responsive. This approach also highlights a useful model to investigate natural products with multiple cellular effects, and could aid in the development of new therapeutics related to natural products.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from Sigma-Aldrich (Dorset, UK): curcumin [(E,E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; C1386], demethoxycurcumin [(E,E)-1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione; D7696], bisdemethoxycurcumin [(1E,6E)-1,7-bis(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione; B6938], EF-24 {(3E,5E)-3,5-bis[(2-fluorophenyl)methylene]-4-piperidinone; E8409}, FLLL31 [(E,E)-1,7-bis(3,4-dimethoxyphenyl)-4,4-dimethyl-1,6-heptadiene-3,5-dione; F9057], tetrahydrocurcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-3,5-heptanedione; 50202], adenosine 3′,5′-cyclic monophosphate (3′,5′-cyclic AMP; A9501, 200 mM stock solution). Curcumin pyrazole {(E)-3,5-bis[β-(4-hydroxy-3-methoxyphenyl)-ethenyl]-1H-pyrazole; SL-318} was obtained from Syninnova. Enaminestore supplied UBS109 [3,5-bis(2-pyridinylmethylidene)-1-methyl-4-piperidone; Z46034963]. All compounds were dissolved in dimethylsulfoxide (DMSO, vehicle).

D. discoideum growth assay

D. discoideum cells were harvested and diluted in axenic medium to 2×10^4 cells/ml. Aliquots of cells (500 µl) were transferred to 24-well plates containing consistent concentrations of solvent (DMSO) in addition to indicated compounds. Cells were grown in 24-well plates, at 22°C, and cell density calculated over 7 days. To provide secondary plots, a rate of exponential growth was calculated (from 72 h to 120 h) at each concentration, and normalised to control (solvent only) conditions.

D. discoideum random cell movement and cell development

D. discoideum random cell movement assays and developmental phenotypes assays were carried out as described (Cocorocchio et al., 2015; Robery et al., 2011). In these experiments, behaviour was monitored in cells undergoing random cell movement by taking images every 15 s over a 15-min period, with 5 min recorded prior to, and 10 min after, compound addition. A minimum of three independent experiments for each drug concentration were used, with ≥10 cells quantified per experiment. Protrusions per cell were averaged over the last 5 min of recording, and normalised to control (solvent only) conditions. In these experiments, average (untreated) protrusions per cell were ∼5.7 with a maximum of 6.4 and a minimum of 5.4, consistent with previous reports (Cocorocchio et al., 2015; Otto et al., 2016a).

D. discoideum restriction enzyme-mediated integration screen

To identify mutants in *D. discoideum* resistant to curcumin and analogues, two libraries of insertional mutants containing 5000 mutants (psrA$^-$) and 11,000 mutants (psenB$^-$) were used. Cells were incubated with different concentrations of each compound over 14 days. Mutants growing in the presence of each compound were identified as previously described (Robery et al., 2011; Waheed et al., 2014).

Mutant growth inhibition assay

Cells were grown in shaking suspensions and harvested in early exponential phase ($1.5\text{-}2.5\times10^6$ cells/ml). Cells were then divided into aliquots and shaken for 24 h in the presence of solvent only or compound, at a concentration which blocked growth by ∼50% in a final volume of 2 ml axenic media. Growth (%) was defined for each wild type and derived mutant (Ax2 and psrA$^-$; or Ax3 and psenB$^-$), with growth normalised to relevant wild-type cell growth in the absence of compound (solvent only). Each condition tested was carried out at least in triplicate.

FRAP assay

FRAP solution was prepared by combining 2 ml TPTZ solution (10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl), 2 ml $FeCl_3$ (10 mM) and 20 ml acetate buffer (300 mM, pH 3.6). The assay was carried out by combining 800 µl FRAP solution with 25 µl of the positive control (1 mM ascorbic acid) or curcumin-related compounds (to give a final concentration of 31.2 µM), and the absorbance was measured at 595 nm. Measurements were obtained in triplicate.

Protein-ligand docking

Protein sequences were obtained from dictybase.org. The tertiary structure of the *D. discoideum* protein was predicted using Phyre2 (Protein Homology/Analogy Recognition Engine V 2.0) (Kelley et al., 2015). Docking analyses were performed using SwissDock to identify the possible binding sites in PsrA. UFCS Chimera was used to display the results obtained from SwissDock (Grosdidier et al., 2011). Results are expressed as deltaG (Gibbs free energy, where a negative value indicates a spontaneous interaction).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.L.R.A., R.S.B.W.; Methodology: M.C.; Formal analysis: M.C.; Resources: A.J.B., B.S., L.K., A.J.H., C.R.L.T.; Data curation: M.C., R.S.B.W.; Writing - original draft: M.C., P.L.R.A., R.S.B.W.; Writing - review & editing: M.C., A.J.B., L.K., A.J.H., C.R.L.T., P.L.R.A., R.S.B.W.; Supervision: P.L.R.A., R.S.B.W.; Project administration: R.S.B.W.; Funding acquisition: P.L.R.A., R.S.B.W.

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Supplementary information

Supplementary information available online at
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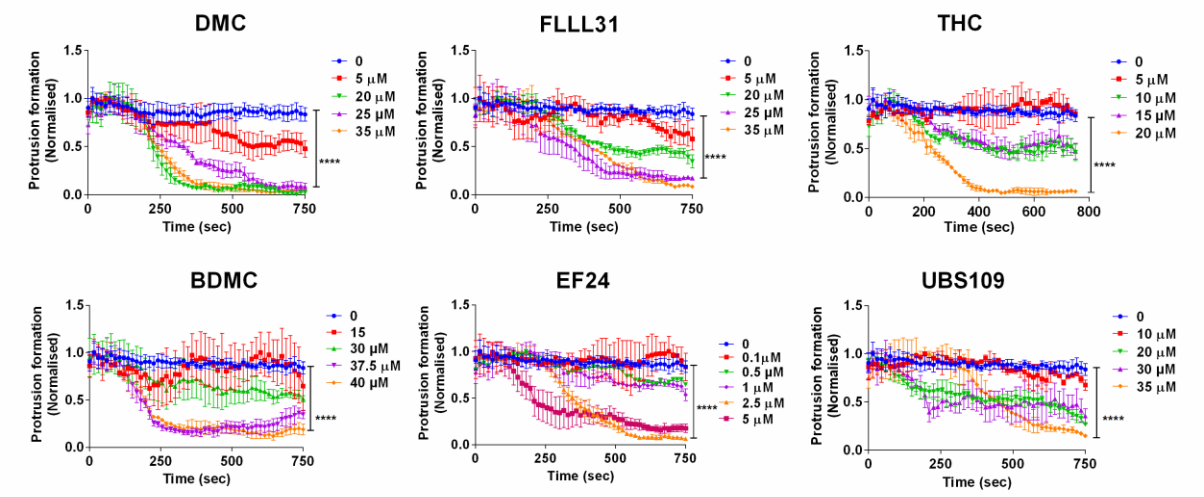
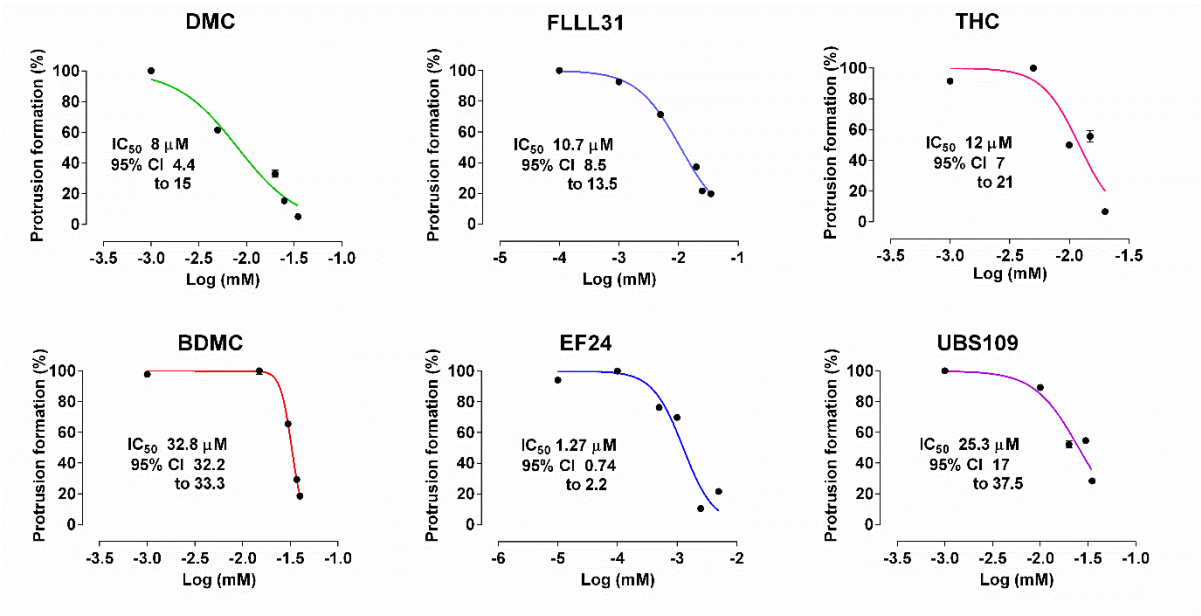
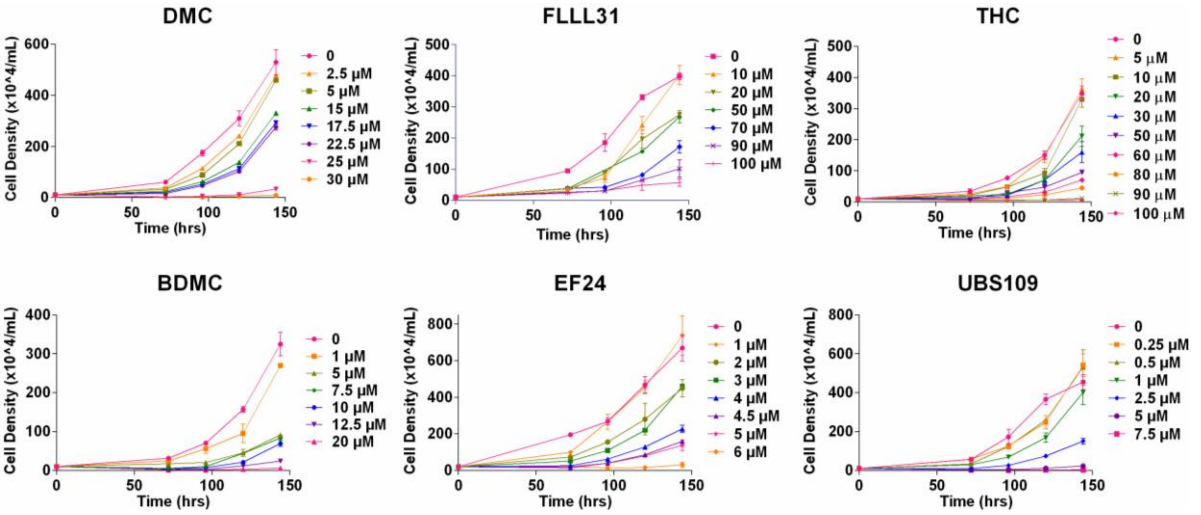
Supplementary information



Supplementary Fig. 1. Raw data of *D. discoideum* acute response to curcumin derivatives. Time-dependent changes in *D. discoideum* cell behaviour (membrane protrusion) were recorded over a 15 minute period for triplicate independent experiments (± SEM) at increasing concentrations of six curcumin derivatives to assess their ability to inhibit protrusion formation. The addition of different concentration of each compound at 210 seconds caused a reduction in protrusion formation. Data is presented as normalised to control (vehicle) conditions. Analysis with Two-tailed t-test showed significant changes after the treatment with: DMC 25 μM, FLLL31 25 μM, THC 20 μM, BDMC 40 μM, EF24 2.5 μM and UBS109 35 μM ($p < 0.0001$ ****).

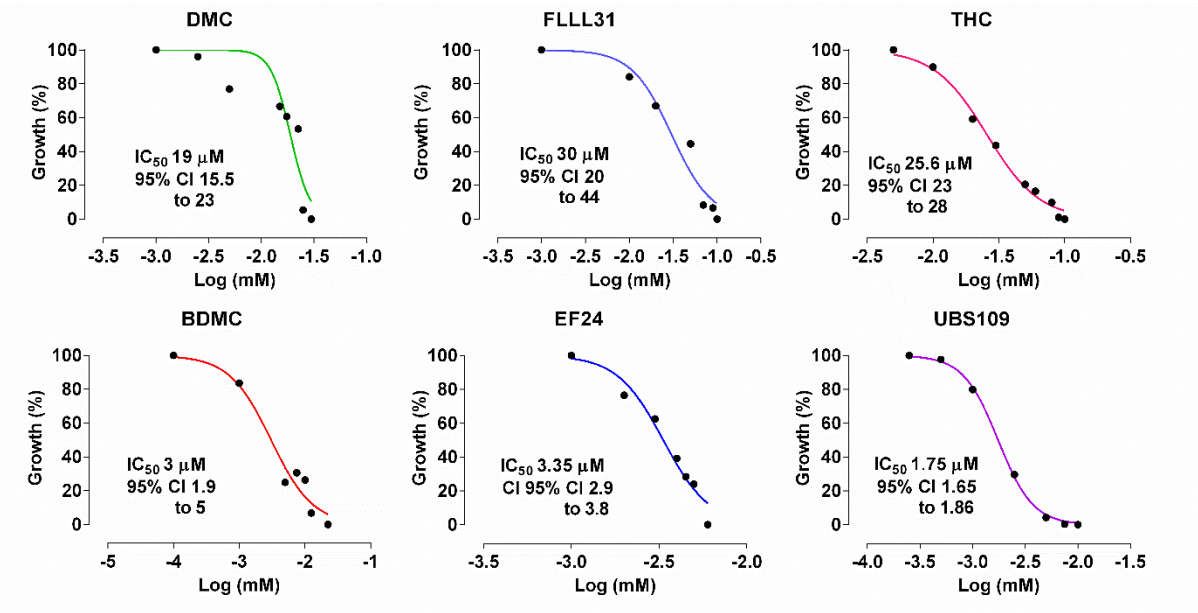


Supplementary Fig. 2. Quantification of the acute effect of curcumin derivatives on *D. discoideum*. Using a range of structurally related compounds, concentration dependent responses were determined for *D. discoideum* cell behaviour (protrusion formation), and illustrated as the normalised reduction in response against the Log (concentration) of each compound (shown with errors based on the 95% confidence intervals), enabling calculation of an IC_{50} values and 95% confidence intervals for each compound.

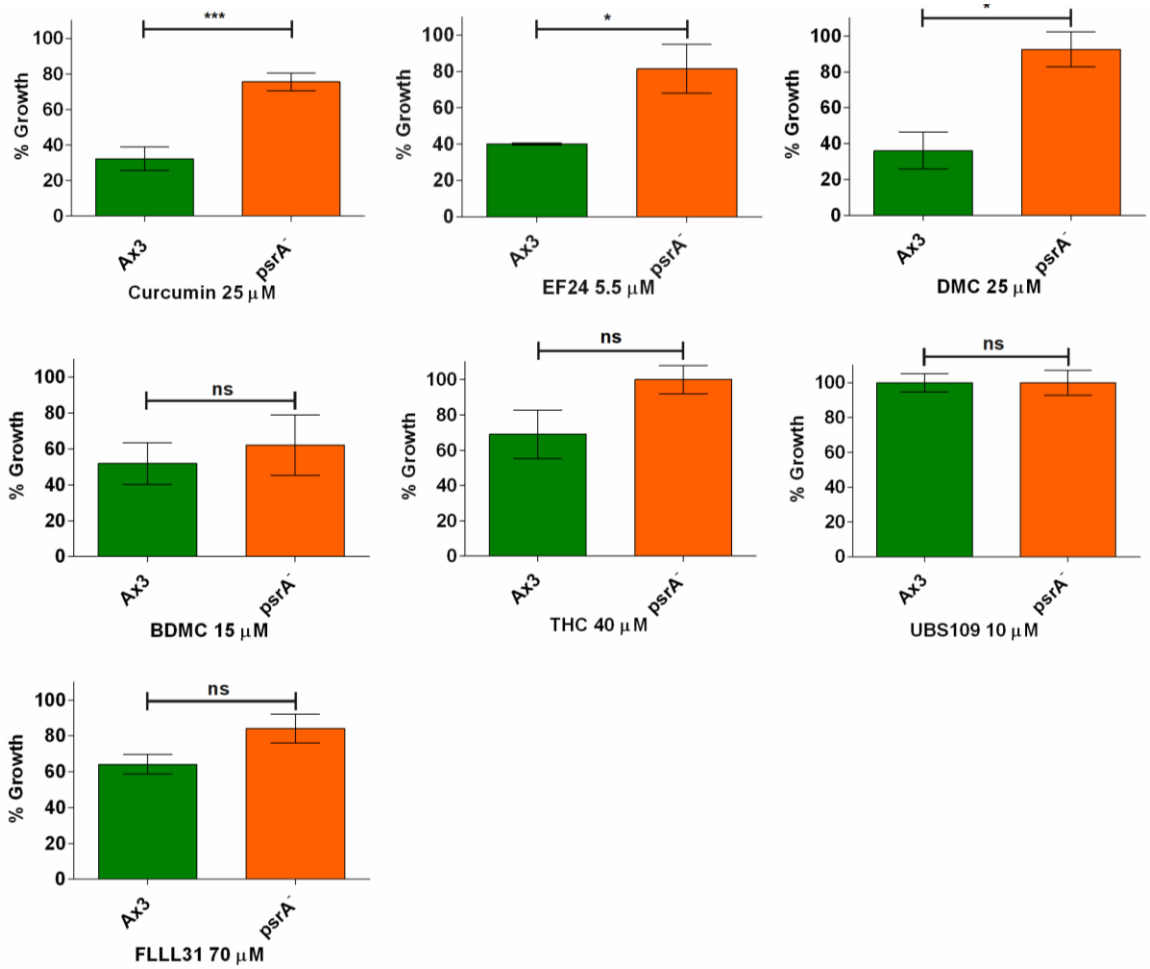


Supplementary Fig. 3. Raw data of *D. discoideum* chronic response to curcumin derivatives. *D. discoideum* cells were grown with increasing concentration of curcumin derivatives in triplicate independent experiments ± SEM. DMC fully blocked growth at 30 µM, FLLL31 and THC at 100 µM,

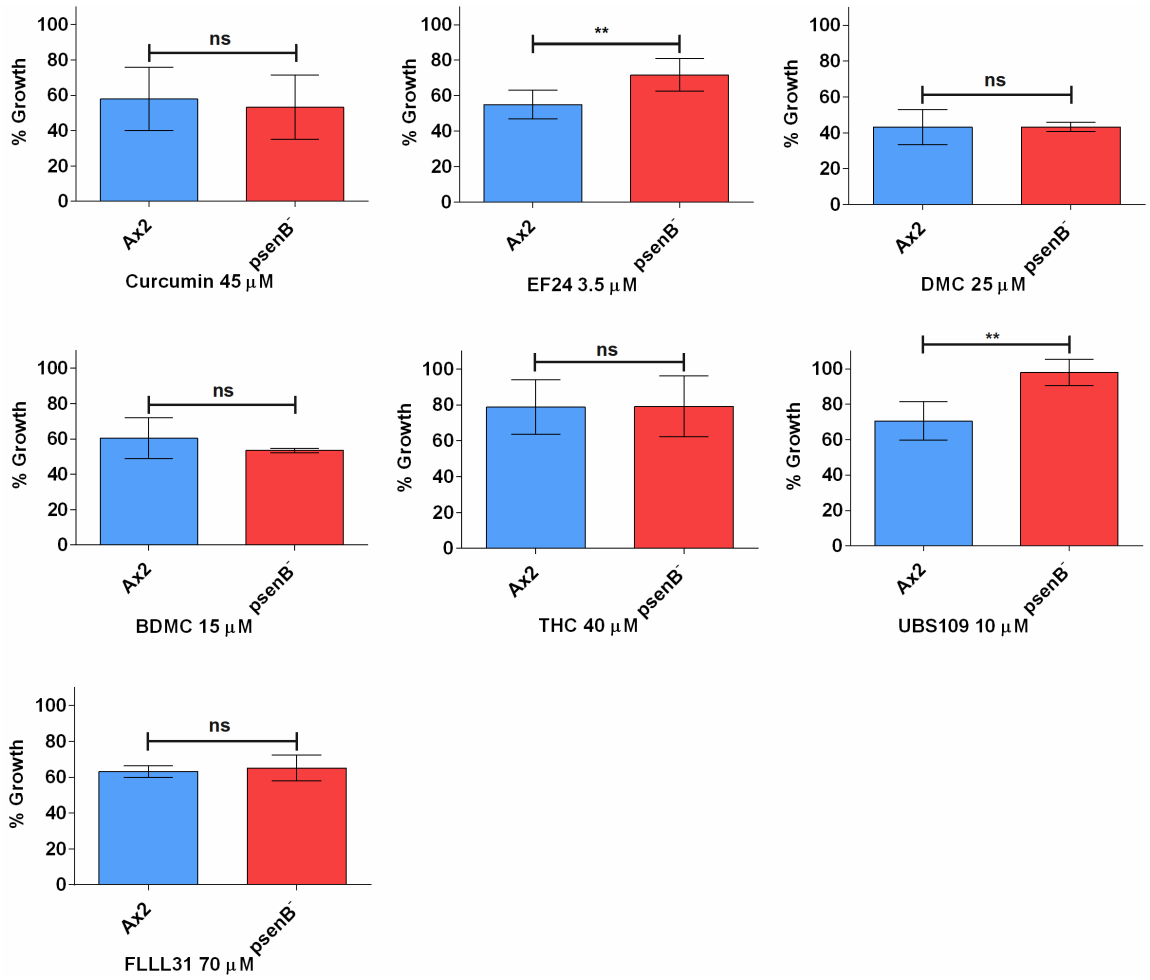
instead BDMC arrested growth at 20 µ, EF24 and UBS109 inhibiteg proliferation at 6 and 5 µM respectively.



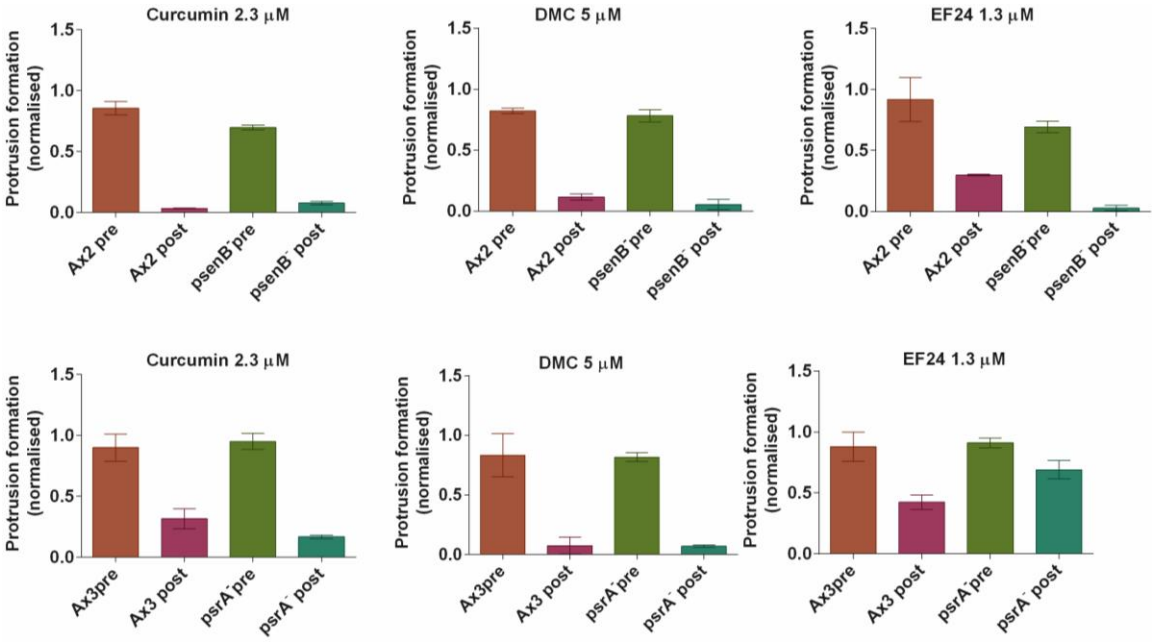
Supplementary Fig. 4. Quantification of the chronic effect of curcumin derivatives on *D. discoideum*. Using a range of structurally related compounds, concentration dependent responses were determined for *D. discoideum* cell growth, and illustrated as the normalised reduction in growth against the Log (concentration) of each compound (shown with errors based on the 95% confidence intervals), enabling calculation of an IC_{50} values and 95% confidence intervals for each compound.



Supplementary Fig. 5. Growth inhibition assay - Ax3 and psrA$^-$ in presence of curcumin and its derivatives. Cells were grown in shaking suspension in presence of different curcumin derivatives. Analysis with Two-tailed t-test showed that psrA- mutants are resistant to curcumin as compared to AX2 (*** $p < 0.001$). psrA- mutants were also resistant to EF24 (* $p < 0.05$), THC (* $p < 0.05$) and DMC (** $p < 0.01$) in comparison to AX2. psrA- mutants were not resistant to BDMC, UBS109 and FLLL31. Data is provided as mean of at least three independent experiments ± SEM.



Supplementary Fig. 6. Growth inhibition assay - Ax2 and psenB$^-$ in presence of curcumin and its derivatives. Cells were grown in shaking suspension in presence of different curcumin derivatives. Analysis with Two-tailed t-test showed that psenB- mutants are resistant to EF24 as compared to AX2 (*** $p < 0.001$). Interestingly psenB- mutants were also resistant to UBS109 (** $p < 0.01$) in comparison to AX2. Results showed that the psenB$^-$ mutants were not resistant to curcumin, DMC, BDMC, THC and FLLL31. Data is provided as mean of at least three independent experiments ± SEM



Supplementary Fig. 7. Assessment of the chronic effect of curcumin and its derivatives on *D. discoideum* mutants. psenB and psrA null mutants were exposed to curcumin, DMC and EF24. The mean of the normalised protrusion formation was calculated for the first and the last 5 min for each cell line. The first set of graphs shows that the psenB$^-$ mutant is not resistant to any of the compounds. The second set of graphs illustrates that psrA is sensitive to this range of molecules. Data is provided as mean of at least three independent experiments ± SEM.

Curcumin and derivatives function through protein phosphatase 2A and presenilin orthologues in Dictyostelium discoideum

Figure 1. (B) N=30 cells analysed per condition from 3 independent technical repeats. (D) N=3 independent technical repeats. (F) N=3 independent technical repeats.

Figure 2. (D) N=3 independent technical repeats.

Figure 3. N=3 independent technical repeats.

Figure 4. N≥3 independent technical repeats.

Supplementary Figure 1. N=30 cells analysed per condition from 3 independent technical repeats.

Supplementary Figure 3. N=3 independent technical repeats.

Supplementary Figure 5. N≥3 independent technical repeats.

Supplementary Figure 6. N≥3 independent technical repeats.

Supplementary Figure 7. N=30 cells analysed per condition from 3 independent technical repeats.

Chapter IV

Critical evaluation

The overall objective of this research is to improve the characterisation of substances that elicit taste response and have medical relevance. The specific project goal has been the development of *D. discoideum* as a model for the evaluation of structurally different tastants, and it is divided into three main milestones.

1) To develop a method for the assessment of the effects of tastants on cell behaviour using the pungent alkaloid capsaicin;

2) To employ the aforementioned method to test a range of known and blinded bitter tasting substances on cell behaviour and assess the ability of the compounds to inhibit protrusion formation;

3) To utilise the method to test the effects of the phytotherapeutic tastant curcumin and a range of structurally related compounds on *D. discoideum* growth, movement and development as well as identify novel molecular targets.

The concept of the project is controversial since *D. discoideum* does not possess taste receptors, taste buds or a nervous system able to process taste. However, the use of *D. discoideum* to sense tastants is dependent upon the ability to detect extracellular chemicals and respond to them in different ways (Aufderheide and Janetopoulos, 2016; Bosgraaf and Van Haastert, 2009; Chung and Firtel, 2002).

For instance, *D. discoideum* can detect chemotactic gradients such as cAMP and folic acid (Chung and Firtel, 2002; Jin et al., 2008; King and Insall, 2009; Pan et al., 2016; Segota et al., 2013) and exposure of cells to these chemoattractants causes changes in cell behaviour. These changes can be used to assess the activity and function of tastant chemicals (Iijima et al., 2002; King and Insall, 2009; Robery et al., 2011). *D. discoideum* also possesses a range of proteins with conserved functions to mammalian orthologues, and the identification of a target for tastants in the amoeba may lead to the discovery of targets related to mammalian models (Eichinger et al., 2005). Therefore, the use of *D. discoideum* represents a valid approach for the investigation of the effects of tastants.

Understanding how taste works is essential as several tastants have potential therapeutic qualities, but it remains unclear whether the mechanism of taste are linked to their medicinal properties. Evidence suggests that the biology of tastants is not confined to taste receptors located on the taste cells, as some of these chemicals may also trigger changes in other cell types (e.g. airway smooth muscle cells) (Zhang et al., 2013a). Furthermore, there are many natural and synthetic therapeutic agents which elicit a specific taste and target disease mechanisms. For instance, the bitter compound azelastine is an H_1-receptor antagonist used for the treatment of allergic rhinitis (El-Shaheny and Yamada, 2014), whereas the bitter alkaloid quinine is used as antimalaria and babesiosis's treatment (Achan et al., 2011; Dorman et al., 2000). Therefore, tastants may have both therapeutic and taste properties, and in many cases, it remains unclear if these properties are interconnected. Thus, a better understanding of the cellular mechanisms of taste transduction could enable the prediction of aversive tastants, impacting positively on medical research and drug development process.

4.1 Development of a model for the assessment of tastants

4.1.1 Critical evaluation of the methodology used

Although the methods paper (Otto et al., 2016) presents an innovative approach to assess the effects of tastants, a range of changes would improve the experimental approach. Indeed, capsaicin had a dose-dependent effect on *D. discoideum* cell behaviour, but three concentrations are insufficient to fully characterise a concentration-response curve and to obtain a precise IC_{50} value. To provide an accurate IC_{50} calculation, a concentration range from no effect to complete inhibition of cell movement must be determined, allowing a more accurate comparison of the rank order of potency over a range of compounds.

Although results suggest that the identified mutants lacking RacGEFs proteins showed partial resistance to capsaicin, further experiments would support this proposal. These experiments could involve a double knockout of both RacGEFs (Rivero and Xiong, 2016; Vlahou and Rivero, 2006), that may provide enhanced resistance over single knockouts (Meßling et al., 2017). If the resulting cells were completely resistant to capsaicin, this would indicate these proteins are either the only two targets or a parallel complementary pathway to capsaicin in this model. Further analysis may include the assessment of capsaicin on growth and the rescue of sensitivity with either *D. discoideum* or human RacGEF genes.

The length of time required for the analysis of data derived from video recordings of cell movement has been identified as a limitation of this approach. Typically, nine movies could be recorded in one day, enabling the tracking of 10 cells over 60 frames for each video. From these series of images, cell movement was quantified using the image analysis software Fiji (Schindelin et al., 2012), and the corrections of automated cell tracking errors, caused by cell-cell interaction,

majorly interfere with the analysis process. Therefore, to facilitate the imaging of the cell outline and increase the speed of the analysis following exposure to tastants, a range of fluorescence techniques were explored. These procedures were investigated since it was speculated that using fluorescence microscopy could provide an enhanced contrast of the cells against the background, speeding up the computer-based analysis (Sanderson et al., 2014).

Two attempts were made to improve the cell imaging recording in random movement assays and automated cell tracking. The first approach involved transforming cells with an empty vector used for overexpressing proteins with the green fluorescent protein GFP tag (Levi et al., 2000), allowing the visualisation of the cytosol of *D. discoideum* cells. This approach was expected to provide bright cells with a dark background. However, cells demonstrated sensitivity to the excitation light during the image capture, resulting in cells rounding and photobleaching over the experiment (Hoebe et al., 2007). The second approach to improve cell behaviour imaging analysis was carried out by employing the fluorescent dye TAMRA (5-Carboxytetramethylrhodamine) (Dougherty et al., 2015). During these experiments, cells were incubated with TAMRA (20 µM, 5 minutes), washed twice in phosphate buffer, and resuspended and assessed using the cell behaviour analysis (section 3.1.1). TAMRA labelled cells were visible using fluorescence microscopy; however, photobleaching issues limited the application of this method due to the assay time length.

Experiments were carried out by manually operating the microscope shutter to minimise the exposure of the cells to the fluorescent light and reduce photobleaching (Frigault et al., 2009). However, these techniques were discontinued as they did not provide benefits over the bright field imaging microscopy in terms of improving the precision and reducing the time of the

analysis. This method could be further developed as a medium-high throughput platform for the screening of tastants, with access to automated equipment for fluorescent cell imaging.

The methodology developed here provides restricted information on underlying molecular mechanisms. In fact, chemicals could negatively affect many essential cellular processes regulating cell behaviour such as actin polymerisation (Noegel and Schleicher, 2000), loss of myosin formation (Kollmar, 2006) and deregulation of mitochondrial function (Barth et al., 2007). Thus, further investigation is required to better understand the molecular mechanisms of capsaicin in *D. discoideum*.

4.1.2 Critical evaluation of *D. discoideum* as a suitable model for the acute assessment of tastants

In the first milestone of the project, a method was developed for the assessment of the acute effects of the pungent tastant capsaicin on cell behaviour. Indeed, *D. discoideum* acutely responded to the administration of this hot tastant, and the concentrations used in these experiments are comparable to the concentrations used in mammalian models. For instance, it has been shown that capsaicin is able to block the hyperpolarisation-activated inward currents via TRPV1 in rat neurons with an IC_{50} of 0.7 µM and a maximal inhibition of 10 µM (Kwak, 2012). Similarly, capsaicin at 1 µM can activate the TRPV1 receptor in mice dorsal root ganglion neurons, increasing the current density in a concentration-dependent manner as well as intracellular calcium concentration (Masuoka et al., 2017). Thus, the concentrations employed in *D. discoideum* are comparable to those used in mammalian models, providing partial support for the possible translation of this mechanism to human studies.

In this study, *D. discoideum* has provided a novel methodology for the assessment of the effects of capsaicin using random cell behaviour. Indeed, two mutants containing disrupted RacGEF proteins have been identified, leading to the discovery of a novel cellular mechanism of the pungent tastant capsaicin. RacGEF proteins are involved in a wide range of cellular processes in *D. discoideum*, including actin organisation, chemotaxis and endocytosis (Rivero and Xiong, 2016; Vlahou and Rivero, 2006). Interestingly, these proteins fulfil similar functions in higher organisms, including humans (Bos et al., 2007; Demarco et al., 2012; Pengelly et al., 2016). Therefore, results obtained in this study may suggest the existence of alternative molecular pathways through which capsaicin regulates cell function.

The data provided in this paper suggest the existence of additional mechanisms through which capsaicin works. Research studies have demonstrated that capsaicin functions through the TRPV1 in mammalian cells (Masuoka et al., 2017; Yang et al., 2014). However, it has been also demonstrated that capsaicin can block transient current in capsaicin-insensitive neurons through a TRPV1-independent mechanism (Yang et al., 2014). Thus, this study identifies that capsaicin may also work through a TRPV1-independent mechanism by modulating the function of RacGEF proteins.

4.2 Investigation of the utility of *D. discoideum* as a model for the identification of bitter tastants

4.2.1 Critical evaluation of the methodology used

This study aimed to investigate the potential utility of *D. discoideum* for the identification of structurally diverse bitter compounds. The first step in this study was to examine if *D. discoideum* behavioural responses were specific to any of the five primary taste qualities. Therefore, a range of compounds and physiochemical conditions such as pH, osmolarity and solubility of the compounds were employed to assess whether *D. discoideum* model could discriminate between the compounds which have been identified as bitter, salty, sweet, sour and umami tasting. Finally, the results obtained using *D. discoideum* were compared to the rat BATA assessments and human taste panel studies to evaluate a correlation amongst these models.

It has been demonstrated that *D. discoideum* does not respond to sweet and umami tastants as well as sour and salty conditions (Cocorocchio et al., 2015) but further studies could be used to confirm these data. The use of standard sweet-tasting compounds such as glucose and sucrose did not cause any changes in *D. discoideum* cell movement up to 50 mM. Although the standard compounds that elicit sweet taste in humans were assessed in this study, the use of additional sweet-tasting compounds with diverse structures (e.g. fructose, maltose, aspartame, saccharin, acesulfame K) could strengthen this approach.

A higher concentration of the phosphate buffer was used to mimic the salty taste, which also increased the osmolarity of the solution from 36 mosmol/kg (standard phosphate buffer) to 118 mosmol/kg. It has been shown that cell behaviour was not affected by increased osmolarity (Cocorocchio et al., 2015). Reasonably, the

salt concentration could have been assessed by using sodium chloride. However, a previously published study has shown that *D. discoideum* cell movement is not affected by increasing sodium chloride concentrations (171 mM – 335 mosmol/kg) (Waligorska et al., 2007), indirectly confirming recent results (Cocorocchio et al., 2015).

Similarly, to mimic the sour taste, the pH of the solution was reduced to 5 to increase the H^+ concentration (Ugawa, 2003). Acidic conditions did not affect cell movement, suggesting that the amoeba is not susceptible to sour taste. However, additional experiments should be conducted to assess the effects of other acidifying compounds on *D. discoideum* cellular behaviour. These experiments may include citric acid (citrus fruit) (Penniston et al., 2008), malic acid (sour apples) (Khan et al., 2013), lactic acid (sour milk) (Widyastuti et al., 2014) and butyric acid (rancid butter) (Pituch et al., 2013). Thus, these experiments could further validate the methodology developed if the results are consistent with recent findings (Cocorocchio et al., 2015).

In this project, *D. discoideum* random cell movement was used to assess the effects of a range of structurally diverse substances with a known bitter taste. The method used to carry out this investigation has been refined based on the knowledge acquired during the initial development stage. For example, for each compound, the concentrations assessed started from no effect and were increased until complete inhibition of cell behaviour was achieved, with a minimum of three experimental repeats for each concentration and at least 30 cells analysed at each concentration. In addition, the study was carried out using several compounds that were blinded in terms of their structures and tastes. These improvements led to a better characterisation of the effects of the investigated molecules on cell movement.

The calculation of the IC_{50} values using non-linear regression curve fit equation enabled the generation of a rank order of the compounds' potencies, which in turn allowed comparison of the data obtained in *D. discoideum* with the rat BATA test and human taste panels (Cocorocchio et al., 2015). These results are of particular importance as they show the utility of the amoeba to replace or reduce animal use as well as demonstrating the translation of findings in *D. discoideum* to humans responses. In addition, these findings suggest that the stronger the effect on *D. discoideum* cell behaviour the more likely the compounds are described as bitter in humans and BATA assays (Cocorocchio et al., 2015). Future experiments should include more tastants from a broader range of chemical space, in a blinded manner, and a panel of at least 30 bitter compounds as well as non-bitter tastants, to add more validity to the system and improve the confidence in the model for the prediction of bitter tastant effects. Although more compounds could have been assessed in *D. discoideum*, the insufficient data available on the concentration-response relationship in the standardised rat BATA test and human taste panels represented the main limitation.

The reproducibility of the methodology has been demonstrated by repeating the experiments at different times with the same compound and with different concentrations (Cocorocchio et al., 2015). Repetition of the experiments demonstrated a comparable concentration response for azelastine and caffeine with similar IC_{50} values. However, all the compounds tested should be repeated on different days, to ensure that the results are consistent and to further assess the reproducibility of this method, which, together with stability, is critical in case pharmaceutical companies adopt this methodology.

The methodology used here is based on the analysis of random cell movement and how it is affected by the administration of bitter tastants. This methodology

was introduced in a previous study showing that when *D. discoideum* cells are exposed to bitter compounds, they lose their typical amoeboid shape and round up, and in so doing, membrane protrusion formation is blocked (Robery et al., 2013). However, this approach has its limitations as it only reflects the changes in cell behaviour but does not identify the molecular targets that are affected when a substance is administered to the cells. Therefore, a mutant screen would be useful to investigate the molecular mechanisms of the compounds tested in *D. discoideum*, possibly leading to the identification of novel pathways through which the chemicals tested might work. Further experiments should also include the analysis of compounds metabolism to assess whether the molecules are converted into more active metabolites. Mass spectrometry could help to identify the methsbolites by comparing their molecular weight to that of the parent compound (Permentier et al., 2008).

A previous study has identified a mutant lacking the Grlj protein, responsible for the detection of the bitter-tasting alkaloid PTU (Robery et al., 2013). In fact, cells lacking the Grlj receptor show partial resistance to the effects of PTU. Grlj is a G-protein coupled receptor protein (Prabhu et al., 2007) and homologue of an uncharacterised human GABA-B receptor. Additional experiments showed that the expression of the human GABA-B in *D. discoideum* restored the sensitivity of the cells to PTU, suggesting a conserved function of the GABA-B protein in the amoeba. Since Grlj receptor shares the Gβ subunit (Wu et al., 1995) with other GPCRs (e.g. cAMP receptor-like, $GABA_B$ and Frizzed-like receptors) (Prabhu and Eichinger, 2006) in *D. discoideum*, future investigation of the effects of bitter-tasting compounds on cells lacking the functional Gbeta subunit, responsible for downstream signalling transduction in cell movement, could indicate whether

these receptor proteins are also involved in the transduction of bitter tastants signalling.

4.2.2 Critical analysis of *D. discoideum* as a suitable model for the evaluation of bitter tastants

The bitter taste of medicines is a critical issue in therapeutic efficacy due to aversiveness. Patients, especially children, are affected by the bitterness of medicines, which reduces compliance and therefore may lead to the failure of therapeutic regimens (Anand et al., 2007; Mennella et al., 2013). Pharmaceutical companies are interested in the assessment of the bitterness of candidate drugs in the early stages of compound selection and development, as this is one of the factors that can limit the clinical utilisation of a therapeutic agent (Walsh et al., 2014). Bitter taste research is routinely carried out in animals, human assessment panels and recently also using the electronic tongue (Anand et al., 2007; Palmer et al., 2013; Rudnitskaya et al., 2013; Soto et al., 2015). However, animal studies have their limitations regarding the experimental approaches that are available, cost and ethical concerns. Furthermore, human panels rely on the subjective response to tastants, are expensive to run (Cram et al., 2009; Gittings et al., 2014) and can only test substances with a known safety profile. In addition, there are also ethical concerns with the use of healthy volunteers in taste panel studies when assessing cytotoxic drugs, and this is particularly important when studying substances for paediatric use (Mennella et al., 2013). Finally, the electronic tongue based on electrochemical sensors is able to detect a range of substances with varying intensities, (Anand et al., 2007). However, this system cannot analyse compounds that are not fully soluble or assess their aversiveness (Anand et al., 2007). The development of a tastant assessment system for the identification of the acute aversive effects of novel chemical entities may impact

on the development of pharmaceuticals and would be of considerable interest to medical research and industry.

The accepted model of bitter taste transduction is solely based on TAS2Rs (Meyerhof et al., 2010) and is not sufficient to fully explain bitter taste transduction. For instance, the one hypothesised mechanism for gustducin-independent bitter taste transduction is through ion channel (K^+) interaction with certain bitter compounds (e.g. denatonium), which is comparable to the ion channel interaction for the transduction of sour and salty stimuli (Gulbransen et al., 2008; Spielman, 1998; Wong et al., 1996). Thus, further investigation is needed to identify possible additional TAS2R-independent molecular mechanisms responsible for the transduction of bitter taste.

The results obtained from these studies are very promising as they suggest that *D. discoideum* has the potential to be used for early screening of bitter and pungent taste liability in candidate drugs (Cocorocchio et al., 2015; Otto et al., 2016). Although it is unlikely that *D. discoideum* will be used independently of animal and human models, it may provide an additional, early screen for the prediction of bitter tastants. Thus, providing a broad set of compounds are screened, and derived data are comparable to the rat BATA assay, this model might be used to decide which NCEs would go on to be studied in BATA assay and which would be discontinued. Furthermore, this assay could also be used for the identification and development of strongly bitter compounds to be combined with toxic products that are not intended for human use.

4.3 Employing *D. discoideum* for the investigation of structure-activity relationship of curcumin and congeners

4.3.1 Critical evaluation of the methodology used

In the third and final part of the project, the effects of the bitter tastant curcumin (Hewlings and Kalman, 2017) and a family of structurally related natural and synthetic analogues were examined on *D. discoideum*, using the methodology developed in the first study (Otto et al., 2016). This investigation was extended into a quantitative structure-activity-relationship study in which the effects of curcumin and its derivatives were assessed in cell growth and development, and potential molecular targets for these compounds were investigated (Cocorocchio et al., 2018). The concentrations of curcumin and its derivatives employed in this study were smaller than the concentrations used *in vivo* and *in vitro* studies (Table 1).

Compounds	***In vivo***	***D. discoideum***
Curcumin	180 – 390 mM (Dadhaniya et al., 2011; Marczylo et al., 2007)	0.5 – 100 µM
DMC	35 mM (Agrawal et al., 2012; Boonrao et al., 2010)	2.5 – 35 µM
BDMC	39 mM (Agrawal et al., 2012; Boonrao et al., 2010)	1 – 40 µM
THC	13 mM – 33 mM (Han et al., 2016; Jearapong et al., 2015; Yoysungnoen et al., 2015)	5 – 100 µM
FLLL31	59 µM – 2.7 mM (Yuan et al., 2014)	5 – 100 µM
EF24	16 – 803 µM (Bisht et al., 2016; Kasinski et al., 2008)	0.1 – 6 µM
UBS109	1.7 mM – 13 mM (Nagaraju et al., 2013; Yamaguchi et al., 2014)	0.25 – 35 µM
CuPy	210 µM (Ahsan et al., 2015)	100 µM

Table 1 Concentrations of natural and artificial curcumin derivatives used *in vivo*, *in vitro* and in *D. discoideum*.

All curcumin-related compounds were able to inhibit protrusion formation in a concentration-dependent manner. Therefore, a non-linear regression analysis was used to calculate the shape and the IC_{50} values of the concentration-response curves using data from a concentration range (no effect to complete inhibition) with at least three replicates. Moreover, the effects of curcumin and its derivatives were investigated on cellular proliferation, where cells were grown in still culture at a range of concentrations, from no effect to complete growth inhibition (Garige and Walters, 2015). The concentrations used in the growth assays were higher as compared to those employed in the random cell movement assays. For instance, curcumin concentrations in random cell movement ranged from 0 to 5 µM and with an IC_{50} of 2.3 µM, whereas in the growth assays curcumin concentrations ranged from 0 to 100 µM, with an IC_{50} of 45.7 µM. All measurements were carried out at least in triplicate, and the slope of the graph during exponential growth of each concentration was used to calculate the IC_{50}. This assay may be improved by using automated counting equipment to increase the speed, accuracy, precision and potential user bias of the assay (Stone et al., 2009).

REMI mutant screens were carried out to investigate the molecular targets of curcumin and its derivatives and successfully led to the identification and isolation of several mutants resistant to growth inhibitory effects of these compounds (Kuspa, 2006). REMI is a consolidated method used to generate mutations by random insertion of linearised plasmid DNA into the genome of the organism that leads to gene ablation. However, this approach has some limitations. Firstly, should the primary target for the compounds be vital, a null mutant would not survive and thus not be identified (Journet et al., 2012). Secondly, the random mutagenesis may not cover the entire genome and therefore all the genes without

a cut site within the open reading frame, necessary for integration of the knockout vector, would not be found (Guerin and Larochelle, 2002). Thirdly, this approach would be unlikely to identify mutants lacking proteins that are encoded by multiple genes (e.g. actin), since ablation of one gene would not affect the remaining copies of the targeted protein. Finally, this approach may not be able to identify cells that are more sensitive to the tested compounds, as those will be lost during the screen. To overcome several of these limitations, REMI mutagenesis can be employed to generate large pools of mutants, following compound treatment at a concentration below complete inhibition, to enrich the frequency of partially resistant mutants. Analysis of this pools using Next Generation Sequencing (NGS) would then allow the identification of disrupted genes. Each mutant would possess a unique sequence tag (or “barcode”) providing a quantitative measure of the relative abundance of that mutant. Therefore, when the pool of mutants is subjected to selection conditions, mutants that increase or decrease their frequency can be identified by changes in barcode read counts. Nevertheless, the mutant screening carried out in this study has proven to be a valuable tool for the identification of a number of potentially relevant targets (Cocorocchio et al., 2018). Athough two curcumin and related compound targets were identified and analysed in detail in this study, a range of other targets were also found. These mutants included insertions into:

- LMBR1 family protein (DDB_G0281669);
- NADPH-cytochrome-P450 oxidoreductase (DDB_G0293904);
- Type A von Willebrand factor (VWFA) domain-containing protein (DDB_G0288103);
- Translocon-associated protein TRAP gamma subunit (DDB_G0267524);
- EGF-like domain-containing protein (DDB_G0289907);

- UNC93-like protein MFSD11(DDB_G0291722);
- Dihydropteridine reductase (DDB_G0272684);
- AAA ATPase domain-containing protein (DDB_G0276169);
- LRRK family protein kinase Roco6 (DDB_G0279417);
- Glycine cleavage system H-protein (DDB_G0287861).

Characterisation of these mutants could also lead to the identification of novel mechanisms through which curcumin and structurally related derivatives may function (Cocorocchio et al., 2018). The study identified a range of molecular targets through which curcumin and its derivatives may function but, due to time limitations, these were not validated.

The REMI screen allowed the identification of two mutants, psrA$^-$ and psenB$^-$, resistant to both curcumin and the synthetic curcuminoid EF24. Mutants were identified using iPCR to isolate flanking DNA sequences of the insertional cassette and to determine the DNA sequence; fragments were amplified and then sequenced. Subsequently, BLAST searches were performed to identify the disrupted gene(s). The recapitulated null mutants, psrA$^-$ (Lee et al., 2008) and psenB$^-$ (Ludtmann et al., 2014), were then assessed using a growth inhibition assay. Results from these experiments showed that psrA$^-$ is significantly resistant to curcumin as well as EF24 and DMC, compared to the parent strain, suggesting that these compounds might work through similar pathways. Therefore, the studied derivatives could be investigated as an alternative to curcumin to target the psrA protein. In addition, results also show that psrA$^-$ is resistant to THC, but the difference was not statistically significant. Therefore, increasing the number of experimental repeats may provide a significant difference, confirming that this compound may also work through the same pathway. Moreover, the psenB$^-$

mutant showed resistance to EF24 as well as UBS109, suggesting that both may work through a similar mechanism of action.

Development assays were carried out to evaluate whether curcumin and related compounds were able to inhibit cell development. Curcumin, DMC, BDMC, THC and FLLL31 all blocked the development process, leading to a similar cell morphology, characterised by the formation of tipped mounds; whereas, cells exposed to EF24, UBS109 and CuPy were able to form fruiting bodies. Based upon this common phenotype, it is possible to speculate that these active compounds may have a single target involved in development. For instance, it may be possible to identify this target by employing a development screen, plating a mutant library as single colonies in the presence of each compound (Kuspa and Loomis, 1992). Mutants able to overcome this developmental block can be identified, characterised, and assessed for resistance to the other curcumin-related compounds. Therefore, additional development assays should be carried out exposing the psrA$^-$ mutant to curcumin to assess whether this protein is involved in the fruiting body formation process.

Amongst many cellular roles that have been ascribed to curcumin, its ability to function as either an antioxidant or a pro-oxidant is one of the most well-known properties (Ak and Gülçin, 2008; Thayyullathil et al., 2008). Therefore, experiments were carried out to evaluate the antioxidant properties of curcumin and its derivatives. Here, the ferric reducing activity of plasma (FRAP) assay was used to evaluate the antioxidant power of curcumin and its derivatives. Each molecule possesses a different potency measured in absorbance at 595 nm (THC 103%, ASC 100%, CuPy 75%, Curcumin 56%, DMC 33%, BDMC 13%, FLLL31 3%, EF24 1.6% and UBS109 0%). The activity of the compounds in acute cell behaviour, growth inhibition and development did not reproduce this order of

potency, suggesting that anti-oxidant activity may not control any of these cellular roles. It has been demonstrated that the diketone and methoxy groups are responsible for the antioxidant activity of curcumin and its derivatives (Anand et al., 2008), further validating recent findings (Cocorocchio et al., 2018). However, the FRAP assay cannot evaluate the intracellular antioxidant activity of the compounds, which may regulate the intracellular production of reactive oxygen species. Therefore, additional experiments that measure the ability of the molecules to inhibit the production of reactive oxygen species intracellularly should be carried out. These experiments include the use of Dichlorodihydrofluorescein diacetate, which is an indicator of peroxide and anion superoxides (Garige and Walters, 2015), as well as Dihydroethidium (DHE) assay for the measurement of intracellular superoxide production (Zhang and Soldati, 2013). Molecular docking prediction was used to assess the interaction between curcumin and $psrA^-$ and showed that curcumin binds $psrA^-$ in the region of interaction formed between the intra-repeat loops of the scaffolding A-subunit and the convex side of the regulatory B56 subunit (Cho and Xu, 2007). Although this hypothesis was based on human protein modelling simulation, these results could suggest a novel mechanism through which curcumin functions in regulating PP2A. To further validate this hypothesis, the precise amino acid sequence of the site of interaction with curcumin should be determined using site-directed mutagenesis, which should confer resistance to curcumin.

4.3.2 Critical evaluation of *D. discoideum* as a model for the characterisation of the cellular effects and targets of curcumin and related compounds

Curcumin has been used for thousands of years as medicine to treat human ailments, such as inflammatory diseases and wounds, and there are several published papers documenting its wide range of cellular effects (Aggarwal and Harikumar, 2009; Butler, 2004; Ghosh et al., 2015; Gurib-Fakim, 2006; Newman and Cragg, 2007). Studies have also demonstrated that curcumin has many properties including anti-oxidant, anti-cancer, neuroprotection and anti-inflammatory (Chougala et al., 2012; Ghosh et al., 2015; Jurenka, 2009; Lakey-Beitia et al., 2017a; McClure et al., 2017; Srinivasan, 1972; Tang and Taghibiglou, 2017; Yang et al., 2017; Zhang et al., 2013b). Despite all the research conducted on this phytopharmaceutical, there is no agreement on curcumin's mechanism(s) of action (Ghosh et al., 2015). Nevertheless, curcumin continues to be widely studied and its therapeutic relevance has been proven by several clinical trials (Lopresti et al., 2014; Panahi et al., 2015; Panahi et al., 2017). Therefore, there is a need to develop new approaches for the investigation of its mechanisms of action. In addition, curcumin has some limitations due to its poor bioavailability, low solubility and rapid metabolism (Gupta et al., 2013). Hence, the identification of novel curcumin analogues could lead to more potent and efficacious therapeutic agents. Most importantly, new treatments using curcumin and congeners may help to treat a wide range of conditions such as cancer, neurodegenerative diseases and inflammation.

In conclusion, this study identifies distinct molecular targets of curcumin in regulating different cellular functions such as cell movement, growth and multicellular development in *D. discoideum* (Cocorocchio et al., 2018).

Furthermore, with the methodology employed in this study, results have shown the dependency of specific functional groups of the compound for enhanced or reduced activity in movement and growth assays. Results obtained here also suggest that curcumin has multiple molecular targets in *D. discoideum*, which play distinct cellular roles. Moreover, a genetic screen was carried out, which led to the identification of presenilin and protein phosphatase 2A (PP2A) as potential targets of curcumin activities. Computer docking analysis identified a potential binding site of curcumin and derivatives on PP2A and described the differing efficacies of these molecules based on binding. However, the sites of action of curcumin identified here using *D. discoideum* need to be investigated in mammalian cells before assessing the therapeutic potential of curcumin and its analogues *in vivo*.

Chapter V

Conclusions

This work aimed to develop *D. discoideum* as an animal reduction model for the characterisation of natural and synthetic tastants as their activites at a cellular level and mechanisms of taste are not fully understood. The unpleasant taste of medicines represents a central aspect in the development of oral drugs, as it can affect considerably patient compliance and in turn the progress of NCEs. Hence, the investigation of the biology of tastants and the prediction of their taste are essential requirements in the drug development process. Taste research mainly employs animal models and human assessment panels. However, these studies have their limitations concerning cost and ethical issues and, more importantly, preclude an improved understanding of the molecular mechanisms of tastant function. The results obtained in this study demonstrate that *D. discoideum* has the potential to be used as an early screening platform for bitter tastant research since data show significant correlation with animal and human models. Hence, this single cell analysis could be integrated into the early compound screening conducted by pharmaceutical companies. This methodology could be useful for the elimination of potentially aversive substances but also for the identification and development of bitter compounds to increase the safety of toxic products that are not intended for human use. This thesis also describes that *D. discoideum* can be used to identify and characterise molecular mechanisms of tastants, demonstrated by curcumin and related compounds. For the first time, data show distinct molecular targets of the bitter compound curcumin in regulating different

cellular functions such as cell movement, growth and multicellular development in *D. discoideum*. Furthermore, it has been demonstrated that specific functional groups in the compounds are responsible for increased or reduced potency in blocking cell movement and growth. Genetic screening led to the identification of protein phosphatase 2A (PP2A) and presenilin as potential targets of curcumin activity. Finally, this work further validates *D. discoideum* as an excellent organism for rapid identification of cellular drug targets, providing valuable support for the development of novel treatments.

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