

Dictyostelium discoideum as a pharmacological model system to study the mechanisms of medicinal drugs and natural products

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ABSTRACT Developing novel compounds for the treatment of diseases remains one of the highest priorities in biomedical research, where it is critical to identify their targets and how they work at a cellular level. Most studies in this area employ mammalian models, since rodents or non-human primates are seen as a good approximation for humans. However, using mammalian models can be problematic for a range of reasons, including high genetic redundancy and the essential role for many proteins in development. More importantly, it is very difficult to identify how compounds function at a cellular or molecular level in these models without a previously suggested mechanism or target. So how can we identify targets of medicinal compounds? In this review we outline the use of an innovative and tractable model system, *Dictyostelium discoideum*, to provide useful insight to the cellular and molecular functions of both therapeutic drugs and pharmacologically active natural products. We outline the advantages of using this model, and then provide a range of exemplar studies using *D. discoideum* in pharmacological research to demonstrate breakthroughs in understanding the action and effects of compounds, and the subsequent translational of these advances to mammalian models leading to potential improvements in societal health.

KEY WORDS: *mechanism of action, natural product, drug discovery, pharmacogenomics, pharmacology*

Introduction

Dictyostelium discoideum is a soil-living eukaryotic amoeba that was first described in 1935. *D. discoideum* employs two mutually exclusive life cycles – initially under optimal nutrient conditions existing in a unicellular state consuming bacteria as a food source and multiplying via mitotic cell division (Li and Purugganan, 2011). However, under starvation conditions, cells enter a development life cycle, where they migrate together and form multicellular structures called fruiting bodies. During this aggregation phase, cells initiate a process of differentiation where two distinct cell types are formed.


In the following sections we will introduce *D. discoideum* as a pharmacological research model. We will highlight the characteristics of the model that facilitate its use in this research area, including specific methodologies and approaches that are rapidly employable in the system. We will then outline a range of studies, relating to both pharmaceutical drugs and potential therapeutic natural products, where *D. discoideum* has been used as a research model to advance our understanding of their cellular mechanisms

and targets. We will also outline subsequent translational work to validate these discoveries in mammalian models or in humans.

Pharmacological model systems and the social amoeba *D. discoideum*

The field of pharmacology focuses on understanding the action of drugs (and natural products) at a molecular, cellular, or organ-

Abbreviations used in this paper: AplD, amoebapore-like protein D; DAG, diacylglycerol; DGKA, diacylglycerol kinase; EGCG, epigallocatechin gallate; ERK1/ERK2, extracellular signal-regulated kinase 1/2; GSK3, glycogen synthase kinase 3; MCT, medium-chain triglyceride; MAPK, mitogen-activated protein kinase; MDCK, Madin-Darby Canine Kidney; mTOR, mechanistic target of rapamycin; PIP₃, phosphoinositide trisphosphate; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PKD2, polycystic kidney disease 2; PPK1, polyphosphate kinase 1; PP2A, protein phosphatase 2A; psrA, regulatory subunit of PP2A; QSAR, quantitative structure-activity relationship; SAR, structure-activity relationship; S-1-P, sphingosine-1-phosphate; TPC, two-pore channel; TRPP2, transient receptor potential cation channel; VPA, valproic acid.

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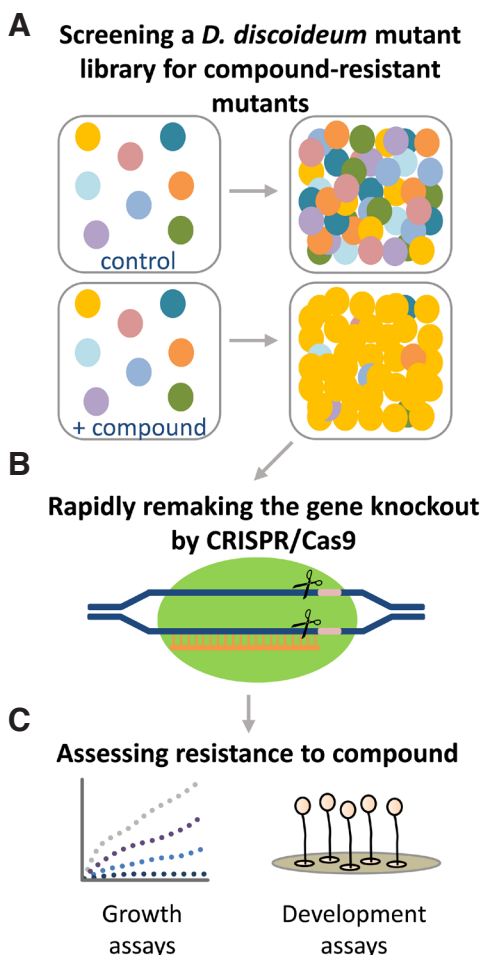


Fig. 1. *D. discoideum* as a pharmacological model. Identifying molecular mechanisms and potential primary targets of medicinal drugs or bioactive natural compounds in *D. discoideum* can employ a standard experimental workflow. **(A)** Providing a compound reduces cell growth at physiological relevant concentrations, a library of mutant cells (represented by different coloured circles) can be grown in the presence of the compound. Resistant mutants (yellow) can be isolated and the mutated gene responsible for the resistance can be rapidly identified. **(B)** The target gene must be ablated in wild type cells, through homologous recombination of a knockout cassette or through CRISPR/Cas9 (pictured). **(C)** The resistant phenotype can then be confirmed, using growth or development assays.

ismal level, and the development of new therapeutic treatments. Research in this area is especially relevant for the treatment of diseases, including for example cancer, epilepsy and Alzheimer's disease where current treatments may not be effective (Laugsand *et al.*, 2011, Yiannopoulou and Papageorgiou, 2013), or may give rise to resistance or cause severe adverse effects (Gaitatzis and Sander, 2013, Kelland, 2007). Thus, understanding the mechanisms of action of current treatments, and the development of new medications remains a research priority. Although animal models provide the industry standard for these studies, it remains highly problematic to identify a drug target in these models without some prior insight to specific targets or mechanisms. Animal-based research approaches are also complicated by the innate complexity of mammalian systems with multiple variants of many proteins

present in widely ranging cell types, and due to metabolism and pharmacokinetics considerations. Thus, new and innovative approaches are needed to help identify drug targets and mechanisms.

Non-animal models have long been employed as innovative, fast, and cost-effective systems for research, often providing fundamental breakthroughs in understanding cellular functions in mammalian systems. These systems traditionally include yeast and fungi, but a range of recent studies have illustrated that *D. discoideum* can now be added to this list (Muller-Taubenberger *et al.*, 2013, Williams *et al.*, 2006). A wide variety of characteristics support the use of *D. discoideum* as an advantageous model for this research. Phylogenetic analysis indicates that *D. discoideum* is more closely related to animals than to plants or bacteria, and contains many orthologues of human proteins that have been lost in yeast (Eichinger *et al.*, 2005), including a range of proteins linked to disease-related processes (Muller-Taubenberger *et al.*, 2013, Williams *et al.*, 2006). Thus, the simple compact genome of *D. discoideum*, with less functional redundancy compared to mammalian cells, provides a useful model for research. In addition, a range of experimental procedures are available in *D. discoideum* that are difficult or impossible in mammalian systems. The haploid nature of *D. discoideum* has allowed the creation of genome wide mutant libraries, which contain thousands of individual mutants having each lost a single gene (Paschke *et al.*, 2018). These libraries can be used to identify mutants with reduced sensitivity to medicinal compounds (Fig. 1), thus suggesting that the encoded proteins are either potential targets or function to revert key changes in drug-sensitive signalling pathways. Mutants that are resistant to a specific compound can be rapidly recapitulated by using either homologous integration of knockout cassettes (Paschke *et al.*, 2018) or CRISPR/Cas9 techniques (Sekine *et al.*, 2018). In these cases, cell growth can provide an effective and rapid means to measuring sensitivity to a compound. Alternately, *D. discoideum* undergoes a simple development cycle upon starvation, where a set of discrete proteins control aggregation and formation of a fruiting body, and this process can also be used to rapidly test compound effects and mutant resistance (Cocorocchio *et al.*, 2018, Kelly *et al.*, 2019). In addition, both the function and the cellular location of identified proteins can be characterised by generating *D. discoideum* mutant cell lines overexpressing the ablated gene or the orthologous human gene (Fischer *et al.*, 2004, Ludtmann *et al.*, 2014, Sekine *et al.*, 2018). On a cellular level *D. discoideum* growth, movement and cell shape can also be used as a rapid readout system to analyse the effects of a compound (Cocorocchio *et al.*, 2018, Robery *et al.*, 2011, Robery *et al.*, 2013). Thus, through identifying and analysing *D. discoideum* mutants lacking potential target proteins, the role of drugs or bioactive natural products can be rapidly explored at a cellular level.

Pharmacological drug analysis and drug discovery

Modern pharmaceutical drug development has tended to focus on the development of novel chemical structures, found through screening libraries of compounds or through iterative cycles of chemical modification and testing (Taylor, 2015). Resulting compounds are thus novel chemicals with efficacy against a known target or for regulating a disease-associated effect. Through this process, it is regularly assumed that the actions of the new drugs

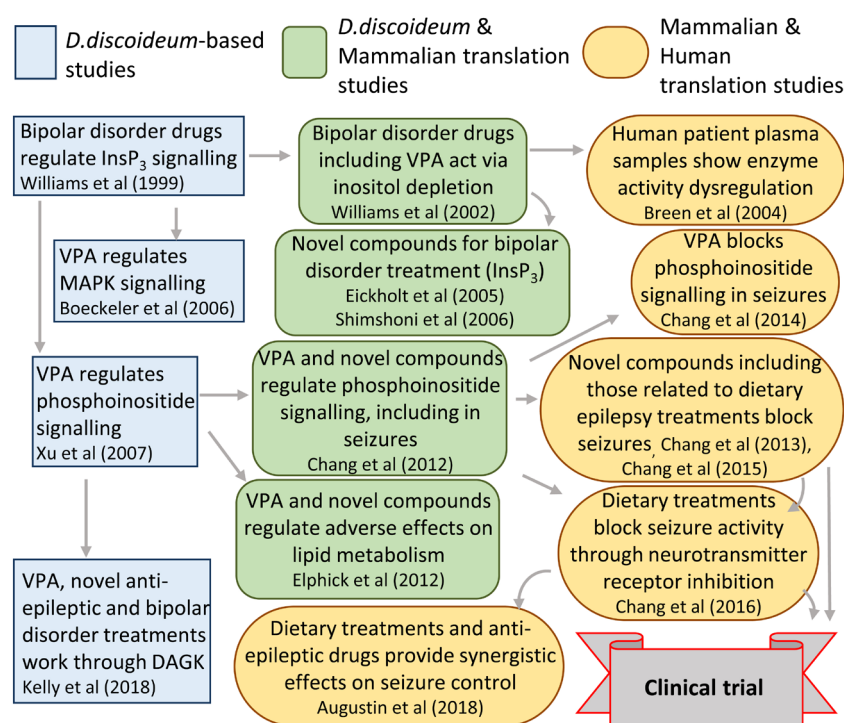


Fig. 2. The use of *D. discoideum* in bipolar disorder and epilepsy research. A range of studies, originating in *D. discoideum* (blue) have investigated the cellular and molecular mechanisms of drugs used as bipolar disorder and epilepsy treatments, including valproic acid (VPA). Some of these studies have led to further research in both *D. discoideum* and animal models (green) to validate drug mechanisms and to identify novel compounds with the potential of therapeutic use. Further studies, in animal models or with human tissue samples (yellow) have further validated these mechanisms and seizure control in pre-clinical models. Currently, this work has led to one clinical trial (grey banner). Relevant papers are cited for each study.

are very specific, and an understanding of potential secondary target effects often remain unknown. Surprisingly, in some cases and despite years of research, the primary drug targets is also unclear. The following sections describe the use of *D. discoideum* as a pharmacological model to study the molecular targets and mechanisms of known drugs and the use of this knowledge to identify potential novel drugs.

Bipolar disorder research in a social amoeba

D. discoideum has been used extensively as a model to investigate therapeutic mechanisms of bipolar disorder treatments. Bipolar disorder is a common neuropsychiatric condition characterised by dramatic changes in mood with potentially devastating impact on individuals and their quality of life, with incidence reports suggesting that 4.4 % of the adult U.S. population experience this illness once in their lives (NIMH, 2017). Current treatment involves the life-long use of moodstabilizing drugs, where the mechanism of action of these treatments remains unclear. Some moodstabilizing drugs, such as lithium and valproic acid (VPA), have been suggested to inhibit glycogen synthase kinase 3 (GSK3) activity (Klein and Melton, 1996; Chen *et al.*, 1999) or alternatively function through the depletion of inositol-containing compounds (the “inositol depletion theory”) (Berridge *et al.*, 1989).

The use of *D. discoideum* as a pharmacological model provided strong evidence that bipolar disorder treatments function through inositol depletion by reducing inositol phosphate signalling (Fig. 2) (Williams *et al.*, 2002, Williams *et al.*, 1999). To arrive at this conclusion, initial experiments were performed that showed that both lithium and VPA blocked the development cycle of *D. discoideum*, suggesting the presence of pharmacological targets in this model. Searching for these targets by screening a pool of insertional mutants, identified a lithium-resistant mutant with an inactivated gene encoding the enzyme prolyl oligopeptidase.

The encoded enzyme has been linked to patients with bipolar disorder (Breen *et al.*, 2004, Williams, 2005). Further studies in *D. discoideum* showed that loss of prolyl oligopeptidase elevated inositol trisphosphate levels to overcome the reduction caused by lithium, thus linking a potential marker for bipolar disorder with a pharmacological mechanism of inositol depletion. This mechanism was then successfully translated to primary mammalian neurons, where lithium, VPA and a third bipolar disorder treatment (carbamazepine) commonly caused an enlargement of the neuronal growth cones, and this effect was reversed by co-treatment with inositol or by inhibiting the mammalian prolyl oligopeptidase (Williams *et al.*, 2002). This discovery also enabled *D. discoideum* to be used to screen for novel chemicals related to VPA that retain this effect of inositol trisphosphate depletion, but were likely to lack serious side effects of VPA, providing a potential new treatment for bipolar disorder (Eickholt *et al.*, 2005, Shimshoni *et al.*, 2007).

Recent studies in *D. discoideum* have provided new mechanistic insight to VPA function linking both epilepsy and bipolar disorder treatment mechanism (Kelly *et al.*, 2018) (Fig. 2). This study focused on the diacylglycerol kinase enzyme (DGKA) that functions in the phosphorylation of diacylglycerol (DAG) to produce phosphatidic acid in the phosphoinositide salvage pathway (Berridge, 2016), and thus is involved in inositol recycling. In *D. discoideum*, loss of this protein reduced the rapid changes caused by VPA on cell movement and on the inhibition of development caused by both VPA and lithium (Kelly *et al.*, 2018). These outcomes suggested a key role for this protein as a regulator of bipolar disorder drugs at a cellular level. The study went on to employ a structure-activity relationship (SAR) approach, where the loss-of-function mutant was shown to be resistant to a range of potential treatments for epilepsy and bipolar disorder (Augustin *et al.*, 2018a, Chang *et al.*, 2012, Chang *et al.*, 2013, Kelly *et al.*, 2018), suggesting that these compounds may therefore function through the same pathway. In

addition, several mammalian and human-based studies have linked DGKA function with epilepsy (Leach *et al.*, 2007, Rodriguez de Turco *et al.*, 2001) and bipolar disorder (Baum *et al.*, 2007, Moya *et al.*, 2010). Furthermore, DAG acts to regulate protein kinase C (PKC) activity that is also elevated during manic episodes (Wang and Friedman, 1996), and lithium is known to increase DAG levels (Brami *et al.*, 1993). Thus, the molecular mechanism of VPA may be evolutionarily conserved from *D. discoideum* to mammals, through DGKA-dependent signalling. These studies have therefore provided key insights into the molecular mechanism of VPA and have proposed DGKA as a new therapeutically-relevant drug target.

Epilepsy research and new therapeutic approaches: from amoeba to humans

The World Health Organization classifies VPA as an essential anti-epileptic medication (WHO, 2017), for the approximately 50 million people worldwide who experience epilepsy. VPA was serendipitously discovered to have anti-convulsant effects in 1963 (Terbach and Williams, 2009), and despite its efficacy and long-term use, its mechanism of action remains to be confirmed. Unfortunately, VPA has several severe side effects, such as hepatotoxicity, pancreatitis and teratogenicity that limit its use (Stadelmaier *et al.*, 2017, Terbach and Williams, 2009). Thus, understanding its underlying molecular mechanisms may help to develop more effective and safer treatments.

D. discoideum has been widely used to explore potential anti-convulsant mechanisms of VPA to identify phosphoinositide signalling as a target (Fig. 2). Initial studies in *D. discoideum*, which focused on analysing VPA-induced biochemical changes that are related to epilepsy treatment, showed that VPA rapidly reduced directional cell movement (chemotaxis) by inhibiting phosphoinositide signalling (Chang *et al.*, 2012, Xu *et al.*, 2007). This observation led to the translation of this effect to mammalian models, where radioactively-labelled inositol was used to monitor phosphoinositides in primary rat neurons, and seizure-like activity was induced, followed by VPA treatment. From this study, seizure activity was found to reduce phosphatidylinositol trisphosphate (PIP₃), and this effect was blocked by VPA (Chang *et al.*, 2014). This effect was verified using *ex vivo* and *in vivo* seizure models, providing strong evidence for this mechanism in the brain.

The VPA-dependent effect of phosphoinositide signalling in *D. discoideum* was also used leading to the identification of potential new anti-epileptic drugs (Chang *et al.*, 2012). Here, more than 100 novel compounds were analysed for inhibitory effects on phosphoinositide signalling to identify novel structures showing increased potency over VPA. From this screen, a new family of active compounds were identified containing branched chain derivatives of medium-chain fatty acids with an eight (octanoic acid) or nine carbon (nonanoic acid) backbone. Several of these compounds were then shown to be effective in seizure control in both *in vitro* and *in vivo* mammalian models (Chang *et al.*, 2016, Chang *et al.*, 2012, Chang *et al.*, 2013, Chang *et al.*, 2015), and as neuroprotective agents (Chang *et al.*, 2015), validating this mechanism of drug discovery. Related compounds are still being developed for ultimate assessment in clinical trials.

Another compound identified in the *D. discoideum*-based anti-epileptic drug screen was decanoic acid (Chang *et al.*, 2012) (Fig. 2), a key component of a therapeutic diet used to treat patients with drug resistant epilepsy (Augustin *et al.*, 2018a, Neal *et al.*,

2009). Dietary treatments for epilepsy are important, since approximately 30% of patients are resistant to anti-epileptic drugs, and specialised diets remain a key approach to treat these individuals (D'Andrea Meira *et al.*, 2019). These diets, commonly called 'ketogenic' diets, involve a low carbohydrate and protein intake, supplemented by long chain fatty acids (in a classical ketogenic diet) or medium-chain fatty acids (in a medium-chain triglyceride (MCT) ketogenic diet) (Augustin *et al.*, 2018a, D'Andrea Meira *et al.*, 2019, Warren *et al.*, 2018). These diets have traditionally been thought to function through the breakdown of fatty acids to produce chemicals called ketones that were thought to be the therapeutically active agents, although evidence for this mechanism remains limited (Augustin *et al.*, 2018a). The identification of decanoic acid in the *D. discoideum* anti-epileptic screen suggested that this medium-chain fatty acid may provide direct seizure control independent of its breakdown to ketones (Chang *et al.*, 2012). This was subsequently validated in multiple *in vitro* seizure models (Chang *et al.*, 2016, Chang *et al.*, 2012, Chang *et al.*, 2013, Chang *et al.*, 2015). Subsequent experiments in mammalian models have shown that decanoic acid directly inhibits a key neurotransmitter receptor and thereby suppresses excitatory neurotransmission (Chang *et al.*, 2016) involved in seizure activity (Augustin *et al.*, 2018a, Augustin *et al.*, 2018b, Chang *et al.*, 2016). Since this mechanism is also shared by the pharmaceutical epilepsy treatment perampanel (Patsalos, 2015), further studies showed a synergistic effect of decanoic acid and perampanel on neurotransmission receptor function and seizure control (Augustin *et al.*, 2018b). Finally, a diet that is rich in decanoic acid has anti-convulsant effects in an *in vivo* seizure model (Tan *et al.*, 2016), independent of ketone generation, supporting a distinct role for decanoic acid (rather than ketones) in seizure control. All these studies provide evidence that some medium-chain fatty acids, including decanoic acid, have anti-convulsant properties. The observations made in *D. discoideum* and in mammalian seizure models have been used to develop a novel MCT-based diet and a clinical trial to test the efficiency of this diet in epileptic patients is currently ongoing (Augustin *et al.*, 2018a).

Other studies in *D. discoideum* have also shown that VPA elevates the activity of the mitogen-activated protein kinase (MAPK) signalling pathway (Boeckeler *et al.*, 2006), consistent with observations made in mammalian studies (Hao *et al.*, 2004). This mechanism may explain the neuroprotective effects of VPA. In addition to analysing positive therapeutic effects of VPA, *D. discoideum* has also been used to assess adverse effects of VPA and related analogues, including teratogenicity and hepatotoxicity (Eickholt *et al.*, 2005, Elphick *et al.*, 2012).

Treating cancer: lessons from *D. discoideum*

In 2018, cancer was responsible for approximately 9.6 million deaths worldwide, which makes it the second leading cause of death (WHO, 2018). One of the most common treatments for cancer are chemotherapeutic drugs, such as the platinum-based drug cisplatin (Kelland, 2007). As a cancer treatment, the anti-proliferative effects of cisplatin were accidentally discovered in 1965 and currently platinum-based drugs are used for the treatment of various solid tumours, in particular testicular or ovarian cancer, where it is assumed that they cause cytotoxicity mainly by covalently binding to DNA molecules (Kelland, 2007). Despite their wide use, cisplatin and related drugs have severe side ef-

fects and patients often become drug resistant. Thus, an improved understanding of the mechanism through which cisplatin functions may help to overcome these adverse effects.

D. discoideum has been used to study the molecular mechanisms underlying cisplatin treatment. Studies into this mechanism initially employed a library of *D. discoideum* mutants that were screened for cells showing resistance to the effect of cisplatin. One cisplatin resistant mutant showed loss of sphingosine-1-phosphate (S-1-P) lyase activity (Li *et al.*, 2000). Based on this, it was shown that sensitivity to cisplatin was regulated by the balance between the two lipids, ceramide and S-1-P (Alexander and Alexander, 2011). In these experiments, increasing the ratio of S-1-P to ceramide through ablation of the S-1-P lyase or through overexpression of the sphingosine kinase decreased the sensitivity of *D. discoideum* cells to cisplatin (Li *et al.*, 2000, Min *et al.*, 2005a). In contrast, increasing the ratio of ceramide to S-1-P by overexpressing the S-1-P lyase or by ablating the sphingosine kinase, increased the sensitivity of *D. discoideum* cells to cisplatin (Min *et al.*, 2004, Min *et al.*, 2005a). Thus, these studies proposed a novel mechanism of action for cisplatin as an anti-cancer agent. The pivotal role of sphingolipid metabolism in controlling sensitivity to cisplatin was confirmed in human-derived cell lines by overexpression of sphingolipid metabolism genes, including S-1-P lyase, ceramide synthase 1 and sphingosine kinase 1 (Min *et al.*, 2007, Min *et al.*, 2005b). Overexpression of S-1-P lyase was confirmed in HEK293 or A549 cells to have the same phenotype as in *D. discoideum* – increased sensitivity to cisplatin (Min *et al.*, 2005b). Overexpressing sphingosine kinase 1 in HEK293 cells decreased cisplatin sensitivity, whereas overexpression of sphingosine kinase 2 has the opposite effect (Min *et al.*, 2007). Similarly, overexpressing ceramide synthase 1, but not ceramide synthase 4 and 5, in cell lines enhanced the sensitivity of cells to cisplatin. These data provide important new insights in the cellular mechanisms regulating cisplatin sensitivity.

D. discoideum has also been used in a range of other cancer-related studies. The role of cisplatin on gene expression in the model has been analysed (Van Driessche *et al.*, 2007), in addition to its regulation of mitogen activated protein kinase phosphatases (Moncho-Amor *et al.*, 2011) and in DNA repair pathways (Gunn *et al.*, 2016). The cellular functions of other anti-cancer treatments, such as bestatin and Poly (ADPribose) polymerase inhibitors have also been analysed in *D. discoideum* (Kolb *et al.*, 2017, Poloz *et al.*, 2012). Finally, *Dictyostelium* strains have also been shown to provide a source of novel compounds with anti-proliferative properties that could thus be used as cancer treatments (Honma *et al.*, 2018, Kubohara and Kikuchi, 2018).

Investigating novel anti-microbial drugs using *D. discoideum*

The discovery of novel anti-microbial compounds for the treatment of infectious diseases is very important. In 2010, approximately 15 million people worldwide died from infectious diseases and infection provides the leading cause of death in low income countries (Dye, 2014), therefore efficient approaches to develop novel antibiotics are needed.

D. discoideum has been proposed as a novel model host for virulent bacteria, enabling the identification of compounds with anti-microbial properties. In one series of studies, *D. discoideum* cells grown on lawns comprising a single bacterial species (*Klebsiella pneumoniae*) as the food source form plaques and subsequently aggregate and develop into fruiting bodies. In contrast, plaque

formation and development is impeded by the presence of the virulent strain *Mycobacterium marinum* (a model organism to study tuberculosis infections) or the pathogenic strain *Pseudomonas aeruginosa* PAO1 (Bravo-Toncio *et al.*, 2016, Ouertatani-Sakouhi *et al.*, 2017). Mechanistic insight into this effect was shown when *D. discoideum* was able to form plaques and aggregate in the presence of a *P. aeruginosa* mutant which lacks the polyphosphate kinase 1 (PPK1). Based on this observation, *D. discoideum* was employed as a host to conduct a screen with thirty compounds that could inhibit the *P. aeruginosa* PPK1 and therefore might have anti-microbial properties (Bravo-Toncio *et al.*, 2016). In a similar manner, a high-throughput screen was performed with a large array of compounds to identify those that allow plaque formation of *D. discoideum* in the presence of *M. marinum*, through either directly inhibiting mycobacterial growth or decreasing *Mycobacteria* virulence (Ouertatani-Sakouhi *et al.*, 2017). These *D. discoideum*-based screens, and others that exploited *D. discoideum* in secondary assays to further characterise the function of compounds (Trofimov *et al.*, 2018; Kicka *et al.*, 2014; Harrison *et al.*, 2015; Slepikas *et al.*, 2016), highlight the value of *D. discoideum* as a host model to quickly screen a large number of potential anti-microbial compounds.

Similarly, *D. discoideum* along with macrophages and *Acanthamoeba castellanii* were also used to investigate the effectiveness of a potential anti-*Legionella pneumophila* drug (Harrison *et al.*, 2015). This study successfully demonstrated that an amoeba can be employed to find novel inhibitors of *L. pneumophila* intracellular replication (Harrison *et al.*, 2015). *D. discoideum* was then further used to determine which mutants of *L. pneumophila* had replication defects (Harrison *et al.*, 2013). Thus, *D. discoideum* provides a successful model for virulent bacterial infection and the discovery of new anti-bacterial drugs.

Intriguingly, *D. discoideum* also represents a novel source of anti-microbial compounds. For instance, it has been shown that the *D. discoideum* protein AplD (amoebaporelike protein D) has anti-microbial effects by destroying the integrity of bacterial membranes (Dhakshinamoorthy *et al.*, 2018). In addition, further *D. discoideum*-based screening assays have been developed to screen for compounds that restore the function of impaired versions of the human phenylalanine hydroxylase enzyme (Kim *et al.*, 2015) or for compounds that inhibit chemotaxis of immune or cancer cells (Liao *et al.*, 2016).

Natural product medicines – learning from nature?

Natural compounds have been used for centuries as medicinal remedies (Taylor, 2015). For many of these remedies, detailed analysis has validated the potential therapeutic efficacies of specifically purified components, although how these compounds work at a molecular or cellular level often remains unclear, making ultimate validation significantly more difficult. Thus, using a tractable biological system such as *D. discoideum* can help to define the molecular and cellular effects of natural compounds, enabling the validation of these mechanisms in mammalian models (Muller-Taubenberger *et al.*, 2013, Warren *et al.*, 2018, Williams *et al.*, 2006). The following sections describe the use of *D. discoideum* as a pharmacological model to study the molecular targets and mechanisms of action of bioactive natural products associated with traditional remedies (Fig. 3).

	Curcumin	Naringenin	Epigallocatechin gallate	Theaflavin	Caffeine	Capsaicin	Quinine
Source	Turmeric	Grapefruit	Green tea	Black tea	Coffee, chocolate, tea	Chilli	Cinchona
Effect	Inhibits cell proliferation, motility and development	Inhibits cell proliferation and behaviour	Blocks aggregation, and cell motility	Delays development and inhibits cell proliferation	Blocks cAMP production, inhibits cell motility, induces autophagy	Blocks cell motility and behaviour	Blocks cell motility and behaviour
Target	PSEN B & PSRA signalling	TRPP2, TPC2 and TPC1	Unknown	Unknown	PI3K, mTORC2	RacGEF Proteins: GXCP and GXCKK	Unknown

Fig. 3. Naturally occurring bioactive compounds have been investigated using *D. discoideum*. A range of studies in *D. discoideum* have focused on natural products that have suggested to have positive effects on health or in disease treatment. These compounds have complex and varied chemical structures, and they are derived from a wide-ranging plants. Analysis of each compound using *D. discoideum* has shown effects on cell proliferation, cell motility or behaviour, and development. In several cases, molecular targets or pathways have been proposed. PSEN B, presenilin B protein; psrA, serine/threonine-protein phosphatase 2A regulatory subunit; TPC1/2, two-pore channel 1/2; TRPP2, transient receptor potential cation channel; PI3K, phosphatidylinositol 3-kinase; mTORC2, mechanistic target of rapamycin complex 2; RacGEF, Rho family GTPase guanine nucleotide exchange factor.

The golden spice - medical insights from Turmeric-derived Curcumin

One naturally occurring bioactive compound that has been characterised using *D. discoideum* is the polyphenol curcumin, derived from the spice turmeric (Fig. 3). Turmeric has been used as a treatment for inflammatory disorders for centuries, and curcumin has been proposed as a treatment for many diseases such as heart disease, cancer, inflammatory disorders, allergies, Alzheimer's disease and asthma (Hewlings and Kalman, 2017).

In both *D. discoideum* (Cocorocchio *et al.*, 2018, Garige and Walters, 2015, Swatson *et al.*, 2017) and mammalian systems (Kunnumakkara *et al.*, 2008), curcumin inhibits cellular growth, allowing *D. discoideum* to be used to explore the molecular mechanisms behind this effect. Curcumin delays the chemotactic response towards cyclic AMP (cAMP), blocks the formation of fruiting bodies during starvation, and reduces the expression of genes essential for development (Cocorocchio *et al.*, 2018, Garige and Walters, 2015). To determine which key features of the curcumin molecule were responsible for these effects, multiple curcumin derivatives were tested in a quantitative structure-activity relationship (QSAR) study (Cocorocchio *et al.*, 2018). From this study, specific regions of the chemical structure (e.g. the diketone group and the α,β -unsaturated carbonyl moiety on the seven-carbon linker) were demonstrated to be essential for the effects on acute cell behaviour. To investigate molecular targets for this compound, a genetic screen was also employed to isolate mutants resistant to curcumin during growth. This approach identified that the protein phosphatase 2A (PP2A) regulatory subunit (psrA) and the presenilin B protein controlled sensitivity to both curcumin and

specific analogues during growth. These targets potentially link the use of curcumin with treatments for cancer (Kiely and Kiely, 2015) and Alzheimer's disease (Ludtmann *et al.*, 2014, Sharma *et al.*, 2019), and further translational studies are necessary to continue this research. Another study has also shown that protein kinase A (PKA) contributes to mediating the effects of curcumin on cell proliferation and transcriptional regulation (Swatson *et al.*, 2017). Interestingly, these *D. discoideum* studies suggest that the cellular mechanisms of curcumin are not solely, as assumed by other studies (Hewlings and Kalman, 2017), based on its anti-oxidant and anti-inflammatory properties, but that curcumin instead functions through specific cell signalling mechanisms and transcription effects. Thus, further insights into the cellular and molecular effects of curcumin may indeed lead to the discovery of molecular targets and mechanisms of action for curcumin in health and medicine.

Finding the function of flavonoids

Flavonoids represent a large group of compounds that are often associated with positive health effects and potential medicinal benefits (Panche *et al.*, 2016). Although most of these compounds are thought to function through anti-oxidative and anti-inflammatory roles, many flavonoids have also been implicated in the modulation of key cell signalling functions, and identifying and characterising these targets and mechanisms should help to validate their therapeutic potential.

D. discoideum has been used to investigate the flavonoid naringenin (Fig. 3), a polyphenol compound found in grapefruit, which has been associated with a wide range of health and medical

benefits (Salehi *et al.*, 2019). In *D. discoideum*, naringenin reduces cell growth, blocks cellular movement and causes cells to lose shape following acute (30 min) exposure (Waheed *et al.*, 2014). Employing this growth effect, a *D. discoideum* mutant library was screened to identify naringenin resistant-cells including mutants lacking the transient receptor potential cation channel (TRPP2), which is encoded by the polycystic kidney disease 2 (PKD2) gene (Waheed *et al.*, 2014), suggesting that this protein may be a direct target of naringenin. To validate this target, the effects of the flavonoid on *D. discoideum* cell movement and cell shape were assessed in cells lacking TRPP2, thus confirming a role for TRPP2 in the cellular function of naringenin in this model. Excitingly, mutations in the human TRPP2 orthologue are associated with polycystic kidney disease where patients develop debilitating kidney cysts, suggesting that naringenin may provide a treatment for this disorder. To examine this, a canine kidney cell line (Madin-Darby Canine Kidney - MDCK cells) was induced to form cysts, and naringenin was shown to block cyst formation through a TRPP2-dependent mechanism (Waheed *et al.*, 2014). These data suggest a conserved function of the *D. discoideum* and canine TRPP2 proteins as a target for naringenin. In a subsequent study exploring the effect of naringenin on proteins related to TRPP2, naringenin impaired the activity of the human two-pore channel 1 (TPC1) and two-pore channel 2 (TPC2) proteins (Pafumi *et al.*, 2017). Thus, the discovery that growth inhibitory effects of naringenin in *D. discoideum* are controlled by TRPP2 has identified a specific molecular target of naringenin in mammalian cells with potential therapeutic implications relating to the treatment of polycystic kidney disease.

Two *D. discoideum* studies have examined other flavonoids contained in different forms of tea, such as epigallocatechin gallate (EGCG) and theaflavins. Surprisingly little is known regarding the cellular and molecular mechanisms of these flavonoids. EGCG is one of the most abundant catechins in green tea (59% of total catechin) (Leung *et al.*, 2001), and has potential health benefits in cancer treatments, autoimmune regulation and metabolic disease therapy (Legeay *et al.*, 2015, Rawangkan *et al.*, 2018). Theaflavins are found predominantly in black tea (Leung *et al.*, 2001) and are thought to have neuroprotective, anti-inflammatory (Anandhan *et al.*, 2013) and anti-hyperlipidaemia effects (Imran *et al.*, 2018). In *D. discoideum* (Fig. 3), EGCG inhibits random cell movement and directional movement (chemotaxis), blocking aggregation and stalling development at a loose mound stage (McQuade *et al.*, 2013). Theaflavins also blocked *D. discoideum* growth, and delayed multicellular development by 5 hours in addition to reducing the formation of fruiting bodies (Ilacqua *et al.*, 2017). Although both these studies did not propose cellular targets for the flavonoids, they establish cellular effects for the compounds and suggest molecular targets for both compounds are present in the model.

What is coffee to an amoeba?

Caffeine is a purine alkaloid that is naturally occurring in coffee, tea and chocolate and is one of the most frequently ingested neuroactive compounds in humans (Saiki *et al.*, 2011, Temple *et al.*, 2017). One mechanism of caffeine is through the non-competitive antagonism of adenosine receptors (Fredholm *et al.*, 1999). In *D. discoideum* (Fig. 3), caffeine blocks activation of cAMP synthesis through blocking adenylyl cyclase activity (Theibert and Devreotes, 1983), thereby preventing the extracellular chemoattractant role of

cAMP in addition to its role as an intracellular second messenger (Reymond *et al.*, 1995). A more recent study in *D. discoideum* shows an effect of caffeine through inhibiting pathways downstream of cAMP signalling, including the phosphatidylinositol 3-kinase (PI3K), mTOR Complex 2 (mTORC2), and protein kinase A (PKA) signalling pathways, as well as the extracellular signal-regulated kinases 1/2 (ERK1 and ERK2) (Tariqul Islam *et al.*, 2019). The caffeine-dependent inhibition of the PI3K/mTORC2 pathway has also been demonstrated in mammalian models, where it has been associated with induction of autophagy (Saiki *et al.*, 2011). Finally, in both *D. discoideum* and mammalian neutrophils, caffeine inhibits chemotaxis (Elferink and De Koster, 1995) as well as the migration of tumour cells in the human gut, through antagonising adenosine receptors (Merighi *et al.*, 2007). Therefore, studies of caffeine in *D. discoideum* complement and extend those in mammalian models.

A broad selection of natural products modify D. discoideum behaviour

D. discoideum has been employed in many other studies to assess the effects of natural compounds. For example, one study employed *D. discoideum* as a model organism to quantify the taste quality of bitterness for a range of natural products and drugs. In these studies, *D. discoideum* cell motility was shown to be strongly inhibited by pungent and bitter tastants, such as capsaicin, a constituent of chilli, and quinine hydrochloride; quinine naturally occurs in the cinchona tree and is used as an antimalarial drug (Achan *et al.*, 2011) (Fig. 3) (Cocorocchio *et al.*, 2016, Otto *et al.*, 2016, Robery *et al.*, 2011, Robery *et al.*, 2013). Within these studies, a strong correlation was found between the effects of bitter tastants on *D. discoideum* cell motility and effects in rat and human taste studies, where compounds that were found most potent in *D. discoideum* cell movement assays were also the strongest bitter tastant in rats and humans (Cocorocchio *et al.*, 2016). This provides the possibility of using *D. discoideum* as an early model for testing compounds for their level of bitterness and whether they have a strong bitter taste before they are developed into human drugs (Williams and Andrews, 2019).

Summary

Improving our understanding of the molecular and cellular actions of therapeutic drugs and bioactive natural products will significantly aid the development of more effective and safer medicinal treatments. Identifying the mechanisms of action and direct targets of medicinal compounds in mammalian model systems can be difficult, unless potential targets and mechanisms can be proposed for focused analysis. In recent years, many studies have demonstrated that *D. discoideum* provides a valuable biomedical model for the study of known drugs and bioactive natural products, as well as in the discovery of novel compounds with medicinal effects. These advances have been possible, since *D. discoideum* offers a range of advantageous research techniques, including genetic screening, rapid targeted gene ablation, and assays for acute cell behaviour and chronic developmental effects that can be used as readouts. Numerous *D. discoideum*-based studies indicate that if a therapeutic drug or natural product has an effect on *D. discoideum* at concentrations related to physiological use, the model provides an excellent platform for identifying potential target proteins and mechanisms of action for these compounds.

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