Letter

Advice on Avoiding the Valley of Death: Insights from a 3Rs Model of Aversive and Emetic Compound Identification

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The gap between the development of new 3Rs (replace, reduce, refine) technologies and their implementation into routine use has been called the "Valley of Death"[1]. Reasons for reluctance in adoption of these approaches, particularly by industry, are often complex and may be related to a lack of awareness of, or confidence in, 3Rs approaches. We report from the Valley of Death on a long-term project developing the social amoeba *Dictyostelium discoideum* as an innovative 3Rs alternative to reduce the use of animals for early identification of novel chemical entities (NCEs) with aversive and emetic properties, to provide guidance so others may learn from our experience.

The side-effects of nausea and vomiting are factors that limit drug development or may reduce patient compliance for established treatments (Holmes et al., 2009); identification of emetic effects of candidate drugs utilizes *in vivo* animal studies. A bitter or pungent taste of a medication can also reduce patient compliance particularly in pediatrics (Mennella et al., 2013) and bitter tastants can be nauseagenic (Peyrot des Gachons et al., 2011).

The taste profile of candidate drugs is assessed by the rodent Brief Aversion Taste Assay (BATA) (e.g., Soto et al., 2015). In these experiments, around 12 animals are housed individually, with some confinement, and with water deprivation, where a specially designed apparatus allows the number of licks taken from bottles containing test substance to be measured (Clapham et al., 2012). Lick number provides an estimation of aversive or bitter taste (Soto et al., 2015). The BATA test falls within the European Union legislation 2010/63/EU (EU, 2010) regulating the use of animals in research (e.g., Soto et al., 2015) as it exceeds the threshold for a regulated procedure. Although this method provides the industry standard, it is time consuming and requires animal experimentation as does testing for emetic liability.

Microorganisms were proposed as an early non-animal methodology (NAM) in a tiered approach to identify emetic liability and aversive properties of NCEs (Holmes et al., 2009). The social amoeba *Dictyostelium discoideum* (Fig. 1) was considered particularly attractive due to its use at the time in pharmacogenetic studies (Waheed et al., 2014; Chang et al., 2012; Kelly et al., 2018), its recognition by the US National Institutes of Health as a biomedical model system, and its use as an innovative model in a range of biomedical-related studies (Muller-Taubenberger et al., 2013).

In a series of papers, we reported the utility of *Dictyostelium* as a model for bitter and emetic substance screening. These studies established the utility of *Dictyostelium* to detect emetic agents and bitter tastants (Robery et al., 2011), including proposing novel molecular targets (Robery et al., 2013). They demonstrated that the model responds (cell movement and shape change) in a concentration-dependent manner to bitter tastants occupying a diverse chemical space, and showed a correlation of response data from eleven bitter tastants between *Dictyostelium* and the rat BATA assay, which itself correlated with human taste panel data (Cocorocchio et al., 2016; Otto et al., 2016). Overall, these results support the use of *Dictyostelium* to estimate the probability that an NCE will have a bitter, aversive taste and hence its continued consideration as a potential 3Rs model in the drug discovery and development pipeline. Comparisons between the human taste panel, rodent BATA and *Dictyostelium* for the detection of potentially aversive (particularly bitter tasting) substances shows some of the key features and advantages of each system (Tab. 1). It is unlikely that one system will provide comprehensive data regarding an NCE, but selective combinations of multiple systems, including *Dictyostelium*, may provide a fast, cheap, and animal reduction approach.

Despite the progress made over the last 10 years, much of it in collaboration with the potential end-user (i.e., industry), we have been unable to obtain further support to finally validate (or not)

[1] https://www.nc3rs.org.uk/news/bridging-3rs-valley-death-nc3rs-strategy-2017-2019

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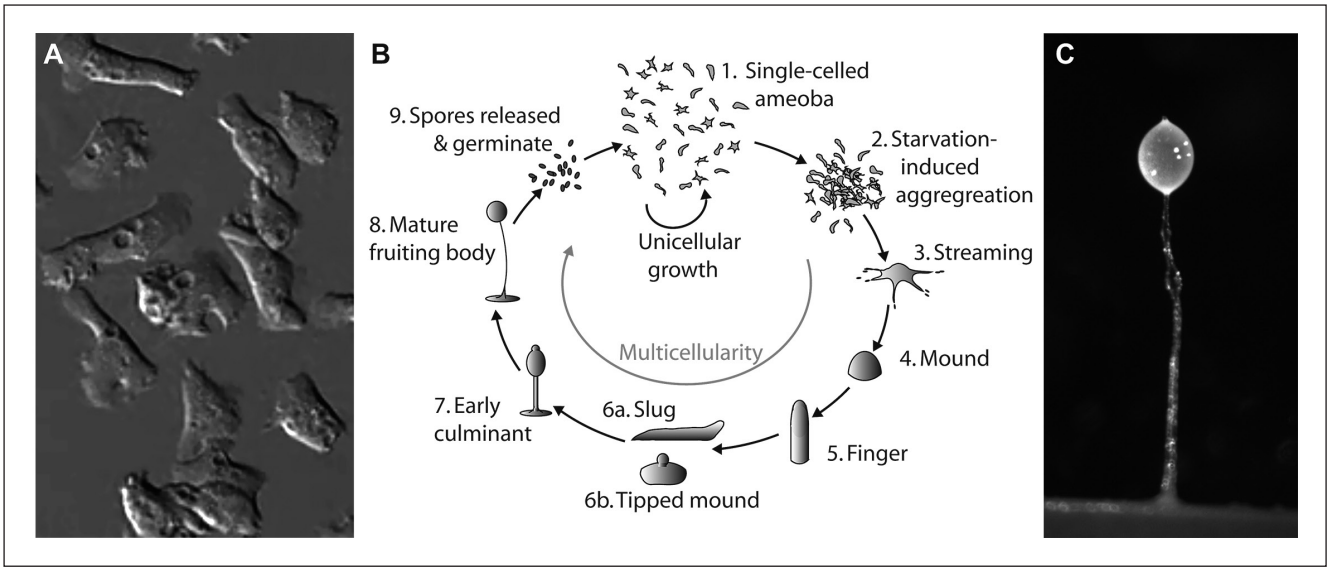

Fig. 1: The social amoeba *Dictyostelium discoideum*

(A) *Dictyostelium* is found in leaf litter of temperate forests, with a single cell's life involving the consumption of microorganisms and division by binary fission. Cells are around 10 µm long, have a structure typical of eukaryotes (with a nucleus, endoplasmic reticulum, and mitochondria), and are haploid. Cells can be grown in the laboratory in nutrient-rich media or in the presence of bacteria as a food source. (B) Following the initiation of starvation, *Dictyostelium* cells progress through a series of stages leading to multicellularity and differentiation in the formation of a mature fruiting body, around 1 mm tall, consisting of a spore head held aloft by a stalk. Spores within this structure are dormant and resistant to dehydration, and upon release germinate to form single-celled amoeba. (C) This development process can be easily reproduced in laboratory conditions, and can be employed as a useful model for development and pharmacogenetic studies.

the *Dictyostelium* assay as a reduction/replacement technology for the BATA assay and exit the Valley of Death. The basis for this lack of support is likely to be multifactorial. Firstly, a single celled organism, without a mouth, gut or nervous system and lacking a range of proteins that may provide a mechanistic target for aversive effects clearly has limited face validity. Secondly, in this area industry seems fundamentally conservative, with a strong focus on maintaining consistent testing using established models. Finally, funding environments remain highly selective, with grant applications often relying upon guidance from experts working in currently established technologies without a drive to supplant these technologies with new approaches, and the smallest criticism can lead to the rejection of a funding application. In an industry setting, significant financial investment in novel technologies without a strategic decision to move into that technology is likely to block the development of new initiatives. Industry commitment to novel 3Rs technologies is sometimes difficult to identify. It is particularly interesting to note that one considered shortcoming of approaches for the development of alternative methods is "*little input from end users*", and that methods were produced that "*did not adequately meet the testing requirements of end users*"[2]. In our study, although we had input from a major industry partner at an early stage in the project to address these points, this did not ensure continued research support to fully assess the potential for industry adoption.

We believe that the barriers we have encountered are not unique and hence the lessons we have learned may have wider applicability; we make some recommendations for researchers looking to develop other NAMs.

1. Develop a relationship with industry partners. Key to implementing a new technology is to address all concerns of the end user, in this case our industrial partner. Thus, listening to their concerns, and addressing these issues is essential for them to ultimately engage with the technology. Relationships with industry can also give access to unpublished animal or human data, enabling comparison with NAM data[2] to facilitate validation. One difficulty with engaging large multinational partners is that it may be hard to find the right person to contact regarding 3Rs-orientated research and ensure that applications for funding reach both the 3Rs coordinator and the decision-maker in the section where the technology will be relevant. In our case, trying to develop a novel technology to improve identification of a potential side effect was not a standard type of novel drug-target or mechanism-related approach regarding a particular therapeutic area. Furthermore, we had developed good working relationships with various industry colleagues, who understood and

[2] ICCVAM (2018). A strategic roadmap for establishing new approaches to evaluate the safety of chemicals and medical products in the United States. doi:10.22427/NTP-ICCVAM-ROADMAP2018

Tab. 1: Comparison between bitter tastant model systems

Parameter	 **Human taste panel**	 **Rat BATA**	 ***Dictyostelium* cell behaviour**
Requires ethical approval/ regulated in EU	Yes	Yes	No
Prior information on *in vivo* toxicity needed	Yes	Yes	No
Throughput capacity	Relatively low	Relatively low	Relatively high
Training required for subjects	Yes	Yes	No
Read out	Perceived sensation and intensity	Licks/unit time	Cell behavior (chemotaxis/cell shape)
Automated data collection and analysis possible	Yes	Yes	Yes
Detects concentration related effects	Yes	Yes	Yes
ED_{50} measurement	Yes	Yes	Yes
Time taken per compound to identify ED_{50}	Hours	Hours/Days	Hours
Face validity	Excellent	Yes, but readout is the response to the taste rather than the sensation	Limited
Construct validity	Yes	Yes	Yes
Predictive validity	Total	Yes, based on relatively limited data	Yes, dependent upon additional validation
Experimental genetic manipulation possible	No	Yes	Yes
Mechanistic studies feasible	Potentially, but complex	Yes, but complex	Yes, relatively straightforward

supported the new technology, but perhaps we should also have focused on developing relationships with those who decide on future research initiatives and subsequent investment.

2. Publish, publish, and publish. Validation of new technologies is considerably strengthened through the peer review process in publishing papers. Publications provide clear evidence of innovation or discovery that has been reviewed independently, increasing trust in the technology. This approach also enhances outreach and supports further funding applications. The journals to publish in may also be worth considering as impact factor may not be as important as access to the target audience, and should this audience be the relevant industry-focused group, or those with specific interest in 3Rs-technology, or a broad readership from all areas of science and society?

3. Network. Develop impact through targeted industry and academic networks. Presenting talks at industry, academic, or government meetings is likely to both improve the potential for engaging industry partners and provide feedback on concerns that still need to be addressed. We gave at least 12 presentations on our *Dictyostelium* research model, including several to predominantly industrial audiences, with subsequent discussions related to the project and funding.

4. Don't give up but recognize the limitations. With the highly competitive state of funding, all potential avenues of support must be investigated to maximize chances of continued investment, so keep looking for alternative mechanisms of support. However, projects such as this, where funding is required at the final step to validate a method for reduction/replacement, are particularly problematic as the final data set (a graph of ID_{50} values for a range of substances in *Dictyostelium vs*. rodent and human data) may look like the pilot data, just with more data points and a better correlation statistic. In addition, the number of animals that would be replaced if *Dictyostelium* exactly matched the predictability of the BATA assay would be relatively small, partially because of repeated use of individual animals. We suspect that a few thousand animals are used globally in the BATA assay but the true number used by industry is impossible to know. In competition with projects developing methods aimed at reducing/replacing a large number of animals in procedures with severity classified as moderate or even severe (particularly involving pain), it seems inevitable that a project aiming to reduce/replace fewer animals in a mild procedure will have a lower priority. Finally, our project focuses on detecting a potential drug side effect rather than investigating disease or drug mechanisms and this may also reduce the priority in competition with other projects.

Thus, following 10 years of research into developing a 3Rs model for screening NCE for emetic and aversive effects, we have arrived in the Valley of Death from which few 3Rs projects seem to emerge. Through highlighting key points in advancing the development of new 3Rs technologies that we have recognized through this time, we hope to help others champion their new technologies into industrial settings.

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Conflict of interest

The authors have no conflicts of interest.

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