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Galleria Mellonella as a model for drug screening of topical steroids against Malassezia Furfur

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ABSTRACT

The process of drug development is a long and laborious system where almost 90% of clinical drug trials end up failing. Therefore, drug screening in the preclinical stage is imperative to ensuring a higher success rate of the newly developed drug. One of the recently proposed model organisms for testing has been the larvae Galleria Mellonella mainly due to its technical and biological advantages. This study investigates the larvae as a potential model for specifically topical treatments against the fungal pathogen Malassezia Furfur. Using an application method, the larvae pre-infected with M.Furfur showed recovery when treated with an antifungal topical treatment. Through this study the G.Mellonella has proven to be an effective model for topical drug testing to conduct an efficient and cost effective testing on a large scale for future pre-clinical research.

Introduction

Drug screening is an imperative process to identify and optimize potential drugs before progressing to clinical trials. Inherently, drug screening models play a vital role in investigating the effectiveness of such antimicrobial drugs against infections. One of the most commonly used models for studying microbial infections is the murine model due to their relatively high similarity to humans about metabolism, body temperature and innate immune response. (Tsai et al., 2016; Cutuli et al., 2019). However, there are limitations that follow the use of rodents for microbial studies. For one the cost of maintenance needed to sustain a large number of organisms is expensive and growing ethical regulation to obtain authorization have made such methods of study laborious (O'Callaghan, 2010; Fuchs, 2006). Additionally, some animal models (e.g., mice and non-human primates) can exhibit significant biological variability within and between experiments, which is further exacerbated due to ethical regulations that limit sample sizes (Fitts, 2011; Zhan et al., 2017). These problems highlight the need to develop a novel, large-scale model for testing antimicrobial drugs. Alternative models that have been proposed in the past include the nematode *Caenorhabditis elegans* (C. elegans) which was the first non-mammalian model to be used as an infection model of a human pathogen (Kaito et al., 2020). Other examples that developed later are the *Drosophila melanogaster* (Fruit Fly), *Danio rerio* (Zebrafish), and more recently the larvae of the greater wax moth *Galleria mellonella* (G. mellonella) (Peterson et al., 2008; Kaito et al., 2020). This recent model as been used to study the virulence of a wide range of pathogens including Gram-positive bacteria, e.g., Streptococcus pyogenes, Staphylococcus aureus, and Clostridium perfringens (Desbois and Coote, 2011; Loh et al., 2013) as well as Gram-negative bacteria Legionella pneumophila, Pseudomonas aeruginosa, and Escherichia coli (Harding et al., 2012; Alghoribi et al., 2014; Asai et al., 2019). This is mainly possible due to the economic and ethical advantages that G.Mellonella provides, along with an innate immune system similar to that of humans, this model organism has been growing in popularity among recent research in infectious virulence and vivo antimicrobial efficacy (Ménard et al., 2021, García-Lara et al., 2005; Champion et al., 2016; Pereira et al., 2020).

I. Advantages of the Greater Wax Moth *Galleria Mellonella*

Various Biological features of the G.Mellonella

Figure 1. Shows that image of the *G.Mellonella*

The *Galleria Mellonella* or *G.Mellonella* is a member of the Galleriinae subfamily within the family Pyralidae of Lepidopteran order (Harding et al., 2013). The medium-white caterpillars can be found invading bee colonies and hence commonly known as the wax worm which refers to different species of larvae/moths that invade, attack, and damage honeybee colonies and hive products (Kwadha et al., 2017). In such natural environments, the exposure to a wider variety to pathogens have prompted the larvae to develop protective defense mechanisms including physical resistant barriers like cuticles as well as effective innate immune systems. But a more intriguing aspect of the *G.Mellonella* immune system is its high functional and structural similarity to mammalian immunity (Binder et al., 2016; Wittweret al., 1999; Trevijano-Contador, 2018; Lavine et al., 2002). Similar to the neutrophils found in mammalian blood, in the cellular response of *G.Mellonella,* immune cells called hemocytes found in hemolymph kill pathogens by the production of superoxides (Pereira et al., 2017). This allows us to gain a better understanding of how certain microbial infections may impact humans.

Similar to a holometabolous insect, the larvae develop through four distinct life stages, namely, egg, larva, pupa, and adult. The completion of the larvae life cycle happens in approximately 40 days depending on biotic and abiotic factors (James et al., 2013). Biotic factors include competition for food and diet quality while abiotic factors such as temperature are crucial to the maintenance of *G.Mellonella*. It has been observed that temperatures averages 29–33 °C and 29–33% relative humidity are optimum for survival (Kwadha et al., 2017). While these are the ideal conditions for larvae maintenance, *G. Mellonella* can sustain incubation at temperatures of 25°C and 37°C which is not easily achievable by other invertebrate models such as *Caenorhabditis elegan* and Drosophila *melanogaster* (Fuchs, 2010; Mowlds et al., 2007). This provides an opportunity to facilitate a number of conditions where microbial infections, specifically fungai, are pathogenic to human hosts.

Additionally, for the study of drug efficacy *G.Mellonella* allows for an in vivo testing model, which is using fully body organisms compared to in vitro testing which uses animal cell cultures for analysis. In fact in some cases of human microbial infections, an in vitro testing is not the best suitable approach to conducting drug efficacy due to the inability to mimic the natural niche. In fact, large amounts of studies show that treatments that show promising in vitro testing do not show the same results in vivo (Szafranska et al., 2014; Chaves-Moreno., 2016). For this reason many studies explore in vivo testing of antimicrobial agents using G.Mellonella.

The G.Mellonella model can overcome Monetary and Ethical obstacles

Contrary to the limitations seen in mammalian models, impediments such as monetary costs and ethical regulations are not an issue. The cost per larvae for *G.Mellonella* is low (less than a dollar) as well as the additional equipment needed for rearing larvae (Kaito et al., 2020). Moreover since the larvae are invertebrates, they do not have nociceptors and are thus insensitive to pain allowing for less control when it comes to ethical regulations (Eisemann

et al., 1984). All of these factors make studies using *G.Mellonella* less challenging for financial, technical and ethical reasons.

II. Previous application of *G.mellonella* to investigate fungal pathogens and antifungal compounds

Investigation of fungal pathogenicity

Invasive fungal diseases have remained a severe and underappreciated cause of illness and death worldwide and mainly those who are immunosuppressed. Apart from a few human fungal pathogens, the majority of fungal species show low virulence in mice and thus *G.Mellonella* has offered an alternative means to investigate fungal pathogens (Trevijano, 2018). More specifically its innate immune response has been recently seen to show great specificity, in addition to having the ability to distinguish between different classes of microorganisms (Hoffmann, 2003). *G.Mellonella* was first described as a model to study the fungal pathogen *Candida spp.* (*C. albicans, C. glabrata, C. tropicalis, C. krusei, C. haemulonii, and C. auris*). One of the early studies titled *"Development of an insect model for the in vivo pathogenicity testing of yeasts"* by Cotter et al. (2000) showed the ability of *Candida species* to kill the larvae *G.mellonella*. The infection was injected in the proleg of the larvae and was monitored over a span of 72 hours. The results showed higher mortality rates in of 90% highly pathogenic species (*C.albicans*) while for the other species were: C. tropicalis: 70% mortality, C. parapsilosis: 45%, C. pseudotropicalis: 45%, C. krusei: 20% and C. glabrata: 0% . The data observed reflects the natural hierarchy observed in the ability of these species to cause disease in a variety of mammals (Cotter et al., 2000). The significance of this study proved that *G.mellonella* is an applicable model to test the pathogenicity within the *Candida species.*

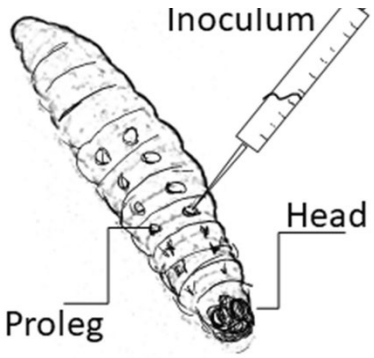

Figure 2. *Shows the homocole injection method performed by Hoffman et al. (2003).*

Using G.Mellonella to study Malassezia Furfur

Since this conclusion, *G.Mellonella* has been used to study a wide range of fungal pathogens including *A. fumigatus, Paracoccidioides lutzii, Histoplasma capsulatum, and C. neoformans* (Trevijano et al., 2018; Mylonakis et al., 2005; Alcazar-Fuoli et al., 2015; Gomez-Lopez et al., 2014; Desalermos., 2014). In this study the focus is on the fungal species *Malassezia Furfur* (*M.Furfur*) which is a member of a monophyletic genus of fungi normally found on human and animal skin (Gao, 2010). This yeast is normally found on the skin, but its abnormal overgrowth can cause several common dermatologic disorders, including seborrheic dermatitis (SD), pityriasis versicolor (PV)(Editorial, 2017; Hunter, 2021). In a recent study by Torres et al. (2020) *G.Mellonella* was shown to be a novel host for fungal *Malassezia furfur.* Using a fungal burden assessment and the characterization of hemocytes, the study showed successful results in establishing a systemic infection of *M.Furfur* (Torres et al., 2020).

Antifungal testing against pathogens using the Larvae model

As discussed, fungal pathogens have been extensively studied in the *G. mellonella* model for pathogenicity. Alongside such studies, there has been an increase in research testing antifungal efficacy using *G.Mellonella* as a host. An article by Rowan et al. (2009), demonstrated an increase in survival of *G.Mellonella* when injected lethal dose of *C.albicans* and followed up 1-4 hours later with the novel antifungal compound $Ag_2(mal)(phen)_3$. The implementation of the laravel model has also been used to explore antifungal treatments including posaconazole, isavuconazole, and nystatin-intralipid (Maurer et al., 2018).

III. Methods of *G.Mellonella* treatment and infection in current literature

In the majority of the studies described above as well in existing literature, *G.mellonella* have been inoculated by an intra-hemocoel injection method. However few have explored the topical methods for infection and treatment. A study titled “Effects of Lemongrass Factor" on Galleria Mellonella Hemocytes " by Rice examines this type of procedure (Rice, 2021). The larvae received 5 microliters of 5% “lemongrass factor” which was applied onto cuticles on the dorsal side of the larvae body. After a 4 hour observation, a mortality rate of 3.3% was observed post infection (Rice, 2021). This study shows successful results by topical means of inoculation against a bacterial infection.

IV. Research Gap and Goals

The research field exploring drug treatments topically using the *G.Mellonella* model is relatively new. Reviewing the current field, there is yet to be research exploring this practice of topical testing for fungal pathogens, specifically *M.furfur*. While the study by Torres et al. “Galleria mellonella as a Novelty in vivo Model of Host-Pathogen Interaction for Malassezia furfur CBS 1878 and Malassezia pachydermatis CBS 1879” has established a successful susceptibility of *M.Furfur* to *G.Mellonella*, it does not dive into testing antifungal treatments against the pathogen. In fact there is little to no research investigating antifungal efficacy against M.Furfur using the G.Mellonella. However, the results from the study have allowed for the opportunity to conduct a more in depth investigation of the pathogen which this study aims to look into. The research goal of this study is to test whether the *G.Mellonella* model can be applied to evaluating the effectiveness of topical antifungal treatments against *M.furfur* using the application method.

Methodology

I. Experimental Setup of larave observation

To achieve the research goal an experimental design was set up to collect the percent survival rate of the larvae. After the arrival of the *G.mellonella* the larvae were incubated for 16 hours. This was to ensure that the larvae settled into their ideal temperatures before conduction of the experiment. Subsequently, a total of 200 healthy larvae were selected based on weight (175–225 mg), uniformity in color (little to no melanization), and responsiveness to touch. The healthy larvae were then divided into 6 experimental groups. These larvae were observed in petri dishes with 5 worms in each.

For the first group, the larvae were not infected nor given any treatment. This control group was to ensure that the possible causes of death were not due to environmental factors or genetic issues in the larvae itself. In the second group, Larave were treated with a 1% butenafine hydrochloride cream. Butenafine topical is used to treat fungal infections of the skin, including tinea (pityriasis) versicolor caused by M. furfur (Mayo Clinic, 2022).

Similarly in the third group larvae were treated only with 1% clotrimazole cream USP. Recent studies have shown that the Culture of Malassezia furfur showed a constant and persistent sensitivity to clotrimazole (Clayton, 2000). These two experimental groups were needed to determine whether the treatments used in this study might have affected the percent survival rates of *G.Mellonella*.

In the last three experimental groups, larvae were pre-treated with the pathogen *M.Furfur*. To ensure that the infection susceptibility of *M.Furfur* has been successfully done, the fourth larvae group was only infected. In the fifth laravel group, to determine whether the antifungal treatment butenafine had an impact on larvae survival.

Table 1. Description of experimental setup and allocation of treatments

n.o	Experimental group	Treatment
1	Control	No treatment
2	Treatment 1 (T1)	Pre-treated with only 1% butenafine hydrochloride cream.
3	Treatment 2 (T2)	Pre-treated with only 1% clotrimazole cream USP.
4	Infected	Pre-infection of M.Furfur
5	Infected + T1	Pre-infection of M.Furfur and post 4 hr treatment of T1
6	Infected + T2	Pre-infection of M.Furfur and post 4 hr treatment of T2

Table 2. Number of petri dishes per experimental group (5 larvae per petri dish)

n.o	Experimental group	Petri dish (Number)
1	Control	5
2	Infected	5
3	Treatment 1 (T1)	5
4	Treatment 2 (T2)	5
5	Infected + T1	10
6	Infected + T2	10

Additionally, the larvae in each petri dish were randomly ID'd in 5 colors including black, red, blue, green, and purple. This step was to determine the individual survival rates of larvae in each group of study.

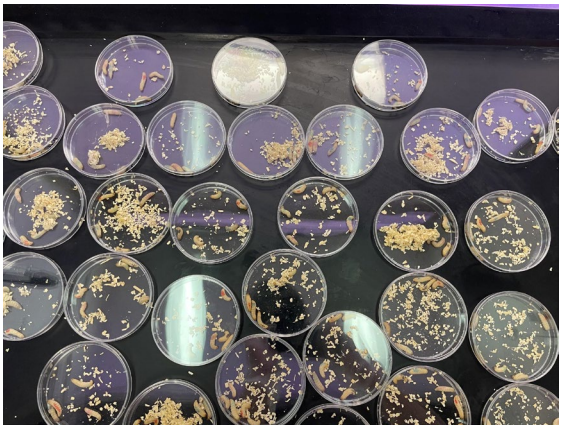

Figure 3. *The image shows the experimental setup*

Figure 4. *The image shows 5 ID'd larvae per dish*

II. Culturing of *Malassezia Furfur* for pre-infection of larvae

For the culturing of *M.Furfur* a special agar was made to accommodate the lipid based dependency of the infection using Dixon's agar recipe. Following the setting of the agar, the *M.furfur* fungal pathogen was streaked using a bent-glass rod and was left to culture overnight at 37°C.

III. Criteria for determining percent survival of larvae

To quantify whether *G.Mellonella* can be used to test the efficacy of antifungal drug treatments, the percent of larvae survival in each petri dish were collected. The criteria to determine whether the larvae were dead or alive was based on the responsiveness as well as melanization. Healthy responsiveness includes movement when stimulated with touch. Similar to the procedure by Cotter et al., (2000) to larvae if the larvae showed no movement, it demonstrated no survival.

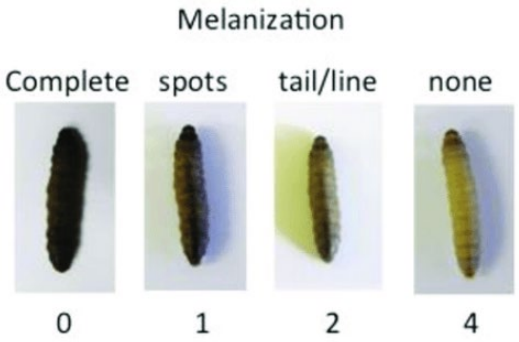

Figure 5. *Images of infected larvae showing different stages of disease.*

Additionally, In this study Melanization was also taken into account when determining survival. On the scale identified by Tsai et al., larvae that showed complete melanization (black larvae, left image) correlates with death of the larvae soon after (Tsai et al., 2016). Using this scale larvae that fell on the scale of 0, were considered dead. Following these two criterias the percent of larvae survival was determined.

IV. Conduction of experiment and collection of data

The larvae were observed over the course of a 120 hour time period. At the 0hr interval, the larvae groups infected, infected (T1), and infected (T2), were infected with *M.Furfur* culture using a swabbing method. Using a sterile swab, the fungal pathogen was applied to the dorsal side of the larvae.

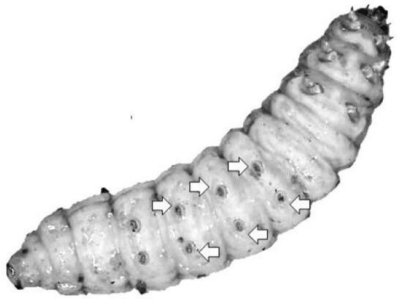

Figure 6. *The image above shows arrows pointing to the dorsal side of the larvae*

Similarly at this interval, the larvae groups T1 and T2 were treated with 1% butenafine and 1% clotrimazole topical antifungal steroids. These 6 larvae groups were observed, and data was collected at the 2nd hr and 4th hr interval. At the 4hr interval the experimental groups infected (T1) and infected (T2) were treated, post the 0hr infection, with 1% butenafine and 1% clotrimazole topical antifungal steroids respectively. The same swab method was used for the application of the treatment. Following the 4hr interval, the percent survival of larvae in each petri dish was collected at the 12hr, 48hr, 72hr and finally 120 intervals.

V. Disposal of larvae

The described experiments followed the ARRIVE guidelines for proposed protocol of handling of the invertebrates post data collection. After the experiment, larvae infected with pathogens were disposed of by placing them in secondary containment and incubating at -20°C for at least 24 hours before sterilization by autoclaving.

Data Analysis and Results

After conducting the experiment, the recorded data over the span of 120 hours was imputed into the SPSS statistics program. Since the experimental groups were independently conducted with each study group treated with a different substance, an independent samples t- test was conducted between the following groups:

Independent samples t-test	Comparing groups
Test 1	Control v/s Infected
Test 2	Control v/s T1
Test 3	Control v/s T2
Test 4	Infected v/s Infected + T1
Test 5	Infected v/s Infected + T2
Test 6	Infected T1 v/s Infected T2

Before determining whether the *G.Mellonella* model can be used to test the drug efficacy of topical steroids against *M.furfur* two main questions had to be answered. Firstly was the infection of *G.mellonella* by *M.furfur* successful? Secondly did any of the treatments used in this study affect the larvae survival rates?

Table 1. Independent sample t-test comparing control and infected group

Treatment	n	mean	SD	SE	t	p
Control	5	100.0	0.000	0.000	-20.58	<0.001
Infected	5	36.00	8.944	4.000		

To determine whether the infected group had been successfully infected by *M.Furfur* after topical application I performed a t-test to compare the mean percent survival rates of the control group and the infected group. Based on Table 1 provided the analysis produced a significant t value ($t_{(10)} = -20.58$, $p < 0.001$). An examination of the means revealed that the control group had higher survival rates (M= 100%) compared to the infected group (M= 36%). These results confirm that the larvae had been infected with the *M.Furfur.*

Table 2. Independent sample t-test comparing control and Treatment 1 (T1) group

Treatment	n	mean	SD	SE	t	p
Control	5	100.0	0.000	0.000	1.633	0.141
T1	5	92.00	10.95	4.890		

Table 3. Independent sample t-test comparing control and Treatment 2 (T2) group

Treatment	n	mean	SD	SE	t	p
Control	5	100.0	0.000	0.000	1.633	0.141
T2	5	92.00	10.86	4.790		

To answer the second question, which was whether the treatments used in this study had an effect on the survival rates of the larvae I performed a t-test to compare the mean percent survival rates of the control group and the treatment groups (T1) and (T2). Based on Table 2 the analysis produced a non- significant t value for the control group and Treatment 1($t_{(10)} = 1.633$, $p=0.141$). Similar results were seen in Table 3 for treatment 2 which produced a non- significant t value ($t_{(10)} = 1.633$, $p=0.141$). Both these results show that the treatments used in this study did not have an effect on the percent survival rates of the larvae.

Table 4. Independent sample t-test comparing infected and infected with post 4hr treatment (T1) group

Treatment	n	mean	SD	SE	t	p
Infected	5	36.00	8.944	4.000	-20.48	<0.001
Infected T1	10	99.00	3.162	1.000		

Finally, I conducted an independent sample t-test to determine whether larvae treated with treatment 1 post infection had higher survival rates than the infected group. Based on Table4 the analysis produced a significant t value

for the infected group and infected group (T1) ($t_{(15)}$ = -20.48, p<0.001). An examination of the means revealed that the infected (T1) group had higher survival rates (M= 99%) compared to the infected group (M= 36%).

Table 5. Independent sample t-test comparing infected and infected with post 4hr treatment (T2) group

Treatment	n	mean	SD	SE	t	p
Infected	5	36.00	8.944	4.000	-20.48	<0.001
Infected T2	10	99.00	3.162	1.000		

I conducted that same t-test to determine whether larvae treated with treatment 2 post infection had higher survival rates than the infected group. Based on Table 5 the analysis produced a significant t value for the infected group and infected group (T1) ($t_{(15)}$ = -20.48, p<0.001). An examination of the means revealed that the infected (T2) group had higher survival rates (M= 99%) compared to the infected group (M= 36%).

Based on the results from both Table 4 and Table 5, the larvae showed an improvement in survival rates when they were treated with antifungal steroids after being exposed to the *M.furfur* pathogen.

Table 6. Independent sample t-test comparing infected T1 and infected T2 groups

Treatment	n	mean	SD	SE	t	p
Infected T1	10	99.00	3.162	1.000	0.000	1.000
Infected T2	10	99.00	3.162	1.000		

Additionally, I wanted to determine whether there was a difference in treatment 1 and treatment 2 used in this study. Based on Table 6 the analysis produced a non-significant t value for the infected (T1) and infected (T2) groups ($t_{(15)}$ = 0.000, p=1.000). The results indicated that both treatments were equally effective in treating infections due to *M.Furfur*.

Discussion and conclusion

I. Obstacles faced with the use of Murine Models

During drug screening several mamalas are used in this process. These mammalian models have been suitable for testing drug efficacy due to their similar responses in metabolism and immune responses to humans. However these models are incredibly expensive, have high ethical regulation and have even shown to have biological variability among and within experimental groups.

For these reasons the use of murine models for large scale and frequent testing can make drug screening a challenging process. An alternative model proposed in this study has been the *Galleria Mellonella* organism for testing topical steroids. Due to biological, financial, and ethical advantages, the *G.mellonella* has become a popular alternative to avoid the obstacles brought by rodent models.

II. Research Goals

As discussed there is still a lack of research testing the *G.mellonella* as a useful model for antifungal testing against *M.furfur*. Additionally there is specifically yet to be research investigating the topical application method for testing

antifungal efficacy against *M.Furfur* using the *G.mellonella* model. This study aims to research both gaps in the current literature and identify whether *G.mellonella* is a suitable model to do so.

III. New understanding and Conclusion

As described, the results of experimental groups infected (T1) and infected (T2) compared to the infected groups showed a significant t value using an independent t-test. This indicates that the survival rates between the groups that were treated with (T1) and (T2) had an increase in survival rates compared to the infected group. The conclusion from this study indicated that *G.Mellonella* is an effective model to test the effectiveness of topical antifungal medications. This is the first study to indicate that the *G.Mellonella* can be used for topical antifungal testing against the fungal pathogen *M.Furfur*.

IV. Limitations and Implications of Findings

One of the limitations for this study was the swab method, which cannot quantitatively identify that exact amount of pathogen or treatment given to the larvae. Thus it may be difficult to identify specific amounts of treatment needed to cure a fungal infection. Additionally, due to the recent establishment of the *G.mellonella* model, there are no stock centers where researchers can purchase larvae that have consistent research methods. Therefore, differences in breeders may affect the genotypes of the larvae, impacting susceptibility to infection as well as survival rates when investigating drug efficacy. However, future studies can address these limitations through a more controlled research environment as well as establishing a consistent stock center to purchase laravel for further studies.

While the *G.Mellonella* model may not be able to completely eliminate the need for the mamilan model for study, it can allow researchers to conduct large scale and more rapid testing of antifungal treatments during the drug screening process. The results from this study mean that researchers can use the G.mellonella model to test possible novel treatments against the Malassezia Furfur pathogen. The initial drug screening using this model can effectively help save time, money, and effort that is invested into this process. Thus, this can help develop more effective and successful treatments against a disease that affects millions of individuals worldwide and in the big picture allows researchers to effectively allocate their resources in the drug development process.

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