REVIEW

Invertebrate Animal Models of Diseases as Screening Tools in Drug Discovery

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ABSTRACT Invertebrate animal models (mainly the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*) are gaining momentum as screening tools in drug discovery. These organisms combine genetic amenability, low cost, and culture conditions compatible with large-scale screens. Their main advantage is to allow high-throughput screening in a physiological context. On the down side, protein divergence between invertebrates and humans causes a high rate of false negatives. Despite important limitations, invertebrate models are an imperfect yet much needed tool to bridge the gap between traditional *in vitro* and preclinical animal assays.

A few years ago, the spectacular development of virtual screening tools combined with the development of molecular genetics techniques to identify biological targets may have left us with the impression that brute-force high-throughput screening (HTS) would soon disappear and give way to intelligence in drug design. Unfortunately, it is now clear that the requirements for virtual screening (a structurally solved biologically relevant target) can be met in only a limited number of therapeutic applications. Therefore, the rest must stick with a strategy that has remained conceptually unchanged since Cinderella: try many candidates one by one and pick the one (or the few) that match.

One of the key issues of HTS is relevance. It is relatively easy to set up robotized screens on cell-free systems. What is slightly more difficult, albeit feasible, is setting up HTS based on mammalian tissue culture cells, bacteria, and yeast. Unfortunately, screening for compounds against numerous pathologies is not amenable to this level of reductionism for three reasons: (i) Some diseases affect organs as a whole, and most organs cannot be reconstituted *in vitro*. This is particularly true for, but not limited to, muscle or nerves. (ii) Cells and organs are physiologically connected, and this interplay may be critical in the development of some diseases. This is an aspect that cannot be reconstituted *in vitro*. (iii) The time component of disease progression is usually not recapitulated *in vitro*. For diseases matching any of these criteria, classical *in vitro* screening can be carried out only by paying a high price in terms of relevance. A fourth and nontechnical reason comes on top of the first three: many diseases, particularly loss-of-function genetic diseases, are still poorly understood and lack validated targets.

Which strategy to choose, then, when the choice seems to be between highly relevant but HTS-incompatible mammalian models on the one hand, and afford-

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TABLE 1. Pros and cons of the invertebrate screening systems

Pros	Cons
Numerous biological processes are conserved between mammals and invertebrates	Some diseases cannot be modeled with invertebrates because the gene or organ does not exist
Many genes are conserved between mammals and invertebrates	Problems of molecule penetration
Studies occur in a physiological context	Concentration within an animal is unknown
Low cost	Protein conservation at the amino acid level is poor
Genetics allows the identification of a drug effector pathway	False negatives

able but poorly relevant *in vitro* systems on the other? One alternative is invertebrate animal models. They may be highly imperfect, but they are quite useful in some instances (Table 1).

***Drosophila melanogaster* and *Caenorhabditis elegans*: Old Players in the Biomedical Field.** The laboratory careers of the fruit fly *D. melanogaster* and the free-living nematode *C. elegans* are 100 and 40 yr old, respectively. These animals have been the laboratory workhorses that have enabled the discovery of a long list of fundamental biological mechanisms. They have been indirectly associated with medical progress and drug discovery for many years because they are the basis from which key fundamental biological principles were discovered (*e.g.*, apoptosis, the cell suicide program that is central to scores of pathological situations). But only recently has their use as direct HTS tools gained momentum.

Several factors may explain this move. First, the sequencing of the *C. elegans* genome (1998) (*1*), the *D. melanogaster* genome (*2*), and eventually the human genome (2004) (*3*) demonstrated that a high degree of homology exists between invertebrates and humans; ~50% of human genes have a counterpart in *D. melanogaster*, and the same is true for *C. elegans* (*2*). Second, inactivation of *C. elegans* or *D. melanogaster* genes generally leads to phenotypes resembling those obtained by inactivating their mammalian homologues (*4*). Third, RNA interference (RNAi) techniques permit genome-wide screens in the animal models *C. elegans* and *D. melanogaster* (*5*). In recent years, such screens have provided a wealth of new putative targets. Fourth, these animals (*C. elegans* more so than *D. melanogaster*) are compatible with HTS formats, a feature of considerable interest overlooked for many years (*6*).

C. elegans, which is 1 mm long as an adult, can be grown in liquid and robotically dispensed into 96-well microplates containing chemical compounds. Although a company (Union Biometrica, Holliston, MA) has developed sorting and analyzing machines for *C. elegans* and *D. melanogaster*, HTS can be performed on these models with standard HTS equipment. *C. elegans* is small enough (adults are ~30 μm in diameter) to be pipetted in liquid with standard pipetting robots. *D. melanogaster* eggs and embryos are larger in size (~100 μm in diameter) but can also be handled by robots equipped with large tips. A large arsenal of genetically modified fluorescent strains provides convenient readouts for HTS. For many applications, the fluorescence readout may be measured globally (on a population of animals) with a standard plate-reader spectrofluorometer. This basic equipment allows for many *C. elegans* and *D. melanogaster* screens. More sophisticated analysis is possible with the Complex Object Parametric Analyzer and Sorter (COPAS) machine from Union Biometrica, which also has a dispenser function (*7*). Alternatively, flash cytometer optical systems (Trophos, Marseilles, France) are rapid image acquisition systems that may be used in HTS. All in all, invertebrate models provide a good trade-off between experimental use and biological relevance for HTS (Figure 1).

First-Pass Filters. Invertebrate-based random compound screens can be separated into two large categories, depending on the aim of the screen (Figure 2). Invertebrate-based screens can be employed to identify new chemical structures and targets of potential interest. For instance, libraries of compounds can be tested on wild-type animals for their toxicity or their ability to produce specific phenotypes, such as paralysis. This strategy is of particular interest for identifying new interesting chemical structures that have not been discovered previously (*8*). Targets and modes of action can often be identified with the use of pre-existing mutants (*8*). Other methods such as affinity chromatography coupled to mass spectrometry are also an option (*9*). This type of screen is of primary interest in the search for new anthelmintics and insecticides (not the subject of this Review). However, the biomedical community may also benefit because new targets may be defined.

The second and most important application of these models is in the search for hits against defined human

KEYWORDS
Invertebrates: Animals that lack a skeleton. The fly *Drosophila melanogaster* and the worm *Caenorhabditis elegans* are invertebrates.
Animal model: An animal species that is studied in research laboratories to elucidate general biological phenomena.
***Drosophila melanogaster*:** A fruit fly species that has been an animal model for a century.
***Caenorhabditis elegans*:** A worm species that has been an animal model for decades.
Animal disease model: An animal on which specific features of a disease have been reproduced.

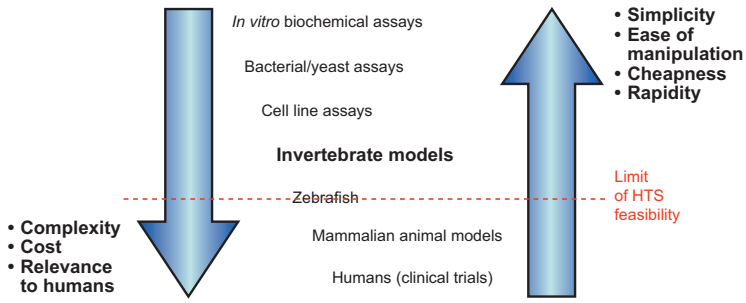

Figure 1. The question of the relevance/efficiency trade-off in HTS. The primary issue in molecule screens against diseases is the relevance/efficiency ratio. The selected option is always a trade-off between relevance to humans and screening efficiency. The best option varies from disease to disease, depending on target, organ, gene conservation, and other variables. Note the position of invertebrate models with respect to the limit of HTS feasibility and relevance to humans. (The zebrafish model is schematically represented over the HTS feasibility limit to illustrate the fact that zebrafish embryos are a convenient HTS system, whereas zebrafish adults are not.)

pathologies. In this case, HTS is not performed on wild-type animals but rather on animals that have been modified genetically or by chemical treatments in order to reproduce relevant traits of a particular disease (Figure 2 and Figure 3). Depending on the disease, genetic modification means either knocking down a given gene (loss-of-function diseases) or expressing a deleterious version of it (gain-of-function diseases). More than a dozen such strains of *C. elegans* and *D. melanogaster* exist, and more are constructed every year. These modified strains are usually called animal models of disease X (*e.g.*, *D. melanogaster* model of Parkinson's disease [PD]). However, one has to keep in mind that the constitution and physiology of invertebrates are significantly different from those of humans and that, as a consequence, these animals can produce only a partial picture of the human symptoms. Whether this partial image overlaps sufficiently with the human situation to make invertebrates valuable screening systems in the search for new drugs is still a matter of debate. The few examples given below illustrate this debate.

Neurodegenerative Diseases. *C. elegans* models of Alzheimer's disease (AD), PD, and Huntington's disease (HD) have been made and are being exploited for the study of these diseases. Amyloid deposits, the hallmark of AD, occur in transgenic *C. elegans* expressing human β-amyloid peptide and cause a partial paralysis of the animals (*10, 11*). Moreover, the transgenic *C. elegans* exhibits increased levels of reactive oxygen species and protein carbonyls, which are also observed in AD patients. Furthermore, DNA microarray experiments revealed that several stress-related genes that were found to be up-regulated in postmortem AD human brains were also up-regulated in *C. elegans* (*12*). PD-like symptoms can be obtained in *C. elegans* by incubation of the animals with the neurotoxic agent 1-methyl-4-phenylpyridinium (MPP+), a compound used to induce mammalian models of the disease. Exposure to MPP+ for 48 h resulted in the death of *C. elegans* dopaminergic neurons, reduced mobility, and increased mortality (*13*). The model was validated when it was shown that these symptoms (also called the phenotypes) were reduced when the animals were also exposed to lisuride, a drug used in PD treatment (*13*). The work done with *C. elegans* for HD may seem more far-fetched because *C. elegans* has no hungtingtin, the neuronal protein that harbors the deleterious polyglutamine repeats at the origin of the disease. However, human huntingtin is able to physically

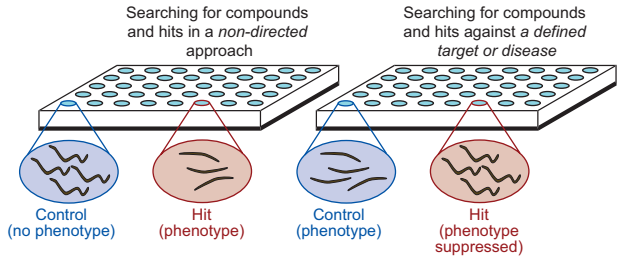

Figure 2. HTS on invertebrate models may be used in two directions. Invertebrates may be used in HTS compound screens for two different purposes. Left: chemical libraries are tested on healthy nematodes to identify drugs that are able to hit nematode targets. Hits are detected by their ability to produce a phenotype, which is a modification of the animals' growth, behavior, morphology, or other detectable trait. In the example shown, the hit induces a paralysis (straight appearance of the worms). Such screens allow for the identification of bioactive molecules. Right: chemicals are tested on nematodes in which a disease-relevant phenotype has been created. Such screens aim at finding molecules able to reverse the phenotype, thereby identifying potential candidates for treating the disease.

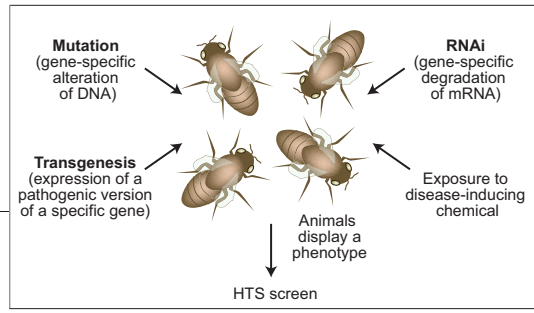

Figure 3. Invertebrate models of diseases may be created in several ways. An invertebrate model of the targeted disease may be created *via* inactivation of genes (by mutation or RNAi), introduction of a pathogenic version of the gene (transgenesis), or exposure to a deleterious chemical. The changes induced by the treatment are called the phenotype. This model will subsequently be used in HTS to identify compounds able to reduce or suppress the phenotype of the animals. The same strategy applies to *D. melanogaster* and to *C. elegans*.

interact with *C. elegans* homologues of its human binding partners (*14*). In addition, transgenic animals expressing the abnormal form of human huntingtin display neuron axonal defects (*15*). This model has been used for pharmacological validation of existing drugs and for further screening (*16*). A number of fly models are also available for HD (*17*) as validation of other assays or for screening purposes (*18, 19*; Figure 4).

Muscle Diseases. Muscles are notoriously difficult to reconstitute *in vitro*. This fact has hindered the exploration of treatments against muscle diseases, especially those that are physiologically complex and poorly understood. Inherited myopathies are a good paradigm for the use of invertebrates in drug discovery. *C. elegans* and *D. melanogaster* both have muscles that are very close in architecture, composition, and function to vertebrate skeletal muscles. Moreover, most genes affected in inherited myopathies have counterparts in those animals. The most famous one is dystrophin, a structural protein whose malfunction is the cause of Duchenne muscular dystrophy (DMD). DMD patients suffer from a progressive necrosis of their skeletal and cardiac muscles. DMD is a slow-evolving disease: it takes a few years for the first symptoms to appear. Despite considerable investment over the past 20 years, the physiopathology of the disease is still poorly understood. Together, these features have hampered the development of drugs against DMD, which still has no efficacious treatment. Mutations of the *C. elegans* dystrophin homologue also result in a progressive muscle necrosis (*20, 21*). Because the *C. elegans* lifespan is much shorter than that of mammals (*C. elegans* becomes an adult in 3 d), the phenotype arises much more quickly than it does in mammals, another sizable experimental advantage. A few years ago, we showed that prednisone, a steroid given as a low-efficiency palliative treatment to DMD patients, was able to slightly reduce the muscle necrosis of dystrophin-deficient worms (*22*). This finding paved the way for a systematic search of new compounds that have a beneficial effect on the muscles of dystrophin-deficient worms. In a first round of screening, 1000 approved drugs of various structures and indications were randomly screened. This work, which was partially published recently, has revealed some unexpected hits and opens new avenues for the treatment of DMD (*23*). Against all expectations, we found that antidepressants are potent suppressors of dystrophin-dependent muscle degeneration in this model (*23*). Preliminary results indicate that some of these hits are also active on the mouse model of the disease (L. Ségalat, unpublished results). This example illustrates how the use of invertebrates may contribute to drug discovery.

Other Diseases. Invertebrate models exist for many other diseases, from diabetes to cancer, depression, and infection. Flies and worms have well-conserved insulin signaling pathways that have been deciphered at the molecular level by genetic analysis, which has provided a detailed understanding of these pathways and their downstream effectors (*24*). One of the most studied pathways is the receptor tyrosine kinase/Ras signaling pathway (*25, 26*), which is over-activated in numerous cancers. Farnesyl transferase inhibitors were shown to reduce the effect of Ras hyperactivation in *C. elegans*, as they do in mammalian cells, thereby validating the model (*27*). *C. elegans* and *D. melanogaster* have also proved to be fairly useful models for studying host–parasite interactions and screening for therapeutic agents (*28, 29*). In a recent study, 6000 synthetic compounds and 1100 natural product extracts were tested for their ability to prevent the death of *C. elegans* infected with the human opportunistic pathogen *Enterococcus faecalis* (*30*). A recent paper on the identification of small molecules able to regulate insulin signaling provides an excellent case study of what can be achieved with *C. elegans* in terms of screens and target identification (*9*).

Limits and Drawbacks of Invertebrate Models in HTS. Invertebrate models have several drawbacks that limit their use. The major drawback is that some diseases cannot be modeled because the animals do not have the corresponding genes and organs. These include acquired immunity diseases, cardiovascular diseases, and non-degenerative behavioral disorders. The second drawback stems from the fact that both *D. melanogaster* and *C. elegans* are surrounded by a thick cu-

ticle that is a physical barrier to the penetration of molecules. The mode of penetration in these animals is a variable combination of limited diffusion through the cuticle and ingestion. As a consequence, the concentration of a given compound within the animal is unknown, varies from compound to compound, and is sometimes null. Two important consequences result from this experimental difficulty: positive results are only qualitative, and negative results cannot be interpreted because it is generally not known whether a negative result is due to poor penetration, docking problems, or a true absence of biological activity in the model. Pharmacologists, who are not used to this level of uncertainty, sometimes regard this drawback as too big to be acceptable. I personally believe that we should use what we can learn from invertebrate-based screening. A partial picture is better than no picture at all.

Conclusions. Although their use is still limited, invertebrate model animals are increasingly being used in disease-oriented molecule screens. They constitute an alternative to *in vitro* systems because their small size and their culture conditions fulfill the requirements for large-scale screens. This is made possible by the fact that flies and nematodes, despite being distant cousins of mammals, share with them a large number of genes and biological pathways. The main advantage of invertebrates over other *in vitro* assays is that they provide a system that is both HTS-compatible and in which the physiological context is preserved. In that respect, they may be comparable to zebrafish embryos, another emerging screening system (*31, 32*). However, invertebrates also have a number of drawbacks that should not be underestimated. Their added value for drug discovery varies from disease to disease and mainly depends on what other options are available.

In the case of rare genetic diseases, especially those in which the physiopathology is poorly understood, the use of invertebrate models may eventually provide important breakthroughs, given that neither massive random screening nor target-driven drug design is currently feasible. The question of relevance to humans remains an important issue. Will any of the hits found in invertebrate models of PD or DMD turn out to be active on mammalian models of these diseases? It's still too early to tell, but the moment of truth will come shortly, because these hits are now being tested on preclinical (mammalian) models.

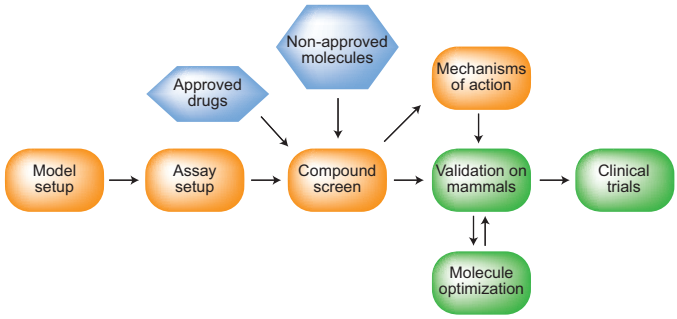

Figure 4. Invertebrate animal models in the drug discovery process. Invertebrate animal models occupy an upstream position similar to that of *in vitro* systems in the drug discovery process. Orange boxes represent steps performed on invertebrates, and green boxes are steps performed on mammals. Model setup refers to the means to produce a phenotype *via* the inactivation of genes or other deleterious treatments relevant to the targeted disease. Assay setup refers to the definition of readouts and procedures that enable the rapid and reliable detection of hits during the screen. Once hits are identified, the genetic amenability of invertebrate models such as *D. melanogaster* and *C. elegans* allows for an identification of the biological functions modulated by the drug (only the most important steps are shown).

In more commonly understood pathologies such as cancer, inflammation, and hypoxia, the shortage of *in vitro* models is less severe, and numerous putative targets have already been identified. The usefulness of invertebrate models as HTS devices for these diseases is more debatable. They nonetheless constitute interesting validation models downstream of traditional *in vitro* HTS and hence help to bridge the gap between *in vitro* and preclinical models. In short, whatever the specifics are, invertebrate models are a promising new addition to the drug discoverer's arsenal. One has to keep in mind that it is often the combination of complementary approaches that is instructive in the end.

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