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Essay

Mechanistic Research for the Student or Educator †

Rehana Leak

Duquesne University; leakr@duq.edu

† Part II of II

Abstract: This two-part series describes how to test hypotheses on molecular mechanisms that underlie biological phenomena, using preclinical drug testing as a simplified example. While pursuing drug testing in preclinical research, it is important for students to understand the limitations of descriptive as well as mechanistic studies. The former does not identify any causal links between two or more variables; it identifies the presence or absence of correlations. The latter has caveats presented in Parts I and II of this series. Part II also describes how to test for a causal link between drug-induced activation of biological targets and therapeutic outcomes. Here, the mechanism of action of the drug is identified with pharmacological or genetic approaches that modify the expression/activity of the drug targets. Without interference with the proposed mechanism of action, a causal link between activation (or inhibition) of the target P and the therapeutic outcomes of drug D cannot be established. Using pharmacological agonists and antagonists, gene knockout and overexpression, or protein knockdown tools, designing a full-factorial three-way ANOVA forces the investigator to include the appropriate control groups, mitigating the risk of false positive or false negative conclusions. Upon completion of this series, the educator and student will have some of the tools in hand to design mechanistic studies and interpret various experimental outcomes, with knowledge of strengths and limitations of preclinical research.

Keywords: mechanistic; hypothesis; physiology; biology; pharmaceutical; biomedicine; preclinical

Introduction

In the biological, biomedical, and pharmaceutical sciences, it is often necessary to determine the physiological mechanism of action of drugs on protein molecules, the chief workhorses of the cell. Part II of this series describes how to test the hypothesis that a drug candidate D protects against a preclinical disease model M by enhancing (or inhibiting) the physiological function of the molecule P. The rationales for employing gene knockout or pharmacological antagonism versus forced gene overexpression or pharmacological agonism to test this hypothesis are presented below, as well as technical limitations of these mechanistic approaches.

Formulating a Hypothesis

Once a candidate biological pathway has been appointed through a literature search, RNA sequencing, proteomics, or other methods, it is helpful to sketch a cartoon of the working hypothesis in the larger context of the cell or tissue, as in **Figure 1**, although the "real life movie would be much more complex," as quoted from Van Mil and colleagues (1).

In our hypothetical example, drug D binds a membrane receptor, and this direct target engagement sets in motion a cascade of downstream events, including the inactivation of a phosphatase (dotted line). With phosphatase activity suppressed, drug D indirectly boosts the function of protein P, which facilitates the dissociation of factor T from its inhibitory partner factor I. When factor I is removed from the complex, ubiquitinated, and degraded by the proteasome, factor T is free to translocate into the nucleus and promote the expression of prosurvival genes. The collective protein products of these genes improve mitochondrial generation of ATP, providing sufficient energy to repair injured tissues, thereby battling disease model M.

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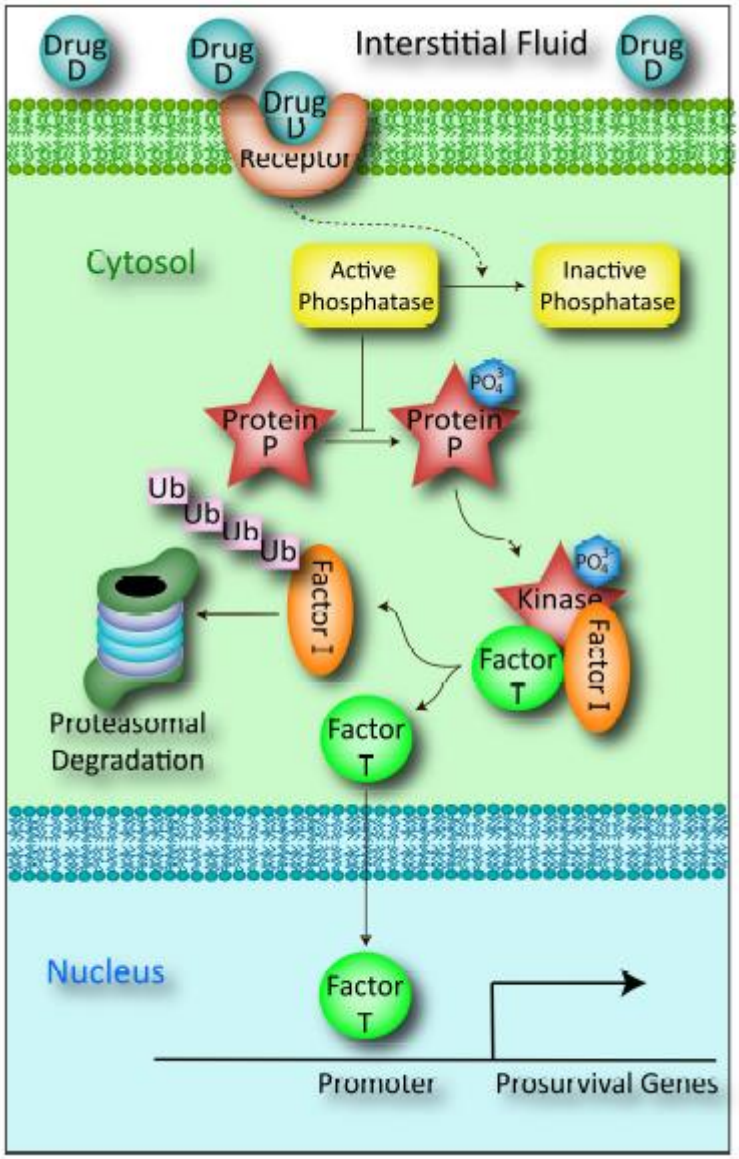

Figure 1. Simplified schematic of a working hypothesis. Drug D binds a plasma membrane-bound receptor protein, which indirectly inactivates a cytosolic protein phosphatase through a series of steps (dotted line). This inactivation step serves to increase the net phosphorylation state of the protein P, shown as a red star with an attached PO_4^{3-} group. Phosphorylated P, in turn, promotes the release of the transcription factor T from its inhibitory partner factor I. Unmoored, the inhibitory partner I is rapidly ubiquitinated and degraded by the proteasome, and its demise permits the activation and translocation of factor T across the nuclear membrane. Once it has entered the nucleus, factor T binds the promoters of genes involved in cellular defense. The products of those prosurvival genes then battle apoptosis to mitigate the toxic consequences of disease model M.

One can measure the binding of drug D to the membrane-bound receptor with radioligand binding assays, but binding of the receptor does not guarantee downstream effects. Rather, the binding assay in this example would be followed by measurements of drug-induced inactivation of the phosphatase, activation (phosphorylation) of protein P and its downstream targets, loss of protein factor I expression (and whether or not this loss is mitigated by proteasome inhibitors), translocation of factor T from cytosol to nucleus, binding of T to the expected promoters, and induction and expression of the correct set of prosurvival genes. All of these events form various segments of the mechanism of action of drug D, even if the membrane receptor is the only direct target of the drug. For the sake of argument, however, we will test the hypothesis that the function of protein P is causally linked to the positive impact of drug D on disease model M.

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An idealized graph of the impact of drug D on protein P is depicted in **Figure 2A**; it shows that disease model M involves a loss of function of the candidate protein P, and that drug D not only prevents this drop, but increases the function of protein P. Even if there were no dramatic upregulation of protein P and drug D simply prevented loss of its function under disease conditions (**Figure 2B**), protein P would still be a suitable candidate for mechanistic follow-up work. Another alternative is that protein P is upregulated as a compensatory response to disease model M, and that drug D simply increases baseline expression and activity of protein P, in the presence or absence of model disease M (**Figure 2C**). There is no statistical interaction between drug D and the disease in **Figure 2C**, and yet, drug D need not be abandoned—protein P may still mediate beneficial effects of drug D by improving overall viability. However, if drug D also increases the activity of protein P under physiological (non-diseased) conditions, as shown by the second green bar in **Figure 2C**, it might have oncogenic potential or other off-target effects on healthy tissues.

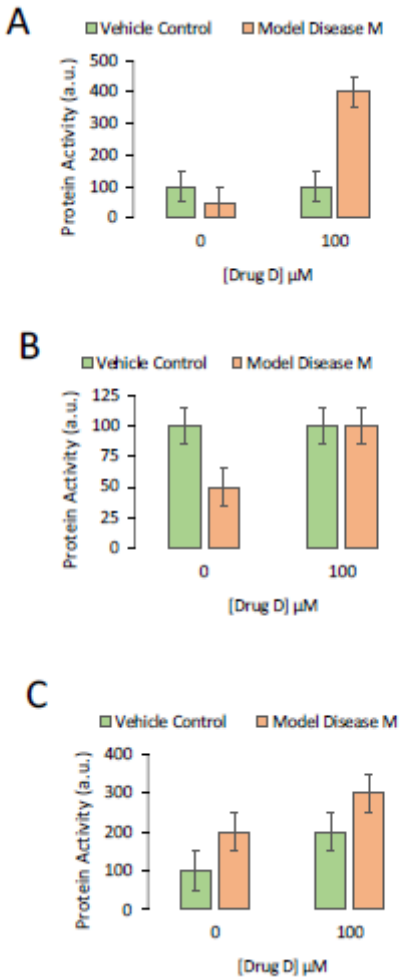

Figure 2. Impact of drug D on the activity of hypothetical prosurvival protein P in disease model M. (A) Drug D reverses the loss of protein activity elicited by disease model M and causes a dramatic increase in this functional measure. (B) Drug D completely prevents the loss of protein activity elicited by disease model M. (C) Drug D increases protein activity in both the vehicle-treated and diseased groups.

Thus far, protein P is *correlated* with prevention of disease M, but the data do not reveal if upregulation of protein P by drug D *causes* prevention of disease M. Even if drug D causes the entire series of events depicted in **Figure 1**, none of the abovementioned measurements guarantee that these proteins form part of the cascade underlying the protective qualities of drug D. We have yet to test the following hypothesis, copied here from Part I:

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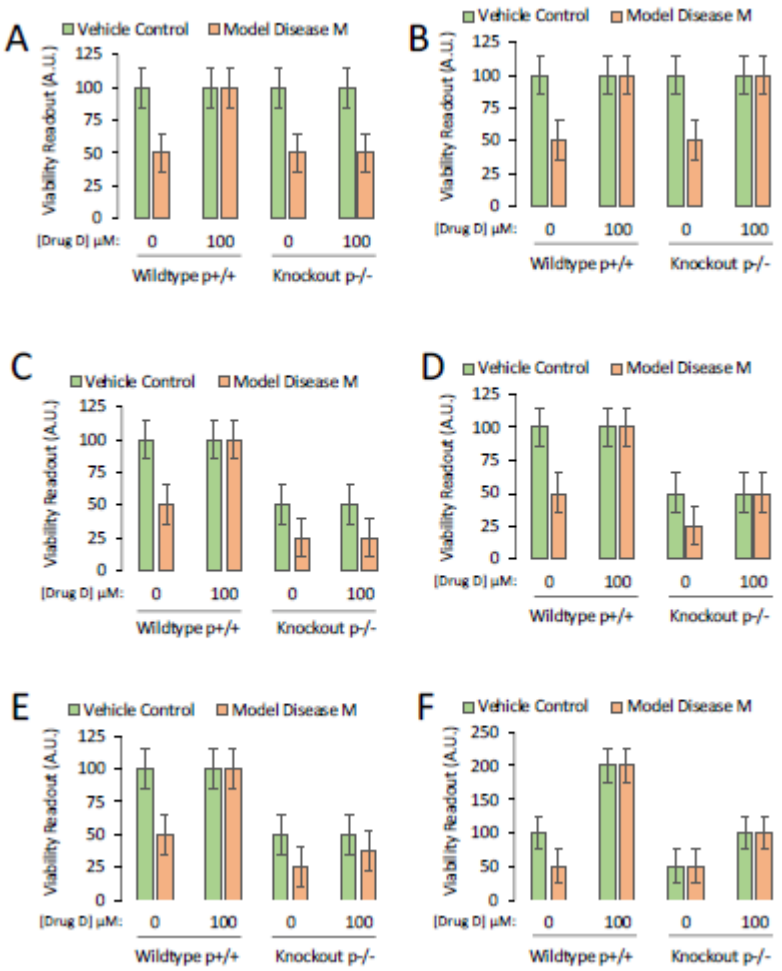
Hypothesis 1: Drug D (or any kind of intervention) prevents the effects of model disease M by upregulating the function of molecule P

Deploying a Full-factorial Three-way ANOVA

In order to claim that a biological pathway is the means whereby the drug acts on the body, the researcher needs to modify the purported mechanism of action, to determine if this interference lessens the protective effects of drug D as expected. In the current example, this involves inhibiting the function of protein P with a number of tools, ranging from a pharmacological antagonist of protein P to deletion of the gene that encodes protein P, or knocking down the translation of protein P from its mRNA. An advantage of employing a full-factorial three-way ANOVA to achieve this goal is that it forces inclusion of all the control groups, each run in parallel (if the lab can afford it). In this example, the three-way ANOVA allows us to test the impact of the following independent variables or factors (**Figure 3**):

Figure 3. Testing the mechanistic hypothesis. (**A**) The protective impact of drug D is completely abolished when the gene coding for prosurvival protein P is knocked out. (**B**) Knocking out expression of protein P does not modify the protective impact of drug D. (**C**) Knocking out the expression of protein P reduces basal viability and also abolishes the protective impact of drug D. (**D**) Knocking out expression of protein P reduces basal viability, but does not abolish, or even reduce, the protective impact of drug D. (**E**) Knocking out expression of protein P reduces baseline viability and partially dampens the protective impact of drug D. (**F**). Practice example; see text for details.



1) Disease state, of which there are two levels: i) vehicle (sham injury) or ii) a stimulus that induces model disease M at LC_{50} values
    - This variable is plotted as green versus orange bars

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2) Treatment, of which there are two levels: i) vehicle or ii) the most effective, nontoxic concentration of drug D (100 μM)
    - This variable is plotted twice on the X axis
    - Inclusion of multiple concentrations of drug D may not be economical or necessary, if the full-fledged concentration-response study for this project has already been completed (see Part I of this two-part series). Exceptions are discussed below.
3) Genotype, of which there are two levels: i) control wildtype cells or ii) cells with knockout of the gene coding for protein P
    - This factor is plotted once on the X axis, as demarcated with the horizontal black lines

Technical Confounds in Mechanistic Research

In general, pharmacological antagonists will be less selective, and, therefore, less desirable than targeted molecular biological approaches. However, if gene deletion is lethal to the embryo in animal studies or lethal to cultured cells, the researcher might be forced to use a pharmacological antagonist or gentle knockdown techniques as the third independent variable (see above). Even if the fetus survives the complete absence of protein P, there might be serious developmental deficits that affect baseline levels of the measurement outcomes. Other potential confounds of embryonic knockout of proteins include the compensatory, developmental upregulation of other proteins with similar functions, which can result in a net lack of change in measurement outcomes (2).

One alternative is to knock down the translation of the protein through RNA interference in adulthood, perhaps by delivering a virus (*e.g.*, lentivirus, adeno-associated virus, *etc.*) directly to the target tissue, but the efficiency of the viral infection may be impractically low. A second alternative is to use a conditional knockout transgenic model, in which, for example, delivery of tamoxifen in adulthood stimulates deletion of a gene flanked by two inserted loxP sites, within cells that express the DNA recombinase Cre (3). The latter approach avoids the potential developmental changes that might compensate against embryonic knockout of a protein and can also be used to target specific cell types. The student will also need to include control groups to avoid confounding actions of tamoxifen, which will bind the estrogen receptor throughout the body after systemic routes of administration (4).

There can be other confounding factors when employing gene knockout techniques. For example, if the researcher only studies a small fraction of cells that manage to survive the deletion of protein P, they might not be representative of the original cellular population as a whole. This can pose a problem during long-term *in vitro* selection of only a few genetically modified cells. As described above for animal studies, knockout of one member of a gene family may also cause compensatory upregulation of other members with similar functions *in vitro*, and no net effect of the knockout would then be observed. Such compensatory changes can lead to the erroneous conclusion that protein P is irrelevant for survival when drug D is administered (*i.e.*, a Type II error). Under those circumstances, it might be wiser to employ a pan-antagonist that non-selectively inhibits all the members of that protein family. In the latter case, the interpretations should reflect not just protein P, but all the pan-antagonist targets.

Putting the Mechanistic Hypothesis to the Test

Idealized data from successful mechanistic tests are illustrated in **Figure 3A**. With deletion of the gene that codes for protein P, there is total loss of efficacy of drug D—it fails to improve viability under diseased conditions. The investigator might then accept the test hypothesis, provided they ensure that the gene is indeed deleted. If the absence of protein P does not mitigate the protective properties of drug D (**Figure 3B**), the researcher would reject the test hypothesis and conclude that drug D protects against model disease M by affecting molecules *other* than protein P.

As protein P is a prosurvival protein, its absence might decrease basal viability, as in **Figure 3C**. Here, protein P exerts a positive impact upon basal viability and *also* mediates the effects of drug D.

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The test hypothesis would be accepted. In **Figure 3D**, protein P is still a prosurvival protein, without which basal viability is lowered, but protein P does not mediate the protective effects of drug D, as its absence has no impact on the latter measure. In **Figures 3B** and **3D**, targets *other* than protein P mediate the protective effects of drug D, and the researcher might have to consult their RNA sequencing or proteomic data sets again.

If protein P only partially mediates the protective impact of drug D, the results may appear as in **Figure 3E**, where loss of protein P reduces, but does not completely abolish the protective effects of drug D. In this event, drug D prevents the toxic consequences of disease M, at least partly, through protein P. As an active learning exercise, the student should reason on their own through the interpretations of **Figure 3F**, before reading the arguments presented below.

A number of possible explanations for data displayed in **Figure 3F** are presented below, by systematically working one's way through the green and orange bars from left to right:

1. Assume that model disease M reduces the function of the prosurvival protein P to near zero in half of the cells present, and thereby kills this vulnerable half, only leaving cells behind that do not express protein P, but survive by means of *other* prosurvival proteins (first two bars in **Figure 3F**).

2. Next, assume that drug D prevents disease M from killing half the cellular population because it dramatically increases the function of those other prosurvival proteins, and their upregulation drives cell division to the maximal levels that the plate size and media nutrients can support (second set of bars in **Figure 3F**).

3. In the third set of bars, protein P is completely absent, and, therefore, half the cells no longer survive. Here, the disease model M exerts *no additional impact* because there is no protein P to inhibit, leaving the investigator with a viability readout of 50 (in arbitrary units) for both the green and orange bars.

4. In the fourth set of bars, drug D improves viability by upregulating the function of *other* prosurvival proteins, even if it fails to do so to maximal levels. The observation that viability is increased by drug D—even in the absence of protein P and *in the absence of disease*—informs the investigator that drug D protects against loss of *basal* viability but does not engage protein P to elicit this effect.

5. The hypothesis states that protein P upregulation is the mechanism of action of the drug *under diseased conditions*. The third set of bars in **Figure 3F** reveal that disease M no longer has any toxic effect when protein P is absent. It is not possible to test the hypothesis that drug D protects against the toxicity of a disease, when the disease cannot even be provoked.

6. The student researcher is forced to try other means to test the hypothesis, as the data collected under conditions of disease are inconclusive. The student might employ mild knockdown of protein P rather than total deletion of the gene, or low concentrations of an antagonist that decrease the function of protein P, but *not to the fullest extent*, such that a reduction in viability with model disease M could still be resolved in the third set of bars.

Complementary Use of Pharmacological and Genetic Approaches

Point number 6 in the above list highlights the importance of employing concentration-response curves. A caveat to the approach employed in **Figure 3** is the lack of concentration-dependent effects of inhibiting protein P, which are more readily achieved with a pharmacological antagonist. Various levels of knockdown of protein P could also be employed to achieve concentration-dependent effects, if the pharmacological antagonists have off-target effects. Another option to defend against confounding off-target effects is to use two antagonists that are in different drug classes and downregulate the function of the same protein P through distinct mechanisms; the statistical probability that their off-target effects also overlap (as much as their effects on protein P) is low.

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Simply put, if two independent antagonists abolish or mitigate the impact of drug D, it seems reasonable to conclude that drug D works fully or partially through upregulation of protein P.

Rather than upregulating prosurvival proteins, drug D might protect against disease model M by reducing the function of a pro-apoptotic protein P. In this scenario, using knockout cells, antagonists, or protein P knockdown is less straightforward. Possible outcomes of the protein assay are illustrated in **Figure 4**. If disease model M is toxic due to an upregulation of a pro-apoptotic protein P, drug D might be expected to prevent the disease-induced increase in protein P function (**Figure 4A**) or to lower the size of the increase (**Figure 4B**). Inclusion of all the controls for the two-way ANOVA forces the investigator to examine the impact of drug D on protein P in normal, non-diseased tissue (second green bar). If drug D encourages a significant rise in the function of pro-apoptotic protein P in the non-diseased control group (**Figure 4C**), this reveals its potential hazards in healthy cells or tissues.

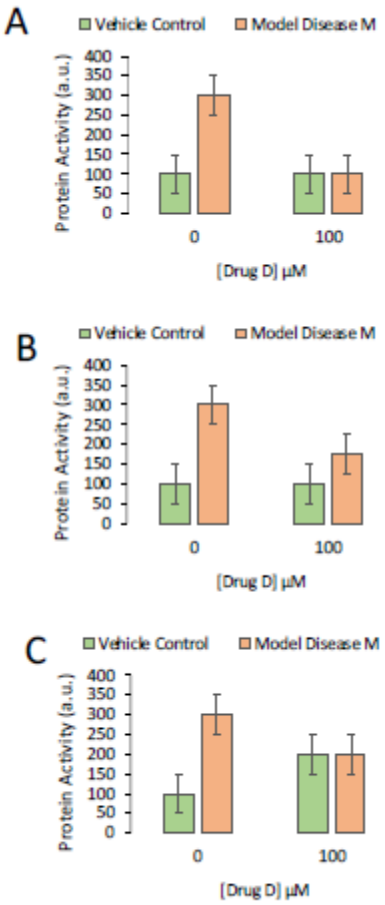

Figure 4. Impact of drug D on the activity of hypothetical prodeath protein P in disease model M. (A) Drug D completely prevents the increase in protein activity elicited by disease model M. (B) Drug D blunts the increase in protein activity elicited by disease model M. (C) Drug D increases protein activity under physiological, baseline conditions (vehicle control group), but abolishes the additional increase in protein activity elicited by disease model M under pathological conditions.

Rather than employing cells with knockout of protein P, *overexpression* of protein P allows researchers to directly interfere with the *downregulation* of protein P by drug D. Alternatively, a pharmacological agonist could be employed. First, the student researcher should ensure that the overexpression of protein P does increase both its levels and function.

Simplified data that allow acceptance or rejection of the test hypothesis are displayed in **Figures 5A** and **5B**, respectively. As protein P is pro-apoptotic, its overexpression might also decrease basal

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viability, but still permit acceptance or rejection of the test hypothesis, as shown in **Figures 5C** and **5D**, respectively. If the downregulation of protein P is only partly responsible for the protective effects of drug D, the data might appear as in **Figure 5E**, where the overexpression of protein P does not fully abolish the protective effects of drug D, but the test hypothesis is accepted.

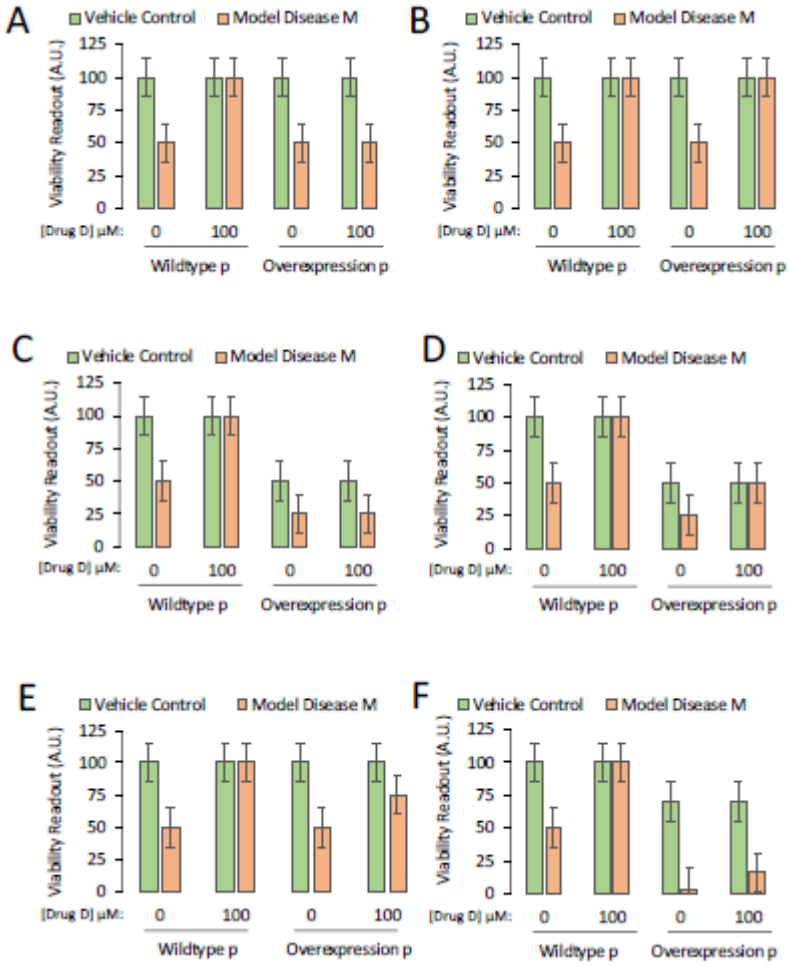

Figure 5. Testing the mechanistic hypothesis. (A) The protective impact of drug D is abolished when prodeath protein P is overexpressed. (B) Overexpression of protein P does not modify the protective impact of drug D. (C) Overexpression of protein P reduces basal viability and also abolishes the protective impact of drug D. (D) Overexpression of protein P reduces baseline viability, but does not abolish the protective impact of drug D. (E) Overexpression of protein P partially dampens the protective impact of drug D. (F) Overexpression of protein P reduces basal viability and synergizes with disease model M to cause massive loss of cellular viability, close to the limits of detection of the assay.

If protein P is strongly pro-apoptotic and its overexpression is overly toxic to cells when it synergizes with disease M, as in **Figure 5F** (third orange bar), it might be too difficult to protect cells with drug D, even if drug D engages other proteins besides P. For example, severely stressed cells might yield to necrotic types of injury that are impossible to reverse even at early stages. Furthermore, even if there is a small increase in viability with drug D in the fourth orange bar of **Figure 5F**, the viability values of the last two orange bars might be too close to background levels, and out of the dynamic range of the assay (see Part I of this series).

Many investigators do not employ the overexpression approach to test whether their drug D is protective by downregulating protein P, but employ knockout cells or animals. The rationale behind the knockout approach in this scenario is that drug D should have no *additional* protective properties in knockout cells compared to wildtype cells, if drug D relies on reducing the function of protein P.

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If a pro-death protein partly responsible for induction of the model disease M is knocked out, one might expect somewhat less toxicity when model disease M is induced, and perhaps also an increase in basal viability (**Figure 6A**). In this scenario, drug D cannot prevent the partial toxicity of model disease M under knockout conditions, if it relies on *further* downregulating protein P (as protein P is already gone), and the investigator would accept the test hypothesis. If, however, drug D is able to fully improve viability relative to vehicle-treated knockout cells, drug D cannot be relying on changing a protein that is absent (**Figure 6B**), and the test hypothesis would be rejected.

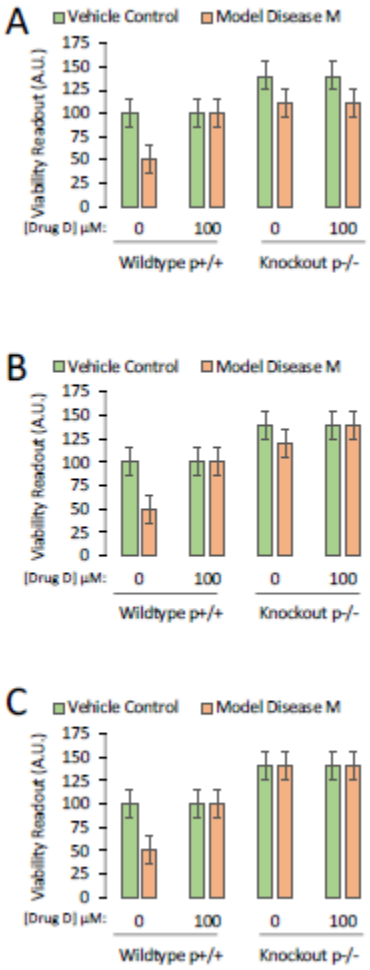

Figure 6. Testing the mechanistic hypothesis. (A) Deletion of the gene that codes for prodeath protein P increases basal viability, mitigates the toxicity of disease model M, and abolishes the protective impact of drug D. (B) Deletion of the gene that codes for protein P increases basal viability, mitigates the toxicity of disease model M, but does not abolish the protective impact of drug D against disease model M. (C) Deletion of the gene that codes for protein P increases basal viability and completely abolishes the toxicity of disease model M. Therefore, in the absence of this protein, the protective impact of drug D can no longer be resolved, and the mechanistic hypothesis cannot be tested.

Interpreting the Unexpected

If removal of the prodeath protein P *completely* abolishes the capacity of the model M to induce cell death, the researcher would not be able to determine if drug D has *additional* protective effects or not, as there is no loss of cells in the third set of bars and, therefore, no toxicity to prevent (**Figure 6C**). In this case, the investigator cannot reject or accept the test hypothesis. As argued above, mild knockdown of protein P or low concentrations of an antagonist against protein P could be employed, to achieve some reduction in viability with model disease M. This approach would allow the

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researcher to determine if drug D continues to mitigate the disease, despite interference with the function of protein P.

Conclusion

There are many more complicated examples of real-life data than depicted here, including data from non-mechanistic experiments. For an example of controls for full-factorial ANOVAs in purely descriptive (non-mechanistic) studies, the student is guided to a report showing an unexpected lack of lasting therapeutic effects of N-acetyl cysteine (and evidence of some toxicity) in an animal model of acute dopaminergic neuron loss (5). Although there are many caveats in this simple study, the inclusion of all control groups in full-factorial ANOVAs mitigated the risk of Type I and II errors.

To conclude, the student and educator now have some of the tools in hand to interpret the outcomes of their research and avoid a few of the more common pitfalls, which can otherwise decelerate research progress and lead to the squandering of human and financial resources.

Abbreviations

None

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