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Chemical Genetics: Where Genetics and Pharmacology Meet

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Genetics and pharmacology can elicit surprisingly different phenotypes despite targeting the same protein. This Essay explores these unexpected differences and their implications for biology and medicine.

Introduction

Genetics and pharmacology are the two dominant approaches for probing protein function in cells. In many cases, however, a small molecule and a genetic mutation can perturb a protein's activity in different ways, leading to different conclusions about the protein's biological function.

For example, the thiazolidinediones (TZDs) are widely prescribed drugs for the treatment of type II diabetes. TZDs were originally discovered based on their ability to enhance insulin sensitivity in obese rats (Kobayashi et al., 1983), but their molecular targets remained elusive. In 1995, these compounds were shown to be selective agonists of PPARγ, a nuclear hormone receptor that is a master regulator of adipogenesis (Lehmann et al., 1995). The identification of PPARγ as the target of TZDs was counterintuitive: these drugs are used to treat diabetes, a disease caused by obesity, yet activate PPARγ, a transcription factor that promotes adipogenesis. Heterozygous deletion of the PPARγ gene, in fact, improves insulin sensitivity in mice (Kubota et al., 1999), suggesting that PPARγ inhibitors, rather than activators, should be developed as antidiabetic drugs. This paradox was ultimately resolved by showing that genetic antagonism and pharmacological agonism of PPARγ both improve glucose metabolism but do so through different mechanisms (Yamauchi et al., 2001). In the case of TZD drugs, enhanced insulin sensitivity in the liver and muscle is achieved at the expense of increased adipose tissue and weight gain.

In this example, TZDs revealed a therapeutic potential of PPARγ agonists that was not predicted from genetic analysis. How often do small molecules reveal unexpected properties of their biological target? This question has not been explored systematically, in part because the selectivity of many pharmacological agents is unknown and difficult to assess. Indeed, less than 5% of the proteome is currently targeted by a potent small molecule; it remains laborious and expensive to identify a single new compound with useful selectivity; and there is no general strategy to inventory the cellular targets of any molecule. By contrast, modern genetic approaches make it possible to link genes to phenotypes efficiently, using robust tools that are widely accessible to the research community. What is the role of pharmacology in a biological universe dominated by the power of genetics?

Here, we highlight recent experiments in which a small molecule and a mutation targeting the same protein have produced radically different phenotypes. Through these examples, we describe the mechanisms by which pharmacology and genetics perturb biological systems in different ways and therefore provide different information about their targets.

Transferring Genetic Selectivity to a Small Molecule

A critical feature of a genetic screen is that it is possible to identify unambiguously the mutation that is responsible for producing a phenotype. By contrast, there is no way to comprehensively identify the cellular targets of a small molecule. This ambiguity confounds direct comparisons between genetic and pharmacological phenotypes because it is always possible that any difference reflects an off-target effect of the drug.

One way to overcome this difficulty is to use a mutation to confer selectivity on a small molecule, an approach referred to as chemical genetics (we use the term chemical genetics in this restricted sense to distinguish it from pharmacology). For the protein kinase superfamily, a single residue in the ATP-binding pocket, termed the gatekeeper, has been shown to control sensitivity to a wide range of small molecule inhibitors (Bishop et al., 2000b). This gatekeeper residue is conserved as a large hydrophobic amino acid among protein kinases, but mutation of the gatekeeper to alanine or glycine creates a novel pocket that can be uniquely accessed by an inhibitor analog (Figure 1A). This inhibitor analog does not inhibit any wild-type kinase, and the gatekeeper mutation typically does not impair the mutated kinase. In this way, the effects of selective pharmacological inhibition of a kinase can be explored in a model system in which the wild-type kinase has been replaced with the drug-sen-

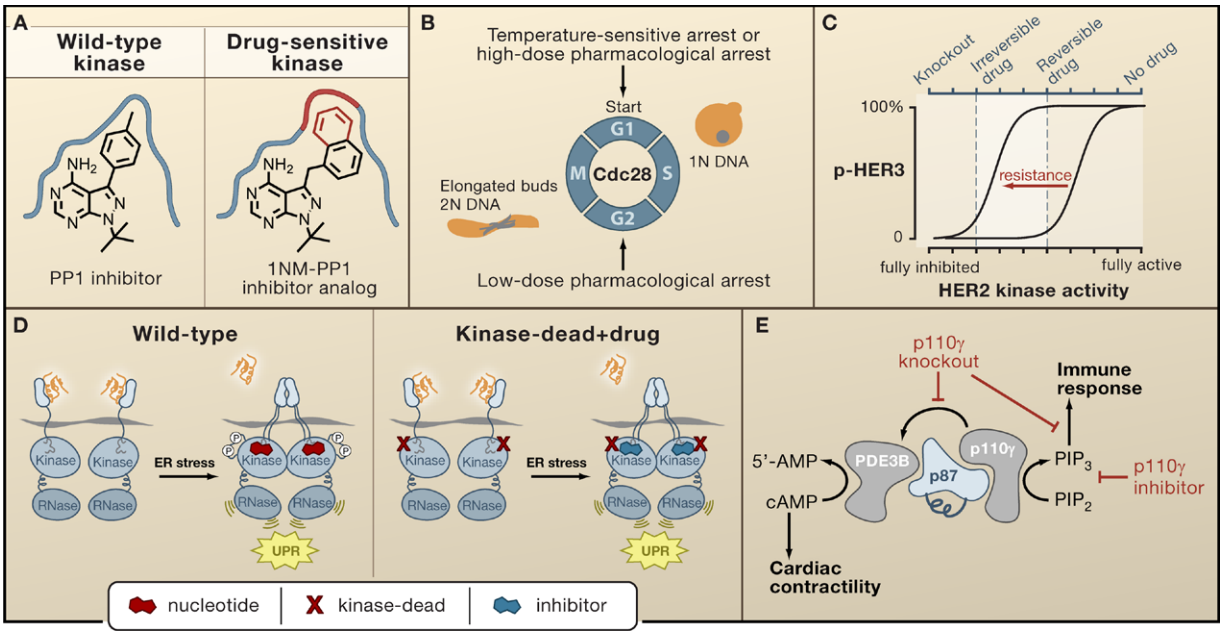

Figure 1. Mechanism of Action of Small Molecule Inhibitors

(A) Mutation of the gatekeeper residue in a protein kinase creates a new pocket (red) that can be uniquely accessed by the small-molecule inhibitor analog 1NM-PP1.

(B) Inhibition of a drug-sensitized yeast cyclin-dependent kinase Cdc28 with a low dose of 1NM-PP1 (0.5 μM) induces cell-cycle arrest at G2/M, whereas a higher dose of 1NM-PP1 (5 μM) induces cell-cycle arrest in G1. Mutant yeast with temperature-sensitive Cdc28 alleles also arrest in G1.

(C) An increase in the sensitivity of the human epidermal growth factor receptor HER3 to phosphorylation by HER2 (for example, by downregulation of a HER3 phosphatase) can induce resistance to small-molecule inhibitors targeting the kinase domains of EGFR/HER2, even though these compounds still bind to the receptors. Reversible inhibitors, irreversible inhibitors, and gene knockouts differ quantitatively in their ability to inhibit the kinase domains of EGFR/HER2, and this is reflected in the differing drug resistance phenotypes observed.

(D) Accumulation of unfolded proteins in the lumen of the endoplasmic reticulum induces the Ire1 transmembrane kinase to dimerize (left). The Ire1 dimer then becomes autophosphorylated resulting in activation of its RNase domain, thereby triggering the unfolded protein response. An Ire1 kinase-dead allele cannot activate its RNase domain and so the unfolded protein response cannot be triggered. The ATP competitive kinase inhibitor 1NM-PP1 rescues the ability of kinase-dead Ire1 to activate its RNase domain such that the unfolded protein response is triggered despite an Ire1 that lacks kinase activity (right).

(E) p110γ allosterically activates PDE3B (the enzyme that catalyzes cAMP destruction) independent of its kinase activity through the p87 adaptor protein. Small-molecule inhibitors of p110γ block kinase activity but do not affect cAMP production, whereas knockout of the p110γ gene results in loss of kinase activity and altered cAMP production.

sitive allele. A similar approach has been used to identify allele-specific ligands for proteins from several families, including GTPases, G protein-coupled receptors, nuclear hormone receptors, motor proteins, and phosphatases (Bishop et al., 2000a). For each of these proteins, a small molecule binding site was reengineered to bind uniquely to a synthetic ligand. As a result, it is possible to perform a critical control experiment in which target-less wild-type cells are treated with the synthetic ligand and thereby confirm that any phenotype requires the drug's target. Here, we focus on how this approach has been used alongside traditional pharmacology to explore cases in which kinase inhibitors and knockouts have produced different functional outcomes (Table 1).

Graded Inhibition and Intermediate Phenotypes

An early application of chemical genetics explored the role of the yeast cyclin-dependent kinase Cdc28 in controlling progression through the cell cycle (Bishop et al., 2000b). Chemical inhibition of a drug-sensitized Cdc28 allele with the inhibitor analog 1NM-PP1 (0.5 μM) induced specific cell-cycle arrest resulting in large hyperpolarized yeast buds and replicated DNA, indicative of failure to enter mitosis (Figure 1B). DNA microarray analysis confirmed that inhibition of Cdc28 blocked expression of key proteins required for the G2/M transition. Treatment of wild-type yeast with 1NM-PP1 had no effect on their morphology, proliferation, or gene expression profile, confirming the selectivity of 1NM-PP1 for the drug-sensitized Cdc28.

The G2/M arrest induced by pharmacological inhibition of Cdc28 contradicts earlier studies using temperature-sensitive yeast mutants, which resulted in arrest of unbudded cells in G1 (Figure 1B). Bishop et al. hypothesized that this discrepancy might reflect a quantitative difference in the level of Cdc28 activity required to drive specific cell-cycle transitions. In this model, entry into mitosis requires a high level of Cdc28 activity and therefore is sensitive to low concentrations of drug, whereas progress through S phase requires less Cdc28 activity and therefore is sensitive only to high drug concentrations or genetic ablation of the protein. To test this hypothesis, the drug-sensitive Cdc28 strain was synchronized by treatment with α factor and then the yeast were released

Table 1. Phenotypic Differences between Kinase Inhibitors and Knockout Models

Kinase	Knockout Phenotype	Inhibition Phenotype	Proposed Explanation	Class
CDC28	Temperature-sensitive alleles arrest in G1 at restrictive temperature	Chemical inhibition induces G2/M arrest (low doses) and G1 arrest (high doses)	The mitotic checkpoint is more sensitive to cyclin-dependent kinase activity than the G1 checkpoint	Phenotypic threshold
Ire1	Knockout of Ire1 or expression of a kinase-dead allele blocks the unfolded protein response (UPR)	An ATP competitive inhibitor of the kinase-dead allele rescues the UPR	An ATP competitive ligand for the Ire1 kinase domain allosterically activates the Ire1 RNAse domain during the UPR	Allosteric drug
Apg1	Cells lacking Apg1 are defective in cytoplasm-to-vacuole targeting (Cvt) and autophagy	Chemical inhibition or expression of Apg1-kinase dead blocks Cvt but not autophagy	Cvt requires catalytic activity of Apg1, whereas autophagy requires its scaffolding function	Disrupted protein complex
Cla4	Cells lacking Cla4 are defective in septin localization	Chemical inhibition has no effect on septin localization	Septin localization may depend on a scaffolding function of Cla4	Disrupted protein complex
Elm1	Cells lacking Elm1 undergo G2/M delay	Chemical inhibition causes G1 delay in bud emergence and Cln2 synthesis, as well as defective G2/M	Elm1 knockout cells accumulate suppressors during culture that compensate for the G1 defect	Cellular compensation
JNK2	JNK2 knockout cells have normal c-Jun phosphorylation and increased proliferation	Chemical inhibition of JNK2 blocks c-Jun phosphorylation and cellular proliferation	Jnk2 knockout causes a compensatory increase in Jnk1 activity and c-Jun expression	Cellular compensation
Aurora B	RNAi targeting impairs cell-cycle arrest in response to both Taxol and Nocodazole	The ZM-447439 inhibitor impairs checkpoint arrest in response to Taxol, but not Nocodazole	RNAi targeting of Aurora B disrupts a centromeric complex, yielding a more severe phenotype than chemical inhibition	Disrupted protein complex
EGFR	RNAi targeting kills tumor cell lines resistant to the EGFR inhibitor gefitinib	Gefitinib cannot kill resistant tumor cells, even though they lack EGFR mutations that block gefitinib binding	Drug resistance is associated with changes that amplify low-level EGFR activity	Phenotypic threshold
p110γ	Knockout mice show increased cardiac contractility and tissue damage	Mice expressing the kinase-dead allele have normal cardiac function	p110γ allosterically activates PDE3B, thereby regulating heart contractility independent of kinase activity	Disrupted protein complex
p110α	Heterozygous knockout mice have normal insulin signaling (homozygous knockout is not viable)	Heterozygous mice expressing the kinase-dead allele or treated with a p110α inhibitor have impaired insulin signaling	Reduced expression of p110α in the knockout triggers compensation, partly by altering its stoichiometry relative to p85	Disrupted protein complex
mTOR	RNAi targeting blocks phosphorylation of Akt at Ser473 in most cell types	The inhibitor rapamycin increases phosphorylation of Akt at Ser473 in most cell types	mTOR-rictor is insensitive to rapamycin and phosphorylates Akt. mTOR-raptor is sensitive to rapamycin and controls a negative feedback loop inhibiting Akt phosphorylation.	Allosteric drug, disrupted protein complex

from cell-cycle arrest in the presence of a higher concentration of 1NM-PP1 (5 μM). Under these conditions, inhibition of Cdc28 induced a uniform G1 arrest in which cells formed small buds but failed to undergo DNA replication (Figure 1B). Thus, complete Cdc28 inhibition induces primary arrest in G1, whereas partial Cdc28 inhibition causes secondary arrest at G2/M. This graded response to pharmacological inhibition is consistent with quantitative models of the cell cycle that suggest that entry into mitosis requires enhanced cyclin-dependent kinase activity (Stern and Nurse, 1996).

The G2/M arrest after partial Cdc28 inhibition represents an intermediate phenotype that was revealed by dosing with a small molecule inhibitor but masked by genetic approaches that resulted in total loss of protein function (such as knockout of the *Cdc28* gene). Such quantitative differences in target inhibition can be critical determinants of the clinical efficacy of a small molecule. For example, gefitinib is a small molecule inhibitor of the tyrosine kinase domain of the epidermal growth factor receptor (EGFR; also called HER1) that has been used clinically to treat various tumors including breast cancer and non-small cell lung cancer. In many cancer patients, the initial response to this drug is followed by drug resistance and disease progression. However, drug resistance is not

always associated with mutations in the kinase domain of EGFR that block binding of the drug, and tumor cell lines selected in vitro for resistance to gefitinib often do not acquire EGFR mutations (Kwak et al., 2005). Nonetheless, these gefitinib-resistant tumor cells remain sensitive to genetic blockade of EGFR signaling (that is, they can be killed by small interfering RNAs that target EGFR or its binding partner HER2). What is the reason for this? Gefitinib resistance is associated with downregulation of cellular tyrosine phosphatase activity and increased expression of EGFR/HER2 substrates, two mechanisms that amplify the EGFR-dependent substrate phosphorylation that remains after drug treatment (Sergina et al., 2007). This suggests that tumor cells can become resistant to gefitinib by increasing their threshold for EGFR inhibition (Figure 1C) without specifically preventing the drug's mechanism of action by, for example, acquiring EGFR mutations that block drug binding. Consistent with this model, gefitinib-resistant cancer cells remain sensitive to irreversible EGFR inhibitors, molecules that are structural analogs of gefitinib but that are more potent due to their covalent mechanism of action (Kwak et al., 2005). Thus, tumor cells can achieve resistance to a kinase inhibitor, despite retaining a genetic requirement for its target, by exploiting quantitative differences in target inhibition.

Allosteric Mechanisms of Small Molecule Action

Ire1 is an endoplasmic reticulum transmembrane kinase that controls the unfolded protein response, the process by which accumulation of unfolded proteins in the lumen of the endoplasmic reticulum activates production of chaperones and related proteins (Patil and Walter, 2001). Unfolded proteins in the endoplasmic reticulum induce dimerization of Ire1, resulting in activation of the Ire1 kinase domains and RNase domains and initiation of the unfolded protein response (Figure 1D). Mutations that disable the Ire1 kinase are known to block the unfolded protein response, but the biochemical function of this kinase has been poorly understood, as it has no known protein substrates.

To investigate signaling by Ire1, wild-type Ire1 kinase was replaced with a drug-sensitive mutant (L745G) (Papa et al., 2003). In this case, the drug-sensitizing mutation markedly impaired Ire1 kinase activity and activation of the unfolded protein response. Paradoxically, when cells expressing the weakened Ire1 (L745G) allele were treated with the inhibitor 1NM-PP1, there was almost complete recovery of the ability of Ire1 (L745G) to induce the unfolded protein response.

How is this possible? Control experiments confirmed that 1NM-PP1 rescued the unfolded protein response through a direct and specific interaction with Ire1(L745G). To explain this, the authors hypothesized that the Ire1 kinase domain allosterically activates the Ire1 RNase domain, which is sufficient to trigger the unfolded protein response, and that this conformational signal is controlled by nucleotide binding to the kinase. In this model, binding of 1NM-PP1 to the Ire1(L745G) kinase mimics nucleotide-dependent allosteric activation of the RNase domain resulting in rescue of Ire1 function (Figure 1D).

This hypothesis was tested by constructing a double mutant form of Ire1(L745G/D828A) that combines kinase-dead and drug-sensitizing mutations. This double mutant had no detectable kinase activity but was able to bind to the kinase inhibitor 1NM-PP1. Yeast expressing Ire1(L745G/D828A) failed to trigger the unfolded protein response in the absence of drug but were able to induce the unfolded protein response to near wild-type levels in the presence of 1NM-PP1 (Figure 1D). Thus, 1NM-PP1 acts as an Ire1 agonist rather than an Ire1 inhibitor, even though this compound binds to the Ire1 active site and directly blocks its kinase activity.

In this case, a kinase inhibitor rescued the phenotype of a kinase-dead mutation, revealing a signaling mechanism in which Ire1 shuttles between active and inactive conformations in response to ligand binding at the ATP site. Is Ire1 unique or do other kinases signal in this way? No functional protein kinase is known to signal in this manner, but the enzyme RNase L contains a kinase domain that lacks essential catalytic residues (referred to as a pseudokinase domain), and binding of nucleotides to this pseudokinase regulates its RNase activity (Dong and Silverman, 1999). The human genome encodes at least 48 pseudokinases, and many of these proteins regulate key signaling pathways or are the target of amplification and mutation in cancer (Boudeau et al., 2006). The biochemical mechanisms that mediate pseudokinase signaling are largely unknown, but it is likely that many use allosteric mechanisms similar to Ire1. Thus, it may be possible to activate or inhibit these pseudokinases by developing drugs that target their vestigial active sites.

Inhibiting Kinase Activity but Leaving Protein Complexes Intact

Small molecules typically do not alter the expression of their target protein. In contrast, knockout of a gene results in complete loss of the target protein, which can disrupt protein complexes or impair functional domains that would be unaffected by a drug. These secondary effects can dominate the phenotype of a knockout, such that a knockout and a small molecule inhibitor targeting the same protein produce very different outcomes.

Studies of the PI3-kinase p110γ illustrate how disruption of a protein complex through knockout of a gene encoding one of the complex subunits can induce a more severe phenotype than the corresponding small molecule inhibitor. p110γ mediates the response of leukocytes to diverse chemotactic and inflammatory stimuli, and mice that lack p110γ have a dampened immune response (Hirsch et al., 2000; Sasaki et al., 2000). This finding has generated significant interest in p110γ inhibitors as anti-inflammatory drugs. However, analysis of p110γ knockout mice

revealed that these animals exhibit elevated contractility of cardiac muscle (Crackower et al., 2002) and suffer from myocardial damage in response to aortic constriction (Patrucco et al., 2004). The cardiac phenotype of the p110γ knockout animals was surprising, in part because overexpression of a kinase-dead p110γ in heart muscle protects mice from myocardial damage (Nienaber et al., 2003). Why does deletion of p110γ produce a phenotype opposite to that obtained by overexpression of a kinase-dead allele? The increased cardiac contractility in the p110γ knockout mice is associated with increased cAMP in cardiomyocytes, suggesting that p110γ may regulate cAMP levels. To address this possibility, Patrucco et al. (2004) generated knockin mice that express a p110γ kinase-dead allele at wild-type levels from the endogenous locus. These animals, which better mimic the effects of a small molecule inhibitor, retain the immune deficits of the original p110γ knockout mice but have normal heart tissue, indicating that the cardiac defect is not due to loss of p110γ kinase activity. Further investigation revealed that p110γ binds to and allosterically activates PDE3B, the enzyme that catalyzes cAMP destruction (Patrucco et al., 2004). Deletion of the gene encoding p110γ disrupts this critical protein-protein interaction, leading to pathological accumulation of cAMP and cardiac failure (Figure 1E). As the regulation of PDE3B by p110γ is independent of kinase activity, it is unaffected by small molecule kinase inhibitors targeting p110γ (Patrucco et al., 2004).

Similar effects have been observed for inhibitors of Aurora kinases. These kinases monitor spindle assembly and chromosome alignment during mitosis, triggering checkpoints that ensure proper chromosome segregation and cytokinesis. Many tumors overexpress Aurora kinases, sparking interest in these enzymes as potential cancer drug targets. Knockdown of Aurora B by RNA interference (RNAi) induces a range of mitotic defects, including failure of cells to arrest in response to paclitaxel (a microtubule-stabilizing agent) or nocodazole (a microtubule polymerization inhibitor) and failure of microtubules to become properly attached to kinetochores, leading to chromosome mislocalization (Ditchfield et al., 2003). In contrast, treatment with the Aurora kinase inhibitor ZM447439 induced a much milder phenotype: the drug blocked paclitaxel-induced mitotic arrest but had no effect on nocodazole-induced arrest or kinetochore-microtubule interactions (Ditchfield et al., 2003). Aurora B binds to the proteins Survivin and INCENP at the centromere, and this complex is required for correct execution of mitosis, suggesting that disruption of this complex may underlie the more severe phenotype resulting from RNAi against Aurora B. Indeed, imaging experiments revealed that Survivin was absent from centromeres after treatment with Aurora B RNAi but was localized correctly after treatment with ZM447439 (Ditchfield et al., 2003). Thus, it is likely that the more dramatic phenotype caused by Aurora B kinase RNAi is due to loss of the scaffolding function of this kinase.

In other cases, disruption of a protein complex can trigger mechanisms of cellular compensation that mask the phenotype induced by knockout of a gene. For example, the PI3-kinases p110α and p110β transduce signals from growth factors such as insulin, but it is unclear which growth factor signaling pathways are controlled by each PI3-kinase isoform. Knockout mice lacking p110α or p110β die early in development, indicating that these kinases are essential and cannot compensate for each other (Bi et al., 2002). Heterozygous deletion of either p110α or p110β leads to no detectable phenotype, and deletion of p85, the binding partner of p110 proteins, causes a paradoxical increase in insulin signaling (Brachmann et al., 2005). However, knockin animals heterozygous for a p110α kinase-dead allele have dramatically impaired responses to insulin (Foukas et al., 2006), and p110α-selective inhibitors cause a similar decrease in insulin signaling (Knight et al., 2006). Why is it that p110α inhibitors or kinase-dead alleles block insulin signaling, whereas animals lacking p110 or p85 have normal or even enhanced insulin sensitivity? Analysis of tissue from heterozygous p110 knockout animals showed that reduced p110 expression is accompanied by a parallel reduction in p85 (Brachmann et al., 2005). Moreover, careful study of p85 knockout animals shows that p85 can function as a negative regulator of PI3-kinase signaling. The model that emerges from these experiments is that PI3-kinase signaling is controlled by the relative stoichiometry of p110 and p85 rather than the absolute expression levels of either protein. For this reason, heterozygous deletion of p110α results in a compensatory decrease in p85 that leaves total PI3-kinase activity unchanged. In contrast, expression of a p110α kinase-dead allele or treatment with a p110α inhibitor blocks the activity of this kinase while leaving p85 levels unperturbed.

Conclusions

There are now many examples where different phenotypes emerge depending on whether a protein is targeted by a small molecule inhibitor or a knockout of the gene (Table 1). These discrepancies reveal the diverse biological functions of a single protein. Given that many therapeutics are small molecules, these examples challenge the general assumption that genetics can act as a surrogate for pharmacology in identifying protein targets for drug development. In the foreseeable future, when every gene in every model organism has been manipulated through genetics, there will still remain a significant fraction of undiscovered biology awaiting the development of the appropriate small molecule tools.

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