Perspectives in Pharmacology

Exploiting Complexity and the Robustness of Network Architecture for Drug Discovery

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ABSTRACT

The issue of complexity stands at the center of contemporary drug discovery and development. The central problem in drug development today is attrition of drug candidates identified by the modern molecular target-based discovery approach, due to two related features of complex metabolic networks: their fundamentally unpredictable response to targeted interventions and their "robustness" (tendency to maintain stable function in the face of internal or external perturbations). Complexity and adaptations are, therefore, generally seen as obstacles to drug discovery. Here, the converse proposition is presented—that the complexity and adaptive responses of highly interconnected metabolic networks can be exploited for therapeutic discovery. Unanticipated connectivity relationships may result in "off-target" changes in metabolic fluxes, leading to unexpected therapeutic actions of agents. Exploiting this approach requires that fully as-sembled living systems (in vivo models) be studied and that informative in vivo biomarkers of the activity of biochemical pathways responsible for disease be available. These biomarkers should be sensitive, predictive of functional endpoints, and have high enough throughput for efficient screening of large numbers of agents. To the extent that such biomarkers unambiguously reflect the activity of pathways that mediate disease or therapeutic response (i.e., are "authentic"), their utility will be increased. Examples are presented of pathway-based screening of approved drugs for unexpected actions. Results support the principle that agents that have one action typically have many actions, including unanticipated actions, reflecting connectivity relationships of complex networks. Pathway-based screening in vivo represents an alternative to the high attrition of the molecular target-based discovery paradigm.

Complexity and Modern Target-Based Drug Discovery

Understanding the complex metabolic networks that control function in cells and organisms is widely seen as the next great challenge in biologic research. In the field of therapeutics, inability to predict the consequences of manipulating molecular targets in complex living systems is the central problem presently limiting the development of effective and safe drugs (Noble, 2001; Duyk, 2003). The problem of complexity, therefore, stands at the center of drug discovery and development (DDD) in the postgenomic era.

The most common perspective on complexity is as an obstacle to successful application of insights generated from reductionist approaches. This perspective was well expressed by Lewis Thomas 30 years ago (Thomas, 1979): "It is essentially impossible to just step in and fix a complex social system, like an urban center or a hamster . . . if there are things you are dissatisfied with and anxious to fix, you cannot set about fixing them with much hope of helping. This realization is one of the sore discouragements of our century . . . whatever you propose to do based on common sense will almost inevitably make matters worse than better . . . you cannot meddle within one part of a complex system from the outside without . . . setting off disastrous events that you hadn't counted on in other remote parts. Intervening is a way of causing trouble".

If Thomas is correct that "meddling" mostly causes trouble in complex systems, this would indeed be very bad news for anyone interested in medical interventions (i.e., physicians, the pharmaceutical industry, public health workers). This review presents an alternative view of complexity, emphasizing its inherent creative possibilities and uses in drug discovery. The role of complexity in the high attrition rate of

ABBREVIATIONS: DDD, drug discovery and development; ALS, amyotrophic lateral sclerosis.

drug candidates is discussed first, as this provides the heuristic model for understanding how the complexity and adaptations of biologic systems can be an ally in drug discovery.

Attrition and the Unpredictability of Functional Outcomes

It is clear that the modern DDD system is not working as advertised (Duyk, 2003) (Food and Drug Administration, www.fda.gov/oc/initiatives/criticalpath; Government Accountability Office, www.gao.gov/new.items/d0749.pdf). Approvals of new chemical entities are at historic lows, despite increasing research budgets and powerful new tools for identifying therapeutic targets and for generating compounds with activity against these targets.

Attrition is the Achilles' heel of molecular target-based drug discovery (Duyk, 2003) (Food and Drug Administration, www.fda.gov/oc/initiatives/criticalpath). Never have there been as many molecular targets or drug candidates as the present, but more than 98% of drug candidates fail and >90% of candidates entering phase II fail to gain approval. These failure rates are higher than 20 years ago (Duyk, 2003) (Food and Drug Administration, www.fda.gov/oc/initiatives/criticalpath; Government Accountability Office, www.gao.gov/new.items/d0749.pdf). Modern drug discovery research is primarily carried out ex vivo and in intentionally simple systems—e.g., using high-throughput enzyme assays, cultured cells for screening, etc. These tools are highly efficient at generating targets and candidates but have low power for predicting the in vivo efficacy or toxicity of agents and targets that are identified. Lacking a reliable understanding of the full range of activities (both beneficial and toxic) of novel compounds in living organisms and having no reliable way to link molecular actions to physiologic or pathophysiologic processes, it is not possible to predict which interventions will be effective and safe in humans.

This inability to predict the consequences of modulating molecular targets should hardly have been a surprise. Although drugs interact with specific physical elements in an organism (e.g., an active site on a protein, a ligand-dependant transcription factor), the actual therapeutic target in vivo is distal and much more complex. What typically matters for phenotype is the output or flow of molecules through a functionally important pathway, which in turn operates within a highly connected and interactive biochemical network (Fig. 1). Thus, functionally important biochemical pathways embedded in larger cellular and organismal networks represent the true targets of drugs. In a fibrotic or cirrhotic liver, for example, there can be no reduction of fibrosis (accumulated collagen in the extracellular matrix) without a reduction in the synthesis rate or an increase in the breakdown rate of collagen. The synthesis and breakdown rates of collagen in the liver, therefore, represent pathways with intrinsic functional significance for fibrosis. In contrast, molecular targets, such as transforming growth factor-β or matrix metalloproteinases, for example, may appear attractive in fibrogenic disorders, but their activities have no intrinsic functional significance; that is, activity of any isolated element may or may not influence global output of or flux through the pathway. In technical terms, the control strength for any component of a pathway in a complex network may range from zero to unity (Kaczer and Burns, 1973;
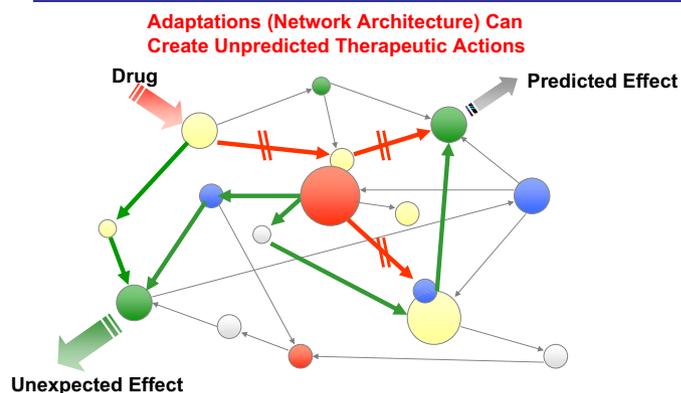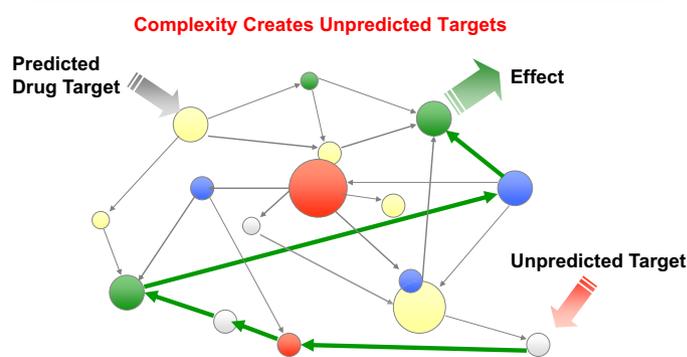

Fig. 1. Fundamental reason for high attrition of drug candidates: outputs of complex biochemical networks are unpredictable and are the true therapeutic targets. This may be exploited for drug discovery, however. A, adaptations (network architecture) can create unpredicted actions of agents. B, complexity may create unpredicted molecular targets with therapeutic utility.

Crabtree and Newsholme, 1987) and is not generally predictable a priori by analyzing the components in isolation.

The notion that alterations in isolated nodes of complex networks have unpredictable consequences is also important in modern genetics. A clear embodiment of this principle is the phenotype of numerous inborn errors of metabolism. In glycogen storage disease-type I (glucose-6-phosphatase deficiency), for example, the ultimate phenotype in adults is not hypoglycemia but includes liver tumors, growth failure, and platelet dysfunction (Chou et al., 2002). These would not be anticipated a priori. Indeed, if glucose-6-phosphatase had been discovered by modern "reverse biology", it might have been classified as a liver tumor suppressor.

These principles of metabolic control also have fundamental implications concerning evolutionary biology and the selection of traits. If genetic mutations affect metabolic phenotype in an unpredictable manner and with the possibility of multiple effects at a distance, it is evident that very different phenotypes can emerge from random alterations at critical nodes. The notion that higher level control over network interactions (i.e., control architecture) is the main determinant of function, rather than the genes or proteins in isolation, of course implies that the same biochemical maps can have very different macroscopic phenotypes.

In context of pharmaceutical discovery, it is worth emphasizing that metabolic fluxes refer to the flow of molecules

through endogenous pathways, regardless of the function of the pathway, and do not include xenobiotic metabolism (i.e., drug metabolism). Thus, anabolic pathways (e.g., protein synthesis, lipogenesis, DNA replication), catabolic pathways (e.g., β oxidation of lipids, proteolysis, RNA degradation), as well as intermediary metabolic pathways (e.g., tricarboxylic acid cycle, glycolysis, ribonucleotide synthesis, amino acid metabolism), other biosynthetic pathways (modification of lipids, glycosaminoglycan synthesis, etc.), and cellular development pathways (e.g., proliferation, differentiation and death of epithelial cells or other cell types) are all included.

Robustness of Flux Distributions

A second fundamental issue for therapeutics that arises from biological complexity is the tendency of evolved metabolic networks to resist external manipulation. Several lines of evidence indicate that evolved biological networks actively maintain and defend stable function (flux distributions) in the face of internal or external perturbations. Technically, this feature is termed robustness (Stephanopoulos and Vallino, 1991; Fischer and Sauer, 2005)

The best characterized examples of robustness come from the field of metabolic engineering (Stephanopoulos et al., 1991; Fischer and Sauer, 2005). Despite the capacity to control the expression of essentially every gene and the level of every protein in bacterial cells (the simplest of organisms), metabolic engineers have learned how difficult it is to direct cells to efficient production of desired molecules (proteins, biofuels, etc.).

The failure to redirect fluxes by targeted manipulation is explained by concepts such as "control architecture" (the system of feed-back and feed-forward connectivity relationships that maintain a characteristic pattern of fluxes within a biochemical network) and "network rigidity" (the degree to which characteristic flux distributions at different nodes are fixed and defended). Metabolic engineers appreciate the importance of the internal control systems that connect pathways and thwart simplistic attempts to redirect flux (Bailey, 2001; Stephanopoulos et al., 2004).

Recently, the robustness of flux distributions in the face of single gene deletions has been explicitly quantified. Stable isotope labeling methods were used to establish the effects on metabolic fluxes in over 130 bacterial mutants, each lacking a specific gene (Fischer and Sauer, 2005). It turned out that very few genes, when absent, alter flux distributions through central metabolic pathways. Even without killing a bacterial cell, classic linear metabolic control models would predict that a reasonably high percentage of gene deletions in central metabolic pathways should alter flux through their cognate pathways. Sauer's results point out the redundancy of complex networks and the degree to which flux distributions are defended by cells and organisms. By extension, it is likely that most gene products in complex mammalian systems also do not exert significant control strength over fluxes through key pathways, even when their activity is reduced substantially (except in trivial cases, such as linear pathways with a single entry route).

Interestingly, however, the few gene products that did alter flux distributions altered more than one flux (Fischer and Sauer, 2005). Accordingly, a less recognized consequence of the rigidity of metabolic networks is that changing one flux will probably induce numerous secondary adaptive changes. This experimental observation is congruent with general theoretical analyses of network behavior. Barabasi (2002) has emphasized that targeted disruptions against the most connected nodes in complex adaptive networks can have profound effects. For instance, disabling a central airport such as Chicago or Dallas can have ripple effects throughout the entire air traffic within the United States. The key is to identify those nodes that induce large effects on flux distributions as these will be the targets most likely to have not just one but many therapeutic (and perhaps undesirable) actions. This principle is discussed in greater detail below.

Implications of Network Rigidity for Therapeutic Discovery

These insights from metabolic engineering have several implications for drug discovery. First, most molecular targets, particularly those in signaling or regulatory pathways, are highly unlikely a priori to have functional consequences or therapeutic utility (Bailey, 2001; Hellerstein, 2008). This discovery might be quite troublesome for many contemporary biologists if they were aware of it (which may explain why this principle is not widely appreciated).

Robustness has more optimistic implications for therapeutics. Gene products that exert an effect on any flux probably affect many fluxes (Fischer and Sauer, 2005). This observation emerges from the same connectivity relationships that prevent most interventions from having any effect. Once any significant flux rate is altered, a cascade of other adaptive flux changes will almost certainly be induced, with unpredictable consequences. This principle has recently been exploited in the field of metabolic engineering for "strain improvement" (Stephanopoulos et al., 2004). Random genetic alterations are introduced, and resulting metabolic fluxes are measured. Improvements in metabolite production from unanticipated molecular targets have resulted, driven by the network's internal adaptive programs.

Historical Context: The History of Drug Discovery

It is also worth reflecting on the history of 20th century drug discovery to provide a context for the notion that complexity can be the ally of therapeutic discovery. The current model of DDD is different from the approach used to discover the most useful drugs of mankind (Le Fanu, 1999). The unappreciated fact about most of our powerful drugs is the role of empirical observation in their discovery.

Antipsychotic drugs, for example, have profoundly altered modern society. The phenothiazine antipsychotic drugs were discovered without any basic understanding of their mechanism of action or biological target. A researcher testing chlorpromazine (Thorazine) as an analgesic agent noted that rats appeared to be calmed by the drug and unperturbed by painful stimuli. Human volunteers given chlorpromazine exhibited a "profound quietue". The first patient with psychiatric disease given chlorpromazine had been institutionalized for more than 20 years and was unable to take care of himself. Within 4 weeks, he was helping to "plan the institution's Christmas party" (Le Fanu, 1999).

Even less rational or targeted is the serendipitous obser-

vation of useful actions of drugs after they were tested and approved for entirely different purposes. Some of our most widely used drugs were discovered in this matter. Sildenafil citrate (Viagra) was originally tested as an antianginal treatment. The observation of stimulated erectile function was serendipitous and only detected because it was hard to miss. No one set out with the goal of overcoming erectile dysfunction by modifying this pathway. A similar story applies to minoxidil (Rogaine), the treatment for male-pattern baldness. Both of these unexpected activities were discovered only because of the physical visibility of the phenotype. Other classic examples of secondary observations that led to important drugs include the development of antimalarials, diuretics, and antidiabetic agents from sulfa antibiotics; antidepressants from the antituberculosis drug isoniazid; and many others. These examples all fit the theme of "one action, many actions" for agents in highly connected systems.

The other key point here is the relative lack of importance of pre-existing mechanistic understanding or therapeutic intent in the development of many drugs. Although extremely inefficient for testing and identifying candidates, the combination of serendipity or trial-and-error based on functional outcome measures was more highly predictive of clinical success and resulted in much lower attrition rates than the molecular target-based approach.

The classic approach has serious limitations, of course (inefficiency, failure to predict toxicities, low throughput), and the "low-lying fruit" may have been found already, so a return of DDD to crude macroscopic phenotypic measures is not possible or desirable. The key question is whether modern technologies exist that can combine the capacity of macroscopic phenotypic measures to predict successful clinical outcomes with the throughput, breadth, and creativity of molecular target-based discovery.

Navigating the Unpredictability and Robustness of Complex Networks

To answer this question, it is worth considering the intuitively familiar problem of driving a car through traffic. There exists no computer program that can drive through traffic, but every 16-year-old learns to do it. What a teenager has that a computer does not have is vision—the ability to see and monitor where they are going. Having vision, it is a simple matter to apply feedback control and navigate through traffic, stop when a pedestrian is in the cross-walk, and so on. In systems theory, this is termed "observability". A key point is that prediction (of the ambulatory behavior and location of each pedestrian in the city) is not necessary when there is real-time observability. This is the notion that led Norbert Weiner to coin the term "cybernetics" (from Greek *kubernetike*, "the art of the steersman", or *kubernetes*, "the pilot of a ship, a helmsman, a guide, a governor") for the science of automated control systems (Weiner, 1948). The helmsman is the key to ensuring that a desired destination or outcome is reached.

In the context of biologic control and drug development, what would need to be observed to allow efficient navigation toward desired outcomes? The key functional output in biologic systems is the flow of molecules through relevant pathways (Kaczser and Burns, 1973; Crabtree and Newsholme, 1987; Noble, 2001). Thus, to understand and control complex systems, one might monitor the molecular fluxes that mediate phenotype, function, and disease (Crabtree and Newsholme, 1987; Hellerstein, 2004, 2008; Hellerstein and Murphy, 2004; Turner and Hellerstein, 2005). Optimally, these fluxes should represent the critical pathways that drive disease or that mediate therapeutic response.

Some examples of outputs of pathways that have intrinsic significance in disease include synthesis and breakdown of tissue collagen in fibrotic disorders or of axonal myelin in multiple sclerosis, mobilization of cholesterol from atherosclerotic lesions in the vessel wall, proliferation of prostate epithelial cells in benign prostatic hyperplasia, insulin-mediated glucose utilization by peripheral tissues in obesity and prediabetes, the proliferation of pancreatic β cells in response to insulin resistance, the formation of new brain cells in the hippocampus in disorders of cognition, and so on. An agent that altered the flow through any of these pathways would be hard to ignore because such pathways can be said to have intrinsic functional significance for disease. These metabolic or cellular pathways provide a conceptual framework for designing authentic biomarkers of disease activity and therapeutic response.

Inventories of Individual Elements of Networks: "-omics" Approaches

Considerable effort has been spent in recent years analyzing the transcriptome (i.e., gene expression microarrays), the proteome (global patterns of protein expression), and the metabolome (metabolite profiles) (Debouck and Metcalf, 2000; Berry, 2001; Weston and Hood, 2004). Statistical approaches have been developed for interpreting gene expression data, including gene set enrichment analysis, significance analysis of microarray to gene-set analyses, and others (Subramanian et al., 2005; Dinu et al., 2007). These approaches do not, however, provide a fundamental solution to the problem of complexity and the interconnectedness of metabolic pathways. Gene expression data, proteomics, or other such inventories of the individual elements of complex networks provide no information about the central control feature of these systems: namely, the connectivity relationships among the components. It is precisely these feed-back and feed-forward interactions that carry the higher level organizational rules responsible for defending or altering flux distributions in the network. For this reason, inventories of individual elements cannot reveal flux through pathways, particularly through specific pathways that drive disease pathogenesis or therapeutic response. The latter can only be learned by measuring actual fluxes that are present in the assembled system. RNA levels, for example, often exhibit little or no correlation with actual fluxes through pathways [e.g., gene expression data are misleading with regard to lipogenic and adipogenic fluxes in adipose tissue of obese mice (Turner et al., 2007a)].

These principles of metabolic control suggest that inventories of network components, whether rational or unbiased, can not result in definitive functional interpretation. It is notable, in this context, that the practitioners of microarray analysis and other -omics approaches implicitly recognize that flux through pathways is the bottom line. The authors who described gene set enrichment analysis of microarray data (Subramanian et al., 2005), for example, state that "an

increase of 20% in all genes encoding members of a metabolic pathway may dramatically alter the flux through the pathway and may be more important than a 20-fold increase in a single gene", or maybe not. Catalogs of all elements of a system can not, in principle, provide definitive functional interpretations

Accordingly, the most interpretable and, thus, the optimal strategy for characterizing the dynamics of pathways in complex networks is to directly measure fluxes through the key pathways, rather than relying on gene expression profiling or other indirect indices.

Exploiting Complexity for Therapeutic Discovery

Taken together, these considerations have potentially profound, although largely unexplored, implications for drug discovery. Although expected flux alterations may not occur in response to a targeted intervention, because of the complexity and adaptations of biologic networks, unexpected flux changes may result and may have therapeutic utility. This is shown schematically in Fig. 1A. Alternatively, interventions at unexpected sites (Fig. 1B) may induce the metabolic output initially sought, thereby uncovering new drug targets. By extension, the more complex and interactive a system is, the more likely it is that unanticipated therapeutic targets and agents will exist.

Accordingly, if we had reliable ways of observing the activities of pathways that are intrinsically important in and predictive of disease progression, these measurements would allow the potential therapeutic benefit of network connections to be exploited. Having a direct readout of the impact of any intervention or perturbation within the network could make complexity our therapeutic ally.

What Is Required to Exploit Complexity?

There are several basic requirements for systematic exploitation of complexity for drug discovery. First, the model system studied clearly must comprise the fully assembled system of interest. To exploit connectivity relationships within a network, the complete repertoire of connections has to be intact. Ex vivo or reductionist approaches can provide little help.

Second, it is optimal if the outputs measured as markers exhibit intrinsic functional significance, as defined above. The less able a metric is to be dissociated from the disease itself—i.e., the more authentic it is as a biomarker (Turner and Hellerstein, 2005; Hellerstein, 2008)—the better it will predict successful clinical response and the more value it will have as a screening tool.

Third, it is optimal if relatively high-throughput measurements are possible to allow broad screening approaches. Finally, the capacity to monitor in real time through noninvasive sampling and rapid analysis is a plus.

Together, these features would allow true observability for diseases of interest and would simplify systematic attempts to discover unexpected targets and drugs.

Stable Isotope-Mass Spectrometric Methods for Measuring the Dynamics of Critical Pathways in Vivo

From an operational standpoint then, the question can be reduced to the following: can functionally important outputs of complex metabolic networks be characterized in vivo in such a manner that efficient and predictive characterization of unexpected drug actions is made feasible?

Recent developments in stable isotope-mass spectrometric techniques may provide this opportunity. A wide variety of intermediary metabolic, biosynthetic, and cellular pathway flux rates can be measured in vivo by use of new kinetic techniques (some are shown in Table 1). Stable isotope labels have no toxicity or risk and can be used safely in humans, so the identical measurements can typically be translated from animal models into man. A stable isotope-labeled tracer is administered, and the pattern or rate of incorporation or loss of label is monitored in molecules of interest by mass spectrometry. By applying simple biochemical rules (e.g., precursor-product relationship, dilution principle, combinatorial analysis of polymerization biosynthesis) or more complex mathematical models, quantitative flux rates of molecules can be measured through anabolic, catabolic, intermediary metabolic, and cellular developmental (birth, differentiation, and death) pathways (Hellerstein, 1995; Hellerstein and Neese, 1999; Wolfe, 2005; Busch et al., 2006, 2007).

Some examples of dynamic pathway fluxes that we have measured using stable isotope-mass spectrometric techniques in vivo include synthesis and turnover rates of proteins [e.g., liver collagen in fibrogenic states (Gardner et al.,

TABLE 1
Some pathway-based drug targets (diseases within parentheses)

Metabolism	Insulin-mediated glucose utilization (pre-diabetes/metabolic syndrome; diabetes mellitus)
	Pancreatic β-cell proliferation (diabetes mellitus)
	Reverse cholesterol transport (cardiovascular disease)
	De novo lipogenesis (hepatic steatosis, hyperlipidemia)
	Mitochondrial proliferation (obesity, rehabilitation)
	Muscle protein synthesis and breakdown (frailty, wasting)
Neurobiology	Axonal microtubule dynamics (ALS, Parkinson's disease)
	Myelination (multiple sclerosis)
	Neurogenesis (depression, cognition, brain injury)
	Microglia proliferation (Alzheimer's and Parkinson's diseases)
	Amyloid-beta turnover (Alzheimer's disease)
	Dendrite microtubule synthesis (synaptic plasticity, learning)
Inflammation	Collagen synthesis (fibrosis of liver, lung, kidney, skin, heart)
	Keratinocyte/keratin turnover (psoriasis, atopic dermatitis)
	Joint-space dynamics (osteoarthritis, rheumatoid arthritis)
Oncology	Angiogenesis (tumor growth)
	Tumor cell proliferation (prognosis, therapeutic response)
	Lymphangiogenesis (metastatic spread)
	DNA-cytosine methylation (gene silencing)
	Ribonucleotide synthesis (cancer cell proliferation)

2007), neuronal microtubule turnover in neurodegenerative diseases (Fanara et al., 2007), turnover of skin keratin in hyperproliferative disorders of the skin (Lindwall et al., 2006)]; lipid fluxes [e.g., reverse cholesterol transport rates in atherosclerosis (Turner et al., 2007b), lipogenesis in obesity and in response to dietary factors (Hellerstein et al., 1996), assembly and secretion of triglycerides by the liver in hyperlipidemic states (Vedala et al., 2006)]; intermediary metabolic and carbohydrate-related fluxes [e.g., whole-body glycolysis rates in insulin resistance (Beysen et al., 2007), hepatic gluconeogenesis, and glycogenolysis in diabetes (Christiansen et al., 2000)]; and the dynamics of cells as reflected in DNA replication and breakdown [e.g., birth and death rates of tumor cells in chronic lymphocytic leukemia (Messmer et al., 2005), lymphocyte kinetics in human immunodeficiency virus infection (Hellerstein et al., 2003), and hippocampal neurogenesis in response to antidepressants (Shankaran et al., 2006)]. These mass spectrometric measurements are capable of relatively high throughput, which is essential for any broad screening initiative.

As a proof-of-concept test of this approach for exploiting complexity, we recently screened a number of approved drugs for their effects on multiple pathways in vivo. The drugs tested were selected for pluripotency and included statins, salicylates, retinoids, calcium-channel blockers, glitazones, and others. The in vivo pathways studied included insulin-mediated glucose utilization, hippocampal neurogenesis, liver collagen synthesis, antigen-driven lymphocyte proliferation, brain microglial proliferation (Table 1), and others.

It is interesting that we observed a "hit rate" (i.e., frequency of discovering previously unknown therapeutic actions) of roughly one for every 10 pathways studied per drug. That is, if three drugs were tested against 7 to 10 pathways, there would be two to three new indications discovered. In view of the fact that the agents that we screened had been approved for years or even decades, this is a remarkably high discovery rate for new indications. For example, Shankaran et al. (2006) found that a widely used class of cardiovascular agents (statins such atorvastatin and simvastatin) has potent neurogenic stimulatory actions in the hippocampus (Fig. 2); that a ligand for the retinoid receptor (isotretinoin, Accutane) has inhibitory effects on microglial proliferation and neuroinflammation (Shankaran et al., 2007); that a cardiovascular drug alters lymphocyte kinetics and has immunosuppressive actions; and so on. All of these actions were previously unknown and could be claimed to be new and patented accordingly.

The extremely high frequency of unanticipated actions of approved drugs that we have observed by screening against complex pathways supports the model, derived from the principle of robustness (see above) that agents that do anything are likely to do many things (Stephanopoulos et al., 2004; Fischer and Sauer, 2005). A recent embodiment of this approach is the work of Chong et al. (2006), who looked for antimalarial activity by repurposing approved drugs. These investigators found antimalarial actions of several approved drugs not previously known or expected to alter host—parasite interactions, congruent with the notion of one action, many actions.
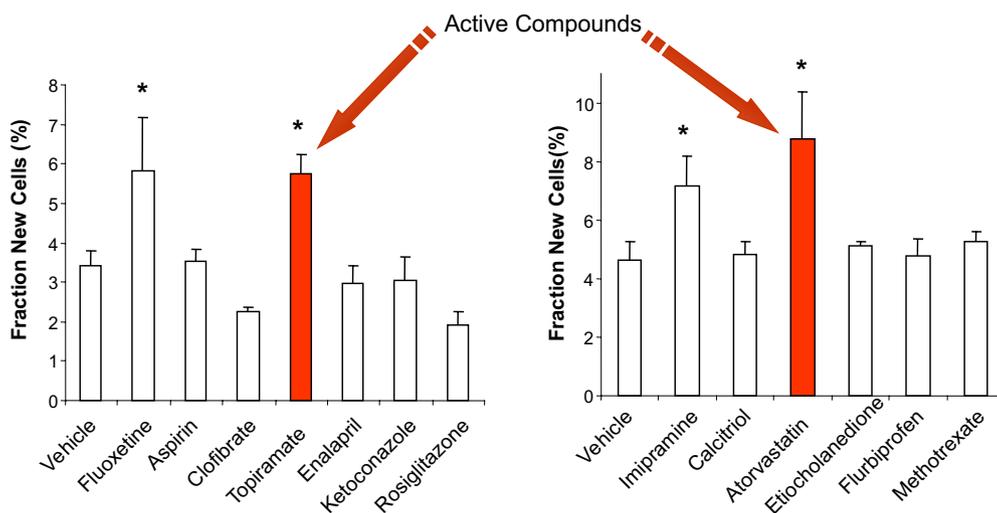
Pathways as Therapeutic Targets in Neurobiology: Prediction of Clinical Response

It is particularly instructive that several pathway measurements in the field of neurobiology have proven to be predictive of clinical response in animal models of disease. Neurobiologic diseases have not traditionally been monitored through biochemical kinetics but are generally investigated using endpoints such as behavior, electrophysiology, neurotransmitters, or histopathology. We recently measured the pathway of hippocampal neurogenesis in adult animals (Shankaran et al., 2006). Adult neurogenesis involves the proliferation and maturation of progenitor cells in selected areas of the brain and is essential for learning and formation of new memories, as well as being implicated in the therapeutic action of antidepressant drugs. By screening a wide variety of compounds in mice, we discovered that several classes of drugs, including statins and antiepileptics, exhibited previously undescribed stimulatory effects on hippocampal neurogenesis (Fig. 2). When compounds were further evaluated for functional activity in standard behavioral models of antidepressant activity or cognition (the forced swim and novel object recognition tests, respectively), improvements were confirmed. Likewise, measurement of microglial proliferation rates in rat brain in response to lipopolysaccharide provided a screening approach for identifying agents that suppress neuroinflammation (Shankaran et al., 2007). Several drugs were identified that had unexpected inhibitory actions on this pathway, and an agent identified by this means (the retinoid isotretinoin) was subsequently shown to delay symptoms in the EAE-MOG (experimental allergic encephalitis-myelin oligodendrocyte glycoprotein) mouse model of multiple sclerosis.

More recently, a novel pathway was identified (microtubule dynamics in neurons) that has potent functional consequences in neurodegenerative conditions (Fanara et al., 2007). Microtubules are essential as the "conveyer belts" used for the transport of molecular cargo along axons. When the dynamics of microtubule assembly/disassembly was measured in neurons, the SODG93A mouse model of amyotrophic lateral sclerosis (ALS) exhibited extraordinary hyperdynamicity of microtubules in peripheral nerves and the central nervous system. This hyperdynamicity was present most strikingly in the microtubule subpopulations that are typically the most stable; it was present before symptoms of disease; it was associated with abnormalities of cargo transport along axons; and it worsened as the disease progressed (Fanara et al., 2007). Moreover, treatment with agents that reduced microtubule turnover rates improved transport of cargo, prevented the death of spinal cord neurons, delayed the onset of neurologic signs of disease, and prolonged life remarkably in these mice ($\sim$30% extension of life span, which is greater than previous reports for any agent). Most importantly, the degree of normalization of this pathway by drugs accurately predicted clinical signs of disease and survival.
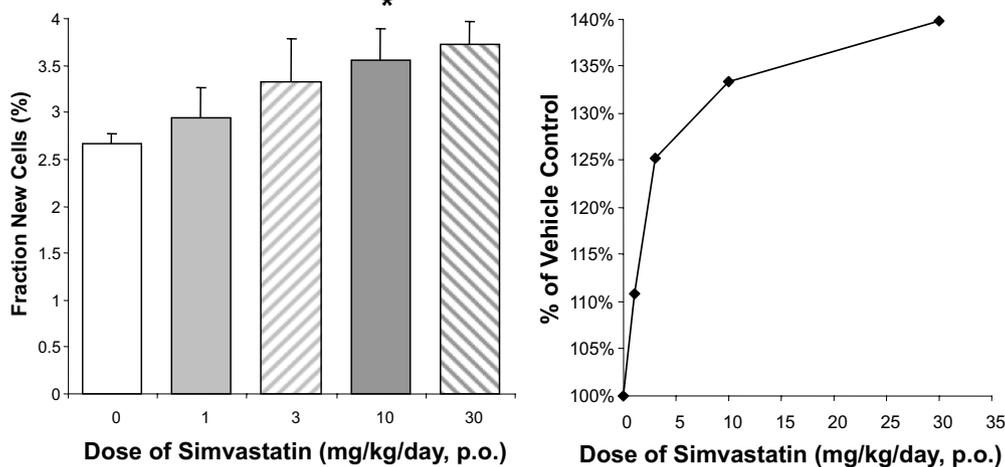
A point about translating results from animal models to humans is worth noting. It is true that one can never be sure whether the control systems discovered in an animal model will apply to humans. A key feature of the stable isotope measurements described here, however, is that most of the techniques for measuring flux rates through pathways in vivo in animal models can be used in identical fashion in human subjects. This

A

Active Compounds

Fraction New Cells (%)

Vehicle · Fluoxetine · Aspirin · Clofibrate · Topiramate · Enalapril · Ketoconazole · Rosiglitazone

Vehicle · Imipramine · Calcitriol · Atorvastatin · Etiocholanedione · Flurbiprofen · Methotrexate

Data: Mean ± SEM * p < 0.05 vs Vehicle. Fluoxetine and Imipramine were used as positive controls

B

Fraction New Cells (%)

Dose of Simvastatin (mg/kg/day, p.o.)

% of Vehicle Control

Dose of Simvastatin (mg/kg/day, p.o.)

Data: Mean +/- SEM * p < 0.05 vs Vehicle

Fig. 2. A, screening approved drugs for stimulation of hippocampal neurogenesis (Shankaran et al., 2006). B, dose-response of simvastatin on hippocampal neurogenesis (Shankaran et al., 2006).

translatability of methods results in the capacity to test immediately, and with modest expense, time, and number of subjects, whether data from preclinical models apply in man.

Implications for Indications Discovery

These results in living animals provide experimental support for the theoretical prediction based on fundamental features of metabolic networks that systematic attempts to identify new therapeutic actions of agents ("indications discovery") are likely to be rewarding if tools for measuring the effects of agents on pathway fluxes are available and capable of relatively high throughput in living organisms.

Challenges and Obstacles for Pathway-Based DDD

To move pathway-based approaches into general use for indications discovery, several challenges must be faced. The most obvious is to identify which pathways should be measured as biomarkers for DDD. Unlike molecular targets, which in a general sense exist as unambiguous physical units that do not require editorial decisions, key pathways exist in the eye of the beholder. There exist hundreds of pathways that might be targeted for therapeutic modulation. Criteria of intrinsic functional significance, authenticity in a disease process, measurability in vivo, capacity for translation into

humans, and medical significance of the disease(s) influence the attractiveness of different pathways for DDD.

Perhaps the most difficult challenge in practice will be to prove that information from pathway measurements predicts functional outcomes. In preclinical models, this is a relatively straightforward process. Agents that are discovered to modulate a pathway flux in an animal model can be tested rapidly against standard animal models of the intended disease. Some examples of this were mentioned above [e.g., normalizing the hyperdynamicity of microtubules in neurons improved transport of molecular cargo along axons in vivo and markedly delayed symptoms and death in the mouse model of ALS (Fanara et al., 2007)]. Other examples have been demonstrated [e.g., infusion of reconstituted HDLs increases cholesterol efflux rate from tissues in vivo in rodents (Turner et al., 2007b) and appears to prevent atherosclerosis in animal models as well as people (Nissen et al., 2003)]. Demonstrating the predictive strength of a pathway for human disease outcomes is more difficult. The prognostic value of tumor cell proliferation rate in chronic lymphocytic leukemia patients is currently being explored in a multicenter prospective clinical trial based on a strong cross-sectional correlation between chronic lymphocytic leukemia tumor cell proliferation rates and aggressiveness of disease (Messmer et al., 2005). Lower turnover rate of T-cells in human immunodeficiency virus-1-infected humans with "virologic failure" while on antiretroviral treatment correlates with attenuated viral pathogenicity and was better associated with clinical progression than plasma viral load (Deeks et al., 2002). We have also reported that whole-body glycolytic utilization of an oral deuterated-glucose load correlates very closely with the results of hyperinsulinemic-euglycemic glucose clamps (Beysen et al., 2007), which in turn predict the efficacy of insulin-sensitizing agents.

Summary and Conclusions

Contemporary DDD is based on a molecular target-based paradigm, which suffers from some fundamental limitations. These are predictable and understandable from principles of metabolic control theory. In a general sense, the disappointing output of new drugs in recent years reflects the failure of molecular target-based DDD to account for the dynamic systems aspects of drug actions in complex biological networks.

This review has focused on a related consequence of biologic unpredictability and robustness that has potential value for therapeutic discovery: namely, the occurrence of unexpected, off-target therapeutic activities of drugs. Unexpected off-target effects arise from the same network of connectivity relationships in biochemical systems that thwart intended actions of candidates from being achieved. To the extent that these unanticipated actions are beneficial in disease states and can be systematically identified, this consequence of unpredictability may be exploited for discovery of new therapies.

The most direct strategy for systematic discovery of actions of agents is to measure the flow of molecules through critical pathways, as metabolic engineers have been forced to do for altering outputs of bacterial systems (Stephanopoulos and Vallino, 1991; Bailey, 2001; Stephanopoulos et al., 2004; Fischer and Sauer, 2005). Moreover, because the flow of molecules through selected pathways may have intrinsic functional significance, biomarkers of metabolic pathway fluxes are probably more predictive of clinical response than static metrics, such as the expression level or activity of proteins or genes in isolation. If this general strategy can be efficiently reduced to practice, it represents an alternative to the high attrition, high cost, and long time-lines of the contemporary molecular target-based drug discovery approach.

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