IN FOCUS

A Systems Biology Approach to Personalizing Therapeutic Combinations

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Summary: The identification of evidence-based, efficacious drug combinations for each cancer, among thousands of potential permutations, is a daunting task. In this perspective, we propose a systematic approach to defining such combinations by molecularly benchmarking a drug against a desired state of efficacy using model systems.
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INTRODUCTION

Although targeted therapies have revolutionized cancer treatment, the lack of durable responses has highlighted the need for combination regimens to overcome primary or acquired drug resistance. To date, the rational design of effective drug combinations has relied on knowledge-based assessments, high-throughput screens, or identification of presumed compensatory pathways after single-drug administration. More recently, we have reported on a data-driven strategy based on the concept of benchmarking against a desired phenotype. In an inducible *NRAS* mouse model of melanoma, the genetic extinction of oncogenic *NRAS* results in complete tumor regression, hence defining a desired "ideal" state. Benchmarking against such a molecular state allowed for identification of a drug combination that more closely simulates the efficacy of genetic *NRAS* extinction. In this perspective, we discuss the potential of generalizing this methodology to enable data-driven cotargeting therapeutic strategies against undruggable cancer targets, both oncogenes and tumor suppressors. Furthermore, we speculate on how this approach can be applied clinically to address the challenge of heterogeneity in patient responses, namely, a systematic approach to the designing of a combination strategy customized to a patient, by benchmarking the patient's unique response to a drug against a predefined molecular state representing the desired efficacy. Such an adaptive approach to personalizing therapeutic combinations has the potential to delineate the clinical paths for durable complete responses in the clinic.

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A BENCHMARK-DRIVEN APPROACH FOR THE DISCOVERY OF COTARGETING STRATEGIES

Systems biology—the data-driven network modeling of complex biologic systems—holds promise to identify novel cancer therapies in an unbiased manner. One recent study applied such modeling to predict that the apoptotic response in breast cancer is optimized by the sequential rather than simultaneous application of chemotherapy and an EGF receptor (EGFR) inhibitor (1). In another study, computational modeling identified ErbB3 as the most effective therapeutic target across the ErbB–PI3K axis, leading to the development of a novel and effective therapeutic ErbB3 antibody (2). Similarly, modeling of EGFR phospho-signaling identified MET plus EGFR inhibition as synergistic (3).

We have recently published (4) another example of a data-driven approach to the development of evidence-based therapeutic combinations (Fig. 1A). This study leveraged a mouse model of melanoma, engineered so that the expression of mutant *NRAS* can be extinguished via withdrawal of doxycycline; loss of mutant *NRAS* expression resulted in a rapid and complete tumor regression—the desired state. In contrast, pharmacologic inhibition of the RAS downstream effector MAP–ERK kinase (MEK), given at maximum-tolerated doses, was unable to phenocopy this response, failing to induce tumor regression and achieving only transient growth arrest. Global transcriptional and targeted proteomic profiling, validated by tumor histopathologic analyses, illuminated the differential molecular effects of genetic *NRAS* extinction versus pharmacologic MEK inhibition. Not surprisingly, mutant *NRAS* proved to be a tumor-maintenance target, as its activities were required for both growth and survival of an established tumor and its genetic extinction resulted in complete tumor regression. However, a potent and specific MEK inhibitor (hereafter MEKi) was only able to block the survival signal by mutant *NRAS*, consequently activating apoptosis, but failing to inhibit the proliferation signal. Therefore, drug(s) that can inhibit proliferation represented a possible rational combination with MEKi against mutant *NRAS*. To that end, network modeling was applied to discover key regulators underpinning these molecular differences in an unbiased manner. Here, we used TRAP (Transcriptional

Regulatory Associations in Pathways; ref. 4), a network model built upon thousands of published transcriptomic profiles in mice to establish key regulators of transcriptomic features. TRAP identified CDK4 as the top regulator responsible for the differential molecular states between genetic extinction of *NRAS* and pharmacologic inhibition of MEK in the iNRAS system (Fig. 1B). Indeed, the combination of MEKi with a cyclin-dependent kinase (CDK)4/6 inhibitor resulted in tumor regression in both mouse models and human xenografts. The synergy arose from a complementary induction of both apoptosis (via MEKi) and cell-cycle arrest (via CDK4/6i). Importantly, CDK4/6i monotherapy induced only cell-cycle arrest and not apoptosis—unlike the majority of targeted antiproliferative drugs (5)—suggesting that systems biology approaches can potentially molecularly distinguish between related drug targets. Finally, in contrast to MEKi alone, transcriptomic analysis confirmed that the drug combination more closely mimicked *NRAS* extinction in terms of target gene modulation (L.N. Kwong and L. Chin; unpublished data).

In summary, by benchmarking against the "ideal or desired" state (i.e., complete tumor regression) achieved through genetic extinction of an undruggable tumor maintenance target (i.e., mutant *NRAS*) in a model system, this data-driven approach identified two drugs (MEK and CDK4 inhibitors) that synergize in combination to approximate the therapeutic efficacy of *NRAS* extinction. This general paradigm of benchmarking against a desired state to delineate potential synergistic combinations can be applied broadly (Fig. 1C). Indeed, various genetically engineered mouse models (GEMM) of oncogene addiction have already been established (6). The proof-of-concept example described above provides a potential path to define novel therapeutic strategies to target other "undruggable" oncogenes (e.g., Myc). Taken a step further, one could envisage using a similar approach to inform on strategies to reactivate tumor suppressor functions; for example, multiple laboratories have generated mouse models (7) in which restoration of wild-type p53 can induce tumor regression. In addition, Premsrirut and colleagues (8) have described a novel *in vivo* short hairpin RNA (shRNA) system capable of systematically generating such reactivation mice, using reversible knockdown of endogenous tumor suppressor genes to model robust tumor regression. These models can be used to define the "ideal states" which rational drugs such as Prima-1 can be benchmarked against to discover cotargets/therapies.

Data-Driven Approaches Are Complementary

As a prime example of an undruggable target, the exact signaling circuits and functions of RAS—30 years after its discovery as an oncogene—are coming into focus, but much remains enigmatic. Pathway diagrams usually involve an elaborate network of various molecules, with only the RAF–MEK–ERK and PI3K–AKT–mTOR arms generally remaining constant. Often included are pathways regulated by the small GTPases RAL, RAC, and RHO. Additional complexity is observed further downstream of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways with respect to downstream effectors and their own complicated networks. The difficulties are compounded by the emergent recognition of differences between KRAS, NRAS, and HRAS, by cell-type and developmental stage–specific activities, and by mutations in other genes that can directly or indirectly have an impact on RAS activity. Because clinically effective pharmacologic approaches to directly target RAS have so far been unsuccessful, comprehensive analyses of RAS/MAPK signaling are required to define vulnerabilities that are shared across tumor types.

Here, we highlight three recent studies that exemplify data-driven approaches to rationally designing anti-MAPK drug combinations. First, Held and colleagues (9) conducted a large-scale combinatorial screen, testing more than 7,000 pairwise combinations of 40 drugs in 19 melanoma cell lines. Among a number of context-specific synergistic combinations, they identified simvastatin (an HMG–CoA reductase inhibitor) plus flavopiridol (a pan-CDK inhibitor) as highly efficacious in *NRAS*-mutant lines both *in vitro* and *in vivo*. Second, Corcoran and colleagues (10) performed an shRNA screen to identify genes that, when inhibited, could cooperate with MEKi to target *KRAS*-mutant cell lines. They validated BCL-XL knockdown and its associated inhibitor, the BH3 mimetic ABT263, as synergistic with MEKi *in vivo*. Finally, Duncan and colleagues (11) described a multiplatform approach to study kinome reprogramming of breast cancers in response to MEK inhibition. Multiple oncogenic kinases including AXL, and ErbB and PDGF family members were upregulated because of feedback mechanisms, and the combination of MEKi with the multikinase inhibitor sorafenib was synergistic *in vivo*.

From a clinical perspective, this wealth of novel potential combination therapies represents a potential boon, and the next step is to thoroughly and systematically evaluate them preclinically to bring forward into the clinic the most promising ones, linked to strong science. Indeed, the complexity and diversity of the results highlight the challenges in identifying and pursuing the most optimal strategies. *In vivo* data reveal that signaling downstream of pharmacologic MEK inhibition is highly complex; even with a conservative cutoff, significant changes are seen in more than 1,500 genes (4), many of which are known oncogenes or tumor suppressors. Even smaller-scale targeted assays such as phospho–receptor tyrosine kinase or pathway-specific protein arrays identify multiple potential cotargeting/combination drug targets, as described above and elsewhere (11, 12). A popular analogy is that of a game of "Whack-a-Mole," where hitting one oncogenic target causes others to pop up and compensate (12). For example, in colorectal cancer, BRAF inhibitor monotherapy is rendered ineffective in part through compensatory activation of EGFR (13). But with hundreds of potential moles popping up after MEK inhibition, the options are either to pick the most likely targets based on prior knowledge combined with functional screening (e.g., EGFR, AKT, etc.), or to select the most statistically significant targets for screening and validation. The strength of the benchmarking approach is that the tumor regression phenotype offers the "ideal" state against which these otherwise noisy expression and posttranslational adaptive changes can be compared in an unbiased manner. This approach informs whether a given gene may be a likely cotarget or not and prioritizes the most promising strategies for preclinical validation.

For example, we and others have noted that phospho-Akt is enhanced upon MEK inhibition (14). Given the known

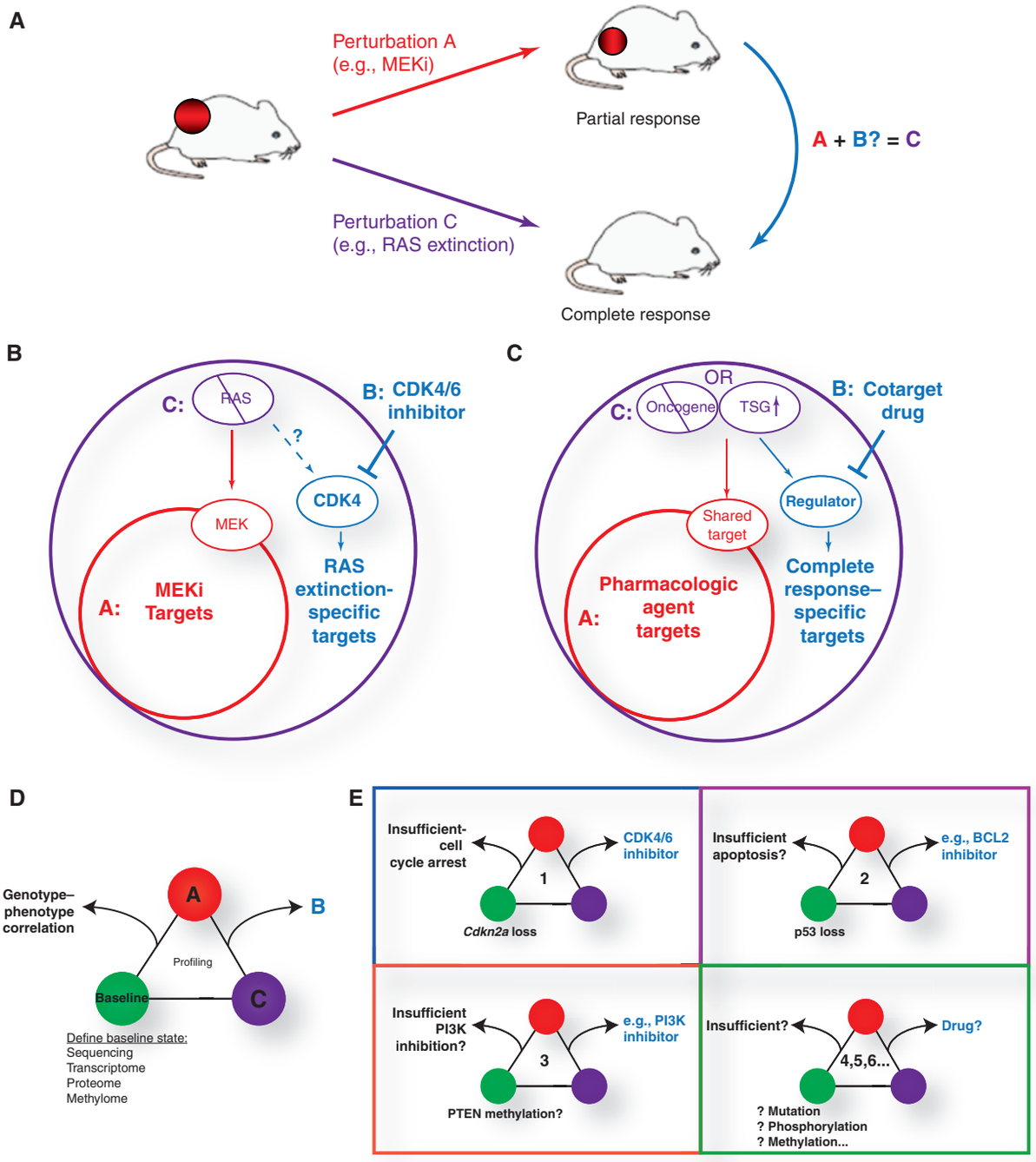

Figure 1. Overview of the benchmark-driven analysis and the creation of cotarget databases. **A,** schematic comparison of a pharmacologic agent showing a partial response (Perturbation A, red) against a genetically engineered model designed to generate a complete response (Perturbation C, purple). Identifying what is missed by A allows for the identification of a cotarget drug B (blue) that, together with drug A, approximates the complete response seen in C. **B,** specific results of the iNRAS study (4). The large circles represent information obtained from expression and protein arrays. The RAS extinction space (purple) identified RAS-specific targets (blue) that were "missed" by the MEKi space (red). The TRAP network algorithm identified CDK4 as a cotarget regulator. The MEK plus CDK4/6 inhibitor combination thus complementarily targets the RAS extinction space. **C,** a generalized schematic. Oncogene extinction or tumor suppressor gene reactivation (i.e., Perturbation C, purple) generates a complete response. A pharmacologic agent (i.e., Perturbation A, red) is compared, and a key regulator(s) of pathways "missed" by A is identified via a systems biology approach. A cotarget drug B against this regulator (blue), when combined with A, would target the full space (purple). **D,** triplets of information are generated by the comparative analysis. Comprehensive characterization of the baseline tumor allows for correlations to be drawn between key baseline elements (e.g., mutations and epimutations) and the observed treatment responses. **E,** a hypothetical database of genotype–phenotype correlations, matching baseline tumor characteristics to preclinically optimized drug combinations. Such databases would inform clinical trial designs and serve as a reference for genotyped patient biopsies to rapidly predict cotargets. *NRAS*-mutant melanoma is depicted here as an example.

oncogenic role of AKT, especially in RAS signaling, it is reasonable to assume that this activation might represent a rational point of coextinction. Indeed, MEK plus AKT/PI3K inhibition has been shown to be effective preclinically (15). However, in the iNRAS model system, extinguishing NRAS genetically also results in an increase in AKT phosphorylation after 4 days, despite the full engagement of tumor regression (4). This observation suggests that AKT activation within days of MEK inhibition may not necessarily be compensatory, at least in this short-term experimental time frame. Thus, one must be cautious in interpreting the upregulation of oncogenes in response to therapy as necessarily compensatory; monitoring tumor responses provides one way to help sort the true compensatory mechanisms (i.e., those seen after pharmacologic treatment, but not in the regression state) from red herrings (i.e., those that are seen in both states, but which, by definition, do not rescue the regression state).

We note that immune therapies might also be amenable to such an approach. Data from several laboratories attest to the human relevance of GEMM immune reactions to therapy, including the influx of $CD8^+$ T-cells seen in both a *Braf*^{V600E};*Pten*$^{-/-}$ melanoma model undergoing BRAF inhibition (16) and in human patients treated with BRAF inhibitors (17). Thus, one could envision benchmarking various immune therapies (e.g., anti-CTLA4, anti–PD-1, etc.) and determining whether mechanisms of immune suppression can be overcome via cotargeting approaches. Indeed, the concept of inducible costimulator (ICOS) induction as boosting anti-CTLA4 therapy illustrates the types of synergistic pathways that might be uncovered (18). Overall, the different data-driven methods of therapy design complement one another, with the strength of the benchmarking approach lying in its ability to highlight unanticipated pathways most relevant to the complete regression phenotype.

Signaling Activity Is Not Binary

One other aspect of the iNRAS study supports the perspective that oncogene activity is not binary, but rather acts like a rheostat to activate various phenotypes at different thresholds of activity (4, 19). Incomplete extinction of NRAS, at an intermediate time point of doxycycline withdrawal, recapitulates MEK inhibition in both the quantitative activity of extracellular signal-regulated kinase (ERK) effectors (e.g., FOSL1, MAFF, etc.) and in the phenotypic outputs of activated apoptosis but not cell-cycle arrest. In other words, one reason that MEKis are inefficient in the setting of *NRAS*-mutant melanoma is that they do not completely or adequately inhibit the flux through downstream signaling pathways, likely due to their biophysical characteristics as a small-molecular inhibitor with issues with inherent affinity, pharmacokinetics/pharmacodynamics, etc. Insights from other mouse models also weigh in on this aspect of RAS biology. Blasco and colleagues (20) have demonstrated that MEK1/2 and ERK1/2 pairs are each necessary for Ras-induced cancer, by genetically ablating each kinase pair specifically in *KRAS*-mutant cells of a mouse model of lung cancer. These data imply that, if MEK and/or ERK inhibitors could be specifically targeted to a cancer and made sufficiently potent to completely inhibit the activities of their respective targets, they might be as effective as genetic ablation. Practically speaking, this level of specificity is challenging in the face of likely toxicities incurred by over-inhibiting a single pathway, although one hopes that it could be achieved by disruptive delivery methods such as nanotargeted delivery of highly potent and efficient inhibitors. In the current context, the rheostat nature of oncogene signaling could be taken advantage of, as the decoupling of therapy-pertinent phenotypes (e.g., apoptosis and proliferation) provides an opportunity to orthogonally target nonredundant pathways. Such complementary drug combinations would therefore avoid over-inhibiting a single pathway (e.g., RAS–MEK–ERK) and might spare a patient the associated toxicity.

Bridging to the Clinic

In the data-driven example above, the desired phenotype and associated molecular state are provided by a tractable, genetically engineered mouse system that approximates, but does not fully capture, the complexities of human cancer. How can we then build on this initial step forward to develop a systematic approach that benchmarks against a desired molecular state for designing clinically effective combinations? We suggest here that a comprehensive and coordinated (i.e., consortium-type) effort to apply the benchmark-driven paradigm to an array of preclinical models of cancer can serve as a bridge toward the clinic. Such an effort not only would enlist inducible GEMM mouse models, but also would use patient-derived, *in vitro*/*ex vivo* engineered xenograft benchmarks to identify optimal drug combinations across a wide array of tumors and their diverse genotypes. Such a preclinical knowledge base of optimized drug combinations would provide a valuable resource for launching rationally designed clinical trials that use target engagement biomarkers and real-time genomic profiles before and during treatment.

In achieving the goal of establishing such a knowledge base, several important aspects must be taken into consideration. First is accounting for the broad mutational heterogeneity within each cancer subtype. For example, even among the 25% of melanomas driven by *NRAS* mutations, other significant and often mutually exclusive mutations, such as in *CDKN2A, TP53, RAC1, PPP6C,* and *NF1* (21), could differentially determine MEKi cotargets for a given tumor. Indeed, preliminary data suggest that *CDKN2A*- and *TP53*-mutant cells may respond differently to MEK and/or CDK4/6 inhibition in their ability to undergo cell-cycle arrest (L.N. Kwong; unpublished data). Fortunately, the benchmark approach precisely allows one the flexibility to adapt the design of combinations to address such mutational heterogeneity. Specifically, the output consists of unbiased triplets of information that compare tumor characteristics across baseline, treatment, and regression (desired phenotype) cohorts (Fig. 1D). Importantly, characterization of the baseline tumor, including whole-exome and RNA transcriptome sequencing as well as proteome analyses, establishes a way to model mutational heterogeneity in the laboratory, allowing for matching baseline genotypes to optimal drug combinations (Fig. 1E).

To further enhance human relevance in this effort, we propose that systems involving human cell lines and *ex vivo* patient-derived xenografts be included: by carefully selecting a diverse set of cell lines and patient-derived cultures to represent genetic heterogeneity, a first view of potential broad drug combination categories can be defined. These can then be expanded for refinement, iteratively. To model

oncogene extinction or tumor suppressor reactivation, inducible shRNA or expression plasmids could be used to define the molecular state corresponding to a desired efficacy, such as tumor regression. Ultimately, correlations between the known baseline genotypes of tumors and their optimal drug combination will allow us to generate a database of therapies tailored to specific mutational profiles both within and across tumor types (Fig. 1E).

A second aspect to bridging toward the clinic is in anticipating how to consistently model complete regression in the laboratory. For example, not all *KRAS*-mutant cancers rely on KRAS: when *KRAS* expression is extinguished by potent shRNAs in human lung and pancreatic cell lines, nearly half of the lines tested were found to be KRAS-independent, as shRNA knockdown failed to induce apoptosis (22), reflecting a differential dependence on mutant KRAS signaling to maintain growth. This spectrum of responses may be generally true for most, if not all, oncogenes and tumor suppressors. In such cases, it will be necessary to identify and overcome the underlying independence-conferring mechanism(s) to properly generate complete regression phenotypes. In the above-referenced KRAS study, epithelial–mesenchymal transition (EMT) was identified as a pervasive signature throughout the KRAS-independent cell lines (22). Indeed, many recent publications have highlighted a similar correlation between EMT status and a wide range of drug resistances. However, it is likely that this represents only one of many mechanisms of oncogene independence. Overall, resensitizing agents would be necessary in oncogene-independent samples for generating the tumor regression state, additionally acting as a cotargeting agent.

In summary, accounting for mutational heterogeneity and consistent benchmarking will require a large-scale investment that a consortial effort can create, with the capacity to potentially provide a clinic-ready, biologically significant knowledge base of tumor genotypes and their matched, optimized drug combination therapies.

Personalizing Therapeutic Combinations in the Clinic

The coclinical trial concept represents an attractive transition from laboratory to clinic (23). In such a trial, biopsies of a patient's tumor are passaged through preclinical model systems (e.g., mouse), and the response of the tumor to the same targeted therapy being administered to the patient is monitored. From there, genomic analyses—sequencing and expression profiling—can make several important determinations. First, expansion and drug dosing of patient-derived xenografts will provide models that can be quickly cross-referenced to the preclinical knowledge base to predict rational cotargeting strategies. Second, such a coclinical trial can provide early predictions of response by allowing for a rapid molecular assessment of response and/or biologic biomarkers. Third, the expression assessment can determine whether the patient xenograft is behaving as predicted by the preclinical knowledge base, or whether novel combinations need to be quickly uncovered. In this last regard, it should also be possible to establish patient-derived cell cultures *ex vivo*, transfect with the appropriate vector (oncogene knockdown or tumor suppressor expression), and perform xenografting to establish a truly personalized drug combination by benchmarking. Ultimately, the coclinical information will be routed back to engage the optimal course of treatment for the patients.

The ultimate clinical application of this approach to designing personalized combination therapy is to be able to monitor, noninvasively and in real time, the response of a patient and his/her tumor to a targeted pharmaceutical compared with a benchmark model. Guided by the preceding preclinical and coclinical data, physicians would be able make informed decisions about efficacious, rational drug combinations in both predictive and reactive manners. The ability to longitudinally monitor important phenotypes (such as various hallmarks of cancer in a tumor) in response to a treatment would provide the rational basis to customize additional drug(s) in combination for each individual patient. For example, if proliferation and apoptosis could be monitored in a clinically relevant time frame, one can imagine treating a *NRAS*-mutant melanoma patient first with a MEKi, followed by evidence-based selection of a CDK4 inhibitor as a combination, based on the observation that proliferation was not inhibited. Alternatively, it is also possible that, in a separate patient with a different genomic makeup of a *NRAS*-mutant melanoma, MEKi is able to shut off proliferation but apoptosis is not activated; in such a patient, the personalized combination could be an AKT or BCL2 inhibitor. The capability to longitudinally monitor a patient's response as envisioned means a data-driven approach to customized combinations can address both the passive and adaptive mechanisms of drug resistance to initial therapies in a timely manner. Although it is not yet available today, we believe such real-time clinical monitoring is on the horizon, given the rapidly advancing technologies in imaging and noninvasive biomarker discovery.

CONCLUSIONS

The advent of targeted therapies has allowed for the tailoring of clinical care based on driver genetic lesions in a patient's DNA; for example, vemurafenib in melanoma targets mutant BRAF, imatinib mesylate in various cancers targets *ABL* or *PDGFR* translocations or *KIT* amplifications, and trastuzumab in breast cancer targets *HER2* amplifications. However, the full promise of such targeted therapy has not yet been realized in part due to the powerful adaptive responses of the tumors. The benchmark-driven paradigm to define cotargeting strategies has the potential to design optimized combinations that are customized to individual patients. Leveraging model systems to establish benchmarks for tumor regression or other desired states in a preclinical or coclinical trial context offers a first step toward translating such a paradigm—and associated *in vivo* systems biology approaches—to clinical application. This will require a coordinated and systematic effort among preclinical modelers and biologists, genomic scientists, and computational modelers, similar to those represented by the National Cancer Institute's (NCI) MMHCC (Mouse Model of Human Cancer Consortium), TCGA (The Cancer Genome Atlas), and ICBP (Integrative Cancer Biology Program). Furthermore, such an effort will require a new model of cooperation and collaboration between academia and industry to ensure that these studies are conducted to industry standards and that the results are clinically

actionable and available as a public resource. Finally, this must be accompanied by educational efforts so that our clinical colleagues can optimize their care of patients and, importantly, feedback to teach and improve these exploratory studies conducted by the research communities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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