Individualized Systems Medicine Strategy to Tailor Treatments for Patients with Chemorefractory Acute Myeloid Leukemia


ABSTRACT

We present an individualized systems medicine (ISM) approach to optimize cancer drug therapies one patient at a time. ISM is based on (i) molecular profiling and ex vivo drug sensitivity and resistance testing (DSRT) of patients’ cancer cells to 187 oncology drugs, (ii) clinical implementation of therapies predicted to be effective, and (iii) studying consecutive samples from the treated patients to understand the basis of resistance. Here, application of ISM to 28 samples from patients with acute myeloid leukemia (AML) uncovered five major taxonomic drug-response subtypes based on DSRT profiles, some with distinct genomic features (e.g., MLL gene fusions in subgroup IV and FLT3-ITD mutations in subgroup V). Therapy based on DSRT resulted in several clinical responses. After progression under DSRT-guided therapies, AML cells displayed significant clonal evolution and novel genomic changes potentially explaining resistance, whereas ex vivo DSRT data showed resistance to the clinically applied drugs and new vulnerabilities to previously ineffective drugs.

SIGNIFICANCE: Here, we demonstrate an ISM strategy to optimize safe and effective personalized cancer therapies for individual patients as well as to understand and predict disease evolution and the next line of therapy. This approach could facilitate systematic drug repositioning of approved targeted drugs as well as help to prioritize and de-risk emerging drugs for clinical testing. Cancer Discov; 3(12); 1–14. © 2013 AACR.
INTRODUCTION

Adult acute myeloid leukemia (AML) is a prototype example of the challenges of modern cancer drug discovery, development, and patient therapy. With the exception of the retinoic acid–sensitive acute promyelocytic leukemia subtype, molecularly targeted therapeutic approaches for AML are yet to be translated into clinical advances. The disease has traditionally been subdivided into different subtypes (M0–M7) based on cellular lineage and biomarkers (1). Current World Health Organization classification reflects the fact that a growing number of AML cases can be categorized on the basis of their underlying genetic abnormalities that define distinct clinicopathologic entities (2). Genomic changes in AML are now relatively well understood, with each AML sample containing roughly 400 genomic variants, of which an average of 13 reside in coding regions (3, 4). Recurrent changes have highlighted potential driver genes, including *NPM1*, *CEBPA*, *DNMT3A*, *TET2*, *RUNX1*, *ASXL1*, *IDH2*, and *MLL*, with mutations in *FLT3*, *IDH1*, *KIT*, and *RAS* genes modifying the disease phenotype (5).

Although several of the recurrent genetic alterations link to tractable drug targets, genetic testing of patients with AML has yet to result in effective personalized or stratified therapies.

Patients with AML have a poor outcome, with a 5-year survival of 30% to 40% (6, 7). The standard therapy for most adult patients with AML is conventional chemotherapy consisting of the nucleoside analog cytarabine combined with a topoisomerase II inhibitor (8, 9). A number of second-line treatment options have been applied in patients with AML after relapse, but the response rates have remained low. Furthermore, patients with AML at relapse exhibit an increased number of genetic alterations, which can be attributed to disease progression and/or DNA-damaging agents used for routine chemotherapy (10). In light of the genomic and molecular diversity of AML, and its continuous evolution in response to chemotherapy, it is important to better understand the potential utility of all targeted cancer drugs that are already available in the clinic. These drugs could be systematically repurposed as off-label indications to responding subgroups of AML. Furthermore, comparative information on the efficacy of the hundreds of emerging targeted anticancer agents, as well as their potential combinations, in patient-derived *ex vivo* samples could dramatically help prioritize clinical development of such agents.

To facilitate testing of already clinically available drugs as well as emerging targeted inhibitors for patients with AML, we undertook a comprehensive functional strategy to directly determine the drug dependency of cancer cells based on *ex vivo* drug sensitivity and resistance testing (DSRT). First, we applied a systematic large panel of drugs covering both cancer chemotherapeutics and many clinically available and emerging molecularly targeted drugs. Second, we developed a new way to score for differential drug response in AML cells as compared with the efficacy of these drugs in normal bone marrow cells. Third, we verified DSRT predictions *in vivo* by treating patients with AML with off-label drugs. Fourth, we assessed the molecular mechanisms underlying development of cancer progression and drug resistance by repeat sampling from relapsed patients, followed by genomic and transcriptomic profiling as well as a new DSRT analysis to understand both coresistance as well as new vulnerabilities. Taken together, we term this approach individualized systems medicine (ISM; Fig. 1).

Here, we demonstrate that the ISM strategy made it possible to (i) create a taxonomy of comprehensive drug responses in AML, (ii) identify clinically actionable AML-selective targeted drugs, (iii) clinically apply such therapies for individual chemorefractory AML patients predicted to be sensitive to targeted drugs,
and (iv) follow individually optimized therapies in patients by analysis of the clonal evolution of leukemic cells and molecular profiling to understand mechanisms of drug resistance.

RESULTS

ISM Strategy for Personalized AML Therapy

To uncover the mechanisms of drug response and resistance as well as to monitor therapy response at the level of individual AML subclones, we combined DSRT and deep molecular profiling data. DSRT was implemented to AML blast cells \textit{ex vivo} using a comprehensive set of 187 drugs, consisting of conventional chemotherapeutics and a broad range of targeted oncology compounds (Table 1 and Supplementary Table S1). Each drug was tested over a 10,000-fold concentration range, allowing for the establishment of accurate dose–response curves for each drug in each patient and control sample with identification of half-maximal effective concentrations and maximal responses (Supplementary Table S2). Although these responses reflect the drug–sample interaction, the detailed interpretation of curve parameters is difficult. To overcome this issue, we found that the most informative way to assess quantitative drug sensitivities was to convert the information to a Drug Sensitivity Score (DSS), a metric used to determine the area under the dose–response curves. Importantly, we developed a new scoring system for a real-time continuous cycle of learning and optimization of therapies, one patient at a time, thereby creating an individualized systems medicine process for improving cancer care.

DSRT Identified Signal Transduction Inhibitors as Putative AML-Selective Drugs

\textit{Ex vivo} DSRT was performed on 28 samples obtained from 18 patients with AML (Supplementary Table S3). Eighteen samples were collected at relapse and 10 at diagnosis, mainly from patients with adverse or intermediate cytogenetic risk (according to European LeukemiaNet; ref. 9). Out of the 187 drugs tested, sDSS indicated the most selective \textit{ex vivo} effective drugs for each individual patient, with a focus on leukemia-specific efficacies by comparing responses with normal bone marrow mononuclear cells. The results were expressed as a patient-specific waterfall plot (Fig. 2A). Several targeted drugs exhibited a selective response in a subset of the AML samples, with only a minimal response in the control samples (Fig. 2B and C and Supplementary Fig. S2), suggesting that these AML samples were addicted to the signaling pathways inhibited by the drugs. In contrast, the average sensitivity to conventional chemotherapeutics did not significantly differ between the patient samples and controls (Fig. 2B and D). This reflects the known limited therapeutic window for these drugs and the difficulty in predicting their

Figure 1. Functional ISM platform for improved AML therapy. The platform involves (i) comprehensive direct DSRT of 187 approved and investigational oncology compounds in \textit{ex vivo} primary cells from serial AML samples; (ii) clinical implementation of testing results in individual patients with relapsed and refractory disease; (iii) deep molecular and genomic profiling of the patients with AML from consecutive samples before and after relapse and drug resistance for monitoring disease progression and clonal evolution; and (iv) integrating drug sensitivity, next-generation sequencing, and clinical follow-up data to understand the biology of disease, drug sensitivity, and resistance that can lead to rapid introduction of novel therapies to the clinic. DSS, Drug Sensitivity Score.
ISM Approach to Therapy Selection

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clinical efficacy based on ex vivo testing. Interestingly, cytarabine, an established and effective AML drug, showed higher selective efficacy against AML cells than other cytotoxic agents (Fig. 2B and Supplementary Fig. S2), suggesting the feasibility of combining cytarabine with promising molecularly targeted drugs in the future clinical testing of relapsed or primary AML.

Significant anticancer-selective effects were observed for the tyrosine kinase inhibitors (TKI) dasatinib in 10 of the AML patient samples (36%) and sunitinib in 10 (36%), MAP–ERK kinase (MEK) inhibitors such as trametinib in 10 (36%), rapalogs such as temsirolimus in 9 (32%), foretinib in 9 (32%), ponatinib in 7 (25%), ruxolitinib in 7 (25%), dactinomycin in 7 (25%), MK-2206 in 6 (21%), sorafenib in 6 (21%), and quizartinib in 5 (18%; Fig. 2E). Thus, we identified several highly (ex vivo) AML-selective drugs that are not currently approved for AML but that are approved for other cancer indications, and would therefore be available in the clinic. Furthermore, a number of effective investigational drug classes were seen, such as AKT inhibitors and ATP-competitive mTOR inhibitors, which could be prioritized for future clinical studies of chemorefractory AML.

Table 1. Drug classes and drugs represented in the DSRT screening platform

<table>
<thead>
<tr>
<th>Classes of drugs</th>
<th>Drugs</th>
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<tbody>
<tr>
<td>Alkylating agents</td>
<td>Altretamine, azacitidine, busulfan, carboplatin, Carmustine, Chlorambucil, cyclophosphamide, dacarbazine, ifosfamide, lomustine, pipobroman, procarbazine, streptozocin, temozolomide, thioTEPA, uracil mustard</td>
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<td>Antimetabolites</td>
<td>Allopurinol, capecitabine, cladribine, clofarabine, cytarabine, decitabine, fludarabine, fluorouracil, gemcitabine, mercaptopurine, methotrexate, nelarabine, pentostatin, thioguanine</td>
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<td>Antitumor antibiotics</td>
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<td>BCL-2 inhibitors</td>
<td>Navitoclax, obatoclax</td>
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<td>HDAC inhibitors</td>
<td>Belinostat, CUDC-101, entinostat, panobinostat, tacedinavine, vorinostat</td>
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<td>Hormone inhibitors</td>
<td>Abiraterone, aminoglutethimide, anastrozole, exemestane, finasteride, flutamide, fulvestrant, goserelin, letrozole, megestrol acetate, nilutamide, raloxifene, tamoxifen</td>
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<td>HSP90 inhibitors</td>
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<td>Immunomodulators</td>
<td>Celecoxib, dexamethasone, fingolimod, imiquimod, lenalidomide, levamisole, methylprednisolone, plexaarof, prednisolone, prednisone, tacrolimus, thalidomide</td>
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<td>Kinase inhibitors, AGC</td>
<td>Alisertib, AT9283, AZD1152-HQPA, BIZ236, bryostatin 1, danusertib, enzastaurin, fasudil, midostaurin, MK-2206, ruboxistaurin, sotrastaurin, UCN-01</td>
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<td>Smoothened (Hh) inhibitors</td>
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<td>Topoisomerase I/II inhibitors</td>
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<tr>
<td>Miscellaneous antineoplastics</td>
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<tr>
<td>Other</td>
<td>2-Methoxyestradiol, anagrelide, bimatoprost, pilocarpine, Prima-1 Met, serdemetan, tarenflurbil, tipifarnib, XAV-939, YM155</td>
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Abbreviations: AGC: PKA, PKG, and PKC kinase group; CAMK: calcium/calmodulin-dependent protein kinase group; CK1: casein kinase group; CMGC: CDK, MAPK, GSK3, and CLK kinase group; PIKL: phosphoinositide 3-kinase (PI3K)-like (PI3K inhibitors and inhibitors of related atypical kinases: mTOR, DNA-PK, ATM, ATR); STE: sterile kinase group; TK: tyrosine kinase group; TKL: tyrosine kinase-like group.
Taxonomy of AML Based on the Comprehensive Drug-Response Profiles

Overall drug-response patterns of the patient samples were visualized with unsupervised hierarchical clustering. Although each individual sample showed a unique leukemia-selective drug-response profile, the overall drug-response profiles segregated the AML patient samples into five robust functional subgroups (Fig. 3; Supplementary Figs. S3 and S4). Thus, despite the underlying genomic and phenotypic variability in AML, similar drug sensitivity patterns were observed among the AML patient samples for certain drug classes. Compared with controls, all five groups showed increased sensitivity to navitoclax, aBCL-2/BCL-XL inhibitor, HSP90 inhibitors, and histone deacetylase (HDAC) inhibitors. Group I exhibited a strong selective response to navitoclax and lack of sensitivity to the remainder of the tested compounds. Group II AMLs were largely nonresponsive to receptor TKIs, but instead showed a potential inflammatory signal-driven phenotype as seen by selective responses to a group of immunosuppressive drugs (e.g., dexamethasone or prednisolone), Janus-activated kinase (JAK) family kinase inhibitors, and MEK inhibitors. Group III, IV, and V AMLs were selectively sensitive to a broad range of TKIs, indicating that they were driven or addicted to receptor tyrosine kinase signaling pathways. Group III AMLs displayed a similar sensitivity pattern to HSP90, HDAC, and phosphoinositide 3-kinase (PI3K)/mTOR inhibitors as group IV and V, albeit with lower selectivity. Group IV AMLs were especially sensitive to MEK and PI3K/mTOR inhibitors, whereas group V AMLs showed selective responses to receptor TKIs targeting ABL, VEGF receptor (VEGFR), platelet derived growth factor receptor (PDGFR), FLT3, KIT, PI3K/mTOR as well as topoisomerase II inhibitors (Fig. 3). Overall, 19 of 28 samples were sensitive to tyrosine kinase inhibition, correlating well with the findings in the recent study by Tyner and colleagues (11).
**Taxonomy of Cancer Drugs Based on the Comprehensive Drug-Response Profiles in AML**

The hierarchical clustering also stratified the drugs based on the variability of responses among the patients with AML (Supplementary Fig. S3). In this unsupervised analysis, drugs with similar modes of action clustered together, such as the PI3K/mTOR inhibitors, MEK inhibitors, HSP90 and HDAC inhibitors, VEGFR-type TKIs, PDGFR inhibitors, and ABL-like kinase inhibitors, antimototics, and topoisomerase II inhibitors. Thus, the unsupervised clustering of drugs into subgroups defined by their intended targets strongly supports the consistency and reproducibility of the DSRT analysis as well as its ability to acquire biologically and medically relevant information. However, there were also notable deviations from the expected patterns. Importantly, the FLT3 inhibitor quizartinib clustered with the topoisomerase II inhibitors but not with other TKIs. Furthermore, the recently approved BCR–ABL1 and FLT3 inhibitor ponatinib clustered with cytarabine, HSP90, and HDAC inhibitors and not with other TKIs. These unexpected links may represent underlying key molecular mechanisms of these drugs in the AML context, including unexpected off-target effects. Furthermore, these drug-clustering patterns from human AML patient specimens ex vivo may in the future be critically important in designing novel therapeutic combination strategies for clinical trials in AML. Therefore, this provides new combinatorial possibilities that could not have easily been discovered without the unbiased DSRT data.

**Genomic and Molecular Findings Underlying the Drug-Response Profiles**

To test whether the molecular profiles of the patient samples correlated with the overall drug responses, we compared the distribution of significant AML mutations and recurrent gene fusions (4) with the DSRT-driven clustering (Fig. 3). FLT3-mutated samples appeared in several different functional groups, but all patient samples in group V, the most tyrosine kinase-dependent group, carried these mutations. Hence, the group V drug-response pattern is a strong indicator of driver FLT3 mutations, and, as expected, an FLT3 mutation is an indicator that the cells are likely to respond to TKI treatment. Several FLT3 inhibitors, such as quizartinib, sunitinib, and foretinib, were among the most selective drugs for group V. Importantly, TKIs without FLT3 inhibitory activity, such as dasatinib, were highly effective in this group (P = 0.03), suggesting that FLT3-driven AMLs are also dependent on other tyrosine kinase signals. Furthermore, mutations in RAS genes correlated significantly with ex vivo sensitivity to MEK inhibitors (P = 0.001). Samples from two patients with MLL fusions clustered together in group IV, possibly linking MLL fusions with sensitivity to MEK inhibitor sensitivity. In addition, an enrichment of TP53 mutations in groups I and II was observed (3 of 4 cases), and all patient samples in taxonomic group II were associated
with adverse karyotypes. Beyond these examples, the majority of the clustering of drug sensitivities could not be attributed to obvious alterations in the AML tumor genomes.

**DSRT Was Predictive of Clinical Responses and Recapitulates Acquisition of Resistance In Vivo**

The results of DSRT were considered to be therapeutically actionable if (i) a distinct leukemia-selective response pattern was seen, (ii) drugs showing sensitivity were available for compassionate or off-label use without significantly delaying the treatment, and (iii) no standard therapy was available. According to European LeukemiaNet (9) response criteria, 3 of 8 evaluable patients had a response to DSRT-guided therapy [Supplementary Table S4; patient 600 (dasatinib–sunitinib–tepsiroimus): complete remission with incomplete platelet recovery (CRI)]; patients 718 (sorafenib–clofarabine) and 800 (dasatinib–clofarabine–vinblastine): morphologic leukemia-free state]. Four other patients had responses that did not meet the European LeukemiaNet criteria. Patient 560 showed a rapid clearance of blasts in peripheral blood after 5 days of treatment (dasatinib–sunitinib), after which therapy was discontinued because of gastrointestinal toxicity. Patient 252 (AML with three prior relapses) had an 8-week progression-free period during dasatinib monotherapy (bone marrow blasts 65%–40%–70%). Patient 784 achieved a transient response with dasatinib–sunitinib–tepsiroimus therapy: bone marrow blasts decreased from 70% to 35%, but the treatment response was lost due to selection of a resistant clone. Patient 1145 had hematologic improvement during ruxolitinib–dexamethasone therapy. Even patients with partial or transitional clinical responses had a profound effect on the clonal composition of the tumors, including selection of potential drug resistance-associated mutations. Therefore, detailed genomic analysis of such cases is important to measure the impact of therapy and to understand the potential mechanisms of response and resistance.

Here, we present in detail two clinical examples on the implementation of DSRT results in patients with AML, including the consecutive sampling and serial monitoring of drug-sensitivity profiles and clonal evolution in vivo. In the first case, the bone marrow cells from a relapsed and refractory 54-year-old patient (sample 600_2) with a normal-karyotype AML FAB M5 were subjected to DSRT and deep molecular profiling. The patient had previously failed three consecutive induction therapies (Fig. 4A). The DSRT results highlighted dasatinib, sunitinib, and temsirolimus among the top five most selective approved drugs. In an off-label compassionate- use setting, the patient received a combination of these targeted drugs, resulting in rapid reduction of the bone marrow blast count and marked improvement in the poor performance status. Concomitantly, the blood counts rapidly normalized resulting in CRI. However, 30 days after achieving the CRI response, resistance emerged. A new DSRT analysis from the relapsed sample (600_3) showed that the drugs used in patient treatment exhibited remarkably reduced anticancer activity as compared with the pretreatment sample (Fig. 4B), demonstrating a match between ex vivo and in vivo responses. In this patient, the ex vivo responses to many other drugs were also strongly reduced in the relapsed sample (Fig. 4C).

A fusion transcript joining NUP98 exon 12 and NSD1 exon 6 resulting from a cryptic chromosome translocation t(5;11) (q35;p15.5) was detected by RNA sequencing in sample 600_2. This oncogenic fusion (12) is relatively common in cytogenetically normal pediatric AML (13, 14), but relatively rare in adult AML (15). The NUP98–NSD1 fusion was also detected in the diagnostic sample (600_0) and all follow-up samples, suggesting that this fusion was the initiating event in the development of the patient’s disease. Exome sequencing revealed a diverse subclonal architecture highlighted by two FLT3-ITD (Supplementary Fig. S5A and S5B) and four different WTI mutations (Supplementary Fig. S6A and S6B; Supplementary Table S5; and Supplementary Methods). After induction chemotherapy, the predominant FLT3-ITD harboring subclone was no longer detectable. Instead, subclones containing WTI mutations and a second FLT3-ITD emerged (Fig. 4D and E). The sensitivity to quizartinib, sunitinib, and other FLT3 inhibitors indicated FLT3 as a disease driver in the 600_2 sample. In the DSRT-relapsed 600_3 sample, the dasatinib, sunitinib, and temsirolimus therapy had further selected a specific subclone still containing the second FLT3-ITD even though the response to FLT3 inhibitors and other TKIs was lost. The broad loss in drug response in the 600_3 sample was also accompanied with decreased phosphorylation of AKT (S473), CHK2 (T68), CREB (S133), ERK1/2 (T202/Y204, T185/Y187), FAK (Y397), p38α (T180/Y182), and STAT1 (Y701; data not shown).

**DSRT and Molecular Profiling Defined Key Oncogenic Signals and Mechanisms of Drug Resistance**

A second clinical case was a 37-year-old patient (784_1) who was diagnosed with a recurrent t(11;19)(q23;p13.1) translocation and corresponding MLL–ELL fusion gene. The patient had relapsed from three previous rounds of conventional therapy. Initial DSRT results (784_1) showed selective responses to MEK inhibitors, rapalogs, and several TKIs, including dasatinib (Fig. 5A). This patient was also treated with dasatinib, sunitinib, and temsirolimus, which led to a rapid decrease in both peripheral leukocytosis and bone marrow blast counts, but the effect was short-lived. The ex vivo drug sensitivity of the resistant sample (784_2) revealed that the cells had lost their response to dasatinib, rapalogs, but preserved their response to ATP-competitive mTOR inhibitors (such as dactolizin and AZD8055). Interestingly, the resistant sample gained sensitivity to a TKI that was previously ineffective in the DSRT, BMS-754807 [insulin-like growth factor-1 receptor (IGF-1R)/Trk inhibitor], as well as crizotinib (TKI) and tipifarnib (farnesyltransferase inhibitor), several topoisomerase II inhibitors, and immunomodulatory and differentiating compounds (Fig. 5B).

Using the DSRT data on kinase inhibitors with published comprehensive biochemical profiling data (16), we identified putative kinases to which the cells were addicted. Importantly, a comparison between the first and the relapsed sample identified a major switch in kinase addiction with a loss of addiction to Src family kinases, PI3K and p38 mitogen-activated protein kinases (MAPK), and a gain of addiction to ALK and Trk family receptor tyrosine kinases (Fig. 5C).

The resistance to the dasatinib, sunitinib, and temsirolimus treatment in this patient was not associated with any novel detected mutations or other genetic alterations, but coincided with more than 1,000-fold enrichment of two fusion transcripts, ETV6–NTRK3 and STRN–ALK (Supplementary Fig. S7), suggesting that resistance emerged from the selection of...
Figure 4. Clinical implementation of DSRT predictions and clonal evolution analysis in a heavily refractory AML patient. A, clinical follow-up of patient 600 from diagnosis through relapse, depicting percentage of bone marrow blasts and number of neutrophils. B, initial DSRT results from relapsed and refractory patient 600 showed that the patient cells ex vivo exhibited sensitivity to several kinase inhibitors (including dasatinib and sunitinib) and rapalogs, and as a result, the patient was treated with a combination of dasatinib, sunitinib, and temsirolimus. The patient achieved complete remission under this drug regimen, but relapsed 5 weeks later. Bar graphs show the before and after relapse sDSS for sensitivity to dasatinib, sunitinib, and temsirolimus. C, comparison of DSRT responses of the initial (600_2) and relapsed sample (600_3), revealing the loss of sensitivity to the majority of tested compounds. Drugs for which the DSS decreased greater than 10 from 600_2 to 600_3 are marked in blue, other drugs with an sDSS greater than 10 in 600_2 are marked in pale blue, and drugs with an sDSS greater than 10 in both 600_2 and 600_3 are marked in green. D, clonal progression of the disease in the patient from diagnosis to relapse; further information is included in the Supplementary Data and Supplementary Table S5. E, summary heatmap illustrating the key putative oncogenic genetic alterations in the clones depicted in D.

preexisting small subclones. The ETV6-NTRK3 encodes for the oncogenic fusion protein TEL-TrkC, whereas the STRN-ALK fusion was out of frame and therefore did not result in a functional fusion protein (Fig. 6A and Supplementary Fig. S8). These genetic events were accompanied by increases in p70S6 kinase (T389) and CREB (S133) phosphorylation (Fig. 6B), suggesting that mTORC1 was hyperactivated, a known mechanism of resistance toward rapalogs (17). The fusion gene MLL-ELL was detected from the diagnostic and subsequent samples, suggesting that this was an initiating driver event in this leukemia. Similar to patient 600, patient 784 initially also had FLT3-ITD mutations (Supplementary Fig. S9A and S9B) that were lost after induction chemotherapy, and a mutation to WT1 augmented by LOH that persisted throughout the course of the disease (Fig. 6C and D and Supplementary Table S6). The gained sensitivity to the dual IGF-IR/TrkC inhibitor BMS-754807 fits with the model of the TEL-TrkC fusion protein as a new driver, because the oncogenic potential of this fusion has been shown to be dependent on the activity of IGF-IR (18, 19) and lead to hyperactivation of mTORC1 (20, 21). Thus, we predict that in this patient, the mechanism of resistance involved...
TEL–TrkC-mediated activation of IGF-IR signaling, which promoted hyperactivation of mTORC1. Supporting this hypothesis, we observed synergistic activities between the BMS-754807 IGF-IR/TrkC inhibitor and dactolisib, an ATP-competitive mTOR inhibitor (Fig. 6E). The combination of BMS-754807 and the MEK inhibitor trametinib, on the other hand, did not result in synergism, indicating that the TEL–TrkC/IGF-IR/mTORC1 dependency represents a separate signal than the one leading to addiction to MEK signaling (Fig. 6F). Taken together, these results indicate how ISM strategy helps to identify not only the mechanisms of resistance, but also potential ways to counteract it with combinatorial therapies.

**DISCUSSION**

We present here an ISM strategy based on the systematic functional testing of patient-derived primary cancer cell sensitivity to targeted anticancer agents coupled with genomic and molecular profiling. Importantly, the intent is to guide treatment decisions for individual cancer patients coupled with monitoring of subsequent responses in patients to measure and understand the efficacy and mechanism of action of the drugs. The ISM strategy allows for an iterative adjustment of therapies for patients with cancer, one patient at a time, with repeated sampling playing a major role in understanding and learning from
ISM Approach to Therapy Selection

**Figure 6.** IGF-IR and mTOR inhibition has a synergistic effect in ETV6-NTRK3-driven AMLs. **A,** validation sequencing and resulting predicted protein structure of two fusions identified in this patient with RNA sequencing. The MLL-ELL fusion was present throughout the disease, whereas the ETV6-NTRK3 (TEL-TrkC) fusion was detected in the dasatinib-temsirolimus resistant sample. **B,** phosphoproteomic profiling of the initial and resistant samples displayed a signaling switch in the leukemic cells. **C,** clonal evolution of the AML from diagnosis to relapse. **D,** summary heatmap illustrating the clonal evolution of the AML from diagnosis to relapse. **E,** combinatorial treatment of the patient cells with the IGF-IR/TrkC inhibitor BMS-754807 and either dactolisib (ATP-competitive mTOR inhibitor) or trametinib (MEK inhibitor) revealed that there is a synergistic effect between mTOR and IGF-IR/TrkC inhibition. **F,** a model of kinase driver switch and drug resistance in this patient.

Each success and failure. Furthermore, ISM facilitates learning by discovering molecular or functional patterns from past patient cases to help therapeutic assessment of new patients.

Application of the DSRT technology to 28 AML patient samples identified effective molecularly targeted compounds inducing selective toxic or inhibitory responses in AML cells over normal bone marrow mononuclear cells. Our data suggest the intriguing possibility that anticancer agents already in clinical use for other diseases, such as dasatinib (current approved indications are chronic myeloid leukemia and Ph+ acute lymphoblastic leukemia), sunitinib (renal cell cancer and gastrointestinal stromal tumors), and temsirolimus (renal cell cancer), could be repositioned for subsets of AML patients. Although we did not achieve long-term cures in patients with AML receiving DSRT-guided therapies, the clinical responses seen are encouraging, given the fact that most of the patients with AML studied here had complex chemorefractory, end-stage disease. Obviously, the clinical results arise from a nonrandomized setting and need to be verified. However, the clinical implementation of ISM in individual patients is a powerful way to create hypotheses to be tested in systematic clinical studies, both for existing and emerging drugs. Indeed, we also identified several investigational oncology drug classes, such as ATP-competitive PI3K/AKT/mTOR pathway inhibitors, MEK inhibitors, and JAK inhibitors, which deserve attention as drugs to prioritize for future clinical trials in AML. Furthermore, the ISM approach could also help to identify effective drug combinations based on associating drug sensitivities.

Unsupervised clustering of the patient samples identified five functional taxonomic groups in AML based on **ex vivo** drug responses. FLT3 mutations were highly enriched among the most TKI-sensitive functional group (group V), with FLT3 inhibitors being the most selective class of drugs for this group. However, these samples were also selectively sensitive to other kinase inhibitors, such as dasatinib, that lack FLT3 activity, suggesting that oncogenic FLT3 signaling may be dependent on the
signaling of other tyrosine kinases whose inhibition may synergize with the therapeutic effects of FLT3 inhibitors. Given that the clinical implementation of FLT3 inhibitors has proven very challenging, the identification of druggable synergetic kinase signals or drugs could be extremely important. Furthermore, we observed a significant association between activating mutations in RAS genes and sensitivity to MEK inhibitors, in line with previous results showing that trametinib exhibited favorable clinical responses in patients with RAS-mutated refractory AML (22).

Finally, we identified a tendency of clustering of TP53 mutations in the two least-responsive functional groups (groups I and II) and mutations and fusions linking to epigenetic modulation in groups III and IV. However, most of the drug-response classifications and variabilities could not yet be attributed to the main mutations detected in the patient samples. Such drug responses may arise from nongenomic causes and complex combinatorial molecular pathways, and these findings therefore highlight the value of DSRT in (i) functionally validating suggestions arising from the genomic profiles and (ii) discovering other drug dependencies that have yet to be deduced from genomic or transcriptomic data. The relationship between genomic changes and drug response may also be more complicated in the chemotherapy patient samples studied here.

Despite multiple efforts over several decades, the direct prediction of cancer cell chemosensitivity in the clinical setting has remained an elusive goal. Compared with previously published approaches, the ISM approach presented here has distinct differences. First, our studies focused on targeted drugs, whereas most of the previous efforts of ex vivo drug testing have focused on conventional chemotherapeutics (23–27). The ex vivo responses to these agents are often nonselective and more difficult to interpret and translate to clinical patient care. Second, we focused on leukemias, whereas many previous studies have focused on solid tumors (28–33), where representative samples are difficult to acquire and consecutive samples from recurrent disease are typically not available. Third, we measured selective anticancer effects as compared with normal bone marrow mononuclear cells, which makes it possible to identify cancer-selective drugs with potential for less systemic toxicity. Fourth, we performed a rapid analysis of the in vivo effects of the drugs from consecutive samples. Our novel endpoint for therapy efficacy in patients was an impact on the clonal composition of the cancer sample.

The different types of responses in our clinically translated patient cases highlight the difficulty in predicting mechanisms of resistance and support the importance of repeated functional testing such as DSRT during disease progression to identify changes in drug sensitivities. In patient 600, the leukemic cells carried a FLT3-ITD mutation and showed addiction to this oncogene based on highly selective responses to FLT3 inhibitors, such as quizartinib and sunitinib. Interestingly, the same FLT3-ITD variant remained in the resistant cells, but the response to quizartinib, sunitinib, and other FLT3 inhibitors was lost, and the cells acquired pan-resistance to almost all agents tested. In contrast, in patient 784, resistance to dasatinib, sunitinib, and temsirolimus treatment was linked to enrichment of clonal populations carrying a tyrosine kinase fusion gene that mediated resistance. The ETV6–NTRK3 fusion and the resulting TEL–TrkC fusion protein is a known oncogenic driver in AML and other cancers (34–36) and is dependent on IGF-IR kinase activity (18–20, 37, 38). This hypothesis is supported by the acquisition of sensitivity, based on ex vivo testing, to the dual IGF-IR/TrkC inhibitor BMS-754807 exclusively in the relapsed sample.

In conclusion, we present an individual-centric, functional systems medicine strategy to systematically identify drugs to which individual patients with AML are sensitive and resistant, implement such strategies in the clinic, and learn from the integrated genomic, molecular, and functional analysis of drug sensitivity and resistance in paired samples. ISM strategy provides a powerful way to create hypotheses to be tested in formal clinical trials, for existing drugs, emerging compounds, and their combinations. In the future, ISM may pave a path for routine individualized optimization of patient therapies in the clinic.

METHODS

Study Patients and Material

Twenty-eight bone marrow aspirates and peripheral blood samples (leukemic cells) and skin biopsies (noncancerous cells for germline genomic information) from 18 AML and high-risk [according to the WHO classification-based Prognostic Scoring System (WPSS); ref. 39] patients with myelodysplastic syndromes (MDS) and 7 samples from different healthy donors (controls) were obtained after informed consent with approval (No. 239/13/03/00/2010, 303/13/03/01/2011). Patient characteristics are summarized in Supplementary Table S3. Mononuclear cells were isolated by Ficoll density gradient (Ficoll-Paque PREMIUM; GE Healthcare), washed, counted, and suspended in Mononuclear Cell Medium (MCM; PromoCell) supplemented with 0.5 μg/mL gentamicin and 2.5 μg/mL amphotericin B. One sample from patient 393, a secondary AML after MDS with 20% myeloblasts, was enriched for the CD34+ cell population (sample 393-3, corresponding to the blast cell population) using paramagnetic beads according to the manufacturer’s instructions (Miltenyi Biotech).

Development of the Compound Collection

The oncology compound collection covers the active substances from the majority of U.S. Food and Drug Administration/European Medicines Agency (FDA/EMA)–approved anticancer drugs (n = 123), as well as emerging investigational and preclinical compounds (n = 64) covering a wide range of molecular targets (Supplementary Table S1). The compounds were obtained from the National Cancer Institute Drug Testing Program (NCI DTP) and commercial chemical vendors: Active Biochem, Axon Medchem, Cayman Chemical Company, ChemieTek, Enzo Life Sciences, LC Laboratories, Santa Cruz Biotechnology, Selleck, Sequoia Research Products, Sigma-Aldrich, and Tocris Biosciences.

DSRT

Ex vivo DSRT was performed on freshly isolated primary AML cells derived from patient samples as well as mononuclear cells derived from healthy donors. The compounds were dissolved in 100% dimethyl sulfoxide (DMSO) and dispensed on tissue culture–treated 384-well plates (Corning) using an acoustic liquid handling device, Echo 550 (Labcyte Inc.). The compounds were plated in five different concentrations in 10-fold dilutions covering a 10,000-fold concentration range (e.g., 1–10,000 nmol/L). The predrugged plates were kept in pressurized StoragePods (Roylan Developments Ltd.) under inert nitrogen gas until needed. The compounds were dissolved with 5 μL of MCM while shaking for 30 minutes. Twenty microliters of single-cell suspension (10,000 cells) was transferred to each well using a MultiDrop Combi (Thermo Scientific) peristaltic dispenser. The plates were incubated in a humidified environment at 37°C and 5% CO2, and after 72 hours cell viability was measured using CellTiter-Glo luminescent assay (Promega) according to the manufacturer’s instructions with a
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Molecular Devices Paradigm plate reader. The data were normalized to negative control (DMSO only) and positive control wells (containing 100 μmol/L benzethonium chloride, effectively killing all cells).

Generation of Dose–Response Curves and Analysis of Data

The plate reader data were uploaded to Dotmatics Browser/Studies software (Dotmatics Ltd.) for a normalized calculation of percentage survival for each data point and generation of dose-response curves for each of the drugs tested. The dose–response curves were fitted on the basis of a four-parameter logistic fit function defined by the top and bottom asymptote, the slope, and the inflection point (EC50).

In the curve fitting, the top asymptote of the curve was fixed to 100% viability, whereas the bottom asymptote was allowed to float between 0% and 75% (i.e., drugs causing <25% inhibition were considered inactive).

Scoring and Clustering of DSRT Data

To quantitatively profile individual patient samples in terms of their DSRT-wide drug responses, as well as to compare drug responses across various AML patient samples, a single measure was developed: DSS. The curve fitting parameters were used to calculate the area under the dose–response curve (AUC) relative to the total area (TA) between 10% threshold and 100% inhibition. Furthermore, the integrated response was divided by a logarithm of the top asymptote (a). Formally, the DSS was calculated by:

$$\text{DSS} = \frac{100 \times \text{AUC}}{\text{TA} \times \log a}$$

We scored for differential activity of the drugs in AML blast cells in comparison with control cells, sDSS. Clustering of the drug sensitivity profiles across the AML patient and control samples was performed using unsupervised hierarchical complete-linkage clustering using Spearman and Euclidean distance measures of the drug and sample profiles, respectively. Reproducibility of the clustering and the resulting drug-response subtypes detected was evaluated using the bootstrap resampling method with the Pclust R-package (40).

Prediction of Kinase Addictions

sDSSs of kinase inhibitors were further used to predict sample-specific kinase addictions. Sample-specific sDSS responses were compared with target profiles for 35 kinase inhibitors overlapping between our compound panel and the panel profiled by Davies and colleagues (16). More specifically, for each kinase target, we calculated a Kinase Inhibition Sensitivity Score (KISS) by averaging the sDSS values among those compounds that selectively target the kinase. These putative selective kinases were compared with gene expression to exclude nonexpressed targets, and the remaining kinases defined a putative “kinaddictome” for each patient sample. For displaying purposes, the resulting kinase subtypes detected was visualized by using a 6-carboxyfluorescein (FAM)-labeled forward primer. The PCR products were separated on an agarose gel and in capillary electrophoresis with an ABI3500Dx Genetic Analyzer and sequenced using MiSeq sequencing technology.

DNA Sequencing

Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen). Exome sequencing was performed on the patient samples highlighted in Fig. 3. In addition, whole-genome sequencing was performed using DNA from the skin and AML cells from sample 784.2. DNA (3 μg) was fragmented and processed according to the NEBNext DNA Sample Prep Master Mix protocol. Exome capture was performed using the NimbleGen SeqCap EZ v2 capture Kit (Roche NimbleGen). Sequencing of exomes and genomes was done using HiSeq1500, 2000, or 2500 instruments (Illumina). For genome samples 4 × 106 and for tumor samples 10 × 106 2 × 106 bp paired-end reads were sequenced per sample. The leukemia DNA sample from patient 1497 was sequenced using the Illumina TruSeq Amplicon Cancer Panel and the MiSeq sequencer (Illumina).

Somatic Mutation Calling and Annotation from Exome-Sequencing Data

Sequence reads were processed and aligned to the reference genome as described previously (41, 42). Somatic mutation calls were made for exome capture target regions of the NimbleGen SeqCap EZ v2 Capture Kit (Roche NimbleGen) and the flanking 500 bps. High confidence somatic mutations were called for each tumor sample using the VarScan2 somatic algorithm (43) with the following parameters:

- -strand-filter 1
- -min-coverage-normal 8
- -min-coverage-tumor 1
- -somatic-P-value 0.01
- -normal-purity 0.95
- -min-var-freq 0

Mutations were annotated with SnpEff (44) using the Ensembl v68 annotation database. To filter out false-positive calls due to genomic repeats, somatic mutation calls in regions defined as repeats in the RepeatMasker track obtained from the University of California, Santa Cruz (UCSC) Genome Browser were removed from the analysis. To filter out misclassified germline variants, population variants included in dbSNP (Single Nucleotide Polymorphism database) version 130 were removed. Remaining mutations were visually validated using the Integrated Genomics Viewer (Broad Institute).

Analysis of Mutation Frequencies in Serial Samples

To examine frequencies of the identified mutations in samples where the mutations did not pass the criteria for high confidence mutations, variant frequencies and read counts for each mutation were retrieved from a set of unfiltered variant calls generated by VarScan2 with the following parameters:

- -strand-filter 0
- -min-coverage-normal 8
- -min-coverage-tumor 1
- -somatic-P-value 1
- -normal-purity 1
- -min-var-freq 0

In addition, we used variant allele frequencies from control-leukemia pairs to identify regions of LOH.

FLT3-ITD Detection by Capillary Sequencing and qPCR

For determination of patients’ FLT3-ITD status, genomic DNA was extracted from the bone marrow mononuclear cell fraction. Qualitative PCR was performed as described by Kottaridis and colleagues (45) by using a 6-carboxyfluorescein (FAM)-labeled forward primer. The PCR products were separated on an agarose gel and in capillary electrophoresis with an ABI3500Dx Genetic Analyzer and sequenced using MiSeq-tailed direct sequencing. Assessment of minimal residual disease (MRD) level was performed with real-time quantitative PCR (qPCR). A patient ITD-specific ASO (allele-specific oligonucleotide) primer was designed at the ITD junction region and used together with a downstream TaqMan probe and reverse primer (primer sequences available upon request). Albumin gene qPCR was additionally performed to normalize the variability in DNA quality in the follow-up samples.

Amplion Sequencing

Amplicons were amplified using locus-specific PCR primers carrying Illumina compatible adapter sequences, grafting sequences (PS and P7), and an amplicon-specific 6-bp index sequence (Supplementary Table S7). The PCR reaction contained 10 ng of sample DNA, 10 μL of 2X Phusion High-Fidelity PCR Master Mix (Thermo Scientific).
and 0.5 μmol/L of each primer. Following PCR amplification, samples were purified using Performa V3 96-Well Short Plate and QuickStep2 SOPE Resin (EdgeBot). Sequencing of PCR amplicons was performed using the Illumina HiSeq2000 instrument (Illumina). Samples were sequenced as 101-bp paired-end reads and one 7-bp index read.

**Library Preparation, Sequencing, and Data Analysis of Transcripts**

Total RNA (2.5 to 5 μg) was used for depletion of rRNA (Ribo-Zero rRNA Removal Kit; Epicentre), purified (RNeasy clean-up-kit; Qiagen), and reverse transcribed to double-stranded cDNA (SuperScript Double-Stranded cDNA Synthesis Kit; Life Technologies). Random hexamers (New England BioLabs) were used for priming the first-strand synthesis reaction. Illumina-compatible Nextera Technology (Epicentre) was used for preparation of RNAseq Libraries. High Molecular Weight (HMW) buffer and 50 ng of cDNA were used for tagmentation as recommended by the manufacturer. After the tagmentation reaction, the fragmented cDNA was purified with SPRI beads (Agencourt AMPure XP, Beckman Coulter). The RNAseq libraries were size selected (350–700 bp fragments) in 2% agarose gel followed by purification with QIAquick gel extraction kit (Qiagen).

Each transcriptome was loaded to occupy one third of the lane capacity in the flow cell. C-Bot (TruSeq PE Cluster Kit v3, Illumina) was used for cluster generation, and an Illumina HiSeq2000 platform (TruSeq SBS Kit v3-HS reagent kit) was used for paired-end sequencing with 100-bp read length. Nextera Read Primers 1 and 2 as well as Nextera Index Read Primer were used for paired-end sequencing and index read sequencing, respectively. RNA-seq data analysis, such as fusion gene identification, mutation calling, and gene expression quantification (Tophat and Cufflinks), was done as described previously (46). Primers used to validate as well as quantify the fusion genes detected are listed in Supplementary Tables S8 and S9.

**Proteomic Analysis**

Phosphoproteomic analysis of the AML patient samples was performed using Proteome Profiler antibody arrays (R&D Systems) according to the manufacturer’s instructions. Lysates containing 300 μg of protein were applied to the arrays, and fluorescently labeled streptavidin (IRDye 800 CW streptavidin; LI-COR) and an Odyssey in sensitivity was considered statistically significant when

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**Disclosure of Potential Conflicts of Interest**

S. Mustjoki has honoraria from the Speakers Bureau of Novartis and Bristol-Myers Squibb and is a consultant/advisory board member of the same. O. Kallioniemi has received commercial research support from IMI-Project Predect (a consortium of pharmaceutical companies) and is a consultant/advisory board member of Medisepas. No potential conflicts of interest were disclosed by the other authors.

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