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A screening based approach to circumvent tumor microenvironment-driven intrinsic resistance to BCR-ABL+ inhibitors in Ph+ acute lymphoblastic leukemia

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Abstract

Signaling by the BCR-ABL fusion kinase drives Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL) and chronic myelogenous leukemia (CML). Despite their clinical activity in many patients with CML, the BCR-ABL kinase inhibitors (BCR-ABL-KIs) imatinib, dasatinib, and nilotinib provide only transient leukemia reduction in patients in Ph+ ALL. While host-derived growth factors present in the leukemia microenvironment have been invoked to explain this drug resistance, their relative contribution remains uncertain. Using genetically-defined murine Ph+ ALL cells, we identified Interleukin 7 (IL-7) as the dominant host-factor that attenuates response to BCR-ABL-KIs. To identify potential combination drugs that could overcome this IL-7-dependent BCR-ABL-KI-resistant phenotype, we screened a small molecule library including FDA-approved drugs. Among the validated hits, the well-tolerated anti-malarial drug dihydroartemisinin (DHA) displayed potent activity *in vitro* and modest *in vivo* monotherapy activity against engineered murine BCR-ABL-KI-resistant Ph+ ALL. Strikingly, co-treatment with DHA and dasatinib *in vivo* strongly reduced primary leukemia burden and improved long-term survival in a murine model that faithfully captures the BCR-ABL-KI-resistant phenotype of human Ph+ ALL. This co-treatment protocol durably cured 90% of treated animals, suggesting that this cell-based screening approach efficiently identified drugs that could be rapidly moved to human clinical testing

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Introduction

Targeted BCR-ABL inhibition with imatinib has improved outcome for CML¹. However, the situation is starkly different for pediatric and adult Ph+ ALL². While continuous BCR-ABL-inhibition therapy -- using imatinib, nilotinib, or dasatinib -- potently kills Ph+ ALL cells *in vitro*, these drugs only induce transient remissions in patients with Ph+ ALL, ultimately allowing relapse and giving poor long-term outcomes. Although additional chemotherapy and hematopoietic stem cell transplantation (HSCT) improve the remission rate in Ph+ ALL patients, relapses remain common³, with an 18- to 24-month survival rate of 64% and an even worse relapse-free survival rate³. Differences in primary responses to BCR-ABL-KIs in patients with Ph+ ALL and CML remain poorly understood, and cannot be attributed solely to mutations in the ABL kinase domain (BCR-ABL^{MUTANTS})⁴.

We previously developed a murine leukemia model of Ph+ ALL, using primary, polyclonal *Arf*^{-/-} p185^{BCR-ABL} luciferase-expressing (Luc+) Leukemia-Initiating pre-B Cells (LICs), which captures key genetic features of human Ph+ ALL (i.e. BCR-ABL expression and *CDKN2A* deletion) and enables *in vivo* luminescent imaging to monitor response to therapy⁵. Such BCR-ABL^{WT} LICs retain phenotypic and signaling properties essential for suspended leukemic blasts to interact with the surrounding host-environment⁶ not typically present in established leukemic cell lines and remain responsive to cytokines implicated in murine pre-B cell development both *in vitro* and *in vivo*⁶. These polyclonal BCR-ABL^{WT} LICs can very efficiently initiate leukemias in syngeneic, immune-competent mice.⁶

In 'recipient'; host mice engrafted with these LICs, residual BCR-ABL^{WT} cells evade treatment with BCR-ABL inhibitors, paralleling what is seen in human Ph+ ALL patients⁴. BCR-ABL kinase domain (KD) mutations, including BCR-ABL^{T315I} (resistant to imatinib, dasatinib and nilotinib), emerge only after long-term exposure to BCR-ABL-KIs^{5, 7}. In contrast to the *in vivo* setting, BCR-ABL^{WT} murine and human cells are quite sensitive to BCR-ABL-KIs *in vitro*⁸. This disparity between *in vitro* and *in vivo* sensitivity implicates the host microenvironment in attenuating the therapeutic response to BCR-ABL kinase inhibition. We previously reported that common gamma chain receptor host cytokines modulate primary imatinib resistance⁶, and other host-derived growth factors have been shown to attenuate BCR-ABL kinase inhibition⁹⁻¹¹. Currently, no consensus exists on the relative importance of host cytokines or growth factors that attenuate the cellular responses to BCR-ABL-targeted agents.

We used a naive screening approach to identify IL-7 as the most potent among candidate host growth factors in inducing resistance to BCR-ABL-KIs in Ph+ ALL. Following on that study, we used a repurposing screen of existing drugs and clinical candidates to identify drugs that blocked proliferation of BCR-ABL^{WT} LICs cultured with murine IL-7. This study identified both the majority of known anti-leukemic drugs and a few novel agents that could overcome cytokine-dependent cell survival signaling. Testing of combinations of these validated hits with dasatinib revealed that the antimalarial drug dihydroartemisinin (DHA) provides strong synergy with BCR-ABL-KI's against Ph+ALL cells responding to the tumor microenvironment. When used *in vivo*, DHA-dasatinib combination therapy eradicates dasatinib-refractory leukemia *in vivo* providing near complete long-term survival.

Materials and Methods

Development of LIC-based screening assay

Before use in any *in vitro* assay, LICs had to pass the following quality control parameters: test thawing (benchmark: 95% viability at 24 h); PCR genotyping to confirm *Arf*^{-/-} status;

mycoplasma testing (negative by Takara Bio Mycoplasma PCR assay, Clontech, Madison WI); sequence verification of BCR-ABL allele; and *in vitro* dasatinib potency confirmation.

The maximum DMSO concentration tolerated by LICs in culture was determined to be 0.2% (by volume) and was maintained at 0.1 % (by volume) in all subsequent work. LICs were plated in 384-well micro-titer plates at a cellular density of 5×10^4 per mL (1250 LICs per well in 25 μ L BCM10) and confirmed to give exponential growth during first 72 h (Supplementary Fig S1). To select assay reagents, 3 high throughput screening (HTS) relevant cellular assays were evaluated according to manufacturer's instructions (see Supplementary Fig S3). The luminescent CTG (CellTiter-Glo; Promega Madison, WI) assay, which monitors cellular ATP content, accurately detected low number of LICs producing results comparable to those obtained by flow cytometry, and provided ease of use in HTS (no prolonged incubation step required between reagent addition and assay readout). When carried out after 72 h, the CTG assay had a 10,000-fold dynamic range, low signal variance (Z' = 0.9 in semi-automated mode, and Z' =0.8 in fully automated mode), displayed minimal drift or edge effects, and had robust inter-plate and inter-day reproducibility.

Forward cytokine phenotypic screen

Lyophilized murine and human recombinant cytokines (R&D Systems, Minneapolis, MN) were diluted with 1 mg/mL BSA in sterile PBS to a final concentration 20 μ g/mL and stored at 4°C. Anti-leukemia drugs, BCR-ABL-KIs, and non-BCR-ABL-specific-KIs (LC labs, Sigma), were solubilized in DMSO to obtain 10 mM stocks concentrations. Subsequent serial titrations were prepared using DMSO in 0.5 mL vials or 384-well drug master plates, and stored at -20°C.

To study the impact of murine cytokines on dasatinib action, LIC stocks containing different concentrations of candidate cytokines were prepared in multi-channel sterile reservoirs and plated into 384-well plates. Drug delivery to cell suspensions was accomplished with a V & P Scientific (San Diego, CA) pin tool (Supplementary Table 1). For murine-human comparisons, human SUP-B15 Ph+ ALL cells (DSMZ cell culture collection, Germany) in McCoy's media containing 20% fetal calf serum and penicillin/streptomycin (Invitrogen, Carlsbad, CA), were plated at 12,500 cells in 25 μ L per well in black clear-bottom 384-well microtiter plates. Drugs were transferred by pin-transfer. After 72 h incubation at 37°C, 20% (by vol) MTS assay reagent (CellTiter 96 Aqueous One Solution, Promega, Madison, WI) was added, microtiter plates were incubated for 75 min at 37°C, and read colorimetrically at 490 nm.

High-throughput (HT) phenotypic drug screening

The IL-7 concentration of 0.85 ng/mL was confirmed (cytokine titration assays similar to Fig. 1a for dasatinib) to confer near-maximal and reproducible resistance against nilotinib and imatinib in BCR-ABL^{WT} LICs (Fig. 1b and Supplementary Table 1). Primary screening of a collection of 5600 compounds (see Supplementary Information for details) highly enriched for approved drugs, development candidates, and compounds of known cellular activity from a variety of signaling mechanisms was carried out at a fixed dose (10 μ M). Active compounds were retested to establish potency using a 10-step, 2-fold dilution scheme (see main text and Supplementary Table 1). In addition to BCR-ABL^{WT} and BCR-ABL^{T315I} LICs, secondary drug-screens were also performed against BCR-ABL^{F317L} LICs, another clinically-known dasatinib-resistant ABL KD mutation, which gave identical results to that for BCR-ABL^{T315I} (not shown). Multiple control assay plates were set-up during all screening runs to allow for inter-plate and inter-day comparisons at 0, 24, 48, and 72 hrs.

Supplementary Data

Supplementary data includes: 1) Supplemental Information (PDF), which contains materials and methods for generation of LICs, cytokine screening, FACS assessment of viability and cell cycle percentages, detailed description of chemical library screened, data processing, quality control and hit scoring criteria for HT drug screens, cluster analysis using therapeutic drug classes, Supplementary figures S1-S11, table 1 and references; and an Excel Supplement containing complete data-sets for cytokine and HT drug screens.

Results

Determining which leukemia-microenvironment cytokines induce drug-resistance against BCR-ABL-KIs

In the absence of drug treatment, both *Arf*^{-/-} p185^{WT} and *Arf*^{-/-} p185^{T315I} pre-B cells (referred to as BCR-ABL^{WT} leukemia initiating cells (LICs)¹² and BCR-ABL^{T315I} LICs, respectively) have identical growth properties *in vitro* (Supplementary Fig. S1), and demonstrate similar, potent leukemia initiation capacity and aggressive progression *in vivo* (Supplementary Fig. S2). They retain both phenotypic and signaling characteristics of leukemic blasts suggesting that they are a ready model for the effects of cytokines upon blasts. Fifteen leukemia-microenvironment cytokines were evaluated for their ability to protect murine BCR-ABL^{WT} LICs from dasatinib *in vitro*.

IL-7 provided the strongest protection against dasatinib (Fig. 1a). In addition, IL3, thymic stromal lymphopoietin (TSLP, IL-7-like cytokine), and IL4 conferred some resistance, but with greatly reduced potency. In contrast, TGF- β enhanced dasatinib activity. Additionally, IL-7 significantly attenuated the potency of all FDA-approved BCR-ABL-KIs against murine BCR-ABL^{WT} LICs (Fig. 1b) and the human Ph⁺ ALL cell line SUP-B15 (Fig. 1c). However, IL-7 did not affect the potency of tested non-BCR-ABL-targeted drugs (Supplementary Fig. S4). Thus, IL-7 drives a specific cellular response that provides resistance to all clinically-available BCR-ABL-KIs. The specificity was further confirmed by three *in vitro* findings: 1) the presence of IL-7 during prolonged dasatinib treatment significantly increased viability of *Arf*^{-/-} BCR-ABL^{WT} LICs (Supplementary Fig. S5); 2) transient BCR-ABL inhibition dramatically increased LIC responsiveness to IL-7 (Supplementary Fig. S6c-d)¹³; and 3) removing IL-7 completely reversed the protection effect and restored dasatinib's potency (data not shown).

Physiological levels of IL-7 confer an aggressive, dasatinib-resistant phenotype to Ph⁺ ALL *in vivo*

To confirm the physiological relevance of this finding, we studied the effect of IL-7 on leukemia progression and dasatinib responsiveness *in vivo*. Implantation of IL-7^{+/+}, but not IL-7^{-/-}, host mice with relatively small numbers of BCR-ABL^{WT} LICs (200, low burden model) led to decreased survival (Fig. 1d), suggesting that murine Ph⁺ ALL progression accelerates in response to IL-7. Injecting higher numbers of LICs (200,000) overcame this trend (200,000, high burden model, Fig. 1e), suggesting the phenomena is most important during expansion of the initial seeding leukemia cell population. Treatment of animals in the high burden model revealed a significantly shortened survival benefit from dasatinib monotherapy of BCR-ABL^{WT}-driven leukemias in IL-7-deficient hosts (Fig. 1e). From a therapeutic perspective, these findings show that *in vivo* responses to dasatinib therapy in Ph⁺ ALL are modulated by IL-7, independent of BCR-ABL KD mutations. These studies also suggest that using the BCR-ABL^{WT} LIC's in the presence of IL-7 as a screening system could identify physiologically relevant pharmacological approaches to reversing this cytokine-dependent drug resistance.

High-throughput screens against primary (IL-7-induced) and acquired (BCR-ABL-mutation) BCR-ABL-KI drug-resistant phenotypes

We hypothesized that pharmacologic agents could be identified that would overcome IL-7 induced BCR-ABL-KI-resistance. A collection of 5600 compounds highly enriched for approved drugs, development candidates, and compounds of known cellular activity from a variety of signaling mechanisms, including all active ingredients from approved oncology and anti-infective drugs, was screened against BCR-ABL^{WT} and BCR-ABL^{T315I} LICs in the presence of IL-7 (0.85 ng/mL) at a fixed drug concentration (10 μ M). The quality of the screen and minimum significant activity (> 10% growth inhibition) was confirmed by receiver operating characteristic analysis and other statistical metrics (Supplementary Fig. S7-S10). 627 compounds showed statistically significant activity (> 10% growth inhibition). These hits were augmented with available structural analogs and drugs of the same pharmacologic classes to yield 706 active compounds. Concentration–response relationships were established for these compounds against BCR-ABL^{WT} and drug-resistant BCR-ABL^{T315I} LICs in the presence of IL-7, giving 229 validated hits with reasonable potency, representing a final hit rate of 4%. While a high hit rate, this is congruent with our historical experience screening this collection in growth inhibitory assays against cancer cell lines and primary cultures. All validated hits were equipotent in inhibiting the growth of BCR-ABL^{WT} and BCR-ABL^{T315I} LICs, suggesting they are not acting through BCR-ABL. This set included 15 conventional anti-leukemic drugs (see Excel Supplement), some of which enhance BCR-ABL-KI-therapy *in vivo*^{3, 5}, thus validating the detection of appropriate phenotypic responses. Furthermore, cluster analysis indicated that this set effectively sampled the space of all existing therapeutic classes (Fig. 2). See Excel Supplement for a detailed annotation of validated hits.

In vitro and *in vivo* anti-Ph+ ALL activity of dihydroartemisinin

Although our validated hits included compounds with varying mechanisms of action and compound in varying stages of development, we focused on approved drugs with favorable human pharmacokinetic and toxicity profiles that have previously been used with pediatric populations but not for leukemia, which quickly narrowed focus to the artemisinin class of antimalarials (ARTs), which includes artemisinin, artesunate, and dihydroartemisinin (DHA)¹⁴. As a class, the ARTs were equipotent against BCR-ABL^{WT} and BCR-ABL^{T315I} LICs (Fig. 3a), but the anti-LIC potency of artemisinin (ca. 1 μ M EC₅₀) was weaker than that of artesunate or DHA (ca. 200 nM EC₅₀). DHA and artesunate also killed the human Ph + ALL cell line SUP-B15 with similar potency in the presence of IL-7 (Fig. 3a, 3b). Studies have shown that DHA kills other genotypes of leukemic cells while selectively sparing normal human lymphocytes^{15, 16}. Therefore, DHA was chosen for further analysis because of its potency, cellular selectivity, oral formulation, favorable pharmacokinetics, known bioavailability in the hematopoietic system, and tolerability in all human age groups.

Due to our interest in modeling the approved drug DHA, which is given orally for malaria, followup studies *in vivo* utilized the oral route rather than the parenteral route more commonly used in oncology studies. DHA monotherapy conferred a significant dose-dependent survival benefit to IL-7^{+/+} host mice bearing low-burden BCR-ABL^{WT} leukemia (Fig. 3c) although this required escalation to high doses (300 mg/kg) for maximal effect. During these studies no significant changes in body weight were observed. This dose falls within the tolerated and bioavailable doses used in prior animal modeling for malaria.¹⁷

By conventional allometry this dose corresponds to roughly 45 mg/kg in a human, which is 4-fold higher than the current efficacious dose used for malaria in humans (up to 12 mg/kg). The limited murine pharmacokinetic data for DHA¹⁸ indicates that it is more rapidly cleared in mice and has lower bioavailability than in humans suggesting that lower relative doses

would be efficacious in humans. Additionally, existing human and murine pharmacokinetic data indicate that plasma concentrations that should be achieved in this model would be well above concentrations efficacious *in vitro* against LIC's, even at doses more closely aligned with those used for malaria.¹⁹ BCR-ABL mutations, which are often selected with dasatinib treatment in this model^{5, 7}, were absent in leukemic samples collected from terminally moribund DHA-treated mice. DHA monotherapy also gave a similar response in mice with low-burden BCR-ABL^{T315I} leukemia, which is completely refractory to dasatinib (Fig. 3d). Thus, unlike BCRABL-KIs, DHA acts against Ph+ ALL via a BCR-ABL kinase-independent mechanism.

DHA synergistically enhances response to dasatinib

Because BCR-ABL^{WT} leukemias in IL-7-deficient mice were more sensitive to dasatinib (Fig. 1e), and DHA displayed anti-Ph+ ALL activity in IL-7^{+/+} host mice (Fig. 3c, 3d), the potential for synergy between the two drugs was evaluated. Cohorts of IL-7^{+/+} immune-competent mice bearing high burdens of *Arf*^{-/-} BCR-ABL^{WT}-driven leukemia were treated with dasatinib alone (10 mg/kg), DHA alone (200 or 300 mg/kg) or binary combinations (Fig. 4a). Despite continuous monotherapy, dasatinib induced only weak initial responses, and all animals later relapsed (Fig. 4b) with complete mortality within 6 weeks of initiating therapy (Fig. 4a). DHA alone was completely ineffective (200 mg/kg) or poorly effective (300 mg/kg) in the high-burden leukemia model (Fig. 4a). However, the combination of DHA and dasatinib provided a strong and rapid initial response, and led to significantly increased overall survival (Fig. 4a & Fig. 4b), in comparison to the treatment with either of the two drugs alone. Of the 12 mice given 200 mg/kg DHA and 10 mg/kg dasatinib, 3 survived long-term. All mice given 300 mg/kg DHA and 10 mg/kg dasatinib (*n*=12) survived the treatment period. As discussed above, the plasma concentrations expected to be reached with such doses are likely providing exposure well above that efficacious against LIC's and within those that could be reached in humans with good tolerability.^{17, 19}

4 weeks after completion of combination therapy, 1 mouse succumbed to an isolated CNS relapse, which may be related to the poor CNS availability of dasatinib²⁰. Remarkably, the 14 surviving mice taken off dasatinib-combined therapy (3/12 at 200 mg/kg and 11/12 at 300 mg/kg DHA) showed no leukemia for one year, the longest period monitored. In this leukemia model, all animals left untreated succumb to full blown leukemia within 4 weeks of injecting a very low numbers of LICs (Supplementary Fig. S2), strongly suggesting that the drug combination cured the disease. All dasatinib-DHA regimens were well tolerated in leukemic and non-leukemic mice, with no noticeable changes in weight gain or clinical signs, in congruence with prior rodent work utilizing similar dosages of DHA¹⁷.

Discussion

We identified IL-7 as the dominant (most potent) cytokine that confers resistance to all FDA-approved BCR-ABL-inhibitors. IL-7 is essential for murine T cell and B cell development²¹, has recently been implicated in human post-fetal B cell development^{21, 22}, and is a major contributor to the regulation and maintenance of mature T lymphoid cells²³. IL-7-rich niches such as bone marrow harbor early persistent drug-refractory leukemias in dasatinib-treated mice, at which point the vast majority of leukemic cells do not harbor BCR-ABL KD mutations⁵ Ultimately, BCR-ABL mutations emerge under continuous drug exposures^{5, 24}, causing secondary drug resistance. Our findings suggest that IL-7 maintains leukemic cell viability, probably through induction of phosphorylation of STAT5, despite continuous BCR-ABL inhibition *in vivo*, thereby facilitating the subsequent acquisition of drug resistance conferring kinase domain mutations. There is a well appreciated relationship between phosphorylation of STAT5 and resistance to imatinib in cells.²⁵⁻²⁷ Additionally,

human Ph+ ALL patients with increased levels of pSTAT5, whose phosphorylation is triggered by IL-7, respond poorly to imatinib therapy.²⁷

The demonstration of IL-7-induced drug resistance provided a rationale for 'complementing' pharmacological BCR-ABL-inhibition with a second drug that induces IL-7-independent leukemic cell death. We assayed 5600 compounds enriched with approved drugs to identify such agents. Our studies confirmed the activities of the majority of known anti-leukemia drugs and also identified several new classes of the compounds with activity against both IL-7-cultured wild-type and BCR-ABL mutant LICs. We characterized the anti-leukemia activity of one such drug: the orally-bioavailable, well-tolerated, antimalarial agent dihydroartemisinin (DHA) that selectively kills leukemic cells while sparing normal lymphocytes^{15, 16}. Mechanistically, DHA acts independently of BCR-ABL-KIs (Fig. 3). In combination with dasatinib, DHA enhanced the depth of remission induction (Fig. 4b) and achieved near-complete long-term survival in a robust murine model of human Ph+ ALL. It is noteworthy that the combination of DHA and dasatinib therapy is significantly more efficacious than the clinically-used triple combination between dasatinib, dexamethasone, and asparaginase when assessed in the same model⁵.

Distinct from its anti-malarial mechanism of action, induction of cell death by ARTs in transformed cells is associated with accelerating the degradation of c-MYC²⁸. c-MYC expression is downstream of activated STAT5²⁹, which is maintained by IL-7 signaling, and STAT5 activation³⁰ and c-MYC expression^{31, 32} are necessary to sustain BCR-ABL transformed cells. This phenomena has recently been implicated in other stroma-induced drug resistance¹⁰ as well as used as an explanation for resistance to imatinib^{10, 33}. However, we do not see changes in pSTAT5 levels in response to treatment with artemisinins (data not shown). Thus, DHA may synergize with BCR-ABL-KI therapy by accelerating the degradation of c-MYC, thereby countering the protective effects of IL-7, as has been reported by others. Alternatively, as has also been reported DHA may affect BCR-ABL levels directly.^{34, 35}

We have shown that phenotypic screening using primary leukemia cells selected to maintain responsiveness to host cytokines allows identification of therapeutic agents or developmental compounds that hold promise for the treatment of Ph+ leukemias. This system provides an unbiased platform for the discovery of such new therapies and to evaluate the potential utility of such agents by demonstrating proof-of-concept *in vivo* using a murine model that closely recapitulates human disease. Finally, our results strongly support further study of BCR-ABL-KI/DHA combination therapy for the treatment of patients with high-risk Ph+ ALL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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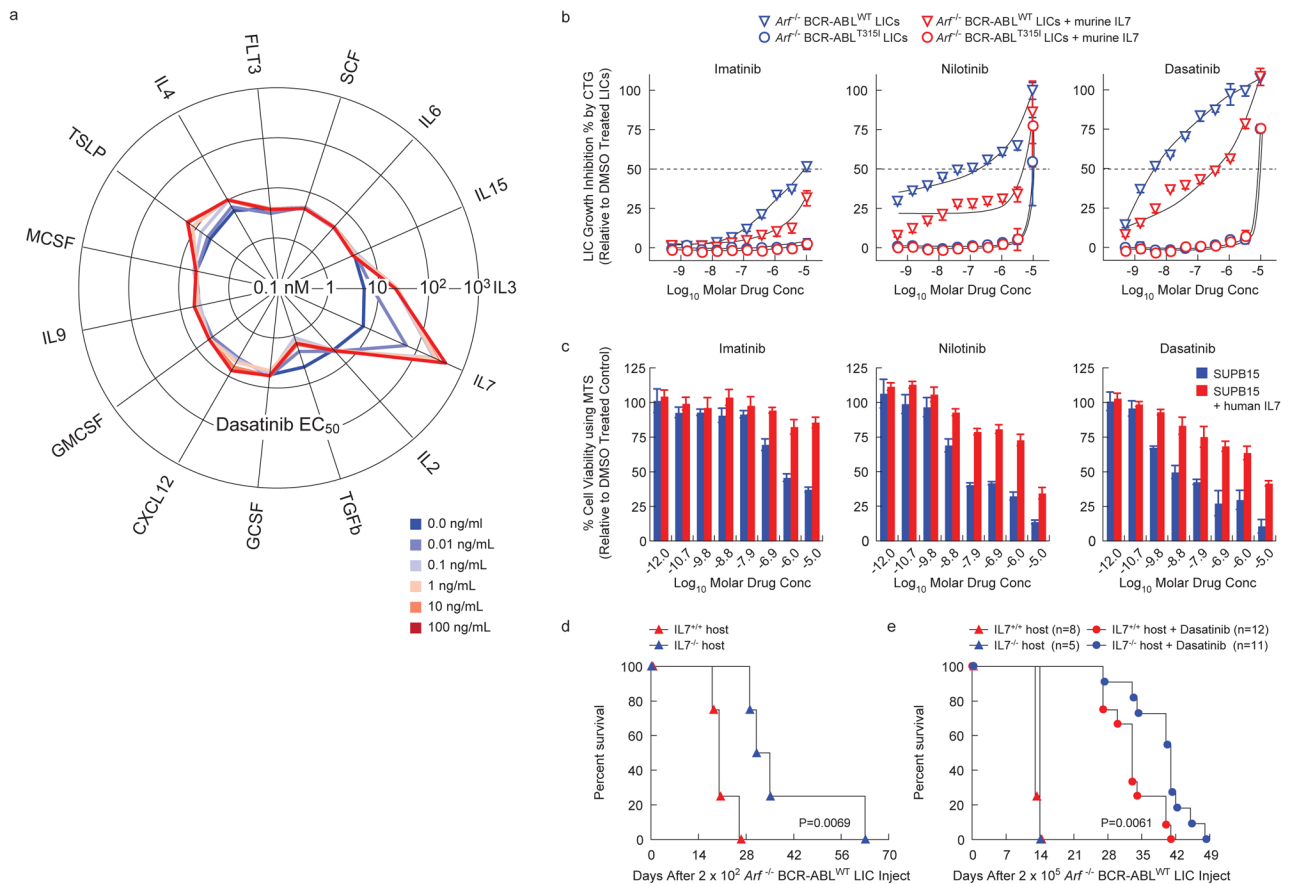


Figure 1. IL-7 induces resistance to BCR-ABL-KIs in Ph+ ALL without mutation of BCR-ABL
(a) Radar plot representing the effect of 15 hematopoietic cytokines on dasatinib potency (EC₅₀) against Arf^{-/-} BCR-ABL^{WT} LICs. CTG assay measured LIC populations after 72-h dasatinib exposures in presence of increasing concentrations of indicated cytokines. Each spoke represents an individual cytokine; each line graph represents dasatinib proliferation inhibition EC₅₀ (nM) at indicated titration of the parent cytokine (see Excel Supplement).
(b) Arf^{-/-} BCR-ABL^{WT} (triangles) and Arf^{-/-} BCR-ABL^{T315I} (circles) LICs were treated with drug or DMSO for 72-h in the absence (blue) or presence (red) of 1 ng/mL mIL-7, in triplicates. For each drug concentration, LIC growth measured by CTG assay was normalized to DMSO-treated LICs and fit using nonlinear regression (+/- sd).
(c) Viability of human Ph+ ALL SUPB15 cells after 72-h drug-treatment in absence or presence of 25 ng/mL hIL-7, was measured by MTS assay. Values were normalized to DMSO-treated cells.
(d, e) Kaplan-Meier survival curves showing overall survival in IL-7^{+/+} and IL-7^{-/-} B16 host-mice receiving day 0 tail vein injection of **(d)** 2 × 10² Arf^{-/-} BCR-ABL^{WT} LICs (vehicle treated), with median survival of 20 days in IL-7^{+/+} (n=4) versus 33 days in IL-7^{-/-} mice (n=4), P=0.0069, or **(e)** 2 × 10⁵ Arf^{-/-} BCR-ABL^{WT} LICs. Data are an average of 2 independent experiments in which vehicle or dasatinib therapy was initiated on day 7 or 9. Survivals of vehicle-treated IL-7^{+/+} and IL-7^{-/-} mice were comparable. Dasatinib treatment caused a median survival of 33 days in IL-7^{+/+} (n=12) versus 41 days in IL-7^{-/-} (n=11) mice, P=0.0061.

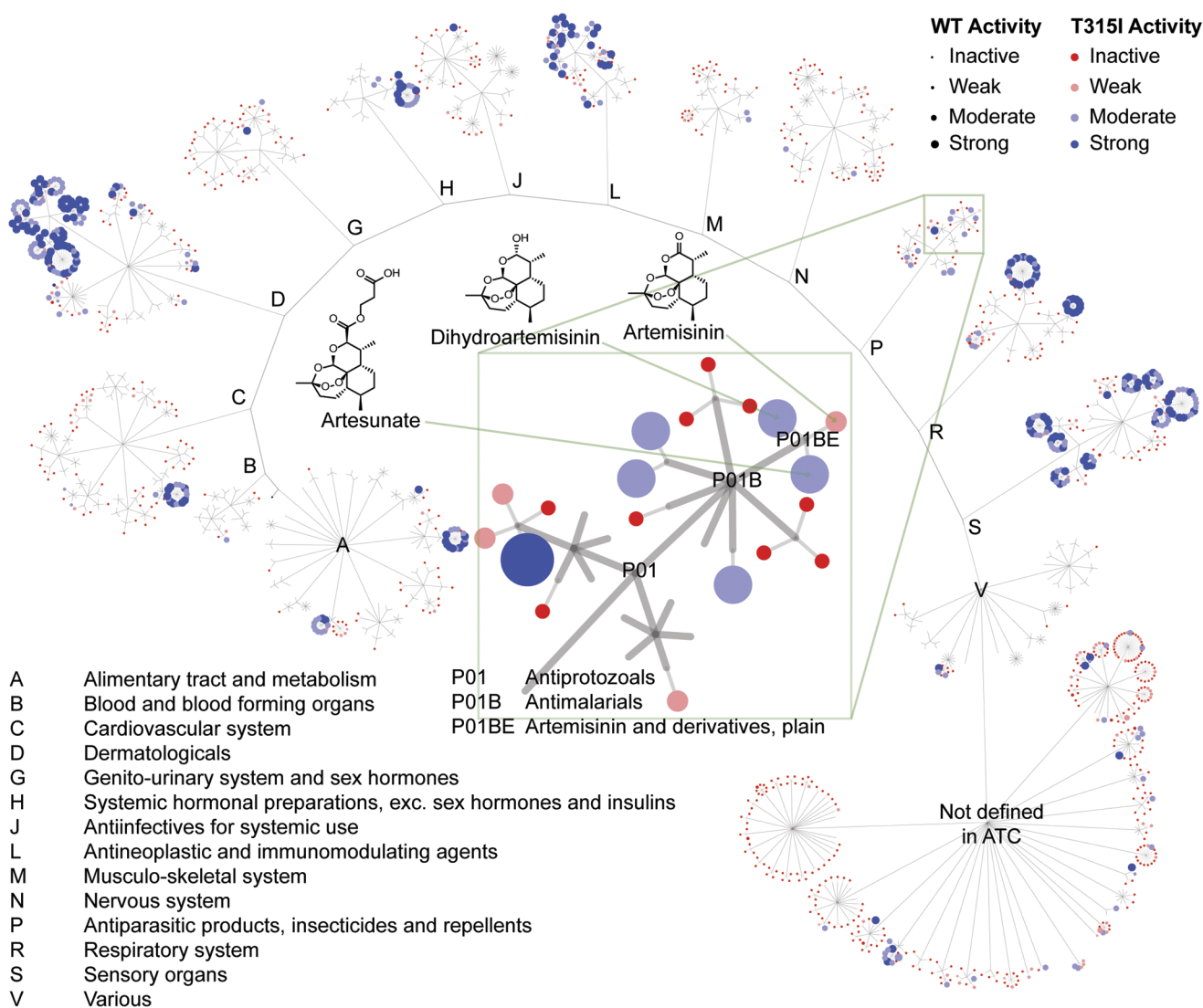


Figure 2. Screen to identify compounds preventing growth of wild type and BCR-ABL^{MUTANT} Ph+ ALL cells in the presence of IL-7

Several hundred validated hits were identified. The hits are shown as a graph with clustering hits using ATC therapeutic classification (http://www.whocc.no/atc/structure_and_principles/). A single compound can be represented more than once because of multiple therapeutic indications, as is the case for the corticosteroids (highly active clusters in A, C, D, H, R, and S). This demonstrates that the active compounds come from drugs used to treat many indications. See Supplemental materials and methods and Supplementary Figure S11 for details on clustering methodology and analysis.

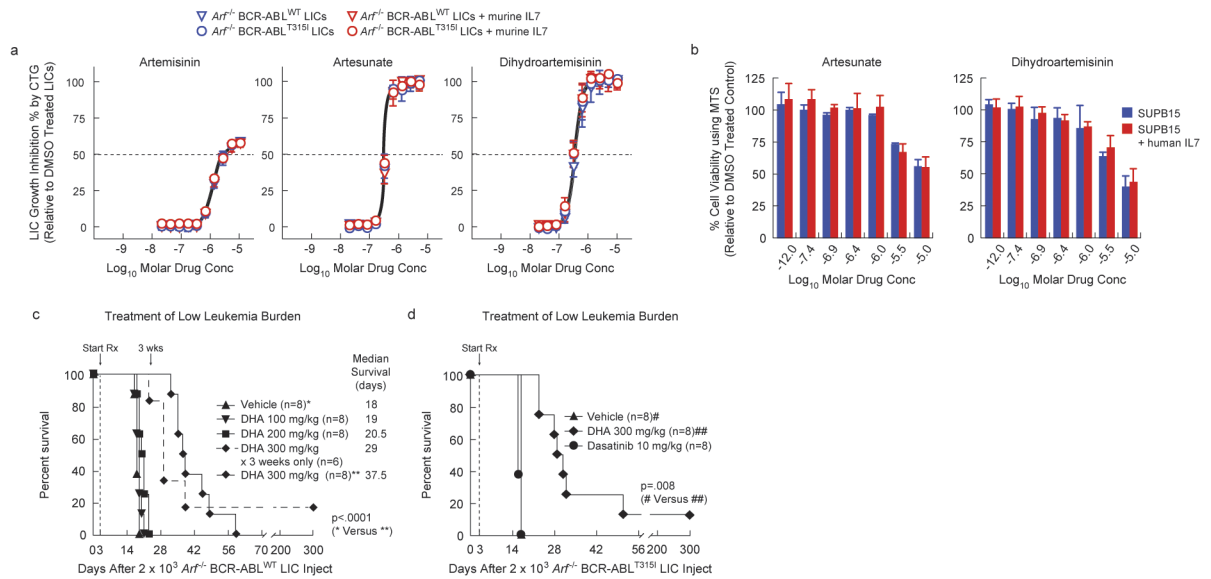


Figure 3. The artemisinin family of antimalarial drugs circumvents IL-7 induced resistance to dasatinib in Ph+ ALL cells in vitro and Ph+ ALL in IL-7^{+/+} host mice

(a) Arf^{-/-} BCR-ABL^{WT} (triangles) and Arf^{-/-} BCR-ABL^{T315I} (circles) LICs were treated with artemisinin, artesunate, dihydroartemisinin, or DMSO for 72 h in the absence (blue) or presence (red) of 1 ng/mL murine IL-7, in triplicates. For each drug concentration, LIC growth (measured by the CTG assay) was normalized to DMSO-treated LICs and fit using nonlinear regression (+/- sd). (b) Human Ph+ ALL SUPB15 cells were treated with artesunate, dihydroartemisinin, or DMSO (no drug) for 72 h in the absence (blue) or presence (red) of 25 ng/mL human IL-7. Viability was measured by the MTS assay. Values were normalized to DMSO-treated cells. (c, d) Kaplan-Meier survival curves showing the overall survival in response to therapy (survival benefit) in IL-7^{+/+} B16 host mice that received day 0 tail vein injection of (c) 2×10³ Arf^{-/-} BCR-ABL^{WT} Luc+ LICs; on day 3, once daily for 5 days of each week, vehicle, 100 mg/kg DHA, 200 mg/kg DHA, 300 mg/kg DHA, or 300 mg/kg DHA (for 3 weeks only) therapy was initiated in mice bearing low burdens of BCR-ABL^{WT}-driven leukemia, or (d) 2×10³ Arf^{-/-} BCR-ABL^{T315I} Luc+ LICs; on day 3, once daily for 5 days of each week, vehicle, 300 mg/kg DHA, or 10 mg/kg dasatinib was given to mice bearing low burdens of BCR-ABL^{T315I}-driven leukemia.

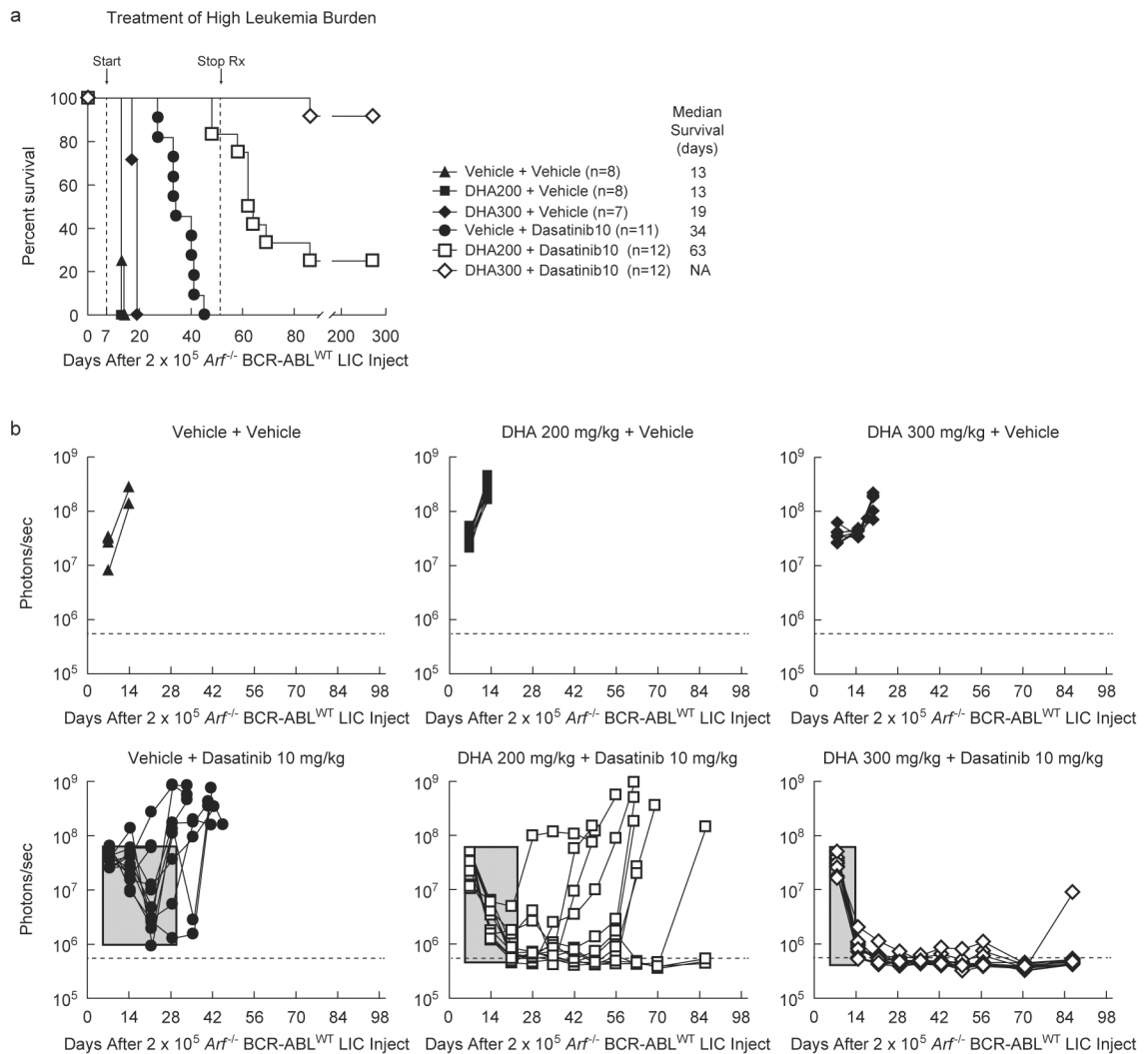


Figure 4. Dihydroartemisinin works synergistically with dasatinib to cure Ph+ ALL

(a) Kaplan-Meier survival curves showing the survival benefit in response to therapy in IL-7^{+/+} Bl6 host mice that received day 0 tail vein injection of 2×10^5 Arf^{-/-} BCR-ABL^{WT} Luc⁺ LICs. On day 7, six mouse groups bearing high burdens of BCR-ABL^{WT}-driven leukemia were started on daily treatment for 5 days of each week, using two-dose regimens - 2 vehicles or 1 vehicle and 1 agent or 2 agents (see label), with 2 doses of each regimen given separately, 8 h apart, by oral gavage. On day 52, all therapy was terminated. (b) For 6 mouse groups in (a), separate graphs represent quantitative assessment of serial whole-animal luminescent signals (p/s/cm²/sr) acquired weekly. Solid lines represent imaging intensity of signal (y-axis) as a measure of leukemia burden from individual mice in the group, which is traced in real time on the x-axis. On day 7, before initiating therapy, all 6 groups had comparable and high burden of leukemia. The horizontal dotted line represents the empirically determined lower limit of sensitivity for luminescent signal as an experimental cut-off for detectable levels of persistent leukemia. Rectangular gray box quantifies experimental primary therapeutic response to indicated regimens; box-width depicts approximate time (days) to induce maximal initial response and box-depth represents maximal decrease achieved in leukemia burden signal. Increasing depth and decreasing width indicate stronger and quicker primary responses, respectively.