The National Cancer Institute ALMANAC: A Comprehensive Screening Resource for the Detection of Anticancer Drug Pairs with Enhanced Therapeutic Activity


Abstract

To date, over 100 small-molecule oncology drugs have been approved by the FDA. Because of the inherent heterogeneity of tumors, these small molecules are often administered in combination to prevent emergence of resistant cell subpopulations. Therefore, new combination strategies to overcome drug resistance in patients with advanced cancer are needed. In this study, we performed a systematic evaluation of the therapeutic activity of over 5,000 pairs of FDA-approved cancer drugs against a panel of 60 well-characterized human tumor cell lines (NCI-60) to uncover combinations with greater than additive growth-inhibitory activity. Screening results were compiled into a database, termed the NCI-ALMANAC (A Large Matrix of Anti-Neoplastic Agent Combinations), publicly available at https://dtp.cancer.gov/ncialmanac. Subsequent in vivo experiments in mouse xenograft models of human cancer confirmed combinations with greater than single-agent efficacy. Concomitant detection of mechanistic biomarkers for these combinations in vivo supported the initiation of two phase I clinical trials at the NCI to evaluate clofarabine with bortezomib and nilotinib with paclitaxel in patients with advanced cancer. Consequently, the hypothesis-generating NCI-ALMANAC web-based resource has demonstrated value in identifying promising combinations of approved drugs with potent anticaner activity for further mechanistic study and translation to clinical trials. Cancer Res; 77(13); 3564–76. ©2017 AACR.

Introduction

Despite recent advances in translational oncology (1), new therapies for cancer are urgently needed, as are new approaches to facilitate the rapid translation of promising preclinical data to clinical evaluation. Recognizing that combining anticancer drugs, whether cytotoxic or molecularly targeted, with distinct mechanisms of action is the approach most likely to overcome single-agent resistance and produce sustained clinical remissions (2, 3), we determined that it would be of value to systematically screen pairwise combinations of 104 FDA-approved oncology drugs in the NCI-60 panel of human tumor cell lines. Our objective was to create a database, the NCI-ALMANAC (A Large Matrix of Anti-Neoplastic Agent Combinations), that could be searched to identify combinations with greater antitumor activity than either agent alone. Promising combinations could then be selected for further evaluation in vitro or in human tumor xenografts models. By screening only approved drugs with proven activity and established safety profiles, combinations identified from the NCI-ALMANAC database offer potential for rapid translation into Investigational New Drug–exempt clinical trials.

The NCI-60 panel is one of many drug development resources that are, and will continue to be, supported by the NCI; each of the cell lines has been extensively characterized at the molecular level; exome sequence, mRNA expression, miRNA expression, protein quantification, protein modification, DNA methylation, enzyme activity, and metabolomics data are all publicly available (4, 5). These datasets enable molecular data mining in the context of combination drug studies to assess the engagement of multiple potential targets and/or downstream markers of drug action, both in animal model systems and in patients during early-phase clinical trials (6). They also support research into the multiple mechanisms driving resistance to cancer therapeutic agents, including mutation or amplification of the gene encoding the drug target, enhanced drug efflux or metabolism, activation of compensatory signaling networks that bypass the effects of target engagement, changes in DNA damage response or epigenetic pathways, or alterations in the tumor microenvironment (7).

Although a variety of active clinical trials are testing drug combinations based on accepted precepts of tumor cell biology,
the preclinical hypotheses supporting many of these studies may be difficult to validate in patients without clearly defined mechanisms of action, resistance, and toxicity for each component of the combination, as well as for the combination itself (8). Indeed, the heterogeneity observed across individual tumors hints at the possibility of distinct cooccurring resistance mechanisms within a patient, such that different regions of the same malignancy may vary in their sensitivity (or resistance) to an individual agent at the time that treatment is initiated (9–11). Hence, developing new combination strategies to overcome drug resistance in patients with advanced cancer is a therapeutic imperative (8, 12). Promising drug combinations may be discovered with synthetic lethal screens (13) utilizing siRNA or shRNA libraries, or CRISPR-Cas9 genome editing (14), to identify targets for commonly used anticancer drugs (15, 16); however, in this study, we utilized an unbiased, "hypothesis-free" phenotypic screening approach (8).

The results of our comprehensive combination screening effort are available in the NCI-ALMANAC database, a web-based resource intended for hypothesis-generating assessments of oncology drugs combinations. On the basis of the outcome of this screening program, several promising drug combinations were examined in human tumor xenografts developed from NCI-60 cell lines. From our in vitro-to-in vivo translation effort, two promising drug combinations never before tested in human studies, clofarabine with bortezomib and paclitaxel with nilotinib, were chosen for clinical evaluation.

Materials and Methods

Cell lines and drug screening

NCI-60 cell lines were obtained from the NCI Developmental Therapeutics Program Tumor Repository in the mid-1990s to early 2000s and distributed for drug screening and xenograft experiments in 2010–2012. For each lot of cells, the Repository performed Applied Biosystems AmpFLSTR Identiﬁer testing with a synthetic control to conﬁrm consistency with the published Identiﬁer STR proﬁle (17) for the given cell line. Each cell line was tested for mycoplasma when it was accepted into the repository, routine mycoplasma testing of lots was not performed. Cells were kept in continuous culture at NCI for no more than 20 passages.

Oncology drugs were obtained through commercial sources. Experiments were performed at three locations (NCI's Frederick National Laboratory for Cancer Research, SRI International, and University of Pittsburgh). At NCI, each drug was tested at 5 concentrations as single agents and in 3 combination matrices, in individual wells. Drug concentrations were selected based on clinical relevance; where possible, concentrations were selected to be below the human peak plasma concentration \( C_{\text{max}} \) at the FDA-approved clinical dose and also to yield measurable activity in our assay systems. In a few cases, one or more concentrations above the \( C_{\text{max}} \) were selected to achieve some growth inhibition in vitro. Growth percent was calculated as described in the standard NCI-60 testing protocol. Inclusion of a time zero measurement in all experiments allowed for determination of cell killing and growth inhibition. Fifty-nine of the NCI-60 cell lines passed predefined quality control parameters for >90% of the drug pairs. The SK-MEL-2 melanoma cell line was an outlier; data for only 65% of the drug pairs met quality control standards as the cells grew poorly in 384-well plates at both testing locations.

Development and use of the NCI ComboScore

Determination of combination beneﬁt ("ComboScore") utilized a modiﬁcation of Bliss independence (18), as follows: Let \( Y_{i}^{A} \) be the growth fraction for the \( i \)th cell line exposed to the \( p \)th concentration of drug A and the \( q \)th concentration of drug B, defined as:

\[
Y_{i}^{A,B} = \frac{T_{i}^{A,B} - T_{i}^{A}}{T_{i}^{B} - T_{0}}
\]

where \( T_{0} \) is the time zero endpoint measurement, \( T_{i}^{A,B} \) is the endpoint measurement after 2 days, and \( T_{i}^{B} \) is the endpoint measurement after 2 days for the control well. Define \( Y_{i}^{A,B} \) as the growth fractions when only exposed to drug A or drug B, respectively. The expected growth fraction for the combination is:

\[
Z_{i}^{A,B} = \begin{cases} 
\min\left(Y_{i}^{A}, Y_{i}^{B}\right) & \text{if } Y_{i}^{A,B} \leq 0 \\
100 \left( \frac{Y_{i}^{A,B}}{Y_{i}^{A} \cdot Y_{i}^{B}} \right) & \text{otherwise}
\end{cases}
\]

where \( \bar{Y}_{i} = \min(Y_{i}, 100) \) truncates the growth fraction at 100.

The final combination score for the cell line and the drug combination is the sum of the differences in expected versus observed growth fractions:

\[
Y_{i}^{\text{CB}} = \sum_{p,q \neq i} Z_{i}^{A,B} - Z_{i}^{A,B}
\]

These calculations set a ceiling of 100% growth, that is, the expected combination growth percentage cannot be greater than the control. One screening site had a high incidence of ‘reversals,’ where the growth percent increased as the concentration of one or both drugs was increased, that is, apparent enhancement of growth by drug treatment. While the phenomenon of hormesis can be real, the frequency with which this was observed, at only one of the screening locations, argued against this being actual hormesis. All data for a drug pair/cell line were removed from the dataset if the growth percent increased by 50% or greater between any 2 adjacent doses of either drug. ComboScores are available for download at https://dtp.cancer.gov/ncialmanac.

Evaluation of combination therapies registered with clinicaltrials.gov

For initial analysis of the clinical status of all NCI-ALMANAC combinations, a drug pair was designated “clinically tested” if the two names were provided as an intervention in the same trial listed in the clinicaltrials.gov database (see Supplementary Methods for further detail).
Heatmap

JMP 11 (SAS institute) software was utilized for preparation of heatmaps and statistical analyses. Two-way hierarchical clustering utilized the Ward method with data standardization and without imputation of missing values.

Human tumor xenografts

Animal experiments were performed at both SRI International and Frederick National Laboratory for Cancer Research. Both SRI International and Frederick National Laboratory are accredited by AAALAC International and follow the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the “Guide for Care and Use of Laboratory Animals” (National Research Council; 2011; National Academies Press; Washington, D.C.).

For mouse inoculation, tumor cells from the NCI-60 panel were used at the 4th to 6th in vitro passage of cryopreserved cell stocks. Cells (1 × 10^5 cells/0.1 mL/injection) were subcutaneously inoculated bilaterally into female athymic nu/nu NCr mice, and therapeutic studies were initiated upon reaching a target volume of 100 mm^3. Sample sizes were n = 10–20 mice for vehicle-treated groups and n = 5–10 mice for single-agent- or combination-treated groups. Mice were treated with drug doses that had been demonstrated to be the MTD for the single agents and the combination in prior experiments by the NCI Developmental Therapeutics Program (see Supplementary Data for dosage regimens); in some studies, treatments at lower doses were also performed. For each mouse, the time from treatment initiation to tumor volume doubling was estimated using exponential growth interpolation when the event was between two time points. Mice for which tumor volume had not doubled by the end of study were considered censored at the last observation. The distribution of time-to-tumor-volume-doubling was estimated for each treatment cohort using the Kaplan–Meier method, and the log-rank test was used to determine significant differences between combination and single-agent treatment groups.

Biomarker analyses of human tumor xenografts

Analyses of apoptosis (19) and DNA damage response biomarkers (20, 21) were performed as described previously (see Supplementary Methods for details).

Results

The NCI-ALMANAC dataset and web tools

Pairwise combinations of 104 FDA-approved anticancer drugs (Supplementary Table S1) were screened in each of the cell lines of the NCI-60 panel to discover those with enhanced growth inhibition or cytotoxicity profiles compared with either single agent (Fig. 1A). Combination activity was reported as a “ComboScore” (22); positive ComboScores indicated greater than additive in vitro activity and negative scores indicated less-than-additive activity (see the Materials and Methods). A total of 5,232 drug pairs were evaluated in each of the cell lines; 304,549 experiments were performed to test each drug at either 9 or 15 combination dose points, for a total of 2,809,671 dose combinations. The NCI-ALMANAC is publicly available at https://dtp.cancer.gov/ncalmanac; the data can be visualized as a heatmap summarizing the entire dataset, as a bar graph of the ComboScores for a particular drug pair in all cell lines, or as dose–response curves for one drug combination in a given cell line (Supplementary Fig. S1). Finally, users can obtain an overview of which drugs or mechanistic categories of drugs perform best in combination with a given drug of interest.

We first researched what fraction of the NCI-ALMANAC drug pairs appeared on clinicaltrials.gov for an estimation of clinical experience (see Materials and Methods for details). At the start of this study in June 2011, nearly 75% of the >5,000 pairs of FDA-approved drugs examined had not been reported to clinicaltrials.gov as being part of an ongoing or completed study (Fig. 1B). Next, to identify patterns in cellular sensitivity and combination activity, we conducted a two-dimensional hierarchical clustering analysis. The resulting heatmap of ComboScores across cell lines and drug combinations revealed that most drug combinations have selective activity for a subset of cell lines, as indicated by the blue–red heterogeneity throughout most of the heatmap, although some combinations did exhibit patterns of predominantly greater than additive (highly red) or predominantly less-than-additive (highly blue) activity across most of the NCI-60 cell lines (Fig. 2A and B). In addition, except for leukemia cell lines, sensitivity to drug combinations appeared to be independent of cellular histology, as cell lines from the same source tissue did not cluster together.

Examination of promising anticancer drug combinations from the NCI-ALMANAC in human tumor xenografts

Because it was not feasible to examine all drug combinations in follow-up animal model experiments, a subset of combinations with greater than additive activity was selected for in vivo analysis based on the ComboScore, the ability of relevant cell lines to grow as xenograft implants, and clinical investigator input regarding clinical utility. An initial study was performed using doses at or near the MTD of each single agent to eliminate those combinations with excessive toxicity (requiring dose reduction >50%). For the remaining combinations, the more clinically pertinent criterion of “greater-than-single-agent” activity was applied to the follow-up xenograft analyses to identify combinations that could potentially confer additional efficacy in patients. We also differentiated between “novel” combinations (those that had not been previously tested together exclusively) and “non-novel” combinations (those tested together exclusively in a clinical trial arm), according to clinicaltrials.gov and the literature.

Various methods have been used to assess drug efficacy in xenograft studies, although to our knowledge, no method has been validated for comparing results of a high-throughput xenograft screen. For our purposes, we chose Kaplan–Meier and log-rank analyses of time-to-tumor-volume-doubling. Out of 13 “non-novel” combinations that were selected for xenograft analysis based on high in vitro ComboScores (Supplementary Table S2), 5 yielded greater-than-single-agent efficacy in at least one of the xenograft models tested. Furthermore, each of these 5 combinations has demonstrated appreciable efficacy (partial and/or complete responses) in clinical trials (Supplementary Table S3). Despite the small number of combinations examined, this analysis suggested that the NCI-ALMANAC results may be used as a starting point to select novel combinations for further study and potential clinical use.

Given these promising results, 20 novel combinations were evaluated for greater-than-single-agent efficacy in one or more xenograft models derived from NCI-60 cell lines, yielding a total of 44 unique “combination + model” experiments (in several
cases, multiple schedules and/or doses were examined for each "combination + model" set; in these cases, only the best outcome was scored. The analysis revealed that 14 of the 44 experiments yielded significantly improved antitumor efficacy for the combination compared with both single-agents (P < 0.05; Fig. 3; Supplementary Table S4). In addition, a panel of xenograft experts designated experiments for which the tumor regression appeared superior for the combination compared with the respective single agents, despite lack of statistical significance in log-rank analysis of time-to-tumor-doubling; 7 such experiments were identified (Fig. 3; Supplementary Table S4). For the remaining experiments, the combination did not yield significantly improved efficacy compared with either single agent (Supplementary Table S4; Fig. 3); in 2 experiments, this was due to substantial single-agent efficacy (i.e., ≤ 20% of mice treated with the single agent experienced a tumor doubling). Following this analysis, two of the combinations that exhibited significantly improved antitumor activity compared to their respective single agents were selected for further efficacy and mechanistic analysis.

**Clofarabine and bortezomib**

The purine nucleoside analogue clofarabine and the proteasome inhibitor bortezomib play important roles in the treatment of hematopoietic malignancies; however, neither agent had previously been demonstrated to exhibit appreciable activity against human solid tumors (23, 24). On the basis of greater than additive activity *in vitro* (Fig. 4A), we tested this combination in five solid tumor xenograft models and determined that the combination caused significant tumor regressions in two colon cancer models, HCT-116 (P < 0.01) and HT29 (P < 0.001), and in the non–small cell lung cancer model NCI-H522 (P < 0.01; Fig. 4B; Supplementary Table S4). In HCT-116 xenografts treated with bortezomib (0.75 mg/kg), clofarabine (60 mg/kg), or both agents in combination on an every two day (Q2D) dosing schedule for two treatment cycles, the combination had significantly greater efficacy than either single agent (P < 0.01) as determined by log-rank analysis of the time-to-tumor-doubling (Supplementary Table S4); in a second experiment, a single treatment cycle at these doses yielded slightly greater-than-single-agent efficacy (P < 0.1; Fig. 4B; Supplementary Table S4). This combination was well tolerated, with no observed median body weight loss > 30% (data not shown). In contrast, although the clofarabine and bortezomib combination had greater than additive efficacy in the ovarian cancer cell line OVCAR-3 and the melanoma line M14 *in vitro* (Fig. 4A), neither single-agent nor combination treatment elicited antitumor effects in the corresponding xenograft models (Fig. 4C; Supplementary Table S4).

Because both bortezomib and clofarabine have been demonstrated to induce apoptosis in a variety of human tumor cell lines (25, 26), we examined whether the activity of the combination in...
the treatment-responsive HCT-116 xenograft models might be explained by an enhanced modulation of apoptotic cascade proteins. To this end, tumor samples from one responsive (HCT-116) and one nonresponsive (M14) model were collected at various time points after treatment with clofarabine, bortezomib, or the combination, and tumor extracts were surveyed for changes in the apoptotic pathway using a 15-biomarker apoptosis multiplex panel (ref. 19 and Supplementary Methods). On the basis of results from a pilot time-course study (data not shown), we selected time points at 6 and 24 hours postdose-5 for subsequent experiments.

In the responsive HCT-116 model, bortezomib alone had virtually no effect compared with vehicle on apoptosis markers at 6 hours, while clofarabine alone significantly altered 3 of the 5 examined apoptosis markers (cleaved caspase-3 and mitochondrial/nuclear lamin-B and survivin) relative to vehicle (Fig. 5A). In contrast, compared with both vehicle and bortezomib alone, the bortezomib–clofarabine combination significantly altered levels of all 5 of the measured apoptosis markers: cytosolic survivin, cleaved caspase-3, and mitochondrial/nuclear lamin-B, BAX, and survivin (Fig. 5A). These trends persisted at the 24-hour posttreatment time point (Supplementary Fig. S2A). In addition, Western blot analysis showed that treatment of HCT-116 xenograft models with clofarabine alone or with the combination yielded high levels of p53 upregulation/stabilization relative to vehicle control (Supplementary Fig. S2B). These results in the sensitive HCT-116 model contrasted with those observed for the nonresponsive M14 model, in which bortezomib–clofarabine combination treatment did not produce this apoptosis-associated biomarker signature (Fig. 5B; Supplementary Fig. S2C). Overall,
in the sensitive HCT-116 xenograft model, the intrinsic apoptotic pathway was activated primarily by clofarabine, and the addition of bortezomib enhanced this activation.

Clofarabine is known to induce DNA damage response signaling (27); therefore, we evaluated the effects of these treatments on markers of DNA damage by immunofluorescence analysis of tumor segments using a validated assay (20, 21). The combination yielded a significantly greater induction of γH2AX compared with both bortezomib alone and clofarabine alone at the 6-hour time point (Fig. 5C and F), the latter indicating a role for bortezomib in potentiating clofarabine-induced DNA damage. Such substantial changes in γH2AX levels were not observed in the nonresponsive M14 model (Fig. 5C, right). The DNA damage marker pNbs1 was significantly increased in the clofarabine- and combination-treated groups relative to the vehicle-treated group as early as 2 hours (data not shown), at 6 hours, and at 24 hours in HCT-116, but not M14, models (Fig. 5D and F). Finally, the mitotic marker histone H3–pSer10 (pHH3) was lower in both the clofarabine-only and combination groups relative to vehicle at 6 and 24 hours in the HCT-116 model (Fig. 5E and F), but was not consistently lower in M14 xenografts (Fig. 5E), suggesting that sustained cell-cycle arrest was specific to the responsive model. As expected, treatment with bortezomib alone did not induce markers of DNA damage or cell-cycle arrest (Fig. 5C–F). Together, these data provide mechanistic evidence that DNA damage, cell-cycle arrest, and apoptosis contribute to the efficacy of the bortezomib-clofarabine combination in the responsive HCT-116 xenograft model.

Paclitaxel and nilotinib

The combination of the microtubule-stabilizing agent paclitaxel and the BCR-ABL kinase inhibitor nilotinib also had not been explored clinically. All 6 hematopoietic tumor cell lines tested had positive ComboScores for the paclitaxel–nilotinib combination, and this drug combination gave positive ComboScores in three of the triple-negative breast cancer NCI-60 cell lines (Hs578T, MDA-MB-468, and BT-549), but negative scores in the two estrogen receptor–positive breast cancer lines (MCF7 and T-47D; Fig. 6A). On the basis of these data, the effects of the nilotinib–paclitaxel combination on breast cancer xenografts derived from triple-negative cell lines were examined. Nilotinib–paclitaxel was well tolerated and highly efficacious in the MDA-MB-468 xenograft model; the combination (at either of the examined paclitaxel doses) was superior to both single agents (Fig. 6B; Supplementary Table S4). Strikingly, the combination treatment resulted in complete tumor regression, with no tumor regrowth observed for over 80 days following the end of therapy (day 134; Fig. 6B).

Single-agent nilotinib had no significant effects on tumor growth compared with vehicle, and single-agent paclitaxel yielded only partial tumor control, with tumor regrowth commencing around day 65–75 (Fig. 6B). Hs578T and BT-549 grew poorly as xenografts and were therefore not assessable in vivo, so we instead examined two additional triple-negative models derived from non–NCI-60 cell lines, SUM-52 PE and SUM-149PT. In both of these models, the nilotinib–paclitaxel combination yielded greater-than-single-agent efficacy (Supplementary Table S4). An additional triple-negative NCI-60 cell line, MDA-MB-231, did not yield valid in vivo screen results for the nilotinib–paclitaxel combination due to a "reversal" profile (see Methods) and also did not yield greater-than-single-agent efficacy when tested as a xenograft model (Supplementary Table S4). Beyond breast cancer models, the nilotinib–paclitaxel combination also had greater-than-single-agent efficacy in the RXF 393 renal carcinoma model, in line with its greater than additive activity in the corresponding cell line in vitro (Supplementary Table S4). In contrast, the U251 cell line, where the combination was less efficacious in vitro (ComboScore of 21), yielded no greater-than-single-agent efficacy when tested as a xenograft model (Supplementary Table S4).

To identify potential biomarkers of response to this combination, we first queried the NCI-60 molecular markers database to look for associations between in vitro ComboScore and drug-related molecular signatures. We found no significant correlations between nilotinib–paclitaxel ComboScores and either (i) protein and RNA expression levels for the non-BCR-ABL kinases known to be inhibited by nilotinib (28), or (ii) activity and RNA expression levels of the ABC family efflux pumps that have been reported to
Figure 4.
Therapeutic activity of the NCI-ALMANAC–derived combination of bortezomib and clofarabine. A, The combination of bortezomib and clofarabine in vitro yielded positive ComboScores across several cell types. Tumor tissue derivations for the NCI-60 cell lines are indicated by bar color: red, leukemia; green, colon; blue, non-small cell lung; gray, CNS; orange, melanoma; purple, ovarian; yellow, renal; turquoise, prostate; and pink, breast. A leukemia cell line for which no ComboScore was available for this combination is indicated by gray text. Models for which xenograft experiments revealed the presence or absence of in vivo greater-than-single-agent activity are indicated by filled triangles or empty triangles, respectively. B and C, Clofarabine–bortezomib combination treatment exhibits enhanced efficacy relative to the respective single-agent treatments in the human colon cancer HCT-116 xenograft model (B) but not in the M14 melanoma xenograft model (C). Median tumor volumes are shown for mice treated with vehicle, single-agent bortezomib [5 × every two days (Q2D), intraperitoneal injection], single-agent clofarabine [5 × every two days (Q2D), oral administration], or the combination (schedule and administration for each agent as indicated for the respective single-agent treatments); treatment commenced on day 6 for the HCT-116 experiment (B) and on day 17 for the M14 experiment (C; dosing period indicated by gray-shaded area in both graphs). D, Kaplan–Meier curves for time-to-tumor doubling analysis of HCT-116 xenograft models treated with the clofarabine–bortezomib combination. The y-axis indicates probability of event-free survival, where an event is defined as one tumor doubling or drug-related death (the latter occurred for only one animal in the 60 mg/kg clofarabine + 0.75 mg/kg bortezomib–treated group). #, the combination–treated group exhibiting greater-than-single-agent time-to-tumor-doubling compared with both bortezomib-alone and clofarabine-alone groups at the corresponding doses (P < 0.1), as determined by log-rank tests. Doses (mg/kg) for each treatment group are indicated in the legend (n = 16 mice for the vehicle group; n = 8 mice for all other groups). Error bars, SE of the median.
Figure 5.
Bortezomib–clofarabine combination treatment modulates markers of apoptosis and DNA damage in responsive, but not unresponsive, xenograft models. Mice bearing HCT-116 or M14 xenografts were treated with either vehicle, 0.75 mg/kg bortezomib [5 × every two days (Q2D), intraperitoneally], 60 mg/kg clofarabine [5 × every two days (Q2D), oral administration], or the combination of bortezomib and clofarabine with the same dosage regimen. Tissues were harvested at 6 and 24 hours postdose and processed into cell lysates (for apoptosis marker assays, A and B) or were formalin-fixed and paraffin-embedded for quantitative immunofluorescence analysis of DNA damage response and cell-cycle markers (C–F). Apoptosis markers were measured in the therapeutically responsive HCT-116 (A) or nonresponsive M14 (B) xenograft models at 6 hours following the day 5 dose. Error bars, SEM; n = 6 mice per treatment group. Statistically significant differences between the combination and single-agent treatment groups are indicated in red, while those compared with vehicle are indicated in black (*, P < 0.05; **, P < 0.01; P values derived from unpaired, nonparametric Mann-Whitney tests). One outlier data point for caspase-3 in the clofarabine HCT-116 group was removed. Effects of drug treatment on nuclear levels of γH2AX (C), pNbs1 (D), or pH3-Ser10 (E) were examined in HCT-116 (left) and M14 (right) models at 6 and 24 hours following the day 5 dose. Representative images from the HCT-116 6-hour group are shown (F). Error bars (C–E), SD (n = 6 mice per treatment group, except for the HCT-116 bortezomib 24-hour group [n = 5 due to unsuitable tissue quality and the M14 bortezomib 6-hour group (n = 4)], and an average of 5,000 cells per mouse tumor were analyzed. Statistically significant differences between the combination and single-agent treatment groups are indicated (*, P < 0.05; **, P < 0.01; the sole statistically significant greater-than-single-agent difference between the HCT-116 clofarabine group and the combination group is bolded for emphasis (C). In the HCT-116 experiments (C–E, left), biomarker levels for both the clofarabine group and combination group were significantly different than vehicle at 6- and 24-hour time points (P < 0.01 vs. vehicle for all; P values derived from nonparametric Mann-Whitney tests).
Figure 6.
In vitro activity, in vivo efficacy, and mechanistic analysis of the NCI-ALMANAC–derived nilotinib–paclitaxel combination. A, The combination of nilotinib and paclitaxel in vitro yielded positive ComboScores across several cell types. Tumor tissue derivations for the NCI-60 cell lines are indicated by bar color: red, leukemia; green, colon; blue, non–small cell lung; gray, CNS; orange, melanoma; purple, ovarian; yellow, renal; turquoise, prostate; and pink, breast. Cell lines for which no ComboScore data are available for this combination are indicated by gray text. Models for which xenograft experiments revealed the presence or absence of in vivo greater-than-single agent activity are indicated by filled triangles or empty triangles, respectively. (Continued on the following page.)
be inhibited by nilotinib (29–31) (Supplementary Fig. S3A). To further examine the possibility of nilotinib-mediated reduction in paclitaxel efflux via ABC transporter inhibition, we analyzed tumor paclitaxel levels in MDA-MB-468 xenograft models and found comparable tumor paclitaxel concentrations in animals treated with the nilotinib–paclitaxel combination and those treated with paclitaxel alone (Supplementary Fig. S3B), providing further evidence that the mechanism of action for this combination does not involve modulation of paclitaxel efflux.

Because apoptosis, among other forms of cell death, has been postulated to play a role in tumor cell killing by paclitaxel and/or nilotinib (32, 33), we examined biomarkers of apoptosis in response to single-agent and combination treatment in the MDA-MB-468 breast cancer xenograft model. No increases in the apoptosis marker cleaved caspase-3 were observed in either single-agent or combination treatment groups compared with the vehicle control (Fig. 6D), supporting a nonapoptotic mechanism of antitumor activity for this drug pair. Corroborating this finding, levels of mitochondrial Mcl-1, associated with antiapoptotic activity (34), were higher in the combination and single-agent treatment groups compared with vehicle (Fig. 6D). Although total levels of the canonically antiapoptotic BAX–Bcl-2 complex were higher following nilotinib–paclitaxel combination treatment relative to the vehicle and single-agent treatments (Fig. 6D), subcellular fractionation experiments revealed that this was primarily due to an increase in ER-associated BAX–Bcl-2 (data not shown), which is involved in the perturbation of calcium homeostasis that occurs in both apoptosis and necroptosis (35). Together, these results suggest that the nilotinib–paclitaxel combination likely suppresses tumor growth through a caspase-3-independent mechanism of cell death.

Discussion

More than 100 small-molecule drugs have been approved for cancer therapy by the FDA (36), and are often used in combination with other agents to address the multiple molecular pathway aberrations, and consequent drug resistance, associated with many malignancies. However, a broad range of potential FDA-approved drug combinations has never been evaluated clinically. We determined that it would be valuable to systematically screen all possible two-drug combinations in vitro to identify novel pairs of FDA-approved drugs with greater in vitro growth inhibition than would be expected from the sum of their single-agent activities. A number of combinations identified in the NCI-ALMANAC screen were further evaluated in animal models for greater-than-single-agent efficacy. Although combination experiments have been conducted before (16, 37–39), to our knowledge, the NCI-ALMANAC is the most extensive anticancer drug combination screening effort for which the data and search tools are publicly available.

Our comprehensive in vitro screen identified 1,898 drug pairs with positive ComboScores in at least 1 cell line. Consequently, we performed in vivo testing of a small subset of drug pairs that have not, to our knowledge, been clinically tested (Supplementary Table S4). Agents were tested at or near their MTD to assess whether combination therapy could achieve efficacy superior to that of the single agents. Forty-eight percent of these novel, NCI-ALMANAC–derived combinations exhibited greater-than-single-agent activity in at least one model (Fig. 3), suggesting that the NCI-ALMANAC may be helpful in identifying additional combinations with clinical efficacy in the future.

The bortezomib–clofarabine combination conferred an antitumor activity advantage over single-agent clofarabine or bortezomib in the HCT-116 xenograft model (Fig. 4B). In view of the known induction of apoptosis in response to single-agent bortezomib (40) or clofarabine (27), we investigated the effects of this combination on apoptotic pathway markers. We found that the antitumor activity of the clofarabine–bortezomib combination was accompanied by several significant apoptosis-associated molecular changes relative to the single-agent- and/or vehicle-treated arms, including decreases in cytosolic and mitochondrial/nuclear survivin and mitochondrial/nuclear-associated lamin-B, as well as increases in caspase-3 activation and mitochondrial/nuclear-associated BAX (Fig. 5A). In contrast, for the nonresistant M14 xenograft model, in which no antitumor efficacy advantage was observed for the combination, we observed no consistent alterations in levels of these apoptotic biomarkers, suggesting that the apoptotic pathway activation observed in the responsive HCT-116 model is associated with the antitumor efficacy of the clofarabine–bortezomib combination. The mechanism whereby clofarabine–bortezomib treatment reduces survivin levels in HCT-116 xenografts could be either p53-dependent (41), as indicated by our Western blot analysis (Supplementary Fig. S1E), or p53-independent (42). Regardless, the greater-than-single-agent activity of this combination has some precedent; previous in vitro experiments have demonstrated that bortezomib-induced cytotoxicity and proapoptotic signaling are enhanced by purine nucleoside analogues (43).

Because clofarabine is both a DNA-damaging agent and a DNA synthesis inhibitor (27), we evaluated the role of DNA damage in...
the activity of the bortezomib-clofarabine combination. The combination and clofarabine alone both significantly increased levels of the DNA damage response markers γH2AX and pNB1 and decreased levels of the cell proliferation marker pHH3 in HCT-116 xenografts compared with vehicle and single-agent bortezomib treatment (Fig. 5C–F); γH2AX levels were also significantly higher in the combination arm relative to the clofarabine-only arm at the 6-hour time point (Fig. 5C). Hence, the ability of clofarabine to induce DNA damage (which could then trigger p53-mediated apoptosis) and/or directly alter apoptotic pathway components (27) might lower the apoptosis threshold in HCT-116 xenografts, rendering these tumors more susceptible to the cytotoxic effects of bortezomib-induced proteasome inhibition.

Although the pharmacodynamic effects of clofarabine and bortezomib converge on apoptosis, potential upstream interactions remain to be explored; for example, it is possible that bortezomib-mediated proteasome inhibition stabilizes a protein required for the uptake, recycling/stability, mechanism of action, or activating phosphorylation of clofarabine. A whole-proteome analysis in Jurkat cells revealed that bortezomib treatment yielded a reproducible 12- to 13-fold upregulation of nucleoside diphosphor activating phosphorylation of clofarabine. A whole-proteome required for the uptake, recycling/stability, mechanism of action, bortezomib converge on apoptosis, potential upstream interactions might lower the apoptosis threshold in HCT-116 xenografts, suggesting a potential role for nilotinib in shifting the apoptotic–necroptotic balance in paclitaxel-induced cell death. Regardless, the impressive in vitro activity and in vivo efficacy of this combination have motivated phase 1 evaluation of this drug pair (clinicaltrials.gov identifier: NCT02379416).

Despite the limitations inherent in the translation of in vitro activity into in vivo efficacy (due in part to the size of the screen, which precluded comprehensive investigation of the effect of different drug administration doses and schedules), and the fact that large-scale animal studies could not easily be conducted to validate all of our screening results, the NCI-ALMANAC generated interesting hypotheses that were further addressed with mechanistic biomarker profiling and xenograft efficacy studies. To explore drug combinations of interest identified in the NCI-ALMANAC screen, we enriched our xenograft testing for pairs that had not been studied previously in a clinical trial (as defined by searching clinicaltrials.gov). Because only FDA-approved small molecules were used in the NCI-ALMANAC screen, our results have the potential for rapid clinical translation. Thus, the NCI-ALMANAC should prove to be a valuable resource that will inform the development and selection of novel combinations of FDA-approved anticancer agents for future clinical trials.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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