Quantification of Pathway Cross-talk Reveals Novel Synergistic Drug Combinations for Breast Cancer

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Abstract

Combinatorial therapeutic approaches are an imperative to improve cancer treatment, because it is critical to impede compensatory signaling mechanisms that can engender drug resistance to individual targeted drugs. Currently approved drug combinations result largely from empirical clinical experience and cover only a small fraction of a vast therapeutic space. Here we present a computational network biology approach, based on pathway cross-talk inhibition, to discover new synergistic drug combinations for breast cancer treatment. In silico analysis identified 390 novel anticancer drug pairs belonging to 10 drug classes that are likely to diminish pathway cross-talk and display synergistic antitumor effects. Ten novel drug combinations were validated experimentally, and seven of these exhibited synergy in human breast cancer cell lines. In particular, we found that one novel combination, pairing the estrogen response modifier raloxifene with the c-Met/VEGFR2 kinase inhibitor caboazatinib, dramatically potentiated the drugs’ individual antitumor effects in a mouse model of breast cancer. When compared with high-throughput combinatorial studies without computational prioritization, our approach offers a significant advance capable of uncovering broad-spectrum utility across many cancer types. Cancer Res; 77(2); 459–69. ©2016 AACR.

Introduction

Breast cancer is a very heterogeneous disease regarding the underlying molecular alterations, the cellular composition of tumors, and the different clinical outcomes (1), which hampers the design of effective treatment strategies (2). To account for this intrinsic diversity, drug discovery efforts have shifted toward mechanism-based and target-oriented strategies, particularly aiming at modulating specific molecular pathways, patient-specific genetic alterations, and the tumor microenvironment (3, 4).

Despite the expanding repertoire of new anticancer agents, treatment failure remains a major challenge in the management of most advanced solid cancers, including breast cancer (5, 6). Multiple compensatory mechanisms are known to counterbalance therapeutic efforts, eventually leading to treatment failure (6). One of the most promising strategies for better clinical outcomes is the use of combinatorial therapy to target the distinct adaptive response mechanisms (7, 8), which may also help to overcome toxicity associated with higher doses of single drugs. In addition, synergistic drug combinations are often more specific and therefore improve the therapeutically relevant selectivity (9). However, although becoming the standard care in breast cancer treatment, most approved drug combinations are the result of empirical clinical experience, and often rely on similar mechanisms of action as preexisting drug combinations, which prevented a systematic sampling of the therapeutic space (Supplementary Fig. S1).

Identifying drug combinations with therapeutic effect remains a challenging task given the exhaustive number of possibilities. Different approaches are available for predicting drug combinations for complex diseases, mostly including mathematical modeling, stochastic search techniques, as well as cell context-based methods like global gene expression or targeted phosphoproteomics profiling (10–13). More recently, network-based models have been proposed for identifying drug synergies and for examining the mechanisms of action of efficient combinations (14–16).

Experimental studies have shown that cancer cells are able to adapt signaling pathway circuits upon drug treatment by establishing alternative signaling routes through cross-talk (17, 18). Hence, a critical aspect to improve cancer treatment is not only to inhibit the primary oncogenic pathways that induce abnormal cell proliferation but, simultaneously, to prevent functional redundancies and pathway cross-talk that facilitate survival of cancer cell populations rendering tumors resistant to therapy. Current network pharmacology principles aim for a synergistic multitarget intervention strategy to improve clinical efficacies, while tackling critical aspects such as drug resistance (19, 20). In line with this idea, we have derived a network-based computational method to quantify the cross-talk between signaling pathways involved in breast cancer and we assessed how combinatorial perturbations impact the signaling cross-talk. We then applied...
this measure to a set of approved and experimental breast cancer drugs to identify combinations, which could efficiently diminish pathway cross-talk and thereby increase clinical efficacy. Finally, we experimentally validated novel drug combinations in several human breast cancer cell lines and confirmed the in vivo synergistic effect between two drugs in mouse xenografts, emphasizing the potential clinical relevance of our strategy.

Materials and Methods

Breast cancer drugs

We compiled a comprehensive set of drugs that are either prescribed or in clinical trials for breast cancer treatment. Primary information on FDA-approved breast cancer drugs has been gathered from the National Cancer Institute (NCI). This data have been complemented with information from DrugBank 3.0 (21) and the therapeutic targets database (TTD; ref. 22). Overall, we collected 64 breast cancer agents, of which 32 are approved. The number of experimental compounds used for breast cancer treatment is most likely higher; yet, the data are scattered across the literature, not accessible in an automatic manner, and often there is no available information on the modulated therapeutic target(s).

Each drug has been associated with its therapeutic target(s). For drugs from DrugBank, we considered only primary targets. Only if none of the targets have a known pharmacologic action we consider all targets for that drug. Targets from TTD were treated as primary therapeutic targets. We further extracted pathways and biological processes that are most likely modulated by a drug through its target(s). Pathway information was retrieved from the KEGG database (23).

Depending on the mechanism of action, we divided drugs into cytotoxic and targeted agents. Furthermore, we classified the set of breast cancer drugs according to their therapeutic target(s) into 11 drug classes. The complete set of breast cancer drugs considered can be found in Supplementary Table S1.

Breast cancer drug combinations of clinical relevance

We extracted drug combinations considered for breast cancer therapy by mining the drug combination database (DCDB; ref. 24), the FDA orange book (25), the NCI, and the Clinical-Trials.gov (26). In total, 170 drug combinations were obtained. Some of them are already approved while the majority is currently in clinical trials. Supplementary Figure S1 provides an overview of the current drug combinations with respect to the 11 drug classes.

Therapeutic signaling networks and pathway cross-talk inhibition

To chart the therapeutic networks associated with each drug, we compiled all those KEGG pathways (excluding disease pathways) that include any of the primary targets of the drug. On average each drug affects 6.5 KEGG pathways (median = 3, SD ± 8.3). Given the XML representation of a pathway, we created a directed network including proteins and their interactions, whereas the type of an interaction, such as activation or inhibition, was used to determine the directionality of an edge in the network.

On the basis of the therapeutic signaling networks, we developed a cross-talk inhibition measure to estimate the amount of cross-talk signaling that can be prevented between pathways by inhibiting specific proteins simultaneously. The concept of pathway cross-talk refers to protein interactions shared between distinct pathways. Because these interactions might also influence the downstream signaling within a pathway, the concept also comprises proteins and interactions downstream of the respective cross-talking interactions (i.e., indirect cross-talk; Fig. 1).

Figure 1.

Pathway cross-talk inhibition. A, Cross-talk identified between pathways A and B, defined as shared protein interactions, or those occurring downstream of them, in the individual pathways. The flow of information within the cross-talk network, that is, the network efficiency, is 0.29. B, Drug 1 inhibits signaling through pathway A but does not affect cross-talk inhibition. In consequence, proliferation is not effectively inhibited. C, Drug 2 reduces signaling through the cross-talk network to 0.05, resulting in a PCI of 0.83. D, Cross-talk signaling is completely prevented by using D1 and D2 in combination.
Given two therapeutic signaling networks, we determined the potential cross-talk between them by identifying interactions directly and indirectly involved in the cross-talk, and representing them as a directed cross-talk network. Using this cross-talk representation, we then applied a topology-based measure, namely network efficiency (NE) to determine the information flow within the network. Network efficiency (NE) is defined as the sum of the inverse length of the shortest path between all network elements and can be computed as follows:

\[
NE = \frac{1}{N(N - 1)} \sum_{i \neq j} d(i, j),
\]

with \( N \) representing the number of network elements and \( d \) denoting the shortest distance between two elements \( i, j \in N \). The network efficiency ranges between 0 and 1, where 1 indicates that all proteins communicate directly with each other, that is, a fully connected network.

Using the network efficiency determined for cross-talking pathways, as described above, we simulated the inhibition of specific protein target(s) and measured the amount of signaling that persists (NE\(_X\)) when removing protein interactions affected by a pharmacologic intervention. We determined the relative reduction of network efficiency, that is, the pathway cross-talk inhibition (PCI), as follows:

\[
PCI = 1 - \frac{NE_X}{NE}.
\]

The final PCI for a given pair of breast cancer drugs is the average of the cross-talk inhibitions between each pair of cross-talking pathways forming the respective therapeutic networks.

**Experimental validation of drug combinations**

Drug combination experiments were conducted in five cell lines, four of them, namely MCF-7, MDA-MB-231, SKBR3, and BT474, representing distinct breast cancer subtypes. In addition, we also included U2OS, a bone osteosarcoma cell line, representing a widely used cancer cell line. When analyzing a combination, we tested the activity of the individual drugs \( D_1 \) and \( D_2 \), and the combination at four concentrations, selected from the literature to cover their activity range (Supplementary Table S2). To assess the cytostatic/cytotoxic effects of a single drug or a drug combination at four concentrations, selected from the literature to cover their activity range (Supplementary Table S2). Cell survival was determined using an MTT-based assay. All drug combinations for 72 or 120 hours (Supplementary Table S2).

Further details on cell lines, culture conditions, and drugs can be found in the Supplementary Section S1.

**Drug combination index**

Given the MTT cell viability measurements, we assessed whether a drug combination induces additive, synergistic, or antagonistic effects in cultured cells. To this end, we determined the drug combination index (DCI) using the Loewe additivity as a reference model, assuming that a drug cannot interact with itself (29, 30). This means that if two drugs are the same or very similar, we expect their combined effect at equal concentrations to be comparable to the one observed when administering one drug alone at double concentration.

The DCI of a combination is computed on the basis of the half-maximal effective concentration that is needed to inhibit cell viability by \( X\% \), with \( X \) commonly corresponding to an inhibition level of 50% (i.e., IC\(_{50}\)). Formally, the DCI\(_X\) is defined as follows:

\[
DCI_X = \frac{C_{D1,X}}{IC_{X,D1}} + \frac{C_{D2,X}}{IC_{X,D2}},
\]

where \( C_{D1,X} \) and \( C_{D2,X} \) represent the concentration of drug \( D_1 \) and drug \( D_2 \) used in combination to induce an effect \( X \) while IC\(_{X,D1}\) and IC\(_{X,D2}\) indicate the corresponding concentrations of the single agents required to produce the same effect. In other words, the DCI measures the fractional shift between single and combinatorial concentrations yielding an inhibition of cell survival of \( X\% \). The concept of the DCI is exemplified for one combination in Supplementary Fig. S2. Using this measure, we can quantify synergistic, additive, and antagonistic combinatorial effects, commonly defined as DCI < 0.85, DCI ~1, and DCI > 1.2, respectively (Supplementary Table S3).

IC\(_X\) values can be determined from dose–response curves for any inhibition level \( X \). Here, we used the drc R package to generate sigmoid-fitted dose–response curves, from which we then estimated the IC\(_X\) for single drugs and combinations (31). In some cases, the single agents do not reach the predefined inhibition level, whereas in others the estimated IC\(_X\) corresponds to a value beyond the tested concentration range. In the latter one, we exploited the relative standard error (RSE) associated with each fit to decide whether to consider an IC\(_X\) (16). The influence of using different RSE thresholds for determining the DCI is discussed in Supplementary Section S2 and Supplementary Table S4. No DCI is reported for cases where neither the single nor the combined inhibition induces the desired effect.

**Dose reduction index**

A major aim of synergistic drug combinations is to reduce the dose of a drug, thereby reducing toxicity while maintaining therapeutic efficacy. The dose reduction index (DRI) measures to which extent the concentration of a drug in combination can be reduced at a given inhibition level \( X \) compared with the concentration of an individual drug alone.

\[
DRI_{X,D1} = \frac{IC_{X,D1}}{IC_{X,D1}} \quad DRI_{X,D2} = \frac{IC_{X,D2}}{IC_{X,D2}}
\]

In general, a DRI above 1 is considered to be beneficial. Furthermore, larger DRIs correlate with a larger magnitude of dose reduction for a given therapeutic effect.

**Mouse xenograft model**

MCF-7 human breast cancer cells were prepared in a 1:1 PBS-Matrigel (BD Biosciences) mixture and \( 1 \times 10^6 \) cells were injected directly into the mammary gland. When tumors reached a volume of 120 to 150 mm\(^3\), mice were randomly assigned to different groups and treated for 15 days with cabozantinib (oral gavage, 2 mg/kg), raloxifene (i.p. 6 mg/kg), the combination of both...
(1 mg/kg of cabozantinib and 3 mg/kg of raloxifene), or the corresponding vehicles. At day 15, mice were sacrificed and tumors were formalin-fixed and paraffin-embedded. Sections were stained with hematoxylin and eosin (H&E), Ki67 (Novocastra), and the “In situ Cell Death Detection Kit, Fluorescein” (Roche), following manufacturer’s instructions. Western blot analysis was used to measure the activity of selected proteins in tumor samples of the different groups. Further details can be found in the Supplementary Section S3. For determining the statistical difference between the treatment groups we used the one-sided t test.

Results

PCI as a tool for inferring synergistic drug combinations

Alternative signaling through pathway cross-talk is one of the main mechanisms leading to treatment failure (18). Therefore, we devised a computational strategy to infer drug combinations that specifically addresses this problem. Our approach is based on the quantification of the level of cross-talk between signaling pathways that can be prevented by simultaneously inhibiting specific sets of proteins. The concept of pathway cross-talk refers to shared protein interactions between distinct signaling cascades, and the interactions downstream of the ones that crosstalk (Fig. 1). To determine pathway cross-talk and inhibition, we first built the therapeutic networks associated with each individual drug by considering its set of known primary targets mapped onto well-annotated canonical pathways (23). We found that, on average, each drug can be associated with 6.5 signaling pathways, of which are among the 170 considered clinically relevant.

In general, we find that pathway pairs involved in clinically relevant combinations share a significantly higher portion of proteins and exhibit a higher cross-talk compared with a background of all human KEGG pathways (P < 2.2e–16; Supplementary Section S4 and Supplementary Fig. S3). When specifically assessing the potential PCI achieved by drug combinations, we observed that those in clinical use have a significantly higher impact on PCI than randomly combined pairs of drugs (Fig. 2A), with an average PCI of 0.34 compared with 0.25 (P = 8.96 × 10⁻⁴). Indeed, for the few clinical drug combinations where efficacy data are available, we found that those exceeding the average PCI of 0.34 are more likely to show clinical efficacy (P = 0.03215; Supplementary Section S5 and Fig. 2A and Supplementary Fig. S4). This implies that before and after inhibiting individual or combined drug targets. Drug combinations with a high impact on PCI are expected to present promising drug combinations.

To examine the clinical relevance of the PCI for inferring novel drug combinations, we assessed its applicability on approved or tested breast cancer drug combinations. To this end, we generated all pairwise combinations from the 64 available breast cancer agents (Supplementary Table S1), examining which of them are currently used for therapy or in clinical trials. Of the potential 2,016 combinations, 170 are documented as tested by the ClinicalTrials.gov (26), the FDA orange book, the NCI (http://www.cancer.gov/cancertopics/druginfo/breastcancer), or the DCDB (24). We considered these as clinically relevant combinations for breast cancer. The remaining ones constituted a set of nontested combinations. Given the two sets, we computed the PCI among the combined drugs. Because pathway cross-talk, as defined here, may only occur among related pathways sharing components, we only considered drug pairs whose pathways have at least a common protein, yielding a total of 1,132 combinations, 86 of which are among the 170 considered clinically relevant.

Figure 2.

In silico validation of the PCI index. A, Comparison of the PCI distributions for the populations of clinically relevant breast cancer drug combinations (i.e., currently in use or in clinical trials) and randomly combined breast cancer drugs (P = 8.96 × 10⁻⁴) as well as for combinations with and without confirmed clinical benefit (P = 0.03215). B, Evaluation of the PCI of 13 synergistic combinations with 55 nonsynergistic (P = 0.00341), 35 additive (P = 0.01667), and 20 antagonistic (P = 0.00069) drug pairs identified among 14 targeted compounds within a liposarcoma cell line (96). ** P < 0.01; *** P < 0.001; **** P < 0.0001.
cross-talk inhibition may be one of the molecular mechanisms exploited by a number of successful breast cancer drug combinations.

In addition, we assessed the PCI for 68 synergistic, additive, or antagonistic drug pairs identified in a combination screen performed in a tumor-derived liposarcoma cell line (DDLS817) considering 14 targeted compounds from distinct drug classes (16). This screen resulted in 14.3% synergistic, 38.5% additive, and 22% antagonistic combinations. Figure 2B demonstrates that synergistic combinations have a significantly higher PCI than nonsynergistic drug pairs and particularly antagonistic ones, with average values of 0.35 compared with 0.22 \((P = 0.00341)\) and 0.18 \((P = 0.00069)\), respectively. A significant correlation between PCI and drug combination index can be also observed as shown in Supplementary Fig. S5 (Pearson correlation coefficient = –0.439, \(P = 0.0001829\)). Furthermore, the average PCI of synergistic combinations is comparable to the one determined for clinically relevant combinations.

Overall, the two complementary evaluations support the value of our approach to identify effective drug combinations.

Identification of novel drug combinations

Among the randomly combined drugs, we found a large number of combinations showing a high impact on the cross-talk inhibition between breast cancer pathways (Fig. 2A and Supplementary Table S5). These drug pairs are promising candidates for combinatorial treatment because the pathway cross-talk identified is directly involved in breast cancer-related processes.

**Figure 3.** PCI between pairs of breast cancer therapeutics, both approved and experimental. Drug combinations in use are indicated with a black dot. Combinations selected for experimental validation are marked with a red star (see also Table 1). Drugs are colored according to their therapeutic classes. Drugs not involved in any pathway cross-talk or its inhibition were removed for better illustration. Note that drugs belonging to the same class might still target different sets of proteins, and thus the cross-talk inhibition achieved when combined with other drugs might vary.
To select the most relevant ones, we used as a threshold the mean PCI of 0.34 observed in the drug combination sets that are clinically used. This includes 62.8% of the clinically relevant combinations, exhibiting a higher likelihood of possessing clinical efficacy (Supplementary Fig. S4), but only 37.3% of the random set of combinations (Supplementary Fig. S6). Overall, 390 novel drug combinations showed a PCI > 0.34, including drugs from 10 different classes, which are therefore likely to exhibit synergistic effects. Furthermore, pathway pairs affected by the novel drug combinations showed a significantly higher protein overlap as well as a higher pathway cross-talk compared with all human KEGG pathways with a P < 2.2e−16 (Supplementary Section S4 and Supplementary Fig. S3). Not surprisingly, the majority of these combinations (370) occur between drugs belonging to different therapeutic subclasses, showing the ability of our method to prevent redundant mechanisms of action. Moreover, about 65% of the new combinations include therapeutic classes never tested together before, expanding the sampling of the potential therapeutic space. We believe that these drug pairs have the potential to increase treatment efficacy by inhibiting oncogenic pathways at distinct points, as well as by reducing the concentration needed for inducing a given effect, which consequently improves their therapeutic index. A full description of the suggested drug combinations, together with the therapeutic pathways involved in the cross-talk inhibition, is provided in Supplementary Table S5.

Experimental validation of selected drug combinations

The fundamental aim of any combinatorial strategy is its therapeutic application. Therefore, we selected a subset of drug combinations to experimentally assess their effects on the proliferation of tumor cells, a key process for tumorigenesis. During the selection process, we only considered truly novel drug combinations; hence omitting those which resemble approved or tested ones (Supplementary Fig. S1). Trastuzumab, for instance, is administered in combination with paclitaxel and tamoxifen (32, 33), thus we disregarded combinations including HER2 inhibitors together with microtubule or estrogen receptor modulators. We selected 10 combinations from the remaining pool according to their overall potential for cross-talk inhibition, involving 10 targeted drugs and one cytotoxic agent (Fig. 3 and Table 1). To maximize the sampling of the combinatorial therapeutic space, we only selected the highest PCI per representative drug class combination.

We then studied the effect of the selected drug combinations in five human cancer cell lines, four of them representing distinct breast cancer subtypes (Fig. 4A), namely triple-negative (MDA-MB-231), hormone receptor-positive (MCF-7), HER2-overexpressing (SKBR3), and triple-positive (BT-474) breast cancer, whereas the fifth was derived from osteosarcoma (U2OS). For the quantification of cytotoxic effects induced by individual drugs or drug combinations, we used the MTT assay, which measures cell proliferation and viability (Supplementary Section S1; ref. 28).

To determine if the tested combinations were of synergistic, additive, or antagonistic nature, we computed the Loewe additivity-based drug combination index (DCI<sub>50</sub>) for each combination in each cell line (30). The DCI<sub>50</sub> compares the half-maximal effective concentrations for inhibiting 50% of cell viability of single agents with the concentration derived for a combination. To avoid overestimating the number of synergistic combinations and, to some extent, account for cell line variability, we adopted a more stringent definition of synergy (Supplementary Table S3), considering a DCI<sub>50</sub> below 0.85 as synergistic. A DCI<sub>50</sub> above 1.2 indicates antagonism whereas any value in between depicts additivity (0.85 > DCI < 1.2; ref. 29).

Considering an inhibition level of 50%, we generated sigmoid-fitted dose–response curves based on the MTT assays, from which we then estimated IC<sub>50</sub> and DCI<sub>50</sub> values (Supplementary Fig. S2 for an example and Supplementary Fig. S7 for all dose–response curves). Figure 4B shows the DCI<sub>50</sub> derived for each combination in the five cell lines. Our results showed that seven out of ten combinations tested displayed a synergistic behavior in at least, one cancer cell line. Overall, we found that 32% of the combinations were synergistic, 38% were additive, and 16% exhibited antagonistic effects in human cancer cell lines. For another 14% we could not determine a reliable DCI. Interestingly, the fraction of synergistic drug combinations slightly increased up to 35% when analyzing only breast cancer cell lines. These numbers emphasize the significant enrichment of synergistic drug combinations compared with traditional experimental high-throughput screens (P < 1e−04; Supplementary Fig. S8A and S8B), which detect synergy in 4% to 14% of the drug pairs tested (16, 34, 35). A detailed description of the comparison can be found in the Supplementary Material (Section S6).

The per-combination perspective shows that the selected combinations are synergistic in a broader extent than anticipated a priori (Supplementary Fig. S9), given the heterogeneity of breast cancer (36, 37). For instance, DC07 is synergistic in four cancer cell lines, DC04 and DC09 in three, DC02 and DC10 in two, and DC05 and DC06 in one.

When analyzing drug combinations separately, we observed clear correlations between the degree of synergy and the

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**Table 1. List of drug combinations selected for experimental validation**

<table>
<thead>
<tr>
<th>Combination</th>
<th>Drug 1</th>
<th>Drug class</th>
<th>Drug 2</th>
<th>Drug class</th>
<th>PCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC01</td>
<td>Cabozantinib</td>
<td>VEGFR inhibitor</td>
<td>Erlotinib</td>
<td>EGFR inhibitor</td>
<td>0.60</td>
</tr>
<tr>
<td>DC02</td>
<td>Cabozantinib</td>
<td>VEGFR inhibitor</td>
<td>Raloxifene</td>
<td>SERM</td>
<td>0.50</td>
</tr>
<tr>
<td>DC03</td>
<td>Olaparib</td>
<td>PARP-1 inhibitor</td>
<td>Tanespimycin</td>
<td>HSP inhibitor</td>
<td>0.88</td>
</tr>
<tr>
<td>DC04</td>
<td>Olaparib</td>
<td>PARP-1 inhibitor</td>
<td>Dinaciclib</td>
<td>CDK inhibitor</td>
<td>1.0</td>
</tr>
<tr>
<td>DC05</td>
<td>Olaparib</td>
<td>PARP-1 inhibitor</td>
<td>PD-033291</td>
<td>CDK inhibitor</td>
<td>0.72</td>
</tr>
<tr>
<td>DC06</td>
<td>Cabozantinib</td>
<td>VEGFR inhibitor</td>
<td>PD-033291</td>
<td>CDK inhibitor</td>
<td>0.34</td>
</tr>
<tr>
<td>DC07</td>
<td>Paclitaxel</td>
<td>Microtubule modulator</td>
<td>Tanespimycin</td>
<td>HSP inhibitor</td>
<td>0.38</td>
</tr>
<tr>
<td>DC08</td>
<td>Paclitaxel</td>
<td>Microtubule modulator</td>
<td>Midostaurin</td>
<td>VEGFR inhibitor</td>
<td>0.44</td>
</tr>
<tr>
<td>DC09</td>
<td>Cabozantinib</td>
<td>VEGFR inhibitor</td>
<td>Trastuzumab</td>
<td>HER2 inhibitor</td>
<td>0.57</td>
</tr>
<tr>
<td>DC10</td>
<td>Figitumumab</td>
<td>IGF-1R inhibitor</td>
<td>Raloxifene</td>
<td>SERM</td>
<td>0.80</td>
</tr>
</tbody>
</table>

**Note:** For each combination, we specified the respective drug classes as well as the overall PCI. Abbreviation: SERM, selective estrogen receptor modulator.
molecular features of each cancer cell line. In the case of DC09, trastuzumab combined with cabozantinib, a very strong synergy was identified in the HER2-overexpressing SKBR3 cells. Strong synergy was also observed for triple-positive cancer cells (BT-474), which express in addition to HER2, the estrogen, and progesterone hormone receptors. Yet, in MCF-7 cells that lack HER2 overexpression, DC09 still showed some synergy, although decreased. In turn, complete lack of HER2 in the triple-negative and osteosarcoma cells revealed antagonistic interactions between trastuzumab and cabozantinib. The opposite trend was visible for DC10, raloxifene and NVP-AEW541, which showed the best synergy in MCF-7 cells followed by BT-474 cells, whereas an additive effect was detected for SKBR3 cells. Surprisingly, a strong synergy was obtained for DC04 in MCF-7 cells and SKBR3 cells, although this combination includes a PARP1 inhibitor, which is expected to be effective in triple negative breast cancers (38). Note that, although individual breast cancer drug indications might give insights on the synergistic mechanisms of a combination in a certain cancer subtype (Supplementary Table S6), experiments in larger cell panels would be required to elucidate the mechanism of synergy of a combination regarding a certain cancer subtype.

When considering the combinatorial effects with respect to the individual cancer cell lines, we observed that MCF-7 cells and SKBR3 cells tend to be more sensitive toward the selected drug combinations, with 50% being synergistic and the remaining ones showing at least additive effects (Supplementary Fig. S10). Again, this enrichment is significant with respect
to high-throughput screens ($P = 5e-04$; Supplementary Fig. S8C). In turn, 30% of the combinations were antagonistic in U2OS cells and BT-474 cells, and 20% were antagonistic in MDA-MB-231 cells. These observations might reflect the prevalence of therapeutic strategies, that is, drugs and combinations, for the more common hormone receptor-positive and HER2-overexpressing breast cancer subtypes.

U2OS cells were included as additional cancer cell line to examine the specificity of synergistic effects, because we recently found that individual anticancer drugs designed for a particular cancer (sub)type do not show a significantly higher activity in cancer cell lines derived from that specific tumor type (39).

Although the IC$_{50}$ is the most used index to measure the effectiveness of a compound at inhibiting a specific biological function, we examined the potential effect of concentration-dependent pharmacodynamic interactions, we determined the DCI for a series of inhibition levels ranging from 20% to 80%, which corresponds to DCI$_{20}$ to DCI$_{80}$. Overall, we found our results to be fairly robust, with 68.3% of the combinations showing a consistent interaction behavior, independently of the inhibition level considered, that is, we found neither synergistic–antagonistic nor additive–antagonistic shifts (Supplementary Fig. S11A).

**Effect of the raloxifene and cabozantinib combination in a xenograft mouse model**

The experimental validation in cancer cell lines revealed a clear synergistic therapeutic effect for combination DC02 (raloxifene with cabozantinib) in hormone receptor-positive breast cancer cells (DCI$_{50} = 0.39$). Our computational model suggested that the observed cross-talk inhibition was the result of the simultaneous modulation of estrogen signaling together with the PI3K/AKT and VEGF pathways by the two drugs, which should not only reduce the growth of the primary tumor but also its ability to spread. Interestingly, we found that this particular combination exhibited a DRI of 51.9 and 2.66 for raloxifene and cabozantinib, respectively. In other words, to achieve the same inhibition level, the doses of the individual drugs could be significantly reduced when combined (Fig. 5).

Finally, its DCI showed a remarkably consistent synergistic behavior in MCF-7 cells throughout all the inhibition levels (Supplementary Fig. 5.1.B).

We thus examined the impact of DC02 in vivo, using MCF-7 cells orthotypically implanted in nude mice, which were treated for 15 days with cabozantinib, raloxifene, or the combination of both. We selected concentrations of 1 mg/kg for cabozantinib and 3 mg/kg for raloxifene (low doses) that were previously reported to have a minor or no effect on tumor growth in related conditions (40, 41). However, because mice treated with the combination were exposed to higher overall drug doses, we assessed the effect of the individual drugs on tumor growth doubling their concentrations. Thus, we applied 2 mg/kg for cabozantinib and 6 mg/kg for raloxifene to avoid that a stronger effect observed for the combination is merely induced by the higher drug concentration administered in the combinatorial treatment.

We found that treating tumor-bearing mice with cabozantinib or raloxifene alone induced a cytostatic effect on tumor growth (Fig. 6A). Strikingly, in agreement with our observations in cultured cell lines and the cross-talk inhibition model, the combined treatment of mice with lower doses of cabozantinib and raloxifene showed a clear synergistic effect reducing the size of the tumors by more than 60% (Fig. 6A). Despite its dramatic impact on tumor growth, the combination has no effect on the body weight and none of the animals demonstrated abnormalities in their behavior, indicating that the combinatorial treatment is not more stressful than individual treatments (Supplementary Fig. S12). To explore whether the molecular processes leading to tumor reduction were indeed the ones suggested by the cross-talk inhibition model, we performed immunohistochemical analyses of tumor sections at the end of the treatment (day 15). TUNEL staining assays showed slightly increased cell death levels in tumors from cabozantinib-treated mice but no differences in the raloxifene-treated mice, which is consistent with observations in ER$^+$ human breast tumors (42). Interestingly, when combining both drugs, cell death levels increased 9.5 times compared with the initial or vehicle-treated tumors (Fig. 6B and C). We also found that cell proliferation, based on Ki67 staining, was strongly inhibited in tumors from mice treated with raloxifene and, to an even larger extent, in tumors from mice treated with the combined drugs (Fig. 6D). We did not observe any effect on cell proliferation in tumors from cabozantinib-treated mice.

The effects on cell proliferation and cell death, as detected in the xenograft mouse tumors, were in line with the current knowledge on the mechanism of action of both drugs (40, 42–48). To further assess PCI for DC02 at the molecular level, we performed Western blotting with tumor samples. In particular, we analyzed the activity of proteins involved in the pathways that were predicted to be modulated predominantly by the drug combination, including Akt and Src and the nuclear form of the estrogen receptor (nER; Fig. 6E). We found that the drug combination had a stronger effect than the individual drugs on the activities of both Src and Akt.

**Figure 5.** Pharmacodynamic interaction between raloxifene and cabozantinib. Isobologram showing the interaction behavior between raloxifene and cabozantinib. Blue and green symbols denote the IC$_{50}$ of raloxifene and cabozantinib, respectively, whereas the red symbol represents the combination. The dotted line indicates additivity. Data points below this line display synergy, whereas points above imply antagonism. The DRI exhibits to which extent the concentration of a drug in combination can be reduced at a given inhibition level compared with the single concentrations.
Figure 6.
Effect of raloxifene, cabozantinib, and combinatorial treatment on tumor growth in a MCF-7 xenograft model.
A, Athymic nude mice, orthotopically injected with 1 × 10^6 MCF-7 cells, were treated for 15 days with cabozantinib (2 mg/kg), raloxifene (6 mg/kg), the combination of cabozantinib (1 mg/kg) and raloxifene (3 mg/kg), or vehicle. Single treatments were combined with the corresponding vehicle of the other drug. Tumor size was measured at the indicated times and was normalized according to the original size of each tumor at the start of the treatment. Nine mice with two tumors each were used per condition.
B, Representative TUNEL staining of tumors collected at day 15. Significant increases in cell death levels were measured for cabozantinib (P = 0.0015) and the combination (P = 0.00016) compared with vehicle.
C, Average area of positive TUNEL-stained cells quantified by ImageJ for each treatment.
D, Representative H&E and Ki67 staining of the initial tumor, vehicle, cabozantinib and raloxifene alone, and the combination of both at day 15. Images shown are ×20. The percentage of positive Ki67-stained cells is indicated below each group.
E, Visualization of the simplified cross-talk network between the estrogen and the VEGFR signaling pathway for DC02. Proteins analyzed by Western blot analysis are colored from yellow to blue.
F, Western blot analysis of phospho-Akt, phospho-Src, Bcl-2, and cyclin D1 to assess their activity with respect to PCI. Tubulin was used as a loading control.
Akt, which was also clear analyzing downstream members of the pathways such as Bcl2 and cyclin D1 (Fig. 6F). These results are consistent with the proposed effect of the drug combination on cross-talk signaling and the reduced tumor growth observed in the xenografts.

Taken together, our study confirms that the combination of cabozantinib and raloxifene has a stronger therapeutic effect in vivo than the single drugs at higher doses. Our results indicate that the dramatic, synergistic effect of the combination on tumor growth emerges from the simultaneous induction of cell death by cabozantinib and the inhibition of cell proliferation by raloxifene. These mechanistic insights agree with the cross-talk inhibition calculated for DC02, which mostly comes from the simultaneous modulation of estrogen and prolactin signaling together with the PI3K/AKT and VEGF signaling pathways by the two drugs. Thus, as suggested by our model, inhibiting the cross-talk between these pathways prevents alternative signaling events, which regulate cell proliferation and survival.

Discussion

Combinatorial therapy is a very promising strategy for improving cancer treatment. The combination of drugs allow to interfere with compensatory mechanisms, often related to treatment failure, using drug concentrations that are less toxic than the high doses of single drugs usually required to achieve similar effects. However, despite its great potential, most approved drug combinations are the result of empirical clinical experience and, not being rationally designed, cover only a small fraction of the vast therapeutic space. In this study, we have presented a computational network biology approach to identify potentially synergistic drug combinations against breast cancer. Even though we focus specifically on pathway cross-talk as a major contributor to treatment failure, other oncogenic features, such as gene mutations, might also be helpful for finding combinatorial treatment (45). Overall, our strategy has identified a set of anticancer drug pairs with a large impact on cross-talk inhibition. The experimental validation of ten selected novel combinations confirmed a synergistic behavior for seven of them in, at least, one of the four breast cancer cell lines tested. This represents a significant enrichment compared with combinatorial studies without computational prioritization. Furthermore, we confirmed that raloxifene combined with cabozantinib has a dramatic synergistic effect interfering with tumor growth in vivo using a mouse xenograft model based on MCF-7 human breast cancer cells, supporting the potential clinical relevance of our strategy. Even though further research is required to enable the translation of a promising combination into therapeutic strategies, our results show that approaches focusing on the inhibition of cross-talk between pathways could provide valuable mechanistic information to discover synergistic drug effects. Moreover, we centered our study on breast cancer, but we believe that our approach can be also applied to other complex diseases, in which pathway cross-talk is likely to play an important role.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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