ABSTRACT

Motivation: Prediction of synergistic effects of drug combinations has traditionally been relied on phenotypic response data. However, such methods cannot be used to identify molecular signaling mechanisms of synergistic drug combinations. In this article, we propose an enhanced Petri-Net (EPN) model to recognize the synergistic effects of drug combinations from the molecular response profiles, i.e. drug-treated microarray data.

Methods: We addressed the downstream signaling network of the targets for the two individual drugs used in the pairwise combinations and applied EPN to the identified targeted signaling network. In EPN, drugs and signaling molecules are assigned to different types of places, while drug doses and molecular expressions are denoted by color tokens. The changes of molecular expressions caused by treatments of drugs are simulated by two actions of EPN: firing and blasting. Firing is to transit the drug and molecule tokens from one node or place to another, and blasting is to reduce the number of molecule tokens by drug tokens in a molecule node. The goal of EPN is to mediate the state characterized by control condition without any treatment to that of treatment and to depict the drug effects on molecules by the drug tokens.

Results: We applied EPN to our generated pairwise drug combination microarray data. The synergistic predictions using EPN are consistent with those predicted using phenotypic response data.

Availability: The software implemented in Python 2.7 programming language is available from request.

Contact: stwong@tmhs.org

1 INTRODUCTION

A combination of drugs, or a drug cocktail, is a common therapeutic strategy used in oncology. The strategy reduces drug toxicity caused by high doses of single drugs and generates enhanced effects with lower doses of combined drugs. A combination of two drugs may generate same effect (simple additive), blunted effect (sub-additive) and exaggerated effect (super-additive or synergistic). Drug combination focuses on the relationship between dosages and effects as well as the methodology distinguishing between additive and non-additive combinations. Methods of analysis for distinguishing between simple additivity and other non-additive outcomes include Lowe dose additivity (Chou and Talalay, 1983), Bliss independence (Bliss, 1939), Gaddum’s non-interaction (highest single agent) (Berenbaum, 1989) and Potentiation (Lehar et al., 2007, 2009). Among them, Lowe dose additivity is the most common model used in prediction of drug combination. Lowe dose additivity distinguishes additive and non-additive drug combinations by the combination index (CI), $CI = \frac{CI_{a}}{CI_{b}} + \frac{CI_{b}}{CI_{b}}$ (Chou and Talalay, 1983), where in case of an inhibitory drug, $X$ refers to a specific percent inhibition level (e.g. 50%), $CI_{a}$ and $CI_{b}$ are the concentration of drugs A and B given in a combination of the two drugs, and IC$_{a}$ and IC$_{b}$ are the concentration of drugs A and B yielding the same level effect, when treated alone, as the combination. If CI > 1, the drug combination has a subadditive effect, and if CI < 1, the drug combination has a synergistic effect. However, the existing methods for prediction of synergistic effects of drug combinations restrict to the phenotypic response data, and, therefore, cannot be used to describe the underlying mechanisms of signal transductions or signaling pathways.

The studies on signaling pathways and related interaction molecules would make it possible to recognize downstream effect of a drug or a drug combination on the targets. Exogenous signals are initially imposed on the receptors, which then carry the signals to second messengers, and eventually the signals are led to transcription-related molecules of DNA in the nucleus, such as transcription factors, RNA polymerases or histone modification complexes. Many drugs target on the upstream of signaling pathways, for example, membrane receptors or second messengers. They act as a long-term effect, called off-target effect (Keisier et al., 2009; Lamb, 2007; Lamb et al., 2006; MacDonald et al., 2006), on gene transcription-related molecules through the downstream signals along the pathway. Since the studies on drug targets alone are not sufficient to indicate the off-target effects, we need to develop computational models in order to simulate the effects of drugs on the signaling pathways.

Many models have been established for network modeling on biological systems, such as ordinary differential equations (ODE), Fuzzy logic system (FLS) and Petri net (PN). ODE is to encode a network as a system of differential equations, which generates a detailed and biochemically realistic representation. However, the ODE model suffers the cost of many free parameters, which must be estimated. If the number of the nodes of the network reaches tens or hundreds, parameter estimation would become challenging. FLS modeling has the same parameter issue. Comparing with these quantitative models, PN model is a better choice to describe biological systems qualitatively. PN has been recently applied in metabolic (Reddy et al., 1996; Voss et al., 2003), genetic (Begges et al., 2007) and signaling networks (Ruths et al., 2008) where few accurate kinetic data are accessible.
The output function
parts: a set of output and an output function \( O \). The input function \( I \) is a mapping from a transition \( t_j \) to a place \( I(t_j) \), known as the input place of the transition. A PN structure, \( C \), is defined as a four-tuple, \( C = (P, T, I, O) \).

\[ \text{Enhanced Petri-net model} \]

2 APPROACH

In this study, we propose a new type of PN model, Enhanced PN (EPN), to predict the synergistic effects of drug combinations using the drug-treated microarray data. We extended the basic PN as a multiple-color tokens, places and transitions comparing with the corresponding two microarrays treated by drugs A and B separately. Our hypothesis for synergism is that there exists such a drug combination that there are significant differences between the treated case and the control case.

To verify whether a drug combination shows synergism, we evaluate the effects of the treated profile of genes by the same method. To determine the optimal state is identified, we use the marking of one type of drug A and the other type of drug B, separately. Our hypothesis for synergism is that there exists such a drug combination that there are significant differences between the treated case and the control case.

To test our model, we generate a gene expression profile for breast cancer cell line MCF7 with 16 microarrays for gefitinib with the combination comparing with the summation of the effects generated by the two drugs individually. To predict whether a drug combination shows synergism, we compare the identified drug effects from the microarray treated by pairwise combination of drugs A and B with those from the corresponding two microarrays treated by drugs A and B separately. Our hypothesis for synergism is that there exists such a drug combination that there are significant differences between the treated case and the control case.

3 METHODS

3.1 RNA isolation and gene expression profiling

MCF-7 cells were seeded at \( 2 \times 10^5 \) cells in 6-well plates. After confluence was achieved, the cells were treated with different concentrations of drugs or Dimethyl sulfoxide (DMSO) in duplicate for 48 h. RNA was extracted from the cells using RNAeasy mini kit (Qiagen). The quality of the RNA samples was monitored using 2100 bioanalyzer (Agilent) before gene expression profiling with Agilent human 4 \times 44k microarrays.

3.2 EPN model

An EPN structure, \( C \), is defined as a four-tuple, \( C = (P, T, I, O) \), where \( P = \{P_P, P_D\} \), \( P_P \) is a set of places for drugs, \( P_D \) is a set of places for molecules, such as genes or proteins, sometimes, called as gene places; \( T = \{T_D, T_P\} \), \( T_D \) is a set of transitions for drug tokens, \( T_P \) is a set of transitions for gene tokens; \( I = \{I_D, I_P\} \), \( I_D \) is an input mapping between a drug transition \( t_D \) and the place \( I(D) \); \( I_P \) is an input mapping between a gene transition \( t_P \) and the place \( I(G) \); \( O = \{O_D, O_P\} \), \( O_D \) is an output mapping between a drug transition \( t_D \) and the place \( O(D) \); \( O_P \) is an output mapping between a gene transition \( t_P \) and the place \( O(G) \).

An EPN graph has four types of nodes. A circle \( D \) represents a gene place, a square \( P \) represents a drug place, a bar \( T \) represents a gene transition and a bar \( ( \) represents a drug transition. The arcs \( A_D \) and \( A_P \) have two colors, \( A_D \) is for drugs and \( A_P \) is for genes. Green arcs \( A_P \) represent the transition from input place to output place and \( A_D \) are the transition from output place to input place. The EPN has four types of functions, that is, gene tokens, drug tokens, effect tokens (blasting tokens) and drug transportation tokens. A marking \( \mu \) of an EPN, \( C = (P, T, I, O) \), is a function from the set of places \( P \) to the non-negative integer space, \( N^P \), \( \mu : P \rightarrow N \). The marking \( \mu = (\mu_D, \mu_P, \mu_D, \mu_P) \) can also be defined as a 4n-vector, \( \mu = (\mu_D, \mu_P, \mu_D, \mu_P) \), where \( \mu_D = (\mu_D^{t_D}, \mu_D^{t_D}, \mu_D^{t_D}, \mu_D^{t_D}) \) and \( \mu_P = (\mu_P^{t_P}, \mu_P^{t_P}, \mu_P^{t_P}, \mu_P^{t_P}) \), where \( \mu_D \) is a function from the set of drug transitions \( T_D \) to the non-negative integers \( N \), \( \mu_P \) is a function from the set of gene transitions \( T_P \) to the non-negative integers \( N \).

A marking \( \pi \) can be defined as an m-vector, \( \pi = (\pi_D^{t_D}, \pi_P^{t_P}) \), where \( \pi_D^{t_D} = (\pi_D^{t_D}, \pi_D^{t_D}, \pi_D^{t_D}, \pi_D^{t_D}) \) and \( \pi_P^{t_P} = (\pi_P^{t_P}, \pi_P^{t_P}, \pi_P^{t_P}, \pi_P^{t_P}) \), where \( \pi_D^{t_D} \) is a function from the set of drug transitions \( T_D \) to the non-negative integers \( N \), \( \pi_P^{t_P} \) is a function from the set of gene transitions \( T_P \) to the non-negative integers \( N \).

The execution of an EPN is based on the firing of transitions and the delivery of drug tokens. A drug transition \( t_D \) can only fire if it is enabled. A drug transition \( t_D \) in a marked EPN \( C = (P, T, I, O) \) with marking \( \mu \) is enabled if for all \( p \in P_D \), \( \mu_D(t_D) \geq 0 \).

\[ \text{doses, 0, 5, 10 and 40 \mu M, and docetaxel with 0, 0.15, 0.6 and 1.2 \mu M.} \]

The synergistic effect of the two drugs, gefitinib and docetaxel, has been confirmed by Takahatake et al. (2007). Then, we apply EPN model to the profile. The analysis shows that EPN predicts the synergistic pairwise drug combinations well. Most of the prediction results are consistent with the prediction results based on the phenotypic response data and the published literature.

Comparing with the existing methods, the advantage of using EPN in prediction of synergism is that it explains how two drugs could generate the synergistic effects. For the synergistic combinations of gefitinib and docetaxel, we found that docetaxel assists gefitinib in the synergy, while the synergistic molecules, such as KRT8, play a leading role in this process. The positive feedback loops between EGFR, target of gefitinib and KRT8 enable gefitinib to amplify its effect and dominate the synergism, whereas certain negative feedback loops between BCL2, target of docetaxel and KRT8 reduce its contribution to the synergism.
where $\eta(p, O_t(\xi))$ is the token number transited from $p_j$ to $O_t(\xi)$. Firing an enabled transition $t_j$ results in a new marking $\mu'$ defined by $\mu'_v(p) = \mu_v(p) - \eta(p, O_t(\xi)) + \eta(p, O_t(\xi))$ for every $v \in \mathbb{V}$ and $p \in \mathbb{P}$. The change in state $G \xrightarrow{t_j} G'$ with marking $\mu'$ is defined by $\delta(G, t_j) \triangleq \{ \pi_j \rightarrow \pi'_j \}$. The state变迁$G'$ is enabled if $\delta(G, t_j)$ is enabled, then

\[
\Delta \omega = \sum_{\pi \in \mathbb{P}} |G_{\text{trans}}(G_{\text{trans}})| - \sum_{\pi \in \mathbb{P}} |G_{\text{trans}}(G_{\text{trans}}) - \pi'_j|.
\]

Consider the change function set $\Delta \omega = \{ \Delta \omega_1, \Delta \omega_2, \Delta \omega_3 \}$ with marking $\mu$, the firing of a transition and blasting a place is defined by two change functions $\Delta \omega_1$ and $\Delta \omega_2$. After blasting in the output place, a bomb removes tokens from the input of the drug tokens $D$ and a change function set $\Delta \omega_3$. The EPN outputs the simulated drug effects on molecules.

### 3.3 Apply EPN to identify the drug effects on molecules

The firing of transitions and blasting of bombs result in the change of gene marking $\mu'$. The state of EPN is defined by its gene marking $\mu$. The task for EPN is to mediate the state, $\mu_{\text{trans}}$, before treatment to that, $\mu_{\text{trans}}$, after the treatment of a drug. It is formulated as an optimization problem in (1). The hypothesis is that the final state $\mu_{\text{trans}}$ is a reachable state from the control state $\mu_{\text{Ctrl}}$. There exists a transition set $\mathbb{T} = \{ T_1, T_2, \ldots, T_{n-t} \}$ and a change function set $\{ \delta_1, \delta_2, \ldots, \delta_{n-t} \}$ that satisfies $\delta_1 = \delta_2 = \cdots = \delta_{n-t}$. The EPN outputs the simulated drug effects on molecules.

Simulating drug effects using EPN can be described as follows:

\[
\begin{align*}
\text{min} \quad & \Delta \omega = \sum_{\pi \in \mathbb{P}} |G_{\text{trans}}(G_{\text{trans}})| - \sum_{\pi \in \mathbb{P}} |G_{\text{trans}}(G_{\text{trans}}) - \pi'_j| \\
\text{s.t.} \quad & \pi' = \pi_0 - \eta(p, O_t(\xi)) + \eta(p, O_t(\xi)) \\
& \mu' = \delta_1(p, \pi_0) \\
& \mu_0 \in \mathbb{M} \\
& \pi_0 \in \mathbb{N} \\
& i = 1, 2, \ldots, k \\
& j = 1, 2, \ldots, m
\end{align*}
\]

Input: $\mu_0$: gene expressions before treatment; $\mu_{\text{trans}}$: gene expressions after treatment; $\mathbb{T}$: downstream signaling network of targets; Output: $\mu'_{\text{trans}}$: drug effects on the molecules.

### 3.4 Transition algorithm

To optimize (1), we proposed a greedy transition algorithm, called Final State Oriented Transition. The transitions of drug tokens and gene tokens are implemented by the algorithm. The steps in the algorithm are: (1) shuffling of the interactions in the downstream targets of the signaling network, (2) transition of drug or gene tokens, and (3) decision of termination. The pseudo code is described as follows (2)

**Step 1.** Shuffle all interactions as $\Omega$.

**Step 2.** Token transition for every interaction $(G_1 \rightarrow G_2)$ in $\Omega$:

\[
\begin{align*}
& \text{if } (\text{Grid-chance} < 1 |) \text{ transit drug tokens} \\
& \text{blast effect tokens} \quad \text{update markings of drug, gene, effect, transportation tokens} \\
& \text{if } (\text{Grid-chance} > 1 |) \text{ transit gene tokens} \quad \text{update markings of gene tokens}
\end{align*}
\]

**Step 3.** Termination $\Delta = \sum_{i=1}^{|G_{\text{trans}}|} |G_{\text{trans}}| - \sum_{i=1}^{|G_{\text{trans}}|} |G_{\text{trans}} - \pi'_j|$

if $(\Delta < 10^{-6})$ (Exit), else go back to Step 2.

### 4 RESULTS

We developed an EPN model to predict synergistic effects of pairwise drug combinations using drug-mediated gene expression microarray data. To test the EPN model, we generated a gene expression profile with 16 combinations of two drugs with different dosage pairs. The drug effects on molecules identified by EPN model were used to predict the synergistic effects of the combinations.

### 4.1 Drug combinations for EPN

Sixteen pairwise drug combinations for gefitinib with doses 0, 5, 10 and 40 $\mu$Mol and docetaxel with 0, 0.15, 0.6 and 1.2 $\mu$Mol, are treated on MCF7 breast cancer cell line. We generated the phenotypic response data, that is, the inhibition rates on cell proliferation, and the treatment of microarray data simultaneously. Applying Calculusyn software (Tallarida, 2001) on the phenotypic response data, we identified that the combinations of (5, 1.2), (20, 0.15), (20, 0.6), (20, 1.2), (40, 0.15), (40, 0.6) and (40, 1.2) exhibit synergistic effects. The results are shown in Table 1. Takabatake et al. (2007) also revealed that gefitinib with dosage 19.42 $\mu$Mol and docetaxel with dosage 0.29 ± 0.04 $\mu$Mol are synergistic.

### 4.2 Downstream signaling network of the targets

Before implementation of EPN on the drug-treated microarray data of pairwise drug combinations, we have to identify the downstream signals and related protein–protein interactions (PPIs) of the targets, i.e. epidermal growth factor receptor (EGFR) of gefitinib and B-cell lymphoma 2 (BCL2) of docetaxel. The combined data for signaling pathways were derived from three pathway databases, including KEGG. Kyoto Encyclopedia of Genes and Genomes (Kanehisa et al., 2004), NCI PID: Pathway Interaction Database (Schafer et al., 2009) and BioCarta (Schafer et al., 2009). The PPIs used here are the physical interactions gathered from five PPI databases, IntAct (Kerrien et al., 2007), DIP (Xenarios et al., 2002), MINT (Chatr-aryamontri et al., 2007), MIPS (Mewes et al., 2002) and BioGrid.
The task of EPN is to figure out those molecules with enhanced effects by comparing the effects of pairwise combinations with those of single drugs. The CI identified by phenotypic responses is calculated as the gene places in EPN while the marking of the gene places is denoted as 1. The markings for gene tokens, drug tokens and effect tokens are generated, as described in transition algorithm of (2), we randomly selected one interaction from the downstream signaling network of the targets, then determine to transition either drug tokens or gene tokens. The number of gene tokens for every transition is a randomized number $\mu_{\text{Gamma}}$ derived from a Gamma distribution, $\text{Gamma}(k, \theta)$, where $k, \theta$ are two parameters for shape and scale of the Gamma distribution. The transition of drug tokens is forced by the output place. The transition of gene tokens depends on whether the interaction is activation or dephosphorylation, inhibition or dephosphorylation. Fig. 2 shows the downstream signaling network for targets of the pairwise combination of gefitinib and docetaxel. The related PPI data are not shown.

### Table 1. The CI identified by phenotypic responses

| Doses of gefitinib (µMol) | Doses of docetaxel (µMol) | CI       |
|---------------------------|---------------------------|----------|
| 5                         | 0.15                      | 3.361    |
| 5                         | 0.6                       | 5.121    |
| 5                         | 1.2                       | 0.894    |
| 20                        | 0.15                      | 0.619    |
| 20                        | 0.6                       | 0.687    |
| 20                        | 1.2                       | 0.664    |
| 40                        | 0.15                      | 0.672    |
| 40                        | 0.6                       | 0.753    |
| 40                        | 1.2                       | 0.619    |

CI used here is developed by CalcuSyn. If CI $< 0.7$, strong synergism or antagonism; if 0.7 $< CI < 0.9$, moderate or slight synergism; and if CI $> 0.9$, nearly additive or antagonism.

As described in transition algorithm of (2), we randomly selected an interaction from the downstream signaling network of the targets, then determine to transition either drug tokens or gene tokens. The number of gene tokens for every transition is a randomized number $\mu_{\text{Gamma}}$ derived from a Gamma distribution, $\text{Gamma}(k, \theta)$, where $k, \theta$ are two parameters for scale and shape of the Gamma distribution. The transition of drug tokens is forced by the output place. The transition of gene tokens depends on whether the interaction is activation or dephosphorylation, inhibition or dephosphorylation. The transition for drug tokens is forced by the output place. The transition for gene tokens depends on whether the interaction is activation or dephosphorylation, inhibition or dephosphorylation. Fig. 2 shows the downstream signaling network for targets of the pairwise combination of gefitinib and docetaxel. The related PPI data are not shown.

### 4.3 Apply EPN to the pairwise-combination microarray data

The task of EPN is to figure out those molecules with enhanced effects by comparing the effects of pairwise combinations with those effects of the corresponding single drugs. It involves three steps: (i) initiate the EPN model, (ii) detect the effects on the molecules of the pairwise combinations and of two single drugs, and (iii) identify the molecules with enhanced effects.

#### 4.3.1 Initiate the EPN model

To construct an EPN, we have to add transitions and tokens in the identified downstream signaling network of the targets of the combinations. The signaling molecules with their interacting protein molecules in Figure 2 are considered as the gene places in EPN while the marking of the gene places is determined by the expression values in the treatment microarray data. The drugs are considered as drug places, and the marking of their drug tokens is initiated as relative large numbers, which ensures their related transitions are enabled. The transitions for drugs and genes were assigned to the interactions between the places. If the interaction $(p_1 \rightarrow p_2)$ is activation or phosphorylation or drug $\rightarrow$ target, the new interactions were defined as from $p_1$ to transition, $p_1 \rightarrow T$ and from transition to $p_2$, $T \rightarrow p_2$. If the interaction $(p_1 \rightarrow p_2)$ is inhibition or dephosphorylation, the new interactions were defined reversely, from $p_2$ to transition, $p_2 \rightarrow T$ and from transition to $p_1$, $T \rightarrow p_1$.

As described in transition algorithm of (2), we randomly selected an interaction from the downstream signaling network of the targets, then determine to transit either drug tokens or gene tokens. The number of gene tokens for every transition is a randomized number $\mu_{\text{Gamma}}$ derived from a Gamma distribution, $\text{Gamma}(k, \theta)$, where $k, \theta$ are two parameters for scale and shape of the Gamma distribution. The transition of drug tokens is forced by the output place. The transition of gene tokens depends on whether the interaction is activation or dephosphorylation, inhibition or dephosphorylation. The transition for drug tokens is forced by the output place. The transition for gene tokens depends on whether the interaction is activation or dephosphorylation, inhibition or dephosphorylation. Fig. 2 shows the downstream signaling network for targets of the pairwise combination of gefitinib and docetaxel. The related PPI data are not shown.

### 4.3.2 Detect the effects on the molecules

We implemented EPN on every combination microarray data for 1000 times. A list of effect token markings is generated, $\Lambda = \{\mu_{B,1}, \mu_{B,2}, \ldots, \mu_{B,1000}\}$. For every place or gene $p_i$, the drug effect $E^p$ is defined as

$$E^p = \frac{1}{1000} \sum_j \mu_{B,j}$$

### 4.3.3 Find the molecules with enhanced effects

The synergistic effects of drug combinations are evaluated by the identified drug
We defined the genes with \(s^p/ > 0\) were filtered out as synergistic markers for combination \((a, b)\).

We designed a random process to evaluate to what degree a combination shows synergistic effect. We randomly generated the treatment expression data for the pairwise combinations and single drugs. The expression of each gene was randomly selected from the 16 expression values of this gene in the treatment microarrays with 16 dose pairs. We repeated the random process for a thousand times and generated 1000 randomized microarrays. To evaluate the significance of synergistic effects, we randomly chose three microarray data for pairwise combination \((a, b)\) and single drugs from the generated randomized microarrays so that we could compute the randomized effect on every place. Thus, we found a random distribution \(f\) for the total synergistic effects \(\sum s^p/\) of a combination \((a, b)\). The \(P\)-value is derived from the complementary cumulative distribution function \(\phi\) of \(f\).

We applied EPN model to our generated combination microarrays. Our results are consistent with the prediction results based on the phenotypic data only indicates whether two drugs are synergistic, but cannot explain how the two drugs derive the synergistic effect. EPN fills this void.

### 4.4 Further understanding of synergism

The conventional prediction methods based on phenotypic response data only indicates whether two drugs are synergistic, but cannot explain how the two drugs derive the synergistic effect. EPN fills this void.

#### 4.4.1 How is synergism generated?

Conventional studies on synergism aim to find the combination whose combined dosage is lower than dosages of individual drugs. The CI was defined as (Chou and Talalay, 1983):

\[
CI = \frac{C_{A,R}}{IC_{X,A}} + \frac{C_{B,R}}{IC_{X,B}}
\]

where in case of an inhibitory drug, \(X\) refers to a specific percent inhibition level (e.g. 50%), \(C_{A,R}\) and \(C_{B,R}\) are the concentrations of drugs A and B given in a combination of the two drugs, and \(IC_{X,A}\)

| Comb(a,b) | P-value | Genes with significantly higher synergistic effects |
|-----------|---------|---------------------------------------------------|
| (5, 0.15) | 0.85    | NFKBIA                                           |
| (5, 0.6)  | 0.053   | NFKBIA                                           |
| (40, 0.6) | 10^{-10}| NFKBIA                                           |
| (20, 0.6) | 0.32    | NFKBIA                                           |
| (20, 1.2) | 0.03    | NFKBIA                                           |
| (20, 1.2) | 10^{-10}| NFKBIA                                           |
| (40, 1.2) | 0.44    | NFKBIA                                           |
| (40, 1.2) | 10^{-10}| NFKBIA                                           |

The application of EPN to randomized gene expression microarray data indicates a cutoff for \(s^p/\), i.e. 50.1. The genes with \(s^p/ > 50.1\) are shown. The bold values in the \(P\)-value column indicates the \(P\)-value is significant \((P < 0.01)\).

The application of EPN to randomized gene expression microarray data indicates a cutoff for \(s^p/\), i.e. 50.1. The genes with \(s^p/ > 50.1\) are shown. The bold values in the \(P\)-value column indicates the \(P\)-value is significant \((P < 0.01)\).

The application of EPN to randomized gene expression microarray data indicates a cutoff for \(s^p/\), i.e. 50.1. The genes with \(s^p/ > 50.1\) are shown. The bold values in the \(P\)-value column indicates the \(P\)-value is significant \((P < 0.01)\).

The application of EPN to randomized gene expression microarray data indicates a cutoff for \(s^p/\), i.e. 50.1. The genes with \(s^p/ > 50.1\) are shown. The bold values in the \(P\)-value column indicates the \(P\)-value is significant \((P < 0.01)\).
The predicted effects for gefitinib and docetaxel in single and combination treatments

| Doses (µMol) | Effects                                      |
|--------------|----------------------------------------------|
|              | Single drug                                  |
|              | Combination                                  |
| Gefitinib    | Docetaxel                                   |
| 5            | 0.15                                        |
| 5            | 0.6                                         |
| 5            | 1.2                                         |
| 20           | 0.15                                        |
| 20           | 0.6                                         |
| 20           | 1.2                                         |
| 40           | 0.15                                        |
| 40           | 0.6                                         |
| 40           | 1.2                                         |
| Gefitinib    | 22 819                                      |
| 5            | 150 841                                      |
| 5            | 91 527                                       |
| 5            | 105 586                                      |
| 20           | 90 328                                       |
| 20           | 89 474                                       |
| 20           | 34 687                                       |
| 20           | 136 433                                      |
| 40           | 179 548                                      |
| 40           | 67 537                                       |
| 40           | 97 426                                       |
| 40           | 87 338                                       |
| Docetaxel    | 50 728                                       |
| 5            | 150 841                                      |
| 5            | 88 472                                       |
| 5            | 75 844                                       |
| 20           | 104 450                                      |
| 20           | 136 433                                      |
| 40           | 117 548                                      |
| 40           | 67 537                                       |
| Gefitinib    | 112 439                                      |
| 5            | 59 973                                       |
| 5            | 90 328                                       |
| 20           | 89 474                                       |
| 20           | 49 301                                       |
| 20           | 34 687                                       |
| 20           | 77 516                                       |
| 40           | 67 537                                       |
| 40           | 54 183                                       |
| Docetaxel    | 64 102                                       |
| 5            | 39 987                                       |
| 5            | 50 503                                       |
| 20           | 49 301                                       |
| 20           | 34 687                                       |
| 20           | 77 516                                       |
| 40           | 67 537                                       |
| 40           | 54 136                                       |

The bold value indicates that (i) the dose-pair has a significant P-value in Table 2 and (ii) total of the predicted effects of the two drugs in combinations are higher than that in single drugs.

The combination (19±2.4, 0.29±0.042). The combination (5, 1.2) shows that docetaxel assists gefitinib with 5 µMol to generate a higher effect, 90 328, than that of 20µMol, 50 728, by using a higher dose of 1.2 µMol, instead of 0.3 µMol.

**4.2.2 How does the combination achieve the synergism?** The combination (20, 1.2) is used to illustrate the molecular mechanisms for the synergistic effects. In EPN, we defined a marking πT of the drug transition Tπ, which records how many drug tokens are transited between gene places by drug transitions. The transition marking, πT, helps to find the pathways by which the drug tokens are transited from drug places to the synergistic molecules as shown in Table 2.

We chose KRT8 (CK8, keratin 8) from the list of synergistic molecules as an example to show how docetaxel assists gefitinib to achieve the synergistic effects. KRT8 is a member of the type II keratin family clustered on the long arm of chromosome 12. Type I and type II keratins heteropolymerize to form intermediate-sized filaments in the cytoplasm of epithelial cells. The product of this gene typically dimerizes with keratin 18 to form an intermediate-sized keratin family clustered on the long arm of chromosome 12. Type I and type II keratins heteropolymerize to form intermediate-sized filaments in the cytoplasm of epithelial cells. This protein plays a role in maintaining cellular structural integrity and also functions in signal transduction and cellular differentiation. KRT8 with another synergistic molecule KRT18 has been revealed as prognostic biomarkers for the patients of triple negative breast cancers (Williams et al., 2009) and invasive breast cancer (Takei et al., 1995).

Using the transition marking, πT, we filtered out the transitions T′ whose πT > 0. These identified transitions facilitate us to chase the drug effects from the two combined drugs to the interested synergistic molecule, KRT8. The transitions are also helpful to understand the mechanisms of the synergistic effects. We showed the identified transitions in Figure 3.

The synergistic effect of (20, 1.2) is elucidated by the identified transitions of the treatment pathway in Figure 3. The reason for the synergism is that the two drugs have many common output places that are, for example, KRT8, the locations for the two drugs to exchange their effects and generate the synergistic effects. Besides this, the transitions in Figure 3 also explain why docetaxel assists gefitinib to generate the synergistic effect. The target of gefitinib, EGFR, has a direct effect on KRT8 through the transition, EGFR → KRT8, whereas the target of docetaxel, BCL2, does not. In addition, gefitinib has a number of positive feed-forward loops through RAS pathway, for instance, EGFR → SOS1 → RRAS2 → RAF1 → KRT8, EGFR → SOS1 → RRAS2 → RAF1 → KRT8, and EGFR → PLCG1 → PKCA → RAF1 → KRT8. The feed-forward loops in accelerating transductions of signals have been extensively studied (Milo et al., 2002; Shen-Orr et al., 2002). They are, here, considered as the important paths for accelerating gefitinib to have an effect on KRT8. In contrast, besides positive feedback loops, docetaxel has many negative feedback loops to KRT8 caused by two inhibitory interactions, BCL2 → [TP53 and SOCS3 → JAK2]. The positive feed-forward loops for gefitinib and the negative feedback loops for docetaxel together help us to understand why gefitinib dominates the synergistic effects and the role of docetaxel is to assist gefitinib to generate such effects.

**5 DISCUSSION**

In this article, we developed a new model, EPN, to predict the synergistic effects of pairwise drug combinations using drug-treated gene expression microarray data. The effects of drugs on molecule expression and the associated pathways are simulated by the defined tokens and transitions in different colors in the model. Synergism is predicted using enhanced synergistic molecules recognized by EPN. The activated drug transitions help to interpret the mechanisms of the synergistic effects of the combinations.
The application of EPN to the microarray profile for gefitinib and docetaxel not only predicts the synergistic dose pairs but also illustrates the mechanism for the synergistic effects. We identified that gefitinib dominates the synergistic effects while docetaxel assists gefitinib to achieve that. The results are also confirmed by the phenotypic response data as shown in Table 4. We tested another two dosages, 2.5 and 10 µMol, for gefitinib and another two dosages, 0.3 and 2.4 µMol, for docetaxel in the response data. We could see that the proliferation inhibition rates are significantly decreased along with the increase of dosage of gefitinib, and the tendency is, however, not with docetaxel.

The molecule, KRT8, helps us to make it clear how the two drugs generate the synergistic effect. While the drugs treat the cells separately, gefitinib is known to inhibit the Ras pathway by its target, EGFR, so that the drug prevents the proliferation of cells. On the other hand, docetaxel binds to another target, TUBB1, which stabilizes microtubules and prevents depolymerization from calcium ions, decreased temperature and dilution, preferentially at the plus end of the microtubule. For the mechanisms of the synergistic combination of two drugs, it remains unclear. Our analysis indicates that gefitinib still uses the target EGFR and its downstream Ras pathway, while docetaxel alternatively imposes its effects on the target, BCL2, so that it helps gefitinib generate the synergistic effects on KRT8. KRT8 is also alternatively imposes its effects on the target, BCL2, so that it helps gefitinib generate the synergistic effects on KRT8. KRT8 is also known as cytokeratin-8 (CK-8) or keratin-8 (K8) is a keratin protein that in human is encoded by the KRT8 gene. It is overexpressed in the patients of breast cancer. So it has been a prognostic biomarker for the patients of triple negative breast cancers (Williams et al., 2009) and invasive breast cancer (Takesi et al., 1995). Therefore, it is the synergistic molecules, such as KRT8, to help gefitinib and docetaxel to generate the synergistic effects.

The proposed method of EPN is suitable for studying the target-therapy drugs. The model works best if the targets with their downstream signaling pathways are already known. Otherwise, one has to ask for assistance from other types of computational methods to simulate the downstream signaling network for the targets, for example, drug-target identification or pathway simulation based on Bayesian network, Boolean network or other gene interaction network using the microarray or protein array data from the patients tissues or disease cell lines.

ACKNOWLEDGEMENTS

GJ. and Z.X. proposed the idea for EPN model. GJ. developed the codes for EPN model. GJ. and Z.H. designed the combination experiments and Z.H. conducted the wet lab experiments. S.W. initiated and supervised this work. G.J. and S.W. wrote the paper.

Funding: National Institutes of Health (U54CA149196); John S Dunn Research Foundation.

Conflict of Interest: none declared.

REFERENCES

Borenstein,M.C. (1990) What is synergy? Pharmacol. Rev. 41, 93-141.
Biss.C.C. (1939) The toxicity of poisons applied jointly. Ann. Appl. Biol. 26, 385-615.
Breitkreutz,B.J. et al. (2008) The BioGRID Interaction Database: 2008 update. Nucleic Acids Res. 36, D637-D640.
Chatr-aryamonti,A. et al. (2007) MINT: the Molecular INTeraction database. Nucleic Acids Res. 35, D572-574.
Chua,N.-C. and Talalay,P. (1983) Analysis of combined drug effects: a new look at a very old problem. Trends Pharmacol. Sci., 4, 450-454.
Grauwildd,R. et al. (2008) Peti net modelling of gene regulation of the Duchenne muscular dystrophy. Bioinformatics, 26, 199-205.
Haydy,S. and Robillard,P.J.N. (2008) Peti net-based method for the analysis of the dynamics of signal-propagation in signaling pathways. Bioinformatics, 24, 209-217.
Kanehisa,M. et al. (2004) The KEGG resource for deciphering the genome. Nucleic Acids Res. 32, D277-D280.
Kreiser,M.I. et al. (2009) Predicting new molecular targets for known drugs. Nature, 462, 175-181.
Kernien,S. et al. (2007) IntAct—open source resource for molecular interaction data. Nucleic Acids Res., 35, D66-665.
Koch,J. et al. (2011) Modeling in Systems Biology: The Petri net Approach (Computational biology). Springer, New York.
Lamb,J. (2007) The Connectivity Map: a new tool for biomedical research. Nat. Rev. Cancer, 7, 54-60.
Lamb,J. et al. (2006) The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and diseases. Science, 313, 1929-1935.
Lehaj,J. et al. (2009) Synergistic drug combinations tend to improve therapeutically relevant selectivity. Nat. Biotechnol., 27, 679-686.
Lehaj,J. et al. (2007) Chemical combination effects predict connectivity in biological systems. Mol. Syst. Biol., 3, 10.
MacDonald,M.I. et al. (2006) Identifying off-target effects and hidden phenotypes of drugs in human cells. Nat. Chem. Biol., 2, 329-337.
Mewes,H.W. et al. (2002) MIPS: a database for genomes and protein sequences. Nucleic Acids Res., 30, 31-34.
Milo,R. et al. (2002) Network motifs: simple building blocks of complex networks. Science, 298, 824-827.
Peterson,J. (1981) Peti Net Theory and the Modeling of Systems. Prentice Hall, Englewood Cliffs.
Reddy,V.N. et al. (1998) Qualitative analysis of biochemical reaction systems. Comput. Biol. Med., 26, 9-24.
Rudd,D. et al. (2008) The signaling peti net-based simulator: a non-parametric strategy for characterizing the dynamics of cell-specific signaling networks. PLoS Comput. Biol., 4, e1000005.
Schaber,C.P. et al. (2009) PID: the Pathway Interaction Database. Nucleic Acids Res., 37, D674-D679.
Shen-Or,S.S. et al. (2002) Network motifs in the transcriptional regulation network of Escherichia coli. Nat. Genet., 31, 64-68.
Steggle,L. et al. (2007) Qualitatively modelling and analysing genetic regulatory networks: a Petri net approach. Bioinformatics, 23, 336-343.
Takahashi,K. et al. (2007) Tumor inhibitory effect of gefitinib (ZD1839, Iressa) and taxane combination therapy in EGFR-overexpressing breast cancer cell lines (MCF7/ADR, MDA-MB-231). Int. J. Cancer, 120, 181-188.
Takesi,H. et al. (1995) Immunohistochemical analysis of cytokeratin 86 as a prognostic correlate in invasive breast carcinoma. Am.J.Concuer., 135, 1101-1105.
Tallarida,R.J. (2001) Drug synergism: its detection and applications. J. Pharmacol. Exp. Ther., 290, 865-872.
Vos,K. et al. (2003) Steady state analysis of metabolic pathways using Petri nets. In Silico Biol., 3, 367-387.
Williams,D.J. et al. (2009) Triple-negative breast carcinoma in women from Vietnam and the United States: characterization of differential marker expression by tissue microarray. Hum Pathol., 40, 1176-1181.
Xenarios,I. et al. (2002) DIP: the Database of Interacting Proteins: a research tool for studying cellular networks of protein interactions. Nucleic Acids Res., 30, 303-305.

Table 4. The phenotypic response data for the pairwise drug combinations

| Docetaxel (µMol) | Gefitinib (µMol) |
|------------------|-----------------|
| 0                | 100.00          |
| 0.15             | 87.94           |
| 0.3              | 83.96           |
| 0.6              | 72.68           |
| 1.2              | 74.48           |
| 2.4              | 75.39           |
| 5                | 93.16           |
| 10               | 91.92           |
| 20               | 91.44           |
| 40               | 92.36           |

0.3 83.96 108.54 91.92 87.33 67.75 55.39
0.6 72.68 95.56 73.31 67.25 60.87 44.29
1.2 74.48 90.14 68.35 64.73 58.09 47.83
2.4 75.39 96.33 70.41 68.72 62.78 42.08

The results are confirmed by the KRT8. KRT8 is also known as cytokeratin-8 (CK-8) or keratin-8 (K8) is a keratin protein that in human is encoded by the KRT8 gene. It is overexpressed in the patients of breast cancer. So it has been a prognostic biomarker for the patients of triple negative breast cancers (Williams et al., 2009) and invasive breast cancer (Takesi et al., 1995). Therefore, it is the synergistic molecules, such as KRT8, to help gefitinib and docetaxel to generate the synergistic effects.

The proposed method of EPN is suitable for studying the target-therapy drugs. The model works best if the targets with their downstream signaling pathways are already known. Otherwise, one has to ask for assistance from other types of computational methods to simulate the downstream signaling network for the targets, for example, drug-target identification or pathway simulation based on Bayesian network, Boolean network or other gene interaction network using the microarray or protein array data from the patients tissues or disease cell lines.