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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Señor Superintendente
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

E. S. D.

Trámite : 011
Evento : 378
Actuación : 412

Referencia : Expediente No. 07.109.905
Asunto : Solicitud de patente para : **ASOCIACIÓN ENTRE LA FERROQUINA Y UN DERIVADO DE ARTEMISINA PARA EL TRATAMIENTO DEL PALUDISMO**
Solicitante : SANOFI-AVENTIS
Fecha solicitud : 18 de octubre de 2007

I. RECURSO VÍA GUBERNATIVA

Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad **SANOFI-AVENTIS** solicitante de la patente de la referencia, por el presente escrito atentamente manifiesto a usted que debidamente notificada de la Resolución No. 19379 del 29 de marzo de 2012, interpongo en su contra el recurso de **REPOSICIÓN**, para que sea revocada y en consecuencia se conceda el privilegio de patente de invención para "**ASOCIACIÓN ENTRE LA FERROQUINA Y UN DERIVADO DE ARTEMISINA PARA EL TRATAMIENTO DEL PALUDISMO**" a favor de **SANOFI-AVENTIS**.



II. OPORTUNIDAD DE LA IMPUGNACIÓN

La Resolución No. 19379 del 29 de marzo de 2012, fue notificada mediante edicto No. 9660 desfijado el veintiséis (26) de abril de dos mil doce (2012), por lo que el término para interponer el recurso de reposición vence el cuatro (04) de mayo de dos mil doce (2012). En consecuencia, me encuentro dentro de la oportunidad legal.



III. LA DECISIÓN RECURRIDA

3.1 Mediante la Resolución objeto de la presente impugnación, el Superintendente de Industria y Comercio decidió:

3.1.1 Negar el privilegio de patente para la invención **"ASOCIACIÓN ENTRE LA FERROQUINA Y UN DERIVADO DE ARTEMISINA PARA EL TRATAMIENTO DEL PALUDISMO"**

3.2 En la parte motiva de la providencia, se mencionaron los siguientes fundamentos:

3.2.1 "D1 (W020051023304) da a conocer composiciones (tabletas cubiertas y cápsulas, pág.7, líneas 22 a 30) que comprenden derivados de la artemisinina, como el arteéter, arteméter y el artesunato (más potentes que la artemisinina contra la malaria severa, pág.2, líneas 10-15) y una quinolina como la cloroquina (pág.6, líneas 1 a 4).

3.2.2 "Tal como se observa, la solicitud se diferencia de lo conocido en D1 en que la composición reivindicada comprende ferroquina a cambio de cloroquina.

3.2.3 "Por otro lado, de acuerdo con los datos presentados en la descripción (tabla II, Fl. 2, pág.9) y en la respuesta del 26 de septiembre de 2011 (tabla II y figura I), se observa que el porcentaje de reducción de la parasitemia, obtenido cuando se utilizan ferroquina y el artesunato, individualmente, a dosis de 3mg/kg/día y 6mg/kg/día, son del 22.6% y del 24,3% respectivamente y con la combinación de los dos a las mismas dosis es del 31.24% de donde se concluye que el efecto de reducción de la parasitemia, con la combinación, es menor que el aditivo (46.9%), por lo tanto no existe evidencia de un efecto sorprendente o inesperado (ej: efecto sinérgico).

3.2.4 "No obstante lo anterior, tal diferencia no permite evidenciar un efecto inesperado que se le pueda atribuir a la presencia de la ferroquina, análogo de la cloroquina, en la materia reivindicada.

3.2.5 "De esta manera, el problema técnico objetivo que resuelve la solicitud es "cómo modificar la composición divulgada en D1 para obtener una composición alternativa, con actividad mejorada para el tratamiento de la malaria".





Sin embargo, D2 ya divulgaba que la actividad antimalaria de la ferroquina (quinolona) es superior a la enseñada por la cloroquina de acuerdo a los 1C50 mostrados en el folio 55 (pág 150, tabla 1), presentando por tal razón una mejor actividad, tanto sobre las cepas sensibles como sobre las resistentes a la cloroquina (folio 53, introducción).

3.2.7 “Entonces, de acuerdo a lo divulgado en D1, en cuanto a las combinaciones sinérgicas, y tomando en cuenta que ya se conocía la superior actividad de la ferroquina (D2), una persona del oficio normalmente versada en la materia estaría motivada a intercambiar la cloroquina de las combinaciones de D1, por la ferroquina conocida en D2, para lograr una composición como la ahora reclamada. Por lo anterior y como el efecto de dicha composición demostrado por el solicitante en la tabla (II) de la descripción (pág.9) no es inesperado, se concluye que la presente reivindicación no es inventiva y no cumple lo dispuesto en el artículo 18 de la Decisión 486.

3.2.8 “Por lo demás, comoquiera que las reivindicaciones dependientes 2 a 3 no mencionan características inventivas adicionales, tampoco pueden ser consideradas inventivas.

3.2.9 “De igual manera la reivindicación 4, que consiste en un kit con la ferroquina y al menos un derivado de artemisinina en compartimentos distintos, está reclamada como una reivindicación independiente, pero aún así está ligada directamente a la composición de la reivindicación 1, la cual ya se demostró que no es inventiva; en consecuencia, la reivindicación 4, tampoco cumple el requisito de nivel inventivo.



IV. OBJETO DEL RECURSO

De acuerdo con los argumentos que expongo a continuación, solicito atentamente a su Despacho lo siguiente:

1. Revocar la Resolución No. **19379** del 29 de marzo de 2012.
2. Conceder el privilegio de patente de invención para **“ASOCIACIÓN ENTRE LA FERROQUINA Y UN DERIVADO DE ARTEMISINA PARA EL TRATAMIENTO DEL PALUDISMO”** a favor de **SANOFI-AVENTIS**.

V. FUNDAMENTOS DEL RECURSO



Respetuosamente me permito disentir de lo expuesto por ese Despacho en la Resolución objeto del presente recurso. En particular, nos encontramos en desacuerdo con el análisis de patentabilidad realizado por el Examinador y consideramos que la presente solicitud sí cumple con el requisito de nivel inventivo tal como se demostrará a continuación.

5.1 La presente solicitud sí tiene nivel inventivo

Así entonces, el artículo 18 de la Decisión 486 señala lo siguiente:

Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica.

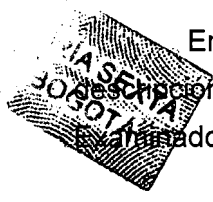
Consecuentemente, una invención se considerará inventiva si la misma no hubiese resultado obvia a partir del arte previo, para una persona del oficio normalmente versada en la materia. Esto a su vez implica que el Examinador a cargo de esta evaluación debe colocarse en el momento exacto en el que el inventor buscaba resolver el problema técnico contemplado en la solicitud y para el cual poseía la literatura y el conocimiento disponibles en dicho momento.

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis del nivel inventivo de una solicitud de patente, se considera que una anterioridad o una combinación de dos anterioridades puede afectar dicho requerimiento de patentabilidad, si una persona medianamente versada en la materia encuentra enseñanzas o sugerencias claras que, a partir de un conocimiento medio en dicho campo, le permitan llegar de manera obvia a la invención reivindicada. Adicionalmente se considera que una mejora sustancial, un efecto inesperado o una ventaja con respecto al arte previo es un indicio de nivel inventivo, por cuanto aporta un salto tecnológico en la regla técnica.

A este respecto manifestamos nuestro desacuerdo con el análisis llevado a cabo por el Examinador y con las conclusiones del mismo y solicitamos atentamente la reconsideración de las razones por las cuales se decidió negar la patente, con base en la siguiente información:



5.1 Efecto sinérgico



En relación con los resultados experimentales reportados en la tabla II de la investigación, se desea analizar y comentar sobre los mismos, teniendo en cuenta la posición del Examinador.

El Examinador establece: *“se observa que el porcentaje de reducción de la parasitemia, obtenido cuando se utilizan la ferroquina y el artesunato, individualmente, a dosis de 3mg/kg/día y 6mg/kg/día, son del 22.6% y del 24,3% respectivamente y con la combinación de los dos a las mismas dosis es del 31.24% de donde se concluye que el efecto de reducción de la parasitemia, con la combinación, es menor que el aditivo (46.9%), por lo tanto no existe evidencia de un efecto sorprendente o inesperado (ej: efecto sinérgico)”*.

En este sentido, es posible determinar que la afirmación realizada por el Examinador no es cierta:

1. Relación no lineal entre dosis-efecto

- Declaración general

Por definición, la relación entre la dosis y el efecto de un ingrediente activo **no es lineal**. En otras palabras, el incremento de la dosis de un ingrediente activo **no significa un incremento proporcional del efecto**.



Lo mismo aplica para la combinación de dos ingredientes activos. Por ejemplo, el efecto de la combinación de dos ingredientes activos no significa la adición de los efectos individuales de dichos ingredientes activos.

El efecto de una combinación no puede ser anticipado.

De hecho, un fenómeno tal como la saturación de ingrediente activo en el medio y/o la interacción ingrediente activo-ingrediente activo pueden ocurrir.

Métodos estadísticos específicos son conocidos **para calcular estos efectos de combinación**; uno de ellos es el **método de independencia de Bliss**¹².



- **Método de independencia de Bliss**¹

Este método se emplea para calcular la relación dosis-efecto esperada para una combinación de ingredientes activos, comparado con la monoterapia.

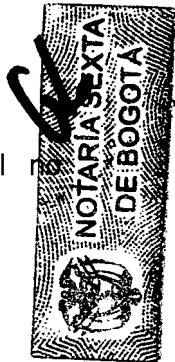
Se asume que los dos ingredientes activos (F_{UA1} y F_{UA2}) actúan por medio de mecanismos independientes. Entonces, la combinación de ingredientes activos puede ser representada como la unión de dos eventos independientes.

El efecto combinado de dos ingredientes activos (F_{UA}) se calcula como el producto de los **efectos individuales de los dos ingredientes activos**, F_{UA1} y F_{UA2} , de acuerdo con la siguiente fórmula:

$$F_{UA} = F_{UA1} \times F_{UA2}$$

En donde F_{UA} es la **actividad medicamentosa remanente** (fraccional no afectado).

Teniendo en cuenta la definición, F_{UA} es el **efecto combinado esperado**.



Si el efecto combinado real de los dos inhibidores ($F_{UA1} \times F_{UA2}$) es **igual** a F_{UA} , se habla de un caso de efecto de adición y **no existe interacción entre los dos inhibidores**.

Si el efecto combinado real ($F_{UA1} \times F_{UA2}$) es **menor** que F_{UA} , se habla de **antagonismo**.

Si el efecto combinado real ($F_{UA1} \times F_{UA2}$) es **mayor** que F_{UA} , se habla de **sinergismo**.

¹ Yan *et al.* BMC systems biology. A formal model for analyzing drug combination effects and its application in TNF-alpha-induced NFkB pathway /2010) -4:50
² Hegreness *et al.*. Proc Nat Acad Sci USA. Accelerated evolution of resistance in multidrug environments (2008) 105:13977-13981.

• La situación de combinación ferroquina-artesunato



Debido a que el efecto relativo a la dosis no es lineal, los porcentajes de reducción del porcentaje de parasitemia de la ferroquina y el artesunato administrados de manera individual **no pueden ser simplemente sumados** (46.9%) con el fin de afirmar cuál es el efecto combinado de los dos ingredientes activos. Este cálculo no refleja la realidad de la situación

En efecto, al observar los valores experimentales de la tabla II, es posible establecer que estos demuestran un efecto sinérgico, confirmado por el análisis del método de Bliss.

(i) Tabla II (página 9 de la descripción)

Parasitemia (en %)	Artesunato (mg/kg/día)	
	0	6
Ferroquina (mg/kg/día)	media	Media
0	31,4	7,1
3	8,8	0,16



(ii) Calculo de F_{UA} , F_{UA1} y F_{UA2}

- Los **efectos individuales** del artesunato (F_{UA1}) y la ferroquina (F_{UA2}) son los siguientes:

- F_{UA1} corresponde al porcentaje (%) de parasitemia después de la administración de una dosis de 6 mg/kg/día de artesunato en monoterapia:

$$F_{UA1} = 7,1/31,4 = 0,226$$

- F_{UA2} corresponde al porcentaje (%) de parasitemia después de la administración de una dosis de 3 mg/kg/día de ferroquina en monoterapia:

$$F_{UA2} = 8,8/31,4 = 0,28$$

- Por lo tanto, el **efecto combinado real** ($F_{UA1} \times F_{UA2}$) = $0,226 \times 0,28 = 0,063$

- El **efecto combinado esperado** (F_{UA}) de la combinación de la ferroquina (3 mg/kg/día) y el artesunato (6 mg/kg/día) corresponde al porcentaje (%) de parasitemia después de la administración de dicha combinación.



$$F_{UA} = 0,16/31,4 = 0,005$$

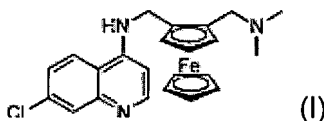
(iii) Resultados y discusión

El **efecto combinado real** $F_{UA1} \times F_{UA2} = 0,063$ es mayor que el **efecto combinado esperado** $F_{UA} = 0,005$.

Este análisis confirma el **efecto sinérgico inesperado** de la combinación de ferroquina (3 mg/kg/día) y artesunato (6 mg/kg/día) respecto a la reducción de parasitemia.

2. Documentos citados por el Examinador

El problema técnico a ser resuelto por la invención es proveer una combinación novedosa de ferroquina (molécula (I) representada a continuación en forma de base libre y en donde Fe representa un anillo de ferroceno) y un derivado de artemisina, útil para el tratamiento y/o prevención de la malaria.



Otro objetivo de la invención es una composición farmacéutica que comprende como ingredientes activos, una combinación de ferroquina (I) y un derivado de artemisina, ventajosamente artesunato (II) o arteméter (II).

- Documento D1: WO 2005/023304: este documento revela una **composición farmacéutica o formulación** que comprende una **combinación** de un derivado de artemisina con un derivado de quinolina en forma de dosis única con uno o más excipientes farmacéuticamente aceptables (página 5, líneas 1 a

4). Este documento revela de manera preferente una composición farmacéutica que comprende un sistema bicapa.



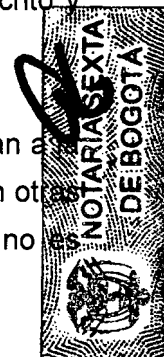
D1 menciona la artemisina y sus derivados tales como dihidroartemisina, arteéter, arteméter y artesunato, así como sus sales y solvatos (página 5, líneas 10 a 14).

Ejemplos de derivados de quinolina son cloroquina, mefloquina, amodiaquina, primaquina y sus sales y solvatos (página 6, líneas 1 a 4).

D1 no menciona o sugiere la ferroquina dentro de la lista de posibles derivados de quinolina. A este respecto, resulta relevante mencionar que la ferroquina es un compuesto organometálico específico (ver estructura (I) arriba).

D1 además no provee información o guía alguna para que la persona medianamente versada en la materia combine específicamente ferroquina con un derivado de artemisina, con el fin de obtener el efecto sinérgico descrito y analizado arriba.

En consecuencia, la información y/o sugerencias incluidas en D1 no llevan a la persona medianamente versada en la materia a la presente invención. En otras palabras, la combinación de los compuestos de la presente invención no es revelada o sugerida en D1.



- Documento D2: Blackie et al, Journal of Organometallic Chemistry, 2003: D2 investiga la síntesis de complejos del tipo $[\text{Au}(\text{L})(\text{PPh}_3)]\text{NO}_3$, $[\text{Au}(\text{C}_6\text{F}_5)(\text{L})]$ y $[\text{Rh}(\text{Cl})(\text{COD})(\text{L})]$, en donde la cloroquina (1) o análogos metálicos de cloroquina y derivados de ferrocenil entre los cuales se incluye ferroquina, identificados en la fórmula como (L) **son combinados con una porción de oro y rodio** (páginas 144 y 145).

Los tres derivados de ferrocenil empleados como ligandos **contienen ya un átomo metálico (Fe)** en su estructura. De este modo, la incorporación de una porción **de oro y rodio** durante la formación de los complejos, con los derivados de ferrocenil conduce a la presencia de **dos centros metálicos** en las moléculas (página 145).

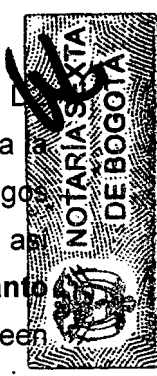


Las pruebas biológicas (tabla 1, página 150) se llevaron a cabo para medir la actividad antimalárica *in vitro* de los nuevos complejos de cloroquina y de derivados de ferrocenil sobre dos cepas sensibles a CQ (D10) y resistentes a CQ (K1) de *P. falciparum*. El objetivo de este estudio es entender si la presencia de los **dos centros metálicos** en la molécula **podría tener un efecto de adición, sinérgico o antagonista** sobre la actividad antimalárica.

D2 así, no provee información o guía alguna para que la persona medianamente versada en la materia combine específicamente ferroquina con un derivado de artemisina, con el fin de obtener un efecto sinérgico como el descrito y analizado arriba.

Adicionalmente, D2 está dirigido a la formación de complejos del segundo metal a los compuestos de ferrocenil 4-aminoquinolinas, entre los cuales se incluye ferroquina. En consecuencia, la persona medianamente versada en la materia, en vista de esta información se vería motivada a probar otras combinaciones metálicas de los derivados de ferrocenil.

En consecuencia, no se entiende cómo la combinación de los documentos D1 y D2 afecta el nivel inventivo de la presente solicitud, ya que la combinación de estos motivaría a la persona medianamente versada en la materia a obtener complejos de cloroquina o análogos metálicos de ésta y derivados de ferrocenil, con una porción de oro o rodio (comprendiendo a los dos centros metálicos) y a su posible unión con un derivado de artemisina. De otro lado, **tanto la combinación como la composición farmacéutica y el kit** de la presente invención proveen un efecto sinérgico (efecto técnico superior) tal como se demostró arriba. Dicho efecto sinérgico no se encuentra sugerido por el arte previo, de manera que resulta inesperado y es otro indicio de nivel inventivo, tal como señala el recientemente publicado INSTRUCTIVO EXAMEN DE SOLICITUDES DE PATENTES DE INVENCION Y MODELO DE UTILIDAD.



Conclusión

El documento D1, el documento D2 o su combinación, no resultan anterioridades válidas capaces de afectar el nivel inventivo de las composiciones farmacéuticas de la presente solicitud.

Por lo tanto, consideramos que el Examinador está pasando por alto el hecho de que el inventor en el momento de solucionar el problema técnico de la solicitud tuvo que emplear habilidad inventiva, pues la presente invención no resulta de la mera extrapolación de las enseñanzas del arte previo, como se afirma. Se está desconociendo el mérito investigativo empleado y que está aportando una ventaja al arte previo.

Dicho mérito ha sido reconocido por oficinas internacionales de patentes tales como la Australiana, quien ha otorgado la patente para una invención equivalente, patente No. AU AU2006238506 (B2).



Por todo lo anterior, consideramos que la presente solicitud cumple a cabalidad con los requerimientos de patentabilidad exigidos por la legislación actual, por lo que atentamente solicitamos en consideración lo siguiente:

VI. PETICIÓN

Por todo lo anterior, solicito a ese Despacho lo siguiente:

6.1 Que al considerar las razones precedentes se revoque la decisión contenida en la Resolución No. **19379** del 29 de marzo de 2012, en cuanto resuelve negar el privilegio de patente de invención.

6.2 Que se otorgue el privilegio de patente de invención para: **"ASOCIACIÓN ENTRE FERROQUINA Y UN DERIVADO DE ARTEMISINA PARA EL TRATAMIENTO DEL PALUDISMO"** a favor de **SANOFI-AVENTIS**.



VII. NOMBRE Y DIRECCIÓN

El nombre y la dirección del solicitante recurrente son los siguientes:

SANOFI-AVENTIS

174 Avenue de France,

F-75013,

Paris

Francia

VIII. NOTIFICACIONES

Atentamente manifiesto a usted que recibiré notificaciones en la Secretaria de su Despacho o en mi oficina de abogada situada en la Carrera 4ª. No. 72-35, piso 4º, de esta ciudad de Bogotá, D.C.

IX. ANEXOS

9.1 Yan *et al.* BMC systems biology. A formal model for analyzing drug combination effects and its application in TNF-alpha-induced NFkB pathway /2010) -4:50

9.2 Hegreness et al.. Proc Nat Acad Sci USA. Accelerated evolution of resistance in multidrug environments (2008) 105:13977-13981.

Señor Superintendente,

Luz Clemencia de Páez

LUZ CLEMENCIA DE PÁEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555



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No. M-2012-216409\JMS

NOTARIA SEXTA DE BOGOTÁ
RECONOCIMIENTO Y PRESENCIA PERSONAL
Bogotá, D.C. 04 MAY 2012
Ante mí AMPARO QUINTERO ARTURO,
NOTARIA SEXTA DEL CIRCUITO DE
BOGOTÁ, D.C.
Comparecieron

Quilones) exhibición) la(s) C.C.
35456344
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Y declaró(aron) que la(s) firma(s) que en el presente documento se(son) la(s) (a) (s) y que el contenido del mismo es cierto.
En constancia se firma esta diligencia.

Suz Clemencia Suárez
TP #23555 @55



RESEARCH ARTICLE

Open Access

A formal model for analyzing drug combination effects and its application in TNF- α -induced NF κ B pathway

Han Yan[†], Bo Zhang[†], Shao Li^{*} and Qianchuan Zhao^{*}

Abstract

Background: Drug combination therapy is commonly used in clinical practice. Many methods including Bliss independence method have been proposed for drug combination design based on simulations models or experiments. Although Bliss independence method can help to solve the drug combination design problem when there are only a small number of combinations, as the number of combinations increases, it may not be scalable. Exploration of system structure becomes important to reduce the complexity of the design problem.

Results: In this paper, we deduced a mathematical model which can simplify the serial structure and parallel structure of biological pathway for synergy evaluation of drug combinations. We demonstrated in steady state the sign of the synergism assessment factor derivative of the original system can be predicted by the sign of its simplified system. In addition, we analyzed the influence of feedback structure on survival ratio of the serial structure. We provided a sufficient condition under which the combination effect could be maintained. Furthermore, we applied our method to find three synergistic drug combinations on tumor necrosis factor α -induced NF κ B pathway and subsequently verified by the cell experiment.

Conclusions: We identified several structural properties underlying the Bliss independence criterion, and developed a systematic simplification framework for drug combination design by combining simulation and system reaction network topology analysis. We hope that this work can provide insights to tackle the challenging problem of assessment of combinational drug therapy effect in a large scale signaling pathway. And hopefully in the future our method could be expanded to more general criteria.

Background

Drug combination therapy is commonly used in clinical practice [1-3]. For example, herbal remedies in traditional Chinese medicine are believed to have synergism effect [4,5]. How to define the drug synergism has been a long-standing controversy amongst pharmacologists, toxicologists and biologists [6,7]. Among existing methods, under the assumption that two drugs acting by independent mechanisms, Bliss independence model is used to define combined effect of two drugs [8]; given that two similar drugs competitively acting on a target, Loewe additivity

model is used to predict the combined effect of two drugs [9]. Chou-Talalay further proposed the Combination Index (CI) theorem, serving as a general expression and quantification of drug interaction based on the mass-action law in biophysics and biochemistry [10-12]. These models are widely used in *in-vitro* and *in-silico* experiments for drug targets design and dose-response relationship analysis to instruct the selection of drugs and design of combination scheme [13-15]. Although improvements in the application scope and sensitivity of synergy evaluation techniques allow a greater exploitation of drug combination studies, it is unlikely that experimental techniques will be sufficient to completely screen the vast space of drug combinations in a cost-effective and timely manner. Hence, finding a way to delimit this space and obtain a manageable set of synergistic combinations is still an ongoing challenge.

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To meet this challenge, we present a new method developed from the original Bliss independence criterion to analyze the relationship between structures and effects for combinational drug targets design from a mathematical aspect. Since Bliss model is relatively simple and still widely used by some researchers recently [16-18], we start our studies from this simple model and operationally provide a combination effect assessment index inspired by the Combination Index theorem [10-12]. With a foundational property of this index, the structure information could be used to help the analysis of drug combinational effects and design of combiantional drug targets. Under this frame, we study two classic structures (serial and parallel structures) in biological signaling systems and propose simplification rules which are helpful for analyzing drug combination effects on the original system. Furthermore, analysis of the feedback structures, which is also very common in signaling pathways, is conducted as an expansion to an original structures without feedback. The usefulness of all the results is demonstrated by numerical experiments.

As a concrete example, we applied our method to an inflammatory angiogenesis-related pathway, the tumor necrosis factor α (TNF- α)-induced NF κ B pathway. The comprehensive research of this pathway has accumulated abundant experimental data. This allows us to construct a TNF- α -induced NF κ B pathway model. Here, we further extended previous model to endothelial cells to construct a more accurate model for drug efficacy prediction. With this new NF κ B model in hand, we simulated the combined effects of three important inhibitors, namely Aldehyde, Geldanamycin and PS-1145, in NF κ B pathway. The simulation results suggested that three inhibitor combinations yielded significant synergism and were validated the simulated results by cell experiments.

Methods

Original Bliss Independence Criterion

Bliss independence [8] or fractional product method [19] is the index for calculating the expected dose-response relationship for drug combination therapy as compared to mono-therapy. It assumes that the two inhibitors act via independent mechanisms. Then drug combination can be represented as the union of two probabilistically independent events. And this criterion is identical to the mutually non-exclusive case [20]. The combined effect of two inhibitors (F_{UA}) is computed as the product of individual effects of the two inhibitors, F_{UA1} and F_{UA2} .

$$F_{UA} = F_{UA1} \times F_{UA2} \quad (1)$$

where F_{UA} is the remaining enzyme activity (fractional unaffected).

Based on the definition, F_{UA} is the expected combined effect. If the actual combined effect of the two inhibitors is equal to F_{UA} , it is additive effect case and there is no interaction between the two inhibitors. If the actual combined effect is lower than F_{UA} , it is called antagonism. If the actual combined effect is higher than F_{UA} , it is called synergism which leads many possible favorable outcomes like increasing or maintaining drug efficacy as decreasing dosage and provides foundations to the combination therapy [20].

Survival ratio

We use survival ratio as representation of the effect (fractional unaffected) and define it as the ratio of component concentrations before and after intervention:

$$r(a, b) = \frac{\text{output}(a, b)}{\text{output}(a_0, b_0)} \quad (2)$$

where a, b are parameters that could be affected. Often they have relationship with the inhibitor doses. a_0 and b_0 represent the normal values, which are the values before being inhibited; a and b represent the values after being inhibited. The output of a system is usually defined as the concentration of some components.

Inspired by the Combination Index theorem offered by Chou-Talalay [10,11], here we introduce an operational concept, "Synergism Assessment Factor", for addressing the interaction of drug combination. Then the Bliss independence criterion could be rewritten as:

$$S = r(a, b) - r(a_0, b) \cdot r(a, b_0) \quad \begin{cases} < 0 & \text{Synergism} \\ = 0 & \text{Additive effect} \\ > 0 & \text{Antagonism} \end{cases}$$

Where $r(a, b)$ is the actual combined effect and $r(a_0, b) \cdot r(a, b_0)$ is the expected combined effect calculated by the product of individual effects. S denotes Synergism Assessment Factor. Eq. 3 is identical to the fraction product equation of Webb [19] and the mutually non-exclusive case in [21]. Compared with the critical point ($CI = 1$) of Chou-Talalay's Combination Index, we used $S = 0$ as the critical point to determine whether there is synergistic effect. Under the first order mutually non-exclusive case, using Eq.3 will get the same conclusion on combination effect as using Combination Index.

Survival ratios of individual intervention and combined intervention can be measured through *in-vitro* or *in-silico* experiments, so it is convenient to verify whether synergism is generated under specific drug combination with this criterion [22]. Therefore, it is widely used in combination therapy design [16-18]. However, it is hard to pre-

dict the combined effect of two inhibitors without experiments according to this model itself. In order to predict the proper dose range to generate synergism, we have to gain the dose-response relationship. The dose-response relationship could be get through series of *in-vitro* experiments costly under different doses. Sometimes the dose-response relationship is assumed to have some special form like Hill equation to reduce the experiment costs [22]. It is feasible when possible targets number is small. As the targets number increases, it will face the combinatorial explosion to choose targets and proper doses, and the experiments cost also increases. New method to narrow down the possibilities in searching targets and doses generating synergism by experiments needs to be developed.

Extended Bliss Independence Criterion

Here, we extend Bliss independence criterion with the sensitivity information of Synergism Assessment Factor. The system with some special structures could be simplified for synergism-generating targets with doses based on this criterion. We define:

$$DS = \frac{\partial^2 r(a,b)}{\partial a \partial b} - \frac{\partial r(a,b_0)}{\partial a} \frac{\partial r(a_0,b)}{\partial b}, \tag{4}$$

where, $r(a, b)$ is the survival ratio of the system; a and b are parameters affected by inhibitor (usually the reaction velocity constants [13]), a_0 and b_0 are separately the value of unaffected. Here DS denotes Synergism Assessment Factor Derivative. Actually DS is the second-order partial derivative of S . Our criterion is stated as

$$DS \begin{cases} < 0 & \text{Synergism} \\ = 0 & \text{Additive effect} \\ > 0 & \text{Antagonism} \end{cases} \tag{5}$$

This criterion is based on the following observations. The connection between DS and S is:

$$S = \int_{a_0}^a \int_{b_0}^b DS db da \tag{6}$$

It is easy to see that if $DS < 0$ (for the interested drug dose ranges), then S is guaranteed to be smaller than 0. It means that synergism is generated over the parameter ranges (a_0, a) , (b_0, b) . Similarly, the condition to generate antagonism is also intuitive.

This extended Bliss independence criterion could be seen as the derivative form of the original Bliss independence criterion.

Fundamental property of synergism assessment factor derivative

The extended Bliss independence criterion introduced in Eq. 5 enables us to find special structures of systems that simplification is possible for synergism-generating targets with doses. Here we provide a basic property of the synergism assessment factor derivative DS . It is the foundation for our main results.

If the inhibitors individually affect some intermediate processes, then the inhibition on these processes could be taken as directly on the products of these processes. That is to say, $x = \Phi(a)$ is the product of the process where parameter a is affected; $y = \Psi(b)$ is the product of the process where parameter b is affected. Then the inhibition effects to the output of system on a and b could be taken as on x and y . The analysis of combination effects on the original system could then be limited on a simplified system with x and y as parameters.

Lemma 1

a and b are system parameters that will be affected by inhibitors. a is of processes that will produce product x ($x = \Phi(a)$), and b is of processes that will produce product y ($y = \Psi(b)$). Then the synergism assessment factor derivative DS of the original system satisfies

$$DS = DS' \frac{\partial x}{\partial a} \frac{\partial y}{\partial b}$$

Where $DS' = \frac{\partial^2 r(x,y)}{\partial x \partial y} - \frac{\partial r(x,y_0)}{x} \frac{\partial r(x_0,y)}{\partial y}$, $x = \Phi(a)$, $y = \Psi(b)$, $x_0 = \Phi(a_0)$, $y_0 = \Psi(b_0)$. Details of proof are given in Additional file 1.

DS is the synergism assessment factor derivative of the original system while DS' could be seen as that of a simplified system. Usually the signs of derivatives $\frac{\partial x}{\partial a}$ and $\frac{\partial y}{\partial b}$ are easy to know, then the sign of DS could be decided on the sign of DS' ; and we only need to analyze the sign of DS' , which is the combination effect of the simplified system. Meanwhile, with the sensitivity information of intermediate process to inhibitors, we can compare the DS values of different inhibitors that have the same structure properties and select proper drug combinations with synergism property.

Methods for the case study of TNF- α -induced NF κ B pathway

Model construction and drug selection (see details in Results and Discussion)

We constructed the TNF- α -induced NF κ B pathway in Human umbilical vein endothelial cell (HUVEC). The model was developed based on literatures [23-26]. We tweaked the parameter values in terms of the experimental data derived from HUVEC [27]. The new pathway model yielded a better simulation of NF κ B activation in HUVEC. The details of Ordinary Differential Equations (ODEs) model of the NF κ B pathway could be found in the Additional file 2.

According to simulation results, we made a short list of NF κ B activity inhibitors covering three key nodes in the pathway including Proteasome Inhibitor II Aldehyde, HSP90 inhibitor Geldanamycin and IKK- β inhibitor PS-1145 (Additional file 3, Table S5). To determine the doses of these inhibitors in our experiment, we refer to the relative IC50 values of these inhibitors taken from the published experimental and clinical data [28-30]. In our model, Intercellular cell adhesion molecule-1 (ICAM-1) is very sensitive to TNF- α stimulation. It is directly regulated by activated NF κ B and become output index of downstream of this pathway.

In the simulation, the changes on relative reaction velocity constants were taken as the inhibition influence on the targets [31,32]. According to Lemma 1, we simplified the system and considered the synergism assessment factor on this simplified system. Through simulations with changing the reaction velocity constants in a wide range that responded to a wide dose range, the drug combination effects of the simplified system were gained. Additional file 3 provides a detailed description of the simulation algorithm.

Cell culture and reagents

To evaluate the computational results of our method, we conducted a cell experiment as follows. HUVECs were isolated from freshly obtained human umbilical cords by established methods [33]. The cells were grown onto gelatin-coated 10 cm² culture dishes in a standard endothelial cell medium (ECM) (ScienCell Research Laboratories). ECM consists of 500 ml of basal medium, 25 ml of fetal bovine serum (FBS), 5 ml of endothelial cell growth supplement (ECGS) and 5 ml of penicillin/streptomycin solution (P/S). Cells used for this study were from passages 4 to 8 in ECM at 37°C in a 5% CO₂/humidified air incubator and starved for 6 hours in 0.1% FBS medium before each assay. All experiments were carried out when the cells were 80% confluent. Proteasome Inhibitor II Aldehyde and HSP90 inhibitor Geldanamycin were purchased from Alexis Biochemicals (San Diego, CA). IkB kinase (IKK- β) inhibitor PS-1145 was from Sigma-Aldrich (St. Louis, MO). These inhibitors were

dissolved in DMSO and stored at -20°C until use. The recombinant human TNF- α (rh-TNF- α) protein was purchased from Cell Signaling Technology Inc. (CST, Beverly, MA). Antibodies to ICAM-1 and β -actin were obtained from CST.

Western blotting

To detect the effect of three combinations on output index of this pathway, ICAM-1 directly regulated by NF κ B was investigated by western blotting as follows. HUVECs were treated with 100 nM Aldehyde, 100 nM Geldanamycin, 100 nM PS-1145 and various combinations of Aldehyde, Geldanamycin and PS-1145 at the dose of 100 nM for 2 hours followed by 10 ng/ml TNF- α . After 6 hours of treatment, whole-cell extracts from treated cells and immunoblotting were prepared as previously described [34]. Whole cell lysates were subjected to SDS-PAGE 10% gels. Proteins were transferred to nitrocellulose blotting membranes (Bio-Rad Laboratories, Hercules, CA), and immunoblotted 4°C overnight with anti-ICAM-1 and anti- β -actin Abs (typically 1:1,000 dilution) followed by secondary antibody conjugated with horseradish peroxidase (1:10,000 dilution). The SuperSignal[®] West Dura (Thermo Fisher Scientific, Rockford, Ill, USA) was used for detection according to the manufacturer's instruction.

Results and Discussion

Most signaling pathways are constructed from three types of structures - serial, parallel and feedback, so our study will be focusing on these three structures. Based on Lemma 1, the simplifications of serial structures and parallel structures are studied. We also analyze the influence of feedback structure on serial structures. Numerical examples are also given; the simulation results of both original and simplified systems showed potency of the method on analyzing the combination effects. All these structures and figures are from Fitzgerald's work [1].

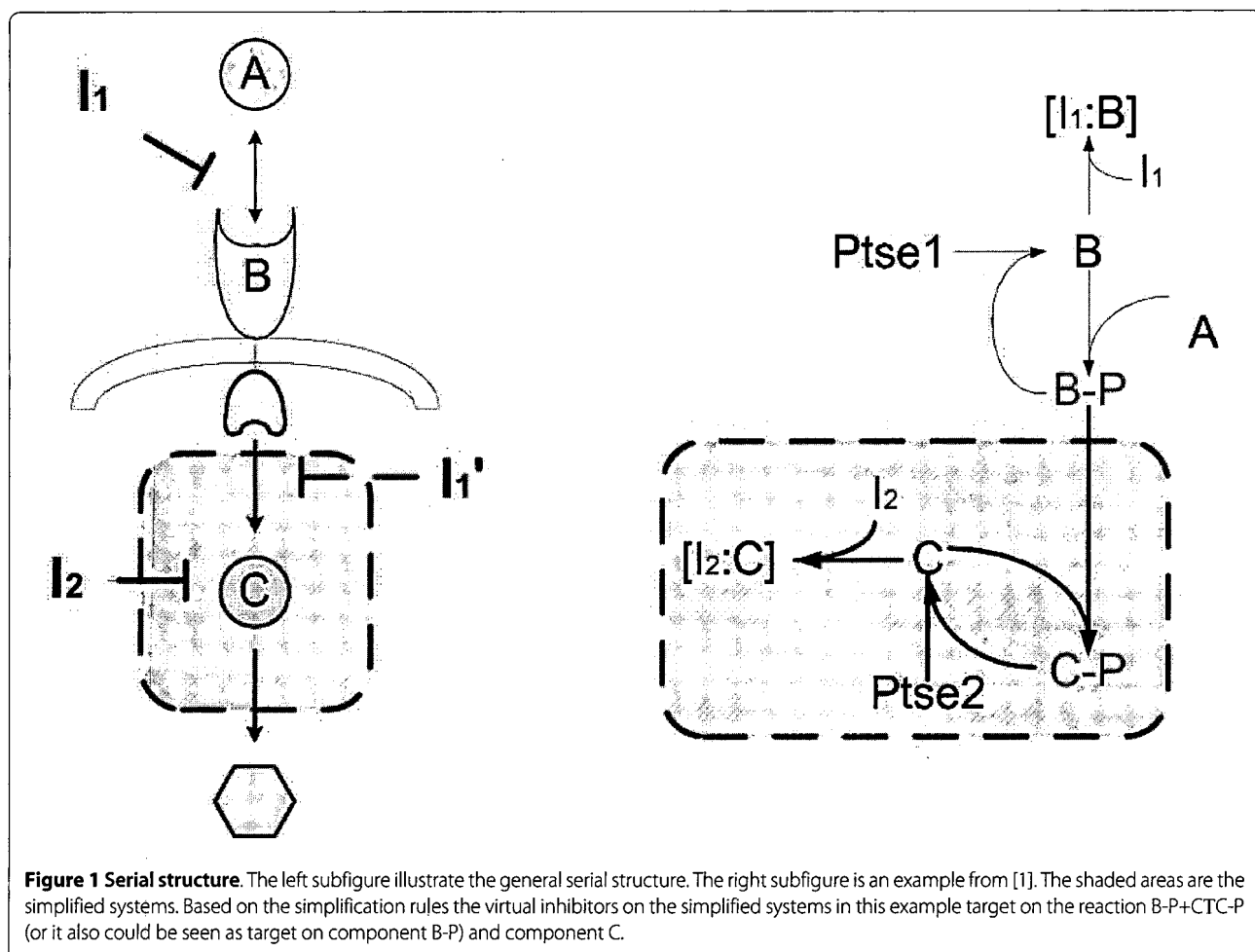
Simplification rule for serial structure

In Figure 1, A activates B, then B activates C. The concentration of activated C is the output of this system. The sequential activation processes construct a typical serial structure. To illustrate the drug combination effects, we designate that I₁ and I₂ are inhibitors affecting the two activation processes separately.

Corollary 1

The sign of the synergism assessment factor derivative DS of original serial structure in Figure 1 is opposite to the sign of the synergism assessment factor derivative DS' of the simplified structure (shaded area in Figure 1). That is to say,

$$\text{sign}(DS) = -\text{sign}(DS')$$



Simplification rule for parallel structure

In Figure 2, there are parallel activation processes: A_1 activates B_1 , A_2 activates B_2 . As in serial structure, I_1 and I_2 are inhibitors affecting those two activation processes separately. Both B_1 and B_2 can activate C . The output of this system is the concentration of activated C . The relation between B_1 and B_2 could be demonstrated as logic OR.

Corollary 2

The sign of the synergism assessment factor derivative DS of original parallel structure in Figure 2 is the same as the sign of the synergism assessment factor derivative DS' of the simplified structure (shaded area in Figure 2). That is to say,

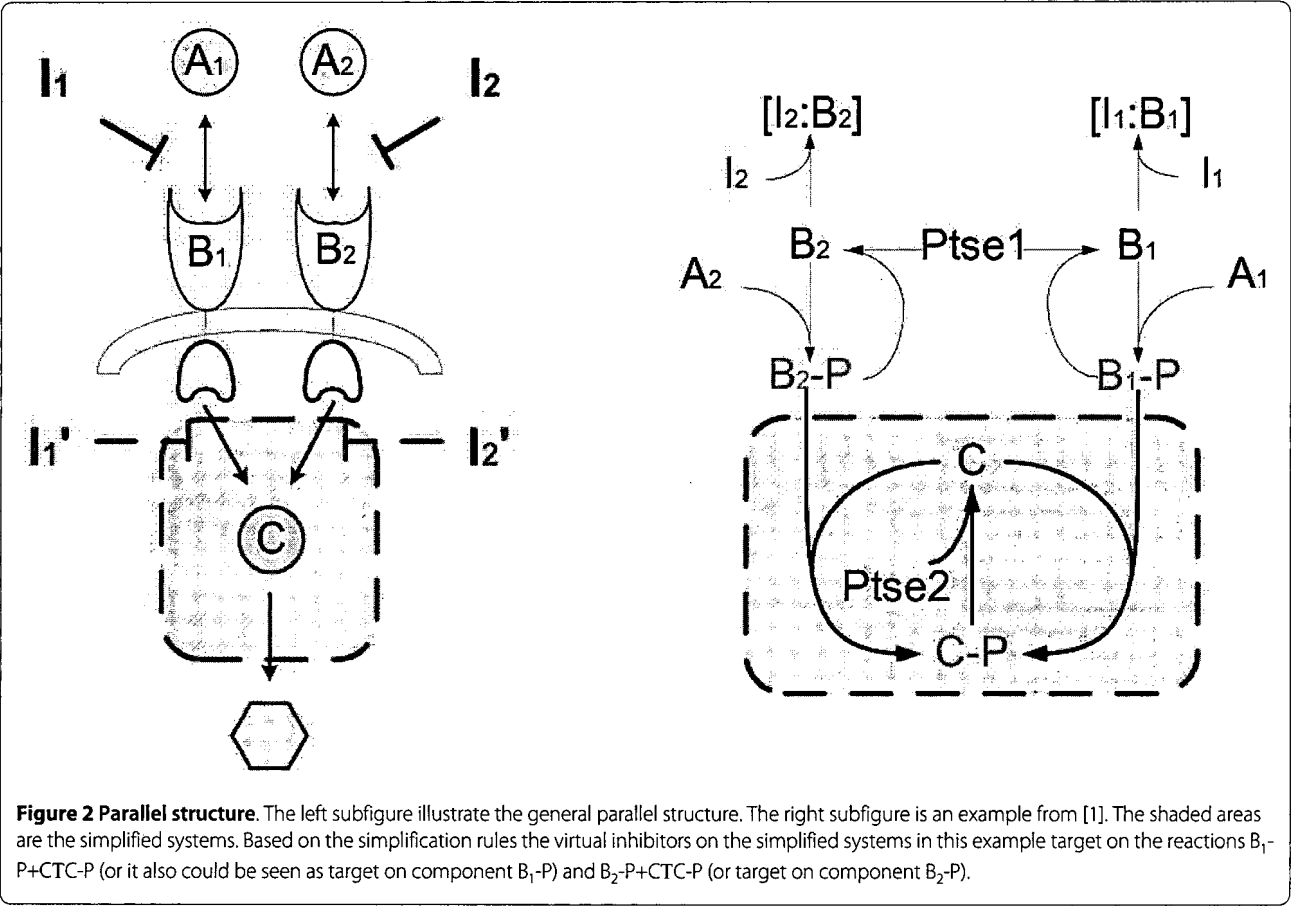
$$\text{sign}(DS) = \text{sign}(DS')$$

By Corollary 1 and 2, whether the simplified systems could generate synergism under equivalent inhibition can help to determine whether the original systems can generate synergism effect under actual inhibition (see details

of proofs in Additional file 1). In Figure 1, the original serial structure under I_1 and I_2 inhibition can be simplified as the system (shaded) under equivalent I_1' and I_2 inhibition from the view of combination effects; It is the same to simplify the original parallel structure under I_1 and I_2 inhibition as a system under equivalent I_1' and I_2 inhibition. This could simplify the system structures, meanwhile simplify the drug combination analysis. Besides, it becomes easier to find drug combinations that could generate synergism on the systems based on these conclusions.

Combination effect preservation rule for systems with feedback

Feedback structures are common regulatory structures in biological systems, especially in signaling pathways. Positive and negative feedback structures could shape the signaling responses in time and space [35], like performing as oscillators or bistable switches. Feedback structures increase the complexity of system structures, and make it more difficult to analyze the drug



combination effects on the systems. From the viewpoint of drug combination therapy, sometimes we just need to know how the feedback structures influence the drug combination effects.

Considering an original system without feedback loop that can generate synergism effect under some drug combinations (left subfigure in Figure 3), if after adding a feedback loop (right subfigure in Figure 3), the output of the new system decreases compared to that of original system, then the feedback loop can be believed that it strengthens the drug combination effects. Upon that, it could only consider combination effect analysis on the original system.

In general feedback structures (as shown in right subfigure in Figure 3) and the system without the feedback loop can be modeled by Ordinary Differential Equations (ODEs) separately as

Feedback structures	System without feedback
$\dot{x}^* = F(R) + g(\gamma^*)$	$\dot{x} = F(R)$
$\dot{\gamma}^* = f(x^*)$	$\dot{\gamma} = f(x)$

Lemma 2

Adding feedback loop to a system will not decrease the drug combination effects of the original system if

$g \cdot \frac{\partial f}{\partial x} < 0$. That is,

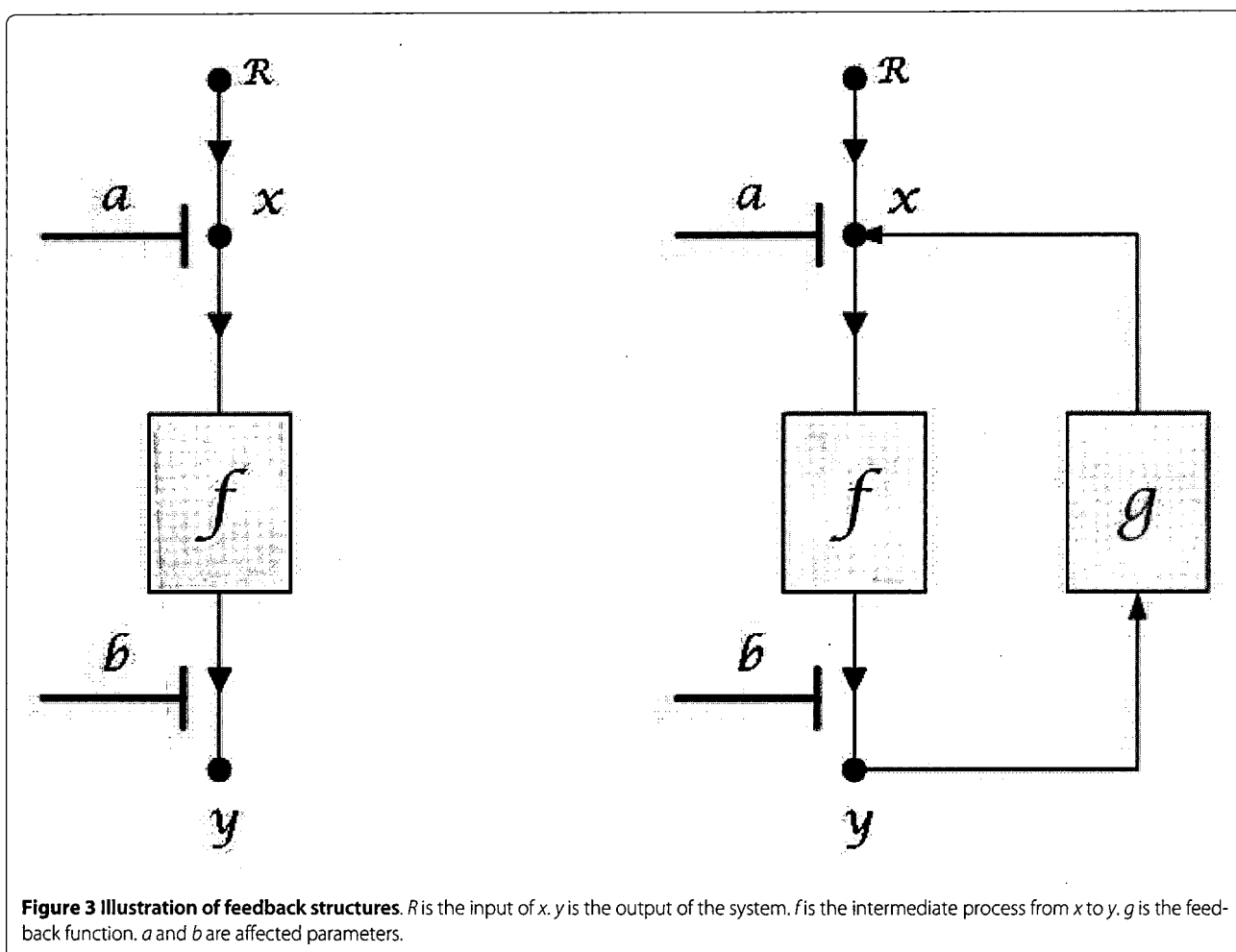
- 1) Negative feedback ($g(\cdot) < 0$), and $\frac{\partial f}{\partial x} > 0$
- or
- 2) Positive feedback ($g(\cdot) < 0$), and $\frac{\partial f}{\partial x} < 0$ is satisfied.

Details of proof are given in Additional file 4.

Examples of serial structure and parallel structure

We adapted numerical examples in [1] as shown in Figure 1 and Figure 2 respectively to verify the Corollary 1 and 2 above. All parameters are referred to [1]. In addition, all the reactions are modeled as Michaelis-Menten equations. Direct evaluation of the synergism assessment factor S by simulation leads to the results shown in Figure 4.

In Figure 4, left figure shows the results of serial structure and right figure shows the results of parallel structure. The blue curves referred to the S curves recovered from results of the simplified systems, while the red lines referred to the results of the original systems. It is obvious



that the recovered *S* curves agreed with the original *S* curves. Actually, the *S* curve of the simplified serial system has opposite sign to that of the original serial system.

S curves with negative signs indicate that both the original serial and parallel structures generate synergistic effect. In left subfigure the absolute values of *S* are smaller (10^{-3} order) than those of the *S* in right subfigure (10^{-1} order). Combination therapy of the parallel structure could be more effective. These results are coincident with the results in [1]. As a comparison from these results, the new method enables us to evaluate the combination effects of original systems by analyzing the effects of the corresponding simplified systems.

An example of negative feedback structure

Figure 5 from [1] shows the example of negative feedback structure. This negative feedback system is constructed based on the serial system in Figure 1. From the results in [1] the influence of feedback on combination effects is verified to have nonnegative impact under the condition 1) in Lemma 2.

Based on the ODEs of the serial structure, the ODEs of this feedback structure are as follows:

$$\begin{aligned}\dot{x}_1 &= \frac{k_1 R(x_{1t} - x_1)}{K_{m1} + x_{1t} - x_1} - \frac{V_1 x_1}{K_{m3} + x_1} - \frac{V_3 x_2 x_1}{K_{m5} + x_1} \\ \dot{x}_2 &= \frac{k_2 x_1 (x_{2t} - x_2)}{K_{m2} + x_{2t} - x_2} - \frac{V_2 x_2}{K_{m4} + x_2} \\ a &= K_{m1} \\ b &= K_{m2}\end{aligned}$$

In the model, x_1 and x_2 are concentrations of B-P and C-P (activated C) respectively. x_2 is the output of the system. x_{1t} and x_{2t} are initial concentrations of B and C respectively. *R* is the concentration of A. K_{m1} and K_{m2} are Michaelis-Menten constants of the BTB-P and CTC-P activation and are affected by I_1 and I_2 , respectively. K_{m5} are Michaelis-Menten constants of B-PTB activation mediated by C-P. From the simulation results in [1], it is obvious that the output of feedback structure example is smaller than the output of serial structure. This compari-

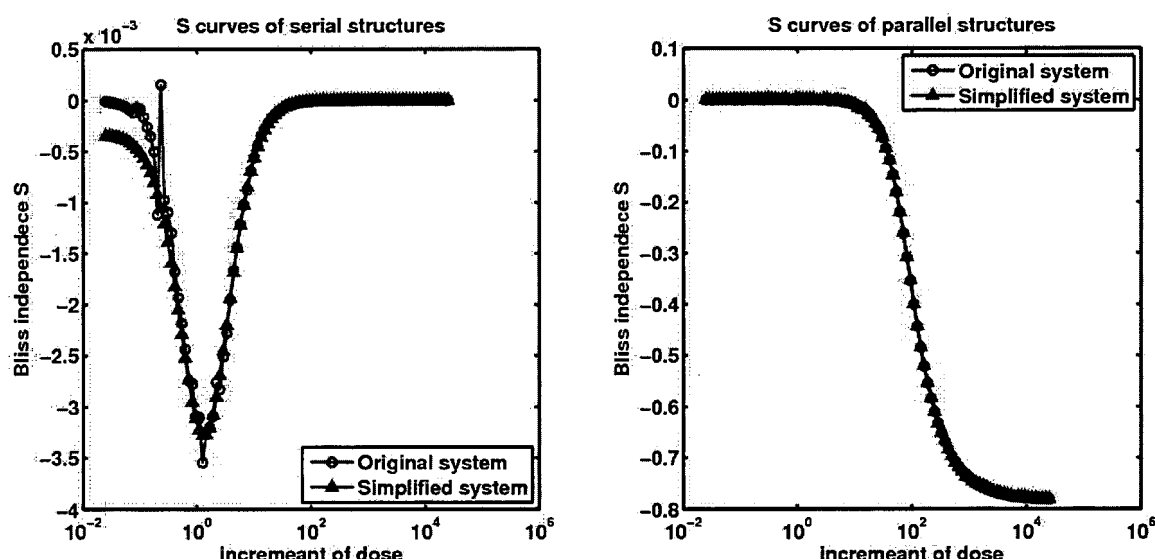


Figure 4 S curves of Figure 1 and 2. In the simulation, we adopted the same doses of both inhibitors like [1]. The left subfigure is the simulation results of the serial structure in Figure 1. The right subfigure is the simulation results of the parallel structure in Figure 2. The S curves of original systems are in red. The blue curves are the S curves recovered from the results of simplified systems based on Lemma 1 and the two corollaries. In fact the S curve of the simplified serial system has opposite sign to that of the original serial system. Obviously the S curves recovered agreed with the original S curves. The signs of S curves are negative showing the synergistic effect of the serial and parallel structures. In left subfigure the absolute values of S are much small (10^{-3} order) to those of the S in right subfigure (10^{-1} order). Combination therapy of the parallel structure could be more effective. These results are coincident with the results in [1].

son verified Lemma 2 that feedback loop can strengthen the drug combination effects.

It should be pointed out, although our method leads to simplified systems, that does not mean the analysis of the simplified systems is easy. Usually simulations or experiments are still needed for analyzing these systems due to their complex dynamics [14,36-38]. Our method also relies on the faithful modeling of the systems which may not be trivial since identifying system structure in general is still a challenging work [39-41].

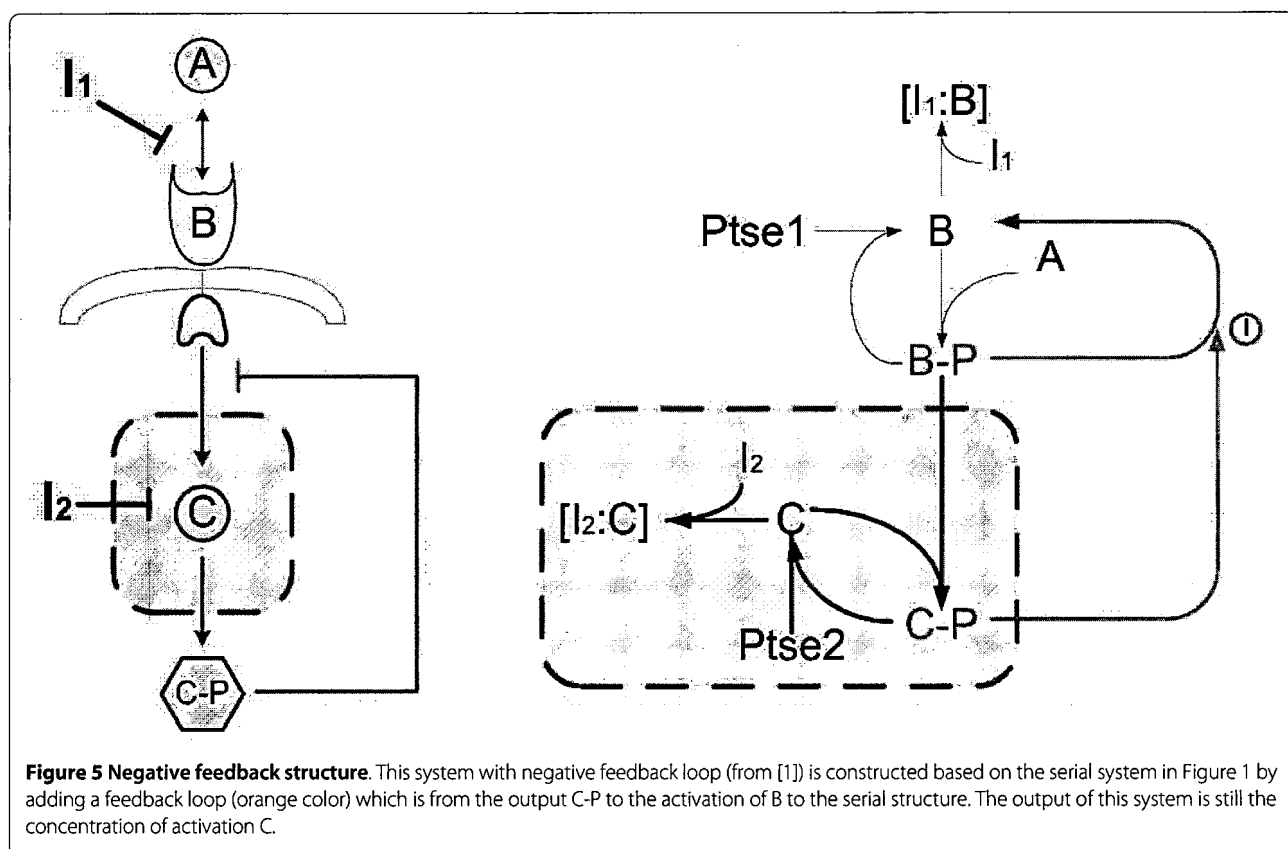
Results and discussion for the case study of TNF- α -induced NF κ B pathway

TNF- α is both a pro-inflammation cytokine and a pro-angiogenic factor [42]. It is responsible for inflammatory angiogenesis and tumorigenesis. The induction of NF κ B signaling pathway by TNF- α can regulate the transcriptional expression of several genes in vascular endothelial cells that lead to angiogenesis [42]. Several standard drug targets such as HSP90 and IKK- β are among the key molecules involved in the pathway responsible for generating angiogenic factors. However, essentially all single-target inhibitors have low therapeutic effects in inflammatory and angiogenic diseases. To find more efficient drug combination solutions, we constructed the model of an inflammatory angiogenesis-related pathway, the TNF- α -induced NF κ B pathway (Figure 6A) and employed our

method in this paper to search for synergistic drug combination solutions within this pathway model.

As a result of our method, I κ B degradation proteasome, HSP90 and IKK- β as important targets in the TNF- α -induced NF κ B pathway are selected. In the simulation, changes on the relative reaction velocity constants were taken as the inhibition influence on targets. It should be pointed out that Geldanamycin as a HSP90 inhibitor dose not target RIP1 directly but could indirectly regulate the binding rate of RIP1, so we changed the reaction velocity constants of RIP1 binding with TNFR1 complex. The change ratio of the reaction velocity constants ranged from 0.9 to 0.0001 fold to cover a wide dose range.

As shown in Figure 6B, the TNF- α -induced NF κ B pathway has a serial structure with feedback. The three targets locate on the serial path (RIP1 and IKK- β) and feedback path (I κ B degradation), especially IKK- β is on the joint of serial path and feedback path. Since TRAF2-RIP1-TRADD-TNFR1-TNF- α activates IKK- β , the inhibition effect on RIP1 could be seen as finally acted on IKK- β . According to Lemma 1, the system could be simplified as the dashed box area in Figure 6A. The corresponding reactions inhibited by the three inhibitors in original and simplified systems were shown in Table 1. We then employed our method to calculate the synergism assessment factor on the simplified system. The sign of synergism assessment factor decides whether there is



synergistic effect or not. The value of synergism assessment factor reflects the efficacy extent of drug combination from Aldehyde, Geldanamycin and PS-1145 (shown in Figure 6B). We screened out the synergistic drug combinations according to the sign of synergism assessment factor in simplified system. Then we did the simulation on the original system for the synergistic drug combinations. The simulation results are shown in Table 2. It shows that all the three drug combinations could generate synergistic effect. And the signs of synergism assessment factors in simplified system were consistent with those of the synergism assessment factors in original system.

ICAM-1, an intercellular adhesion molecule expressed in endothelial cells, is a common cellular readout of TNF- α induced signaling pathway [43]. To verify the simulation results, we observed the effect of Aldehyde, Geldanamycin, PS-1145 and related combinations on ICAM-1 expression level by western blotting analysis. In the experiment, we used Bliss independent model to assess whether three combinations induced synergistic inhibition effect on ICAM-1 expression. As shown in Figure 6C and Figure 6D, all the three drug combinations generate synergistic effect. Taken together, this result suggests that the synergistic combinations predicted by our method are qualitatively consistent with the experimental observations. Currently, the method can only make qualitative

prediction on drug combinational effects. This still could provide some clues for drug combination design based on mechanisms. Besides, as the model we provided here is specific for TNF- α -induced NF κ B pathway in HUVEC, the general applicability of our method still needs further investigation.

Conclusions

In this paper we presented a new method based on an extended Bliss independence criterion to analyze the relationship between structures and effects for combination drug targets design from a mathematical aspect. We analyzed two classic structures, serial structure and parallel structure, and showed in steady state the sign of the synergism assessment factor derivative of the original system can be predicted by the sign of its simplified system. In addition, we analyzed the influence of feedback structure on survival ratio of the system, and showed that the feedback structure could not destroy the drug combination effect of the system without feedback under some conditions. We demonstrated by numerical examples that these results are useful for reducing the amount of computational load if system reaction network topology knowledge is available. In the case study, the effects of inhibitor combinations predicted by our method were experimentally validated by measuring the output (ICAM-1 expression) of TNF- α -induced NF κ B pathway.

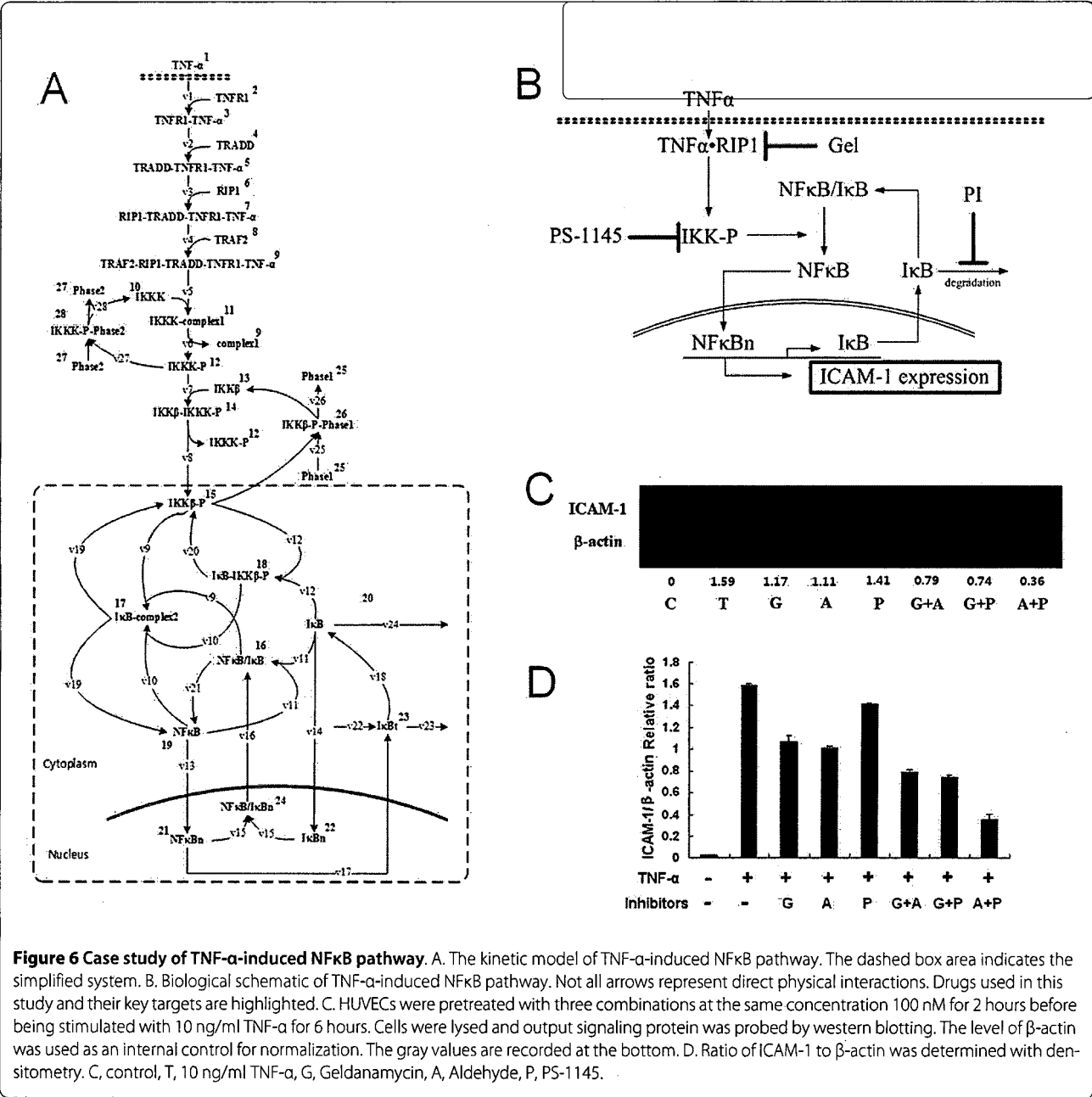


Table 1: Comparison of reactions inhibited by drugs in original and simplified systems.

Inhibitors	Corresponding reactions in original system	Corresponding reactions in simplified system
Geldanamycin	RIP1 binding with TNFR1 complex	IKKK-P activates IKK-β
PS-1145	IKK-β-P phosphorylates IκB	IKK-β-P phosphorylates IκB
Aldehyde	IκB degradation	IκB degradation

In the simulation, we simplified the system according to Lemma 1. Here we list the corresponding reactions inhibited by the three inhibitors in original and simplified systems.

Table 2: Simulation and experimental results for the case study of TNF-α-induced NFκB pathway.

Inhibitor A	Inhibitor B	Synergism Assessment Factor (Simulation)		Predicted interaction	Synergism Assessment Factor (Experiment)
		simplified system	original system		
Geldanamycin	Aldehyde	-0.0632	-0.2678	Synergism	-0.0167
Geldanamycin	PS-1145	-0.1248	-0.2555	Synergism	-0.1878
PS-1145	Aldehyde	-0.1754	-0.1754	Synergism	-0.3931

Here we consider the synergism assessment factors of the three combinations in the simplified and original system. The sign of synergism assessment factor indicates whether the drug combination generates synergistic effect or not. If the sign is negative, it is synergistic effect. Otherwise, it is antagonistic effect. In the experiment, ICAM-1 expression is considered as the measurement readout of the efficacy of drugs or drug combinations. We also evaluate the effects of drug combination by calculating synergism assessment factor (see Eq. 3). The negative values represent synergism since we rewrite calculation formula of Bliss independence model according to survival ratio rather than inhibition ratio.

Hopefully, this work can provide some insights to tackle the challenging problem of assessment of combination drug therapy effect in a large scale signaling pathways. As we point out, Bliss model is relatively simple, so in this paper we focused on this simple model. With deep understanding of dose-effect curves, we hope in the future our method could be expanded to more general criteria such as the law of mass action [10-12,21].

Note

In the following figures (Figure 1, 2, 3, 4, 5), A, B, C, A₁, A₂, B₁, B₂ are components of the systems, I₁ and I₂ are inhibitors. The shaded areas represent simplified systems for the original systems. I₁ and I₂ are virtual inhibitors on the simplified systems equivalent to I₁ and I₂.

Additional material

Additional file 1 Proof of system simplification rules. Proof of Lemma 1 (fundamental property of synergism assessment factor derivative), Corollary 1 (simplification rule for serial structure) and 2 (simplification rule for parallel structure).

Additional file 2 Summary of the model of TNF-α-induced NFκB pathway. Tables S1 - S4 list details of the mathematical model of TNF-α-induced NFκB pathway including reactions and rate equations, initial components concentrations, kinetic parameters and ordinary differential equations.

Additional file 3 Summary of algorithm for TNF-α-induced NFκB pathway. Implementation of the algorithm for TNF-α-induced NFκB pathway.

Additional file 4 Proof of influence of feedback. Proof of Lemma 2 for influence of feedback structure to original serial system based on comparison principle that is used to compute bounds on solutions of differential equations.

Authors' contributions

HY: study conception, research design, mathematical analysis, manuscript writing; BZ: model constructing, experimental study, manuscript writing; SL: directed the design of the model constructing, experimental study, study con-

ception, helped with the bioinformatics analyses, participated the revision of the manuscript; QZ: formulated and directed the design of the study. All authors read and approved the final manuscript.

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Accelerated evolution of resistance in multidrug environments

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The emergence of resistance during multidrug chemotherapy impedes the treatment of many human diseases, including malaria, TB, HIV, and cancer. Although certain combination therapies have long been known to be more effective in curing patients than single drugs, the impact of such treatments on the evolution of drug resistance is unclear. In particular, very little is known about how the evolution of resistance is affected by the nature of the interactions—synergy or antagonism—between drugs. Here we directly measure the effect of various inhibitory and subinhibitory drug combinations on the rate of adaptation. We develop an automated assay for monitoring the parallel evolution of hundreds of *Escherichia coli* populations in a two-dimensional grid of drug gradients over many generations. We find a correlation between synergy and the rate of adaptation, whereby evolution in more synergistic drug combinations, typically preferred in clinical settings, is faster than evolution in antagonistic combinations. We also find that resistance to some synergistic combinations evolves faster than resistance to individual drugs. The accelerated evolution may be due to a larger selective advantage for resistance mutations in synergistic treatments. We describe a simple geometric model in which mutations conferring resistance to one drug of a synergistic pair prevent not only the inhibitory effect of that drug but also its enhancing effect on the other drug. Future study of the profound impact that synergy and other drug-pair properties can have on the rate of adaptation may suggest new treatment strategies for combating the spread of antibiotic resistance.

adaptation | antagonism | synergy | antibiotics | antibiotic resistance

Challenged by rapid emergence of drug-resistant pathogens and limited supply of new antibiotics, clinicians increasingly rely on multidrug treatments to combat infections (1–7). When drugs are applied together the effect of a drug can depend on the presence or absence of the other drug. Such interactions between drugs are classified as additive, synergistic, or antagonistic depending on whether their combined effect on bacterial growth is equal to, greater than, or less than expected based on the inhibitory abilities of the individual drugs (8, 9). Two main goals of drug treatment are stopping bacterial growth and preventing the evolution of drug resistance (5, 10–12). Although synergistically interacting drugs are often favored because of their greater combined ability to inhibit growth (13), little direct evidence for their ability to suppress the evolution of resistance exists, and some studies even suggest the contrary (14–18).

Examining the evolution of resistance in the context of a simple geometric model of drug–drug fitness landscapes, we find that mutations conferring full or partial resistance to one of the individual drugs may be more beneficial to bacteria in synergistic than in antagonistic drug treatments [Fig. 1; the situation for mutations conferring simultaneous resistance to both drugs is illustrated in supporting information (SI) Fig. S1]. This deduction is based on the simplifying assumption, demonstrated in previous work, that mutations conferring resistance to a single drug are effectively equivalent to a reduction in that drug's concentration (16, 17, 19, 20). When drugs amplify each other's effects (synergy) (9), this effective reduction in the concentration

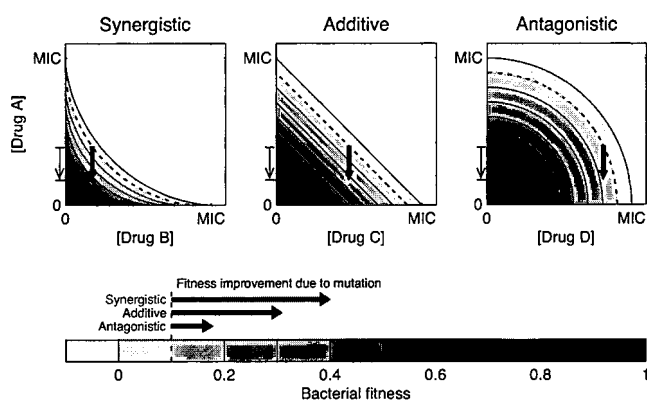


Fig. 1. A simple geometric model shows that a mutation conferring resistance to a single drug is most advantageous in a synergistic drug combination. Shown are isoboles, or lines of equal bacterial growth rate, in the plane of concentrations of drugs A with either drug B (where B interacts with drug A synergistically), drug C (additively), or drug D (antagonistically). The arrows shown on the isobolograms for the three types of interaction all correspond to the exact same mutation (indicated by a thin arrow along the axis of drug A's concentration), which confers partial resistance to drug A by reducing the effective concentration of drug A felt by the resistant mutant. The three arrows' origins represent environments that have the same initial concentration of drug A and the same fitness inhibition (10%, dotted line). Although the mutation changes the effective concentration of drug A by the same amount in all environments, the fitness gain conferred by the mutation is greatest in the synergistic case (it crosses more fitness contour lines).

of one of the drugs not only relieves the effect associated with that drug but also reduces its enhancing influence on the other drug. In contrast, when drugs partially inhibit one another (antagonism), resistance mutations that remove some of the effect of one of the drugs will actually reveal the previously suppressed effect of the other drug (Fig. 1). This scenario has recently been experimentally observed for a hyperantagonistic drug pair in which horizontally transferred alleles that confer resistance to one of the drugs can actually be deleterious in the combined drug environment (17). This intuition applies whether the starting concentration is above or below an organism's minimal inhibitory concentration (MIC). Such expected differences in the selective advantage of resistant mutants in two-drug

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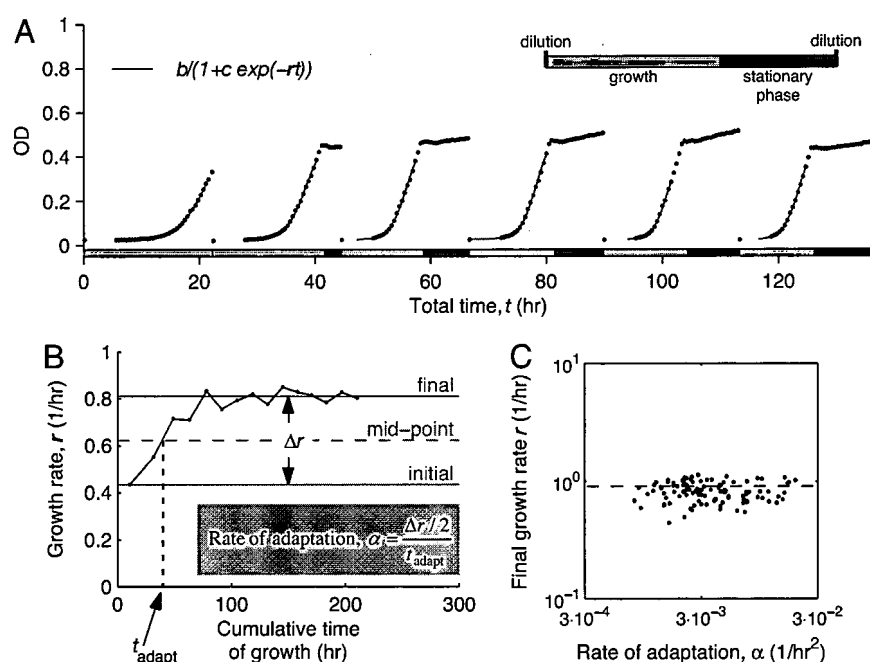


Fig. 2. Parallel quantitative measurement of the rate of adaptation in multidrug environments. (A) Example of growth in one particular dosage of ciprofloxacin (CIP) and doxycycline (DOX), showing measurements of optical density as a function of time (OD, black dots). Cells are propagated in media containing the drugs through daily serial transfers over 15 days (only the first 6 days are shown), resulting in alternating periods of growth and stationary phase (inset). Best fit of the logistic growth curve (red lines; indicated equation) defines the growth rate (r) for this population in each day. The measurement error in the growth rates was estimated to be $\approx 0.06/h$ (see *Materials and Methods* and Fig. S3). (B) Data points corresponding to daily growth rates show how the fitness in the population from A increases over time. Time is measured in hours of growth (time in stationary phase is excluded). The total increase in growth rate of the population is denoted by Δr , and the adaptation time, t_{adapt} , is defined as the time at which the population crosses the midpoint between its initial and final growth rates (see *Materials and Methods* for more details). Measured Δr and t_{adapt} are used for determining the rate of adaptation of each population (α , equation shown). (C) Scatter plot of rates of adaptation and final growth rates for CIP-DOX and ERY-DOX. The point highlighted in magenta corresponds to the population shown in A and B. Most populations that evolve recover the growth rate in a drug-free environment (dashed horizontal line).

environments suggest the hypothesis that the rate of adaptation, the speed with which lineages carrying such mutations spread within evolving populations, might be greater for synergistic than for antagonistic drug combinations.

To experimentally explore the relation between drug interaction and the rate of adaptation, we compared a drug pair that exhibits strong antagonism [doxycycline (DOX), a tetracycline antibiotic, and ciprofloxacin (CIP), a fluoroquinolone] to another pair showing strong synergy [DOX and a macrolide antibiotic, erythromycin (ERY)] (5, 17, 21, 22). For each pair, drugs were mixed in a two-dimensional array of wells in which the concentration of each of the single drugs varied from zero to above its MIC. Populations derived from clonal expansion of a single wild-type, drug-sensitive *E. coli* bacterium were introduced to all wells and propagated through daily serial transfers (see *Materials and Methods*) (23–25). The rates at which bacterial populations developed residual resistance to the various drug combinations were measured by tracking the evolution of these populations over ≈ 170 generations (Fig. 2). The degree of antibiotic resistance acquired by each population is manifest in the increase of its growth rate over time (The changes in MIC that accompanied these accelerations of growth were relatively mild, smaller than a factor of 2 in some cases and up to an 8-fold increase in others; data not shown). From daily growth curves (Fig. 2A) we estimated how the growth rate changed for each population as it evolved (Fig. 2B). Typically, we see a saturation curve with a relatively fast initial increase in fitness followed by a plateau with little or no additional adaptation in later times. We combined the increase in growth rate of each population during evolution, Δr , and the time it took the population to reach the half-way mark of that increase, t_{adapt} , to define the rate of adaptation, $\alpha \equiv (\Delta r/2)/t_{adapt}$ (Fig. 2B). This adaptation rate

shows large variability among the different drug treatments, reflecting variability in both initial growth rates and adaptation times (Fig. 2C). In contrast, much less variability was manifest in the final growth rates attained by the populations, and by the end of the experiment most of the populations were growing at a rate similar to that in the drug-free environment (Fig. 2C).

Results

Our data show that drug combinations have a strong effect on the evolution of drug resistance (Fig. 3). Different drug pairs, however, have profoundly different impacts on the rate of adaptation. For ERY-DOX, which is a strongly synergistic drug pair, we see accelerated adaptation when the drugs are used in combination (center of the 2-D drug grid, arrow in Fig. 3C) relative to drug treatments involving either of the drugs alone (the two edges lying on the drug concentration axes). For the antagonistic drug pair CIP-DOX, such an acceleration is absent in the combinations that we tested. Moreover, the opposite effect—a depression of the rate of adaptation relative to the single-drug environments—is apparent for some CIP-DOX combinations (Fig. 3D). Related to these observations is the fact that resistance to the synergistic drug pair evolves rapidly, not only compared to each drug separately, but also compared to the antagonistic pair. This link between the way drugs interact and the rate of adaptation is consistent with the hypothesis described in Fig. 1, and further exploration of this relation was achieved by the additional experiments and analyses described below.

We expanded on the comparison of strongly synergistic and strongly antagonistic drug combinations by including two additional drug pairs in our study—CIP with the aminoglycoside amikacin (AMI) and AMI with DOX—which interact synergistically at some dose combinations but antagonistically at others.

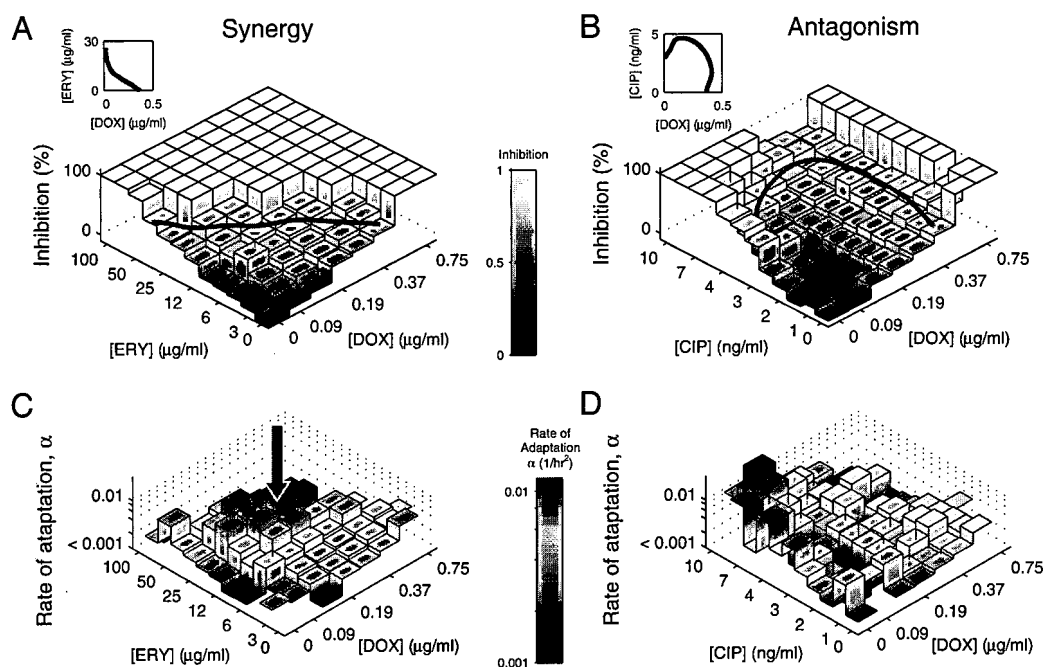


Fig. 3. Different drug pairs vary profoundly in their impact on the rate of adaptation. (A and B) For two pairs of drugs (A, synergistic ERY-DOX; B, antagonistic CIP-DOX), the initial level of inhibition is shown for a matrix of concentrations of the two drugs. The level of inhibition is defined as $1 - r/r_0$, where r is the growth rate of the population in the presence of antibiotics and r_0 is the drug-free growth rate. The solid line corresponds to the line of 50% inhibition and is also shown on a linear scale in the insets (see Fig. 1 for comparison). (C and D) The rate of adaptation for the drug combinations shown in A and B. The arrow in C points to a region of drug concentrations where the rate of adaptation for the synergistic ERY-DOX combination is accelerated relative to the single-drug treatments. This acceleration is surprising because the more expected outcome of combining drugs is for adaptation to slow down.

In addition, we used the fact that for any drug pair, including strongly synergistic or antagonistic ones, the strength of interactions varies for different drug ratios and dosages (26). Therefore, for all drug pairs, for each combination of concentrations of two drugs, we measured the degree of synergy (S , defined in *Materials and Methods* and in Fig. 4), which ranges from +1 for strongly synergistic interactions to -1 for strongly antagonistic ones, and compared it with the rate of adaptation (Fig. 4; see raw data in Fig. S2). We find a positive correlation between synergy and the rate of adaptation ($\rho = 0.683$, $P < 0.001$). Two aspects of the data contribute to this correlation: the correlation among the means of the four drug pairs and the correlation within each pair. To isolate the latter, we calculated the partial correlation between synergy and the rate of adaptation when controlling for drug-pair membership, which yields $\rho = 0.533$, $P < 0.001$ (see *SI Text*). Another factor that is likely to influence the rate of adaptation is the initial inhibition of growth of the ancestral strain in each environment. Again, we have verified that the observed correlation between adaptation rate and synergy remains when accounting for the confounding factor of initial inhibition (see *SI Text*). Indeed, the partial correlation between rate of adaptation and degree of synergy when controlling for initial inhibition supports the same conclusion as before ($P < 0.001$), namely, that synergy promotes faster emergence of drug resistance.

Discussion

Our simple geometric model provides some insight into how synergistic drug interactions may lead to faster adaptation (Fig. 1). It should be noted that the same model also indicates the influence of another factor on the rate of adaptation: the availability of mutations that confer simultaneous resistance to both drugs (27, 28) (Fig. S1). It is certainly possible that the level of pleiotropy in mutational effects contributes to the observed differences in the rates of adaptation between drug pairs in our

experiments. Indeed, drug pairs with similar mechanisms of action, such as ERY and DOX, could have increased pleiotropy. Conversely, pleiotropy could inhibit evolution if mutation con-

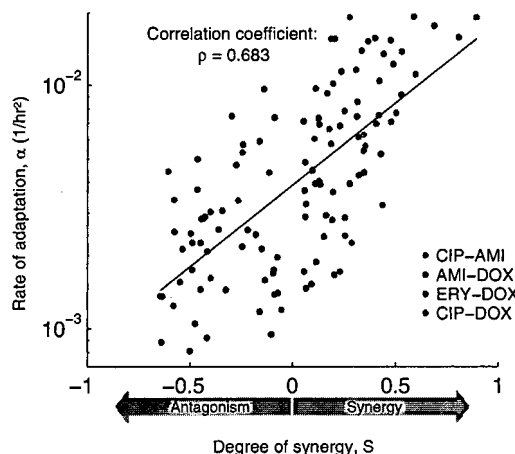


Fig. 4. The degree of synergy and the rate of adaptation are positively correlated: The more synergistically the drugs interact, the faster the bacteria evolve resistance. Shown are data from all four drug pairs in our study (legend). Each dot represents the rate of adaptation for a given drug pair and concentrations versus the degree of synergy (S). The variability in rates of adaptation has two contributions: an error of approx 25% due to errors in measuring growth rates, and an inherent stochasticity of the mutation process (see *Materials and Methods* and Figs. S3 and S4). For a combination (x, y) of drug concentrations, S is measured as the deviation from the neutral expectation defined by Bliss independence: $S = (f_{xy}/f_{00})(f_{0y}/f_{00}) - f_{xy}/f_{00}$, where f_{xy} denotes wild-type growth rates when the concentration of one drug is x and that of the other is y . Following this definition, positive values of S corresponds to synergistic interactions and negative values to antagonistic ones. The solid line is the best fit to all 116 data points from all of the drug pairs.

ferring resistance to one drug reduces resistance to another. Further, the rates of mutation may itself vary with some of the drugs, especially with DNA synthesis inhibitors such as CIP. The contribution of these additional factors to the rate of adaptation and their possible correlation with drug synergy is, however, beyond the scope of this work.

We have shown that the rate at which laboratory populations of bacteria adapt to multidrug treatments is correlated with the degree of synergy between the drugs. This correlation implies that antagonistic drug combinations, although typically avoided in clinical settings, may be more effective than synergistic combinations in forestalling the development of antibiotic resistance. Antagonism between drugs may generate sign epistasis between single-resistant mutations, thereby limiting the mutational paths leading to adaptation (29). Further research is needed, however, to see how these results relate to clinical measurements of success in multidrug chemotherapies, whether with bacteria, viruses, or cancer cells. Success in such treatments is the consequence of a multitude of factors, including pharmacokinetics, heterogeneous drug concentrations, horizontal gene transfer, enhanced bactericidal activity, and microbial species and host interactions. Nevertheless, our findings suggest that the choice of drug combinations may involve a tradeoff between two important factors: immediate inhibition of growth and eventual delay in the evolution of resistance. Synergy, by definition, has the advantage that less drug achieves more inhibition or faster killing of wild-type cells. This enhanced inhibition may be crucial to the efficacy of some drug treatments, especially when it is possible to reach *in vivo* concentrations so high that resistant mutants cannot survive. On the other hand, when treatment dosages fluctuate close to the MIC, which is where antibiotic resistance is most likely to evolve (30), antagonistic drug pairs may be a more effective antimicrobial weapon in forestalling the emergence of resistance. Our work suggests that antagonistic drug combinations may form an important part of treatment strategies designed to achieve this goal.

Materials and Methods

Media and Strains. All experiments were conducted in M9 Minimal Media (Bio101 #3035-012) supplemented with 0.2% glucose and 0.1% Casamino Acids. Drug solutions were made from powder stocks [doxycycline hyclate (Sigma D-9891); ciprofloxacin (Fluka 17850); erythromycin (Fluka 45673); and amikacin disulfate stock (Sigma A-1774)] and diluted in the growth media on a 2-D grid at indicated concentrations in 96-well plates, with 150 μ l per well. To ensure constant drug conditions, all plates of a given drug pair were made from a single master deep-well plate and stored at -20°C (no measurable decay of drugs was observed during the experiment). The populations were founded from an individual colony of strains of fluorescently labeled MC4100 *E. coli* described previously (24). This strain was not preadapted to the growth conditions of these experiments.

Properties of Antibiotics Used. The four antibiotics used in the experiments, DOX, ERY, CIP, and AMI, represent a range of action mechanisms (17). DOX is a tetracycline antibiotic used to treat both Gram-negative and Gram-positive infections; it inhibits protein synthesis by blocking aminoacyl-tRNA binding at the A-site in the 30S ribosomal subunit (22). ERY is a macrolide that binds the 23S rRNA molecule in the 50S subunit and also inhibits protein synthesis (5). CIP is a fluoroquinolone, a derivative of nalidixic acid, and is a broad-spectrum antibiotic that inhibits the action of DNA gyrase in both Gram-positive and Gram-negative bacteria (5). AMI is an aminoglycoside antibiotic and works by inhibiting protein synthesis (5).

Parallel Evolution. Cultures were propagated in parallel for >150 generations with daily serial transfers. OD curves were measured in a fully automated robotic system (Staccato Sciclone Cell Station, Caliper LifeSciences), including a microplate shaker (Liconic LPX40), and a plate reader (Victor3, Perkin-Elmer). The equipment was maintained in an environmental room at constant temperature (30°C) and relative humidity (70%). One initial OD measurement [absorbance at 600 nm (OD_{600})] was taken at the beginning of each day followed by a 5-h gap and then measurements every 24 min. Except for read periods, plates were shaken continuously at ≈ 1000 rpm. At the end of each

day, plates were removed from the robotic systems and cells were transferred from each population to freshly thawed antibiotic gradient plates by using a 96-pin replicator (VP409). Volume transferred by the pins was measured by using fluorescein solution and was found to be 0.04 μ l per pin, with a variability of 20% between pins. These volumes correspond to an $\approx 3,300$ -fold dilution or ≈ 11.7 generations of binary fission.

Determination of Adaptation Rate. We estimated the growth rate, r [as well as the three parameters for the baseline, a , yield, $a + b$, and lag time, $\ln(c)/r$], for each population on each day by the best fit of the function $\text{OD}(t) = a + b/(1 + \exp(-rt))$ to the OD measurements taken during that day. It is also possible to extract growth rates by fitting a linear function to log-transformed and background-subtracted OD measurements during exponential phase, but for our data this alternative method leads to a larger error than the method that we employ (Fig. S3). Because a spreading mutant population will keep growing until the resources are exhausted (stationary phase) or until the time of dilution at the end of the day, we measure time in terms of hours spent in growth. For each population we keep track of the time (in hours) spent in growth (and not in stationary phase) since the beginning of the experiment. Let $t(i)$ be the time spent in growth at the end of day i of the experiment. Then the growth rate calculated from the OD measurements of day i is interpreted as the growth rate of the population, $r(T)$, at time $T = t(i - 1) + [t(i) - t(i - 1)]/2$. The measurements of r versus T for all populations are shown in Fig. S2. All of the populations that survived the antibiotic treatment but were initially inhibited exhibited an increase in their growth rate. The growth rate measured in the 1st day of the experiment was defined as the initial growth rate. The final growth rate was calculated as the average growth rate over the last 6 days of the experiment. From these we calculated the total increase in growth rate, Δr , as the difference between calculated final and initial growth rates. We focused on all populations for which $\Delta r > 0.2$ 1/h, which is above the estimated error in growth rate measurement (this filter was applied for calculating correlations but not for plotting the data shown in Fig. 3). The time of adaptation for each of these populations, t_{adapt} (Fig. 2B), is defined as the interpolated time at which the growth-rate improvement of the population reached half its maximum value, and the rate of adaptation is defined as $\alpha = (\Delta r/2)/t_{\text{adapt}}$. Defining t_{adapt} as the time to traverse a different fraction of the fitness increase, for example $1/4$ or $3/4$ rather than $1/2$, leads only to small changes in the correlations and partial correlations between synergy and the rate of adaptation and does not change their statistical significance. The results are also essentially unchanged if one employs a different definition of the rate of adaptation, namely $\alpha \equiv 1/t_{\text{adapt}}$, a definition that focuses only on adaptation times.

Estimation of Measurement Errors in Growth Rates and Rates of Adaptation. We used replicate measurements to assess the error in the measurement of growth rates. On the 1st day of the evolutionary experiment, when all bacterial populations were primarily composed of the ancestral strain, we compared the growth rates for pairs of wells that contained exactly the same drug concentrations. These wells were: four drug-free wells, one from each plate; three columns containing identical gradients of concentrations of DOX alone from the AMI-DOX, ERY-DOX, and CIP-DOX plates; two rows containing only CIP from the CIP-AMI and CIP-DOX plates; and wells in the column containing AMI alone in the CIP-AMI plate that correspond to wells with the same concentrations of AMI in the AMI-DOX plate. The error was inferred from the standard deviation of the differences between replicates and was found to be $\approx 0.06/\text{h}$ (Fig. S3).

We then used the estimated error in growth rate measurements to assess their contribution to uncertainty in the rates of adaptation. For each growth-rates trajectory, 100 randomly modified trajectories were generated by adding to each daily measured growth rate an independent sample from a normal distribution with a mean of zero and a standard deviation of 0.06/h. The rate of adaptation was then calculated separately from each of these trajectories, providing a distribution of values typically around the measured rate of adaptation, α . The standard deviation of this distribution is interpreted as the error in α that is caused by the error in measuring growth rates. We found the error in α to be roughly proportional to α , corresponding to an average multiplicative error of 25%.

To estimate the role of inherent biological variability in the adaptive process between wells, we compared rates of adaptation between identical wells (the subset of the replicate well-pairs listed above for which a rate of adaptation was determined). In some cases, differences in rates of adaptation between replicate experiments were observed that were significantly larger than the measurement error, indicating the presence of underlying mutational events that led to markedly different adaptive trajectories (Fig. S4).

Definition of Degree of Synergy. Antibiotic interactions—synergy or antagonism—are defined as deviations from neutral or additive expectations (8). We define neutrality by using Bliss independence (9), where the expected fitness of a bacterium in the presence of two antibiotics is the product of its fitness in the presence of each of the antibiotics separately:

$$f_{xy}/f_{00} = (f_{x0}/f_{00})(f_{0y}/f_{00}),$$

where f_{xy}/f_{00} is the fitness of the bacteria in the two-drug environment relative to its drug-free fitness. Note that when two drugs show Bliss independence, the fitness advantage of a single-drug resistance mutant does not depend on the presence or absence of the other drug. Synergy is defined as the difference between the neutral expectation and the actual measured value of the bacterial fitness under antibiotic combinations:

$$S = (f_{x0}/f_{00})(f_{0y}/f_{00}) - f_{xy}/f_{00}.$$

The value of S is calculated based on inhibition of the wild-type in the single and double perturbed environments, as measured by the growth rates in the 1st day of the experiment. Synergy is not defined when the growth rate is zero in the combined drug environment and in at least one of the single drug environments.

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