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When two are better than one: Modeling the mechanisms of antibody mixtures

Tal Einav[1], Jesse D. Bloom[1,2*]

[1] Basic Sciences Division and Computational Biology Program, Fred Hutchinson Cancer Research Center, Seattle, WA, United States of America
[2] Howard Hughes Medical Institute, Seattle, WA, United States of America

* jbloom@fredhutch.org

Abstract

It is difficult to predict how antibodies will behave when mixed together, even after each has been independently characterized. Here, we present a statistical mechanical model for the activity of antibody mixtures that accounts for whether pairs of antibodies bind to distinct or overlapping epitopes. This model requires measuring n individual antibodies and their $\frac{n(n-1)}{2}$ pairwise interactions to predict the 2^n potential combinations. We apply this model to epidermal growth factor receptor (EGFR) antibodies and find that the activity of antibody mixtures can be predicted without positing synergy at the molecular level. In addition, we demonstrate how the model can be used in reverse, where straightforward experiments measuring the activity of antibody mixtures can be used to infer the molecular interactions between antibodies. Lastly, we generalize this model to analyze engineered multidomain antibodies, where components of different antibodies are tethered together to form novel amalgams, and characterize how well it predicts recently designed influenza antibodies.

Author summary

With the rise of new combination antibody therapeutic regimens, it is important to understand how antibodies work together as well as individually. Here, we investigate the specific case of monoclonal antibodies targeting a cancer-causing receptor or the influenza virus and develop a statistical mechanical framework that predicts the effectiveness of a mixture of antibodies. The power of this model lies in its ability to make a large number of predictions based on a limited amount of data. For example, once 10 antibodies have been individually characterized, our model can predict how any of the $2^{10} = 1024$ combinations will behave. This predictive power provides ample opportunities to test our model and paves the way to expedite the design of future therapeutics.

Introduction

Antibodies can bind with strong affinity and exquisite specificity to a multitude of antigens. Due to their clinical and commercial success, antibodies are one of the largest and fastest growing classes of therapeutic drugs [1]. While most therapies currently use monoclonal antibodies (mAbs), mounting evidence suggests that mixtures of antibodies can behave in fundamentally different ways [2]. There is ample precedent for the idea

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that combinations of therapeutics can be extremely powerful—for instance, during the past 50 years the monumental triumphs of combination anti-retroviral therapy and chemotherapy cocktails have provided unprecedented control over HIV and multiple types of cancer [3, 4], and in many cases no single drug has emerged with comparable effects. However, it is difficult to predict how antibody mixtures will behave relative to their constitutive parts. Often, the vast number of potential combinations is prohibitively large to systematically test, since both the composition of the mixture and the relative concentration of each component can influence its efficacy [5].

Here, we develop a statistical mechanical model that bridges the gap between how an antibody operates on its own and how it behaves in concert. Specifically, each antibody is characterized by its binding affinity and potency, while its interaction with other antibodies is described by whether its epitope is distinct from or overlaps with theirs. This information enables us to translate the molecular details of how each antibody acts individually into the macroscopic readout of a system's activity in the presence of an arbitrary mixture.

To test the predictive power of our framework, we apply it to a beautiful recent case study of inhibitory antibodies against the epidermal growth factor receptor (EGFR), where 10 antibodies were individually characterized for their ability to inhibit receptor activity and then all possible 2-Ab and 3-Ab mixtures were similarly tested [6]. We demonstrate that our framework can accurately predict the activity of these mixtures based solely on the behaviors of the ten monoclonal antibody as well as their epitope mappings.

Lastly, we generalize our model to predict the potency of engineered multidomain antibodies from their individual components. Specifically, we consider the recent work by Laursen *et al.* where four single-domain antibodies were assayed for their ability to neutralize a panel of influenza strains, and then the potency of constructs comprising 2-4 of these single-domain antibodies were measured [7]. Our generalized model can once again predict the efficacy of the multidomain constructs based upon their constitutive components, once a single fit parameter is inferred to quantify the effects of the linker joining the single-domain antibodies. This enables us to quantitatively ascertain how tethering antibodies enhances the two key features of potency and breadth that are instrumental for designing novel anti-viral therapeutics. Notably, our models do *not* posit complex molecular synergy between antibodies. Our results therefore show that many antibody mixtures function without synergy, and hence that their effects can be computationally predicted to expedite future experiments.

Results

Modeling the mechanisms of action for antibody mixtures

Consider a monoclonal antibody that binds to a receptor and inhibits its activity. Two parameters characterize this inhibition: (1) the dissociation constant K_D quantifies an antibody's binding affinity (with a smaller value indicating tighter binding) and (2) the potency α relates the activity when an antibody is bound to the activity in the absence of antibody. A value of $\alpha = 1$ represents an impotent antibody that does not affect activity while $\alpha = 0$ implies that an antibody fully inhibits activity upon binding. As derived in S1 Text Section A, for an antibody that binds to a single site on a receptor, the activity at a concentration c of antibody is given by

$$\text{Fractional Activity} = \frac{1 + \alpha \frac{c}{K_D}}{1 + \frac{c}{K_D}}. \tag{1}$$

To characterize a mixture of two antibodies, we not only need their individual

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dissociation constants and potencies but also require a model for how these antibodies interact. When two antibodies bind to distinct epitopes, the simplest scenario is that their ability to bind and inhibit activity is independent of the presence of the other antibody, and hence that their combined potency when simultaneously bound equals the product of their individual potencies (Fig 1A). Alternatively, if the two antibodies compete for the same epitope, they cannot both be simultaneously bound (Fig 1B).

We also define the general case of a synergistic interaction where the binding of the first antibody alters the binding or potency of the second antibody (Fig 1C, purple text). This definition encompasses cases where the second antibody binds more tightly ($K_{D,\text{eff}}^{(2)} < K_D^{(2)}$) or more weakly ($K_{D,\text{eff}}^{(2)} > K_D^{(2)}$) in the presence of the first antibody, as well as when the potency of the second antibody may increase ($\alpha_{2,\text{eff}} > \alpha_2$) or decrease ($\alpha_{2,\text{eff}} < \alpha_2$). This also includes cases where two epitopes slightly overlap and partially inhibit one another's binding, and the competitive binding model can be viewed as the extreme limit $K_{D,\text{eff}}^{(2)} \to \infty$ where one antibody infinitely penalizes the binding of the other.

While the synergistic model in Fig 1C has the merit of being highly general, an important feature of the independent and competitive models (Fig 1A,B) is that they predict all antibody combinations with few parameters. In both of these latter models, once the $K_D^{(j)}$ and α_j of 10 antibodies are known (which requires $2 \cdot 10$ experiments) and their epitopes are mapped ($\frac{10 \cdot 9}{2}$ additional experiments), the potency of all $2^{10} = 1024$ possible mixtures of these antibodies can be predicted without recourse to fitting. In contrast, because the synergistic model allows arbitrary interactions between each combination of antibodies, the behavior of a mixture exhibiting synergy cannot be predicted without actually making a measurement on that combination to quantify the synergy.

For these reasons, in this work we focus on the two cases of independent or competitive binding and show how we can combine both models to transform our molecular understanding of each monoclonal antibody's action into a prediction of the efficacy of an antibody mixture. Deviations from our predictions provide a rigorous way to measure antibody synergy by computing $\frac{K_{D,\text{eff}}^{(2)}}{K_D^{(2)}}$ and $\frac{\alpha_{2,\text{eff}}}{\alpha_2}$.

To mathematize the independent and competitive binding models, we enumerate the possible binding states and compute their relative Boltzmann weights. The fractional activity of each state equals the product of its relative probability and relative activity divided by the sum of all relative probabilities for normalization (see S1 Text Section A). When two antibodies bind independently as in Fig 1A, this factors into the form

$$\text{Fractional Activity}_{(\text{distinct epitopes})} = \left(\frac{1 + \alpha_1 \frac{c_1}{K_D^{(1)}}}{1 + \frac{c_1}{K_D^{(1)}}}\right)\left(\frac{1 + \alpha_2 \frac{c_2}{K_D^{(2)}}}{1 + \frac{c_2}{K_D^{(2)}}}\right). \tag{2}$$

If these two antibodies compete for the same epitope as in Fig 1B, the activity becomes

$$\text{Fractional Activity}_{(\text{overlapping epitopes})} = \frac{1 + \alpha_1 \frac{c_1}{K_D^{(1)}} + \alpha_2 \frac{c_2}{K_D^{(2)}}}{1 + \frac{c_1}{K_D^{(1)}} + \frac{c_2}{K_D^{(2)}}}. \tag{3}$$

These equations are readily extended to mixtures with three or more antibodies (see S1 Text Section A).

Antibody mixtures against EGFR are well characterized using independent and competitive binding models

To test the predictive power of the independent and competitive binding models, we applied them to published experiments on the epidermal growth factor receptor (EGFR)

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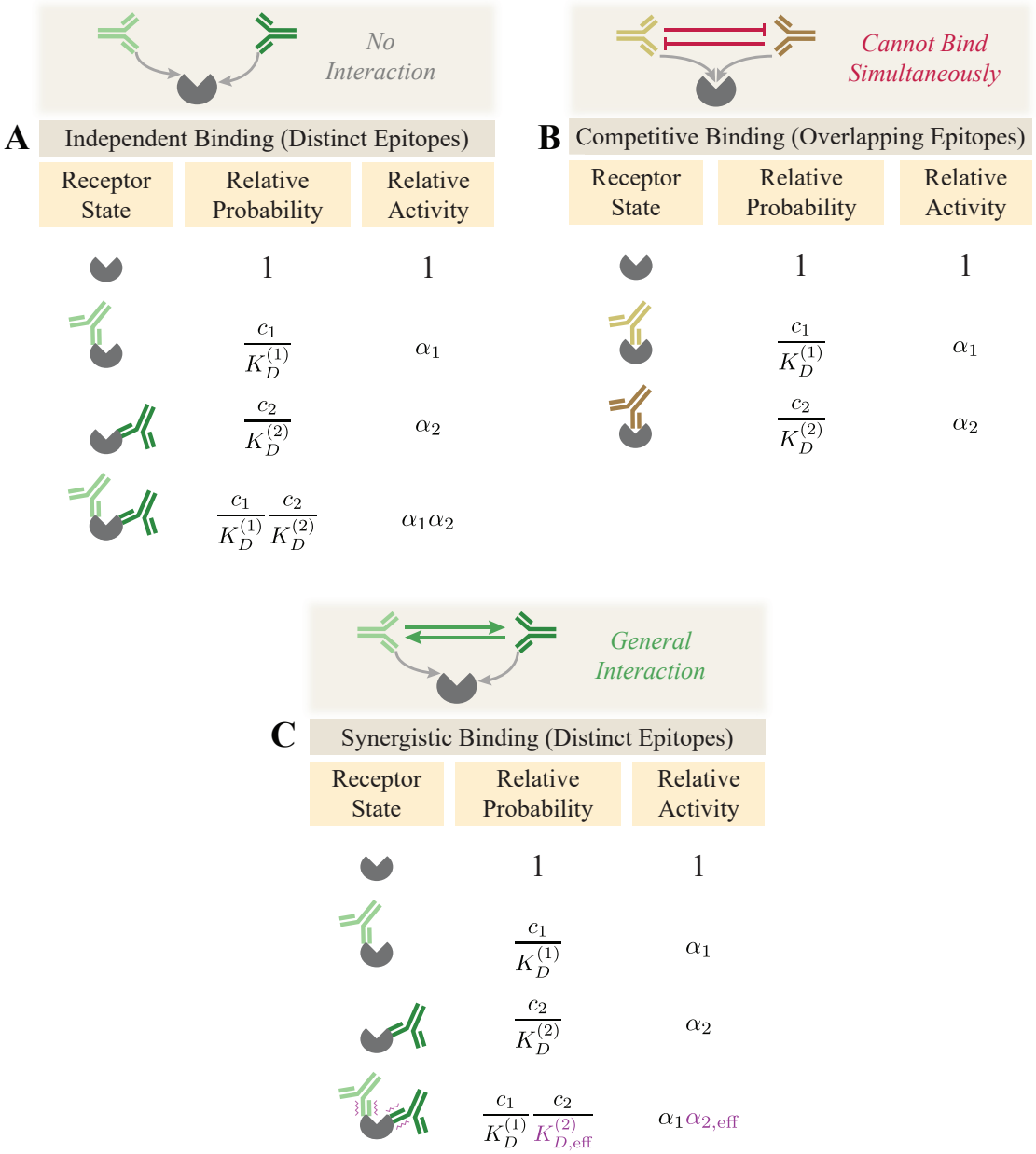

Fig 1. Binding modes for a 2-Ab mixture. Two antibodies with concentrations c_1 and c_2 can bind (A) independently to different epitopes or (B) competitively to the same epitope. (C) Antibodies bind synergistically if either the product of binding affinities ($K_D^{(j)}$) or potencies (α_j) are altered when both antibodies bind.

where ten monoclonal antibodies were individually characterized and then the activity of all 165 possible 2-Ab and 3-Ab mixtures was measured [6]. We first use each monoclonal antibody's response to infer its dissociation constant K_D and potency α. We then utilize surface plasmon resonance (SPR) measurements to determine which pairs of antibodies bind independently and which compete for the same epitope. These data enable us to use the above framework and predict EGFR activity in the presence of any mixture.

EGFR is a transmembrane protein that activates in the presence of epidermal

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growth factors. Upon ligand binding, the receptor's intracellular tyrosine kinase domain autophosphorylates which leads to downstream signaling cascades central to cell migration and proliferation. Overexpression of EGFR has been linked to a number of cancers, and decreasing EGFR activity in such tumors by sterically occluding ligand binding has reduced the rate of cancer proliferation [6].

Koefoed *et al.* investigated how a panel of ten monoclonal antibodies inhibit EGFR activity in the human cell line A431NS [6]. They then measured how 1:1 mixtures of two antibodies or 1:1:1 mixtures of three antibodies affect EGFR activity. All measurement were carried out at a total concentration of $2\frac{\mu g}{mL}$, implying that each antibody was half as dilute in the 2-Ab mixtures and one-third as dilute in the 3-Ab mixtures relative to the monoclonal antibody measurement.

The 45 possible 2-Ab mixtures (35 binding to distinct epitopes; 10 binding to overlapping epitopes) and the 120 possible 3-Ab mixtures (50 binding to distinct epitopes; 70 binding to overlapping epitopes) were assayed for their ability to inhibit EGFR activity. Fig 2A shows the experimental measurements for mixtures of two antibodies, with the monoclonal antibody measurements shown on the diagonal, the measured activity of 2-Ab mixtures shown on the bottom-left and the predicted activity on the top-right. Each antibody is labeled with its binding epitopes inferred through SPR [6], so that antibodies binding to overlapping epitopes are predicted using Eq (3) (pairs within the dashed gray boxes) while mixtures binding to distinct epitopes use Eq (2).

For example, antibodies #1 and #4 bind to distinct epitopes (III/C and III/B, respectively). Hence, the predicted activity of their mixture (0.50) very nearly equals the product of their individual activity ($0.65 \times 0.75 = 0.49$), with the slight deviation arising because each antibody concentration was halved in the mixture ($c_1 = c_2 = 1\frac{\mu g}{mL}$ for the 2-Ab mixture characterized by Eq (2), whereas the individual mAbs were measured at $c = 2\frac{\mu g}{mL}$ using Eq (1)). The predicted activity roughly approximates the measured value 0.43 of the mixture.

On the other hand, antibodies #1 and #2 bind to the same epitope (III/C), and hence their predicted combined activity (0.67) lies between their individual activities (0.65 and 0.69) since both antibodies compete for the same site. The measured activity of the mixture (0.65) closely matches the prediction of the overlapping epitope model, but is very different than the prediction of 0.45 made by the distinct-binding model.

Fig 2B shows the measured EGFR activity in the presence of all 2-Ab and 3-Ab mixtures is highly correlated with the predicted activity ($R^2 = 0.90$) Notably, the predictions are made solely from the monoclonal antibody data and epitope measurements, and do not involve any fitting of the 2-Ab or 3-Ab measurements. The strong correlation between the predicted and measured activities suggests that EGFR antibody mixtures can be characterized with minimal synergistic effects in either their binding or effector functions. If we did not have the epitope mapping through SPR and assumed that all antibodies bound to distinct epitopes (Fig 2C, $R^2 = 0.85$) or competed for the same epitope (Fig 2D, $R^2 = 0.86$), the resulting predictions are slightly more scattered from the diagonal, demonstrating that properly acknowledging which pairs of antibodies vie for the same epitope boosts the predictive power of the model.

That said, the predictions incorporating the SPR mapping display a consistent bias towards having a slightly lower measured than predicted activity, suggesting that several pairs of antibody enhance one another's binding affinity or potency. To quantify this, if we recharacterize the activity from the 2-Ab mixtures to a synergistic model where each $\alpha_{2,\text{eff}}$ is fit to exactly match the data, we find an average value of $\frac{\alpha_{2,\text{eff}}}{\alpha_2} = 0.9$, showing that when pairs of antibodies are simultaneously bound they typically boost their collective inhibitory activity by ∼10%.

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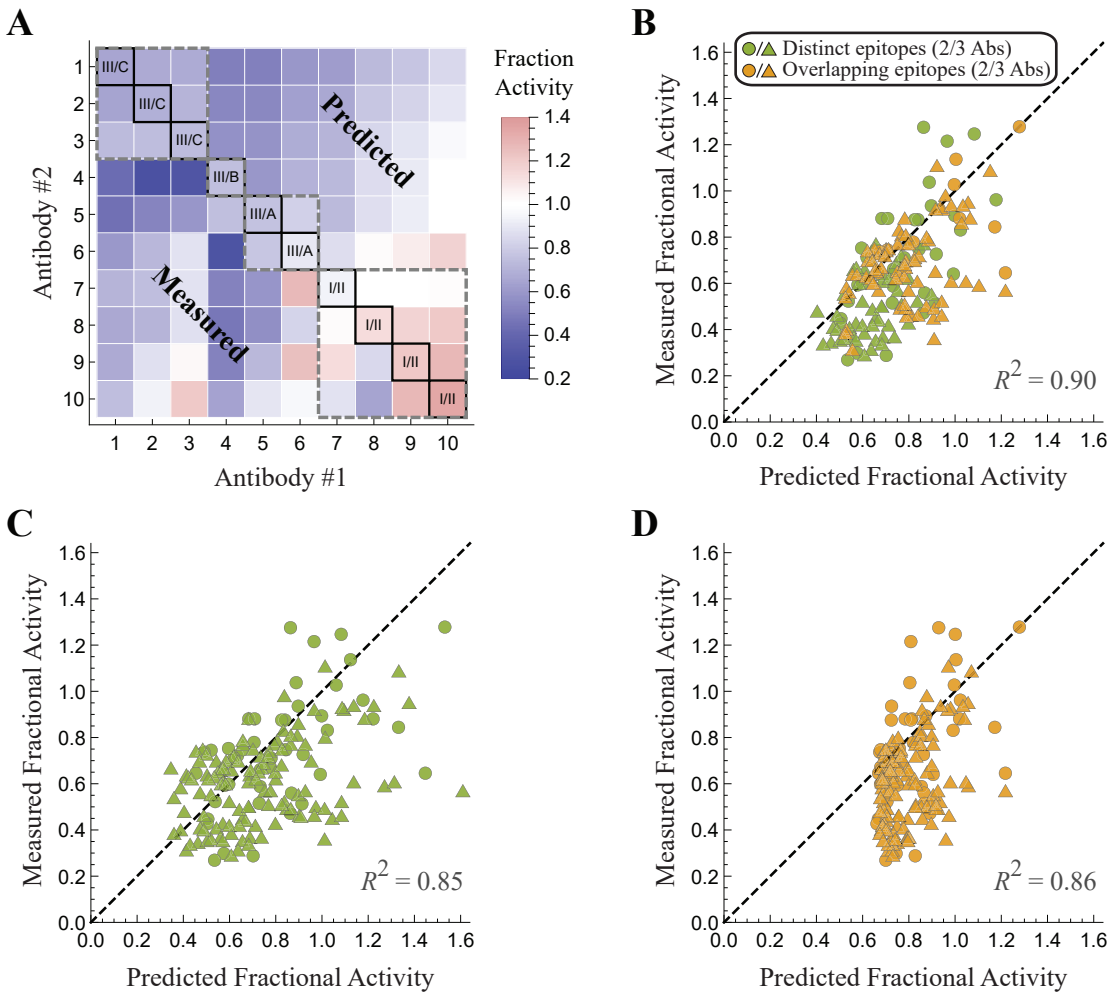

Fig 2. Predicting how antibody mixtures affect the epidermal growth factor receptor (EGFR). (A) The fractional activity of EGFR in the presence of monoclonal antibodies (diagonal) together with the measured (bottom-left) and predicted (top-right) activity of all 2-Ab combinations. The dashed gray boxes enclose antibody pairs that compete for the same epitope while all other pairs bind independently. (B) The predicted versus measured fractional activity for all 2-Ab and 3-Ab mixtures using the same epitope mapping as in Panel A inferred by SPR. Without the epitope map, the activity of the mixtures could alternately be predicted by assuming that all antibodies either (C) bind independently or (D) compete for the same epitope; in either case, the resulting predictions fall further from the diagonal line, indicating poorer predictive power.

Differentiating distinct versus overlapping epitopes using antibody mixtures

In the previous section, we used SPR measurements to quantify which antibodies compete for overlapping epitopes, thereby permitting us to translate the molecular knowledge of antibody interactions into a macroscopic quantity of interest, namely, the activity of EGFR. In this section, we do the reverse and utilize activity measurements to categorize which subsets of antibodies bind to overlapping epitopes. This method can be applied to model antibody mixtures in other biological systems where SPR measurements are not readily available.

For the remainder of this section, we ignore the known epitope mappings discerned by Koefoed *et al.* and consider what mapping best characterizes the data. For example,

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given the individual activities of antibody #1 (0.65) and #2 (0.69), the predicted activity of their combination (at the concentration of $1\frac{\mu g}{mL}$ for each antibody dictated by the experiments) would be 0.45 if they bind to distinct epitopes and 0.67 if they bind to overlapping epitopes. Since the measured activity of this mixture was 0.65, it suggests the latter option. We note that such analysis will work best for potent antibodies (whose individual activity is far from 1), since only in this regime will the predictions of the distinct versus overlapping models be significantly different. Therefore, the activity measurements of each individual antibody would optimally be carried out at saturating concentrations (where Eq (1) is as far from 1 as possible).

Proceeding to the other antibodies, we characterize each pair according to whichever model prediction lies closer to the experimental measurement. To account for experimental error, we left an antibody pair uncategorized if the two model predictions were too close to one another (within $4\sigma = 0.16$ where σ is the SEM of the measurements) or if the experimental measurement was close (within 1σ) to the average of the two model predictions (see S1 Text Section B).

Fig 3A shows how this analysis compares to the experimental measurement inferred by SPR. While the model predictions are much sparser (with the majority of antibody pairs uncategorized because the two model predictions were too close to one another), the classifications only disagreed with the SPR measurements in two cases (claiming that antibodies #7-8 overlap with antibody #10; notice that antibodies #7-8 have individual activities close to 1, making them difficult to characterize).

Using these classifications, we defined unique EGFR epitopes by grouping together any antibodies that bind to overlapping epitopes. In this way, we split the ten antibodies into four distinct groups (antibodies #1-3, #4-5, #6, and #7-10 indicated by the dashed gray rectangles in Fig 3A), enabling us to distinguish which antibodies bind independently or competitively and hence predict the activity of the 2-Ab and 3-Ab mixtures. Note that it is not the pairwise classification between two antibodies that determines whether we apply the distinct or competitive models, but rather these four groupings of antibody epitopes. For example, although antibodies #7 and #8 are uncategorized through their 2-Ab mixture, they fall within a single epitope group and hence are considered to bind competitively. Similarly, antibody #1 and #4 are modeled as binding independently because they belong to two distinct epitope groups. Antibody #6 is considered to be in its own epitope group since it did not overlap with any other antibody.

Surprisingly, the results shown in Fig 3B have a coefficient of determination $R^2 = 0.90$ that is on par with the results obtained using the SPR measurements (Fig 2B). Since the inferred epitope map relied on the 2-Ab activity data, we compared the predicted activity of the 3-Ab mixtures using the epitopes inferred through SPR with those inferred through the activity data and showed that they are nearly identical ($R^2 = 0.997$, see S1 Text Section A). This suggests that there is no loss in the predictive power of the model when an epitope mapping is inferred through activity measurements.

In summary, whether antibodies bind independently or competitively can be determined either: (1) directly through pairwise competition experiments or (2) by analyzing the activity of their 2-Ab mixtures in light of our two models. When this information is combined with the potency and dissociation constant of each antibody, the activity of an arbitrary mixture can be predicted. The Supplementary Information contains a Mathematica program that can analyze either form of the pairwise interactions to determine the epitope grouping. If the characteristics of the individual antibodies are also provided, the program can predict the activity of any antibody mixtures at any specified ratio of the constituents.

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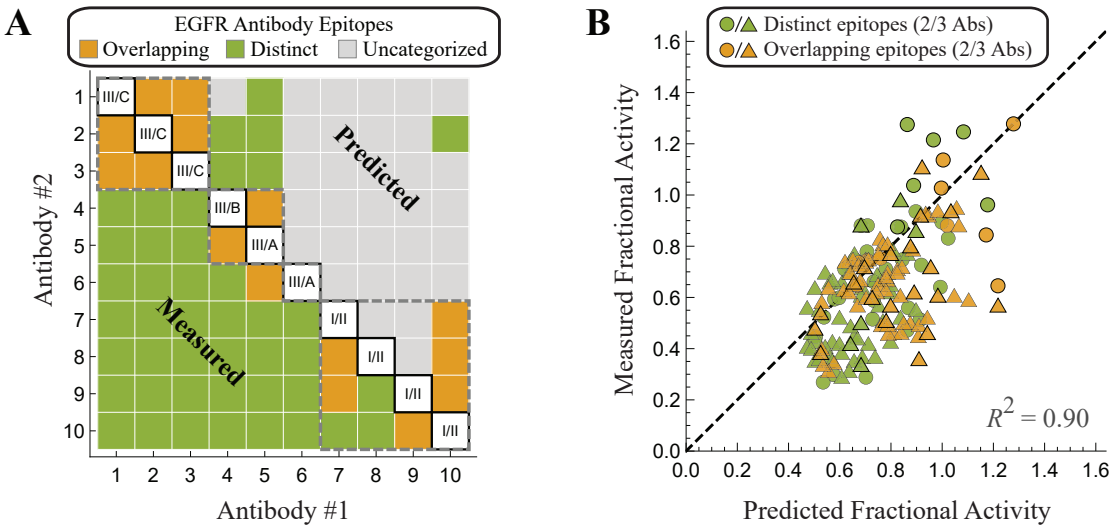

Fig 3. Classifying antibody epitopes as overlapping or distinct. (A) Comparing the experimentally measured activity to the overlapping or distinct epitope models enables us to characterize each antibody pair (provided the two models predict sufficiently different activities). (B) The resulting predictions for the 2-Ab and 3-Ab mixtures have the same predictive power ($R^2 = 0.90$) as a model that relies on epitope groupings given by SPR measurements (Fig 2B).

Multidomain antibodies boost breadth and potency via avidity

While the previous sections analyzed combinations of whole, unmodified antibodies, we now extend our framework to connect with the rising tide of engineering efforts that genetically fuse different antibody components to construct multi-domain antibodies [8]. Specifically, we focus our attention on recent work by Laursen *et al.* who isolated single-domain antibodies from llamas immunized with H2 or H7 influenza hemagglutinin (HA) [7]. The four single-domain antibodies isolated in this manner included one antibody that preferentially binds influenza A group 1 strains ($\mathrm{Ab_{A1}}$), another that binds influenza A group 2 strains ($\mathrm{Ab_{A2}}$), and two antibodies that bind to influenza B strains ($\mathrm{Ab_B^{(1)}}$ and $\mathrm{Ab_B^{(2)}}$). Fig 4A,B shows data from a representative influenza A group 1 strain (blue dot, only bound by the blue $\mathrm{Ab_{A1}}$), influenza A group 2 strain (green dot, only bound by the green $\mathrm{Ab_{A2}}$), and influenza B strain (gold dot, bound by both of the yellow $\mathrm{Ab_B^{(1)}}$ and $\mathrm{Ab_B^{(2)}}$ antibodies).

In the contexts of rapidly evolving pathogens such as influenza, two important characteristics of antibodies are their potency and breadth. Potency is measured by the inhibitory concentration $\mathrm{IC_{50}}$ at which 50% of a virus is neutralized, where a smaller $\mathrm{IC_{50}}$ represents a better antibody. Breadth is a measure of how many strains are susceptible to an antibody.

In an effort to improve the potency and breadth of their antibodies, Laursen *et al.* tethered together different domains using a flexible amino acid linker (right-most columns of Fig 4A,B) and tested them against a panel of influenza strains. To make contact with these multidomain constructs, consider a concentration c of the tethered antibody $\mathrm{Ab_{A1}}$–$\mathrm{Ab_{A2}}$. Relative to the unbound HA state, the $\mathrm{Ab_{A1}}$ or $\mathrm{Ab_{A2}}$ portions of the antibody will neutralize the virus with relative probability $\frac{c}{\mathrm{IC_{50,A1}}}$ or $\frac{c}{\mathrm{IC_{50,A2}}}$, respectively. Although neutralization is mediated by antibody binding, the two quantities may or may not be proportional [9–11], and hence we replace dissociation constants with $\mathrm{IC_{50}}$s in our model (see S1 Text Section C).

Laursen *et al.* determined that their tethered constructs cannot intra-spike crosslink two binding sites on a single HA trimer, but they can inter-spike crosslink adjacent

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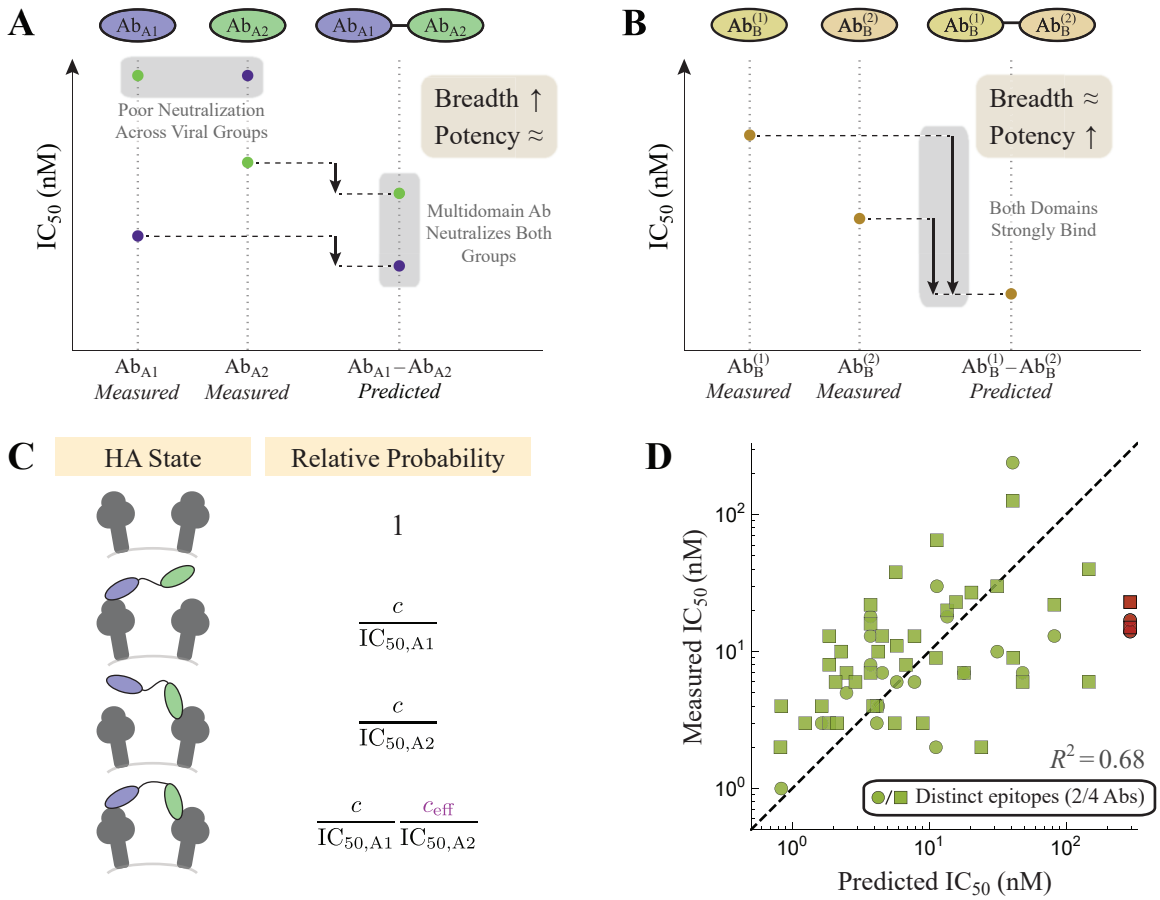

Fig 4. Tethering influenza antibodies increases breadth and potency. (A) The influenza A antibodies $\mathrm{Ab_{A1}}$ and $\mathrm{Ab_{A2}}$ were tethered together to form $\mathrm{Ab_{A1}}$–$\mathrm{Ab_{A2}}$ while (B) two influenza B antibodies formed $\mathrm{Ab_B^{(1)}}$–$\mathrm{Ab_B^{(2)}}$. Representative data shown for an influenza A group 1 (blue), influenza A group 2 (green), and influenza B (gold) strains. Strong potency is marked by a small $\mathrm{IC_{50}}$ while large breadth implies that multiple strains are controlled by an antibody. (C) Representative states of HA and their corresponding Boltzmann weights for multidomain antibodies, where crosslinking between adjacent spikes boosts neutralization via avidity ($c_{\mathrm{eff}} = 1400\,\mathrm{nM}$ in Eq (5)). (D) Theoretical predictions of the potency of all multidomain antibodies versus their measured values. The red points denote two outlier influenza strains discussed in the text that are not neutralized by $\mathrm{Ab_{A1}}$ or $\mathrm{Ab_{A2}}$ individually but are highly neutralized by their combination.

HA [7]. The linker connecting the two antibody domains facilitates such crosslinking, since when one domain is bound the other domain is confined to a smaller volume around its potential binding sites. This effect can be quantified by stating that the second domain has an effective concentration c_{eff} (Fig 4C, purple), making the relative probability of the doubly bound state $\frac{c}{\mathrm{IC_{50,A1}}}\frac{c_{\mathrm{eff}}}{\mathrm{IC_{50,A2}}}$. Therefore, the fraction of virus neutralized by two tethered antibody domains is given by

$$\text{Fraction Neutralized} = \frac{\frac{c}{\mathrm{IC_{50,A1}}} + \frac{c}{\mathrm{IC_{50,A2}}} + \frac{c}{\mathrm{IC_{50,A1}}}\frac{c_{\mathrm{eff}}}{\mathrm{IC_{50,A2}}}}{1 + \frac{c}{\mathrm{IC_{50,A1}}} + \frac{c}{\mathrm{IC_{50,A2}}} + \frac{c}{\mathrm{IC_{50,A1}}}\frac{c_{\mathrm{eff}}}{\mathrm{IC_{50,A2}}}}. \tag{4}$$

Note that this equation assumes that influenza virus is fully neutralized at saturating concentrations of antibody ($\alpha = 0$ in Eq (1), with Fraction Neutralized analogous to $1 -$ Fractional Activity).

The $\mathrm{IC_{50}}$ of the tethered construct is defined as the concentration c at which half of

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the virus is neutralized, which can be solved to yield

$$\mathrm{IC}_{50,\mathrm{A1\text{–}A2}} = \frac{\mathrm{IC}_{50,\mathrm{A1}}\,\mathrm{IC}_{50,\mathrm{A2}}}{c_{\mathrm{eff}} + \mathrm{IC}_{50,\mathrm{A1}} + \mathrm{IC}_{50,\mathrm{A2}}}, \tag{5}$$

with an analogous expression holding for the $\mathrm{Ab}_{\mathrm{B}}^{(1)}$–$\mathrm{Ab}_{\mathrm{B}}^{(2)}$ construct. Using the measured IC_{50}s of $\mathrm{Ab}_{\mathrm{A1}}$–$\mathrm{Ab}_{\mathrm{A2}}$ and $\mathrm{Ab}_{\mathrm{B}}^{(1)}$–$\mathrm{Ab}_{\mathrm{B}}^{(2)}$ against the various influenza strains, we can infer the value of the single parameter $c_{\mathrm{eff}} = 1400\,\mathrm{nM}$ (see S1 Text Section C). This result is both physically meaningful and biologically actionable, as it enables us to predict the IC_{50} of the tethered multidomain antibodies against the entire panel of influenza strains. Fig 5A,B compares the resulting predictions to the experimental measurements, where plot markers linked by horizontal line segments indicate a close match between the predicted and measured values.

The two tethered antibodies display unique trends that arise from their compositions. Since the two domains in $\mathrm{Ab}_{\mathrm{A1}}$–$\mathrm{Ab}_{\mathrm{A2}}$ bind nearly complementary strains, the tethered construct will increase breadth (since this multidomain antibodies can now bind to both group 1 and group 2 strains) but will only marginally improve potency. Mathematically, if $\mathrm{Ab}_{\mathrm{A1}}$ binds tightly to an influenza A group 1 strain while $\mathrm{Ab}_{\mathrm{A2}}$ binds weakly to this same strain ($\mathrm{IC}_{50,\mathrm{A2}} \to \infty$), their tethered construct has an $\mathrm{IC}_{50,\mathrm{A1\text{–}A2}} \approx \mathrm{IC}_{50,\mathrm{A1}}$. Said another way, $\mathrm{Ab}_{\mathrm{A1}}$–$\mathrm{Ab}_{\mathrm{A2}}$ should be approximately as potent as a mixture of the individual antibodies $\mathrm{Ab}_{\mathrm{A1}}$ and $\mathrm{Ab}_{\mathrm{A2}}$. Note that since the experiments could not accurately measure weak binding ($> 1000\,\mathrm{nM}$), the predicted IC_{50} for the multidomain antibodies represents a lower bound.

On the other hand, tethering the two influenza B antibodies yields a marked improvement in potency over either individual antibody, since both domains can bind to any influenza B strain and boost neutralization via avidity. The process of engineering a multivalent interaction is reminiscent of engineered bispecific IgG [8], and adding additional domains could yield further enhancement in potency, provided that all domains can simultaneously bind.

While the model is able to characterize the majority of tethered antibodies, it also highlights some of the outliers in the data. For example, the H3N2 strains A/Panama/2007/99 and A/Wisconsin/67/05 were poorly neutralized by either $\mathrm{Ab}_{\mathrm{A1}}$ or $\mathrm{Ab}_{\mathrm{A2}}$ ($\mathrm{IC}_{50} \geq 1000\,\mathrm{nM}$), but the tethered construct exhibited an $\mathrm{IC}_{50} = 14\,\mathrm{nM}$ and $\mathrm{IC}_{50} = 17\,\mathrm{nM}$, respectively, far more potent than the 300 nM lower limit predicted for both viruses (red circles in Fig 4D and red lines in Fig 5A). Interestingly, Laursen *et al.* found that mixing the individual, untethered antibodies $\mathrm{Ab}_{\mathrm{A1}}$ and $\mathrm{Ab}_{\mathrm{A2}}$ also resulted in shockingly poor neutralization ($\mathrm{IC}_{50} \geq 1000\,\mathrm{nM}$), suggesting that the tether is responsible for the increase in potency [7]. From the vantage of our quantitative model, this outlier cries out for further investigation.

To further boost neutralization, Laursen *et al.* created two additional constructs that combined all four antibody domains, the first being the linear chain ($\mathrm{Ab}_{\mathrm{A1}}$–$\mathrm{Ab}_{\mathrm{A2}}$–$\mathrm{Ab}_{\mathrm{B}}^{(1)}$–$\mathrm{Ab}_{\mathrm{B}}^{(2)}$). Since the influenza A antibodies do not bind the influenza B strains (and vise versa), this construct should have the same IC_{50} as $\mathrm{Ab}_{\mathrm{A1}}$–$\mathrm{Ab}_{\mathrm{A2}}$ for the influenza A strains and as $\mathrm{Ab}_{\mathrm{B}}^{(1)}$–$\mathrm{Ab}_{\mathrm{B}}^{(2)}$ for the influenza B strains, as was found experimentally (compare the *Predicted* columns in Fig 5A-C). For example, the two H3N2 strains (A/Panama/2007/99 and A/Wisconsin/67/05) were again found to have measured IC_{50}s (15 nM and 23 nM) far smaller than their predicted lower bound of 300 nM (red squares in Fig 4D, red lines in Fig 5C).

A second construct containing all four antibody domains attached two copies of $\mathrm{Ab}_{\mathrm{A1}}$–$\mathrm{Ab}_{\mathrm{A2}}$–$\mathrm{Ab}_{\mathrm{B}}^{(1)}$–$\mathrm{Ab}_{\mathrm{B}}^{(2)}$ through an IgG backbone (Fig 5D). Since the identical domains in both arms of this construct should be able to simultaneously bind, the new antibody should markedly boost potency through avidity. Surprisingly, the

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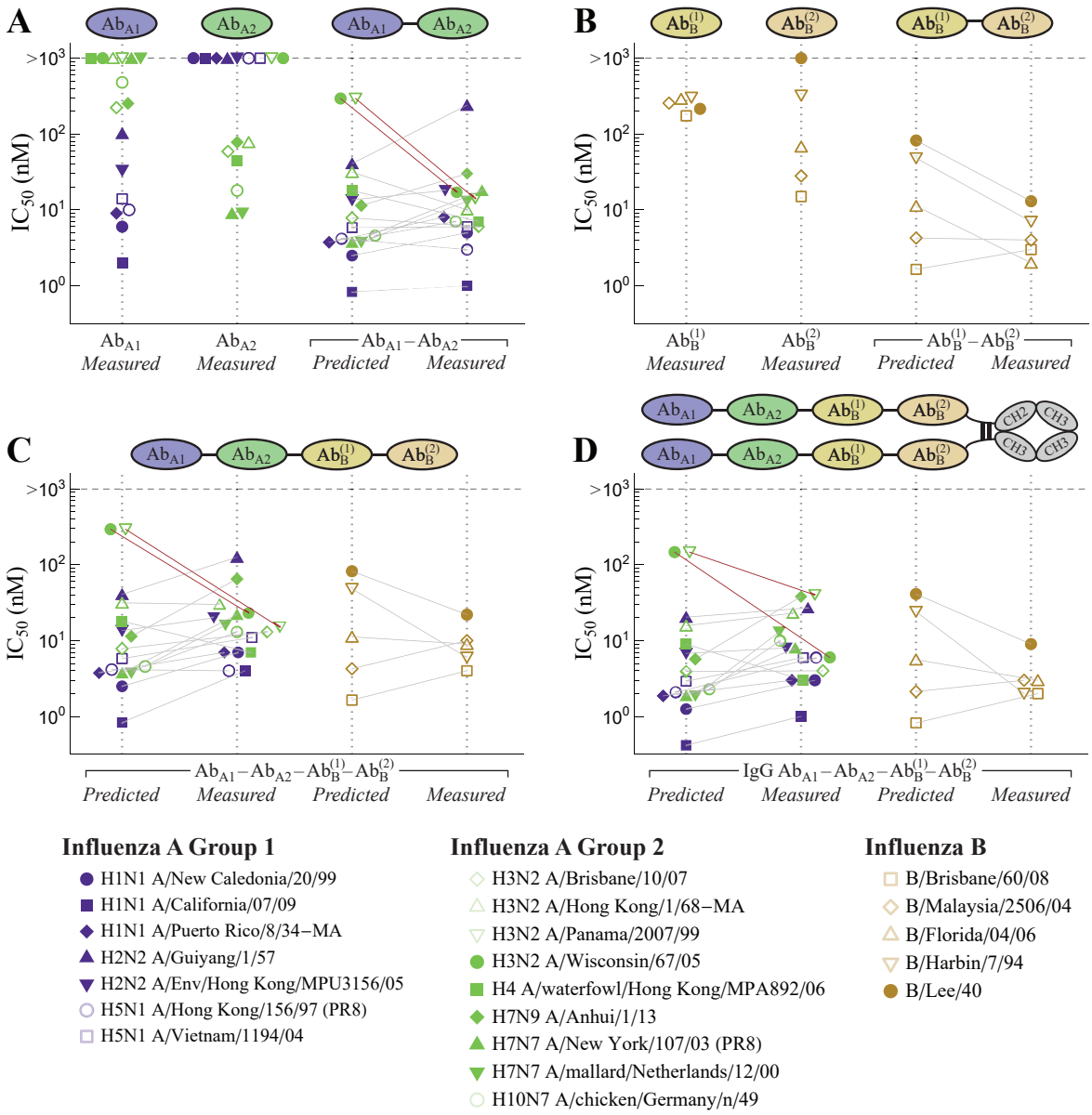

Fig 5. Neutralization of multidomain antibodies. (A,B) The potency of the 2-Ab constructs and their constitutive antibodies against a panel of influenza strains. Ab_{A1} primarily binds influenza A group 1 (blue), Ab_{A2} to influenza A group 2 (green), and the two Ab_B antibodies to influenza B strains (gold). (C) All four antibodies were tethered to form the linear chain Ab_{A1}–Ab_{A2}–$Ab_B^{(1)}$–$Ab_B^{(2)}$ and (D) two copies of this chain were placed on an IgG backbone. The model suggests that the two arms of the IgG are not capable of simultaneously binding a virion. Red lines indicate two outlier influenza strains discussed in the text that are not neutralized by Ab_{A1} or Ab_{A2} individually but are highly neutralized by their combination. Data was digitized from Figs 1 and 3 of Ref [7].

neutralization of this final construct was well characterized as half the IC_{50} of an individual Ab_{A1}–Ab_{A2}–$Ab_B^{(1)}$–$Ab_B^{(2)}$, suggesting that there was no noticeable avidity and that the increase in neutralization only arose from having twice as many antibody domains. As above, this intriguing result presents an opportunity to both quantitatively check experimental results and to advocate for future studies in potentially highly

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promising directions. In this particular instance, it suggests that the IgG backbone used did not permit simultaneous binding of both arms. If a different multivalent scaffold (perhaps with greater flexibility or with longer linkers) enabled bivalent binding of both arms, it could potentially increase the neutralization of this construct by 100-fold as seen in the influenza B constructs.

Discussion

In this work, we developed a statistical mechanical model that predicts the collective efficacy of an antibody mixture whose constituents are assumed to bind to a single site on a receptor. Each antibody is first individually characterized by its ability to bind the receptor (through its dissociation constant K_D) and inhibit activity (via its potency α) as per Eq (1). Importantly, this implies that the activity of each monoclonal antibody must be measured at a minimum of two concentrations in order to infer both parameters, and additional measurements would further refine these parameter values and the corresponding model predictions.

After each antibody is individually characterized, the activity of a combination of antibodies will depend upon whether they bind independently to distinct epitopes or compete for overlapping epitopes. Theoretical models often assume for simplicity that all antibodies bind independently, and in the contexts where this constraint can be experimentally imposed such models can accurately predict the effectiveness of antibody mixtures [12]. Yet when the antibody epitopes are unknown or when a large number of antibodies are combined, it is likely that some subset of antibodies will compete with each other while others will bind independently, which will give rise to a markedly different response. Our model generalized these previous results to account for antibody mixtures where arbitrary subsets can bind independently or competitively (Eqs (2) and (3), S1 Text Section A).

We showed that in the context of the EGFR receptor, where every pairwise interaction was measured using surface plasmon resonance, our model is better able to predict the efficacy of all 2-Ab and 3-Ab mixtures than a model that assumes all antibodies bind independently or competitively (Fig 2). This suggest that mixtures of antibodies do not exhibit large synergistic effects. More generally, similar models in the contexts of anti-cancer drug cocktails and anti-HIV antibody mixtures also found that the majority of cases that were described as synergistic could instead be characterized by an independent binding model [12, 13]. This raises the possibility that synergy is more the exception then the norm, and hence that simple models can computationally explore the full design space of antibody combinations.

While it is often straightforward to measure the efficacy of n individual antibodies, it is more challenging to quantify all $\frac{n(n+1)}{2}$ pairwise interactions and determine which antibodies bind independently and which compete for an overlapping epitope. We demonstrated that after each antibody is individually characterized, our model can be applied in reverse by using the activity of 2-Ab mixtures to classify whether antibodies compete or bind independently (Fig 3). Surprisingly, while the resulting categorizations were much sparser than the direct SPR measurements, the classifications produced by this method predicted the efficacy of antibody combinations with an $R^2 = 0.90$, comparable to the predictions made using the complete SPR results (Fig 2B). This suggests that key features of how antibodies interact on a molecular level can be indirectly inferred from simple activity measurements of antibody combinations.

Modern bioengineering has opened up a new avenue of mixing antibodies by genetically fusing different components to construct multi-domain antibodies [8]. Such antibodies can harness multivalent interactions to greatly increase binding avidity by over 100x (e.g. comparing the IC_{50}s of the A/Wisconsin/67/05 and B/Harbin/7/94

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strains of the 4-fused domains on an IgG backbone in Fig 5D to the corresponding IC_{50}s for the individual antibody domains in Panels A and B). For such constructs, the composition of the linker can heavily influence the ability to multivalently bind and neutralize a virus [11, 14], although Laursen *et al.* surprisingly found little variation when they modified the length of their amino acid linker (see Table S11 in Ref [7]). Another curious feature of their system was that placing their linear 4-domain antibody (Fig 5C) on an IgG backbone (Fig 5D) only resulted in a 2x decrease in IC_{50}, suggesting that the two "arms" of the IgG could not simultaneously bind. We would expect that a different backbone that allows both arms to simultaneously bind would markedly increase the neutralization potency of this construct. In this way, quantitatively modeling these multidomain antibodies can guide experimental efforts to design more potent constructs.

To close, we mention that two possible avenues of future work. First, although our model classifies antibody epitopes as either distinct or overlapping, SPR measurements indicate that there is a continuum of possible interactions. It would be fascinating to translate this more nuanced level of interaction into more precise dissociation constants when two antibodies are bound. Second, while our model focused on mixtures of antibodies, it can be applied equally well to small molecule drugs where the number of distinct combinations may be prohibitively large to measure experimentally but straightforward to explore computationally.

Methods

The coefficient of determination used to quantify how well the theoretical predictions matched the experimental measurements (Fig 2B-D, Fig 3B, Fig 4D) was calculated using

$$R^2 = 1 - \frac{\sum_{j=1}^{n} \left(y_{\text{measured}}^{(j)} - y_{\text{predicted}}^{(j)} \right)^2}{\sum_{j=1}^{n} \left(y_{\text{data}}^{(j)} \right)^2} \tag{6}$$

where y_{measured} and $y_{\text{predicted}}$ represent a vector of the measured and predicted activities for the n mixtures analyzed. In Fig 4D, we computed the R^2 of $\log_{10}$(activity) to prevent the largest activities from dominating the result (since the IC_{50} values span multiple decades).

Data from the EGFR antibody mixtures was obtained by digitizing Ref [6] Fig S1 using WebPlotDigitizer [15]. Data for the influenza multidomain antibodies was obtained from the authors of Ref [7].

The EGFR antibody epitopes experimentally characterized through SPR (Fig 3A, bottom-left) were categorized as overlapping if the average of the two antibody measurements (with preincubation by either antibody) were > 50 and as distinct if the average was < 50.

The original nomenclature for the antibodies used in Koefoed *et al.* and Laursen *et al.* are given in S1 Text Table S1.

Author contributions

T.E. and J.D.B. both participated in the conceptualization, investigation, and writing of this work.

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