Discovering Anti-platelet Drug Combinations with an Integrated Model of Activator-Inhibitor Relationships, Activator-Activator Synergies and Inhibitor-Inhibitor Synergies

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Abstract

Identifying effective therapeutic drug combinations that modulate complex signaling pathways in platelets is central to the advancement of effective anti-thrombotic therapies. However, there is no systems model of the platelet that predicts responses to different inhibitor combinations. We developed an approach which goes beyond current inhibitor-inhibitor combination screening to efficiently consider other signaling aspects that may give insights into the behaviour of the platelet as a system. We investigated combinations of platelet inhibitors and activators. We evaluated three distinct strands of information, namely: activator-inhibitor combination screens (testing a panel of inhibitors against a panel of activators); inhibitor-inhibitor synergy screens; and activator-activator synergy screens. We demonstrated how these analyses may be efficiently performed, both experimentally and computationally, to identify particular combinations of most interest. Robust tests of activator-activator synergy and of inhibitor-inhibitor synergy required combinations to show significant excesses over the double doses of each component. Modeling identified multiple effects of an inhibitor of the P2Y12 ADP receptor, and complementarity between inhibitor-inhibitor synergy effects and activator-inhibitor combination effects. This approach accelerates the mapping of combination effects of compounds to develop combinations that may be therapeutically beneficial. We integrated the three information sources into a unified model that predicted the benefits of a triple drug combination targeting ADP, thromboxane and thrombin signaling.

Author Summary

Drugs are often used in combinations, but establishing the best combinations is a considerable challenge for basic and clinical research. Anti-platelet therapies reduce thrombosis

and heart attacks by lowering the activation of platelet cells. We wanted to find good drug combinations, but a full systems model of the platelet is absent, so we had no good predictions of how particular combinations might behave. Instead, we put together three sources of knowledge. The first concerned what inhibitors act on what activators; the second concerned what pairs of activators synergise together (having a bigger effect than expected); and the third concerned what pairs of inhibitors synergise together. We implemented an efficient experimental approach to collect this information from experiments on platelets. We developed a statistical model that brought these separate results together. This gave us insights into how platelet inhibitors act. For example, an inhibitor of an ADP receptor showed multiple effects. We also worked out from the model what further (triple) combinations of drugs may be most efficient. We predicted, and then tested experimentally, the effects of a triple drug combination. This simultaneously inhibited the platelet's responses to three stimulants that it encounters during coronary thrombosis, namely ADP, thromboxane and thrombin.

Introduction

Cells are subject to diverse stimuli *in vivo*, and combine these inputs to generate appropriate biological responses. Activators and inhibitors of various targets work together in different configurations to elicit valuable and sometimes unpredictable outcomes, both natural and therapeutically induced. Many therapeutic approaches combine multiple agents acting on different targets, for example in cardiovascular disease[1], cancer[2–4], and infection[5]. Ideally, we would have a full systems model of every clinically important signaling process, helping us to predict and define potent combinations. However, in many systems, such a model is largely absent. Accordingly many workers seek to simply study the combination effects without considering additional information regarding the signaling network. Thus, screens for novel agents can take a systematic approach[6,7], but are limited usually to comparing the inhibitor combinations to the effects of single agents, without considering wider aspects of the signaling system.

 However, the discovery of synergistic effects is not trivial. There is a large set of compounds that target distinct proteins, and considering the pairwise or higher order combinations of all of these is a very substantial task. Accordingly, such screens are frequently performed under a very limited set of experimental conditions. However, in many physiological contexts, cells may be subject to diverse challenges, and it would therefore be ideal for a synergistic combination of drugs to be effective under not just one, but under many alternative conditions. To meet this challenge, systems biology approaches seek to develop integrated computational predictive models of an entire signaling process, and ultimately of a cell, tissue or organism. These models are valuable but often challenging, since their construction requires extensive experimental data, and for this reason they are often developed under relatively limited and controlled settings, such as that of a well characterized cell line. Thus, there is still a requirement to develop more efficient screening methods that by-pass the need for a complete model of a given system, but which capture the essential functional components of that system, as might be relevant in a therapeutic or other practical setting. In order to accelerate the discovery of critical combinations of factors, scientists can either take a bottom-up approach, starting with pairwise combinations and making combinations more complex, or a top-down approach starting with a set of factors and winnowing down the system to the essential components,

such as was done to successfully choose 4 transcription factors from 24 that govern the generation of pluripotent stem cells.[8]

High intracellular levels of cAMP maintain platelets in a resting state[9], with prostaglandin I2 (PGI2) and nitric oxide (NO), sustaining the production of cAMP via G_s[10] or limiting its degradation through the cGMP-dependent action of phosphodiesterase III[11]. On the other hand, platelet activators inhibit adenyl cyclase and reduce cAMP via $G\alpha_I$, while βγ subunits of G_i type proteins activate PLC and phosphoinoside 3-kinase (PI3K). The coordinated activity of different types of G proteins is required to modulate platelet behaviour. Platelet activation through G proteins involves $G\alpha_i$ $G\alpha_q$ and $G\alpha_{12/13}$[12], with the thrombin receptor, PAR1, acting through all three [13–15] and favouring $G\alpha_q$-mediated calcium mobilization over $G\alpha_{12/13}$ signaling when stimulated with thrombin-receptor activating peptide (TRAP) [16]. TxA_2 receptors couple to $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ [14,17,18]. Platelet responses to epinephrine are mediated by the α_{2A}-adrenergic receptors[19], acting in mice through the $G\alpha_i$ family member $G\alpha_z$[20]. ADP signalling in platelets, important for sustained aggregation[21], is via GPCRs P2Y1 (coupled to $G\alpha_q$ in mice[22]), and P2Y12 (coupled to $G\alpha_{i2}$ in mice[20]). The activation of GPVI (the only non-GPCR receptor targeted in our study) by Collagen or CRP leads to Lyn and Fyn phosphorylation of the FcR gamma-chain[23], allowing Syk docking[24] and activation of phospholipase C (PLC)γ2 [25] and Phosphoinoside 3 kinase (PI3K) [26,27].

Our goal was to develop efficient and practical methods to identify combinations of platelet inhibitors that would be robust in inhibiting platelets under multiple conditions, and would provide insights into platelet signaling networks. We sought to expand inhibitor combination screening by the incorporation of additional information that might give some insights into the performance of the platelet as a system.

The first step in developing our method was to investigate which inhibitors act against which activators[28]. Intuitively combinations of inhibitors are likely to be markedly synergistic when they are acting on parallel pathways. However, it has been shown that under certain feedback conditions, strong synergistic effects will be seen between upstream and downstream points that are located serially along a pathway [7]. Thus, we had no strong expectations of which combinations might show the strongest synergy. We noted that the available consensus that defines the relationships among activators and inhibitors of most signaling systems is frequently based on primary observations that are accumulated in the scientific literature in a piece-meal fashion. Since separate studies may often apply either subtly or grossly different experimental conditions, it is not ideal to simply take the accepted consensus of opinion to pair activators and inhibitors together on the basis of their literature defined targets, but it is of interest to re-evaluate these relationships in a systematic way. The second step in identifying useful combinations was to experimentally evaluate synergistic effects[29,30]. Synergy is defined as a functional interaction between two reagents that shows a much greater effect than expected, based on the known effects of the two reagents alone. There are multiple different definitions of what is precisely meant by synergy[31], and these different definitions may be considered to lie on a spectrum of tests, ranging from weak tests that provide only a suggestion of synergy, and strong tests that provide more robust evidence for such synergy. Typically, the more robust tests rely on the analysis of multiple doses of the two compounds alone and in various combinations. Such synergy studies may rely on analysis of synergies among inhibitors [1,6,7]. However, synergy studies are not confined to examine synergy among inhibitors, even when inhibition is the primary therapeutic goal. Investigation of synergies among activators [32] can assist in defining the profile of inhibitory effects of single and combination inhibitors, which reduce not only the main effects of the activators, but also provide information regarding their synergistic effects.

Since activator-inhibitor relationships, activator-activator synergy and inhibitor-inhibitor synergy each provide insights into the complex network of interacting factors that help in choosing inhibitor combinations, we set out to develop a practical framework integrating all three approaches (S1 Fig.). We integrated this information into a predictive model, and evaluated whether predictions of the model could accelerate the discovery of compound combinations effective at targeting platelet inhibition. This approach predicted a triple combination of compounds that was experimentally validated.

Methods

Ethics Statement

Informed consent was obtained from all subjects for the donation of blood samples for the purpose of platelet function analysis, with study approval obtained from the Royal College of Surgeons in Ireland Research Ethics Committee (REC679b).

Experimental Methods

Experimental methods followed a previous study[33]. Washed platelets were prepared from venous blood of consenting healthy donors drawn via phlebotomy into 15% (v/v) acid-citrate-dextrose (ACD) anticoagulant (38mM citric acid anhydrous, 75 mM sodium citrate, 124mM dextrose). Blood was centrifuged at 150 x g for 10 minutes at room temperature and platelet rich plasma (PRP) was collected and acidified to pH 6.5 with ACD. 1 μM prostaglandin E1 (PGE1) was added prior to centrifuge PRP at 720 x g for 10 minutes. The resulting pellet was resuspended in JNL buffer (6 mM dextrose, 130 mM NaCl, 9 mM NaHCO$_3$, 10 mM sodium citrate, 10 mM Tris base, 3mM KCl, 0.81 mM KH$_2$PO$_4$ and 0.9 mM MgCl$_2$6H$_2$O, pH 7.35) adjusting the concentration to 3×10^5 platelets/ μl. Washed platelets were supplemented with 1.8 mM CaCl$_2$ immediately prior to the experiment.

The ADP release assay used white 96-well plates (white plates with white wells; Sigma-Aldrich, Ireland). Platelets were incubated with inhibitors for 10 minutes at 37°C on orbital slow shake using a Wallac 1420 Multilabel Counter (Perkin Elmer). 10 μl cocktail (K) or activators were then added and allowed to activate platelets for 10 minutes in the same conditions used with the inhibitors. 10 μl of the detection reagent Chrono-lume (Chronolog; Labmedics Limited, UK) were added and sample luminescence detected after an additional 5 seconds with rapid shaking measuring arbitrary absorbance units (AAU).

The compounds used as platelet activators were CRP (*Ca*, triple-helical Collagen-related peptide from 0.013 to 30 μg/ml; purchased from Dr Richard Farndale, Cambridge, UK), U46619 (*Xa*, from 0.003 to 6 μM; Santa Cruz Biotechnology, Germany), TRAP (*Ta*, Thrombin Receptor Activator Peptide sequence SFLLRN from 0.25 to 16 μM; Sigma-Aldrich, Ireland), Epinephrine (*Ea*, from 0.001 to 30 μM; Chronolog, Labmedics Limited, UK), and ADP (*Aa*, from 0.137 to 100 μM; Chronolog, Labmedics Limited, UK). Hill coefficients and response to single agents was evaluated in 4 donors. EC50s and EC90s were determined with GraphPad Prism software, which uses the equation Y = Bottom + (Top-Bottom)/(1+10$^{(\text{LogEC50-\% inhibition})^*\text{HillSlope}}$). The 2xEC50s were obtained by simply doubling the EC50s. In the case of ADP, to avoid doses higher than 20 μM that might interfere with the assay (S4 Fig.), 10 μ M was used instead of the actual EC50 (∼ 50 μM). The letter used to represent each compound denoted the selected dose for each, the letter followed by "2" to denote a dose that is double the selected dose, and the letter followed by "90" to denote a dose that causes the 90% activation (S1 Table).

A mother solution of the "activator cocktail" (K), which is all the activators at their selected doses (0.025 μ M Epinephrine (Ea), 0.5 μ M U46619 (Xa), 1 μg/ml CRP (Ca), 4 μM TRAP (Ta), and 10 μM ADP (Aa)) was prepared and serial 1:2 dilutions were used to stimulate platelets. Its

EC50, was found to be 0.1636 fold the concentration of the mother solution (S2 Fig.), and this dose was used for cocktail activation in tests of inhibitor synergy. The rationale for choosing this dose was that this was the dose that gave a 50% activation of platelets, which should be relatively sensitive to inhibition by inhibitors or inhibitor pairs: if a higher concentration of the cocktail had been used, it is possible that the platelets would be consistently activated in a way that masked many inhibitory effects or inhibitor combination effects. It is slightly less than the five-fold reduction that would be obtained were the doses to be crudely divided by the number of activators. These doses lie below the individual EC20 values for all five activators (S2 Fig.).

To determine inhibitor IC50s, we evaluated ADP release induced by different doses within a range specified in parentheses. Inhibitors used were Wortmannin (*Pi*, from 0.137 to 100 nM; Sigma-Aldrich, Ireland), SQ29548 (*Xi*, from 2.195 nM to 1.6 μM; Enzo Life Sciences, UK), BMS200261 (*Ti*, from 0.000685 to 0.5 nM; Sigma-Aldrich, Ireland), Yohimbine (*Ei*, from 15.625 nM to 2 μM; Sigma-Aldrich, Ireland), and MRS2395 (*Ai*, from 0.137 to 100 μM; Sigma-Aldrich, Ireland). All were dissolved in water except MRS2395, which was dissolved in ethanol, where the ethanol proportion was equal to or less than the 0.37% of the total volume. Platelets were pre-incubated with the inhibitors and then stimulated with the activator cocktail. Cocktail-stimulated platelets were almost completely insensitive to Wortmannin inhibition and therefore the IC50 for Wortmannin was determined on platelets stimulated with 1 μg/ml of CRP.

The 10 consenting healthy donors were all Caucasian between 24 and 42 years of age. Each plate harboured four types of treatments (single agents, activator/activator combinations, inhibitor/inhibitor combinations, activator/inhibitor combinations) and two types of controls (resting and cocktail-activated platelets). Two different arrangements of wells were used in order to limit position effects and, since the results for the two plate layouts broadly correlated, a dataset was assembled from 10 consenting healthy volunteers.

Statistical Modeling

To account for donor/plate variation, analysis was of the rank within each donor of the observed ADP level. Statistical analysis was performed using STATA version 12.0 [34] and the fitting of the final models confirmed using R [35]. Visualisations of data for Fig. 1 and for S3 Fig. (below), were constructed using R[35].

The visualizations were performed using either the basic visualization package or the *gplots* package in R. The clustering (S3 Fig.) was performed using the *hclust* function of R, which performs hierarchical clustering (each object is assigned to a cluster, and then the two most similar objects/clusters are joined in one cluster; and so on iteratively until one cluster is created). A one-tail Wilcoxon test was used to test the significance of whether activator-activator and inhibitor-inhibitor combinations were superior to either of the double doses of the component reagents. Raw data were converted to logarithms to the base 10 for visualisation. A small number of duplicate treatments within an individual (ADP for group 1 and Epinephrine for group 2) were replaced by their respective means.

Main effect terms were held fixed, while interaction terms were added using a forward step-wise multiple regression approach (adding terms that significantly improved the model, $p < 0.05$). The pair-wise interactions were tested by fitting pair-wise interaction terms, along with main effect terms. We present results for synergies of inhibitors (the two inhibitors together inhibit much more strongly than expected) or activators (the two activators activate much more strongly than expected); other significant synergistic interactions were not seen.

We defined significant interaction as observation that the double doses of the activators on their own BOTH have significantly less activating effects than the combination in single doses

DEFINITION OF SYNERGY

For each reagent, D is the dose typically leading to a 50% activation (for an activator)

or 50% inhibition of cocktail-induced activation (for an inhibitor).

2D represents a doubling of that dose. This provides a test of Loewe additivity.

Inhibitor-Inhibitor

$$X_{Dx} Y_{Dy} < X_{2Dx} \text{ AND } X_{Dx} Y_{Dy} < Y_{2Dy}$$
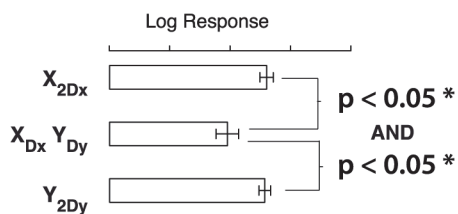
Activator-Activator

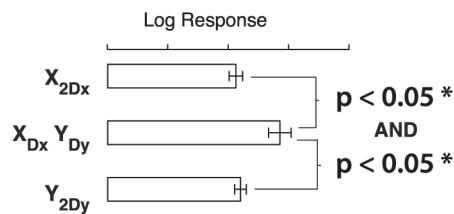$$X_{Dx} Y_{Dy} > X_{2Dx} \text{ AND } X_{Dx} Y_{Dy} > Y_{2Dy}$$

Fig 1. Robust tests of synergy for activator-activator and inhibitor-inhibitor combinations. These tests correspond to a particular case of Loewe additivity.

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(two Wilcoxon one-tailed tests with P<0.05 for each, Fig. 1). This approach may be beneficial when reagents lack clear dose response relationships[31]. It is equivalent to a limiting case of Loewe additivity, effectively sampling a single point on the isobole when activators have similar potency [30,31].

To integrate the three strands of information, we took the significant interactions identified in the double Wilcoxon test for synergy, and the significant activator-inhibitor combination terms identified from the stepwise linear regression modelling. We brought those forward into an integrated model, including the main effects for each activator and inhibitor.

The inhibitor-inhibitor and activator-activator testing component of the statistical study design was based on a sequential test, namely to test inhibition combination first against one double dose (one-tailed test, $p < 0.05$), and then against the second double dose (second one-tailed test, p<0.05). No algorithms are available to calculate the power of this approach. Nevertheless, the study design may be informed by the assumption, when two inhibitors each confer a roughly equivalent effect, that this test is equivalent to a test of the inhibitor combination versus either double dose. Assuming a log ADP intensity of 5.2 for a double dose of inhibitor, and 4.9 for a dual inhibitor combination (s.d. = 0.2), in order to have 90% power to detect a significant difference (two-tailed, $p < 0.05$), a sample size of 10 subjects is required.

Supplemental Data, Program, Output Files Description

Input, analysis code and output is given in two alternative statistical analysis environments, R and STATA. The same results are obtained using either. The input is the complete analysis dataset presented in the main paper.

Results

We investigated reagents thought to act primarily on six proteins in pathways of major therapeutic interest in the inhibition of platelet function[33], denoted by single letters as follows: Thromboxane Receptor (X), Thrombin PAR1 Receptor (T), P2Y12 ADP receptor (A),

Epinephrine Receptor (*E*), PI3 Kinase (*P)*, and GPVI Collagen Receptor (*C)*. The suffix "a" was used to indicate a reagent that activated the protein, and "i" for a reagent thought to inhibit it (so that *Xa* denotes Thromboxane Receptor activator and *Xi* its inhibitor). There was no inhibitor available for GPVI, and an inhibitor of PI3 kinase was included because of its inhibitory effects on GPVI stimulated activation. Dose response curves for the activators and inhibitors used in the study (S2 Fig.) were used to select doses for use in combination studies (S1 Table). Visualization of the assay results indicated strong donor variability (Fig. 2). Accordingly, subsequent analysis was performed on the rank of the assay result within each donor dataset, thus correcting for donor effects during analysis.

Activator-Inhibitor Combinations Highlight Multiple Actions of an ADP Inhibitor

Activator-inhibitor combinations are summarized in Fig. 3A, with more detailed plots in Fig. 4. The expectation was that effects would largely be seen along the diagonal, corresponding to the *a priori* pairing of activators and inhibitors. In order to make it easier to see to what extent pairings match or depart from that expectation, we adjusted the data for visualisation purposes, where the values represent the mean values in panel A, minus the mean value observed for the single dose activator alone. Two of the combinations strongly match our expectations (Xa/Xi, and Ta/Ti). However, any combinations involving the ADP inhibitor (Ai) showed a marked departure from expectation, since its extent of inhibition of ADP activation (Aa) was markedly less than that of Ca and Xa (Fig. 3A and 3C). In spite of markedly inhibiting Ca and Xa, Ai did not manage at that same dose to prevent some activation by Aa (Fig. 3A) This suggests that it is not acting as a very efficient inhibitor of its intended target, but may be acting via other mechanisms. Overall, epinephrine (Ea) had weak activatory effects and its inhibitor yohimbine[36] (Ei) had weak inhibitory effects, which may explain why the model did not detect synergies involving this activator-inhibitor pair. It is possible that the doses of epinephrine defined in advance were inappropriate for the particular donors in this study. To evaluate the significance of the observed combination effects, we carried out multiple regression modelling. The regression model was fitted by including a parameter for the main effect for each of the activators and inhibitors. Each additional significant activator-inhibitor combination term (given a value of 1 if the experiment included both the activator and inhibitor; zero otherwise) between a particular inhibitor and a particular activator was added as a parameter in a stepwise fashion until no additional significant terms (p<0.05) could be added. An initial model that included only activator and inhibitor effects alone explained 68% of the variance (S2 Table). This rose to 73% when specificity of action was considered, by including four additional significant activator-inhibitor combination terms (S3 Table). We considered whether a Boolean representation of activator-inhibitor relationships (e.g. that inhibitor *Ai* cancels out entirely the effect of activator *Ta*) would model the data adequately. However, a Boolean model of the activator-inhibitor relationships explained less of the variance in the data and provided a significantly poorer fit (p<10^{-5}; S4 Table).

Significant inhibition (Fig. 3B and 3D) was observed for two activators by the inhibitors normally associated with their receptors (*Ti/Ta* and *Xi/Xa*). While *GPVI* Collagen receptor activation (*Ca*) is thought to be strongly mediated by PI3K [33], inhibiting PI3K (with *Pi*) had similar effects on *Ca* as it had on *Xa* and *Ta* responses, indicating that *Pi* is not highly specific for *GPVI* inhibition, and that its target PI3K may be a convergence point for different signalling routes. Most strikingly, the presumed ADP *P2Y12* inhibitor *Ai* (MRS2395) inhibited other activators (*Ai/Ca*; *Ai/Ta*, and *Ai/Xa*) significantly, and more strongly than it inhibited ADP activation. This may be consistent with either a central role for the *P2Y12* receptor in mediating
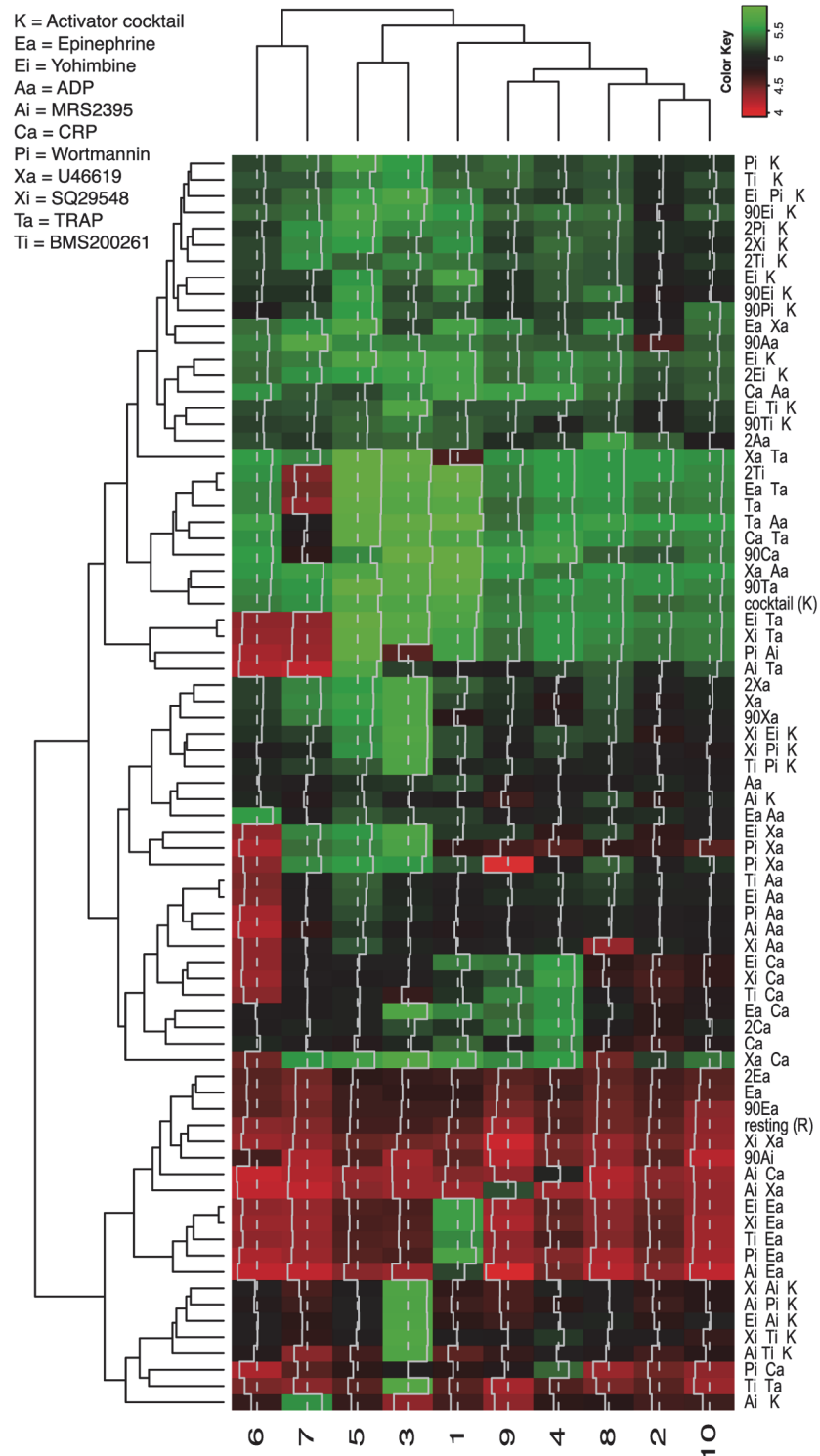
Fig 2. Heatmap of platelet activation (log ADP release) in each donor for each reagent combination.
Columns: 10 donors. Rows: different experimental conditions. Green: activated platelets with high ADP release, measured in $\log_{10}$ Arbitrary Absorbance Units (AAU); red: non-activated platelets. White vertical line: actual value of $\log_{10}$ (AAU). The white vertical dashed lines across each column represent the middle value between the maximum and minimum values observed for the entire dataset. Data were grouped by hierarchical clustering. Any technically replicated results were represented by their means. The five activators used were used at doses typically corresponding to their EC50 (see text): 0.025 μM Epinephrine (Ea), 0.5 μM

U46619 (Xa), 1 μg/ml CRP (Ca), 4 μM TRAP (Ta), and 10 μM ADP (Aa), respectively intended to activate the epinephrine, thromboxane, collagen, thrombin and ADP receptors; K represents a cocktail comprising all five activators combined at a dilution corresponding to their combined EC50 (the individual concentrations shown, multiplied by 0.1636). The five inhibitors used at their IC50 values were 1uM Yohimbine (Ei), 68.39 nM SQ29548 (Xi), 16.5 nM Wortmannin (Pi), 2.85 uM BMS200261 (Ti), and 36.77 uM MRS2395 (Ai), respectively intended to inhibit the epinephrine receptor, thromboxane receptor, PI3K, thrombin receptor and ADP receptor. For comparison purposes, the double doses of individual activators and inhibitors were included, which are shown preceded by the number "2"; EC90 and IC90 doses (see text) were also included for comparison, with the prefix "90".

signalling via many receptors, or with an alternative target of action of the drug. Regardless of the mechanism of the observed effect, this first strand of evidence highlights the influence of Ai on multiple activators. This suggests that Ai is a promising candidate to include in a set of compounds to inhibit platelets in combination.

Activator-Activator Synergies

Significant synergy was defined here as a much greater effect of a combination of two reagents than the double doses of either reagent (requirement to pass two one-tailed Wilcoxon tests, each with $p < 0.05$). While more conservative than other approaches[37], it avoids statistical difficulties when effect sizes of different reagents are imbalanced, sampled from non-equivalent points on their respective dose response curves, or where reagents do not have standard dose response curves. Activator-activator synergies are summarized in the bottom left triangle of Fig. 3B, and the same observations after adjustment for differences in main effects of activators in the bottom left triangle of Fig. 3D. The detailed results are shown in Fig. 5. Fig. 3D displays the difference of the activation or inhibition from the most effective double dose of either the first or the second agent within the combination. Two significant activator-activator synergies were identified: activators of the ADP and collagen receptors (Aa and Ca) synergised significantly, and activators of the ADP and thromboxane receptors (Aa and Xa) synergised significantly. This second strand of evidence suggests that concurrent inhibition of platelets activation elicited by Aa, Ca and Xa may be useful in lowering the activation of platelets in the presence of multiple activators. Again, it particularly points to an important role for the ADP receptor in activation.

Inhibitor-Inhibitor Synergies

We tested the effects of inhibitors on the activation of platelets by a cocktail of all five activators, since such a cocktail may be physiologically relevant, and may be more sensitive to inhibitor synergies. The cocktail activation of platelets showed a steep dose response consistent with likely cooperative (synergistic) activity (S2 Fig.). We chose a dose of this cocktail that yielded 50% activation (see Methods), intended as a non-saturating combination activator to be used in inhibitor experiments. While it is likely that this cocktail is more dominated by particular activators, it was notable that, while double doses for four of the five inhibitors had difficulty overcoming the activatory effect of this cocktail, eight of the ten inhibitor combinations lowered platelet activation somewhat (Fig. 3D). This indicated that the doses of activators used in the cocktail were showing sensitivity to inhibitor combinations, but much less sensitivity to double doses of single inhibitors. Thus, the dose of cocktail employed in the study appeared to be appropriate for the purpose of detecting synergies among inhibitors, avoiding saturation effects.

As before, synergy was defined for each pair of inhibitors whenever the combination of inhibitors had a significantly greater effect than either of the inhibitors in a double concentration
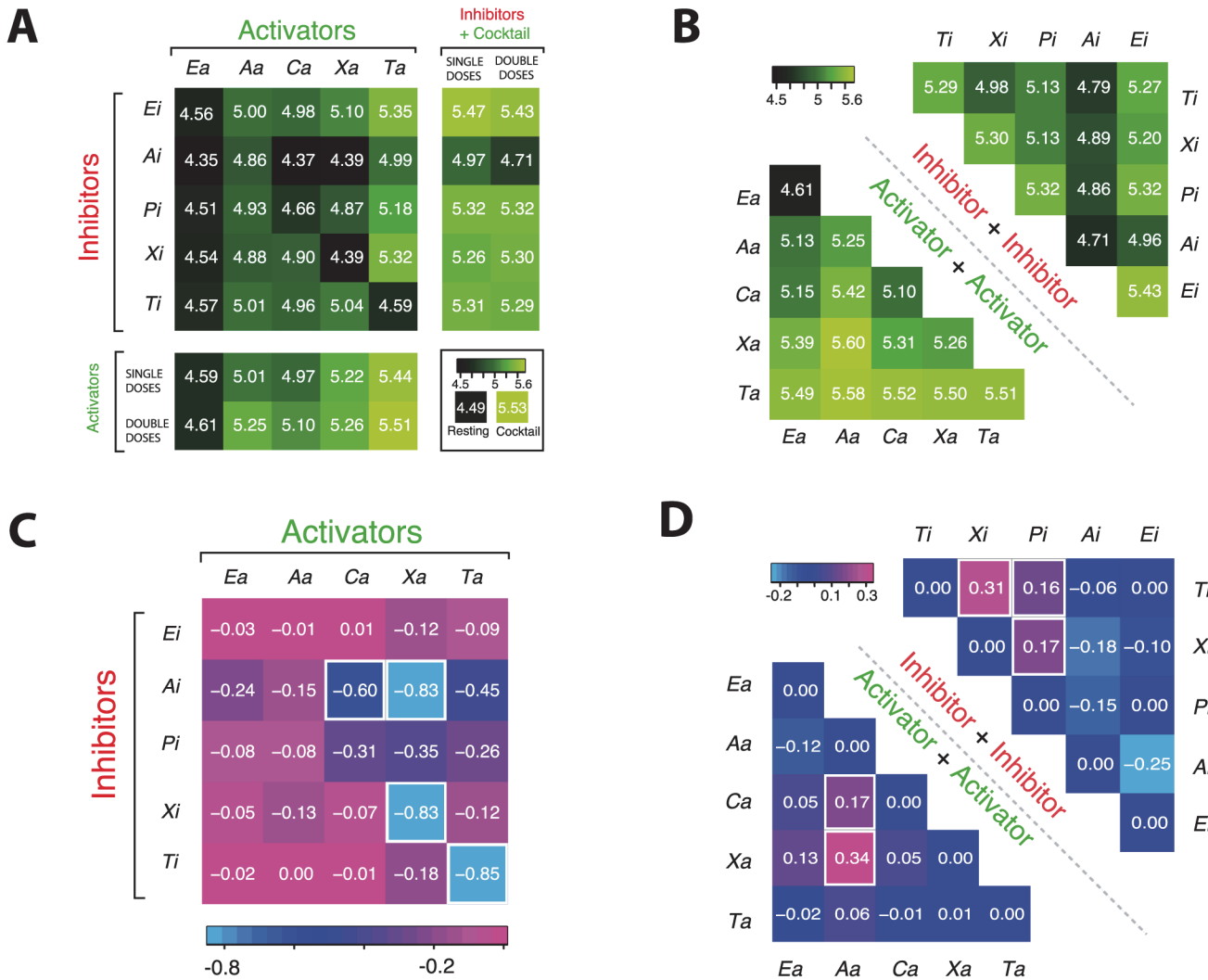
Fig 3. Identification of activator-activator synergy, inhibitor-inhibitor synergy, and activator-inhibitor combination effects. Combination experiments of activators and inhibitors. **(A)** Mean $\log_{10}$ ADP release across platelets from 10 blood donors are shown, with green indicating platelet activation. Combinations of activator and inhibitors. Single and double doses (concentrations) of each activator alone are shown at the bottom; single and double doses of each inhibitor in the presence of a cocktail of all five activators are shown to the right; resting and cocktail are shown bottom right **(B)** Activator-activator combinations and inhibitor-inhibitor combinations, log(AAU) ADP release. Inhibitor-inhibitor data represents the inhibition of a cocktail of all five agonists. **(C)** To more easily visualize the data allowing for the differences in levels of activation among the five activators, a simple correction of the data is shown, with the values in panel A subtracted by the value of the single dose activator alone (thus, for CaXi the value is 4.90–4.97 = −0.07). Four significant activator-inhibitor combinations identified by statistical modeling (see text) are highlighted within a white box. Two of these lie on the diagonal, as expected *a priori*. **(D)** As for panel B, but calculated to display the difference of the activation or inhibition from the most effective double dose of either the first or the second agent within the combination (thus, for PiTi the value is 5.29–5.13 = 0.16). Positive synergy corresponds to more combined stimulation for the activator-activator pairs, indicated in magenta, and also to less combined stimulation for the inhibitor-inhibitor pairs, which are also indicated in magenta (i.e. magenta implies strong positive synergy of either activation, or of inhibition).

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(Wilcoxon $p < 0.05$ for both comparisons). We observed three significant inhibitor-inhibitor synergies, which involved the pairwise combinations of the inhibitors of Thromboxane Receptor, Thrombin Receptor and PI3K (Fig. 3B and 3D; Fig. 6; *Xi/Ti*, *Xi/Pi*, *Pi/Ti*). This third strand of evidence provides a different perspective from the activator-inhibitor and activator-activator combinations, raising the question of how to reconcile these findings into a single model that makes useful predictions.
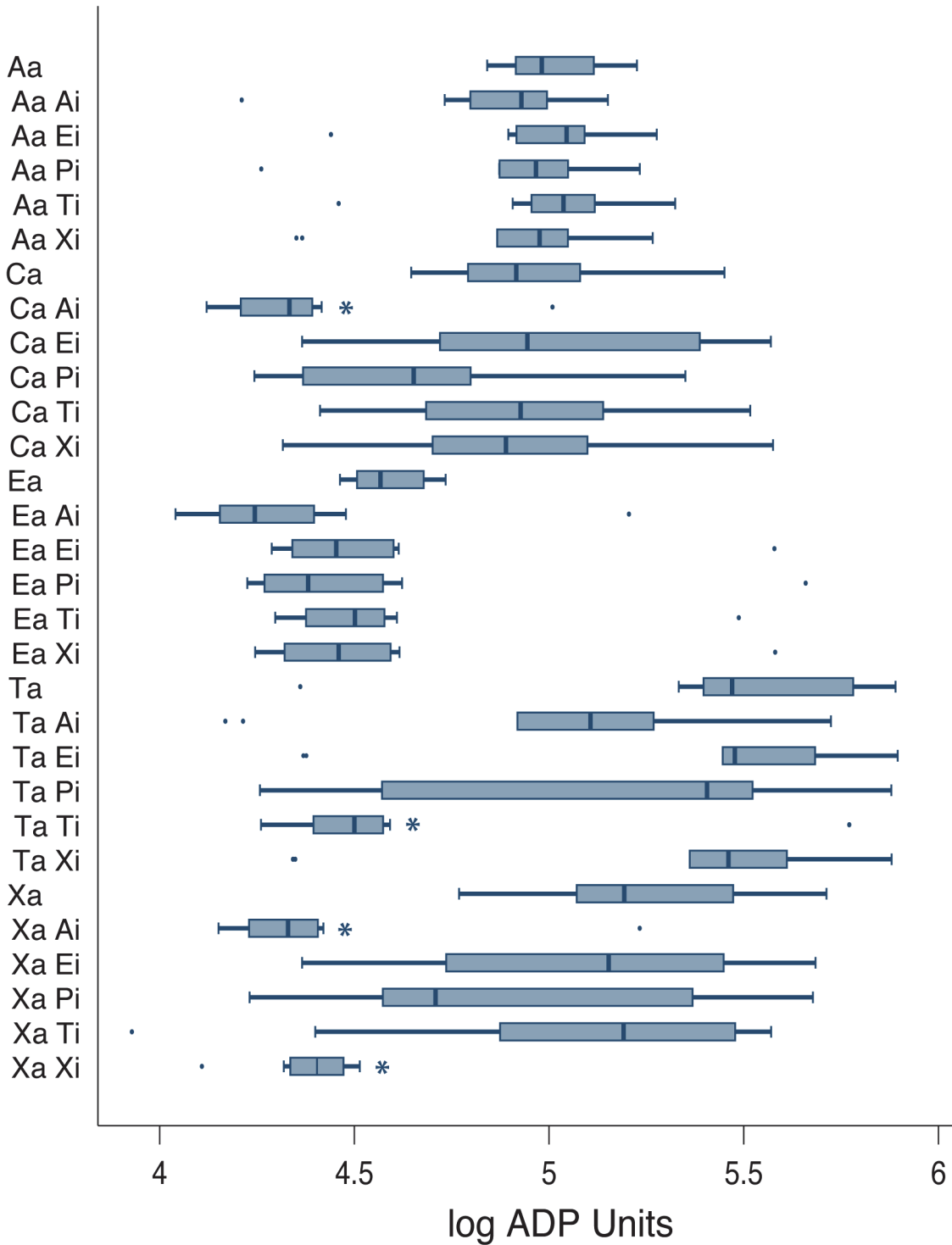
Fig 4. Combinations of activators and inhibitors. Boxplot indicating the effects of the five inhibitors on the five activators. Activators are indicated on their own in single dose (see text) and in combination with inhibitors at single dose. The four significant effects highlighted in the statistical model (see text) and in Fig. 3C are indicated by asterisks. The central grey box represents the 25%–75% percentile of each distribution.

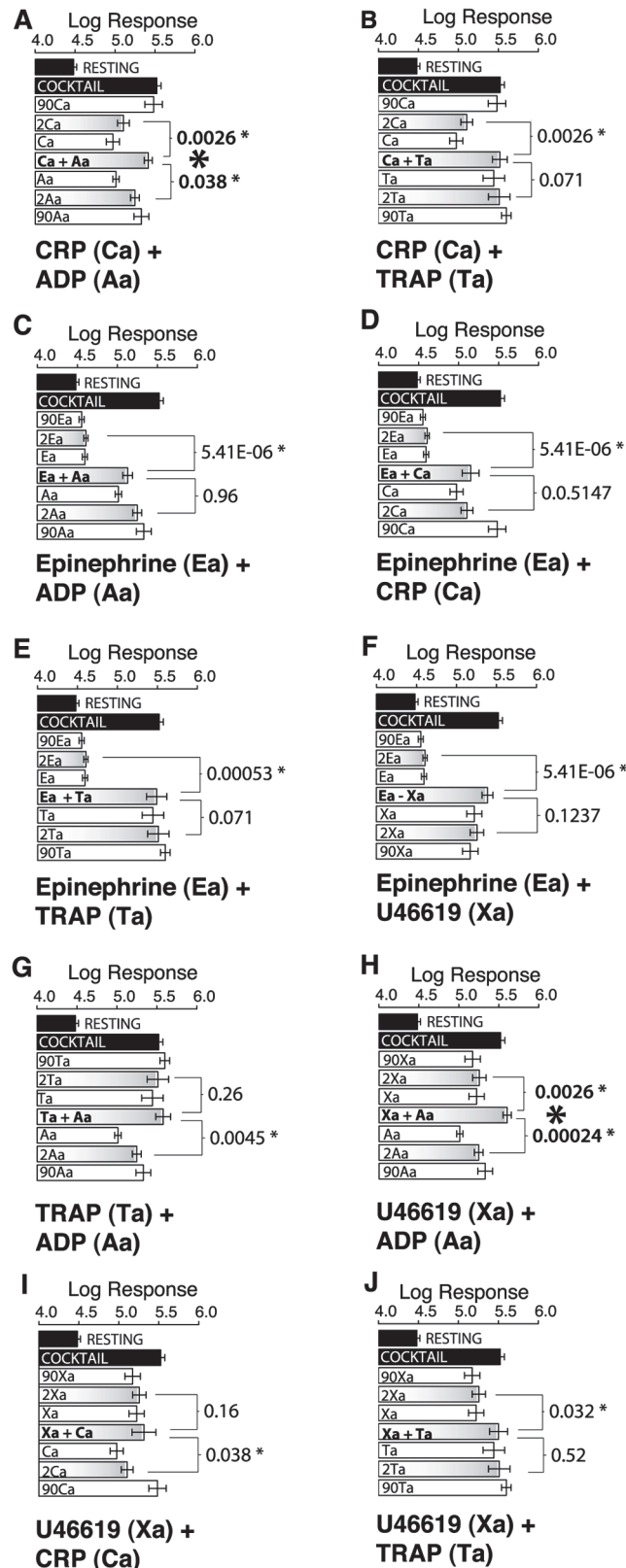doi:10.1371/journal.pcbi.1004119.g004

Fig 5. Combinations of activators. Synergy is defined as occurring where the double dose of either of the two individual activators are significantly less effective than the combination of single doses of both reagents.

Reagents are labelled as with the suffix "a" indicating activator. Label without a number indicates the chosen (typically 50% activation) dose for the activator. The prefix "2" indicates a doubling of this dose. The prefix "90" indicates the dose chosen to approximate 90% activation by the reagent. "Log response" on the horizontal axis refers to ADP release, as measured by the $\log_{10}$ luminescence of the measured arbitrary absorbance units (AAU). Small *: significant difference from the indicated double dose activator, by one-tailed Wilcoxon test $P < 0.05$. Large *represents where both tests are significant (**a** and **h**). Combinations are shown for the following activator pairs (A) Ca and Aa (B) Ca and Ta (C) Ea and Aa (D) Ea and Ca (E) Ea and Ta (F) Ea and Xa (G) Ta and Aa (H) Xa and Aa (I) Xa and Ca (L) Xa and Ta.

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Integrated Model

The goal of anti-platelet therapy is to effectively inhibit platelet activation exposed to multiple challenges. We wished to define what combination of inhibitors would most effectively inhibit platelet activation brought about by several stimuli. In particular, a researcher faced with all the visually displayed information in Fig. 3 would typically find it hard to anticipate what the likely effect of three way combinations might be. Ideally, the different strands of information should be weighted in a sensible way, that is proportional to the degree of evidence supporting each set of data, to predict an outcome of interest to the investigator. To address this, we created an integrated model. The primary data we used in building the model involved pairwise and main effects, but does not provide direct experimental information regarding three-way or higher order synergies. While pairwise synergies are typically the most important [38,39], it is still of interest to investigate further synergy. To combine the three strands of information, we took (i) the linear regression model derived from the activator-inhibitor combination analysis, that already included all main effects and four activator-inhibitor combination effects, and added (ii) the two significant activator-activator synergy and (iii) the three significant inhibitor-inhibitor synergy terms identified above. These parameters were then fitted together in a unified multiple regression model predicting platelet activation. The resulting "integrated model" thus considers simultaneously all the platelet activation data, comprising resting and cocktail activated controls, single doses, and the various combinations of activators and inhibitors (Fig. 7A; S4 Table). As expected, adding the two additional strands of synergy data resulted in a significantly better fit to the data (p<0.0001, S4 Table).

Fig. 7A provides a visual representation of the model that can help advance understanding and interpretation of drug combination effects in platelets. We set out to exploit this integrated model to make predictions of the most effective trios of platelet inhibitors. We considered the scenario where a platelet is challenged by all five activators: collagen, epinephrine and activated thrombin, plus ADP and thromboxane release from adjacent platelets, as may occur during coronary arterial platelet plug formation in the presence of a ruptured atherosclerotic plaque. The integrated model (S4 Table) was applied to predict the ADP release for each of the 32(2^5) possible three-way combinations of the single dose inhibitors. This enabled us to predict how well each combination could inhibit platelet activation (S5 Table). The most effective predicted combinations all included *Ai* (the ADP receptor inhibitor). Of these combinations, the most effective trio of inhibitors identified was a combination therapy targeting ADP, thrombin and thromboxane signalling (*Ai*, *Xi* and *Ti*). We experimentally tested whether *Ai*, *Xi* and *Ti* together strongly inhibit the five-activator cocktail. As a comparison, we also considered whether adding a PI3K inhibitor (*Pi*) to *Ai* and *Xi* would be as efficient; this acts as a control combination, since the integrated model predicted that it would not result in such a strong inhibition of platelet activation (S5 Table). Fig. 7B indicates that while the *Ai/Xi/Ti* combination favoured by the model exhibited a marked inhibition of platelet activation, the less favoured *Ai/Xi/Pi* combination showed much less inhibition (p = 0.0003). This experimental validation of the model indicates that the integration of these three sources of data into a single model can aid in
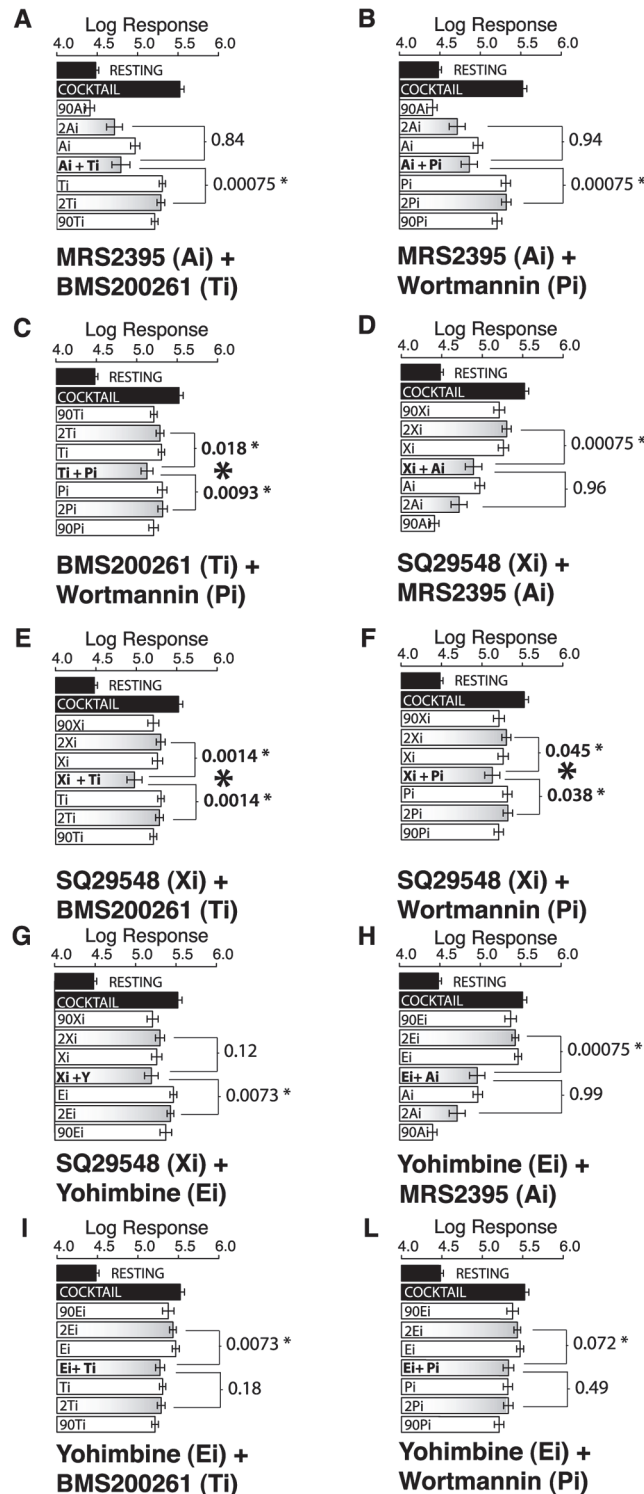
Fig 6. Combinations of inhibitors. Synergy is defined as occurring where the double dose of either of the two individual reagents result in significantly less inhibition than the combination of single doses together. Reagents are labelled as with the suffix "i" indicating inhibitor. Label without a number indicates the chosen (typically 50% activation) dose for the inhibitor. The prefix "2" indicates a doubling of this dose. The prefix "90" indicates the dose chosen to approximate 90% activation by the reagent. "Log response" on the horizontal axis refers to the $\log_{10}$ luminescence of the measured arbitrary absorbance units (AAU). The cocktail of activators is included in each experiment with the indicated inhibitors (excluding the "Resting" control of

unactivated platelets). Small *: significant difference from the double dose of the indicated inhibitor, by one-tailed Wilcoxon test P < 0.05. Large *represents where both tests are significant(**c, e** and **f**). Combinations are shown for the following inhibitor pairs (A) Ai and Ti (B) Ai and Pi (C) Ti and Pi (D) Xi and Ai (E) Xi and Ti (F) Xi and Pi (G) Xi and Ei (H) Ei and Ai (I) Ei and Ti (L) Ei and Pi.

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pinpointing higher order effective drug combinations. The model is also useful when trying to determine how much of the pattern of platelet activation in the system remains unexplained, for example by assessing model fit and exploring donor response variability (See S1 Text).

Discussion

Our method demonstrates that a systematic approach to considering pairwise reagent interactions can lead to the discovery of particular combinations of importance in modulating biological activity, identifying a triple combination of platelet inhibitors that is particularly effective. It is of interest to also integrate our findings with what is known previously of platelet signaling (Fig. 8), so that we not only identify useful combinations of inhibitors, but also advance understanding of platelet signaling. TXA2R and PAR1 are the only known activators of $G_{12/13}$ in platelets. PI3K is not a downstream effector of $G_{12/13}$ and co-activation of both Gi and $G_{12/13}$ is sufficient to activate platelets[40]. Thus, the synergy of *Pi* with both *Xi* and *Ti* makes sense, as two independent pathways ($G_{12/13}$ and PI3K transmitted) are being targeted in parallel. This suggests that the engagement of both pathways may be required for full activation. By the same logic, since they share a common effector pathway, it is not surprising that there is no significant synergy between *Xa* and *Ta*. However, paradoxically, the inhibitors *Xi* and *Ti* synergise strongly. This suggests that activation and inhibition states of these two receptors are not simple on-off switches. In endothelial cells TRAP causes the engagement of Gq prior to the
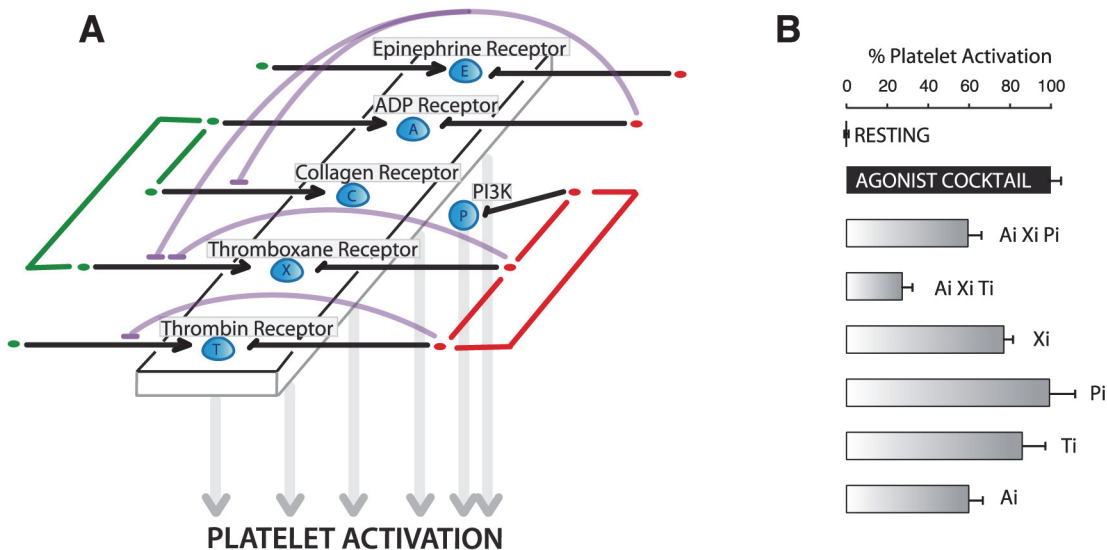

Fig 7. Integrated modelling and validation of synergy and activator-inhibitor combination effects. (A) a schematic of the integrated model (S4 Table), investigating the influence of five activators (green dots) and five inhibitors (red dots) on platelet activation. Each solid line (10 black main effects, 4 purple activator-inhibitor combination effects, 3 red inhibitor-inhibitor synergy effects, 2 green activator-activator synergy effects) represents a parameter within the multiple regression model predicting platelet activation. The five receptors and the kinase shown in the model are not explicitly modelled since there is no direct data on their activation states. The predictions of this model were used to assess the impact of all possible three way combinations of inhibitors on platelets activated by a cocktail of five activators (S5 Table). **(B)** testing the most strongly predicted inhibitor triple combination. This shows that the most strongly predicted three-way combination of *Xi, Ai, Ti* had a clearly stronger effect than the alternative *Xi, Ai, Pi* combination which was ranked more weakly by the predictive model (p = 0.0003).

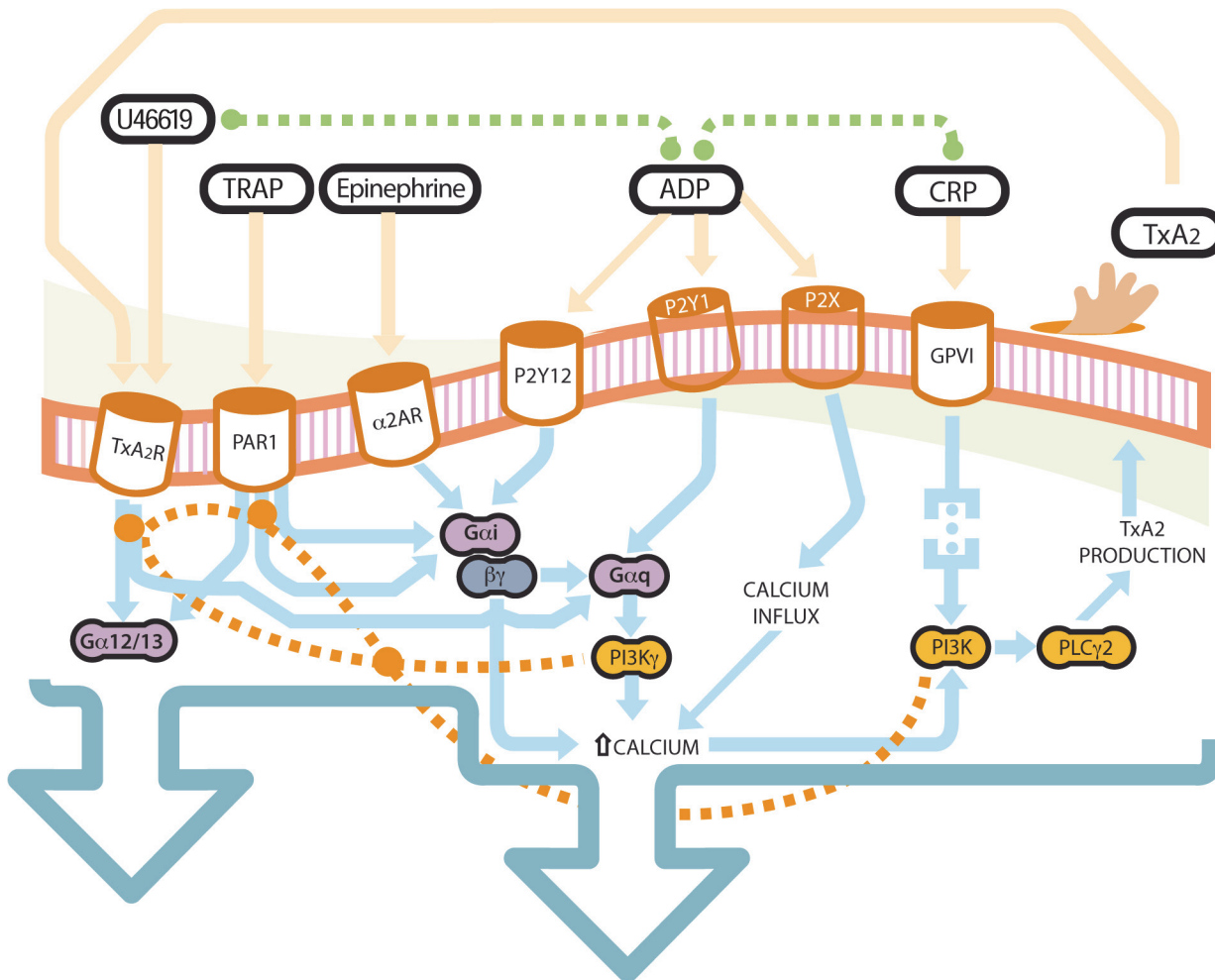doi:10.1371/journal.pcbi.1004119.g007

Fig 8. Interpreting the integrated model in the context of platelet signaling pathways. As in Fig. 7, activatory synergies are represented by green lines, inhibitory synergies by red lines. Activators U46619 (Xa), TRAP (Ta), Epinephrine (Ea) ADP (Aa) and CRP (Ca) are indicated extracellularly, acting on their receptors, namely the thromboxane receptor (TXA2R), the thrombin receptor (PAR1), the Epinephrine receptor (α2AR), the ADP receptors (P2Y12, P2Y1 and P2X), and the collagen receptor (GPVI).

engagement of $G_{12/13}$ [16]. There may be relatively subtle dose dependent effects, such that the spectrum of $G_{12/13}$ and G_α inhibition by a single versus a double concentration of Ti is not resulting in a balanced increase in the inhibition of both pathways. Alternatively, the difference between the lack of activator synergy and the presence of inhibitor synergy could reflect the presence of more than two conformational states of a receptor being induced by activators and inhibitors. This would be consistent with a multiple state model for the thromboxane receptor studied in a platelet-like cell system [41] where certain inhibitors, including Xi, act as inverse agonists, actually downregulating constitutive activation of the receptor. One explanation for the multiple inhibitory effects seen with Ai (MRS2395) is that it is a "dirty" compound with multiple targets, that is not as efficiently targeting P2Y12 as might be expected. Dirty compounds in principle may have the potential to exhibit multiple synergisms resulting from their diverse targets, but we noted that Ai did not synergize significantly with any of the other four inhibitors. Finally, in our activator-inhibitor screen we observed that while Pi(Wortmannin) predictably inhibited Ca (CRP-induced) response [26,27], its inhibitory effects were seen across multiple activators, most notably Xa (U46619-induced) response, in spite of the fact that the

existing literature suggests that TXA2 mediated signalling might not immediately involve PI3K (Fig. 8). This paradox may potentially be explained by a second wave of signalling and secretion via PI3K following the initial induction of activation [42]. It is also possible that the platelet signalling network is altered in the inhibition experiments by the presence of the three additional activators (*Ea*, *Ca* and *Aa*), thus potentiating the synergy of the two inhibitors. The two most plausible explanations, of alternative receptor states versus alternative network wiring, may not necessarily be mutually exclusive, since alternative receptor states are likely to represent responses to alternative states of the signaling networks either intracellularly or extracellularly.

Linear modeling defined the activator-inhibitor effects, and in general such model parameterisation needs to be approached with some care to ensure that statistically sensible parameters correspond to biologically interpretable ones. The linear statistical modeling was then used to integrate the different effects of activator-inhibitor, activator-activator, and inhibitor-inhibitor effects only after synergistic activator-activator and inhibitor-inhibitor effects were predefined in a manner consistent with Loewe isobole analysis, comparing combinations to double doses of both constituents. This avoids some of the dangers of linear modeling in inferring statistically significant synergies under some model which does not correspond robustly to Loewe additivity. Overall, the combined experimental and modeling approach may miss some important interactions that would be detected if we had performed the analysis across the dose response curves of each reagent combination. Given the complexity of platelet signaling, we think it likely that other synergies will emerge at different doses, and with larger sample sizes, or different stimulatory or inhibitory conditions. Nevertheless, we believe our approach is a relatively efficient way of establishing the most critical features of the signaling system, particularly when ensuring that all assays are carried out on the limited material provided by each donor in the study. Statistically, our approach appears relatively robust but clearly is open to further development, in particular moving away from a two-stage analysis (defining synergy effects separately from activator-inhibitor effects, and then combining these). Future models that estimate the synergism simultaneously with the activator-inhibitor effects may increase the efficiency of such studies, and widen the applicability to a wider set of scenarios, for example testing the effects of genetic activatory and inhibitory factors on a phenotype.

Integrated modelling of activator-activator, inhibitor-inhibitor and activator-inhibitor combinations may accelerate the discovery of compound and drug combinations that will more effectively target disease states, not only in platelet signalling, but in other potential applications, including cancer therapeutics. Many drugs that are highly successful in the clinic may have a broader mechanism of action than initially hypothesised, often contributing to their clinical efficacy. The systematic approach implemented here provides direct observations of activator-inhibitor relationships that ignores pre-conceived notions regarding the specificity or generality of action of drugs. Thus, in our study, we had prior beliefs concerning the specificity of particular agents in preventing the activation of platelets by certain activators. However, the fact that these pre-conceptions were partly disproved under the particular conditions of our study did not prevent the study design and the computational modelling from identifying a useful triple combination. Clinically used anti-thrombotic regimens provide partial support for the proposed combination identified here, routinely combining inhibition of both ADP and thromboxane signalling[43]. Adding a thrombin receptor inhibitor to these two, as suggested by the integrated model and its experimental validation, is also indicated as a useful three-way combination by a separate study which indicated its apparent synergistic advantages[44]. Clearly, this experimental test of our prediction is relatively limited, considering only two three-way combinations for comparison. Applying modeling to define higher order combinations is likely to be of particular value in experiments with larger numbers of agonists and antagonists, where the number of three-way combinations becomes impractical to screen efficiently.

One approach to screening for synergy that has the potential to actually define whether the reagents are acting in serial or in parallel, is to investigate the response profile of synergy derived from investigating the compounds at different concentrations[7]. While our approach cannot resolve whether factors are in serial or in parallel, it does appear to be efficient at identifying interesting combinations. To get a deeper understanding of how the combinations work, they could be studied in combination with analyses of intermediate components in platelet signaling, such as the phosphorylation states of various proteins. Full systems modelling of the dynamics of intermediate signalling factors may more exquisitely and accurately achieve a similar goal to this study, but would need to model the activation states and kinetics of the "hidden" layer of receptors in Fig. 8, However, this requires collecting quantitative information on the states of these receptors in the presence of multiple combinations of activators and inhibitors. In many clinical contexts such data is difficult to collect, and thus a useful systems model is absent, and may be difficult to develop. Accordingly, synergy modelling integrated with activator-inhibitor combination screens provides a key step in moving beyond the capabilities of current synergy screens[32]. When novel therapeutic inhibitors of blood associated targets are likely to be prescribed in combination with existing therapies, and there are manipulable agonists of the multiple pathways targeted, we advocate initial *ex vivo* studies to define the combinatorial landscape and make predictions to help in the design of *in vivo* synergy combination trials in human subjects.

Supporting Information

S1 Text. Supplementary results, tables and data file description.
(DOCX)

S1 Fig. Simplified theoretical illustration of a system illustrating the value of integrating activator-inhibitor, activator-activator, and inhibitor-inhibitor combination effects.
(TIFF)

S2 Fig. Dose-response curves for the single agents and the activator cocktail.
(EPS)

S3 Fig. Effects of ADP inhibitor Ai on platelet activators.
(EPS)

S4 Fig. ADP does not interfere with the assay.
(EPS)

S5 Fig. Plots of residuals from the statistical model.
(EPS)

S1 Table. Doses of activators and inhibitors used, including coding used in main text (second value) and the single letter coding using in S2 Fig. (third value).
(DOCX)

S2 Table. Multiple regression model with main effect terms (assumes no activator-inhibitor specificity) for baseline comparison.
(DOCX)

S3 Table. Stepwise linear modelling of Activator-Inhibitor combinations.
(DOCX)

S4 Table. Boolean modelling of Activator-Inhibitor combinations.
(DOCX)

S5 Table. Integrated model.
(DOCX)

S6 Table. Using the integrated model to predict effects of inhibitor combinations on platelets activated by all five activators.
(DOCX)

S1 Data File. Dataset_R_format.csv.
(CSV)

S2 Data File. Dataset_STATA_format.csv.
(CSV)

S1 Code File. R_code.r.
(R)

S2 Code File. STATA_code.do.
(DO)

S3 Code File. Fig. 1.R (sample code for generation of heatmaps).
(DOCX)

S1 Output File. R_output.txt.
(TXT)

S2 Output File. STATA_output.log.
(LOG)

Author Contributions

Conceived and designed the experiments: FL DCS NM FPC. Performed the experiments: FL KG. Analyzed the data: FL DCS DJF. Wrote the paper: FL DCS NM KG FPC DJF.

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