Simulating BRAFV600E-MEK-ERK signalling dynamics in response to vertical inhibition treatment strategies

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Abstract

In vertical inhibition treatment strategies, multiple components of an intracellular pathway are simultaneously inhibited. Vertical inhibition of the BRAFV600E-MEK-ERK signalling pathway is a standard of care for treating BRAFV600E-mutated melanoma where two targeted cancer drugs, a BRAFV600E-inhibitor, and a MEK-inhibitor, are administered in combination. Targeted therapies have been linked to early onsets of drug resistance in clinics, and thus treatment strategies of higher complexities and lower doses have been proposed as alternatives to current clinical strategies. However, finding optimal complex, low-dose treatment strategies is a challenge, as it is possible to design more treatment strategies than are feasibly testable in experimental settings.

To quantitatively address this challenge, we develop a mathematical model of BRAFV600E-MEK-ERK signalling dynamics in response to combinations of the BRAFV600E-inhibitor dabrafenib (DBF), the MEK-inhibitor trametinib (TMT), and the ERK-inhibitor SCH772984 (SCH). From a model of the BRAFV600E-MEK-ERK pathway, and a set of molecular-level drug-protein interactions, we extract a system of chemical reactions that is parameterized by *in vitro* data and converted to a system of ordinary differential equations (ODEs) using the law of mass action. The ODEs are solved numerically to produce simulations of how pathway-component concentrations change over time in response to different treatment strategies, *i.e.,* inhibitor combinations and doses. The model can thus be used to limit the search space for effective treatment strategies that target the BRAFV600E-MEK-ERK pathway and warrant further experimental investigation. Moreover, the results demonstrate that whilst DBF monotherapies and DBF-TMT-SCH combination therapies show marked sensitivity to intracellular BRAFV600E concentrations *in silico*, TMT and SCH monotherapies do not.

Introduction

Cancer cells have properties that differentiate them from normal cells of the human body. Targeted cancer drugs work by specifically targeting those properties.[1] The clinical use of small-molecule targeted cancer drugs, which began in the early 2000s, has improved survival rates for multiple cancers.[2] However, targeted cancer drugs have been linked to early onsets of drug resistance in patients,[3,4,5] which poses a major clinical question: How do we avoid or delay resistance to targeted cancer drugs? Proposed strategies to approach this question include altering (i) drug combinations, (ii) drug doses, and (iii) drug schedules. By altering (i-iii), it is possible to design more treatment strategies than can be feasibly tested. Therefore, optimal strategies cannot be found by pre-clinical experiments and clinical trials alone. To limit the search space for effective treatment strategies that warrant pre-clinical and clinical investigation, rational and quantitative approaches can be used. Rational treatment strategies refer to strategies informed by mechanistic understandings of how treatments target cancer cells' abilities to survive and proliferate.[4,6] One such rational treatment strategy is vertical inhibition, where multiple components of an intracellular signalling pathway are simultaneously inhibited.[5]

For treating BRAFV600E-mutated melanoma, for instance, vertical inhibition is now a standard of care.[7] The BRAFV600E mutation causes hyper-activated signalling in the intracellular BRAFV600E-MEK-ERK pathway, in which BRAFV600E activates MEK via phosphorylation events, which in turn activates ERK via phosphorylation events.[8] Activated ERK promotes cell survival and cell proliferation by activating numerous substrates in the cell nucleus and cytoplasm. Thus, hyper-activated BRAFV600E-MEK-ERK signalling may result in high ERK-activity and, by extension, uncontrolled cell proliferation.[9,10] Vertical inhibition of the BRAFV600E-MEK-ERK pathway ultimately acts to suppress ERK's ability to activate substrates. The rationale is that by inhibiting not only the BRAFV600E oncogene but also downstream pathway components such as MEK and ERK, treatments should be more effective and drug resistance should be impeded. In support of this rationale, previous experimental results indicate that when multiple components in a signalling pathway are inhibited, the risk for cancer cells evading drug effects via adaptive feedback reactivation of pathway signalling is reduced.[11,12,13,14] Moreover, experimental results show that high-dose mono therapies of inhibitors that target the BRAFV600E-MEK-ERK pathway exacerbate the onset of drug resistance *in vivo*, by creating evolutionary pressures that select for drug-resistant cell clones.[15] These evolutionary pressures decrease with increased treatment complexities, enabled via vertical inhibition.[15] The risks of high-dose drug treatments are important to note, as cancer drugs are commonly clinically administered at doses that are determined in the early, dose-escalation stages of clinical trials. This is the case for the BRAFV600E-specific inhibitor dabrafenib (DBF), and the MEK-inhibitor trametinib (TMT) which are administered together for treating BRAFV600E-mutated melanoma.[16,17,18]

Low-dose vertical inhibition strategies have been shown to provide promising alternatives to high-dose strategies *in vitro* and *in vivo*.[19] Further, mathematical models can be used to simulate and analyze pathway signalling dynamics,[20,21,22,23,24] and quantitatively identify vertical inhibition, low-dose drug combinations that yield synergistic treatment responses *in silico*.[25] Accordingly, we (some of the authors) recently developed a mathematical model and a computational framework for simulating signalling dynamics in the BRAFV600E-MEK-ERK pathway in response to vertical inhibition with two drugs: DBF and TMT.[25] In the previous study, we calculated ERK activities in response to varying concentrations of drugs, adenosine triphosphate (ATP), and pathway components. The model provided a mechanistic understanding on a molecular level of when DBF-TMT synergy occurs and identified intracellular BRAFV600E and ATP levels as factors in drug resistance. Beyond two-component vertical inhibition strategies, combining BRAFV600E, MEK, and ERK inhibitors has shown promising results in pre-clinical experiments.[15] Therefore, ERK-inhibitors have been proposed to be used in combination with DFB and TMT, as part of a three-drug vertical inhibition strategy.[15] To investigate such strategies *in silico* we here extend our previous mathematical model[25] to also include an ATP-competitive ERK-inhibitor. The model is parameterized by data pertaining specifically to the ERK-inhibitor SCH772984,[26] but the parameter values can be adjusted to study other ATP-competitive ERK-inhibitors. We use the model to simulate BRAFV600E-MEK-ERK signalling dynamics in response to various combinations and doses of BRAFV600E, MEK, and ERK inhibitors. Our results also identify two and three-component vertical inhibition dose combinations that yield synergistic treatment responses *in silico*, in terms of inhibiting the activation of a general ERK-activated substrate.

Model and Methods

Our mathematical model is composed of a pathway model of the intracellular BRAFV600E-MEK-ERK pathway (Fig 1a), and a set of possible interactions that can occur between pathway components and inhibitors (Fig 1b). From the pathway model and the set of possible interactions, we extract a system of chemical reactions that describe pathway signalling in response to vertical inhibition (Fig 1c). Using a computational framework[25] that builds on the law of mass action, we convert the system of chemical reactions into a system of ordinary differential equations (ODEs) that are solved numerically to produce simulations of pathway signalling dynamics. These simulations show how concentrations of the pathway components change over time, in response to different inhibitor combinations and concentrations. The model is adapted from previous studies,[21,25] and is extended to include a general substrate downstream of ERK and an ERK-inhibitor. The next subsections provide detailed descriptions of the mathematical model and the computational methods used in this study. Information on how to access, run, and modify the code files is available in the Supplementary Material (SM2).

Modelling the BRAFV600E-MEK-ERK pathway in the absence of inhibitors

The pathway model consists of a three-tiered cascade comprising BRAFV600E, and various forms of MEK, ERK, and a general substrate S (Fig 1a). Throughout this study, single (p) and double (pp) p-prefixes respectively indicate that one or two phosphate groups are bound to the pathway component of interest. At the top of the cascade is the BRAFV600E-mutated BRAF oncogene, which we model as always activated to simulate hyper-activated pathway signalling. BRAFV600E can transform MEK into its activated form ppMEK, which can transform ERK into its activated form ppERK, which in turn can activate the substrate S. More precisely, all activation events downstream of BRAFV600E are mediated via two sequential phosphorylation events, where an ATP molecule binds to an enzyme (*i.e.* BRAFV600E/ppMEK/ppERK) and donates a phosphate group to a substrate (*i.e.* MEK/pMEK/ERK/pERK/S/pS) (Fig 1a,b). After the phosphate group donation, the ATP molecule converts to adenosine diphosphate (ADP). In our model, dephosphorylation is mediated by the phosphatases phosph1, phosp2, and phosph3 which, respectively, remove phosphate groups from the molecules pMEK/ppMEK, pERK/ppERK, and pS/ppS (Fig 1a,c).

The pathway model used in this study is an extension of our recently published model of the BRAFV600E-MEK-ERK patwhay,[25] which is an adaptation of a more general pathway model presented by Huang and Ferrell in 1996.[21] All components downstream of ppERK in the pathway model are introduced in the current study (Fig. 1a).

Modelling interactions between pathway components and inhibitors

We model interactions between pathway components and inhibitors on a molecular level (Fig 1b). To do this, we distinguish between ATP-competitive and ATP-allosteric inhibitors. DBF[27] and SCH[26] are ATP-competitive inhibitors, meaning that they compete with ATP for a binding site on their target enzyme, *i.e.* BRAFV600E, and ppERK, respectively. As such, DBF prevents ATP from binding to BRAFV600E and, by extension, inhibits ATP-mediated MEK phosphorylation. Similarly, SCH prevents ATP from binding to ppERK and, by extension, inhibits ATP-mediated S phosphorylation. On the other hand, TMT is an ATP-allosteric inhibitor,[28] and thus TMT binds to a MEK-binding site that is adjacent to, but distinct from, the ATP-binding site. MEK-bound TMT prevents MEK-bound ATP from donating a phosphate group to MEK-bound ERK or pERK. The modelled interactions pertaining to BRAFV600E and MEK inhibition (Fig. 1b, i-x) were presented in one of our previous studies, whereas the interactions pertaining to ERK inhibition are introduced in this study (Fig. 1b, xi-xv). Note that we have made the simplifying modelling assumption that the drugs TMT and SCH only bind to activated, *i.e., doubly phosphorylated*, forms of MEK and ERK, respectively.

Formulating the system of chemical reactions that describes pathway dynamics

From the pathway model (Fig. 1a) and the set of possible interactions between pathway components and inhibitors (Fig. 1b), we extract a system of chemical reactions (Fig. 1c). These reactions are associated with rate constants that we obtain from published *in vitro* data, as is described in the Supplementary Material (SM3).

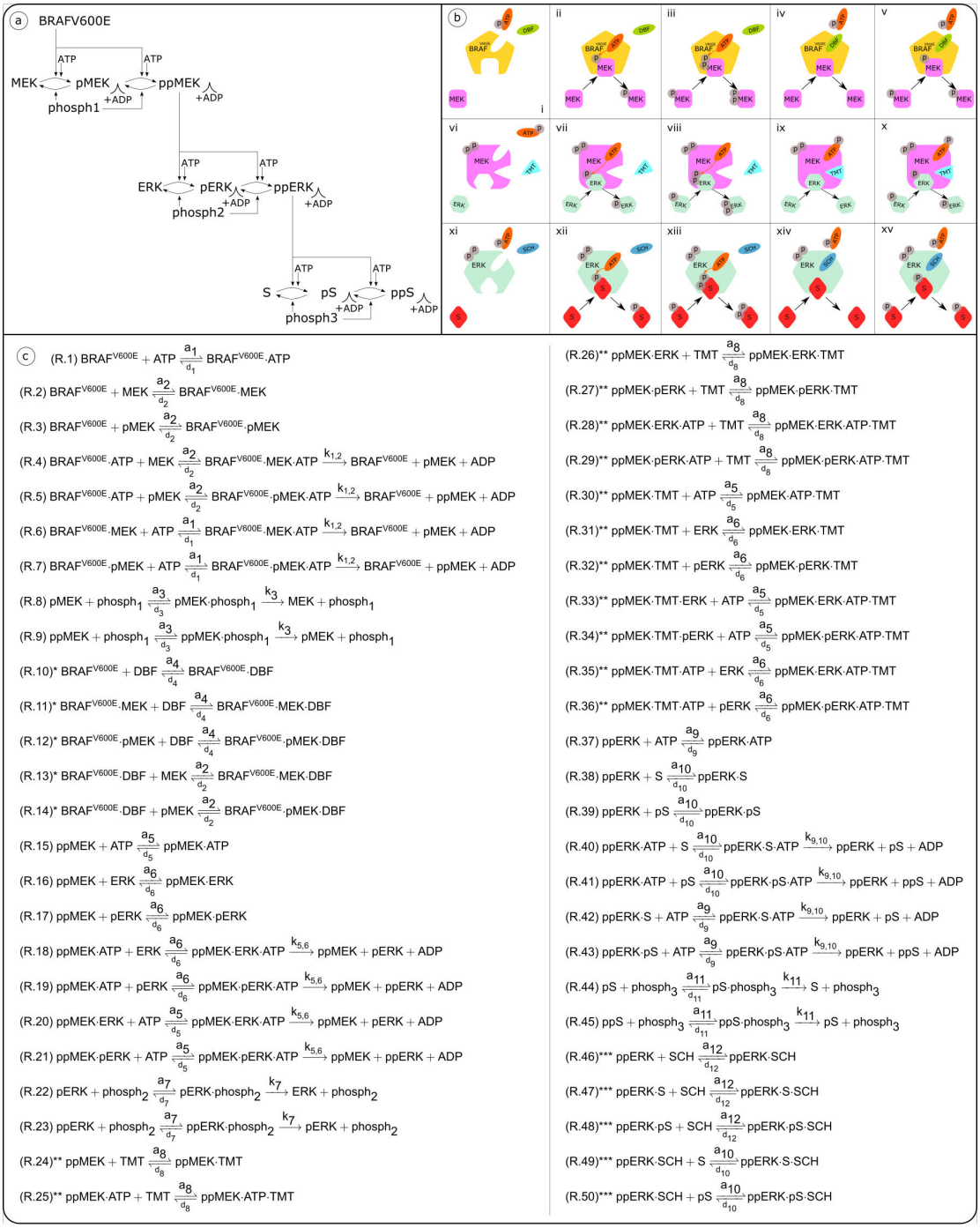

Fig. 1: **The mathematical model used in this study is composed of (a) a model of the BRAFV600E-MEK-ERK signalling pathway, (b) a set of possible interactions that can occur between pathway components and inhibitors, and (c) a system of chemical reactions that is extracted from (a) and (b). (a)** The pathway model comprises the BRAFV600E-MEK-ERK signalling cascade and a general substrate S. Singly and doubly phosphorylated pathway components are, respectively, indicated by the prefixes (p) and (pp). Via two sequential ATP-meditated phosphorylation events, in which ATP donates a phosphate group to a substrate and turns into ADP, BRAFV600E activates MEK to the form ppMEK. Similarly, ppMEK activates ERK to the form ppERK, which in turn activates the general substrate S. Dephosphorylation is mediated by phosphatases (phosph1, phosph2, phosph3). **(b)** The model includes a set of possible interactions that can occur between pathway components and the BRAFV600E-inhibitor DBF(i), the MEK-inhibitor TMT(vi), and the ERK-inhibitor SCH(xi). If inhibitors are not bound to the enzymes, enzyme-bound ATP donates a phosphate group to the substrate MEK(ii)/pMEK(iii)/ERK(vii)/pERK(viii)/S(xii)/pS(xiii). The ATP-competitive inhibitor DBF binds to the same site as ATP on the BRAFV600E molecule and thus inhibits ATP from binding to BRAFV600E and donating a phosphate group to MEK(iv)/pMEK(v). The ATP-allosteric inhibitor TMT binds to a binding site adjacent to the ATP-binding site on the ppMEK molecule and inhibits ATP from donating a phosphate group to ERK(ix)/pERK(x). The ATP-competitive inhibitor SCH binds to the same site as ATP on the ppERK molecule and thus inhibits ATP from binding to ppERK and donating a phosphate group to S(xiv)/pS(xv). **(c)** A system of chemical reactions is extracted from (a) and (b). Reactions marked by one (*), two (**), or three (***) stars involve the inhibitors DBF, MEK, and SCH, respectively, and can be omitted when modelling pathway dynamics in the absence of drugs. This figure had been adapted from one of our previous publications[25]

Converting chemical reactions to equations, and simulating pathway dynamics

Using the law of mass action, we convert the system of chemical reactions to a system of ODEs. The law of mass action states that the rate of a chemical reaction is proportional to the product of the reactants' concentrations.[29] The reactions-to-ODEs conversion process is automated by a computational MATLAB[30] framework, that we (some of the authors) previously developed.[25] The full system of ODEs for our model (Fig. 1) is available in the Supplementary Material (SM1.2). After formulating the equations, we also use the computational framework to numerically solve the equations and thus produce simulations of intracellular pathway dynamics. The simulations show how concentrations of the pathway components change over time. In this study, we are interested in studying vertical inhibition strategies in which up to three components of the BRAFV600E-MEK-ERK signalling pathway are simultaneously inhibited. Therefore, a specific output of interest is the fraction of activated (*i.e.*, free and singly or doubly phosphorylated) substrate S in the system which we call S_{act} and define as

$$S_{act}(t) = \frac{[pS](t) + [ppS](t)}{S_{tot}}, \tag{1}$$

where we have used the slanted bracket notation $[x](t)$ to denote the concentration of substrate x at time t, and S_{tot} is the total amount of substrate S in the system. Our mathematical model obeys a set of conservation laws that ensures the total amount of various pathway components is constant throughout the simulations. For example, the conservation law for S_{tot} reads

$$\begin{aligned} S_{tot} = [S](t) + [pS](t) + [ppS](t) + [ppERK \cdot S](t) + [ppERK \cdot pS](t) + \\ [ppERK \cdot S \cdot ATP](t) + [ppERK \cdot pS \cdot ATP](t) + [ppERK \cdot S \cdot SCH](t) + \\ [ppERK \cdot pS \cdot SCH](t) + [S \cdot phosph_3](t) + [pS \cdot phosph_3](t). \end{aligned} \tag{2}$$

where the left-hand-side of Eq. 2 corresponds to the denominator in Eq. 1. The full list of conservation laws is available in the Supplementary Material (SM1.3).

Using *in vitro* data to parameterise the model

The system of reactions (Fig. 1c, R.1-R.51) includes 12 forward rate constants $a_1, a_2, ..., a_{12}$, 12 reverse rate constants $d_1, d_2, ..., d_{12}$ and 6 catalytic rate constants $k_{1,2}, k_3, k_{5,6}, k_7, k_{9,10}, k_{11}$. The rate constants are listed in the Supplementary Material (Table SM3.T1) and are derived from published data[21,26,31,32,33,34,35,36,37,38,39] using binding data calculations[40,41,42] (SM3.1,3). The initial and total concentrations of all pathway components are listed in the Supplementary Material (Table SM3.T.2) and are obtained from published data[21,37] (SM3.2).

Results and Discussion

We simulate signalling dynamics in the BRAFV600E-MEK-ERK pathway using the previously mentioned computational framework.[25] By altering the initial inhibitor concentrations, we simulate treatment responses to vertical inhibition strategies that target one, two, or three components of the pathway. To simulate pathway dynamics in the absence of a specific inhibitor, we simply set the initial concentration of that inhibitor to zero. Throughout this study, the total amount of inhibitor concentrations is constant over the time courses of the simulations.

Simulating treatment responses to monotherapies that inhibit the BRAFV600E-MEK-ERK pathway

We first simulate pathway dynamics in response to monotherapies that inhibit BRAFV600E, MEK, or ERK activity. Our main simulation output of interest is $S_{act}(t)$, the fraction of free and activated substrate S in the system (Eq. 1). We are interested in investigating how $S_{act}(t)$ changes in time, in response to different doses of the BRAFV600E-inhibitor DBF, the MEK-inhibitor TMT, or the ERK-inhibitor SCH. We are also interested in understanding how intracellular concentrations of BRAFV600E and ATP impact treatment responses *in silico*.

We choose an initial condition in which only free and non-phosphorylated MEK, ERK, and S are present in the system, in line with previous studies[21,25] (Supplementary Material, SM3.2). Hence the substrate S is not immediately activated when the simulations start, instead, S activation occurs after MEK and ERK activation, as regulated by the pathway dynamics. This transient behaviour of S activation is illustrated in Fig. 2, where monotherapy responses to DBF, TMT, and SCH are plotted over increasing inhibitor concentrations for four different simulation time points. With the baseline values of total BRAFV600E and ATP concentrations, which are estimated to correspond to physiological values,[25] TMT and SCH monotherapies yield steady-state activated substrate levels within 24 hours for all doses (Fig. 2, left). These steady-state levels are reached within 8 hours for TMT, and within 3 hours for SCH. In contrast, no steady-states are reached in response to DBF monotherapies.

By comparing the two left-most columns in Fig. 2, we can study what happens when the total BRAFV600E concentration is increased from the 3 nM baseline value to 10 nM. This comparison shows that the increased BRAFV600E concentration raises the dose-response curves and thus desensitizes the pathway to DBF monotherapies. The simulation results show that the steady-state treatment responses to TMT and SCH monotherapies are not affected by the BRAFV600E concentrations, although the time durations to reach the steady-states do decrease with increasing BRAFV600E concentrations. This latter result is visible in Fig. 2 for TMT monotherapies. We note that the transient system behaviour of responses to SCH monotherapies is not visible from Fig. 2, as steady-state levels are reached within 3 hours. However, this transient system behaviour is observable from supplementary simulation results where we plot activated substrate levels over time (Supplementary Material, SM4).

Next, to investigate the role of ATP in treatment responses, we increase the total ATP concentration from the baseline value of 1 mM to 5 mM in the third column. We then compare the first and third columns in Fig. 2. Doing this, we see that the increase in ATP levels render the pathway less sensitive to DBF, TMT, and SCH monotherapies at all regarded time points. As such, the steady-state levels of the activated substrate are increased for both TMT and SCH monotherapies when ATP is increased. These results follow from the fact that ATP is needed for the phosphorylation of MEK, ERK, and S. Hence, when intracellular ATP concentrations increase, so does the probability that ATP-binding sites on the catalyzing enzymes will be occupied by ATP molecules.

In summary, from the simulation results, we find that both BRAFV600E and ATP levels impact transient and absolute values of $S_{act}(t)$ in response to DBF monotherapies. Conversely, in response to TMT and SCH doses, ATP concentrations predominately impact the steady-state levels of $S_{act}(t)$, whilst BRAFV600E concentrations impact the time until steady-state levels are reached. This finding is substantiated by Fig. 2 (right), in which both total BRAFV600E and ATP concentrations have been elevated from the baseline values.

Notably, the model predicts that DBF monotherapies are initially effective in suppressing substrate activation, but that this efficacy decreases over time. For instance, at 3 hours and baseline BRAFV600E and ATP concentrations, 0.58 μM DBF suffices to achieve 90% inhibition of activated substrate, whilst the same dose inhibits less than 10% substrate activation at 24 hours. On the other hand, a 2.7 μM SCH dose achieves 90% inhibition of activated substrate for all time points at 3 hours and over. Even more strikingly, when the BRAFV600E concentration is increased to 10 nM, 90% inhibition of activated substrate is achieved by *e.g.,* 2.0 μM DBF at 3 hours, 8.1 μM DBF at 24 hours, and 2.7 μM SCH for time points at 3 hours and over. These results suggest that, due to (i) DBF-leakage, and (ii) DBF and TMT sensitivity to BRAFV600E concentrations, SCH monotherapies may be effective in complementing DBF and DBF-TMT therapies in inhibiting substrate activity.

Simulating treatment responses to two-component vertical inhibition of the BRAFV600E-MEK-ERK pathway

We next simulate pathway dynamics in response to vertical inhibition treatment strategies that target two components of the BRAFV600E-MEK-ERK pathway. Activated substrate levels (S_{act}, Eq. 1) in response to BRAFV600E and MEK inhibition (DBF-TMT), BRAFV600E and ERK inhibition (DBF-SCH), and MEK and ERK inhibition (TMT-SCH) are shown in the heatmaps for three time points in Fig. 3. These heatmaps demonstrate the dynamic behaviour of the system through the time-varying S_{act} levels. We use these heatmaps to visually identify dose combinations of two inhibitors that yield synergistic treatment responses *in silico*.

In this study, we categorize a drug combination as being synergistic, or not, using the method of isoboles.[43] To understand this method, consider an N-dimensional space where N denotes the number of drugs that we want to include in a drug combination. A drug dose combination can be describes as a point $\bar{d} = (d_1, d_2, ..., d_N)$ where d_i denotes the dose of drug i. In this space, we can create a synergy-isobole that connects all monotherapy doses that achieve some desired drug effect $E*$. If a drug dose combination $\bar{d}$ yields a drug effect that is higher than $E*$, and $\bar{d}$ lies under the synergy-isobole, the drug combination is categorized as synergistic. Otherwise, the drug combination is categorized as non-synergistic.

Here, we measure drug effects in terms of S_{act} levels, and in Fig. 3 we draw out isoboles between monotherapy doses that yield 90% S_{act} inhibition at 24 hours, such that $S_{act}(t =$ 24 hours$)$ =0.1. Thus, we categorize two-drug combination drug doses that achieve $S_{act}(t =$ 24 hours$) <$ 0.1 and lie under the isobole lines as synergistic. The 0.1 threshold value is chosen to reflect an inhibition level that we assume to be effective from a treatment perspective. This assumption is extrapolated from previous studies, where 80%,[44] and 90%[45] inhibition of ERK phosphorylation has been observed in patients with tumour regression. Importantly, the model can identify drug combinations that achieve any inhibition level of interest.

With the chosen synergy categorization, our results identify a broad range of synergistic DBF-TMT combinations (Fig. 3). These results are in line with our previous mathematical study,[25] in which we identified DBF-TMT combinations that synergistically inhibit activated ERK levels (as opposed to activated substrate S levels which are the focal simulation output in the current study). However, compared to DBF-TMT combinations, our results only identify a narrower range of synergistic DBF-SCH or TMT-SCH combinations (Fig. 3). These ranges are close to the monotherapies that yield 90% substrate inhibition. Note that these results do not mean that DBF-SCH and TMT-SCH combinations are generally non-synergistic in practice, but rather that our pathway model alone cannot explain DBF-SCH or TMT-SCH synergies, according to our chosen synergy-categorization. We hypothesize that the inclusions of alternative pathway components or treatment-induced feedback loops in the pathway model might capture *eg.,* potential DBF-SCH synergies. We also note that, from a treatment perspective, drug combinations need not be synergistic in order to be beneficial. Moreover, potential synergies might result from mechanisms on the cell population or tissue scale, rather than from pathway-intrinsic mechanisms on the intracellular scale. To capture such synergies mathematically, our intracellular model could, for example, be migrated to a cell population or tissue model via a multi-scale modelling approach.[46,47]

Simulating treatment responses to three-component vertical inhibition of the BRAFV600E-MEK-ERK pathway

We, lastly, simulate BRAFV600E-MEK-ERK pathway dynamics in response to simultaneous BRAFV600E, MEK, and ERK inhibition. We are especially interested in identifying synergistic drug dose combinations of the BRAFV600E-inhibitor DBF, the MEK-inhibitor TMT, and the ERK-inhibitor SCH. Using the same isobole-based synergy categorization as in the previous subsection, a three-drug combination strategy is here classified as synergistic if the strategy achieves more than 90% S_{act} inhibition at some time t and lies under a plane that connects the three monotherapy doses that yield $S_{act}(t) = 0.1$.

In Fig. 4, three-drug combination therapies that yield S_{act} =0.1 at 8, 16, and 24 hours are plotted on a surface. By picking any point on this surface, and using the axes to read out where in DBF-TMT-SCH space the regarded surface point lies, drug-dose combinations that yield 90% inhibition can be identified. To aid readability, we have included a numerical martrix of these surface point drug-dose combinations in the Supplementary Material (SM5). Also included in Fig. 4 are synergy-isoboles for 90% inhibition. Thus, we can use the plots to graphically identify synergistic combinations of three drugs as any part of the $S_{act}(t)$ =0.1 surface that lies under the synergy-isobole. Using this graphical method, we can observe a range of drug combinations yield synergistic treatment responses. This builds on the synergistic *in silico* relationships that were observed for two-component vertical inhibition strategies in Fig. 3, and in our previous mathematical study.[25] Note that the choice of using 90% inhibition in the drug-dose surfaces and synergy planes in Fig. 4 is guided by our assumption that 90% inhibition corresponds to an effective treatment objective, following our discussion in the previous subsection. In the Supplementary Material (SM5), we have repeated this graphical method to find drug

combinations that achieve 25%, 50%, and 75% inhibition of substrate activity. More generally, the methodology used in this study can be used to find a range of one, two, and three-component vertical inhibition strategies that target the BRAFV600E-MEK-ERK pathway and achieve selected activity levels of any chosen pathway components.

The simulation results in Fig. 4 show that both S_{act} levels and the synergy-isoboles change over time and are sensitive to the total amount of BRAFV600E and ATP concentrations in the system. Increasing the total BRAFV600E and ATP concentrations pushes the S_{act} =0.1 surface towards higher drug doses. In other words, increasing BRAFV600E and ATP concentrations make DBF-TMT-SCH treatment strategies less effective *in silico*. This result suggests that high BRAFV600E and ATP concentrations may result in drug resistance to DBF-TMT-SCH vertical inhibition, mediated by intrinsic mechanisms of the BRAFV600E-MEK-ERK pathway. These observations are in line with the discussions that followed the monotherapy simulations, where we examined the impact of BRAFV600E and ATP concentrations on pathway dynamics.

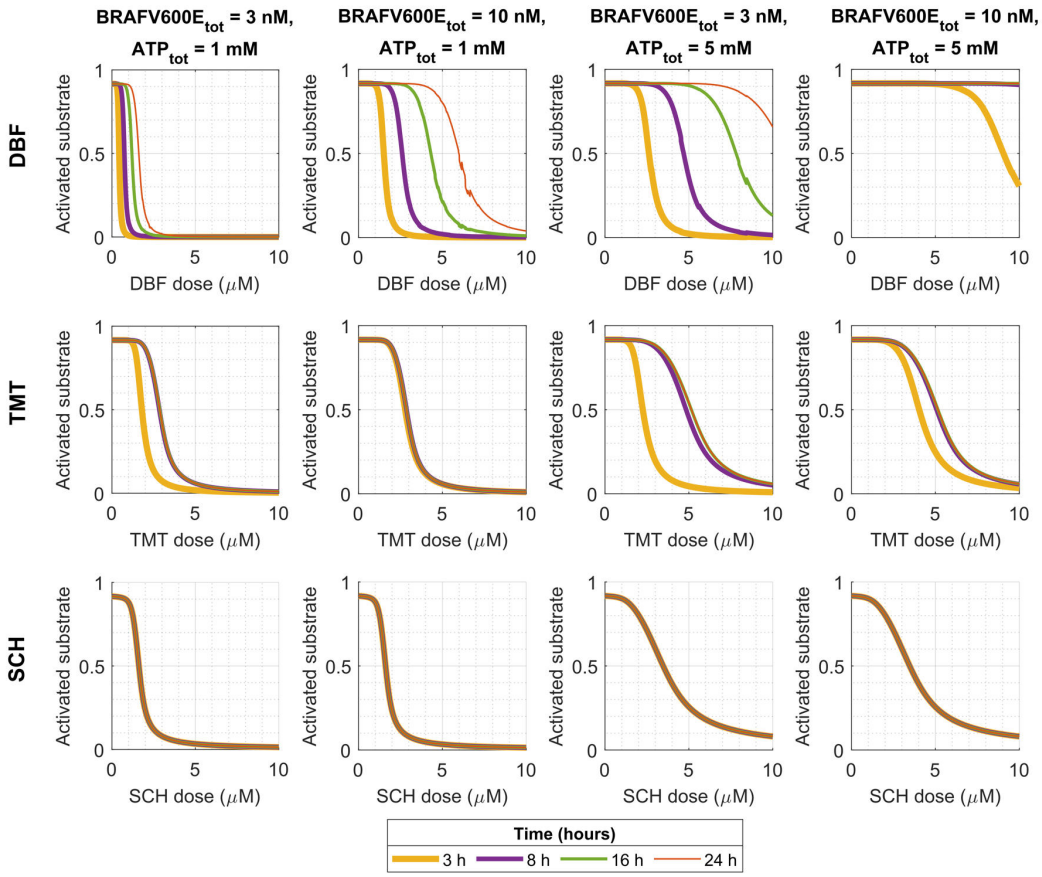

Fig. 2: **Mathematical modelling quantifies *in silico* treatment responses to monotherapies that target components of the BRAFV600E-MEK-ERK pathway.** The plots show activated substrate levels (S_{act}, Eq. 1) over time in response to different doses of the BRAFV600E-inhibitor DBF (top row), the MEK-inhibitor TMT (middle row), and the ERK-inhibitor SCH (bottom row). The plots show activated substrate levels at four simulation time points (3, 8, 16, and 24 hours). The total BRAFV600E and ATP concentrations vary between the columns, where baseline values (BRAFV600E$_{tot}$=3 nM, ATP$_{tot}$=1 mM) are used in the left-most column

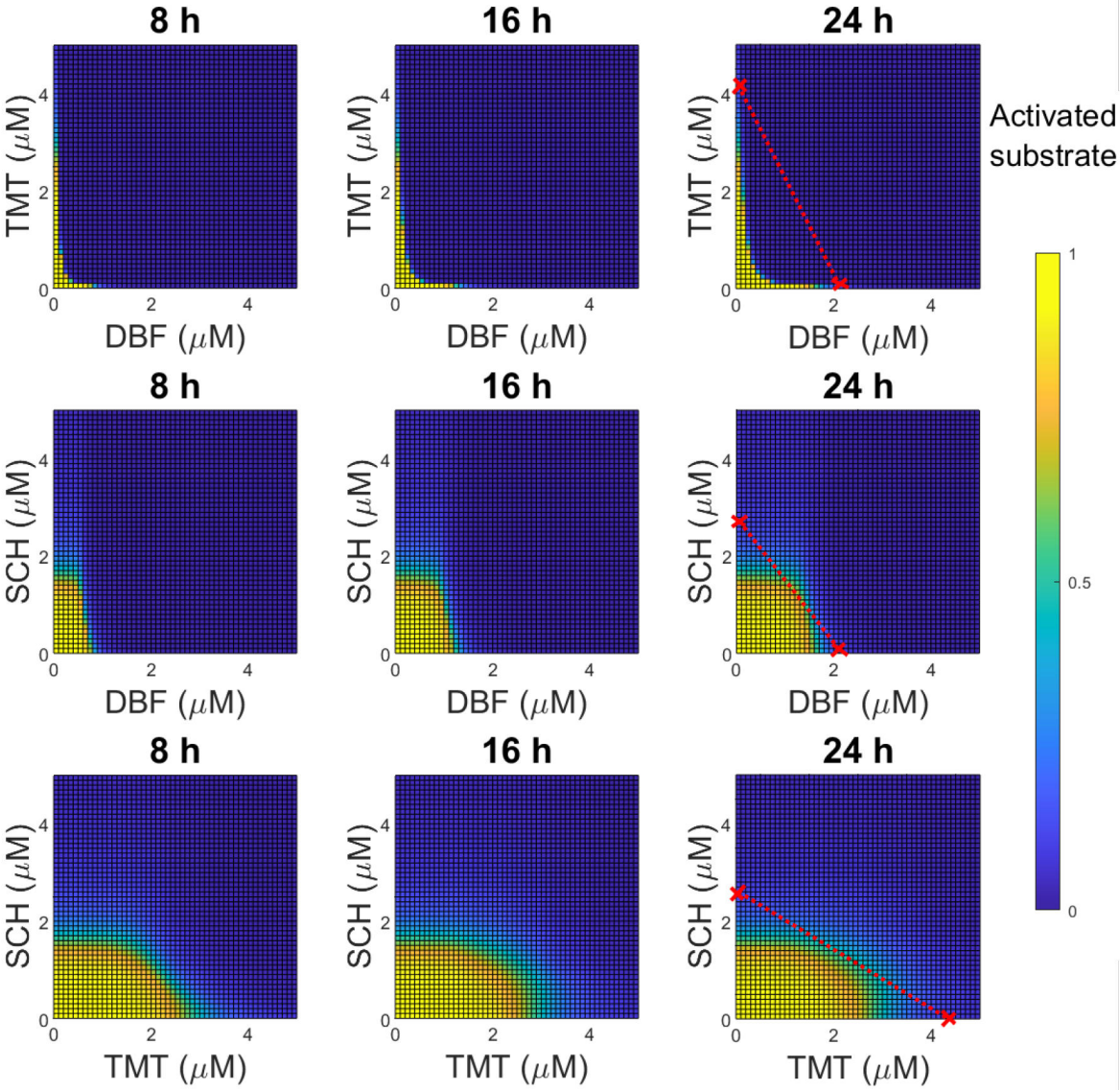

Fig. 3: **Mathematical modelling quantifies *in silico* treatment responses to vertical inhibition treatment strategies that target up to two components of the BRAFV600E-MEK-ERK pathway.** The heatmaps show levels of substrate activity (S_{act}, Eq. 1) in response to combination treatments of BRAFV600E and MEK inhibitors (DBF-TMT, top row), BRAFV600E and ERK inhibitors (DBF-SCH, middle row), and MEK and ERK inhibitors (TMT-SCH, bottom row) at three different simulation time points (8, 16 and 24 hours). Baseline values of total BRAFV600E (3 nM) and ATP (1 mM) concentrations are used in the simulations. The right-most plots include isoboles (lines) that connect the relevant monotherapy doses that yield 90% inhibition of S_{act} at 24 hours. Two-drug combination doses under the isoboles that achieve more than 90% inhibition of S_{act}, such that $S_{act}(t =$ 24 hours$)$ <0.1, are here categorised as synergistic

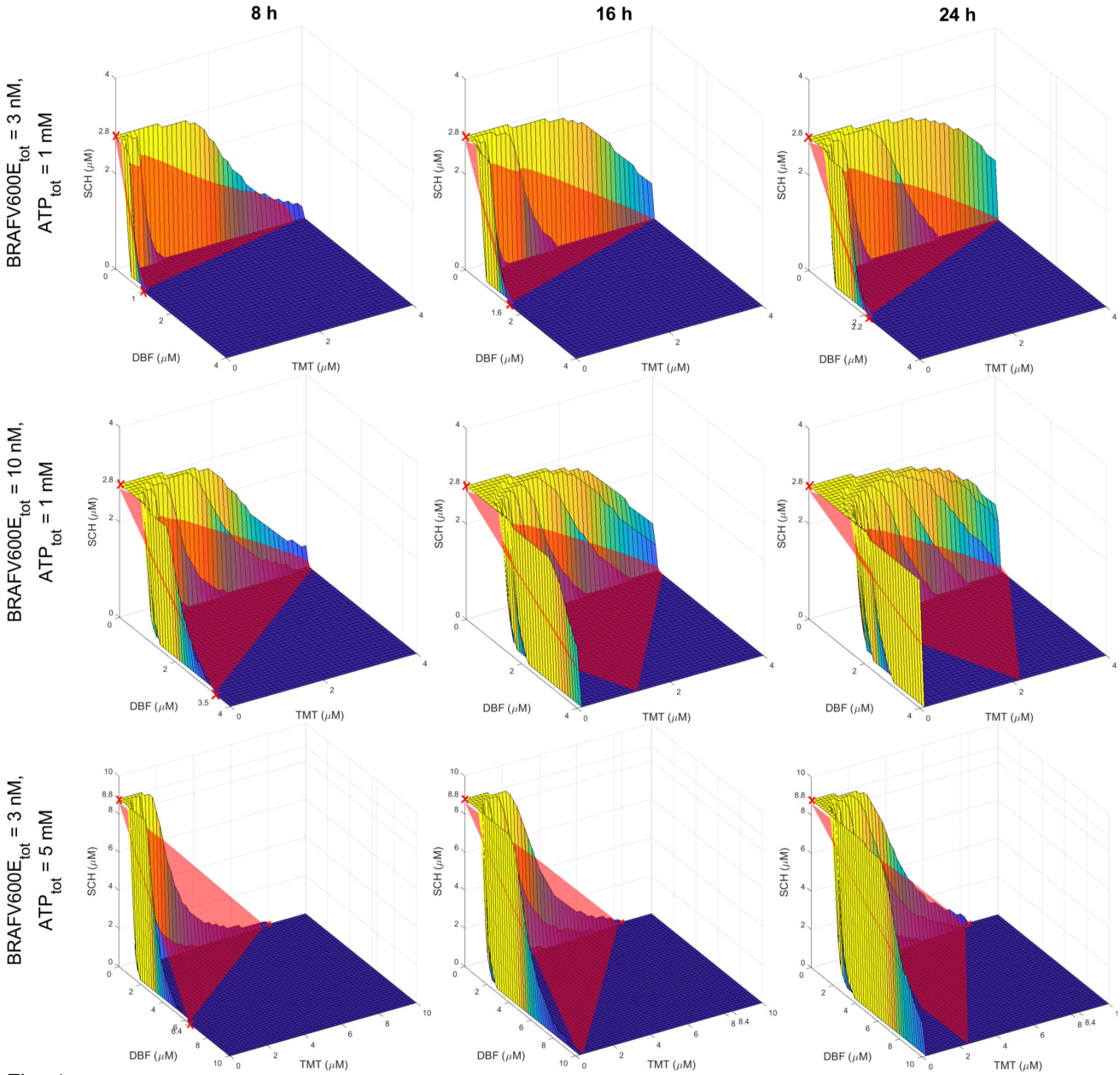

Fig. 4: **Mathematical modelling quantifies *in silico* treatment responses to vertical inhibition treatment strategies that target up to three components of the BRAFV600E-MEK-ERK pathway.** The yellow-to-blue surface plots show DBF-TMT-SCH combination therapies that yield 90% substrate activation ($S_{act}(t)$ =0.1, Eq. 1). The red synergy-isoboles (planes) connect the monotherapy doses that yield a 90% inhibition of substrate activity. Monotherapy doses that yield 90% substrate activity are marked with crosses. Treatment combinations, *i.e.,* parts of the $S_{act}(t)$ =0.1 surfaces, that lie under the synergy-isoboles are here categorised as synergistic. Results are plotted at 8 (left column), 16 (middle column) and 24 (right column) hours for baseline, total ATP, and BRAFV600E concentrations (top row), and for elevated BRAFV600E concentrations (middle row) and ATP concentrations (bottom row)

Conclusion

In this study, we present a mathematical model of signalling dynamics in the intracellular BRAFV600E-MEK-ERK pathway. The model can be used to simulate pathway dynamics in response to combinations of the BRAFV00E-inhibitor DBF, the MEK-inhibitor TMT, and the ERK-inhibitor SCH. In its versatility, the model can be used to identify a range of one-, two-, and three-component vertical inhibition treatment strategies that achieve some desired treatment response, such as suppressing the activity of selected pathway components to chosen levels. In this article, we specifically use the model to identify treatment strategies that achieve a 50% activity level of an un-specified substrate S downstream of ERK. We argue that this *in silico* methodology of identifying viable treatment strategies can be used to inform which treatment strategies warrant further investigation *in vitro* and *in vivo*. This is important, as it is possible to design more treatment strategies than are feasibly testable in experimental and clinical settings by varying *e.g.,* drug combinations, drug doses, and drug schedules (although the latter is not discussed in this article). As such, our study contributes an approach to limit the search space for effective vertical inhibition strategies.

The methodology outlined in this study can also be used to identify vertical inhibition treatment strategies that comprise two (Fig. 3) or three (Fig. 4) inhibitors and are synergistic *in silico*. Here, the model identified a broad range of synergistic DBF-TMT dose combinations, but a narrower range of synergistic DBF-SCH and TMT-SCH doses (Fig. 3). However, drug combinations of DBF-TMT,[27] DBF-SCH,[48] and DBF-TMT-SCH984[15] (where SCH984, like SCH772984, is an ATP-competitive ERK-inhibitor) have all shown markedly improved treatment effects, compared to the respective monotherapies alone, in pre-clinical experiments.

The fact that the mathematical model presented in this paper predicts striking DBF-TMT synergies, but not so striking synergies between other inhibitor combinations, suggests that, whilst the pathway model (Fig. 1a) suffices to explain DBF-TMT synergies, a model extension might be needed to explain experimentally observed DBF-SCH synergies. Such an extension could, for instance, include drug-protein interactions with un-phosphorylated or singly phosphorylated proteins, so that TMT could bind to MEK, and pMEK (not solely ppMEK), and SCH could bind to ERK and pERK (not solely ppERK). Another model extension would be to include alternative pathway components that feed into MEK, or feedback loops in the BRAFV600E-MEK-ERK signalling pathway that are activated by DBF monotherapies but suppressed by combination therapies. On this note, one of the working hypotheses for why vertical pathway inhibition yields better treatment responses than DBF alone is that BRAFV600E-inhibitor monotherapies can activate alternative signalling programs to phosphorylate MEK and ERK.[49] This type of alternative pathway activation has been recognized as a mechanism of drug resistance to BRAFV600E-inhibitors.[49] Building on this, Kholodenko et al.[50] investigated drug resistance mediated by the reactivation of initially inhibited signalling pathways in a recent publication. They analyzed how pathway topologies affect pathway signalling in responses to drug treatments. As an example, they considered RAF-MEK-ERK signalling in BRAFV600E mutated melanoma subjected to vertical inhibition treatment strategies.

Another intracellular mechanism for resistance to BRAFV600E-inhibitors is elevated BRAFV00E activity.[51,52] This mode of drug resistance is captured by our model, as our simulation results show that increasing BRAFV600E concentrations yield decreasing sensitivity to inhibitors that target the BRAFV600E-MEK-ERK pathway, as illustrated in Figs. 2 and 4. These simulation results also show that ATP concentrations impact drug resistance *in silico*, although we note that the clinical relevance of this finding is not straightforward to interpret. This is because tumour cells in physiological oxygen conditions decrease their oxidative phosphorylation in favour of increased use of glycolysis, according to the Warburg effect.[53,54,55] Though both processes produce ATP, which generates energy for cellular processes, oxidative phosphorylation generally generates ATP more effectively than glycolysis.[56] The complex relationship between drug resistance, ATP, and ATP production in tumour cells is beyond the scope of this study but has been investigated in other works.[56,57,58] We also remark that the discrepancy in ATP levels between experimental models and *in situ* tumours has been suggested to contribute to the discrepancy in drug performance between laboratory and physiological settings.[57] Our results, which show that ATP levels may impact treatment responses to ATP-competitive and ATP-allosteric inhibitors *in silico*, support this argument.

One limiting factor of this study is that the model parameter values are gathered and derived from multiple literature sources (Supplementary Material, SM3). As such, the parameter values are based on results from different studies with different experimental settings, which we need to consider when evaluating the values' correctness. We anticipate that access to a complete set of cell-line-specific model parameter values, derived from one baseline experimental setting, would improve the predictive ability of the model. Just as importantly, such a setting would (a) provide an experimental system in which to test and evaluate the model's predictive performance, and (b) inform model updates. In our previous study, we evaluated model-predicted levels of activated ERK in response to DFB monotherapies against clinical data (Fig. 6 in Hamis et al., 2021[25]). The results of this evaluation were promising, which indicates that the model used in our previous study, and by extension the model used in our current study, has the potential to be of clinical relevance. On the topic of clinical relevance, we note that beyond BRAFV600E-mutant melanoma, the BRAFV600E-MEK-ERK pathway is also a target for treating non-small-cell lung carcinoma, thyroid cancer, and paediatric high-grade glioma with the BRAFV600E-mutation.[59,60] The mathematical model presented in this article could be modified, ideally by cancer-type specific model parameter values, to simulate BRAFV600E-MEK-ERK pathway dynamics and vertical inhibition treatment responses in other cancers.

To summarise, the translational value of this bio-mathematical study is two-fold. Firstly, the mathematical model increases our understanding of BRAFV600E-MEK-ERK signalling dynamics in response to vertical inhibition. Notably, the model and provides molecular-level mechanistic explanations for why resistance to DBF monotherapies and DBF-TMT-SCH combination therapies markedly increases with intracellular BRAFV600E concentrations, whilst resistance to TMT and SCH monotherapies does not. Secondly, the methodology presented in this study can be used as a tool to inform the design of pre-clinical studies that aim to find a range of drug dose combinations that target the BRAFV600E-MEK-ERK signalling pathway and achieve desired treatment responses. These mathematically identified dose combinations could offer alternatives to high-dose combinations which are currently used in clinics. As such, this bio-mathematical study constitutes a calculated step towards rational treatment strategies.

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Authors' contributions

ADC and SH produced the results and drafted the manuscript. All authors revised the manuscript. All authors have approved of the manuscript, and have agreed to be accountable for all aspects of the work presented in the manuscript.

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

Data availability

All code files and simulation data are available on the code-hosting platform GitHub (`https://github.com/SJHamis/MAPKcascades`). Instructions on how to access, run, and modify the code files are available in the Supplementary Material (SM2).

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