Kinase Inhibition Leads to Hormesis in a Dual Phosphorylation-Dephosphorylation Cycle

Peter Rashkov¹, Ian P. Barrett², Robert E. Beardmore¹, Claus Bendtsen²*, Ivana Gudelj¹*

¹ School of Biosciences, University of Exeter, Exeter, United Kingdom, ² Discovery Sciences, Innovative Medicines and Early Development, AstraZeneca, Cambridge, United Kingdom

* Claus.Bendtsen@astrazeneca.com (CB); I.Gudelj@exeter.ac.uk (IG)

Abstract

Many antimicrobial and anti-tumour drugs elicit hormetic responses characterised by low-dose stimulation and high-dose inhibition. While this can have profound consequences for human health, with low drug concentrations actually stimulating pathogen or tumour growth, the mechanistic understanding behind such responses is still lacking. We propose a novel, simple but general mechanism that could give rise to hormesis in systems where an inhibitor acts on an enzyme. At its core is one of the basic building blocks in intracellular signalling, the dual phosphorylation-dephosphorylation motif, found in diverse regulatory processes including control of cell proliferation and programmed cell death. Our analytically-derived conditions for observing hormesis provide clues as to why this mechanism has not been previously identified. Current mathematical models regularly make simplifying assumptions that lack empirical support but inadvertently preclude the observation of hormesis. In addition, due to the inherent population heterogeneities, the presence of hormesis is likely to be masked in empirical population-level studies. Therefore, examining hormetic responses at single-cell level coupled with improved mathematical models could substantially enhance detection and mechanistic understanding of hormesis.

Author Summary

Hormesis is a highly controversial and poorly understood phenomenon. It describes the idea that an inhibitor molecule, like an anti-cancer or anti-microbial drug, can inadvertently stimulate cell growth instead of suppressing it. This can have a profound effect on human health leading to failures in clinical treatments. Therefore, getting at the mechanistic basis of hormesis is critical for drug development and clinical practice, however molecular mechanisms underpinning hormesis remain poorly understood. In this paper we use a mathematical model to propose a simple and yet general mechanism that could explain why we find hormesis so widely in living systems. In particular, we discover that hormesis is present within a fundamental structure that forms a basic building block of many intracellular signalling pathways found in diverse processes including control of cell reproduction and programmed cell death. The benefits of our study are two-fold. Having simple molecular understanding of the causes of hormetic responses can greatly improve the design of new drug compounds that avoid such responses. Moreover, due to the...
fundamental nature of the newly proposed mechanism, our findings have a potential broad applicability to both anti-cancer and anti-microbial drugs.

Introduction

Hormesis is a phenomenon describing biphasic dose response relationships that exhibit low-dose stimulation and high-dose inhibition [1]. Many medical agents such as antibacterials, antifungals, and anti-tumour drugs have been found to display hormeric response [2] with the earliest observations dating back to 1800s. In particular, low concentrations of certain antifungals were found to stimulate fungal growth [3] or metabolism [4] while inducing toxicity at high concentrations. From the early 1920s the concept of low-dose stimulation and high-dose toxicity of various chemical elements with respect to bacterial growth was widely recognised [5]. We now know that bacteria can exhibit hormeric response to a wide range of antibiotic drugs, regardless of their mode of action [6]. This phenomenon is also found in tumour cells exposed to anti-tumour drugs. In fact, hormesis has been observed in an astonishingly broad range of tumour types including pancreatic, colon and breast (reviewed in [7]).

Despite the overwhelming body of research, some dating back a century, that documents hormeric responses to a broad range of compounds, their clinical significance has only relatively recently come to the fore [2]. The consequence of hormesis could have a profound effect for human health [8, 9]. Drug concentration generally varies substantially within the human body and as drug gets cleared, the associated low concentration can in turn stimulate pathogen or tumour growth. Therefore understanding the mechanistic basis of hormesis is vital for both drug development and clinical practice.

The vast majority of targets for antibiotics, antifungals and anti-tumour drugs fall into the following categories: enzymes, receptors, transporters and DNA/RNA and the ribosome [10]. However how such drug-target interactions lead to hormesis remains poorly understood. The biological explanations put forward are overcompensation after a disruption of homeostasis (reviewed in [11]), direct stimulatory response [12], superimposition of different monotonic dose-response curves [13], or heterogenic susceptibility of different tissues to the same stimuli [14]. These explanations provide understanding of hormesis at a phenotypic level but lack understanding at the molecular level. Some inroads have also been made with respect to mammalian cells focusing on drug mechanisms mediated via receptor and/or cell signalling pathways (reviewed in [7]). For example, biphasic dose response could occur through interaction of two different receptor subtypes that mediate/activate opposing stimulatory and inhibitory pathways via the same antagonist [15]. However, hormeric response is a built in feature of such receptor mediated mechanisms rather than an emergent property of the underlying biological system.

An area of research where understanding of the mechanisms giving rise to hormesis is particularly lacking involves enzyme-targeting drugs. Known as enzyme inhibitors, they are designed to block enzyme activity leading to disruption of bacterial cell wall [16], fungal membranes [17] and fungal cell wall [18] as well as programmed tumour cell death [19], to name a few. With regards to hormeric dose-responses to antibiotics, a recent study focusing on inhibition of a specific enzyme, Dihydropteroate synthase, suggested the involvement of bacterial quorum sensing [20]. To our knowledge, mechanisms behind hormeric dose-response to enzyme-inhibiting antifungals are not known.

In recent years kinase inhibitors, a subset of enzyme inhibitors, have been shown to be very effective therapeutic agents in a broad range of diseases, including cancers. Amongst other
enzyme inhibitors, significant attention has been focused on those inhibiting the mitogen-activated protein kinase (MAPK) pathway [21–24], which is of fundamental importance to human health as abnormal regulations of MAPK contribute to tumour progression [25].

The observations of hormesis in MAPKs as a result of inhibition of BRAF oncogene are widespread: low doses of RAF inhibitors designed to cease tumour proliferation [26] can cause a paradoxical activation of tumour cell activity through undesired MAPK up-regulation [8, 9, 27–32]. Current explanations of hormetic responses induced by RAF kinase inhibition involve complex phenomena affecting regulatory mechanisms, feedback pathways or enzymatic activity [33], making them difficult to generalise. More generally, enzyme competition for the same substrate was recently proposed as a simpler mechanism giving rise to hormetic effects of enzyme-targeting Alzheimer’s drugs [34].

In this paper we put forward a novel, simple but general mechanism driving hormetic responses in systems where an inhibitor acts on an enzyme. We develop a mathematical model based on a basic building block in intracellular signalling, namely a dual phosphorylation-dephosphorylation motif, to which a kinase inhibitor is applied. In a broader context, dual-phosphorylation can be found in diverse processes such as circadian rhythms [35], virulence regulation [36, 37], mitotic entry [38], transcription [39, 40], cytokine production [40], as well as in MAPK pathways which regulate primary cellular activities in eukaryotes including proliferation and programmed cell death [41, 42].

The model demonstrates that under certain conditions the steady state amount of the double-phosphorylated protein substrate in the cycle can substantially increase at low inhibitor doses compared to the base level without inhibition. Therefore the dose-response curve of the double-phosphorylated substrate possesses a hallmark of hormesis: it is upward sloping at low inhibitor doses and downward sloping at high inhibitor doses. The existence of hormesis in our model depends on the mechanism of inhibition and the dissociation rates of the kinase-substrate-inhibitor complexes. We also found that the magnitude of hormetic responses depends on the substrate-kinase ratio in a non-monotone way.

The benefits of our study are two-fold. Our mechanism is based on a principal component of intracellular signalling pathways, and as such has a potential broad applicability. Moreover having simple molecular understanding of the causes of hormetic responses can greatly improve the design of new drug compounds that avoid such responses.

**Materials and Methods**

**The mathematical model**

We consider a simple dual phosphorylation-dephosphorylation motif, whereby a distinct kinase protein is phosphorylating a separate protein substrate. Multiple phosphorylations can occur in close proximity or in diverse sites on a protein and here we focus on the former, instances of which can be found in activation of conventional MAPK enzymes [43], cell-cycle regulation via cyclin-dependent kinase 1 [44], regulation of other non-MAPK kinases [45] and ion channel trafficking [46]. The motif we consider is a subset of futile cycles [47, 48] also known as a single stage module in the context of MAPK pathways [49, 50]. Based on the experimental evidence for MAPK pathways [51–53] we assume that our motif follows a distributive mechanism consisting of two sequential phosphorylation steps and two sequential dephosphorylation steps that share the same intermediate mono-phosphorylated form. In particular, the protein substrate (C) is first converted into a mono-phosphorylated form (C_P) and subsequently into a double-phosphorylated form (C_PP), through a chain of reactions facilitated by a kinase (kin). Conversely C_PP is converted back to C_P which is subsequently converted to C, through a chain of reactions facilitated by a phosphatase (pho). In the distributive mechanism,
the kinase (phosphatase) facilitates at most one phosphorylation (dephosphorylation) in each molecular encounter [48].

Therefore our dual phosphorylation-dephosphorylation motif can be described by the following reaction kinetic equations, which are a simplification of the reaction scheme described in [54]:

\[
\begin{align*}
C + \text{kin} & \xrightarrow{k_1} C \cdot \text{kin} \xrightarrow{k_2} C_p + \text{kin}, \\
C_p + \text{kin} & \xrightarrow{k_3} C_p \cdot \text{kin} \xrightarrow{k_4} C_{pp} + \text{kin}, \\
C_{pp} + \text{pho} & \xrightarrow{k_5} C_{pp} \cdot \text{pho} \xrightarrow{k_6} C_p + \text{pho}, \\
C_p + \text{pho} & \xrightarrow{k_7} C_p \cdot \text{pho} \xrightarrow{k_8} C + \text{pho}.
\end{align*}
\]

(1)

Next we describe the assumptions behind the introduction of an inhibitor into Eq (1), based on the general modifier mechanism also known as hyperbolic or partial competitive inhibition [55]. We assume that the inhibitor (inh) is able to react with the kinase and the substrate-kinase intermediate complexes \(C \cdot \text{kin}\) and \(C_p \cdot \text{kin}\) according to the following inhibition scheme:

\[
\begin{align*}
\text{kin} + \text{inh} & \xrightarrow{d_i} \text{kin} \cdot \text{inh}, \\
C \cdot \text{kin} + \text{inh} & \xrightarrow{e_{i1}} C \cdot \text{kin} \cdot \text{inh} \xrightarrow{e_{i2}} C + \text{kin} \cdot \text{inh}, \\
C_p \cdot \text{kin} + \text{inh} & \xrightarrow{e_{i3}} C_p \cdot \text{kin} \cdot \text{inh} \xrightarrow{e_{i4}} C_p + \text{kin} \cdot \text{inh}.
\end{align*}
\]

(2)

The first- and second-order rates \(k_i\) and \(e_i\) in Eqs (1) and (2) and the association and dissociation rates \(d_i\) and \(e_i\) in Eq (2) are considered dimensionless. In our system intermediate substrate-kinase-inhibitor complexes are able to dissociate into a substrate and kinase-inhibitor complex with forward \(e_{i2}, e_{i4}\) and backward \(e_{i-2}, e_{i-4}\) rates [56].

The model describing the time evolution of the substrate, kinase, phosphatase and inhibitor concentrations is based on the law of mass action and assumes the total conservation of mass holds for all four compounds. The details of the system of 9 differential equations and the corresponding analysis are presented in S1 Appendix. (with Supplementary Tables A1 and A2 containing model parameter values). This model system is studied under steady state conditions, that is, when all concentrations of reactants have reached a dynamic equilibrium. Numerical simulations are conducted with Matcont, a continuation package in MATLAB used for numerical bifurcation analysis of ODEs [57].

**Results**

In the absence of an inhibitor, the double phosphorylation motif Eq (1) can possess either a single or two stable steady states of the doubly-phosphorylated form of the substrate \(C_{pp}\) [50, 54, 58, 59]. Therefore in our study we consider two cases: first, when the motif Eq (1) is mono-stable and second, when this motif is bi-stable.

In the case of a single stable steady state (\(C_{pp}^*\)) in the absence of an inhibitor, we find that \(C_{pp}\) can exhibit biphasic (or hormetic) response to an inhibitor as illustrated in Fig 1. In particular, the observed dose-response curve in the presence of an inhibitor has an inverted U-shape: for sufficiently low inhibitor doses the computed steady-state values of \(C_{pp}\) increase
monotonically, while for sufficiently large inhibitor doses, the computed steady-state values of $C_{PP}$ monotonically decrease.

Moreover, by making simplifying assumptions that $e_{-2} = e_{-4} = 0$, $e_2 > e_1$, $e_4 > e_3$ and the inhibitor has fast off rate, we can analytically derive the slope of the dose-response curve, in other words the slope of the relationship between the steady-state value of $C_{PP}$ and the total amount of inhibitor at low doses (see S1 Appendix for details). This allows us to identify two primary factors necessary for the hormesis to be observed:

(C1) the strong dissociation effect of intermediate substrate-kinase-inhibitor complexes $C_{kin \cdot inh}$ and $C_P \cdot kin \cdot inh$, corresponding to $e_2$, $e_4 > 0$,

(C2) large dissociation rate of kinase-inhibitor complexes.

Note that the hormesis is still observed in numerical simulations when $e_{-2}$, $e_{-4} > 0$ (Fig A1 in S1 Appendix).

In addition the above conditions (C1·C2) can also be used to forecast the presence of a hormetic dose response in the second case under our consideration, namely when in the absence of an inhibitor the motif Eq (1) has two stable steady states $C_{PP,1}^*$ (Fig 2A) and $C_{PP,2}^*$ (Fig 2B). In this case the numerical simulations predict that cells with high base level of double-phosphorylated substrate will respond differently to inhibition from the cells with low base level of double-phosphorylated substrate. In particular, cells with initially high levels of $C_{PP}$ (at steady state $C_{PP,1}^*$) will exhibit a monotone decreasing dose-response (Fig 2A) while cells with low initial levels of $C_{PP}$ (at steady state $C_{PP,2}^*$) will exhibit a hormetic response (Fig 2B).

The magnitude of hormetric response can differ between the mono- and bi-stable cases under consideration as illustrated in Figs 1 and 2B. In the mono-stable case the $C_{PP}$ value at dose $inh^*$ is approximately two-fold higher compared to the base level $C_{PP}^*$ value in the
absence of an inhibitor (Fig 1). In the bi-stable case the $C_{PP}$ value at dose $inh^*$ is approximately six-fold higher than the base level $C_{PP,2}^*$ value in the absence of an inhibitor (Fig 2B).

In general, we find that the ratio of total mass of protein substrate to kinase mass influences the magnitude of hormetic response in a non-monotone way as shown in Fig 3. For sufficiently small substrate-kinase ratio, a hormetic response is not observed (absence of hormesis is labelled as 100% response in Fig 3 because the maximal response is equal to the baseline of no inhibition). However, the hormetic response increases sharply as the substrate-kinase ratio increases. Further increases of this ratio lead to a sharp decline in the magnitude of hormetic response, which continues to increase slowly for sufficiently large substrate-kinase ratios (see Fig 3 inset). Therefore, the magnitude of hormetic response peaks at intermediate values of the substrate-kinase ratio, as frequently observed in the MAPK pathway [60] for example, while hormesis is not observed for low substrate-kinase ratios.

Discussion

Hormetic responses to enzyme-targeting drugs have been observed in both prokaryotes [20, 61, 62] and eukaryotes [8, 9, 27, 31, 32] but the mechanistic understanding behind such responses is still lacking. In this paper we focus on eukaryotic cells and propose a novel, simple but general mechanism that could give rise to hormesis in systems where an inhibitor acts on an enzyme.

At the core of our newly-proposed mechanism is one of the basic building blocks in intracellular signalling, the dual phosphorylation-dephosphorylation motif, found in diverse regulatory processes including MAPK pathways which control cell proliferation and programmed cell death in eukaryotes [41, 42]. We analytically derive conditions that lead to hormetic dose-response of the doubly-phosphorylated substrate in the presence of a kinase inhibitor. The conditions required for hormesis to be observed are surprisingly simple and involve two main...
factors: (C1) strong dissociation effect of intermediate substrate–kinase–inhibitor complexes and (C2) large dissociation rate of kinase–inhibitor complexes. Crystallographic studies of kinase inhibitors bound to their targets demonstrate that a number of different conformational states can be induced. Type 1 kinase inhibitors are defined as binding the kinase in its active conformation and crystal structures of ternary complexes of ATP analogues bound with substrate peptides are reported (for review see [63, 64]). Indeed it is not uncommon for crystal systems of substrate peptide complexes to be used in Structure Based Design campaigns to develop Type 1 kinase inhibitors [65].

Given the fundamental nature of the dual phosphorylation–dephosphorylation motif and the relative simplicity of the derived conditions necessary to observe hormesis, why was this mechanism previously overlooked in theoretical literature? A further examination of the (C1) condition could provide a potential answer. In general, when considering partial competitive enzyme inhibition [55] as we do here, classical enzyme kinetics literature [55, 56] assumes not only equilibrium concentrations of different enzyme species but it also assumes that at those equilibrium concentrations there is no flux through substrate–kinase–inhibitor complexes. However, we find that in our study as flux decreases the maximum hormetic response also decreases (Fig 4) indicating that under the no-flux assumption, hormetic responses could be overlooked.

Once a new mechanism is proposed to explain a particular biological phenomenon, ideally it should be put to test. However, there are a number of difficulties associated with in vitro tests of our model predictions. First, biochemical assays involved with in vitro studies are not standardised and vary between research groups, making comparisons between already published observations difficult. Second, testing our model predictions requires measurements of single and double phosphorylation outputs, this could be problematic as antibody specificity required to distinguish these outputs might not readily be available. This would particularly be relevant for systems where phosphorylation sites are situated close together. Third, ensuring that the condition for observing hormesis $e_2, e_4 > 0$ is satisfied experimentally is challenging as...
kinase biochemical assays would not usually include phosphatase activity. Furthermore varying rates of reactions individually or measuring fluxes in such systems is equally difficult.

Having discussed difficulties associated with testing our model in reductionist in vitro systems, we next consider whether these difficulties could be overcome with a cell-based experimental systems. In particular, our model predicts that hormetic dose-response could be a wide-spread feature of MAPK pathways when exposed to enzyme inhibitors. However we argue here that the non-trivial biphasic dose-response associated with hormesis might often be overlooked when performing experiments at cell population level, as we now discuss.

Consider the case where in the absence of an inhibitor, the double phosphorylation motif Eq (1) possesses two stable steady states of the doubly-phosphorylated form of the substrate C_{pp}. This means that tumour cells within a population can be grouped into two types: type-1 cells with ‘high’ C_{pp} and type-2 cells with ‘low’ C_{pp}. In reality these heterogeneous cell phenotypes can emerge not only due to multistability of the system [50, 54] but also due to stochastic fluctuations which lead to different concentrations of the the total protein substrate [66, 67]. In general, an untreated tumour is likely to harbour different proportions of cells in different phenotypic states [68].

We show that different cell types can respond differently to the presence of an inhibitor. Namely, our model predicts that in certain cases cells with initially high levels of C_{pp} (at steady state C_{pp,1^*}) will exhibit a monotone decreasing dose-response (Fig 2A) while cells with low
initial levels of $C_{PP}$ (at steady state $C_{PP,2}^{+}$) will exhibit a hormetric response (Fig 2B). This has an important consequence for measuring $C_{PP}$ at a population level as it is frequently done [69], as well as determining inhibitory concentrations (IC). Such consequences are best illustrated with the following example.

Let us assume, for example, that 88% of the tumour cells are type-1 cells and 12% of the tumour cells are type-2 cells. We can then simulate our model to generate dose response curves of $C_{PP}$ for both type-1 (Fig 5, green line) and type-2 (Fig 5, blue line) phenotypes. In addition, we can also numerically generate sampled values of the combined dose response of the entire population as would be measured, for instance, in a western blot or population-based imaging assay for $C_{PP}$ (Fig 5, red dots). By fitting a logistic curve to the sampled values of the combined dose response (Fig 5, red dashed line) we can estimate the inhibitor concentration causing 50% inhibition of the entire population, denoted $IC_{50}$. However, the same inhibitor concentration has the opposing effects on the two sub-populations: while it inhibits type-1 cells, it actually stimulates type-2 cells. This can be observed by comparing steady-state values of $C_{PP}$ in the absence of inhibition ($C_{PP,1}^{+}$ for type-1 and $C_{PP,2}^{+}$ for type 2) to the steady-state values of $C_{PP}$ in the presence of the inhibitor ($C_{PP,1}^{+}$ for type-1 and $C_{PP,2}^{+}$ for type 2) at the $IC_{50}$ concentration estimated for the entire population (Fig 5). In particular, the inhibition of type-1 cells can be seen from $C_{PP,1}^{+} > C_{PP,1}^{+}$ while the stimulation of type-2 cells can be seen from $C_{PP,2}^{+} < C_{PP,2}^{+}$. Such unexpected stimulatory effects of the population-level $IC_{50}$ exerted on type-2 sub-population could be further amplified when taking into account the imperfect drug penetration in a tumour [70]. In that case tumour cells would actually experience a lower inhibitor concentration $IC_{p}^{+} < IC_{50}$, which could lead to significant increases in steady-state values of $C_{PP}$ (denoted by $C_{PP,2}^{+}$ in Fig 5), compared to the steady-state values of $C_{PP}$ in the absence of inhibition (denoted by $C_{PP,2}^{+}$ in Fig 5). A numerical example with balanced type-1 and type-2 cell populations is presented in Fig A5 of S1 Appendix, showing that in this case it is also possible to mask the hormetric response at the population level, although the maximal hormetric response of the type-2 cells at the corresponding $IC_{50}$ is substantially lower.

The presence of hormetric responses to an inhibitor which are masked at a population level could, therefore, complicate the interpretation of, and understanding gained from, preclinical models. Such complex sub-population effects have been noted for example in the NF-$\kappa$B pathway, controlling DNA transcription, cytokine production and cell survival [71]. In particular, studies have shown that observing non-synchronous cells at a population level may under-represent oscillatory behaviour of nuclear shuttling [40, 72–74].

Examining hormetric responses at single-cell level could substantially improve detection rates as well as help identify mechanisms driving hormesis. However, while measuring and analysing single-cell bacterial dose response to antibiotics is already feasible [75], such methodology has rarely been implemented for studying dose-responses of tumour cells. Therefore, a wider application of single-cell dose-response techniques used for prokaryotes to tumour cells will greatly enhance our understanding of hormesis in cancer settings.

The conclusions of our study are based on the assumption that the dual phosphorylation-dephosphorylation motif presented in Eq (1) follows a distributive mechanism, whereby kinase (phosphatase) facilitates at most one phosphorylation (dephosphorylation) in each molecular encounter. This is motivated by the experimental evidence for MAPK pathways [51–53]. However, phosphorylation and dephosphorylation cycles can also follow a processive mechanism in which the kinase (phosphatase) facilitates two or more phosphorylations (dephosphorylations) before the final product is released [48]. In addition, a quasi-processive mechanism has been recently proposed to operate under the physiological condition of molecular crowding, which is a critical factor converting distributive into processive phosphorylation [76–78]. Our model can readily be extended to consider these alternative scenarios.
The findings presented here are relevant to applications in drug discovery relating to MAPK inhibition. Whereas inhibitors are specifically designed to target and suppress various stages in the MAPK pathways, the hormesis phenomenon leads to the opposite effect lowering the effectiveness of the compound and potentially leading to failure in the clinic [8, 9, 32]. Therefore, understanding mechanisms that lead to this undesired effect is important for designing inhibitors that would avoid them. Indeed, a recent study proposed a novel inhibitor, designed specifically to avoid MAPK activation at low-doses [79].

Our study could help achieve a similar goal. In particular, a straightforward approach to mitigate the risk of hormetic response is to favour inhibitor mechanisms of action for which this is impossible under our model. Protein substrate competitive inhibitors is one such example as these would generally, through steric hindrance, prohibit the formation of the necessary tertiary complex. In practice, structural biology can be employed to confirm that substrate and inhibitor complexes are mutually exclusive.

Overall, we argue that mathematical models are particularly useful tools in the drug-discovery process. Given the difficulties associated with measuring hormetic responses empirically be it with reductionist in vitro biochemical assays or cell based systems, the involvement of mathematical models in this process is of paramount importance. What we demonstrate here is that theoretical models classically make assumptions that immediately discount the...

---

Fig 5. Heterogeneous populations. Dose response curves of type-1 (green line) and type-2 (blue line) cells in the presence of an inhibitor (inh\textsubscript{tot}), in the case when motif Eq (1) is bistable. A logistic curve (red line) is fitted to sample points (red dots) generated numerically from a population containing 88% type-1 cells and 12% type-2 cells. The logistic fit to data is used to estimate IC\textsubscript{50} of the total population (see Supplementary Information). In the absence of the inhibitor type-1 cells are at C\textsubscript{PP,1}\textsuperscript{*} stead state while type-2 cells are at C\textsubscript{PP,2}**. In the presence of the inhibitor at IC\textsubscript{50} while the total population exhibits 50% inhibition, the same concentration has the opposing effects on the two sub-populations. In particular, type-1 cells are inhibited, which can be deduced from the observation that the steady state C\textsubscript{PP,1}** calculated at the population-level IC\textsubscript{50} is lower than the steady state C\textsubscript{PP,1}** calculated in the absence of the inhibitor. Contrary to this type-2 cells are stimulated since the steady state C\textsubscript{PP,2}** calculated at the population-level IC\textsubscript{50} is higher than the steady state C\textsubscript{PP,2}** calculated in the absence of the inhibitor. This stimulatory effect is amplified even further for IC\textsuperscript{*}<IC\textsubscript{50}, as seen by comparing the relatively high values of the steady state C\textsubscript{PP,2}** at IC\textsuperscript{*} to the relatively low values of the steady state C\textsubscript{PP,2}** in the absence of the inhibitor.

doi:10.1371/journal.pcbi.1005216.g005
possibility of observing hormetic responses in cell signalling pathways in the presence of inhibitors. Namely the assumption of no flux through substrate-kinase-inhibitor complex in motif Eq (2) is widespread in theoretical literature despite the lack of empirical support. It is, therefore, crucial that model assumptions are regularly challenged so that important behaviours are not overlooked.

Supporting Information

S1 Appendix. The file contains a detailed mathematical model describing the time evolution of the substrate, kinase, phosphate and inhibitor concentrations, alongside the corresponding analysis and model parametrisation. (PDF)

Author Contributions

Conceptualization: IG CB IPB REB.
Formal analysis: PR.
Funding acquisition: IG REB.
Methodology: PR IG IPB CB.
Software: PR.
Supervision: IG REB IPB CB.
Writing – original draft: IG REB IPB CB PR.

References

1. Calabrese EJ, Baldwin LA. Chemotherapeutics and hormesis. Crit Rev Toxicol. 2003; 33(3-4):305–353. doi: 10.1080/713611041 PMID: 12809428
2. Calabrese EJ. Hormesis: a revolution in toxicology, risk assessment and medicine. EMBO Rep. 2004; 5 (Special Issue):S37–S40. doi: 10.1038/sj.embor.7400222 PMID: 15495733
3. Raulin J. Études chimiques sur la végétation. Ann Sci Nat Paris, sér 5. 1869; 11:93–299.
4. Schulz H. Über Hefegifte. Pflügers Arch Gesamte Physiol Menschen Tiere. 1888; 42(1):517–541. doi: 10.1007/BF01669373
5. Hotchkiss M. Studies on salt action VI. The stimulating and inhibitive effect of certain cations upon bacterial growth. J Bacteriol. 1923; 8:141–162. PMID: 16558991
6. Davies J, Spiegelman GB, Yim G. The world of subinhibitory antibiotic concentrations. Curr Opin Microbiol. 2006; 9(5):445–453. doi: 10.1016/j.mib.2006.08.006 PMID: 16942902
7. Calabrese EJ. Hormetic mechanisms. Crit Rev Toxicol. 2013; 43(7):580–606. doi: 10.3109/10408444.2013.808172 PMID: 23875765
8. Callahan MK, Rampal R, Harding JJ, Klimmek VM, Chung R, Merghoub T, et al. Progression of RAS-mutant leukemia during RAF inhibitor treatment. N Engl J Med. 2012; 367(24):2316–2321. doi: 10.1056/NEJMoa1208958 PMID: 23134356
9. Su F, Viros A, Milagre C, Trunzer K, Bollah G, Spleiss O, et al. RAS mutations in cutaneous squamous-cell carcinomas in patients treated with BRAF inhibitors. N Engl J Med. 2012; 366(3):207–215. doi: 10.1056/NEJMoa1105358 PMID: 22256804
10. Imming P, Sinning C, Meyer A. Drugs, their targets and the nature and number of drug targets. Nat Rev Drug Discov. 2006; 5:821–834. doi: 10.1038/nrd2132 PMID: 17016423
11. Garzon CD, Flores FJ. Hormesis: biphasic dose-responses to fungicides in plant pathogens and their potential threat to agriculture. In: Nita M, editor. Fungicides—showcases of integrated plant disease management from around the world. InTech; 2013. pp. 311–328.
12. Calabrese EJ, Baldwin LA. Hormesis: a generalizable and unifying hypothesis. Crit Rev Toxicol. 2001; 31(4-5):353–424. doi: 10.1080/20014091111730 PMID: 11904172
13. Conolly RB, Lutz WK. Nonmonotonic dose-response relationships: mechanistic basis, kinetic modeling, and implications for risk assessment. Toxicol Sci. 2004; 77(1):151–157. doi: 10.1093/toxsci/kfh007 PMID: 14600281

14. Bae ON, Lim KM, Han JY, Jung BI, Lee JY, Noh JY, et al. U-shaped dose response in vasomotor tone: A mixed result of heterogenic response of multiple cells to xenobiotics. Toxicol Sci. 2008; 103(1):181–190. doi: 10.1093/toxsci/kfn023 PMID: 18281258

15. Szabadi E. A model of two functionally antagonistic receptor populations activated by the same agonist. J Theor Biol. 1977; 69(1):101–112. doi: 10.1016/0022-5193(77)90390-3 PMID: 592862

16. Nicolau KC, Boddy CNC, Bräse S, Winsinger N. Chemistry, biology, and medicine of the glycopeptide antibiotics. Angew Chem Int Ed Engl. 1999; 38(15):2096–2152. doi: 10.1002/(SICI)1521-3773(19990802)38:15<2096::AID-AIE20963E3.0.CO;2-F PMID: 10425471

17. Maertens Ja. History of the development of azole derivatives. Clin Microbiology Infect. 2004; 10 Suppl 1:1–10. doi: 10.1111/j.1470-9465.2004.00841.x PMID: 14748798

18. Denning DW. Echinocandin antifungal drugs. The Lancet. 2003; 362(9380):1142–1151. doi: 10.1016/S0140-6736(03)14472-8 PMID: 14550704

19. Nitiss J. Targeting DNA topoisomerase II in cancer chemotherapy. Nat Rev Cancer. 2009; 9(5):338–350. doi: 10.1038/nrc2607 PMID: 19375006

20. Deng Z, Lin Z, Zou X, Yao Z, Tian D, Wang D, et al. Model of hormesis and its toxicity mechanism based on quorum sensing: A case study on the toxicity of sulfonamides to Photobac terium phosphorum. Environ Sci Technol. 2012; 46(14):7746–7754. doi: 10.1021/es203490f PMID: 22715968

21. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med. 2011; 364(26):2507–2516. doi: 10.1056/NEJMoa1103782 PMID: 21639808

22. Sosman JA, Kim KB, Schuchter L, Gonzalez R, Pavlick AC, Weber JS, et al. Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. N Engl J Med. 2012; 366(8):707–714. doi: 10.1056/NEJMoa1112302 PMID: 22356324

23. Hauschild A, Grob JJ, Demidov LV, Jouary T, Gutzmer R, Millward M, et al. Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. The Lancet. 2012; 380(9839):358–365. doi: 10.1016/S0140-6736(12)60868-X PMID: 22735384

24. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med. 2010; 363(9):809–819. doi: 10.1056/NEJMoa1002011 PMID: 20818844

25. Roberts P, Der C. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene. 2007; 26:3291–3310. doi: 10.1038/sj.onc.1210422 PMID: 17496923

26. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature. 2002; 417(6892):949–954. doi: 10.1038/nature00766 PMID: 12068308

27. Hall-Jackson CA, Eyers PA, Cohen P, Goedert M, Boyle FT, Hewitt N, et al. Paradoxical activation of RAF by a novel RAF inhibitor. Chem Biol. 1999; 6(8):559–568. doi: 10.1016/S1074-5521(99)80088-X PMID: 10421767

28. Poulikakos PI, Zhang C, Bollag G, Shokat KM, Rosen N. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. Nature. 2010; 464(7287):427–430. doi: 10.1038/nature08833 PMID: 20130576

29. Heidorn SJ, Milagre C, Whittaker S, Nourry A, Niculescu-Duvas I, Dhomen N, et al. Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell. 2010; 140(2):209–221. doi: 10.1016/j.cell.2009.12.040 PMID: 20141835

30. Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, et al. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature. 2010; 464(7287):431–435. doi: 10.1038/nature08892 PMID: 20179705

31. Andrews MC, Behren A, Chionh F, Mariadason J, Vella LJ, Do H, et al. BRAF inhibitor-driven tumor proliferation in a KRAS-mutated colon carcinoma is not overcome by MEK1/2 inhibition. J Clin Oncol. 2010; 31(35):e424–e451. doi: 10.1200/JCO.2010.30.0114 PMID: 21403036

32. Zimmer L, Hillen U, Livingstone E, Lacouture ME, Busam K, Carvajal RD, et al. Atypical melanocytic proliferations and new primary melanomas in patients with advanced melanoma undergoing selective BRAF Inhibition. J Clin Oncol. 2012; 30(19):2375–2383. doi: 10.1200/JCO.2011.41.1660 PMID: 22614973

33. Holderfield M, Nagel TE, Stuart DD. Mechanism and consequences of RAF kinase activation by small-molecule inhibitors. Br J Cancer. 2014; 111(4):640–645. doi: 10.1038/bjc.2014.139 PMID: 24642617
34. Ortega F, Stott J, Visser SAG, Bendtsen C. Interplay between α-, β-, and γ-secretases determines biphasic amyloid-β protein level in the presence of a γ-secretase inhibitor. J Biol Chem. 2013; 288(2):785–792. doi: 10.1074/jbc.M112.419135 PMID: 23152503

35. Nishiwaki T, Satomi Y, Kitayama Y, Terauchi K, Kiyohara R, Takao T, et al. A sequential program of dual phosphorylation of KaIC as a basis for circadian rhythm in cyanobacteria. EMBO J. 2007; 26(17):4029–4037. doi: 10.1038/sj.emboj.7601832 PMID: 17717528

36. Horstmann N, Saldana M, Sahasrabhojane P, Yao H, Su X, Thompson E, et al. Dual-site phosphorylation of the control of virulence regulator impacts group A streptococcal global gene expression and pathogenesis. PLoS Pathog. 2014; 10(5):e1004088. doi: 10.1371/journal.ppat.1004088 PMID: 24788524

37. Whitmore SE, Lamont RJ. Tyrosine phosphorylation and bacterial virulence. Int J Oral Sci. 2012; 4(1):1–6. doi: 10.1038/ijos.2012.6 PMID: 22388693

38. Bulavin DV, Higashimoto Y, Demidenko ZN, Meek S, Graves P, Phillips C, et al. Dual phospho rylation of the control of virulence regulator impacts group A streptococcal global gene expression and pathogenesi s. PLoS Pathog. 2014; 10(5):e1004088. doi: 10.1371/journal.ppat.1004088 PMID: 24788524

39. Martin-Verstraete I, Charrier V, Stülke J, Galinier A, Erni B, Rapoport G, et al. Antagonistic effects of dual PTS-catalysed phosphorylation on the Bacillus subtilis transcriptional activator LevR. Mol Microbiol. 1998; 28(2):293–303. doi: 10.1046/j.1365-2958.1998.00781.x PMID: 9622354

40. Hoffmann A, Levchenko A, Scott ML, Baltimore D. The IκB-NF-κB signaling module: temporal control and selective gene activation. Science. 2002; 298(5596):1241–1245. doi: 10.1126/science.1071914 PMID: 12424381

41. Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev. 1999; 79(1):143–180. PMID: 9922370

42. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature. 2001; 410(6824):37–40. doi: 10.1038/35065000 PMID: 11242034

43. Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev. 2011; 75(1):50–83. doi: 10.1128/MMBR.00031-10 PMID: 21372320

44. Ayeni JO, Varadarajan R, Mukherjee O, Stuart DT, Sprenger F, Srayko M, et al. Dual phosphorylation of Cdk1 coordinates cell proliferation with key developmental processes in Drosophila. Genetics. 2014; 196(1):197–210. doi: 10.1534/generics.113.156281 PMID: 24214343

45. Fu Z, Larson KA, Chitta RK, Parker SA, Turk BE, Lawrence MW, et al. Identification of yin-yang regulators and a phosphorylation consensus for male germ cell-associated kinase (MAK)-related kinase. Mol Cell Biol. 2006; 26(22):8639–8654. doi: 10.1128/MCB.00816-06 PMID: 16954377

46. Kilisch M, Lytvovenko O, Arakel EC, Bertinetti D, Schwappach B. A dual phosphorylation switch controls 14-3-3-dependent cell surface expression of TASK-1. J Cell Sci. 2016; 129(4):831–842. doi: 10.1242/jcs.180182 PMID: 26743085

47. Samoilov M, Plyasunov S, Arkin AP. Stochastic amplification and signaling in enzymatic futile cycles through noise-induced bistability with oscillations. Proc Nat Acad Sci USA. 2005; 102(7):2310–2315. doi: 10.1073/pnas.0406841102 PMID: 15701703

48. Wang L, Sontag ED. On the number of steady states in a multiple futile cycle. J Math Biol. 2008; 57(1):29–52. doi: 10.1007/s00285-007-0145-z PMID: 18008071

49. Huang CY, Ferrell JE. Ultrasensitivity in the mitogen-activated protein kinase cascade. Proc Nat Acad Sci USA. 1996; 93(19):10078–10083. doi: 10.1073/pnas.93.19.10078 PMID: 8816754

50. Qiao L, Nachbar RB, Kevrekidis IG, Shvartsman SY. Bistability and oscillations in the Huang-Ferrell model of MAPK signaling. PLoS Comput Biol. 2007; 3(9):e1819–1826. doi: 10.1371/journal.pcbi.0030184 PMID: 17907797

51. Ferrell JE, Bhatt RR. Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase. J Biol Chem. 1997; 272(30):19008–19016. doi: 10.1074/jbc.272.30.19008 PMID: 9228083

52. Burack WR, Sturgill TW. The activating dual phosphorylation of MAPK by MEK is nonprocessive. Biochemistry. 1997; 36(20):5929–5933. doi: 10.1021/bi970535d PMID: 9166761

53. Zhao Y, Zhang ZY. The mechanism of dephosphorylation of extracellular signal-regulated kinase 2 by mitogen-activated protein kinase phosphatase 3. J Biol Chem. 2001; 276(34):32382–32391. doi: 10.1074/jbc.M103369200 PMID: 11432864

54. Markevich NI, Hoek JB, Kholodenko BN. Signaling switches and bistability arising from multisite phosphorylation in protein kinase cascades. J Cell Biol. 2004; 164(3):353–359. doi: 10.1083/jcb.200308060 PMID: 14744999

55. Cornish-Bowden A. Fundamentals of enzyme kinetics. London: Portland Press; 1995.
56. Segel I. Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems. Wiley; 1993.

57. Dhooge A, Govaerts W, Kuznetsov YA. MATCONT: A MATLAB package for numerical bifurcation analysis of ODEs. ACM Trans Math Software. 2003; 29:141–164. doi: 10.1145/779359.779362

58. Ortega F, Garcés JL, Mas F, Kholodenko BN, Cascante M. Bistability from double phosphorylation in signal transduction: kinetic and structural requirements. FEBS J. 2006; 273(17):3915–3926. doi: 10.1111/j.1742-4658.2006.05394.x PMID: 16934303

59. Conradi C, Mincheva M. Catalytic constants enable the emergence of bistability in dual phosphorylation. J R Soc Interface. 2014; 11(95):20140158. doi: 10.1098/rsif.2014.0158 PMID: 24647909

60. Wang M, Weiss M, Simonovic M, Hergarter G, Schrimpf SP, Hengartner MO, et al. PaxDb, a database of protein abundance averages across all three domains of life. Mol Cell Proteomics. 2012; 11(8):492–500. doi: 10.1074/mcp.O111.014704 PMID: 22535208

61. Yoh M, Frimpong E, Voravuthikunchai S, Honda T. Effect of subinhibitory concentrations of antimicrobial agents (quinolones and macrolide) on the production of verotoxin by enterohemorrhagic Escherichia coli O157:H7. Can J Microbiol. 1999; 45(9):732–739. doi: 10.1139/w99-069 PMID: 10526400

62. Seyedsayamdost MR. High-throughput platform for the discovery of elicitors of silent bacterial gene clusters. Proc Nat Acad Sci USA. 2014; 111(20):7266–7271. doi: 10.1073/pnas.1400019111 PMID: 24808135

63. Brown NR, Noble ME, Endicott JA, Johnson LN. The structural basis for specificity of substrate and recruitment peptides for cyclin-dependent kinases. Nat Cell Biol. 1999; 1(7):438–443. doi: 10.1038/15674 PMID: 10559988

64. Zhang J, Yang PL, Gray NS. Targeting cancer with small molecule kinase inhibitors. Nature Rev Cancer. 2009; 9(1):28–39. doi: 10.1038/nrc2559 PMID: 19104514

65. Bullock AN, Debreczeni J, Amos AL, Knapp S, Turk BE. Structure and substrate specificity of the Pim-1 kinase. J Biol Chem. 2005; 280(50):41675–41682. doi: 10.1074/jbc.M5071200 PMID: 16227028

66. Feinerman O, Veiga J, Dorfman JR, Gerstel P, Altan-Bonnet G. Variability and robustness in T cell activation from regulated heterogeneity in protein levels. Science. 2008; 321(5892):1081–1084. doi: 10.1126/science.1158013 PMID: 18719282

67. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144(5):646–674. doi: 10.1016/j.cell.2011.02.013 PMID: 21376230

68. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med. 2012; 366(10):883–892. doi: 10.1056/NEJMoa1113205 PMID: 22397650

69. Little AS, Balmanov K, Sale MJ, Newman S, Dry JR, Hampson M, et al. Amplification of the driving oncogene, KRAS or BRAF, underpins acquired resistance to MEK1/2 inhibitors in colorectal cancer cells. Sci Signal. 2011; 4(166):ra17. doi: 10.1126/sci signal.2001752 PMID: 21447798

70. Meads MB, Gatenby RA, Dalton WS. Environment-mediated drug resistance: a major contributor to minimal residual disease. Nat Rev Cancer. 2009; 9(9):665–674. doi: 10.1038/nrc2711 PMID: 19693095

71. Gerondakis S, Grossmann M, Nakamura Y, Pohl T, Grumont R. Genetic approaches in mice to understand Rel/NF-κB and IκB function: transgenics and knockouts. Oncogene. 1999; 18:6888–6895. doi: 10.1038/sj.onc.1203236 PMID: 10602464

72. Kearns JD, Basak S, Werner SL, Huang CS, Hoffmann A. IκB- provides negative feedback to control NF-κB oscillations, signaling dynamics, and inflammatory gene expression. J Cell Biol. 2006; 173(5):659–664. doi: 10.1083/jcb.200510155 PMID: 16735576

73. Mothes J, Busse D, Koehl B, Wolf J. Sources of dynamic variability in NF-κB signal transduction: a mechanistic model. BioEssays. 2015; 37(4):452–462. doi: 10.1002/bies.201400113 PMID: 25640005

74. O’Dea EL, Barken D, Peralta RQ, Tran KT, Werner SL, Kearns JD, et al. A homeostatic model of IκB metabolism to control constitutive NF-κB activity. Mol Syst Biol. 2007; 3:111. doi: 10.1038/msb4100148 PMID: 17486138

75. Millard BL, Niepel M, Menden MP, Muhlich JL, Sorger PK. Adaptive informatics for multifactorial and high-content biological data. Nat Methods. 2011; 8(6):487–492. doi: 10.1038/nmeth.1600 PMID: 21516115

76. Aoki K, Yamada K, Kunida K, Yasuda S, Matsuda M. Processive phosphorylation of ERK MAP kinase in mammalian cells. Proc Nat Acad Sci USA. 2011; 108:12675–12680. doi: 10.1073/pnas.1104030108 PMID: 21763338

77. Aoki K, Takahashi K, Kaizuka K, Matsuda M. A quantitative model of ERK MAP kinase phosphorylation in crowded media. Sci Rep. 2013; 3:1541. doi: 10.1038/srep01541 PMID: 23526948
78. Sun J, Yi M, Yang L, Wei W, Ding Y, Jia Y. Enhancement of tunability of MAPK cascade due to coexistence of processive and distributive phosphorylation mechanisms. Biophys J. 2014; 106(5):1215–1226. doi: 10.1016/j.bpj.2014.01.036 PMID: 24606945

79. Zhang C, Spevak W, Zhang Y, Burton EA, Ma Y, Habets G, et al. RAF inhibitors that evade paradoxical MAPK pathway activation. Nature. 2015; 526(7574):583–586. doi: 10.1038/nature14982 PMID: 26466569