Computational study of the mechanism of Bcl-2 apoptotic switch

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Abstract

Programmed cell death - apoptosis is one of the most studied biological phenomenon of recent years. Apoptotic regulatory network contains several significant control points, including probably the most important one - Bcl–2 apoptotic switch. There are two proposed hypotheses regarding its internal working - the indirect activation and direct activation models. Since these hypotheses form extreme poles of full continuum of intermediate models, we have constructed more general model with these two models as extreme cases.

By studying relationship between model parameters and steady-state response ultrasensitivity we have found optimal interaction pattern which reproduces behavior of Bcl-2 apoptotic switch. Our results show, that stimulus-response ultrasensitivity is negatively related to spontaneous activation of Bcl-2 effectors - subgroup of Bcl-2 proteins. We found that ultrasensitivity requires effector’s activation, mediated by another subgroup of Bcl-2 proteins - activators. We have shown that the auto-activation of effectors forms ultrasensitivity enhancing feedback loop, only if mediated by monomers, but not by oligomers. Robustness analysis revealed that interaction pattern proposed by direct activation hypothesis is able to conserve stimulus-response dependence and preserve ultrasensitivity despite large changes of its internal parameters. This ability is strongly reduced as for the intermediate to indirect side of the models.

Computer simulation of the more general model presented here suggest, that stimulus-response ultrasensitivity is an emergent property of the direct

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activation model, that cannot originate within model of indirect activation. Introduction of indirect-model-specific interactions does not provide better explanation of Bcl-2 functioning compared to direct model.

Keywords: programmed cell death, apoptosis, ultrasensitivity of biological regulatory network, Bcl-2 family of proteins, robustness analysis of biological switch

1. Introduction

Apoptosis is the most ubiquitous type of programmed cell death, that has been observed and studied for more than one century [1]. It is a physiological phenomenon common for all multicellular organisms, necessary to tissue and body genesis and homeostasis [2, 3]. Defects of its regulation may cause numerous diseases, including cancer, autoimmunity and neurodegenerative disorders [2, 4]. Recent research suggests, that apoptosis is distinct process known not only by its characteristic morphology and genomic destruction, but also as a process of progressive cellular disassembly with remarkably high level of complexity and flexible, multifaceted regulation. Apoptosis may be initiated by triggering events from within the cell or from outside the cell [4]. Apoptosis signaling then proceeds through multiple independent pathways [4].

Most of the apoptotic signaling pathways converge to mitochondria and may cause rapid increase in mitochondrial membrane permeabilization [5, 6]. Mitochondrial outer membrane permeabilization (MOMP) leads to release of several pro-apoptotic factors, including cytochrome c and Smac/DIABLO [7, 8] to cytosol. Cytosolic cytochrome c then associates with pro-apoptotic factors, such as adaptor protein Apaf-1, to form Apoptosome complex [8, 9]. At the same time, Smac/DIABLO inhibits IAPs - the inhibitors of apoptosis [8, 9]. Thus, mitochondria play pivotal role in apoptosis signaling by integrating and sensing incoming apoptotic signals and eventually responding by permeabilization of their outer membrane.

Growing evidence demonstrate that MOMP is regulated by proteins of Bcl-2 family [10, 11], which includes both pro-apoptotic and anti-apoptotic members. Proteins of the bcl-2 family are categorized by their relation to the apoptotic process and according to their Bcl-2 homology (BH) domains in their α-helical regions [12, 13, 14]. Anti-apoptotic members such as Bcl-2 itself, Bcl-xL and Bcl-w exhibit four BH domains (BH1-4) [14], pre-
vent MOMP and protect cells from a wide range of cytotoxic impacts [13]. Pro-apoptotic members lack the BH4 domain and can be further divided to BH3-only proteins, such as Bid, Bik, Bim, Bad, Noxa and PUMA and multidomain proteins, such as Bax, Bak and Bok [13, 11]. BH3-only proteins can be activated by multiple pro-apoptotic signals and cytotoxic conditions, including cytokine deprivation or DNA damage [15]. Multidomain Bcl-2 proteins, also termed ‘effectors’, once activated, permeabilize mitochondrial outer membrane (MOM) by allowing formation of oligomerized pores (MAC – mitochondrial apoptosis-induced channel) [16, 17]. The interplay between the three groups of Bcl-2 family determines MOMP commitment and forms so-called Bcl-2 apoptotic switch [18, 11, 19].

Although there is general agreement that Bcl-2 proteins regulate MOMP, details of the Bcl-2 apoptotic switch mechanism remain controversial. It is still not clear, how various members of the Bcl-2 family interact between each other, and which of these interactions are decisive for MOMP induction. In particular, it is not resolved, whether the BH3-only proteins are able to activate multidomain effectors such as Bax and Bak directly, or whether they act indirectly, through neutralization of Bcl-2 anti-apoptotic sentinels to initiate MOMP [15, 20, 19]. The mechanism, based on the neutralization of the anti-apoptotic proteins, is known as indirect model [12, 10, 21, 22]. Another, based on direct activation of effectors Bax and Bak by BH3 only proteins corresponds to the direct model [10, 21, 22]. Another closely related question, deals with the manner by which anti-apoptotic proteins inhibit activity of effectors [15, 20, 19]. Do the anti-apoptotic proteins bind non-activated Bax/Bak to prevent their activation, or do they bind and neutralize activated Bax/Bak?

In the indirect model (see Fig. 1, Indirect model), effectors are activated spontaneously by conformational changes without presence of external factors [23, 19]. In indirect model, the anti-apoptotic Bcl-2 proteins are bound to effectors blocking their spontaneous activation [24]. Signals for MOMP commitment activate BH3-only proteins, which can bind anti-apoptotic Bcl-2 proteins. Active BH3-only proteins can displace effectors from their anti-apoptotic relatives, thus promoting MOMP [25, 24, 23, 21]. Nevertheless, spontaneous activation of Bax and Bak, suggested in the indirect model, still lack convincing experimental confirmation [26, 19].

In the direct model (see Fig. 1, Direct model), effectors are activated solely by interactions with some members of the BH3-only proteins [27, 28, 29, 30, 31]. Such members (e.g. Bid and Bim) are therefore called activators.
Other members of BH3-only proteins (such as Bad and Bik), are called ‘enablers’, ‘de-repressors’ or ‘sensitizers’, because they can bind anti-apoptotic Bcl-2 proteins, but do not activate Bax or Bak directly. Thus, enablers can help to promote MOMP by displacing the activators from anti-apoptotic Bcl-2 proteins [21].

Cellular switches such as Bcl-2 apoptotic switch are molecular mechanisms converting continuous incoming signals to two mutually distinct outputs. Their role is to ensure unambiguous transitions between two different cellular states [32]. One of the necessary requirements to generate this behavior is ultrasensitive reaction mechanism [33, 34, 32]. Systems with ultrasensitive responses are defined as systems which are more sensitive to stimulus changes than hyperbolic systems (systems with response given by the Michaelis-Menten equation) [35, 36, 32].

Comparison of the indirect and the direct model of Bcl-2 apoptotic switch, based on measuring of ultrasensitivity has already been done in work of Chen et al. [20]. Chen et al. concludes that the direct model is more plausible compared to its indirect alternative, since it leads to greater robustness, especially with respect to ultrasensitivity preservation.

In the present work, we take more general approach. Our approach originates from a different point of view on the Bcl-2 apoptotic switch controversy. Since interactions contained in the model of indirect activation may take their place in addition to those contained in the direct activation model [22], hypotheses of indirect and direct activation are not entirely contradictory. Since models of indirect and direct activation can be considered as special cases of the more general continuum of models, we came out with the hybrid model that merges the interactions from both indirect and direct models. We used the hybrid model to browse space between hypotheses of indirect and direct activation, looking for parameter settings and interaction patterns under which model would preserve expected behavior.

The idea of combining indirect and direct activation into hybrid model is not entirely new, it has been previously discussed [26, 22]. However, it has never been examined whether such hybrid model could provide a better explanation of Bcl-2 apoptotic switch functioning compared to the models of indirect/direct activation, nor whether any new system properties may emerge from combining of those models. We have tried to answer these questions here, as well as to comprehensively investigate importance of individual interactions between particular Bcl-2 family for the proper switch functioning.
1.1. Mathematical model and its biological relevance

We constructed hybrid model of biological switch formed by interactions of Bcl-2 family of proteins (scheme of this model is depicted in Fig. 2). Since the hybrid model combines the models of indirect activation and direct activation, by setting particular reaction rates to zero (effectively omitting corresponding interactions) we can reduce hybrid model to indirect, or direct one. To reduce the overall complexity of the generalized model, while preserve fundamental features of Bcl-2 family proteins, our models include only individual members representing whole class of Bcl-2 proteins with similar properties. Anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-w, Bcl-xL and Mcl-1 [21, 14] are represented by Bcl-2. Moreover, pro-apoptotic Bcl-2 family members are grouped with respect to their composition of BH domains. The members containing multiple BH domains - effectors, for example Bax and Bak [15, 14, 21], are represented by Bax. BH3-only members of Bcl-2 family - Bid, Bim, Bad, Bik, Noxa, PUMA and others, are all assumed to be able to interact with effector proteins and thus induce their activation. Therefore they are represented by Act (Activators).

Biological mechanism formed by Bcl-2 family of proteins processes multitude input signals into a single output signal - permeability of mitochondrial outer membrane. Activation of effectors leads to mitochondrial outer membrane permeabilization [7, 37, 27, 38]. Among incoming signals are truncation of Bid by caspase-8 and activation of other BH3-only proteins by diverse cytotoxic conditions, for example cytokine deprivation or DNA damage [13, 23]. These input signals are represented in our model by single input stimulus - E, reaction rate of Act conversion to Act–a (reaction - 1).

\[
1. \text{Act} \xrightarrow{E} \text{Act–a}
\]

Inhibition of activation of effector proteins Bax by anti-apoptotic Bcl-2 proteins is essential element of both models of Bcl-2 apoptotic switch. While the indirect model implies binding of non-activated Bax by anti-apoptotic Bcl-2 proteins (reaction - 2) [39, 24], the direct activation hypothesis assumes that Bax activity is inhibited solely by reversible binding and neutralization of activated Bax by anti-apoptotic Bcl-2 proteins (reaction - 3).

\[
2. \text{Bcl-2} + \text{Bax} \rightleftharpoons \text{Bcl-2} \sim \text{Bax}
\]

\[
3. \text{Bcl-2} + \text{Bax–a} \rightleftharpoons \text{Bcl-2} \sim \text{Bax–a}
\]

Activated Act–a also reversibly bind anti-apoptotic Bcl-2, leading to their mutual neutralization (reaction - 4) [39, 31].
Moreover, Act–a is able to displace effectors Bax (indirect model) (reaction - 5), Bax–a (direct model) (reaction - 6) or both (hybrid model) from their complexes with Bcl-2 [24, 23]. In the hybrid model, both Bax and Bax–a can compete to form the complex with Bcl-2 (reaction - 7).

Where the indirect model includes spontaneous activation of effector (reaction - 8), the direct model assumes that effectors are activated by Act–a (reaction - 9) [28, 29, 30, 31]. Both of these reactions are incorporated in the hybrid model. Spontaneous neutralization of Bax–a (reaction - 10) corresponds to unmediated conformational changes that suppress its activity.

The activity of effectors results in formation of mitochondrial apoptosis-induced channels. In accordance with conclusions of Martinez et al. [40] we have modeled assembly of these channels as incremental growth of homooligomers constituted from monomeric effector units (reactions 11 and 12). Since MACs typically contain around 9 monomers, for simplicity we delimit oligomers growth up to 20 monomer units. We have verified that further growth of oligomer size does not qualitatively influence results proposed in this work (data not shown).

Additionally, all of the present compounds are continuously degraded. Bcl-2, Bax and Act are at the same time produced to balance their degradation flows.

13. (All) →
14. → Bcl-2
15. → Bax
16. → Act
1.2. Initial concentration and reaction rates

Initial concentrations of particular species have been estimated in accordance with experimentally obtained data published in literature [10, 41, 42, 20]. Concentrations of anti-apoptotic Bcl-2 and effector proteins (represented by Bcl2 and Bax, respectively) are considered to be in range of hundreds of nanomols, concentration of effectors was reported to be two times higher than that of Bcl-2 [10, 20]. Concentration of BH3-only proteins (represented by Act) is considered to be much lower, in range of 1-20 nanomols [41, 42] (Table 1).

The rates of binding of activators by anti-apoptotic Bcl-2 proteins has been estimated according to corresponding dissociation constants $K_d$ published in literature [43, 44], assuming reverse reaction rate $10^{-3} \text{s}^{-1}$. The rest of reaction rates (binding of activated and non-activated Bax by anti-apoptotic Bcl-2 proteins, direct activation of Bax and its inactivation) have been chosen in accordance with previously published models [20, 45, 19]. All the species are continuously degraded with degradation rate corresponding to half-life $t_{1/2} = 180 \text{min}$. Productions of inactive Bax, Act and Bcl-2 proteins are modeled by zero-order reactions parametrized to balance degradation under initial conditions (Tables 2 and 3).

We used values of input stimulus $E$ from $10^{-4}$ to $10^{-1} \text{min}^{-1}$. These values correspond to caspase-8 catalytic activity in Bid truncation, referred as $\sim 10^6 \text{M}^{-1}\text{s}^{-1}$ [46], assuming the number of active caspase-8 in range from $10^0$ - $10^3$ molecules per cell volume [47].

1.3. Implementation details

All models were expressed in the CMDL (chemical model definition language) as well as SBML (the systems biology markup language) format (SBML format is available in supplementary). All the simulations were done by using CMDL/SBML ODE solver - ODEtoJava-dopr54-adaptive within Dizzy - Chemical kinetics simulations software [48].

2. Results

In this work we focused on ultrasensitivity of Bcl-2 apoptotic switch and its dependence on particular interactions between the members of Bcl-2 family. To quantify the sensitivity of particular models, we used approach proposed in the work of Legewie et al. [36] (brief summary can be found in
As the reference response, used to calculate the relative amplification coefficient, we used Michaelis-Menten (hyperbolic) equation. Models with parameter sets, exhibiting relative amplification coefficient $n_R$ higher than unity were classified as ultrasensitive [36].

First of all, we compared steady-state stimulus-response dependence (Fig. 3) of hybrid, indirect and direct models (using reference setup, parameters from Table 3). The input stimulus is represented by the value of the parameter $E$ - rate of Act to Act-a conversion.

In most of the previously published modeling works response have been quantified by the relative activity of effectors. In contrast, in this work we quantified response as the sum of amounts of individual oligomers that contains more than 6 monomer units, weighted by their size (Eq. 1). Such calculation is reflecting the fact that MAC pore complex needs to contain a minimum 6 Bax/Bak monomer units to allow transport of cytochrome c (the major pro-apoptotic factor released from mitochondria), also taking into account that the larger pores contribute to permeabilization more than smaller ones [40].

$$\text{Response} = \sum_{i=6}^{20} i \cdot MAC_i$$ (1)

For all three models we evaluated the dependence of the response coefficient on the stimulus, and the activated fraction [49, 36]. Then we calculated corresponding relative amplification coefficients $n_R$.

While the indirect and hybrid models were proved to be strongly sub-sensitive ($n_R \sim 10^{-2}$), the direct model resembled ultrasensitive behavior ($n_R \approx 2.0$).

2.1. Variations of pivotal parameters and their effects on sensitivity

In order to investigate the influence of individual reaction parameters on sensitivity of the Bcl-2 apoptotic switch models, we plotted relative amplification coefficient of hybrid model as a function of variation of individual reaction parameters ($k_1, k_{i1}, k_{i2}, k_{in}, k_c, k_s, k_o$). The same parameter variation was then performed after the reduction of the hybrid model into its limiting direct/indirect cases.

Our results demonstrate, how dramatically can variation of single reaction parameter effect the sensitivity of the modeled system (Fig. 4). The variation of the parameter $k_s$ (spontaneous activation of Bax) change the relative amplification coefficient of hybrid model by two orders of magnitude.
As ks is 100× decreased hybrid model reaches the ultrasensitivity. Less apparent is the influence of parameters kc (activation of Bax by Act–a) and kin (inactivation of Bax–a). The dependence of sensitivity to parameter’s variation is monotonic for most of the parameters. One notable exception is non-monotonic dependence on reaction parameter ki (binding of Bax by Bcl-2). We found local maxima of sensitivity around \( ki = 12.0 \cdot 10^{-6} \) and \( ki = 7.0 \cdot 10^{-6} \), for the hybrid and indirect model respectively. Similarly, the dependence of relative amplification coefficient of the direct model on variation of parameters contains several local extreme values. By comparing our models, we can see how different is their capability to preserve ultrasensitive response. While direct model keeps ultrasensitive response under wide range of variations, indirect model yields response only with relative amplification coefficient far below unity.

Although single point variation of reaction parameters may reveal certain interdependence of system properties on the value of chosen parameter, it provides only very limited view on relationships between these properties and system parameters.

To get a deeper understanding of the impact of the individual Bcl-2 proteins interactions and their interplay on the switching behavior of the modeled system, we performed an additional analysis. We have generated 2000 sets of randomly changed values of reaction rates ki, ki1, kc and ks related to controversial interactions. At the same time the rest of the parameters remained unchanged at their reference values. Then we calculated relative amplification coefficient of the hybrid model corresponding to each set of parameters. To obtain picture about how the particular parameters affect sensitivity of hybrid model, we separated all the parameter sets to intervals, according to the variation of individual parameters. Then, for each interval we calculated the mean of relative amplification coefficients - \( \bar{n}_R \) given by the parameter sets within the interval.

As can be seen from Fig. 5, the mean relative amplification coefficient of parameter sets, in which the value of ks is decreased (compared to its reference value), is much higher than the mean relative amplification coefficient of parameter sets in which the ks is increased. This means, that the decreased value of ks yield higher relative amplification coefficient more often than the increased ones. In case of the parameter kc, the highest mean relative amplification coefficient (almost \( \bar{n}_R \approx 0.8 \)) was obtained for parameter sets in which kc was varied from 17.8 to 31.6 × its reference value. There is no such apparent dependency observed in case of parameters ki and ki1. The
mean relative amplification coefficient is approximately the same, regardless of variation of these parameters (data not shown).

Our results indicate that the presence of spontaneous activation of effectors (with corresponding parameter $k_s$) adversely influence sensitivity of hybrid model. On the contrary, activation of effectors by activators (parameter $k_c$) seems to be beneficial for ultrasensitive, switching behavior of the model. These findings are unfavorable for the hypothesis of indirect activation, that rely on the spontaneous activation of effectors without activation by activators.

Taking into account the findings above, we set parameters $k_s$ and $k_c$ of hybrid model to $10^{-2}$ and $20.0 \times$ their reference values, respectively. In next step we investigated how the interplay between two alternatives of effectors inhibition may affect sensitivity of the hybrid model. We have simultaneously varied values of parameters $k_i$ and $k_{i1}$ (corresponding to inhibition of effectors before activation, and after activation, respectively) and measured the relative amplification coefficient.

Results shows (Fig. 6) that sensitivity of the hybrid model is only weakly affected by changing the values of $k_i$ and $k_{i1}$. Relative amplification coefficient of hybrid model is changed only marginally, even as we changed the values of $k_i$ and $k_{i1}$ by several orders of magnitude. It clearly appears that the highest relative amplification coefficient is reached when the value of the parameter $k_i$ is reduced and of the parameter $k_{i1}$ is increased, indicating the importance of neutralization of active effectors by anti-apoptotic proteins. The lowest relative amplification coefficient was found as we reduced values of both parameters.

2.2. Addition of the auto-activation of Bax

Works of Ruffolo et al. [50] and Tan et al. [51] provided experimental evidence that activated effectors Bak, as well as Bax can directly interact with, and positively influence activity of non-activated Bak and Bax, respectively. This interaction, called Bax/Bak auto-activation can enhance activity of effectors, initially activated by spontaneous activation (as is proposed by hypothesis of indirect activation) or by BH3-only activators (hypothesis of direct activation).

Such auto-activation forms positive feedback loop, that can dramatically change the stimulus-response steady-state properties of the model. This could make our search for ultrasensitive setup even more difficult, therefore we have not involved this interaction into initial stages of our model. On
the other hand, addition of such positive feedback to ultrasensitive reaction mechanism may lead to increase of the ultrasensitivity.

It is still not determined whether auto-activation of effectors is mediated by monomeric, or by oligomerized effectors [26]. In most of the previous modeling works, auto-activation of effectors was modeled as arrangement of the inactive effectors into oligomerized pores. In contrast to these works, we have tried to investigate, which of the two alternatives of auto-activation has the most beneficial effect on the switching properties of the Bcl-2 apoptotic switch. We have modeled the auto-activation mediated by monomeric effectors as the following reaction (reaction auto1):

\[
\text{auto1: } \text{Bax} + \text{Bax–}a \rightarrow \text{Bax–}a + \text{Bax–}a
\]

Auto-activation mediated through oligomerization was modeled as an arrangement of the inactive Bax into oligomerized pore (reaction auto2), similarly to previous models of Bcl-2 apoptotic switch [19, 52].

\[
\text{auto2: } \text{Bax} + MAC_i \rightarrow MAC_{i+1}
\]

We have added auto-activation of Bax into the hybrid model and measured relative amplification coefficient for different values of corresponding reaction rate - \(k_a\). We tested first the impact of the auto-activation of Bax mediated by monomeric units of active Bax–a. We have found that for narrow area of \(k_a\) values rapid rise of the relative amplification coefficient is followed by dramatic decline as value the of \(k_a\) exceeds the threshold \(\sim 2.0 \cdot 10^{-6}\) (see Fig. 7). Increasing the value of the parameter \(k_a\) amplifies the strength of the positive feedback. As we take a closer look on the stimulus-response curves (Fig. 8), we can see that the positive feedback narrows the intermediate part of the sigmoidal stimulus response curve, making it steeper.

There are numerous works discussing how the addition of positive feedback into an ultrasensitive reaction system may influence its steady-state properties [53, 54, 55]. Such addition is often associated with emergence of the bistable behavior assuming the feedback strength exceeds certain threshold. In contrast with these suggestions, we observe rapid fall of the relative amplification coefficient for overly strong feedback. The inflection point of the stimulus-response curve shifts to the left along the stimulus axis as we increase the \(k_a\) value, and when the certain threshold value is reached, the inflection point leaves reasonable range of input stimuli. The auto-activation then causes high mitochondrial membrane permeability even at basal level of incoming stimuli.
We have replaced auto-activation mediated by monomers - reaction auto1, by reaction auto2 and evaluated the relative amplification coefficient for different values of corresponding reaction parameter - ka. Relative amplification coefficient was only slightly affected by variation of the ka value, indicating that auto-activation mediated by oligomerized effectors has only marginal effect on the sensitivity of the hybrid model.

These results show, that although both forms of auto-activation may efficiently enhance the effectors activity, only the auto-activation mediated by monomers interaction can amplify the steady-state ultrasensitivity of the hybrid model. Although, it should be noted that sensitivity increase occurs only within the narrow range of values of auto-activation reaction rate. Overly fast auto-activation of effectors causes rapid onset of permeability even if the input stimulus is weak, thus disabling switching behavior.

2.3. Robustness analysis

All essential biological mechanisms should exhibit considerable level of robustness against parameter changes [56], since effects of environmental differences, polymorphism or mutations needs to be compensated [57]. Biological switches such as Bcl-2 apoptotic switch should preserve ultrasensitivity as their pivotal feature, in spite of variation of parameters.

First, we have adjusted the parameter setup of the hybrid model in accordance to all the previous results, to obtain desirable level of sensitivity (adjusted values of parameters are summarized in Table 4). To test the robustness of our models, we measured relative amplification coefficient for multiple sets of varied reaction rates (ki, ki1, ki2, kc, ks, kin) and initial concentrations (Act, Bax, Bcl-2). Similar to the work of Barkai and Leibler [58] and some later works [57, 20], we generated set of modified parameters, by multiplying each of reference parameters by $10^q$, where q is random real number taken from Gaussian distribution (mean = 0.0, variance = 1.0). Relative amplification coefficient of each set was then plotted as a function of total parameter variation ($T$), which is defined as total order of magnitude of parameter variation [58, 57, 20]

$$T = \sum_{i=1}^{n_p} \log_{10} \left| \frac{p_i}{p_{i,\text{ref}}} \right|$$

(2)

where $n_p$ is number of varied parameters, $p_i$ is varied parameter and $p_{i,\text{ref}}$ is corresponding reference parameter. Robustness was quantified by frequency
of occurrence of ultrasensitive responses among 1000 random parameter sets. Similar approach was previously used in the work of Chen et al. [20], where direct and indirect model were compared. It needs to be remarked that our model considerably differs to those proposed by Chen et al., therefore robustness analysis results are not directly comparable.

We have found around 35% from 1000 hybrid model parameter sets which yield sigmoidal response curve with corresponding relative amplification coefficient higher than 1.0 (see the scatter plot in Fig. 9). The values of the relative response coefficient mostly occurred below the 2.5, in around 90% of all sets. As we reduced the hybrid model to the indirect model, none of the thousand sets yield sigmoidal response curve with relative amplification higher than 1.0, strongly opposing the suggestion, that the indirect model gives rise to ultrasensitive response. On the other hand, as we reduced the hybrid model to the direct model, we have obtained sigmoidal response curve with relative amplification coefficient higher that one in around 60% of parameter sets. This shows that omitting the spontaneous activation of effectors, and their preventive inhibition makes response more model more sensitive.

Frequency of occurrence of relative amplification coefficient higher than unity, is a measure of model’s capability to preserve ultrasensitive stimulus-response dependence despite changing its internal parameters. Although ability to preserve ultrasensitive response is directly related to switching robustness, there is something else we would expect from the robust ultrasensitive switch. In fact, besides preserving ultrasensitivity of stimulus-response dependence, robust switch should be able to conserve the stimulus-response dependence itself. With respect to subsequent processes regulated by ultrasensitive switch, we consider, that proper functioning of such switch requires reaction mechanism that is able to conserve its stimulus-response characteristics despite small changes of its internal parameters. We hypothesized, that spontaneous activation of effectors, or the preventive inhibition of effectors may possibly strengthen this capability. To characterize the model’s ability to preserve the stimulus-response dependence, we have measured the position of its inflection point.

We took parameter sets, which yield relative amplification coefficient higher than one, and plotted the corresponding stimulus-response curves and stimulus-response slope dependencies (see Fig. 10). For each response dependence, we localized the inflection point at which response slope reaches its maximum. Then, we plotted statistical distribution of inflection point
coordinates (Fig. 11). As can be seen from Fig. 11, for the direct model, there are two statistically significant peaks located around stimulus values $1.8 \times 10^{-3}$ and $9.5 \times 10^{-3}$. This indicates, that the interaction pattern of direct model is efficiently conserving stimulus coordinate of the inflection point in close proximity of these values. Hereby, stimulus coordinate of inflection point changes only a little as we change internal parameters of the direct model. We do not observe such conservation of stimulus coordinate in the case of the hybrid model (Fig. 11 - Hybrid model), showing that stimulus coordinate of inflection point varies proportionally to variation of internal parameters.

The distributions of the response coordinate of the inflection point of both models show, that the inflection point occurs typically around 0.1 to 0.2 % of the highest response reached. The response coordinate of the inflection point is strongly conserved in both hybrid/direct models.

Taken together, we found that the direct model is, compared to the hybrid one, more robust with respect to ultrasensitivity, as well as regarding the switching threshold behavior of inflection point of its stimulus-response dependence.

3. Discussion

3.1. Steady-state ultrasensitivity originates from Bax/Bak activation by BH3-only activators, not from spontaneous activation

Our aim was to identify those interactions between Bcl-2 subgroups, which are responsible for emerging switching behavior. Therefore we examined how the variations of the model’s reaction rates can affect properties in the modeled system. Single parameter variations, as well as simultaneous variations of multiple parameters revealed interesting relations between parameter setup and steady-state sensitivity of the hybrid model and reduced indirect/direct models.

Based on these results we can conclude that spontaneous activation of effectors Bax/Bak plays minor if any role in activation, allowing their further oligomerization and mitochondrial permeabilization. Similarly, it seems that the neutralization of activated effectors contributes to switching behavior far more than preventive inhibition of inactive forms. Taken together, the interaction pattern corresponding to hypothesis of direct activation forms switching mechanisms that yields ultrasensitive behavior. Alternative interaction pattern - the indirect model, does not lead to this behavior.
3.2. Monomers mediated auto-activation of Bax/Bak may strongly enhance ultrasensitivity of the Bcl-2 apoptotic switch

As was reported in work of Tan et al. [51], activated Bax may activate non-activated Bax. Similar finding was reported for Bak in work of Ruffolo et al. [50]. The mechanism of this auto-activation is still not fully understood. It is discussed, whether auto-activation is mediated by monomers or by oligomerized Bax/Bak. Despite the lack of knowledge, auto-activation of effectors is sometimes referred as responsible for switching properties of Bcl-2 apoptotic switch. The relevance of the auto-activation with respect to the switching function of Bcl-2 regulatory mechanism has never been examined.

Such autocatalytic activations of effectors form positive feedback loop, that can strengthen ultrasensitivity of the reaction mechanism or even lead to bistable behavior [54]. We have modeled the influence of auto-activation on the switching properties of the studied system, by measuring sensitivity after addition of auto-activation and variation its reaction rate.

We have added autocatalytic activation of effectors into adjusted model and varied its reaction rate. In case of monomers mediated auto-activation, within certain values of reaction rate we observed rapid increase of sensitivity, followed by its sudden fall. This is due to the withdrawal of the inflection point of sigmoid stimulus-response curve for the reasonable range of input stimuli. This means that overly strong auto-activation may easily cause massive effectors activation and high MOM permeability even by basal stimuli. Auto-activation mediated by oligomers has no significant effect on the ultrasensitivity of the modeled system.

Although hybrid model of the Bcl-2 apoptotic switch exhibits sufficient level of ultrasensitivity even without presence of any auto-activation feedback loop, addition of monomers-mediated auto-activation may strongly amplify ultrasensitivity. Ultrasensitivity amplification is attributed to monomers mediated auto-activation only. Auto-activation by oligomerized effectors can only enhance activity of effectors but does not contribute to ultrasensitive behavior.

3.3. The direct model is more robust, compared to the hybrid or indirect ones

Upon addition of the auto-activation into the hybrid model, we have adjusted reaction rates of this model to obtain strongly ultrasensitive response. Then we analyzed its robustness with respect to its ability to preserve ultrasensitivity against variations of its reaction parameters and initial conditions. We measured frequency of occurrence of the ultrasensitive response among
thousand of randomly generated parameter sets. There were around 35% of the parameter sets of the hybrid model which yielded sigmoidal stimulus response with relative amplification coefficient higher than one. We have then reduced the hybrid model to the indirect/direct ones and compared robustness of the hybrid model with robustness of indirect and direct models. We have found that indirect model is unable to provide ultrasensitive behavior, since none of the thousand parameter sets have yield sigmoidal stimulus response dependence with relative amplification coefficient higher than one. In opposite, the direct model compared to the hybrid model was found much more robust (70% of the parameter sets), indicating that the model’s ability to preserve ultrasensitivity is negatively affected by spontaneous activation of effectors and their preventive inhibition.

We have hypothesized that these interactions may possibly increase model’s ability to conserve stimulus-response dependence. In order to examine this assumption, we have compared stimulus response curves of ultrasensitive responses obtained from previous analysis. We have plotted the distribution of the threshold stimulus (the stimulus corresponding to inflection point of the stimulus response sigmoid) and the corresponding threshold response. In case of the direct model, we have observed two significant peaks of that distribution. This interesting finding indicates that the direct model exhibits the capability to conserve threshold stimulus within area around those peaks. We found no such effect in case of the hybrid model. This rules out our assumption about robustness supporting role of the spontaneous activation and preventive inhibition of effectors.

3.4. Indirect model interaction may provide some other system properties, but not steady-state ultrasensitivity

Taken together, all the results from this work point to following conclusions: interaction pattern as described by the hypothesis of indirect activation is very unlikely to give arise to the switching behavior as is expected from Bcl-2 apoptotic switch. On the other hand, its alternative - the direct model hypothesis gives much more plausible explanation of functioning of Bcl-2 apoptotic switch. By merging these interaction patterns into one, we obtained hybrid model. Hybrid model, compared to direct one, is less robust with respect to ultrasensitivity preserving, and seems to be less efficient in providing switching function.

Despite these conclusions related to steady-state ultrasensitivity, we cannot completely rule out the indirect model interactions. Since the require-
ments for ultrasensitivity suggest that these interactions must proceed at very low rates, but these interactions still may exist and provide other important system properties. Low rate spontaneous activation of effectors can make response to changed stimuli more abrupt, while still preserving desirable steady-state ultrasensitivity. At certain affinity, continuous binding and inhibition of inactive effectors by anti-apoptotic proteins may possibly help to attenuate the noise of the incoming signal. However these ideas need further examination. Therefore, the study of transient system properties of the Bcl-2 apoptotic switch is aim of our further modeling work.

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5. Appendix

In metabolic control analysis, the response coefficient is defined as steady state fractional change of the system response $\frac{\Delta X}{X}$ divided by fractional change of stimulus $\frac{\Delta S}{S}$ in limit as $\Delta S$ tends to zero \cite{59, 49}:

$$R^X_S = \lim_{\Delta S \to 0} \frac{\Delta X/X}{\Delta S/S} = \frac{d\ln X}{d\ln S}$$

(A.1)

The global sensitivity is measured through relative amplification coefficient using definition of Legewie et al. \cite{36} as:

$$n_R = \frac{\int_{f_L}^{f_H} R^X_S \, df}{\left| \int_{f_L}^{f_H} R^X_{S_r} \, df \right|}$$

(A.2)
Where $f$ is activated fraction defined as:

$$f = \frac{X - X_{\text{min}}}{X_{\text{max}} - X_{\text{min}}}$$  \hspace{1cm} \text{(A.3)}$$

$f_H$ and $f_L$ are margins of the activated fraction range and $R_{X_{r}}^X$ is the response coefficient of the reference response $X_r$, which can be any monotonically increasing or decreasing function.

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Figures

Tables
Figure 1: Layout of the interplay between particular members of the Bcl-2 family as described by the indirect activation model (top) and direct activation model (bottom).

Figure 2: Simplified scheme of the hybrid model as proposed in this work (production and degradation flows, as well as reverse reaction are not depicted).
Figure 3: Plot of the stimulus-response dependence (response is scaled to one) of hybrid model, as well as indirect and direct models, using reference parameter setup (see Table 3).

Figure 4: Dependence of relative amplification coefficient of indirect hybrid (left) and reduced - indirect (center), direct (right) models, on variation of individual parameters.
Figure 5: Dependence of the mean relative amplification coefficient on variation of parameter as used in randomly chosen parameter sets.
Figure 6: Dependence of relative amplification coefficient on simultaneous variation of reaction parameters. Parameters $k_i/k_{i1}$ are reaction rates of binding and inhibition of inactive/active effectors by their anti-apoptotic relatives.

Figure 7: Dependence of relative amplification coefficient on the variation of reaction rate of Bax auto-activation. Black line corresponds to auto-activation mediated by monomers (as described by reaction auto1), gray line corresponds to auto-activation mediated by oligomerized Bax.
Figure 8: Changing shape of stimulus-response curves upon addition of monomers mediated Bax auto-activation (auto1) with increasing reaction rate (ka values are displayed beside each curve).

Figure 9: Scatter plots of relative amplification coefficient with dependence on the value of total parameter variation. Dots localized over gray dashed line corresponds to randomly generated parameter sets that yield ultrasensitive response, with relative amplification coefficient higher than one.
Figure 10: For each randomly generated parameter set yielding ultrasensitive response (see Fig. 9, dots over gray dashed line), corresponding stimulus-response dependence was plotted (gray lines). Stimulus-response dependencies are normalized, and merged to common inflection point. Similarly, corresponding stimulus-response slope dependencies are plotted. Black lines correspond to response produced under adjusted parameter setup (see Table 4).
Figure 11: For each stimulus-response dependence (Fig. 10, gray lines) we have measured its threshold stimulus and corresponding threshold response. Histograms shows distribution of these values.
| Species      | Initial concentration | Notes & Ref. |
|--------------|------------------------|--------------|
|              | # · cell⁻¹ | nM |          |
| Bcl-2        | 60 000     | 100 | [10, 20] |
| Bax          | 120 000    | 200 | [10, 20] |
| Act          | 6000       | 10  | [41, 42] |
| Bax–a        | 0          | 0   |          |
| Act–a        | 0          | 0   |          |
| Bcl-2~Bax    | 0          | 0   |          |
| Bcl-2~Bax–a  | 0          | 0   |          |
| Bcl-2~Act–a  | 0          | 0   |          |
| MACi         | 0          | 0   |          |

Table 1: List of initial concentrations. Concentrations are listed as numbers of molecules per reference cell volume 1 pl, and in more common units - nanomols.

| No. | Reaction                          | $k_+$ | $k_-$ |
|-----|-----------------------------------|-------|-------|
| 1.  | Act $\xrightarrow{E} \text{Act-a}$ | E     |       |
| 2.  | Bcl-2 + Bax $\rightleftharpoons$ Bcl-2~Bax | ki    | kmi   |
| 3.  | Bcl-2 + Bax–a $\rightleftharpoons$ Bcl-2~Bax–a | ki1   | kmi1  |
| 4.  | Bcl-2 + Act–a $\rightleftharpoons$ Bcl-2~Act–a | ki2   | kmi2  |
| 5.  | Bcl-2~Act–a + Bax $\rightleftharpoons$ Bcl-2~Bax + Act–a | ki    | ki2   |
| 6.  | Bcl-2~Act–a + Bax–a $\rightleftharpoons$ Bcl-2~Bax–a + Act–a | ki1   | ki2   |
| 7.  | Bcl-2~Bax–a + Bax $\rightleftharpoons$ Bcl-2~Bax + Bax–a | ki    | ki1   |
| 8.  | Bax $\rightarrow$ Bax–a           | ks    |       |
| 9.  | Act–a + Bax $\rightarrow$ Act–a + Bax–a | kc    |       |
| 10. | Bax–a $\rightarrow$ Bax           | kin   |       |
| 11. | Bax–a + Bax–a $\rightleftharpoons$ MAC₂ | ko    | kmo   |
| 12. | Bax–a + MACᵢ $\rightleftharpoons$ MACᵢ₊₁ | ko    | kmo   |
| 13. | (All) $\rightarrow$               | kd    |       |
| 14. | $\rightarrow$ Bcl-2             | kpBcl-2 |       |
| 15. | $\rightarrow$ Bax                | kpBax |       |
| 16. | $\rightarrow$ Act                | kpAct |       |

Table 2: List of reactions and corresponding reaction parameters. Reversible reactions are listed with forward/reverse reaction parameters.
of parameters remained unchanged. 

Table 4: List of adjusted values of reaction parameters. In order to obtain desirable level of ultrasensitivity, listed parameters have been adjusted according to previous results. Rest of parameters remained unchanged.

| Param. | Value       | Unit                   | Notes & Ref.                      |
|--------|-------------|------------------------|----------------------------------|
| ki     | $1.0 \cdot 10^{-6}$ | (1.0 $\cdot 10^4$) | cell $\cdot$ min$^{-1}$ $(M^{-1} \cdot s^{-1})$ | (in direct) $= 0$, estimated |
| kmi    | 0.06        | (0.001)                | min$^{-1}$ $(s^{-1})$            | = kmi1                          |
| ki1    | $1.0 \cdot 10^{-6}$ | (1.0 $\cdot 10^4$) | cell $\cdot$ min$^{-1}$ $(M^{-1} \cdot s^{-1})$ | (in indirect) $= 0$, estimated |
| kmi1   | 0.06        | (0.001)                | min$^{-1}$ $(s^{-1})$            | same as in [19]                  |
| ki2    | $1.0 \cdot 10^{-6}$ | (1.0 $\cdot 10^4$) | cell $\cdot$ min$^{-1}$ $(M^{-1} \cdot s^{-1})$ |                     |
| kmi2   | 0.06        | (0.001)                | min$^{-1}$ $(s^{-1})$            | same as in [19]                  |
| kc     | $1.0 \cdot 10^{-6}$ | (1.0 $\cdot 10^4$) | cell $\cdot$ min$^{-1}$ $(M^{-1} \cdot s^{-1})$ | (in indirect) $= 0$,          |
| ks     | 0.06        | (0.001)                | min$^{-1}$ $(s^{-1})$            | estimated                       |
| kin    | 0.06        | (0.001)                | min$^{-1}$ $(s^{-1})$            | estimated                       |
| ko     | $1.0 \cdot 10^{-6}$ | (1.0 $\cdot 10^4$) | cell $\cdot$ min$^{-1}$ $(M^{-1} \cdot s^{-1})$ | estimated                       |
| kmo    | 0.06        | (0.001)                | min$^{-1}$ $(s^{-1})$            | estimated                       |
| kd     | 0.0039      | (6.5 $\cdot 10^{-5}$) | min$^{-1}$ $(s^{-1})$            | $t_{1/2} = 180 \text{ min}$, estimated |
| kpBcl-2| 234.0       | (6.4 $\cdot 10^{-12}$)| $\cdot$ cell$^{-1}$ min$^{-1}$ $(M^{-1} \cdot s^{-1})$ | = Bcl$_{2\text{init}}$ : kd |
| kpBax  | 468.0       | (1.3 $\cdot 10^{-11}$)| $\cdot$ cell$^{-1}$ min$^{-1}$ $(M^{-1} \cdot s^{-1})$ | = Bax$_{\text{init}}$ : kd     |
| kpAct  | 23.4        | (6.4 $\cdot 10^{-13}$)| $\cdot$ cell$^{-1}$ min$^{-1}$ $(M^{-1} \cdot s^{-1})$ | = Act$_{\text{init}}$ : kd     |

Table 3: List of reference values of reaction parameters. By setting parameters ki1 and kc equal to zero, the hybrid model is reduced to the indirect one. Similarly, the direct model can be obtained by setting parameters ki and ks of the hybrid model equal to zero. Production rates kpBcl-2, kpBax and kpAct has been set to balance degradation of corresponding species under initial conditions.