Mathematical Modeling reveals threshold behaviour of CD95-induced Apoptosis

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One-sentence summary

Mathematical modeling and quantitative numerical simulations reveal a threshold behaviour of CD95-induced apoptosis.

Abstract

Mathematical modeling is required for understanding the complex signaling behavior of large signal transduction networks. Previous attempts to model signal transduction pathways have been either limited to small systems or have been based on qualitative data only. Here, we address the complexity of a large signal transduction network by combining subsystems of different information qualities, i.e. mechanistically well-understood network parts and ‘black boxes’ defined by the observed input-output behavior. The sensitivity analysis of the mathematical model was key for the identification of critical system parameters and two essential system properties: modularity and robustness. We used programmed cell death, in particular CD95-induced apoptosis, as a prototype application. The resulting data-based model provides new insight into CD95-mediated apoptosis and allows predictions like for the threshold of life and death.

Cells process information by biochemical interactions between molecules. While most of the research on cellular signaling has been focused on deciphering the molecular components of signaling pathways[1], more recently theoretical models for describing the complex signaling behavior on system level have been developed[2, 3,4]. Models of signal transduction networks are either based on discrete models describing signaling as information processing[5] or on continuous models where the information flux is modeled by a biochemical reaction network. In the latter case, the reaction network is translated into a system of ordinary differential equations[6,7,8].

A robust and reliable mathematical simulation of complex networks requires quantitative information on reaction rates and molecule concentrations. For most reactions and molecules, these parameters are not directly accessible *in vivo*. Existing data usually refers to different experimental settings, cell types and states of cells and can therefore not be used for quantitative models. Further, signaling processes are described on various different levels of information quality ranging from mechanistically well-understood interactions to purely semantic relations like activation or inhibition.

Accordingly, mathematical simulations of signal transduction networks have been restricted to well-investigated pathways where most biochemical mechanisms are well understood[9,10]. In a recent data-based study on the JAK-STAT pathway[11] robustly measured data and parameter estimation[12,13] have been suggested as key components for model identification and reliable quantitative simulations[14]. However, the number of assessable parameters and therefore the maximum size of the model is very limited due to

the large amount of experimental data required for high-dimensional parameter estimation problems and the curse of dimensionality[15].

In our study, we present an approach overcoming the present obstacles in large-scale modeling of signal transduction networks. Our approach integrates information on various different levels in a unified form and allows robust parameter estimation even in high-dimensional parameter space. As a case-study, we applied our system to programmed cell death (apoptosis), which is one of the most complex signaling pathways and an essential property of all living organisms.

Defects in apoptosis result in a number of serious diseases such as cancer, autoimmunity and neurodegeneration. To develop efficient therapies fundamental questions about its molecular mechanisms and regulation remain to be answered. The question of a threshold for induction of apoptosis plays a central role in our understanding of the sensitivity and resistance of cells towards various chemotherapeutic agents. Apoptosis is triggered by a number of factors, including UV-light, γ-radiation, chemotherapeutic drugs, growth factor withdrawal (‘death by neglect’) and signaling from the death receptors[16,17,18]. During the last decade many of the molecular mechanisms of apoptosis signaling have been examined and elucidated[19]. Diverse apoptosis pathways are generally divided into signaling via the death receptors (extrinsic pathway) or the mitochondria (intrinsic pathway). Both pathways imply caspases as effector molecules[20,21]**.** Various caspases are involved in both the initiation of the apoptotic process and the execution of the final apoptotic program.

CD95 (APO-1/Fas) induced apoptosis is one of the best studied apoptosis pathways. CD95 is a member of the death receptor family, a subfamily of the TNF-R superfamily. Crosslinking of CD95 either with its natural ligand CD95L or with agonistic antibodies such as anti-APO-1 induces apoptosis in sensitive cells. Upon CD95 stimulation the Death-Inducing Signaling Complex (DISC) is formed[22]. As a result of the CD95 DISC formation active caspase-8 is autocatalytically cleaved at the DISC resulting in the formation of active caspase-8 which starts the apoptotic cascade[23].

In a first attempt to theoretically describe apoptotic signaling a mathematical model including more than 20 reactions with ad hoc fixed parameters was proposed[24]. In contrast to that work, we decided to establish a data-based approach for a generalized model of CD95-induced apoptosis where parameters are estimated on the basis of quantitative experimental data. In a first step, we reconstructed the network topology of CD95-induced apoptosis by searching databases[25] and the literature. Molecules and reactions directly or indirectly interacting with the known players of this pathway were incorporated leading to a model with about 70 molecules, 80 reactions and more than 120 unknown parameters (data not shown). This complexity cannot be matched by experimental data at present.

To reduce the complexity of the model without sacrificing essential components of the network, we incorporated subunits of different information qualities: reactions with well-understood biochemical mechanisms were modeled mechanistically. For all other

interactions, ‘black boxes’ were introduced, defined by their experimentally observed input-output behavior. Notably, these black boxes do not assume any knowledge on the exact underlying mechanism. A great advantage of the so-obtained ‘Structured Information Models’ is that they combine heterogeneous information in one model (supp. Fig. 1) instead of dealing with isolated models[26].

The resulting model of CD95-induced apoptosis consists of 41 molecules[27], 36 reactions, and 54 parameters (Fig. 1). Thus, even the simplified model contains more than 50 missing parameters, which is still too high for robust parameter estimation given the limited number of data points. For identification of the most critical system parameters, sensitivity analysis was applied. Sensitivities describe the relative changes of molecule concentrations (and therefore of the system behavior) as a result of changes of the parameters. Since in general sensitivities can be determined for specific sets of parameters only (local sensitivity analysis), the usefulness of sensitivity analysis is limited if most parameters are unknown at first. In a virtual experiment, we therefore determined sensitivities for a large number of randomly chosen points in parameter space within specified ranges, covering more than 3 orders of magnitude (see supp. online material). Surprisingly, the distribution of most sensitivities showed distinct and narrow peaks (Fig. 2) indicating that most sensitivities of the system are highly robust to large variations in parameter values.

The sensitivity analyses led us to a further inherent system property, the modularity of the apoptotic signaling pathway. Apparently, clusters of molecules and parameters can be

identified by subsets of molecules whose concentrations depend on a subset of parameters only (Fig 3a). In addition to these parameters that can be efficiently estimated locally there are global parameters belonging to more than one cluster. The problem of global parameters is addressed by a hierarchical approach where parameter estimation is performed on two levels. On the upper level, the global parameters are estimated by optimising all clusters: For each cluster parameter estimation is recursively called (lower level), depending on the global parameter values proposed by the algorithm on the upper level, but independent of the local parameters of other clusters (see supp. online material). As a result, reduction of the system dimensionality is achieved. In a second step, we introduced a sensitivity-control within the parameter estimation algorithm (see supp. online material), which calculates the local sensitivities after each step in parameter space to determine a subset of parameters relevant for the next estimation step[28].

As a result of the sensitivity analysis, we designed a set of experiments to measure time series of concentrations of 15 different molecules after activation of CD95 (see above Fig. 1). For our experiments, we chose human B-lymphoblastoid SKW 6.4 cells, which are type I cells and are highly sensitive to CD95-mediated apoptosis. Cells were stimulated with different concentrations of agonistic anti-APO-1 antibody for various periods of time (from 5 minutes to 4 days). Each sample was evaluated by three independent approaches. Cell death was determined by flow cytometry analysis, the caspase activity was measured by fluorometric caspase activity assays, and the change of concentration of major apoptotic molecules was evaluated by western blot analysis. For

all measurements, standardization of experiments was crucial for robust quantitative measurements[29] (supp. Fig. 4).

In a first experiment, time series were measured for a 'fast' activation scenario with an oversaturated ligand concentration corresponding to more than one ligand per CD95 receptor[30]. A good fit of the caspase activities, PARP, BID etc. could then be achieved (supp. Fig. 3) reproducing the fast cleavage of procaspase 8 into its active form, followed by activation of the executioner caspases, Bid and PARP (Fig. 4A-D and supp. Fig. 5). We conclude that the mathematical model is well suited to fit the experimental data. However, the model is still underdetermined, i.e. many different model parameter settings are able to match the same experimental data. Accordingly, the generalization ability of the model and its usefulness for biological predictions are very much limited. Therefore, we decided to gain more information about the system by measuring different activation scenarios with lower initial ligand concentrations and to base the parameter estimation on these multiple conditions (Fig. 3b and supp. Fig. 6). Therefore, parallel models, each representing one activation scenario, were automatically generated based on a common set of biochemical parameters but different initial values of ligand concentration. Our model is able to predict several activation scenarios as a result of one combined parameter estimation step (Fig 4E-H). The estimated parameters also show accordance with *in vitro* data where available (see supp. online material).

Both the model predictions and the experimental data show that with decreasing ligand concentration apoptosis is slowed down considerably; however, cell death is achieved for

all activation strengths. To address the question whether the apoptotic process slows down continuously with lower ligand concentrations or whether there is a threshold for induction of apoptosis at a distinct receptor-ligand ratio, we simulated induction of apoptosis for very low ligand concentrations. Our model predicts that below a critical concentration corresponding to a receptor-ligand ratio of about 100, apoptosis is completely stopped (Fig. 4I-K). This prediction was validated by experiments (Fig. 4M-N).

It remains puzzling that even for the below-threshold scenario a sufficient number of receptors is apparently activated to cleave procaspase-8, thereby triggering all subsequent caspases[31]. In an intuitive interpretation, one would assume that even for very low ligand concentration apoptosis should not be entirely stopped but only delayed. This apparent contradiction between model prediction and intuitive considerations can be only resolved by elucidating the exact mechanism of this threshold behaviour and by revealing the responsible molecules and molecular interactions.

The binding of the short and the large splice variants of c-FLIP to the DISC constitutes a competitive process to the activation of caspase-8[32]. As a result of our parameter estimation, we conclude that there are more receptors in total than c-FLIP molecules are able to bind within a reasonable time frame. The cleavage rate of caspase-8 is dependent on the number of active receptors. Whenever c-FLIP binds to a DISC, the respective binding site will be irreversibly blocked. The simulation of the below-threshold scenario shows a steady decrease of active DISCs until all of them are blocked by c-FLIP. As a

consequence, it shows a limited generation of the intermediate caspase-8 cleavage product p43/p41, mainly cleaved via c-FLIP$_L$ (Fig. 4L), but no significant generation of active caspase-8 as a result of the early and complete DISC-blockage. In contrast, the simulation for a ligand-receptor ratio above the threshold shows a qualitatively completely different behavior: due to the higher number of active receptors, the amount of c-FLIP is not sufficient to block all DISCs before active caspase-8 can be generated in a quantity that is sufficient to trigger apoptosis.

In contrast to the presently controversial discussion about threshold mechanisms involving downstream inhibitors like IAP or XIAP[33,34], our model suggests that the threshold of CD95-induced apoptosis is included upstream in the DISC. The ratio between active receptors to c-FLIP as well as the ratio between the binding rates of c-FLIP to DISC and procaspase-8 to DISC seem to be highly relevant parameters for this threshold. We experimentally verified the proposed mechanism by systematically scanning the activity of downstream molecules. Our results confirm that a low amount of p43/41 was generated and that neither the executioner caspases (e.g. caspase-3) nor PARP cleavage showed any increased activity for activation strengths beyond the threshold (supp. Fig. 7).

We showed that the mathematical model of CD95-induced apoptosis provides insights into important biochemical mechanisms and predictions like the threshold of life and death. The problem of the high number of unknown parameters could be approached by incorporating parameter sensitivities into the parameter estimation, which allowed to

drastically reduce the complexity of the problem. Two inherent system properties, i.e. the modularity and the extremely high robustness[35,36] of sensitivities, were essential here. Different levels of information were incorporated by introduction of 'black boxes' simulating observed input-output-behavior where exact knowledge on biochemical reactions is missing. Additional information about the parameters could be gained by incorporation of different activation scenarios into one parameter estimation step.

The developed framework provides a general basis for large-scale modeling and simulation of complex biochemical networks including signal transduction pathways and metabolic networks. The proposed method for automatic model reduction can be readily applied to other applications. The widely used approach of manually simplifying models in advance is time-consuming and potentially introduces a user bias into the model. In contrast, the intrinsic reduction of the model dimensionality proposed here is systematic and adaptive to both the original model and the experimental data. Further, techniques like the combination of heterogeneous information levels or the modularization of parameter estimation are based on very general properties of biochemical networks and are well-adapted to the presently limited availability of robust kinetic data.

The established loop between model and experiment was an essential component of this study. Outcomes of experiments performed for different scenarios and different molecules are used to verify, to refine and to adapt the theoretical model, which in return was used for experimental planning. We are now able to simulate the mechanism of CD95 induced apoptosis under different conditions (e.g. for different expressions of c-

$FLIP_L$, c-$FLIP_S$ or FADD), thereby predicting a higher or lower resistance to apoptosis. Abnormal c-FLIP expression has been identified in various diseases connected with dysregulation in CD95 signaling such as multiple sclerosis (MS), Alzheimer's disease (AD), diabetes mellitus, rheumatoid arthritis (RA), Hodgkin's disease (HD) and different cancers[37,38]. It was shown that c-$FLIP_S$ has a short half-life and c-$FLIP_S$ might be downregulated by inhibitors of protein synthesis resulting in sensitization of tumors to apoptosis. Our modeling framework is a powerful tool for predicting potential interaction partners of chemotherapeutics in the apoptotic pathway and for studying the mechanism behind the regulation of apoptosis by drugs in treatment of cancer and other diseases. This is of outmost clinical relevance since there is strong evidence showing a highly complex and dynamic pattern of multiple resistance mechanisms in particular after challenging tumor cells by chemotherapeutic drugs. The challenge is even more increasing, once the *in vivo* situation of resistance mechanisms will be attempted to be functionally dissected[39].

In summary, the modular and hierarchical structure of our framework provides a high degree of flexibility for model extensions in various ways, either by adding additional pathways and systems like proliferation or gene expression, or by adding more detailed biochemical mechanisms with more information becoming available. An important model extension will be the incorporation of stochastic effects, especially close to critical receptor-ligand ratios, where we expect a continuous decrease of the death rate for a population of many cells due to fluctuations. A further challenge will be to describe differences between type I/II cells and to understand different sensitivities to various

drugs interacting with the apoptotic pathway. This work is presently underway in our laboratories.

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Notes

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on homophilic contacts. The DD of CD95 interacts with the DD of FADD, while the death effector domain (DED) of FADD interacts with the N-terminal tandem DED of procaspase-8. Two CD95-signaling pathways were established. Type I cells are characterized by intensive DISC formation and mitochondria–independent caspase-3 activation. In Type II cells the formation of DISC complex is reduced and the activation of caspase-3 occurs downstream of the mitochondria. Namely, in Type II cells the active form of caspase-8 cleaves Bid, followed by tBid translocation to mitochondria which results in the release of cytochrome C, apoptosome formation and the activation of caspase-9. caspase-9 then activates caspase-3 trigerring the following apoptotic events.

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[27] As molecules we also consider complexes like DISC.

[28] The system dimensionality was reduced from 60 unknown parameters to 18 by the hierarchical approach. As a result of the sensitivity control within the parameter estimation the objective function related to the optimal fit could be decreased by factor of 10 to 100.

[29] To standardize the assays, SKW 6.4 cells for all experiments were taken from the logarithmic growth phase. The calibration of quantitative western blot analysis is required to obtain robust measurements of protein concentrations. To ensure the linear relation between the antigen and the strength of the signal, serial dilutions of recombinant proteins or cell lysates were probed by western blotting with antibodies to be used in the experiments. Quantification of chemiluminisense showed excellent linearity in proportion to the amount of an antigen for the concentration of the lysates and used recombinant proteins (suppl. Fig. XXX). Thus, the following western blot experiments were done within the same concentration range for the lysates and the antibody solutions.

30 In this fast activation scenario oversaturation was achieved by 5 µg anti-APO-1 per ml corresponding to a ligand-receptor ratio of about 5:1. The ratio was determined under the assumption that there are about 40000 CD95 receptors per cell.

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Figure legends

Figure 1: Structured information model of CD95-induced apoptosis.

In the mechanistic part (DISC, Caspases, IAP), interactions are modeled as elementary reactions (law of mass action, black arrows), enzymatic reactions (red arrows) and competitive inhibitions (black arrows). Receptors are activated by ligands initiating the DISC formation. After binding to the DISC binding site (DISCbs), procaspase-8 is cleaved (initiator caspase), followed by the activation of executioner caspases (3, 6, 7). PARP cleavage was chosen as experimental end-point of the pathway. The mitochondria and the degradation process, which influences all molecules, are modeled as black boxes defined by their input-output behavior (see supp. online material). Each reaction contains one or more unknown parameters. Since the black boxes are based on observations, the number of unknown parameters is low here. Experimental time series exist for all molecules framed by red lines.

Figure 2: A: Sensitivity matrix of parameters and molecules.

The sensitivity matrix (s_{ij}) shows the relative changes of the concentrations of molecule i (left to right) due to a change of parameter j (front to back). Sensitivities are low in general (<<1) indicating high robustness. The sensitivities of the executioner caspases (supp. Fig. 2) are extremely low indicating the extreme robustness of the core functionality of the apoptotic system. **B**: Sensitivity of Sensitivities: each box shows one histogram for a specific sensitivity, calculated for 10^5 randomly chosen points in parameter space. X-axis: Sensitivity, Y-axis: density of occurrence (see supp. online material). The histograms shown here are representative for the complete matrix. They

show distinct and narrow peaks in most cases. Sensitivities with a clear peak close to zero indicate that the respective molecule concentration is insensitive to the respective parameter – an important property for further modularization.

Figure 3: Framework for modeling and simulation of large signal transduction networks. **A:** Prior to parameter estimation, sensitivities are determined for randomly chosen points in parameter space. All sensitivities with a distinct peak close to zero are considered irrelevant (compare Fig. 2). In the next step ('clustering'), irrelevant sensitivities are removed (white squares), and the matrix is rearranged in a way that provides 'independent clusters' (see supp. online material). On this basis, the parameter estimation is performed for each cluster independently by minimization of the respective objective function. In case of 'global parameters', the parameter estimation for the single clusters is recursively called within the parameter estimation for the global parameters. The right-hand side displays the core part of the computiational system. Whenever sensitivity analysis is applied or the objective function has to be determined for parameter estimation, the simulation is started with a certain parameter set. The simulation is based on the biochemical reaction equations and on the definition of the black boxes, which are automatically translated into a system of differential equations (model generation). The result of the simulation is used to compute sensitivities or the objective function by comparing model predictions with experimental data. **B:** For those parts of the systems that are experimentally not accessible additional information can be gained by measuring the dynamic system behavior under different initial conditions. The unknown parameters are estimated for all different initial conditions at once. These models are simulated in

parallel. The maximum likelihood estimation minimizes the sum of the respective objective functions depending on the corresponding experimental data sets.

Figure 4: Model predictions and experimental validation.

(**A-E**) Parameter estimation for the fast and the slow activation scenario is in good agreement with experimental data. **(A-C)**: The high ligand concentration (fast activation) leads to an early activation of receptors, followed by a fast DISC formation, resulting in a high cleavage capacity of procaspase-8 via the intermediate product (p43/p41) (A). The early caspase-8 generation is followed by the cleavage of caspase-3, -7, and -2 (B) and PARP as well as by cleavage of Bid (C). After PARP cleavage, the degradation process starts. **(D,E)** The model computed on the basis of fast and slow activation (200 ng anti-APO-1/ml) using the same set of biochemical parameters fits well for both the fast and the slow activation scenario. In the slow case, the capacity of caspase-8 cleavage is much lower due to the smaller percentage of receptors activated by ligands. However, there is still a cleavage of 100% of the executioner caspases and PARP resulting in cell death. (**F,G,L**) To test the hypothesis of a threshold behaviour of CD95 induced apoptosis, the activation was simulated for even lower ligand concentrations (100 ng/ml, 10 ng/ml, 1 ng/ml) using the previously estimated parameter set. As expected, caspase-8 cleavage is slowing down. However, for 1 ng/ml (F,G) the death process was completely stopped. According to the model, c-FLIP is blocking the low number of active DISCs (see red and green curve in **H**) before caspase-8 can be generated in a sufficiently high number. For higher ligand concentrations, c-FLIP is not sufficient to block all active DISCs, resulting in a steady caspase-8 cleavage capacity. Experimental data of both the caspase activities

(**M**) and the death rates (**N**) well agree with the model predictions (The death rate for 10 ng/ml was below 100%, due to stochastic effects since the death rates were measured for a population of many cells). The standard deviation of the experimental data were 20% on average.